Host-virus interactions: Innate immunity in wild-type and drug-resistant HIV-1 infections

Diane Nadia Singhroy

Department of Microbiology & Immunology
McGill University, Montréal, Québec, Canada
April 2016

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

“The best way to find yourself is to lose yourself in the service of others”

- Mahatma Gandhi

Table of contents

Abstract

The innate immune system is the first line of defence against HIV, activated within minutes of transmission. HIV must replicate and diversify fast enough to overcome the harsh environment it faces within its target immune cells. The enormous selective pressure that the immune system exercises on replicating HIV species would favor certain phenotypes that permit HIV to replicate despite intrinsic immune factors. Importantly, drug resistance mutations have been shown to be archived and to re-emerge rapidly when failing treatments are re-initiated.

It is widely established that resting memory CD4+ T-lymphocytes represent an important latent reservoir for HIV. Monocytes and macrophage also contribute to the latent reservoir. In addition, monocytes can infiltrate and differentiate in tissues where they reside for decades. They can also penetrate into privileged drug sanctuary sites and they are largely resistant to the cytopathic effects of HIV infection. Wild type (WT) HIV-1 have difficulty infecting dendritic cells (DC), monocytes, macrophage and resting CD4+ T-cells in part because of an intrinsic immune factor called SAMHD1 that keeps dNTP pool low, thus limiting the availability of this substrate for HIV reverse transcription. Previous work in our laboratory has demonstrated that HIV-1 reverse transcriptase (RT) enzyme harboring specific drug resistance mutations (DRMs) can overcome low dNTP compared to WT. This led us to hypothesis that such DRMs, specifically E138K, may help the virus to overcome SAMHD1 restriction and to replicate better in the above mentioned immune cells.

The research I performed during my thesis is divided in 3 parts. First I studied PRMT6 and arginine methylation which we consider to be part of the intrinsic immune system. We had previously showed in the laboratory that PRMT6 is able to restrict HIV replication. My work centered on the regulation of PRMT6 through automethylation that I characterized and demonstrated that PRMT6 automethylation is crucial for its stability and anti-HIV activity.

The second intrinsic immune factor I studied was SAMHD1 where I looked at the replication profile of different drug resistant mutants in primary immune cells and cell lines that express or not SAMHD1. I did in fact find that different drug resistant viruses grew differently in different cell types. Most remarkably, some of the single mutants replicate better in DC and resting CD4+ T-cells whereas E138K mutant replicate as well as the WT virus in macrophages.

We extended our study to integrase drug resistant mutants given that selection studies in cord blood mononuclear cells indicated that cell types might influence the genotype that are selected for under the integrase inhibitor dolutegravir (DTG) pressure. We found that R263K- and H51Y/R263K-containing HIV replicated better than WT in resting CD4+ T-cells. This showed both RT and IN drug resistant viruses have improved fitness in specific cells. Drug resistant virus emerging from long-lived reservoir immune cells could also contribute to the chronic immune activation that is seen in some patients.

Résumé

Le système immunitaire inné est la première ligne de défense contre le VIH, activée seulement quelques minutes après la transmission. Le VIH doit se répliquer et se diversifier suffisamment rapidement pour surmonter l'environnement hostile auquel il fait face à l'intérieur de ses cellules immunitaires cibles. L'énorme pression sélective que le système immunitaire applique sur le virus pourrait paradoxalement favoriser certains phénotypes qui permettent la réplication du VIH en dépit des facteurs immunitaires intrinsèques. Les mutations de résistance aux médicaments sont ainsi archivées dans le réservoir et peuvent réapparaître rapidement lorsque les traitements défaillants sont ré-initiées.

Il est largement établi que les lymphocytes CD4+ quiescent représentent un réservoir latent important pour le VIH. Les monocytes et les macrophages contribuent également au réservoir latent. De plus, les monocytes peuvent infiltrer et se différencier dans les tissus où ils résident pendant des décennies. Ils peuvent également pénétrer dans les sites sanctuaires qui sont peu accessibles aux médicaments et ils sont largement résistants aux effets cytopathogèniques du VIH. Le type sauvage du VIH-1 a de la difficulté à infecter les cellules dendritiques, les monocytes, les macrophages et les lymphocytes CD4+ quiescents, en partie à cause d'un facteur immunitaire intrinsèque appelé SAMHD1 qui maintient des concentrations de dNTP faibles, limitant ainsi la disponibilité de ce substrat pour la transcription inverse du VIH. Des travaux antérieurs dans notre laboratoire et d'autres ont démontré que le VIH-1 hébergeant des mutations spécifiques de résistance aux médicaments dans la transcriptase inverse (RT) peut surmonter les faibles concentrations de dNTP mieux que le type sauvage. Cela nous a

conduit à l'hypothèse que ces mutations, spécifiquement E138K, peuvent aider le virus à surmonter l'effet négatif de SAMHD1 et à se répliquer mieux dans les cellules immunitaires susmentionnées.

La recherche que j'effectué au cours de ma thèse est divisée en 3 parties. Tout d'abord, j'ai étudié PRMT6 et la méthylation de l'arginine que nous considérons comme faisant partie du système immunitaire intrinsèque. Il avait été montré précédemment dans le laboratoire que PRMT6 est en mesure de limiter la réplication du VIH. Mon travail a été centré sur la régulation de PRMT6 par automethylation que j'ai caractérisé et j'ai démontré que l'automethylation de PRMT6 est cruciale pour sa stabilité et son activité anti-VIH.

Le deuxième facteur immunitaire intrinsèque que j'ai étudié est SAMHD1. J'ai mesuré le profil de réplication de différents mutants résistants aux médicaments dans les cellules immunitaires primaires et des lignées cellulaires qui expriment ou non SAMHD1. J'ai découvert que certains virus résistants aux médicaments se répliquent différemment dans différents types de cellules. De manière remarquable, certains de ces mutants simples répliquent mieux dans les cellules dendritiques et les lymphocytes CD4+ quiescents, tandis que le mutant E138K se réplique aussi bien que le virus de type sauvage dans les macrophages.

Nous avons élargi notre étude à des mutants dans l'intégrase qui sont aussi résistants aux médicaments étant donné que les études de sélection dans les cellules mononucléées du sang de cordon ont indiquées que différents types de cellules peuvent influencer les génotypes qui

sont sélectionnés en présence de l'inhibiteur d'intégrase dolutégravir (DTG). Nous avons constaté que les virus qui contiennent R263K et H51Y/R263K se répliquent mieux que le type sauvage dans les lymphocytes CD4 + quiescents. Cela montre que les virus résistants aux médicaments qui ont des mutations dans la RT ou dans intégrase peuvent dans les deux cas se répliquer efficacement dans des cellules spécifiques. La réplication persistante de certains de ces mutants résistants aux médicaments dans les cellules immunitaires réservoirs pourrait également contribuer à l'activation immunitaire chronique que l'on voit chez certains patients.

Acknowledgements

I would first like to thank my Ph.D. supervisor Dr. Mark Wainberg for his support and patience throughout my graduate journey. You have been a role model to me since AIDS 2006 as a scientist speaking out to help reduce the stigma against HIV/AIDS; as a bridge between science, communities and policy; and as an educator of scientists and the public. Thank you for giving me the freedom to pursue my doctoral studies, along with my own advocacy in access to medicines, which allowed me to grow and address HIV in a larger societal context. From you I have learned to integrate advocacy and science and to engage all stakeholders from different sides of an issue to establish and accomplish common goals. I am also humbled to have been given the opportunity to mentor bright young students in your lab and these are experiences I will always treasure.

Second, I would like to thank my dear friend and mentor Dr. Thibault Mesplède. Through your example I learned to always approach my work with the highest standards of impartiality, rigor, dedication and due diligence. As this important chapter in my life comes to a conclusion, I remember all the ups and downs and you've had my back since day one. I'm glad we wrote this chapter together. 3'.

I would like to acknowledge Dr. Chen Liang and Dr. Lawrence Kleiman as my Ph.D. committee advisors, providing me with constructive feedback, experiment suggestions and advice. I would also like to acknowledge those who helped in the editing and critique of this manuscript,

including Dr. Mark Wainberg, Dr. Thibault Mesplède, Dr. Robert Scarborough, and Dr. Robert Biskin.

Thank you to all my colleagues at the Wainberg lab, past and present for their camaraderie, technical help and troubleshooting, scientific (and political) discussions, and for making the lab environment a pleasant one to come to everyday. In particular, I would like to thank Dr. Victor Kramer, Dr. Peter Quashie, Sue Germinario, Cesar Collazos, Estrella Moyal, Maureen Oliveira, Shalom Spira and Bonnie Spira for their help and generosity throughout. It takes a village...to form a Ph.D.

Thank you to the UAEM family for the inspiration and friendships.

Thank you Robert B. for encouragement, hope and masonry.

Finally I would like to thank my extended family who has been just as vital to the completion of this thesis;

I would like to express my deepest gratitude to Steve and Salma Soroka for your unwavering support and kindness . Thank you to Anastasia Dzhun, Noah Soroka and Arlene 'Popo' Singroy for your love and encouragement.

Finally to Maman: you have always been an example of strength, feminism and grace. You have taught me to think independently and persevere no matter what. Your compassion, your altruism and your concern for others is what inspired me to pursue a carrier related to health.

To my mother Hélène, my sister Céline and my brothers Éric and Richard, this manuscript is dedicated to you. Merci.

I would like to acknowledge the Canadian Institutes of Health Research (CIHR), McGill University's department of Microbiology and Immunology and Faculty of Medicine, and the Lady Davis Institute for Medical Research for providing me with the various scholarships to fund my graduate studies.

Preface

This thesis was written in accordance with McGill University's "Guidelines for preparation of a thesis." Additionally, the format of this thesis is "Manuscript- based", of which chapters 2 and 4 are based on two published manuscripts and chapter 3 and 5 are based on two manuscripts in perpetration for submission. The contributions of the co-authors are stated at the beginning of each chapter.

The work presented in this manuscript is the product of my original scholarship and distinctly contributes knowledge to the field.

Bellow is listed other manuscripts not included in this thesis but to which I have made contributions over the course of my graduate studies.

Mesplède T, Quashie PK, Hassounah S, Osman N, Han Y, Liang J, **Singhroy DN**, Wainberg MA. *The R263K substitution in HIV-1 subtype C is more deleterious for integrase enzymatic function and viral replication than in subtype B*. AIDS. 2015 Jul 31;29(12):1459-66.

Mesplède T, Osman N, Wares M, Quashie PK, Hassounah S, Anstett K, Han Y, **Singhroy DN**, Wainberg MA. *Addition of E138K to R263K in HIV integrase increases resistance to dolutegravir, but fails to restore activity of the HIV integrase enzyme and viral replication capacity*. J Antimicrob Chemother. 2014 Oct;69(10):2733-40

Quashie PK, Mesplède T, Han YS, Oliveira M, **Singhroy DN,** Fujiwara T, Underwood MR, Wainberg MA. *Characterization of the R263K mutation in HIV-1 integrase that confers low-level resistance to the second-generation integrase strand transfer inhibitor dolutegravir*. J Virol. 2012 Mar;86(5):2696-705.

Mesplède T, Quashie PK, Osman N, Han Y, **Singhroy DN**, Lie Y, Petropoulos CJ, Huang W, Wainberg MA. *Viral fitness cost prevents HIV-1 from evading dolutegravir drug pressure.* Retrovirology. 2013 Feb 22;10:22.

Kramer VG, Schader SM, Oliveira M, Colby-Germinario SP, Donahue DA, **Singhroy DN**, Tressler R, Sloan RD, Wainberg MA*. Maraviroc and other HIV-1 entry inhibitors exhibit a class-specific redistribution effect that results in increased extracellular viral load.* Antimicrob Agents Chemother. 2012 Aug;56(8):4154-60

Nguyen TL, Tumilasci VF, **Singhroy DN**, Arguello M, Hiscott J. *The emergence of combinatorial strategies in the development of RNA oncolytic virus therapies.* Cell Microbiol. 2009 Jun;11(6):889-97

List of Figures and Tables

List of abbreviations

AIDS	acquired immunodeficiency syndrome
APOBEC	apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like
APP	acute phase protein
ARM	arginine rich motif
ARV	antiretroviral
AGS	Aicardi-Goutieres syndrome
AZT	zidovudine
CA	capsid protein
cART	combination antiretroviral therapy
CBP	CREB- binding protein
CCD	catalytic core domain
CCR	chemokine C-C Motif Receptor
cDC	conventional dendritic cell
CDK2	cyclin dependent kinase 2
CD	cluster of differentiation
cGAS	cyclic GMP-AMP synthase
CRF	circulating recombinant form
CTD	C-terminal domain
CypA	cyclophilin A
CXCR4	C-X-C chemokine receptor type 4
DC	dendritic cell
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DTG	dolutegravir
EC	elite controller
Env	envelope
ESCRT	endosomal sorting complexes required for transport
gag	group-specific antigen
GMCSF	granulocyte-macrophage colony-stimulating growth factor

HAART	Highly Active Antiretroviral Therapy
HAT	histone acetyltransferase
HIV	Human Immunodeficiency Virus
HRF	host restriction factor
HrF	host resistance factor
IDU	injection drug users
IFI16	interferon gamma inducible protein 16
IFNAR	type 1 IFN receptor
IFN-1	type 1 interferon
IL	interleukin
IN	integrase
INSTI	integrase strand transfer inhibitor
IRF	interferon regulatory factor
ISG	interferon-stimulated gene
LEDGF/p75	lens epithelium-derived growth factor
LRM	leucine rich motif
LTNP	long-term non-progressor
LTR	long terminal repeat
MA	matrix
mDC	myeloid dendritic cell
MDDC	monocyte-derived DC
MDM	monocyte derived macrophage
MT	microbial translocation
MyD88	myeloid differentiation primary response gene 88
NC	nucleocapsid
Nef	negative Regulatory Factor
NES	nuclear export signal
NFκB	nuclear factor kappa B
NHEJ	non-homologous end joining

NK	natural killer
NLS	nuclear localisation signal
NNRTI	non-nucleoside reverse transcriptase inhibitor
NRTI	nucleos(t)ides reverse transcriptase inhibitor
NTD	N-terminal domain
PBMC	peripheral blood mononuclear cell
PBS	primer binding site
pDC	plasmacytoid dendritic cell
PAMP	pathogen-associated molecular pattern
PIC	pre-integration complex
pol	polymerase
PPT	polypurine track
PR	protease
PRR	pattern recognition receptor
PRMT6	protein arginine methyltransferase 6
P-TEFb	positive transcription elongation factor
RRE	Rev response element
RNApol II	RNA polymerase II
RT	reverse transcriptase
RTI	reverse transcriptase inhibitor
SAMHD1	sterile alpha motif- and HD-domain containing protein 1
SOCS2	suppressor of cytokine signaling protein 2
STING	stimulator of interferon genes
TAM	thymidine analogue mutations
Tat	transactivator of transcription
TAR	transactivating response elements
TLR7	Toll-Like Receptor 7
TREX1	three prime repair exonuclease 1
TRIF	TIR-domain-containing adapter-inducing interferon-β

TRIM5α	tripartite motif containing protein 5 alpha
UNAIDS	Joint United Nations Programme on HIV/AIDS
Vif	viral infectivity factor
Vpr	viral protein R
Vpu	viral protein U
Vpx	viral protein X
WHO	World Health Organization

Chapter 1

Introduction

In 1981 reports began to emerge of men who presented with a newly acquired cellular immunodeficiency, marked by multiple infections or who developed a previously rare form of cancer called Kaposi's sarcoma [1, 2]. This disease was termed Acquired Immunodeficiency Syndrome (AIDS). By mid 1982, the number of individuals who reported having multiple infections rose to 452 [3]. This mysterious disease was emerging globally when Dr. Françoise Barré-Sinoussi, along with Dr. Luc Montagnier, identified Human Immunodeficiency Virus (HIV) as the causative agent of AIDS in lymph node biopsies from a French patient afflicted with lymphadenopathy in 1983 [4, 5]. Further isolation of the virus from AIDS patients by Dr. Jay Levy and Dr. Robert Gallo confirmed the earlier observation, leaving no doubt that HIV was indeed the etiological agent of AIDS [6, 7].

Today there are approximately 36.7 million individuals living with HIV globally and AIDS-related deaths have claimed the lives of an estimated 35 million people since the beginning of the pandemic [8, 9]. Treatment access is still a major hurdle to curbing the pandemic. In 2015, only 46% of adults living with HIV had access to treatment. Treatment access is similar for pediatric populations where only 49% of infected children have access to antiretroviral therapy [9].

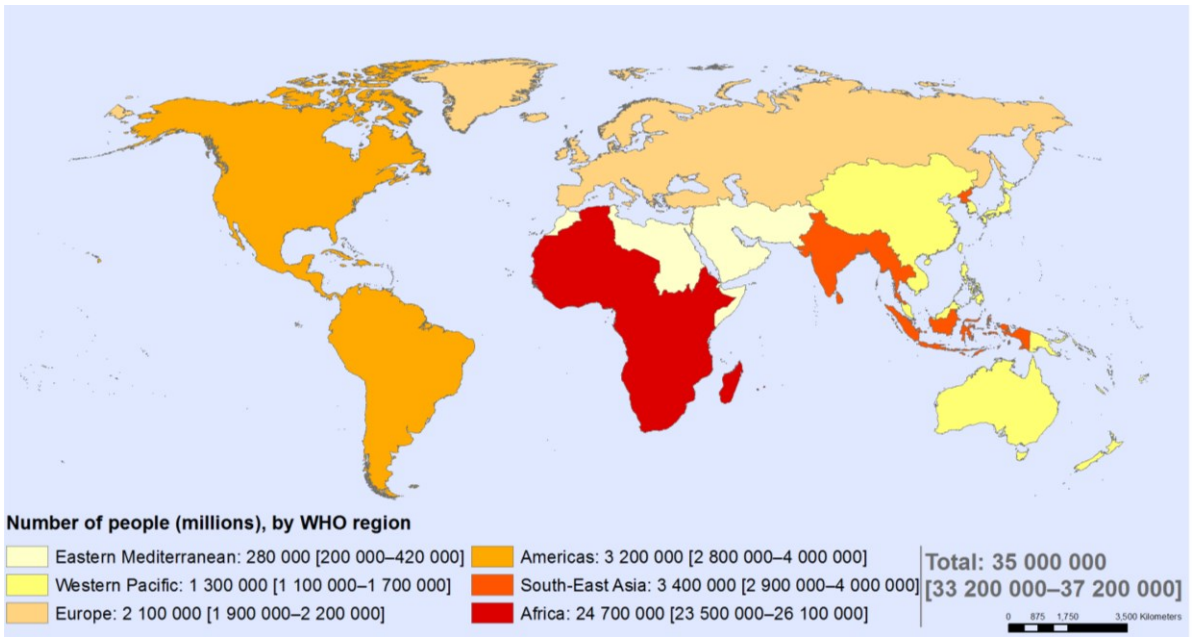


Figure 1.1: 2013 WHO estimate of the number of people living with HIV by region. From [10]

New World Health Organization (WHO) guidelines state that treatment should be initiated at any cluster of differentiation 4 (CD4)+ T-cell count upon the confirmation of HIV infection [11]. This is not only critical for the overall long-term health of people living with HIV, but treatment can be used as prevention by keeping viral loads at undetectable levels, thus preventing transmission. With the ambitious goals proposed by the Joint United Nations Programme on HIV/AIDS (UNAIDS) to have 90% of people living with HIV on treatment and virally suppressed by 2020, we still have to overcome many systemic hurdles to expand treatment to the over 21 million of individuals remaining untreated [12]. Treating HIV as a chronic disease, that is, taking medication for one's entire life, continues to be the only option to control the virus. HIV is the most studied human pathogen, yet a cure or a vaccine is still elusive. The 'Berlin Patient', Timothy Ray Brown, is the only known person who has been cured of HIV. As a leukemia patient also diagnosed with HIV, he received a stem cell transplant from a donor with the HIV-

resistant delta32 CCR5 homozygotic gene deletion [13]. However, such risky treatment is not realistic to employ on a large scale, especially in low-resource settings where HIV hits hardest.

Even though antiretroviral therapy is efficacious in inhibiting HIV replication to undetectable RNA levels in the plasma of infected individuals, HIV infection is associated with persistent immune activation even under treatment [14]. The etiological causes of this persistent immune dysregulation are not entirely understood but may be due in part to low levels of replication that are undetectable by traditional methods (i.e. RNA levels below 50 copies/ml of plasma but not null in the blood or other anatomic compartments) or irreversible changes that occur before the initiation of therapy. Importantly, persistent immune activation is associated with various HIV co-morbidities including cardiovascular, kidney, and liver diseases and neurological disorders. As HIV-infected individuals grow older, these health issues become more and more pressing.

Whether low levels of replication contribute to the maintenance of viral reservoirs is also still unknown [15]. Viral reservoirs are long-lived cells and are the source of viral rebound upon treatment cessation. In order to attain long-term remission or a sterilizing cure, scientists will need to come up with creative solutions and utilize immune approaches to restore the balance in the innate immune system of HIV-positive individuals in order to eradicate the reservoirs. The possibility to distribute cure strategies to low-resource settings should be carefully examined as well. Our laboratory has formulated the hypothesis that the use of dolutegravir may help to attain a cure for HIV by cornering the virus into an evolutionary dead-end [16].

Whether this approach may work remains unknown but illustrates the necessity of out-of-the-box thinking behind curative strategies. Solutions that bring new ways of employing old concepts, such as monotherapy, or using the body's own defences should be examined. Until a cure is found, it is clear that a multifaceted approach is needed to treat people living with HIV. This involves educating, reducing stigma, encouraging HIV testing, and investing in infrastructure and mechanisms that allow for everyone to access the best new highly effective antiretroviral therapy, no matter where they live or their socio-economic status.

The focus of my studies has been on HIV-1 interactions with components of the innate immune system. My first project centred on the regulation of protein arginine methyltransferase (PRMT6) anti-viral activity, an intrinsic immune factor identified in Dr. Wainberg's lab. The Wainberg lab is a world leader in antiretroviral (ARV) drug resistance research and it is in this context that I studied a second intrinsic immune factor, Sterile Alpha Motif- and HD-domain containing protein 1 (SAMHD1). Drug resistance mutations often appear under selective pressure with ARVs. However, *in vivo*, host factors can drive viral adaptation, as seen with hypermutations caused by Apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like (APOBEC3G). It is thus conceivable that SAMHD1 favors the appearance of certain drug resistant mutations. In this project, I studied reverse transcriptase inhibitor (RTI) resistant HIV-1 in SAMHD1-expressing immune cells, ie. monocytes, macrophages, and dendritic cells. More recently, the Wainberg lab has done extensive work with the integrase inhibitor dolutegravir (DTG). In particular, we were the first to identify the DTG-specific resistance arginine to lysine mutation R263K, which is now recognized as the most common resistance mutation against this

drug in individuals experiencing treatment failure. My third project investigated the effect of combining R263K with RTI-resistance mutations on new drug combination therapies. Concomitantly, in a fourth project I investigated the innate immune response behind a curative hypothesis developed in the laboratory involving DTG use by investigating how DTG resistant viruses behave in long-lived reservoir cells such as macrophages.

The following chapter introduces the reader to required information needed to interpret my research and to understand its larger implications. The first part presents general HIV virology and the natural history of HIV infection. The second part focuses on the innate immune factors that I studied. Finally, I give an overview of HIV-1 antiviral therapies, drug resistance, with a focus on specific reverse transcription (RT) and integrase (IN) inhibitors that are pertinent to my research.

1.1. HIV epidemiology

HIV is a retrovirus from the genus *lentivirus* containing several overlapping open reading frames coding for enzymes, structural protein and accessory proteins that it needs to navigate within the host cell. This genus is characterized by the ability to enter a latency state after integrating its genome into that of the host cell. HIV is highly genetically diverse due to its error-prone reverse transcriptase ($3X10^5$ mutations per replication cycles), a high frequency of recombination, and the fact that it can produce up to 10^9 new viral particles per day [17-19]. The result of this dynamic and high genetic heterogeneity, with variants within a single

individual known as quasispecies, is the basis for HIV's ability to develop drug resistance and one of the reasons why a cure or vaccine remains elusive. The geographic diversity of HIV is reflected in the many subgroups and circulating recombinant forms (CRF) of the virus.

HIV is classified into two types, HIV-1 and HIV-2, originating from zoonotic transmissions of simian immunodeficiency viruses (SIV) from various non-human primates. HIV-1 is further divided into four groups M, N, O and P. Based on sequence analyses, HIV-1 M and N originates from SIV*cpzPtt* in chimpanzees (*Pan troglodytes troglodytes)* from southern regions of Cameroon [20]. The ancestor of Group P and O HIV-1 was found to be the Western Lowlands Gorilla (Gorilla *gorilla gorilla)* SIV*gor* from South and Southwest Cameroon [21, 22]. The pandemic HIV-1 group M is responsible for over 90% of worldwide infections and is subdivided into 9 subtypes (A-D, F-H, J, K) and 48 CRFs, the most common being CRF01_AE, CRF02_AG and CRF11_cpx [23]. Subtype B is most prevalent in the Americas, Western Europe, Australia, China and some North African countries [24]. Subtype C affects Southern and Eastern Africa and India, whereas Subtype A is found mostly in Sub-Saharan Africa, Russia and Western Europe. The circulating recombinant form CRF02_AG in found in Central Africa and CRF01_AE (or CRF01_AU) in South East Asia [24].

HIV-2 is less pathogenic than HIV-1 and is predominantly found in West African countries. It emerged by cross species infection from SIV*smm* infected Sooty Mangabeys (*Cercocebus atys*) [25]. Compared to HIV-1, HIV-2 has an additional accessory factor viral protein X (Vpx), which is also found in many known SIV strains. HIV-2 acquisition of Vpx is the result of an evolutionary

race that occurred between simian Vpr and SAMHD1, an innate immune protein, in primates. Unlike HIV-2 Vpx, HIV-1 Vpr lacks the ability to degrade SAMHD1 and this reflects the zoonotic transmission of HIV-1 from the chimpanzee SIV lineage; SIV*cpzPtt* Vpr also never acquired the ability to degrade SAMHD1 [26]. Although it has not been tested, it is likely that HIV-1 group O Vpr, like the SIV*gor* Vpr, does not degrade SAMHD1. Interestingly both HIV-M and HIV-O cause similar pathogenesis [27].

Studying HIV counterparts in non-human primates has been indispensable for our *in vivo* understanding of immune controls that relate to the virus, especially when it comes to innate and mucosal immunity and host restriction factors (HRF). Indeed, much of the early work on pathogenesis was first elucidated while studying SIV infections in macaques.

1.2. HIV pathogenesis

The natural development of HIV-1 infection varies widely from person to person and can range from rapid disease progression to AIDS to an asymptomatic long-term non-progression (LTNP) (treatment naive individuals who maintain a normal CD4+ T-cell levels for long period of times, upwards of 5 to 10 years). The reasons for this are still obscure; however, the recent discovery of host restriction factors that function to inhibit retroviral activities may shed light on this process.

HIV is most often transmitted sexually though mucosal membranes [28]. Other epidemiologically important sources of HIV transmission occur though the use of contaminated needles among injection drug users (IDUs) and from mother-to-child where proper treatment is not readily available.

The rate of transmission is highly dependent on the viral load of the donor. Studies in serodiscordant heterosexual couples found that approximately 1,500 HIV-1 RNA copies per mL of serum is the minimum concentration of virus for transmission to occur and this was independent of subtype, presence of co-infection or sex of the donor [29, 30]. A watershed moment in HIV prevention came when the HPTN-052 study found that when the HIV positive partner was virologically suppressed to an average of 400 copies of RNA per mL with early anti-retroviral treatment, the risk of transmission was reduced by 96% compared to a cohort where treatment was initiated only once CD4+ T-cell counts were less than 250 cells/ml^3 or upon the development of AIDS, thus strongly supporting the practice of treatment as prevention [31].

Once HIV reaches the subepithelial cells in the genital tract, its interaction with the early innate immune response will determine the nature of the disease progression. For example, although the molecular mechanisms are still unknown, high levels of inflammation in the genital tract of women positively correlate with the risk of HIV acquisition [32]. HIV infects immune cells that express CD4 and Chemokine (C-C Motif) Receptor (CCR5) or C-X-C chemokine receptor type 4 (CXCR4) receptors such as T-lymphocytes, monocytes, macrophages and dendritic cells (DC) that can all be found at the mucus membrane of the genitals.

Only 1:200 female or 1:700 male exposure events results in an established infection [33]. 76% of infections are caused by a single founder virus in heterosexual intercourse and by less than 5 founder viruses in the other 24% [34]. Several factors cause this bottleneck, including tropism and viral fitness. Phenotypic studies using Affinofile cell lines have shown that founder viruses are typically CCR5/CD4+ T-Cell tropic [35]. Resting CD4+ T-cells are 5 times more abundant than CCR5 expressing macrophages and dendritic cells in the lamina propria (the layer just below the epithelial tissue and above the mucosa) and these resting CD4+ T-cells are the most vulnerable to HIV-1 infection [36, 37].

The acute phase of infection is divided into three stages: the eclipse phase, the expansion phase, and the containment phase [38]. The acute phase, or primary infection, is critical in determining the long-term trajectory of the illness, including rapid or slow progression to AIDS. The eclipse phase occurs for about the first 10 days post-transmission during which viral RNA is undetectable in the plasma. When viral transmission occurs at the genital mucosa, the local expansion of a small founder population at the locus of infection is key for subsequent dissemination [39]. This, however, is not the case for rectal transmission or direct infection in the blood through contaminated needles [40]. The establishment of systemic infection, however, is mainly driven by the replicative rate (R_0) of the founder virus such that a fitter virus, will be more successful where $R_0 \geq 1$, if it can outcompete the hostile environment at the exposure site [41].

At the mucosal foci, HIV-1 can be detected by the innate immune system through Toll-Like Receptor 7 (TLR7) and triggers an inflammatory response recruiting plasmacytoid dendritic cells (pDC) followed by an influx of CD4+ T-cells. Although pDCs are potent producers of type 1 interferon (IFN-1) and other antiviral cytokines, HIV-1 can undergo cell-to-cell spread in CD4+ T-cells, a propagation mechanism which is less susceptible to inhibition by IFN-1 [42]. Additionally, transmitted founder viruses were also found to be more efficient at binding to conventional dendritic cells (cDC) compared to chronic phase viruses, allowing for their spread to CD4+ T-cells in draining lymph nodes, where a productive infection is more likely to occur [43].

The viral expansion phase is marked by a sharp elevation in viral load, an increase of acute phase proteins (APP) (i.e.: the cytokine storm), and depletion of CD4+ memory T-cells. Macrophages and DCs have been identified as a source of APPs. At this point the infected person develops flu-like symptoms that clear up after about 3 to 4 weeks. Once the infection reaches the lymphoid tissue, HIV has an abundance of target CD4+CCR5+ T-cells to infect and can propagate. The Gut Associated Lymphoid Tissue (GALT) is particularity badly affected during acute infection since 20% of the CD4+ T-cell population becomes infected, and 60% of uninfected T-cells become activated by soluble factors released by nearby activated immune cells and can die by apoptosis through a process known as the “bystander effect” [44-46]. The presence of activated CD4+ effector memory T-cells in the intestinal mucosa supplies the virus with host cells in which to actively replicate. Some believe that when the acute infection leaves the intestinal mucosa weakened, this allows microbial matter, such as lipopolysaccharide (LPS)

from bacterial cell wall and toxins from the gut to sustain chronic immune activation and inflammation [47]. However, there is no clear consensus that microbial translocation (MT) drives chromic immune activation as seen in longitudinal observational studies that did not find changes in MT markers in HIV positive cohorts over time [48]. Finally, the containment phase is characterized by a partial immunological control of HIV replication, a decrease in viral load and eventually leads to the chronic phase of HIV infection.

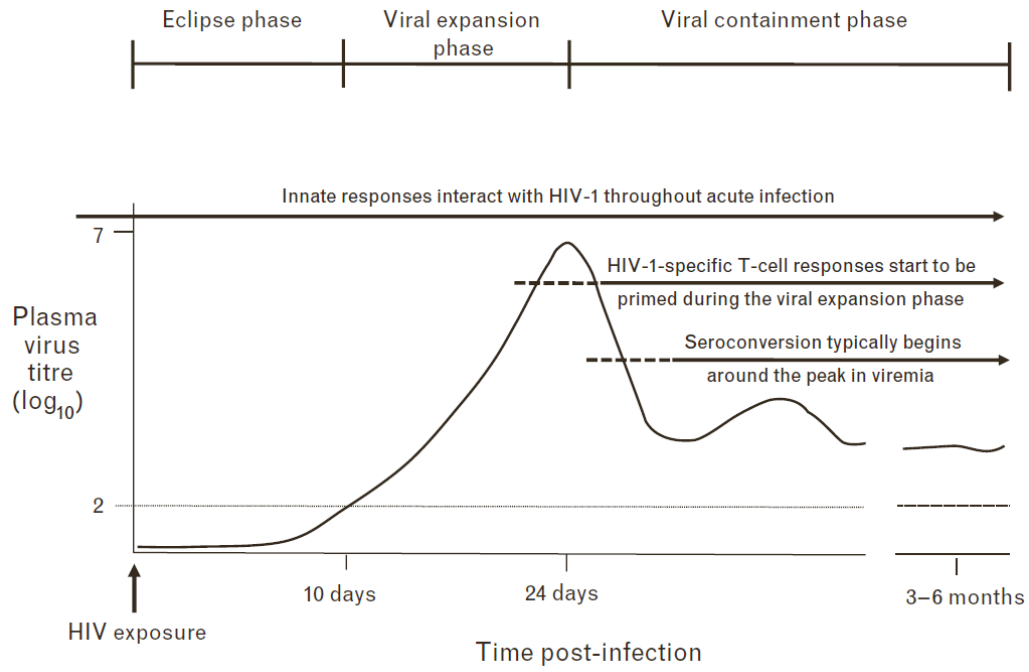


Figure 1.2: Steps in the acute phase of HIV infection. General immune response in the 3 phases of acute HIV-1 infection and its effect on vial load. From [38]

The importance of chronic inflammation on the progression to AIDS is underlined by studies in long-term non-progressors. Long-term non-progressors are HIV-positive individuals who stay disease free for many years after infection despite expressing varying levels of viral loads [49, 50]. Importantly, a subset of these individuals has low or normal levels of immune activation

compared to healthy individuals. The same is true for chimpanzees infected with virulent HIV-1 that seem immune to chronic immune activation and do not develop the disease [51]. Similarly, individuals infected with HIV-2 have a lower state of immune activation compared to HIV-1 positive individuals, and can remain asymptomatic for many years [52].

Although the exact causes of the chronic inflammation associated with HIV-1 are still a much debated issue, its effect is seen in the aging HIV infected persons who are more susceptible to non-HIV-1 related comorbidities such as, atherosclerosis and other cardiovascular diseases, cancer, diabetes, and lymph node fibrosis [53].

Premature T-cell exhaustion resembling the aging of the immune system is accelerated in HIV-1 positive individuals, caused by prolonged immune activation and inflammation due to continuous HIV infection [54]. This immuno-senescence in aging individuals with HIV is similar to the state of the immune system seen in HIV-negative people nearly 20 years older. The term "inflame-aging" was coined to describe the precocious onset of pro-inflammatory diseases such as cognitive impairment, heart disease, decreases in bone density, and aging-related "frailty" that would otherwise occur in the rest of the population two decades later.

The chronic phase results in a slow but steady decrease in blood-borne CD4+ T-cells and eventually to the last phase of HIV infection, AIDS, if left untreated. AIDS is characterized by an abrogation of host immunity and the occurrence of various and eventually fatal opportunistic infections or AIDS related cancers.

Altogether, this illustrates the importance of the immune response for the establishment and the course of HIV infection.

1.3. The immune response to HIV

When the viral load reaches its peak, the infected person develops flu-like symptoms that clear up after about 3 to 4 weeks. This is symptomatic of the activation of the innate immune system and is observed in numerous viral infections, including those involving influenza and other viruses.

During HIV infection, the innate immune response is activated early in the acute phase and primes the adaptive immune response. The innate immune response involves production and release of soluble cytokines and chemokine that mediate the activation of innate immune cells such as macrophages and natural killer (NK) cells. Whereas macrophages are able to engulf infected and/or apoptotic cells, NK cells can kill cells that are virally infected.

The adaptive immune response peaks later during the infection and involves cytotoxic CD8+ T-lymphocytes responsible for the killing of 15-35% of infected cells [55]. HIV is able to escape the cytotoxic T-cell response by developing mutations that affect the epitopes that are recognized by CD8+ cytotoxic T-cells [55]. HIV can also evolve rapidly to escape another arm of the adaptive immune response: the humoral, or antibody response. Neutralizing antibodies

directed against HIV can be detected as early as 12 weeks after transmission [56]. It can take as few as 10 days for HIV to develop the multiple mutations that are needed to escape adaptive recognition of immune cells and antibodies [55]. Importantly, the acute phase of infection allows the virus to dramatically increase its genetic diversity in a short period of time and escape mutations are then positively selected for by the various immune responses. The peak viral load can reach above 1 million RNA copies/ml of plasma and usually occurs within 3 to 4 weeks. The viral load will then tail off owing in part to the CD8+ cytotoxic response which also contributes to the ongoing control of HIV replication during the chronic phase of the infection, under combination antiretroviral therapy (cART), in long-term non-progressors and in non-human primate models [57].

In a healthy person, the immune response is self-regulating; however, diseases such as HIV can tip the immune system off balance. Chronic inflammation and ongoing immune activation associated with HIV was documented as far back as 1988, shortly after the discovery of the virus [58]. However, scientists are only now beginning to document the causes and consequences of this chronic immune activation. Progression to AIDS is caused by depletion of CD4+ T-cell initially at the mucosal level and subsequently in the blood stream [59]. Persistent immune activation correlates with CD4+ T cell depletion but paradoxically it is not correlated with viral load [59-61]. Even if the virus is subdued by treatment, immune activation persists.

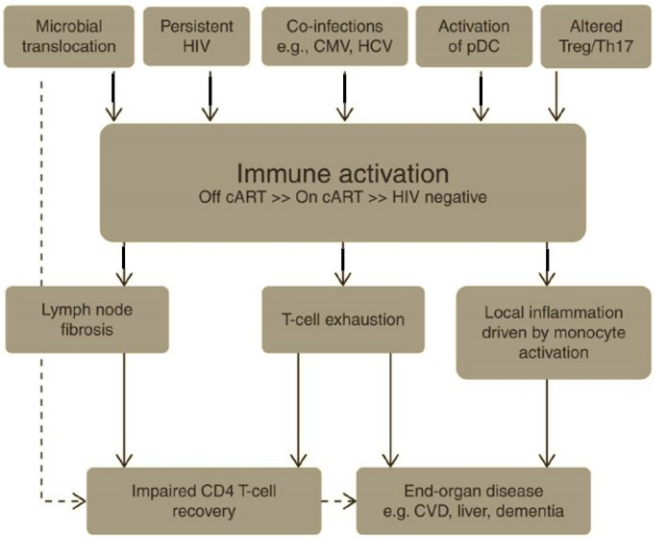


Figure 1.3: Factors that may contribute to chronic immune activation. Adapted from [62]

The interplay between HIV infection and the immune system is thus complex and does not stop upon treatment initiation. This is due to the ability of the virus to infect immune cells through a precisely coordinated replicative cycle. In the next sections, I will describe the HIV replication cycle, including the role of accessory proteins.

1.4. HIV replication cycle

The HIV replication cycle contributes to pathogenesis through its ability to usurp crucial immune cells and host factors. Since my thesis focuses on viral replication in the context of the innate immune system, I will highlight interactions between the virus and key intrinsic immune factors.

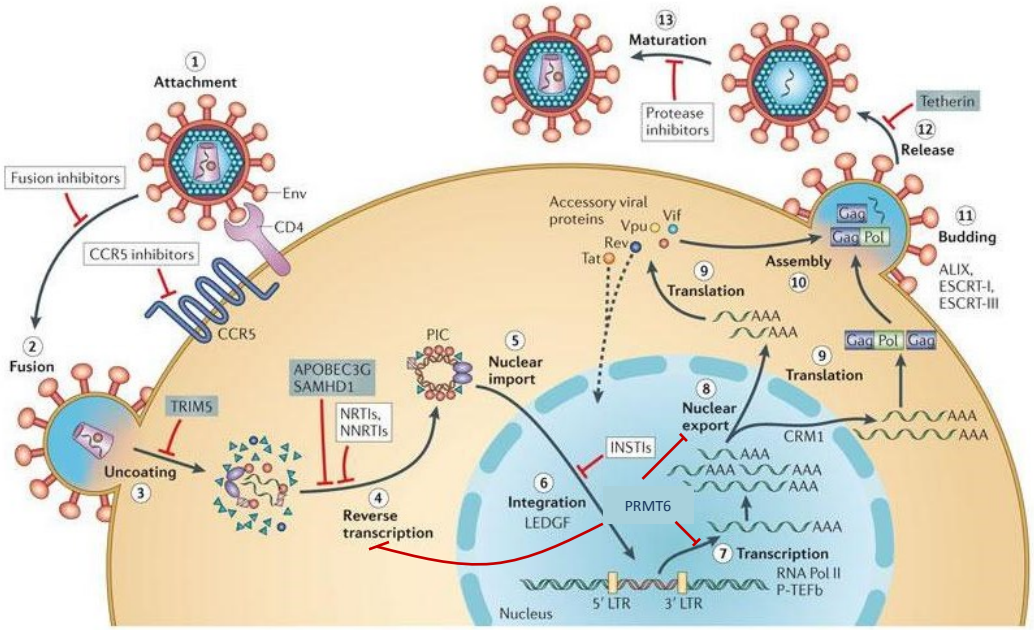


Figure 1.4: Steps in the HIV replication cycle. As HIV progresses though its replication cycle, it encounters Host restriction factors, in grey (see section 1.5.3). Also indicated are the targets of current antiretroviral therapy. From [63]

1.4.1. Entry

Most free virus that enters the cell is degraded at a rate of about 10^3 to 10^5 virions for every successful infection [19, 64]. Although viral fusion typically occurs at the cell surface, entry through endosomal compartments may also contribute to infection and in part help to explain cell-to-cell spread of HIV [65, 66]. Regardless of the entry point, precise sequential interactions of HIV envelope (env) proteins expressed at the surface of viral particles with specific host cellular receptors are essential for the viral payload to be delivered into the cytoplasm. Env is a trimer made up of the gp120 surface glycoprotein non-covalently bound to the transmembrane gp41 protein. Heavy glycosylation of gp120 reduces the antigenicity of HIV [67]. The sequential

molecular events necessary for entry at the cell surface are as follows. Gp120 first binds to the CD4 receptor on the surface of immune cells such as CD4+ T-lymphocytes, monocytes, macrophages and dendritic cells. Once bound to CD4, gp120 undergoes a conformational change exposing its co-receptor binding sites [68]. The binding to co-receptors CCR5 or CXCR4 is needed to initiate fusion [69]. Various HIV viruses display different affinity for either of these two co-receptors and are characterized accordingly. HIV that infects through the β-chemokine receptor CCR5 is termed R5 or macrophage (M)-tropic [70]. There is much debate over the viral tropism of *primo* infection, but for now the paradigm states that most new infections through sexual contacts are initially established though M-tropic viruses [71]. X4 or T-cell (T)-tropic viruses bind to the α-chemokine receptor CXCR4, and largely emerge upon rapid progression to AIDS [72, 73]. HIV tropism is determined by the V3 loop of gp120 and although some HIV strains can be dual tropic, alternative co-receptor usage such as CCR8 and CCR3 is also possible, albeit, less common [74, 75]. Co-receptor engagement by gp120 triggers conformational changes in gp41 that results in the fusion of the viral and cellular membranes. Once fusion occurs, the viral core is released into the cytoplasm through the fusion pore and proceeds to uncoating.

1.4.2. Uncoating

The viral core injected into the cytoplasm is composed of a conical p24 capsid protein (CA) shell that encloses two copies of the HIV positive-sense single-stranded RNA genome, the viral enzymes reverse transcriptase (RT), protease (PR) and integrase (IN), the accessory viral protein R (Vpr), a structural nucleocapsid (NC) protein, and various cellular proteins [76]. The CA is a

highly helical protein made up of an N-terminal domain connected to a C-terminal domain by a short flexible linker. The cone-shaped CA core is the result of 12 pentamers incorporated into the highly structured hexagonal lattice [77].

Uncoating is a process whereby the protective CA disassembles in order for reverse transcription of the viral RNA to proceed. Although much is still unknown about this step, uncoating is considered to be highly regulated, and requires many cellular factors to complete. As soon as decapsidation begins, viral components become susceptible to being detected by the innate and intrinsic immune systems. Consequently, it is hypothesised that HIV has evolved to use CA to camouflage its genetic payload from DNA and RNA sensors. CA's C-terminal major homology domain, the highly ordered lattice structure of the core and the tightly regulated decapsidation process are important to modulate immune recognition but also restrict CA genetic evolution [78]. In fact, several host restriction factors, such as Tripartite motif containing protein 5 alpha (TRIM5α), have been found to target CA proteins, although HIV-1 is able to evade these defences.

Although it is unclear why, proper uncoating is crucial for successful reverse transcription [79]. Indeed, premature uncoating of the viral core has been associated with abortive reverse transcription, i.e. production of incomplete reverse transcripts [80]. CA protein is also implicated in nuclear import of the pre-integration complex (PIC) and in integration itself [81, 82]. Uncoating immediately precedes or happens concomitantly with the initiation of reverse transcription.

