Transforming Growth Factor-Beta (TGF-β) Induces HIV-1 Restriction in Langerhans Cells

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Summary

Transforming growth factor-beta (TGF-β) drives the development of immature LC from hematopoietic progenitor cells and shapes the cells functions. Here I showed that two LC model cells, MuLC and MDLC, used exchangeably in the research, differ significantly in their phenotype and immune responses. Discrepancies between these models were specifically visible during stimulation with type-I IFN, where MuLC failed to up-regulate ISG levels. Yet both MuLC and MDLC demonstrated low susceptibility to HIV-1 infection, even in the absence of SAMHD-1. This post-entry restriction was conferred by the action of TGF-β on differentiation cells as indicated by our study. Indeed, in the absence of TGF-β supplementation, derived cells showed MDDC phenotype related to high susceptibility of the cells to HIV-1 infection during co-infection with SIV-Vpx. Additionally blocking of the TGF-β signalling, reversed the restrictive phenotype of LC. Importantly this pattern was also confirmed in skin extracted real epidermal LC versus dermal DC, suggesting that SAMHD-1-independent restriction activity operates in TGF-β derived cells. Accordingly to PCR analysis virus replication in LC is interrupted prior to integration, suggesting the role of additional restriction factors at early stages of virus infection or lack of essential viral dependency factors such as dNTPs. Interestingly maturation of MDLC with a synthetic bacterial triacylated lipopeptide or TNF-alpha significantly increased their susceptibility to HIV-1 infection, which may explain why HIV-1 acquisition is increased during co-infection with other STIs. In summary, our study strongly supports the action of SAMHD-1-independent HIV-1 restriction mechanisms in LC. A better understanding of the balance between HIV-1 restriction and propagation from LC to CD4+ T cells may help in the development of new microbicides or vaccines to curb HIV-1 infection at its earliest stages.

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Abbreviations

AGS	Aicardi-Goutieres syndrome
AIDS	acquired immunodeficiency syndrome
APOBEC	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like
APC	antigen presenting cells
ARV	anti-retroviral drugs
CCR5	C-C chemokine receptor 5
CD	cluster of differentiation
cDNA	complimentary DNA
CLR	C-type lectin receptor
CXCR4	C-X-C chemokine receptor type 4
DC	Dendritic cells
DC-SIGN	dendritic cell-specific intracellular adhesion molecule-3-grabbing non-integrin
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleoside triphosphates
EDTA	ethylenediaminetetraacetic acid
Env	envelope
FACS	fluorescence-activated cell sorting
FBS	foetal bovie serum
FITC	fluorescein isothiocyanate
Gag	group specific-antigen
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage colony-stimulating factor
gp120/41	glycoprotein 120/41
HAART	Highly active antiretroviral therapy
HEK 293T cells	human embryonic kidney 293T cells

HIV	Human immunodeficiency virus
HRP	Horseradish peroxidise
IL-10	Interleukin-10
IL-2	Interleukin-2
IL-4	Interleukin-4
IFN	Interferon
ISG	interferon-stimulated genes
LC	Langerhans cells
LB agar	Lauria-Bertani agar
LTNP	Long-term non progressors
LTR	Long terminal repeat
MDDC	monocyte-derived Dendritic Cells
MDLC	monocyte-derived Langerhans Cells
MuLC	MUTZ-3 derived Langerhans Cells
MUTZ-3	CD34+ human acute myeloid leukemia cell line
MHC	major histocompatibility complex
mRNA	messenger RNA
MX	interferon-inducible myxovirus resistance protein
Nef	negative factor
NFκβ	nuclear factor kappa Beta
NNRTIs	Non-Nucleoside Reverse Transcriptase Inhibitors
NRTIs	Nucleoside/nucleotide Reverse Transcriptase Inhibitors
PAM3CSK4	synthetic triacetylated lipopeptide
PMBC	Peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PFA	paraformaldehyde

PI	protease inhibitors
PIC	pre-integration complex
pol	polymerase
Rev	regulator of expression of virion proteins
RNA	ribonucleic acid
RPMI 1640	Roswell Park Memorial Institute 1640 medium
R5	CCR5-tropic HIV
SAMHD-1	Sterile alpha motif and HD domain-containing protein-1
SIV	Simian immunodeficiency virus
STI	Sexually transmitted infections
STD	Sexually transmitted disease
Tat	Trans-activator
TGF-β	Transforming Growth Factor-β
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor-α
TRIM5α	tripartite motif containing 5 alpha
Vif	viral infectivity factor
VLP	viral-like particles
Vpr	Viral protein R
Vpu	Viral protein U
Vpx	Viral protein X
VSV-G	Vasicular stomatitis virus G glycoprotein envelope
X4	CXCR4-tropic HIV
WT	wild type

1. General Introduction

1.1. HIV Pathogenesis and AIDS

1.1.1. HIV Origins and Subsets

Human immunodeficiency virus (HIV) was first described in humans in 1983 as a cause of a threatening disease spreading at the time in the homosexual men population (Barre-Sinoussi et al. 1983; Popovic et al. 1984). Acquired Immuno Deficiency Syndrome (AIDS), as the disease was named, resulted in unusually high susceptibility of affected individuals to opportunistic infections (Greene 2007). A few routes of transmission of the virus were identified, of which sexual transmission remains the most common (Hladik and McElrath 2008; Kaul et al. 2011).

In 1986 Clavel et al. (Clavel et al. 1986) described HIV type 2 (HIV-2) as a causative agent of AIDS in human. The virus was closely related to Simian Immunodeficiency Virus (SIV) affecting macaques (Chakrabarti et al. 1987). Not long afterwards, scientists were able to link HIV-1 and HIV-2 origin in human to zoonotic transfer between primates in Africa and their human hunters (Hahn et al. 2000; Peeters et al. 2002). Four independent SIVcpz transmissions from chimpanzees to human are believed to give rise to presently occurring groups of HIV-1: group M, N, O and P. Group M is without a doubt the most commonly diagnosed, estimated to affect 60 million people worldwide over the decades (Gupta and Towers 2009; Sauter et al. 2009). The remaining groups appear in a handful of individuals; group O affects estimated 100,000 individuals while groups N and P were diagnosed in less than 20 patients, mostly restricted to South African regions of Cameroon and neighbour countries (De Leys et al. 1990; Simon et al. 1998; Mintsa-Ndong et al. 2009).

HIV-2 infections do occur in people but the transmission rates as well as progression to AIDS in infected individuals are very low compared to HIV-1 infections (Popper et al. 2000; Rowland-Jones and Whittle 2007). The HIV-2 genome phylogenetically is closely related to SIVmac/SIVsmm suggesting that the origin of HIV-2 is different to HIV-1. In fact, SIVsmm does not cause AIDS in its natural host, sooty mangabeys (Rey-Cuillé et al. 1998; Silvestri et al. 2003). Observed discrepancies in successful spread of HIV-1 and HIV-2 rely on the presence of the effective immune responses generated against HIV-2, but not HIV-1. In fact, more effective antibody and $CD8^+$ T cell responses against HIV-2 are present in infected individuals (Gillespie et al. 2005; Duvall et al. 2008), which limit CD4+ T cell depletion. Additionally, HIV-2 shows less resistance to interferon responses, which could explain why this virus avoids infection of dendritic cells despite the presence of Vpx protein in its genome. The function of Vpx in HIV-2 thus is not to increase virus replication in DC, as HIV-2 does not efficiently enter these cells. Instead, Vpx enhances HIV-2 propagation in macrophages and T cells. As an obligate intracellular parasite, HIV depends heavily on host factors for its replication. Additionally, virus has to deal with both host immune responses and cellular restriction factors (see Section 1.3.2), which can be a significant obstacle to virus replication. The absence of *Vpx* from HIV-1 genome could be seen as a disadvantage in terms of adaptation of the virus to its host. About 60 million people have been infected with HIV-1 in the last 30 years, compared to 2 million with HIV-2, suggesting that HIV-1 evolved to infect humans more successfully than HIV-2.

Although the date of the initial identification of HIV-1 is recorded as 1983, phylogenetic and statistical analyses suggest the presence of HIV-1 and -2 in the west central African population up to 70 years before that date (Korber et al. 2000;

Worobey et al. 2008). Leopoldville, a city in Africa where HIV-1 is believed to have originated, harbours evidence of the earliest strains of HIV-1 group M (Zhu et al. 1998). The spread of HIV-1 from this location to a worldwide distribution was almost certainly related to human migration, development of cities and trading.

1.1.2. Simian Immunodeficiency Virus

Simian Immunodeficiency Virus (SIV) is a lentivirus affecting over a half of known African primates species. Estimated to appear in primates over 77,000 years ago (and rather closer to a million years point) (Sharp et al. 2000; Worobey et al. 2010) SIV had enough time to spread within different species and co-evaluate with its host. As a consequence of host immune pressure, point mutations introduced by error prone reverse transcription and recombination between viruses, SIV acquired characteristics specific for each natural host it infects. SIVcpz, identified primarily in Chimpanzees has arisen as a result of recombination between SIV from red-capped mangabeys (SIVcm) and spot-nosed monkeys (SIVsn) co-infecting these primates (Bailes et al. 2003). SIVcpz was further transmitted to human in last 100 years resulting in the catastrophic pandemic of HIV-1 group M (Keele et al. 2006; Worobey et al. 2008). Similarly to HIV-1 in human, SIVcpz infection of chimpanzees causes mucosal depletion of CD4+ T cells and consequently AIDS and host death (Keele et al. 2009). In contrast, CD4+ T cells depletion is not observed in SIVsmm natural host sooty mangabeys (SM) despite detectable levels of virus replication (Rey-Cuillé et al. 1998; Duvall et al. 2008). The reason for this "tolerance" to SIVsmm in SM is thought to be a consequence of low SIV-specific T-cell immune response and lack of chronic immune activation acquired over thousands years of infection of the specie (Dunham

et al. 2006; Wang et al. 2006; Paiardini et al. 2009). The importance of virus-host co-evolution is further highlighted by the fact that SIVmac strains, originating from SIVsmm are pathogenic to their non-natural host rhesus macaque. Similarly, HIV-2 that was acquired in human from SM causes AIDS in infected individuals. However, the pathogenicity of HIV-2 is much lower compared to HIV-1, suggesting changes to SIVsmm acquired during evolution in its primate host.

1.1.3. HIV-1 Epidemic

Since its first identification, HIV-1 has spread around the globe infecting millions of people. In 2012, the number of infected individuals worldwide reached 35.3 million, including an estimated 100,000 people living in the United Kingdom. The most affected Sub-Saharan African region sees as many as 25 million people living with HIV-1 compared to 860,000 cases in Western and Central Europe (UNAIDS 2015). Novel prevention programmes introduced by governments have dramatically decreased the number of new HIV infections. Additionally, introduction of antiretroviral therapies (Yi et al. 2011), voluntary male circumcision (Auvert et al. 2005; Bailey et al. 2007; Gray et al. 2007) and pre-exposure prophylaxis (Anderson et al. 2010) have proven to be a promising strategy to fight HIV-1 transmission in most affected regions. Yet, an estimated 2.3 million new HIV infections were recorded worldwide in 2013 (UNAIDS 2013), suggesting that additional steps have to be taken before the progression of HIV epidemics can be stopped and reversed.

1.1.4. Stages of HIV-1 Infection and Host Immune Responses

Three distinctive stages of HIV-1 infection have been identified in patients: acute phase, chronic phase and AIDS.

The acute infection phase takes place within 2-4 weeks of HIV-1 acquisition and is characterised by flu-like symptoms including fatigue, nausea, fever and skin rashes. When the fist symptoms manifest in a patient, HIV-1 already successfully reaches lymph nodes and gut associated lymphoid tissue (GALT) where it infects CD4+ CCR5+ T cells. The number of CD4+ T cells drops rapidly, in particular in GALT in relation to intense HIV-1 replication and bystander cell death effect (Brenchley et al. 2004; Doitsh et al. 2010; Monroe et al. 2014). Depletion of CD4+ T cells is a characteristic feature of early HIV infection and can be triggered by virus replication in target cell or cell lysis by cytotoxic T lymphocytes. Non infected CD4+ T cells that constitute the majority of depleted cells die through apoptosis induced by pro-inflammatory cytokines, cytophatic effect of viral gp120 protein (Cao et al. 1996) and Fas Ligand-mediated apoptosis (Gasper-Smith et al. 2008). Pyroptosis of abortively infected cells was also suggested to play an important role in tonsillar CD4+T cell depletion, however this study remains to be confirmed in different T cells subsets (Doitsh et al. 2014). Activation of other immune cells by the presence of HIV-1 infection triggers activation and release of pro-inflammatory cytokines from DC, macrophages, Natural killer cells and monocytes that decelerates the HIV-1 replication rate, but does not clear the virus. HIV-1 infection of activated or resting CD4+ T cells leads to establishment of latent reservoirs in patients (Chavez et al. 2015). This integrated provirus produces minimal or no viral transcripts due to resting state of the host cell, meaning it cannot be detected by the immune system or cleared with the available antiretroviral drugs. Thus, proviruses can survive undetected in

resting CD4+ T cells for prolonged periods of time until new viral progeny is produced in response to cell activation (Finzi et al. 1997).

Eventually the immune response settles and signs of illness disappear. By the end of the acute phase, the $CD4^+$ T cell count increases and then normalizes usually at lower level compared to before infection (**Figure 1.1**). This set point of $CD4^+$ T cells as well as the HIV-1 viral load is used to predict the timeline of progression to AIDS for the patient. Because symptoms of early HIV-1 infection are brief and very similar to those associated with a cold or flu, identification of HIV-1 acquisition is often difficult. Following normalization of the infection, the immune system will constantly battle with slowly replicating HIV-1 during *chronic phase*. During that time, further decrease in $CD4^+$ T cell numbers caused by the above-mentioned mechanisms is observed in patients. There are no symptoms of HIV-1 infection in the *chronic phase* and therefore the virus can remain undetectable in patients for a prolonged time. This also gives a great risk for HIV-1 to spread from unaware carriers to their uninfected partners.

As the CD4+ T cell count drops below 350 cells/mm^3 of blood, the last stage of HIV infection, *Acquired Immuno Deficiency Syndrome (AIDS)* (see Section 1.1.4) begins. Once a clinical diagnosis is made and signs of AIDS are apparent, progression to death is quite rapid and certain in the absence of antiretroviral therapy.

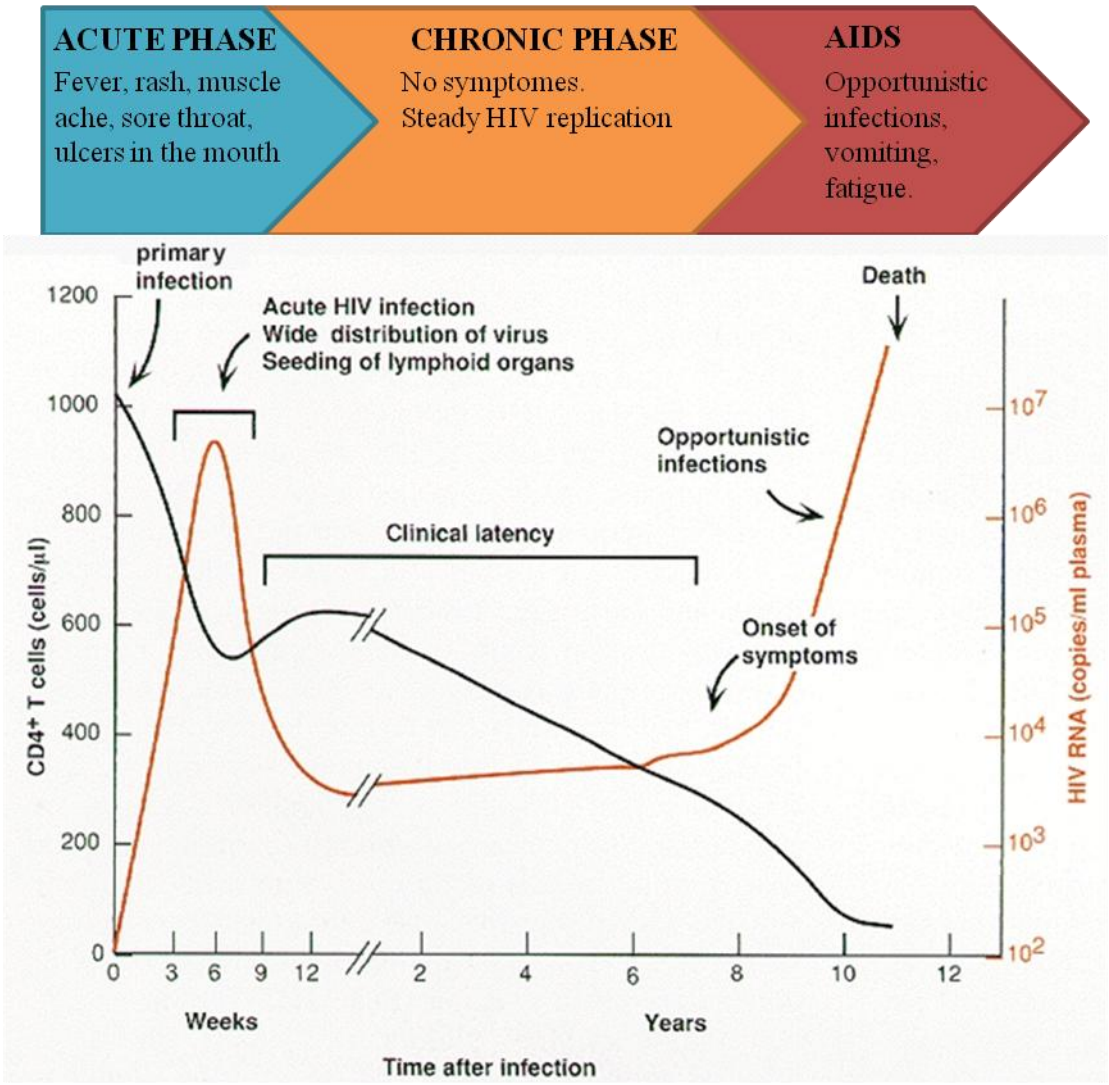

Figure 1.1. HIV Infection Timecourse. (taken from Coffin JM, Hughes SH, Varmus HE, editors. Retroviruses. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 1997. Course of Infection with HIV and SIV).

1.1.4. Acquired Immuno-Deficiency Syndrome (AIDS)

Long-term HIV-1 infection inevitably leads to a decrease of CD4+ T cell numbers to a level that can no longer adequately battle against normally harmless infections. This state is termed Acquired Immuno-Deficiency Syndrome (AIDS), which used to be a death sentence. However, advantages of current treatments allow HIV-1 positive patients to keep their CD4+ T cell count within the normal range (500 – 1,000

cells/mm^3). A drop to below 350 T cells/mm^3 of blood increases the risk of opportunistic infections, and is a main indication of AIDS. At this stage, virus load in a patient's blood is high, and the risk of sexual transmission increases (Maartens et al. 2014).

The timeline from initial HIV-1 infection towards development of AIDS differs between individuals. On average, it takes about 10 years, however in some rapid progressors, it may develop within 3 years from sero-conversion. On the other hand, about 5% of HIV-1 positive individuals do not develop AIDS and these patients are referred to as long-term non-progressors (LTNP) (Zeller et al. 1996). This ability to control virus propagation in LTNP was proposed to depend on multiple factors such as genetic factors, cell surface receptors, and the extent of immune response (Pereyra et al. 2008; Ntale et al. 2012). However, the exact mechanism of virus inhibition has not been yet elucidated. This knowledge could potentially bring on new treatment strategies to be used in the group of patients progressing to AIDS.

With a weakened immune system, AIDS patients are very prone to opportunistic infections, which would normally not cause significant disease in a healthy individual. Among the most common AIDS-defining conditions classified by CDC (AIDS.gov 2010) are *Candidiasis*, Kaposi's sarcoma, Herpes simplex, pneumonia and more than a dozen others. Additionally, those opportunistic infections trigger a positive feedback loop by boosting HIV-1 replication (see Section 1.6.). Consequently, constant opportunistic infections result in AIDS patient death.

Despite proof that HIV-1 causes AIDS, sceptics actively try to diminish HIV-1 research and the existence of the virus itself (Society 2013). Such unscientific statements mislead the readers and promote ignorance of HIV-1 prophylaxis among

susceptible people. Therefore, accurate education of vulnerable communities is also an essential step required for prevention of new HIV infections.

1.1.5. HIV-1/AIDS Treatment

Current CDC guidelines suggest starting Highly Active Antiretroviral Therapy (HAART or ART) when the blood CD4+ T-cell count drops below 350 cells/mm^3 (AIDS.gov 2014). Other recommendations apply to pregnant women and children. The standard medication combines 3 different antiretroviral drugs (ARV) acting on 2 separate HIV-1 replication steps. Based on their mode of action ARV can be divided into 5 groups: Nucleoside/Nucleotide Reverse Transcriptase Inhibitors (NRTIs), Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs), Protease Inhibitors (PI), Entry/Fusion Inhibitors and Integrase Inhibitors. Examples of each type of drug and their mode of action can be found on the AIDS website (http://www.aidsmeds.com/list.shtml).

Regular and conscientious taking of prescribed antiviral medication is essential for successful therapy. Failure to do so may not only lead to an increased viral load in the patient's blood, but also potentiate the risk of HIV-1 resistance to the treatment scheme. Healthy lifestyle involving daily exercise, a balanced diet and sufficient rest is also recommended to accompany ART therapy.

Apart from HAART, HIV positive patients, who are prone to opportunistic infections, are offered prophylactic treatment to avoid acquisition of such diseases. Prevention of co-infections in those patients is important, because opportunistic pathogens endanger the life of an immune-compromised person, while potentially also increasing HIV-1 viraemia. More detail of this interchangeable mechanism can be found in section 1.6.

1.2. HIV-1 Virion Structure and Genome

HIV-1 is the most significant member of the *Retroviridae* subfamily, genus *Lentivirus*. The virus consists of a diploid, single-stranded RNA genome enclosed in a capsid core. A lipid membrane layer surrounds the viral capsid and other viral proteins form the particle (**Figure 1.2**). The trimeric viral envelope glycoprotein (composed of gp120 and gp41) protrudes from the membrane and allows HIV-1 entry into target cells. The HIV-1 receptor (CD4) and either one of the co-receptors (CCR5 or CXCR4) are required for gp120 interaction with target cells and productive virus entry. Long Terminal Repeats (LTRs) flank the RNA genome of HIV-1 at both 5' and 3' ends. LTR regions are particularly important for HIV-1 integration and transcription enhancement. Three major genes (gag, pol and env) are located between both LTRs and encode structural proteins (p17, p24, p9, p6), viral enzymes (protease, reverse transcriptase, RNase H, and integrase) and envelope glycoproteins (gp120, gp41) respectively (**Figure 1.2**). Viral enzymes are essential for different steps of HIV-1 replication within cells. For instance, reverse transcriptase is not normally present in cells but is required to convert the HIV-1 RNA genome into DNA. This is followed by integration of HIV-1dsDNA into the host cell genome mediated by viral integrase. On the other hand, the protease acts at the post-translational stage of HIV-1 replication, and cleaves the pol polypeptide into single functional enzymes. In addition to essential genes, HIV-1 encodes accessory proteins: vif, vpr, vpu, nef and tat (and vpx present in HIV-2 and SIV). Although called accessory proteins, these non-structural proteins are critical requirement to HIV-1 replication. Accordingly, Vpu, Vif and Vpx counteract the function of cellular anti-viral restriction factors (see **Section 1.4**), Tetherin, APOBEC3G and SAMHD-1, respectively. Thus, Vpu enhances budding and release of new viral particles from infected cells by internalization and degradation of tetherin

(Neil et al. 2008; Lehmann et al. 2011). Vif on the other hand prevents packaging of APOBEC3G to newly formed particles that ensure effective reverse transcription of HIV in the consequent target cells (Sheehy et al. 2003). The reverse transcription process is also augmented by the function on Vpx, described in detail in Section 1.4.2.

Viral protein R, Vpr, is present in both HIV-1 and HIV-2 particles and serves multiple functions in cellular immune evasion and it operates at several steps of HIV replication cycle (reviewed in (Guenzel et al. 2014). Vpr induces G2 cell cycle arrest in cells and mediates nuclear entry of HIV pre-integration complex in cooperation with viral matrix, integrase proteins and capsid (He et al. 1995; Re et al. 1995; Jenkins et al. 1998; Suzuki et al. 2009). Vpr also increases the activity of various gene promoters, mainly LTR of HIV (Sawaya et al. 1998; Yao et al. 1998; Cui et al. 2006). In context of immune evasion, Vpr induces apoptosis in bystander T cells while acting anti-apoptotic in infected cells (Conti et al. 1998; Moon and Yang 2006). Vpr also inhibits interferon induction in infected cells preventing expression of interferon-inducible restriction factors (Mashiba et al. 2014; Harman et al. 2015) On the systemic level, Vpr promotes Th2 responses and prevents maturation and activation of macrophages and dendritic cells thus supporting viral propagation in the individual (Ayyavoo et al. 2002). Therefore Vpr protein has a wide repertoire of functions, all aimed at suppression of immune responses and increase of viral replication. The importance of this protein in virus life cycle is highlighted by the fact that SIV defective in Vpr/Vpx does not cause AIDS in rhesus monkeys (Gibbs et al. 1995). Similarly, defects in Vpr of HIV-1 decrease infection rate by 50% in macrophages (Eckstein et al. 2001).

While Vpr and Vpx are both packed into viral particle, Tat and Nef, the other accessory proteins are synthesised immediately after provirus integration. The early

expression of these proteins relates to their role in stimulating transcription of full length virus genome from LTR (function of Tat) and decrease of CD4 and MHCI from the cell surface (the role of Nef)(Piguet et al. 1998; Piguet et al. 1999; Piguet et al. 2000). Thus, the appearance of accessory proteins is timely regulated depending on their function in the viral life cycle. These accessory proteins modify the environment of the cell in order to hide the virus from cellular immune responses, counteract cellular restriction factors, and promote viral replication and spread (Malim and Emerman 2008).

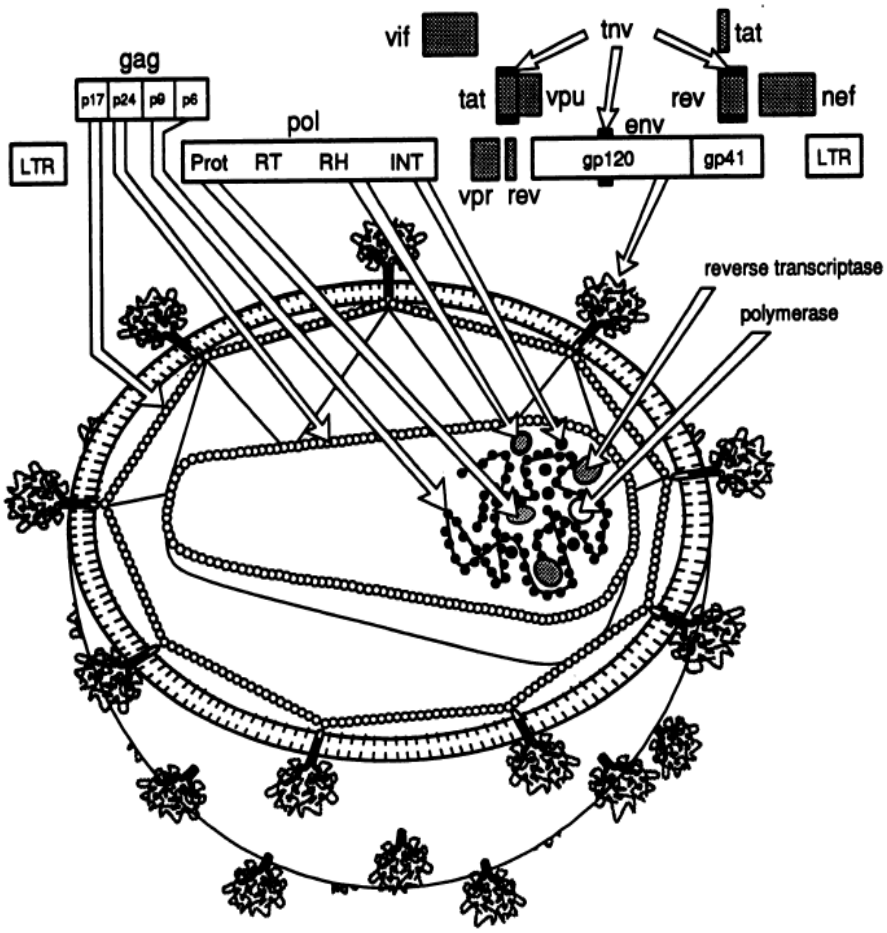

Figure 1.2. Structure of HIV-1 Particle and Genome Composition. HIV-1 particle comprise membrane with embedded envelope glycoprotein spikes. Matrix underlies membrane layer, and sheds capsid core. Two copies of the viral RNA are contained inside the capsid. Each RNA strand encodes 3 main genes, gag, pol and env, as well as additional and accessory genes. Differential expression of genes and splicing of translated proteins forms various viral components**.**

1.3. HIV-1 Replication Cycle

The HIV-1 replication cycle starts with the attachment of virus to the CD4 receptor and co-receptor on the target cell surface (**Figure 1.3.** 1). Envelope components gp120 and gp41 mediate CD4 and co-receptor binding and fusion of the viral and cell

membranes, respectively. Gp41 is shed from the environment by the structure of gp120. Conformational shift of gp120, triggered by binding of this protein to CD4 and either of the co-receptors, reveals gp41 and induces its fusogenic capacity (Sattentau and Moore 1991; Doms and Trono 2000). According to co-receptor usage, HIV-1 strains are divided into R5, X4 or R5/X4 viruses (binding CCR5, CXCR4 or both, respectively). Release of the HIV-1 capsid into the cell cytoplasm follows the fusion of the virus at the cell surface (**Figure 1.3** 2). At this point viral capsid does not uncoat but it is bound by cleavage and polyaddenylation specificity factor 6 (CPSF6) and cyclophilin A (CypA) proteins present in the host cell cytoplasm (Schaller et al. 2011; Price et al. 2012; Bichel et al. 2013; Rasaiyaah et al. 2013). The attachment of the CPSF6 and CypA stabilize the capsid, participate in timing of reverse transcription process and mediate particle translocation towards the nuclear pore (Yamashita et al. 2007; Bichel et al. 2013). The process of reverse transcription, during which reverse transcriptase converts single stranded viral RNA into double-stranded DNA (cDNA), is a characteristic stage of retrovirus replication (**Figure 1.3.** 3). Nucleotides present in the cytoplasm of the infected cell are required for this process. Subsequent to CPSF6 binding, interaction of capsid with Nup358 at the nuclear pore triggers isomerisation of the capsid and a release of viral cDNA. TNPO3 transportin 3 and Nup153 further orchestrate its entry to the nucleus and determine the integration site (Diaz-Griffero 2012; De Iaco et al. 2013) (**Figure 1.3.** 4). Thus by "hiding" its nucleic acids in the capsid, HIV can avoid recognition by cellular DNA sensors, cGAS and IFTIM16 (Gao et al. 2013; Rasaiyaah et al. 2013; Sun et al. 2013). In addition, HIV utilizes the function of cellular DNase TREX1 to destroy any excess reverse transcription products that would otherwise trigger interferon responses in the cells (Yan et al. 2010). Although the capsid provides a safe and compact environment perfect for the

action of reverse transcription enzyme, at the same time the virus is risking restriction by TRIM5α (see Section 1.4).

Following nuclear entry, viral integrase inserts HIV cDNA into the host genome and the cellular machinery transcribes it into mRNA (**Figure 1.3** 5).Firstly, a short early transcript encoding Tat, Rev and Nef is translated. Both Tat and Rev proteins migrate back into the nucleus where Tat enhances the transcription of integrated HIV-1, and Rev facilitates transport of unspliced transcripts from the nucleus to the cytoplasm. Nef, triggers down-regulation of the cell surface marker CD4, and other surface proteins required for triggering an immune response to infection such as MHC class I (Piguet et al. 2000; Janvier et al. 2001; Malim and Emerman 2008). Smaller transcripts of unspliced viral RNA serve as a temple for Tat, Vpu, Vif, Vpr and envelope proteins. The second larger transcript encodes gag and pol proteins. Gag polyprotein interacts with viral and cellular proteins and moves toward the viral assembly point at the cytoplasmic side of the cell membrane. During budding, the polyprotein undergoes a series of controlled cleavages and its components form the mature viral core. Full-length unspliced RNA represents the genome of new HIV particles (**Figure 1.3** 6 and 7) (Bell and Lever 2013).

It is important to realize that defined host cellular factors actively participate in HIV-1 replication and are therefore imperative for this process. Down-regulation, or inactivation of any cellular factors involved in virus infection may decrease or completely abolish replication. Thus, the biology and activation status of the cell, commonly influenced by the local environment, determine cell ability to support HIV-1 replication. This subject will be further deliberated later when the differences in HIV-1 replication kinetics in dendritic cell subsets are considered.

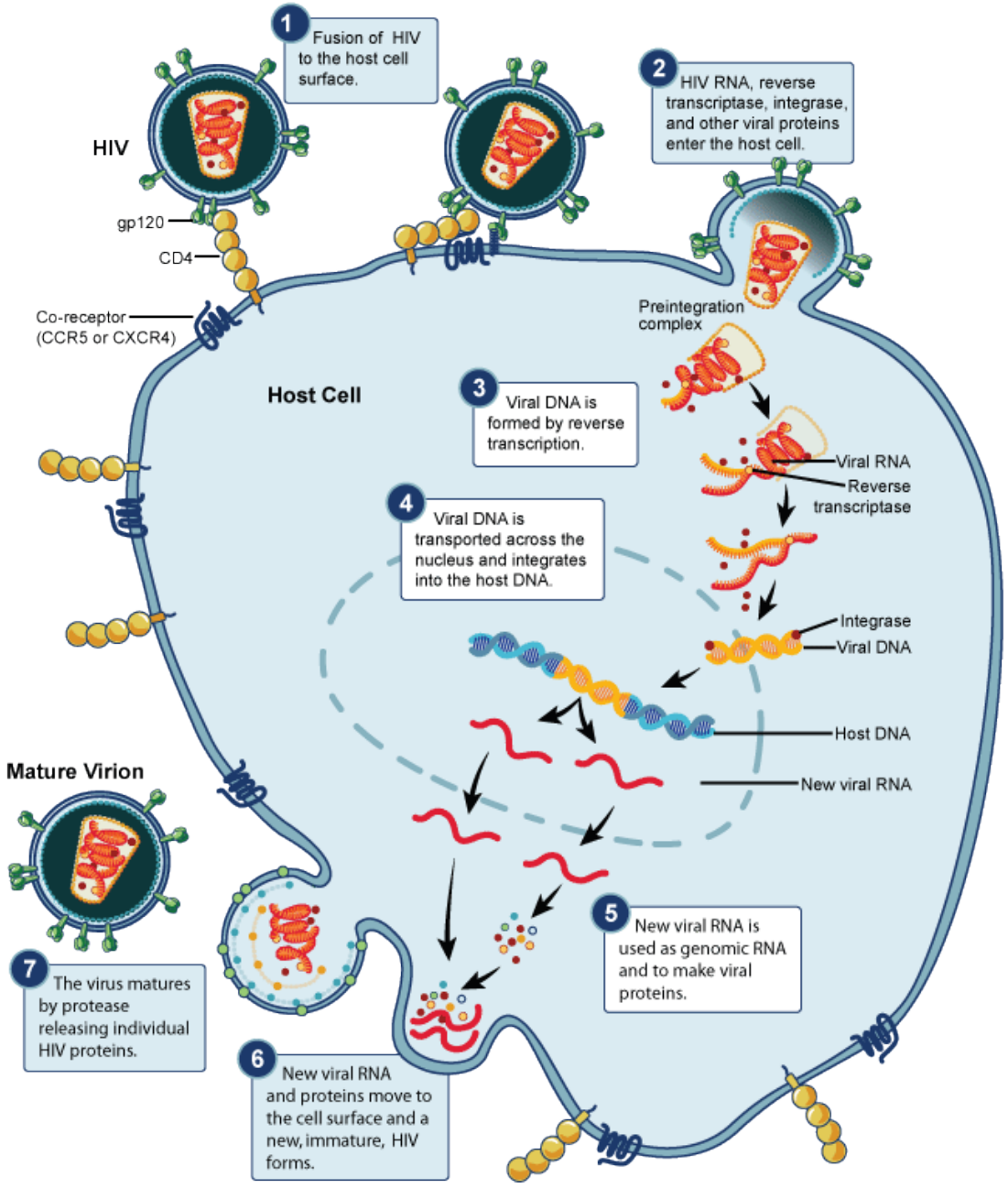

Figure 1.3. Simplified Overview of HIV-1 Replication Steps. The steps of HIV-1 replication in the cells are shown and include **1**) Attachment of HIV to receptor/co-receptor and fusion with the host cell membrane. **2**) Entry of HIV capsid, RNA and enzymes to host cell cytoplasm. **3**) Reverse transcription process. **4**) Nuclear entry and integration of provirus in cell DNA. **5**) Transcription of integrated provirus and **6**) formation of new viral particles at the host cell membrane.**7**) HIV particle budding and maturation. (Adapted from National Institute of Allergy and Infectious Diseases).

1.4. HIV-1 Restriction Factors

A number of cellular proteins, termed restriction factors, interfere with HIV-1 replication. The most investigated are tripartite motif-containing 5α (TRIM5α), apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3G (APOBEC3G) and SAM domain HD domain-containing protein 1 (SAMHD-1) that inhibit early steps of HIV-1 replication before integration and tetherin (also called Bst-2 or CD317), reported to act during the release of new HIV-1 particles from the infected cells (**Table 1.2**). These restrictions are constantly expressed in cells and, for some of them, their levels are additionally increased upon interferon-alpha (IFN-α) treatment.

At early stages of infection in human cells, HIV-1 is restricted by the action of APOBEC3G. This restriction factor binds viral reverse transcriptase or viral genomic RNA and stalls the synthesis of complementary DNA (Mangeat et al. 2003; Newman et al. 2005). Also by introducing G to A hypermutations in newly formed reverse transcription products, APOBEC3G renders virus non-infectious (Harris et al. 2003; Lecossier et al. 2003). Interestingly, APOBEC3G does not mediate this protective effect in infected cells, but it has to be incorporated into the forming virus particle to inhibit HIV-1 reverse transcription in the subsequent cells. The fact that the levels of APOBEC3G are interferon inducible suggests predominant anti-viral function of this protein. Reverse transcription of HIV-1 is also controlled by the presence of SAMHD-1 in cells. This predominant block to virus replication in myeloid cells is described in detail in Section 1.4.1.

TRIM5α is another stably expressed restriction factor present in the cell cytoplasm. Similarly to APOBEC3FG, TRIM5α levels can be induced by IFN signalling.

Although expressed in human, this restriction factor is thought to be weakly effective against HIV-1 and more likely to have a function in inhibition of SIVrm (Kirmaier et al. 2010). TRIM5α. binds viral capsid and stabilizes it preventing the process of reverse transcription and uncoating (Stremlau et al. 2004). The capsid binding sites of TRIM5α dictate the susceptibility of different HIV strains to this restriction. Interestingly, CypA binding HIV-1 capsid protects viral particle from Ref-1 restriction, this mechanism could also explain resistance of HIV-1 to human TRIM5α.

In contrast to aforementioned restriction factors, tetherin (also referred to as Bst2) acts at a very late stage of HIV-1 replication. Indeed, tetherin binds viral envelope in endoplasmic reticulum and at the cell surface and prevents release and maturation of virus progeny (Neil et al. 2008). Two isomers of tetherin are present in human cells, short and long and these arise from the alternative translation. Short tetherin is more resistant to Vpu-mediated degradation, but lacks cytoplasmic signalling sequence. In contrast, the long isomer can induce NFκβ signalling, but is an easier target for Vpu (Gupta and Towers 2009; Mangeat et al. 2009; Miyagi et al. 2009). As described in Section 1.2., HIV-1 and HIV-2 down regulate tetherin with the accessory protein Vpu. The conservation of this gene in HIV-1 and HIV-2 subsets suggest that down modulation of this restriction factor is an important step in ensuring successful propagation of HIV.

Table 1.1. Summary of Cellular Restriction Factors acting on HIV-1 Replication.

Restriction factor	Stage of HIV replication affected	Mechanism of action	IFN inducible	HIV anti-protein	Reference
APOBEC3G	Reverse transcription	Introduces T→U supermutation in HIV-1 genome leading to abortive reverse transcription.	Yes	Vif	(Sheehy et al. 2003; Zhang et al. 2003).
Trim5α	Uncoating	Binds HIV-1 capsid and prevents uncoating.	Yes	Unknown	(Stremlau et al. 2004)
SAMHD-1	Reverse transcription	Depletes dNTP pool; directly degrade HIV-1 RNA.	No/Yes	Vpx	(Goldstone et al. 2011; Beloglazova et al. 2013; Ryoo et al. 2014)
Tetherin	Budding	Anchors newly produced HIV-1 particles to cell membrane, preventing virus maturation and release.	Yes	Vpu	(Neil et al. 2008)
MX2	Prior integration	affects nuclear entry of viral cDNA or its stability in the nucleus	Exclusively expressed after IFN stimulation	Not known	(Goujon et al. 2013)

In addition to constitutively expressed restriction factors described above, other antiviral proteins are induced only in response to interferon stimulation of the cell.

IFN-α is a warning cytokine expressed by immune cells in response to pathogen sensing. It plays a crucial role in fighting viral infections by inducing an antiviral state in cells and expression of interferon-stimulated genes (ISG). The cellular levels of APOBEC3G, TRIM5α and tetherin are sensitive to IFN stimulation, but other ISG are also expressed upon STAT signalling, such as myxovirus resistance-2, MX2 (Goujon et al. 2013). Although the exact mechanism of MX2 mediated inhibition of HIV remains to be elucidated, this protein was found to inhibit HIV prior to integration, possibly affecting nuclear entry of viral cDNA or its stability in the nucleus (Fricke et al. 2014). Interestingly viral capsid governs sensitivity to MX2 and disruption of this interaction renders MX2 inactive against HIV-1 infection (Liu et al. 2015). The functions and importance of ISG in terms of HIV-1 infection is only now being discovered and appreciated.

1.4.1. SAMHD-1

Recently (2011), two research laboratories identified SAMHD-1 a s a potent viral restriction factor (Hrecka et al. 2011; Laguette et al. 2011). SAMHD-1 acts against a wide range of pathogens including DNA viruses such as vaccinia virus, herpex simplex virus and Hepatitis B virus (Hollenbaugh et al. 2013; Kim et al. 2013; Chen et al. 2014), retroviruses including HIV-1 and SIV (Hrecka et al. 2011; Laguette et al. 2011), as well as retro-elements (Zhao et al.). SAMHD-1 is a triphosphohydrolase expressed in myeloid cells such as dendritic cells, macrophages, and is present in quiescent $CD4^+$ T cells. In cells, SAMHD-1 and related DNase TREX1 deplete RNA and DNA, respectively, present in the cytoplasm which prevents activation of immune sensing by endogenous retroviruses or gene transcription products. SAMHD1/TREX1

deficiency in human leads to development of Aicardi-Goutieres syndrome (see Section 1.4.3.). SAMHD-1 is a nuclear protein (Rice et al. 2009; Brandariz-Nunez et al. 2012; Guo et al. 2013), also present at lower concentrations in the cytoplasm (Baldauf et al. 2012). It consists of two main domains: the sterile alpha motif (SAM) domain, and the histidine-aspartic (HD) domain. The SAM domain is involved in nucleic acid binding and protein-protein interactions (Qiao and Bowie 2005), whereas the phosphor-hydrolase enzymatic activity of the protein localizes to the HD domain (Zimmerman et al. 2008). A mutational study performed by White et al., (White et al. 2013a) showed that the HD domain alone is sufficient for SAMHD-1 restrictive properties and SAM is dispensable for that function. The same authors additionally demonstrated that nuclear localization of SAMHD-1 is not required for its HIV-1 restriction function.

