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ON HIV-1 RESTRICTION IN HUMAN DENDRITIC CELLS AND PERIPHERAL BLOOD MONONUCLEAR CELLS

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Stockholm 2012

Published by Karolinska Institutet.
Printed by Larserics Digital Print AB, Sundbyberg, Sweden

ISBN 978-91-7457-610-8

To my family

ABSTRACT

Dendritic cells (DCs) are one of the first cells to encounter HIV-1 during sexual transmission. They may transmit the virus to $CD4^+$ T-cells either locally or in the draining lymph nodes. In the present work, we have focused the studies on monocyte derived DCs. Upon DC maturation, HIV-1 replication is restricted in these DCs. DC maturation can be triggered by pathogens, danger signals and pro-inflammatory mediators such as TNF-α and IFN-α. The maturation signal results in several functional and phenotypic changes in the DCs. In this thesis, we have studied the influence of apoptotic cells and pro-inflammatory cytokines in their capacity to induce DC maturation and inhibit HIV-1 replication. We found that antiviral host APOBEC3 molecules were restricting HIV-1 replication in the DCs. We, furthermore, studied the effect of proteasome inhibitors on HIV-1 replication in primary cells.

We demonstrated that apoptotic activated $CD4^+$ T-cells (ApoAct) can trigger DC maturation, which was quantified in terms of expression of co-stimulatory molecules. In addition, we detected a reduced frequency of HIV-1 infection in DCs. A prerequisite, for inducing DC maturation and inhibition of HIV-1 replication by the apoptotic cells, was the activation of $CD4^+$ T-cells before inducing apoptosis. Hence, apoptotic resting $CD4^+$ T-cells (ApoRest) did not exert these effects on the DCs. We also found that DCs exposed to ApoAct (either HIV-1 infected or uninfected) secreted MIP-1α, MIP-1β, MCP-1, and TNF-α. Blocking of TNF-α using monoclonal antibodies, partially abrogated induction of co-stimulatory CD86 molecules and reduction of HIV-1 infection in DCs co-cultured with ApoAct. Expression of APOBEC3G in DCs was increased after co-culture with ApoAct, but not ApoRest. Silencing of APOBEC3G in DCs abrogated the HIV-1 inhibitory effect mediated by ApoAct. Sequence analyses of an *env* region revealed significant induction of G-to-A hypermutations in the context of GG or GA dinucleotides in DNA isolated from DCs/ApoAct co-cultures exposed to HIV-1, which are signs of functional APOBEC3 activities.

We found that both the cellular and supernatant fractions of apoptotic activated peripheral blood mononuclear cells (PBMC) were involved in triggering DC maturation. More specifically, the TNF-α present in the supernatant was involved and the cell-cell contact dependent signaling engaged beta-2 integrins, DC-SIGN and TLR4. We also found multiple signaling pathways and transcription factors being activated in DCs when they were co-cultured with ApoAct. These molecules include p38, JNK, PI3K-Akt, Src family of tyrosine kinases, NFκB p65 and AP1 transcription factor family members, c-Jun and c-Fos.

We showed that DCs upon treatment with TNF-α up-regulated co-stimulatory molecules and were able to restrict HIV-1 replication in DCs without inducing the expression of APOBEC3 mRNA (A3G, A3A or A3F). However, when the DCs were treated with low quantities of IFN-α2b they failed to up-regulate co-stimulatory molecules but significantly induced A3G, A3A and A3F mRNA expression and restricted viral replication in DCs. Sequence analyses of the *env* region from HIV-1 infected DCs treated with low quantities of IFN-α2b, showed an induction of high frequency of G-to-A hypermutations.

In addition, we also demonstrated that proteasome inhibitors can effectively reduce transcription from the HIV-1 LTR-promoter. Treatment of PBMCs with proteasome inhibitors showed reduced replication of HIV-1 in PBMC. The results were similar when the PBMCs were treated with proteasome inhibitors alone or in combination with other antiretroviral drugs. Futhermore, proteasome inhibitors reduced expression of IL-2 inducible T-cell kinase (Itk), a Tec-family kinase that is involved in HIV-1 replication.

In conclusion, we have showed that activated apoptotic lymphocytes and pro-inflammatory mediators can induce maturation in DCs and reduce HIV-1 infection, at least in part by inducing APOBEC3 molecules. Low quantities of IFN-α2b restricted HIV-1 replication in DCs while keeping an immature phenotype. We also identified some of the molecules and signaling pathways involved in DC response to ApoAct. Finally, proteasome inhibitors inhibit HIV-1 replication in PBMCs by targeting host factors essential for HIV-1 replication. These finding can be employed in therapeutic and/or prevention strategies.

LIST OF PUBLICATIONS

I. **Venkatramanan Mohanram***, Ulrika Johansson*, Annette E. Sköld, Joshua Fink, Sushil Kumar Pathak, Barbro Mäkitalo, Lilian Walther-Jallow[¶], Anna-Lena Spetz[¶]. Exposure to Apoptotic Activated $CD4^+$ T Cells Induces Maturation and APOBEC3G- Mediated Inhibition of HIV-1 Infection in Dendritic Cells. *PLoS One, June 2011, 6, e21171.* *, ¶ Equal Contribution

II. Sushil Kumar Pathak, Annette E. Sköld, **Venkatramanan Mohanram**, Cathrine Persson, Ulrika Johansson, Anna-Lena Spetz. Activated Apoptotic Cells Induce Dendritic Cell Maturation via Engagement of TLR4, DC-SIGN and Beta-2 integrins. *Manuscript*.

III. **Venkatramanan Mohanram**, Annette E. Sköld, Sushil Kumar Pathak, Anna-Lena Spetz. Low quantities of interferon-alpha2b induce APOBEC3 family proteins and restrict HIV-1 replication but do not mature dendritic cells. *Manuscript*.

IV. Liang Yu*, **Venkatramanan Mohanram***, Oscar E. Simonson, C.I. Edvard Smith, Anna-Lena Spetz[¶], Abdalla J. Mohamed[¶]. Proteasome inhibitors block HIV-1 replication by affecting both cellular and viral targets. *Biochemical and Biophysical Research Communications, May 2009, 385, 100-105*. *, ¶ Equal Contribution

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LIST OF ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
APOBEC	Apolipoprotein B mRNA-editing Enzyme
ART	Anti-Retroviral Therapy
ATP	Adenosine Triphosphate
AZT	Azidothymidine
CCR	CC Chemokine Receptor
CD	Cluster of Differentiation
CLR	C type Lectin Receptors
CTS	Central Termination Signal
CXCR	CXC Chemokine Receptor
DAMP	Danger Associated Molecular Pattern
DC	Dendritic Cell
DC-SIGN	Dendritic cell Specific ICAM-3 Grabbing Nonintegrin
DCIR	Dendritic cell immunoreceptor
DNA	Deoxyribonucleic Acid
DR	Death Receptor
FACS	Fluorescence - Activated Cell sorting
GALT	Gut-Associated Lymphoid Tissue
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
gp	Glycoprotein
HMGB1	High Mobility Group Box 1
HIV-1	Human Immunodeficiency Virus-1

HSA	Human Serum Albumin
ISGF	IFN-Stimulated Gene Factor
IL	Interleukin
Itk	IL-2 inducible T cell Kinase
IND	Indinavir
ICAM	Intercellular Adhesion Molecule
IFN	Interferon
IRF	Interferon Regulatory Factor
JAK	Janus Kinase
LPS	Lipopolysaccharide
LMM	Low Molecular Mass
LTR	Long Terminal Repeats
MIP	Macrophage Inflammatory Protein
MHC	Major Histocompatibility Complex
MCP	Monocyte Chemotactic Protein
MDDC	Monocyte Derived Dendritic Cell
mDC	Myeloid Dendritic Cell
NF-κB	Nuclear Factor kappa B
PAMP	Pathogen Associated Molecular Pattern
PRR	Pattern Recognition Receptors
PBMC	Peripheral Blood Mononuclear cell
PEG	Polyethylene Glycol
PHA	Phytohaemagglutinin
pDC	Plasmacytoid Dendritic Cell

PARN	Poly (A)-specific Ribonuclease
RLR	Retinoic Acid-Inducible Gene-like Receptor
NLR	Nucleotide-Binding Oligomerization Domain-like Receptor
RRE	Rev-Response Element
RNA	Ribonucleic Acid
RT	Reverse Transcriptase
SAMDH	SAM domain HD domain-containing Protein
STAT	Signal Transducers and Activators of Transcription
SIV	Simian Immunodeficiency Virus
TAT	Trans-Activator of Transcription
TGF	Transforming Growth Factor
TNF	Tumor Necrosis Factor
TLR	Toll Like Receptor
TAR	Transactivation Response Element
TRIM	Tripartite Motif-containing Protein
TYK	Tyrosine Kinase
UPP	Ubiquitin-Proteasome Pathway
ZAP	Zinc-finger Antiviral Protein

1 INTRODUCTION

1.1 HIV-1

Since its discovery, HIV-1 infection has caused approximately 25 million deaths worldwide (www.unaids.org). Today very efficient antiretroviral therapy that can keep the viral load under detection level is available, although there is not yet a solution for an absolute cure or a preventive vaccine for HIV-1 infection. However, therapy is not always accessible to everyone. In the third world, where the majority of infected people live, therapy is lacking due to high cost and poor infrastructure. Several efforts with innovative strategies are being pursued to curb HIV-1 infection. These efforts have contributed to progressively turn HIV-1 infection from an inevitably fatal condition into a chronic manageable disease. In this thesis, attempts were made to inhibit viral replication in primary cells by modulating host factors.

1.1.1 HIV-1 discovery

HIV-1, the causative agent of AIDS, was discovered two and a half year [1] after this disease was first identified [2]. It took not more than two years for blood tests to become commercially available, reducing the transmission of AIDS through blood transfusion in developed countries to almost to zero. It was the work of two groups that let to the discovery of HIV-1, Robert C. Gallo in the US, Luc Montagnier and Françoise Barré-Sinoussi in France, were trying to isolate retroviruses from cancer cells. When these groups came across the medical reports of a novel immunodeficiency syndrome in 1981[2], the search for a causative agent was on. Both groups were convinced that a human retrovirus was the causative agent. In 1983, Françoise Barré-Sinoussi and Luc Montagnier reported the first true isolate of HIV-1 from a patient with lymphadenopathy [1, 3]. Later in the same year, Robert Gallo at the national cancer institute in the US also isolated HIV-1 [4]. The progress made from the discovery of the virus to providing treatment for AIDS was the fastest in the medical history.

Despite the fast development of efficient, there is still no cure or effective vaccine available, although significant progress has been made in understanding the viral transmission and pathogenesis. HIV-1 still remains a challenge for the scientific community. According to the UNAIDS report from 2010 an estimated 25 million people have died of AIDS and 33 million are living with HIV-1 infection (www.unaids.org).

1.1.2 HIV-1 genes and proteins

HIV-1 is a lentivirus belonging to the Retroviridae family. It has two copies of single stranded RNA that consists of 9 genes that encodes 15 different proteins [5]. The HIV-1 genome encodes the major structural and non-structural proteins common to all replication-competent retroviruses. From the 5'- to 3'-end of the genome is the *gag* (group-specific antigen), *pol* (polymerase), and *env* (envelope glycoprotein) genes. The *gag* gene encodes a polyprotein precursor $Pr55^{Gag}$, which is cleaved by the viral protease to generate Gag proteins matrix (p17), capsid (p24), nucleocapsid (p7), p6 and two spacer peptides, p2 and p1. The Gag proteins drive the assembly and release of

virus particles and encapsulate the viral RNA genome. The *pol*-encoded enzymes are initially synthesized as part of a large polyprotein precursor $Pr160^{GagPol}$. The Gag and Pol precursors are cleaved by the viral protease. The *pol*-encoded enzymes- protease involved in cleaving the viral polypeptide precursor, reverse transcriptase (RT) converts the viral RNA genome into double stranded DNA, and integrase catalyze the integration of viral DNA into the host cell chromosome.

The Env precursor, known as gp160, is processed by a cellular protease during Env trafficking to the cell surface. gp160 processing results in the generation of gp120 the surface Env glycoprotein and gp41 the transmembrane glycoprotein. gp120 interacts with receptor and co-receptor, and gp41 anchors the gp120/gp41 complex in the membrane and also contains domains that are critical for catalyzing the membrane fusion reaction between viral and host lipid bilayers during virus entry. In addition to the *gag*, *pol*, and *env* genes, HIV-1 also encodes a number of regulatory and accessory proteins. Tat is critical for transcription from the HIV-1 LTR and Rev plays a major role in the transport of viral RNAs from the nucleus to the cytoplasm. Vpu, Vif, Vpr and Nef are accessory proteins [5-7]. Vpu mediates degradation of CD4 and down regulation surface expression of MHC class 1 molecules [8, 9]. Nef is also involved in down regulation of CD4 molecules and MHC class I molecules [10, 11]. Vpr and Vif have implications in cytopathicity and G_2 cell cycle arrest [12-14] Vif also mediates degradation of APOBEC3G molecules through the ubiquitin proteasome pathway [15].

1.1.3 HIV-1 Replication cycle

The HIV-1 replication cycle can be divided into an early and a late phase. In the early events, the virus binds to its main receptor CD4, expressed primarily on the cell surface of T-cells and macrophages, as well as a co-receptor [5]. In dendritic cells, HIV-1 gp120 binds to the C-type lectin DC-SIGN [16]. The main co-receptors are the chemokine receptors CCR5 and CXCR4. After the binding, the virus uses the host cholesterol rich lipid rafts for the membrane fusion between the lipid bilayers of the viral envelope and the host cell plasma membrane. Upon entry into the host cytoplasm, the viral RT facilities the reverse transcription of the viral RNA into DNA. The DNA is subsequently imported into the nucleus as preintegration complex. Viral DNA integrates into the host cell chromosome and the reaction is catalyzed by the viral integrase. The late events include the expression of the provirus, transport of the viral mRNA to the cytoplasm by Rev and assembly and budding with the aid of the Gag protein.