1.4.3 Reverse transcription

Retroviruses are a subgroup of viruses that convert their RNA genomes into cDNA that can then be integrated into the host genome. The HIV RT is the enzyme responsible for converting the two positive single-stranded genomic RNA templates into double-stranded proviral DNA. RT is a DNA polymerase that is both RNA dependent and DNA dependent and also possesses RNaseH activity.

The RT enzyme is a heterodimer composed of 66kDa (p66) and a 51kDa (p51) subunits. The p66 and p51 polypeptides both contain 4 subdomains termed thumb, finger, palm and connection but p66 has an extra RNaseH domain at its C-terminus [83]. p51 acts as a rigid structural support stabilizing p66 as it carries out both DNA polymerase and RNaseH enzymatic activity [84].

Reverse transcription is a discontinuous process that occurs in the cytoplasm after viral entry in parallel with or slightly after uncoating starts. Host $tRNA_{lys3}$ act as a primer for RT to initiate reverse transcription by annealing to the primer binding site (PBS) at the 5' end of the viral plus-strand RNA [85]. NC is involved in $tRNA_{Lys3}$ annealing to the PBS of viral genomic RNA by means of two basic regions that flank the first Zn^{2+}-finger motif [86]. Our lab discovered that NC can be arginine methylated by PRMT6 in these basic regions [87]. *In vitro* studies revealed that arginine methylation significantly decreased the annealing capacity of NC, thereby diminishing HIV-1 replication capacity.

RNA-dependent DNA synthesis advances towards the 5’ end of the template. RNaseH concomitantly degrades the plus-stand RNA template creating a single minus stranded strong-stop DNA ((-)ssDNA) segment [88]. The strand transfer of the (–)ssDNA from the 5’ end to the 3’ end of the RNA template is then accomplished through complementary R terminal repeats [88]. Once hybridized, viral DNA synthesis continues and RNA degradation occurs from the 3’ end of the viral RNA following the newly synthesized DNA, except for polypurine tracks (PPT) which are highly resistant to RNaseH degradation. These PPTs will then act as primers for DNA-dependent plus-strand DNA synthesis. A second strand transfer event occurs when the $tRNA_{lys3}$ is degraded exposing the complementary PBS of the plus and minus nascent DNA strands [89]. The final proviral DNA is flanked by two long terminal repeats (LTR) and along with MA, IN, Vpr and cellular co-factors makes up the PIC. Both MA and IN harbor nuclear localisation signals (NLS) whereas Vpr also has non-canonical NLS [90]. The PIC navigates along microtubules from the cytoplasm to the nucleus that it enters through nuclear pores, thereby giving HIV the ability to infect non-dividing cells [91]. The susceptibility of reverse transcription and its products to intrinsic immune factors and innate immune detection has been intensely studied over the last decade. Host restriction proteins that interact functionally with RT are described below.

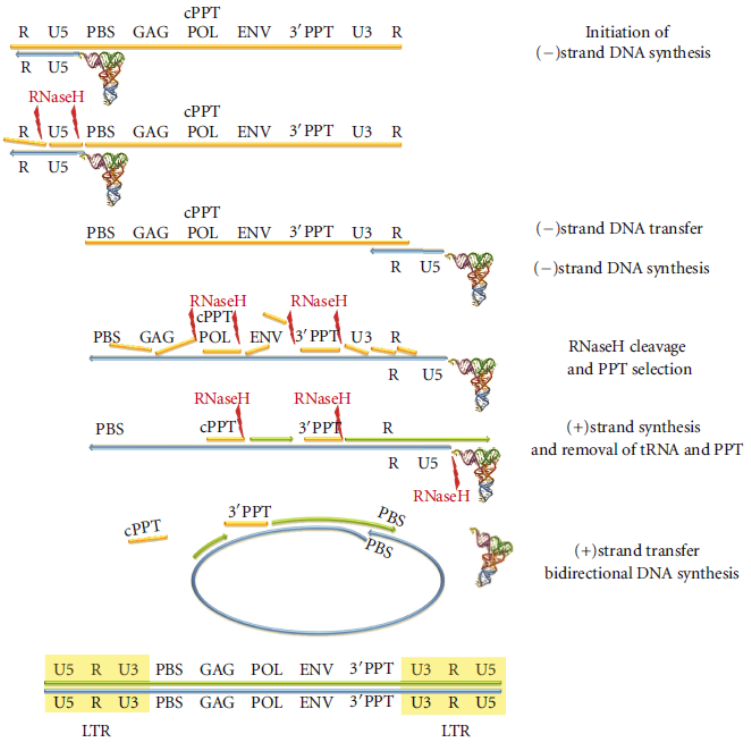


Figure 1.5: The steps of HIV-1 Reverse Transcription. Schematic of the mains steps in HIV-1 RT. The yellow strand represents the genomic plus strand viral RNA. The blue strand is the minus strand strong stop DNA and the product of RNA dependent DNA polymerase. The green strand depicts the positive complimentary DNA resulting from DNA dependent DNA polymerase. The From [92]

Reverse transcription is very error-prone with a mutation rate of $3x10^5$ mutations per cycle of replication [17]. The resulting quasispecies have a high chance of harboring mutations that can help the virus to evade the immune system and antiretroviral drugs. The development of G to A mutations are thought to be driven by an HRF termed APOBEC3G. APOBEC3G is a cytidine deaminase that causes G to A hypermutations in newly synthesized proviral DNA [93]. APOBEC3G can cause genetic instability or introduce lethal mutations. However, sublethal G to A hypermutations were found to increase HIV progeny's genetic diversity. In fact, hypermutation was associated with increases in treatment failure and APOBEC3G-mediated G to A mutations can lead to appearance of M184I/E138K highly drug resistant viruses in

individuals treated with etravirine and rilpivirine [94, 95]. HIV, however, can counter APOBEC3G through its Vif-mediated proteasomal degradation ability [96].

The recently discovered SAMHD1 is a deoxynucleoside triphosphate hydrolase effectively depleting nucleotide stores essential for reverse transcription [97]. SAMHD1 is expressed in myeloid cells and resting CD4+ T-cells and this in part explains the low permissiveness of HIV infection in these cells [98, 99]. SAMHD1 is unique among other host restriction factors in that it does not act directly on the virus, but rather it limits the building blocks that HIV needs to replicate. More information in regard to SAMHD1 is provided below (section 1.5.3.5).

Other cellular factors facilitate reverse transcription. For example, the RT enzyme is stabilized and rendered more efficient through phosphorylation by host cyclin-dependent kinase. Recently the p21 host protein was found to be upregulated in CD4+ T-cells of elite controllers and to inhibit cyclin dependent kinase 2 (CDK2) phosphorylation through a yet unknown mechanism [100].

Finally, both the substrate and the end product of reverse transcription are important ligands for the innate immune system. Indeed, in addition to countering intrinsic immune factors, HIV viral RNA and proviral cDNA must also evade detection by pathogen-associated pattern recognition receptors (PRR) of the innate immune system, namely TLR7/8, *Cyclic GMP-AMP synthase* (cGAS), and interferon gamma inducible protein 16 (IFI16) [101].

1.4.4. Integration

Stable integration of HIV cDNA into the host chromosome driven by the viral enzyme IN is crucial for the establishment of infection. *In vivo*, integration is made up of three steps that begin in the cytoplasm with the formation of the PIC. The first two steps, 3' processing and stand transfer, are directly catalyzed by IN. The final step, gap repair, is accomplished by host cell factors. IN is a 288 amino-acid long protein composed of 3 domains, N-terminal domain (NTD), catalytic core domain (CCD) and C-terminal domain (CTD), and is active as a tetramer for strand-transfer [102]. Residues 1 to 47 of IN make up its NTD that include the conserved HCCH Zn^{+2}- binding motif responsible for stabilizing IN tertiary structure and promoting multimerization [103]. The RNaseH-like CCD is located between residues 53 to 184 and contains the conserved catalytic triad D,D-35-E motif that directs the Mg^{2+} or Mn^{2+} ions indispensable for IN activity [104, 105]. Both the CCD and CTD contain DNA biding sites [106].

Once reverse transcription is complete, IN hydrolyses the viral cDNA at the 3' end of both terminally-located LTRs and generates two nucleotide overhangs at the 5' end as a consequence. When the PIC reaches inside the nucleus, a cellular co-factor termed lens epithelium-derived growth factor (LEDGF/p75) tethers it to the cellular chromosomal DNA that IN can then cleave through a nucleophilic attack using divalent ions and the 3'-OH ends of both LTRs, and into which it inserts the viral cDNA giving rise to proviral DNA [107]. Additionally, in the presence of LEDGF/p75, HIV integrates preferentially into actively transcribed genes [108].

Single-stranded DNA gaps flanking the provirus caused by the strand transfer process are repaired by cellular DNA repair mechanisms [106].

However, unintegrated viral DNA is in fact the predominant form of HIV cDNA found in infected cells [109]. The 2-LTR circle forms of unintegrated viral cDNA are generated by the non-homologous end joining (NHEJ) DNA repair mechanism and may be clinically relevant since they can be sequenced to reveal the emergence of drug resistance in patients [110, 111]. The circularization of HIV cDNA by NHEJ is thought to be part of a cellular defence mechanism [109]. APOBEC3F and APOBEC3G have also been found to inhibit integration by interfering with 3' processing and IN binding to the viral DNA substrate, respectively [112, 113].

1.4.5. HIV gene expression

The HIV-1 genome is approximately 9.5 kb long and codes for 15 proteins through alternative splicing and overlapping reading frames. Structural matrix (MA), CA, p6 and NC proteins are coded by the group-specific antigen (*gag)* gene. The viral enzymes RT, IN, and PR are encoded in the polymerase (*pol)* gene and *env* codes for the envelope gp120 and gp41 proteins. Regulatory or accessory proteins Tat, Rev, negative regulatory factor (Nef), Vpu, Vpr, and viral infectivity factor (Vif) are individually coded by their respective genes. Efficient gene expression from the integrated provirus is driven by the binding of HIV Trans-activator of transcription (Tat) to the LTR promoter.

The 640bp-long LTR is the transcriptional promoter of HIV and is subdivided into the U3, R and U5 regions. The core promoter is found in the U3 domain directly downstream of the transcription start site located at the border between the U3 and R regions. The U3 domain contains many binding sites for host transcription factors whereas the transcription initiation site is found within the R domain.

Viral gene expression is dependent on the physiologic state of the host cells which has a direct consequence on, *inter alia,* the chromatin structure surrounding the viral promoter, the availability of cellular transcriptional factors, and the presence of epigenetic factors [114]. The integrated provirus is precisely structured into nucleosomes such that nuc-1 is positioned immediately downstream of the HIV transcription initiation site in the LTR, thus inhibiting transcription in the absence of activating signals [115]. Chromatin remodelling at the site of the viral promoter can be triggered by numerous cellular events and initiates basal proviral transcription by the binding of cellular transcription factors such nuclear factor kappa B (NFκB), NFAT, or SP1 [116]. In the absence of Tat, viral transcription is inefficient and results mostly in the synthesis of abortive short mRNA due to RNA polymerase II (RNApol II) stalling near the HIV promoter. However, early multiply spiced genes products that are eventually generated result in the production of Tat, Rev, and Nef. Newly transcribed Tat can then translocate to the nucleus, thereby increasing viral RNA synthesis by a hundred fold [117].

Tat is a small 14kDa protein made up of 5 domains. The first 3 domains are responsible for its transactivation function [118]. Tat's forth domain (aa49-72) houses an arginine rich motif

(ARM) and directs its RNA binding [118]. Tat can induce nucleosome acetylation by recruiting histone acetyltransferase (HAT) like the CREB- binding protein (CBP)/p300 to the HIV promoter [119]. However, it is Tat binding to the transactivating response elements (TAR) on 5' end of nascent viral mRNA that most enhances transcription efficiency and the production of full length viral RNA. Acetylated Tat binds to the positive transcription elongation factor (P-TEFb) composed of CDK9 and cyclin T1 and this complex is recruited to the elongation complex by binding to TAR through Tat [120]. CDK9 hyper-phosphorylates RNApol II as well as other transcription elongation factors. This reaction releases RNApol II from the TAR element in addition to increasing RT polymerase processivity [121, 122]. Tat activity has been shown to be fine-tuned by posttranslational modifications. Studies demonstrated that the K50 residue of Tat is acetylated by the histone acetyltransferase (HAT) p300 [123, 124]. Previously we showed that Tat can undergo another posttranslational modification involving PRMT6 that specifically methylates arginine residues in the ARM on R52 and R53 [125, 126]. In contrast to acetylation, methylation of arginine residues in Tat leads to decreased transactivation activity as determined in MAGI cell assays and hence, down-regulates HIV replication. Methylation of Tat leads to reduced TAR binding and severely diminished cyclin-T1 recruiting. So far, nothing is known about a possible interplay between closely located acetylation and methylation events taking place in the ARM of Tat, but these events may be similar to what has been reported for histones whereby a so-called "histone code" has been established that in part involves arginine methylation [127]. Recently it was showed that lysine K51 contained in the ARM domain of Tat is monomethylated by Set7/9-KMT7 [128]. Interestingly, this methylation was found to

positively regulate Tat transactivation. Lysine methylation could contribute to the fine-tuning of Tat transactivation or possibly be suppressed by neighbouring methylated arginines.

Initially only fully spliced mRNA molecules are exported from the nucleus and used to produce Tat, Rev and Nef. But eventually each of Env proteins, Vif, Vpr, and Vpu are translated from partially spliced RNA and Gag and Pol polyproteins are translated from the unspliced transcripts [129]. The nuclear export of these intron-containing mRNAs is mediated by the HIV Rev protein and is delayed compared to other transcripts. They are thus referred to as late genes. Rev is a 19kD phosphoprotein that binds to the cis-acting secondary RNA structure Rev response element (RRE) present only in unspliced or partially spliced viral RNA [130, 131]. Different sequence motifs of Rev are important for its activity: the leucine rich motif (LRM) located in the C-terminal domain contains a nuclear export signal (NES), whereas the ARM within the N-terminal portion of Rev harbours a NLS and is responsible for binding to the RRE. In addition, the Rev ARM is flanked by multimerization sites at which interaction between multiple Rev proteins is thought to take place during the binding of a single molecule of viral RNA. When a threshold amount of Rev has multimerized on the RRE, Crm1 binds to the NES and mediates viral mRNA translocation to the cytoplasm through the nuclear pore complex [132]. Recently, we revealed Rev to be arginine-methylated by PRMT6 and to be modified in the ARM, as we showed for Tat [87]. Rev methylation has a negative effect on Rev function by means of reduced binding affinity to its target RNA sequence RRE [87].

1.4.6. Assembly, budding and maturation

The assembly of the various elements that make up HIV virions at the host plasma membrane is mediated by Gag. The Gag polyprotein makes up the structural core of the virus and is composed of MA, CA, NC, and p6. MA is located at the N-terminal of Gag and its myristylation recruits the polyprotein to areas enriched in phosphatidylinositol-(4,5)-bisphosphate ($PI(4,5)P_2$) phospholipid at the plasma membrane [133]. The two zinc-finger motifs of NC are involved in the recruitment of the two single-stranded RNA viral genomes. The HIV full-length RNA transcript contains a phi element that is recognised by NC zinc fingers.

The incorporation of HIV Env proteins to the virion is still poorly understood. However, their trafficking to the plasma membrane may be mediated through a combination of cellular secretory pathways and Gag [134]. Budding of the virion is catalyzed by the host cell endosomal sorting complexes required for transport (ESCRT) machinery which is recruited by p6. Maturation of the viral particle occurs only once protease from the GagPol polyprotein cleaves Gag and GagPol polyproteins. HIV then adopts an enveloped, bound spherical shape with a distinctive conical CA core.

1.4.7 HIV accessory proteins

HIV has evolved to use host dependency factors that are host proteins necessary for the completion of the replication cycle [135]. HIV accessory proteins are involved in hijacking these

cellular proteins in processes that are indispensable for HIV replication and its pathogenesis. This is particularly important given that several HIV accessory proteins also target host restriction factors. Below, I will describe the HIV accessory proteins Vpr/Vpx, Vpu, Vif and Nef.

1.4.7.1 Viral protein R (Vpr) and viral protein X (Vpx)

Vpr is a 96 amino acid polypeptide with a molecular weight of 12.7 kDa [136]. Structurally, Vpr has an N-terminal helix-turn-helix domain, and an alpha helix in the C terminal that is involved in its binding to many cellular host proteins through its hydrophobic core [137, 138]. The importance of Vpr for HIV virulence has been highlighted by the identification of defective Vpr sequences from patients with slow disease progression [139-141]. Vpr has many roles during the replication cycle, including facilitating tRNAlys incorporation into newly synthesized virions [142]. Vpr also contributes to PIC shuttling into the nucleus [143]. One of its roles most relevant to this thesis is its ability to regulate the cell cycle. Indeed, Vpr can induce cell cycle arrest of proliferative T cells in G2 through its ability to interact with and regulate the cullin4-DDB1 (DCAF1) E3 ubiquitin ligase [144-146]. In fact, Vpr directly regulates Cullin4 E3 ubiquitin ligase activity [147]. Vpr is absent from HIV-2 in which most of its functions are performed by the analogous Vpx. Strikingly, Vpx from HIV-2 also interacts with the Cul4-DDB1 (DCAF1) ubiquitin ligase complex to degrade SAMHD1, suggesting that one of the consequences of SAMHD1 degradation might be to regulate the cell cycle [148] . HIV-1 Vpr also interacts with CypA through two proline residues 14 and 35 [149]. Interestingly, Vpr induces the expression of interferon-stimulated genes (ISG) in macrophages [150].

1.4.7.2 Viral infectivity factor (Vif)

The 192 aa Vif is structurally a very unique protein that resembles the suppressor of cytokine signaling protein 2 (SOCS2) and is stabilized with a zinc finger moiety [151]. Vif is important in mediating the proteasomal degradation of APOBEC3G. It does this by binding to the HRF and recruiting an E3 ubiquitin ligase complex [152]. In the absence of Vif, HIV-1 does not replicate efficiently in primary CD4+ T-cells, its main target.

1.4.7.3 Viral protein U (Vpu)

The 16kDa, 81 aa protein Vpu is composed of a short luminal 4 amino-acid domain, a transmembrane domain and an intracellular domain. Interestingly, Vpu is absent in HIV-2. During HIV-1 infection, Vpu decreases CD4 expression at the surface of the infected cells through direct interaction causing the retention of the receptor in the endoplasmic reticulum (ER) and its degradation through a so-called ER-associated degradation pathway[153]. Vpu is also important for the downregulation of the bone marrow stromal cell antigen 2 (BST2, also known as Tetherin) from the cell surface [154]. BST2 is described in further detail below but is a restriction factor that tethers newly produced Vpu-negative viral particles at the cell surface, preventing release and propagation of infection [154]. In some cases, Vpu favors HIV-1 infection without downregulating BST2; this has been linked with the capacity of Vpu to repel BST2 from the membrane sites of viral assembly [155, 156]. Notably, Vpu was also shown to assemble into transmembrane structures that are similar to ion channels and is thus considered

as a viroporin [157]. However, the importance of this observation for HIV-1 replication and pathogenesis remains unknown.

1.4.7.4 Nef protein

Nef is another membrane-associated protein but in contrast to Vpu, its association with the membrane depends on its myristoylation [153]. The 30kDa Nef protein acts as a homodimer to address cellular protein targets to endosomes and other intracellular vesicles. Viruses with mutations that render Nef non-functional can be identified *in vivo* in HIV-positive individuals and are associated with slow disease progression and better clinical outcomes [158]. Nef also downregulates CD4 from the cell membrane and promotes its degradation through endocytosis followed by lysosomal degradation [159].

1.5. Innate and intrinsic immunity

The innate immune response is defined as a general anti-microbial response that does not involve maturation of the immune system into specialized recognition of specific pathogens. Consequently, the innate immunity is devoid of memory. The autonomous defensive effector proteins within cells are termed intrinsic immunity. Although there can be overlap between these artificially defined responses, HIV host restriction factors are usually part of the intrinsic immune arm. Both innate and intrinsic immune responses contribute to the regulation of HIV replication.

1.5.1. Cells of the innate immune system

Cells that are exclusively part of the antiviral innate immune system include monocytes, macrophages and dendritic cells that all express higher levels of pattern recognition receptors (PRR) than other cells. They represent the cellular component of the first line of defense against invading pathogens and link innate and adaptive immunity. My thesis focuses on two important immune cell types that are part of a sentinel immune system: myeloid dendritic cells (mDCs) and macrophages. Both cell types play a crucial role in the initial phase of response against HIV but are also thought to contribute to the dissemination of infection, to be part of the latent viral reservoirs, and to play a role in the chronic immune activation associated with HIV infection.

1.5.1.1. Macrophages and monocytes

Macrophages are sentinel phagocytes generated from monocytes that originate from the bone marrow. Monocytes circulate from the blood to tissue where they can differentiate to macrophages or DCs. Macrophage differentiation and classification are highly dependent on their anatomical setting. Macrophages can be polarized in two ways. M1 macrophages are associated with a proinflammatory response that contributes to viral control. However, the large amounts of proinflammatory cytokines that are secreted by M1 macrophages result in tissue damage. Conversely, the M2 macrophages secrete anti-inflammatory cytokines that are involved in tissue repair. *In vitro*, monocytes stimulated with GM-CSF will give rise to

macrophages resembling the M1 polarization. In vivo, macrophages are highly concentrated in the mucosal epithelium, but also important are macrophages that are located in the secondary lymphoid tissues and peripheral blood mononuclear cells (PBMC), which all have distinct phenotypes that may participate in antimicrobial immunity, inflammatory response, homeostasis, tissue repair and tumor control [160].

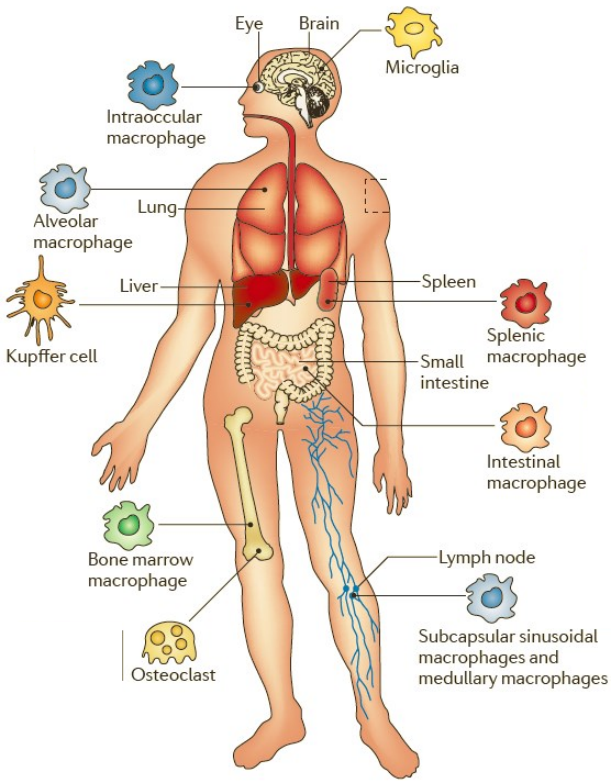


Figure 1.6: Tissue specific macrophages. Monocytes migrate to tissue where they differentiate to mature macrophages and carry out their various immune and homeostatic functions. From [161]

Although macrophages express the necessary surface receptors, CD4 and CCR5, their permissiveness to HIV infection is highly variable among macrophage tissue class localization [162]. Similar to DCs, macrophages express C-type lectins that help in the trans-infection of HIV from macrophages to CD4+ T-cells. In addition, macrophages are believed to be an important

part of the viral reservoir since they are resilient against HIV-1 cytopathic effects and HIV infection has been shown to prevent macrophages from undergoing apoptosis [163-165]. For example, microglia are long-lived macrophages of the CNS that live up to several decades and are also HIV reservoir cells [166].

In the context of HIV research, macrophages have generally been isolated in one of two ways. The simplest way is to plate primary PBMCs on support and allow adherence of monocytes which then differentiate into macrophage through “activation by adherence”. Alternatively, higher yields can be obtained by isolating CD14+ monocytes and differentiating them *in vitro* using granulocyte-macrophage colony-stimulating growth factors (GMCSF) to produce monocyte derived macrophages (MDMs).

Although monocytes are less susceptible to HIV infection in vitro than T-lymphocytes, 1% of circulating monocytes were found to be infected by HIV in untreated individuals. These cells have a circulating half-life of approximately 72h and once they invade a tissue, they can promote the infection of resident lymphocytes, differentiate, and become long-lived resident cells, thus contributing to the persistent viral reservoir [167]. Furthermore, it has been hypothesized that monocyte precursors in the bone marrow are infected with HIV and will persistently give rise to infected monocytes in the blood that are, in turn, persistently seeding tissues with new viruses, constantly infusing the body with virus [168].

1.5.1.2. Dendritic cells

HIV *trans*-infection occurs when the virus is simply internalised or captured whole by a host dendritic cell without initiating viral replication. When a suitable host cell, such as a CD4+ lymphocyte, is in proximity, the virus is then presented to this new target though an infectious synapse. In the new cells, HIV can undergo *cis*- infection to potentially actively replicate and produce viral progeny. The sub-mucosal region is typically rich in immature forms of dendritic cells acting as sentinels and constantly sampling their environment through endocytosis. Dendritic cells play a crucial role in initiating adaptive immunity by capturing pathogens, processing them, and presenting their antigens to CD8+ or CD4+ T-cell through their class I or class II major histocompatibility complexes. They do this either by recruiting these cells inside the infected tissue or by migrating outside of it to draining secondary lymphatic organs. Thus, *trans*- infection by conventional DC (cDC) to CD4+ T cells is thought to be an important route of viral dissemination.

cDC capture of HIV via endocytosis once HIV-1 gp120 can bind to the Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN).Other possible C-type lectin receptors have also been suggested to mediate capture and internalization [169] . Maturity of cDC has an impact on how HIV-1 particles are treated once in the cells and on their ability to transmit the virus to CD4+ T-cells. When immature cDC capture HIV-1 there is a greater tendency for the virus to be degraded [170]. There is also debate on mature trafficking virus-containing endosomes (reviewed in [171]) . In mature cDC some HIV-1 particles do not proceed

to the degradation pathways and remain close to the cell surface in virus-containing compartments [172]. Alternatively, HIV-1 virions were found to accumulate in invaginations on the surface of cDC membranes [173]. Cell-to-cell transfer of HIV-1 occurs through the infectious synapse. The captured HIV is shuttled to the contact zone between the cDC and the target CD4+ T-cells where the virus can then infect the target host [174]. Other models have also suggested that this transfer to the secondary target cell is mediated by exosomes [175]. Productive cis-infection of cDC is rare because of intrinsic host factors such as SAMHD1, and innate immune sensors such as cGAS (discussed sections 1.5.2.2.) that trigger an antiviral immune response.

Dendritic cells are extremely hard to study because they represent only a small fraction (<0.5%) of circulating blood cells PBMCs. Because of their residence in tissues, immature DCs are even more complex to isolate in great number and to work with. Most of what we know about human DC has been extrapolated from studies of murine and simian infections. The increased phenotypic capability we have with new flow cytometry-based techniques allows us to better align our animal finding to humans. Dendritic cells may be classified based on morphology, tissue localization, prototypical function, differentiations state and now increasingly via surface markers and gene expression. With advancements in flow cytometry and the development of ever more performing single cell identification tools, there are now at least 28 DC subsets [176]. Although the classical nomenclature for dendritic cells and macrophages overlooks numerous functional roles, this system is sufficient for the scope of this thesis. Classically we consider that there are four major DC subsets: conventional DC (cDC) also referred to as myeloid DC (mDC), plasmacytoid DC (pDC), Langerhans cells, and monocyte-derived DC (MDDC).

Dendritic cells develop from hematopoietic cells in the bone marrow. The round shaped pDCs can be of both lymphoid and myeloid origin and predominantly circulate in the blood [177]. HIV rarely replicates in pDCs. When HIV viral particles are taken in by pDC, they are rapidly degraded in early endosomes where viral ssRNA can be detected by TLR7 to trigger high production of type 1 IFN and pro-inflammatory cytokines such as IL-6 [178]. Although pDCs only make up less than 0.2% of PBMCs, they contribute significantly to the systemic concentration of type 1 IFN during an infection and are able to release up to 1,000 times more of these cytokines than any other human cell type [179, 180]. Importantly, the production of IFN or inflammatory cytokines does not seem to inhibit HIV replication and may actually promote HIV pathogenesis by recruiting target cells to the initial site of infection, allowing their infection. Later on in chronic infection continued type 1 IFN production contributes to bystander depletion of CD4+ T-cell subsets. In addition, HIV infection results in the recruitment of pDCs to secondary lymphoid organs and these cells are vastly depleted in the blood.

1.5.2. Innate immunity

The innate immune system is the first to respond to an invading pathogen, and this occurs within minutes of transmission. Conserved viral factors termed pathogen-associated molecular pattern (PAMP) are sensed by host pattern-recognition receptors (PRRs) that are membrane-bound, cytoplasmic or nuclear. Although all cells express low levels of some PRRs and are able to detect viral infections and mount an innate immune response, cells of the innate immune system, such a plasmacytoid dendritic cells (pDCs) and myeloid cells, are particularly sensitive

because they express higher levels of PRRs. This first line of defense triggers the production of type 1 interferon (IFN) and pro-inflammatory cytokines, including interleukin (IL)-6, that induce an autonomous antiviral response and modulate the subsequent adaptive immune response. Clinically, IL-6 and IFNs are important markers of immune activation and inflammation.

The pro-inflammatory cytokine IL-6 is produced upon viral infection and is also a marker of disease progression in untreated HIV-infected individuals. Progression is marked by a shift from a predominance of the T-helper Th1 lymphocyte in the blood to Th2 cells, the latter producing higher levels of IL-6 than the former [181]. In addition, monocytes are also producers of IL-6 and high levels of this cytokine is a predictor of serious non AIDS-associated co-morbidities and death in treated HIV-positive individuals [182]. This predictive association is also independent of CD4+ or CD8+ T-lymphocyte activation.

The interferon family is made up of three types. Type 1 interferons (IFN-1) are glycosylated cytokines that were characterized over 50 years ago for their ability to interfere with viral replication in various models [183]. Upon binding of their viral ligands, PRRs initiate a signal cascade that induces the production of Type 1 IFN. In particular, the phosphorylation-induced nuclear translocation of the interferon regulatory factors (IRFs) and NFkB transcription factors allows their binding to the IFN promoters and initiates their transcription followed by their secretion. Type 1 IFNs act in an autocrine, paracrine, and systemic ways to induce a cellular and physiological antiviral state. All cells express type 1 IFN receptors (IFNARs), though at various levels, and are thus able to respond to this cytokine through the transcriptional regulation of

hundreds of genes. Genes that are actively transcribed upon IFN stimulation are called interferon-stimulated genes (ISGs) and include resistance and restriction factors, inflammatory and co-stimulatory cytokines that serve to induce an antiviral state but can be harmful if chronically upregulated.

Indeed, in recent years numerous studies have shown that prolonged elevated systemic type 1 IFN production is linked to immune and inflammatory diseases known as interferonopathies such as systemic lupus erythematous, multiple sclerosis, dermatomyositis, Sjogren's disease, scleroderma, rheumatoid arthritis, and neuropsychiatric pathology [184, 185]. In the context of HIV infection, a well-balanced immune response is an important determinant of clinical outcomes.

In this regard, SIV infections of non-human primate models have been useful in studying the mechanisms behind viral control and immunopathogenesis caused by the innate immune response to the infection. SIVs are also especially useful in the study of HRFs. Limitations of non-human primate models include differences in HRF such as TRIM5α but also genomic differences between SIV and HIV such as vpx/vpr coding sequences.

With the exception of chimpanzees, SIV infections in their natural hosts are generally nonpathogenic despite the presence of high viral load. Interestingly, chronic immune activation, marked by persistent type 1 IFN and ISG expression, is absent in the natural course of these SIV infections. It is hypothesised that progression to AIDS is averted if the innate and adaptive

immune responses return to baseline shortly after the acute phase of infection [186]. In these animal models, it was also proposed that CD4+ T-cell preservation could be linked to viral restriction in macrophage and central memory T-cell, two cells in which SAMHD1 is found to restrict HIV in humans [186, 187]. In humans, natural control of progression to AIDS is seen in individuals who are identified as elite controllers (EC) and long-term non-progressors (LTNP). However, the role of innate immunity in chronic inflammation or viral control in these two populations remains unclear. Chronic immune activation can be seen in some LTNP and EC but to a lower extent than in natural progressors who are under ART [188] . Subsets of LTNPs can maintain low levels of inflammation [189].

Observations of the innate immune response in these 'natural' experiments paired with systems biology analyses indicate that the dynamics and timing of the innate immune response are key in controlling the virus and averting chronic immune activation [190]. It seems that a strong IFN-mediated inflammatory response would be important at the time of and shortly after transmission. If this first wave catches the virus at just the right time, infection might trigger only a transient innate immune response that includes type 1 IFN and ISG expression.

Like all organisms, HIV requires replication of its genome to propagate. Therefore, nucleic acids present an appealing target for innate immune sensing and antiviral strategies. Due to this vulnerability, the virus has evolved mechanisms to minimise host detection of its RNA or DNA. In terms of HIV-1 nucleic acid detection, there are several important innate immune receptors: TLR7 and TLR8 that are localized in the endosomes and can detect ssRNA molecules; cGAS that

detects viral DNA in the cytoplasm; and IFI16 that can recognize viral DNA in both the cytoplasm and nucleus. These immune sensors are detailed below.

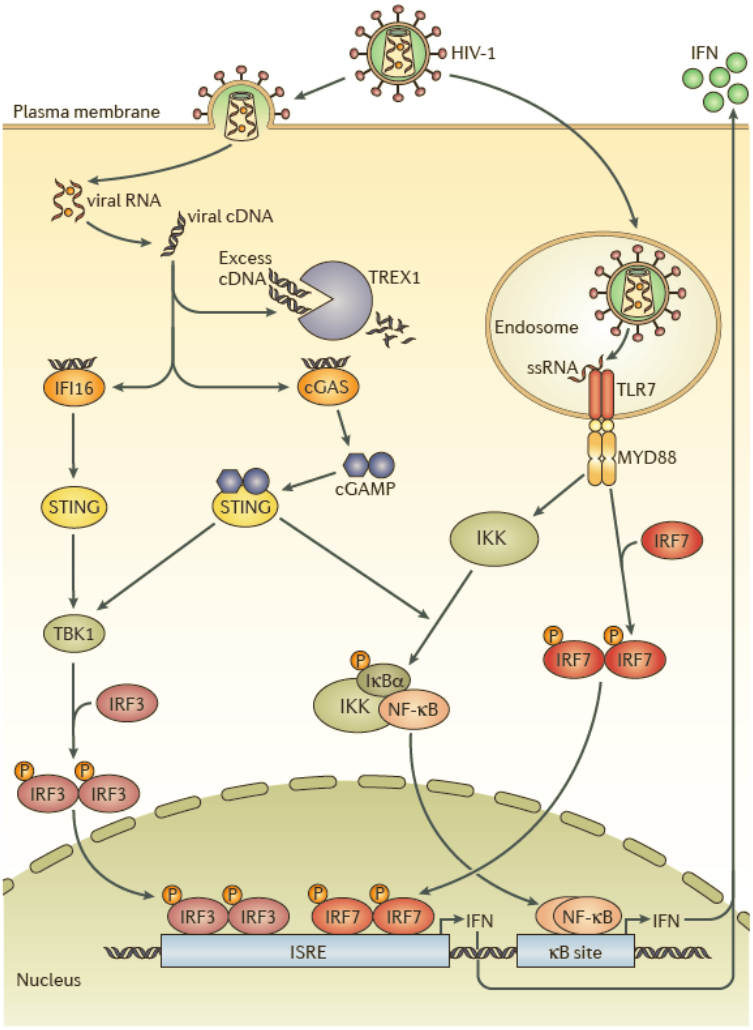


Figure1.7: Nucleotide innate immune sensors. Detection of exogenous DNA in the cytoplasm through cGAS and IFI16 triggers signal transduction down to IFR3 nuclear translocation promoting the expression of IFN-1 and other anti-viral cytokines. Endoplasmic foreign RNA signals thought TLR 7 or 8 and results in the recruitment of NFkB and IRF7 to the nucleus and the production of IFN-1 [191]

1.5.2.1 Toll-like receptor 7 (TLR7) and TLR8

Toll-like receptors are transmembrane proteins found on the cell surface or in endosomal compartments. In humans, there are 10 known TLRs and all but TLR3 can recruit the myeloid differentiation primary response gene 88 (MyD88) to induce IFR- and NFkB-dependent interferon production [192]. Another adaptor called TIR-domain-containing adapter-inducing interferon-β (TRIF) mediates TLR3 signalling. Early detection of HIV-1 during acute infection is most often achieved by pDCs that are rich in TLR7 [193]. When HIV-1 is endocytosed, TLR7 located in endosomal compartments can detect HIV-1 ssRNA [178]. Active replication is not necessary for detection since endosomal acidification degrades the viruses, exposing the genome to innate sensing. Recognition of viral ssRNA via TLR7 in pDC triggers a strong IFN production. TLR8 can also detect ssRNA in macrophages. In addition, the detection of ssRNA by TLR7 and TLR8 also results in the production of interleukin 6 (IL-6) when it occurs in monocytes and MDDC [194]. Importantly, HIV is able to hijack this signalling pathway and it has been shown that HIV replication in dendritic cells requires signalling from both TLR8 and DC-SIGN [195].

1.5.2.2 Cyclic GMP/AMP synthase (cGAS)

One of the most recently discovered members of the PRR family of molecules, cGAS, binds specifically to foreign double-stranded DNA in the cytoplasm of infected cells, including HIV-1 dsDNA reverse transcripts. Upon HIV-1 dsDNA binding, cGAS produces cyclic GMP/AMP

dinucleotides (cGAMP) that are recognized by the endoplasmic reticulum adapter protein Stimulator of interferon genes (STING) that subsequently activates the transcription of type 1 interferon and pro-inflammatory genes [196]. Remarkably, cGAS contains a conserved nucleotidyltransferase domain that is also found in DNA polymerases and 2'-5'-oligoadenylate synthase (OAS), which is a known IFN-inducible protein that also activates interferon and pro-inflammatory genes through its interaction with RNaseL [197]. Notably, cGAS recognition of HIV-1 DNA is active in MDDC. To avoid detection, HIV actively targets its own DNA for degradation, effectively eliminating incomplete reverse transcripts in most cells through the use of the cytosolic exonuclease three prime repair exonuclease TREX1. TREX1 is the most abundant exonuclease in human cells and part of the endoplasmic reticulum-associated SET complex that participates in the control of the replication of endogenous retroviruses. TREX1 prevents the expression of interferon during HIV-1 infection whereas abrogating its expression restores the innate immune response. Although not fully proven, it is possible that TREX1 contributes to the ability of HIV to escape cGAS and/or IFI16 detection in some cell types [198].

1.5.2.3. Gamma interferon-inducible protein 16 (IFI16)

IFI16 is unique amongst PRR that detect DNA because it is localised both in the cytoplasm and the nucleus. All other DNA-specific PRRs described so far are exclusively localized within the cytoplasm where they are more likely to detect foreign DNA. It was shown very recently that IFI16 discriminates between human and pathogenic DNA within the latter's cellular compartment by binding naked foreign DNA while ignoring chromatinized human DNA [199].

IFI16 is expressed in lymphoid cells and is IFN-γ-inducible in myeloid cells. It binds dsDNA or ssDNA and signals through STING to activate the innate immune response against viral infections, including HIV-1. Importantly, IFI16 is thought to be critical for HIV pathogenesis. Indeed CD4+ T-lymphocytes death during HIV infection is not systemically associated with active HIV replication. In fact, most killed CD4+ T-cells are negative for HIV markers through the so-called bystander effect. Following the earlier demonstration that IFI16 specifically binds HIV-1 ssDNA, two recent major publications demonstrated that IFI16 is indispensable for the death of uninfected bystander CD4+ T-lymphocytes through the induction of a specific form of apoptosis called pyroptosis [200-202]. Furthermore in treatment naive patients, increased IFI16 expression was found to correlate with higher chronic immune activation of CD4+ memory T-cells [203].

1.5.3. Intrinsic immunity

Intrinsic immunity is composed of constitutively expressed antiviral effectors of the innate immune system. They include host restriction factors and host resistance factors or cellular anti-viral inhibitors. Throughout its replication cycle, HIV interacts with a plethora of host proteins that will support or curb its replication. The 'Brass study' aimed to map the dependency factors through a siRNA screen, and identified over 250 cellular proteins needed for HIV infection [135]. Soon after, several screens sought to identify the protein factors that impede the virus directly [204, 205]. While these studies add to the list of intrinsic effector

proteins, they did not allow the identification of some major HRFs, in part because they were performed using cells that were susceptible to HIV infection.

In contrast, most established HRFs were discovered when studying HIV infection in non-permissive cells. HRFs act as important barriers against zoonotic transmission of viruses since they are effective against orthologous viruses. Although HRFs are constitutively expressed, in some cell types these pre-mobilized responses can be further upregulated by interferon signaling [206]. But by being able to act autonomously and immediately, they place a dominant selection pressure on the virus to escape them. Consequently, HRF can often be overcome by substrate saturation and most are counteracted by HIV-1 accessory proteins. At present, four HRF against HIV-1 have been identified: APOBEC3G, Trim5α, Tetherin and SAMHD1. The very recently discovered serine incorporator SERINC3 and SERINC5 will not be detailed in this thesis because of the lack of extensive information about them but they are known to be counteracted by Nef [207, 208].

A second but still important class of intrinsic immune effectors proteins are called host resistance factors (HrF). This class of effectors are not limited to only ISGs. They apply an inhibitory pressure on HIV but it may not be enough to render a cell completely non-permissive. As such the virus may simply be able to tolerate attenuations without necessarily countering it directly. Host resistance factor can also be a cellular factor whose antiviral activity has not yet been characterised *in vivo*. Host resistance factors of note are IFITM and MxB. In 2005, our lab was the first to identify viral inhibitory activities of PRMT6 [87, 125, 126, 209]. During my thesis,

I have worked at characterizing the role of PRMT6 in the context of an HIV-1 infection. I also examined whether the ability of SAMHD1 to hamper HIV-1 infection in myeloid cells by limiting dNTP levels contributes to the resilience of certain HIV-1 drug resistant mutations.

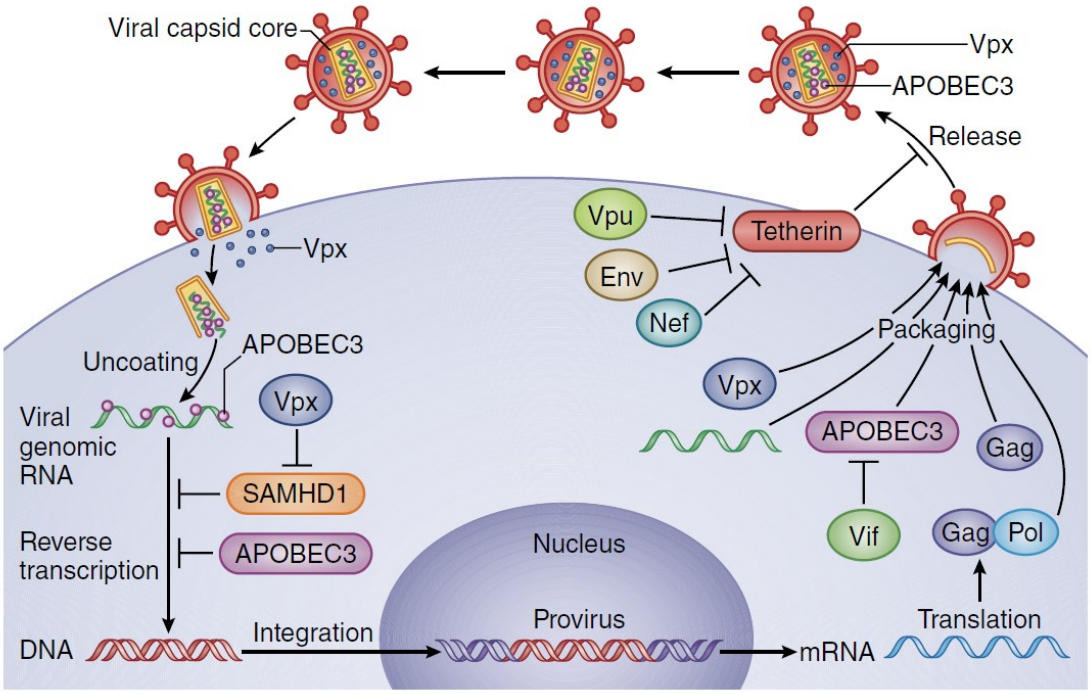


Figure 1.8: Established host restriction factors against HIV-1 infection and viral proteins antagonists. APOBEC3G must be packaged in budding virions to take effect in the subsequent round of infection. Tetherin is localized to the plasma membrane and prevent the release of viral particles. The effect of SAMHD1 are experience by HIV-1 RT in the nucleus by restricting the levels of available dNPT. TRIM5a not shown because hTRIM5a does not act on HIV-1. From [210]

1.5.3.1 APOBEC proteins

Apolipoprotein B editing complex catalytic subunit 3G (APOBEC3G) was the first host factor to be identified as an inhibitor of HIV-1 replication in human cells [211]. Of the seven human APOBEC3 proteins, APOBEC3F and APOBEC3DE are the only other ones capable of inhibiting HIV-1 *in vivo*, albeit to a lesser extent than APOBEC3G [212]. APOBEC3G codes for a cytidine

deaminase that, when expressed, prevents the replication HIV-1 mutants lacking Vif (HIV-1 ΔVif) [211]. Vif counters APOBEC3G by inducing its proteasomal degradation through ubiquitination. Cells expressing APOBEC3G were termed non-permissive and those lacking APOBEC3G and thus conducive to HIV-1 viral replication in the absence of Vif were termed permissive. APOBEC3G is an ISG, and type 1 IFN considerably stimulates its expression in myeloid cells [213]. In order for APOBEC3G to act on HIV-1 it must be packaged into virions from the producer cells. When the virus containing APOBEC3G infects another target cell, APOBEC3G will during reverse transcription mediate deoxycytidine to deoxyuridine changes in the negative cDNA, resulting in G-to-A hypermutations. These mutations may be lethal to the virus. However, sublethal substitutions can give rise to viral escape mutations, thus making the virus more adaptable.