The mechanism of action of SAMHD-1 was proposed to rely on the cleavage of deoxynucloside triphosphates into deoxynucleosides and triphosphate (Goldstone et al. 2011; Powell et al. 2011). SAMHD-1 mediated depletion of the nucleosides pool from cells renders HIV-1 reverse transcription very inefficient (Hrecka et al. 2011; Laguette et al. 2011; Lahouassa et al. 2012). The levels of dNTP in SAMHD-1 expressing macrophages range in very low concentrations between 20 and 50nM. This is in contrast to the higher levels of 2-30 μM dNTP concentration observed in activated CD4+ T-cells that do not express SAMHD-1 (Nguyen et al., 2014). Interestingly, research has shown that SAMHD-1 is present in quiescent T-cells, which may have a direct incidence on the formation of HIV-1 reservoirs within these cells (Gao et al. 1993; Baldauf et al. 2012; Descours et al. 2012).

New data are now emerging to suggest that the dNTPase activity of SAMHD-1 may not be the only mechanism of HIV-1 restriction by this protein. An alternative mode

of action proposed for SAMHD-1 includes direct interaction of the protein with HIV-1 genomic ssRNA (Beloglazova et al. 2013; Ryoo et al. 2014). Subsequently, RNase activity of SAMHD-1 leads to cleavage and degradation of viral ssRNA. This activity is regulated by phosphorylation of SAMHD-1 at T592 which renders SAMHD-1 inactive against HIV-1 infection, without lowering dNTP levels (Cribier et al. 2013; Welbourn et al. 2013; White et al. 2013b) (**Figure 1.4**). SAMHD-1 is phosphorylated by cyclin dependent kinase (CDK). As CDK is also involved in regulation of cell cycle, inactivation of SAMHD-1 and induction of cell cycle can be related processes. In fact, SAMHD-1 activity concentrates at degradation of cystolic RNA and depletion of dNTP, therefore inhibition of this protein could be a pre-requisite for efficient mitosis. Thus, SAMHD-1 uses 2 separate mechanisms to inhibit early steps of HIV-1 replication: it depletes dNTP in the cell cytoplasm by dNTPase activity and directly destabilizes or degrades the HIV-1 genome using its RNase activity (**Figure 1.4**.). The switch between these mechanisms was proposed to be dependent on SAMHD-1 oligomerization and the presence of dGTP (Ryoo et al. 2014). In the presence of dGTP, SAMHD-1 proteins form tetramers, a requirement for its dNTPase function (Ji et al. 2013; Yan et al. 2013; Zhu et al. 2013). On the other hand, in low dGTP conditions, SAMHD-1 exists as a dimer or a single protein, which favours its RNase activity (**Figure 1.4**). However, this new model still requires further confirmation.

It is unknown how SAMHD-1 imposes its RNase activity on viral genomic RNA while capsid proteins protect it. If we assume that cytoplasmic sensors and proteins have no access to viral nucleic acids, then the RNase function of SAMHD-1 should be limited as well. SAMHD-1 is also present in the nucleus however; viral reverse transcription product (cDNA) present at this stage of HIV replication cycle is no longer a substrate for an RNase, such as SAMHD-1. In that case, SAMHD-1

restriction activity on HIV-1 would be limited to depletion of dNTP pool. Degradation of HIV-1 RNA could take place if the viral RNA is released from the capsid protection or when SAMHD-1 enters a confided area within the viral capsid. If the latter is the case then cGAS, which is 10 kDa smaller protein than SAMHD-1, could theoretically also gain access to HIV-1 nucleic acids. Consequently, induction of interferon response is likely. However, the exact correlation between SAMHD-1 function and the presence of CPSF6 is unknown.

SAMHD-1 successfully keeps HIV-1 replication at the lowest levels in myeloid cells and when expressed in virus susceptible T-cells. However, this powerful barrier has its dark sides, too. As a "double edged sword", SAMHD-1 by its action prevents sensing of HIV-1. Therefore, the immune system does not respond to the initial virus invasion until it is too late to stop its systemic spread. In the case of HIV-2 infection, Vpx-mediated degradation of SAMHD-1 results in an initial boost of virus replication followed by cDNA sensing and release of type-I interferon from infected cells. As a result, type-I interferon production generates an antiviral state on neighbouring cells, therefore impairing the propagation of infection (Manel et al. 2010; Baldauf et al. 2012). Other reports suggest that low dNTP levels in SAMHD-1 positive $CD4^+$ T-cells can promote formation of incomplete strands of HIV-1 cDNA during reverse transcription. This abortive HIV-1 infection of bystander T-cells consequently triggers immune responses in these cells leading to activation of capsase 3 and T-cell death by pyroptosis. Pro-inflammatory molecules released from dying T cells additionally attract HIV-1 susceptible cells and set chronic inflammation in infected patients (Doitsh et al. 2014; Hansen et al. 2014). Accordingly, a drop in T-cell levels happens at a quicker rate during HIV-1 infection, compared to Vpx expressing HIV-2 infection.

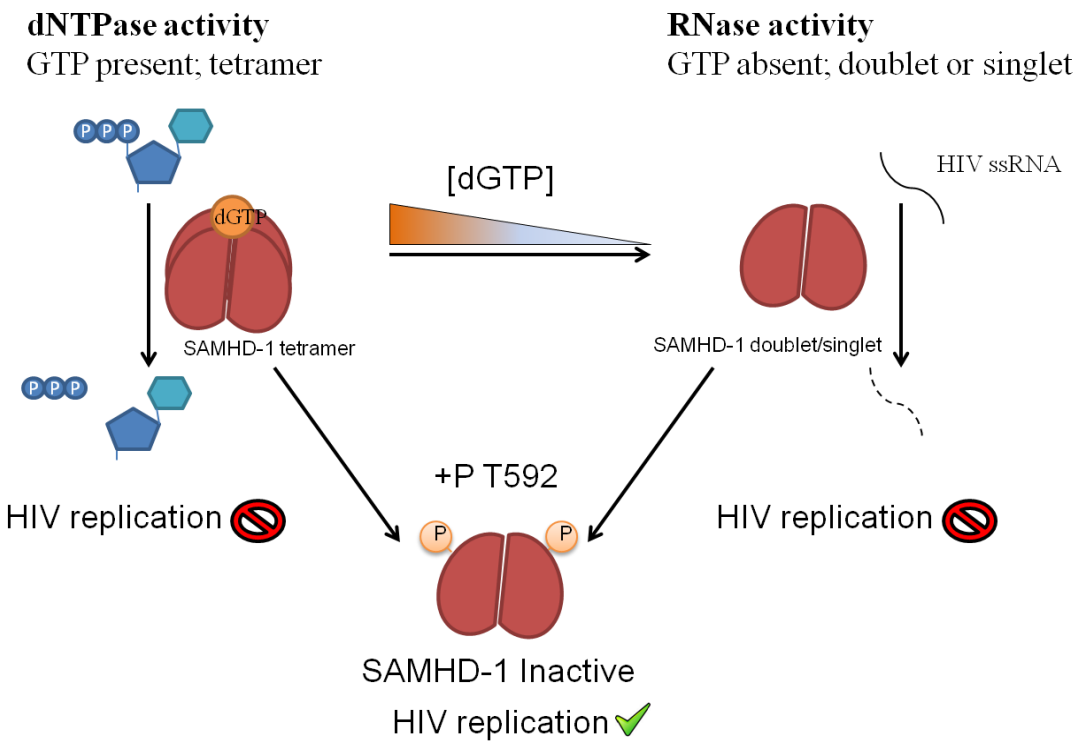

Figure 1.4. Different Mechanisms of SAMHD-1 Restriction of HIV-1 Replication. In the presence of GTP, SAMHD-1 exists in tetramer form able to hydrolase nucleosides to nucleotides and triphosphate, simultaneously blocking HIV-1 reverse transcription. In low GTP conditions, dimeric form of SAMHD-1 binds and degrades HIV-1 single stranded RNA. Phosphorylation of SAMHD-1 at Threonine 592 renders both mechanisms inactive and allows HIV-1 replication in the cell.

1.4.2. Vpx Counteracts SAMHD-1-mediated Viral Restriction

Vpx is a 12-16 kDa accessory protein encoded by HIV-2 and some SIV genomes, but absent in the HIV-1 (Zhang et al. 2012; Etienne et al. 2013). Vpx is packaged into virions during their assembly (Wu et al. 1994; Singhal et al. 2006a; Singhal et al. 2006b) and it shuttles between the nucleus and cytoplasm in target cells (Belshan and Ratner 2003; Singhal et al. 2006a; Singhal et al. 2006b). Similarly to other accessory proteins, Vpx has been proposed to serve multiple functions it mediates SAMHD-1 degradation and enhances nuclear import of viral genome and reverse transcription

independently of SAMHD-1 (Berger et al. 2010; Pertel et al. 2011; Reinhard et al. 2014). Most likely, the low susceptibility of HIV-2 reverse transcriptase to dNTP prompted acquisition of Vpx in the genome. Interestingly, Vpx originates from duplication of Vpr that itself has no effect on SAMHD-1 levels in the cells (Lim et al. 2012).When expressed in target cells, Vpx binds SAMHD-1 and leads to its proteasomal-mediated degradation. Vpx was reported to interact with $CRL4^{DCAF1}$ E3 ubiquitinin ligase (VPRBP, a cullin-RING ubiquitin ligase) thereby recruiting SAMHD-1 to the complex (Hrecka et al. 2011). The C-terminal domain of SAMHD-1 is required for this interaction as showed by mutational studies (Ahn et al. 2012). Ubiquitination of SAMHD-1 is followed by its proteasomal degradation, which results in an observed HIV-1 replication boost. Recently, it has been suggested that neddylation of SAMHD-1 is also required for protein degradation (Hofmann et al. 2013) but these results have not yet been confirmed.

Interestingly, mutational studies showed that some modifications in Vpx could decrease HIV-2 replication in MDM or MDDC, although SAMHD-1 expression was efficiently down regulated (Goujon et al. 2008), which suggest additional roles for this protein during HIV-2 infection. One such functions involves transport of HIV-2/SIV pre-integration complexes (PIC) to the nucleus (Belshan and Ratner 2003; Belshan et al. 2006).

The absence of Vpx in the HIV-1 genome may be of benefit for successful viral spread. Low replication levels imposed by SAMHD-1 restriction in antigen presenting cells prevents immune sensing of the infection and induction of type-I interferon (Nobile et al. 2005; Manel et al. 2010; Lahaye et al. 2013). Therefore, HIV-1 in the absence of Vpx expression remains unnoticed by the immune system until it is too late and virus spreads to susceptible cells. Acute immune activation at this point

temporarily restrains further HIV-1 dissemination but does not allow clearance of integrated viruses. In the case of HIV-2 infection, Vpx-dependent SAMHD-1 degradation boosts virus replication at early transmission sites at the cost of a strong immune activation. This allows virus control and seemingly decreases the rate of $CD4^+$ T-cell death (Manel et al. 2010; Baldauf et al. 2012). Other studies imply that high levels of type-I IFN produced by innate immune cells play a part in chronic immune activation associated with progression to AIDS (Boasso et al. 2008; Ganesan et al. 2010). However, this effect refers to later stages of HIV-1 infection and might not be directly related to SAMHD-1 down regulation. The lack of the *vpx* gene in the HIV-1 genome might have triggered a specific adaptation of HIV-1 to replicate in an environment with negligible dNTP levels. Accordingly, reverse transcriptase of HIV-1 shows increased affinity for dNTP, compared to HIV-2 and other viruses reported to down-regulate SAMHD-1 expression (Lahouassa et al. 2012). Whether vpx loss is a trigger or a result of improved reverse transcriptase function remains unknown. Nonetheless, the absence of *vpx* in the HIV-1 genome may have settled a *sine qua non* condition for its successful propagation.

1.4.3. Vpx as a Tool to Facilitate Genetic Modification of Primary Cells

Delivery of Vpx via transduction of cells with virus-like particles (VLP) (SIV3-Vpx) is a successful way for SAMHD-1 down regulation employed by researchers (Goujon et al. 2006; Berger et al. 2011b; Laguette et al. 2011). SIV3-Vpx is acquired via co-transfection of HEK293T cells with pMD.G plasmid and pSIV3+ packaging construct (see Materials and Methods Table 2.1 and Section 2.6.4.). pMD.G is a source of Vesicular Stomatitis Virus envelope (VSV-G) (Naldini et al. 1996) that is

incorporated into new SIV3-Vpx particles. VSV-G binds to low density lipoprotein receptor (LDLR) and fuses with the cell membrane only after a pH change in lysosomes (Sun et al. 2005; Finkelshtein et al. 2013). As LDLR is abundantly expressed on the cell surface membrane, the entry of pseudotyped VSV-G lentivectors is efficient and independent of CD4 or CCR5/CXCR4 receptors.

pSIV3+ construct has been derived from SIV (SIVmac251) through elimination of env and 3'LTR only. 5'LTR and other leader sequences were replaced with Cytomegaloma Virus (CMV) early promoter/enhancer sequence that ensures strong gene expression in comparison to LTR (Nègre et al. 2000). Thus, in addition to our gene of interest, *vpx*, pSIV3+ also encodes *gag*, *pol*, *vif*, *vpr*, *rev* and *tat* genes. As described above (see Section 1.2), both Vpr and Vpx are packed into new virions, as they function at early stages of virus infection. Therefore, SIV3-Vpx particles derived from pSIV3+ also contain Vpr protein. Vpr is a multifunctional involved in systemic immune envision and persistence of HIV-1 infection (Ayyavoo et al. 1997; Ayyavoo et al. 2002; Mashiba et al. 2014; Harman et al. 2015). Among its functions Vpr is recognised as an important inducer of HIV-1 LTR, particularly in macrophages that promotes viral pathogenesis (Varin et al. 2005; Mashiba et al. 2014). A multiple transcription binding site at viral LTR are required for this function of Vpr, suggesting that replacing LTR with another promoter may cause loss of function. However, on top of direct binding to LTR, Vpr also induces NFκβ, which has a pronounced effect on gene transcription downstream of other promoters such as CMV and EF-1α (Yurochko et al. 1997; Roux et al. 2000; Gangwani et al. 2013). Thus, usage of SIV3-Vpx in *in vitro* studies could result in boost to expression of the gene downstream of LTR or NFκβ-sensitive promoter. Such effects could then be wrongly accredited to the function of Vpx or lack of SAMHD-1 in the cells. Similarly, investigation of

cytokine milieu or cell survival in SIV3-Vpx treated cells should be analysed with caution as Vpr is a known inducer of TNF-α in DC and a modulator of cell apoptosis (Nakamura et al. 2002; Busca et al. 2012).

In this study I showed that SIV3-Vpx transduction of the cells efficiently depletes SAMHD-1 without activating the cells (see Chapter 4). I have exploited this method in our research and demonstrated efficient and reproducible Vpx-mediated decrease of SAMHD-1 expression in monocyte derived Langerhans cells and monocyte derived dendritic cells (see **Chapter 4**). The effect of the presence of Vpr on the experimental outcomes is considered in result chapters.

1.4.4. Aicardi-Goutieres Syndrome (AGS)

Mutation of the SAMHD-1 gene causes a rare disorder called Aicardi-Goutieres syndrome (AGS). AGS can also result from mutations or malfunctions of other nucleases including TREX1, RNASEH2A, RNASEH2B and RNASHE2C (Crow et al. 2006; Rice et al. 2007). Nuclease breaks up unneeded cellular DNA and RNA molecules after transcription, replication or other cellular processes. Any abnormality in these nuclease triggers accumulation of nucleic acid molecules and may be mistaken for viral infection. Thus, immune activation in AGS is related to increased systemic levels of IFN-α (Crow and Rehwinkel 2009; Stetson 2012; Lee-Kirsch et al. 2014). In cases of SAMHD-1 mutations in AGS patients, IFN-α is triggered by high dNTP levels and consequent DNA damage (Kretschmer et al. 2014) . AGS onset is early, affecting babies in their first year of life. The manifestation of disease includes encephalopathy, psychomotor retardation, hepatosplenomegaly, thrombocytopenia and in some cases death (Rice et al. 2007) .

1.5. Sexual Transmission of HIV-1

The majority of HIV-1 transmission happen via sexual transmission. The risk of acquiring HIV-1 from an infected partner by receptive penile-vaginal intercourse is less than 0.1% (CDC.gov 2014) although the probability depends on multiple factors described further (see Section 1.6.). This risk of transmission significantly increases for man who have sex with man due to easier passage of the virus through the rectal and gut mucosa (see Section 1.5.3). In contrast, the physical structure of the female reproductive tract poses a very potent barrier to HIV-1 passage, if intact. Additionally, mucus and the vaginal environment are unfavourable to HIV-1.

1.5.1. Mucosal Surface: Female Reproductive Tract Biology

The female reproductive tract anatomically consists of ectocervix, cervix and endocervix. Vagina and ectocervix are covered with multi-layered squamous epithelium, and a single layered of columnar epithelium lining endocervix (**Figure 1.5**). The reproductive tract is covered in dense, acidic mucous that captures pathogens and prevents growth of bacteria and fungi. Penetration of SIV-1 through the mucus after direct uterine SIV inoculation was demonstrated in monkeys (Joag et al. 1997; Hladik and Hope 2009), but the efficiency of the process was very low, confirming its protective role against SIV-1 acquisition. However, the main transmission route of SIV in primates is via contact with infected blood or body fluids taking place during fights and hunting. Additionally, the risk of vertical transmission in infected primates is lower compared to HIV-1 mother to child transmission in human suggesting different adaptation of HIV-1 and SIV to its hosts.

Underneath the top layer of the reproductive tract lies a connective tissue layer, called the lamina propria. This submucosal epithelium contains dense population of immune cells including dendritic cell subsets, macrophages and memory T-lymphocytes (**Figure 1.5**). Additionally, high numbers of CD4+ T-cells locate to the zone of conversion where ectocervix changes into endocervix, referred to as the transformation zone (**Figure 1.5**).

Immune cells present at the mucosal surfaces act as a barrier to infection and are the first cells to sample invading pathogens. Unfortunately, in the case of HIV-1 infection, these cells can become targets and carriers for the virus from the initial infection site to more susceptible target cells in lymph nodes (see Section 1.5). High concentration of target CD4+ T-cell in the conversion zone also supports virus entry and settlement of founder cells (Haase 2010). Thus, the relatively low transmission rate of HIV-1 during receptive penile-vaginal intercourse results from the physical barriers posed by the epithelial lining as well as the obstructive environment provided by the mucous layer. Nonetheless, HIV-1 is able to penetrate genital mucosa and cause an infection. Several mechanisms may be involved in helping the virus to cross the epithelial layers (see Section 1.5). Furthermore, other factors may increase susceptibility of the individual to HIV-1 acquisition.

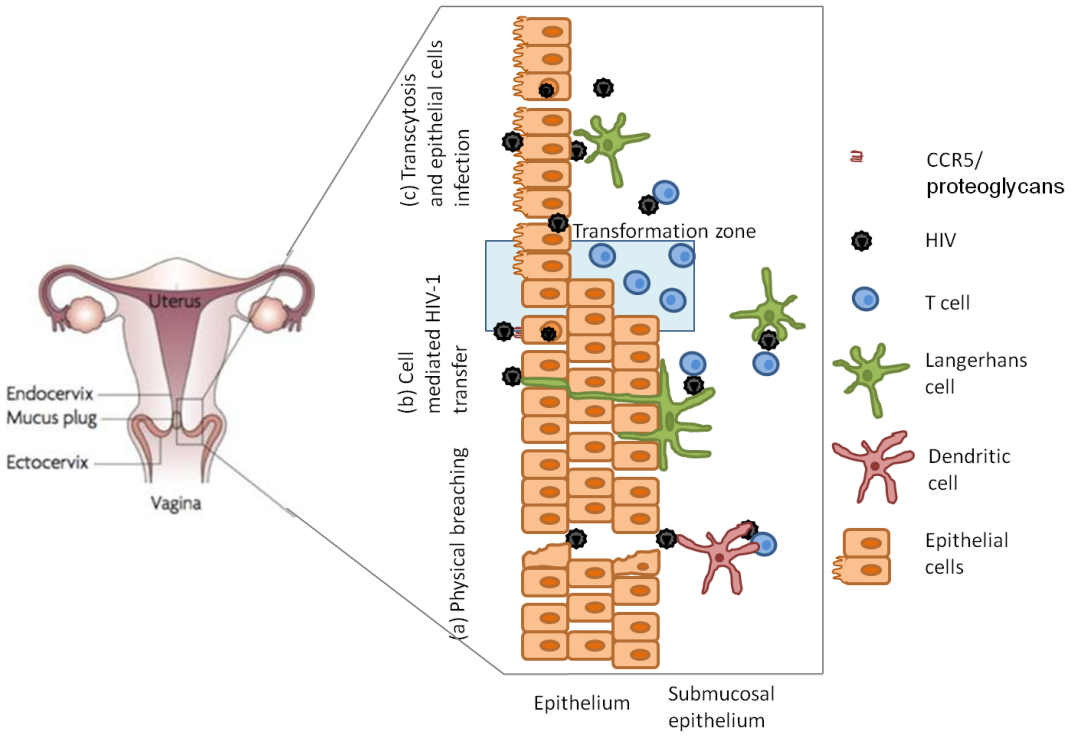

Figure 1.5. Schematic Overview of HIV-1 Transmission Mechanism trough Female Genital Tract. The non-permissive epithelial layer in female reproductive tract stops passage of HIV-1 into submucosal epithelium. **(A)** However, a physical breaching of epithelium allows passage of free HIV-1 particles and infection of the immune cells. **(B)** Langerhans cell mediated transfer of HIV-1 from the epithelial surface and subsequent transmission to T cells promotes virus acquisition. **(C)** Transcytosis or infection of epithelial cells also increase chances of viral passage into susceptible T cells located in the transformation zone and submucosa.

1.5.2. HIV-1 Transmission Mechanisms in Vaginal Mucosa

According to a study in a non-human primate model, it takes 30 to 60 minutes for SIV to penetrate the cervico-vaginal epithelium *in vivo* (Hu et al. 2000). How HIV-1 bypasses the mucous and epithelial barriers remains a subject of debate as several mechanisms have been suggested. These include physical breaching, transcytosis and

infection of the epithelial cells due to penetration through cervico-vaginal epithelium breaks and uptake by Langerhans and Dendritic cells.

1.5.2.1. Factors Increasing Epithelial Permeability to HIV-1

The composition and pH of mucous and thickness of the epithelium changes accordingly to hormones released at different stages of the menstrual cycle. Rise in oestrogens during ovulation makes mucous less viscous and less acidic, to allow sperm cells to penetrate. Those changes simultaneously weaken protective barriers of the reproductive tract and amplify the chance of acquiring sexually transmitted infections (STIs), including HIV-1 (reviewed in (Wira and Fahey 2008). Among other factors increasing the permeability of the epithelial barrier to HIV-1 are some components of semen (Munch et al. 2007) and co-infections with other STIs. The importance of the latter has been emphasized in numerous reports, and will be further discussed in Section 1.6.

1.5.2.2. Epithelial Cells Transcytosis and Infection

The efficiency of HIV-1 transmission during sexual intercourse is relatively low, suggesting that epithelial cells form a relatively successful barrier to the pathogens (Gray et al. 2001). However, mucosal breaching arising during sexual intercourse allows free virus passage and infection of cells in the submucosal epithelium (**Figure 1.5A**). Mechanisms that are more complex are required for passage through an intact epithelium. These include transcytosis or productive infection of the epithelial cells (**Figure 1.5.C**), although productive infection of epithelial cells remains controversial

as the presence of CD4 on these cells is disputable (Dezzutti et al. 2001; Yeaman et al. 2004).

The presence of CCR5 receptors and transmembrane proteoglycans on genital tract epithelia is involved in HIV-1 uptake (Bobardt et al. 2007). Bound virus particles are sequestered into intracellular compartments where they can survive for a prolonged period before the polarized release into submucosal areas (Dezzutti et al. 2001; Wu et al. 2003). Thus, the transcytosis process allows free virion passage through the epithelium and consequent infection of the underlying immune cells. The exact contribution of transcytosis to HIV-1 transmission has not been quantified but it is expected to be rather infrequent.

1.5.2.3. Immune Cells Contribution to HIV-1 Transmission

The immune cell network at mucosal surfaces is complex, comprising a number of cell subsets. Focusing on the female reproductive tract, the top layer of the epithelium accommodates professional antigen presenting cells Langerhans cells (LC). Macrophages, subsets of dendritic cells (DC) and T-cell are also located in the submucosa. HIV-1 infection of LC and DC is inefficient, predominantly due to high expression of SAMHD-1 in these cells. However, both LC and DC could be transport vehicles for the virus to reach sites containing susceptible T-cells. Accordingly, to a common model, LC and DC capture HIV-1 at mucosal surfaces and rapidly migrate towards the proximal lymph nodes. While in the lymph node, the high concentration of T-cells allows for efficient transmission of the virus and its consequent systemic spread following active viral replication. Alternatively, LC/DC can transmit HIV-1

directly to the mucosal resident T-cells, which then become a virus factory and founder cells.

1.5.2.3.1. Langerhans Cells

Langerhans cells are professional antigen presenting cells situated at top layers of the mucosal and skin epithelia. Due to their location, Langerhans cells are believed to be the first targets for HIV-1 during sexual transmission (Zaitseva et al. 1997; Collins et al. 2000; Hu et al. 2000; Kawamura et al. 2003). Although LC play an imperative role in HIV-1 transmission, the detailed mechanisms involved in this process remains debatable.

As remarkably demonstrated by (Hladik et al. 2007), LC extend and retract their dendrites through the epithelial sheet to sample the environment. HIV-1 can be found attached to these protrusions via interactions with a C-type lectin, Langerin (Turville et al. 2002a). Despite a large amount of research, the fate of the virus from this point is still unknown. In one scenario, Langerin binding leads to endocytosis and subsequent degradation of HIV-1 in Birbeck granules (de Witte et al. 2007) (**Figure 1.6**). Although this setting would explain the low infection rates of LC, it does not clarify how these cells transfer the virus to T cells.

New data suggests that attachment to the major HIV-1 receptors (CD4 and CCR5) on LC accounts for virus uptake and transmission (Hladik et al. 2007; Kawamura et al. 2008) (**Figure 1.6**). Endocytosed virus remains infectious in intracellular compartments for several days until successfully passed onto T-cell (Hladik et al. 2007). In line with that statement, it takes about 4 days for subepithelial LC to reach susceptible T cells in the lymph nodes (Merad et al. 2002). In addition, LC challenged with HIV-1 efficiently transmits the virus even after that time. Studies with mucosal

models demonstrated that in the absence of LC/DC infection HIV-1 transmission to CD4+ T cells is inefficient (Pope et al. 1994). Also increased replication of HIV in T cells is observed if virus is delivered from LC or DC (Pope et al. 1994; Granelli-Piperno et al. 1998), highlighting an important role of LC and DC in HIV-1 sexual transmission.

It is curious that following HIV-1 binding to its entry receptors on LC, HIV-1 is internalized rather than causing productive infection. Only low levels of infection of LC have been demonstrated (Kawamura et al. 2003; de Witte et al. 2007; Ballweber et al. 2011) despite the presence of CCR5 and CD4 on these cells (Hladik et al. 2007).

Such a strict restriction of HIV-1 in immature LC may result from virus capture by Langerin, or possibly may be due to a replication block imposed by SAMHD-1. Nevertheless, some studies speculate that low ongoing productive infection of LC is sufficient for HIV-1 transmission, particularly by activated LC (Reece et al. 1998; de Jong et al. 2008). In agreement with this, maturation of LC by bacterial antigens down regulated langerin expression (de Jong et al. 2008; Ogawa et al. 2009) and increases HIV-1 replication in these cells (Hrecka et al. 2011), which could also promote virus passage to T cells (see Section 1.6).

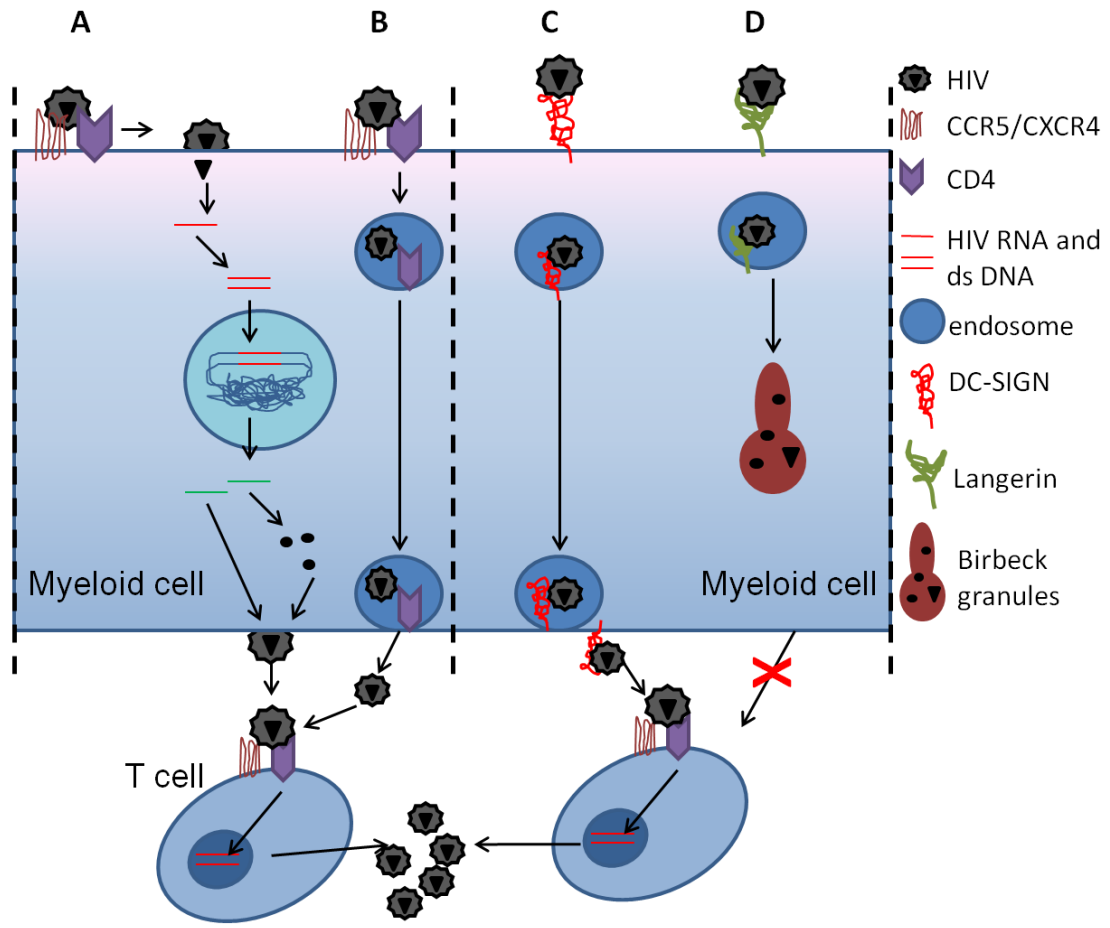

Figure 1.6. Possible Mechanisms of HIV-1 Transmission from DC and LC to T Cells. (A) Receptor mediated entry of HIV-1 settles productive infection and further transmission to t cells. **(B)** Eventually, binding of HIV-1 to its receptors results in its uptake into intracellular compartments and release of intact particle at the site of contact of infected cell with T cell. **(C)** HIV-1 virions readily attach to surface expressed DC-SIGN. DC-SIGN bound particles are being directly transmitted to T cells. **(D)** In contrast, Langerin attached HIV-1 is degraded in Birbeck granules, which prevents HIV transmission to T cells. HIV-1 efficiently replicates in susceptible T cells.

In summary, LC efficiently transmit HIV-1 to T-cells in the submucosa or after migration to lymph nodes (Shen et al. 2011). Tenofovir containing gel is a pre-exposure prophylaxis soon to be introduced to clinics with hope to prevent new HIV-1 transmission (Abdool Karim et al. 2010; Rohan et al. 2010; Johnson et al. 2012;

Cohen et al. 2013). Tenofovir is a reverse-transcription inhibitor already used in HIV-1 treatment that efficiently blocks virus multiplication in infected individuals.

However, if productive infection of LC is not required for successful transmission of HIV-1 from mucosa to lymph nodes, LC contribution to systemic spread of HIV-1 will not be blocked with tenofovir gel. Therefore, detailed understanding of the interactions between LC and HIV-1 are necessary for development of new preventative measures against HIV-1 acquisition that work against viral replication and spread.

1.5.2.3.2. Dendritic Cells

Other dendritic cell subsets reside in the lower layers of the epithelium and submucosa. Together with other immune cells such as macrophages, they provide a second line of defence against pathogens. In response to stimuli DCs migrate toward lymph nodes, providing an opportunity for HIV transmission. DCs express great quantities of a C-type lectin receptor (CLR) called DC-SIGN that functions as an HIV-1 attachment site. In contrast to langerin, DC-SIGN facilitates virus binding to CCR5 and its productive entry to DC (Lee et al. 2001), or direct transmission of the virus to CD4+ T cells (**Figure 1.6.**). Alternatively, DC-SIGN bound HIV-1 particles are internalized and degraded for antigen presentation (Moris et al. 2004; Moris et al. 2006). Surprisingly, however, a substantial fraction of internalized virions escape lysosomal degradation and exist in the endosomal compartments, from where they are transmitted to T-cells (**Figure 1.6.**) (Tacchetti et al. 1997; Turville et al. 2004). The escape mechanism and viral transmission to CD4+ T-cells was suggested to be DC-SIGN-dependent (Geijtenbeek et al. 2000; Kwon et al. 2002), although DC-SIGN-

dependent internalization could be dispensable (Arrighi et al. 2004a; Arrighi et al. 2004b; Granelli-Piperno et al. 2005).

1.5.2.3.3. Other Immune Cells

Apart from LC and DC, HIV-1 also targets T-cells and macrophages at submucosal surfaces. Infection of resident T-cells results in robust replication of the virus in these cells. However, mucosal cells get probably infected significantly later compared to DC, therefore underlining the role of DC subsets in the initial spread of HIV-1 (Shen et al. 2011).

In conclusion, LC and DC are among the very first cells facing HIV-1 during sexual transmission. They capture the virus and efficiently transfer HIV-1 to susceptible $CD4^+$ T-cells in submucosa or lymph nodes. APC-mediated activation of T-cells additionally supports virus replication in these cells. Moreover, co-infections with other STI significantly increase HIV-1 transmission from LC/DC to $CD4^+$ T cells kinetic.

1.5.3. HIV-1 Transmission in Men Having Sex with Men

While male to female risk of HIV-1 transmission is 8 in 10,000 exposures (the estimate may vary depending on viral load and the presence of co-infection), the risk of HIV-1 acquisition during receptive anal intercourse, with the same health conditions, rises by almost 18 fold (CDC.gov 2014). For years, this increased frequency of transmission was entitled to risky sexual behaviour from men having sex with men (MSM), including lack of condom use and multiple partners. Although these factors increase the chances for HIV-1 acquisition, biological differences between gut and vaginal mucosa remain an important issue. While the female reproductive tract is

protected from pathogens by the mucus, pH and thick layer of epithelial cells (see Section 1.5.2.), the gastrointestinal tract lacks these features.

Importantly, the Gastrointestinal tract harbours high proportions of immune cells, including activated CD4+ CCR5+/CXCR4+ T cells that are contained in T cell zone in Payer's patches. HIV-1 can gain access to these target cells via transcytosis across M cells, (cells that transport antigens from intestine lumen to CD4+ T cells), via interactions with intestinal DC or during breaching of epithelial layer (reviewed in (Brenchley et al. 2004). Infection with HIV-1 in gut mucosa leads to irreversible depletion of CD4+ T cells in this compartment and disturbance of the gut homeostasis. In consequence, HIV-1+ individuals develop diarrhoea and other multiple gut dysfunctions. Interestingly, irrespectively of the route of HIV-1 acquisition, the gastrointestinal tract seems to be a preferential target for the virus due to high number of activated target cells (Poles et al. 2001).

1.6. The Effect of Co-infections on HIV-1 Transmission

As discussed above, HIV-1 passage through mucosal membrane is rather ineffective. However, high viral inoculums, semen components and hormones can positively influence viral transmission (see Section 1.5). Nevertheless, the pre-existence of sexually transmitted disease (STD) at mucosal sites is the most commonly recognized trigger of increased HIV-1 transmission. Some of these mechanisms are summarized in **Table 1.2**. STDs can be of bacterial or viral origin. Chlamydia and Gonorrhoea are the most common bacterial infections of the lower genital tract, whereas Herpes Simplex Virus (HSV) and Human Papilloma Virus (HPV) account for the majority of viral infections.

Table 1.2. The impact of STD co-infection on HIV-1 transmission and systemic spread.

STD	Cell	Effect	Reference
Treponema pallidum (syphilis)	macrophages	Increased CCR5 expression	(Sellati et al. 2000)
	U937 human promonocytic cells	Increased HIV-1 transcription via NFκβ stimulation	(Theus et al. 1998)
H. Ducreyi (chancroid)	T cells	Cell activation	(Van Laer et al. 1995)
	DC	TNF-α release	(Banks et al. 2007)
Chlamydia Trachomatis	Epithelial cells Mononuclear cells	Increased CCR5 expression. Increased HIV-1 replication	(Schust et al. 2012)
Neisseris gonorrhoea	LC	Cell stimulation, TNF-α release	(de Jong et al. 2008)
Bacterial vaginosis	LC	Increased HIV replication	(Ogawa et al. 2009)
HSV-1/-2	Epidermis, T cell (indirectly)	Increased HIV-1 shedding and mucosal barrier permeability. Enriched target cell population.	(Schacker et al. 1998)

1.6.1. HIV-1 Mucosal Barrier Passage during STD Co-infection.

Sexually transmitted infections increase the possibility of both HIV-1 transmission and acquisition. Considering transmission, increased levels of virus particles in semen or vaginal fluids are observed during the acute phase of HIV-1 infection, and it correlates with elevated risk of transmitting the virus to a sexual partner. Keeping this in mind, any co-infections which enhance HIV-1 viral load in the patient's blood, such as malaria (Hoffman et al. 1999), HSV (Mole et al. 1997), and some STD (Galvin and Cohen 2004), significantly contribute to sexual transmission of HIV-1. The risk of horizontal transmission, from mother to child, is also higher when the mother is co-infected with human cytomegalovirus (CMV). However this effect, seemingly relies on an elevated proliferation and maturation of HIV-1 target CD4+ cord blood mononuclear cells, rather than increased virus replication per se (Johnson et al. 2014).

STI play an equally important role in boosting HIV-1 acquisition. First, STDs cause damage to genital mucosa and therefore increase permeability, which can favour virus entry. For instance, ulcers that arise during HSV-1 infection strongly disrupt mucosal uniformity and promote HIV-1 passage (**Figure 1.7**) (Schacker et al. 1998; Schacker et al. 2002). Mucosal epithelia cell death set off by *Haemophilu ducreyi* infection has the same effect (Banks et al. 2007). Interestingly, Patterson et al (1998) showed that STI can increase the expression of CCR5 in cervical epithelial cells and could therefore potentially promote HIV-1 binding and transcytosis (**Figure 1.7**).

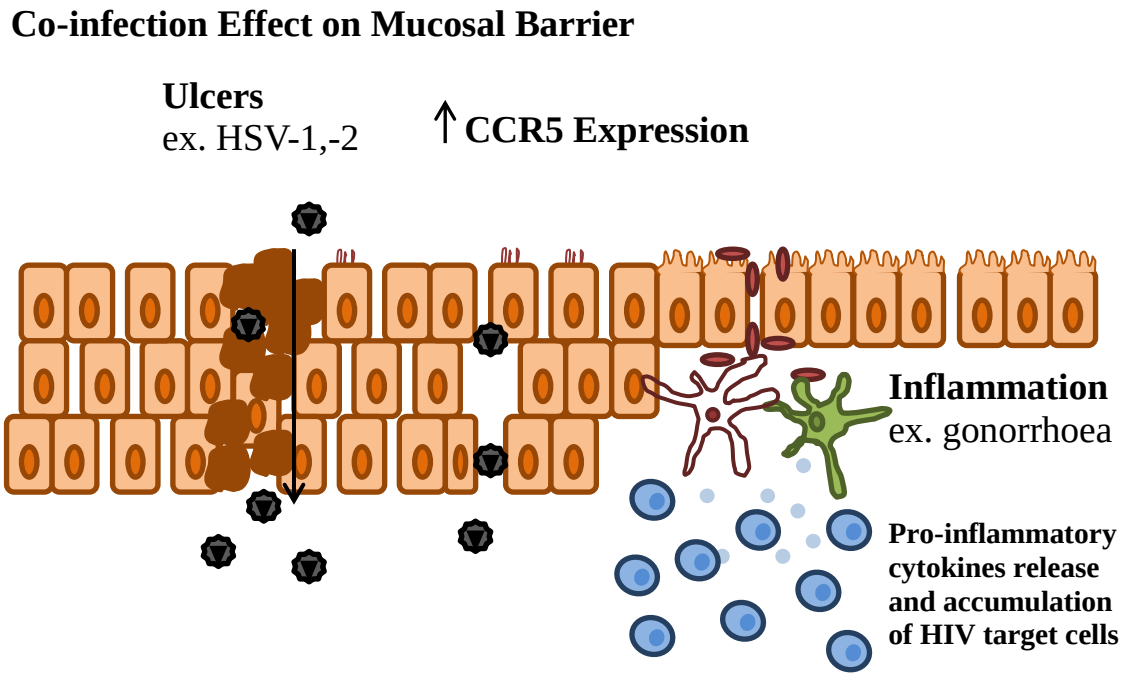

Figure 1.7. Mechanism Involved in Increased HIV-1 Acquisition during Co-infections with Other Pathogens. Ulcers formed during HSV-1, or HSV-2 infection of reproductive tract epithelia breech the mucosal barrier and allow HIV-1 passage to underlying target cells. Additionally, co-infections may increase the expression of CCR5 on the epithelial cells allowing virus transcytosis. Inflammation induced by bacterial infections weakens muscosal barrier and attracts HIV-1 target cells.

STD antigens are recognised by Toll-like receptors (TLR), which are particularly well expressed by epithelial cells and mucosal immune cells. Each of 9 TLR present in human immune cells recognise a specific pathogen associated molecular pattern (PAMP) such as bacterial cell wall components or viral nucleic acids (See Appendix 1). TLR are transmembrane proteins distributed accordingly to their ligand specificity. And so, TLR recognising intracellular pathogens (viruses and some bacteria) are localised to intracellular compartments such as endoplasmic reticulum. Examples of these TLRs include TLR3 (ligand: dsRNA), TLR7 (ssRNA), TLR8 (ssRNA) and TLR9 (unmethylated CpG oligodeoxynucleotide DNA present in some viruses and

bacteria). The remaining TLRs, TLR-1, -2, -4, -5 and -6 are distributed on the cell surface and engage specifically with bacterial signatures such as peptidoglycan of gram+ bacteria and lipopolysaccharide (LPS) of Gram negative bacteria. Apart from specific distribution of TLR to cell compartments, different immune cells show specific repertoire of these PRR. For instance, located at the mucosal surfaces, Langerhans cells lack TLR4 as these cells are constantly exposed to commensal bacteria (Flacher et al. 2006). In contrast, professional IFN producing plasmacytoid DC predominantly respond to TLR7 and TLR9 expressed in these cells (Hemmi et al. 2002).