1.1.3.1 Entry and membrane fusion

The CD4 molecule is the principal HIV-1 receptor [17]. A small segment of the N-terminal extracellular domain of CD4 has a high-affinity for binding to gp120. There are five conserved domains (C1-C5) and five variable domains (V1-V5) in gp120. CD4 binding site in gp120 is deeply recessed and flanked by heavily glycosylated variable regions [18].

CD4 alone is not sufficient for HIV-1 Env-mediated membrane fusion and virus entry. This conclusion came from the observation that primary virus isolates from HIV-1 infected individuals display variable tropism for $CD4^+$ T-cells. Certain isolates, referred to as macrophage-tropic replicated efficiently in primary

macrophage cultures, whereas other isolates, referred to as T-cell-line tropic did not productively infect macrophages but replicated efficiently in T-cell lines. Both isolates replicated in activated peripheral blood mononuclear cells (PBMC). Members of the G protein-coupled receptor superfamily of seven-transmembrane domain proteins act as a co-receptors for HIV-1 entry [19, 20]. These molecules are receptors for the α- and β-chemokines. The two most important co-receptors are the α-chemokine receptor CXCR4 and the β-chemokine receptor CCR5. The viral strains are named based on the co-receptor usage, the strains that preferentially use CXCR4 are named as X4 viruses and the strains that utilized CCR5 are termed as R5 viruses, and dual-tropic strains that utilized both CCR5 and CXCR4 are called as R5X4 viruses.

The V3 loop of gp120 plays a major role in determining HIV-1 tropism, exchanging the V3 region between isolates can confer macrophage tropism upon T-cell line-tropic clones [21]. The V1/V2 region also appears to influence co-receptor usage. In addition to the V1/V2 and V3 variable loops, highly conserved regions of gp120 take part in co-receptor binding. The binding of gp120 to CD4 changes the conformation of gp120 so that its affinity for co-receptor is increased. gp120 first binds CD4 to form a complex composed of gp120, CD4, and co-receptor and then there is a conformational change in gp41, which triggers membrane fusion [19-21]. The importance of co-receptors *in vivo* were shown by a number of studies indicating that genetic heterogeneity at co-receptor alleles can affect the susceptibility of an individual to HIV-1 infection. The best characterized example of this phenomenon is the CCR5/Δ32 mutation. The individuals homozygous for this mutant allele encodes for a truncated form of the CCR5 protein are almost completely resistant to HIV-1 infection [22-24].

The membrane fusion that takes place between the lipid bilayers of the viral envelope and the host cell plasma membrane enables the viral core to gain access to the cytoplasm. The membrane fusion is catalyzed by the gp120/gp41 Env glycoprotein complex. The gp41 contains a highly hydrophobic N-terminus also known as the fusion peptide and two motifs N-helix and the C-helix. These helixes are organized in an antiparallel fashion to generate a six-helix bundle [25, 26]. Mutations in these helixes can inhibit membrane fusion and the helixes also undergo rearrangements following CD4/co-receptor binding to ensure that the N-terminal fusion peptide inserts directly into the target membrane.

1.1.3.2 Reverse transcription and integration

Reverse transcription is initiated using a tRNA molecule that is bound to the primer binding site [27, 28]. DNA synthesis proceeds to the 5' end of the RNA molecule, generating a DNA/RNA hybrid. The RNA portion of this hybrid is degraded by the RNaseH, which is part of the RT holoenzyme, leading to the generation a DNA fragment known as the minus-strand strong stop DNA. The minus-strand strong stop DNA has short regions of homology called the R regions. Using these R regions the minus-strand strong stop DNA jumps from the 5' to the 3' end of the genome. This step is referred as the first strand transfer. Minus-strand synthesis occurs by using the 3' end of the minus-strand strong stop DNA as a primer. The RNA remaining from minus-strand synthesis acts as a primer for plus-strand synthesis. The priming occurs at a purine rich sequence known as the central polypurine tract. The tRNA bound to

the primer binding site is removed by RNaseH, thereby allowing second-strand transfer to take place. Plus-strand synthesis proceeds to the end of the minus strand. An additional termination site, referred to as the central termination signal, is located near the center of the genome. Since the CTS are 3' of the central polypurine tract, approximately 100 nucleotides of plus-strand DNA is displaced which results in the formation of a DNA flap. It has been reported that this central flap plays a crucial role in the import of the viral preintegration complex to the nucleus [29, 30].

The RT/template interaction is of a relatively low affinity because reverse transcription involves "jumps" from one template to another [31]. As a consequence of frequent template switch, novel recombinant DNA genome containing sequences derived from both parental RNAs are generated [32]. The high frequency of genetic recombination, together with the high mutation rate of HIV-1 RT (3×10^{-5} per cycle of replication)[33] results in HIV-1 populations being highly heterogeneous in sequence forming quasi-species. This gives HIV-1 an upper hand in rapidly evading the host immune response and developing resistance to antiviral drugs.

The viral DNA is transported to the nucleus as part of the preintegration complex. The viral integrase protein catalyzes the insertion of the linear, double-stranded viral DNA into the host cell chromosome. The integrated DNA, which is referred to as the provirus, behaves essentially as a cellular gene. Integration is an essential step in retrovirus replication. An integrase mutant generally fails to establish spreading infections. The integrase clips off several nucleotides from the 3' termini of both strands of linear viral DNA. This reaction is known as 3'-end processing and generates a double-stranded DNA with 3'-recessed ends. In the nucleus, integrase makes a staggered cleavage in the cellular target DNA. The 3'recessed ends of viral DNA formed in the 3'-end processing reaction are joined to the ends of the cleaved cellular DNA by a process known as strand transfer. Cellular repair enzymes fill in the gaps between the integrated viral DNA and the host target DNA to complete the integration process [7].

1.1.3.3 Provirus expression and budding

New viruses are made from the provirus that has been integrated into the host genome. The provirus makes use of host cellular machineries to produce new viruses. Transcription of HIV-1 is initiated at LTR (Long Terminal Repeat), which has cis-acting elements required for RNA synthesis [5]. The HIV-1 LTR has three regions - U3, R and U5. Transcription is initiated at the U3/R junction. U3 contains the required elements that direct the binding of RNA polymerase II to the DNA template. The transcription factor II D binds to the TATA box that is located approximately 25 nucleotides upstream of the transcription start site. There are three Sp1 and two NF-κB binding sites at the 5' of the TATA box. There is a modulatory region upstream of the NF-κB sites, which contains binding sites for several additional transcriptional factors. The basal transcriptional activity from the HIV-1 LTR is very low but the RNA synthesis is greatly increased when the viral protein transcriptional transactivator protein Tat is present [34, 35]. Tat stimulates transcription of HIV-1 by interacting with a stem-loop structure known as the transactivation response element (TAR) [36]. Tat do not act alone, it directly interacts with a host cell factor cyclin T, which in turn recruits a cyclin-dependent kinase (CDK9) to the TAR element [37]. Cyclin T and CDK9 form a complex known as positive transcription elongation factor b (P-TEF-b). This results in hyper-phosphorylation of the RNA polymerase II, which in turn stimulates transcriptional elongation. HIV-1 structural proteins are

encoded by unspliced or partially spliced mRNAs and the viral RNA genome itself is derived from a full-length RNA. The unspliced mRNA cannot be exported to the cytoplasm and HIV-1 uses the viral protein Rev to overcome this problem. Rev binds to a cis-acting RNA element known as the Rev-response element (RRE) to facilitate the export of unspliced or partially spliced mRNA to the cytoplasm [38]. Rev uses the cellular machinery to shuttle back and forth to the nucleus. It binds to the cellular nuclear import receptor importin b to enter the nucleus. To export the RNA to the cytoplasm, Rev interacts with the nucleocytoplasmic transport factor Crm1 [39]. Once all the necessary proteins are made, the new virion is packed with two copies of single stranded RNA in the cytoplasm and budded out. Lipid rafts are involved in budding of the virus [40-42]. The p6 domain of Gag plays an important role in budding of the newly assembled virions [43, 44].

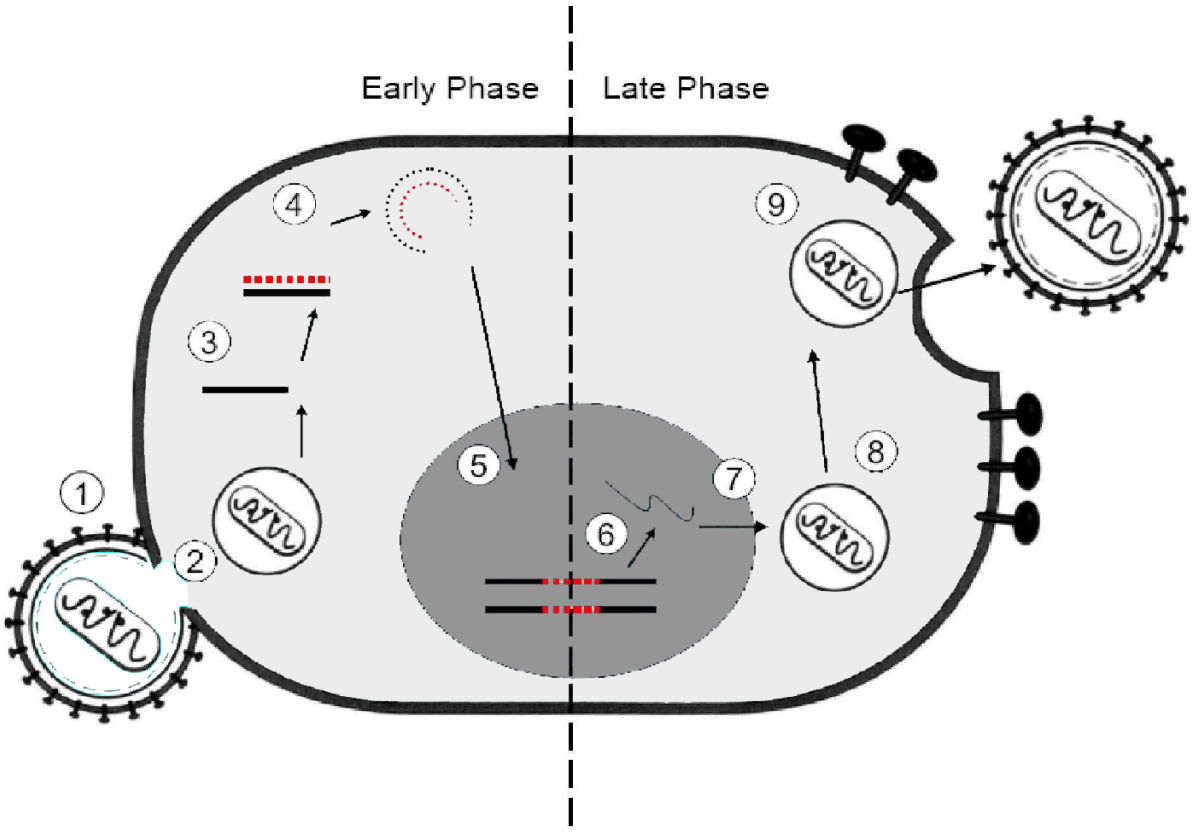

Figure 1. HIV-1 replication cycle (1) The early phase begins with viral attachment, quickly followed by fusion and entry (2) Uncoating of the viral core (3) Reverse transcription converts the single-stranded viral RNA genome into double-stranded DNA (4) The preintegration complex of viral and cellular proteins is transported to the nucleus (5) Integration of viral DNA into the host chromosomal DNA. (6) Late phase of the cycle, Initiation of transcription by RNA Pol II generates spliced and unspliced mRNA templates. (7) Translation produces accessory proteins and polyproteins (Gag and Gag-Pol) encoding structural and enzymatic proteins (8) Core particle assembly is initiated and (9) Final assembly and release of progeny virions. Figure adapted from [5].

1.1.4 NF-κB

NF-κB is a ubiquitous transcription factor and has five members, NF-κB1 (p50), NF-κB2 (p52), RelA (p65), c-RelA and RelB. In a steady state condition, NF-κB stays in the cytosol as heterodimers (p65/RelA, p50/NF-κB1). NF-κB is maintained as a heterodimer by an inhibitory protein IκB [45]. Following cellular stimulation, these inhibitory proteins are phosphorylated and subsequently degraded by the ubiquitin-proteasome pathway (UPP) leading to the release of NF-κB. Free NF-κB translocates to the nucleus and binding to the NF-κB binding site of the viral LTR and initiates provirus transcription [46, 47]. NF-κB activation and signalling is known to be strictly dependent on the activity of the 26S proteasome [48, 49].

1.1.5 HIV-1 transmission and pathogenesis

Most of the HIV-1 infections occur after HIV-1 exposure on a mucosal surface. The infection risk varies according to the route of infection [50] and there are many factors associated with mucosal integrity. In addition, genetic constitution influences the susceptibility and resistance to HIV-1 infection during sexual transmission [51]. The knowledge of initial events of HIV-1 infection are obtained by studying the human tissue explants [52, 53] and vaginal inoculation of macaque with SIV [54-57]. These studies suggest that $CD4^+$ T-cells and dendritic Langerhans cells are the first targets in HIV-1 infection [52, 53]. In addition, other DC populations may also play an important role [58]. The virus crosses the mucosal barrier by transcytosis - a vesicular transport from one side of the cell to another or through intercellular space in the epithelium or by binding to the DCs in the intraepithelial to access the target cells [59]. Viral evolution studies on viruses present early after viral transmission have shown that HIV-1 infection often occurs from a single founder virus [60, 61].