1.5.3.2 Tripartite motif containing protein 5 alpha (TRIM5α)

TRIM5α is found in the cells of most primates and confers resistance to HIV-1 in many old world monkey cells [214]. TRIM5α recognizes incoming viral capsid and interferes with the uncoating process [215]. Orthologues of Tripartite-motif TRIM5α were among the first host restriction factors against retroviruses to be discovered. The exact mechanism by which TRIM5α proteins inhibits retroviral infection is still highly debated. Firstly TRIM5α could impede retroviral replication by promoting rapid uncoating through ubiquitin-dependent proteasomal degradation of the viral CA [216]. Recently it was shown that TRIM5α binding of CA regulates innate immune signalling [217]. Human TRIM5α however has limited restriction activity against

HIV-1 due to the interference of another host protein, the peptidyl-prolyl *cis-trans* isomerase cyclophin A (CypA) [218]. CypA binds to the N-terminal of the CA protein, isomerizes CA's proline rich domain and is packaged into newly-assembled HIV viral particles [219, 220]. The conformational change that CypA catalyzes is thought to prevent the recognition of CA by TRIM5α [221]. However it is the CypA present in target cells that contributes most to HIV-1 infectivity [222]. In addition, in dendritic cells, CypA interaction with CA seems to upregulate type 1 interferon response through cGAS [223, 224].

1.5.3.3 Tetherin

Tetherin has been identified as a host restriction factor that "tethers" HIV-1 particles to the cell surface preventing their release [154]. This restriction factor is antagonized by Vpu resulting in its degradation via the β-TrCP2 dependent pathway [225]. Viruses that are lacking or defective for Vpu are captured at the cell surface during budding. These virions are then endocytosed and degraded [226]. By using a flow cytometry-based co-culture assay, our lab has shown that tetherin also restricts cell-to-cell spread of HIV-1 infection [227].

1.5.3.4 Protein arginine methyl-transferase 6 (PRMT6)

Arginine methylation is a posttranslational modification that involves the addition of one or two methyl groups to the nitrogen atoms of the guanidino group of arginine. These S-adenosyl-L-

methionine (AdoMet)-dependent methylations are carried out by PRMTs, a series of enzymes found only in eukaryotes. Arginine methylation has been implicated in RNA processing, transcriptional regulation, signal transduction, DNA repair and contributes to the "histone code". Three major types of arginine methylation have been described: type I methyltransferases catalyze the formation of ω-N^G-monomethylarginine and ω-N^G,N^G-dimethylarginine (asymmetric); type II enzymes produce ω-N^G-monomethylarginine and ω-N^G,N'^G-dimethylarginine (symmetric); type III enzymes only generate monomethylarginines. In humans, eleven different PRMTs have been described: PRMT1, PRMT2, PRMT3, PRMT4, PRMT6 and PRMT8 are all type I enzymes, whereas PRMT5, and PRMT9 are type II enzymes. PRMT7 is the only known type III methyltransferase identified. The classification and activity of PRMT10 and PRMT11 have not yet been established. Although arginine methylation does not change the overall charge of arginine, the methyl groups are thought to impose steric constraints and disrupt hydrogen bonds. Thus, arginine methylation can modulate intra- and inter-protein interactions [228, 229].

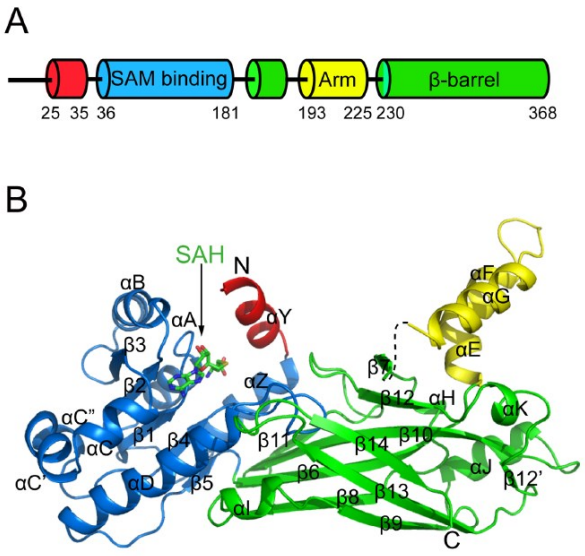


Figure1.9: Crystal structure of *Trypanosoma brucei* PRMT6. A) Organisation of different functional domains of PRMT6. B) The crystal structure of *Trypanosoma brucei* PRMT6 is highly homologous to that of the human. From [230]

PRMT6 has some degree of tissue specificity seen by higher expression levels in the brain, testis and placenta [231]. This enzyme is localised in the nucleus and is an important epigenetic regulator. Di-methylation of Histone H3 on arginine-2 (H3R2) by PRMT6 prevents the concomitant methylation of the Lysine-4 of H3 (H3K4), both residues being important in the histone code [232]. A consequence of H3R2 methylation is the direct transcriptional repression of TP53, the gene encoding the tumor suppressor protein p53 [233]. Additionally, PRMT6 methylates the AT-hook of HMGA1a, a transcription factor involved in chromatin architecture [234]. Methylation of DNA polymerase β on Arg-83 and Arg-152 by PRMT6 increases the activity of the former and implicates PRMT6 in DNA base excision repair [235]. When PRMT6 is knocked down, cyclin-dependent kinase inhibitors accumulate and the cells stall at the G2 replication phase [236]. It is therefore unsurprising that deregulation of PRMT6 may result in oncogenesis as shown in such cancers as bladder and lung [237].

As a transcriptional regulator in the context of host immunity, PRMT6 downregulates the expression of MHC class II HLA-DQ by methylating the DNA bind AT-hook of its transcription factor RFX5 [238]. Finally, PRMT6 acts as a coactivator for NFkB in IL-6 protein expression [239]. When PRMT6 was discovered to regulate Tat, this meant that it could also play a role in intrinsic immunity.

Human PRMT6 compromises HIV-1 protein function by methylating HIV-1 Tat, Rev, and NC. Methylation of arginine residues at position R52 and R53 in the ARM domain of Tat leads to decreased transactivation activity as determined in MAGI cell assays and therefore down-regulation of HIV replication [125]. Methylation of Tat also leads to reduced TAR binding and severely diminished cyclin-T1 recruiting [126]. PRMT6 methylation of Tat was also shown to increase the Tat protein half-life by 4.7 fold [240]. Recently, it was shown that methylation of Tat residues R52/53 prevents it from localizing to the nucleolus [241]. As in Tat, Rev is arginine-methylated by PRMT6 in the ARM domain. Rev methylation has a negative effect on Rev function by means of reduced binding affinity to its target RNA sequence RRE [87]. NC was the third HIV-1 protein that is recognized by PRMT6 as a substrate and is arginine methylated in the basic regions flanking the first Zn^{2+}-finger motif. Further *in vitro* studies revealed that arginine methylation decreased the annealing capacity of NC [209].

The importance of PRMT6 in HIV-1 was further accentuated in a Genome Wide Association Study (GWAS) comparing a HIV-1 subtype B positive LTNP cohort to a cohort of rapid HIV-1 subtype B progressors to AIDS, that found a novel association between rapid progression and

PRMT6 SNPs [242]. Moreover, haplotype analysis showed that the best signal for progression to AIDS was a SNP in the PRMT6 gene. Therefore, variations in PRMT6 SNPs might, in part, determine disease progression in HIV-positive individuals. Possibly, some natural PRMT6 isoforms are more adept at controlling HIV-1 infections.

PMRT6 auto-methylation has also been reported [231]. However, little is known about the role of this post-translational modification on PRMT6 activity.

1.5.3.5. SAMHD1

SAMHD1 was first discovered in murine macrophages as a GTP-binding IFN-γ-induced Mg21 protein [243]. Later, in humans, several mutations causing SAMHD1 dysfunction were associated with Aicardi-Goutieres Syndrome (AGS), a genetically associated childhood encephalopathy due to chronically high levels of IFN type 1 in the cerebrospinal fluid that resembles congenital viral infection [244]. This interferonopathy is thought to be triggered, possibly through the DNA sensor cGAS, by the abnormal accumulation of nucleic acids due to defective nucleases such as TREX1 or SAMHD1, although the exact downstream pathway has yet to be elucidated [245] .

Non-dividing human myeloid cells such as macrophages and dendritic cells are marginally susceptible to productive HIV-1 infection. In order to render these cells more permissive to HIV-1 infection or HIV-1-derived lentiviral particle transduction, scientists need to supply vpx *in*

trans. Since the late 1980s it has been known that vpx facilitates SIV infections in monocytes, macrophage and dendritic cells but did not affect permissiveness in activated CD4+ T-cells [246, 247]. In fact, the presence of vpx could increase viral infectivity in myeloid cells 100-fold [248]. Vpx likely countered myeloid restriction by causing a still unknown host factor to undergo proteasomal degradation via Cullin4 ubiquitin ligase [249]. Using mass spectrometry, SAMHD1 was discovered to co-immunoprecipitate with the vpx-ubiquitin-ligase complex and subsequently identified as a HIV-1 host restriction factor [250, 251].

SAMHD1 is a 626 amino acid long enzyme, 72.2 kDa, and composed of 2 domains. The SAM domain, implicated in protein-protein interactions, is located near the N-terminal domain between amino acids 45 to 110 [252]. The HD domain between residues 162 and 335 is responsible for the protein's catalytic activity, oligomerization and nucleic acid binding [210]. The exact mechanism of HIV-1 restriction in myeloid or resting CD4+ T cells is a highly debated question in regard to dNTPase or possible RNAse activity [253].

Initially, SAMHD1 was characterized as a regulator of intracellular dNTP pools, and the evidence that points to this is its primary mechanism of restriction . Non-dividing cells that are refractory to HIV-1 infection are low in intracellular pools of dNTP. In fact, dNTP levels in macrophage are 130 to 250 fold lower than in activated CD4+ T cells [254]. Similarly, dNTP levels in delta-SAMHD1 THP1 cells that were differentiated with PMA were considerably higher than in SAMHD1-expressing THP1 cells [255]. *In vivo*, CD14+ myeloid cells from AGS patients were uncharacteristically susceptible to HIV-1 infection [256]. SAMHD1 was also found to restrict the

early stages of HIV-1 replication in resting or quiescent CD4+ T-cells that also have low dNTP levels [99, 187]. Differences in kinetics between vpx-mediated SAMDH1 degradation, dNTP levels and HIV replication have been reported [254]. Exposure of primary MDMs to vpx followed by western blot analyses showed SAMHD1 levels to decrease within 2 hours, which correlated with proviral synthesis, as measured by the formation of 2LTR circles [257]. This study along with others showed that wild type HIV-1 RT processivity is stoichiometrically dependent on dNTP concentration [254, 258, 259].

An early model of SAMHD1 enzymatic function was first proposed by Goldstone *et al*. [97]. Through anion exchange HLPC and x-ray crystallography, they found that SAMHD1 forms dimers whose catalytic activity is induced by allosteric binding of dGTP [97]. The enzyme then goes on to hydrolyse dNTPs into deoxynucleoside and inorganic triphosphate [97]. This dGTP-dependent mechanism was further supported by Powell et al. by varying individual dNTP concentration in *in vitro* enzymatic assays measuring HPLC retention times of the 4 nucleotides [260]. It was later shown that both dGTP or GTP binding to primary allosteric sites induced SAMHD1 to form an active homotetrameric complex and this is the currently accepted model [261, 262].

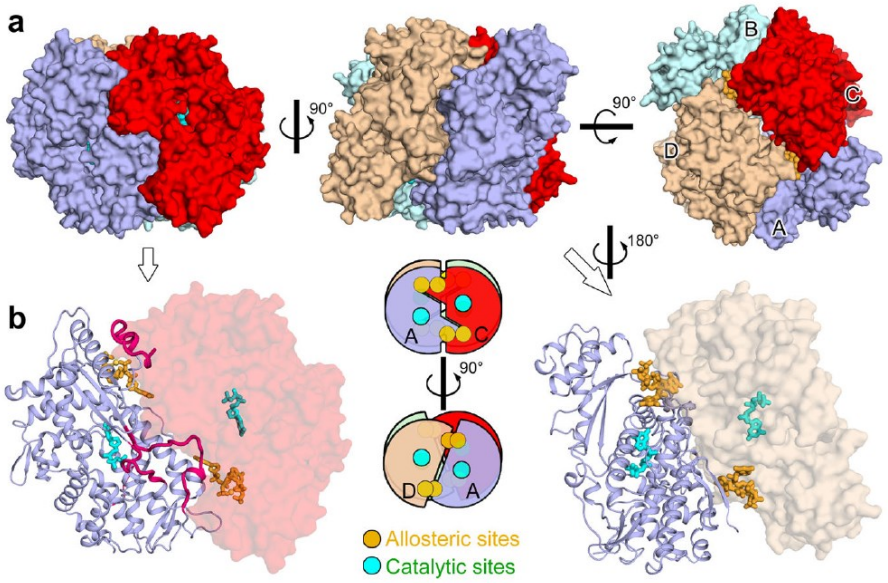


Figure 1.10: Crystal structure of hSAMHD1. A) Represented here is the crystal structure of the catalytic core of the SAMHD1 tetramer bound to dGTP. B) SAMHD1 dimers are seen here with bound dGTP in orange and cyan. Adopted from [263]

The first evidence that pointed to SAMHD1 RNAse activity came from *in vitro* enzymatic assays. SAMHD1 was purified and shown to be a 3' to 5' exonuclease [264]. This implied a direct degradation of HIV-1 RNA as another potential means of viral restriction. Another group has confirmed this RNAse activity but only with regards to retroviruses despite SAMHD1 not exhibiting nucleic acid sequence specificity [265]. However, the RNAse hypothesis was put in question when an important study found contaminating endonuleases to coprecipitate with SAMHD1 during protein purification [266].

Although SAMHD1 is expressed in the majority of human tissues, it has not been observed to restrict HIV in proliferating cells [267]. It is therefore no surprise that the enzymatic activity of SAMHD1 is linked to cell cycle and that this activity is regulated by phosphorylation. Several

cyclin dependent kinases (CDK), including CDK1 CDK2 and CD6, have been suggested to phosphorylate SAMHD1 [268-272]. However, RNAi knockdowns of CDK1, CDK2, CDK4, CD5 or CDK6 in proliferating macrophages revealed that CDK2 and CDK6 are the most likely candidates [270]. Studies with a CDK inhibitor corroborated CDK6-induced CDK2 phosphorylation as the principal SAMHD1 kinase cascade [273]. Moreover, p21, an inhibitor of CDK1 and CDK2, and cyclin-D3, a partner of CDK6, were also found to modulate SAMHD1 activity [274, 275]. Threonine 592 (T592) was identified as the phosphorylated residue located in the HD domain [268, 276]. Dephosphorylation stabilizes SAMHD1 as an active tetramer at low dNTP levels allowing it to maintain these reduced dNTP levels in resting or terminally-differentiated cells. Conversely, phosphorylation destabilises the SAMHD1 tetramer and although the phosphorylated protein is still somewhat active at very high dNTP concentrations, it cannot efficiently degrade dNTPs at low dNTP concentration [277].

HIV-2 and SIVsmm Vpx targets Human SAMHD1 for proteasomal degradation by binding to its C-terminal and recruiting it to the Cul4A-DECAF1 E3 Ubiquitin ligase complex [265]. The targeting of SAMHD1 to proteasomal degradation occurs in the nucleus, since removal of the NLS of SAMHD1 prevents its degradation [278]. The protein degradation of host SAMHD1 is not the same across all primate immunodeficiency viruses. In fact, vpr from some non-human primates can also degrade SAMHD1 [26]. Interestingly, when analysing the pathogenicity of primate lentiviruses towards their natural host, the trend that emerges is that the virus is more pathogenic when SAMHD1 is degraded [26].

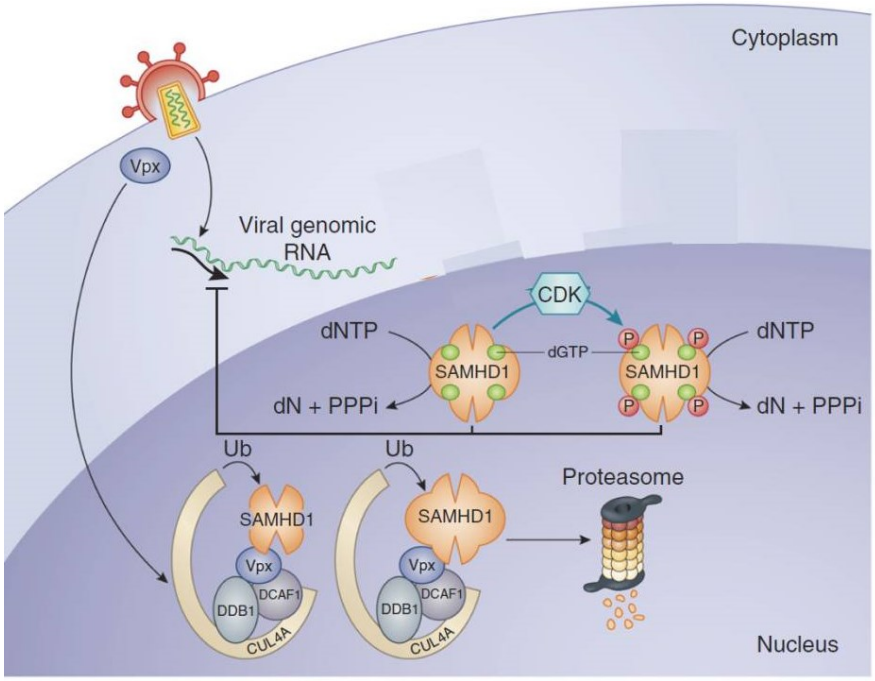


Figure 1.11: SAMHD1 antiviral restriction: Upon phosphorylation of SAMHD1 by CDK2 or CDK6 induced tetramerization permitting SAMHD1 to efficiently hydrolyse dNTP, keeping these pools low and inhibiting HIV-1 reverse transcription. Vpx however can target SAMHD1 for proteasomal degradation. Adapted from [210].

Accordingly, SAMHD1 is only weakly induced in cDC from elite controllers (EC), as opposed to a significant induction in control groups [279]. Although there is no consensus regarding SAMHD1 expression in studies that report mRNA levels in elite controllers, mRNA levels do not indicate the activation state of SAMHD1 and so only limited conclusions can be made [204, 280, 281]. While understanding this limitation, these observational studies in humans and NHP may still provide clues as to what role SAMHD1 plays in disease progression or chronic inflammation.

Each of the timing, dynamics and nature of innate activation have been shown to be important in viral control and subsequent chronic immune activation. In cases in which SAMHD1 is

degraded, the virus may be detected early by cGAS by somehow bypassing TREX1. When this happens in key innate immune cells, namely macrophages or DC cells, infection may trigger a direct type of innate immune response and may lead to an outcome whereby the virus is less pathogenic. Of course this is a simplified model, but it may open the door to interesting therapeutic considerations.

The first HIV antiretroviral drugs came from molecules that were previously used to treat cancer. In oncology and orphan diseases new forms of targeted immunotherapy, such as autologous transplantation of chimeric antigen receptor (CAR)-modified T-cells or antibody drug conjugates (ADC) like Kadcyla, are being developed. Ongoing clinical trials are testing the use of classical small molecule immune modulators such as metformin. Understanding the innate immune system is not only important for curative strategies but also for preventing or managing the toxic chronic inflammatory response in aging HIV patients. Going forward, we can look to development of cancer immunomodulation therapies and to adapting these strategies for HIV to complement HAART. Therapies could involve activating a strong innate immune response, through specific DNA sensors during acute infection. Curative strategies could involve ADCs directed to receptors of long-lived latently infected cells such as macrophages.

1.6. Antiretroviral drugs

After having detailed the immune consequences of HIV infection and some of the host factors that partially limit viral replication, I will now describe the antiretroviral drugs that efficiently inhibit viral infection and pathogenesis.

1.6.1 Antiretroviral drugs and resistance overview

The first drug to demonstrate anti HIV properties was suramin in 1984, initially used to treat parasitic infections in Africa, but this was not further pursued due to its toxicity [282]. The first class of HIV drugs to be approved by the FDA were nucleoside analogues that inhibit reverse transcriptase. These drugs are termed nucleos(t)ides reverse transcriptase inhibitors (NRTI). Zidovudine (AZT) demonstrated efficacy against HIV, and was rapidly approved by the FDA in 1987. However, doctors observed that AZT lost its potency after only 6 month of use and it became clear that patients undergoing monotherapy would quickly fail therapy as drug resistance emerged. In 1988, the hyperadaptibiliy of HIV to treatment was discovered to be related to the lack of a proofreading exonuclease in RT, resulting in a high genetic variability of RT transcripts [283, 284]. Soon after, specific drug resistance mutations were found to be associated with AZT treatment failure [285]. Other drugs, such as non-nucleoside reverse transcriptase inhibitors (NNRTI), were developed but drug resistance would soon emerge after weeks to months of use.

A turning point came when researchers combined a protease inhibitor with two RTI that would produce a drug regimen that was highly effective at inhibiting the virus but would be less susceptible to drug resistance. Highly Active Antiretroviral Therapy or HAART signaled a new hope for patients that HIV was no longer a death sentence. In fact a recent study found that a 20-year-old HIV positive individual initiating HAART in 2007 should have a life expectancy of 70 years, that is as great as that of the average American or Canadian [286].

There are currently 6 classes of medicines that inhibit HIV replication. NRTI and NNRTI inhibit RT; protease inhibitors (PI) prevent the cleaving of polyproteins in budding virions, thus preventing their maturation into fully infectious viral particles; fusion inhibitors prevent fusion of the HIV envelope and host membrane; entry inhibitors bind to CCR5 to prevent HIV from binding to this coreceptor; and integrase strand transfer inhibitors (INSTI) inhibit the integration of HIV into the host genome.

Table1.1: Antiretroviral therapy by drug class as of December 2015. Adopted from[287]

Brand name	Generic name	Approval date
Multi-class combination products		
Atripla	Efavirenz (EFV), emtricitabine (FTC)and tenofovir disoproxil fumarate (TDF)	July 6th, 2006
Complera	Emtricitibine (FTC), rilpivirine (RPV) and tenofovir disoproxil fumarate (TDF)	August 8th, 2011
Stibild	Elvitegravir (EVG)/ cobicistat(c)/ emtricitabine (FTC)/ tenofovir disoproxil fumarate (TDF)	**August 27, 2012**
Triumeq	Dolutegravir (DTG), lamivudine (3TC), abacavir (ABC)	**August 22, 2014**
Genvoya	Elvitegravir (EVG)/ cobicistat(c)/ emtricitabine(FTC)/ tenofovir alafenamide fumarate (TAF)	**November 5, 2015**
Nucleos(t)ide reverse transcriptase inhibitors (NRTIs)		
Combivir	Lamivudine (3TC) and zidovudine (AZT)	September 27th, 1997
Emtriva	Emtricitabine (FTC)	July 2nd, 2003
Epivir	Lamivudine (3TC)	November 17th, 1995
Epzicom	Abacavir (ABC) and lamivudine (3TC)	August, 2nd, 2004
Hivid	Zalcitabine (ddC)	June 19th, 1992
Retrovir	Zidovudine (AZT)	March 19th, 1987
Trizivir	Abacavir (ABC), zidovudine (AZT), and lumivudine (3TC)	November 14th, 2000
Truvada	Tenofovir disoproxil fumarate (TDF) and emtricitabine (FTC)	August, 2nd, 2004
Videx	Didanosine (ddI)	October 9th, 1991
Videx EC	Enteric coated didanosine (ddI)	October 31st, 2000
Viread	Tenofovir disoproxil fumarate (TDF)	October 26th, 2001
Zerit	Stavudine (d4T)	June 24th, 1994
Ziagen	Abacavir sulphate (ABC)	December 17th, 1998
Nonnucleoside reverse transciptase inhibitors (NRTIs)		
Edurant	Rilpivirine (RPV)	May 20th, 2011
Intelence	Etravirine (ETR)	January 18th, 2008
Rescriptor	Delavirdine (DLV)	April 4th, 1997
Sustiva	Efavirenz (EFV)	September 17th, 1998
Viramune	Nevirapine (NVP)	June 21st, 1996
Viramune XR (extended release)	Nevirapine (NVP)	March 25th, 2011
Protease inhibitors (Pis)		
Agenerase	Amprenavir (APV)	April 15th, 1999
Aptivus	Tipranavir (TPV)	June 22nd, 2005
Crixivan	Indinavir (IDV)	March 13th, 1996
Fortovase	Saquinavir (SQV)	Novemebr 7th, 1997
Invirase	Saquinavir mesylate (SQV)	Decemebr 6th, 1995
Kaletra	Lopinavir (LPV) and ritonavirin (RTV)	September 15th, 2000
Lexiva	Fosamprenavir calcium (FOS-APV)	October 20th, 2003
Norvir	Ritonavir (RTV	June 23rd, 2006
Prezista	Darunavir (TMC-114)	June 23rd, 2006
Reyataz	Atazanavir sulphate (ATV)	June 20th, 2003
Viracept	Nelfinavir mesylate (NFV)	March 14th, 1997
Fusion inhibitors		
Fuzeon	Enfuvirtide (T20)	March 13th, 2003
CCR5 antagonists		
Selzentry	Maraviroc (UK-427857)	August 6th, 2007
Integrase strand transfer inhibitors		
Isentress	Raltegravir (MK-0518)	October 12th, 2007
Vitekta	Elvitegravir (EVG)	**September 24, 2014**
Tivicay	Dolutegravir (DTG)	**August 12, 2013**

For the purpose of this thesis, I detail below reverse transcriptase and integrase inhibitors and resistance against some of these antiretroviral drugs.

1.6.2. Reverse transcriptase inhibitors (RTIs)

Also know as chain terminators, NRTI are prodrugs that lack a 3'hydoxyl that, once phosphorylated, competes with dNTP in the cell and binds to the growing DNA chain during reverse transcription. The absence of a 3'hydroxyl group prevents the addition of the subsequent nucleotide, thus stopping elongation. NNRTI inhibit allosterically by binding to the hydrophobic pocket near the catalytic site of RT to induce a conformational change [288].

A purely Darwinian process, HIV drug resistance is caused by the selection under drug pressure of variants that are more fit in the presence of inhibitor. Drug resistance substitutions, are archived within the viral reservoir and can also be transmitted from one individual to another. Importantly, the use of combination therapy dramatically reduces the emergence of resistance mutations compared to monotherapy with reverse transcriptase inhibitors. Some drug resistant mutations will prevent or delay the emergence of resistance mutations to another drug whereas some mutations can confer cross-resistance to several inhibitors of the same class.

NRTI resistance has been found to occur in one of two ways. Thymidine analogue mutations (TAMs) in RT, selected by AZT and d4T, enhance the enzyme's ability to excise the nucleoside analogue through pyrophosphorolysis [288]. The second mechanism that leads to NRTI resistance are mutations that improve RT discrimination between the nucleotide analogue and

proper dNTP. In this regard, an M184V/I mutation in RT will cause the virus to be highly resistant to 3TC and FTC and somewhat resistant to ABC [289]. Developing M184V/I carries with it a fitness cost resulting from a lower RT processivity. Furthermore, M184I is less fit than M184V; therefore, M184V outcompetes M184I and M184V is more prevalent [290]. Although M184V/I confer strong resistance to 3TC, these mutations possess properties that are beneficial in combination therapy with other NRTI. M184V/I RT mutants have higher transcription fidelity, therefore slowing down the emergence of random mutations that could lead to additional drug resistance. The relationship between mutations can be neutral or additive or synergistic or antagonistic; and knowing when these functional interactions occur can be exploited for clinical benefit. TDF and 3TC are prescribed together because if M184V confers resistance against 3TC, it can also increase susceptibility to TDF [291]. This illustrates that drug strategies can be designed to improve treatment without the necessity of developing new drugs.

However, the large distribution of NRTI inhibitors also has consequence for the generation of resistant variants that can be transmitted and that are cross-resistant to several drugs of the same class. In addition, since NRTI are analogous to host dNTP, this drug class, especially the early forms, have higher toxicity because they can affect endogenous replication.

Like NRTI, first generation NNRTI have been an indispensable part of the standard of care of HIV treatment since the advent of HAART. However, the development of resistance and cross-resistance against these agents in the population of treated and naïve HIV-positive individuals has pushed researchers to develop better second-generation drugs. In particular, the K103N

resistance mutation against the NNRTI efavirenz (EFV) is commonly found in cohorts of HIV-positive individuals [292]. This is likely due to its low impact on viral fitness and transmission as well as the fact that EFV was a recommended treatment for many individuals over many years while being relatively toxic. Etravirine (ETV) was developed to have a higher genetic barrier to resistance in hopes of decreasing susceptibility to development of common NNRTI resistance mutations such as K103N and Y181C [293]. Based on favorable results from the DUET trials, ETV was approved by the FDA for use in treatment-experienced individuals [294]. Like ETV, Rilpivirine (RPV) is a diarylpyrimidine and a second-generation NNRTI that was also developed as part of a rational drug design [295]. The ECHO and THRIVE phase 3 clinical trials led to the approval of RPV and demonstrated the emergence of E138K or K101E resistance mutations in individuals experiencing virological failure.

Drug class		NRTI				NNRTI
Drug		3TC	FTC	ABC	TDF	RPV
Consensus	Position					
E	138					K
M	184	I	I	I	*	
M	184	V	V	V	*	

Table 1.2: Clinically significant RT resistance substitutions examined in this thesis. Consensus and positions are defined based on NL4.3/HXB2. 3TC, lamivudine; FTC, emtricitabine; ABC, abacavir; TDF, tenofovir; RPV, rilpivirine. * indicates increased susceptibility

Interestingly, the E138K and M184I/V mutations possess properties that would make them a dangerous combination that would confer both resistance and restored replicative fitness to HIV-1. More about this interaction is detailed in Chapter 3.

1.6.3. Integrase inhibitors

Although non-catalytic inhibitors are under development, only integrase strand-transfer inhibitors (INSTIs) are currently used for therapy. These inhibitors specifically inhibit the second step of integration (strand-transfer) and are largely innocuous in regard to 3'-processing. INSTIs bind within the integrase catalytic pocket and inhibit integration through three processes: first, interaction with amino-acid residues that are important for integrase catalytic activity; second, chelation of Mg2+/Mn2+ divalent ions; and third by pi-stacking interaction with the nucleotide located immediately at the 5'end of the 'CA' dinucleotide that signals 3'-processing.

Three INSTIs are currently in clinical use: raltegravir (RAL), elvitegravir (EVG) and dolutegravir (DTG). A fourth INSTI termed cabotegravir is expected to be approved in the coming year. INSTIs are safe and well tolerated compared to most RTIs and PIs, likely because in contrast to RT or protease, human cells do not express any proteins that resemble integrase. They are efficacious against various HIV subtypes and viruses that are resistant against other inhibitors.

Several randomized clinical trials have demonstrated the efficacy of INSTIs: STARTMRK and Protocol 004 for RAL, Studies 102 and 103 for EVG and SPRING, SINGLE and FLAMINGO for DTG (reviewed in [296]). There are several differences between these three drugs. The most notable is that because of unfavourable pharmacokinetic profiles, RAL has to be taken twice daily and EVG has to be administered with a booster called cobicistat that inhibits the elimination of EVG by the cytochrome P450 3A enzymes, whereas DTG is a once daily unboosted drug. In any case,

viral suppression rates for INSTIs in treatment-naïve individuals were high, between 85.6 and 90% at 48 weeks, and between 76% and 83% after 96 weeks weeks (analyses were conducted using an intention to treat methodology). Treatment-experienced individuals who have previously suffered from treatment failure with other antiretroviral drugs can also use INSTIs but the efficacy of these drugs is reduced in this vulnerable population.

Although RAL, EVG, and DTG are very similar in terms of efficacy at suppressing viral loads in the plasma of treated individuals, there are differences in regard to HIV drug resistance. Whereas any individuals who use RAL or EVG can experience treatment failure with emergent integrase and/or reverse transcriptase drug resistance mutations, drug resistance mutations that are exclusively in integrase have been reported so far for DTG only in treatment-experienced patients. There has been no report so far of a treatment-naïve individual who failed DTG-based therapy with resistance mutations in either RT or integrase. Furthermore, treatment-experienced individuals who have experienced treatment failure with DTG and who have developed resistance mutations have done so exclusively in integrase and no resistance substitution in RT has been reported. The unique resistance profile of DTG has raised interest toward employment of this compound in curative strategies [16].

Amongst 6 treatment-experienced individuals who experienced treatment failure with DTG, 4 possessed the R263K substitution in integrase. This same substitution was first identified in pre-clinical tissue culture selection studies and its activity on integrase catalytic activity and viral fitness was characterized. R263K decreases integration in cell-free assays and in tissue

culture as well as viral fitness and the capacity of the virus to evolve rapidly [297-299]. In tissue culture, but not *in vivo*, H51Y was found to emerge following R263K but it did not compensate for integration and fitness defects [298].

In recent years, HIV/AIDS has been redefined as a persistent inflammatory disease in high resource countries. This change in paradigm came about when studying people living with HIV for decades. Although viral load may often be controlled, many of these persons are now developing non-AIDS related inflammatory-linked illnesses. Consequently, this has reinvigorated the interest in the innate immune system and its role in inflammation. Treatments to attenuate inflammation in HIV positive individuals are currently been investigated. It is therefore important to understand the role of the innate immune system in chronic inflammation and also in individuals who have been under HAART for some time and who could harbor drug resistant mutations.

1.8 Objective

In many parts of the world where effective antiretrovirals are readily accessible, people living with HIV-1 can expect to live as long as the general population. Some patients are now on cART for over 20 years and the consequences of chronic immune activation are evident through the premature development non-AIDS related illness. Additionally, with treatment experience, the threat of drug resistance still remains. The over-reaching theme of my doctoral research was to study how drug-resistance and chronic immune activation are related in the context of innate immunity. Drawing from my understanding of HRFs by studying PRMT6 and based on previous

work in the lab on RT and drug resistance, I hypothesized that particular drug resistant viruses would replicate better in immune cells that display analogous dNTP conditions as seen in the lab and *in vivo* these conditions would be regulated by a then newly discovered HRF, SAMHD1. Similar hypotheses were being investigated in other laboratories, but did not study the different drug resistant viruses in the spectrum of primary immune HIV-1 target cells [255, 300, 301]. We then extended our scope to IN resistant mutants based on selection studies we had performed in the lab.

Altogether the observations from my research encourage further investigation of drug resistant viruses interaction with host immune cells. The objectives specific to each project are described in the preface section of the chapters. The preface also provides more contexts and highlights the studies that informed individual hypothesis and/or study rational.

In seeking to better understand components of the innate immunity, the results of this doctoral research would have significance towards viral reservoirs, the emergence and perhaps the control of drug resistance, the causes of chronic immune activation and viral eradication strategies.

1.9.REFERENCES

1. Gottlieb, M.S., et al., *Pneumocystis carinii pneumonia and mucosal candidiasis in previously healthy homosexual men: evidence of a new acquired cellular immunodeficiency.* N Engl J Med, 1981. **305**(24): p. 1425-31.
2. Centers for Disease, C., *Kaposi's sarcoma and Pneumocystis pneumonia among homosexual men--New York City and California.* MMWR Morb Mortal Wkly Rep, 1981. **30**(25): p. 305-8.
3. Susan M. Schader and M.A. Wainberg., *Insights into HIV-1 pathogenesis through drug discovery: 30 years of basic research and concerns for the future.* HIV & AIDS Review, 2011. **10**(4): p. 91-98.
4. Barre-Sinoussi, F., *HIV: a discovery opening the road to novel scientific knowledge and global health improvement.* Virology, 2010. **397**(2): p. 255-9.
5. Barre-Sinoussi, F., et al., *Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS).* Science, 1983. **220**(4599): p. 868-71.
6. Levy, J.A., et al., *Isolation of lymphocytopathic retroviruses from San Francisco patients with AIDS.* Science, 1984. **225**(4664): p. 840-2.
7. Gallo, R.C., et al., *Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS.* Science, 1984. **224**(4648): p. 500-3.
8. UNAIDS, *UNAIDS Report: How AIDS changed everything.* 2014.
9. Organization, W.H., *UNAIDS Fact Sheet 2016.* 2016.
10. WHO, *Adults and children estimated to be living with HIV, 2013 By WHO region.* 2014.
11. in *Guideline on When to Start Antiretroviral Therapy and on Pre-Exposure Prophylaxis for HIV*. 2015: Geneva.
12. UNAIDS, *UNAIDS Fact sheet 2015.* 2015.
13. Hutter, G., et al., *Long-term control of HIV by CCR5 Delta32/Delta32 stem-cell transplantation.* N Engl J Med, 2009. **360**(7): p. 692-8.
14. Younas, M., et al., *Immune activation in the course of HIV-1 infection: Causes, phenotypes and persistence under therapy.* HIV Med, 2016. **17**(2): p. 89-105.
15. Archin, N.M., et al., *Eradicating HIV-1 infection: seeking to clear a persistent pathogen.* Nat Rev Microbiol, 2014. **12**(11): p. 750-64.
16. Mesplede, T. and M.A. Wainberg, *Resistance against Integrase Strand Transfer Inhibitors and Relevance to HIV Persistence.* Viruses, 2015. **7**(7): p. 3703-18.
17. Mansky, L.M. and H.M. Temin, *Lower in vivo mutation rate of human immunodeficiency virus type 1 than that predicted from the fidelity of purified reverse transcriptase.* J Virol, 1995. **69**(8): p. 5087-94.
18. Smyth, R.P., M.P. Davenport, and J. Mak, *The origin of genetic diversity in HIV-1.* Virus Res, 2012. **169**(2): p. 415-29.
19. Fields, B.N., et al., *Fields virology*. 6th ed. 2013, Philadelphia: Lippincott Williams & Wilkins.
20. Keele, B.F., et al., *Chimpanzee reservoirs of pandemic and nonpandemic HIV-1.* Science, 2006. **313**(5786): p. 523-6.
21. Plantier, J.C., et al., *A new human immunodeficiency virus derived from gorillas.* Nat Med, 2009. **15**(8): p. 871-2.
22. D'Arc, M., et al., *Origin of the HIV-1 group O epidemic in western lowland gorillas.* Proc Natl Acad Sci U S A, 2015. **112**(11): p. E1343-52.

23. Tebit, D.M. and E.J. Arts, *Tracking a century of global expansion and evolution of HIV to drive understanding and to combat disease.* Lancet Infect Dis, 2011. **11**(1): p. 45-56.
24. Santos, A.F. and M.A. Soares, *HIV Genetic Diversity and Drug Resistance.* Viruses, 2010. **2**(2): p. 503-31.
25. Peeters, M., M. Jung, and A. Ayouba, *The origin and molecular epidemiology of HIV.* Expert Rev Anti Infect Ther, 2013. **11**(9): p. 885-96.
26. Lim, E.S., et al., *The ability of primate lentiviruses to degrade the monocyte restriction factor SAMHD1 preceded the birth of the viral accessory protein Vpx.* Cell Host Microbe, 2012. **11**(2): p. 194-204.
27. Mourez, T., F. Simon, and J.C. Plantier, *Non-M variants of human immunodeficiency virus type 1.* Clin Microbiol Rev, 2013. **26**(3): p. 448-61.
28. Society, C.A., *HIV Transmission: Factors that Affect Biological Risk.* 2013.
29. Quinn, T.C., et al., *Viral load and heterosexual transmission of human immunodeficiency virus type 1. Rakai Project Study Group.* N Engl J Med, 2000. **342**(13): p. 921-9.
30. Gray, R.H., et al., *Probability of HIV-1 transmission per coital act in monogamous, heterosexual, HIV-1-discordant couples in Rakai, Uganda.* Lancet, 2001. **357**(9263): p. 1149-53.
31. Cohen, M.S., et al., *Prevention of HIV-1 infection with early antiretroviral therapy.* N Engl J Med, 2011. **365**(6): p. 493-505.
32. Masson, L., et al., *Genital inflammation and the risk of HIV acquisition in women.* Clin Infect Dis, 2015. **61**(2): p. 260-9.
33. Hladik, F. and M.J. McElrath, *Setting the stage: host invasion by HIV.* Nat Rev Immunol, 2008. **8**(6): p. 447-57.
34. Keele, B.F., et al., *Identification and characterization of transmitted and early founder virus envelopes in primary HIV-1 infection.* Proc Natl Acad Sci U S A, 2008. **105**(21): p. 7552-7.
35. Ping, L.H., et al., *Comparison of viral Env proteins from acute and chronic infections with subtype C human immunodeficiency virus type 1 identifies differences in glycosylation and CCR5 utilization and suggests a new strategy for immunogen design.* J Virol, 2013. **87**(13): p. 7218-33.
36. Pudney, J., A.J. Quayle, and D.J. Anderson, *Immunological microenvironments in the human vagina and cervix: mediators of cellular immunity are concentrated in the cervical transformation zone.* Biol Reprod, 2005. **73**(6): p. 1253-63.
37. Haase, A.T., *Targeting early infection to prevent HIV-1 mucosal transmission.* Nature, 2010. **464**(7286): p. 217-23.
38. Borrow, P., *Innate immunity in acute HIV-1 infection.* Curr Opin HIV AIDS, 2011. **6**(5): p. 353-63.
39. Zhang, Z., et al., *Sexual transmission and propagation of SIV and HIV in resting and activated CD4+ T cells.* Science, 1999. **286**(5443): p. 1353-7.
40. Miyake, A., et al., *Rapid dissemination of a pathogenic simian/human immunodeficiency virus to systemic organs and active replication in lymphoid tissues following intrarectal infection.* J Gen Virol, 2006. **87**(Pt 5): p. 1311-20.
41. Joseph, S.B., et al., *Bottlenecks in HIV-1 transmission: insights from the study of founder viruses.* Nat Rev Microbiol, 2015. **13**(7): p. 414-25.
42. Vendrame, D., et al., *Partial inhibition of human immunodeficiency virus replication by type I interferons: impact of cell-to-cell viral transfer.* J Virol, 2009. **83**(20): p. 10527-37.
43. Parrish, N.F., et al., *Phenotypic properties of transmitted founder HIV-1.* Proc Natl Acad Sci U S A, 2013. **110**(17): p. 6626-33.
44. Bangs, S.C., A.J. McMichael, and X.N. Xu, *Bystander T cell activation--implications for HIV infection and other diseases.* Trends Immunol, 2006. **27**(11): p. 518-24.
45. Bangs, S.C., et al., *Human CD4+ memory T cells are preferential targets for bystander activation and apoptosis.* J Immunol, 2009. **182**(4): p. 1962-71.