Engagement of TLR with its specific ligand leads to induction of NFκβ signalling cascade through intracellular domain of TLR receptor and MyD88 adaptor protein. Stimulation of TLRs results in release of pro-inflammatory cytokines and chemokines, such as TNFα, IL6, IL-12 and type I IFN in case of TLR 3, 7, 8 and 9 activation. In respect to HIV-1 infection, chemokines can recruit HIV-1 target cells to the point of infection and enhance the risk of HIV-1 transmission (Zhu et al. 2009; Lavelle et al. 2010). Cytokines, on the other hand, directly stimulate HIV-1 replication in infected cells as described subsequently. Although, inflammation is a protective response to pathogens, in case of HIV-1 infection this has a dramatic outcome (see Sections 1.6.2).

1.6.2. Inflammation and HIV-1 Transmission

Persistent inflammation likely plays an imperative role in HIV-1 transmission facilitated by STI. One of the pro-inflammatory cytokines released by DC in response to H. ducreyi infection is TNF-α (Banks et al. 2007). TNF-α is produced by a number

of cells including LC, T-cells, epithelial cells and others, in response to bacterial lipopolysaccharides. Although this cytokine provides cell-mediated immunity against bacteria pathogens, in terms of HIV-1 infection it has a deleterious effect. TNF-α activates NF-κβ signalling in macrophages and this subsequently promotes HIV-1 transcription from the LTR (Mellors et al. 1991; Chang et al. 1994; Herbein et al. 2008). The same mechanism enhances HIV-1 replication in LC and its further transmission to T-cells (de Jong et al. 2008). The ability to process antigens in mature LC exposed to TNF-α is impaired, which could be another mechanism involved in increased viral transfer from LC to T-cells. However, other reports suggest that TNF-α suppresses HIV-1 replication in macrophages via stimulation of RANTES production and a decrease in CCR5 expression on these cells (Lane et al. 1999). Other inflammatory cytokines differently affect HIV-1 propagation (see Chapter 4). In Chapter 4, the role of cytokines, particularly Transforming Growth Factor Beta (TGF-β), on HIV-1 susceptibility of immune cells will be discussed further.

1.7. Summary

HIV-1 has effectively propagated in the human population for over 90 years. It has successfully spread around the globe and collectively infected more than 60 million individuals. HIV-1 treatments that are available today, robustly silence virus replication and prolong the life of patients. However, treatment itself has significant side effects, which themselves can decrease the quality of life.

The human body has a few barriers against HIV-1 acquisition. These include physical blocks, such as the thick vaginal epidermal layer with mucus; the microenvironment of the reproductive tract that negatively affects virus infection capability, and also

directly modulates local cell biology functions. On a molecular level, cells are equipped with restriction factors, which counteract HIV-1 replication processes. Out of a few such identified restrictions, SAMHD-1 potently blocks HIV-1 at an early stage of cellular infection. This restriction factor is present in various immune cells. SAMHD-1 is constitutively expressed by dendritic cell subsets and macrophages, and in resting T-cells. SAMHD-1 mediated depletion of cytoplasmic nucleosides blocks HIV-1 reverse transcription. Additionally, SAMHD-1 directly interacts with HIV-1 genomic ssRNA, leading to its cleavage and degradation.

The sexual route of transmission is the predominant way of HIV-1 acquisition. Risk of HIV-1 transmission is particularly high in men having sex with men due to high permeability of gut mucosa to the virus. Male to female transmission is more challenging for the virus, as it has to overcome vaginal mucus, physical barriers such as thick cell layers, and an unfavourable environment. Yet, the number of female carriers increases every year. Langerhans cells are likely the very first immune cells, which encounter HIV-1 during sexual transmission. Previously these cells were described as not permissive to HIV-1 infection. This function was assigned to Langerin, a lectin that captures virus particles and leads to their degradation. However, the exact role of SAMHD1 and other restriction factors in HIV-1 resistance is poorly investigated in Langerhans cells. As Langerhans cells are the very first cells encountering HIV-1 during sexual transmission, it is important to understand the events accompanying this process. Mechanisms behind HIV-1 restrictions in Langerhans cells, if recognized, may possibly offer the opportunity for development of preventative measures against the sexual transmission of HIV-1.

1.8. Thesis Aims

The main aim of this work is to investigate the interaction of HIV-1 with Langerhans cells. Tissue resident LC are the first immune cells that are likely to meet HIV-1 in the mucosal surfaces as they carry the HIV receptor/co-receptors CD4/CCR5. Some studies show these cells can become productively infected with HIV-1 especially in the context of co-infections. However, LC are difficult to infect by HIV-1 due to the presence of Langerin (de Witte et al. 2007). Langerin binds viral particles and mediates their degradation in lysosomal compartments called Birbeck granules (de Witte et al. 2007). However, saturation of Langerin achieved by high viral doses allows viral infection of these cells. The current available literature does not consider post entry restrictions operating in LC despite recognized role of these cells in initial viral dissemination during sexual acquisition.

The principle objectives for this study comprised:

1. Development of Langerhans cells model systems including skin isolated epidermal LC and monocyte derived Langerhans cells (MDLC).
2. Characterization of the post-entry HIV-1 restriction mechanism operating in Langerhans Cells.
3. Understanding the role of Transforming Growth Factor β (TGF-β) in modulation of Langerhans cells susceptibility to HIV-1 infection.

Accordingly, Chapter 3 of this thesis concentrates on the biology and properties of the Langerhans cells systems, describes the methodology and limitations to each LC model system in particular in the context of HIV-1 infection. The other two research questions are addressed in Chapter 4, which explores the HIV-1 infection pattern of

Langerhans cells and describes the novel mechanism of virus restriction operating in these cells.

2. Materials and Methods

2.1. Equipment

Centrifuge 5415R (Eppendorf)

Heraeus Megafuge 40R (Thermo Fisher, Scientific)

Beckman Coulter Optima™ L-100XP Ultracentrifuge

15 ml tubes (Corning Centristar™)

50 ml tubes (Corning Centristar™)

Tissue flasks (Nunc™)

Water Bath (Grant)

Incubator (Nuaire, Triple Red, Lab Technology)

pH meter Jenway model 3540, Scientific Laboratories supplies

Nikon TMS-F Microscope

2.2. Cell Isolation

2.2.1. Buffers

MACS buffer – 2.5g Bovine Serum Albumin (BSA) (Sigma-Aldrich), 0.5M EDTA (Gibco) in 500 ml Phosphate buffered saline (PBS) (Sigma-Aldrich).

2.2.2. Isolation of Peripheral Blood Mononuclear Cells (PBMC)

Buffy coats from anonymous, healthy donors were obtained from the Welsh Blood Service. Blood was diluted with sterile PBS (Sigma-Aldrich) to a total volume of 180 ml and gently pipetted onto a Ficoll (Ficoll-Paque Plus; Fisher (GE)) layer. The gradient was centrifuged for 30 minutes at 2000rpm (800xg) without the break at 4°C. The Peripheral blood mononuclear cells (PBMC) fraction was carefully collected and washed 3 times with sterile PBS. Healthy PBMC were counted using a Bright-Line

hemacytometer (Hasser Scientific, Harsham) in the presence of Trypan Blue Stain 0.4% (Gibco Life Technologies), and then subjected to MACS isolation of CD14+ cells (see Section 2.2.3).

2.2.3. Isolation of Peripheral Blood Monocytes (CD14+)

CD14+ monocytes were isolated from blood derived PBMC using CD14 MicroBeads (Miltenyi Biotec) according to manufacturer protocol. PBS washed PBMC were counted and resuspended in 80µl of MACS buffer per 10^7 total cells. 20µl of CD14 MicroBeads per 10^7 total cells was added and cells were incubated at 4°C for 15 minutes. Cells were then washed with cold MACS buffer and spun at 1800rpm for 10 minutes. After re-suspension in 500µl of MACS buffer per 10^8 total cells the cells were applied onto the isolation column and left to flow through with gravity. Column was washed 3 times with 3ml of MACS buffer. Column was removed from separator and magnetically labelled CD14+ cells were retrieved from the column by flushing the column with 5ml of MACS buffer. CD14+ cells were counted and used for generation of MDDC and MDLC (see Section 2.3.1 and 2.3.2).

2.2.4. Isolation of CD1a+ MUTZ-3 Derived Langerhans Cells (MuLC)

CD1a+ MuLC were purified from the MUTZ-3 derived culture (see Section 2.3.3. and 2.4.1.) using CD1a MicroBeads (Miltenyi Biotec) according to manufacturer protocol. Immature MuLC were counted and resuspended in 80µl of MACS buffer per 10^7 total cells. 20µl of CD1a MicroBeads per 10^7 total cells was added and cells were incubated at 4°C for 15 minutes. Cells were then washed with cold MACS buffer and spun at 1800rpm for 10 minutes. After re-suspension in 500µl of MACS buffer (per 10^7 total cells) cells were applied onto the isolation column and left to flow through

with gravity. Column was washed 3 times with 3ml of MACS buffer. Column was removed from separator and magnetically labelled CD1a+ cells were retrieved from the column by flushing the column with MACS buffer. Enriched CD1a+ Langerin+ cells population was used for the experiments.

2.3. Cell Differentiation

2.3.1. Generation of Monocyte Derived Dendritic Cells (MDDC)

Blood isolated CD14+ cells were cultured for 6 days in 6 well plates at a density of 2 x 10^6 cells per well at 37°C as described before (Blanchet et al. 2013). Iscove's Modified Dulbecco's Medium (IMDM; Life Technology Ltd., Paisley, United Kingdom) containing 10% Fetal Bovine Serum (FBS; SIGMA), 100U/ml penicillin and 100µg/ml streptomycin (Gibco, Life Technologies), 2mM L-glutamine (Gibco, Life Technologies), 10mM HEPES Buffer (Sigma-Aldrich), 1% minimum essential medium non-essential amino acids (Gibco, Life Technologies), and 1mM sodium pyruvate (Gibco, Life Technologies) was supplemented with 50µM β-2-Mercaptoethanol (β-2M) (Sigma, Life Science) (added only on day 0), 500U/ml GM-CSF (MACS, Miltenyi Biotec) and 500U/ml IL4 (MACS, Miltenyi Biotec). At days 2, 4 and 6 of culture a third of the culture medium was replaced by fresh medium containing GM-CSF and IL4. Differentiated MDDCs were analyzed by flow cytometry (see Section 2.10) for expression of cell specific markers at day 7.

2.3.2. Generation of Monocyte Derived Langerhans Cells (MDLC)

Blood isolated CD14+ monocytes were seeded at 1x10^6 cells/ml in RPMI1640 medium (Life Technologies) supplemented with 10% FBS, 100U/ml penicillin, 100µg/ml streptomycin, 2mM L-glutamine in tissue culture flask. MDLC were

generated over 6 days with GM-CSF (500IU/ml), IL4 (500IU/ml) and TGF-β (10ng/ml) (Peprotech).Complete medium with cytokines were refreshed at day 3. Differentiated MDLC were phenotyped and used at day 6 or 7.

2.3.3. Induction of Immature Langerhans-like Cells from MUTZ-3 Cell Line (MuLC)

MUTZ-3 cell line (see Section 2.4.1) was used to generate Langerhans cells (MuLC) as described previously (Masterson et al. 2002). MUTZ-3 cells were harvested and seeded in 12 well tissue culture plates at a concentration of $1x10^5$ cells/ml. For induction of Langerhans cells phenotype MUTZ-3 culture medium MEM-α (see Section 2.4.1) was additionally supplemented with GM-CSF (100ng/ml), TGF-β (10ng/ml) and TNF-α (2.5ng/ml) (Peprotech). Cytokines were refreshed at day 4 and 8. Cells were collected and subjected to phenotyping at day 10.

2.3.4. Generation of $CD141^+$ DC Cells

Blood isolated $CD14^+$ cells were cultured for 6 days in 6 well plates at density of 2 x 10^6 cells per well at 37°C as described before (Blanchet et al. 2013). Fully supplemented IMDM medium (Life Technology Ltd., Paisley, United Kingdom) (see section 2.3.1) was supplemented with 50μM β-2-Mercaptoethanol (β-2M) (Sigma, Life Science) (added only on day 0), 500U/ml GM-CSF (MACS, Miltenyi Biotec) and 500U/ml IL4 (MACS, Miltenyi Biotec). At days 2, 4 and 6 of culture a third of the culture medium was replaced by fresh medium containing GM-CSF and IL4. 100nM of active Vitamin D3 ($1,25(OH)_2D3$) (Sigma) was additionally supplemented to cells at day 4 to induce $CD141^+$ DC phenotype. Differentiated cells were analyzed by flow cytometry (see Section 2.10) for expression of cell specific markers at day 7.

2.4. Cell Lines Culture and Maintenance

2.4.1. MUTZ-3 Cell Line Culture

An immortalized human acute myeloid leukaemia-derived cell line (MUTZ-3) was a kind gift from Dr Tania de Gruijl (Dept Medical Oncology, VU University Medical Centre, Amsterdam, Holland). For routine culture, MUTZ-3 cells were maintained at a concentration of $2x10^5$ cells/ml to $1x10^6$ cells/ml in 12 well tissue culture plates (Sigma-Aldrich, Corning Costar). Every 2-3 days cells were collected, spun at 1500rpm for 5 minutes and reseeded at a density of $2x10^5$ cells/ml in Minimum Essential Media alpha with ribonucleosides and deoxyribonucleosides (MEM-α GlutaMAX™ nucleosides; Life Technologies) supplemented with 20% heat inactivated FBS, 100U/ml penicillin, 100µg/ml streptomycin, 2mM L-glutamine, 50µM 2-mercaptoethanol and 10% conditioned medium from renal carcinoma cell line 5637 (see Section 2.4.3).

2.4.2. 5637 Cell Line Culture

Renal carcinoma cell line 5637 was a kind gift from Dr Tanja de Gruijl (Dept Medical Oncology, VU University Medical Centre, Amsterdam, Holland). Cells were maintained in RPMI 1640 medium supplemented with 10% heat inactivated FBS, 100 U/ml penicillin, 100µg/ml streptomycin, and 50µM 2-mercaptoethanol. At 70-80% confluency, cells were removed from tissue flask with 0.05% Trypsin-EDTA (Gibco, Life Technologies) treatment and spun at 1500rpm for 5 minutes. Cells were seeded at $5x10^5$ cells/ml in tissue culture flasks and incubated at 37°C, 5% CO_2.

2.4.3. Generation of 5637 Conditioned Medium

5637 conditioned medium for MUTZ-3 culture was generated from 5637 culture medium. Briefly, 5637 cells were seeded in 180cm^2 tissue culture flask at 5x10^5 cells/ml in 30ml of fully supplemented RPMI1640 medium. After overnight culture, medium was replaced with 30ml of fresh RPMI1640 medium. Cultured medium was collected after 40 hours and filtered with sterile 0.2µm filter. Aliquots were stored at -30°C and used within 14 days.

2.4.4. HEK293 Cell Line Culture

HEK293 cells were maintained at 40-80% confluency in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies) supplemented with 10% heat inactivated FBS, 100 U/ml penicillin, 100µg/ml streptomycin and 2mM L-glutamine. Cells were harvested from tissue culture flask with short trypsin treatment at 37°C. After spinning at 1500rpm for 5 minutes, cells were counted and seeded at 3x10^5 cells/ml.

2.5. Epidermal Langerhans Cells and Dermal Dendritic Cells Isolation from Skin Explants

Human skin samples were obtained from female patients undergoing mastectomy or breast reduction surgery with informed written patient consent and local ethical committee approval (South East Wales Research Ethics Committees Panel C, Reference: 08/WSE03/55). Skin was transported following surgery as previously described (Pearton et al. 2010). Subcutaneous fat and excess lower dermis were removed by blunt dissection. The upper layers of the skin were subsequently removed using a dermatome set to a depth of 300µm to collect the epidermis and upper papillary dermis. Skin sheets were cut into 1cm² pieces and incubated with agitation in

a shaking water bath (at 175 strokes/minute) in RPMI containing collagenase A (10 mg/ml), DNase I (20 U/ml) and Dispase II (10 mg/ml) for 30 minutes at 37°C, after which the epidermis was mechanically separated from the dermis using forceps. Epidermal and dermal sheets were cultured separately in RPMI with 10% human AB serum (Invitrogen, USA) and 1% Penicillin/Streptomycin/Fungizone (P/S/F) solution (DC-RPMI) for 48 hours, after which migratory cells were collected from the media.

2.6. Bacteria Protocols

2.6.1. Bacteria Culture Media and Reagents

Luria-Bertani (LB) broth consisted of 10g/l tryptone (Fisher Scientific), 5g/L yeast extract (Fisher Scientific), 5g/L NaCl (Fisher Scientific) resuspended in H_2O and autoclaved before use. LB-broth was supplemented with 100µg/ml ampicillin.

2.6.2. Bacteria Transformation

Escherichia coli DH5α chemocompetent cells (Promega) were defrosted and heat shock transformed in a water bath (at 42°C for 45 seconds followed by 15 seconds on ice) with 1ng of relevant plasmid (**Table 2.1**). Transformed bacteria were left to rest at room temperature for 2 minutes. After addition of 130µl of S.O.C medium (Invitrogen), bacteria were incubated for 1 hour at 37°C. Bacteria suspension was then transferred to 2mls of LB-broth (see Section 2.6.1) supplemented with selective antibiotic and shaken overnight. The following day, bacteria were transferred to 400mls of LB-broth with selective antibiotic and shaken (Orbital Incubator, Gallenkamp) at 37°C overnight. After incubation it was spun at 4000xg for 30 minutes and the resulting bacteria pellet was used for purification of plasmid using Maxipreps (see Section 2.7).

2.6.3. Plasmids Table

Table 2.1. List of Plasmids

Plasmid name	Product	Antibiotic resistance	Reference/obtained from
pMD.G	VSV-G envelope	ampicillin	(Naldini et al. 1996)
pR8.91	gag-pol	ampicillin	(Naldini et al. 1996)
plox.EW.delta.Sal GFP	GFP	ampicillin	(Salmon et al. 2000)
SIV3-Vpx	Vpx, Vpr	ampicillin	(Nègre et al. 2000)
pR8Bal	R5/X4 dual tropic HIV-1	ampicillin	

2.6.4. Plasmids Maps

pMD.G plasmid encodes VSV-G envelop protein downstream of Cytomegaloma Virus (CMV) promoter (Naldini et al. 1996). Plasmid is used in delivery of lentivectors and viral like particles transduced with VSV-G envelop.

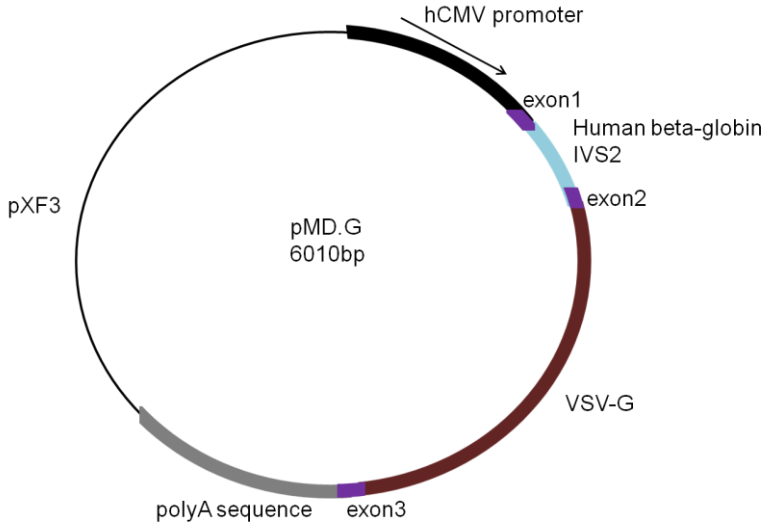

Figure 2.1. Schematic Representation of pMD.G Construct. pXF3 (poison sequence minus pBR322, low copy plasmid); hCMV human Cytomegaloma Virus (CMV) promoter; human beta globin sequence, VSV-G envelop, poly A sequence.

pR8.91 is a packaging construct used for production of lentivirus, such as VSV-G HIV-GFP used in this study. Plasmid encodes Gag-Pol proteins under control of CMV promoter.

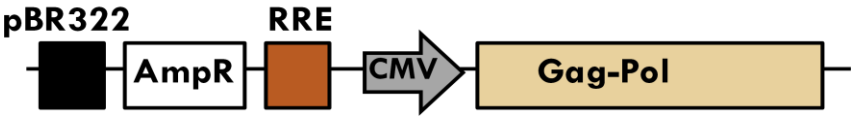

Figure 2.2. Schematic Representation of pR8.91 Construct. AmpR Ampicilin resistance gene; RRE Rev-responsive element; CMV promoter; Gag-Pol genes of HIV. (Adapted from http://plasmid.med.harvard.edu/PlasmidRepository/file/map/dR8.91.pdf)

plox.EW.delta.Sal.GFP encodes GFP protein downstream of EF-1α promoter. EF-1α promoter ensures robust, constitutive and long-term expression of downstream genes (Kim et al. 1990; Kim et al. 2007). EF-1α is often used where CMV promoter is silenced. pLox.EW.delta.Sal.GFP plasmid is co-transfected with pMD.G and pR8.91 when producing VSV-G HIV-GFP lentivirus (see Section 2.8.1).

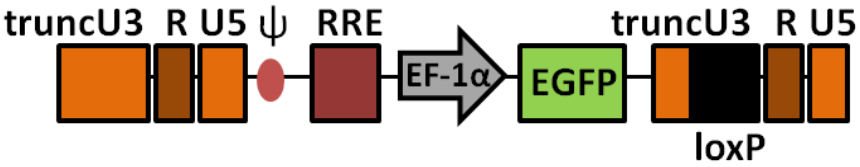

Figure 2.3. Schematic Diagram of pLox.EW.deltaSal.GFP. ψ packaging signal; RRE Rev-responsive element; CMV promoter, GFP green fluorescent protein; Adapted from (Salmon et al. 2000).

SIV3-Vpx – SIV3-Vpx construct (**Figure XX**) originate from SIV (SIVmac251) and was derived through elimination of env and 3'LTR, and replacement of 5'LTR promoter with more potent CMV early promoter/enhancer sequence (Nègre et al. 2000). Therefore, SIV3-Vpx construct encoded *gag*, *pol*, *vif*, *vpr*, *rev* and *tat* genes, of which *vpr* and *vpx* gene products are packed into viral like particles. Vpx protein delivered in SIV3-Vpx particles acts on SAMHD-1 leading to its ubiquitinin-mediated degradation (Hrecka et al. 2011). Vpr has not known effect on SAMHD-1, however it suppresses innate immune in macrophages and DC (Mashiba et al. 2014; Harman et al. 2015) (see Introduction, Section 1.4.3). Due to these properties of Vpr, SIV3-Vpx should perhaps be considered to be used in parallel with SAMHD-1 siRNA/shRNA. The consequences of the presence of Vpr protein on the experiments performed in this study will be discussed in the following Chapters.

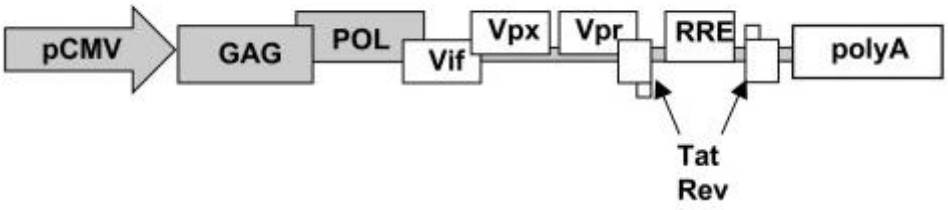

Figure 2.4. Schematic Diagram of SIV3-Vpx Construct. pCMV Cytomegaloma Virus promoter; Gag – Tat – SIV structural and accessory proteins; RRE Rev-responsive element; poly A sequence. Adapted from (Nègre et al. 2000).

pR8-Bal is a culture adapted construct derived from pR8 plasmid by inserting Bal envelope sequence (C.Aiken, Vanderbilt University, Nashville, Tenn). Construct encodes full-length HIV-1 virus.

2.7. Plasmid DNA Isolation

2.7.1. Maxiprep Procedure

QIAGEN® Plasmid Purification Kit (QIAGEN) was used to perform Maxipreps. The procedure was done according to the manufacturer instructions. Briefly, transformed, centrifuged bacteria were resuspended and lysed in 10ml Buffer P1. 10ml of buffer P2 was added to lysates and samples were mixed vigorously by inverting tubes 4-6 times. Bacteria lysates were incubated for 5 minutes at room temperature. After incubation, 10ml of chilled Buffer P3 was added to lysates and mixed. The mixture was incubated for 20 minutes on ice and then centrifuged at 4540xg at 4°C for 30 min. Precipitated material was separated from the supernatant using a QIAfilter filter. A QIAGEN-tip 500 was equilibrated with 10ml Buffer QBT and filtered lysates were added onto the resin and left to pass through by gravity. The QIAGEN-tip was washed twice with 2 x 30ml of Buffer QC in order to remove the contaminants. Elution of resin bound DNA was achieved using 15ml of Buffer QF. 12ml of room temperature isopropanol was added to DNA-buffer QF mix and centrifuged for 30min at 4540xg at 4°C. Formed pellet was washed with 5ml of room temperature 70% ethanol and centrifuged at 4540xg for 10min at 4°C. After spin, the pellet was air dried for 10-20 minutes and re-dissolved in TE buffer (Invitrogen, Life Technologies). Plasmids were stored at -30°C.

2.7.2. Quantification of Plasmid Preparations

Plasmid quantification was performed using Nano-drop spectrophotometer (ND-1000, Labtech International) blanked with TE buffer. Three readings were taken for each Maxiprep samples and the mean value was noted for future use.

2.8. Virus Production

2.8.1. Viral Strains

All viruses were produced by calcium phosphate transfection of HEK293T cells with corresponding plasmids as described previously (Blanchet et al. 2013). VSV-G HIV-GFP was obtained by co-transfection of 30µg pMD.G (vesicular stomatitis virus envelope protein, VSV-G) expression vector (Naldini et al. 1996), 32µg pR8.91 (gag-pol expression vector; (Naldini et al. 1996) and 45µg plox.EW.delta.Sal GFP (a retroviral expression vector encoding green fluorescent protein) per 1 flask of HEK293T cells. VSV-G-pseudotyped SIV3 lentivector encoding the Vpx gene (Nègre et al. 2000; Goujon et al. 2003; Goujon et al. 2007) was produced by co-transfection of 20µg pMD.G and 40µg $SIV3^{+}$ packaging construct. Proviral plasmid pR8BaL, encoding HIV-1 R5 strain provirus was used for wild type HIV-1 virus production at 90µg per HEK293T flask.

2.8.2. Buffers

0.5M $CaCl_2$ was prepared by dissolving 36.75g of $CaCl_2$ (SigmaUltra C5080) in 500ml of distilled H_2O and it was stored at -70°C until use; **2x HeBS** was prepared by mixing 16.36g NaCl (SigmaUltra S7653) (final 0.28M), 11.9g HEPES (SigmaUltra H7523) (final 0.05M), and 0.213g anhydrous Na_2HPO_4 (SigmaUltra S7907) (1.5mM final) in 1000ml distilled H_2O. pH was adjusted to 7.00 with NaOH solution. Solution was stored at -70°C until use; **HEPES H_2O** was prepared by adding 125µl of 1M HEPES (Gibco-BRL. Ref 15630-056) (final 2.5mM) to 50 ml of distilled H_2O. HEPES H_2O was stored at 4°C until use.

2.8.3. HEK293T Cells Transfection: Virus Production Protocol

HEK293T cells were seeded in 180cm^2 tissue flask at 8x10^5 cells/ml in 15 ml of fully supplemented DMEM medium (see Section 2.4.4) and left overnight to reach 70-80% confluency. Transfection mix was prepared by mixing required amount of plasmids DNA (see Section 2.8.1) with HEPES buffered dH_2O (2.5mM) to a final volume of 750µl per tissue culture flask. After 5 minutes incubation at room temperature, DNA-HEPES mix was added to 750µl $CaCl_2$ per flask, and the resulting mix was added drop-by-drop into a tube containing 1.5ml of 2xHBS per flask while continuously vortexing at a low speed. Following 30 minutes incubation at room temperature, 3ml of transfection mix was distributed equally on growing HEK293 cells in a tissue culture flask using disposable transfer pipettes (VWR International). Cells were then incubated at 37°C for 6 hours. Afterwards, all medium from flask was carefully removed without disturbing HEK293 layer, and cells were washed with sterile PBS. Fresh 15ml of DMEM medium was added to each flask of HEK293 and cells were left for 48 hours at 37°C.

Cell supernatant was collected, filtered using 0.45µm sterile millex®GP filter (Millipore Ireland Ltd.) and overlaid on top of 20% sucrose (Sigma) gradient in Beckman ultracentrifuge conical tubes (Beckman Coulter). The gradient was centrifuged in ultracentrifuge at 26000rpm, 4°C for 90 minutes. The supernatant and sucrose were then aspirated using VACUSAFE™ Vacuum aspiration system (INTEGRA Biosciences), avoiding the viral pellet. Tubes were inverted for 10 minutes and viral particles were resuspended in 300µl DMEM per tube. The pellet in medium was left at room temperature for 20 minutes and resuspended viral particles were aliquoted into sterile o-ringed screw tubes (Fisher Scientific). Viral preparations were stored until use at -80°C.

2.8.4. Virus Quantification by p24 ELISA

A Lenti-X p24 rapid titre ELISA kit (Clontech Laboratories, Inc) was used to quantify HIV-1 Gag p24 according to the manufacturer's instructions. Briefly, virus preparations were lysed with Triton x10 (BDH Limited) at 1:10 ratio. A serial dilution of the sample was prepared using DMEM culture medium ranging from 1:1000 – 1:100000 depending on the virus production. Dilutions of 0 – 200pg/ml for the p24 standard curve were prepared by diluting the p24 control (provided with the kit) in complete DMEM culture medium.

For the p24 ELISA assay 20µl of lysis buffer was aliquoted into each well designated for the samples. 200µl of p24 standard curve dilutions and virus production samples were aliquoted into appropriately labelled duplicate wells and incubated at 37 (±1)°C for 60 (±5) minutes. The content of the wells was aspirated and the plate was washed with 1x wash buffer. 100µl of Anti-p24 (Biotin conjugate) detector antibody was added into each well and plate was incubated at 37 (±1)°C for 60 (±5) minutes. Plate was washed again, as described above. 100µl of Streptavidin-HRP conjugate was dispensed into each well and the plate was incubated at room temperature for 30 (±5) minutes. Plate was washed once again and 100µl of Substrate Solution was added into each well. After incubation at room temperature for 20 (±2) minutes, the reaction was stopped by addition of 100µl of Stop Solution to each well. The absorbance values at 450nm of each well were measured using a microtitre plate reader (Fluostar Optima) blanked on the negative control well. The standard curve was constructed based on the values acquired for a p24 control dilution, which allowed the calculation of p24 content in virus samples.

2.8.5. Vpx-expressing SIV-derived Lentivectors Efficiency Assessment

MDLC and MDDC were used to investigate the efficiency of new Vpx-expressing SIV-derived lentivectors (SIV3-Vpx) production to degrade SAMHD-1. Accordingly, cells were seeded in 96 well plates (100,000 cells/well) in RPMI or IMDM for MDLC and MDDC, respectively. New SIV3-Vpx stock was added to cells at different doses raging from 3-20µl. After 4 hours, cells were lysed (see Sections 2.11.3) and SAMHD-1 levels were investigated by western blotting (see Section 2.11.4). The lowest amount of Vpx-expressing SIV-derived lentivectors resulting in complete degradation of SAMHD-1 in both cells was used for further experiments. SIV3-Vpx stock efficiency was further controlled every 3-4 weeks.

2.9. Cell Infections and Assays

2.9.1. HIV-1 Infection and VSV-G HIV-GFP Transfection of Cells

Cells were seeded in U-bottom 96-well plates (Thermo Scientific) at $1x10^5$cells/well in 100µl of differentiating medium. Where indicated, AZT (1µM) and Vpx-expressing SIV-derived lentivectors were added at least 4 hours prior to infection. Cells were infected with 20-40ng p24gag of R5 HIV-1 (R8Bal) or 17-63ng p24 of VSV-G HIV-GFP. If indicated, cells were pre-treated with compounds before infections.

The following reagent was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: Zidovudine (AZT).

2.9.2. Cell Stimulation with Interferon

For IFN treatment, cells were incubated with 1000U/ml of one of IFN-α2a (Sigma-Aldrich), human IFN-α2a, human IFN-β1a, human IFN-β1b, or human IFN-γ1b (all

MACS, Miltenyi Biotec) for 24 hours or 48 hours in 96 well plate. Consequent SIV3-Vpx treatment and infections were carried out when required.

2.9.3. Cell Stimulation with Toll-like Receptors (TLR) Agonists and TNF-α

For TLR stimulation experiments, $1x10^5$cells/well in 100µl of medium were treated in 96-well plates for indicated times with the following agonists: **TLR1** – Synthetic triacylated lipoprotein Pam3CSK4 (1µg/ml) (InvivoGen); **TLR2** – Peptidoglycan from *Bacillus subtilis* (10µg/ml) (SIGMA-Aldrich); **TLR3** – Poly I:C (HMW) (2.5 µg/ml) (Invivo Gen); **TLR4** – Lipopolysaccharide (LPS, 1µg/ml) (InvivoGen); **TLR5** –Flagelling (1µg/ml) (InvivoGen); **TLR6** – Synthetic diacylated lipoprotein FSL1 (1µg/ml) (InvivoGen); **TLR7** – ssRNA40 (1µg/ml) (Ivivo Gen); **TLR 8** – R848 (1µg/ml) (Ivivo Gen); **TLR9** – *E.coil* ssDNA (5µg/ml) (InvivoGen). For maturation assays, $1x10^5$cells/well in 100µl of medium were treated in 96-well plates for indicated times with human recombinant human TNF-α (Peprotech).

2.9.4. MDLC Delivery in the Presence of TGF-β Signalling Inhibitor LY2109761

Delivery of MDLC in the presence of LY2109761, TGF-β signalling inhibitor, was performed in 12 well plates (1mln cells/well in 1 ml of RPMI1640) for 7 days in the presence of TGF-β, GM-CSF and IL-4 (see Section 2.3.2). Prior to addition of cytokines, monocytes were pre-treated with 5 µM or 10µM of LY2109761 for 5-10 minutes. Medium was replaced every second day in addition to LY2109761 and cytokines. Experiments were performed at day 7.

2.10. Flow Cytometry

2.10.1. Buffers

FACS buffer used for cell surface staining and sample storage consists of 1% bovine serum albumin BSA (Sigma-Aldrich) and 0.025% sodium azide (Sigma) resuspended in sterile PBS.

Phosflow Perm/wash buffer (BD biosciences) was prepared according to manufacturer indications. 10x Phosflow Perm/wash buffer stock was diluted 1:10 in sterile H_2O and stored at 4°C until use.

Paraformaldehyde (PFA) (Fisher Scientific) was prepared by diluting PFA stock in H_2O to reach desirable final concentration. 1% solution was used for fixing experiments performed in category 2 laboratory, whereas 2% solution was used for experiments involving HIV-1 infections and treatment.

2.10.2. Flow Cytometry Antibodies Table

Table 2.2. List of Flow Cytometry Antibodies

Marker	Fluorochrome	Company	clone	dilution
Isotypes				
mIgG (isotype)	FITC	BD Pharmingen	X40	1/200
mIgG (isotype)	PE	BD Pharmingen	X40	1/200
mIgG (isotype)	APC	BD Pharmingen	X40	1/200
Cell phenotype				
CD1a	FITC	BD Pharmingen	HI149	1/50
DC-SIGN	PE	eBiosciences	eB-h209	1/50
CD14	FITC	ANCELL	UCHM1	1/50
CD83	PE	Immunotech	HB15a	1/50
HLA-DR	APC	BD Bioscience	G46-6	1/50
CD19	FITC	DAKO	HD37	1/50
CD16	PE	BD Pharmingen	3G8	1/50
CD1a	APC	BD Pharmingen	H1149	1/50
CD3	FITC	DAKO	UCHM1	1/50
HLA-ABC	PE	Pharmingen	G46-2.7	1/50
CD4	APC	Immunotech	13B8.2	1/50
Langerin	PE	Immunotech	DCGM4	1/50
CCR5	FITC	Pharmingen	2D7/CCR5	1/50
CD141	PE	MACS	AD5-14H12	1/50
CD45	APC	eBioscience	2D1	1/50
Cytokines				
TNF-α	FITC	Pharmingen	MAb11	1/50
IL-10	APC	Biolegend	JS3-19FI	1/50
IL6	APC	BioLegend	MQ2-13A5	1/50
IL8	APC	BioLegend	E8N1	1/50

2.10.3. Cell Surface Antibody Staining

Cell surface staining was performed by mixing required antibodies in FACS buffer (see Section 2.10.1). 50µl of prepared staining mix was applied per $1x10^5$ cells in U-bottom 96 well plate (Cell Star). Staining was performed over 30-40 minutes at 4°C in

dark. Afterwards, 100µl of FACS buffer was added to each staining well and cells were spun at 15000rpm for 5 minutes. Washing step was repeated with additional 200µl FACS buffer. Cells were resuspended in 300µl FACS buffer and transferred to FACS tubes (Gosselin).

2.10.4. Cell Intracellular Antibody Staining

Intracellular staining was performed similarly to surface staining (see Section 2.10.3) with use of 1x Perm buffer (see Section 2.10.1) instead of FACS buffer for antibody mix preparation and the first wash. Second wash and final sample resuspension was done with FACS buffer.

2.10.5. Flow Cytometry Acquisition and Analysis

Acquisition of the samples was carried out on a BD FACS Canto™II (BD Biosciences) instrument. Isotype control was set up and used for each experiment. At least 5000 events were recorded. Collected data was analysed using FlowJo programme (FlowJo Enterprise).

Recorded cells were gated on the side and forward scatter C (SSC and FSC, respectively) and selected population was analysed for expression of the desirable markers. Gates were set on isotype controls for each of the individual fluorochrome.

2.11. Immunoblotting

2.11.1. Buffers

Lysis Buffer combined 20 mM Tris (pH 7.5) (Fisher), 150 mM NaCl (Fisher), 1% Tergitol-type NP40 (Sigma-Aldrich), 1 mM $MgCl_2$, 1mM EGTA (Sigma), 1 mM

$NaVO_4$, 10mM $Na_4P_2O_7$, 1% n-dodecyl-β-D-Maltoside (Sigma-Aldrich) and 1x Protease Inhibitor (Roche).

MOPS SDS Running buffer (20x) (NuPAGE Novex, Life Technology) was diluted 1:20 in distilled H_2O before use and stored in the fridge.

Pierce® Western Blotting Transfer Buffer (10x) (Thermo Scientific) was stored at 4°C and made up to 1x with H_2O before use.

Washing buffer was made using 1 tablet of phosphate buffered saline (Fisher Scientific, UK) and 100µl of Tween®20 (Sigma, Life Science) per 100ml of H_2O.

Restore™ Western Blot Stripping Buffer (Thermo Scientific) was used as described below (see Section 2.11.5).

2.11.2. Immunoblotting Antibodies List and Preparation

Table 2.3. List of Immunoblotting Antibodies

antibody	dilution	host	company	clone
NFκβ signalling				
IKKα	1:1000	rabbit	Cell Signalling	3G12
IKKβ	1:1000	rabbit	Cell Signalling	D30C6
p-IKKα/β (Ser176/180)	1:1000	rabbit	Cell Signalling	16A6
NFκβ	1:1000	rabbit	Cell Signalling	D14E12
p-NFκβ(Ser536)	1:1000	rabbit	Cell Signalling	93H1
IKβa	1:1000	rabbit	Cell Signalling	L35A5
p-Ikβ-α (Ser32)	1:1000	rabbit	Cell Signalling	14D4
Restriction factors				
APOBEC3F	1:1000	rabbit	abnova	
APOBEC3G	1:1000	rabbit	abcam	mAbcam 75560
SAMHD1	1:1000	mouse	abcam	
TRIM5α	1:1000	mouse	ImmunoDiagnostics	Clone 4.1
Tetherin /BST2	1:1000	rabbit	Strebel & AIDS reagent program, 2009.	
MX2	1:1000	mouse	Santa-Cruz	H-7
TGF-β signalling				
p-SMAD3 (Ser423/425)	1:1000	rabbit	Cell Signalling	C25A9
SMAD3	1:1000	rabbit	Cell Signalling	C67H9
SMAD2/3	1:1000	rabbit	Cell Signalling	D7G7
SMAD2/3	1:1000	rabbit	Cell Signalling	D43B4
p-SMAD2 (Ser465/467)	1:1000	rabbit	Cell Signalling	138D4
SMAD4	1:1000	rabbit	Cell Signalling	
Controls				
ACTIN	1:2000	mouse	Millipore	MAB1501R

RIG-I	1:1000	mouse	Alexis Biochemicals	Alme-1
Secondary antibodies				
polyclonal anti-rabbit - HRP	1:3000	Goat	Dako	PO448
polyclonal anti-mouse -HRP	1:5000	Goat	Dako	PO447

Antibodies were diluted in PBS containing 1% BSA and 0.025% NaN_3. Prepared antibody solution was stored at 4°C and used 2-4 times depending on antibody efficiency.

2.11.3. Cells Lysis

For western blot analysis 2 x 10^5- 3 x 10^5 cells per condition were washed with PBS and spun at 1500rpm for 5 minutes in 1.5 ml o-ringed screw tubes (Simport). Cell pellet was lysed for 20 minutes with 24µl of lysis buffer (see Section 2.11.1). Samples were then spun at 13200 rpm for 18 minutes at 4°C. Samples were transferred to fresh tubes avoiding cell debris pellet. 6µl NuPage® Sample reducing agent (Invitogen) (10x) and NuPage® LDS Sample buffer (Novex, Life Technologies) (4x) mix was added to each sample and the proteins were denatured for 10 minutes at 80°C in analog heat block (VWR). Prepared samples were stored at -30°C until use.

2.11.4. Immunoblotting Protocol

Prepared samples were loaded onto pre-cast, polyacrylamide NuPAGE® 4-12% Bis-Tris gels (Novex, Life Technologies) using Gel saver tips (Star Lab). SeeBlue® pre-stained standard ladder (Novex, Life Technologies) and Mark12™ unstained standard ladder (Novex, Life Technologies) were loaded on the gel for molecular size reference. Gel was run in X Cell SureLock™ running system (Invitrogen, Novex Mini-cell) in MOPS running buffer (see Section 2.11.1) at 150 V for 60 minutes in

Power350 system (Fisher Scientific). Proteins were transferred from the gel into 0.45µm Nitrocellulose Blotting Membrane (Amersham™ Hybond ECL, GE Healthcare, Life Sciences) using Mini ProteanII™ transfer system (Bio-RAD) in Pierce® Western Blotting Transfer Buffer (see Section 2.11.1). Transfer was performed at 90 V for 50 minutes, and transfer efficiency was confirmed by 0.4% Ponceau red solution stain. Membrane was blocked in 5% milk for 30 minutes prior to overnight incubation with primary antibody (see Section 2.11.2).