During vaginal transmission, the virus can be transported from the mucosa to the draining lymph node by the DCs where the virus infects $CD4^+$ $CCR5^+$ T-cells [16]. Studies using SIV-infected rhesus macaque (both intrarectal and intravenous inoculation) has shown that primary infection is characterized by a selective loss of $CD4^+$ T-cells within mucosa-associated lymphoid tissues that coincides with the massive increase of viral load in the plasma [62-64]. In the intravenously SIV-infected rhesus macaque, depletion of GALT $CD4^+$ T-cells occurs relatively rapidly within 1–2 weeks post-infection and mainly at effector sites, i.e., the lamina propria [65]. Recent studies have provided evidence that this massive mucosal $CD4^+$ T-cell depletion is not a specific characteristic of the SIV-macaque model, but is also observed in the gut of acutely HIV-1-infected patients 4–6 weeks post infection [66-68]. The SIV-macaque model with different route of inoculation reveals that this trait is independent of the route of transmission [57, 69-72]. High levels of CCR5 and CXCR4 co-receptor expression on intestinal $CD4^+$ T-cells, and their predominantly activated state make them highly susceptible to the viral infection and this may, in part, explain the severity of $CD4^+$ T-cell depletion seen in GALT [73]. When HIV-1 vigorously replicates in the GALT and other lymphoid tissues, the viral load in the plasma increases exponentially to reach a peak, usually more than a million RNA copies per ml of blood, but the $CD4^+$ T-cell decreases in number and later returns to near normal levels in the blood but not in the GALT [64, 66, 74]. After the acute phase of infection there is an asymptomatic period of HIV-1 infection were the viral load decreases to reach a more stable level, known as the viral set point. This viral set

point is believed to occur when there is equilibrium between viral replication and immune control [75-77].

Knowledge on HIV-1 life cycle has not only enabled the discovery of antiretroviral drugs but may also pave the way for better understanding of the host factors involved in the viral life cycle that can be novel targets for inhibiting viral replication.

1.2 Host restriction factors – A defence against HIV-1 infection

Humans and other mammals have developed a system of antiviral activities mediated by intracellular proteins referred to as restriction factors. These proteins can block infection of sensitive viruses after they have entered the host cell. They are species specific in that each species can restrict a subset of viruses with exquisite sensitivity [78]. TRIM5α, tetherin and APOBEC3 molecules and recently dendritic and myeloid cell specific restriction factor called SAMHD1 were all identified to have antiviral activity. In recent studies, human genes were screened for identification of HIV-1 restriction factors and factors involved in HIV-1 replication using siRNA genomic screens. These studies revealed numerous genes involved in restriction of HIV-1 replication during the early stages of the viral life cycle [79-83]. However, these studies did not always reveal the same target genes. This may be due to the fact that different cellular targets were used, different viral strains grown in different target cells *in vitro* or the use of pseudoviruses, differences in kinetics, doses of viruses used, to mention some factors that can explain the differential outcome. Hence, a lot of work remains to be done to validate the target genes discovered in the siRNA genomic screens. Here short descriptions are made for the so far most studied HIV-1 restriction molecules.

1.2.1 TRIM5α

Tripartite motif-containing protein 5 or TRIM5α mediates low level of HIV-1 replication inhibition in humans, but they play a very important role in the antiviral activity in non-human primates [84]. TRIM5α binds as multimer to the incoming virus capsid, which leads to a premature uncoating of the capsid thereby restricting reverse transcription and nuclear transport of the viral genome [85, 86]. The exact mechanism of action of TRIM5α, has not been shown conclusively, but the capsid protein from restricted viruses, is ultimately removed by proteasome-dependent degradation [87].

1.2.2 Tetherin

Tetherin is another human cellular protein, which contributes to retroviral inhibition [88]. Tetherin is also known as bone marrow stromal antigen 2 or CD317. Tetherin is a type 2 integral membrane protein. When the virus buds from the surface of the cell, tetherin tethers the virions to the producer cell, by means of its N-terminal transmembrane domain and its C-terminal glycosyl-phosphatidylinositol anchor there by inhibits the release of the virus from the producer cell. The viral protein Vpu antagonizes tetherin [88]. Tetherin also cross-links the virions to each other resulting in the inhibition of the release of the virus from the infected cell.

1.2.3 SAMHD1

The cell specific SAM domain HD domain-containing protein 1 or SAMHD1 is a protein involved in Aicardi–Goutieres syndrome, a genetic encephalopathy with symptoms mimicking congenital viral infection. SAMHD1 negatively regulates interferon responses [89]. A recent study has shown that SAMHD1 acts as an antiretroviral factor [90]. The quantification of total viral DNA species after infection in SAMHD1 silenced cells resulted in the accumulation of total viral DNA shows that the restriction is at the reverse transcription level. The report concludes that SAMHD1 is expressed in cells of the myeloid lineage and inhibits viral replication by interfering at an early step of the viral life cycle. Viral protein Vpx counter acts SAMHD1 by inducing proteasomal degradation [90, 91].

1.2.4 Zinc-finger antiviral protein (ZAP)

A recent report have demonstrated that ZAP inhibits HIV-1 by recruiting both the 5' and 3' mRNA degradation machinery to specifically promote the degradation of multiply spliced HIV-1 mRNAs. ZAP selectively recruits cellular poly(A)-specific ribonuclease (PARN) to shorten the poly(A) tail of target viral mRNA and recruits the RNA exosome to degrade the RNA body from the 3' end. In addition, ZAP recruits cellular decapping complex through its cofactor RNA helicase p72 to initiate degradation of the target viral mRNA from the 5' end. Depletion of each of these mRNA degradation enzymes reduced ZAP's activity [92].

1.2.5 APOBEC3 molecules

APOBEC3 proteins or apolipoprotein B mRNA-editing enzyme 3 belongs to cellular polynucleotide cytidine deaminases family. Initially, Vif was considered to be important for the production of infectious virus from the proviral genome [93, 94]. The APOBEC3G antiretroviral factor was initially named CEM15. CEM-SS is a T-cell line that supports replication of Δ*vif* virus. When these cells, were stably transduced with CEM15 encoding retroviral vector that enforced expression of APOBEC3G (CEM15), these cells restricted the replication of Δ*vif* virus. With this experiment it was demonstrated that APOBEC3G is a restriction factor and was responsible for restricting HIV-1 infection [15].

Six members of APOBEC3 family have been identified to be involved in HIV-1 restriction. Studies on APOBEC3G (A3G) have shed lights on the molecular mechanism by which the APOBEC3 family member act on the virus and how viral Vif counteracts them. A3G has a DNA deaminase activity with distinct sequence specificity [95]. A3G molecules have to be present in the virus producing cells to exert antiviral function. To do so, A3G molecules get packaged into viral particles and when the virus enters a fresh cell A3G heavily deaminate cytosines into uracils in minus-strand viral DNA during the viral reverse transcription [96-100]. Adenine is incorporated on the opposite plus-strand as a result of uracil introduction in the minus-strand viral DNA. The deaminase activity on the minus-strand can be deduced by the accumulation of G-to-A hypermutations mainly on the plus-strand viral DNA following second-strand synthesis. The frequent detection of G-to-A hypermutation in samples from HIV-1 patients constitutes clear evidence that A3G does act to deaminate the HIV-1 DNA during natural infection [101, 102]. These hypermutations can be lethal to the virus in two ways. First, the G-to-A hypermutation would get fixed in the provirus, which leads to truncated protein production or disturbed viral

gene expression. Second, uracil residues can be recognized by cellular DNA repair enzymes, leading to the degradation of the unpaired DNA strands as a part of repair mechanism [103] (Fig.2).

A study showed that in resting $CD4^+$ T-cells, A3G exists predominantly in the low molecular mass (LMM) configuration and was shown to impose a potent post-entry restriction on HIV-1infection [104]. But this report was later retracted, as another study failed to confirm the above mentioned claim about involvement of A3G in the post-entry restriction of HIV-1 [105]. Thus, the relative contribution of A3G to the resistance of quiescent T-cells to HIV-1 infection remains to be resolved.

Apart from the cytidine deaminases activity, A3G is proposed to interfere with reverse transcription by inhibiting tRNA3Lys from binding to the HIV-1 primer-binding site [106]. Removal of primer tRNA from the minus-strand results in an insufficient substrate for the integrase that inhibits proviral integration in the host genome [107].

Vif counteracts APOBEC3G by recruiting E3 ubiquitin ligase complex through the interaction of Vif BC box motif and elongin C [108-111]. Vif acts as a bridge to target APOBEC3G for ubiquitination through its N-terminal domain and subsequent degradation of APOBEC3G via the proteasomal degradation pathway [112-115].

Apart from A3G there are other APOBEC3 members that play a role in HIV-1 inhibition. APOBEC3F (A3F) is a strong inhibitor of Vif deficient HIV-1. It inhibits viral replication in the same fashion as A3G by introducing G-to-A mutation in the viral genome, though the degree of mutagenesis has been noted to be lower than A3G in some cell culture experiments. [116-120]. A3F also undergo Vif-mediated proteasomal degradation [121]. APOBEC3B (A3B) is another member of the APOBEC3 family, which is resistant to Vif and it has been shown to have significant antiviral effect but is poorly expressed in $CD4^+$ T-cells so it may not have a significant effect on HIV-1 during natural infection. A3G, A3F and A3B have two CDA domains [116, 122, 123]. APOBEC3A (A3A) contains a single CDA domain, is expressed in immature monocytes and in other myeloid cells. A3A has been reported to restrict HIV-1 infection during the early phase of its life cycle. The antiviral effect of A3A is exerted at the reverse transcription step through a mechanism that may involve deamination. [124, 125]. APOBEC3C (A3C) also contains a single CDA domain, is expressed in $CD4^+$ T-cells and can act in target cells to introduce infrequent G-to-A mutations at sublethal levels during infection by some HIV-1 strains [126]. Lastly, a single domain protein, APOBEC3D/E (A3D/E), can also modestly inhibit HIV-1 infection in a Vif-sensitive manner when overexpressed in virus-producing cells [127].

During natural infection, the virus has an active Vif to counter act APOBEC3 and one can argue that Vif might mediate low effectiveness of APOBEC3 molecules, which in turn will result in introduction of sub lethal mutations which might add up to the viral genetic diversity. Population studies have shown that there is a possibility of variations in the functionality of different *vif* alleles [128], mRNA level of A3G and A3F are associated with infection status, as well as with differences in rates of disease progression, plasma virus loads, and transmission [129-131]. In addition, increased $CD4^+$ T-cell counts and decreased virus loads have each been correlated with the hypermutated HIV-1 sequences [101, 132]. Strategies to enhance or modulate the expression of host restriction factors could be vital in preventing HIV-1 transmission.

Discovery of more restriction factor will shed light on host defense mechanism against retroviruses and may also lead to new therapeutic interventions.

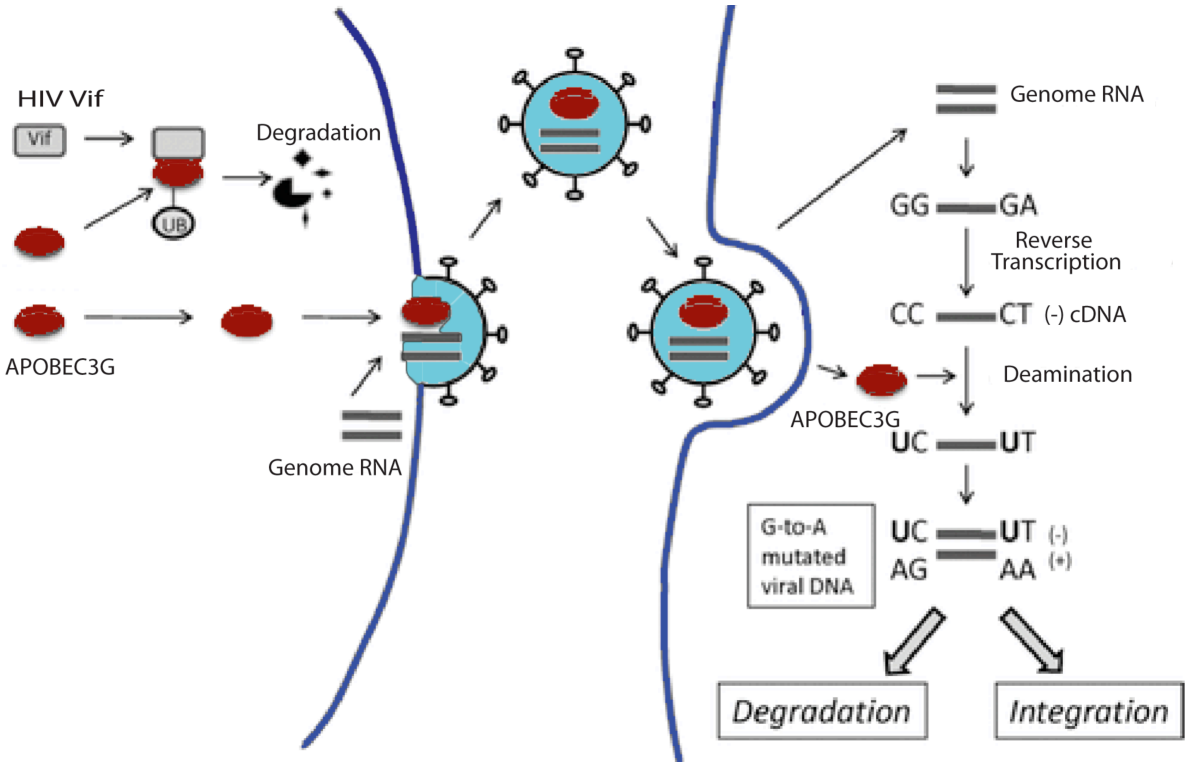

Figure 2. Induction of G-to-A hypermutation in the provirus by APOBEC3G - Adapted from [133].

1.3 Dendritic cells

Dendritic cells are sparsely distributed antigen-presenting cells (APCs), which play a critical role in bridging the innate and adaptive immune response. Dendritic cells (DCs) have been referred to as "professional" APCs. The principal function of DCs is to present antigens, and to induce a primary immune response in resting naive T-cell. To perform this function, DCs capture antigens, process them, and present them on the cell surface as peptides on the major histocompatibility complexes (MHC). DCs can also present self-antigens to T-cells to regulate T cell activation or in other words induce tolerance [134].

DCs were first observed in human skin by Paul Langerhans in 1868 and were named Langerhans cells (LCs). LCs were mistaken as nerve cells due to their morphology. Steinman and Cohn established the term dendritic cell when they discovered these cells in mouse spleen [135-137]. Later LCs and DCs have proved to be originated from hematopoietic progenitors in the bone marrow [138].