46. McMichael, A.J., et al., *The immune response during acute HIV-1 infection: clues for vaccine development.* Nat Rev Immunol, 2010. **10**(1): p. 11-23.
47. Brenchley, J.M., et al., *Microbial translocation is a cause of systemic immune activation in chronic HIV infection.* Nat Med, 2006. **12**(12): p. 1365-71.
48. Redd, A.D., et al., *Microbial translocation, the innate cytokine response, and HIV-1 disease progression in Africa.* Proc Natl Acad Sci U S A, 2009. **106**(16): p. 6718-23.
49. Cadogan, M. and A.G. Dalgleish, *HIV induced AIDS and related cancers: chronic immune activation and future therapeutic strategies.* Adv Cancer Res, 2008. **101**: p. 349-95.
50. Choudhary, S.K., et al., *Low immune activation despite high levels of pathogenic human immunodeficiency virus type 1 results in long-term asymptomatic disease.* J Virol, 2007. **81**(16): p. 8838-42.
51. Gougeon, M.L., et al., *Lack of chronic immune activation in HIV-infected chimpanzees correlates with the resistance of T cells to Fas/Apo-1 (CD95)-induced apoptosis and preservation of a T helper 1 phenotype.* J Immunol, 1997. **158**(6): p. 2964-76.
52. Machuca, A., et al., *HIV type 2 primary isolates induce a lower degree of apoptosis "in vitro" compared with HIV type 1 primary isolates.* AIDS Res Hum Retroviruses, 2004. **20**(5): p. 507-12.
53. Aberg, J.A., *Aging, inflammation, and HIV infection.* Top Antivir Med, 2012. **20**(3): p. 101-5.
54. Desai, S. and A. Landay, *Early immune senescence in HIV disease.* Curr HIV/AIDS Rep, 2010. **7**(1): p. 4-10.
55. Goonetilleke, N., et al., *The first T cell response to transmitted/founder virus contributes to the control of acute viremia in HIV-1 infection.* J Exp Med, 2009. **206**(6): p. 1253-72.
56. Wei, X., et al., *Antibody neutralization and escape by HIV-1.* Nature, 2003. **422**(6929): p. 307-12.
57. Migueles, S.A. and M. Connors, *Success and failure of the cellular immune response against HIV-1.* Nat Immunol, 2015. **16**(6): p. 563-70.
58. Ascher, M.S. and H.W. Sheppard, *AIDS as immune system activation: a model for pathogenesis.* Clin Exp Immunol, 1988. **73**(2): p. 165-7.
59. Grossman, Z., et al., *Pathogenesis of HIV infection: what the virus spares is as important as what it destroys.* Nat Med, 2006. **12**(3): p. 289-95.
60. Chevalier, M.F. and L. Weiss, *The split personality of regulatory T cells in HIV infection.* Blood, 2013. **121**(1): p. 29-37.
61. Soghoian, D.Z., et al., *HIV-specific cytolytic CD4 T cell responses during acute HIV infection predict disease outcome.* Sci Transl Med, 2012. **4**(123): p. 123ra25.
62. Rajasuriar, R., et al., *Persistent immune activation in chronic HIV infection: do any interventions work?* AIDS, 2013. **27**(8): p. 1199-208.
63. Engelman, A. and P. Cherepanov, *The structural biology of HIV-1: mechanistic and therapeutic insights.* Nat Rev Microbiol, 2012. **10**(4): p. 279-90.
64. Melikyan, G.B., *HIV entry: a game of hide-and-fuse?* Curr Opin Virol, 2014. **4**: p. 1-7.
65. Permanyer, M., E. Ballana, and J.A. Este, *Endocytosis of HIV: anything goes.* Trends Microbiol, 2010. **18**(12): p. 543-51.
66. Clotet-Codina, I., et al., *HIV endocytosis after dendritic cell to T cell viral transfer leads to productive virus infection.* Antiviral Res, 2009. **83**(1): p. 94-8.
67. Wyatt, R., et al., *The antigenic structure of the HIV gp120 envelope glycoprotein.* Nature, 1998. **393**(6686): p. 705-11.
68. Kwong, P.D., et al., *Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody.* Nature, 1998. **393**(6686): p. 648-59.
69. Wu, L., et al., *CD4-induced interaction of primary HIV-1 gp120 glycoproteins with the chemokine receptor CCR-5.* Nature, 1996. **384**(6605): p. 179-83.

70. Rottman, J.B., et al., *Cellular localization of the chemokine receptor CCR5. Correlation to cellular targets of HIV-1 infection.* Am J Pathol, 1997. **151**(5): p. 1341-51.
71. Duncan, C.J. and Q.J. Sattentau, *Viral determinants of HIV-1 macrophage tropism.* Viruses, 2011. **3**(11): p. 2255-79.
72. Weinberger, A.D. and A.S. Perelson, *Persistence and emergence of X4 virus in HIV infection.* Math Biosci Eng, 2011. **8**(2): p. 605-26.
73. Verani, A., et al., *CXCR4 is a functional coreceptor for infection of human macrophages by CXCR4-dependent primary HIV-1 isolates.* J Immunol, 1998. **161**(5): p. 2084-8.
74. Pollakis, G. and W.A. Paxton, *Use of (alternative) coreceptors for HIV entry.* Curr Opin HIV AIDS, 2012. **7**(5): p. 440-9.
75. Speck, R.F., et al., *Selective employment of chemokine receptors as human immunodeficiency virus type 1 coreceptors determined by individual amino acids within the envelope V3 loop.* J Virol, 1997. **71**(9): p. 7136-9.
76. Arhel, N., *Revisiting HIV-1 uncoating.* Retrovirology, 2010. **7**: p. 96.
77. Pornillos, O., B.K. Ganser-Pornillos, and M. Yeager, *Atomic-level modelling of the HIV capsid.* Nature, 2011. **469**(7330): p. 424-7.
78. Ambrose, Z. and C. Aiken, *HIV-1 uncoating: connection to nuclear entry and regulation by host proteins.* Virology, 2014. **454-455**: p. 371-9.
79. Forshey, B.M., et al., *Formation of a human immunodeficiency virus type 1 core of optimal stability is crucial for viral replication.* J Virol, 2002. **76**(11): p. 5667-77.
80. Fassati, A., *Multiple roles of the capsid protein in the early steps of HIV-1 infection.* Virus Res, 2012. **170**(1-2): p. 15-24.
81. Yamashita, M. and M. Emerman, *Capsid is a dominant determinant of retrovirus infectivity in nondividing cells.* J Virol, 2004. **78**(11): p. 5670-8.
82. Vozzolo, L., et al., *Gyrase B inhibitor impairs HIV-1 replication by targeting Hsp90 and the capsid protein.* J Biol Chem, 2010. **285**(50): p. 39314-28.
83. Le Grice, S.F., *Human immunodeficiency virus reverse transcriptase: 25 years of research, drug discovery, and promise.* J Biol Chem, 2012. **287**(49): p. 40850-7.
84. Sarafianos, S.G., et al., *Crystal structure of HIV-1 reverse transcriptase in complex with a polypurine tract RNA:DNA.* EMBO J, 2001. **20**(6): p. 1449-61.
85. Gotte, M., X. Li, and M.A. Wainberg, *HIV-1 reverse transcription: a brief overview focused on structure-function relationships among molecules involved in initiation of the reaction.* Arch Biochem Biophys, 1999. **365**(2): p. 199-210.
86. De Rocquigny, H., et al., *Viral RNA annealing activities of human immunodeficiency virus type 1 nucleocapsid protein require only peptide domains outside the zinc fingers.* Proc Natl Acad Sci U S A, 1992. **89**(14): p. 6472-6.
87. Invernizzi, C.F., et al., *PRMT6 diminishes HIV-1 Rev binding to and export of viral RNA.* Retrovirology, 2006. **3**: p. 93.
88. Freed, E.O., *HIV-1 replication.* Somat Cell Mol Genet, 2001. **26**(1-6): p. 13-33.
89. Smith, C.M., J.S. Smith, and M.J. Roth, *RNase H requirements for the second strand transfer reaction of human immunodeficiency virus type 1 reverse transcription.* J Virol, 1999. **73**(8): p. 6573-81.
90. Matreyek, K.A. and A. Engelman, *Viral and cellular requirements for the nuclear entry of retroviral preintegration nucleoprotein complexes.* Viruses, 2013. **5**(10): p. 2483-511.
91. McDonald, D., et al., *Visualization of the intracellular behavior of HIV in living cells.* J Cell Biol, 2002. **159**(3): p. 441-52.

92. Esposito, F., A. Corona, and E. Tramontano, *HIV-1 Reverse Transcriptase Still Remains a New Drug Target: Structure, Function, Classical Inhibitors, and New Inhibitors with Innovative Mechanisms of Actions.* Mol Biol Int, 2012. **2012**: p. 586401.
93. Zhang, H., et al., *The cytidine deaminase CEM15 induces hypermutation in newly synthesized HIV-1 DNA.* Nature, 2003. **424**(6944): p. 94-8.
94. Neogi, U., et al., *Human APOBEC3G-mediated hypermutation is associated with antiretroviral therapy failure in HIV-1 subtype C-infected individuals.* J Int AIDS Soc, 2013. **16**: p. 18472.
95. Fourati, S., et al., *E138K and M184I mutations in HIV-1 reverse transcriptase coemerge as a result of APOBEC3 editing in the absence of drug exposure.* AIDS, 2012. **26**(13): p. 1619-24.
96. Sheehy, A.M., N.C. Gaddis, and M.H. Malim, *The antiretroviral enzyme APOBEC3G is degraded by the proteasome in response to HIV-1 Vif.* Nat Med, 2003. **9**(11): p. 1404-7.
97. Goldstone, D.C., et al., *HIV-1 restriction factor SAMHD1 is a deoxynucleoside triphosphate triphosphohydrolase.* Nature, 2011. **480**(7377): p. 379-82.
98. Ruffin, N., et al., *Low SAMHD1 expression following T-cell activation and proliferation renders CD4+ T cells susceptible to HIV-1.* AIDS, 2015. **29**(5): p. 519-30.
99. Baldauf, H.M., et al., *SAMHD1 restricts HIV-1 infection in resting CD4(+) T cells.* Nat Med, 2012. **18**(11): p. 1682-7.
100. Leng, J., et al., *A cell-intrinsic inhibitor of HIV-1 reverse transcription in CD4(+) T cells from elite controllers.* Cell Host Microbe, 2014. **15**(6): p. 717-28.
101. Landau, N.R., *The innate immune response to HIV-1: to sense or not to sense.* DNA Cell Biol, 2014. **33**(5): p. 271-4.
102. Quashie, P.K., et al., *Structural Studies of the HIV-1 Integrase Protein: Compound Screening and Characterization of a DNA-Binding Inhibitor.* PLoS One, 2015. **10**(6): p. e0128310.
103. Zheng, R., T.M. Jenkins, and R. Craigie, *Zinc folds the N-terminal domain of HIV-1 integrase, promotes multimerization, and enhances catalytic activity.* Proc Natl Acad Sci U S A, 1996. **93**(24): p. 13659-64.
104. Craigie, R., *HIV integrase, a brief overview from chemistry to therapeutics.* J Biol Chem, 2001. **276**(26): p. 23213-6.
105. Dyda, F., et al., *Crystal structure of the catalytic domain of HIV-1 integrase: similarity to other polynucleotidyl transferases.* Science, 1994. **266**(5193): p. 1981-6.
106. Turlure, F., et al., *Human cell proteins and human immunodeficiency virus DNA integration.* Front Biosci, 2004. **9**: p. 3187-208.
107. Engelman, A. and P. Cherepanov, *The lentiviral integrase binding protein LEDGF/p75 and HIV-1 replication.* PLoS Pathog, 2008. **4**(3): p. e1000046.
108. Shun, M.C., et al., *LEDGF/p75 functions downstream from preintegration complex formation to effect gene-specific HIV-1 integration.* Genes Dev, 2007. **21**(14): p. 1767-78.
109. Sloan, R.D. and M.A. Wainberg, *The role of unintegrated DNA in HIV infection.* Retrovirology, 2011. **8**: p. 52.
110. Li, L., et al., *Role of the non-homologous DNA end joining pathway in the early steps of retroviral infection.* EMBO J, 2001. **20**(12): p. 3272-81.
111. Sharkey, M., et al., *Episomal viral cDNAs identify a reservoir that fuels viral rebound after treatment interruption and that contributes to treatment failure.* PLoS Pathog, 2011. **7**(2): p. e1001303.
112. Mbisa, J.L., W. Bu, and V.K. Pathak, *APOBEC3F and APOBEC3G inhibit HIV-1 DNA integration by different mechanisms.* J Virol, 2010. **84**(10): p. 5250-9.
113. Mbisa, J.L., et al., *Human immunodeficiency virus type 1 cDNAs produced in the presence of APOBEC3G exhibit defects in plus-strand DNA transfer and integration.* J Virol, 2007. **81**(13): p. 7099-110.

114. Van Lint, C., S. Bouchat, and A. Marcello, *HIV-1 transcription and latency: an update.* Retrovirology, 2013. **10**: p. 67.
115. Verdin, E., P. Paras, Jr., and C. Van Lint, *Chromatin disruption in the promoter of human immunodeficiency virus type 1 during transcriptional activation.* EMBO J, 1993. **12**(8): p. 3249-59.
116. Coiras, M., et al., *Understanding HIV-1 latency provides clues for the eradication of long-term reservoirs.* Nat Rev Microbiol, 2009. **7**(11): p. 798-812.
117. Sodroski, J., et al., *Trans-acting transcriptional regulation of human T-cell leukemia virus type III long terminal repeat.* Science, 1985. **227**(4683): p. 171-3.
118. Gatignol, A., *Transcription of HIV: Tat and cellular chromatin.* Adv Pharmacol, 2007. **55**: p. 137-59.
119. Hottiger, M.O. and G.J. Nabel, *Interaction of human immunodeficiency virus type 1 Tat with the transcriptional coactivators p300 and CREB binding protein.* J Virol, 1998. **72**(10): p. 8252-6.
120. Karn, J. and C.M. Stoltzfus, *Transcriptional and posttranscriptional regulation of HIV-1 gene expression.* Cold Spring Harb Perspect Med, 2012. **2**(2): p. a006916.
121. Fujinaga, K., et al., *Dynamics of human immunodeficiency virus transcription: P-TEFb phosphorylates RD and dissociates negative effectors from the transactivation response element.* Mol Cell Biol, 2004. **24**(2): p. 787-95.
122. Isel, C. and J. Karn, *Direct evidence that HIV-1 Tat stimulates RNA polymerase II carboxyl-terminal domain hyperphosphorylation during transcriptional elongation.* J Mol Biol, 1999. **290**(5): p. 929-41.
123. Dassonneville, L., et al., *Binding of Hoechst 33258 to the TAR RNA of HIV-1. Recognition of a pyrimidine bulge-dependent structure.* Nucleic Acids Res, 1997. **25**(22): p. 4487-92.
124. Kiernan, R.E., et al., *HIV-1 tat transcriptional activity is regulated by acetylation.* EMBO J, 1999. **18**(21): p. 6106-18.
125. Boulanger, M.C., et al., *Methylation of Tat by PRMT6 regulates human immunodeficiency virus type 1 gene expression.* J Virol, 2005. **79**(1): p. 124-31.
126. Xie, B., et al., *Arginine methylation of the human immunodeficiency virus type 1 Tat protein by PRMT6 negatively affects Tat Interactions with both cyclin T1 and the Tat transactivation region.* J Virol, 2007. **81**(8): p. 4226-34.
127. Strahl, B.D. and C.D. Allis, *The language of covalent histone modifications.* Nature, 2000. **403**(6765): p. 41-5.
128. Pagans, S., et al., *The Cellular lysine methyltransferase Set7/9-KMT7 binds HIV-1 TAR RNA, monomethylates the viral transactivator Tat, and enhances HIV transcription.* Cell Host Microbe, 2010. **7**(3): p. 234-44.
129. Morrow, C.D., J. Park, and J.K. Wakefield, *Viral gene products and replication of the human immunodeficiency type 1 virus.* Am J Physiol, 1994. **266**(5 Pt 1): p. C1135-56.
130. Kalland, K.H., et al., *Subcellular distribution of human immunodeficiency virus type 1 Rev and colocalization of Rev with RNA splicing factors in a speckled pattern in the nucleoplasm.* J Virol, 1994. **68**(3): p. 1475-85.
131. Pollard, V.W. and M.H. Malim, *The HIV-1 Rev protein.* Annu Rev Microbiol, 1998. **52**: p. 491-532.
132. Suhasini, M. and T.R. Reddy, *Cellular proteins and HIV-1 Rev function.* Curr HIV Res, 2009. **7**(1): p. 91-100.
133. Ono, A., et al., *Phosphatidylinositol (4,5) bisphosphate regulates HIV-1 Gag targeting to the plasma membrane.* Proc Natl Acad Sci U S A, 2004. **101**(41): p. 14889-94.
134. Freed, E.O., *HIV-1 assembly, release and maturation.* Nat Rev Microbiol, 2015.
135. Brass, A.L., et al., *Identification of host proteins required for HIV infection through a functional genomic screen.* Science, 2008. **319**(5865): p. 921-6.

136. Tristem, M., et al., *Evolution of the primate lentiviruses: evidence from vpx and vpr.* EMBO J, 1992. **11**(9): p. 3405-12.
137. Schuler, W., et al., *NMR structure of the (52-96) C-terminal domain of the HIV-1 regulatory protein Vpr: molecular insights into its biological functions.* J Mol Biol, 1999. **285**(5): p. 2105-17.
138. Morellet, N., et al., *NMR structure of the HIV-1 regulatory protein VPR.* J Mol Biol, 2003. **327**(1): p. 215-27.
139. Caly, L., et al., *Impaired nuclear import and viral incorporation of Vpr derived from a HIV long-term non-progressor.* Retrovirology, 2008. **5**: p. 67.
140. Somasundaran, M., et al., *Evidence for a cytopathogenicity determinant in HIV-1 Vpr.* Proc Natl Acad Sci U S A, 2002. **99**(14): p. 9503-8.
141. Zhao, Y., et al., *Functional conservation of HIV-1 Vpr and variability in a mother-child pair of long-term non-progressors.* Virus Res, 2002. **89**(1): p. 103-21.
142. Stark, L.A. and R.T. Hay, *Human immunodeficiency virus type 1 (HIV-1) viral protein R (Vpr) interacts with Lys-tRNA synthetase: implications for priming of HIV-1 reverse transcription.* J Virol, 1998. **72**(4): p. 3037-44.
143. de Noronha, C.M., et al., *Dynamic disruptions in nuclear envelope architecture and integrity induced by HIV-1 Vpr.* Science, 2001. **294**(5544): p. 1105-8.
144. Belzile, J.P., et al., *HIV-1 Vpr-mediated G2 arrest involves the DDB1-CUL4AVPRBP E3 ubiquitin ligase.* PLoS Pathog, 2007. **3**(7): p. e85.
145. DeHart, J.L., et al., *HIV-1 Vpr activates the G2 checkpoint through manipulation of the ubiquitin proteasome system.* Virol J, 2007. **4**: p. 57.
146. Wen, X., et al., *The HIV1 protein Vpr acts to promote G2 cell cycle arrest by engaging a DDB1 and Cullin4A-containing ubiquitin ligase complex using VprBP/DCAF1 as an adaptor.* J Biol Chem, 2007. **282**(37): p. 27046-57.
147. Hrecka, K., et al., *Lentiviral Vpr usurps Cul4-DDB1[VprBP] E3 ubiquitin ligase to modulate cell cycle.* Proc Natl Acad Sci U S A, 2007. **104**(28): p. 11778-83.
148. Romani, B. and E.A. Cohen, *Lentivirus Vpr and Vpx accessory proteins usurp the cullin4-DDB1 (DCAF1) E3 ubiquitin ligase.* Curr Opin Virol, 2012. **2**(6): p. 755-63.
149. Planelles, V. and S. Benichou, *Vpr and its interactions with cellular proteins.* Curr Top Microbiol Immunol, 2009. **339**: p. 177-200.
150. Zahoor, M.A., et al., *HIV-1 Vpr induces interferon-stimulated genes in human monocyte-derived macrophages.* PLoS One, 2014. **9**(8): p. e106418.
151. Guo, Y., et al., *Structural basis for hijacking CBF-beta and CUL5 E3 ligase complex by HIV-1 Vif.* Nature, 2014. **505**(7482): p. 229-33.
152. Salter, J.D., G.A. Morales, and H.C. Smith, *Structural insights for HIV-1 therapeutic strategies targeting Vif.* Trends Biochem Sci, 2014. **39**(9): p. 373-80.
153. Sugden, S.M., et al., *Remodeling of the Host Cell Plasma Membrane by HIV-1 Nef and Vpu: A Strategy to Ensure Viral Fitness and Persistence.* Viruses, 2016. **8**(3).
154. Neil, S.J., T. Zang, and P.D. Bieniasz, *Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu.* Nature, 2008. **451**(7177): p. 425-30.
155. Lewinski, M.K., et al., *Membrane Anchoring by a C-terminal Tryptophan Enables HIV-1 Vpu to Displace Bone Marrow Stromal Antigen 2 (BST2) from Sites of Viral Assembly.* J Biol Chem, 2015. **290**(17): p. 10919-33.
156. Rollason, R., et al., *Expression of HIV-1 Vpu leads to loss of the viral restriction factor CD317/Tetherin from lipid rafts and its enhanced lysosomal degradation.* PLoS One, 2013. **8**(9): p. e75680.
157. Gonzalez, M.E., *Vpu Protein: The Viroporin Encoded by HIV-1.* Viruses, 2015. **7**(8): p. 4352-68.

158. Kirchhoff, F., et al., *Brief report: absence of intact nef sequences in a long-term survivor with nonprogressive HIV-1 infection.* N Engl J Med, 1995. **332**(4): p. 228-32.
159. Garcia, J.V. and A.D. Miller, *Serine phosphorylation-independent downregulation of cell-surface CD4 by nef.* Nature, 1991. **350**(6318): p. 508-11.
160. Royce, R.A., et al., *Sexual transmission of HIV.* N Engl J Med, 1997. **336**(15): p. 1072-8.
161. Murray, P.J. and T.A. Wynn, *Protective and pathogenic functions of macrophage subsets.* Nat Rev Immunol, 2011. **11**(11): p. 723-37.
162. Rich, E.A., et al., *Increased susceptibility of differentiated mononuclear phagocytes to productive infection with human immunodeficiency virus-1 (HIV-1).* J Clin Invest, 1992. **89**(1): p. 176-83.
163. Alexaki, A., Y. Liu, and B. Wigdahl, *Cellular reservoirs of HIV-1 and their role in viral persistence.* Curr HIV Res, 2008. **6**(5): p. 388-400.
164. Cobos-Jimenez, V., et al., *Macrophages and HIV-1.* Curr Opin HIV AIDS, 2011. **6**(5): p. 385-90.
165. Collman, R., et al., *Infection of monocyte-derived macrophages with human immunodeficiency virus type 1 (HIV-1). Monocyte-tropic and lymphocyte-tropic strains of HIV-1 show distinctive patterns of replication in a panel of cell types.* J Exp Med, 1989. **170**(4): p. 1149-63.
166. Lassmann, H., et al., *Bone marrow derived elements and resident microglia in brain inflammation.* Glia, 1993. **7**(1): p. 19-24.
167. Shen, L. and R.F. Siliciano, *Viral reservoirs, residual viremia, and the potential of highly active antiretroviral therapy to eradicate HIV infection.* J Allergy Clin Immunol, 2008. **122**(1): p. 22-8.
168. Alexaki, A. and B. Wigdahl, *HIV-1 infection of bone marrow hematopoietic progenitor cells and their role in trafficking and viral dissemination.* PLoS Pathog, 2008. **4**(12): p. e1000215.
169. Kijewski, S.D. and S. Gummuluru, *A mechanistic overview of dendritic cell-mediated HIV-1 infection: the story so far.* Future Virol, 2015. **10**(3): p. 257-269.
170. Turville, S.G., et al., *Immunodeficiency virus uptake, turnover, and 2-phase transfer in human dendritic cells.* Blood, 2004. **103**(6): p. 2170-9.
171. Ahmed, Z., et al., *The role of human dendritic cells in HIV-1 infection.* J Invest Dermatol, 2015. **135**(5): p. 1225-33.
172. Garcia, E., et al., *HIV-1 trafficking to the dendritic cell-T-cell infectious synapse uses a pathway of tetraspanin sorting to the immunological synapse.* Traffic, 2005. **6**(6): p. 488-501.
173. Yu, H.J., M.A. Reuter, and D. McDonald, *HIV traffics through a specialized, surface-accessible intracellular compartment during trans-infection of T cells by mature dendritic cells.* PLoS Pathog, 2008. **4**(8): p. e1000134.
174. Izquierdo-Useros, N., et al., *HIV-1 capture and transmission by dendritic cells: the role of viral glycolipids and the cellular receptor Siglec-1.* PLoS Pathog, 2014. **10**(7): p. e1004146.
175. Izquierdo-Useros, N., et al., *Capture and transfer of HIV-1 particles by mature dendritic cells converges with the exosome-dissemination pathway.* Blood, 2009. **113**(12): p. 2732-41.
176. Guilliams, M. and L. van de Laar, *A Hitchhiker's Guide to Myeloid Cell Subsets: Practical Implementation of a Novel Mononuclear Phagocyte Classification System.* Front Immunol, 2015. **6**: p. 406.
177. Zuniga, E.I., et al., *Bone marrow plasmacytoid dendritic cells can differentiate into myeloid dendritic cells upon virus infection.* Nat Immunol, 2004. **5**(12): p. 1227-34.
178. Beignon, A.S., et al., *Endocytosis of HIV-1 activates plasmacytoid dendritic cells via Toll-like receptor-viral RNA interactions.* J Clin Invest, 2005. **115**(11): p. 3265-75.
179. Howell, D.M., et al., *Decreased frequency of functional natural interferon-producing cells in peripheral blood of patients with the acquired immune deficiency syndrome.* Clin Immunol Immunopathol, 1994. **71**(2): p. 223-30.
180. Siegal, F.P., et al., *The nature of the principal type 1 interferon-producing cells in human blood.* Science, 1999. **284**(5421): p. 1835-7.

181. Kedzierska, K. and S.M. Crowe, *Cytokines and HIV-1: interactions and clinical implications.* Antivir Chem Chemother, 2001. **12**(3): p. 133-50.
182. Mehraj, V., et al., *Immune Suppression by Myeloid Cells in HIV Infection: New Targets for Immunotherapy.* Open AIDS J, 2014. **8**: p. 66-78.
183. Hughes, R., G. Towers, and M. Noursadeghi, *Innate immune interferon responses to human immunodeficiency virus-1 infection.* Rev Med Virol, 2012. **22**(4): p. 257-66.
184. Pisetsky, D.S., *The role of innate immunity in the induction of autoimmunity.* Autoimmun Rev, 2008. **8**(1): p. 69-72.
185. Nasi, M., et al., *Persistent inflammation in HIV infection: established concepts, new perspectives.* Immunol Lett, 2014. **161**(2): p. 184-8.
186. Chahroudi, A., et al., *Natural SIV hosts: showing AIDS the door.* Science, 2012. **335**(6073): p. 1188-93.
187. Descours, B., et al., *SAMHD1 restricts HIV-1 reverse transcription in quiescent CD4(+) T-cells.* Retrovirology, 2012. **9**: p. 87.
188. Saez-Cirion, A. and G. Pancino, *HIV controllers: a genetically determined or inducible phenotype?* Immunol Rev, 2013. **254**(1): p. 281-94.
189. Pancino, G., et al., *Natural resistance to HIV infection: lessons learned from HIV-exposed uninfected individuals.* J Infect Dis, 2010. **202 Suppl 3**: p. S345-50.
190. Benecke, A., M. Gale, Jr., and M.G. Katze, *Dynamics of innate immunity are key to chronic immune activation in AIDS.* Curr Opin HIV AIDS, 2012. **7**(1): p. 79-85.
191. Doyle, T., C. Goujon, and M.H. Malim, *HIV-1 and interferons: who's interfering with whom?* Nat Rev Microbiol, 2015. **13**(7): p. 403-13.
192. Blasius, A.L. and B. Beutler, *Intracellular toll-like receptors.* Immunity, 2010. **32**(3): p. 305-15.
193. O'Brien, M., et al., *Spatiotemporal trafficking of HIV in human plasmacytoid dendritic cells defines a persistently IFN-alpha-producing and partially matured phenotype.* J Clin Invest, 2011. **121**(3): p. 1088-101.
194. Meier, A., et al., *MyD88-dependent immune activation mediated by human immunodeficiency virus type 1-encoded Toll-like receptor ligands.* J Virol, 2007. **81**(15): p. 8180-91.
195. Gringhuis, S.I., et al., *HIV-1 exploits innate signaling by TLR8 and DC-SIGN for productive infection of dendritic cells.* Nat Immunol, 2010. **11**(5): p. 419-26.
196. Diner, B.A., K.K. Lum, and I.M. Cristea, *The emerging role of nuclear viral DNA sensors.* J Biol Chem, 2015. **290**(44): p. 26412-21.
197. Cai, X., Y.H. Chiu, and Z.J. Chen, *The cGAS-cGAMP-STING pathway of cytosolic DNA sensing and signaling.* Mol Cell, 2014. **54**(2): p. 289-96.
198. Hasan, M. and N. Yan, *Safeguard against DNA sensing: the role of TREX1 in HIV-1 infection and autoimmune diseases.* Front Microbiol, 2014. **5**: p. 193.
199. Stratmann, S., et al., *The innate immune sensor IFI16 recognizes foreign DNA in the nucleus by scanning along the duplex.* Elife, 2015. **4**.
200. Doitsh, G., et al., *Cell death by pyroptosis drives CD4 T-cell depletion in HIV-1 infection.* Nature, 2014. **505**(7484): p. 509-14.
201. Jakobsen, M.R., et al., *IFI16 senses DNA forms of the lentiviral replication cycle and controls HIV-1 replication.* Proc Natl Acad Sci U S A, 2013. **110**(48): p. E4571-80.
202. Monroe, K.M., et al., *IFI16 DNA sensor is required for death of lymphoid CD4 T cells abortively infected with HIV.* Science, 2014. **343**(6169): p. 428-32.
203. Nissen, S.K., et al., *Innate DNA sensing is impaired in HIV patients and IFI16 expression correlates with chronic immune activation.* Clin Exp Immunol, 2014. **177**(1): p. 295-309.
204. Abdel-Mohsen, M., et al., *Expression profile of host restriction factors in HIV-1 elite controllers.* Retrovirology, 2013. **10**: p. 106.

205. Liu, L., et al., *A whole genome screen for HIV restriction factors.* Retrovirology, 2011. **8**: p. 94.
206. Neil, S. and P. Bieniasz, *Human immunodeficiency virus, restriction factors, and interferon.* J Interferon Cytokine Res, 2009. **29**(9): p. 569-80.
207. Usami, Y., Y. Wu, and H.G. Gottlinger, *SERINC3 and SERINC5 restrict HIV-1 infectivity and are counteracted by Nef.* Nature, 2015. **526**(7572): p. 218-23.
208. Rosa, A., et al., *HIV-1 Nef promotes infection by excluding SERINC5 from virion incorporation.* Nature, 2015. **526**(7572): p. 212-7.
209. Invernizzi, C.F., et al., *Arginine methylation of the HIV-1 nucleocapsid protein results in its diminished function.* AIDS, 2007. **21**(7): p. 795-805.
210. Simon, V., N. Bloch, and N.R. Landau, *Intrinsic host restrictions to HIV-1 and mechanisms of viral escape.* Nat Immunol, 2015. **16**(6): p. 546-53.
211. Sheehy, A.M., et al., *Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein.* Nature, 2002. **418**(6898): p. 646-50.
212. Chaipan, C., et al., *APOBEC3G restricts HIV-1 to a greater extent than APOBEC3F and APOBEC3DE in human primary CD4+ T cells and macrophages.* J Virol, 2013. **87**(1): p. 444-53.
213. Koning, F.A., et al., *Defining APOBEC3 expression patterns in human tissues and hematopoietic cell subsets.* J Virol, 2009. **83**(18): p. 9474-85.
214. Stremlau, M., et al., *The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys.* Nature, 2004. **427**(6977): p. 848-53.
215. Sebastian, S. and J. Luban, *TRIM5alpha selectively binds a restriction-sensitive retroviral capsid.* Retrovirology, 2005. **2**: p. 40.
216. Stremlau, M., et al., *Specific recognition and accelerated uncoating of retroviral capsids by the TRIM5alpha restriction factor.* Proc Natl Acad Sci U S A, 2006. **103**(14): p. 5514-9.
217. Pertel, T., et al., *TRIM5 is an innate immune sensor for the retrovirus capsid lattice.* Nature, 2011. **472**(7343): p. 361-5.
218. Mascarenhas, A.P. and K. Musier-Forsyth, *The capsid protein of human immunodeficiency virus: interactions of HIV-1 capsid with host protein factors.* FEBS J, 2009. **276**(21): p. 6118-27.
219. Franke, E.K., H.E. Yuan, and J. Luban, *Specific incorporation of cyclophilin A into HIV-1 virions.* Nature, 1994. **372**(6504): p. 359-62.
220. Zhou, D., et al., *Cyclophilin A and viral infections.* Biochem Biophys Res Commun, 2012. **424**(4): p. 647-50.
221. Towers, G.J., *The control of viral infection by tripartite motif proteins and cyclophilin A.* Retrovirology, 2007. **4**: p. 40.
222. Hatziioannou, T., et al., *Cyclophilin interactions with incoming human immunodeficiency virus type 1 capsids with opposing effects on infectivity in human cells.* J Virol, 2005. **79**(1): p. 176-83.
223. Manel, N., et al., *A cryptic sensor for HIV-1 activates antiviral innate immunity in dendritic cells.* Nature, 2010. **467**(7312): p. 214-7.
224. Lahaye, X., et al., *The capsids of HIV-1 and HIV-2 determine immune detection of the viral cDNA by the innate sensor cGAS in dendritic cells.* Immunity, 2013. **39**(6): p. 1132-42.
225. Mangeat, B., et al., *HIV-1 Vpu neutralizes the antiviral factor Tetherin/BST-2 by binding it and directing its beta-TrCP2-dependent degradation.* PLoS Pathog, 2009. **5**(9): p. e1000574.
226. Neil, S.J., et al., *HIV-1 Vpu promotes release and prevents endocytosis of nascent retrovirus particles from the plasma membrane.* PLoS Pathog, 2006. **2**(5): p. e39.
227. Kuhl, B.D., et al., *Tetherin restricts direct cell-to-cell infection of HIV-1.* Retrovirology, 2010. **7**: p. 115.
228. Krause, C.D., et al., *Protein arginine methyltransferases: evolution and assessment of their pharmacological and therapeutic potential.* Pharmacol Ther, 2007. **113**(1): p. 50-87.

229. McBride, A.E. and P.A. Silver, *State of the arg: protein methylation at arginine comes of age.* Cell, 2001. **106**(1): p. 5-8.
230. Wang, C., et al., *Crystal structure of arginine methyltransferase 6 from Trypanosoma brucei.* PLoS One, 2014. **9**(2): p. e87267.
231. Frankel, A., et al., *The novel human protein arginine N-methyltransferase PRMT6 is a nuclear enzyme displaying unique substrate specificity.* J Biol Chem, 2002. **277**(5): p. 3537-43.
232. Guccione, E., et al., *Methylation of histone H3R2 by PRMT6 and H3K4 by an MLL complex are mutually exclusive.* Nature, 2007. **449**(7164): p. 933-7.
233. Neault, M., et al., *Ablation of PRMT6 reveals a role as a negative transcriptional regulator of the p53 tumor suppressor.* Nucleic Acids Res, 2012. **40**(19): p. 9513-21.
234. Sgarra, R., et al., *The AT-hook of the chromatin architectural transcription factor high mobility group A1a is arginine-methylated by protein arginine methyltransferase 6.* J Biol Chem, 2006. **281**(7): p. 3764-72.
235. El-Andaloussi, N., et al., *Arginine methylation regulates DNA polymerase beta.* Mol Cell, 2006. **22**(1): p. 51-62.
236. Kleinschmidt, M.A., et al., *Cell cycle regulation by the PRMT6 arginine methyltransferase through repression of cyclin-dependent kinase inhibitors.* PLoS One, 2012. **7**(8): p. e41446.
237. Yoshimatsu, M., et al., *Dysregulation of PRMT1 and PRMT6, Type I arginine methyltransferases, is involved in various types of human cancers.* Int J Cancer, 2011. **128**(3): p. 562-73.
238. Stavride, P., P. Arampatzi, and J. Papamatheakis, *Differential regulation of MHCII genes by PRMT6, via an AT-hook motif of RFX5.* Mol Immunol, 2013. **56**(4): p. 390-8.
239. Di Lorenzo, A., et al., *A gain-of-function mouse model identifies PRMT6 as a NF-kappaB coactivator.* Nucleic Acids Res, 2014. **42**(13): p. 8297-309.
240. Sivakumaran, H., et al., *Arginine methylation increases the stability of human immunodeficiency virus type 1 Tat.* J Virol, 2009. **83**(22): p. 11694-703.
241. Fulcher, A.J., et al., *The protein arginine methyltransferase PRMT6 inhibits HIV-1 Tat nucleolar retention.* Biochim Biophys Acta, 2016. **1863**(2): p. 254-62.
242. Le Clerc, S., et al., *Genomewide association study of a rapid progression cohort identifies new susceptibility alleles for AIDS (ANRS Genomewide Association Study 03).* J Infect Dis, 2009. **200**(8): p. 1194-201.
243. Lafuse, W.P., et al., *Cloning and characterization of a novel cDNA that is IFN-gamma-induced in mouse peritoneal macrophages and encodes a putative GTP-binding protein.* J Leukoc Biol, 1995. **57**(3): p. 477-83.
244. Rice, G.I., et al., *Mutations involved in Aicardi-Goutieres syndrome implicate SAMHD1 as regulator of the innate immune response.* Nat Genet, 2009. **41**(7): p. 829-32.
245. Crow, Y.J. and N. Manel, *Aicardi-Goutieres syndrome and the type I interferonopathies.* Nat Rev Immunol, 2015. **15**(7): p. 429-40.
246. Guyader, M., et al., *VPX mutants of HIV-2 are infectious in established cell lines but display a severe defect in peripheral blood lymphocytes.* EMBO J, 1989. **8**(4): p. 1169-75.
247. Yu, X.F., et al., *The vpx gene of simian immunodeficiency virus facilitates efficient viral replication in fresh lymphocytes and macrophage.* J Virol, 1991. **65**(9): p. 5088-91.
248. Sharova, N., et al., *Primate lentiviral Vpx commandeers DDB1 to counteract a macrophage restriction.* PLoS Pathog, 2008. **4**(5): p. e1000057.
249. Srivastava, S., et al., *Lentiviral Vpx accessory factor targets VprBP/DCAF1 substrate adaptor for cullin 4 E3 ubiquitin ligase to enable macrophage infection.* PLoS Pathog, 2008. **4**(5): p. e1000059.
250. Hrecka, K., et al., *Vpx relieves inhibition of HIV-1 infection of macrophages mediated by the SAMHD1 protein.* Nature, 2011. **474**(7353): p. 658-61.

251. Laguette, N., et al., *SAMHD1 is the dendritic- and myeloid-cell-specific HIV-1 restriction factor counteracted by Vpx.* Nature, 2011. **474**(7353): p. 654-7.
252. Laguette, N. and M. Benkirane, *How SAMHD1 changes our view of viral restriction.* Trends Immunol, 2012. **33**(1): p. 26-33.
253. Ballana, E. and J.A. Este, *SAMHD1: at the crossroads of cell proliferation, immune responses, and virus restriction.* Trends Microbiol, 2015. **23**(11): p. 680-92.
254. Diamond, T.L., et al., *Macrophage tropism of HIV-1 depends on efficient cellular dNTP utilization by reverse transcriptase.* J Biol Chem, 2004. **279**(49): p. 51545-53.
255. Lahouassa, H., et al., *SAMHD1 restricts the replication of human immunodeficiency virus type 1 by depleting the intracellular pool of deoxynucleoside triphosphates.* Nat Immunol, 2012. **13**(3): p. 223-8.
256. Berger, A., et al., *SAMHD1-deficient CD14+ cells from individuals with Aicardi-Goutieres syndrome are highly susceptible to HIV-1 infection.* PLoS Pathog, 2011. **7**(12): p. e1002425.
257. Kim, B., et al., *Tight interplay among SAMHD1 protein level, cellular dNTP levels, and HIV-1 proviral DNA synthesis kinetics in human primary monocyte-derived macrophages.* J Biol Chem, 2012. **287**(26): p. 21570-4.
258. Xu, H.T., et al., *Effects of the K65R and K65R/M184V reverse transcriptase mutations in subtype C HIV on enzyme function and drug resistance.* Retrovirology, 2009. **6**: p. 14.
259. Jamburuthugoda, V.K., P. Chugh, and B. Kim, *Modification of human immunodeficiency virus type 1 reverse transcriptase to target cells with elevated cellular dNTP concentrations.* J Biol Chem, 2006. **281**(19): p. 13388-95.
260. Powell, R.D., et al., *Aicardi-Goutieres syndrome gene and HIV-1 restriction factor SAMHD1 is a dGTP-regulated deoxynucleotide triphosphohydrolase.* J Biol Chem, 2011. **286**(51): p. 43596-600.
261. Hansen, E.C., et al., *GTP activator and dNTP substrates of HIV-1 restriction factor SAMHD1 generate a long-lived activated state.* Proc Natl Acad Sci U S A, 2014. **111**(18): p. E1843-51.
262. Zhu, C., et al., *Structural insight into dGTP-dependent activation of tetrameric SAMHD1 deoxynucleoside triphosphate triphosphohydrolase.* Nat Commun, 2013. **4**: p. 2722.
263. Ji, X., et al., *Mechanism of allosteric activation of SAMHD1 by dGTP.* Nat Struct Mol Biol, 2013. **20**(11): p. 1304-9.
264. Beloglazova, N., et al., *Nuclease activity of the human SAMHD1 protein implicated in the Aicardi-Goutieres syndrome and HIV-1 restriction.* J Biol Chem, 2013. **288**(12): p. 8101-10.
265. Choi, J., et al., *SAMHD1 specifically restricts retroviruses through its RNase activity.* Retrovirology, 2015. **12**: p. 46.
266. Seamon, K.J., et al., *SAMHD1 is a single-stranded nucleic acid binding protein with no active site-associated nuclease activity.* Nucleic Acids Res, 2015.
267. Schmidt, S., et al., *SAMHD1's protein expression profile in humans.* J Leukoc Biol, 2015. **98**(1): p. 5-14.
268. White, T.E., et al., *The retroviral restriction ability of SAMHD1, but not its deoxynucleotide triphosphohydrolase activity, is regulated by phosphorylation.* Cell Host Microbe, 2013. **13**(4): p. 441-51.
269. Cribier, A., et al., *Phosphorylation of SAMHD1 by cyclin A2/CDK1 regulates its restriction activity toward HIV-1.* Cell Rep, 2013. **3**(4): p. 1036-43.
270. Pauls, E., et al., *Cell cycle control and HIV-1 susceptibility are linked by CDK6-dependent CDK2 phosphorylation of SAMHD1 in myeloid and lymphoid cells.* J Immunol, 2014. **193**(4): p. 1988-97.
271. St Gelais, C., et al., *Identification of cellular proteins interacting with the retroviral restriction factor SAMHD1.* J Virol, 2014. **88**(10): p. 5834-44.
272. Yan, J., et al., *CyclinA2-Cyclin-dependent Kinase Regulates SAMHD1 Protein Phosphohydrolase Domain.* J Biol Chem, 2015. **290**(21): p. 13279-92.

273. Pauls, E., et al., *Palbociclib, a selective inhibitor of cyclin-dependent kinase4/6, blocks HIV-1 reverse transcription through the control of sterile alpha motif and HD domain-containing protein-1 (SAMHD1) activity.* AIDS, 2014. **28**(15): p. 2213-22.
274. Allouch, A., et al., *p21-mediated RNR2 repression restricts HIV-1 replication in macrophages by inhibiting dNTP biosynthesis pathway.* Proc Natl Acad Sci U S A, 2013. **110**(42): p. E3997-4006.
275. Ruiz, A., et al., *Cyclin D3-dependent control of the dNTP pool and HIV-1 replication in human macrophages.* Cell Cycle, 2015. **14**(11): p. 1657-65.
276. Tang, C., et al., *Impaired dNTPase activity of SAMHD1 by phosphomimetic mutation of Thr-592.* J Biol Chem, 2015. **290**(44): p. 26352-9.
277. Arnold, L.H., et al., *Phospho-dependent Regulation of SAMHD1 Oligomerisation Couples Catalysis and Restriction.* PLoS Pathog, 2015. **11**(10): p. e1005194.
278. Hofmann, H., et al., *The Vpx lentiviral accessory protein targets SAMHD1 for degradation in the nucleus.* J Virol, 2012. **86**(23): p. 12552-60.
279. Martin-Gayo, E., et al., *Potent Cell-Intrinsic Immune Responses in Dendritic Cells Facilitate HIV-1-Specific T Cell Immunity in HIV-1 Elite Controllers.* PLoS Pathog, 2015. **11**(6): p. e1004930.
280. Buchanan, E.L., M.A. McAlexander, and K.W. Witwer, *SAMHD1 expression in blood cells of HIV-1 elite suppressors and viraemic progressors.* J Antimicrob Chemother, 2015. **70**(3): p. 954-6.
281. Riveira-Munoz, E., et al., *Increased expression of SAMHD1 in a subset of HIV-1 elite controllers.* J Antimicrob Chemother, 2014. **69**(11): p. 3057-60.
282. Mitsuya, H., et al., *Suramin protection of T cells in vitro against infectivity and cytopathic effect of HTLV-III.* Science, 1984. **226**(4671): p. 172-4.
283. Roberts, J.D., K. Bebenek, and T.A. Kunkel, *The accuracy of reverse transcriptase from HIV-1.* Science, 1988. **242**(4882): p. 1171-3.
284. Preston, B.D., B.J. Poiesz, and L.A. Loeb, *Fidelity of HIV-1 reverse transcriptase.* Science, 1988. **242**(4882): p. 1168-71.
285. Larder, B.A. and S.D. Kemp, *Multiple mutations in HIV-1 reverse transcriptase confer high-level resistance to zidovudine (AZT).* Science, 1989. **246**(4934): p. 1155-8.
286. Samji, H., et al., *Closing the gap: increases in life expectancy among treated HIV-positive individuals in the United States and Canada.* PLoS One, 2013. **8**(12): p. e81355.
287. Schader, S.M. and M.A. Wainberg, *Insights into HIV-1 pathogenesis through drug discovery: 30 years of basic research and concerns for the future.* HIV & AIDS Review, 2011. **10**(4): p. 91-98.
288. Kohlstaedt, L.A., et al., *Crystal structure at 3.5 A resolution of HIV-1 reverse transcriptase complexed with an inhibitor.* Science, 1992. **256**(5065): p. 1783-90.
289. Turner, D., et al., *Rationale for maintenance of the M184v resistance mutation in human immunodeficiency virus type 1 reverse transcriptase in treatment experienced patients.* New Microbiol, 2004. **27**(2 Suppl 1): p. 31-9.
290. Petrella, M. and M.A. Wainberg, *Might the M184V substitution in HIV-1 RT confer clinical benefit?* AIDS Rev, 2002. **4**(4): p. 224-32.
291. Das, K. and E. Arnold, *HIV-1 reverse transcriptase and antiviral drug resistance. Part 2.* Curr Opin Virol, 2013. **3**(2): p. 119-28.
292. Yang, W.L., et al., *Assessing the Paradox Between Transmitted and Acquired HIV Type 1 Drug Resistance Mutations in the Swiss HIV Cohort Study From 1998 to 2012.* J Infect Dis, 2015. **212**(1): p. 28-38.
293. Minuto, J.J. and R. Haubrich, *Etravirine: a second-generation NNRTI for treatment-experienced adults with resistant HIV-1 infection.* Futur HIV Ther, 2008. **2**(6): p. 525-537.
294. Sax, P.E., *FDA approval: etravirine.* AIDS Clin Care, 2008. **20**(3): p. 17-8.
295. Schrijvers, R., B.A. Desimmie, and Z. Debyser, *Rilpivirine: a step forward in tailored HIV treatment.* Lancet, 2011. **378**(9787): p. 201-3.