2.11.5. Immunoblotting Results Acquisition

After overnight primary antibody incubation at 4°C, the membrane was incubated for 1 hour at room temperature with the relevant secondary antibody conjugated to HRP. Following extensive washing, bands were revealed on high performance chemiluminescence film (Hyperfilm ™ ECL, GE Healthcare Amersham) using SuperSignal West Pico Solution (Thermo Scientific) in AGFA SRX 101A (Konica). An Unstained Standard Mark12™ Ladder was used for molecular weight identification. Where required antibodies were removed from membranes using Restore™ Western Blot Stripping Buffer (Thermo Scientific) by 5-15 minutes treatment at room temperature. Before re-use of the membrane it was blocked on the rocking platform with 5% milk for 30-40 minutes. Band intensity was analysed using image processing and analysis software Java ImageJ 1.48 (National Institute of Health).

2.12. Quantitative Real Time PCR

2.12.1. RNA Extraction from Cells

$6x10^5$ of MDDC and MDLC per condition, were used for RNA extraction using RNeasy Mini Kit (Qiagen) according to manufacturer instructions. Cells were centrifuged at 1500pm for 5 minutes, the supernatant was removed and the pellet lysed in RLT buffer (provided). After homogenization of samples by pipetting, lysates were added directly to a QIAshredder spin column and spun for 2 minutes at full speed. Passed through lysates were mixed with 1ml of 70% ethanol. The mix was applied to an RNeasy spin column and centrifuged for 15 seconds at 10,000rpm. RNA trapped in spin column was successively washed with 700µl of Buffer RW1 and twice with 500µl of Buffer RPE with 15second spin at 10,000rpm between 1 wash and with 2 minutes spin at the same speed after second wash. RNA was extracted from spin column to a 1.5ml clean tube using 50µl RNase-free water applied to the column before 1 minute spin at 10,000rpm. Pure RNA was quantified using nano-drop system (see Section 2.7.2) and was used for cDNA synthesis (see Section 2.12.2).

2.12.2. cDNA Synthesis Reaction

RNA isolated from cells (see Section 2.12.1) was used as a template for cDNA synthesis using qPCR BIO cDNA synthesis kit (PCR BIOSYSTEMS). Mastermix for the reaction was prepared according to manufacturer protocol and comprised of reagents indicated in **Table 2.4**.

Table 2.4. Components of cDNA synthesis reaction mix

Reagent	20µl reaction
5c cDNA synthesis mix	4.0µl
20x RTase	1.0µl
Sample RNA (1µg)	xµl
PCR grade H_2O	Up to 20µl final volume

Total 20µl reaction mix for each sample was incubated at 42°C for 30minutes, followed by 10minutes incubation at 85°C. The products were quantified using Nano-drop system (see Section 2.7.2) and used for qPCR reaction (see Section 2.12.4).

2.12.3 DNA Extraction from Cells

DNA extraction from MDLC and MDDC was achieved using QIAamp DNA Mini Kit (QIAGEN). Accordingly, 200µl of cells suspension ($4x10^5$ cells/sample) was added to 20µl of Proteinase K (provided) and mixed using a pipette. 200µl of Buffer AL was added to homogenous sample and pulse-vortexed for 15 seconds. The samples were then incubated at 56°C for 10 minutes before addition of 200µl of ethanol (96-100%). Pulse-vortexed samples were applied to the QIAamp spin column and centrifuged for 1 minute at 8000rpm. Then spin column containing DNA was washed with 500µl of Buffer AW1 and centrifuged at 8000rpm for 1 minute. 500µl of Buffer AW2 was added to spin column and centrifuged at 14,000rpm for 3 minutes. DNA was extracted from QIAamp spin column after 1 minute room temperature incubation in Buffer AE (200µl) and centrifugation at 8000rpm (1 minute). DNA was quantified using nano-drop system (see Section 2.7.2) and was used for qPCR (see Section 2.12.4).

2.12.4 Quantitative PCR

Real-time PCR was performed on ViiA7 Real-Time PCR system (ΔΔCt methods) using qPCRBio SyGreen Mix Lo-Rox (PCR Biosystems).

For analysis of IFN-β and MX2 mRNA products:

50ng of sample cDNA (section 2.12.2) was used with primers:

IFN-β forward primer 5'-AGCACAGGATGAACTTTGAC-3', and

IFN-β reverse primer 5'-TGATAGACATTAGCCAGGAG-3' (Eurofins MWG Operon, Germany).

MX2 forward primer 5'-AAGCAGTATCGAGGCAAGGA-3'

MX2 reverse primer 5'-TCGTGCTCTGAACAGTTTGG-3' (Eurofins MWG Operon, Germany).

For GFP DNA quantification:

10ng of sample DNA (section 2.12.3) was used with primers:

GFP forward primer 5'-aagttcatctgcaccaccg-3'

GFP reverse primer 5'- tccttgaagaagatggtgcg-3'

(Eurofins MWG Operon, Germany).

PCR was performed in the conditions indicated in **Table 2.5**.

Table 2.5. Quantitative PCR settings

Step	**Denature**	**PCR**	
	HOLD	Cycle (40)	
		Denature	Anneal/Extend
Time	20 sec	1 sec	20 sec
Temp	95°C	95°C	60°C

Results of qPCR were analysed using ExpressionSuite Software v1.0.3, and data were normalized to β-actin expression (QuantiTect human ACTB2SG primer, QIAGEN) or GAPDH expression (Hs_GAPDH_1_SG QuantiTect Prime primer, QIAGEN).

2.13. Statistical Analysis

Student's t-tests were used to evaluate the significance of differences between experimental groups. *p*-values <0.05, <0.01 or <0.001 were considered significant and marked with *, ** or ***, respectively. NS indicated no significant difference.

2.14. Ethics Statement

Human skin samples were obtained from female patients undergoing mastectomy or breast reduction surgery with informed written patient consent and local ethical committee approval (South East Wales Research Ethics Committees Panel C, Reference: 08/WSE03/55).

3. Immunological Properties of Langerhans Cell Model Systems: Cell Lines, Monocyte-derived LC and ex vivo Human LC

3.1 Introduction

Dendritic cells were first described in 1973 in mice by Steinman and Cohn (Steinman and Cohn 1973), who observed "an adherent nucleated cell [population] whose morphological features are quite distinct [to granulocytes, lymphocytes and mononuclear phagocytes]". In general, DC are antigen presenting cells (APC) and critical immune regulatory cells which are important in innate immunity and specialized in stimulating T cells responses as well as in promoting tolerance. According to their phenotype, location, function and origin, dendritic cells populating the human body are divided into different subsets, which also include Langerhans cells (Ziegler-Heitbrock and Hofer 2013).

For research purposes, model cell lines have been developed to mimic LC and DC subsets in laboratory and culture conditions. Some recognized model systems include monocyte-derived Langerhans Cells (MDLC) and Dendritic Cells (MDDC), and described MUTZ-3 derived cells (Masterson et al. 2002; Santegoets et al. 2006). MDLC and MDDC show the closest resemblance to their *in vivo* counterparts, perhaps due to their origin from blood-isolated monocytes. In fact, MDDC used in this work express CD1a, HLA-DR, DC-SIGN, and other markers characteristic of some tissue-resident myeloid DC. Responses of MDDC to interferon or pathogen were also similar to those described in the literature for blood isolated DC. Although MDLC were positive for Langerin and presented an immature phenotype, these cells were also expressing significant amounts of DC-SIGN, a notion known already but, in fact,

poorly relayed in scientific publications (Turville et al. 2002b; Ganor et al. 2013). This could be, however, of particular importance considering that langerin and DC-SIGN were reported to have seemingly opposing roles on HIV-1 replication and transfer toward target $CD4^+$ T cells (Geijtenbeek et al. 2000). Maturation of these cells occurred when induced with a specific set of Toll Like Receptors (TLR) agonists and correlated with the simultaneous release of pro-inflammatory or tolerogenic cytokines. Importantly, MDLC responded to Interferon-α treatment as evidenced by the increase of expression of Interferon-inducible restriction factors and to our surprise, Langerin. The interferon response was much less pronounced in the MUTZ-3 derived LC population, although their phenotype was closely related to LC.

Furthermore, a successful method for isolation of primary dermal DC and epidermal LC from skin samples is detailed in this Chapter. This method allowed isolation of antigen presenting cells from the heterogeneous population that also included tissue-supporting cells, such as keratinocytes. Immune cells consisted of a population of dermal DC or epidermal LC, obtained from dermis and epidermis, respectively.

Thus, in the context of this research, the cell model systems are validated and their properties and limitations discussed.

3.2 Results

3.2.1. MUTZ-3 Cell Line Shows Characteristics of Monocytes

The MUTZ-3 cell line used in my studies was a kind gift from Dr Tania de Gruij (Dept. Medical Oncology, VU University Medical Centre, Amsterdam, Holland). Flow cytometry analysis demonstrated that this cell line was composed of mixed populations of $CD14^+$ (mean 41.6%, SEM=15.9) and $CD14^-$ (mean 59.4%, SEM=15.9) cells (**Figure 3.1A and B**), similar to published observations (Santegoets et al. 2006; de Jong et al. 2010b). Remarkably, MUTZ-3 revealed the trend of higher proportions of $CD14^+$ cells at lower MUTZ-3 culture densities, suggesting that culture conditions may influence the proficiency of MuLC differentiation (data not shown).

On average 40.5% (SEM=2.72) MUTZ-3 cells stained for HLA-DR, but no expression of CD1a was detected (**Figure 3.1A and B**). Furthermore, the mean fluorescent intensity analysis revealed the presence of approximately 200 molecules of HLA-DR per cell (MFI=211.6 +/- 17.7) supporting the notion that these cells might be differentiated into APC (**Figure 3.1. C**). Further analysis failed to identify the presence of CD83 marker in total MUTZ-3 population, suggesting that this cell line remains immature during culture propagation. The undifferentiated state of MUTZ-3 was also confirmed by the absence of cell lineage specific markers for Langerhans cells, Langerin (mean 0.07%), Dendritic cells, DC-SIGN (mean 0.05%, MFI= 5.79SEM=0.34), Natural killer cells (CD16) (mean 0.5%) and B cells (CD19) (mean 0.1%) (**Figure 3.1**). 5.3% (SEM=2.02, MFI=34.2) of MUTZ-3 expressed HIV-1 entry receptor, CD4, and 1.2% were positive for HIV-1 co-receptor (CCR5) (**Figure 3.1B**), suggesting that undifferentiated MUTZ-3 could be productive targets of HIV-1 infection.

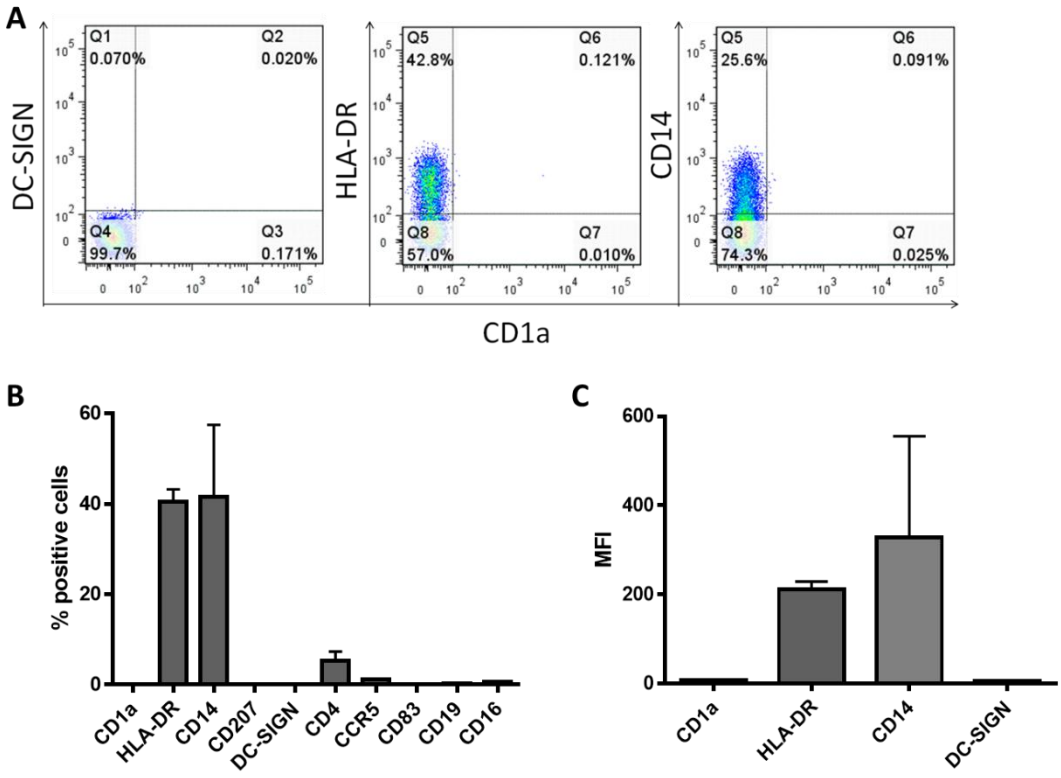

Figure 3.1. **Undifferentiated MUTZ-3 Cell Line Express Only a Few Markers. (A)** Representative flow cytometry analysis for chosen markers and **(B)** summarized data (*n=5*) are shown. **(C)** Mean fluorescent intensity for selected markers is showed (*n≥2*). Error bars represent ±SEM.

Taken together, MUTZ-3 showed a naïve, monocyte-like phenotype profile, with predominant and exclusive expression of CD14 and HLA-DR. Similar to monocytes, MUTZ-3 cells also have a potential to differentiate into cell types resembling Langerhans cells or Dendritic cells, when cultured with the appropriate differentiation medium. MUTZ-3 derived LC used in my study are characterized in the following section (see Section 3.2.2).

3.2.2. MUTZ-3 Derived LC Phenotypically Resemble Langerhans Cells

Production of MuLC from MUTZ-3 cells is done over 10 days in medium supplemented with GM-CSF, TGF-β and TNF-α (see Material and Methods Section 2.3.3). Once fully differentiated, MuLC almost completely down-regulated expression of CD14 (7.5%, SEM=2.146, SD= 4.79) and increased the levels of HLA-DR (80.2%, SEM=4.96, SD=8.59) (**Figure 3.2A and B**). MuLC also became positive for CD1a (63.7%, SEM=6.01, SD=13.4) and Langerin (61.687%, SEM=3.144, SD=7.701), and stained weakly with anti-DC-SIGN antibody (6.68%, SEM=1.45, SD=3.254) (**Figure 3.2A and B**). Thus, differentiated MuLC acquired Langerhans cells phenotype while losing monocytic/MUTZ-3 characteristic features.

Only a small proportion of MuLC (4.63%, SEM=1.879, SD=3.785) underwent culture-induced maturation, as demonstrated by the low occurrence of CD83 marker (**Figure 3.2B**), suggesting immature, or semi-matured phenotype of MuLC.

MuLC derived in our laboratory expressed substantial levels of HIV-1 entry receptor CD4, comprising a mean of 60.4% (SEM=5.22) CD4 positive cells (**Figure 3.2B**). MuLC also stained for HIV-1 co-receptors, CCR5 and CXCR4, however at lower levels; 3.6% (SEM=1.51, SD=3.38) and 2.3% (SEM=0.485, SD= 1.086), respectively.

Apart from phenotypic similarity to primary LC, differentiated MuLC also acquire a star-like appearance similar to their *in vivo* counterparts (**Figure 3.2C**). Additionally, previous studies demonstrated occurrence of Birbeck granules in MuLC (Santegoets et al. 2006).

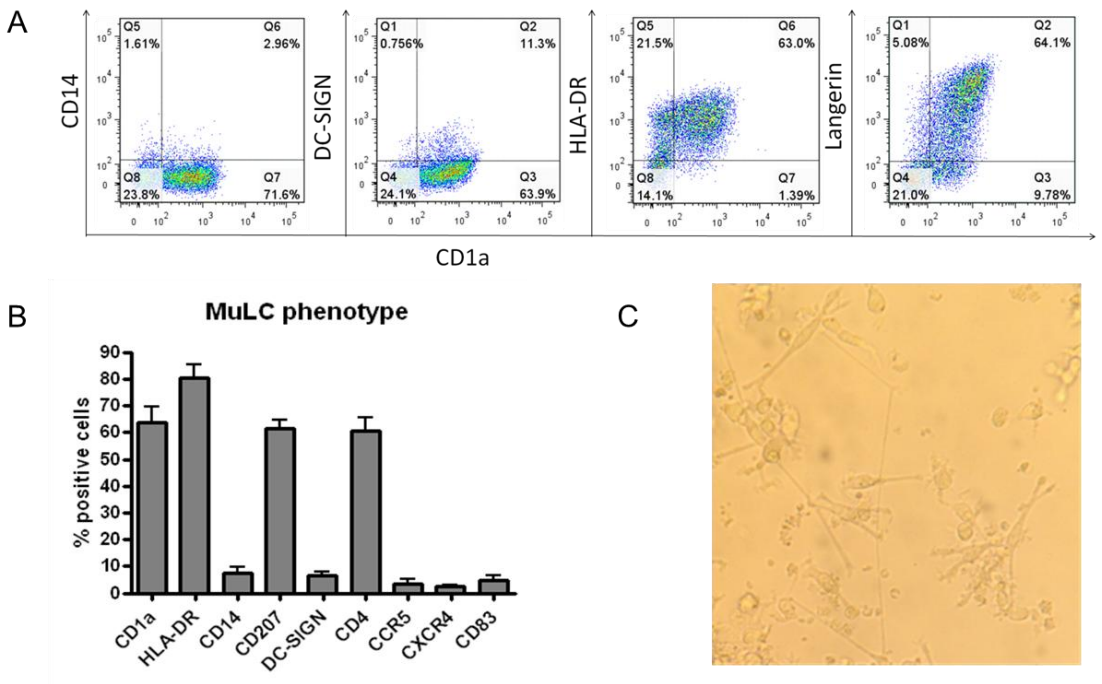

Figure 3.2. **MuLC Phenotype Resembles this of Langerhans Cells.** MuLC were isolated and stained for surface expression of selected markers. **(A)** Representative flow cytometry analysis for chosen markers and **(B)** summarized data (*n=5*) are shown. **(C)** Light microscope (40x) view on differentiated MuLC, showing extended dendrites protruding from the cells. Error bars represent ±SEM.

3.2.3. MDLC are Double Positive for Langerin and DC-SIGN

In contrast to the high percentage of Langerin^{+} MuLC, around one third (26.8% (SEM=5.6)) of monocyte-derived Langerhans cells (MDLC) obtained routinely were positive for langerin, as recorded by intracellular staining, and also expressed significant amounts of DC-SIGN (53.4%, SEM=6.4) (**Figure 3.3A and B**). A higher amount of Langerin was observed inside MDLC, when analysed with intracellular staining, compared to surface staining (**Figure 3.3A**). Interestingly, CD14 expression on MDLC remained relatively high (mean 60.5%, SEM=23.5) after differentiation, although CD1a and HLA-DR markers were present on most of the MDLC population

(97.2%, SEM=0.7; 69.58% SEM=11.7, respectively) (**Figure 3.3B**). MDLC derived in our lab were immature, comprising 1% (SEM=0.8) $CD83^+$ and 2.9% (SEM=1.4) $CD86^+$ population. Thus, differentiation of MDLC in IL-4, GM-CSF and TGF-β cytokine cocktail results in immature phenotype of the cells. Further analysis of MDLC evidenced low percentage of $CD11c^+$ population (32.7%, SEM=18.9), and high number of CD11b (99.3%, SEM=0.3) and e-cadherin (98.6%, SEM=0.5) positive cells (**Figure 3.3B**). MDLC were also analysed for expression of HIV entry receptors/co-receptors. The results demonstrated that an average of 15.6% (SEM=11.3) of these cells were positive for CD4, 6.8% (SEM=2.6) expressed CCR5, and less than 1% stained for CXCR4 (0.9%, SEM=0.2), making them a likely target for R5 tropic HIV infection (**Figure 3.3C**).

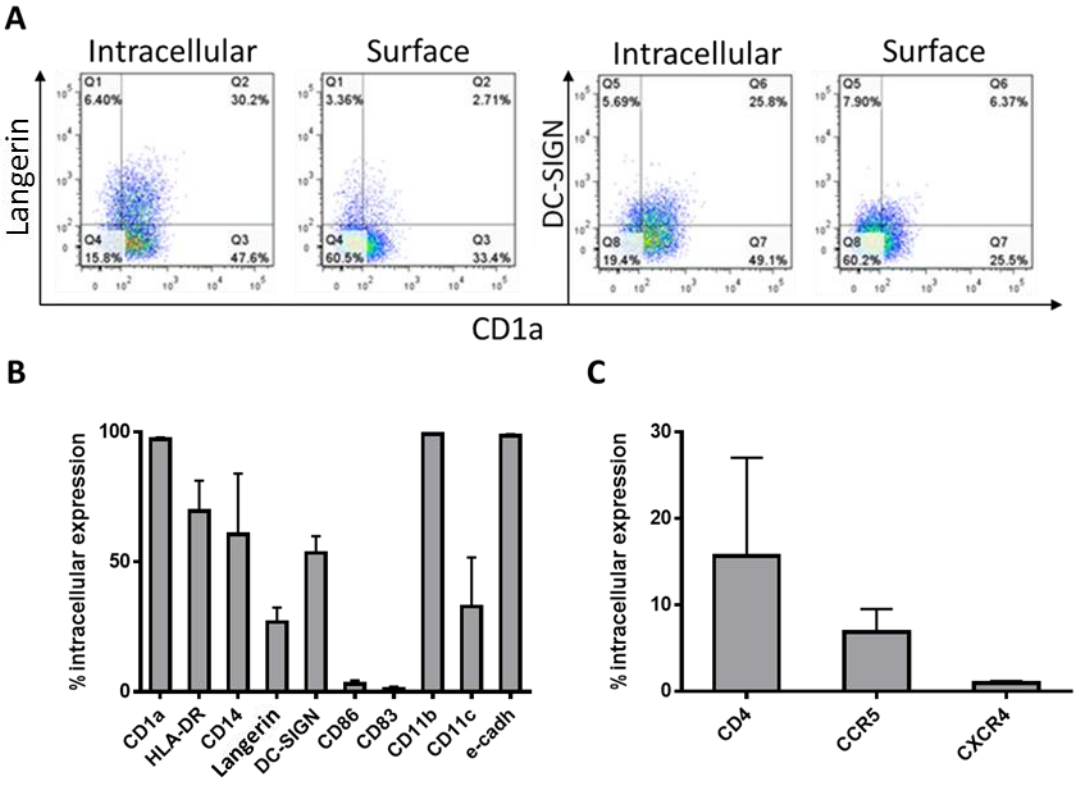

Figure 3.3. MDLC are Double Positive for Langerin and DC-SIGN. Intracellular and surface expression markers were investigated by flow cytometry in fully differentiated MDLC. **(A)** Representative dot plots of Langerin, CD1a and DC-SIGN staining in MDLC are shown, and **(B)** combined data for cells specific markers is illustrated (CD83 *n=2*, the rest *n≥3*). **(C)** Graph represents maturation status of MDLC and expression of HIV-1 entry receptor and co-receptors (CD4 *n=2*, the rest *n≥3*). Error bars represent ±SEM.

3.2.4. Phenotype of MDDC Shows Characteristics of Myeloid Dendritic Cells

Analysis of monocyte-derived Dendritic Cells (MDDC) revealed a phenotype similar to that observed from blood isolated myeloid DC. Indeed, MDDC presented high expression of CD1a (74.0%, SEM=8.7%), HLA-DR (87.0%, SEM=4.9%), DC-SIGN (93.3%, SEM=1.6), and lacked Langerin (mean=4.43%, SEM=3.998) (**Figure 3.4A and C**). Upon differentiation, MDDC showed an immature phenotype (CD83 5.5%,

SEM=1.5) and, in contrast to their MDLC counterparts, they almost completely lost expression of CD14 (2.8%, SEM=1.6) (**Figure 3.4B**).

DC are known to support transmission and replication of HIV-1 during early stages of virus acquisition (Cameron et al. 1992; Pope et al. 1994). R5 tropic HIV-1 particles are predominantly found in newly infected individuals, most likely due to selection of these viruses by the availability of entry co-receptors on mucosal DC. Accordingly, substantial expression of main HIV receptor, CD4 (43.2%, SEM=16.9), and low levels of CCR5 (3.46%), but no CXCR4 (0.4%) were detected on MDDC (**Figure 3.4B and C**). Therefore, MDDC could be a target for infectious R5-tropic HIV-1.

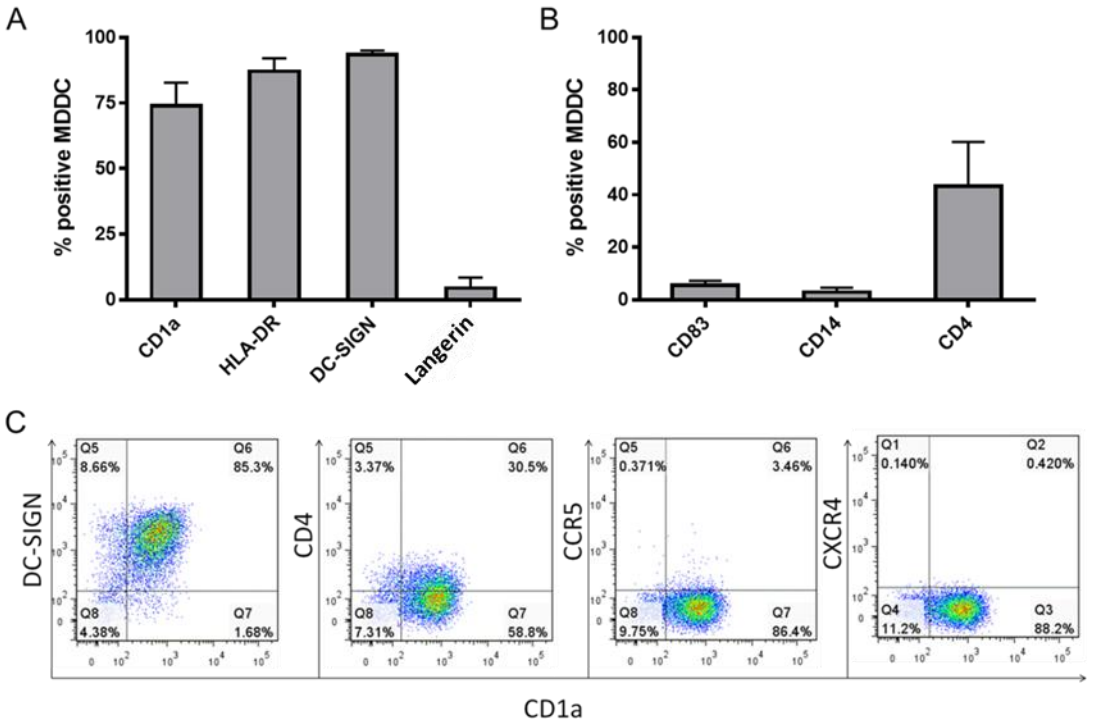

Figure 3.4. MDDC Resemble the Phenotype of Myeloid DC. MDDC were collected after differentiation and were surface stained with a panel of antibodies for phenotypic characterization. **(A)** Mean expression of markers in different donors is represented in graph (Langerin *n=2,* the rest *n≥ 3*), and **(B)** (*n≥ 3)*. **(C)** Example of flow cytometry analysis is shown. Error bars represent ±SEM.

3.2.5. Cell Walkout Method for Isolation of Skin Immune Cells

Primary cells were obtained from skin samples from healthy donors undergoing breast or abdominal reduction. Cells were collected as described in materials and methods (see Section 2.5), and as explained below. The isolation protocol and further experiments with primary cells were performed with the help of Matt Ivory (PhD student, Cardiff School of Pharmacy and Pharmaceutical Sciences, Cardiff University, Redwood Building, King Edward VII Avenue, Cardiff, CF10 3NB, United Kingdom) and under supervision of Professor James Birchall.

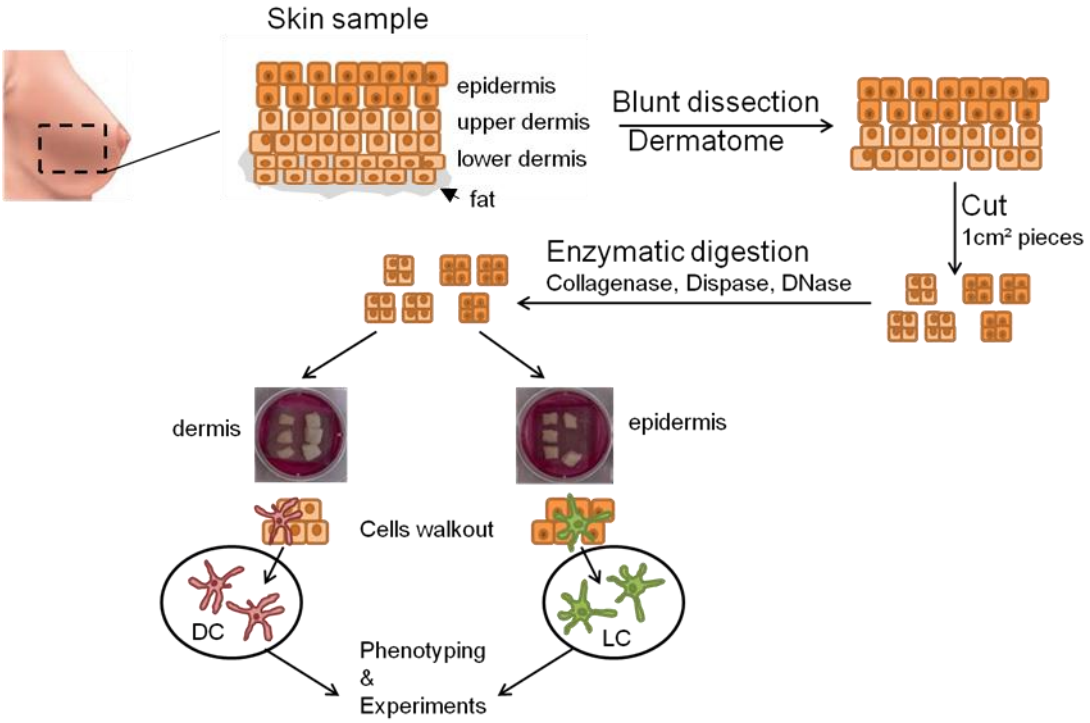

Figure 3.5. Skin Epidermal LC and Dermal DC Isolation Method. Isolation of epidermal LC and dermal DC from healthy donor skin explant was performed as shown in the diagram. Removal of fat and excess lower dermis was followed by enzymatic digestion of skin collagen with Collagenase and Dispase. After 30 minutes incubation in a shaking water bath, epidermis dermis were separated using forceps and cultured separately in RPMI medium for 48 hours. Walkout cells were then phenotyped and used for the experiments.

Upon patient consent and local ethical committee approval, the skin sample was taken to the laboratory within a few hours post operation and processed (**Figure 3.5**). Primary, subcutaneous fat and excess lower dermis were removed by blunt dissection, followed by separation of upper layers of the skin with a dermatome. When possible, the dermatome was used directly before excision of the skin from the patient. The upper layers of skin were incubated in the presence of dispase and collagenase, in order to facilitate dissociation of dermis and epidermis. These two layers were then separated from each other using forceps, and independently cultured in supplemented medium (see Materials and Methods Section 2.5). After 24 hours, Langerhans cells migrating from epidermis, and dermal DC leaving dermis were collected and used for phenotyping and functional studies.

3.2.6. Skin Isolated Dermal DC Express CD141 Marker

Total population of dermal sheet walkout cells was phenotypically analyzed to identify emigrated antigen presenting cells. Dermal T cells were gated out during analysis on the basis of their size/granularity (lower than dDC FSC and SSC values) and based on their HLA-DR negative staining. Dermal DC were detected by selection of CD141 positive population within HLA-DR and CD45 positive cells (**Figure 3.6A**). On average, 66.7% (SEM=3.8) of total dermal walkout cells were HLA-DR and CD45 double positive (**Figure 3.6B**), of which 20.6% (SEM=6.1) expressed both CD11c and CD141 (**Figure 3.6C**). Dermal DC were previously characterized by expression of CD141, CD11c and the presence of substantial amount of CD14 (Chu et al. 2012). In agreement, CD14 surface expression was observed on most of $CD141^{+}$ population (**Figure 3.6A**).

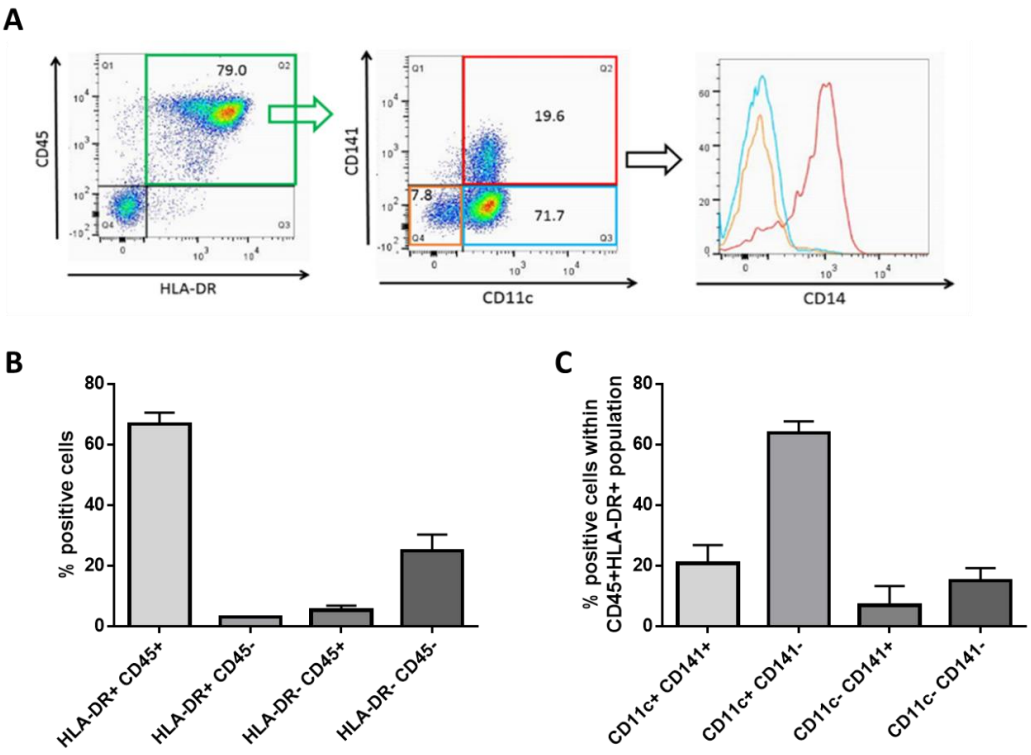

Figure 3.6. Dermal DC Express CD141 and CD14. Dermal walkout cells (Figure 3.5, materials and methods 2.5) were stained with panel of antibodies to distinguish dendritic cells population. **(A)** Total walkout cells were gated on live and single cells, and HLA-DR, CD45 double positive population was further analysed for expression of CD141 and CD11c. Expression of CD14 within populations was investigated. **(B)** Expression profiles for CD45 and HLA-DR markers for 3 skin samples are shown (*n=4*). **(C)** Expression of CD11c and CD141 within HLA-DR+CD11c+ cells is represented in graph (*n=4*). Error bars represent ±SEM.

Dermal DC were reported to be from myeloid origin, therefore expression of monocytic markers on their surface is not surprising. Interestingly, these $CD14^{+}$ dermal DC have demonstrated the potential to differentiate into Langerhans cells in the presence of TGF-β (Klechevsky et al. 2008) thus may represent progenitors for inflammatory LC.

A large proportion of HLA-DR^{+} CD45^{+} cells included CD141^{-} CD11c^{+} positive cells (63.83%, SEM=3.7) (**Figure 3.6B**). These cells may represent undifferentiated dermal DC or cells that have lost their CD141 expression during migration from tissue sample. In fact, monocyte-derived dermal DC *in vitro* (Chu et al. 2012) stain highly for CD141 after differentiation, but lose expression of this marker within short time span of experiment (data not shown). Alternatively, CD141^{-} population may represent different subsets of antigen presenting cells within this tissue.

3.2.7. Epidermal Langerhans Cells Constitute only a Small Fraction of Epidermis in the Skin Walkout System

Phenotypic profiling of epidermis walkout cells identified a minor population of HLA-DR and CD1a double positive cells (**Figure 3.7A**). Proportions of these HLA-DR^{+}CD1a^{+} cells varied from 3.94% to 6.26% (mean=4.85%, SEM=0.7) between donors (**Figure 3.7B**). The majority of HLA-DR^{+} CD1a^{+} cells were langerin^{+} (mean= 81.6%, SEM=1) and the variability between donors was low (**Figure 3.7A and C**). Two conclusions can be drawn from these results: 1) Langerhans cells exist at low density in epidermis, and 2) Langerhans cells represent the major professional antigen presenting cells in the epidermis. Keratinocytes and some epidermal cells most likely accounted for the remaining HLA-DR and CD1a double negative cells (**Figure 3.7B**). Further information provided by Matt Ivory suggests that walkout LC were semi-matured as they evidenced expression of some maturation markers such as CD83 or CD86.

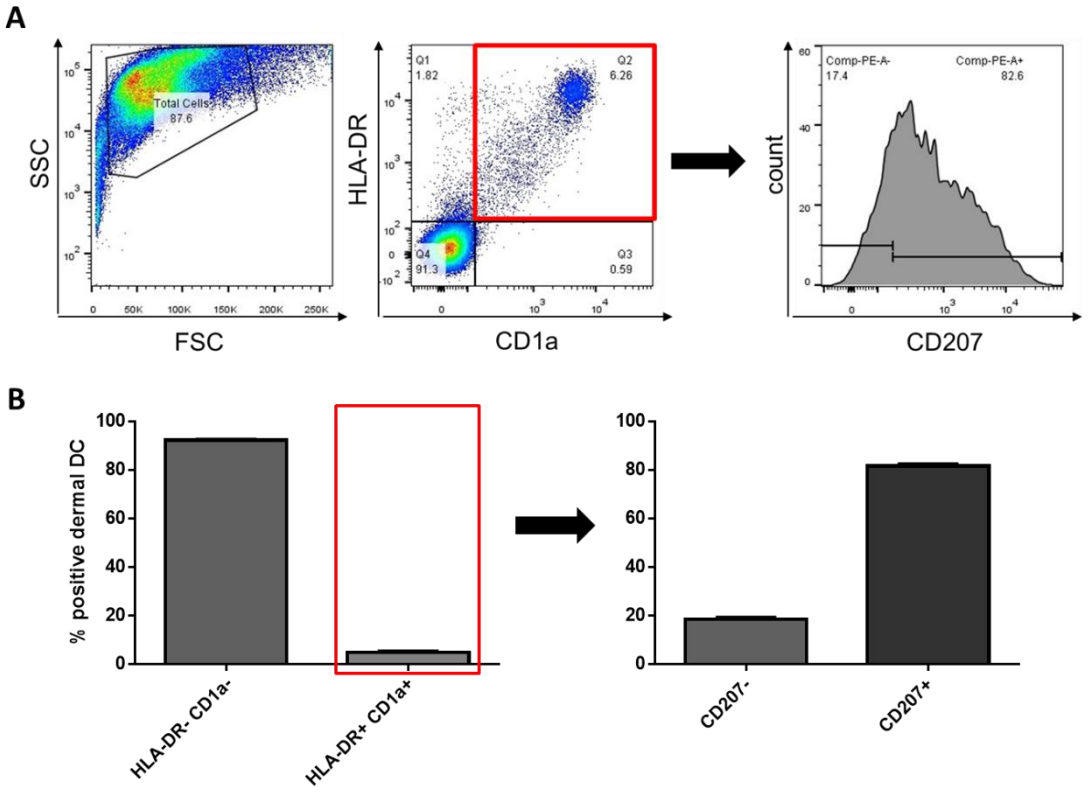

Figure 3.7. Epidermal Langerhans Cells Constitute Small Fraction of Epidermal Walkout Cells, but Express High Levels of Langerin and CD1a. Epidermis walkout cells (see Figure 3.5, Material and Methods 2.5) were fixed and stained with panel of antibodies for determining Langerhans cells population. **(A)** Total walkout cells were gated on live and single cells, and HLA-DR, CD1a double positive population was further analysed for expression of Langerin. **(B)** Proportion of total CD1a HLA-DR double positive and double negative populations for 3 skin samples (n=2), and a fraction of Langerin positive and negative cells within CD1a+HLA-DR+ population (n=2) are shown. Error bars represent ±SEM.

3.2.8. Interferon alpha Up-regulates Expression of Restriction Factors in MDLC and MDDC, but not in MuLC

Interferon (IFN), particularly type I, is released from virally infected cells and induces an anti-viral state in surrounding cells. A wide array of immune defence genes, including restriction factors, such as APOBEC family members, BST-2/tetherin and myxovirus resistance members (MX1 and MX2) (Pavlovic et al. 1990; Haller and Kochs 2011; Goujon et al. 2013) are all induced by type-I IFN. Consequently, expression of those cellular factors, concomitantly with other defence mechanisms, interferes with viruses' replication and transmission.

To further characterize my cell models, the response of MuLC, MDLC and MDDC to type-I IFN-α treatment was investigated. Cells were pre-treated for 24 hours with 1000U/ml of IFN-α, and restriction factors expression was investigated by western blot (**Figure 3.8**). Relative quantification of proteins was performed by IMAGE J and normalized to the same actin value between cells for comparison.

The results showed a significant level of SAMHD-1 protein expression in MDDC (16847.2) and MDLC (14956.28) that was almost unchanged in response to IFN-α (MDDC=13958.58; MDLC=19785.3) (**Figure 3.8A**). Correspondingly, SAMHD-1 expression in MDDC seemed to insignificantly decrease after IFN-α stimulation, while a moderate increase was noted in MDLC. Steady state MDDC and MDLC, both expressed a minor amount of APOBEC3G and BST-2/Tetherin (APOBEC3G: MDDC 4515.57, MDLC 3609.79; BST-2/tetherin: MDDC 10629.5; MDLC 15463.42) (Figure 3.8A). However, in contrast to SAMHD-1, the level of expression of these restriction factors was strongly up-regulated upon IFN-α treatment in both cell types, although

the increase of APOBEC3G expression in MDLC (1.39 fold increase) was much lower compared to MDDC (6.29 fold increase).

IFN-inducible RIG-I protein served as a positive control for our experiments, and its expression was significantly up-regulated after IFN-α challenge in MDDC (ns=7451.642, +IFN=30385.32) and MDLC (ns=4661.21, +IFN= 8225.6) (**Figure 3.8A**). Actin detection served as a loading control and was used for normalization purposes.

Western blot analysis of differentiated, untreated MuLC showed constitutively high expression of BST-2/tetherin (40468.91), which was not drastically changed in response to IFN-α (45557.07) (**Figure 3.8B**). High expression of BST-2/Tetherin on MuLC was unexpected and may be a characteristic acquisition for these cells during differentiation. Also, cell activation with TNF-α, present in the differentiation medium, might contribute to this phenotype, although this hypothesis was not tested.