1.3.1 DC subsets

DCs constitute a heterogeneous population of cells located throughout the blood, tissues, and lymphoid organs and are derived from different lineages [139]. They are involved in the generation of both innate and acquired immune responses, including release of cytokines such as IL-12, IL-10, and IFN-α, stimulation of naive T-cell

clonal expansion [140, 141], natural killer (NK) cell stimulation [142] and plays an important role in peripheral tolerance to self peptides [143]. DCs are derived from $CD34^+$ stem cells via at least two generation pathways: myeloid and lymphoid [144]. Myeloid DCs (mDCs) also known as conventional DCs (cDC), characterized by CD1a and CD11c expression, are found in most tissues except the brain and testes [141]. DCs of lymphoid lineage, commonly called plasmacytoid DCs (pDCs), have a more restricted distribution being found predominantly in the blood and lymphoid tissues [145, 146]. DCs can also be generated from blood monocytes (known as monocyte derived DCs or MDDCs) or bone marrow precursors [147, 148].

Dendritic cells express different pattern recognition receptors (PRR), depending on the cell subset and to some extent on location. These PRR are able to recognize motifs unique to different classes of pathogen [149, 150]. The PRRs includes C type lectin receptors (CLR), which recognize glycosylated carbohydrate domains [151] and Toll like receptors (TLR), which have a high degree of specificity for individual pathogen associated molecular patterns [152], retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) [153].

1.3.2 DC maturation and migration

DCs exist as precursors, immature and mature DCs, and their location in the body depends on the state of maturation. Immature DCs are very efficient in picking up antigens as they look for pathogens in the surrounding area, express moderate amounts of MHC class II and low levels of co-stimulatory molecules. They also express the chemokine receptors CCR1 and CCR5. DCs are matured in response to stimuli such as antigen uptake or viral replication in DCs as well as exogenous stimuli such as lipopolysaccharide (LPS) or cytokines such as TNF-α. Maturation results in many functional and phenotype changes in DCs. 1) Up-regulation of DC co-stimulatory molecules CD40, CD80, CD83, CD86, and also MHC class II are all up-regulated as well as adhesion molecules ICAM-1 and VLA-4, which enables more prolonged DC-T-cell interaction [154, 155]. 2) Chemokine receptors CCR7 expression on DCs are increased, which induces migration to the secondary lymphoid tissue [156] in response to CCL21 and CCL4 produced in the lymph node [157, 158]. 3) Secretion of cytokines IL-12, IL-15, IL-18 and chemokines MDC, TARC, ELC, stimulates DC-T-cell interactions, which helps in helper and cytotoxic T lymphocytes activation [159] and 4) C-type lectin receptor DC-SIGN, mannose receptor (MR) and to a lesser degree, Langerin are all down-regulated by maturation. DC maturation can be triggered by recognition of pathogen associated molecular patters (PAMPs) that engage for example TLRs, NLRs, or C-type lectins expressed in DCs. DCs can also respond to endogenous danger associated molecular patterns (DAMPs) from injured cells [160]. The High mobility group box 1 (HMGB1) protein is released from necrotic and late apoptotic cells [161, 162]. HMGB1 has been shown to induce DC maturation *in vitro* and to have an adjuvant effect when added to together with soluble antigen [163].

1.3.3 DCs during HIV-1 infection

DCs are one of the first cells to meet HIV-1 during sexual transmission. They detect viruses in peripheral tissue sites and following viral uptake and activation they migrate to draining lymph nodes, where they trigger adaptive immune responses and promote NK cell activation.

Reports from animal studies suggest that HIV-1 transmission by DCs can occur either by direct infection (cis) or through binding of the virus to DCs without infection (trans) [164-166]. Apart from the CD4 and chemokine receptors expressed on DCs HIV-1 also uses C-type lectin receptor DC-specific ICAM3-grabbing non-integrin (DC-SIGN), Langerin (also known as CLEC4K or CD207) and DC immunoreceptor (DCIR; also known as CLEC4A) to enter DCs [16, 167]. HIV-1 also uses lipid drafts to bind and internalize into DCs [168]. Expression of these receptors varies according to the DC subtype, localization and maturation status. The gp120 of HIV-1 envelope binds to DC-SIGN of DCs and enters early endosomal compartment where the virus stays intact. When the DCs reach the draining lymph nodes they promote infection to the T-cells through viral synapses or cell-cell fusion or other mechanisms [169]. There is also a report that the transfer of HIV-1 to T-cells can occur independently of DC-SIGN [170]. DCIR was also recently found to bind to HIV-1 and promotes transmission of infectious virus to $CD4^+$ T-cells in a similar fashion to DC-SIGN [171]. Langerin can bind and internalize HIV-1 and migratory Langerhans cells are believed to enhance HIV-1 infection by promoting the spread of the virus to T-cells in draining lymph nodes. In-vitro-derived Langerhans cells can transmit infectious HIV-1 to T-cells [172].

HIV-1 does not induce maturation of cDCs by activating TLRs [173] however there are reports claiming that HIV-1 matures MDDCs and Langerhans cells [174]. Replication of integrated HIV-1 in immature cDCs can be initiated by TLR8 and DC-SIGN mediated signal transduction events [175]. HIV-1 is targeted to TLR8 containing endosomal compartments by DC-SIGN, and this initiates the transcription of integrated HIV-1 DNA through TLR8 activation and subsequent nuclear factor-κB (NF-κB) signaling. The binding of gp120 to DC-SIGN leads to RAF1-mediated phosphorylation of the p65 subunit of NF-κB, which results in productive transcription within infected DCs [175]. Following HIV-1 infection, fusion between endosomes and autophagosomes is inhibited and this prevents autophagy-mediated viral degradation [176].

There is a progressive reduction in blood DC numbers after HIV-1 infection that correlates with increasing plasma viral load and disease progression [177-180]. This decrease in DC frequency occurs early during acute HIV-1 infection and is sustained in the later stages of infection. Patients with chronic HIV-1 infection also have fewer blood DCs compared with uninfected controls [181, 182]. It was observed that DC numbers returned to normal levels following antiretroviral therapy (ART) [183].

DCs undergo several functional changes during HIV-1 infection. Their maturation is impaired, they have an altered cytokine profile and also altered ability to stimulate T-cells [184, 185]. HIV-1 proteins Vpr and Nef are shown to be involved in the reduction of co-stimulator molecules CD80 and CD86, they are also involved in the reduction of HLA-DR in DCs, this in turn reduces the ability of the DCs to stimulate $CD8^+$ T-cell activation [186-189]. Studies from our group has reveled that HIV-1-infected DCs up-regulate cell surface markers but fail to produce IL-12 p70 in

response to CD40 ligand stimulation [190].

cDCs from patients with acute HIV-1 infection produce increased levels of IL-12p70, IL-6, TNF-α and MIP-1α compared with cDCs from uninfected controls [181]. pDCs isolated from patients with acute HIV-1 infection has shown decreased IFN-α production during the early stages of infection, but increased IFN-α production at later stages of infection [181]. Although IFN-α produced by pDCs can inhibit viral replication they do not inhibit transmission of the virus to $CD4^+$ T-cells [191]. The altered cytokine production and down regulation of co-stimulatory molecules, MHC class I and CD4 of DCs by HIV-1 could explain, at least in part, the severe impairment of T-cell function during HIV-1 infection [192]. Upregulation of DC-SIGN can favor the transmission of the virus to the $CD4^+$ T-cells through DC-T cell contact. The close interaction of $CD4^+$ T-cells with APCs has been shown to favor HIV-1 replication [193] this can be the reason why HIV-1 specific memory $CD4^+$ T-cells are readily infected compared to memory $CD4^+$ T-cells with other specificities [194]. DC when co-cultured with activated $CD4^+$ T-cells enhances viral replication in a cell-cell contact dependent manner [195].

Although HIV-1 can induce DC dysfunction, the DCs are capable of initiating effective T-cell response during acute and early infection [196]. These DCs are likely to be uninfected and prime T-cells by cross-presentation or by direct presentation of exogenously derived viral antigen that are derived from non-infectious virus or from previously infected apoptotic cells or from immune complexes. The cross-presentation of HIV-1 antigen derived from apoptotic cells has been shown to induce both $CD4^+$ and $CD8^+$ memory T-cell response [197, 198]. Cross-presentation of infected apoptotic cells has been shown to be more effective than the direct presentation of non-infectious virus or infectious virus [199].

DCs are being investigated as candidates for immunotherapeutic strategies to augment T-cell immunity to HIV-1 infection, but more work has to be done to get a clear insight on the viral entry and replication in DCs.

1.4 Apoptosis

Apoptosis and necrosis are two major processes by which cells die. During necrosis, the cell ruptures and releases its intracellular content, which activates macrophages leading to the release of pro-inflammatory cytokines. These cytokines may provide the co-stimulation required for T-cell activation and immunity. Apoptosis is a controlled process, in organogenesis and tissue remodelling during development, to eliminate used-up, damaged, or misplaced cells during the embryonic development and the tissue homeostasis of multicellular organisms. Apoptotic cell death also occurs during infections and wound healing and in different disease states [200, 201].

There are many stimuli and conditions, both physiological and pathological, which can trigger apoptosis. It is an energy-dependent process, which implicates the activation of a group of cysteine proteases called caspases in a complex cascade of events [202-204]. Depending on the initiating stimuli, two major mechanisms could be defined: the intrinsic pathway or mitochondrial pathway [205], that involves members of the bcl-2 family and mitochondrial functions and the extrinsic pathway which is activated by

extracellular signals that act via death receptors (DR) [206, 207]. Several DRs have been described that all belong to the TNF receptor superfamily. Each pathway activates its own initiator caspase, which, in turn, will activate the executioner caspase-3. There are evidences that the two pathways are linked and that molecules in one pathway can influence the other [208]. A third pathway, involving T-cell mediated cytotoxicity and perforin/granzyme dependent killing of cell works in a caspase-independent fashion.

1.4.1 Uptake of apoptotic cells by phagocytes

DCs can take up pathogens as well as apoptotic cells. The ingestion of pathogens often triggers a pro-inflammatory response, whereas the engulfment of apoptotic cells is generally anti-inflammatory or immunologically silent [209]. The recognition of apoptotic cells involves the 'eat-me' signals that they display on the cell surface, and many of these signals are recognized by engulfment receptors that are expressed by the phagocyte [210]. Uptake of apoptotic bodies involve sensing, recognition of the apoptotic cells by the DCs, cytoskeletal reorganization and internalization of the target, processing and degradation of the ingested apoptotic cell and post-engulfment responses of the DCs.

Sensing of apoptotic bodies by phagocytes can be promoted by the “find me” signal molecules like lysophosphatidylcholine (LPC) [211] and nucleotides such as ATP and UTP [212]. These molecules, which are diffusible extracellular factors, promote the recruitment of phagocytes. The molecules that are involved in the “eat me” signal are found in the surface of the apoptotic cell. Phosphatidylserine is one such molecule that is found on the plasma membrane of the apoptotic cell. Other eat-me signals include changes in the surface charge of glycoproteins and lipids resulting in addition of sugars, the binding of thrombospondin or the complement component C1q to the apoptotic-cell surface, and the expression of intercellular adhesion molecule 3 (ICAM3) and oxidized low-density lipoprotein (LDL)-like moiety [213-216].

There are several receptors expressed by the phagocyte that recognize these eat-me signals. Lectins that bind altered sugars [217], the vitronectin receptor that binds thrombospondin [218], scavenger receptors that bind oxidized LDL-like moieties [219], CD14 that binds ICAM3 [220], CD91 also known as LRP1 binds C1q through calreticulin [221] [222] and the receptor tyrosine kinase MER binds to the serum-derived protein GAS6 growth-arrest-specific 6 [223]. Apoptotic cell recognition appears to depend on the cell that is being engulfed, the receptors that are expressed by the phagocyte and the state of activation of the phagocyte during the process [224-226].

The engulfment of apoptotic cells by phagocytes often results in non-inflammatory and non-immunogenic response [209]. Engulfment of apoptotic cells by monocytes and macrophages induces reduced secretion of pro-inflammatory cytokines such as TNF-α and IL-1 when treated with LPS [227-229]. This was partially mediated through the secretion of anti-inflammatory cytokines like IL-10, TGF-β, platelet activating factor and prostaglandin E2 [228, 229]. This above mentioned effect is applicable to DCs, however, the damping of immune response is not entirely coupled to the anti-inflammatory cytokines [230-233]. Engulfment of infected apoptotic cells by DCs results in secretion of both pro-inflammatory (IL-6) and anti-inflammatory (TGF-β) that leads to a Th17 response [234]. Many studies have also shown proofs of immune stimulatory effect of apoptotic cells on DCs [234-241]. HMGB1 and caspase

activation can influence immunogenicity of apoptotic cells [242].

It has been shown that activated apoptotic PBMC (ApoAct) induced DC up-regulation of co-stimulatory molecules, release of pro-inflammatory cytokines and proliferation of autologous T cells recognizing allo-antigen from engulfed apoptotic material. However, resting apoptotic PBMC (RestApo) were unable to generate these DC responses [240]. It has also been shown that murine activated apoptotic splenocytes can provide endogenous adjuvant activity when used in combination with a DNA vaccine to augment both cellular and humoral immune responses after immunization *in vivo* [243].

The immunogenicity of apoptotic cells differs depending on the type of phagocytes, the type of apoptotic stimuli and the presence of pathogenic antigens. More knowledge is needed to understand the role played by apoptotic cells during pathological conditions. Nevertheless, the use of apoptotic cells or altering of pathways inducing apoptosis as therapeutic agent may be considered.

1.5 Type I Interferons

Interferon (IFN) was first described as a product of influenza virus infected chick embryo cells, capable of inducing resistance to infection [244]. Some years later, a functionally related protein, which is now called IFN-γ or type II IFN was described as an IFN-like virus-inhibitory protein produced by mitogen-activated human T-cells [245]. Interferons are expressed in low levels in physiological conditions but their expression is enhanced when the cells are exposed to virus or other stimuli. Apart from being a potent antiviral factor they also have immunomodulatory effect on immune cells. The interferons are divided into three types according to their receptor usage - type I IFNs (IFN-α, IFN-β, and IFN-ω), which bind to a specific cell surface receptor complex known as the IFN-α/-β receptor (IFNAR), The type II IFN (IFN-γ) binds to the IFN-γ receptor and type III IFNs, which signal through a receptor complex consisting of IL10R2 also called CRF2-4 and IFNLR1 also called CRF2-12. Twelve separate functional IFN-α genes and proteins and a single IFN-β gene exist in humans [246]. Type I interferons will be discussed in this thesis as we have used IFN-α to modulate HIV-1 infection in DCs.