296. Mesplede, T., et al., *Integrase strand transfer inhibitors in the management of HIV-positive individuals.* Ann Med, 2014. **46**(3): p. 123-9.
297. Quashie, P.K., et al., *Characterization of the R263K mutation in HIV-1 integrase that confers low-level resistance to the second-generation integrase strand transfer inhibitor dolutegravir.* J Virol, 2012. **86**(5): p. 2696-705.
298. Mesplede, T., et al., *Viral fitness cost prevents HIV-1 from evading dolutegravir drug pressure.* Retrovirology, 2013. **10**: p. 22.
299. Mesplede, T., et al., *Dolutegravir inhibits HIV-1 Env evolution in primary human cells.* AIDS, 2015. **29**(6): p. 659-65.
300. Jamburuthugoda, V.K., et al., *Reduced dNTP binding affinity of 3TC-resistant M184I HIV-1 reverse transcriptase variants responsible for viral infection failure in macrophage.* J Biol Chem, 2008. **283**(14): p. 9206-16.
301. Le Douce, V., et al., *Molecular mechanisms of HIV-1 persistence in the monocyte-macrophage lineage.* Retrovirology, 2010. **7**: p. 32.

Chapter 2

Automethylation of protein arginine methyltransferase 6 (PRMT6) regulates its stability and its anti- HIV-1 activity

The following chapter was adapted from the published manuscript entitled "Automethylation of protein arginine methyltransferase 6 (PRMT6) regulates its stability and its anti-HIV-1 activity" authored by Diane N. Singhroy, Thibault Mesplède, Arielle Sabbah, Peter K. Quashie, Jean-Pierre Falgueyret, and Mark A. Wainberg. Approximately 90% of the experiments and data analysis was performed by me under the supervision of Dr. Mark Wainberg. Dr. Thibault Mesplède helped with the project design and analysis. Samples for mass spectrometry were prepared by me and Pierre Falgueyret operated the mass spectrometer. Peter K. Quashie and Pierre Falgueyret assisted with the technical interpretation of the MS spectra. Ariel Sabbah helped generate the PRMT6 mutant plasmids under my supervision. Dr. Wainberg and Dr. Mesplède critiqued the manuscript.

2.1 Preface

PRMT6 automethylation was initially observed when this protein was first discovered [1]. However, little is known about the role of this post-translational modification on PRMT6 cellular activities or what residues are targeted by this activity. Automethylation is not exclusive to PRMT6 as it has also been shown to occur with PRMT1, PRMT4, PRMT3, PRMT7 and PRMT8 [2, 3]. However PRMT4 and PRMT8 are the only other PRMT proteins whose automethylation has been characterised. For PRMT8, automethylation increases its affinity for AdoMet, thus regulating its enzymatic activity [4].

When conducting proof- of- concept experiments for an earlier PRMT6 project idea, I noticed that enzymatically inactive PRMT6 (PRMT6-KLA), used at the time as a control, was being degraded upon cyclohexamide (CHX) (an inhibitor of protein synthesis) treatment in a cell based assay (figure 2.1) whereas the catalytically active WT PRMT6 was remarkably stable under the same conditions.

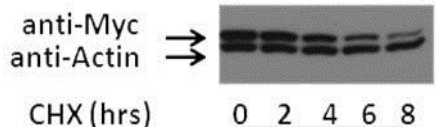


Figure 2.1: Stability of inactive PRMT6. Western blots for transfected Myc tagged PRMT6-KLA (upper bands) following treatment with CHX in HeLa cells. Actin was used as a loading control (lower bands). Unpublished data, for purposes of illustration only.

The cellular functions of PRMT6 arginine methylation are varied and substrate-dependent, and include regulation of gene expression, DNA base excision repair and intrinsic immunity [5]. But

importantly, PRMT6 was previously shown to control the protein stability of Tat [6]. The role of arginine methylation in protein stability and turnover has been documented for PRMT4/CARM1 [7]. Specifically, in this study PRMT4/CARM1 methylation of the p300/CBP interacting protein (p/CIP) led to protein degradation. Very little is known about the regulation of PRMT6 activity and expression. Given that (i) arginine methylation is involved in the regulation of protein stability and (ii) PRMT6-KLA seems less stable than WT PRMT6 in tissue culture, we investigated the role of PRMT6 automethylation on its stability. It is our hypothesis that PRMT6 protein stability may impact HIV-1 replication through changes in the levels of methylation of its viral protein substrates. Notably, as explained above, HIV inhibition by host restriction factors is a measurable activity.

We studied how PRMT6 automethylation affects its ability to restrict HIV-1 replication. This represents an important question as several inhibitors of PRMT proteins are currently in development for cancer therapy and could represent a new therapeutic target for HIV-1 treatment as well.

2.2 Abstract

Protein arginine methyltransferase 6 (PRMT6) is a nuclear enzyme that methylates arginine residues on histones and transcription factors. In addition, PRMT6 inhibits HIV-1 replication in cell culture by directly methylating and interfering with the functions of several HIV-1 proteins, i.e. Tat, Rev and nucleocapsid (NC). PRMT6 also displays automethylation capacity but the role of this post-translational modification in its antiretroviral activity remains unknown.

Here we report the identification by liquid chromatography-mass spectrometry of R35 within PRMT6 as the main target residue for automethylation and have confirmed this by site-directed mutagenesis and *in vitro* and *in vivo* methylation assays. We further show that automethylation at position 35 greatly affects PRMT6 stability and is indispensable for its antiretroviral activity, as demonstrated in HIV-1 single-cycle TZM-bl infectivity assays.

These results show that PRMT6 automethylation plays a role in the stability of this protein and that this event is indispensible for its anti-HIV-1 activity.

2.3 Introduction

Throughout its replication cycle, HIV interacts with a plethora of cellular proteins that can either restrict or aid infection. Recent genome-wide screens have identified many of the factors that contribute to HIV pathogenesis, in particular through the regulation of HIV protein function [8-13]. These interactions can sometimes result in post-translational modifications that are important in the regulation of HIV proteins. For examples, acetylation of Tat regulates its transcriptional activity [14] and serine phosphorylation is essential for the activity of Vif [15].

Arginine methylation is a posttranslational modification in eukaryotes that results in the covalent addition of one or two methyl groups to the terminal nitrogen atom of arginine [16]. This reaction is catalyzed by protein arginine methyltransferases (PRMTs) and has been implicated in transcriptional regulation, epigenetics, DNA repair, mRNA splicing and signal transduction [17, 18]. Arginine methylation is dependent on the methyl donor S-adenosyl-L-

methionine (SAM) to yield the methylated arginine and an S-adenosylhomocysteine [19]. Eleven PRMTs have been characterized to date and are classified as type I, II or III [20]. Both type I and type II produce ω-N^G- monomethylated arginine (MMA) intermediates, however type I enzymes further catalyze the formation of ω-N^G- N^G- asymmetric dimethylarginines (aDMA), while type II enzymes catalyze N^G- N^G- symmetric dimethylarginines (sDMA) [19, 21]. Type III PRMTs only catalyze the formation of MMA residues. The addition of a methyl group does not change the overall charge of a protein, but can reallocate hydrogen bond sites, thus affecting protein-protein interactions [22]. The importance of arginine methylation in the cell is further supported by the fact that over 200 proteins contain putative dimethylated arginines [23].

PRMT6 is a 41.9 kDa Type I methyltransferase found in the nucleus [1]. It typically targets arginine in glycine and arginine rich (GAR) motifs. Thrombospondin-1 (TSP-1), H3R2 and H2A were all found to be arginine methylated by PRMT6 [24-26]. Our group has found that PRMT6 methylates and restricts the function of the HIV proteins Tat, Rev, and Nucleocapsid (NC) [27-30], resulting in restriction of HIV-1 replication by PRMT6. Methylation of Tat by PRMT6 leads to a disruption of the Tat-TAR-cyclin T1 complex and decreases Tat specific transcriptional activation [27]. Once NC is methylated by PRMT6, it is less able to promote RNA annealing and to initiate reverse transcription [30].

The HIV-1 Rev protein is a 19 kDa protein found in the nucleolus, the perinuclear zone, and the cytoplasm of HIV infected cells. It mediates viral protein expression at the level of viral RNA splicing and nuclear export of unspliced and single-spliced viral RNA by binding to the *cis*-acting

Rev response element (RRE) [31, 32]. Rev is arginine methylated by PRMT6 in its arginine rich motif (ARM) located within the Rev nuclear localization signal (NLS) [29]. Rev-mediated export of viral RNA is decreased when Rev is methylated by PRMT6 because methylated Rev is unable to bind efficiently to the RRE [29].

Automethylation of PMRT6 has been reported [1], but the site and role of automethylation have not been identified. PRMT6 requires homodimerization to transfer a methyl group from SAM to the protein substrate and this could favour automethylation [33]. As documented for CARM1 (PRMT4), another member of the PRMT family, we have hypothesized that automethylation could modulate PRMT6 function [34]. Mutagenesis of the automethylation site of CARMI did not affect its catalytic activity but impaired its transcriptional and RNA-processing capacity. Additionally, arginine methylation of the HIV protein Tat by PRMT6 and of the cellular protein axin by PRMT1 increase the stability of these proteins [6, 35]. We now report that R35 is crucial for PRMT6 automethylation and that arginine automethylation is important for PRMT6 stability and its ability to inhibit HIV-1 replication.

2.4 Material and methods

2.4.1 Reagents

Recombinant glutathione-S-transferase (GST)-tagged wild-type PRMT6 (GST-PRMT6-WT), methyltransferase inactive V86K/D88A PRMT6 (GST-PRMT6-KLA) and histidine-tagged HIV-1 Rev protein (His-Rev) were prepared as described previously [29]. Myc-tagged wild-type PRMT6

(myc-PRMT6-WT) and inactive Myc-tagged methyltransferase V86K/D88A PRMT6 (Myc-PRMT6-KLA) have also been described previously [28]. GST- and Myc-tagged PRMT6-R35A mutants were generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) through use of a 5'-GCGGCCCTGGAGGCACCCCGGAGGAC-3' forward primer and a 5'-GTCCTCCGGGGTGCCTCCAGGGCCGC-3' reverse primer (Invitrogen). To quantify viral genomic RNA, quantitative real-time polymerase chain reaction (QPCR) primers and probe used were 5'-CCGTCTGTTGTGTGACTCTGG-3' forward Primer, 5'-GAGTCCTGCGTCGAGAGATCT-3' reverse primer and 5' FAM-TCTAGCAGTGGCGCCCGAACAGG-TAMRA-3' probe (Invitrogen) [36]. Anti-PRMT6 rabbit polyclonal antibody was purchased from Imgenex and anti-Myc mouse monoclonal antibody was purchased from Invitrogen. Anti-actin mouse monoclonal antibody was purchased from MP Biomedicals.

The following reagents were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: TZM-bl from Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc and pNL4-3 from Dr. Malcolm Martin [37]

2.4.2 Liquid chromatography-mass spectrometry analysis

Recombinant GST-PRMT6-WT and GST-PRMT6-KLA (5 µg each) were incubated with 25 µM S-adenosylmethionine (Sigma) in 25 mM Tris-HCl (pH 7.4) for 3 hours at 37°C in a final volume of 30 µl. The reactions were stopped by adding 6 µl of 5× Läemmli buffer, followed by boiling for 5 minutes and centrifugation at 16,000 g for 2 minutes. The samples were then run on 10% SDS

polyacrylamide gels. Bands corresponding to GST-PRMT6-WT and GST-PRMT6-KLA were cut out, processed, and analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Prior HPLC the polyacrylamide gel plug containing GST-PRMT6 was subjected to in-gel tryptic digestion for protein ID and identification of the dimethylated arginines. Briefly the polyacrylamide gel plug was destained with 25mM ammonium bicarbonate (NH_4HCO_3) and 50% acetonitrile (ACN). The gel particle was then incubated with 100% ACN. Reduction was accomplished with 10mM DTT and alkylation with 55mM iodoacetamide. After washing with 50 mM NH_4HCO_3, the gel particles were shrunk with 100% ACN and dried down in a SpeedVac. In order to detect methylated peptides, samples underwent partial in-gel digestion with 1.6 µg of Trypsin (Sigma), an enzyme that does not digest the C-terminus of methylated arginine. Gel particles were vortexed and sonicated in 5% formic acid (FA)/50% ACN to extract sample from the gel. The volume of the sample was reduced to 15ul in the SpeedVac and cleaned using a C18 ZipTip (Millipore).

The samples were dried down in a Speed vacuum apparatus and resolubilized in 50 µl of acetonitrile (ACN) 5% /formic acid (FA) 0.1%. 2 µl of each sample were directly injected onto a C18 analytical column (75 µm i.d. x 100 mm) using the Proxeon EASY nLC system. A 21-min gradient was used to elute peptides at a flow rate of 300 nl/min. The gradient started at 3% acetonitrile/0.2% formic acid and a linear gradient to 35% acetonitrile/0.2% formic acid was achieved in 13 min, then ramped up to 92% acetonitrile 0.2% formic acid in 2 minutes.

The liquid chromatography (LC) system was coupled to a LTQ-Orbitrap mass spectrometer (MS) (Thermo Fisher). A full MS spectrum was collected at the level of the Orbitrap (FT-MS); then, the ten most abundant multiply charged ions (threshold > 5000 counts) were selected for MS/MS sequencing at the level of the linear trap and stored in an exclusion list for 30 seconds. Tandem MS experiments were performed using a collision-induced dissociation set at 35% with activation time of 10 msec. The data were processed using Proteome Discoverer (version 1.3) running the SEQUEST search engine. Database searching against a FASTA file containing 136 sequences (mostly bacterial, yeast and mammalian contaminants) including PRMT6 WT and mutant sequences was performed allowing differential modification on cysteine residues (carbamidomethylation: +58), methionine (oxidation: + 16) and arginine (dimethylation: +28). MS/MS spectra were searched for protein tryptic digests allowing a maximum of two missed cleavage sites per peptide. Only peptides with Xcorr values greater than 3.2, equivalent to a false discovery rate lower than 1%, were retained to assess coverage of PRMT6 (WT and Mutant) and dimethylation sites.

2.4.3 In vitro methylation assay

Recombinant GST-tagged PRMT6 proteins (3-4 µg) were incubated with 1–2 µg of recombinant histidine-tagged Rev with 0.55 µCi of [methyl-3H]-S- adenosyl-L-methionine (Perkin Elmer life sciences) and 25 mM Tris-HCl (pH 7.4) for 3 hours at 37°C in a final volume of 25 µl. Reactions were stopped by adding 5 µl of 5 × Läemmli buffer followed by boiling for 5 minutes and

centrifugation at 16,000 g for 2 minutes. Samples were loaded onto 10% SDS polyacrylamide gels. Gels were stained with Coomassie brilliant blue R-250 solution (Bio-Rad Laboratories) and, after destaining, soaked in 1x ENH^{3}ANCE (Perkin Elmer life sciences) for 45 minutes. Gels were then dried and exposed on HyBlot CL autoradiography film (Denville Scientific) for 1 to 3 days. Gels and films were quantified with the ImageJ software [38]. Automethylation assays were similarly performed; however, the incubation period was increased to between 14 and 21 days for reasons of enhanced sensitivity.

2.4.4 In vivo methylation assay

HeLa cells were transfected with Lipofectamine 2000 reagent according to the manufacturer's guidelines (Invitrogen) with 1 µg myc-tagged PRMT6 WT DNA. At 24 hr after transfection, S-adenosyl-L-methionine (New England Biolabs)) was added to the cells. Cells were collected at 48h post transfection and lysed with RIPA buffer, and 30 µl of lysate was then incubated with myc-conjugated agarose beads (Sigma). After several washes, beads were boiled in the presence of Läemmli buffer and centrifuged. The supernatant containing the immunoprecipitated proteins was run on an SDS-PAGE gel. The appropriate band was processed for LC-MS/MS as described above.

2.4.5 Cell culture

HeLa, 293T and TZM-bl cells were all cultured in Dulbecco Modified Eagle medium (DMEM) (Gibco), supplemented with 10% foetal bovine serum, 50 IU of penicillin/ml, 50 µg of streptomycin/ml, and 2 mM L-glutamine at 37ºC in 5% CO_2.

2.4.6 Cycloheximide treatment and immunoblotting

HeLa cells were transfected with Lipofectamine 2000 reagent according to the manufacturer's guidelines (Invitrogen) with 1 µg myc-tagged PRMT6 WT, KLA or R35A plasmid. At 24 hr after transfection, the cells were treated with 100 µg/ml of cycloheximide (Sigma) and collected after 0, 2, 4, 6 and 8 hr. Cells were lysed with RIPA buffer containing protease inhibitor cocktail (Sigma). Protein concentrations of whole cell extracts were quantified using Bradford assays (5x BioRad Protein assay). 15 µg of protein was boiled with Läemmli buffer for 5 minutes and samples were loaded onto a 10% SDS polyacrylamide gel. Western blots were performed on a PVDF membrane (BioRad). Bands were quantification with ImageJ (http://rsb.info.nih.gov/ij/).

2.4.7 Viral production and quantification

Human Embryonic Kidney (HEK)-293T cells were transfected as described above. Cells (3×10^6) were co-transfected with 4 µg of pNL4.3 and 4 µg of myc-PRMT6 plasmids; at 48 hours post

transfection, culture supernatants were collected, centrifuged at 1200 rpm and passed through a 0.45 μm filter. Viral particles in the supernatants were quantified by quantitative real-time PCR (QPCR) as described previously [39] and by HIV reverse transcriptase (RT) activity assay, also previously described [40]. The same supernatants were then used to measure infectivity in TZM-bl, as described below. Experiments were repeated 3 separate times and data were analysed using GraphPad Prism software.

2.4.8 Infectivity assay

Viruses produced in 293T cells, as described above, in the presence of myc-PRMT6 plasmids were normalized on the basis of QPCR or HIV RT activity and were 5-fold titrated onto TZM-bl luciferase reporter cells and incubated for 48 hours. TZM-bl cells were lysed using the luciferase assay system (Promega) and luminescence was read with a MicroBeta Trilux Luminescence counter over a period of 1 second (Perkin Elmer). Experiments were repeated 3 times and data were analyzed using GraphPad Prism software.

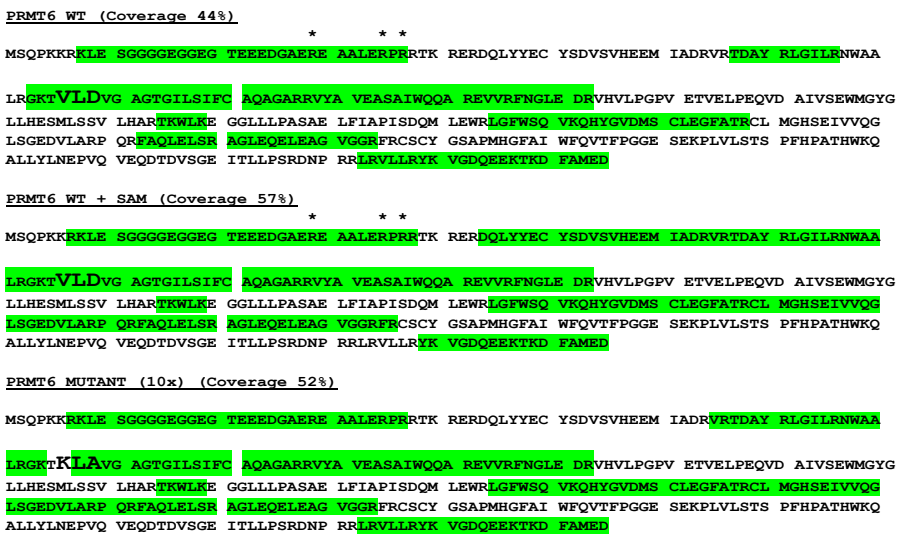
2.5 Results

2.5.1 Identification of PRMT6 automethylation sites *in vitro* and *in vivo*

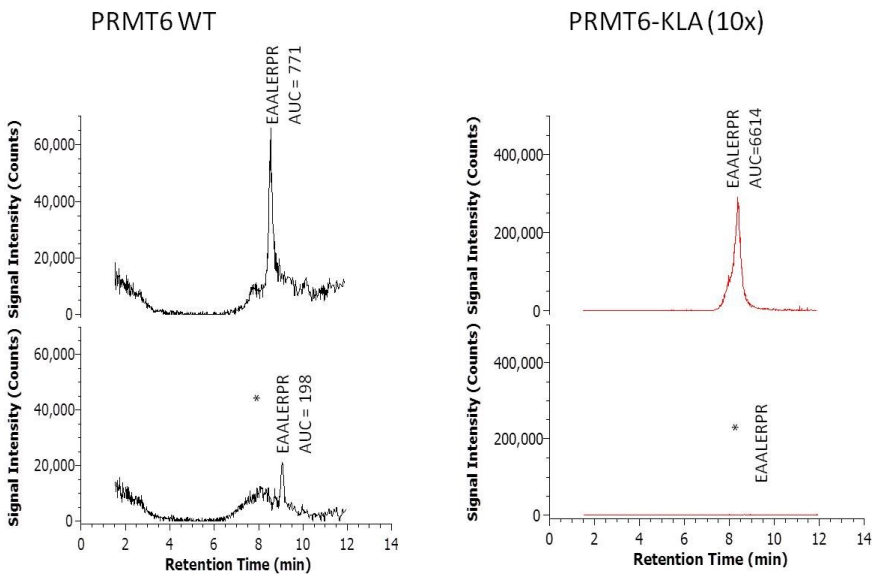
PRMT6 was previously shown to be automethylated [1]. Now, we wished to identify the arginine residues that are involved and, therefore, used purified recombinant PRMT6 in an *in vitro* methylation assay in the presence and absence of the methyl group donor S-adenosyl-

methionine (SAM) with catalytically inactive recombinant PRMT6 V86K/D88A (PRMT6-KLA) serving as a control. Post-translational methylation was determined by mass spectrometry and showed that R29, R35 and R37 in the N-terminal region of the protein were methylated in the PRMT6 wild-type protein with and without SAM but not in the KLA inactive mutant (Figure 2.2A). Extracting ions from the peptides that contain R35 from both proteins confirmed the absence of methylation on this residue within the KLA mutant (Figure 2.2B). The observation that PRMT6-WT was methylated in the absence of added substrate suggested that most of the active protein was automethylated during bacterial expression and also provided an explanation for the low levels of automethylation observed in previous studies [1]. We also confirmed that R35 is methylated *in vivo* by conducting an *in vivo* methylation assay (Figure 2.2E).

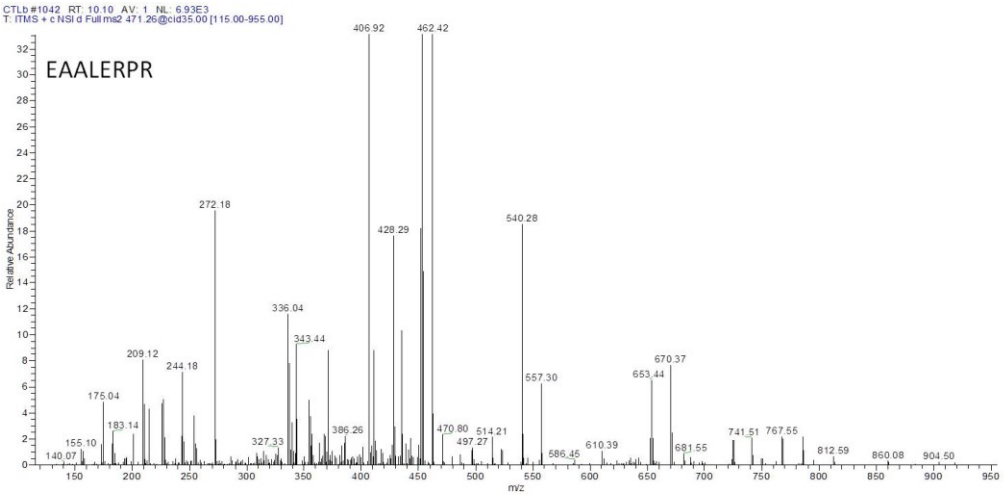
A



B

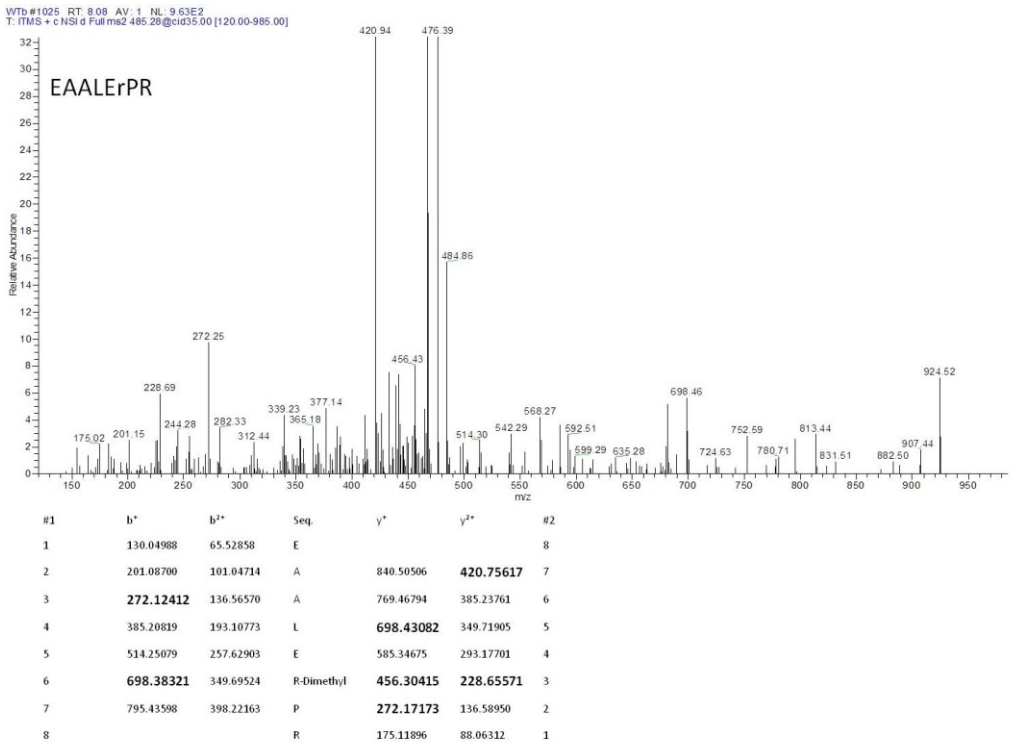


C



#1	b*	b²*	Seq.	y*	y²*	#2
1	130.04988	65.52858	E			8
2	201.08700	101.04714	A	812.47376	**406.74052**	7
3	**272.12412**	136.56570	A	741.43664	**371.22196**	6
4	385.20819	193.10773	L	**670.39952**	**335.70340**	5
5	514.25079	257.62903	E	**557.31545**	279.16136	4
6	**670.35191**	**335.67959**	R	**428.27285**	214.64006	3
7	767.40468	384.20598	P	**272.17173**	136.58950	2
8			R	175.11896	88.06312	1

D



#1	b+	b2+	Seq.	y+	y2+	#2
1	130.04988	65.52858	E			8
2	201.08700	101.04714	A	840.50506	**420.75617**	7
3	**272.12412**	136.56570	A	769.46794	385.23761	6
4	385.20819	193.10773	L	**698.43082**	349.71905	5
5	514.25079	257.62903	E	585.34675	293.17701	4
6	**698.38321**	349.69524	R-Dimethyl	**456.30415**	**228.65571**	3
7	795.43598	398.22163	P	**272.17173**	136.58950	2
8			R	175.11896	88.06312	1

E

PRMT6 in vivo (Coverage 63%)

```
                                  *       * **
MSQPKKRKLE SGGGGEGGEG TEEEDGAERE AALERPRRTK RERDQLYYEC YSDVSVHEEM IADRVRTDAY RLGILRNWAA
 *
LRGKTVLDVG AGTGILSIFC AQAGARRVYA VEASAIWQQA REVVRFNGLE DRVHVLPGPV ETVELPEQVD AIVSEWMGYG
LLHESMLSSV LHARTKWLKE GGLLLPASAE LFIAPISDQM LEWRLGFWSQ VKQHYGVDMS CLEGFATRCL MGHSEIVVQG
LSGEDVLARP QRFAQLELSR AGLEQELEAG VGGRFRCSCY GSAPMHGFAI WFQVTFPGGE SEKPLVLSTS PFHPATHWKQ
ALLYLNEPVQ VEQDTDVSGE ITLLPSRDNP RRLRVLLRYK VGDQEEKTKD FAMED
```

Figure 2.2: Mass spectrometric analysis of PRMT6 arginine methylated residues. Following *in vitro* and *in vivo* methylation, methylated arginine residues in recombinant PRMT6 were mapped by LC-MS/MS. **(A)** Percent coverage obtained for PRMT6 WT (-/+ SAM) and the PRMT6 KLA mutant. Recombinant PRMT6 was digested with Trypsin in an ammonium bicarbonate buffer and peptides were separated onto a C18 column and sequenced by LC-MS as described in Methods. In order to evaluate trace amounts of the dimethylated arginine in the mutated protein, a 10-fold concentrated peptide solution was injected. Percentages of coverage obtained for PRMT6, PRMT6 + SAM and the PRMT6 mutant were 44%, 57%, and 52%, respectively, and are highlighted in green. Dimethylated arginine was identified only with PRMT6 WT +/- SAM (R29; R35; R37). Dimethylated residues are represented with an (*) above

the residue. **(B)** In order to evaluate the percentage of dimethylated arginine on PRMT6, ions corresponding to EAALERPR (m/z 471.26) and EAALER*PR (m/z 485.27) were extracted. The dimethylated form of the peptide was only observed in the WT protein and in the WT protein + SAM (data not shown), and there was no detectable signal at m/z 485.27 in the PRMT6 KLA mutant even when used at 10 fold the concentration of the WT protein. Areas under the curve (AUC) were studied for both peptides from the PRMT6 WT protein; assuming no differences in ionization efficiency between the two peptides, the methylated protein apparently represents 10 to 20 % of the total amount of protein present. MS/MS spectra observed for the methylated **(C)** EAALERPR and non-methylated **(D)** peptide of wild type PRMT6. Observed ions are indicated in bold. **(E)** *In vivo* methylation assays were performed in HeLa cells with transfected myc-tagged PRMT6. Samples were digested and processed in the same manner as described for the *in vitro* methylation assays. The percent coverage obtained for PRMT6-WT was 63% and is highlighted in green. Dimethylated arginines were identified for residues R29, R35, R38, R39 and R82. Dimethylated residues are represented with an (*) above the residue. Amino acids that would be mutated for PRMT6-KLA are indicated by enlarged letters.

Alignment studies show that the R35, R37, and R38 residues are well conserved among mammals e.g. *Homo sapiens, Pan troglodytes, Macaca mulatta, Sus scrofa, Mus musculus, Rattus norvegicus,* and *Bos Taurus* (Figure 2.3), but the region is not conserved in some other organisms, e.g. *Arabidopsis thaliana* and *Danio rerio* (data not shown), suggesting that its emergence followed the divergence that occurred between these branches in evolution. R29 is poorly evolutionary conserved.

Homo sapiens	AEREAALE**R**PRRTKRERDQLY	47
Pan troglodytes	AEREAALE**R**PRRTKRERDQLY	47
Macaca mulatta	AEREAALE**R**PRRTKRERDQLY	47
Sus scrofa	GEQEAALP**R**PRRTRRERDQLY	47
Mus musculus	GEQEAAPP**R**PRRTKSERDQLY	50
Rattus norvegicus	GEQEAAPP**R**PRRTKRERDQLY	47
Bos taurus	GELEVAVP**R**PRRTRRERDQLY	47
Consensus	-E-E-A--**R**PRRT--ERDQLY	

Figure 2.3: The R35 residue is conserved in evolution. Sequence alignment of PRMT6 proteins from various organisms showing conservation of the arginine residue at position 35 (*H. sapiens*). The consensus sequence was produced using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). R35 is bolded and the underlined text refers to the arginine rich motif.

2.5.2 PRMT6 is automethylated at R35

To characterize the role of automethylation in the activity of PRMT6, the central residue R35 was mutated to an alanine residue (PRMT6-R35A) and automethylation was measured *in vitro* in the presence of ^{3}H-SAM (Figure 2.4). Mutant PRMT6-KLA was used as a negative control. Weak but consistent automethylation was measured for the wild-type recombinant PRMT6 protein but not for either KLA or R35A, suggesting that R35 plays a critical role in automethylation. Therefore our study focused on the role of this residue.

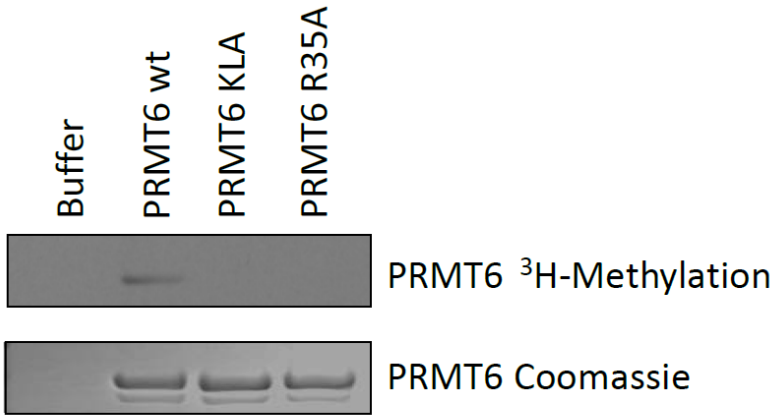


Figure 2.4: PRMT6-R35A does not automethylate. Cell-free automethylation assays were performed using the indicated PRMT6 recombinant proteins in the presence of ^{3}H-SAM. Autoradiography (upper) and Coomassie staining (lower) are shown. ^{3}H-SAM was contained in all wells. The first lane contains methylation buffer only, without the presence of PRMT6. The other lanes contain the indicated recombinant proteins. This experiment was performed three times with similar results being obtained each time; a representative result is shown.

In a similar experiment, PRMT6-R35A was shown to be able to methylate the purified HIV-1 Rev protein *in vitro*, indicating that this mutated derivative protein retains its ability to arginine methylate other proteins but not itself (Figure 2.5). In the same experiment, PRMT6-KLA was, as expected, completely unable to methylate Rev. Thus, R35 is a PRMT6 primary automethylation site and automethylation may not be essential for the methylation of HIV-1 Rev *in vitro*. We performed similar *in vitro* methylation experiments using HIV-1 Tat as a substrate and found that PRMT6-R35A retains methylation activity (data not shown).

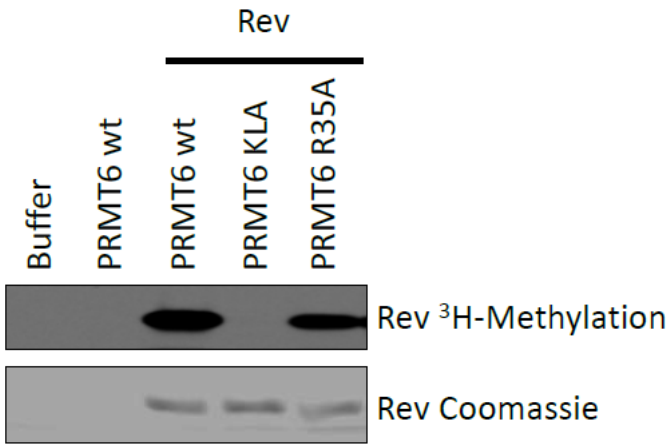


Figure 2.5: PRMT6-R35A is catalytically active. PRMT6 cell-free methylation assay was performed with HIV-1 Rev protein, a known substrate of PRMT6. The upper frame represents the autoradiograph of Rev methylation by PRMT6. The lower frame represents a Coomassie stained gel of Rev. This experiment was performed three times with similar results obtained each time; a representative result is shown.

2.5.3 PRMT6 automethylation regulates its stability

We next measured the stability of the wild-type, KLA, and R35A PRMT6 proteins (Figure 2.6). Plasmids coding for the various forms of myc-tagged PRMT6 were transfected into HeLa cells, and, at 24h after transfection, cycloheximide (CHX) was added to culture supernatants. Since CHX inhibits protein synthesis, it can be used to study protein degradation over time in studies in which expression levels of PRMT6 protein are measured by Western-blot. The results show that PRMT6 is very stable (Figure 2.6A, first panel), but that the mutant R35A protein and the catalytically inactive KLA form of PRMT6 were less stable, with their expression levels decreasing over time. Densitometric quantification of the Western-blot confirmed that the

expression levels of both mutant proteins decreased faster than the WT protein following the addition of CHX (Figure 2.6B). Thus, PRMT6 automethylation is important for its stability.

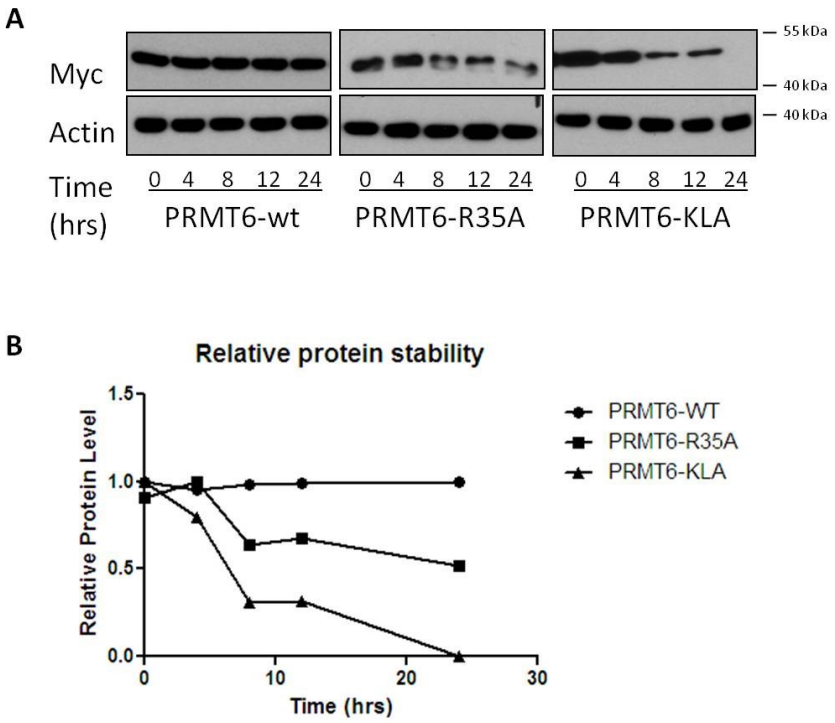


Figure 2.6: PRMT6-R35A is less stable than PRMT6-WT. (A) Western blots for Myc-PRMT6-WT, -PRMT6-R35A and -PRMT6-KLA, following treatment with CHX (upper panels). Actin was used as a loading control (lower panels). 15 μg of protein from whole cell extract were loaded into each well. (B) Densitometric analysis of Myc-PRMT6-WT and -R35A degradation over time following the addition of CHX. Myc expression was normalized to levels of actin. Each experiment was performed three times with similar results obtained each time; a representative blot is shown.

2.5.4 PRMT6 automethylation is required for inhibition of HIV-1 replication

We have shown that the expression level of PRMT6 is important for inhibition of HIV-1 replication, and now wished to assess the impact of the R35A mutation on this activity (Figure 2.7). Proviral DNA coding for pNL4-3 virus was co-transfected into 293T cells together with

either a control empty plasmid or plasmids coding for PRMT6-WT or R35A mutant proteins. The resulting viruses were quantified by RT activity assay and by QPCR (Figure 2.7A and 2.7B) and their infectiousness was tested in TZM-bl reporter cells by normalizing the amount of infecting virus for either RT activity or viral RNA (Figure 2.7C and 2.7D). p24 was not used for normalization since PRMT6 affects virion size [41]. Since PRMT6-WT and R35A display differences in their stability (Figure 2.6), we verified the proper expression of these proteins in these experiments by measuring PRMT6 protein expression at 24 hours post transfection by Western blot (Figure 2.7E). Densitometric quantification indicated that PRMT6-WT expression levels were 20% lower than those of PRMT6-R35A in these experiments, measures that are independent from differences in stability. The data show that expression of PRMT6-WT decreased HIV-1 infectivity, but that the R35A mutation restored viral infectivity to levels similar to those of virus grown in the absence of PRMT6. This indicates that prevention of PRMT6 automethylation results in a significant decrease in PRMT6 anti-HIV activity of approximately 90% (Figure 2.7).

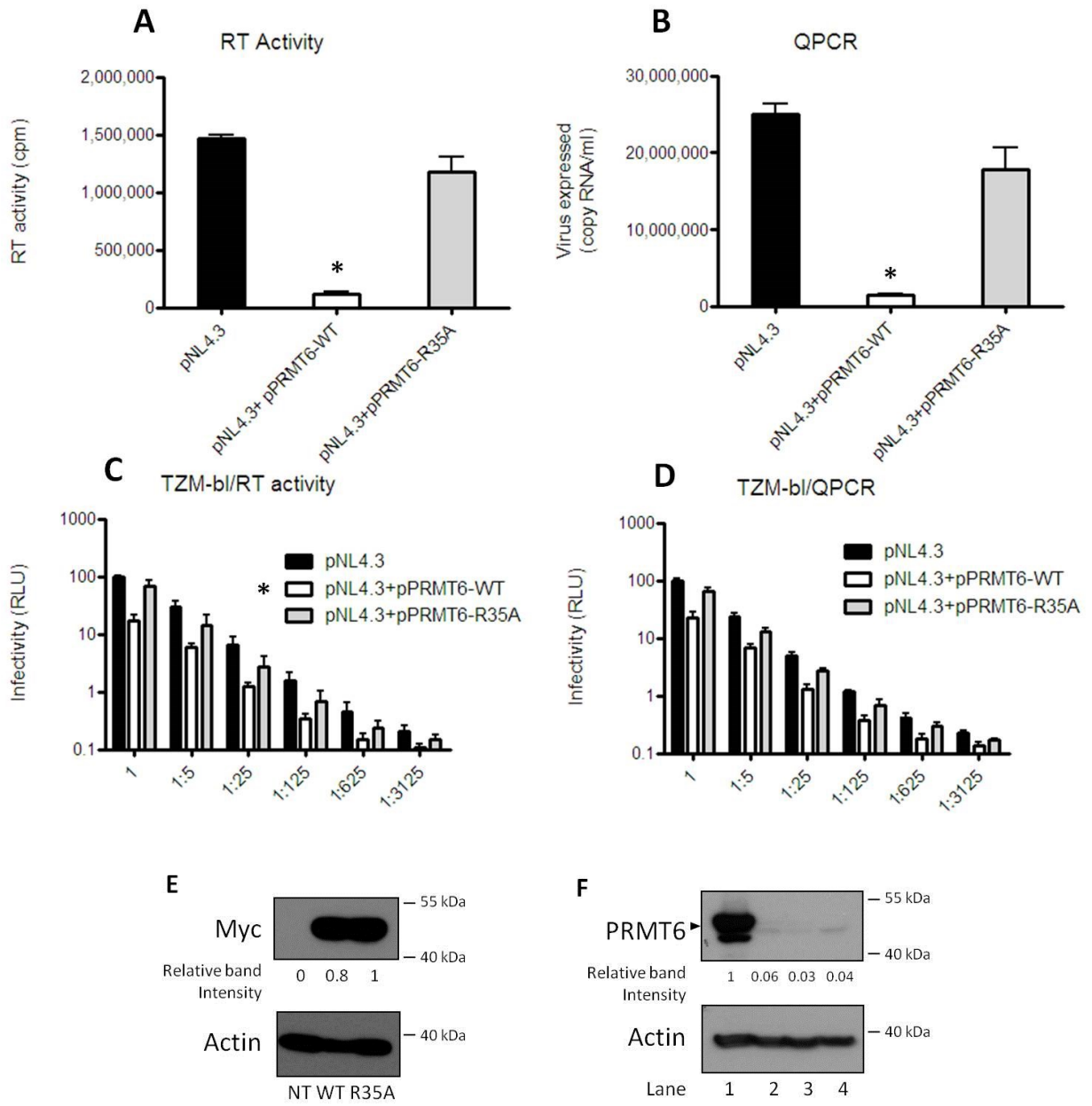


Figure 2.7: PRMT6 automethylation is necessary for its HIV-1 restriction activity. The indicated forms of PRMT6 plasmid were co-transfected with HIV-1 pNL4.3 proviral DNA into 293T cells. At 48 hours after transfection, cell culture fluids containing virus were collected, quantified by QPCR (A) or by HIV RT activity assay (B), and titrated onto TZM-bl cells (C, D) Luciferase activities are expressed as relative light units (RLU). The amount of transfected PRMT6 expression, i.e. not protein stability, was quantified by Western blots with anti-Myc and anti-actin antibodies followed by densitometric analysis normalized against actin (E). Endogenous levels of PRMT6 in HeLa cells (lane 2), TZM-bl cells (lane 3) and 293T cells (lane 4) were compared to transfected PRMT6 in HeLa cells (lane 1) (F). 15 μg of protein from whole cell extracts were loaded into each well used for analysis in the Western blots in (E) and (F). Actin was used as a loading control. Infectivity was measured by luciferase assay at 48 hours after infection. The control experiment was transfection of pNL4.3 with an empty plasmid (referred to on the figure as pNL4.3 only).