MuLC exposed to IFN-α also showed an increase in APOBEC3F expression (5628) compared to non-stimulated cells (2166). Although western blot representation inferred only an apparent marginal enhancement of this protein, IMAGE J analysis revealed 2.59 fold increase in APOBEC3F between untreated and treated MuLC (**Figure 3.8B and C**). Although expression of this restriction factor was slightly induced in MuLC upon IFN-α treatment, all tested interferon-stimulated genes (ISG) did not evidence any signal, like MX2 and positive control RIG-I whose expression were not up-regulated (**Figure 3.8B and C**). In fact, higher levels of RIG-I constitutive expression (13880.74) were observed in unstimulated MuLC, compared to MDLC (see above), and were unchanged upon IFN-α treatment (12104.73). In comparison, activated MDLC showed higher levels of all investigated ISG products,

namely MX2, APOBEC3F, BST-2/tetherin and RIG-I (**Figure 3.8B**). Again, SAMHD-1 levels were not, or only slightly increased after treatment in both MDLC and MDDC.

From this data, it is possible to conclude that both MDDC and MDLC are fully responsive to type-I IFN treatments as evidenced by the significant increase in expression of many ISG, therefore supporting that these cells could be highly effective during an antiviral immune response. In contrast, the cell line model MuLC, although being phenotypically and morphologically related to LC, are seemingly impaired in their capacity to respond to IFN-α as evidenced by the lack of increased expression of ISG such as RIG-I, BST-2/Tetherin or MX2. However, these cells presented elevated expression levels of RIG, BST-2/Tetherin, even when unstimulated. This would nevertheless correlate with the observation that MuLC could be semi-matured maybe due to the presence of the pro-inflammatory cytokine TNF-α in the differentiation medium.

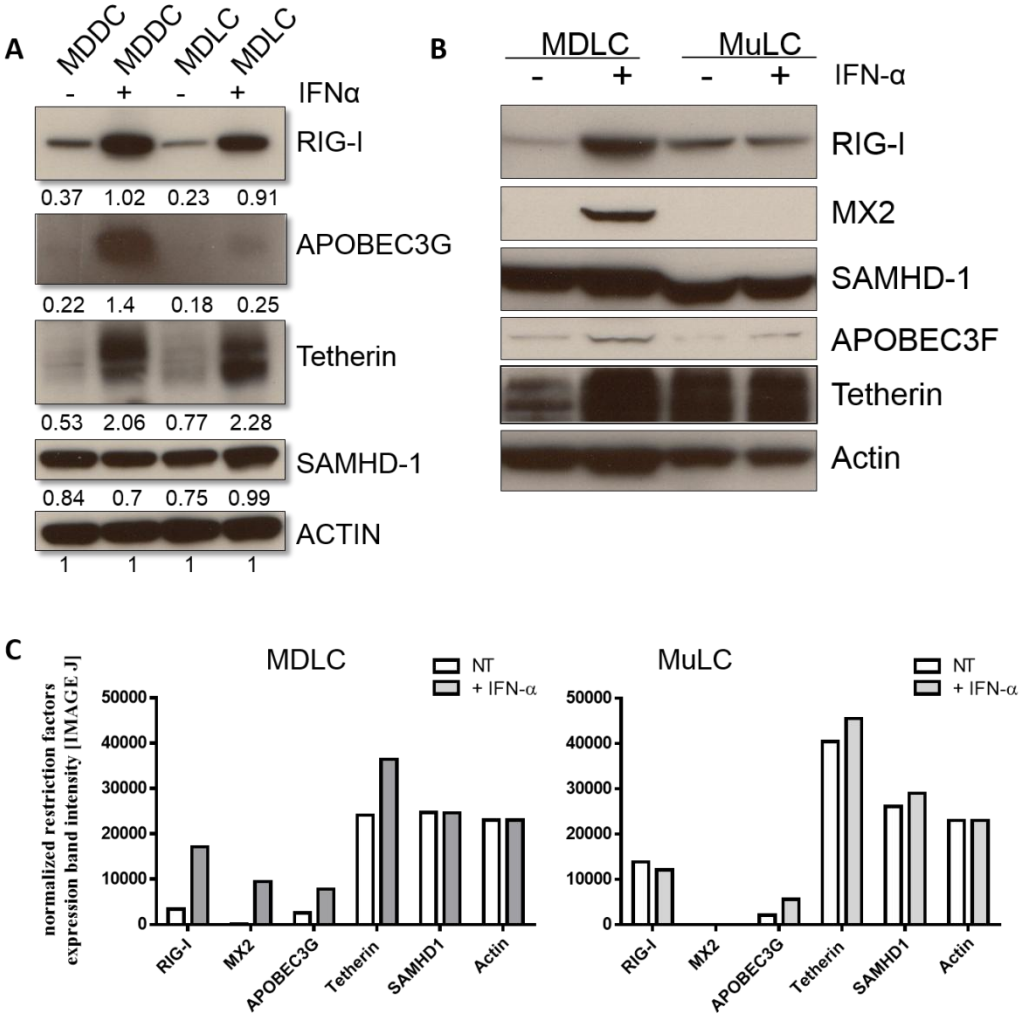

Figure 3.8. IFN-α Induces Expression of Restriction Factors in MDDC, MDLC, but not MuLC. MDDC, MDLC and MuLC were treated with IFN-α for 24 hours, and total lysates was analysed by Western blot for expression of restriction factors. MDDC and MDLC respond to IFN by up regulating IFN-inducible restriction factors, APOBEC3G and BST-2/tetherin, but not SAMHD-1. IMAGE J quantification was performed on all samples and normalized to actin. **(A)** Relative values are shown. **(B)** MuLC failed to respond to IFN, compared to MDLC, and showed constant high expression of BST-2/Tetherin. Relative quantification of bands was performed with IMAGE J and normalized to the same actin level in all samples. **(C)** Representation of quantified levels are demonstrated. RIG-I served as a positive control for IFN response. Actin was a loading control.

3.2.9. Langerin is a New Type I Interferon Inducible Factor in MDLC

In addition to the above IFN-α-induced changes in cells, stimulated MDLC also showed a notable and significant enhancement of surface (mean 32.4%, SEM=6.12, p=0.01), and intracellular (mean 60.5%, SEM=5.6, p=0.019) levels of Langerin (**Figure 3.9A and B**). In steady-state MDLC, the percentage of intracellular Langerin was considerably higher compared to surface expression of this lectin, as expected. However, the fold increase of surface langerin expression on IFN-α treated MDLC was equal to 6.5 (SEM= 2.02) and was greater than that observed inside the cell (mean 3.5 fold, SEM=1.5) (**Figure 3.9C**).

Further experiments demonstrated that enhanced langerin expression was most apparent and significant in response to type-I interferons, that is IFN-α2a (p=0.006), IFN-α2b (p=0.02) and IFN-β1a (p=0.02) (**Figure 3.10A and B**). The effect of IFN-β1b on Langerin was also evident (almost a 100% increase), but remained just above statistical significance (p=0.074) for the number of experiments performed. Type-II interferon treatment (IFN-γ), in contrast, did not induce any significant effect as langerin expression remained similar to untreated MDLC (mean langerin = 12.6%, SEM=1.64) (p=0.48). Thus the above results suggest that Langerin is a type I interferon inducible gene and it might account to the defence mechanism in the mucosa. Verification of the results with freshly isolated epidermal LC would have to be undertaken to confirm this phenomena.

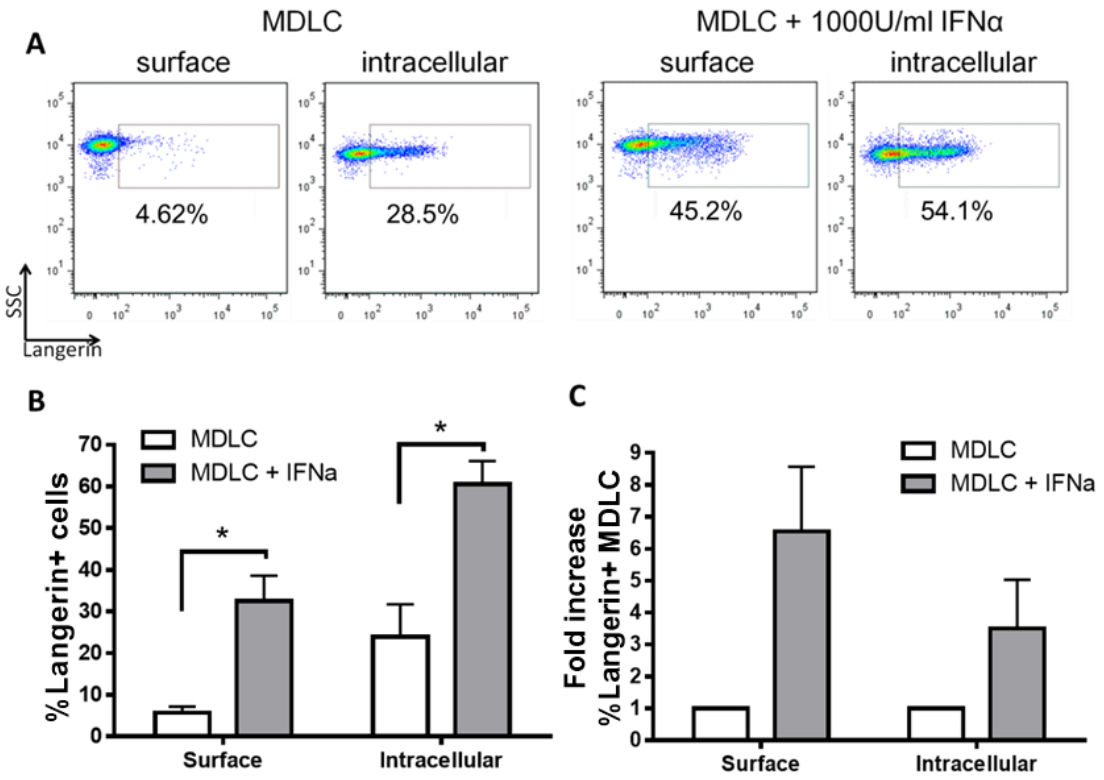

Figure 3.9. Interferon-α Stimulates Surface and Intracellular Expression of Langerin in MDLC. MDLC were treated for 24 hours with 1000U/ml of IFN-α, fixed and stained for langerin. **(A)** A representative experiment showing surface and intracellular levels of langerin in unstimulated cells (MDLC) and in IFN-α treated cells (MDLC + 1000U/ml IFN-α) is shown in, and **(B)** pooled data is represented in graph (*n=3*). **(C)** The fold increase of surface and intracellular langerin is shown in graph (*n=3*). MDLC indicate non treated cells. Error bars represent ±SEM. * $p \leq 0.05$.

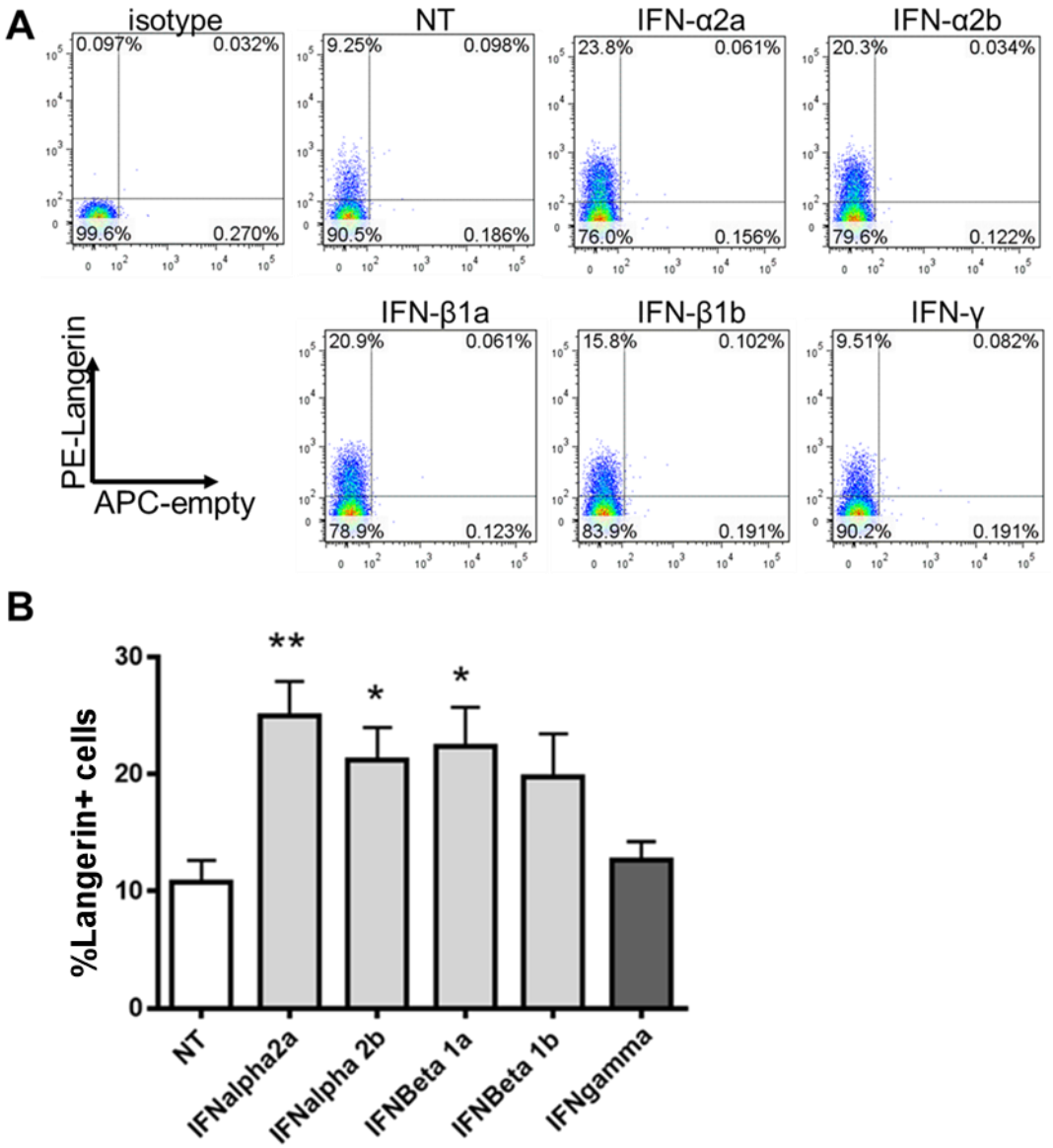

Figure 3.10. Intracellular Langerin Expression in MDLC Treated with Type-I or Type-II IFN. MDLC were treated for 24 hours with 1000U/ml of different IFNs, fixed and stained intracellularly for langerin. **(A)** Flow cytometry analysis of representative experiment is shown in. **(B)** Pooled data for langerin positive MDLC is represented in graph (*n=4*). Error bars represent ±SEM. * $p \leq 0.05$, ** ≤ 0.01.

3.2.10. MuLC Fail to Significantly Up-regulate Langerin after IFN-α Stimulation

Consistent with the viral restriction factors expression analysis reported above, MuLC did not significantly respond to IFN-α treatment in terms of langerin levels (**Figure 3.11A and B**). Indeed, the slight 5.25% increased expression of total langerin expression observed was not significant (p=0.22).

Although langerin levels on MuLC in these experiments were lower compared to above results, MuLC usually express high levels of langerin which again correlates with a possible increased semi-matured status of these cells. Also, as a cell line, Langerin levels and responsiveness could be significantly influenced by the duration of MUTZ-3 cell line maintenance in culture. This would likely affect the differentiation efficiency of MUTZ-3 into MuLC.

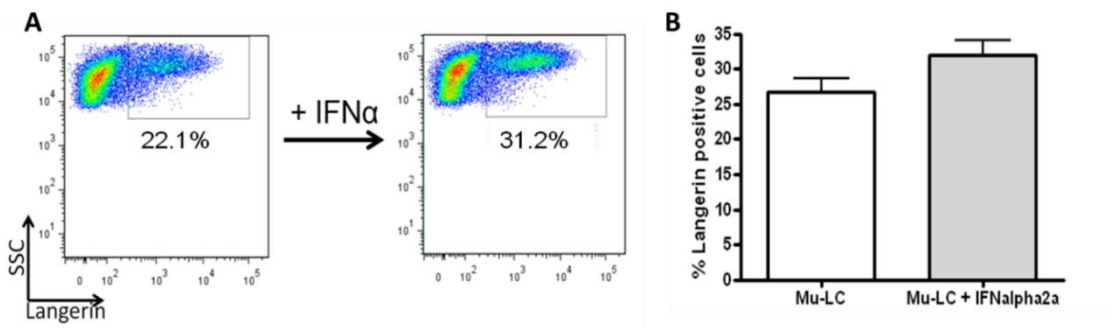

Figure 3.11. IFN-α Does Not Induce Significant Increase of Langerin in MuLC. MuLC were treated for 24 hours with 1000U/ml of IFN-α, fixed and stained intracellularly for langerin. **(A)** Flow cytometry analysis of representative experiment is shown in. **(B)** Pooled data for MuLC stimulation is represented in graph (*n=2*). Error bars represent ±SEM.

Therefore, the results demonstrate that IFN-α triggers up-regulation of restriction factor expression in MDLC and MDDC. In contrast, stimulated MuLC remained unresponsive to IFN type-I treatments suggesting a defect of these cells in integrating IFN type-I-mediated signalling. In fact impaired IRF8 and STAT responses were

described in MUTZ-3 cell line derived DC confirming perturbation of immune responses in this model (Rasaiyaah et al. 2009). Thus, despite phenotypical resembles of MuLC to epidermal LC, the immortalized biology of MUTZ-3 poses significant limitations to the range of studies these cells could be used for.

3.2.11. Responses of MDLC to TLR Agonists are Limited

Finally, responses of MDLC and MDDC to microbial components were investigated. For that reason, MDLC and MDDC were treated with agonists to TLR 1–TLR 9 (see Materials and Methods Section 2.9.3 and Appendix 1 (p.241)) and the levels of cytokines produced and expression of maturation markers were measured by flow cytometry after 24 or 48 hours, respectively.

The results showed a significant stimulation of MDLC with TLR2 (peptidoglycan) ($p=0.02$), TLR4 (LPS) ($p=0.002$), TLR6 (FSL1) ($p=0.005$) and TLR8 (ssRNA) ($p=0.01$) agonists, a weaker response upon TLR1 (Pam3CSK4), TLR3 (poly I:C) and TLR5 (flagellin) stimulation and no response at all when TLR7 (Imiquimod) or TLR9 (E.coli ssDNA) agonists were used (**Figure 3.12A**). A previous report claimed that MDLC coming into contact with peptidoglycan released mainly the supposed anti-inflammatory cytokine IL-10 (Flacher et al. 2006). Indeed, after TLR2 (peptidoglycan) agonist stimulation 28.2% (SEM=7.3) of *in vitro* MDLC stained positive in FITC channel which included anti-IL-10 and anti-TNF-α antibodies. Closer analysis showed that an average of 15.4% (SEM=5.2) of MDLC produced TNF-α in response to TLR-2 (peptidoglycan) challenge (Figure 3.12.B), and 62.2% (SEM=5.9, $p=0.012$) expressed CD86. Less noticeable maturation of MDLC was observed after challenge with TLR-6 agonist, FSL1. Typically, 33.9% (SEM=0.45, $p=0.0017$) of

these cells expressed CD86 (**Figure 3.12C**), and 28.6% (SEM=5.0) produced TNF-α/IL-10. In agreement, a study by Rose and colleagues (Rose et al. 2009) demonstrated that vaginal application of FSL-1, in mice, triggers significant production of pro-inflammatory cytokines, including TNF-α.

Surprisingly, the addition of LPS (TLR4 agonist) to MDLC triggered production of IL-10/TNF-α (25.3%, SEM=3.4%, compared to 1.2% NT, p=0.002) (**Figure 3.12A**). It was an unexpected result as previous research claimed the lack of TLR-4 transcripts in primary LC (Flacher et al. 2006). Addition of TLR8 agonist (ssRNA) to MDLC resulted in a 59.5% (SEM=4.05, p=0.0048) increase in CD86 expression and functional activation of these cells (**Figure 3.12C and D**). Production of TNF-α from ssRNA stimulated MDLC was at the base level (0.347%, SEM=0.09, p=0.87), suggesting that IL-10 might be a major cytokine expressed upon TLR8 agonist challenge in MDLC (48.5%, SEM=10.4%, p=0.01).

Viral mimicking TLR3 agonist, poly I:C, induced little production of cytokines (12.6%, SEM=4.5, p=0.06) (**Figure 3.12A**), and pronounced increase of CD86 expression (mean CD86=50.1%, SEM=39.1) (**Figure 3.12C**). Perhaps other cytokines are released in MDLC in response to TLR3 stimulation, although the efficiency and cytokine profile reported appeared quite controversial particularly when considering the origin of LC ($CD34^+$ or monocyte-derived)(Flacher et al. 2006; Renn et al. 2006; Rozis et al. 2008). According to the literature, type-I interferons would be prospective candidates, as they are readily produced in viral infections. However, as demonstrated in Chapter 4, poly I:C does not trigger a major production of IFN in MDLC (see Chapter 4 Figure 4.13), and in agreement with previous reports shows that LC are not type-I IFN producer cells. TLR3-mediated activation of LC was, nevertheless, shown to produce some inflammatory cytokines (IL-6, IL-8, TNF-α…) (Flacher et al. 2006)

and interestingly, IFN-inducible chemokines, such as CXCL9 and CXCL11, therefore suggesting a role for LC in IFN- mediated antiviral responses (Renn et al. 2006).

Challenge of MDLC with TLR1 and TLR5 agonists (Pam3CSK4 and flagellin, respectively) caused partial maturation of cells (mean CD86, TLR1=12.0%, TLR5=14.9%), which appeared statistically significant in the case of TLR5 stimulation (p=0.02). Release of cytokines from Pam3CSK4-treated MDLC was moderate (mean=16.29, SEM=7) and not significant (p=0.097). On the other hand, little IL-10/TNF-α production was recorded for the TLR5 agonist, Flagellin (mean=3.32, SEM=1.14), despite an apparent maturation of the cells (**Figure 3.12**).

Further analysis demonstrated that neither Imiquimod (TLR7 agonist), nor *E.coli* DNA (TLR9 agonist) induced activation of MDLC (IL-10/TNF-α production: mean=1.0%, SEM=0.29, p=0.6; mean=1.69, SEM=0.15, p=0.07, respectively) (**Figure 3.12A, C and D**). Maturation of MDLC, measured by CD86 expression, was also low in both conditions (**Figure 3.12C and D**), suggesting unresponsiveness of MDLC to these TLR agonists which is reminiscent of results obtained with MDDC. Of note, LC were described as lacking mRNA encoding for TLR7 and TLR9 (Kadowaki et al. 2001), which would explain the above results.

Thus, the data shows a limited and specific TLR response profile of MDLC. While some of the results match those described in the literature, some inconsistencies were evident. Although the *in vitro*-derived LC progenitors should be considered ($CD34^+$ versus monocytes), nevertheless, only a limited array of cytokines and maturation markers in the presented experiments have been analysed, compared to other studies.

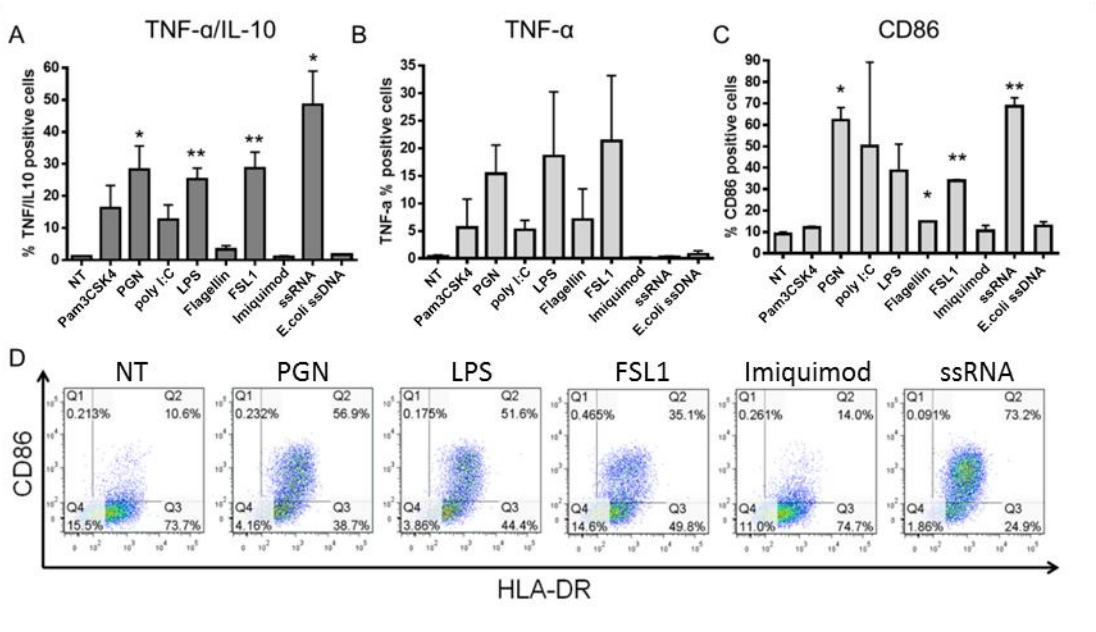

Figure 3.12. MDLC Mature and Become Activated after TLR Stimulation. MDLC were treated with TLR agonists (see Appendix 1) for 24h in the presence of brefeldin A (BfA) for cytokine production analysis, and for 48h for monitoring of maturation. Following the treatment, cells were fixed and stained, and production of TNF-α and IL10 and expression of CD86 and HLA-DR was measured by flow cytometry. **(A)**Pooled data for TNF-α/IL-10 and **(B)** TNF-α only release are shown in graphs (*n=3*). **(C)** Up-regulation of CD86 in MDLC is presented in graph (*n=3*). **(D)** A representative stimulation of MDLC for selected TLR agonist treatment is shown. NT indicates non treated cells. Error bars represent ±SEM. * $p \leq 0.05$, ** ≤ 0.01.

3.2.12. MDDC Recognise Wide Range of Pathogen Molecules Through Toll-like Receptor Activation

Similarly to MDLC, MDDC did not respond to TLR7 (Imiquimod) (mean=1.76%, SEM=0.6, p=0.78) and TLR9 (E.coli ssDNA) (mean=1.58%, SEM=0.4, p=0.9) challenge as measured by TNF-α/IL-10 production (**Figure 3.13A and B**). The TLR repertoire of MDDC does not include TLR7, and these cells are found unresponsive to Imiquimod (Cunningham et al. 2013).

In contrast, only a minor increase in cytokine release was observed in MDDC after stimulation with flagellin (TLR5 agonist) (mean=9.25%, SEM=5.5, p=0.23). Although weakly, TLR5 agonist activated MDDC, suggesting the presence of this PRR in the cells. Contradictory information is being published about the TLR range in different dendritic cell subsets. Most notably, cells differentiated from monocytes may differ between laboratories, likewise experimental settings and readouts. Nevertheless, a consensus, supporting a role for TLR5 in MDDC, is emerging from the literature and is in line with my results.

The addition of Pam3CSK4 (TLR1 agonist) into MDDC culture did not generate a significant response, although the mean percentage of TNF-α/IL-10 positive cells was high (mean=37.6%, SEM=13.9, p=0.053). Variations in TLR1 expression and responses from MDDC derived from different donors may explain the standard deviation. Nevertheless, these results tend toward a functional presence and activation of TLR1, which could also contribute to the production of different molecules once engaged. Indeed, TLR1 mRNA was detected in MDDC (Rozis et al. 2008) and agonists for TLR1 (Pam3CSK4) (and TLR5 – Flagellin) demonstrated robust production of IL-23 (Deifl et al. 2014), a cytokine not considered in my profiling.

Therefore, it is apparent that different PAMPs trigger various cytokine release profiles in MDDC and a wide array of cytokine production upon TLR stimulation would be necessary to conclude on the exact functionality for each TLR.

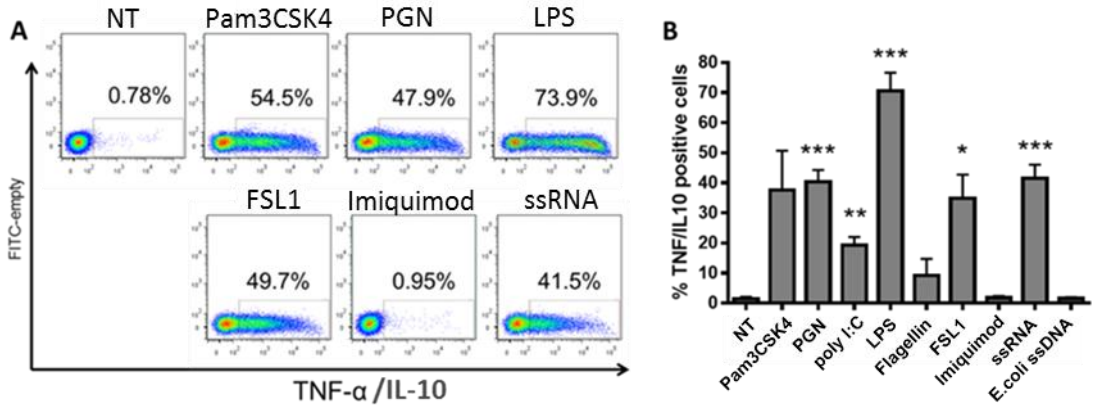

Figure 3.13. MDDC Respond to Selective TLRs Agonists' Repertoire. MDDC were stimulated with TLR agonists for 24h in the presence of brefeldin A (BfA). Following the treatment, cells were fixed and stained, and production of TNF-α and IL10 was measured by flow cytometry. **(A)** A representative stimulation of MDDC for selected TLR agonist treatment is shown**. (B)** Pooled data for MDDC in represented in graph (*n=3*). NT indicates non treated cells. Error bars represent ±SEM. * $p \leq 0.05$, ** ≤ 0.01, *** ≤ 0.001.

Furthermore, a significant stimulation of MDDC with TLR2 agonist peptidoglycan (mean=40.3%, SEM=3.9, p=0.0006), TLR4 agonist LPS (mean=70.5%, SEM=6.0, p=0.0003) and TLR8 agonist ssRNA (mean=41.6%, SEM=4.4, p=0.0009) were recorded, supporting the notion that MDDC are readily prone to highly respond to these components. Although at a lower level, viral-mimicking compound, poly I:C also significantly activated MDDC (p=0.0026). On average, 19.4% (SEM=2.6) of cells stained positive for TNF-α/IL-10 after 24 hours stimulation with poly I:C (**Figure 3.13B**). As demonstrated in the following Chapter 4, poly I:C also prompted type-I interferon production in MDDC.

The aim of the above study was to investigate responsiveness of MDLC and MDDC to TLR agonists. TNF-α/IL-10 are the cytokines usually released during the pathogen infection therefore these were used in the above profiling. The ability of my model systems to respond to TLR agonists will be further employed in Chapter 4 in the context of HIV-1 and co-infections.

3.3. Discussion

The complex system of skin immunology provides both tolerance and inflammatory responses, and involves several subsets of DC and Langerhans cells. Although an efficient method for isolation of these cells from skin samples was developed, the obtained skin layers often present mixed populations of epidermal or dermal antigen presenting cells in addition to keratinocytes (Chu et al. 2012). Accordingly, antigen-presenting cells obtained from the epidermal and dermal walkout represented the minority of the cells among heterogeneous populations in our settings. Low density of DC and, in particular, LC in skin samples requires usage of high number of walkout cells in order to obtain a good quantity of Langerin^{+} cells and CD141^{+} population. Additionally, depending on the nature of the experiment, an immune-based isolation of LC, such as MACS CD1a-beads, might be required in order to avoid contamination of results with residual keratinocytes or epithelial cells (de Jong et al. 2010a). Despite low numbers, immune walkout cells presented a typical and expected phenotype, including the presence of CD14, CD141 and CD11c markers on dermal DC, and langerin and CD1a on epidermal LC. The validity of this system is further confirmed by other studies demonstrating immunological functions of these cells, such as antigen processing and presentation, up-regulation of MHC class I and II in the presence of maturation stimuli, and T cell stimulation (Peña-Cruz et al. 2001; Chu et al. 2012).

However, the semi-matured status of primary skin isolated immune cells, possibly obtained during skin processing, was observed here (data not shown) and by others (Chu et al. 2012). LC are known to spontaneously mature in the steady-state epidermis and migrate to the lymph nodes (Kel et al. 2010; Bobr et al. 2012). Similarly, $CD141^+$ dermal DC take part in elimination of auto-reactive T lymphocytes via the process of homeostatic maturation (Probst et al. 2003; Waithman et al. 2007). Although LC migrating from steady state tissue increase expression of CD86, those cells display no stimulatory capacities, and lead to induction of tolerance and prevention of hypersensitivity development (Kaplan et al. 2005; Cumberbatch et al. 2006; Bobr et al. 2010). Thus, the semi-matured phenotype of walkout cells might be a natural behaviour of these cells. Importantly, matured DC show different susceptibility to HIV-1 infection, and the virus itself can block full maturation of these cells to prevent immune activation. Thus, matured status of primary LC and DC requires consideration when analysing the results, especially in the context of HIV-1 infection. Although results obtained in primary cells are desirable, working with these cells is problematic, time consuming and often provides variable results. It does not come as a surprise then that laboratory models for these cells have been developed (Romani et al. 1994; Sallusto and Lanzavecchia 1994). Monocyte-derived cells, mimicking dendritic cells and Langerhans cells phenotypes and functions, have definitely become the most reliable and invaluable tools used in related research. In fact, MDDC partially solved the problems related to isolation of blood DC from patient samples, and skin explants, although inherent fluctuations in variability of the results could still occur due to the primary nature of these cells. MDDC are widely accepted as a relevant *in vitro* DC model, quite similar to some blood or dermal $DC\text{-}SIGN^+$ subsets and used for equivalent studies. The results presented above confirm that MDDC

display the phenotype observed in some DC subsets, including expression of CD1a, HLA-DR and DC-SIGN. Absence of CD14 in MDDC distinguish these cells from inflammatory DC, a monocyte-derived DC subset differentiating at the site of inflammation (Hespel and Moser 2012; Mildner and Jung 2014). On the other hand, the majority of MDLC stain positive for this molecule, possibly suggesting their relationship to short-lived LC. Short-lived LC derive *in vivo* from monocytes that repopulate epidermis during infection and tissue inflammation (Romani et al. 2012; Seré et al. 2012). Both, MDLC and inflammatory LC originate from monocytes, express low levels of Langerin and survive for a relatively short time, in contrast to steady-state LC (Merad et al. 2002; Kanitakis et al. 2011; Seré et al. 2012). However, MDLC are immature in our settings, while inflammatory LC would be expected to have an activated phenotype due to their localization in inflamed tissues. Importantly, MDLC expressed higher levels of DC-SIGN than Langerin, which could skew the interpretation of HIV-1 entry in these cells, as both lectins were reported to have the opposite role in this respect (Geijtenbeek et al. 2000; Kwon et al. 2002; de Witte et al. 2007; van den Berg et al. 2014). The reverse DC-SIGN/Langerin ratio was observed in MuLC. MuLC are derived from an acute myelomonocytic leukemia cell line, MUTZ-3, and were used in this work. Langerin was expressed well by MuLC and was accompanied by a high expression of CD1a. According to the literature, MuLC were found to be semi-matured, although I recorded low levels of CD83 expression in these cells. Although the phenotyping of MuLC was limited, levels of expression of other common maturation markers, such as CD80 or CD86, might indeed be higher and therefore lead to an underestimation of matured cells in this study. In fact, MuLC are derived in the presence of TNF-α, and this cytokine triggers maturation of cultured MDDC. Depending on the maturation trigger, activated cells respond differently to

HIV infection, which could be translated into higher or lower virus replication. Some pro-inflammatory stimuli, like TNF-α, were previously described to increase HIV-1 productive infection of LC (de Jong et al. 2008; Ogawa et al. 2009). In agreement, MuLC have higher susceptibility to HIV-1 infection, when compared to immature MDLC, yet the total infection remained significantly lower than in MDDC (see Chapter 4). All together, the interpretation of results obtained with MuLC should take into account an activation status of these cells and their potential consequences on the experimental data.

Several technical challenges are associated with primary cells work. Firstly, acquisition of skin or blood samples involves setting of reliable and tested sources. Even if these conditions are met, the quality of sample can vary from donor to donor providing insufficient numbers or unsatisfactory quality of the cells. Genetic variability between primary cells samples additionally results in variation with the results. Although these donor specific differences reflect the situation normally occurring in the population, it can significantly prolong the number of experiments required to obtain statistically significant results. Limited life-span and non-proliferative properties of terminally differentiated MDLC additionally add on to the limitations of these systems. MuLC obtained from MUTZ-3 monocytic cell line were described before (Masterson et al. 2002; Santegoets et al. 2006; de Jong et al. 2010b) and were employed in this study for a reliable comparison across available LC models. Infections studies with these cells uncovered some limitation in these models including limited IFN response (Rasaiyaah et al. 2009) and activated state upon full differentiation (see Chapter 3), which could make them unsuitable for some experiments (see Chapter 4).

Importantly for this research, all investigated cell models including MUTZ-3, expressed HIV-1 entry receptor CD4, and co-receptor CCR5, at variable levels. Yet, a recent study challenged the presence of CCR5 in MuLC (Nasr et al. 2014), although these cells were reported to be susceptible to productive HIV-1 infection (de Jong et al. 2010a). The levels of HIV-1 infection of MDDC, MDLC and MuLC remain low, suggesting post-entry restriction mechanisms operating in these cells (Pion et al. 2006; Coleman and Wu 2009; Hrecka et al. 2011; Laguette et al. 2011)(see Chapter 4). Similarly, research on blood monocytes demonstrated that these cells are not permissive to HIV-1 infection *in vitro* (Filion et al. 1990; Sonza et al. 1996; Naif et al. 1998), suggesting the same profile in monocytic cell line, MUTZ-3.

Functional analysis of MuLC showed fundamental alteration of their immune responses, including lack of response to type-I interferon and consequently failure to up-regulate antiviral cellular factors such as MX2 and RIG I. It is plausible that an "activation priming" of these cells occurs during differentiation and therefore influences their responsiveness toward a second inflammatory "recall". Indeed, the modulation of gene expression upon type-I IFN in myeloid cells, previously treated with TNF-α, was recently reported to be different compared to untreated cells, with a significant decrease in expression of genes related to immune responses, cell migration and proliferation (Henig et al. 2013). Previous studies described the impaired expression of genes encoding immune sensing and signalling in MUTZ-3 derived cells (Rasaiyaah et al. 2009). Among other genes such as STAT family members, interferon regulatory factor 8 (IRF8) was down regulated at the mRNA and protein levels, possibly affecting regulation of interferon inducible genes (Rasaiyaah et al. 2009). In such cases, the observed irresponsiveness of MuLC to IFN-α stimulation could be a consequence of disturbed type-I interferon signalling in these

cells. Another IRF transcription factor, IRF9, also locates downstream from the Interferon Receptor (Platanias 2005) and its function might explain the observed up-regulation of APOBEC3F expression in MuLC. While the lack of expression or functionality of IFN receptor(s) or IFN signalling proteins cannot be excluded, it is conceivable that MuLC have reached a saturating activated state upon differentiation that would therefore dampen a subsequent re-stimulation.

In contrast, interferon stimulation of MDLC and MDDC resulted in up-regulation of all investigated Interferon-inducible proteins, however, a marked difference was seen in the amount of APOBEC3G expression upon IFN-α challenge of MDLC and MDDC. Both MDLC and MDDC derive from monocytes isolated from the same donor, and differ only in the growing conditions by the presence of TGF-β in the medium of MDLC. TGF-β was reported to exert a strong immunosuppressive effect on immune cells (Letterio and Roberts 1998; Kobie et al. 2003; Li et al. 2006b), which may facilitate the progression of HIV-1 infection in patients (Card et al. 2012). It shapes the function and phenotype of LC (Letterio and Roberts 1998; Li et al. 2006b) and was also demonstrated to decrease the ability of DC to mature in response to TLR agonists (Sewankambo et al. 1997). Perhaps, due to these properties, TGF-β also exerts a direct or indirect negative effect on APOBEC3G expression in MDLC. However, this theory requires verification.

Unexpectedly, Langerin was identified as a type-I, not type-II, interferon inducible molecule, suggesting an antiviral role for this receptor. IFN-α/β and IFN-γ share some common signalling components and induce similar genes. Nonetheless, they also differ in several aspects, including binding receptor, promoter and in the nature of the immune responses they induce and contribute to (Goodbourn et al. 2000). Therefore, modulation of Langerin may be dependent on type-I Interferon induced signalling, but

irresponsive, or weakly responsive, to type-II Interferon stimulation. It would be interesting to see whether other pro-inflammatory signals trigger similar induction of Langerin in these and other myeloid cells. The possible increase in total Langerin during inflammation might imply a role of this molecule as yet another IFN-inducible defence mechanism in the mucosal area. This aspect is novel, as Langerin has never been classified as an interferon-stimulated gene. Nevertheless, Langerin was already known for its ability to bind pathogens and target them toward Birbeck granules, specific LC structures thought to participate in the degradation of internalized foreign entities (de Witte et al. 2007). Thus, a more pronounced Langerin expression observed on MDLC cell surface could be of importance regarding viral infection and inflammation. Such a mechanism would reinforce the binding of microbes to this C-type lectin receptor and their consequent degradation. Accordingly, short lived LC populating the epidermis during inflammation express higher amount of Langerin (Romani et al. 2012; Seré et al. 2012). Thus, the ability to up-regulate Langerin could be an adaptation of Langerhans cells acquired during infection. Intracellular Langerin possibly serves as a recycling source or contributes to formation of Birbeck granules. Further investigation of this phenomenon would be interesting to decipher the role of this CLR in infection and inflammation.

Other TGF-β-dependent differences distinguishing MDLC from MDDC were reflected in TLR profiles, as well as HIV-1 infection susceptibility (see Chapter 4). Regarding TLR agonist responses, both MDLC and MDDC displayed TLR-induced activation similar to those described in the literature (Flacher et al. 2006), but slightly different to their *in vivo* counterparts (Kadowaki et al. 2001; Zarember and Godowski 2002). The main inconsistencies between MDLC and primary LC were observed in their response to TLR4 agonist lipopolysaccharide (LPS). Skin and mucosal LC are

thought to lack TLR4 receptor in their repertoire, due to constant exposure of these cells to commensal bacteria. In contrast, a significant production of pro-inflammatory cytokines from MDLC upon TLR4 engagement was observed in this work. TLRs can form heterodimers and extend their repertoire and responses (Triantafilou and Triantafilou 2002; Zarember and Godowski 2002). In the case of LPS, several clusters of receptors are involved in sensing and signalling. One of the potent LPS binding molecules is CD14 (Wright et al. 1990), which forms activation clusters with other components, usually including TLR4. However, recognition of *Leptospira interrogans* in macrophages is independent of TLR4 expression, but instead requires CD14 and TLR2 (Werts et al. 2001). Perhaps similarly, CD14-dependent mechanisms of LPS recognition operate in MDLC, as these cells express high amounts of this molecule. Whether sensing is TLR4-dependent, and involves TLR2 or other receptors, was not investigated further.