All type I IFNs are genetically and structurally very similar. They all lack introns, they are located on the short arm of chromosome 9 and utilize the same receptor subunits [247] but different type I IFNs stimulate different antiviral, anti-proliferative, and clinical responses [248] and are likely to stimulate different immunoregulatory responses as well. The expression of type I IFNs are regulated by an intracellular signaling pathway that is activated by PRRs. These receptors recognize molecular patterns specific to microorganisms, such as viral genome nucleic acids [153]. Recent studies have revealed that the PRRs including TLRs, RLRs, and NLRs are involved in discriminating self from non-self nucleic acid [249]. Early type I IFN themselves provide a positive autocrine feedback loop for the production of later appearing IFN types [250]

The responses to type I IFNs are initiated by their interactions with a specific cell-surface receptor, which has an extracellular ligand binding domain and an intracellular kinase domain, activated after ligand-induced dimerization [251]. The receptor has two chains, IFNAR1 and IFNAR2c. Both are necessary for most functions and, in the absence of either, there is no high-affinity ligand binding and leads to little biological

effect. Each receptor subunit binds constitutively to a single specific member of the Janus kinase (JAK) family. IFNAR1 binds to tyrosine kinase 2 (TYK2) and IFNAR2 binds to JAK1. Ligand binding induces the phosphorylation of JAK1, TYK2, intracellular tyrosine residues of each receptor chain and signal transducers and activators of transcription (STATs). Activated STATs dimerize, dissociate from the receptor, and translocate to the nucleus to induce the expression of interferon-stimulated genes (ISGs) [252]. In many cells, type I IFNs activate a major transcription factor, IFN-stimulated gene factor 3 (ISGF3), a complex of phosphorylated STAT1, STAT2, and unphosphorylated interferon regulatory factor 9 (IRF-9 or p48), which binds to IFN-stimulated response elements (ISREs) present in the promoters of many ISGs. Type I IFNs can activate all seven STAT family members in different cell types, leading to the formation of many hetero- and homodimer pairs, as well as complexes with other transcription factors.

In the presence of IFN-α monocyte-derived DCs get activated and express co-stimulatory molecules and enhances T- and B-cell functions in a variety of *in vitro* and *in vivo* models [253-261]. Treatment of several different cell types with IFN-α leads to higher levels of APOBEC3 expression [262, 263]. IFN-α exhibits strong immune adjuvant activity in mice characterized by induction of long-term antibody production and immunological memory against a poorly immunogenic reference antigen [264]. Moreover in this study, they showed that DCs were responsible for mediating the adjuvant effect of IFN-α *in vivo*. Studies on IFN-α -receptor deficient mice showed that the adjuvant activity of IFN-α involves a direct effect of IFN-α on both B- and T-cells [265, 266]. Notably, a powerful adjuvant activity of IFN-α for commercial human vaccines was demonstrated in a prophylactic vaccination mouse model against influenza virus [267]. IFN-α can enhance recruitment of activated APCs mainly mDCs and pDCs at the site of vaccination [268].

The goal of "pegylating" a therapeutic protein, that is, the addition of polyethylene glycol (PEG) residues, is to delay clearance and increase the area under the time concentration curve, thus potentially enhancing efficacy compared with the native non-pegylated protein. Pegylation also helps to ease administration by reducing the frequency of dosing. Several factors influence the pharmacokinetic properties of a pegylated molecule, including the number of ethylene glycol residues (molecular weight), the configuration (linear or branched) of the PEG chain, and the amino acid to which the PEG is conjugated [269]. Such modification of protein tertiary structure with pegylation may alter efficacy and immunogenicity.

IFN-α is the first biotherapeutic agent that is extensively used in clinic specially used in cancer treatment and in combination with Ribavirin in hepatitis C treatment [270-273], A study showed that a low daily dose of IFN-α monotherapy exhibits potent anti-HIV-1 activity *in vivo* in treatment naïve HIV-1 infected individuals without serious adverse effects [274]. A study on the efficacy and tolerance of IFN-α treatment of chronic hepatitis C in HIV-seropositive patients suggested that the treatment was associated with higher $CD4^+$ cell count with no severe toxicity [275]. In natural hosts of SIV (such as African Green Monkeys and Sooty Mangabeys), the infection is often non-pathogenic despite the presence of high viral load [276]. Experiments showed that type I IFN production was only transient after viral inoculation in natural hosts that do not develop disease [277-279]. In contrast, in macaques where SIV infection is pathogenic, there was a local accumulation of pDCs producing high quantities of IFN-α that can fuel the infection. It is possible that similar events take place during HIV-1 transmission and acute infection, which may be associated with high production of

IFN-α and pro-inflammatory cytokines [59].

1.6 HIV-1 therapies and prevention strategies

The first drug that was discovered to have an effect on HIV-1 viral replication was AZT (zidovudine or azidothymidine), AZT is a nucleoside reverse transcriptase inhibitor that blocks the conversion of viral RNA to DNA [280]. AZT initially had some clinical benefits, but this was short lived, as the virus evolved and produced resistant strains to AZT. Then the use of triple-combination therapy was found to be efficient against HIV-1 infection. An important breakthrough, that has changed the life expectancy for HIV-1 patients were the discoveries of additional classes of drugs and the introduction of combination therapy [281]. The initial combination of ART was one protease inhibitor and two nucleoside reverse transcriptase inhibitors. This treatment had profound effect clinically but it had many problems like side effects, high cost and most importantly sticking to the medication regimen to near perfection as even missing one dose per week could give rise to drug resistant HIV-1 strains [281]. Several advancements have been made with ART and the problems associated with these drugs have been addressed, till date ART has increased the life expectancy of the patients and greatly reduced the transmission of the virus. A randomized controlled trial that called the HPTN052 study directly examined the ability of ART to interrupt HIV-1 transmission from an index patient with HIV-1 to his or her sexual partner reveled that an early start of ART has reduced HIV-1 transmission by 96% [282].

Several studies using ART as pre-exposure prophylaxis are ongoing. The aim is to investigate the protective effect of these drugs as microbicide (oral or topical pre-exposure of the drugs) on HIV-1 acquisition [283]. One such promising study is the CAPRISA 004 study in South Africa, where 1% tenofovir gel was delivered into the vaginal vault up to 12 hours before and 12 hours within a sexual intercourse. In 2010, the investigators reported the first report of this study. They show a 39% reduction in overall acquisition of HIV-1 and a 54% in the most adherent women [284]. In another study, called the iPrEx study [285], HIV-1 negative men who have sex with men were given two oral antiviral drug daily, emtricitabine and tenofivir disoproxil fumarate (TDF + FTC) for up to 2.8 years. The study recorded a 44% reduction in HIV-1 acquisition. Eight larger trials with oral antiretroviral agents for pre-exposure prophylaxis are currently ongoing [282]. Substantial progresses were made in prevention of mother-to-child transmission by using ART as a prophylaxis [282].

The focus for development of an HIV-1 vaccine is to elucidate innate, cell-mediated and antibody-mediated HIV-1 restriction. Initial efforts in HIV-1 vaccine development were aiming to induce neutralizing antibodies. Two-phase III trials of a vaccine composed of gp 120 (AIDSVAX B/E), did not find any protection of healthy individuals against HIV-1 infection and the vaccine failed to induce high levels of gp120 specific antibodies [286, 287]. A T-cell vaccine called the V520 was the first T-cell vaccine tested in phase IIB trial and the trial is known as the STEP trial. The vaccine was composed of a replication-defective adenovirus type-5 vector expressing three HIV-1 genes (*gag*, *pol* and *nef*). Pre-clinical data in macaques had showed promising results [288-290]. 3000 individuals had been enrolled, when an interim analysis showed that the vaccine neither prevented HIV-1 acquisition nor reduced the initial viral load despite induction of T-cell responses. Moreover, there was an increased risk of HIV-1 acquisition in study participants who had higher pre-existing

levels of immunity against adenovirus type-5 [291] [292, 293]. In a trial conducted in Thailand, a canarypox vector vaccine (ALVAC-HIV) boosted with a recombinant glycoprotein vaccine (AIDSVAX B/E) led to a 31% reduction of HIV-1 incidence in vaccine recipients [294], which is the first large HIV-1 trial that gave a hint that it may be possible to develop an effective HIV-1 vaccine. Many studies have confirmed that male circumcision largely reduce HIV-1 transmission up to 60% [295-297]. Studies on HIV-discordant couples provided initial evidence of the effectiveness of the male condom in prevention of HIV infection [298]. Hence, it is conceivable that future HIV-1 preventive strategies are likely to entail a combination of advocacy for condom use, male circumcision, microbicides, oral pre- or post exposure prophylaxis as well as a HIV-1 vaccine. For ethical reasons all these preventive strategies have to be offered when conducting clinical trials, which will result in very high numbers of participants required to prove a prophylactic effect by a HIV-1 vaccine.

2 OBJECTIVE OF THE THESIS

The overall objective of the thesis was to study ways to restrict HIV-1 replication in dendritic cells and peripheral blood mononuclear cells.

Specific objectives were as follows;

1) To determine the influence of apoptotic $CD4^+$ T-cells on HIV-1 replication in dendritic cells.
2) To identify the molecules and the signaling pathways involved in dendritic cell maturation induced by apoptotic activated $CD4^+$ T-cells.
3) To investigate the antiviral effect of TNF-α and IFN-α on HIV-1 exposed dendritic cells.
4) To inhibit HIV-1 replication in peripheral blood mononuclear cells using proteasome inhibitors.

3 RESULTS AND DISCUSSION

3.1 Induction of maturation, cytokine and chemokine secretion in MDDC upon contact with apoptotic activated uninfected and/or HIV-1 infected $CD4^+$ T-cells (paper I)

DCs are likely to be one of the first cells to encounter HIV-1 during sexual transmission and their migratory effect can contribute to the spread of the virus as they can act as a vehicle for capturing virus and transmitting them to $CD4^+$ T-cell in the lymphoid tissues [50]. Reducing HIV-1 infection in DCs may therefore greatly reduce viral transmission to other susceptible cells. DC maturation suppresses HIV-1 infection at different stages of viral life cycle - post integration restriction at the transcription level [299], decreased viral fusion [300] and inhibition during reverse transcription [301]. Previous studies from our group suggested that apoptotic anti-CD3/CD28 - activated PBMCs, but not apoptotic resting PBMCs, can induce DC maturation [240]. In paper I, we first investigated whether apoptotic HIV-1 infected activated $CD4^+$ T-cells were able to induce DC maturation. For the experimental set up, we used anti-CD3/CD28 mAbs to activate $CD4^+$ T-cells for 24 hours followed by infection with HIV-1_{BaL}. Cells were γ-irradiated to induce apoptosis and added to the immature DC cultures (in vitro–differentiated monocytes cultured for 6 days in the presence of IL-4 and GM-CSF) immediately after radiation exposure, to allow early apoptotic events to occur in the co-cultures [240, 302-304]. Longer exposures to activation, or activation induced cell death, as well as resting apoptotic cells do not promote DC maturation and such cells are instead involved in peripheral tolerance mechanisms [240, 305-307]. This is the reason why we induced apoptosis no longer than 3-4 days after activation of the T-cells.

Immature DCs co-cultured with apoptotic activated (ApoAct) or apoptotic HIV-1 infected (ApoInf) $CD4^+$ T-cells where analyzed for expression of the co-stimulatory molecule CD86 after 72 hours or 7 days. DCs co-cultured with ApoAct or ApoInf induced CD86 expression significantly as compared with medium control both after 72 hours and 7 days of culture. DCs exposed to HIV-1_{BaL} viral particles did not significantly up-regulate CD86 after 72 hours or 7 days of culture as compared with the medium control. DC encounters infected or uninfected apoptotic cells that contain viral particles *in vivo*. We therefore investigated whether the induction of DC maturation was functional in the presence of viral particles. We observed that the maturation signal provided by the ApoAct or ApoInf occurred even in the presence of free HIV-1_{BaL}, and the efficiency of induced CD86 expression was comparable to that of the positive control. We also studied the expression of CD83, another molecule associated with DC maturation and functional antigen-presenting capacity whose expression was similar to that of CD86 but with a tendency of lower expression than CD86.

DC maturation results in secretion of cytokines [159]. To asess the cytokine production in our culture system, we collected supernatants from the co-cultures after 4, 8 and 24 hours. Multiplexed cytokine assay, was used for simultaneous analyses of MIP-1α, MIP-1α, TNF-α, IL-10, IL-12p70, and MCP-1. Supernatants from DCs co-cultures with apoptotic anti-CD3- and anti-CD28-activated $CD4^+$ T-cells, either uninfected or HIV-1 infected, showed a significant induction of TNF-α as compared with medium control after 24 hours of co-culture. Also supernatants from DC co-cultured with ApoAct or ApoInf that contained exogenous HIV-$1_{BaL,}$ showed a

significant induction of TNF-α. IL-12p40 or IL-12p70 molecules were not found in any of the above mentioned culture supernatants. There was no induction of the above mentioned cytokines in the DCs co-cultured with apoptotic resting $CD4^+$ T-cells. We also detected a significant induction of chemokines MCP-1, MIP1-α and MIP-1β secretion in the supernatants from the DC co-cultured with ApoAct regardless of infection with HIV-1_{BaL} and also in the ApoAct and ApoInf co-cultures with exogenous HIV-1_{BaL}. There was no significant secretion of these cytokines from the $CD4^+$ apoptotic T-cells *per se*.

These findings show that both ApoAct and ApoInf are able to provide a maturation signal to immature DCs even in the presence of exogenous HIV-1. Moreover, that the free viruses alone do not induce maturation in DCs showing that the added viruses do not contain any bystander soluble factors influencing DC maturation. The HIV-1 infection of activated CD4+ T-cells does not seem to interfere with the DC maturation signal. However, a requirement for the induction of maturation signal is that the $CD4^+$ T-cells have to be activated before apoptosis induction. In addition, ApoAct and ApoInf induce pro-inflammatory cytokines and chemokines production in DCs.