Each experiment was performed in triplicate on three separate occasions, with similar results being obtained each time. T-tests were performed for both (A) and (B), showing that only PRMT6-WT was significantly different than the control *: $p < 0.05$. Two-way ANOVA was performed for (C) and (D), showing that only PRMT6-WT statistically inhibited HIV replication *:$p<0.001$

2.6 Discussion

Although PRMT6 is known to be able to restrict the activity of the HIV-1 Tat, NC, and Rev proteins as well as viral replication, the role of PRMT6 automethylation in this process has been unknown. Additionally, the identity of the residue(s) targeted for automethylation has remained elusive. Here, we have shown that R29, R35, and R37 of PRMT6 are auto-dimethylated, as demonstrated in a cell-free reaction. PRMT6 automethylation sites were confirmed *in vivo.* Mutating R35 to an alanine resulted in the inhibition of PRMT6 automethylation. We have further shown that automethylation is required for PRMT6 protein stability and its anti-HIV-1 activity. This is important as several inhibitors of PRMT proteins are currently being developed for cancer therapy and could also have potential for treatment of HIV infection [41].

Major host restriction factors (HRFs) that control HIV replication in specific cell types include TRIM5α, APOBEC3G, tetherin and SAMHD1. Some of these HRFs are regulated by post-translational modifications, i.e. phosphorylation and ubiquitination for APOBEC3G [42-44]. The current study reinforces the fact that arginine methylation can play a role in regulation of anti-HIV activity, and, in addition, is the first to identify R35 as a major residue targeted for automethylation within PRMT6. Finally, preventing PRMT6 automethylation by introducing the

R35A antagonized the antiretroviral effects of this protein, and also demonstrating that PRMT6-mediated anti-HIV activity is both specific and that this activity is regulated *in vivo*.

To identify potential methylated arginines, we performed mass spectrometric analysis on recombinant PRMT6-WT and PRMT6-KLA. These studies led to the identification of three arginine residues, i.e. R29, R35, and R37 in the N-terminal region of PRMT6 that are specifically methylated in the WT protein but not in the KLA mutant. This indicated that these residues are modified through automethylation. Additionally, we show here that PRMT6 is automethylated in the absence of added SAM, suggesting that most of PRMT6 was automethylated during bacterial expression. This may provide an explanation for the low levels of PRMT6 *in vitro* automethylation reported previously [1]. Other related enzymes including PRMT1, CARM1 and PRMT8 have all been described as possessing automethylation activity [2, 34, 45]. In similar fashion to PRMT6, as shown here, CARM1 automethylation has been shown to be dispensable for its enzymatic activity *in vitro,* but can still affect the ability of the enzyme to activate transcription and to regulate pre-mRNA splicing [34].

Following CHX treatment, both the methyltransferase deficient PRMT6-KLA mutant and the PRMT6-R35A mutant displayed considerably less stability than did WT PRMT6. Arginine methylation has been linked to protein stability in several previous studies. For example, PRMT1 arginine methylation of axin increases the stability of the latter by blocking ubiquitination [35]. PRMT3 methylation of the 40S ribosomal protein S2 (rpS2) also increases protein stability by reducing rpS2 ubiquitination [46]. Importantly, PRMT6 methylation has

been shown to increase the half-life of the HIV-1 Tat protein without changing its ubiquitination pattern [6]. We are currently trying to determine whether the enhanced degradation of PRMT6-KLA and PRMT-R35A may also be due to ubiquitination.

Our findings further show that methylated PRMT6 is able to restrict HIV-1 replication whereas the ability of PRMT6-R35A to play this role is diminished by as much as 90% (Figure 2.7A and B). Since PRMT6-R35A has intact catalytic activity *in vitro*, we believe that its diminished restriction activity may be due to a reduced availability of mutated PRMT6 within the cell, as a result of its poor stability. Experiments using a proteasome inhibitor such a lactacystin should potentially result in increased viral restriction. When viral input was normalized by QPCR or RT activity, WT PRMT6 restriction was carried over to a second cycle of infection, as shown in a TZM-bl infectivity assay (Figure 2.7C and D). This suggests that PRMT6 methylation affects both initial virus production as well as subsequent rounds of replication. Future experiments should include assays of the replication capacity of HIV-1 in the presence or absence of PRMT6 in T-cells.

A recent study reported that arginine methylation of antigenic peptides displayed on human leukocyte antigens (HLA) resulted in specific recognition by the immune system and elicited a T-cell response [47]. It would be interesting to determine whether PRMT6 methylated HIV-1 proteins can trigger an equivalent response, as this might represent a means of eliciting an anti-HIV-1 immunological response. Although it is clear that arginine methylation plays an important regulatory role in protein stability, further investigation is needed to clarify the role that PRMTs

play in regard to proteasomal degradation pathways. In conclusions, PRMT6 is automethylated at position R35 and this event plays an important role in regard to the stability of this protein. Due to problems of degradation of non-automethylated PRMT6, the ability of the non-automethylated protein to restrict viral replication is greatly reduced.

2.7 References

1. Frankel, A., et al., *The novel human protein arginine N-methyltransferase PRMT6 is a nuclear enzyme displaying unique substrate specificity.* J Biol Chem, 2002. **277**(5): p. 3537-43.
2. Sayegh, J., et al., *Regulation of protein arginine methyltransferase 8 (PRMT8) activity by its N-terminal domain.* J Biol Chem, 2007. **282**(50): p. 36444-53.
3. Feng, Y., et al., *Mammalian protein arginine methyltransferase 7 (PRMT7) specifically targets RXR sites in lysine- and arginine-rich regions.* J Biol Chem, 2013. **288**(52): p. 37010-25.
4. Dillon, M.B., et al., *Automethylation of protein arginine methyltransferase 8 (PRMT8) regulates activity by impeding S-adenosylmethionine sensitivity.* J Biol Chem, 2013. **288**(39): p. 27872-80.
5. Morales, Y., et al., *Biochemistry and regulation of the protein arginine methyltransferases (PRMTs).* Arch Biochem Biophys, 2016. **590**: p. 138-52.
6. Sivakumaran, H., et al., *Arginine methylation increases the stability of human immunodeficiency virus type 1 Tat.* J Virol, 2009. **83**(22): p. 11694-703.
7. Naeem, H., et al., *The activity and stability of the transcriptional coactivator p/CIP/SRC-3 are regulated by CARM1-dependent methylation.* Mol Cell Biol, 2007. **27**(1): p. 120-34.
8. Krause, C.D., et al., *Protein arginine methyltransferases: evolution and assessment of their pharmacological and therapeutic potential.* Pharmacol Ther, 2007. **113**(1): p. 50-87.
9. Konig, R., et al., *Global analysis of host-pathogen interactions that regulate early-stage HIV-1 replication.* Cell, 2008. **135**(1): p. 49-60.
10. Brass, A.L., et al., *Identification of host proteins required for HIV infection through a functional genomic screen.* Science, 2008. **319**(5865): p. 921-6.
11. Le Clerc, S., et al., *Genomewide association study of a rapid progression cohort identifies new susceptibility alleles for AIDS (ANRS Genomewide Association Study 03).* J Infect Dis, 2009. **200**(8): p. 1194-201.
12. Zhou, H., et al., *Genome-scale RNAi screen for host factors required for HIV replication.* Cell Host Microbe, 2008. **4**(5): p. 495-504.
13. Liu, L., et al., *A whole genome screen for HIV restriction factors.* Retrovirology, 2011. **8**: p. 94.
14. Kiernan, R.E., et al., *HIV-1 tat transcriptional activity is regulated by acetylation.* EMBO J, 1999. **18**(21): p. 6106-18.
15. Yang, X., J. Goncalves, and D. Gabuzda, *Phosphorylation of Vif and its role in HIV-1 replication.* J Biol Chem, 1996. **271**(17): p. 10121-9.
16. Gary, J.D. and S. Clarke, *RNA and protein interactions modulated by protein arginine methylation.* Prog Nucleic Acid Res Mol Biol, 1998. **61**: p. 65-131.
17. Bedford, M.T. and S.G. Clarke, *Protein arginine methylation in mammals: who, what, and why.* Mol Cell, 2009. **33**(1): p. 1-13.

18. Miranda, T.B., et al., *PRMT7 is a member of the protein arginine methyltransferase family with a distinct substrate specificity.* J Biol Chem, 2004. **279**(22): p. 22902-7.
19. Bedford, M.T. and S. Richard, *Arginine methylation an emerging regulator of protein function.* Mol Cell, 2005. **18**(3): p. 263-72.
20. Bedford, M.T., *Arginine methylation at a glance.* J Cell Sci, 2007. **120**(Pt 24): p. 4243-6.
21. Lakowski, T.M. and A. Frankel, *A kinetic study of human protein arginine N-methyltransferase 6 reveals a distributive mechanism.* J Biol Chem, 2008. **283**(15): p. 10015-25.
22. Lee, Y.H. and M.R. Stallcup, *Minireview: protein arginine methylation of nonhistone proteins in transcriptional regulation.* Mol Endocrinol, 2009. **23**(4): p. 425-33.
23. Boisvert, F.M., et al., *A proteomic analysis of arginine-methylated protein complexes.* Mol Cell Proteomics, 2003. **2**(12): p. 1319-30.
24. Michaud-Levesque, J. and S. Richard, *Thrombospondin-1 is a transcriptional repression target of PRMT6.* J Biol Chem, 2009. **284**(32): p. 21338-46.
25. Hyllus, D., et al., *PRMT6-mediated methylation of R2 in histone H3 antagonizes H3 K4 trimethylation.* Genes Dev, 2007. **21**(24): p. 3369-80.
26. Waldmann, T., et al., *Methylation of H2AR29 is a novel repressive PRMT6 target.* Epigenetics Chromatin, 2011. **4**: p. 11.
27. Xie, B., et al., *Arginine methylation of the human immunodeficiency virus type 1 Tat protein by PRMT6 negatively affects Tat Interactions with both cyclin T1 and the Tat transactivation region.* J Virol, 2007. **81**(8): p. 4226-34.
28. Boulanger, M.C., et al., *Methylation of Tat by PRMT6 regulates human immunodeficiency virus type 1 gene expression.* J Virol, 2005. **79**(1): p. 124-31.
29. Invernizzi, C.F., et al., *PRMT6 diminishes HIV-1 Rev binding to and export of viral RNA.* Retrovirology, 2006. **3**: p. 93.
30. Invernizzi, C.F., et al., *Arginine methylation of the HIV-1 nucleocapsid protein results in its diminished function.* AIDS, 2007. **21**(7): p. 795-805.
31. Suhasini, M. and T.R. Reddy, *Cellular proteins and HIV-1 Rev function.* Curr HIV Res, 2009. **7**(1): p. 91-100.
32. Tange, T.O., T.H. Jensen, and J. Kjems, *In vitro interaction between human immunodeficiency virus type 1 Rev protein and splicing factor ASF/SF2-associated protein, p32.* J Biol Chem, 1996. **271**(17): p. 10066-72.
33. Thomas, D., et al., *Forster resonance energy transfer measurements of cofactor-dependent effects on protein arginine N-methyltransferase homodimerization.* Protein Sci, 2010. **19**(11): p. 2141-51.
34. Kuhn, P., et al., *Automethylation of CARM1 allows coupling of transcription and mRNA splicing.* Nucleic Acids Res, 2010.
35. Cha, B., et al., *Methylation by protein arginine methyltransferase 1 increases stability of Axin, a negative regulator of Wnt signaling.* Oncogene, 2011. **30**(20): p. 2379-89.
36. Yamamoto, N., et al., *Analysis of human immunodeficiency virus type 1 integration by using a specific, sensitive and quantitative assay based on real-time polymerase chain reaction.* Virus Genes, 2006. **32**(1): p. 105-13.
37. Adachi, A., et al., *Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone.* J Virol, 1986. **59**(2): p. 284-91.
38. Rasband, W.S., *ImageJ*, N.I.o. Health, Editor. 1997-2011: Bethesda, Maryland, USA.
39. Donahue, D.A., et al., *Stage-dependent inhibition of HIV-1 replication by antiretroviral drugs in cell culture.* Antimicrob Agents Chemother, 2010. **54**(3): p. 1047-54.

40. Schader, S.M., et al., *In vitro resistance profile of the candidate HIV-1 microbicide drug dapivirine.* Antimicrob Agents Chemother, 2012. **56**(2): p. 751-6.
41. Willemsen, N.M., et al., *Protein methylation is required to maintain optimal HIV-1 infectivity.* Retrovirology, 2006. **3**: p. 92.
42. Shirakawa, K., et al., *Phosphorylation of APOBEC3G by protein kinase A regulates its interaction with HIV-1 Vif.* Nat Struct Mol Biol, 2008. **15**(11): p. 1184-91.
43. Dussart, S., et al., *APOBEC3G ubiquitination by Nedd4-1 favors its packaging into HIV-1 particles.* J Mol Biol, 2005. **345**(3): p. 547-58.
44. Douaisi, M., et al., *The tyrosine kinases Fyn and Hck favor the recruitment of tyrosine-phosphorylated APOBEC3G into vif-defective HIV-1 particles.* Biochem Biophys Res Commun, 2005. **329**(3): p. 917-24.
45. Lakowski, T.M., et al., *Neta-substituted arginyl peptide inhibitors of protein arginine N-methyltransferases.* ACS Chem Biol, 2010. **5**(11): p. 1053-63.
46. Choi, S., et al., *PRMT3 inhibits ubiquitination of ribosomal protein S2 and together forms an active enzyme complex.* Biochim Biophys Acta, 2008. **1780**(9): p. 1062-9.
47. Jarmalavicius, S., U. Trefzer, and P. Walden, *Differential arginine methylation of the G-protein pathway suppressor GPS-2 recognized by tumor-specific T cells in melanoma.* FASEB J, 2010. **24**(3): p. 937-46.

Chapter 3

Reverse transcriptase inhibitor-resistance substitutions can change the HIV-1 replication profile in primary human cells

The following chapter was adapted from the manuscript in preparation entitled “Reverse transcriptase inhibitor-resistance substitutions can change the HIV-1 replication profile in primary human cells” authored by Diane N. Singhroy, Thibault Mesplède, Christina Qian, and Mark A. Wainberg . Approximately 90% of the experiments and data analysis were performed by me under the supervision of Dr. Mark Wainberg. Dr. Thibault Mesplède helped with the project design and analysis. Christina Qian helped generate the some of the pSF162R3_Nef+ drug resistant mutant plasmids under my supervision. Dr. Wainberg and Dr. Mesplède critiqued the manuscript.

3.1 Preface

We next examined another intrinsic immune factor, SAMHD1. The ability of SAMHD1 to decrease dNTP pools in macrophages, dendritic cells and resting CD4+ T-cells is important for its HIV-1-restriction activity [1-3]. By limiting the pool of dNTPs available during HIV-1 reverse transcription, SAMHD1 could prevent the completion of this crucial step, leading to an abortive infection in cells expressing SAMHD1 [4]. The role of SAMHD1 in HIV-1 restriction in peripheral blood mononuclear cells (PBMC) was unambiguously proven through HIV-1 infection of monocyte-derived CD14+ cells from Aicardi-Goutières syndrome (AGS) patients with mutations in the *SAMHD1* gene, since these cells are naturally resistant to infection in healthy subjects [5].

In the past, we have characterized multiple mutations in reverse transcriptase (RT) that confer resistance to either nucleoside reverse transcriptase inhibitors (NRTI) or non-nucleoside reverse transcriptase inhibitors (NNRTI). M184V and M184I are known to confer high-levels of resistance to emtricitabine (FTC) and lamivudine (3TC) [6, 7]. Alone, these mutations also cause reduced viral replicative fitness because they result in diminished RT processivity and decreased binding affinity of dNTP substrates for RT [8]. Also, E138A/G/K/Q mutations in RT were shown to confer resistance to etravirine (ETR) [9, 10]. Recently, two phase III clinical trials, -ECHO and THRIVE-, found that the combination of E138K and M184I was common in patients who failed treatment with the second generation NNRTIs ETR or rilpivirine (RLV) while also on background regiments of FTC and tenofovir (TDF) [11, 12]. Importantly, biochemical characterization of

purified recombinant RT proteins showed that E138K restored the loss in enzyme processivity associated with M184V/I alone [8]. Moreover, when E138K and M184V/I occurred in combination, the virus showed increased fitness due to increased RT processivity. These effects were observable only in the presence of low concentrations of dNTPs. In the presence of high concentration of dNTPs, no differences were observed between the different WT or mutated enzymes (Table 3.1).

Table 3.1. Summary of RTI resistance phenotypes of mutated viruses

	High dNTP concentration		Low dNTP concentration		MT2 cells 9wks	CBMC 19wks		TZM-bl
Genotype	**RT Processivity**	**DNA polymerase rate**	**RT Processivity**	**DNA polymerase rate**	**Mutation(s) selected Any drug**	**Mutation(s) selected Any drug**	**Binding affinity (Km)**	**Replication capacity**
WT	Norm	Med	low	Med	M184I/V, E138K	E138K	High	high
E138K	Norm	Low	Very High	Low	M184I	M184V	Very High	Low
M184I	Norm	Med	Very low	Very low	E138K	E138K	Very Low	Low
M184V	Norm	Med	Very low	Very low	E138K	E138K	Med	Med/Low
E138K/M184I	Norm	High	High	High	NA	NA	Med	High
E138K/M184V	Norm	Med	high	high	NA	NA	High	High
Reference	[8]	[8]	[8]	[8]	[13]	[13]	[8]	[8]

Considering that SAMHD1 is a host restriction factor that reduces the dNTP pool in myeloid cells and resting CD4+ T-cells and that we have characterized mutations in HIV-1 RT that allow this enzyme to be more active in the presence of limited amounts of dNTPs, I hypothesized that HIV-1 viruses harboring these NRTI/NNRTI resistance mutations may overcome SAMHD-1 restriction. Our objective was to determine if viruses bearing E138K/M184V/I mutations grow better in cells that have naturally low dNTP concentrations, such as macrophages and dendritic cells, and the role that this may play in overcoming SAMHD1 restriction.

The potential implications of this study are considerable. If our hypothesis is true, some viruses may have acquired drug-resistance mutations that may have improved their ability to infect macrophages and dendritic cells, because they would be more naturally resistant to the host restriction factor SAMHD1. This is of great concern since M184I/V mutations are among the most prevalent NRTI-resistant mutations. Furthermore, E138K is selected by ETR and RPV, second generation NNRTIs that otherwise show excellent efficacy as antiviral drugs. SAMHD1 is expressed in non-proliferating cells such as macrophages and dendritic cells but also in resting CD4+ T-cells; thus, this observation could have important repercussions for the constitution of viral reservoirs, viral tropism, and the establishment of latency.

3.2 Abstract

We have previously characterized the enzymatic properties and phenotypes of mutated viruses containing M184I/V and E138K, alone or in combination, that are resistant to second generation NNRTIs such as ETR, as seen in the clinical trials ECHO and THRIVE. These RTI resistant mutant viruses display different enzymatic processivity and polymerization rates under high and low dNTP levels, respectively. SAMHD1 is a recently identified host restriction factor that inhibits viral replication by limiting dNTP stores in myeloid cells and resting CD4+ T-lymphocytes. It is therefore conceivable that cells possessing different dNTP levels would be more susceptible to drug resistant mutants. Accordingly, we infected various primary immune cells with RTI resistant virus and measured viral infection by qRT-PCR. Additionally, with the same viruses we infect THP-1 stable cells lines that express or are down-regulated for SAMHD1.

We found that viruses harboring M184V replicated better than wild type in resting CD4+ lymphocytes and MDMs. M184I containing viruses replicated better in cells with higher dNTP levels such as activated CD4+ lymphocytes and THP-1 lacking SAMHD1.

3.3 Introduction

Human immunodeficiency virus 1 (HIV-1) has a limited ability to infect monocyte derived dendritic cells and macrophages [2]. However, transduction of Vpx from HIV-2 or *Sooty mangbey* SIV_{smm} can alleviate this restriction [14, 15]. The cellular protein targeted by Vpx for degradation and responsible for HIV-1 restriction in these cells was recently identified through mass spectrometry as the SAM and HD domain containing protein 1 SAMHD1 [2, 14]. Mutated SAMHD1 is one of the known causative genes of the rare autosomic disease Aicardi-Goutières Syndrome (AGS), whose symptoms resemble a congenital viral infection [16]. Trex1, another noteworthy protein contributing to this syndrome, was recently shown to participate in the degradation of HIV-1 reverse transcribed DNA [17]. SAMHD1 HIV-1 restriction is based on its deoxynucleotide triphosphohydrolase activity that degrades dNTP stores in the host cell [1, 18]. Drug resistance emerges under selective pressure through random mutation in the viral transcript due to an error prone RT. Previous work in our lab has demonstrated that E138K can compensate for the poor RT processivity of M184I/V enzymes at low dNTP concentrations *in vitro* [8]. Importantly, these mutations emerged clinically in patients who failed drug regimens containing the second generation NNRTI RLV [19].

Here, we investigated the consequence of these mutations in primary cells in order to determine whether E138K/M184I/V also possesses an evolutionary advantage in ability to grow in cells with low dNTP pools, that is also important for establishment of the HIV-1 reservoir and, therefore, for the long term archiving of drug resistant mutated viruses. Our results show that RT-resistant viruses display differences in their susceptibility to SAMHD1 inhibition and in their ability to replicate within different primary human cells.

3.4. Materials and Methods

3.4.1. Stable SAMHD1 shRNA THP-1 cell line

THP-1 cells (ATCC# TIB-202) were transduced with SAMHD1-shRNA lentiviral particles or Control- shRNA lentiviral particles (both from Santa Cruz Biotechnology) and selected with puromycin (Invitrogen). Stable cell lines were established and cultured in Roswell Park Memorial Institute media (RPMI) (Gibco) with 10% FBS and 3µg/mL puromycin (Invitrogen) and will be referred to as THP1-dSAM or THP1-ctl. Before infections, THP1 stable cell lines were differentiated into macrophage-like cells through use of 20ng/ml of PMA (Invitrogen) over 3 days.

3.4.2. Primary cell isolation

PBMCs were isolated from donor whole blood using Ficoll gradient separation and used for the CD4+ T-cell and CD14+ monocyte isolations. Primary resting CD4+ T-cells were isolated from

PBMC using a Dynabeads magnetic bead negative CD4+ T-cell isolation kit (Invitrogen). Resting CD4+ T-cells were cultured in RPMI-1640 culture medium (Gibco) supplemented with 10% FBS and 20 U/ml human interleukin-2 (IL-2). Activated CD4+ T-cells were activated with 10 µg/ml phytohemagglutinin A (PHA) for 72 h. Primary CD14+ monocytes were isolated from fresh PBMCs using the CD14+ magnetic bead isolation kit (Miltenyi Biotec) and cultured in monocyte medium (Iscove's Modified Dulbecco's Medium (IMDM) from Gibco supplemented with 10% FBS). Monocytes were used to generate monocyte-derived macrophages (MDM) and monocyte-derived dendritic cells (MDDC). To produce MDM, monocytes were stimulated with 50ng/ml of GM-CSF in monocyte medium for 6 days. MDDM were generated by stimulation with 10ng/ml GM-CSF and 50ng/ml IL4 (Invitrogen) in monocyte medium. Cells were maintained at 37°C under 5% CO_2.

3.4.3. Virus production

The viral R5-tropic plasmids pSF162R3_Nef+ (donation from Dr. Amanda Brown) and X4-tropic plasmid pBR-NL43-IRES-eGFP (NIH AIDS Research and Reference Reagent Program) were used to generate the HIV-1 drug resistant mutant plasmids $pSF162_{RT(E138K)}$, $pSF162_{RT(M184I)}$, $pSF162_{RT(M184V)}$, $pSF162_{RT(E138K/M184I)}$, $pSF162_{RT(E138K/M184V)}$, and $pBR\text{-}NL43_{RT(E138K)}$, $pBR\text{-}NL43_{RT(M184I)}$, $pBR\text{-}NL43_{RT(M184V)}$, $pBR\text{-}NL43_{RT(E138K/M184I)}$, $pBR\text{-}NL43_{RT(E138K/M184V)}$, through site directed mutagenesis using the following primers: in pSF162R3_Nef+, $E138K_{(RT)}$ sense (5'-ctgcatttaccatacctagtataaacaataagacaccagggatta-3') and antisense (5'-taatccctggtgtcttattgtttatactaggtatggtaaatgcag-3'), $M184I_{(RT)}$ sense (5'-

gacatagttatctatcaatacatagatgatctgtatgtaggatctga-3') and antisense (5'-tcagatcctacatacagatcatctatgtattgatagataactatgtc-3'), M184V$_{(RT)}$ sense (5'-cagacatagttatctatcaatacgtggatgatctgtatgtaggatct-3') and antisense (5'-agatcctacatacagatcatccacgtattgatagataactatgtctg-3'); in pBR-NL43-IRES-eGFP E138K$_{(RT)}$ same as pSF162$_{RT(E138K)}$, M184I$_{(RT)}$ sense (5'-gacatagtcatctatcaatacatagatgatttgtatgtaggatctga-3') and antisense (5'-tcagatcctacatacaaatcatctatgtattgatagatgactatgtc-3'), M184V$_{(RT)}$ sense (5'-cagacatagtcatctatcaatacgtggatgatttgtatgtaggatct-3') and antisense (5'-agatcctacatacaaatcatccacgtattgatagatgactatgtctg-3').

Virus was produced as previously described [20]. Briefly, 293T cells were transfected with Lipofectamine 2000 reagent according to the manufacturer's guidelines (Invitrogen) with 8µg of HIV-1 mutant or wild type plasmids. After 48 hours, culture supernatants were harvested, centrifuged at 1,200 rpm and passed through a 0.45 µm filter and treated with Benzonase (Sigma-Aldrich). Viral particles in the supernatants were quantified by p24 ELISA as described previously. Viral stocks were aliquoted and stored at -80°C.

3.4.4. Infections

At 24h before infection, puromycin was removed from differentiated THP-1 stable cells. Stable cells were infected with 300ng of p24 of HIV-1 BR-NL43-IRES-eGFP wild type or mutant virus for 200,000 cells through spinoculation. Cells were incubated with virus for 2hr at 2,700 rpm at 25 °C, followed by 3 hours of incubation at 37 °C. Virus was then removed by 2 rounds of washes

and RPMI 10% FBS supplemented media was added. Cells were collected after 5 days and pellets were stored at -80°C until needed.

Cells were infected as described above with 300ng of p24 for 200,000 cells. Infection was optimized based on the level of p24 needed for productive infection in MDMs. CD4+ T-cells were infected with BR-NL43-IRES-eGFP wild type or mutant virus while MDM and MDDM were infected with pSF162R3_Nef+ wild type or mutant virus. Cells were collected and pelleted after 5 days of infection. All experiments were done in duplicate.

3.4.5. Multiplex quantitative real time PCR

Total RNA was extracted using AllPrep DNA/RNA mini kit (Qiagen). qRT-PCR was performed with a SuperScript III Platinum one-step quantitative RT-PCR system (Invitrogen) using a Corbett Rotor-Gene 6000 thermocycler (Corbett). The samples were normalized for their GAPDH mRNA content. Primers/probe set IDs for GAPDH and SAMHD1 were GAPDH Hs03929097_g1 and Hs00210019_m1, respectively (Invitrogen, Thermo Fisher Scientific). The GAPDH probe was tagged with VIC and MGB_pl. The SAMHD1 probe was tagged with FAM and MGB. HIV-1 Gag primer sequences were: sense (5'-AGTGGGGGGACATCAAGCAGC-3') and antisense (5'-TACTAGTAGTTCCTGCTATGTC-3'); and probe (FAM 3'-ATCAATGAGGAAGCTGCAGAATGGGA-3'BHQ) [21]. Cycling conditions were 50°C for 15 min, 95°C for 10 min, and 70 repeats of 95°C for 15 sec and 60°C for 30 sec. Multiplex qPCR was analyzed using the $2^{-\Delta\Delta CT}$ method and plotted using GraphPad Prism 4.0 Software.

3.5 Results

3.5.1 SAMHD1 inhibits HIV replication in macrophage-like THP-1 cells

To test the role of SAMHD1 in the restriction of NRTI-resistant HIV-1 viruses, THP1-dSAM or THP1-ctl stable cells lines were infected with viruses containing the E138K, M184V and/or M184I substitutions (Figure 3.1). First, we verified that knockdown was efficacious in THP1 and observed a >100 fold reduction in the levels of SAMHD1 mRNA under each condition, as measured by quantitative PCR (Figure 3.1A). Next we quantified the replication of BR-NL43$_{WT}$, BR-NL4.3$_{E138K}$, BR-NL4.3$_{M184V}$, BR-NL4.3$_{M184I}$, BR-NL4.3$_{E138K/M184V}$ and BR-NL4.3$_{E138K/M184I}$ viruses in THP1-dSAM or THP1-ctl cells by measuring the levels of cell-associated HIV RNA (Figure 3.1B). This analysis showed that replication levels of the BR-NL4.3$_{E138K}$, BR-NL4.3$_{M184I}$, BR-NL4.3$_{M184V}$, and BR-NL4.3$_{E138K/M184I}$ viruses were lower than that of the BR-NL4.3$_{WT}$ virus when SAMHD1 was present. Under these conditions, the BR-NL4.3$_{WT}$ and BR-NL4.3$_{E138K/M184V}$ viruses attained similar levels of replication.

SAMHD1 knockdown resulted in a 2-fold increase in the level of replication of the BR-NL4.3$_{WT}$ virus. Replication levels were increased to a greater extent for the BR-NL4.3$_{M184I}$ mutant virus. Replication levels of the BR-NL4.3$_{E138K}$, BR-NL4.3$_{M184V}$, and BR-NL4.3$_{E138K/M184V}$ viruses were also increased by abrogating SAMHD1 expression but remained lower than that of BR-NL4.3$_{WT}$ in the absence of SAMHD1. All mutant viruses replicated better in the absence of SAMHD1 than did the BR-NL4.3$_{WT}$ virus in the presence of this protein. SAMHD1 knockdown had no effect on the replication of the BR-NL4.3$_{E138K/M184V}$ mutant virus.

When replication levels were normalized to those measured in the presence of SAMHD1 (Figure 3.1C), we observed a 4-fold increase for the E138K and M184V viruses, and a 6-fold increase for the M184I virus. E138K/M184I benefited from a 3-fold increase in replication when SAMHD1 expression was inhibited.

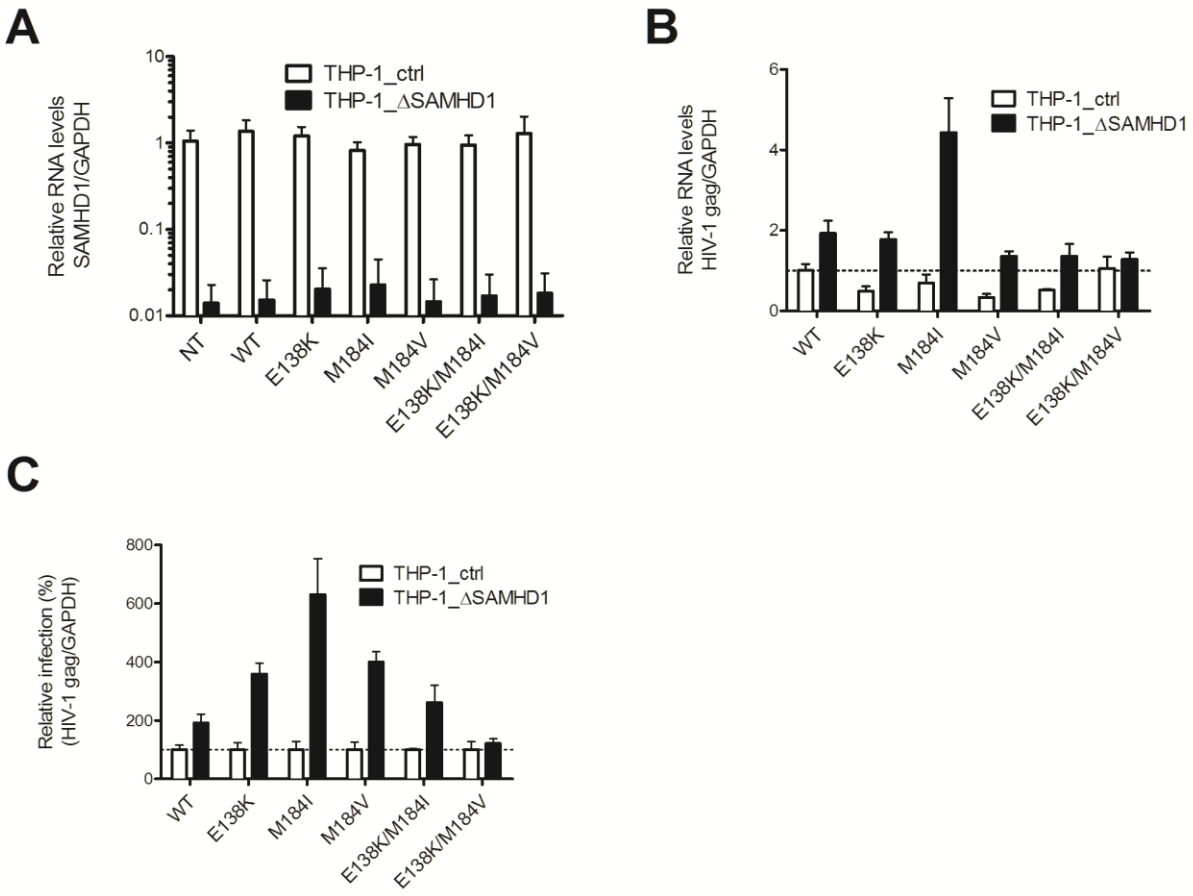


Figure 3.1: SAMHD1 knockdown alters the replication of RTI-resistant HIV-1. SAMHD1 mRNA levels (A), HIV-1 replication levels quantified by Q-PCR of viral RNA normalized by the quantity of GAPDH mRNA (B), and HIV infection relative to WT (C), were measured in THP1 cells transfected with a control siRNA (THP1-ctl, white bars) or with a shRNA targeting SAMHD1 (THP1-dSAM, black bars). Note that the y-axis for Figure 3.1A is on a log scale to accommodate very small THP-1_dSAM values. Experiments were performed in triplicate.

3.5.2 CD4+ T-lymphocyte activation changes the tropism of HIV drug resistant viruses

CD4+ T-lymphocyte activation increases dNTP levels, at least in part through CDK2-mediated phosphorylation and inhibition of SAMHD1 [22]. Accordingly, we investigated levels of replication of RTI-resistant viruses in quiescent or activated CD4+ T-lymphocytes (Figure 3.2). In activated CD4+ T-lymphocytes, levels of replication were similar between BR-NL4.3$_{WT}$ and all the mutated viruses with the exception of the BR-NL4.3$_{M184I}$ and BR-NL4.3$_{E138K/M184V}$ viruses that displayed increased levels of replication. Compared to resting CD4+ T-lymphocytres, activation resulted in increased viral RNA levels in cells infected with BR-NL4.3$_{WT}$, BR-NL4.3$_{M184I}$, BR-NL4.3$_{E138K/M184I}$ and BR-NL4.3$_{E138K/M184V}$. In contrast, the presence of the E138K substitution rendered HIV insensitive to the positive effects of T-cell activation. In addition, levels of replication of the BR-NL4.3$_{M184V}$ virus were only doubled by activation but, in contrast to BR-NL4.3$_{E138K}$, this was due to high levels of replication in quiescent resting CD4+ T-lymphocytes. BR-NL4.3$_{M184V}$ levels of replication in resting CD4+ T-lymphocytes were similar to those attained by the WT virus in activated cells.

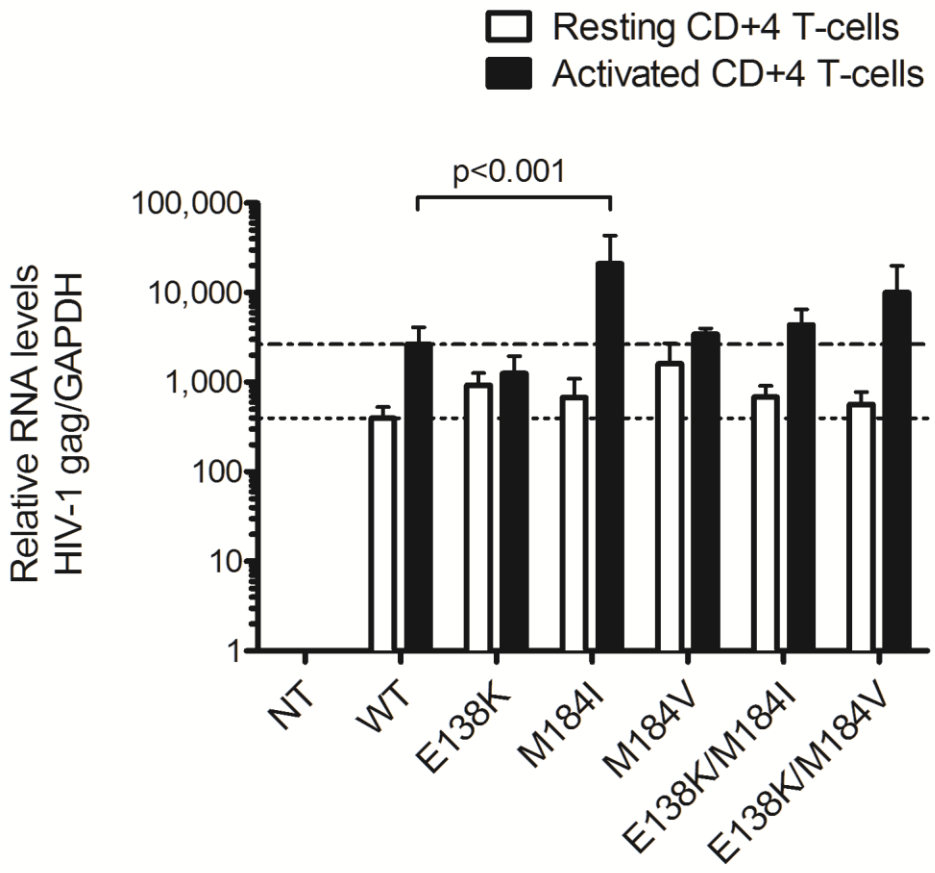


Figure 3.2: CD4+ T-lymphocyte activation alters the replication of RTI-resistant HIV-1. HIV-1 replication levels quantified by Q-PCR of viral RNA normalized by the quantity of GAPDH mRNA were measured in resting (white) and activated (black) primary human CD4+ T-lymphocytes. Note that the y-axis is log scale to accommodate for smaller values in resting T-lymphocytes. Experiments were repeated at least three times.

3.5.3 NRTI-resistance substitutions can alter HIV-1 replication profiles in myeloid cells

SAMHD1 has been shown to inhibit HIV replication in CD14+ myeloid cells. Accordingly, we investigated the effects of the E138K, M184I and M184V substitutions alone or in combination on HIV replication in MDDC and MDM (Figure 3.3). Our results show that $SF162_{M184V}$ was the

only NRTI-resistant virus to replicate better than the WT virus in MDDC whereas replication of other resistant viruses was diminished (Figure 3.3A). In primary human MDM, replication levels of $SF162_{M184I}$, $SF162_{M184V}$, $SF162_{E138K/M184I}$ and $SF162_{E138K/M184V}$ were decreased compared to WT whereas the E138K substitution had no significant effect on HIV-1 replication (Figure 3.3B).

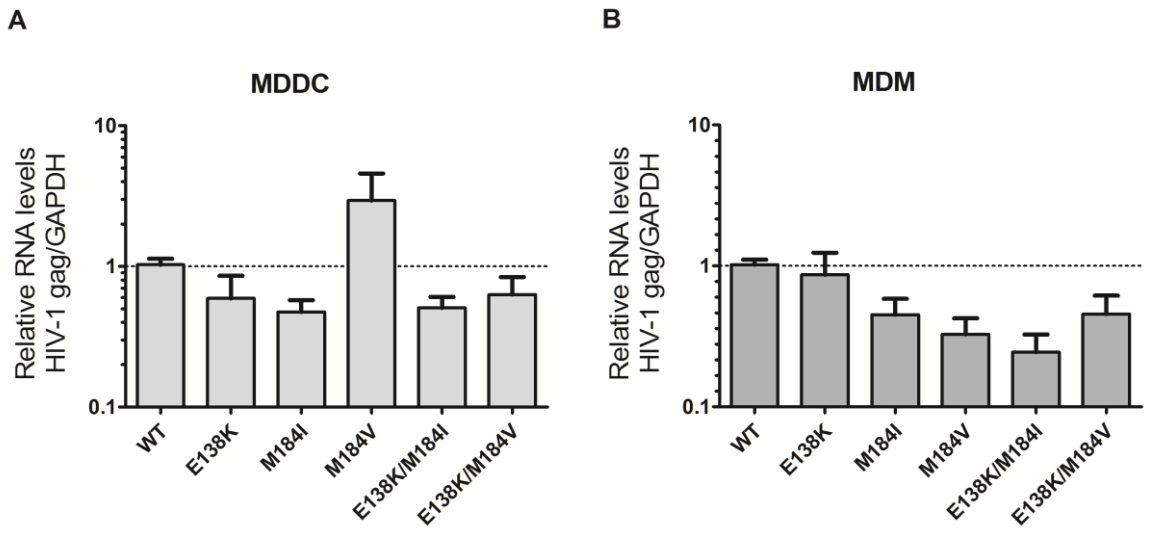


Figure 3.3: HIV-1 replication in myeloid cells is affected by NRTI-resistance. HIV-1 replication levels quantified by Q-PCR of viral RNA normalized by the quantity of GAPDH mRNA in primary CD14+ monocyte derived dendritic cells (A) and terminally CD14+ monocyte- differentiated macrophages (B). Y-axis is in log scale. Experiments were performed in triplicate.

3.6 Discussion

SAMHD1 has been shown to inhibit HIV-1 replication at least in part through the degradation of cellular dNTPs that are necessary for efficient reverse transcription. Some HIV viruses have evolved to become resistant against reverse transcriptase inhibitors through changes in their

reverse transcriptase enzymes that improve dNTP use. Here we investigated if such changes can affect HIV-1 susceptibility to SAMHD1 inhibition and on viral ability to replicate in various cell types in which SAMHD1 has been deactivated. Our results show that RT-resistant viruses display differences in their susceptibility to SAMHD1 inhibition and in their ability to replicate within different primary human cells.

As expected, $NL4.3_{WT}$ replicated better in THP1 cells in which SAMHD1 expression was knocked down than in SAMHD1-positive cells (Figure 3.1) and in activated CD4+ T-lymphocytes vs. resting T-lymphocytes (Figure 3.2). Although the E138K substitution has been previously associated with decreased K_M in biochemical reverse transcriptase assays with purified recombinant enzymes [8], our current study shows that the replication of the $NL4.3_{E138K}$ virus was inhibited by SAMHD1 and was increased following SAMHD1 knockdown in THP1 cells (Figure 3.1). Given that the calculated K_M for the RT_{E138K} enzyme was 2.59µM, compared to 5.63µM for RT_{WT}, this suggests that THP1 dNTP levels in the presence of SAMHD1 were <2.59µM. The increased ability of RT_{E138K} to bind dNTP may also explain the fact that the $SF162_{E138K}$ virus attained levels of replication similar to those of $SP162_{WT}$ in terminally differentiated human macrophages (Figure 3.3B), that have been shown previously to contain between 0.04 and 0.3µM dNTP [23, 24]. In contrast with SAMHD1 knockdown in THP1 cells, CD4+ T-lymphocyte activation did not result in increased levels of replication for the $NL4.3_{E138K}$ virus vs. $NL4.3_{WT}$ (Figure 3.2). Given that $NL4.3_{E138K}$ replication was modestly increased in resting CD4+ T-lymphocytes compared to WT ($p>0.05$, Student's *t* test) (Figure 3.2), this suggests that dNTP levels in these cells may be >2.59µM but <5.63µM (Figure 3.4). Importantly,

this values correlates with previously published dNTP levels in resting CD4+ T-lymphocytes that were found to be approximately 3.4µM [25].