MDLC stimulated with TLR8 agonist, ssRNA, led to predominant IL-10 production, suggesting a tolerogenic response to this molecule. However, simultaneous up-regulation of CD86 on these MDLC might indicate concomitant maturation and stimulatory properties of these cells. According to the literature, LC-induced tolerance occurs despite up-regulation of co-stimulatory molecules CD86 and CD80, and is consistent with the failure to translocate NF-κB family member RelB from the cytoplasm to the nucleus (Shklovskaya et al. 2011). Based on this information and the above results, it seems that TLR8 stimulation of Langerhans cells might result in tolerogenic functions of these cells. Data available on TLR8 in MDLC are contradictory in terms of expression of this receptor, therefore interpretation of this result should be supported by a TLR8 mRNA expression profile.

Interestingly, neither MDLC nor MDDC were activated after challenge with *E.coli* ssDNA, which could have perhaps resulted from problems with agonist stock, or its dose used in our research. Other TLR9 agonists, such as CpG-A ODN D19 might have been a better choice as it has been previously reported to activate MDDC (Hoene et al. 2006). Intracellular localization of TLR9 might also make it challenging to successfully deliver the agonist, therefore could be responsible for observed lack of activation in MDDC and MDLC. The reports on TLR9 expression in some primary DC subsets and LC are contradictory, but most indicate absence of this PRR in these cells. Therefore, the results might indicate a normal response observed in cells.

MDDC mimicked their primary counterparts in all, but one, response to TLR agonists. Accordingly, flagellin stimulation of MDDC induced cytokine release from these cells, which is generally not observed in dendritic cells. However, the cytokine release in MDDC in response to TLR5 agonist was low, suggesting that MDDC may express only low levels of this PRR.

The above results demonstrate that MDDC provide a reliable model for DC in context of their phenotype, TLR responses, and Interferon induced up-regulation of viral restriction factors. On the other hand, MDLC have to be used with greater caution due to their conflicting C-type lectin receptor phenotype (expressing both DC-SIGN and Langerin), and a different TLR repertoire. Interferon responses of these cells seem to be unaffected and match those expected from primary LC. The opposite situation applies to MuLC, for which the phenotype is like the one observed in skin LC, but Interferon responses are distorted. Thus, depending on the nature and requirements of the study, MuLC or MDLC may be a preferable system. Primary cells isolated from tissue samples still provide a great tool, however due to difficulties related to their

acquisition, these cells are mostly used to confirm the findings acquired in cell models, MuLC or MDLC.

4. TGF-β Induces HIV-1 Restriction in MDLC

4.1. Introduction

Due to their mucosal distribution, Langerhans cells (LC) are likely early cellular targets for HIV-1 during sexual transmission (Kawamura et al. 2005; Hladik et al. 2007; Piguet and Steinman 2007). In common with other myeloid dendritic cell (DC) subsets, LC do not readily support virus infection. However, LC are also potential carriers promoting HIV-1 transfer to susceptible CD4+ T cells (Niedecken et al. 1987; Miller and Shattock 2003). Low HIV-1 infection of LC was previously attributed to the presence of Langerin (a LC-specific C-type lectin receptor), which forms a protective barrier against the virus (Kawamura et al. 2000; de Witte et al. 2007). Indeed, Langerin expressed on LC surface efficiently binds incoming viral particles leading to their internalisation and degradation in LC lysosomal compartments called Birbeck granules. Other DC subsets restrict HIV-1 infection due to the presence of cellular restriction factors, such as SAMHD1 (Berger et al. 2011a; Hrecka et al. 2011; Laguette et al. 2011), APOBEC3G (Pion et al. 2006), Bst-2/tetherin (Neil et al. 2008) and the Interferon-inducible MX2 protein (Goujon et al. 2013; Kane et al. 2013). SAMHD1 was shown to be highly expressed in cells of myeloid origin, as well as in quiescent $CD4^+$ T cells, in which it mediates depletion of the cellular deoxynucleoside triphosphate (dNTP) pool, leading to a drastic impediment to HIV-1 reverse transcription (Goldstone et al. 2011; Lahouassa et al. 2012). Additionally, SAMHD1 imposes an antiviral activity independent of its effect on deoxynucleotide levels but by direct binding and cleavage of HIV-1 ssRNA (Beloglazova et al. 2013; Reinhard et al. 2014; Ryoo et al. 2014). The primate lentivirus auxiliary protein (Vpx*),* which is expressed in HIV-2 and some SIV viral genomes, triggers degradation of SAMHD1 and consequently rescues viral infection in resistant myeloid cells and quiescent T

cells (Hrecka et al. 2011; Laguette et al. 2011; Descours et al. 2012). Therefore, the absence of a Vpx gene in the HIV-1 genome makes SAMHD1 a major restriction to HIV-1 in myeloid cell lineages (Berger et al. 2011a) but it also prevents efficient viral sensing in these cells (Manel et al. 2010). The only cellular restriction described so far in LC involves Langerin, which induces viral degradation after capture and internalization of incoming virions (de Witte et al. 2007), although particularly functional at low viral doses (de Jong et al. 2008). Little is known about post-entry restrictions to HIV-1 in LC. Previous work indicates that high viral titres of HIV-1 can lead to productive LC infection, despite the presence of Langerin, and consequently, increased HIV-1 transmission to T cells (Collins et al. 2000; de Witte et al. 2007; de Jong et al. 2008; Kawamura et al. 2008). Importantly, *de novo* HIV-1 production in LC is also reported in cells that are matured in response to sexually transmitted infections (STIs), in particular gram-positive bacteria (de Jong et al. 2008; Ogawa et al. 2009; Ogawa et al. 2013) or herpes simplex virus (HSV) (Ogawa et al. 2013). STIs are strongly associated with increased risk of HIV-1 acquisition (Sewankambo et al. 1997; Galvin and Cohen 2004; Peretti et al. 2005), and LC susceptibility to HIV-1 infection might play a role in the context of co-infections. Productive HIV-1 infection of LC also promotes viral transfer to $CD4^+$ T cells (Kawamura et al. 2000; de Jong et al. 2008; Ogawa et al. 2009; Ganor et al. 2013). Therefore understanding the balance between cellular restrictions and viral dissemination by LC may be central to better understand the early events of HIV-1 infection, especially during co-infection with other bacterial or viral pathogens.

4.2. Results

4.2.1. SIV3-Vpx-mediated Degradation of SAMHD1 Uncovers a Novel HIV-1 Restriction Mechanism in Immature Langerhans Cells

To explore the role of SAMHD1 in LC I used two well-established cell models of LC: monocyte-derived Langerhans cells (MDLC) and Mutz-3-derived Langerhans cells (MuLC) (Masterson et al. 2002; Santegoets et al. 2006; de Jong et al. 2010a) (see Chapter 3). Both models were previously reported to be relevant to analyze interactions between LC and HIV-1, and both display significant phenotypic and functional similarities to skin-resident Langerhans cells, as verified by us (see Chapter 3) and others (Geissmann et al. 1998; Ginhoux et al. 2006). First, I demonstrated that MuLC, and autologous MDLC and MDDC express the SAMHD1 protein. Of note, expression in MDDC was slightly more abundant than in LC, as quantified by ImageJ (**Figure 4.1A and B**). I then confirmed that SAMHD1 expression was down-regulated in MuLC and MDLC after transduction with Vpx-expressing SIV-derived lentivectors (SIV3-Vpx), to a similar extent compared to MDDC (**Figure 4.1A and B**) for which Vpx-mediated SAMHD-1 degradation was previously described (Miyagi et al. 2009; Hrecka et al. 2011; Laguette et al. 2011). Indeed densitometry quantification on western-blotting experiments showed that the SAMHD1 signal was down-regulated on average by 87.6% (SD=12, SEM=8.5) in MDDC, 94.6% (SD=2.2, SEM=1.9) in MDLC and 94.6% (SD=2.7 SEM=1.55) in MuLC (**Figure 4.1B**). Vpx-mediated SAMHD1 degradation in DC and LC was highly reproducible and very rapid, as shown by the down-regulation of expression already observed 30 minutes after transduction with Vpx-expressing SIV-derived lentivectors and almost complete degradation after 3h (**Figure 4.1C**). In addition, SAMHD-1 was absent from MDLC

and MDDC even after 6 days post transduction with SIV3-Vpx, suggesting a long-lasting down-regulation of this restriction factor in cells (**Figure 4.1D**). Therefore, the system of SAMHD-1 degradation used in my study was effective, reproducible, time-dependent and long lasting. SAMHD-1 down modulation was achieved in all cell systems at the comparable level proving the reliability of this method. To assure the consistent performance, SIV3-Vpx stocks were rigorously calibrated throughout its storage with satisfactory outcome. Accordingly, the efficiency of SIV3-Vpx to down regulate SAMHD-1 in MDLC and MDDC was tested every few weeks in these cells by western blot (see **Materials and Methods, Section 2.8.**5). For that reason, SAMHD-1 western blot controls were not run with each individual experiment. Although the outcomes were reproducible between donors, I cannot exclude incomplete degradation of SAMHD-1 in some untested samples.

Apart from Vpx, SIV Vpr protein is packed to the SIV3-VLP particles during stock production and it is therefore delivered to target cells (see **Introduction, Section 1.2**). Although Vpr is not known to have an effect on SAMHD-1 in the cells, this accessory protein is a potent modulator of immune biology (see **Introduction, Section 1.2**). Thus, the presence of Vpr in the SIV3-Vpx could have some unwanted effects on the experimental system, such as increasing the viral infection of cells and dampening of cell immune activation (Ayyavoo et al. 2002; Majumder et al. 2005; Kogan et al. 2013; Harman et al. 2015). Therefore, the potential impact of the Vpr on my experimental outcomes will be considered in this Chapter.

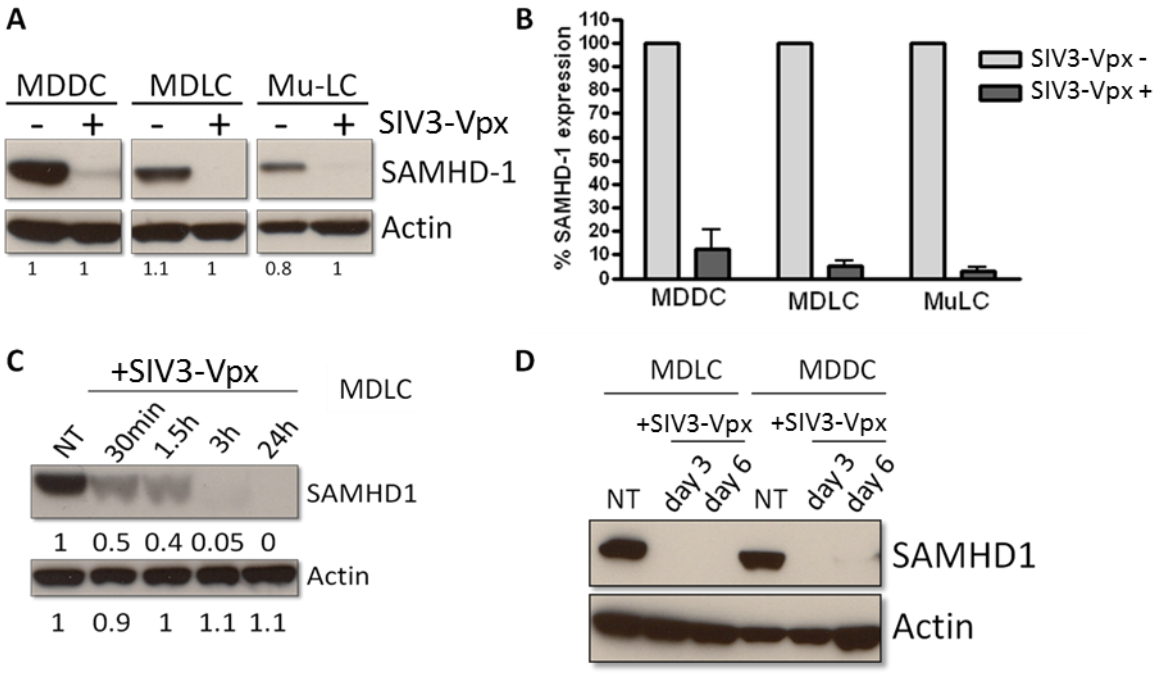

Figure 4.1. Effective Vpx-mediated Degradation of SAMHD-1 in Different Myeloid Cellular Models. MDDC, MDLC and MuLC were treated with Vpx-expressing SIV-derived lentivectors for 4 hours, unless stated otherwise on the figure, followed by cell lysis and western blot analysis of SAMHD-1 expression. **(A)** A representative experiment and **(B)** pooled data for SAMHD-1 down-regulation are shown (*n=3*). **(C)** Lysates of MDLC treated with Vpx-expressing SIV-derived lentivectors for 30min, 1.5hour, 3hours and 24hours were analysed by western blot, and the results are shown. **(D)** Similarly, MDLC and MDDC treated as above for 3 and 6 days were analysed for SAMHD-1 expression. In all experiments actin served as a loading control. Quantification of SAMHD-1 was performed by densitometry analysis using IMAGEJ software, and was normalized to actin levels. Error bars represent ±SEM.

Although comparable Vpx-mediated degradation of SAMHD1 was evident in both MDDC and MDLC (**Figure 4.1B**), I noticed a significantly lower HIV-1 (R5) infection of MDLC (5.9%, SD=3.2%, SEM=1.84) compared to MDDC (55.5%, SD=11.9, SEM=6.84) (**Figure 4.2A** (a representative experiment) **and B** (pooled data)). SIV3-Vpx delivery into MDDC caused a 9.3 fold enhancement of HIV-1

infection (SEM=1.0) compared to non-transduced cells similar to the effect observed in other studies (Hrecka et al. 2011; Laguette et al. 2011). In contrast, MDLC remain in part refractory to HIV-1 infection, even in the absence of SAMHD-1 as demonstrated by the average 5.5 fold increase (SEM=3.3) of gag positive MDLC (**Figure 4.2C**) when compared to the 9.3 fold increased observed in MDDC.

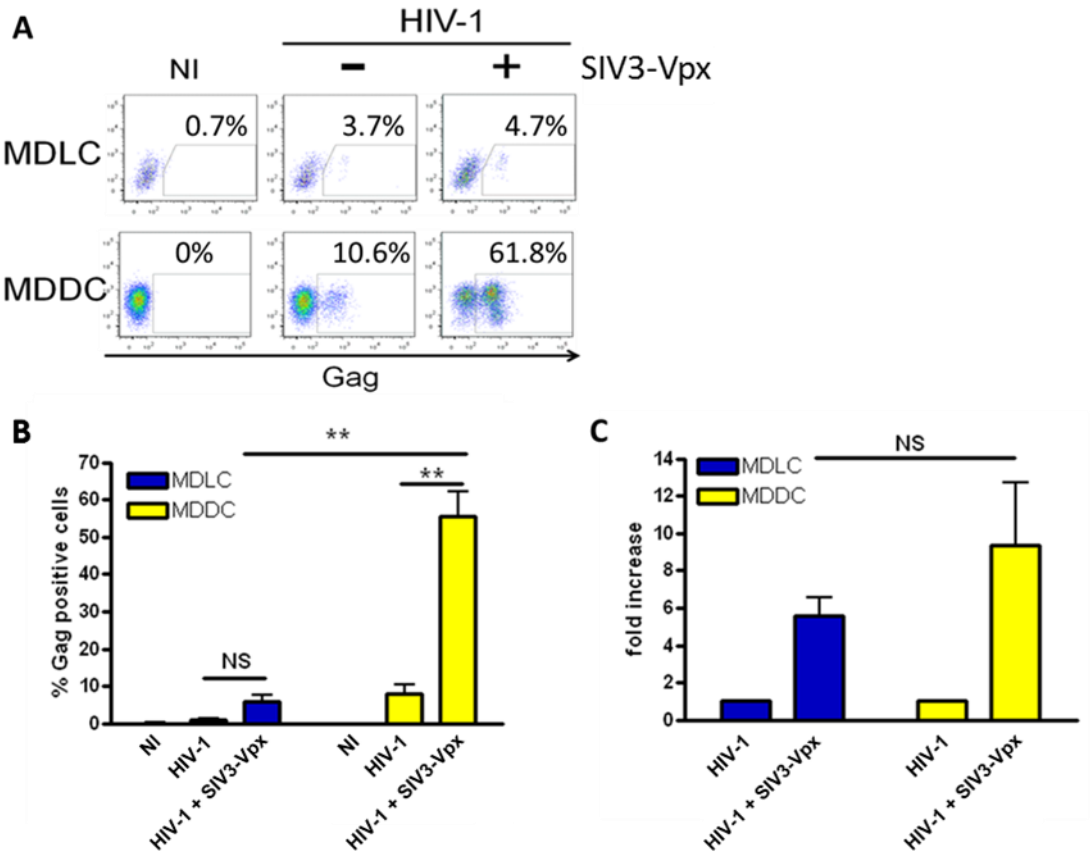

Figure 4.2. SAMHD-1-Independent Restriction Activity in HIV-1-R5 Infected MDLC. Autologous MDLC and MDDC were infected with full length HIV-1 (R5) (strain R8Bal at 25ng p24/10^5 cells), both with (HIV+SIV3-Vpx) and without (HIV) pre-treatment for 4 hours with VSV-G pseudotyped Vpx-encoding SIV3 lentivector (SIV3-Vpx), or left uninfected (NI). Four days later, cells were fixed, washed, stained with anti-HIV-1-Gag and infection levels were measured by flow cytometry. **(A)** A representative experiment and **(B)** quantification of pooled data for MDLC and MDDC are shown (*n=3*). **(C)** Fold increase of HIV-1 infection in MDLC and MDDC in the presence of SIV3-Vpx is showed in graph (*n=3*). The statistical significance of the results was calculated using student t-test and significant values indicated (*) as described in Materials and Methods. NS= insignificant. Error bars represent ±SEM.

The same resistance to HIV-1 infection was observed when using the MuLC cell line, with only 6% (SEM=0.866) of cells infected in the presence of SIV3-Vpx (**Figure**

4.3A and B). In contrast, an average 66% (SEM=8.079) of MDDC stained positive for HIV-1 p24 gag-FITC (**Figure 4.3B**). Interestingly, while infected MDDC appear as a separate population on flow cytometry analysis, Gag^{+} MuLC do not present a clearly distinguished infected population suggesting that gag expression is also low at the cell level or that gag staining in case of MuLC infection is a result of pseudotransduction and not a productive infection (**Figure 4.3A**) (and see Figure 4.7). Low percentage (0.2-0.3%) of uninfected MuLC and MDDC stained positive for gag despite the lack of HIV-1 in experimental conditions. These obvious false positive populations may represent autofluorescent cells or cells that uptake KC57 gag antibody. Due to use of primary cells that often form complex population in terms of side and forward scatter (SSC and FSC, respectively) minimal background staining and GFP expression appear in the non infected populations. Possibly, a stricter gating strategy could be applied when necessary. However, the drawback of a stricter gating could be a significant loss of detection of truly infected population. For that reason, the cut off point was set as close to the main population as it allows for the accurate measurement of infection. I assessed that my gating strategy has no significant impact on the patterns of infection observed in the experiments, and it provides the optimal balance between low background and accurate detection of Gag/GFP positive populations.

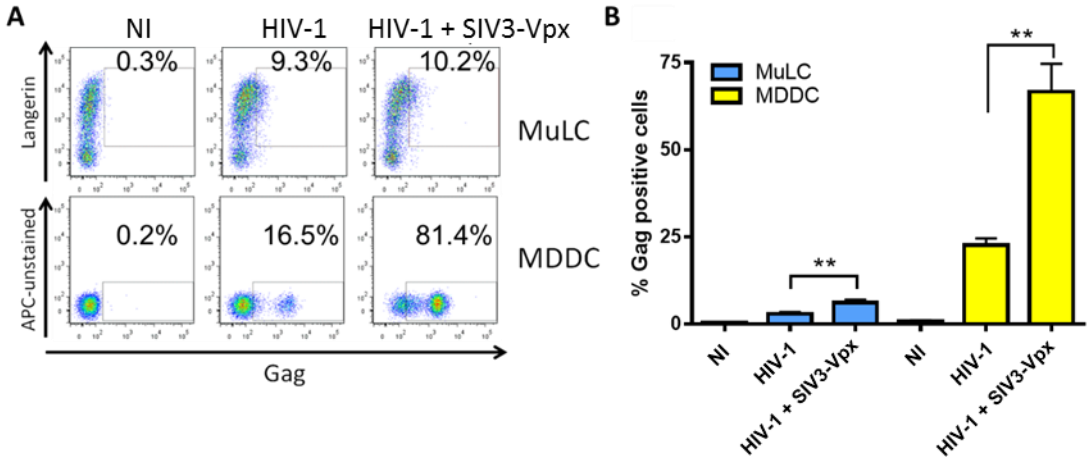

Figure 4.3. Non-premissiveness of MuLC to HIV-1 Infection. MuLC and MDDC were infected with full length HIV-1 (R5) (strain R8Bal at 25ng p24/10^5 cells), both with (HIV+ SIV3-Vpx) or without (HIV) pre-treatment for 4 hours with VSV-G pseudotyped Vpx-encoding SIV3 lentivector (SIV3-Vpx), or left uninfected (NI). Four days later cells were fixed, washed, stained with anti-HIV-1-Gag and infection levels were measured by flow cytometry. **(A)** A representative experiment and **(B)** quantification of pooled data for MuLC and MDDC are shown (*n=3*). The statistical significance of the results was calculated using student t-test and significant values indicated (*) as described in Materials and Methods. Error bars represent ±SEM.

In order to verify MDDC as an appropriate infection control used alongside MDLC and MuLC I infected MDDC and a Vitamin D induced monocyte-derived dermal DC model ($CD141^+$ DC) (see **Materials and Methods Section 2.3.4**) with full length HIV-1 in the presence or absence of SIV3-Vpx. I observed similar constitutive and Vpx-mediated down-regulation levels of SAMHD-1 expression in both cell types after 24 hours (**Figure 4.4A**). Although the control for SAMHD-1 expression at 6 days time point has not been performed for this specific experiment, the results demonstrated in **Figure 4.1.D** show successful SIV3-Vpx mediated SAMHD-1 depletion in MDDC up to at least 6 days.

Flow cytometry analysis of Gag^+ cells revealed a significantly higher HIV-1 infection of $CD141^+$ DC compared to MDDC ($p=0.0387$) 4 days post infection when SAMHD1 expression was previously shut-down (**Figure 4.4B**). However, at day 6, the average infection of $CD141^+$ DC in the absence of SAMHD-1 reached 73.8% (SEM=9.82) and was comparable to that observed in MDDC (75.1%, SEM=11.88) (**Figure 4.4C**). Of note, I could observe a significant HIV-1 infection in MDDC occurring at day 6, even when expressing SAMHD-1 (p24 Gag^+ MDDC= 36.0%, SEM=12.6), but still susceptible to AZT inhibition (p24 Gag^+ MDDC= 0.07%, SEM=0.032). All together, these data demonstrate similar high predisposition of both, MDDC and CD141+ DC to HIV-1 infection in the absence of SAMHD-1.

Based on these results I confirmed that MDDC could therefore represent a relevant dendritic cell model for our studies of HIV-1 infection, particularly when considering mucosal DC subsets.

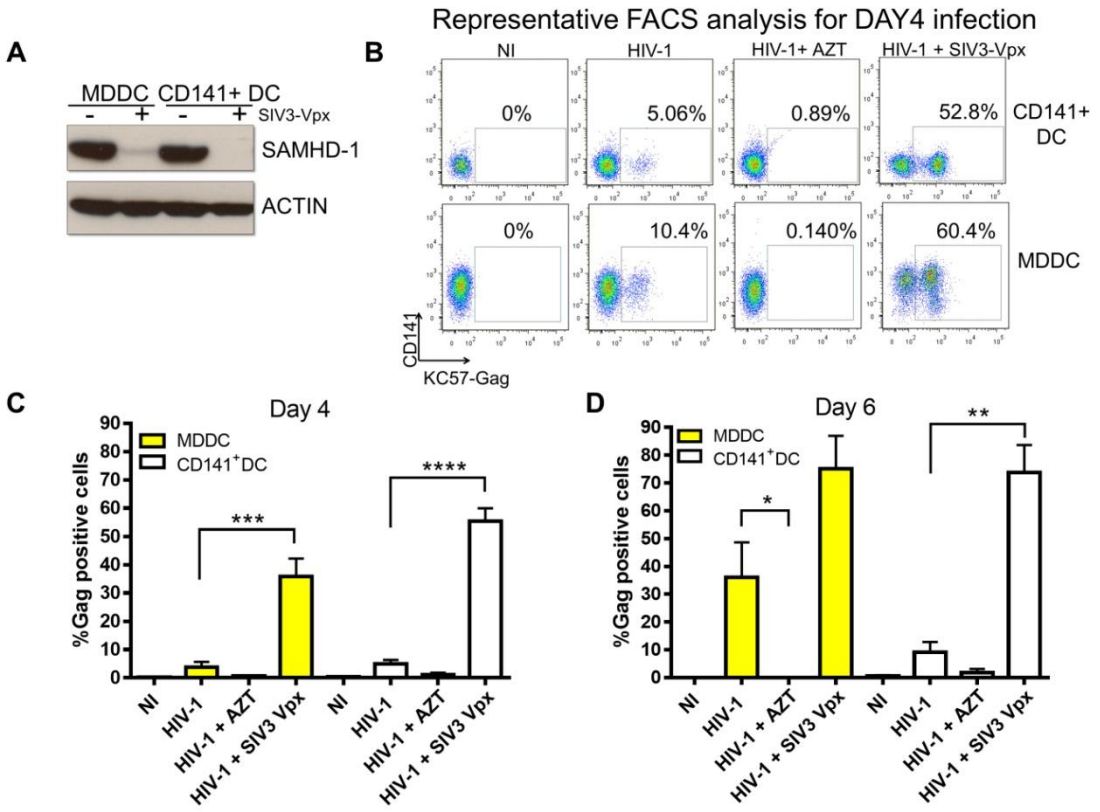

Figure 4.4. HIV-1 Infection is Comparable between MDDC and CD141$^+$ Dendritic cells. Homologous MDDC and CD141$^+$ cells were treated or not with SIV3-Vpx for 4 hours and infected with 25ng p24 HIV-1 (R8Bal). AZT [1µM] was added to indicated wells 1 hour prior to addition of HIV-1. **(A)** SAMHD-1 down-regulation in treated cells was analysed by western-blotting after 16 hours post SIV3-Vpx addition. The levels of p24 in cells at days 4 and 6 post infection were measured. **(B)** A representative FACS analysis is shown. **(C)** Pooled data for days 4 and **(D)** 6 are shown in ($n \geq 5$) and ($n \geq 3$), respectively. NI indicates non-infected cells, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Error bars represent ±SEM.

The HIV-1 envelope glycoprotein was shown to bind Langerin, which has been previously reported to protect LC from HIV-1 infection by capturing incoming virions and targeting them for degradation in Birbeck granules (de Witte et al. 2007). Moreover the efficiency of viral entry into LC could be affected by differential expression of cell surface HIV receptors/co-receptors (CD4 and CCR5) compared to

other viral target cells. Therefore in order to evaluate if HIV-1 restriction occurred at the entry or post-entry level I transduced MDDC and MDLC with Vesicular Stomatitis Virus G protein (VSV-G)-pseudotyped HIV-1 lentiviral vectors encoding green fluorescent protein (GFP) (VSV-G HIV-GFP) (Dull et al. 1998). Vesicular Stomatitis Virus binds to low density lipoprotein receptor (LDLR) and fuses with the cell membrane only after pH change in lysosomes (Sun et al. 2005; Johannsdottir et al. 2009; Cureton et al. 2010; Finkelshtein et al. 2013). Therefore, VSV-G pseudotyped lentivector would overcome entry receptor limitations imposed normally on HIV-1 envelope and ensure effective and comparable entry into MDLC, MDDC and MuLC. The entry efficiency of VSV-G pseudotyped constructs is validated in this study by almost complete down regulation of SAMHD-1 in cells treated with VSV-G pseudotyped SIV3-Vpx (see **Figure 4.1**).

Both MDDC and MDLC infected with VSV-G HIV-GFP demonstrated low transduction efficiency (**Figure 4.5A and B**). Surprisingly when these cells were previously transduced with Vpx-expressing SIV-derived lentivectors, only MDDC were strongly and significantly infected with VSV-G HIV-GFP (**Figure 4.5B**), reminiscent of the productive infection observed with full length HIV-1 R5 (see **Figure 4.2**). Indeed after down-regulation of SAMHD1 expression I observed that 53.8% (SD=22.1, SEM =9.874, MFI=2292) of MDDC were expressing GFP compared to only 5.9% (SD=1.6, SEM=0.816, MFI=628) of MDLC (**Figure 4.5B**). Addition of SIV3-Vpx to MDDC prior to infection with VSV-G HIV-GFP, triggered 72.6 fold (SEM=12.1, SD=34.25) increase percentage of GFP+ cells (p=0.0001), while susceptibility of MDLC increased by 16.4 fold (SEM=5.6, SD=15.0) (p=0.0187) (**Figure 4.5C**). As mentioned above, poor permissiveness of MDLC to VSV-G envelope can be ruled out as a reason for the observed discrepancies in

MDLC and MDDC susceptibility to infection with VSV-G HIV-GFP. This statement is based on the ability of VSV-G pseudotyped SIV3-Vpx to down modulate SAMHD-1 levels to comparable extend in both cell types. Additionally, as these cells originate from the same precursors, the LDLR composition of the cell membrane would not be expected to vary, thus making VSV-G HIV-GFP entry comparable in both MDLC and MDDC. However, as VSV-G HIV-GFP fuses with endosomal membrane, it bypasses most of the cytoplasm. This can differentially affect interaction of lentiviral capsid with cytoplasmic proteins in MDLC and MDDC, possibly resulting in different kinetics of the infection.

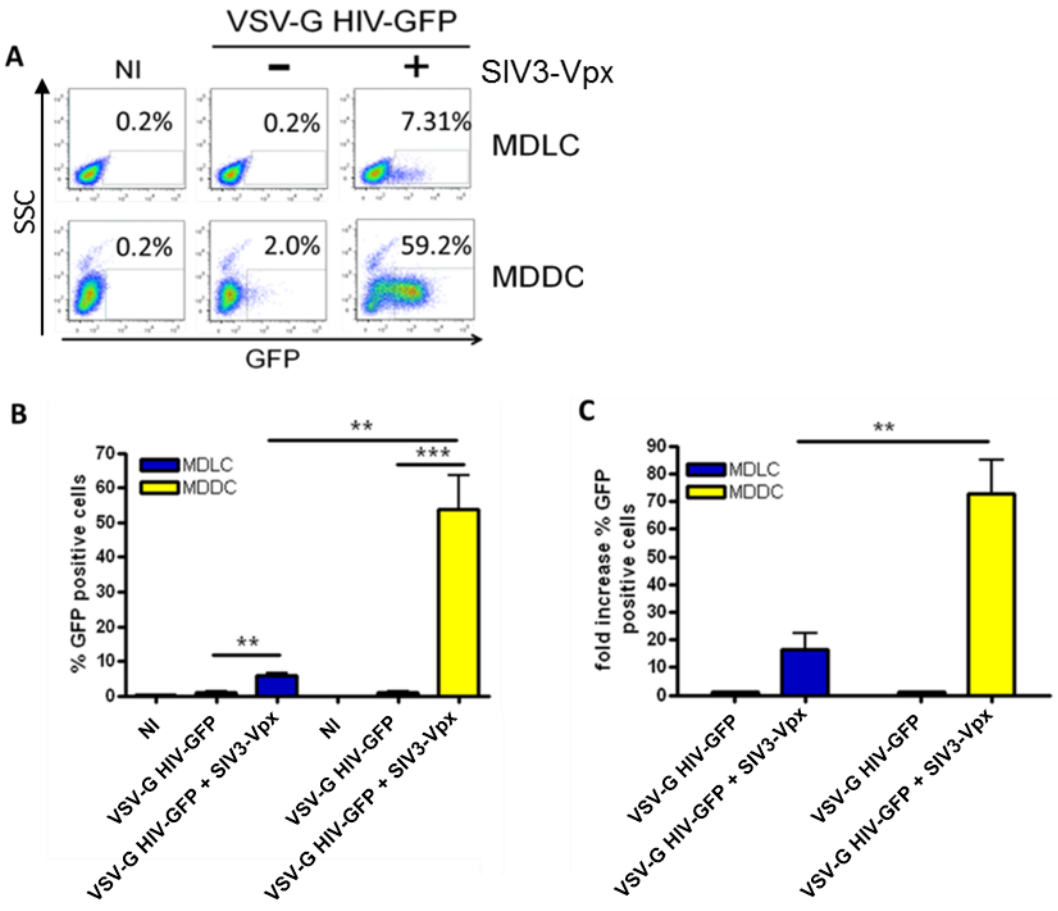

Figure 4.5. MDLC Infection with VSV-G HIV-GFP Lentivirus. Autologous MDLC and MDDC were transduced (or not) with VSV-G pseudotyped Vpx encoding SIV3 lentivector (SIV3-Vpx) prior to challenge with VSV-G HIV-GFP (VSV-G HIV-GFP) (30ng p24/10^5 cells) and the percentage of GFP positive cells was analyzed by flow cytometry after 3 days. **(A)** A representative experiment and **(B)** quantification of pooled data for MDLC and MDDC are shown (*n=7*). **(C)** Fold increase of HIV-1 infection in MDLC and MDDC in the presence of SIV3-Vpx is shown (*n=7*). The statistical significance of the data was calculated using student t-test and significant values indicated (*) as described in Materials and Methods. Error bars represent ±SEM.

This low infection pattern of MDLC was further confirmed using MuLC, which were only 2.2 fold (SD=1.31, SEM=0.655) more sensitive to VSV-G HIV-GFP when

SAMHD1 expression was down regulated (p=0.1545) (**Figure 4.6A**). In contrast, in the presence of SIV3-Vpx, susceptibility of MDDC to infection increased 55 fold (SD=25.512, SEM=12.756) (p=0.0241). This profile was reflected in significantly higher infection of MDDC compared to MuLC (p=0.011). On average 63% (SD=25.1, SEM=12.593) of MDDC became GFP positive after VSV-G HIV-GFP + SIV3-Vpx challenge, however MDDC remained refractory to transduction in the presence of SAMHD-1 (**Figure 4.6B** (representative experiment) **and C** (pooled data)). When compared to MDDC, MuLC appeared less permissive to VSV-G HIV-GFP if pre-treated with SIV3-Vpx (mean=17.1%, SD=3.517, SEM=1.758), yet small percentage of MuLC (8.6%, SD=2.344, SEM= 1.172) seemed to support lentivirus transduction even when SAMHD-1 remained in the cells.

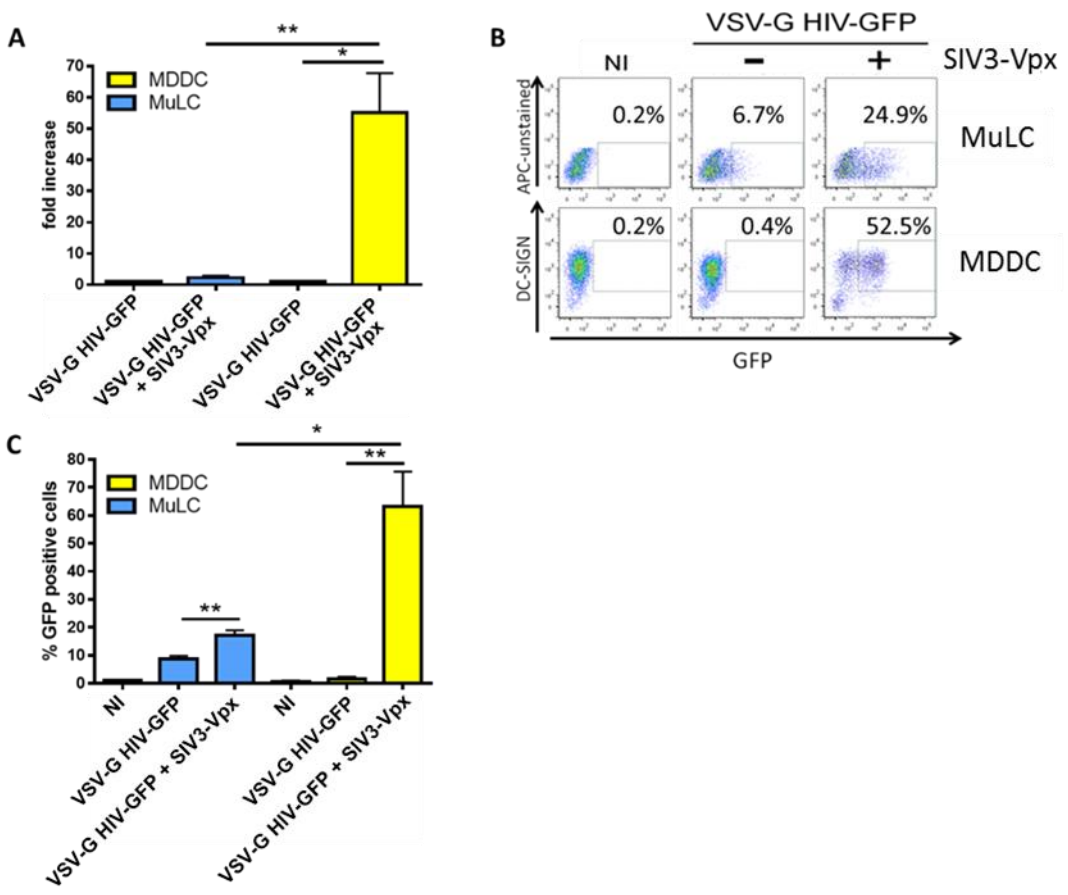

Figure 4.6. Low VSV-G HIV-GFP Infection of SAMHD-1 Depleted MuLC. MuLC and MDDC were transduced (or not) with VSV-G pseudotyped Vpx encoding SIV3 lentivector (SIV3-Vpx) prior to challenge with VSV-G HIV-GFP (VSV-G HIV-GFP) (30ng p24/10^5 cells) and the percentage of GFP positive cells was analyzed by flow cytometry. **(A)** Fold increase of HIV-1 infection in MuLC and MDDC in the presence of Vpx is showed in graph (*n=7*). **(B)** A representative experiment and **(C)** quantification of pooled data for MuLC and MDDC are shown (*n=7*). The statistical significance of the results was calculated using student t-test and significant values indicated (*) as described in Materials and Methods. Error bars represent ±SEM.

Further analysis of VSV-G HIV-GFP transduced MDDC, previously treated with Vpx-encoding lentivectors, revealed a significant effect of Zidovudine (AZT) on the

GFP expression in MDDC ($p<0.01$), which was, however, absent in MuLC ($p=0.859$) (**Figure 4.7. A and B** (representative FACS plot)). Indeed GFP expression in AZT pre-treated MuLC decreased from 12.8% to 12.45% only (SEM=1.69% and 1.06, n=4) compared to inhibition of 50.1% GFP positive MDDC (SEM==6.01%, n=3). In contrast, AZT pre-treatment of MDLC successfully stalled lentivector infection from 4.4% (SEM=0. n=1) to 0.2% (SEM=0, n=1) in these cells (**Figure 4.7. A and B**). The lack of AZT effect on GFP expression in MuLC was unexpected since this pharmacological compound used in the clinics is a potent HIV-1 reverse transcriptase inhibitor. Although I have not gone into the details of such phenotype, a possible explanation could be that MuLC derived from tumour cell line harbour higher levels of nucleotides in their cytoplasm that renders AZT weakly efficient. Alternatively, infection "positive" cells could represent a passive transfer of GFP that may be present in the producer cells (HEK293T) supernatant (Nash and Lever 2004). This process termed pseudotransduction can especially influence the readout of infection at early time points of the experiments. However, passively transferred GFP does not amplify and is degraded within a few days (Nash and Lever 2004). Therefore, infection readout at later time points of experiment indicate newly synthesised GFP protein and is unlikely "contaminated" by pseudotransduced molecules. However, this does not explain why GFP levels are increased in SIV3-Vpx pre-treated MuLC (see **Figure 4.6**) and why they remain insensitive to AZT treatment even after 3 days of infection.

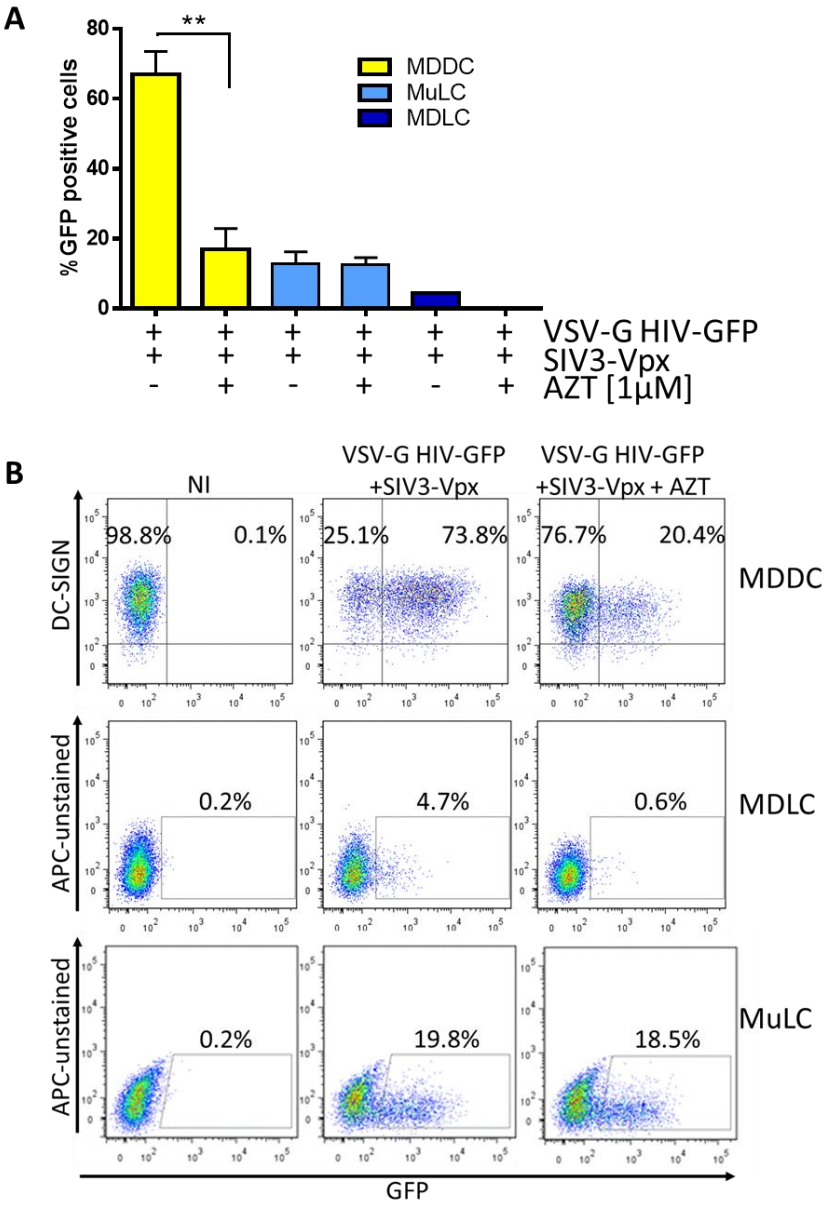

Figure 4.7. AZT Inhibits Virus Infection in MDDC and MDLC but not MuLC. MuLC, MDLC and MDDC were pre-treated with VSV-G pseudotyped Vpx encoding SIV3 lentivector (SIV3-Vpx) and AZT [1µM] (where indicated) for 3 hours before being transduction with VSV-G HIV-GFP (30ng of p24). After 3 days of infection, cells were collected and analyzed by FACS for GFP expression. **(A)** AZT-mediated inhibition of GFP expression in MDDC (p=0.0049) (*n=3*), MDLC (*n=1*) but not MuLC is shown (*n=4*). **(B)** Representative FACS plot for VSV-G HIV-GFP + SIV3-Vpx infection of MDDC, MDLC and MuLC in the absence and presence of AZT treatment is shown. Error bars represent ±SEM.