3.2 APOBEC3G- and TNF-alpha contribute to reduction of HIV-1 infection in MDDC when co-cultured with apoptotic activated $CD4^+$ T-cells (paper I)

HIV-1 infection in the co-cultures was determined by quantifying the percentage of p24 expressing DCs using flow cytometry [190, 302]. Co-culture of DCs with ApoAct with and without HIV-1 infection, resulted in a significantly reduced percentage of $p24^+$ DCs as compared with DCs exposed only to HIV-1_{BaL}. There was no reduction of $p24^+$ DCs when co-cultured with ApoRest. Both autologous and allogeneic ApoAct co-cultures showed similar reduction in HIV-1 p24 and similar induction of CD86 expression. Similar results were obtained when infection efficiency was measured using quantitative PCR, which showed a ten fold decrease in viral DNA copies when ApoAct (infected or uninfected) were co-cultured with DCs and there was a decrease in the relative proportion of integrated proviral DNA when assessed using Alu-PCR. There was no effect on viral infection when the DCs were co-cultured with ApoRest. Serial dilution of ApoAct demonstrated that the increase in numbers of ApoAct in the co-culture resulted in decrease in percentage of $p24^+$ DCs. Similar results were obtained when kinetic experiments were performed. We detected a reduction in p24 when the DCs were exposed to HIV-1_{BaL} for 30 minutes, 1 hour or 2 hours prior to the addition of ApoAct, similarly p24 expression was reduced when DCs were first co-cultured with ApoAct for 30 minutes, 1 hour or 2 hours prior to infection. The inhibition effect of the ApoAct was detected with three other isolates (HIV_{IIIB} and two other primary isolates).

As there was significant amount of pro-inflammatory cytokines and chemokines released into supernatants, we want to assess if there was any soluble factors involved in the reduction of HIV-1 infection. We therefore collected supernatants after 24 hours of DC/ApoAct co-culture to be used as Conditioned medium (CM). There was reduction in p24 expression in DCs exposed to HIV-1_{BaL} and CM as compared with DCs exposed to HIV-1_{BaL} alone. There was also a dose-dependent response in the reduction of p24 expression in HIV-1_{BaL} infected DCs after exposure to different

volumes of CM. The addition of blocking antibody to TNF-α restored p24 expression but the addition of blocking antibodies to MIP-1α, MIP-1β, IFN-γ and RANTES to the CM did not restore p24 expression. The HIV-1 inhibitory effect by ApoAct was partially lost in the presence of TNF-α mAb. In addition, the TNF-α mAb blocked CM-mediated up-regulation of CD86 in DCs. There was a dose dependent inhibition of HIV-1 infection in DCs when treated with different concentrations of recombinant TNF-α. The HIV-1 inhibitory effect by TNF-α was lost in the presence of TNF-α mAb. These findings provide evidence for a partial HIV-1 inhibitory effect mediated by TNF-α upon ApoAct and DC co-cultures.

CM added 2 hours or 16 hours after HIV-1 exposure did not significantly reduce p24 expression in DCs but ApoAct added 2 hours or 16 hours after HIV-1 exposure reduced HIV-1 p24 expression in DCs. This data suggested that there might be intrinsic factors involved in restricting HIV-1 replication in the DCs. We therefore assessed the mRNA level of the host antiviral factor APOBEC3G in the DCs before and after co-culture. We observed an induction of APOBEC3G mRNA in the DCs co-cultured with ApoAct (irrespective of autologous or allogeneic origin of apoptotic cells) and also in LPS stimulated DCs after 6 hours. We did not detect APOBEC3G mRNA after 2 hours, this shows that the mRNA detected was expressed in DCs but not in ApoAct. To investigate the role played by the APOBEC3G in inducing the reduction of HIV-1 infection, we silenced the APOBEC3G expression by using siRNA. There was a significant increase in p24 expression after silencing of APOBEC3G in DCs co-cultured with ApoAct. To verify that APOBEC3 proteins exerted a function to restrict HIV-1 infection in the DC, we sequenced a region in *env* from the DNA obtained from HIV-1 infected DC co-cultured with ApoAct or ApoRest and measured the frequencies of G-to-A mutations using the program HYPERMUT 2.0. Three clones had significant $p<0.05$, Fisher exact p-value in co-cultures with ApoAct, while none of the clones sequenced from ApoRest co-cultures were found to have significant induction of G-to-A hypermutations. G-to-A mutations were detected both in the GG and GA context. There was a significant difference in the mutation frequency in clones obtained from ApoAct DC co-cultures compared with ApoRest.

The above data suggest that ApoAct can induce maturation and pro-inflammatory cytokine secretion in DCs. Alloantigen-induced immune responses may reduce HIV-1 susceptibility in $CD4^+$ T-cells [308, 309]. Here we show that both allogeneic and autologous ApoAct are able to provide DCs with a maturation signal and upregulation of APOBEC3G mRNA. TNF-α treatment can inhibit HIV-1 infection in these DCs and the HIV-1 inhibitory effect by ApoAct can be partially explained by release of TNF-α. TNF-α can inhibit or promote viral replication depending on the target cell type [310]. CM added 2 hours or 16 hours after HIV-1 exposure did not significantly reduce p24 expression in DCs. It is therefore possible that the soluble factors in the CM are restricting the viral replication only at a very early stage of viral life cycle. Further studies are required to understand the mechanism by which the TNF-α exerts inhibition of viral replication in DCs. Several other viral restriction factors could play a role in TNF-α mediated HIV-1 inhibition. One such candidate worth studying is SAMHD1, which is expressed in cells of the myeloid lineage, which inhibits viral replication by interfering at an early step of the viral life cycle [90]. The siRNA silencing results demonstrated that antiviral factor APOBEC3G is crucial for exerting the HIV-1 inhibition effect. Vif counteracts APOBEC3G [15, 311], in this study we used a complete virus that is capable of producing active Vif, still we detected an induction of G-to-A hypermutation. One possible explanation

could be that other APOBEC3 molecules might be involved in reduction of viral replication in the present system. Recently it has been shown that MDDC express APOBEC3A and it is involved in the restriction of viral replication [125]. The high level of expression of these molecules might have overcome the Vif mediated degradation. In addition, DCs do not replicate HIV-1 as effectively as activated $CD4^+$ T-cells, therefore the level of Vif is also reduced and the antiviral activity of APOBEC may prevail. The pool of APOBEC3 molecules already present in the target cells can efficiently restrict replication of the incoming virus as the Vif is absent in this scenario. The expression levels of these molecules vary greatly between the cell types. Based on the results from an experiment where CM was added 2 hours or 16 hours after HIV-1 exposure did not significantly reduce p24 expression in DCs showing that the CM restriction happens before integration but ApoAct added 2 hours or 16 hours after HIV-1 exposure reduced HIV-1 p24 expression in DCs shows the viral inhibition occurs after integration. In addition the data from the Alu-PCR revealed the presence of integrated DNA in DCs co-cultured with ApoAct, from the above data it is possible to conclude that the inhibition is likely to occur both at pre- and post-integration level.

3.3 Both cellular and supernatant fractions from activated apoptotic lymphocytes are required for stimulation of DC maturation signal (paper II)

Data from our previous reports [240] and paper 1 suggested that ApoAct (PBMC or $CD4^+$ T-cells) can induce DC maturation. Here we investigated the molecules and the signaling pathways involved in the DC maturation by ApoAct. In this study, we used activated apoptotic PBMC (ApoAct PBMC). We started investigating both the cellular and soluble factors, as theoretically, both of them can induce DC maturation. We co-cultured immature DCs with cellular or soluble fractions derived from ApoAct PBMC. We detected partial up-regulation of the maturation markers CD80 and CD86 on DCs under both conditions with a more prominent effect by the cellular fraction. When we used a transwell system to co-culture DC / ApoAct PBMC, we observed a partial up-regulation of maturation markers on DCs. This effect was not detected in co-cultures containing ApoRest PBMC. Cultures containing neutralizing antibodies against TNF-α, showed reduced up-regulation of co-stimulatory molecules, while the cultures with neutralizing antibodies against HMGB1 did not affect the up-regulation of co-stimulatory molecules.

To investigate molecules engaged in the cellular fraction, DCs / ApoAct PBMC co-culture was incubated with neutralizing antibodies against the integrins CD18, CD11a and CD11b, which resulted in inhibition of up-regulation of co-stimulatory molecules. αvβ5 is an integrin previously shown to be involved in uptake of apoptotic cells [312] but we were not able to block DC maturation by adding anti- αvβ5 antibodies. Blocking phagocytosis of apoptotic cells with cytochalasin D did not inhibit induction of maturation signal, showing that phagocytosis of apoptotic cells is not required, but rather that cell-cell-contact is sufficient to trigger DC maturation signaling. Inhibition of up-regulation of co-stimulatory molecules was observed in the presence of

neutralizing antibodies against DC-SIGN and also in the presence of anti-TLR4 antibodies. The role of TLR4 was further conformed by the silencing of TLR4 using siRNA. The role of TLR4 in DC maturation in our system was assessed *in vivo* using $TLR4^{-/-}$ mice and $MyD88^{-/-}$ mice. Wild-type and TLR4 knockout mice were injected with activated apoptotic splenocytes in combination with human serum albumin (HSA) as a model protein antigen. A previous report from our laboratory showed an adjuvant effect by activated apoptotic splenocytes in combination with a DNA vaccine in mice [243]. We detected significant induction of anti-HSA antibodies in wild-type mice when activated apoptotic splenocytes were used as adjuvant in combination with HSA. However, we did not detect significant induction of anti-HSA antibodies in the knockout mice demonstrating a role for TLR4 in providing the adjuvant effect *in vivo*. Resting apoptotic splenocytes did not provide any adjuvant effect neither in wild-type nor in the knockout mice.

3.4 Involvement of multiple signaling pathways in DC co-cultured with activated apoptotic PBMC (paper II)

Several intracellular signaling molecules and pathways have been suggested to be involved in DC maturation signaling and available reports clearly indicate that the role of each pathway is stimulus specific having both positive and negative regulatory role. Therefore, in order to get a clear picture, it is important to characterize the role of these pathways in specific contexts. p38, ERK and JNK MAPKs were rapidly activated in a dose dependent manner in the DC / ApoAct PBMC co-cultures. NFκB and AP-1 transcription factor family are involved in DC activation, pro-inflammatory cytokine secretion and also in up-regulation of co-stimulatory molecules [313]. Both ApoAct and ApoRest induced degradation of inhibitory IκB protein, but full activation of NFκB p65 was achieved only in DCs co-cultured with ApoAct. In addition, ApoAct, but not ApoRest, induced activation of AP-1 transcription factor family members c-Jun and c-Fos in a time dependent manner. Inhibition of p38, ERK and JNK MAPKs using specific inhibitors revealed that p38 and JNK MAPK were required for maturation signaling in DCs. ERK MAPK did not seem to be involved in DC maturation by ApoAct PBMC. Inhibition of PI3K-AKT, Src family of tyrosine kinases and Raf-1 suggested that PI3K-AKT and Src family of tyrosine kinases were involved in the DC maturation signaling upon contact with ApoAct. However, the up-regulation of co-stimulatory molecules in DC remained unaffected when a Raf-1 inhibitor was used suggesting that the activation signal is not DC-SIGN dependent. Activation of AKT/protein kinase B (PKB), a major effector of PI3K, has been reported to be a critical factor in both activation and survival of DCs [314, 315]. We observed that both release of pro-inflammatory cytokines and expression of CD80 and CD86 were significantly inhibited in PP1 and PP2 pretreated DCs after co-cultured with ApoAct. This shows that apart from regulating the release of pro-inflammatory cytokines, Src family of tyrosine kinase could also regulate the expression of maturation markers on DCs. Treatment of DC / ApoAct PBMC with neutralizing antibodies against *TLR4 and β2-integrins reveled that* AKT activation is dependent on *TLR4 and β2-integrins.* β2-integrin dependent cell-contact, TLR4-PI3K/Akt, and Src family of tyrosine kinases were also required for activation of p38 MAPK dependent activation of NFκB whereas neutralizing antibody against DC-SIGN had no effect on ser-536 phosphorylation of p65 which further supports that NFκB was not activated by DC-SIGN. Further, we observed that TLR4, DC-SIGN and Src family of tyrosine kinases regulated JNK

MAPK activation and activation of c-Jun and c-Fos was regulated by p38 MAPK, JNK MAPK and Src family of tyrosine kinases (Fig. 3).

The above data suggest that activation of p38 MAPK occurred in a DC-SIGN independent but TLR4 and CD18 dependent manner. However, activation of JNK MAPK was dependent on TLR4, DC-SIGN and CD18. These findings suggest a cooperative signaling between these molecules upon DC/ApoAct contact. It has been reported that DC-SIGN and TLR4 cooperate to induce an inflammatory phenotype in human DCs in response to *S. manosi* worm glycolipids [316]. In this study we could decipher some of the molecules involved the DC maturation signaling by ApoAct PBMCs. But it is not clear from the data whether the integrins involved in triggering the signaling were expressed on the DCs and/or the apoptotic cells.