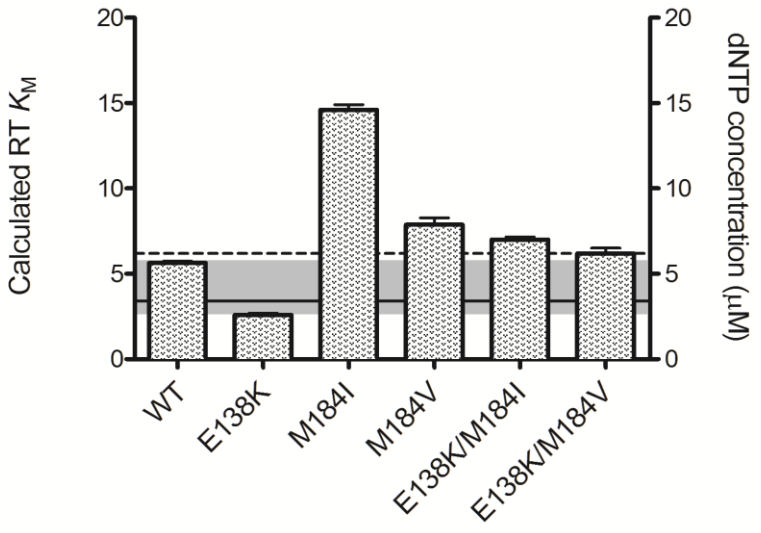


Figure 3.4: Calculated RT K_Ms help to explain replication levels in resting and activated CD4+ T-lymphocytes. Previously reported K_Ms for WT, E138K, M148I, M184V, E138K/M184I, and E138K/M184V RT enzymes are presented [8]. Plain and dashed bars represent previous estimates of dNTP levels in resting and activated CD4+ T-lymphocytes, respectively [25]. The shaded area indicates our estimate of dNTP levels in resting CD4+ T-lymphocytes.

The same study estimated dNTP levels to be ~6.2µM in activated CD4+ T-lymphocytes [25]. However, we show here that $NL4.3_{M184I}$ replicated at higher levels than $NL4.3_{WT}$ in these cells. Given that we have previously shown the Michaelis constant (K_M) of the RT_{M184I} enzyme to be 14.6µM [8], our results suggest that dNTP levels may actually be >6.2µM in activated CD4+ T-lymphocytes. This hypothesis is supported by previous estimates for dNTP levels in activated CD4+ T-lymphocytes that varied from 3 to 30µM [23].

Neither of the E138K, M184I/V or E138K/M184I substitutions increased the RT K_M as much as M184I [8]. This indicates that RT_{M184I} required larger amounts of dNTP than RT_{WT} and other mutant enzymes to reach half-maximal reverse transcription activity. Accordingly, BR-NL4.3$_{M184I}$ was most favourably impacted by SAMHD1 knockdown in THP1 cells compared to BR-NL4.3$_{WT}$ and other mutant viruses (Figure 3.1). BR-NL4.3$_{M184I}$ replication levels were also most highly induced following CD4+ T-lymphocyte activation compared to resting cells (Figure 3.2).

In THP1 cells, the replication of viruses containing either of the E138K or M148V substitutions was increased as a consequence of SAMHD1 knockdown whereas replication of the virus containing both substitutions in combination (E138K/M184V) was unaffected (Figure 3.1). In contrast, BR-NL4.3$_{E138K/M184I}$ replication was increased by shRNA against SAMHD1 (Figure 3.1). Given that K_M values for $RT_{E138K/M184I}$ and $RT_{E138K/M184V}$ are similar, i.e. 7µM and 6.2µM, respectively (Figure 3.4), this suggests that an uncharacterized restriction mechanism specifically inhibited BR-NL4.3$_{E138K/M184V}$ replication in THP1 cells. All other observations with doubly mutated viruses can be explained by a dNTP-dependent inhibition of HIV-1 replication. In particular, replication of both BR-NL4.3$_{E138K/M184I}$ and BR-NL4.3$_{E138K/M184V}$ was also increased in activated CD4+ T-lymphocytes compared to resting cells (Figure 3.2).

In THP1 cells expressing SAMHD1 and primary macrophages that both contain low dNTP levels, replication of NRTI-resistant viruses was lower than BR-NL4.3$_{WT}$ (Figures 3.1 and 3.3B). Similar observations were made in dendritic cells, with the exception of the BR-NL4.3$_{M184V}$ virus that replicated at statistically non-significantly higher levels than BR-NL4.3$_{WT}$ (Figure 3.3A). This observation cannot be explained by dNTP levels that were estimated to be between 0.001µM

and 0.4μM and seemingly contradicts previous studies [23, 26]. This apparent contradiction may be due to differences in the methods used to isolate dendritic cells between our study and those of others. In the current study, dendritic cells were isolated using a CD14+ isolation kit whereas previous studies have differentiated unsorted PBMCs into dendritic cells. Another factor that may especially affect dNTP levels in MDMs is ecto-5'-nucleotidase (ref:Battastini). MDM typically polarize to M1 when stimulated with GM-CSF and have decreased amounts of nucleotidase compared to M2. As a result, M2 should be more refractory to HIV-1 infection and it would be interesting to measure if there is redundancy with SAMHD1 controlled dNTP pools.

In conclusion, our study demonstrates that the ability of SAMHD1 to inhibit the replication of NRTI-resistant HIV strains correlates with the affinity of reverse transcriptase to interact with dNTPs in THP1 cells The catalytic activity of RTI-resistant RT also helps to explain differences in the ability of the corresponding viruses to infect primary human cells.

3.7. References

1. Goldstone, D.C., et al., *HIV-1 restriction factor SAMHD1 is a deoxynucleoside triphosphate triphosphohydrolase.* Nature, 2011. **480**(7377): p. 379-82.
2. Laguette, N., et al., *SAMHD1 is the dendritic- and myeloid-cell-specific HIV-1 restriction factor counteracted by Vpx.* Nature, 2011. **474**(7353): p. 654-7.
3. Baldauf, H.M., et al., *SAMHD1 restricts HIV-1 infection in resting CD4(+) T cells.* Nat Med, 2012. **18**(11): p. 1682-7.
4. Kim, B., et al., *Tight interplay among SAMHD1 protein level, cellular dNTP levels, and HIV-1 proviral DNA synthesis kinetics in human primary monocyte-derived macrophages.* J Biol Chem, 2012. **287**(26): p. 21570-4.
5. Berger, A., et al., *SAMHD1-deficient CD14+ cells from individuals with Aicardi-Goutieres syndrome are highly susceptible to HIV-1 infection.* PLoS Pathog, 2011. **7**(12): p. e1002425.
6. Turner, D., B. Brenner, and M.A. Wainberg, *Multiple effects of the M184V resistance mutation in the reverse transcriptase of human immunodeficiency virus type 1.* Clin Diagn Lab Immunol, 2003. **10**(6): p. 979-81.
7. Petrella, M., et al., *Differential maintenance of the M184V substitution in the reverse transcriptase of human immunodeficiency virus type 1 by various nucleoside antiretroviral agents in tissue culture.* Antimicrob Agents Chemother, 2004. **48**(11): p. 4189-94.
8. Xu, H.T., et al., *Compensation by the E138K mutation in HIV-1 reverse transcriptase for deficits in viral replication capacity and enzyme processivity associated with the M184I/V mutations.* J Virol, 2011. **85**(21): p. 11300-8.
9. Tambuyzer, L., et al., *Characterization of genotypic and phenotypic changes in HIV-1-infected patients with virologic failure on an etravirine-containing regimen in the DUET-1 and DUET-2 clinical studies.* AIDS Res Hum Retroviruses, 2010. **26**(11): p. 1197-205.
10. Tambuyzer, L., et al., *Effect of mutations at position E138 in HIV-1 reverse transcriptase on phenotypic susceptibility and virologic response to etravirine.* J Acquir Immune Defic Syndr, 2011. **58**(1): p. 18-22.
11. Cohen, C.J., et al., *Rilpivirine versus efavirenz with two background nucleoside or nucleotide reverse transcriptase inhibitors in treatment-naive adults infected with HIV-1 (THRIVE): a phase 3, randomised, non-inferiority trial.* Lancet, 2011. **378**(9787): p. 229-37.
12. Molina, J.M., et al., *Rilpivirine versus efavirenz with tenofovir and emtricitabine in treatment-naive adults infected with HIV-1 (ECHO): a phase 3 randomised double-blind active-controlled trial.* Lancet, 2011. **378**(9787): p. 238-46.
13. Xu, H.T., et al., *Subunit-selective mutational analysis and tissue culture evaluations of the interactions of the E138K and M184I mutations in HIV-1 reverse transcriptase.* J Virol, 2012. **86**(16): p. 8422-31.
14. Hrecka, K., et al., *Vpx relieves inhibition of HIV-1 infection of macrophages mediated by the SAMHD1 protein.* Nature, 2011. **474**(7353): p. 658-61.
15. Bobadilla, S., N. Sunseri, and N.R. Landau, *Efficient transduction of myeloid cells by an HIV-1-derived lentiviral vector that packages the Vpx accessory protein.* Gene Ther, 2013. **20**(5): p. 514-20.
16. Rice, G.I., et al., *Mutations involved in Aicardi-Goutieres syndrome implicate SAMHD1 as regulator of the innate immune response.* Nat Genet, 2009. **41**(7): p. 829-32.
17. Yan, N., et al., *The cytosolic exonuclease TREX1 inhibits the innate immune response to human immunodeficiency virus type 1.* Nat Immunol, 2010. **11**(11): p. 1005-13.

18. Powell, R.D., et al., *Aicardi-Goutieres syndrome gene and HIV-1 restriction factor SAMHD1 is a dGTP-regulated deoxynucleotide triphosphohydrolase.* J Biol Chem, 2011. **286**(51): p. 43596-600.
19. Zaharatos, G.J. and M.A. Wainberg, *Update on rilpivirine: a new potent non-nucleoside reverse transcriptase inhibitor (NNRTI) of HIV replication.* Ann Med, 2013. **45**(3): p. 236-41.
20. Singhroy, D.N., et al., *Automethylation of protein arginine methyltransferase 6 (PRMT6) regulates its stability and its anti-HIV-1 activity.* Retrovirology, 2013. **10**: p. 73.
21. Kondo, M., et al., *Quantitation of HIV-1 group M proviral DNA using TaqMan MGB real-time PCR.* J Virol Methods, 2009. **157**(2): p. 141-6.
22. Pauls, E., et al., *Cell cycle control and HIV-1 susceptibility are linked by CDK6-dependent CDK2 phosphorylation of SAMHD1 in myeloid and lymphoid cells.* J Immunol, 2014. **193**(4): p. 1988-97.
23. St Gelais, C., et al., *SAMHD1 restricts HIV-1 infection in dendritic cells (DCs) by dNTP depletion, but its expression in DCs and primary CD4+ T-lymphocytes cannot be upregulated by interferons.* Retrovirology, 2012. **9**: p. 105.
24. Kennedy, E.M., et al., *Ribonucleoside triphosphates as substrate of human immunodeficiency virus type 1 reverse transcriptase in human macrophages.* J Biol Chem, 2010. **285**(50): p. 39380-91.
25. Diamond, T.L., et al., *Macrophage tropism of HIV-1 depends on efficient cellular dNTP utilization by reverse transcriptase.* J Biol Chem, 2004. **279**(49): p. 51545-53.
26. Pingen, M., et al., *Diminished transmission of drug resistant HIV-1 variants with reduced replication capacity in a human transmission model.* Retrovirology, 2014. **11**: p. 113.

Chapter 4

Combination of the R263K and M184I/V Resistance Substitutions against Dolutegravir and Lamivudine Decreases HIV Replicative Capacity

The following chapter was adapted from the published manuscript entitled "Combination of the R263K and M184I/V Resistance Substitutions against Dolutegravir and Lamivudine Decreases HIV Replicative Capacity" authored by Diane N. Singhroy, Thibault Mesplède, and Mark A. Wainberg. Approximately 90% of the experiments and data analysis was performed by me under the supervision of Dr. Mark Wainberg. Dr. Thibault Mesplède helped with the project design and analysis. Dr. Wainberg and Dr. Mesplède critiqued the manuscript.

4.1 Preface

The integrase inhibitor dolutegravir (DTG) is currently formulated as a single pill or in combination with the reverse transcriptase inhibitors lamivudine (3TC) and abacavir (ABC). There is also a great interest in dual therapy that will combine only DTG+3TC either for treatment simplification for individuals who are currently successfully treated with any ART regimen or for treatment initiation for newly diagnosed individuals. Importantly, DTG has a high genetic barrier to resistance, as demonstrated by the absence of resistance mutations in treatment-naive individuals who have experienced treatment failure with this drug. In treatment-experienced individuals exposed to DTG, treatment failure-associated mutations were rare although 4 out of 6 such individuals developed a R263K substitution in integrase. R263K confers low levels of resistance against DTG and decreases HIV replicative capacity. Similarly, the M184I/V resistance substitutions in reverse transcriptase that confer high level resistance against 3TC are also associated with decreases in HIV fitness. In fact, replicative defects associated with these mutations are usually sufficient to decrease viral loads in individuals who suffer from treatment failure. Given that (i) the therapeutic options described above both involve combining DTG with 3TC and (ii) resistance pathways against both drugs have been shown to decrease viral fitness, we investigated the impact of combining the M184I/V and R263K substitutions on drug resistance and viral replicative capacity. Our results show that combining M184I/V with R263K did not change resistance levels associated with either drug but further decreased HIV-1 replicative capacity compared with either mutation

alone. This suggests that therapeutic regimens combining DTG with 3TC may provide benefits even in cases of treatment failure.

4.2. Abstract

We investigated the effect of combining the dolutegravir-specific R263K integrase resistance substitution with either M184I or M184V, two reverse transcriptase drug resistance substitutions that are frequently detected in individuals failing therapeutic regimens containing either lamivudine or emtricitabine. The presence of R263K and M184I/V in a single virus resulted in substantial further decreases in viral replicative capacity compared to the presence of single substitutions alone.

4.3 Introduction

In treatment-naïve individuals, human immunodeficiency virus (HIV) has been able to develop resistance mutations against all new antiretrovirals (ARV) tested to date with the exception of the strand-transfer integrase inhibitor (INSTI) dolutegravir (DTG) [1, 2]. Virological failure in ARV-naïve individuals receiving DTG was not associated with the emergence of drug resistance mutations against either DTG or other drugs that were co-administered with it [3-6]. By contrast, drug resistance has been reported against other integrase inhibitors, i.e. raltegravir (RAL) and elvitegravir (EVG), in both treatment-naïve and -experienced individuals [7]. The

emergence of resistance substitutions against reverse transcriptase inhibitors co-administered with RAL and EVG has also been reported [4, 8, 9] as has the occurrence of relevant resistance substitutions in patients failing protease inhibitors [10]. The robustness of DTG is likely due, in large part, to a longer residency time of DTG within the HIV integrase catalytic pocket compared to RAL and EVG [11], an observation that is supported by structural modeling [12].
In contrast, resistance against DTG has been reported in treatment-experienced individuals [13, 14], who have previously failed RAL- or EVG-containing regimens [13, 15, 16] and the pre-existence of mutations in integrase at positions Q148H/K/R together with two or more secondary resistance mutations is associated with high rates of virological failure with DTG in second-line therapy [15-18].

Tissue culture selection experiments with DTG have yielded a R263K substitution in integrase [19, 20] that decreases HIV-1 replicative capacity [19] and such studies performed with DTG for more than five years have so far failed to yield any secondary substitution that might compensate for the replicative defects associated with R263K, even though substitutions at positions M50I, H51Y and E138K were identified in these experiments [21-23]. This has led us to hypothesize that the R263K substitution may represent an evolutionary dead-end for HIV and that this may explain the absence of resistance to DTG in treatment-naïve individuals [2]. Furthermore, the R263K substitution was present in 2 highly treatment-experienced individuals treated with DTG who had not previously received an integrase inhibitor and the detection of R263K at week 24 was not followed by the development of additional changes in integrase between weeks 24 and 48, although DTG treatment was maintained [14]. This observation is in

contrast with the rapid genetic diversification of the integrase coding region following virological failure with either RAL or DTG [13, 24]. In addition, the presence of R263K delayed the emergence of the M184I/V resistance mutation in tissue culture selection experiments performed with 3TC [20]. DTG is now co-formulated with two nucleoside reverse transcriptase inhibitors (NRTIs), 3TC and abacavir (ABC) as the once daily, single dose combination Triumeq, and treatment failure with the latter NRTIs in the absence of DTG has commonly led to appearance of the M184I/V resistance substitutions that are associated with resistance against 3TC and emticitabine (FTC) [25, 26]. M184V is also associated with a lower incidence of thymidine analogue mutations and with increased susceptibility to some reverse transcriptase inhibitors such as tenofovir and zidovudine [27, 28]. Both M184I and M184V are also detrimental to HIV replication capacity [26, 29].

It is important to design drug combinations that will limit the likelihood of drug resistance. Since the R263K and M184I/V substitutions decrease HIV replication capacity, we wished to investigate the effect that combining these mutations might have on viral replicative capacity and drug resistance. Here we show that the combination of R263K with M184I/V further decreases HIV replication capacity compared to the presence of the single mutations alone, without altering levels of drug resistance.

4.4.Materials and methodes

4.4.1. Cells and reagents

PM1, 293T and TZM-bl reporter cells were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH, from Dr. Marvin Reitz [30], from Dr. Andrew Rice [31] and Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc. [32-36], respectively. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood obtained from the Jewish General Hospital, Montréal, QC, Canada, as previously described [21]. 293T and TZM-bl cells were grown in DMEM supplemented with 10% FBS, 2mM L-glutamine, 50 U/ml penicillin and 50µg/ml streptomycin. PM1 cells were grown in RPMI that was similarly supplemented. Dolutegravir (DTG) was kindly provided by ViiV Healthcare Ltd. (Research Triangle Park, North Carolina, USA). Lamivudine (3TC) was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH.

4.4.2 Viruses

The pNL4-3 infectious molecular clone bearing the R263K mutation in the integrase coding region has been described previously [19]. The original pNL4-3 plasmid was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH from Dr. Malcolm Martin [37]. The M184I and M184V mutations in reverse transcriptase were introduced within pNL4-3 or pNL4-$3_{IN(R263K)}$ by site-directed mutagenesis using the following primers: M184I-sense (5'-

GACATAGTCATCTATCAATACATAGATGATTTGTATGTAGGATCTGA-3'), M184I-antisense (5'-TCAGATCCTACATACAAATCATCTATGTATTGATAGATGACTATGTC-3'), M184V-sense (5'-CAGACATAGTCATCTATCAATACGTGGATGATTTGTATGTAGGATCT-3') and M184V-antisense (5'-AGATCCTACATACAAATCATCCACGTATTGATAGATGACTATGTCTG-3'), as previously described [19]. The presence of the mutations was confirmed by sequencing (McGill University and Génome Québec Innovation Centre, Montréal, QC, Canada) and two molecular clones for each construct were isolated and used subsequently. Genetically homogenous viruses were produced by transfecting 12.5 µg of plasmid coding for pNL4-3, pNL4-$3_{IN(R263K)}$, pNL4-$3_{RT(M184I)}$, pNL4-$3_{RT(M184V)}$, pNL4-$3_{RT(M184I)/IN(R263K)}$ or pNL4-$3_{RT(M184V)/IN(R263K)}$ into 293T cells using Lipofectamine 2000 and OptiMEM (Life Technologies, Burlington, ON, Canada). Medium was renewed 6 h after transfection. Forty-eight hours later, culture fluids were harvested, purified by centrifugation and filtration at 0.45 µM, treated with Benzonase to remove plasmid DNA (Millipore, Etobicoke, ON, Canada), aliquoted and stored at -80°C. Viral stocks were quantified and normalized by measuring levels of p24 antigen using the Alliance HIV-1 p24 Antigen ELISA kit (Perkin Elmer, Woodbridge, ON, Canada).

4.4.3 Infectivity and resistance assays in TZM-bl cells

Relative infectivity of the recombinant viruses was measured using a non-competitive short-term infectivity assay in TZMbl cells, as previously described [21]. Briefly, 30,000 TZMbl cells were infected with 1:10 serial dilutions of recombinant virus in triplicate. Twenty-four hours later, culture fluids were removed, cells were lysed using the Luciferase Cell Culture

LysisReagent (Promega, Madison, WI, USA), and luciferase activity from the lysate was measured using the Luciferase Assay System kit (Promega, Madison, WI, USA) and a Micro-Beta2 luminometer (Perkin Elmer, Woodbridge, ON, Canada). Resulting luciferase activities were plotted relative to p24 levels. Resistance assays were performed using similar methods except that TZMbl cells were infected with 400ng p24/ml in the presence of 1:3 serial dilutions of either 3TC or DTG.

4.4.4. Replication capacity in PM1 cells

To monitor long-term viral growth in tissue culture, 30,000 PM1 cells were infected with 400ng p24/ml of each recombinant virus in a final 200 µl volume, and p24 levels from 100 µl culture fluids were measured at 3, 5, 7 and 15 days after infection by ELISA as described above. Ten thousand uninfected PM1 cells in 100µl fresh media were added to the culture at each time point to allow continuous viral growth.

4.4.5. Integration by Alu-mediated Q-PCR

Levels of HIV-1 DNA integration into PBMC DNA were measured using a two-step quantitative PCR as previously described [19, 23]. Briefly 250,000 PBMCs were infected with 400ng p24/ml recombinant viruses. Seventy-two hours after infection, cells were harvested, washed twice with PBS and DNA was extracted using the DNeasy Blood and Tissue Extraction kit (Qiagen, Toronto, ON, Canada). Five hundred ng of DNA were amplified by PCR using Alu-sense (5'-

GCCTCCCAAAGTGCTGGGATTACAG-3') and Out-antisense (5'-GTTCCTGCTATGTCACTTCC-3') primers. The cycling conditions were 95°C for 5 min and 50 repeats of 95°C for 15 s, 50°C for 15 s, and 72°C for 3 min 30 s, followed by 72°C for 5 min. Amplification products were purified using the QIAquick PCR Purification kit (Qiagen, Toronto, ON, Canada) and used for quantitative PCR with *int*-sense (5'-TTAAGCCTCAATAAAGCTTGCC-3') and *int*-antisense (5'-GTTCGGGCGCCACTGCTAGA-3') primers and the *int*-probe (5'-FAM-CCAGAGTCACACAACAGAGGGGCACA-TAMRA-3'). Bglo-sense (5'-GGTACGGCTGTCATCACTTAGAC-3') and bglo-antisense (5'-AACGGCAGACTTCTCCTCAG-3') primers and the bglo-probe (5'-FAM-CTCACCCTGTGGAGCCACACC-BHQ1-3') were used to quantify the abundance of the beta-globin gene. All quantitative PCR were performed using the Platinum qPCRSuperMix-UDG kit (Life Technologies) on a Corbett Rotor-Gene 6000 thermocycler (Corbett Life Science, Qiagen). The cycling conditions were 50°C for 2 min, 95°C for 2 min, and 50 repeats of95°C for 10 s, 60°C for 10 s, and 72°C for 30 s. Levels of integrated DNA were normalized to the abundance of the beta-globin gene.

4.4.6. Statistical analysis

Student's t-tests were performed to measure statistical significance using the open-source statistics package OpenEpi (http://www.openepi.com/). Significance was defined as $p<0.05$.

4.5. Results

4.5.1. Effects of combining the M184I/V and R263K substitutions on HIV-1 susceptibility to DTG and 3TC

M184I/V and R263K individually confer high-level and low-level resistance against 3TC and DTG, respectively. However, it is not known what effect combining these substitutions has on drug resistance. Here we measured the susceptibility to 3TC and DTG of NL4.3 viruses bearing the M184I, M184V, R263K, M184I/R263K and M184V/R263K substitutions in TZM-bl cells (Table 4.1). The resulting luciferase activity was plotted relative to the p24 levels, taking into account the basal production of luciferase. The results confirm that both M184I and M184V confer high-level resistance against 3TC by 820- and >1,000-fold, respectively. As expected, R263K did not confer resistance against 3TC and the addition of R263K to M184I or M184V did not significantly change levels of resistance against 3TC (Student's t-test). The M184I/R263K and M184V/R263K combinations of substitutions conferred 677-fold and >1,000-fold resistance against 3TC, respectively. Reciprocally, R263K conferred a low 2.75-fold level of resistance against DTG whereas neither M184I nor M184V had a significant effect on HIV-1 susceptibility to this drug. As expected, the addition of M184I or M184V to R263K did not significantly increase levels of resistance against DTG compared to the R263K substitution alone (Student's t-test).

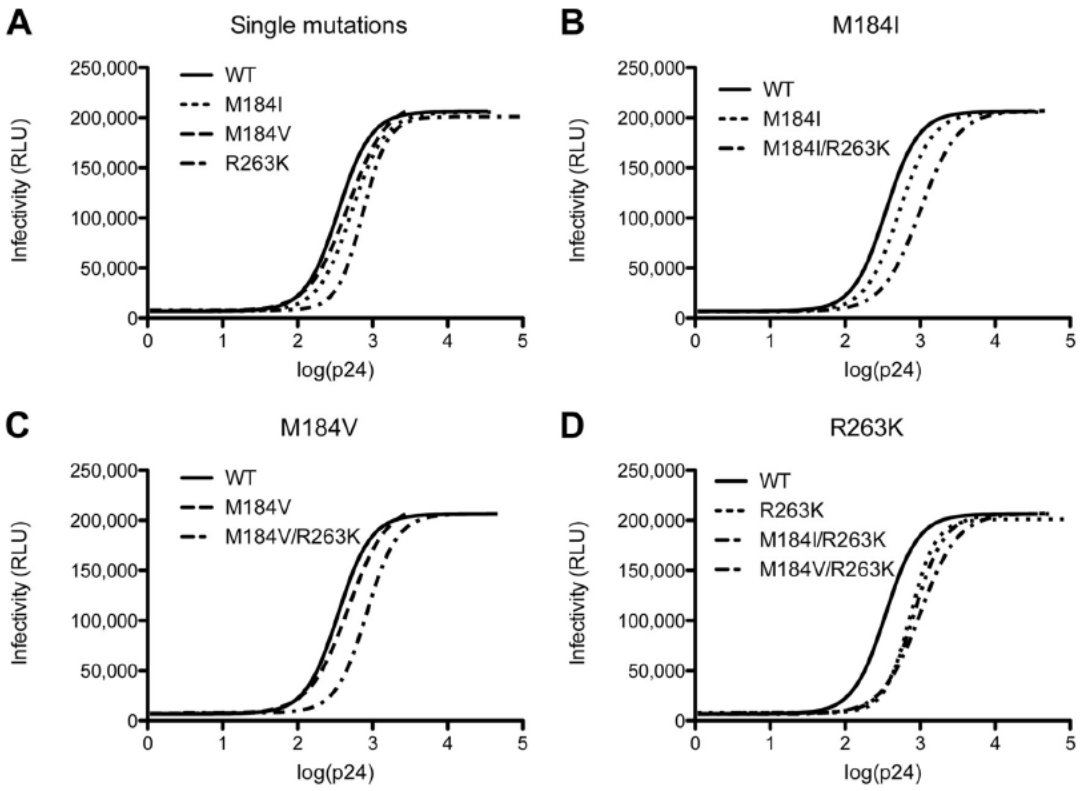
Table 4.1. Effects of the M184I, M184V, and R263K substitutions in isolation or in combination on HIV-1 susceptibility to 3TC and DTG

	Susceptibility to 3TC			Susceptibility to DTG		
Genotype	EC_{50} (nM)	95% confidence interval	Fold change[a]	EC_{50} (nM)	95% confidence interval	Fold change[a]
WT	0.95	0.6075–1.490	1	0.8	0.4383–1.467	1
M184I	780.8	242.0–2,519	820	1	0.4199–2.352	1.25
M184V	3,596	1,534–8,426	3,780	1.1	0.4652–2.412	1.4
R263K	0.37	0.2750–0.5009	0.4	2.2	0.4002–11.71	2.75
M184I-R263K	644.2	296.4–1,400	677	4.2	2.353–7.474	5.25
M184V-R263K	2,099	1,043–4,225	2,206	4.4	2.948–6.640	5.5

[a] Fold changes were calculated by arbitrarily setting the WT EC_{50} at 1.

4.5.2. Combining the M184I/V and R263K resistance mutations decreases viral infectivity.

Since M184I/V and R263K, in isolation, are associated with decreased viral infectivity, we measured the effects of these substitutions both individually and in combination on HIV-1 infectivity using TZM-bl reporter cells (Figure 4.1). The presence of the R263K substitution was more detrimental to HIV-1 infectivity than M184I or M184V (2.1-fold compared to 1.4-fold and 1.5-fold decrease in infectivity, respectively). Differences in infectivity between M184I and M184V were not significant (Student's t-test). Combining M184I or M184V with R263K further decreased infectivity compared to R263K alone (2.9-fold and 2.4-fold for M184I/R263K and M184V/R263K, respectively).



E

Genotype	EC_{50} (pg p24)	95% confidence intervals	Fold change in infectivity
WT	348	330 to 367	**1.0**
M184I	527	498 to 557	**1.5***
M184V	472	432 to 516	**1.4***
R263K	737	685 to 793	**2.1***
M184I/R263K	998	937 to 1063	**2.9*#o**
M184V/R263K	831	770 to 897	**2.4*#$**

Figure 4.1.: The combination of M184I/V with R263K decreases HIV-1 infectiousness. Effects of single M184I/V or R263K (A), M184I and/or R263K (B), M184V and/or R263K (C), or R263K and/or M184I/V (D) substitutions on HIV-1 infectivity in TZM-bl reporter cells are expressed relative to p24 levels. (E) Half effective viral concentrations (EC_{50}s) and 95% confidence intervals were calculated from the data presented in panels A through D. Fold changes were calculated by arbitrarily setting the WT EC50 at 1. *, statistically significant difference from WT; #, statistically significant difference from R263K; o, statistically significant difference from M184I; $, statistically significant difference from M184V (*n* = 6, Student's *t* test, *P* <0.05 for all).

4.5.3. Combining the M184I/V and R263K resistance mutations decreases viral replicative capacity.

To confirm the above results, we measured the effects of the 3TC- and DTG-resistance mutations on HIV-1 replication capacity in PM1 cells over 15 days by quantifying levels of p24 in culture fluids (Figure 4.2). The results confirm that the individual resistance mutations decrease viral replication capacity by approximately 30% compared to WT after 7 days of infection, at which time there was no statistically significant difference between the replication levels of viruses containing either of the M184I, M184V or R263K substitutions. In contrast, the M184I/R263K and M184V/R263K combinations of mutations resulted in approximate 50% decreases in maximal viral production at day 7.

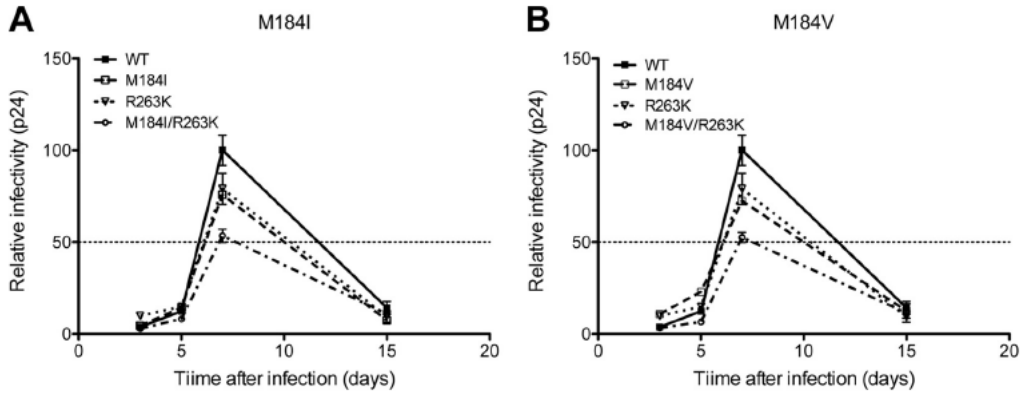


Figure 4.2.: The combination of M184I/V with R263K decreases HIV-1 replicative capacity. Effects of M184I and/or R263K (A) and M184V and/or R263K (B) on viral replication in PM1 cells measured by p24 levels over 15 days.

4.5.4. Combining the M184I/V and R263K resistance substitutions decreases viral DNA integration.

We have previously shown that the R263K substitution results in an approximate 50% decrease in viral DNA integration into PBMC DNA [19, 21], and this result is confirmed here (Figure 4.3). Now, we tested the effect of adding the M184I/V mutations to R263K on viral DNA integration in primary PBMCs (Figure 4.3). The M184I and M184V single mutations decreased viral integration by approximately 35% due to their effects on viral replication whereas integration levels associated with M184I/R263K and M184V/R263K were diminished by 67% and 64% relative to WT, respectively. Thus, the addition of M184I/V to R263K decreased integration levels compared to R263K only.

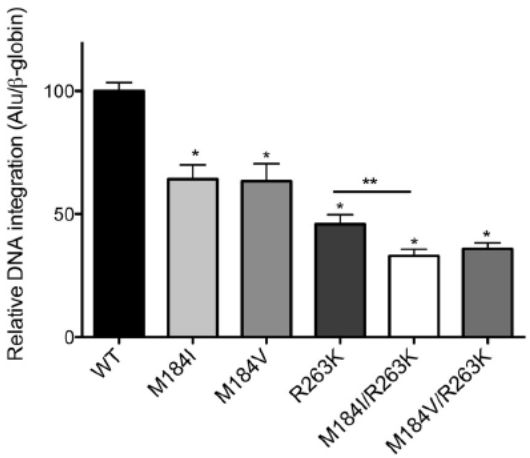


Figure 4.3.: Combining M184I/V with R263K decreases HIV-1 genomic integration. Effects of the M184I, M184V, and R263K substitutions on relative HIV-1 DNA integration into human PBMC genomic DNA were measured by *Alu* mediated PCR. *, statistically significant difference from WT (Student's t test, P<0.05). **, M184I-R263K levels of integration were statistically significantly different from those of R263K alone (Student's t test, P < 0.05).

4.6. Discussion

The recent approval of a single daily pill regimen of DTG/3TC/ABC motivated us to study the effects of 3TC- and DTG-resistance mutations in combination. Our results show that the presence of M184I or M184V with R263K further decreased viral infectiousness and replication capacity compared to the effects of the individual substitutions alone. Given that viral load in individuals who have failed treatment with 3TC in association with the emergence of M184I/V mutations are often lower than baseline levels [26, 29], our results suggest that the combined presence of M184I/V and R263K may have a strong effect on viral load, even though these combinations of mutations have not been reported in clinical settings.

Studies to evaluate DTG resistance mutations could potentially be performed in non-human primate models. We have generated SIV_{mac239} and simian tropic HIV (stHIV) viruses that contain integrase resistance mutations and shown that these viruses possess similar phenotypes as in HIV [38].

Our findings suggest that the use of 3TC in combination with DTG may be beneficial for treatment of HIV-positive individuals. Ongoing clinical trials currently evaluate the antiviral activity of DTG+3TC as dual therapy.

In treatment-naïve individuals, DTG has demonstrated robustness against the emergence of resistance mutations and the use of DTG has even been shown to be superior to that of an

excellent boosted protease inhibitor [6]. This observation may be related to the fact that the most common resistance substitution that has been selected by DTG in tissue culture and in INSTI-naive patients has been R263K, which is associated with a decrease in viral replicative capacity. Moreover, no compensatory mutation for R263K has yet been reported [2, 14, 39], and the appearance of R263K in two treatment-experienced individuals who failed DTG treatment has not led to the occurrence of other resistance substitutions within integrase, despite the fact that therapy with DTG was continued in these patients even after detection of the R263K substitution [14]. This contrasts with the generation of multiple integrase mutations in some patients who have failed DTG after having previously failed either RAL or EVG [13].

Previous tissue culture selection studies have shown that the M184I/V substitution can emerge after 8 weeks of culture under 3TC pressure in R263K-containing viruses and that this represents a delay compared to the occurrence of M184I/V in WT viruses [20]. Given the absence of compensatory secondary resistance mutations to R263K, both in tissue culture and in the clinic [2, 14, 19], it is possible that the presence of M184I/V substitutions will help to prevent the emergence of such compensatory mutations in individuals co-treated with 3TC/FTC and DTG.

4.7 References

1. Wainberg, M.A., G.J. Zaharatos, and B.G. Brenner, *Development of antiretroviral drug resistance.* N Engl J Med, 2011. **365**(7): p. 637-46.
2. Wainberg, M.A., T. Mesplede, and F. Raffi, *What if HIV were unable to develop resistance against a new therapeutic agent?* BMC Med, 2013. **11**(1): p. 249.
3. Walmsley, S.L., et al., *Dolutegravir plus abacavir-lamivudine for the treatment of HIV-1 infection.* N Engl J Med, 2013. **369**(19): p. 1807-18.
4. Raffi, F., et al., *Once-daily dolutegravir versus raltegravir in antiretroviral-naive adults with HIV-1 infection: 48 week results from the randomised, double-blind, non-inferiority SPRING-2 study.* Lancet, 2013. **381**(9868): p. 735-43.
5. Raffi, F., et al., *Once-daily dolutegravir versus twice-daily raltegravir in antiretroviral-naive adults with HIV-1 infection (SPRING-2 study): 96 week results from a randomised, double-blind, non-inferiority trial.* Lancet Infect Dis, 2013. **13**(11): p. 927-35.
6. Clotet, B., et al., *Once-daily dolutegravir versus darunavir plus ritonavir in antiretroviral-naive adults with HIV-1 infection (FLAMINGO): 48 week results from the randomised open-label phase 3b study.* Lancet, 2014. **383**(9936): p. 2222-31.
7. Mesplede, T. and M. Wainberg, *Integrase Strand Transfer Inhibitors in HIV Therapy.* Infec Dis and Therapy, 2013. **2**(2): p. 83-93.
8. Wohl, D.A., et al., *A randomized, double-blind comparison of single-tablet regimen elvitegravir/cobicistat/emtricitabine/tenofovir DF versus single-tablet regimen efavirenz/emtricitabine/tenofovir DF for initial treatment of HIV-1 infection: analysis of week 144 results.* J Acquir Immune Defic Syndr, 2014. **65**(3): p. e118-20.
9. Molina, J.M., et al., *Efficacy and safety of once daily elvitegravir versus twice daily raltegravir in treatment-experienced patients with HIV-1 receiving a ritonavir-boosted protease inhibitor: randomised, double-blind, phase 3, non-inferiority study.* Lancet Infect Dis, 2012. **12**(1): p. 27-35.
10. Orkin, C., et al., *Final 192-week efficacy and safety of once-daily darunavir/ritonavir compared with lopinavir/ritonavir in HIV-1-infected treatment-naive patients in the ARTEMIS trial.* HIV Med, 2013. **14**(1): p. 49-59.
11. Hightower, K.E., et al., *Dolutegravir (S/GSK1349572) exhibits significantly slower dissociation than raltegravir and elvitegravir from wild-type and integrase inhibitor-resistant HIV-1 integrase-DNA complexes.* Antimicrob Agents Chemother, 2011. **55**(10): p. 4552-9.
12. DeAnda, F., et al., *Dolutegravir interactions with HIV-1 integrase-DNA: structural rationale for drug resistance and dissociation kinetics.* PLoS One, 2013. **8**(10): p. e77448.
13. Eron, J.J., et al., *Safety and efficacy of dolutegravir in treatment-experienced subjects with raltegravir-resistant HIV type 1 infection: 24-week results of the VIKING Study.* J Infect Dis, 2013. **207**(5): p. 740-8.
14. Cahn, P., et al., *Dolutegravir versus raltegravir in antiretroviral-experienced, integrase-inhibitor-naive adults with HIV: week 48 results from the randomised, double-blind, non-inferiority SAILING study.* Lancet, 2013. **382**(9893): p. 700-8.
15. Castagna, A., et al., *Dolutegravir in Antiretroviral-Experienced Patients With Raltegravir- and/or Elvitegravir-Resistant HIV-1: 24-Week Results of the Phase III VIKING-3 Study.* J Infect Dis, 2014.
16. Akil, B., et al., *Dolutegravir versus placebo in subjects harbouring HIV-1 with integrase inhibitor resistance associated substitutions: 48-week results from VIKING-4, a randomized study.* Antivir Ther, 2014.
17. Mesplede, T., et al., *Integrase strand transfer inhibitors in the management of HIV-positive individuals.* Ann Med, 2014. **46**(3): p. 123-9.

18. Hurt, C.B., et al., *Resistance to HIV integrase strand transfer inhibitors among clinical specimens in the United States, 2009-2012.* Clin Infect Dis, 2014. **58**(3): p. 423-31.
19. Quashie, P.K., et al., *Characterization of the R263K mutation in HIV-1 integrase that confers low-level resistance to the second-generation integrase strand transfer inhibitor dolutegravir.* J Virol, 2012. **86**(5): p. 2696-705.
20. Oliveira, M., et al., *Resistance mutations against dolutegravir in HIV integrase impair the emergence of resistance against reverse transcriptase inhibitors.* AIDS, 2014. **28**(6): p. 813-9.
21. Mesplede, T., et al., *Viral fitness cost prevents HIV-1 from evading dolutegravir drug pressure.* Retrovirology, 2013. **10**: p. 22.
22. Wares, M., et al., *The M50I polymorphic substitution in association with the R263K mutation in HIV-1 subtype B integrase increases drug resistance but does not restore viral replicative fitness.* Retrovirology, 2014. **11**(1): p. 7.
23. Mesplede, T., et al., *Addition of E138K to R263K in HIV integrase increases resistance to dolutegravir, but fails to restore activity of the HIV integrase enzyme and viral replication capacity.* J Antimicrob Chemother, 2014. **69**(10): p. 2733-40.
24. Armenia, D., et al., *Study of genotypic and phenotypic HIV-1 dynamics of integrase mutations during raltegravir treatment: a refined analysis by ultra-deep 454 pyrosequencing.* J Infect Dis, 2012. **205**(4): p. 557-67.
25. Keulen, W., C. Boucher, and B. Berkhout, *Nucleotide substitution patterns can predict the requirements for drug-resistance of HIV-1 proteins.* Antiviral Res, 1996. **31**(1-2): p. 45-57.
26. Schuurman, R., et al., *Rapid changes in human immunodeficiency virus type 1 RNA load and appearance of drug-resistant virus populations in persons treated with lamivudine (3TC).* J Infect Dis, 1995. **171**(6): p. 1411-9.
27. Ait-Khaled, M., et al., *M184V is associated with a low incidence of thymidine analogue mutations and low phenotypic resistance to zidovudine and stavudine.* AIDS, 2002. **16**(12): p. 1686-9.
28. Miller, M.D., et al., *Antiviral activity of tenofovir (PMPA) against nucleoside-resistant clinical HIV samples.* Nucleosides Nucleotides Nucleic Acids, 2001. **20**(4-7): p. 1025-8.
29. Devereux, H.L., et al., *Replicative fitness in vivo of HIV-1 variants with multiple drug resistance-associated mutations.* J Med Virol, 2001. **65**(2): p. 218-24.
30. Lusso, P., et al., *Growth of macrophage-tropic and primary human immunodeficiency virus type 1 (HIV-1) isolates in a unique CD4+ T-cell clone (PM1): failure to downregulate CD4 and to interfere with cell-line-tropic HIV-1.* J Virol, 1995. **69**(6): p. 3712-20.
31. Graham, F.L., et al., *Characteristics of a human cell line transformed by DNA from human adenovirus type 5.* J Gen Virol, 1977. **36**(1): p. 59-74.
32. Platt, E.J., et al., *Evidence that ecotropic murine leukemia virus contamination in TZM-bl cells does not affect the outcome of neutralizing antibody assays with human immunodeficiency virus type 1.* J Virol, 2009. **83**(16): p. 8289-92.
33. Takeuchi, Y., M.O. McClure, and M. Pizzato, *Identification of gammaretroviruses constitutively released from cell lines used for human immunodeficiency virus research.* J Virol, 2008. **82**(24): p. 12585-8.
34. Wei, X., et al., *Emergence of resistant human immunodeficiency virus type 1 in patients receiving fusion inhibitor (T-20) monotherapy.* Antimicrob Agents Chemother, 2002. **46**(6): p. 1896-905.
35. Derdeyn, C.A., et al., *Sensitivity of human immunodeficiency virus type 1 to the fusion inhibitor T-20 is modulated by coreceptor specificity defined by the V3 loop of gp120.* J Virol, 2000. **74**(18): p. 8358-67.
36. Platt, E.J., et al., *Effects of CCR5 and CD4 cell surface concentrations on infections by macrophagetropic isolates of human immunodeficiency virus type 1.* J Virol, 1998. **72**(4): p. 2855-64.

37. Adachi, A., et al., *Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone.* J Virol, 1986. **59**(2): p. 284-91.
38. Hassounah, S.A., et al., *Effect of HIV-1 integrase resistance mutations when introduced into SIVmac239 on susceptibility to integrase strand transfer inhibitors.* J Virol, 2014. **88**(17): p. 9683-92.
39. Mesplede, T. and M.A. Wainberg, *Is Resistance to Dolutegravir Possible When This Drug Is Used in First-Line Therapy?* Viruses, 2014. **6**(9): p. 3377-3385.

Chapter 5

Replication profile for dolutegravir-resistant HIV-1 subtype B in various primary immune cells

The following chapter was adapted from the manuscript in preparation entitled “Replication profile for dolutegravir-resistant HIV-1 subtype B in various primary immune cells” authored by Diane N. Singhroy, Thibault Mesplède, Christina Qian, and Mark A. Wainberg. Approximately 90% of the experiments and data analysis was performed by me under the supervision of Dr. Mark Wainberg. Special thanks to Dr. Mesplede whose ideas have been the source of inspiration for this study and have been partially presented in “Resistance against Integrase Strand Transfer Inhibitors and Relevance to HIV Persistence” [1]. Dr. Thibault Mesplède helped with the project design and analysis. Christina Qian helped generate some of the pSF162R3_Nef+ drug resistant mutant plasmids under my supervision. Dr. Wainberg and Dr. Mesplède critiqued the manuscript.