Interestingly, even in the absence of SAMHD1 viral restriction in MDLC could not be overcome with increasing doses of VSV-G HIV-GFP (**Figure 4.8A**). At high virus dose (100ng p24) MDLC infection remained below 12% (SD=9.291, SEM=4.155), while MDDC were on average 35.8% (SD=17.151, SEM=7.67) GFP positive (p=0.024) (**Figure 4.8B**). Furthermore a high inoculation dose of HIV-1 was not enough to pass restriction within MDLC, which even in the presence of SIV3-Vpx reached only 3.95% infection (SEM=1.59). In the same conditions, 33.1% (SEM=4.9) of MDDC became positive for GFP. However, the results suggest that the dose of 100ng HIV-1 p24 is possibly toxic for cells, as a drop in infection of MDDC and MDLC could be observed at this dose (**Figure 4.8C**). Together these results highlight that SAMHD-1-independent post-entry restriction to HIV-1 in MDLC cannot be overcome by increasing dose of viral input.

Interestingly, titration of viral infections in both MDDC and MDLC was quite poor. Doubling of viral dose resulted in less than 10% increase of GFP positive MDDC (**Figure 4.8.B**). This effect was more pronounced in HIV-1 infected MDDC (approximately 15% increase), possibly because in contrast to VSV-G HIV-GFP, HIV-1 is a replication competent virus and can spread in the cell culture. Perhaps, titration effect would be more evident at lower inoculums of p24 (0.1-25ng p24). Additionally, low infection of the cells at high doses of virus could result from incomplete degradation of SAMHD-1.

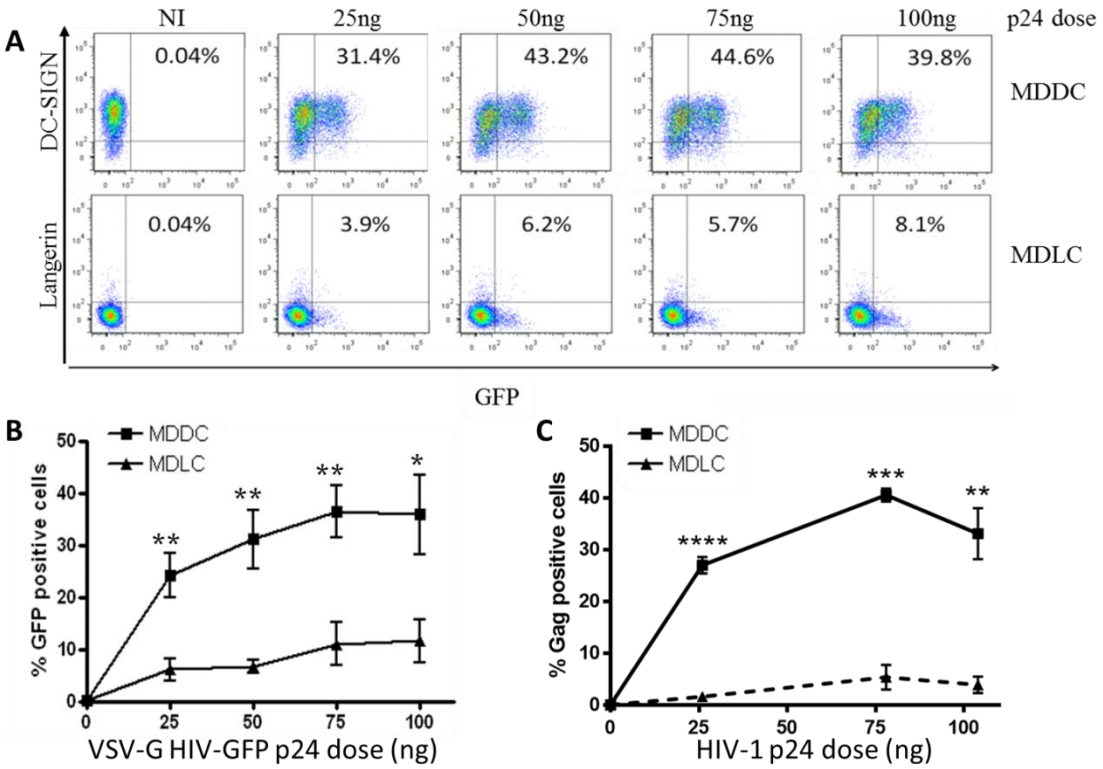

Figure 4.8. Challenge of MDLC and MDDC with Increasing Dose of VSV-G HIV-GFP. Autologous MDLC and MDDC were pre-treated with VSV-G pseudotyped Vpx encoding SIV3 lentivector (SIV3-Vpx) for 3 hours before challenge with various dose of VSV-G HIV-GFP or R5 HIV-1. **(A)** A representative flow cytometry analysis for VSV-G HIV-GFP and **(B)** pooled data for 3 donors are showed. **(C)** Profile of HIV-1 infection of MDDC and MDLC is demonstrated in graph ($n \geq 4$). The statistical significance of the results was calculated using student t-test and significant values indicated (*) as described in Materials and Methods. Error bars represent ±SEM.

In the above experiments, where indicated, SIV3-Vpx was added to cells and the susceptibility of MDDC, MDLC and MuLC to infection with wild type HIV-1 or VSV-G pseudotyped HIV-GFP construct was investigated. As considered before, in addition to Vpx, SIV3-Vpx carries Vpr protein that when present in cells can modulate their biology and immune responses to the infection (Ayyavoo et al. 2002; Muthumani et al. 2002). One of the functions of Vpr *in vivo* is enhancement of

integrated pro-virus transcription from viral LTR, that is achieved by binding of Vpr to LTR and transcription factors (Cohen et al. 1990; Agostini et al. 1996). Similarly, high HIV-1 infection of MDDC and CD141+ MDDC was observed in this study following addition of SIV3-Vpx (see **Figure 4.4**). Although SAMHD-1 down regulation in myeloid cells was demonstrated to have pronounced effect on cell infectability (Hrecka et al. 2011; Laguette et al. 2011; Lahouassa et al. 2012) also confirmed in this work, the additional enhancement of infection by SIV3-Vpx derived Vpr cannot be excluded unless SIV3-Vpx ΔVpr is used as an additional control. A potential effect of Vpr on virus propagation in MDLC and MuLC is more difficult to interpret due to low p24 gag readout in these cells. However, lack of HIV-1/VSV-G HIV-GFP infection stimulation in these cells after SIV3-Vpx addition suggest that despite effective SAMHD-1 down modulation in these cells, neither Vpr or Vpx are able to lift additional cellular restriction to HIV-1 present in these cells.

4.2.2. *Ex Vivo* Infection of Primary Skin Isolated Epidermal LC and Dermal DC with VSV-G HIV-GFP

Importantly, I extended our findings to physiologically more relevant cells by comparing infection levels in primary epidermal LC and dermal DC freshly isolated from skin (see Materials and Methods, Section 2.5, and Chapter 3 Section 3.2.5). Briefly, cells isolated from the dermis or epidermis of freshly excised patient skin explants were transduced with VSV-G HIV-GFP with or without pre-treatment with Vpx-expressing SIV-derived lentivectors. Using antibodies against characteristic lineage markers, I was able to select pure dermal DC and epidermal LC populations and evaluate the efficiency of VSV-G HIV-GFP infection in these cell subsets. T cells

that constitute a significant proportion of dermis walkout cells were gated out during flow cytometry analysis, based on their size and granularity (lower FSC and SCC values compared to derma DC) and HLA-DR negative phenotype.

Western blot analysis of epidermal and dermal walkout cells showed clear but not complete down regulation of SAMHD-1 in the cells after SIV3-Vpx treatment (**Figure 4.9A**). This result demonstrates a successful entry of VSV-G pseudotyped SIV3-Vpx construct in epidermal LC and dermal DC, suggesting that VSV-G HIV-GFP lentivirus can also infect these cells. However, based on the efficiency of SAMHD-1 degradation, it seems that only a small proportion of target cells are not infected by SIV3-Vpx. The presence of keratinocytes and other cells in dermis and epidermis walkout populations could limit the effect of SIV3-Vpx and VSV-G HIV-GFP as VSV-G pseudotyped particles can bind and enter keratinocytes. Thus, incomplete SAMHD-1 degradation in skin samples could result from limited entry of SIV3-Vpx into epidermal LC and dermal DC rather than a defect in SIV3-Vpx particles. Infection experiments with VSV-G HIV-GFP demonstrated low infection efficiency in both immature dermal DC (2.0%, SD=1.7%) and immature epidermal LC (1.9%, SD=1.1). However, SIV3-Vpx-mediated removal of SAMHD1 rendered dermal DC significantly more susceptible to infection (16.5% GFP positive cells, SD=15.6). In contrast, pre-treatment of LC with Vpx-expressing lentivectors did not lead to a significant enhancement of VSV-G HIV-GFP infection (total GFP positive LC 4.9%, SD=3.6%) when compared to dermal DC subsets (p=0.037) (**Figure 4.9B** (representative experiment) **and C** (pooled data)). Quantification confirmed a statistically significant difference in infection rates between skin-derived DC subsets (11.1 fold increase on average upon VSV-G HIV-GFP infection of dermal DC compared to a modest 2.5 fold increase for epidermal LC) (**Figure 4.9D**). However,

remaining low levels of SAMHD-1 present in target cells (Figure 4.9A) or lower entry of VSV-G HIV-GFP into epidermal LC due to low numbers of these cells in epidermal walkout population (see Chapter 3, Figure 3.7) could mediate this effect.

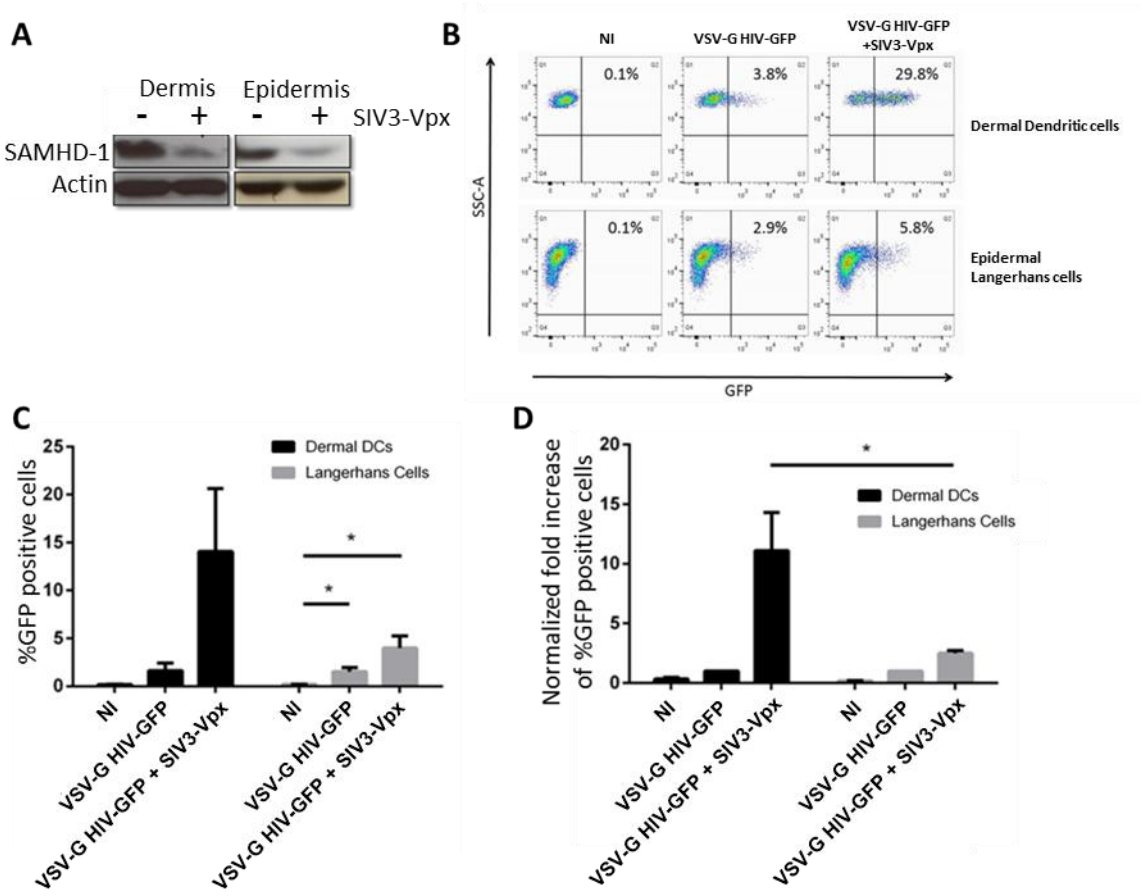

Figure 4.9. **Skin-isolated Epidermal LC Remain Refractory to VSV-G HIV-GFP in the Presence of SIV3-Vpx, in Contrast to Dermal DC.** Primary *ex vivo* DC/LC isolated from dermal and epidermal skin sheets were infected with VSV-G HIV-GFP (40ng p24/10^5 cells) with (VSV-G HIV-GFP + SIV3-Vpx) or without (VSV-G HIV-GFP) SIV3-Vpx pre-treatment and infection levels were measured 3 days post-infection. **(A)** A representative experiment and **(B)** a quantification of GFP^+ cells from all donors (*n=4)* are shown. **(C)** Data were normalised to VSV-G HIV-GFP values in order to eliminate inter-patient variability and to show fold-change in infection occurring in the presence of SIV3-Vpx. NI indicates non-infected cells. Error bars represent ±SEM; * $p < 0.05$.

I have further investigated our results to exclude non-specific infection of skin tissue cells. Accordingly the analysis showed that upon infection with VSV-G HIV-GFP, GFP^+ cells were predominantly among the HLA-DR^+ population of epidermal walkout cells (i.e. Langerhans cells (see **Figure 3.7**)) (**Figure 4.10**) and among CD45/HLA-DR double positive cells acquired from the dermis (**Figure 4.11**). In addition, only marginal levels of GFP expression were recorded in remaining epidermal and dermal walkout cells. Thus, our data suggest that lower VSV-G HIV-GFP infection of epidermal LC, compared to dermal DC, results from block to virus infection in these cells rather than by a limited proportion of LC in the epidermal walkout population. However, an unproductive entry of VSV-G HIV-GFP into skin tissue cells cannot be excluded.

Taken together our data indicate that SAMHD1 is not a major HIV-1 restriction factor in primary epidermal LC, reminiscent of the phenotype obtained with *in vitro* differentiated primary LC and cell lines. However, it is also likely that an additional restriction operating in LC plays a dominant role in viral inhibition, therefore making it impossible to identify SAMHD-1 function in these cells. This restriction is not affected by the function of SIV3-Vpx or SIV3-Vpx derived Vpr, which are both delivered to cells in SIV3-Vpx particles. Therefore, our results clearly demonstrate that the SAMHD1-independent HIV-1 restriction activity found in *in vitro* derived immature LC is apparently operating at a post-entry level in viable primary LC isolated from skin.

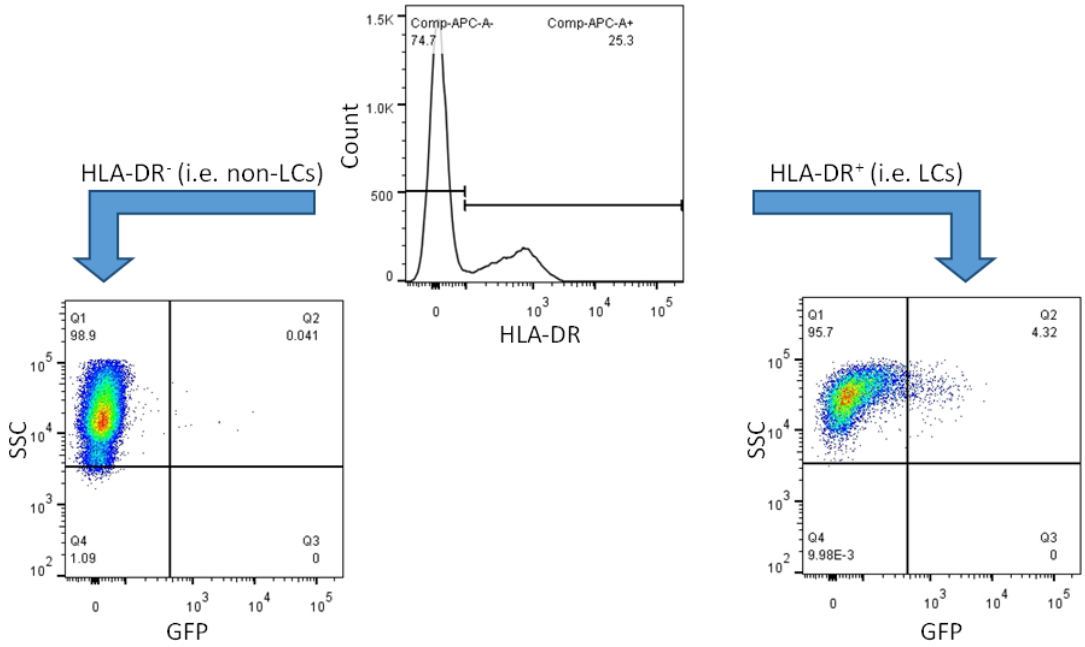

Figure 4.10. GFP Expression in VSV-G HIV-GFP + SIV3-Vpx Transduced Epidermal Walkout Cells is Predominantly Detected in HLA-DR$^+$ Cells. The extended analysis of skin epidermal walkout cells transduced with VSV-G HIV-GFP (40ng p24/10^5 cells) + SIV3-Vpx. HLA-DR negative population (bottom left panel) indicates non-Langerhans cells. Bottom right panel shows HLA-DR positive population consisting of Langerhans cells. Total epidermal walkout population (top panel).

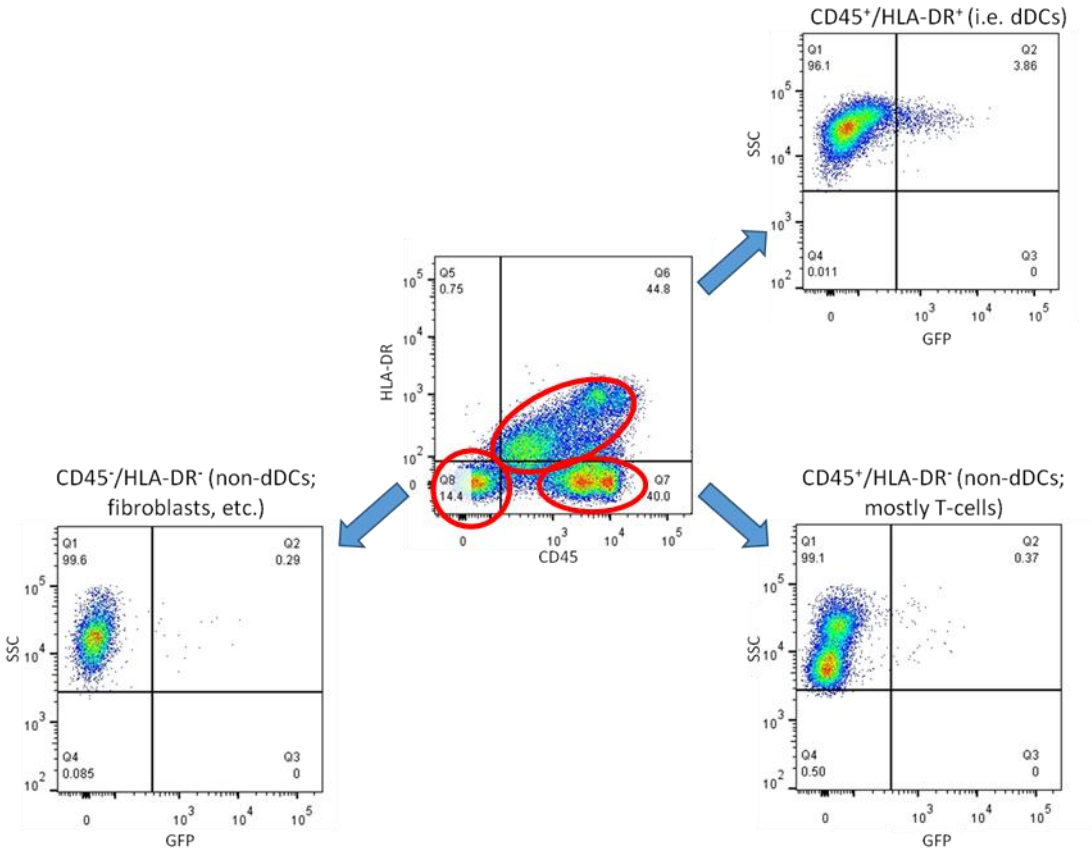

Figure 4.11. GFP Expression in VSV-G HIV-GFP + SIV3-Vpx Transduced Dermal Walkout Cells is Predominantly Detected in CD45$^+$/HLA-DR$^+$ Cells. The extended analysis of skin dermal walkout cells challenged with VSV-G HIV-GFP (40ng p24/10^5 cells) + SIV3-Vpx. Predominant GFP expression in CD45$^+$/HLA-DR$^+$ (top right panel) is shown. Little infection in non-dermal DC populations is illustrated (bottom panel). Total dermal walkout population (central panel).

4.2.3. IFN-α Restricts HIV-1 Infection of LC.

IFN-α is a potent antiviral cytokine which increases expression of several HIV-1 cellular restriction factors, including BST-2/Tetherin (Van Damme et al. 2008), members of the APOBEC3 family (Peng et al. 2006), MX2 (Goujon et al. 2013; Kane et al. 2013) and other Interferon stimulated genes (ISG) (Neil and Bieniasz 2009; Schoggins et al. 2011; Pillai et al. 2012). IFN-α was shown to induce an antiviral state in myeloid cells, thus potently restricting HIV-1 infection. Indeed, I confirmed that VSV-G HIV-GFP infection of IFN-α treated MDDC was restricted even after Vpx-mediated degradation of SAMHD1 (**Figure 4.12A**). While IFN-α pre-treatment also appeared to restrict viral infection in MDLC, the effect was less pronounced, possibly due to the dominant pre-existing antiviral state in immature MDLC (**Figure 4.12B and C**). Interestingly, SIV3-Vpx appears to rescue some GFP expression in MDLC treated with interferon (**Figure 4.12.C**) although the total percentage of infected cells is much lower compared to MDLC infected in the absence of the interferon treatment. Indeed, addition of SIV3-Vpx to MDLC increases VSV-G HIV-GFP infection by 1.7 fold and this effect is amplified in IFN treated cells where the fold induction is 4.15 (**Figure 4.12.**C). As Vpr is also present in SIV3-Vpx particles, it is likely that this accessory protein modulates interferon-induced restriction to HIV lentivectors, allowing partial rescue of VSV-G HIV-GFP infection. In fact, recently Vpr was described to regulate several interferon-stimulated genes in MDDC (Zahoor et al. 2015), many of which were up-regulated. The result described above was limited to one donor in my study and similar pattern was not observed in remaining 2 donors (analysis not shown). As the effect did not reach a statistical significance, no further investigation was undertaken.

In order to control MDLC responsiveness to IFN-α, I analyzed the IFN-α-mediated increase in expression of antiviral restriction factors, in particular A3G, BST-2/tetherin, SAMHD1 and MX2, as well as ISG family members such as retinoic acid inducible gene I (RIG-I) (Kato et al. 2005). In line with our previous observations (see Chapter 3), I observed no significant differences in expression of these restriction factors upon IFN-α treatment of both MDLC and MDDC (**Figure 4.12D**). Accordingly IFN-α could induce expression or up-regulation of BST-2/tetherin, RIG-I and A3G in both MDDC and MDLC (**Figure 4.12D**). The presence of SIV3-Vpx had no effect on APOBEC3G protein levels in MDDC and MDLC, despite recent reports suggesting Vpr mediated decrease of APOBEC3A mRNA in MDDC (Zahoor et al. 2015). APOBEC3G is unlikely restriction to VSV-G HIV-GFP due to its main restriction activity on de novo produced viruses. As a single round construct, VSV-G HIV-GFP is not under pressure from APOBEC3G, although APOBEC3G has been described to block a viral reverse transcription in single round assays in primary cells such as DC and human CD4+ T cells (Pion et al. 2006; Gillick et al. 2013).

Interestingly I observed no changes in SAMHD1 expression in both cell types, in agreement with recent reports showing that its expression is not modulated upon IFN-α treatment of DC (St Gelais et al. 2012; Cribier et al. 2013). Recently the MX2 protein was described to act as an IFN-α-inducible HIV-1 restriction factor in myeloid cells (Goujon et al. 2013; Kane et al. 2013). In agreement, Figure 4.8.D shows that MX2 is also expressed in MDLC, but only after IFN-α stimulation, and this may account for the enhanced IFN-α-mediated HIV-1 restriction activity observed in MDLC, although this was not investigated in our study. While MX2 might play a role in HIV-1 restriction, it is only expressed after IFN-α treatment (**Figure 4.12D**) and thus, unlikely to act in steady-state immature LC. Human Vpr protein was recently

described to increase MX2 levels in Vpr transduced MDDC (Zahoor et al. 2015). I have not observed any significant changes in MX2 levels in MDDC in the presence of SIV3-Vpx, but it appeared to down modulate the levels of MX2 in interferon treated MDLC. The measurement of proteins levels based on western blot readout offers only semi quantitative evaluation. Additional RT-PCR or more quantitative WB assays could be performed in the future to verify any effect of SIV3-Vpx derived Vpr on MX2 levels in MDLC.

As interferon-induced restriction to HIV-1 is manifested usually at early stages of virus replication, in depth evaluation of the steps of the reverse transcription process in MDLC and MDDC would provide important information regarding interferon inducible restriction in these cells. PCR targeting early and late reverse transcription products as well as a total number of integrated proviruses could be used to achieve this goal.

Attempts to reproduce these results in a MuLC cell line were not undertaken because these cells were seemingly unresponsive to IFN-α (see Chapter 3, Figure 3.8). I have previously described the inefficient response of MuLC to type I Interferon in Chapter 3, and concluded that a lack of IFN-α receptor, or impaired downstream IFN-α signalling pathway in these cells may be the cause for the observed phenotype. Taken together these results show that MX2 is exclusively expressed in Interferon stimulated MDLC, suggesting it may not take part in VSV-G HIV-GFP restriction in these cells. However, MX2 expression during cells infection has not been investigated. However, clearly, a further enhanced antiviral state in MDLC could be efficiently induced upon type-I Interferon treatment on top of the naturally refractory phenotype of these cells.

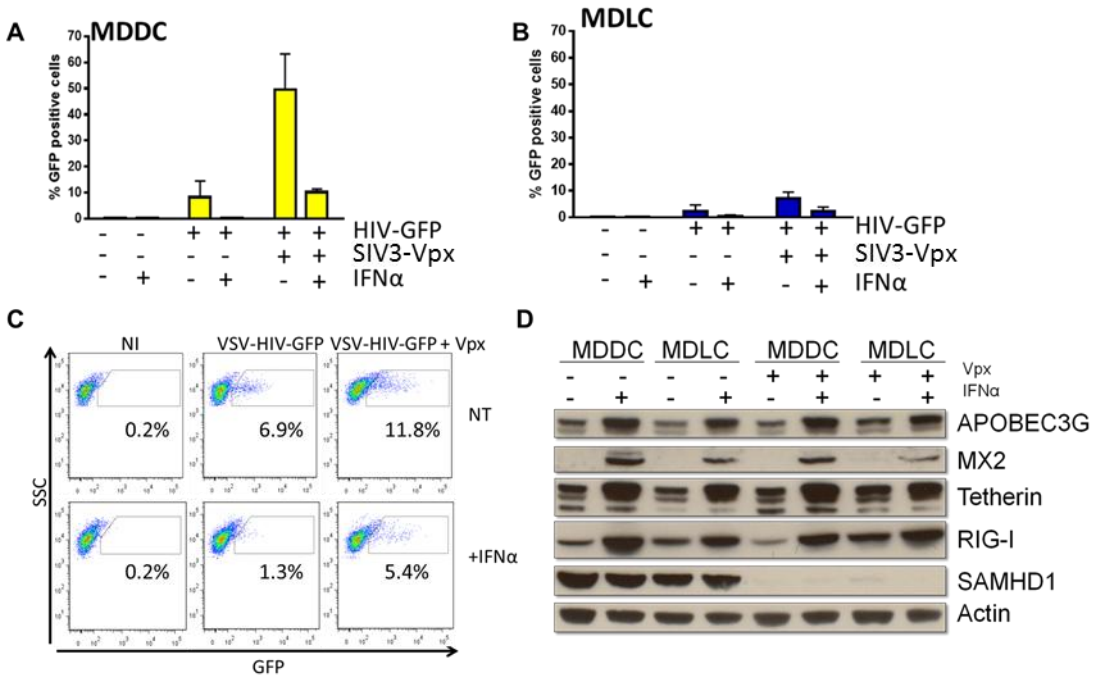

Figure 4.12. IFN-α-mediated Decrease of VSV-G HIV-GFP Infection in MDLC and MDDC. MDLC and MDDC were pre-treated with 1000U/ml IFNα for 24 hours followed by VSV-G HIV-GFP infection with or without SIV3-Vpx pre-treatment. Pooled data for **(A)** MDLC and **(B)** MDDC infection in the presence of IFNα is represented in graphs (*n=4*). **(C)** A representative infection analysis after 4 days is shown for MDLC. **(D)** Analysis of cellular restriction factors expression in MDLC and MDDC after 24 hours IFNα treatment, and SIV3-Vpx transduction where indicated, was performed by western blot analysis. Actin served as a loading control. NI indicates non-infected cells. Error bars represent ±SEM.

4.2.4. HIV-1 Restriction in LC Does not Require IFN type-I Production.

To further investigate whether VSV-G HIV-GFP restriction in MDLC is dependent on type-I Interferon release from infected cells, I transduced MDLC and MDDC with VSV-G HIV-GFP with or without addition of SIV3-Vpx. I observed, that at early points of infection (6-12 hrs), VSV-G HIV-GFP and Vpx-expressing lentivectors do

not significantly stimulate IFNβ mRNA synthesis in MDLC (mean 6 hrs 1.472 and mean 12 hrs 4.09) compared to the negative control (**Figure 4.13A**). The lack of stimulation of IFNβ mRNA at early time points of infection (up to 48 hrs) was also observed in MDDC (Figure 4.13.A). However, significant increase of IFNβ mRNA levels was demonstrated in MDDC at 48 and 72 hours post VSV-G HIV-GFP transduction (mean 48 hrs 9.9-fold, 72 hrs 8.1-fold) and at 24 hours onwards in MDLC (24 hrs mean 17.72). As viral integration takes place in the first 12 hours of infection, the above data suggests limited lentivirus sensing in both cell types prior to integration. Addition of poly dA:dT used to stimulate a strong IFN response induced 272- and 837-fold stronger IFNβ mRNA synthesis in both MDLC and MDDC compared to unstimulated cells ($p<0.0001$) (Figure 4.13.A). In line with IFNβ stimulation in infected MDLC and MDDC at later stages of VSV-G HIV-GFP (+SIV3-Vpx) infection (48h), both cell types showed partial maturation (Figure 4.13.B and C), although the effect was more significant in MDDC, when compared to MDLC, possibly due to partial maturation of MDDC already before infection. Alternatively, lower rates of VSV-G HIV-GFP infection of MDLC may be responsible for total lower expression of CD86 compared to MDDC. Thus, our data suggest that a pre-existing block to virus infection operates in MDLC and it does not require type I IFN stimulation for its action. Accordingly, IFN inducible restriction factor MX2 was not up-regulated in MDLC until 24 hours post infection with VSV-G HIV-GFP (+SIV3-Vpx) (Figure 4.13.D) in line with our IFNβ stimulation results. Late expression of MX2 in MDLC transduced with VSV-G HIV-GFP (+SIV3-Vpx) confirms that an early block to lentivirus replication in MDLC is independent of MX2 and type I IFN function. However, a delayed involvement of sensing mechanisms in

MDLC might further contribute to limiting virus propagation at later stages of infection (e.g. 48hrs post infection).

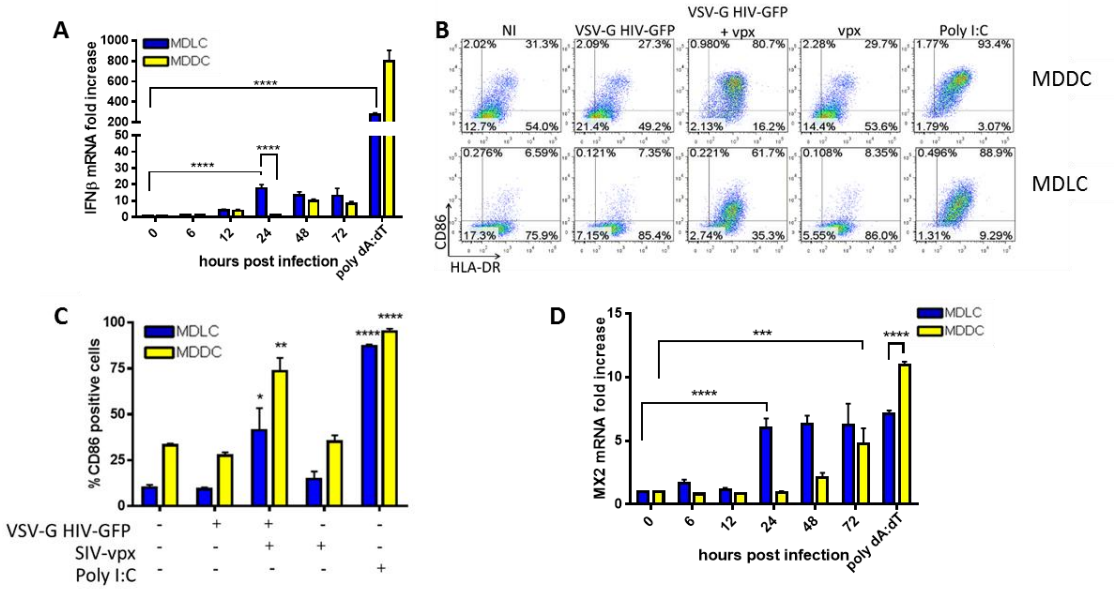

Figure 4.13. Lack of IFN-β Release from MDDC and MDLC after Transduction with VSV-G HIV-GFP Lentivector. (A) Homologous MDDC and MDLC were pre-treated or not with SIV3-Vpx for 4 hours and infected with 30ng p24 VSV-G HIV-GFP for indicated times (0,6,12,24,48 and 72) or were treated with poly dA;dT for 6 hours and IFNβ mRNA levels were measured (*n=3*). **(B)** Similarly, infected or poly I:C (25μg) treated MDLC and MDDC were stained with CD86 and HLA-DR antibody and maturation of the cells was investigated after 48 hours (representative experiment) and **(C)** (pooled data (*n=3*)). **(D)** Expression of MX2 mRNA in VSV-G HIV-GFP + SIV3-Vpx infected MDLC and MDDC was measured after 0,6,12,24,48 and 72 hours (*n=3*). Poly dA:dT served as a positive control for interferon stimulation and MX2 expression. NI indicates non-infected cells. *p < 0.05, ***p < 0.001, **** p < 0.0001. Error bars represent ±SEM.

In confirmation of these assumptions VSV-G HIV-GFP + SIV3-Vpx infection of MDLC induced higher Langerin expression in these cells, similarly to the effect of type-I Interferon treatment (see Chapter 3, Figure 3.9) (**Figure 4.14A**).

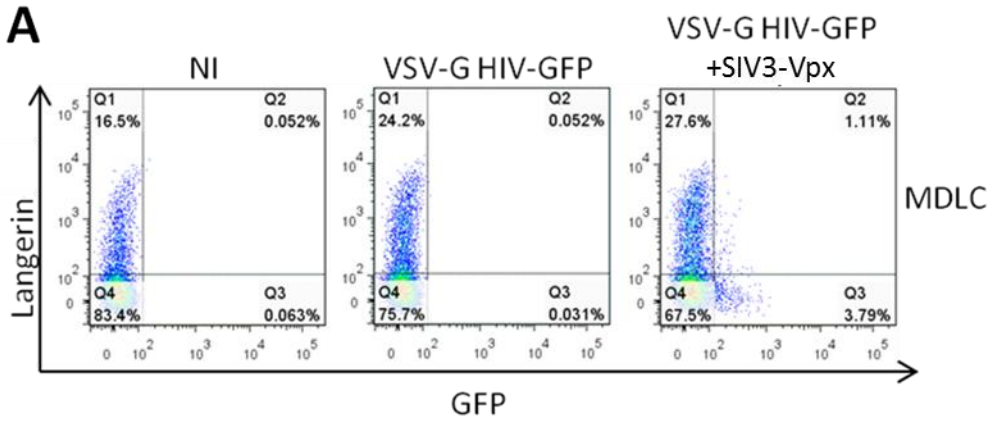

Figure 4.14. Lack of Langerin Up-regulation in MDLC Transduced with VSV-G HIV-GFP + SIV3-Vpx. MDDC and MDLC were transduced with 30ng p24 VSV-G HIV-GFP (±SIV3-Vpx) for 3 days. Langerin levels on infected MDLC are shown. NI indicates non-infected cells.

4.2.5. HIV-1 Post-entry Restriction in LC is Partially Abolished by TLR-2 Agonists and TNF-α, and Correlates with NF-κβ Pathway Activation.

Previous reports suggested that stimulation of LC with TNF-α or TLR2 agonists promoted HIV-1 infection of these cells (de Jong et al. 2008; Ogawa et al. 2009). Such observations are particularly relevant in the context of co-infections between HIV-1 and other STIs, including gram-positive bacteria and HSV-2. The mechanisms of this increase in infection of mature LC are not well understood, but may result from a reduction of Langerin expression in mature LC (Ogawa et al. 2009). Our study therefore aimed at also deciphering if the increased infection of LC observed upon bacterial co-infection or TNF-*α* exposure would correlate with the modulation of a post-entry viral restriction activity in LC. The following experiment was performed in MDLC and MDDC transduced with SIV3-Vpx prior infection, in order to exclude the

role of SAMHD-1 block on HIV-GFP-VSVG infection in a setting mimicking co-infections.

I observed an enhancement of VSV-G HIV-GFP infection of LC pre-treated with Pam3CSK4 (mean 3.28% or 2.4 fold increase) and TNF-α (mean 7% or 4.1 fold increase) (**Figure 4.15A and B**). Pre-treatment with TNF-α also increased MDDC susceptibility to VSV-G HIV-GFP (+SIV3-Vpx) infection by average 11.1% (1.5 fold) (p=0.095 ns) while Pam3CSK4 had no significant effect (mean 0.2% of 2.3 fold decrease) (**Figure 4.15C**). In contrast VSV-G HIV-GFP transduction of poly I:C treated MDDC was 10 fold decreased compared to non-stimulated cells (p=0.022) (**Figure 4.15A and C**). Surprisingly while MDLC were poorly responsive to poly I:C in terms of type-I IFN response (see **Figure 4.13A**), this TLR-3 agonist rendered MDLC significantly more restrictive to VSV-G HIV-GFP infection (**Figure 4.15A and B**). However the involvement of Interferon in poly I:C induced inhibition of VSV-G HIV-GFP infection in MDLC cannot be excluded as maturation of these cells was observed after 48hours treatment (see **Figure 4.13)**.

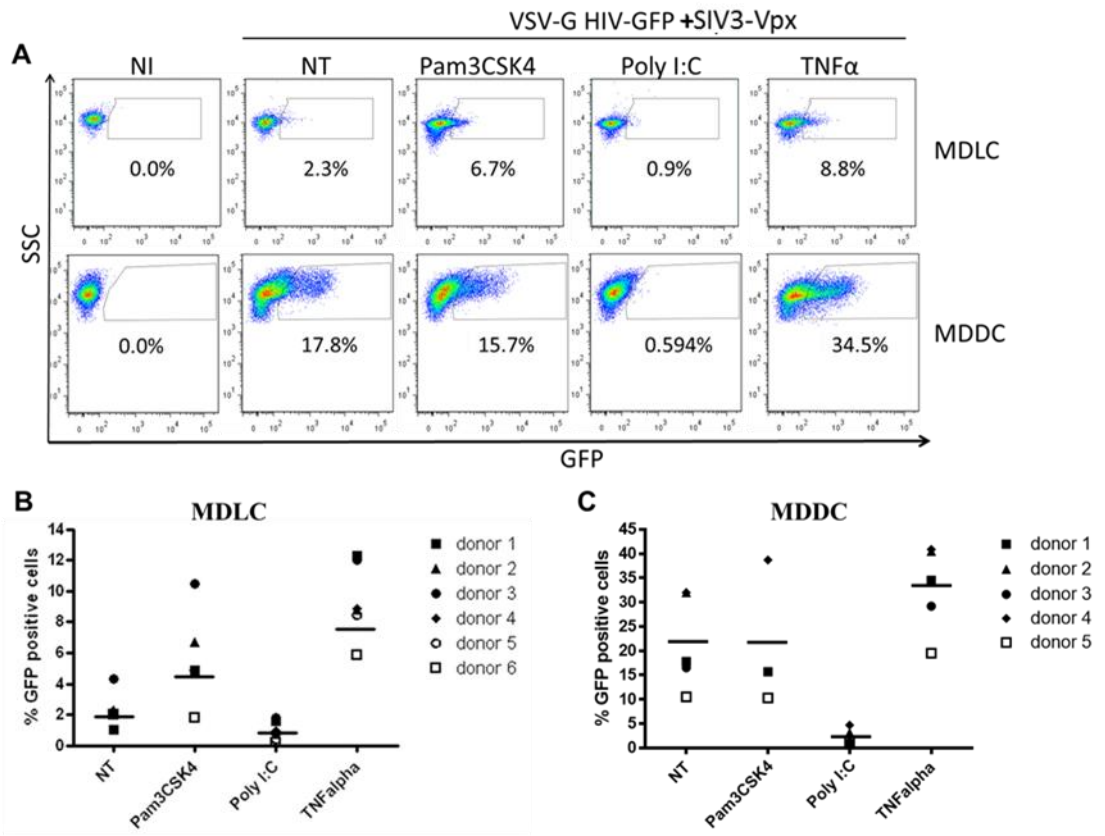

Figure 4.15. TLR Agonists and TNF-α Regulate VSV-G HIV-GFP Infection of MDLC and MDDC. MDLC and MDDC were pre-treated with TLR agonists: Pam3CSK4 (5µg/ml) (TLR-2), Poly I:C (25µg/ml) (TLR-3) or TNF-α (0.1µg/ml) for 8-16 hours, then transduced with SIV3-Vpx for 4 hours and infected with 20ng p24 VSV-G HIV-GFP for 3 days. Cells were fixed, washed and GFP expression was analyzed by flow cytometry. **(A)** A representative infection profiles for MDLC and MDDC**. (B)** A graph representing the infection profile for each of donors in MDLC (*n=6*), and MDDC **(C)** (*n=5*) are depicted. The horizontal line on graphs represents the mean % of GFP positive cells**.** NI indicates non-infected cells. NT indicates non-treated infected cells.