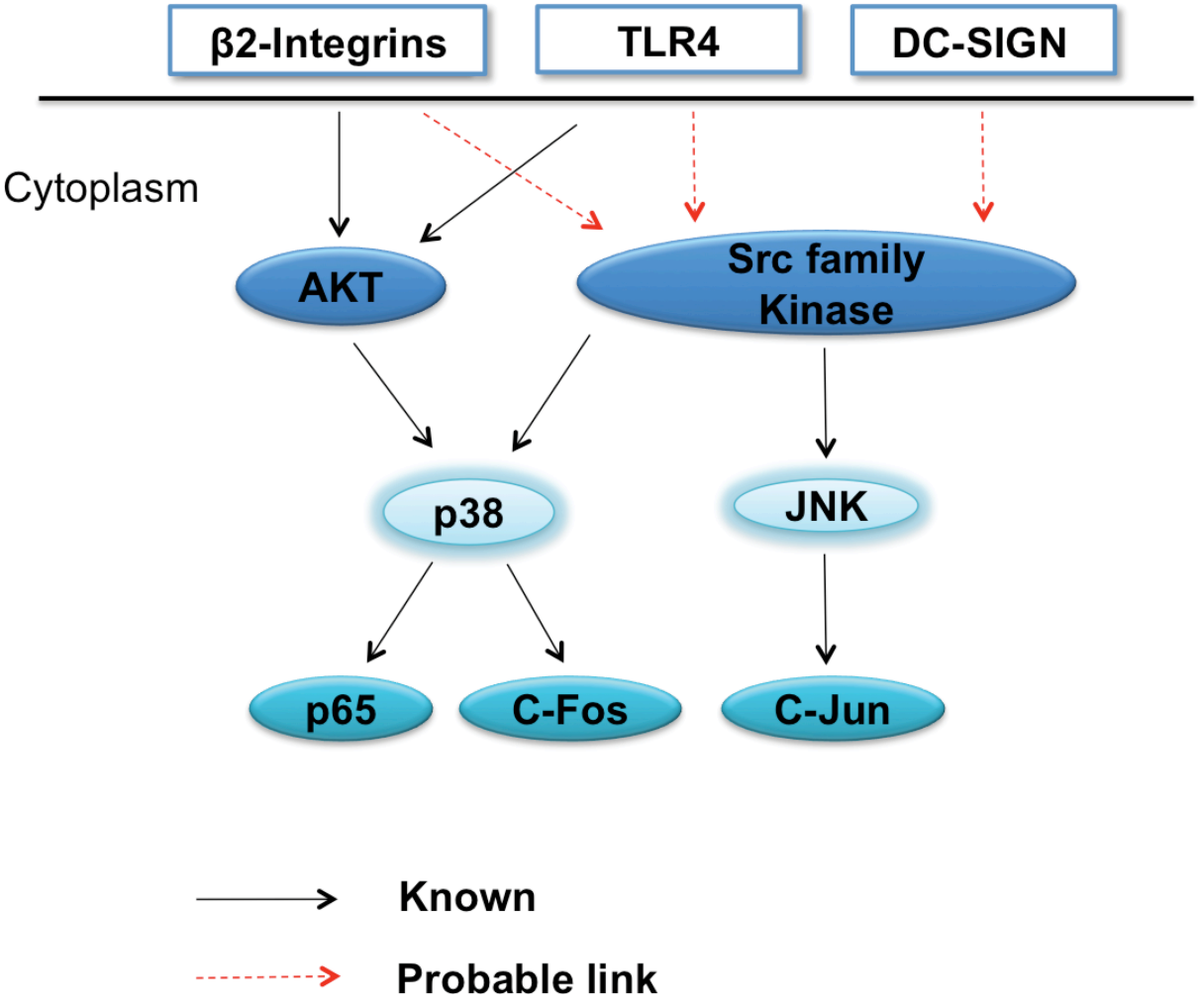

Figure 3. Molecules and signaling pathways involved in DC responses to activated apoptotic PBMC

3.5 IFN-alpha inhibits HIV-1 replication and up-regulates APOBEC3 molecules without inducing maturation in MDDC (paper III)

In paper I we demonstrated that TNF-α plays a role in reducing HIV-1 replication in DCs. In this study, we treated MDDCs with TNF-α or IFN-α and compared their ability to induce maturation (in terms of up-regulation of co-stimulatory molecules) and inhibit viral replication in MDDCs. We treated DCs with 3 concentrations of TNF-α (0.3, 3, 30 ng/ml single-dose increasing concentrations) and 3 concentrations of pegylated IFN-α2b (10^2, 10^3, 10^4 U/ml single-dose increasing concentrations). We used a pegylated IFN-α in our studies as the addition of PEG may delay clearance of the protein. We found that IFN-α2b did not up-regulate co-stimulatory molecules at lower concentrations i.e. (10^2, 10^3 U/ml). At the higher concentration both TNF-α (3, 30 ng/ml) and IFN-α2b (10^4 U/ml) induced up-regulate co-stimulatory molecules.

DC maturation can inhibit viral replication at different stages of viral life cycle - post integration restriction at the transcription level [299], decreased viral fusion [300] and inhibition during reverse transcription [301]. Here, we compared TNF-α and IFN-α for their ability to inhibit HIV-1 replication in DCs. HIV-1_{BaL} exposed DCs were cultured with these cytokines at different concentrations as mentioned above. TNF-α could inhibit HIV-1 replication significantly only at higher concentration (30 ng/ml) but IFN-α2b significantly reduced viral replication at all the concentrations used (10^2, 10^3, 10^4 U/ml). Even the lower concentration of IFN-α2b, which was not able to mature DCs, could significantly inhibit HIV-1 replication. We observed similar results with respect to DC maturation and HIV-1 restriction when we used non-pegylated IFN-α (IFN-α2A and IFN-αA/D) at the same concentration as IFN-α2b.

Then we investigated whether there was an up-regulation of antiviral host factor APOBEC3 molecules in these DCs, as we detected elevated APOBEC3G expression in matured DCs in paper I. DCs treated with TNF-α did not show significant expression of APOBEC3G (A3G), APOBEC3F (A3F) or APOBEC3A (A3A) mRNA even at concentration when viral replication was inhibited. On the other hand, DCs treated with IFN-α2b had a dose dependent induction of expression of all the three APOBEC3 mRNA.

Cloning and sequencing of an *env* region in the DNA obtained from the HIV-1 exposed DC treated with low concentration of pegylated IFN-α2b (10^2 U/ml) revealed significant number of G-to-A hypermutations. Twelve out of the sixty clones had significant mutation rate (Fisher exact p-value <0.05). The G-to-A mutations occurred in both GG and GA contexts.

In summary, we showed that IFN-α can restrict viral replication in DCs without inducing maturation in DCs and that the viral restriction can be partly explained by the expression of APOBEC3 mRNA. Whereas TNF-α can induce maturation and restrict viral replication in DCs, the APOBEC3 molecules do not play a major role in TNF-α mediated viral restriction. However, siRNA experiments are required to fully reveal the role of APOBEC3 molecules in these experimental settings.

HIV-1 infection is characterized by acute and chronic immune activation of which some may be due to dysfunctional cytokine and IFN-α production [59]. SIV infection is often non-pathogenic in African Green Monkeys and Sooty Mangabeys despite the presence of high viral load [276] Experiments showed that type I IFN production was only transient after viral inoculation in natural hosts that do not develop disease [277-279] but in macaques where SIV infection is pathogenic, pDCs accumulates locally and produce high quantities of IFN-α that can fuel the infection. It is possible that similar events can take place during HIV-1 transmission and acute infection that can be associated with high production of IFN-α and pro-inflammatory cytokines [59]. High production of IFN-α and pro-inflammatory cytokines such as TNF-α will induce DC maturation and trigger migration of DCs into lymphnodes facilitating dissemination of the infection [317]. The above data suggests that a window of low IFN-α concentration can inhibit viral replication without inducing DC maturation. We noticed a high induction of A3A mRNA expression by IFN-α2b. In addition, treatment of DCs with IFN-α2b introduced G-to-A hypermutations that occurred in both GG and GA contexts in the proviral DNA. A recent report demonstrated that A3A is preferentially expressed in myeloid cells compared with peripheral blood lymphoid cells and restricts HIV-1 replication in myeloid cells [125]. A3A can induce mutations in GG as well as in GA contexts [311]. Our finding corresponds with the above reports. We have also observed induction of A3G and A3F mRNA by IFN-α2b and these molecules were reported to be involved in viral restriction.

TNF-α on the other hand could only limit replication in MDDCs in a relatively high concentration (30ng/ml) that also induced DC maturation. None of the concentrations of TNF-α (0.3-30ng/ml) that we used in the present study showed induction of APOBEC3 family proteins. TNF-α had been reported to enhance viral replication in monocyte derived macrophages [310]. More experiments has to be done to decipher the mechanism by which TNF-α limits viral replication in MDDCs, which may help understand the differential regulation of viral replication by TNF-α on different cell types.

3.6 Proteasome inhibitors blocked HIV-1 LTR-mediated gene expression both *in vitro* and *in vivo* (Paper IV)

NF-κB is a ubiquitous transcription factor critical in inflammation and immunity. HIV-1 LTR promoter has active NF-κB binding sites [318]. Various reagents targeting the NF-κB signaling pathway have been examined for their inhibitory effects on HIV-1 [47]. Bortezomib is the first FDA-approved proteasome inhibitor, which is used in clinic for the treatment multiple myeloma [319]. Here we studied the effect of two proteasome inhibitors Bortezomib and MG132 on the HIV-1 LTR promoter activity. HIV-1 LTR-luciferase (pLTR-Luc) construct was transiently transfected in different cell lines (A20, Jurkat and HEK239T cells) and were incubated overnight with MG132 or Bortezomib. We observed a reduction in the expression of luciferase in these cell lines in the presence of proteasome inhibitors. We obtained similar results when the HIV-1 LTR-GFP (pLTR-GFP) transfected cells were treated with MG132.

To examine if the proteasome inhibitors can block the HIV-1 LTR- mediated gene expression *in vivo* we employed an *in vivo* gene delivery technique known as the hydrodynamic infusion method. pLTR-Luc was introduced into the liver of mice. The HIV-1 LTR-promoter was functional and highly active in hepatocytes. When these animals were injected with Bortezomib we observed a reduction of reporter gene expression, which is consistent with the
in vitro data.

3.7 Proteasome inhibitors blocks HIV-1 replication and inhibit expression of IL-2 inducible T cell kinase in PBMC (PAPER IV)

A Tec-family tyrosine kinase - IL-2 inducible T cell kinase (Itk), which is involved in T-cell activation and development, has been shown to play a vital role in HIV-1 replication [320]. Inhibition of Itk expression has been shown to block HIV-1 replication at multiple stages of its life cycle [320]. We observed a reduction in base line expression of Itk in resting PBMC by both the proteasome inhibitors in a dose-dependent manner. In addition, the proteasome inhibitors when added during the activation of PBMC with PHA and IL-2 blocked activation-dependent Itk induction in PBMC. However, we observed a moderate effect on the expression of Itk when pre-activated PBMC (activated with PHA- and IL-2 for 72 hours) were treated with these drugs. On the other hand, the steady state levels of NF-κB p65 fell sharply in these pre-activated PBMC when treated with the proteasome inhibitors. Thus, the proteasome inhibitors effectively suppress induction of Itk expression during PHA activation, but only moderately after activation. However, treatment with these drugs downregulate steady-state levels of Itk in resting PBMC.

To examine whether the proteasome inhibitors can inhibit viral replication, we infected PBMCs with HIV-1_{BaL} in the presence of either of the two proteasome inhibitors or in the presence of the antiretroviral drugs Indinavir (IND) or azidothymidine (AZT). When we assessed the supernatant for reverse transcriptase (RT) activity, we found that the proteasome inhibitors significantly restricted HIV-1 replication in PBMCs as efficiently as the antiretroviral drugs that are used in HIV-1 therapy. The HIV-1 exposed PBMCs were treated in combination with proteasome inhibitors (Bortezomib or MG132) and the antiretroviral drugs. The combination of proteasome inhibitor with AZT or IND did not exhibit synergistic effects but they did inhibited viral replication significantly without severely affecting the cell viability.

Proteasome inhibitors effectively suppressed induction of Itk expression in PBMC during PHA activation. But in the PBMC that were pre-activated, the suppression of expression level of Itk by the proteasome inhibitors was moderate. This differential effect on Itk expression by proteasome inhibitors depending on the activation status of the PBMC might also affect the HIV-1 inhibition by proteasome inhibitors differentially. The time point of the treatment of PBMC with proteasome inhibitors might have an impact on the inhibition of viral replication. To investigate this, we employed three different treatment conditions. In “Treatment 1”, the pre-activated PBMC (activated with PHA- and IL-2 for 48 hours) was treated with Bortezomib at the time of infection. We then observed that 2.5 nM of Bortezomib was required for significant reduction of RT-activity and also to reduce viral DNA. In “Treatment 2” - we added Bortezomib at the time of activation (together with PHA and IL-2) and

washed it away during infection. Bortezomib at a concentration of 1.25 nM and 2.5 nM, reduced viral RT-activity. But an efficient reduction in viral DNA was noticed only with 2.5nM of Bortezomib. In "Treatment 3", PBMCs were continuously treated with Bortezomib throughout the experiment. We observed an effective reduction in RT-activity in both the concentration of Bortezomib used. Even though we measured a decrease in viral DNA by both concentrations, we detected a robust reduction in HIV-1 DNA only when 2.5 nM of Bortezomib was used.

Collectively, proteasomes inhibitors can inhibit HIV-1 LTR activity and also restrict viral replication in PBMCs. Most of the current antiviral drugs are directed against viral targets, which can lead to the development of drug resistant viral strains. HIV-1 preferentially replicates in proliferating $CD4^+$ T-cells, partly by taking advantage of the activated host transcription machinery [321]. Proteasome inhibitors targets host transcription factor and it is less likely that the virus develops resistance to the proteasome inhibitors. By using proteasome inhibitors one can inhibit the degradation of IκB and thereby maintain the inactive form of NF-κB in the cytosol, which in turn inhibit viral replication. Apart from NF-κB, proteasome inhibitors reduce the expression of Itk and also interfere with the T-cell activation and thereby affecting viral replication. A possible drawback of targeting Itk can be that it might affect immune functions and is relatively toxic. Reports from Itk $^{-/-}$ mice suggest that these mice are still able to mount an immune response upon infection. The results show that CTL responses are reduced in the absence of Itk whereas antiviral B-cell responses are not affected [322]. Vif counteracts antiviral host factors APOBEC3 molecules by mediating these molecules to undergo proteasome dependent degradation. There are also other host factors, such as SAMDH1 and Tetherin, that undergo degradation through the ubiquitin proteasome pathway. There is a theoretical possibility that the use of proteasome inhibitors may restore antiviral factors in the host. As a plethora, of molecules are regulated by ubiquitin proteasome pathway, restricting these molecules on a long-term basis might not be ideal because of side effects. Proteasome inhibitors may have the possibilities to be used as a intermittent third line treatment in HIV-1 infection or as a microbicide locally at the site of HIV-1 exposure.