5.1 Preface

Dolutegravir (DTG), a second generation INI, is the third drug of this class to be approved for the treatment of HIV infection. Compared to its predecessors, raltegravir (RAL) and elvitegravir (EVG), DTG has a higher genetic barrier to resistance and, similar to RAL and EVG, is very well tolerated in patients and efficacious against HIV infection. To date, no drug resistance mutations have developed in treatment-naïve patients treated with DTG and an optimal background containing other drugs [2]. However, in treatment-experienced individuals, as reported in the SAILING phase 3 clinical trial, four of the six (4/6) patients who failed DTG-based treatment with resistance mutations had developed R263K [3].

We study the emergence of drug resistance in cell culture by growing primary isolates or clonal virus under conditions of sub-optimal drug pressure [4]. As the virus becomes resistant, the drug pressure is increased progressively and resistance mutations and pathways are identified by sequencing purified viral RNAs. We can thus map out resistance pathways that will reveal primary, secondary and/or compensatory mutations. Through this approach, we try to reproduce pharmacological conditions in the body under which the virus may develop drug resistance. These selection assays can be performed in cell lines like MT2 T-cells or primary cells such as CBMCs or PBMCs isolated from healthy donors and they allow researchers and clinicians to predict resistance pathways, the degrees of susceptibility of various antivirals to the emergence of drug resistance, and patterns of drug cross-resistance for patients who have experienced treatment failure.

The R263K drug resistance mutation against DTG was first identified in our lab before it was seen clinically [5]. In cell culture drug selections, R263K was the most frequent mutation to appear after 20 weeks and it persisted for as long as drug pressure was maintained [5]. Although the level of resistance attained by R263K viruses was low (2.3 fold in TZM-bl assays), R263K also resulted in a less fit virus as measured in viral growth assays in PM1 cells. The R263K mutation was found to decrease HIV infectivity to 80% of WT and HIV-1 DNA integration within host DNA by 20 to 30%. Furthermore, secondary mutations that emerged after R263K in tissue culture selection experiments, such as H51Y and E138K, did not restore viral fitness. This is different from EVG and RAL, whereby the appearance of secondary mutations often restores viral fitness and may be more likely to lead to treatment failure (Figure 5.1).

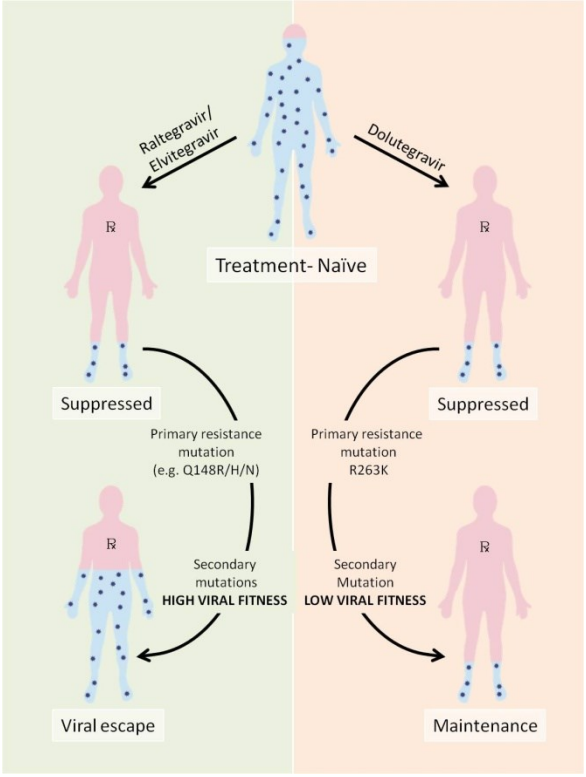


Figure 5.1: A model of resistance that may help to explain the virological robustness of DTG against resistance. When suppressed individuals experience treatment failure with RAL or EVG (left), their virus can sequentially acquire primary and secondary compensatory resistance mutations. In contrast, in the absence of secondary compensatory resistance mutations, DTG (right) will maintain its clinical activity even in the presence of R263K.

In contrast, another tissue culture selection study with DTG by Kobayashi et al. did not select for the R263K mutation [6]. Importantly, our group conducted resistance selection experiments with CBMCs whereas the Kobayashi group used MT2 cells. This key difference has led us to hypothesize that R263K may either emerge in certain cells types or have a higher propensity to infect and replicate in specific cell populations that would be present in the heterogeneous CBMCs but not MT2 cells. It is worth investigating this issue because viruses harboring R263K may then preferentially contribute to the viral reservoir. The MT2 cell line was derived from a case of adult T-cell leukemia and carries HTLV-1 [7]. On the other hand, CBMCs isolated from donor whole blood are a heterogeneous mixture of immune cells consisting of 60% T-cells, 13%

B-cells, 11% monocytes and 13% dendritic cells [8]. Importantly, several of these cells could serve as potential reservoirs.

In addition to allowing the establishment of latent viral reservoirs, viral replication may also persist at low levels in anatomical and cellular reservoirs despite HAART. This has been reported recently via ultradeep sequencing of HIV in the blood and lymph nodes [9]. Whether active replication within viral reservoirs contributes to HIV pathogenesis or not, the cells that constitute the reservoir are a major obstacle to a sterilizing cure.

The resistance profile, anatomical distribution, and pharmacokinetics of DTG opens the door to its use in curative strategies [1]. In our study, we also measured interleukin (IL)-6 expression because this pro-inflammatory molecule is an important inflammatory marker in untreated individuals that also persists under HAART.

Intriguingly, R263K is also a rare natural polymorphism found in less than 5% of HIV-1 sequences in multiple databanks [10]. In addition, the R to K transition in most viral sequences requires only a single G to A nucleotide change. It remains unclear why more individuals treated with DTG have not suffered treatment failure following the common emergence of this mutation.

Our primary objective was to investigate whether DTG resistant viruses would grow better in various primary cells based on the hypothesis stated earlier. The results could direct continuing

work on cellular reservoirs and may be extended to the study of anatomical sanctuary sites of HIV-1 persistence.

5.2 Abstract

HIV infection results in the early establishment of cellular reservoirs that represent a considerable obstacle to cure. Resting CD4+ T-lymphocytes, macrophages and other cells that sometimes reside in specific tissues and organs are all thought to contribute to this reservoir. We have previously formulated the hypothesis that the unique resistance profile of the integrase inhibitor dolutegravir (DTG) may result from its ability to inhibit actively replicating virus. The existence of persistent HIV replication under antiretroviral therapy (ART) has been shown previously but it was only recently demonstrated through sequential ultradeep sequencing experiments that persistent HIV genetic evolution could occur under treatment. We hypothesize that the absence of resistance mutations, either in integrase or in reverse transcriptase, in individuals experiencing treatment failure with DTG may indicate that the latter drug efficaciously inhibits persistent replication. Importantly, tissue culture selection studies with primary human cord-blood mononuclear cells revealed the common emergence of the R263K substitution in integrase under DTG pressure whereas similar experiments with MT2 cells did not lead to the emergence of this substitution. R263K was later confirmed to be the most common resistance substitution in the rare cases of treatment-experienced individuals suffering from treatment failure with DTG and who had *de novo* mutations. Here, we investigated the replication capacity of HIV-1 bearing the R263K, H51Y, E138K and H51Y/R263K

substitutions in primary human CD4+ T-lymphocytes (either resting or activated), in monocytes, monocyte-derived macrophages, and monocyte-derived dendritic cells. As stated above, replication in primary human activated CD4+ T-lymphocytes of R263K-containing virus was decreased by approximately 20% compared to WT. In contrast, primary human resting CD4+ T-lymphocytes supported higher levels of replication for R263K virus than WT. Similar observations were made with the H51Y/R263K combination of mutations. These results help to explain the absence of DTG-resistance in treatment-naïve individuals and have implications for the presence of such viruses in cellular reservoirs.

5.3 Introduction

As explained above, dolutegravir (DTG) is particularly robust against the emergence of drug resistance mutations. Indeed, to date, no treatment-naïve individual has been reported to fail dolutegravir-based therapy with emerging resistance substitutions in integrase. In addition and in contrast to RAL or EVG, no N(t)RTI resistance was detected in either treatment-naïve or treatment-experienced individuals who experienced DTG-based treatment failure when the latter drug was co-administered with RT inhibitors [3, 11-14]. However, several recent reports highlight the emergence of resistance mutations against DTG in treatment-experienced individuals. Importantly, in 4 of 6 reported cases of the use of DTG in treatment-experienced patients, the R263K integrase substitution was found. In most of these cases, there was no additional mutation that emerged, even when individuals experiencing treatment failure were maintained for years under DTG-based therapy. In one paediatric highly treatment-experienced

individual, however, the emergence of R263K was followed 100 weeks later by the addition of several secondary substitutions, including E138K and S147G. Importantly, E138K was also seen secondary to R263K during our pre-clinical tissue culture selection experiments with DTG [5]. Another R263K-associated secondary mutation in tissue culture was H51Y [5]. The addition of either of E138K or H51Y to R263K did not restore integrase catalytic activity, viral infectivity, or HIV DNA integration. Indeed, R263K caused a 20% decrease in integrase strand-transfer activity associated with a decrease in DNA binding *in vitro*, as well as a 2- to 3-fold reduction in infectivity and 20-30% reduction in HIV-1 DNA integration within cellular DNA. The addition of H51Y to R263K led to further decreases in integrase strand-transfer activity (35%), infectivity (7-fold) and DNA integration during infection (40-50%). Simultaneously, HIV-1 resistance against DTG increased from 2-fold with R263K to 7-fold with H51Y/R263K. E138K is a substitution that can be found secondary to various primary resistance mutations that emerge following treatment failure with RAL or EVG, or with DTG following previous treatment failure with RAL [15]. In addition, as mentioned above, E138K was found to emerge secondary to R263K both in tissue culture selection studies and in a paediatric patient. The addition of E138K to R263K increased HIV resistance against DTG to 4.3-fold but did not improved integrase strand-transfer activity nor viral infectivity compared to R263K alone [16].

Given that the emergence of some resistance mutations against DTG seems to be facilitated in CBMCs versus transformed T-cell lines, we investigated how these mutations affect the ability of HIV-1 to infect various types of cells.

5.4 Materials and Methods

5.4.1. Primary cell isolation

Similar to the primary cell isolation described in Chapter 3 (section 3.4.2.), PBMCs were isolated from donor whole blood by Ficoll gradient separation and used for CD4+ T-cell and CD14+ monocyte isolations. Activation and differentiation were also performed as indicated in Chapter 3 (section 3.4.2.). MDMs, MDDCs and monocytes were cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% FBS and 2mM glutamate and maintained at 37°C under 5% CO_2. Resting and activated CD4+ T-lymphocytes were cultured in Roswell Park Memorial Institute medium (RPMI) supplemented with IL-2, 10% FBS and 2mM glutamate and maintained at 37°C, 5% CO_2.

5.4.2. Virus production

Generation of the pNL4.3$_{IN(E138K)}$, pNL4.3$_{IN(H51Y),}$ pNL4.3$_{IN(R263K),}$ pNL4.3$_{IN(H51Y/R263K)}$ viruses was described previously [5, 16, 17]. R5-tropic plasmids pSF162R3_Nef+ (donation from Dr. Amanda Brown) [18] were used to generate HIV-1 drug resistant mutant plasmids pSF162$_{IN(E138K)}$, pSF162$_{IN(H51Y)}$, pSF162$_{IN(R263K)}$, pSF162$_{IN(H51Y/R263K)}$ through site directed mutagenesis using the following primers: E138K$_{(IN)}$ sense (5'-gggcagggatcaagcagaaatttggcattccctac-3') and antisense (5'-gtagggaatgccaaatttctgcttgatccctgccc-3'), H51YI$_{(IN)}$ sense (5'-gctaaaaggagaagccatgtatggacaagtagactgtag-3') and antisense (5'-ctacagtctacttgtccatacatggcttctccttttagc-3'), R263K sense (5'-

tgacataaaagtagtgccaagaaaaaaagcaaagatcattagggatt-3') and antisense (5'-aatccctaatgatctttgctttttttcttggcactactttatgtca-3').

The virus was produced as described in Chapter 3 (section 3.4.3.). Viral particles in the supernatants were quantified by p24 ELISA, described previously. Viral stocks were aliquoted and stored at -80°C.

5.4.3. Infections

All primary cells were infected in the same manner. For 200,000 cells, 300ng of p24 of virus were incubated for 2hr at 2,700 rpm at 25 °C, followed by 3 hours incubation at 37 °C. Virus was then removed by 2 rounds of washes with PBS and the appropriate media was added (see above). Cells were collected after 72 hours and pellets were stored at -80°C until needed. To account for tropism, NL4.3-based clonal virus was used to infect T-cells whereas SF162R3_Nef+ based clonal virus was used to infect monocytes, macrophages and dendritic cells. All experiments were done in duplicate.

5.4.5.Quantitative real time PCR

Total RNA was extracted using an AllPrep DNA/RNA mini kit (Qiagen). qRT-PCR was performed with SuperScript III Platinum one-step quantitative RT-PCR system (Invitrogen) using a Corbett Rotor-Gene 6000 thermocycler (Corbett). The samples were normalized for their GAPDH mRNA

content. Primers/probes set IDs for IL6 and GAPDH are IL6 ID Hs00985639_m1 and GAPDH Hs03929097_g1, respectively (Invitrogen, Thermo Fisher Scientific). HIV-1 Gag primer sequences were: sense (5'-AGTGGGGGGACATCAAGCAGC-3') and antisense (5'-TACTAGTAGTTCCTGCTATGTC-3'); and probe (FAM 3'-ATCAATGAGGAAGCTGCAGAATGGGA-3'BHQ) [19]. The cycling conditions were 50°C for 15 min, 95°C for 10 min, and 70 repeats of 95°C for 15 sec and 60°C for 30 sec. The results were analysed with GraphPad Prism 4.0 Software.

5.6 Results

5.6.1 R263K favours HIV-1 infection in resting CD4+ T-lymphocytes

The R263K integrase substitution commonly emerged during tissue culture selection experiments with DTG using cord-blood mononuclear cells (CBMCs) but not MT-2 transformed T-cells [5, 6]. Given that CBMCs are a mixture of various cell types, we investigated the ability of viruses carrying R263K and other integrase substitutions to infect primary human resting and activated CD4+ T-cells, CD14+ monocytes, MDM and MDDC (Figure 5.2). HIV-1 infections were performed as described in the Methods section for 72h and the presence of HIV RNA was quantified by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and normalised against qRT-PCR results for the mRNA of the GAPDH gene. As previously established, HIV-1 infection was approximately 1-log less efficacious in resting CD4+ T-cells

compared to activated cells and was very low in monocytes, macrophages and dendritic cells [20, 21]. This was also true for the E138K, H51Y, R263K and H51Y/R263K viruses. However, although R263K was associated with diminished HIV-1 replication in activated CD4+ T-cells to approximately 60% of the WT virus, replication of this mutant was actually higher than that of WT virus in resting CD4+ T-cells. This effect was even more pronounced for the H51Y/R263K mutant that replicated approximately 15-fold less efficiently than the WT in activated CD4+ T-cells but 2.3-fold more efficiently in resting CD4+ T-cells. Other interesting changes in the ability of integrase mutated viruses to replicate in various primary cell types included the relative inability of the E138K mutant to replicate in monocyte-derived macrophages (MDM).

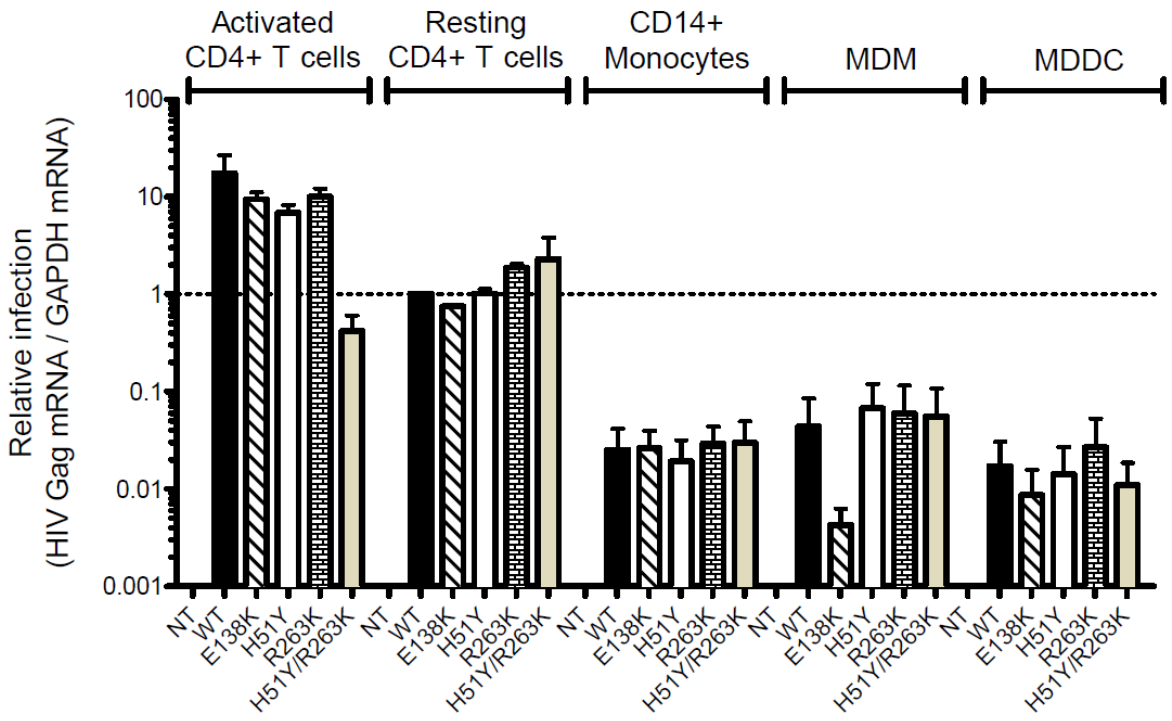


Figure 5.2: Relative HIV infection in various cells. Primary cells isolated from donor PBMC were infected with WT or mutant HIV-1, indicated on x axis. Infection levels were measured by qRT-PCR for HIV gag/GAPDH RNA and normalized to WT CD4 resting T-cells.

5.6.2 E138K increases IL6 transcripts in myeloid cells

Next, we used the same samples to estimate the degree of inflammation associated with HIV-1 infection in these experiments by quantifying the transcription of the interleukin-6 gene (IL6) (Figure 2). Our results show that IL6 mRNA production in activated CD4+ T-cells increased by approximately 100-fold following infection. WT HIV-1 infection also resulted in an increase in IL6 mRNA in monocytes but was innocuous or inhibitory for IL6 gene transcription in resting CD4+ T-cells, macrophages and dendritic cells. In contrast, infections with the E138K mutant resulted in increases in IL6 mRNA levels in myeloid cells, namely monocytes, MDM and MDDC. R263K virus infection also stimulated IL6 expression in macrophages. Increases in infectiousness

of the R263K and H51Y/R263K mutants in resting CD4+ T-cells (Figure 5.2) were not associated with increased IL6 transcripts (Figure 5.3).

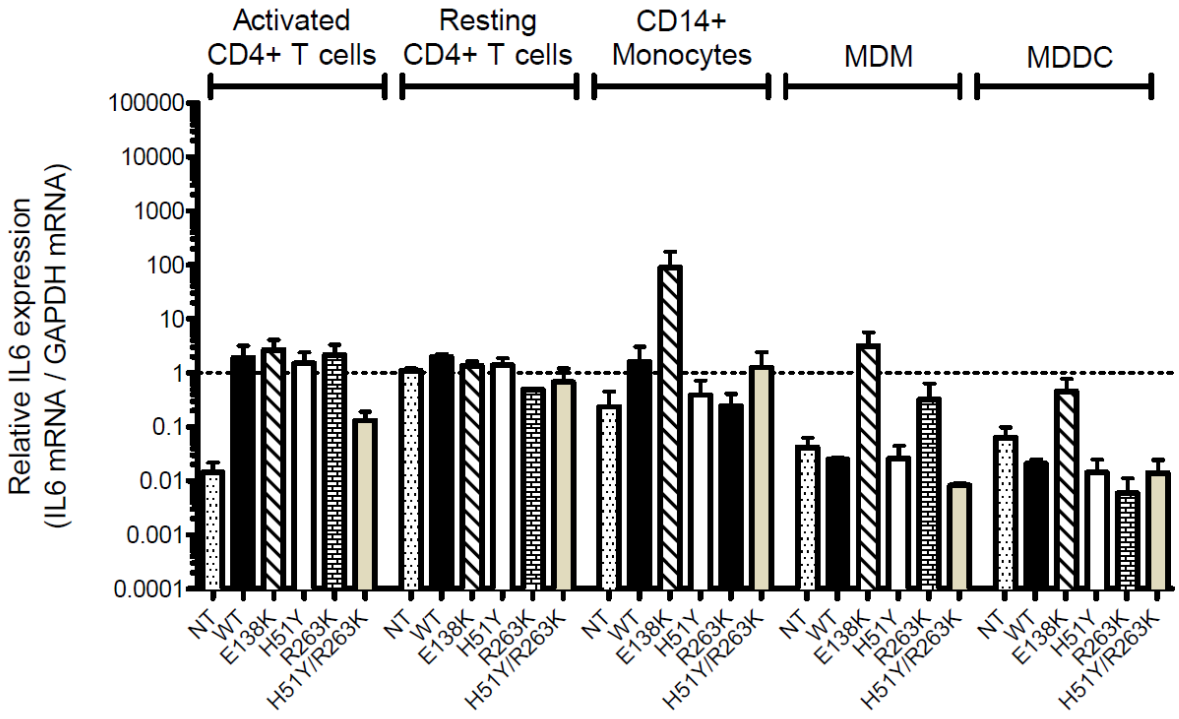


Figure 5.3: Relative IL6 expression in various HIV-infected cells. Il-6 gene expression was measured in primary cells infected with WT or mutant HIV-1 and normalized to GAPDH mRNA content and expressed relative to the levels measured in untreated CD4+ T-lymphocytes.

5.7 Future directions

We sought to determine if certain DTG DRMs would enable better replication than WT in various primary immune cells. This has been observed before in the sense that viruses harboring RAL DRMs, i.e. N155H/S and Q148R/H, grew better in macrophages than in activated

CD4+ T-cells under RAL drug pressure [22]. However, DTG preserved its efficacy against RAL DRM in both cell types [23]. Based on our experiments, we observe that different DRM-containing viruses possess differences in replicative capacity in various primary human immune cells. This could be enough for an escape mutation to be selected for under DTG drug pressure. Importantly in CD4+ T-cells, the augmentation in HIV-1 replication for both R263K-containing viruses (R263K and H51Y/R263K) was not due to problems with sample quality as the level of IL6 transcripts was mostly unchanged or slightly decreased compared to WT. In addition, these experiments were performed using cells isolated from two healthy donors (n=2). Future experiments should be performed with more donors and include the extraction of DNA to measure the levels of HIV DNA integration within the host genome in the various primary cell types. The use of a kit allowing the purification of both DNA and total RNA did not allow the proper recovery of satisfying amounts of DNA in these experiments.

It should also be noted that CBMCs were activated with PMA prior to infection and drug selection in our previous tissue culture experiments [5]. Although this does not completely exclude the existence of a few resting T-lymphocytes in the CBMC population used for selections, it does make it less likely that R263K would have been selected in these particular cells *in vitro.* In contrast*, in vivo* resting CD4+ T-cells in the blood or in tissue and infiltrating lymphocytes, monocytes, macrophages and dendritic cells in tissues may be more susceptible to the emergence of R263K-containing viruses than *in vitro* activated CD4+ T-lymphocytes. Our observations should enable us to further characterize resting CD4+ T-cell sub-populations in which the replication of R263K-containing viruses may be favoured compared to WT. Similar

experiments should be performed to further characterize myeloid cells in which these viruses may also replicate efficaciously.

All cells were collected after 72 hours post inoculation. Myeloid cells are more refractory to HIV-1 infection than CD4+ T-cells and this could account for the lower infection levels in these cells. Future experiments should be run to day 5 as this was a previously established optimal time course for SF162. Studies have demonstrated that RTIs and PIs have different inhibitory efficacy in macrophages versus CD4+ T-cells [24]. It is therefore conceivable that INIs also display different inhibition profiles in macrophages or dendritic cells and this has yet to be determined for DTG. The importance of macrophages in HIV-1 reservoirs is augmented by the fact that they can penetrate to tissues that can represent drug sanctuaries such as the CNS. Latently infected monocytes can also migrate to various tissues, where they can differentiate and contribute to the establishment of long-term reservoirs. Future experiments should thus include tissue culture selection experiments with MDM in the presence of increasing concentrations of DTG. Notably, macrophage cell-to-cell HIV-1 spread is mostly resistant to drug-mediated inhibition [25], although DTG was not tested in these conditions. It would be remarkable to confirm whether myeloid cells are more susceptible to the emergence of resistance mutations against DTG. If this were the case, it would be important to sample the blood or tissues of individuals under DTG therapy to test for the presence of viruses with the R263K substitution within myeloid cells. This would be particularly important because monocytes and macrophages are an important source of IL-6 and could thus contribute to persistent inflammation under treatment [26].

The molecular mechanisms at play in the higher infectivity of both R263K and H51Y/R263K viruses compared to WT in resting CD4+ T-lymphocytes remains to be characterized. Measuring reverse transcripts and integration by Alu-mediated qPCR may provide information in this regard. Future experiments could include the examination of the nuclear localization of integration sites, as WT integration has been shown to happen at the vicinity of the nuclear membrane whereas RAL treatment relocalized integration deeper inside the nucleus [27]. Additionally, the DNA sequence targeted for integration of the various mutants should be examined by ultra-deep sequencing of integration sites, as previously described [28]. Finally, the same analysis would allow us to identify whether integration of R263K-containing viruses is favoured in specific genomic features such as GC-rich regions, near transcription start sites, or in actively transcribed genes, etc. [29, 30]. In particular, it has been shown that WT integration is more common in the proximity of Alu repeat genomic elements in CD4+ T-cells; is this also true for R263K mutants? These experiments should be done in activated CD4+ T-cells and compared with results obtained with resting CD4+ T-cells and myeloid cells. This type of analysis has been used in previous publications [31].

The most striking observation from the IL6 mRNA measurements is the consistent up-regulation of IL6 gene expression by E138K mutant virus in myeloid cells. Given the common emergence of this substitution secondary to primary resistance mutations against RAL and EVG, future work should be undertaken to investigate the inflammatory pathways that are triggered by E138K infection.

5.8 References

1. Mesplede, T. and M.A. Wainberg, *Resistance against Integrase Strand Transfer Inhibitors and Relevance to HIV Persistence.* Viruses, 2015. **7**(7): p. 3703-18.
2. Mesplede, T., et al., *Integrase strand transfer inhibitors in the management of HIV-positive individuals.* Ann Med, 2014. **46**(3): p. 123-9.
3. Cahn, P., et al., *Dolutegravir versus raltegravir in antiretroviral-experienced, integrase-inhibitor-naive adults with HIV: week 48 results from the randomised, double-blind, non-inferiority SAILING study.* Lancet, 2013. **382**(9893): p. 700-8.
4. Oliveira, M., B.G. Brenner, and M.A. Wainberg, *Isolation of drug-resistant mutant HIV variants using tissue culture drug selection.* Methods Mol Biol, 2009. **485**: p. 427-33.
5. Quashie, P.K., et al., *Characterization of the R263K mutation in HIV-1 integrase that confers low-level resistance to the second-generation integrase strand transfer inhibitor dolutegravir.* J Virol, 2012. **86**(5): p. 2696-705.
6. Kobayashi, M., et al., *In Vitro antiretroviral properties of S/GSK1349572, a next-generation HIV integrase inhibitor.* Antimicrob Agents Chemother, 2011. **55**(2): p. 813-21.
7. Miyoshi, I., et al., *Type C virus particles in a cord T-cell line derived by co-cultivating normal human cord leukocytes and human leukaemic T cells.* Nature, 1981. **294**(5843): p. 770-1.
8. Kohler, C., et al., *Phenotypic characterization of mononuclear blood cells from pregnant Gabonese and their newborns.* Trop Med Int Health, 2011. **16**(9): p. 1061-9.
9. Lorenzo-Redondo, R., et al., *Persistent HIV-1 replication maintains the tissue reservoir during therapy.* Nature, 2016. **530**(7588): p. 51-6.
10. Lataillade, M., J. Chiarella, and M.J. Kozal, *Natural polymorphism of the HIV-1 integrase gene and mutations associated with integrase inhibitor resistance.* Antivir Ther, 2007. **12**(4): p. 563-70.
11. Raffi, F., et al., *Once-daily dolutegravir versus twice-daily raltegravir in antiretroviral-naive adults with HIV-1 infection (SPRING-2 study): 96 week results from a randomised, double-blind, non-inferiority trial.* Lancet Infect Dis, 2013. **13**(11): p. 927-35.
12. Raffi, F., et al., *Once-daily dolutegravir versus raltegravir in antiretroviral-naive adults with HIV-1 infection: 48 week results from the randomised, double-blind, non-inferiority SPRING-2 study.* Lancet, 2013. **381**(9868): p. 735-43.
13. Clotet, B., et al., *Once-daily dolutegravir versus darunavir plus ritonavir in antiretroviral-naive adults with HIV-1 infection (FLAMINGO): 48 week results from the randomised open-label phase 3b study.* Lancet, 2014. **383**(9936): p. 2222-31.
14. Walmsley, S.L., et al., *Dolutegravir plus abacavir-lamivudine for the treatment of HIV-1 infection.* N Engl J Med, 2013. **369**(19): p. 1807-18.
15. Eron, J.J., et al., *Safety and efficacy of dolutegravir in treatment-experienced subjects with raltegravir-resistant HIV type 1 infection: 24-week results of the VIKING Study.* J Infect Dis, 2013. **207**(5): p. 740-8.
16. Mesplede, T., et al., *Addition of E138K to R263K in HIV integrase increases resistance to dolutegravir, but fails to restore activity of the HIV integrase enzyme and viral replication capacity.* J Antimicrob Chemother, 2014. **69**(10): p. 2733-40.
17. Cutillas, V., et al., *The R262K Substitution Combined with H51Y in HIV-1 Subtype B Integrase Confers Low-Level Resistance against Dolutegravir.* Antimicrob Agents Chemother, 2015. **59**(1): p. 310-6.

18. Brown, A., et al., *HLA-A2 down-regulation on primary human macrophages infected with an M-tropic EGFP-tagged HIV-1 reporter virus.* J Leukoc Biol, 2005. **78**(3): p. 675-85.
19. Kondo, M., et al., *Quantitation of HIV-1 group M proviral DNA using TaqMan MGB real-time PCR.* J Virol Methods, 2009. **157**(2): p. 141-6.
20. Zack, J.A., et al., *HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure.* Cell, 1990. **61**(2): p. 213-22.
21. Zagury, D., et al., *Long-term cultures of HTLV-III--infected T cells: a model of cytopathology of T-cell depletion in AIDS.* Science, 1986. **231**(4740): p. 850-3.
22. Marsden, M.D., et al., *Single mutations in HIV integrase confer high-level resistance to raltegravir in primary human macrophages.* Antimicrob Agents Chemother, 2011. **55**(8): p. 3696-702.
23. Canducci, F., et al., *In vitro phenotypes to elvitegravir and dolutegravir in primary macrophages and lymphocytes of clonal recombinant viral variants selected in patients failing raltegravir.* J Antimicrob Chemother, 2013. **68**(11): p. 2525-32.
24. Aquaro, S., et al., *Mechanisms underlying activity of antiretroviral drugs in HIV-1-infected macrophages: new therapeutic strategies.* J Leukoc Biol, 2006. **80**(5): p. 1103-10.
25. Sigal, A., et al., *Cell-to-cell spread of HIV permits ongoing replication despite antiretroviral therapy.* Nature, 2011. **477**(7362): p. 95-8.
26. Anzinger, J.J., et al., *Monocytes as regulators of inflammation and HIV-related comorbidities during cART.* J Immunol Res, 2014. **2014**: p. 569819.
27. Marini, B., et al., *Nuclear architecture dictates HIV-1 integration site selection.* Nature, 2015. **521**(7551): p. 227-31.
28. Serrao, E., et al., *Integrase residues that determine nucleotide preferences at sites of HIV-1 integration: implications for the mechanism of target DNA binding.* Nucleic Acids Res, 2014. **42**(8): p. 5164-76.
29. Singh, P.K., et al., *LEDGF/p75 interacts with mRNA splicing factors and targets HIV-1 integration to highly spliced genes.* Genes Dev, 2015. **29**(21): p. 2287-97.
30. Cohn, L.B., et al., *HIV-1 integration landscape during latent and active infection.* Cell, 2015. **160**(3): p. 420-32.
31. Serrao, E., P. Cherepanov, and A.N. Engelman, *Amplification, Next-generation Sequencing, and Genomic DNA Mapping of Retroviral Integration Sites.* J Vis Exp, 2016(109).

Chapter 6

General Discussion

In chapter 2 we investigated a possible mechanism of regulation of the innate immune factor, PRMT6. PRMT6 possesses antiviral activity against HIV-1 and its regulation could have an effect on HIV-1 replication. PRTM6 automethylation was identified early, but the function of this posttranslational modification remained unknown. Here we identified 3 auto-dimethylated arginines in PRMT6's N-terminal domain. Amongst these, R35 was found to be important in PRMT6 stability and by extension its antiviral activity.

The work presented in chapter 3 represents a first attempt to characterize replication of virus carrying drug resistant mutations in specific target immune cells. Previous work in our lab found that RT enzymes containing drug resistance mutations possess different *in vitro* activities when the dNTP concentration is varied. We wanted to see if important NRTI and NNRTI drug resistance mutations would confer to the virus an advantage when growing in cells known to possess low dNTP levels. Except for the replication of virus bearing the M184V drug resistant mutation (DRM) in MDDC, the replication levels of all other mutated viruses correlated with previous *in vitro* RT enzymatic studies conducted in our lab [1]. This suggests that viruses bearing different DRMs replicate differently in different cells types based on their cellular dNTP concentrations, which are known to be regulated by SAMHD1.

In chapter 4, a new single dose combination drug combining DTG, ABC and 3TC prompted us to determine the effect of combining the M184I/V_{RT} DRMs and INSTI R263K_{IN} DRM that are known to individually decrease HIV-1 replicative capacity in proliferative susceptible cells. Our results showed that replicative capacity was further decreased with this combination of mutations in

RT and IN; however this did not translate into increased susceptibility to either drug. Notably, a decrease in replicative capacity should also have lowered the likelihood that compensatory mutations would emerge when NRTIs are used in combination with DTG. Finally, we studied the replication of INSTI DR mutants in primary human immune cells. We found that viruses containing the R263K substitution in IN replicate better than wild type in resting CD4+ T-cells. Interestingly, $E138K_{IN}$ substitutions resulted in a marked increase in IL-6 expression in myeloid cells compared to all other viruses we tested.

Altogether, our results show that viruses bearing certain drug resistance mutations replicate better than the wild type virus in different primary human immune cells. For RT drug resistant mutants, this may be due to the increased ability of these viruses to replicate in the presence of low dNTP levels that are found in specific immune cells such as MDM and are at least in part caused by the activity of the host restriction factor SAMHD1 [2, 3]. Other factors may also contribute to the increased ability of some drug resistant viruses to replicate better than WT in various immune cells. In regard to the ability of some IN drug resistant mutants to replicate better in resting CD4+ T-cells, the underlying molecular mechanism remains unknown but, as stated above, may involve the mobilisation of specific chromatin structures for integration. In either case, one of the most important discoveries of this thesis is that an increased ability to replicate in some immune cells was found for both RT and IN drug resistant mutants.

This raises questions in regard to the emergence of drug resistance mutations itself. Specifically, two hypotheses can explain the emergence of drug resistance mutations from ongoing or

residual viremia: the classical hypothesis posits that drug resistance emerges in actively replicating circulating activated CD4+ T-cells in individuals who for many reason fail to adhere to their drug regimen (e.g.: lack of stable access to treatment, side effects, high pill burden) and that drug resistant viruses are archived within viral reservoirs secondary to this emergence. A second hypothesis, that agrees better with our results, is that drug resistance may emerge continuously from cells, including macrophages, found in drug sanctuaries such as the lymph nodes, the brain, or other tissues, and that such viruses could be transmitted to blood-borne activated CD4+ T-cells, leading to virological failure. In fact, residual viremia can be detected in some patients taking suppressive cART [4]. Also viruses with certain phenotypes may be preferentially selected in certain cell types early on in HIV infection during formation of the quasispecies, before detection and/or treatment.

Our data are consistent with an important study of viral genetic diversity under treatment, emphasising the importance of drug sanctuaries to drive the emergence of drug resistance [5]. We propose that specific cell type in addition to drug pressure positively influences the development of drug resistance.

Whether this occurs *in vivo*, our results unambiguously demonstrate that some drug resistant mutants can replicate better in immune cells that are also part of the HIV reservoir, specifically macrophages and resting CD4+ T-cells. This result is supported by the specific selection of the E138K RT substitution in CBMCs under 3TC+ETR drug pressure whereas MT2 selections led to the development of V90I and M184I substitutions [6]. Similarly, DTG selected for R263K in

CBMCs but not in MT2 cells [7, 8]. These two observations parallel our current results in which viruses mutated in E138K in RT replicated as well as WT in macrophages and R263K mutants replicated better than WT in resting CD4+ T-cells. These observations are particularly important because both macrophages and CD4+ resting T-cells are considered to be important contributors to the HIV reservoir which is itself the main obstacle to a HIV sterilizing cure, and likely contributes to persistent inflammation in HIV-positive individuals under cART. It is conceivable that drug resistant viruses that initially emerged from these cells types would have a greater chance of being archived within them. One of the strategies to surmount the issue of the reservoir is based on a "shock and kill" approach in which latently infected cells are stimulated so that dormant viral species are reactivated and killed either by cART or by additional interventions. Given our results, such an approach could result in the expansion of drug resistant viruses that may already be archived in immune cells dispersed throughout the body. In particular, macrophages have the ability to deeply infiltrate various organs and tissues where they can reside and be resistant to the cytopathic effect of the virus. Altogether, it seems that using DTG 50 mg BID when proceeding to "shock and kill" trials may represent an additional safety net as this drug may be superior to others in regard to resistance and to its activity against viral reservoirs [9]. DTG has a higher genetic barrier to resistance than all other antiretroviral drugs currently in use; should drug resistance emerge, this should result in a less fit virus that can be easily suppressed with other drugs such as HIV protease inhibitors.

Although there is not yet an existing mechanism that would rationalize the ability of some IN-mutant viruses to replicate better in some immune cells, we also noted that the E138K

substitution in IN resulted in elevated IL6 mRNA levels in MDM cells and this corresponded with a marked decrease in replication in these cells. This suggests that this mutation renders the virus susceptible to detection by the immune system, specifically in these cells.

We have previously studied the phenotype that $E138K_{IN}$ confers to the IN enzyme [10]. Specifically, $E138K_{IN}$ mutant IN has a lower Km (0.5) than either the wild type IN or the $E138K_{IN}$/R163K enzyme and along with higher Vmax, the enzymatic efficiency was greater. However, despite these seemingly advantageous enzymatic properties, the $E138K_{IN}$ containing virus exhibited a decreased infectivity as measured in TZM-bl [10]. It would be conceivable that $E138K_{IN}$ virus was less efficient at successfully integrating proviral DNA into the host genome, possibly as a result of a higher binding affinity. Therefore, it would be interesting to know if there is an increase in unintegrated viral DNA, in the form of linear DNA, 1 or 2LTR circles, in cells infected with $E138K_{IN}$ virus. Furthermore, increased foreign unintegrated DNA may trigger innate DNA sensors such as IFI16 [11]. This could then initiate an antiviral response leading to the expression of cytokines such as IL6, as we have observed in our study (Figure 5.3). The better control of $E138K_{IN}$ virus in MDM and MDDC could thus be due to the presence of certain innate immune effectors in these cells [12-16]. Interestingly one study observed that elite suppressors have higher levels of unintegrated DNA and high levels of 2-LTR circles compared to natural progressors, perhaps pointing to an immune factor that contributes to viremia control and that is triggered by unintegrated DNA [17].

It would be fascinating to investigate the innate immune receptor responsible for detecting unintegrated viral DNA forms when E138K impairs integration kinetics or process. Based on the

literature, such a receptor could be TLR9, cGAS or IFI16. Evidently, the receptor in question should act upstream of transcription factor(s) that is/are responsible for IL6 expression, including NFkB [18]. One way to investigate this issue would be to perform infections with E138K viruses in the presence of siRNAs against various TLRs, RIG-I-like receptors and DNA receptors. Unfortunately, macrophages are very resistant to transfection with and silencing by siRNAs so two alternative approaches could be tested. First, similarly to what we did for SAMHD1, we could establish stable THP1 cell lines that express shRNAs against those various receptors. Alternatively, we would have to remove the corresponding genes from THP1 cells using a CRISPR/Cas9 system.

Our experiments and interpretation of these results raise the interesting possibility that drug resistance mutations are not only emerging because they confer resistance against a specific drug but more importantly because they allow viral replication in specific human cells. Although not proven, this concept is in part supported by the observations reported above that specific DRMs emerge in specific cells. In order to confirm this, we could proceed to *in silico* or actual random mutagenesis of RT or IN and test both the activity and the susceptibility to current drugs of relevant mutated viruses. Any mutation that yields sufficient reverse transcriptase or strand transfer activity in an *in vitro* assay should not eliminate HIV replication. Should such a mutation also be associated with resistance to ARV, it should also be selected in tissue culture experiments or *in vivo*.

6.1 References.

1. Xu, H.T., et al., *Compensation by the E138K mutation in HIV-1 reverse transcriptase for deficits in viral replication capacity and enzyme processivity associated with the M184I/V mutations.* J Virol, 2011. **85**(21): p. 11300-8.
2. Jamburuthugoda, V.K., et al., *Reduced dNTP binding affinity of 3TC-resistant M184I HIV-1 reverse transcriptase variants responsible for viral infection failure in macrophage.* J Biol Chem, 2008. **283**(14): p. 9206-16.
3. Kim, B., et al., *Tight interplay among SAMHD1 protein level, cellular dNTP levels, and HIV-1 proviral DNA synthesis kinetics in human primary monocyte-derived macrophages.* J Biol Chem, 2012. **287**(26): p. 21570-4.
4. Dornadula, G., et al., *Residual HIV-1 RNA in blood plasma of patients taking suppressive highly active antiretroviral therapy.* JAMA, 1999. **282**(17): p. 1627-32.
5. Lorenzo-Redondo, R., et al., *Persistent HIV-1 replication maintains the tissue reservoir during therapy.* Nature, 2016. **530**(7588): p. 51-6.
6. Xu, H.T., et al., *Subunit-selective mutational analysis and tissue culture evaluations of the interactions of the E138K and M184I mutations in HIV-1 reverse transcriptase.* J Virol, 2012. **86**(16): p. 8422-31.
7. Kobayashi, M., et al., *In Vitro antiretroviral properties of S/GSK1349572, a next-generation HIV integrase inhibitor.* Antimicrob Agents Chemother, 2011. **55**(2): p. 813-21.
8. Quashie, P.K., et al., *Characterization of the R263K mutation in HIV-1 integrase that confers low-level resistance to the second-generation integrase strand transfer inhibitor dolutegravir.* J Virol, 2012. **86**(5): p. 2696-705.
9. Mesplede, T. and M.A. Wainberg, *Resistance against Integrase Strand Transfer Inhibitors and Relevance to HIV Persistence.* Viruses, 2015. **7**(7): p. 3703-18.
10. Mesplede, T., et al., *Addition of E138K to R263K in HIV integrase increases resistance to dolutegravir, but fails to restore activity of the HIV integrase enzyme and viral replication capacity.* J Antimicrob Chemother, 2014. **69**(10): p. 2733-40.
11. Stratmann, S., et al., *The innate immune sensor IFI16 recognizes foreign DNA in the nucleus by scanning along the duplex.* Elife, 2015. **4**.
12. Makela, S.M., et al., *Multiple signaling pathways contribute to synergistic TLR ligand-dependent cytokine gene expression in human monocyte-derived macrophages and dendritic cells.* J Leukoc Biol, 2009. **85**(4): p. 664-72.
13. Unterholzner, L., et al., *IFI16 is an innate immune sensor for intracellular DNA.* Nat Immunol, 2010. **11**(11): p. 997-1004.
14. Soby, S., et al., *HSV-1-induced chemokine expression via IFI16-dependent and IFI16-independent pathways in human monocyte-derived macrophages.* Herpesviridae, 2012. **3**(1): p. 6.
15. Wang, Y., et al., *RIG-I activation inhibits HIV replication in macrophages.* J Leukoc Biol, 2013. **94**(2): p. 337-41.
16. Pandey, S., T. Kawai, and S. Akira, *Microbial sensing by Toll-like receptors and intracellular nucleic acid sensors.* Cold Spring Harb Perspect Biol, 2015. **7**(1): p. a016246.
17. Graf, E.H., et al., *Elite suppressors harbor low levels of integrated HIV DNA and high levels of 2-LTR circular HIV DNA compared to HIV+ patients on and off HAART.* PLoS Pathog, 2011. **7**(2): p. e1001300.

18. Laird, S.M., et al., *Expression of nuclear factor kappa B in human endometrium; role in the control of interleukin 6 and leukaemia inhibitory factor production.* Mol Hum Reprod, 2000. **6**(1): p. 34-40.