4 CONCLUDING REMARK

Dendritic cells are located in mucosal and lymphoid tissues and their location in the mucosa makes them one of the first cells to encounter HIV-1 during sexual transmission. Apart from priming the T-cells, the DCs can also contribute to the spread of the virus to $CD4^+$ T-cells through mere cell-cell contact and by hijacking the virus from the site of infection and migrating to the lymph nodes were it meets abundance of $CD4^+$ $CCR5^+$ cells. It has been shown that HIV-1 can infect DCs acting as a viral reservoir *in vivo* [323]. Protecting DCs from acquiring infection may therefore theoretically, reduce the transmission of the virus to vulnerable cells. DC maturation reduces HIV-1 infection at different stages of viral life cycle - post integration restriction at the transcription level [299], decreased viral fusion [300] and inhibition during reverse transcription [301]. On the other hand, induction of DC maturation was shown to increase infection to T-cells in *trans*. In our attempt to restrict viral replication in primary cells, we first used apoptotic cells to induce DC activation. Apoptotic cells are know to have a immune-dampening effect on the immune system but many emerging reports and the previous work from our group has suggested that they can be potent immune activators in some circumstances.

Co-culturing of DCs with apoptotic activated $CD4^+$ T-cells resulted in the maturation of DCs and also induced the secretion of pro-inflammatory cytokines and chemokines. But resting apoptotic $CD4^+$ T-cells did not exert this effect on DCs. The capacity to activate DCs was not affected if the apoptotic cells were infected with HIV-1 and also occurred in the presence of free virus in the co-cultures. The apoptotic cells can either be allogeneic or autologous to induce this effect. One requirement is however that the apoptotic cells have to be activated before co-culturing. These findings were in line with previous data from our group using apoptotic PBMCs. DCs maturation can lead to inhibition of HIV-1 replication. We found that apoptotic activated cells, when co-cultured with HIV_{BaL} exposed DCs, reduced the percentage of $p24^+$ DCs. Again, the resting apoptotic cells could not mount this effect. We also observed that two factors played a major role in inhibiting viral replication. The first one was the pro-inflammatory cytokine TNF-α, which is partly involved in the restriction of HIV-1 in DCs exemplifying the differential function of TNF-α during HIV-1 infection. The second, was the host intrinsic factor APOBEC3G, which we demonstrated to be involved in restriction of HIV-1 in DCs / ApoAct co-cultures. Analysis of more intrinsic factors, such as other APOBEC3 molecules (especially A3A and A3F as we observed a significant G-to-A hypermutations in the proviral DNA), TRIM5α, SAMHB 1 might have shed more light on the mechanism by which the ApoAct / DC co-culture inhibits viral replication. The results from the PCR analysis show that there is no complete elimination of the virus and the inhibition occurs both at the pre- and post-integration level. We have also noticed that prolonged activation of $CD4^+$ T-cells prior to the induction of apoptosis reduced its ability to induce maturation in DCs. These above data suggest the apoptotic cells may induce host factors during HIV-1 infection. It has to be noted that HIV-1 infection *in vivo* leads to aberrant immune activation, a thorough investigation on how efficient the lymphocytes obtained form HIV-1 infected individuals can exert an antiviral effect on DCs will provide better understanding of HIV-1 pathogenesis involving interactions between HIV-1, apoptotic cells and DCs.

When we made an attempt to characterize the molecules and the signaling pathway involved in the maturation of DCs by apoptotic cells we found that both soluble and cellular factors were involved. Using blocking antibodies against TNF-α, revealed that TNF-α is involved in the maturation of DCs. ApoAct per se secreted very low amount of TNF-α. Hence, it was the TNF-α secreted by the DCs that acted in an autocrine manner to induce maturation. HMGB1 did not play a role in inducing DCs maturation in our system. When we looked for PRRs and their involvement in DC maturation, we found that DC-SIGN and TLR4 were engaged. It is known that apart from foreign antigen DC-SIGN binds to number of endogenous ligands like ICAM-2 and ICAM-3 to induce DC tolerance [324-326]. Here, our data suggest that DC-SIGN is involved in inducing DC maturation in our co-cultures. Using $TLR4^{-/-}$ and $Myd88^{-/-}$ mice, we found TLR4 involvement for the adjuvant effect *in vivo*. Blocking CD18 using antibodies revealed that β2-integrin is one of the molecules indulged in inducing DCs maturation. In the present study we could not clearly distinguish whether the integrins from the DCs or the apoptotic cells are responsible for the DCs maturation signal. It could be from either side.

We found that both AKT and Src family of tyrosine kinases regulated activation of MAPKs in DCs that were co-cultured with ApoAct. Although we observed a strong activation of MAPK members ERK, p38 and JNK, signaling, only p38 and JNK MAPK is required for ApoAct-induced DC maturation. Activation of ERK MAPK seems to be linked to some other response in DCs. Activation of p38 MAPK occurred in a DC-SIGN independent but TLR4 and CD18 dependent manner, whereas activation of JNK MAPK was dependent on TLR4, DC-SIGN and CD18, suggesting a cooperative signaling between these molecules. NF-κB and AP1 family of transcription factors play an important role in DC activation and release of pro-inflammatory cytokines. Here we show that that ApoAct-induced DC activation involves both NF-κB and AP1 family of transcription factors. We have identified some of the molecules involved during the DC maturation when co-cultured with ApoAct. Several cell surface molecules, signaling molecules and pathways were reported previously in other systems. These previous studies and our data suggest that the molecules and the pathway involved are stimuli specific.

When we treated TNF-α and IFN-α in DCs, we found that both cytokines can induce DC maturation. But in case of TNF-α, the DCs had to be treated at a concentration in which maturation occurred in order to inhibit HIV-1 replication. This was not the case with IFN-α. IFN-α could exhibit antiviral activity at lower concentrations without inducing DCs maturation. The antiviral exerted by IFN-α can be partly explained by the up-regulation of expression of APOBEC3 mRNA (A3G, A3F, A3A) and significant induction of G-to-A hypermutations in the proviral DNA. We also found that DC maturation by TNF-α does not up-regulate the expression of APOBEC3 mRNA. This effect of IFN-α on the DCs may be exploited for HIV-1 prevention. Animal studies suggest that concentrations of IFN-α are crucial for the differential effect of IFN-α in inhibiting viral replication. IFN-α can be used locally at the site of infection at low concentration so that the DCs do not attain the migratory ability and at the same time can restrict viral replication in them. This may prevent the DCs from migrating to the lymph nodes and spreading the infection to the $CD4^+$ T-cells.

We used proteasome inhibitors (one of them is in clinical use to treat multiple myeloma) to reduce viral infection in PBMCs. The proviral gene expression is highly

dependent on host transcription factor NF-κB. Here we showed that proteasome inhibitors reduced the NF-κB activity both *in vitro* and *in vivo* and that they can restrict viral replication in PBMCs without significantly affecting the cell viability. However, the proteasome inhibitors were able exhibit this effect only at a very narrow concentration and also the inhibitors had to be continuously present in the culture for inhibiting the viral replication efficiently. It remains to be investigated whether newer generations of proteasome inhibitors with better toxicity profiles will have effective antiviral activities. The proteasome inhibitors not only affect NF-κB but also Itk, which is critical for T-cell activation and viral replication. Several molecules in the host that are crucial for viral replication is regulated by ubiquitin proteasome pathway, for example the APOBEC3 family molecules. The proteasome inhibitors may restore the level of antiviral factors like APOBEC3 and restrict the expression of proviral factors such as Itk. Most of the current anti-HIV-1 drugs are against viral targets and the virus can efficiently develop resistance strains to these drugs but targeting the host factors can solve this problem. Again, targeting host molecules can disturb the integrity of the host cells and also some of these molecules are involved in the immune activation. Restricting these molecules on a long-term basis might not be ideal as they induce several side effects. Proteasome inhibitors may have the possibilities to be used as a third line treatment in HIV-1 infection or as a microbicide locally at the site of HIV-1 exposure.

Both ApoAct and IFN-α were able to up-regulate expression of APOBEC3 molecules in DCs and proteasome inhibitors may also restrict degradation of APOBEC3 molecules. During natural infection, the virus has an active Vif to counteract APOBEC3 and one can argue that the Vif might lead to low expression of APOBEC3 molecules which in turn will result in introduction of sub lethal mutations which might add up to the viral genetic diversity and subsequently can lead to more potent viral strains. In our system, we used virus that has a fully functional Vif but still we could detect an inhibitory effect exerted by APOBEC3 molecules. There is a possibility that the level of the APOBEC3 molecules induced in our system could have efficiently evaded Vif mediated degradation. Moreover, the pool of APOBEC3 molecules already present in the target cells can efficiently restrict replication of the incoming virus, as the Vif is absent in this scenario. Up-regulation of these molecules at the time of infection or during the viral entry into the target cell can prove lethal for the virus. The evolution of the virus can be in both directions. The virus survives as long as the host survives so the mutations can also push the virus to be less aggressive so that virus has a host to survive in.

Apoptotic cells, IFN-α and proteasome inhibitors all have a possibility to be used in therapeutic and/or prophylactic interventions but the key is to identify the site of administration and the time of administration of these agents and also to be used in ideal combinations with other drugs.

5 ACKNOWLEDGEMENTS

I would like to express my sincere thanks to all the wonderful people, who in their own capacity contributed directly or indirectly to the completion of my thesis. This work was carried out at the center for Infectious Medicine (CIM), Department of Medicine, Karolinska University Hospital Huddinge.

Firstly, A sincere thanks to my supervisor **Dr. Anna-Lena Spetz,** for giving me this rarest of opportunities. Working with you was a great learning experience. Thank you for sharing your valuable ideas and experience, you always had a positive outlook and approachable attitude. This gave me a lot of confidence. Thanks a lot for your guidance and for your endless support all these years. I am deeply indebted to you for this opportunity.

My co-supervisor **Dr. Lilian Walther-Jallow**, since the first day when you picked me up from Arlanda till date you have been very supportive and very kind to me. Thank you for patiently training me in the lab and for all the good discussions. I would also like to thank my other co-supervisor **Prof. Juha Kere** for his guidance.

A special thanks to **Prof. Hans-Gustaf Ljunggren**, for giving a space and letting me work in one of the best working environment that you have created. I consider myself to be very fortunate to work at CIM.

I would also like to thank **Dr. Vinoth Kumar** and **Prof. Ranga Uday Kumar** for introducing me to Science. I also thank my external mentor **Dr. Carani B. Sanjeevi** for his advice.

I thank Karolinska Institutet for funding my PhD salary.

Former and present members of **Spetz' group**: Thank you **Barbro** for your inputs and suggestions. Thank you **Ulrika** for being a very helpful colleague and for all the good discussions. **Annette** you have been a good friend all these years. Your hard work and interest in science has always inspired me. I sincerely thank you for your constant support and advice on and off the lab. Thank you **Sushil** for your guidance and for good scientific discussions.

I also thank all my co-authors **Joshua Fink, Cathrine Persson, Liangh Yu, Oscar E. Simonson, Prof. C.I. Edvard Smith** and **Dr. Abdalla J. Mohamed** for their contribution to the thesis.

I would like to thank all the members of **CIM** (both past and present) for making my stay here a memorable one. Thank you **Carina, Berit** and **Ann** for helping me with the administrative procedures. Thank you **Hernan, Lena, Elisabeth** and **Anette** for your patience and help with all the practical stuffs. Thank you **Oscar** and **Sandra** for your concern, care and friendship. Thanks to **Sofia, Kathrina, Anh Thu, Helena, Heinrich, Jakob, Sam, Terry, Sayma, Erika, Martin, Pär, Monika, Kim, Carlotta, Mattias, Puran, Hannes, Adil, Ramana, Shrikanth, Jubayer, Maroof, Xiang, Anquan, Sanna,** and **Peggy** for your friendship and for all the fun, you have all been amazing

thank you really.

Thank you **Cyril, Robban** for your concern and guidance, the Alps trip was great. Your energy and positive outlook has been an inspiration. Thank you **Julius** for your advice and guidance. Thank you **Lidjya, Yenan, Steph, John** and **Andrew** for the basketball matches and for the nice barbeques, it was fun. Thank you **Steve** and **Markus** for your inputs.

I met many wonderful people Outside CIM as well, **Stefano** Thank you for all that you done for me its been a pleasure knowing you, you made such a big impact in my life. You are an amazing person. **Fran, Lech** and **Sai Kiran** guys you have been great, you guys have been very supportive, caring from day one and still you are, thank you very much. **Nicola, Alice, Sanne, Meral, Nancy, Ling, Wendy, Sarah, Eddy, Jean, Edwardo and Flavia** thanks for all the fun. I thank all the Internovam football team members especially **Robin** for spending a lot of time and energy to organize the matches and for being a wonderful person also **Mohamed, Rui, Tom, Babi, Bea** and **fahad** for all the nice matches.

Thanks **Karthick** and **Meena** for your support especially during Vidya's delivery. Thanks a lot **Dinakar, Kanth, Vivek, Indira, Jayesh, Tina, Senthil, Vipin, Elena, Kavitha** and **Vishal** for your friendship and support.

I would also like to thank my family members, I would not have reached this far without their support. Firstly thanks to my **Amma** and **Appa** for giving me a good education and thanks a lot for your unconditional love and support, you are the best. I also thank my brother **Roshan** for his support and guidance. I also would like to thank my uncle **Barathan** and Aunt **Malika** for their constant support and care. Thanks to my cousins for their support, inspiration and guidance. Thanks to my uncle **Mailarinathan** and aunt **Shanthi** for their support especially when Gautham was born.

Thanks to my son **Gautham** for being my stress buster while writing and also for choosing vidya and me.

Vidya, you are the most special and a very important person in my life, thanks a lot for your understanding, love and care. These two years has been the happiest years of my life. You followed me half way across the world leaving everything behind for me, I am glad that we met and that we are going to spend the rest of our life together. You mean a lot to me. Spending time with you and Gautham makes me happier than ever.

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