As TLR-2 or TNF-α-treated LC were more susceptible to infection I therefore investigated whether expression of known HIV-1 restriction factors could be modulated upon treatment with TLR agonists in mature MDLC and MDDC. I detected no change in APOBEC3G expression in both cell types. However, SAMHD1

expression levels were slightly decreased upon TNF-α treatment in MDLC (**Figure 4.16**), which cannot be clearly confirmed in MDDC due to lower protein content in TNF-α treated sample. Together these results suggest that LC maturation with TLR-1/TLR-2 agonists and TNF-α, but not TLR-3 agonists, promotes HIV-1 infection independently of SAMHD1, A3G or Langerin expression.

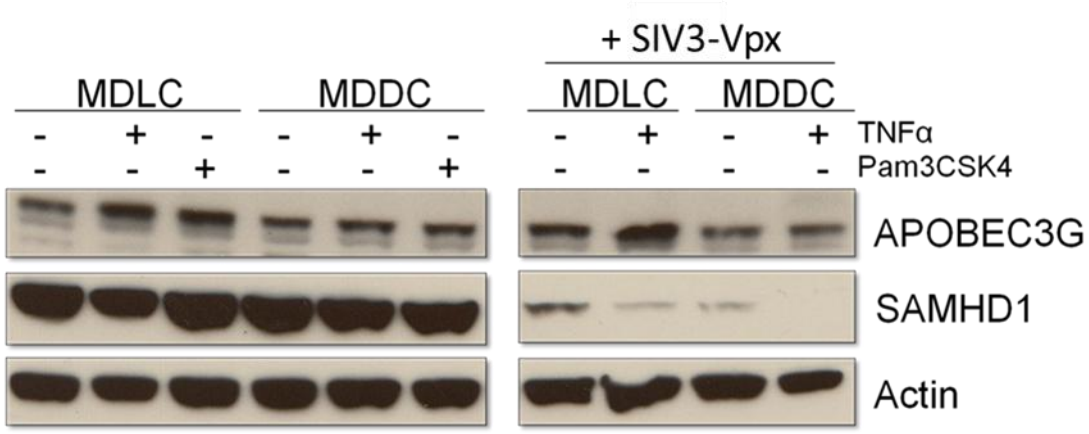

Figure 4.16. The Effect of TNF-α and TLR Agonist Stimulation on Restriction Factors Levels in MDLC and MDDC. Autologous MDDC and MDLC were treated with TLR agonists: Pam3CSK4 (5μg/ml) (TLR-2) or TNF-α (0.1μg/ml) for 8-16 hours with or without SIV-3 Vpx transduction. Expression of APOBEC3G and SAMHD-1 was analysed by western blotting.

Most of the TLR agonists and pro-inflammatory mediators are known to signal through the NF-κβ pathway and I therefore investigated NF-κβ activation status in MDLC and MDDC upon LPS or TNF-α stimulation. I noticed increased levels of the active phosphorylated form of NF-κβ (p-NF-κβ p65) in MDDC and MDLC after both LPS and TNF-α treatments (**Figure 4.17**). These results correlated with a timely decrease of expression of IκB-α, a major negative NF-κβ regulator. Of note, unstimulated MDLC were evidencing less p-NF-κβ, compared to MDDC counterparts, suggesting that at a resting state MDLC might have a lower background activity than MDDC. Although NF-κβ signalling was efficiently induced in MDLC

upon TNF-α stimulation (see **Figure 4.17**, rescue of VSV-G HIV-GFP infection was far from complete, as observed by the remaining low infection level of these cells even after SAMHD1 depletion (see **Figure 4.15**). SIV3-Vpx, that also contains Vpr protein, has been used in this experiment to down modulate SAMHD-1 expression in the cells prior infection. Multiple conflicting descriptions of Vpr effect on NF-κβ are published, some suggesting activation (Varin et al. 2005; Hoshino et al. 2010) others inhibition (Ayyavoo et al. 1997; Mariani et al. 2001; Majumder et al. 2005) of this pathway. In context of this study, recently published research by Kogan M et al (2013) demonstrated that Vpr inhibits NF-κβ signalling induced by TNF-α, but it does not have this effect in LPS treated macrophages and U1 cells (Kogan et al. 2013). If that is also the case in MDLC and MDDC, the effect on GFP expression in TNF-α treated cells may depend on other than NF-κβ signalling pathway induced by this cytokine, such as p28 mitogen-activated protein kinases (p38-MAPK) (see **Chapter 4 Discussion**). Taken together it is plausible that the partial restoration of VSV-G HIV-GFP infection in MDLC treated with TNF-α is mediated by increased cellular activity of the cells, activation of transcription factors or down modulation of present post-entry restrictions.

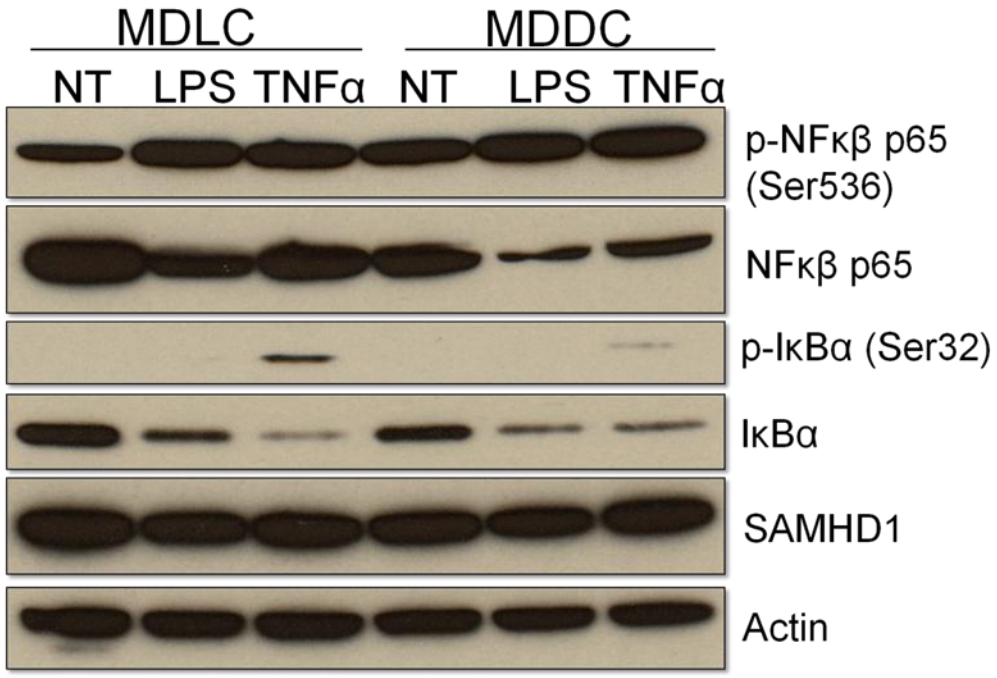

Figure 4.17. The Effect of LPS and TNF-α on NF-κβ Signalling Pathway in MDDC and MDLC. Autologous MDLC and MDDC were treated with LPS (1μg/ml) or TNF-α (0.1μg/ml) and after 1 hour activation of NFκβ pathway was investigated by western blot. Actin served as a loading control.

4.2.6. TGF-β Triggers HIV-1 Restrictive Phenotype in Langerhans Cells.

In this study MDDC and MDLC were derived from the same donors, yet demonstrated high divergence in susceptibility to HIV-1 infection. The unique notable difference between both DC subsets relied on the presence of TGF-β, which when added to monocytes induced cell differentiation into MDLC. Indeed, MDDC and MDLC are both differentiated from monocytes using GM-CSF and IL-4, while TGF-β was only added in MDLC culture medium. In order to investigate the role of TGF-β in the post-entry resistance of MDLC to HIV-1 infection I infected monocytes with VSV-G HIV-GFP, in the presence of SIV3-Vpx, at different time points during

differentiation (**Figure 4.18A**). MDLC progenitors supplemented with TGF-β became more resistant to VSV-G HIV-GFP infection after 1 day of differentiation, reaching 16.3% (SD=3.9) GFP positive cells compared to 24.9% (SD=12.2) in monocytes differentiated to MDDC (**Figure 4.18B and C**). The resistance to HIV-1 infection in

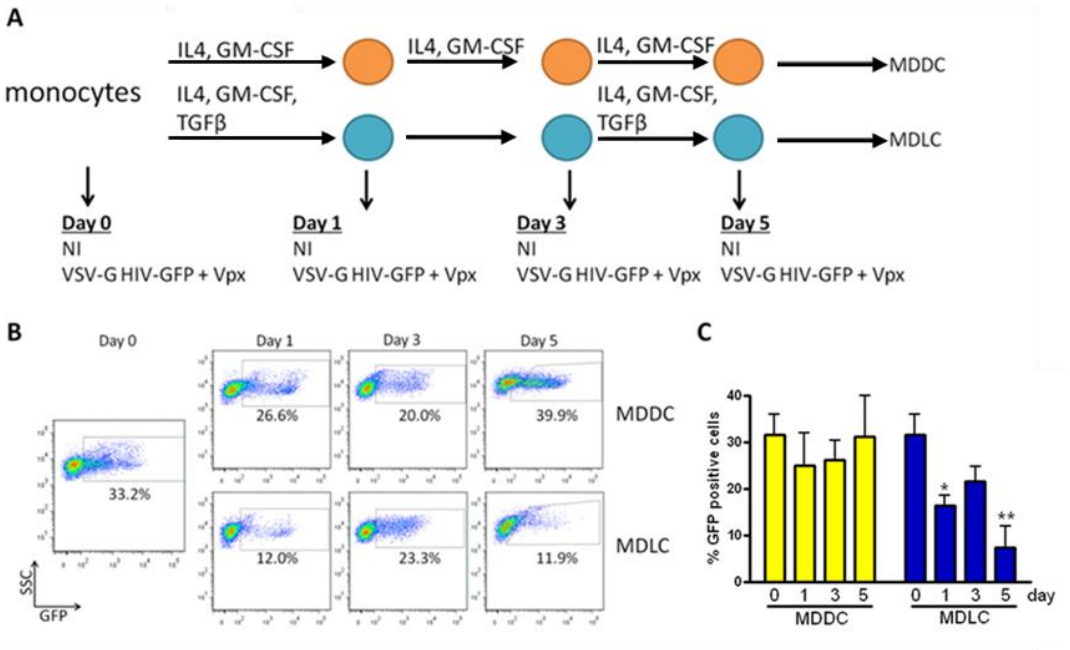

Figure 4.18. TGF-β Triggers a Post-entry Restriction to VSV-G HIV-GFP in MDLC. Monocytes obtained from the same donor were seeded for differentiation into MDDC (GM-CSF+IL-4) and MDLC (GM-CSF+IL-4+TGF-β). At days 0, 1, 3 and 5 of differentiation cells were infected for 4 days with 25ng p24/10^5 cells of VSV-G HIV-GFP + SIV3-Vpx. **(A)** A schematic design of the experiment is represented. **(B)** A representative infection analysis after 4 days is shown for MDDC (top panel) and MDLC (bottom panel). **(C)** Pooled data for MDLC and MDDC is represented in graph (*n=2*). NI indicates non-infected cells. *p < 0.05, ** p < 0.01. Error bars represent ±SEM.

MDLC was even more pronounced after 5 days of differentiation as only 7.4% (SD=6.4) of differentiating MDLC became positive for GFP, in contrast to more than 30% for monocytes derived without TGF-β. Interestingly resistance to HIV-1

infection of differentiating monocytes followed the pattern of TGF-β supplementation with a maximal restriction observed at day 5, which is typical of terminal differentiation to functional LC in this *ex vivo* system.

In agreement with the role of TGF-β in MDLC viral restriction, pharmacological inhibition of its downstream signalling molecule SMAD2 restored infectivity of these cells regardless of the SAMHD-1 (**Figure 4.19**). Signalling studies confirmed that phosphorylation of SMAD2 was successfully inhibited at both lower (5μM) and higher (10μM) concentration of LY2109761 (**Figure 4.19A**). The strongest phenotype was observed using 5μM concentration of LY2109761, where the mean VSV-G HIV-GFP + SIV3-Vpx infection of MDLC reached 80.7% (SEM=1.6) compared to average 15.1% (SEM=5.3) in MDLC (**Figure 4.19B**). Interestingly even in the presence of SAMHD-1, LY2109761 (5μM) induced significant level of GFP expression in the cells ($p=0.0004$, mean=6.3%, SEM=0.45, compared to NT MDLC mean=0.1, SEM=0.05), highlighting the importance of this signalling molecules for TGF-β-mediated effect. Higher doses of SMAD2 inhibitor had less pronounced effect on VSV-G HIV-GFP infection in the presence of SIV3-Vpx (mean=54.5, SEM=13.6), possibly due to toxic effect of the drug on MDLC.

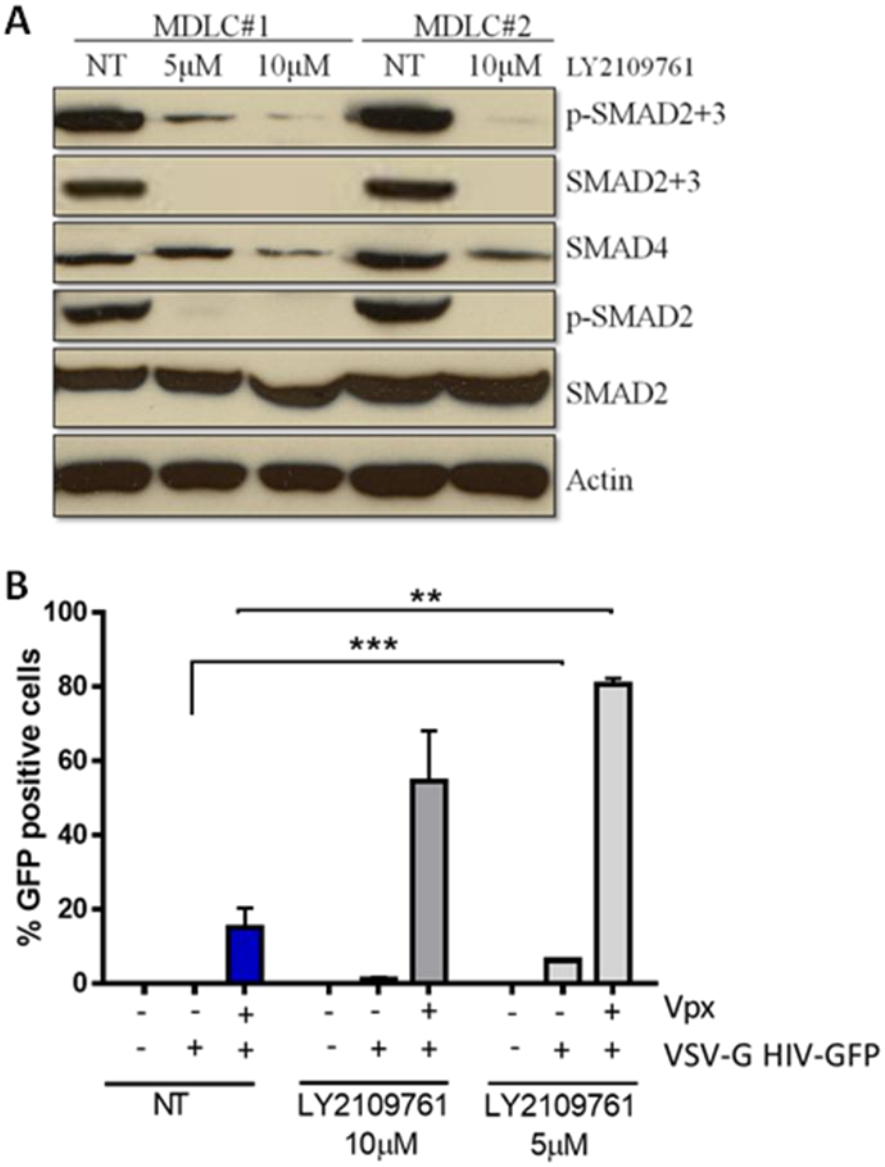

Figure 4.19. Inhibition of SMAD2 Signalling Prevents Development of VSV-G HIV-GFP Restriction in MDLC. MDLC derived in the presence or absence of SMAD2 inhibitor LY2109761 (5 or 10μM final concentration) (see Materials and Methods, Section 2.9.4) were transduced with VSV-G HIV-GFP (±SIV3-Vpx) for 3 days. **(A)** Inhibition of SMAD2 phosphorylation in two donors is showed. **(B)** GFP expression in MDLC (NT) and MDLC derived in the presence of LY2109761 are illustrated in (*n=3*). NI indicates non-infected cells. **p < 0.01, *** p < 0.001. Error bars represent ±SEM.

Interestingly supplementation of TGF-β into fully differentiated MDDC rendered them less permissive to VSV-G HIV-GFP infection (**Figure 4.20. A**) reminiscent of results obtained with MDLC. MDDC pre-treated with TGF-β (24hours) presented 3 fold less susceptibility to VSV-G HIV-GFP infection (mean infection = 2.12%, SEM=1.49, n=2) compared to non-treated MDDC (mean infection = 6.02, SEM=1.24, n=2), although the effect remained statistically insignificant (p=0.090), perhaps due to limited number of replicates. In contrast Vpx-mediated degradation of SAMHD-1 rescued VSV-G HIV-GFP infection in TGF-β treated cells and allowed high GFP expression in MDDC (NT MDDC mean = 82.65%, SEM=4.55, n=2; MDDC + TGF-β mean=85.6%, SEM=3.4, n=2) (**Figure 4.20.B**). Thus suggesting that, although a TGF-β-mediated restriction activity could be induced in MDDC, SAMHD1 remains the most prominent viral restriction factor in these cells and down regulation of SAMHD-1 allows effective high viral infection of MDDC.

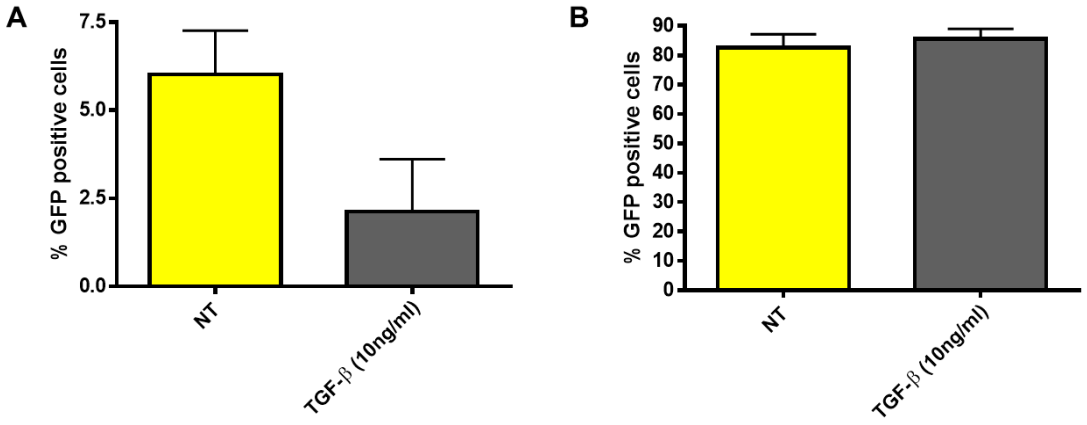

Figure 4.20. TGF-β Enhances Restriction of VSV-G HIV-GFP Infection in MDDC in the Presence of SAMHD-1. At day 6 of differentiation, MDDC were treated with 10ng/ml of TGF-β for 24 hours. Cells were then pre-treated or not with Vpx-expressing lentivectors and infected for 3 days with 25ng p24/10^5 cells of VSV-G HIV-GFP. **(A)** Data for infections in the absence and **(B)** in the presence of SIV3-Vpx are shown (*n=2*). NT indicates cells non-treated with TGF-β. Error bars represent ±SEM.

4.2.7. Virus Infection in MDLC is Affected at the Reverse Transcription Step

To further investigate the step of VSV-G HIV-GFP infection affected in MDLC, I isolated DNA from infected MDLC and MDDC for Quantitative real time PCR analysis. Using established GFP primers, I observed a higher accumulation of GFP DNA in MDDC samples infected with VSV-G HIV-GFP (± SIV3-Vpx) as compared to autologous MDLC. Although the infection susceptibility differed between donors, the trend remained the same, that is GFP DNA in VSV-G HIV-GFP transduced MDDC was quantified to be 1,1 and 257.6 in donor 1, 2 and 3, accordingly (**Figure 4.21A, B and C**). In contrast, the amount of GFP DNA in MDLC ranged between 0.06 and 17.0 (donor 2 and 3 accordingly) (**Figure 4.21B and C)**. Reminiscent of infection profiles, removal of SAMHD-1 by SIV3-Vpx supplementation to cells resulted in a substantial increase of GFP DNA in MDDC that remained marginal in MDLC. Accordingly, relative quantification of GFP DNA in MDDC was the highest in donor 3 and was equal to 1444, while it reached insignificant 98.1 in MDLC (**Figure 4.21 C**). Interestingly a substantial difference in the amount of GFP DNA was recorded between used donors, suggesting unsuccessful degradation of SAMHD-1 in one of the donors or divergent genetic susceptibility of blood samples to infection. If in fact cells were not completely depleted of SAMHD-1, acquired results do not allow to exclude the role of this restriction factor in VSV-G HIV-GFP inhibition, even if it was reduced in both MDDC and MDLC to the same extend. However, the difference in GFP DNA levels between VSV-G HIV-GFP infected MDLC and MDDC in donor 3 reflected this observed in GFP protein expression analysis for the each cell type. Based on this preliminary data I suggest that a block at the RT step upon VSV-G HIV-

GFP infection is present in MDLC and it could be partially lifted by SAMHD-1 degradation.

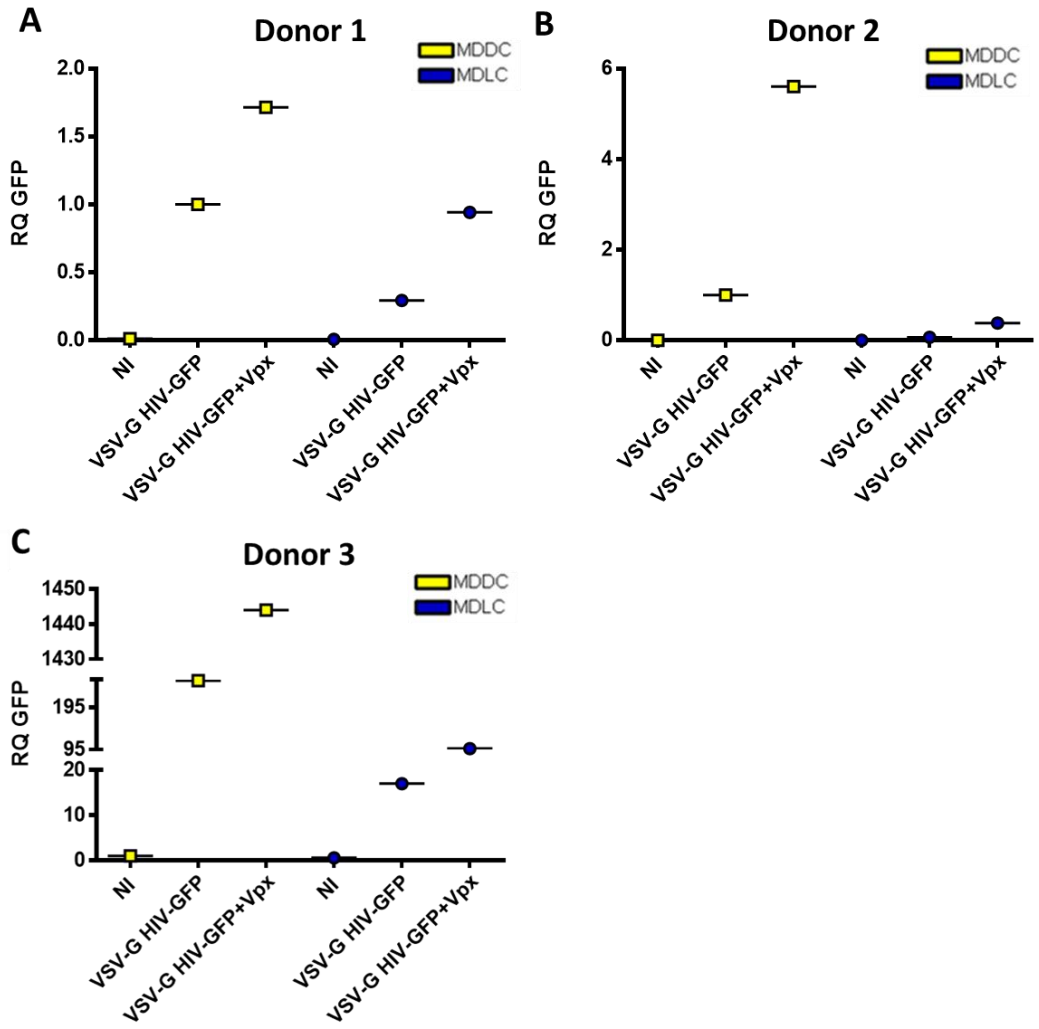

Figure 4.21. Relative Quantification of GFP in VSV-G HIV-GFP Infected MDDC and MDLC. Homologous MDDC and MDLC were pre-treated or not with SIV3-Vpx for 4 hours and infected with 30ng p24 VSV-G HIV-GFP. After 6 hours DNA was extracted from the cells for GFP quantification. **(A, B and C)** Analysis of GFP expression levels by qRT-PCR is shown for 3 donors, donor 1 **(A)**, donor 2 **(B)** and donor 3 (**C**) (*n=2*). NI indicates non-infected cells. Primers used: GFP forward primer 5'-aagttcatctgcaccaccg-3' GFP reverse primer 5'- tccttgaagaagatggtgcg-3' (Eurofins MWG Operon, Germany).

4.3. Discussion

Myeloid cells, such as macrophages and DC subsets, are able to partially restrict HIV-1 infection due to the presence of cellular restriction factors like SAMHD1 (Berger et al. 2011a; Hrecka et al. 2011; Laguette et al. 2011) or A3G (Pion et al. 2006). Whereas a growing body of evidence is available on the role of SAMHD1 in myeloid cells very little is known about restriction of HIV-1 infection of LC. These are among the first immune cells that can encounter HIV-1 during sexual transmission due to their localization in epithelia and mucosal surfaces. In this study, primary skin-resident epidermal LC, MDLC and the LC-like cell line (MuLC) have been used to investigate HIV-1 restriction mechanisms in LC. Our results show that the cellular restriction factor SAMHD1 is expressed in LC and that Vpx mediates its degradation in a time-dependent manner, as expected. However, in contrast to other myeloid DC subsets, SAMHD1 degradation in LC was not associated with increased susceptibility to HIV-1 infection.

Langerin or the availability of entry receptors can restrict entry of HIV-1 to LC (Kawamura et al. 2000; de Witte et al. 2007). Therefore, VSV-G pseudotyped GFP expressing HIV-lentivectors, which can bypass Langerin-mediated binding and the influence of HIV-1 surface receptors/co-receptors to efficiently enter into the cell was used in this study. Exploiting an *ex vivo* model of skin-resident primary epidermal LC, I report restriction of VSV-G HIV-GFP infection in these cells, even upon Vpx-mediated SAMHD1 degradation. The infection pattern was quite similar when using *ex vivo* monocyte-derived LC (MDLC) and the Langerhans-like cell line (MuLC). This is in sharp contrast to dermal DC and MDDC, which became considerably more susceptible to VSV-G HIV-GFP transduction in the absence of SAMHD1 expression.

Based on our results restriction of VSV-G HIV-GFP infection in LC might occur at the reverse transcription step, as lower levels of GFP DNA were detected in MDLC at 6 hours post-infection. Pre-treatment with the reverse-transcription inhibitor AZT led to a strong block of VSV-G HIV-GFP transduction in MDLC and in MDDC. Intriguingly GFP expression in MuLC, although already very low, remained unaffected by this treatment possibly due to unsuccessful inhibition of reverse-transcriptase activity by AZT or, more probably, suggesting that the residual GFP signal would come from the viral input and not related to viral infection, however this is unlikely.

As considered above, SIV3-Vpx particles employed in this study contain SIV Vpr protein. In HIV-1, Vpr promotes virus replication by increasing transcription of provirus and by deregulation of immune responses (Majumder et al. 2005; Mashiba et al. 2014; Reinhard et al. 2014; Zahoor et al. 2015) (see Introduction Section 1.2). Therefore, a possible effect of SIV Vpr present in SIV3-Vpx particles on experimental outcomes has been considered in the result section of this chapter. Accordingly, incoming SIV Vpr could boost transcription of provirus from both LTR by directly binding to LTR and transcription factors and via induction of NFκβ (Roux et al. 2000; Gangwani et al. 2013). Consequently, higher readouts for p24 Gag in SIV3-Vpx treated cells could be due to the presence of SIV Vpr protein as well as due to SAMHD1 depletion. In case of VSV-G HIV-GFP transduction experiments, GFP levels in cells could be elevated by cellular activation triggered by SIV3-Vpx derived Vpr protein. However, the effect of Vpr-mediated transcription activation could be difficult to detect, especially in MDDC, where SAMHD-1 down regulation results in significant boost to infection. Both SIV3-Vpx derived proteins, Vpx and Vpr could influence the level of infection in MDDC, however they fail to achieve similar

outcome in MDLC possibly due to the presence of other dominant restriction factor in these cells. Therefore, I hypothesise, that the presence of SIV Vpr in SIV3-Vpx could have some effect on biology of the cells used in this study and their susceptibility to infection. However, I believe that this effect does not influence the main conclusion of the above study that a very potent restriction, other than SAMHD-1, operates in Langerhans cells. Actually, the fact that two influential HIV accessory proteins cannot overcome this blockage does imply a great effectiveness of the TGF-β induced HIV-1 restriction observed in LC.

Viral infections are sensed by infected cells leading to release of type-I Interferon. In the case of HIV-1, virus "hides" from cell intrinsic sensing through the shielding action of capsid (Lahaye et al. 2013; Rasaiyaah et al. 2013) and by the employment of the cellular DNase, Trex1 (Goldfeld et al. 1991; Yan et al. 2010). Trex1 keeps the amount of viral reverse transcription products in check, which directly prevents sensing of HIV-1 dsDNA by cGas and other cellular DNA sensors. Depletion of Trex1 in cells leads to Interferon production in HIV-1 infected cells (Yan et al. 2010) highlighting the role of this protein in successful HIV-1 progression. Similarly, HIV-2, which encodes the Vpx protein counteracting SAMHD-1 in cells, is less pathogenic in human due to increased immune responses towards the virus. Thus to exclude the involvement of Interferon in VSV-G HIV-GFP restriction in MDLC I investigated the amount of IFN-β transcripts in infected MDLC and MDDC. Importantly, at early time points (up to 24 hours) I could not detect a significant induction of IFN-β mRNA expression in MDLC or MDDC transduced with VSV-G HIV-GFP, in presence or absence of SIV3-Vpx. However, lack of IFN-β detection in cells could be explained by using single round replication VSV-G HIV-GFP lentiviral vectors, which do not encode Gag/Pol viral proteins and the agreement with reports showing that newly

synthesized HIV-1 capsid is required to induce full DC maturation in absence of SAMHD1 expression (Manel et al. 2010). Additionally, HIV-1 Vpr protein was demonstrated to inhibit interferon stimulation in infected dendritic cells and macrophages (Mashiba et al. 2014; Harman et al. 2015). If this function is conserved in SIV Vpr that is present in SIV3-Vpx particles, Vpr could block early release of interferon from infected cells and rescue virus replication in MDDC, but not MDLC. Despite lack of IFN-β stimulation, I did observe, a partial maturation of both MDDC and MDLC at later stages of VSV-G HIV-GFP + SIV3-Vpx infection leading to an increase in cell surface expression of CD86, which suggests that some level of vector sensing could take place in both myeloid subsets. Since even a partial maturation of DC could initiate their migration from mucosa to lymph nodes this suggests that LC could, however, limit HIV-1 transmission to $CD4^+$ T cells (Harman et al. 2006) due to their capacity in potently restricting viral replication.

Other restriction factors can potentially operate in DC subsets, including BST-2/Tetherin and A3G. The former was reported to act on the last stages of HIV-1 replication and more precisely during viral release, therefore making unlikely for BST-2/Tetherin to have any role as a post-entry restriction factor in LC in the context of single round replication lentiviral vectors such as VSV-G HIV-GFP (Berger et al. 2011a). On the other hand A3G was reported to generate lethal editing of nascent reverse transcripts upon infection of target cells (Mangeat et al. 2003) and was also involved in post-entry restriction of HIV-1 infection of DC (Pion et al. 2006) and in the direct inhibition of reverse transcripts elongation (Bishop et al. 2008). Our results showed that A3G levels are seemingly not modulated in immature MDLC compared to MDDC and, as expected, A3G expression increased in both cell types upon IFN-α treatment. APOBEC3G acts at early steps of HIV-1 replication and it exerts its

restriction in new infection only when incorporated into virus particle in producer cells (Mangeat et al. 2003; Newman et al. 2005). For that reason, critical role of A3G in VSV-G HIV-GFP inhibition in LC is unlikely. Recently, IFN-α-inducible MX2 was reported to restrict HIV-1 infection at a pre-integration step of the viral replication cycle (Goujon et al. 2013; Kane et al. 2013). I confirmed in our study that MX2 expression can be, almost exclusively, induced by IFN-α treatment of both MDLC and MDDC, while immature LC are totally lacking MX2 expression. MX2 expression was also previously reported to be stimulated in cells treated with TLR3 agonists (e.g. Poly I:C), independently of IFN-α (Farina et al. 2011). I evidenced in our study that IFN-α and poly I:C further inhibit VSV-G HIV-GFP infection of MDLC, suggesting therefore, that MX2 could be likely involved in the restriction of VSV-G HIV-GFP in IFN-α or poly I:C stimulated MDLC. In fact, I recorded a significant MX2 mRNA increase in synthetic double-stranded DNA sequence (poly dA:dT) treated MDLC and MDDC. However, neither steady-state nor VSV-G HIV-GFP + SIV3-Vpx transduced MDLC express MX2 at protein or mRNA level thus virus restriction activity is unlikely to rely on this ISG.

Interestingly I observed an increased susceptibility of MDLC to VSV-G HIV-GFP infection in MDLC matured with TNF-α or TLR-2 agonists, found on gram-positive bacteria, in agreement with previous results obtained with full length replication-competent virus (de Jong et al. 2008; Ogawa et al. 2009). This is in contrast to other subsets of DC, which become more resistant to HIV-1 infection after maturation. Co-infections with other pathogens, such as Chlamydia or HSV-2, were reported to significantly raise the risk of HIV-1 acquisition; possibly, in part due to increased ability of mature LC to deliver HIV-1 from mucosal surfaces to susceptible $CD4^+$ T cells (Sewankambo et al. 1997; Galvin and Cohen 2004; Peretti et al. 2005; Ogawa et

al. 2013). This enhanced efficiency of virus transmission by matured LC was shown to result from stimulated HIV-1 *de novo* production in matured LC rather than increased viral capture by Langerin (Kawamura et al. 2000; de Jong et al. 2008; Ogawa et al. 2009; Ganor et al. 2013). Our results with VSV-G HIV-GFP lentiviral vectors, which bypass Langerin and HIV-1 receptors/co-receptors (CD4/CCR5), showed that LC stimulated with TNF-α or TLR-2 agonists became more prone to infection, seemingly due to a partial elimination of post-entry viral restriction. Hence the mechanisms by which TNF-α and PAM3CSK4 promote virus infection are seemingly independent of SAMHD1, as the expression of this restriction factor was down regulated with SIV3-Vpx in LC prior to viral infection. TNF-α induces NF-κβ signalling and was previously described to enhance HIV-1 transcription from LTR in cells (Duh et al. 1989). In this study, GFP expression in VSV-G HIV-GFP construct is mediated via human elongation factor-1 alpha (EF-1α) promoter, which is not known to be responsive to NF-κβ. Therefore, TNF-α-mediated increase of GFP expression in the cells results from changes to the cell imposed by general cell activation and unlikely the induction of provirus transcription.

At this point it is worth to consider the presence of Vpr protein in SIV3-Vpx particles, as this protein was demonstrated to have an inhibitory effect of NF-κβ stimulation downstream of TNF-α receptor in macrophages (Kogan et al. 2013). If NF-κβ signal transduction is impaired in my systems due to the presence of Vpr, the results would suggest the involvement of other signalling pathway in stimulation of GFP expression in the cells. Apart from NF-κβ signalling, binding of TNF-α to either of its receptor I or II triggers activation of Erk/MAPK dependent and MAPK-p38-dependent signalling pathways and downstream activation of transcription factors, such as ATF2, c-Jun and Elk (Chang and Karin 2001; Wajant et al. 2002). Additionally, Vpr protein

itself activates infected cells that might consequently partially alleviate the restriction operating in the cells. However, if in fact TNF-α effect on GFP expression in cells relies on enhanced transcription of integrated provirus, the step in virus life cycle prior integration will orchestrate the magnitude of TNF-α effect. For instance, if reverse transcription is lower in MDLC compared to MDDC, less viral dsDNA integrates into host genome resulting in weaker effect of TNF-α. However, that was not the case in this study as about 4-fold increase of GFP positive MDLC was observed in the presence of TNF-α compared to average 2-fold increase in MDDC in the same conditions. Eventually, multiple effect of TNF-α excreted simultaneously on the cells could results in boost to GFP expression observed in MDLC and MDDC. Regardless the mechanism, my results support an increased incidence of HIV-1 transmission during co-infection with other pathogens due to maturation of LC and a greater propensity for HIV-1 infection (de Jong et al. 2008; Ogawa et al. 2009; de Jong et al. 2010c; Ogawa et al. 2013). It is remarkable that maturation of LC with Poly I:C, a TLR-3 agonist, strongly increased further their resistance to HIV-1 infection, while exposure to gram-positive bacteria components (TLR-2 agonists) had the opposite effect. A better understanding of the mechanisms behind these phenomena would undoubtedly benefit the development of new microbicides aimed at decreasing the incidence of HIV-1 acquisition during co-infections.

The variable susceptibility of epidermal LC and dermal DC, derived from the same donor, to HIV-1 infection may be linked to the status of the local environment in each skin layer, such as cytokine levels. However, this hypothesis requires further investigation. In contrast, *ex vivo* monocyte-derived LC and DC are grown in strictly controlled conditions and their differentiation environment differs uniquely by the presence of TGF-β in MDLC culture medium. Our results show that monocytes

differentiating in the presence of TGF-β became more resistant to HIV infection, soon after initial supplementation of medium with this cytokine, compared to monocytes grown in conditions lacking this cytokine. Pharmacological inhibition of TGF-β signalling restores infectability of these cells. Additionally, TGF-β treatment of fully differentiated MDDC renders them less permissive to VSV-G HIV-GFP, suggesting that a TGF-β dependent viral restriction activity could be transferable to other cells. In agreement with these results it has been shown that TGF-β also restrains HIV-1 infection in monocytes and macrophages (Poli et al. 1991). However, the immunosuppressive and anti-inflammatory actions of this cytokine may also support HIV-1 progression in infected hosts, possibly by interfering with endocytic rates and antigen presentation capacities of DC (Cerwenka and Swain 1999; Kobie et al. 2003; Li et al. 2006b). Furthermore elevated plasma levels of TGF-β were described in HIV-1 infected patients with high viremia but not in patients naturally controlling HIV-1 infection (Card et al. 2012). An increased expression of the CXCR4 co-receptor was also proposed to favour HIV-1 entry into macrophages exposed to TGF-β (Chen et al. 2005). Whereas inhibition of T cell activation in a TGF-β environment may support HIV-1 systemic progression initial events of virus acquisition may be affected differently by TGF-β, depending on the nature of immune cells targeted by the virus. In this study, I demonstrated that the presence of TGF-β correlated with an induced natural post-entry resistance to HIV-1 infection of immature LC.

Our data conclusively support the existence of a novel, SAMHD1-independent and Interferon-independent, post-entry HIV-1 restriction mechanism present in immature monocyte-derived and freshly isolated skin LC. Importantly this TGF-β-dependent natural post-entry restriction activity was lowered upon treatment with pro-inflammatory molecules such as TNF-α and TLR-2 agonists, a context relevant to co-

infections with STIs including bacteria or HSV-2. Further characterisation of this restriction mechanism present in LC may offer potent means to control early events of HIV-1 infection and spread.

5. General Discussion

5.1. Restriction of HIV-1/VSV-G HIV-GFP Infection in Langerhans cell Models

Dendritic cell subsets residing at the mucosal surfaces play a crucial role in early events of HIV-1 infection and contribute to viral transmission to $CD4^+$ T cells. HIV-1 poorly replicates in myeloid cells due to the expression of restriction factors while preserving the ability to highjack these cells in order to favour systemic dissemination. Recent reports suggest that the cellular factor SAMHD-1 greatly inhibits the viral reverse transcription step by limiting the availability of the cellular deoxynucleotide triphosphates (dNTP) pool due to its dNTP hydrolase activity (Goldstone et al. 2011; Powell et al. 2011) (see General Introduction, Section 1.4.1). Interestingly, a recent report challenged this finding by demonstrating that SAMHD-1 could directly bind and degrade viral RNA (Ryoo et al. 2014). Indeed, the authors claimed that the SAMHD-1 RNase activity, but not the dNTPase function, was essential for HIV-1 restriction as a specific SAMHD-1 mutant (SAMHD1D137N), possessing a functional RNase activity but not the DNase, was able to restrict HIV-1 infection. In contrast, a mutated form of SAMHD-1 (SAMHD1Q548A) with an active DNase function but lacking RNase activity was defective in HIV-1 restriction (Ryoo et al. 2014). Although Langerhans cells also originate from myeloid precursors the role of SAMHD-1 in these cells has never been investigated. In this study I hypothesise that SAMHD-1 could also operate in Langerhans cells, but it appears that it was not acting as the main HIV-1 restriction in these cells. Using two Langerhans cell models, Monocytes-derived LC (MDLC) and MUTZ-3 derived LC (MuLC), in parallel with primary skin isolated epidermal LC, I discovered that degradation of SAMHD-1 by Vpx-expressing SIV-derived lentivectors does not lead to a major increase in

susceptibility to HIV-1 infection of either Langerhans cell models or primary Langerhans cells, possibly due to a TGF-β-induced HIV-1 restriction in these cells. Therefore, I challenge the notion that SAMHD-1 is the major restriction to HIV-1 infection in Langerhans cells. Instead, I hypothesise that the environmental factors, such as the presence of TGF-β can induce in these cells more potent restriction to HIV-1 infection. Although, SAMHD-1 function would still operate in Langerhans cells, its function may be imperceptible due to the predominant restriction imposed by the action of TGF-β. MDLC and MuLC are commonly used systems to study Langerhans cells functions (Masterson et al. 2002; Santegoets et al. 2006). Given the phenotypic and functional similarities of these cells to real LC, their popularity does not come as a surprise. However, both MuLC and MDLC show some dissimilarity to each other. For instance, considerable amounts of DC-SIGN are found in MDLC while Langerin is a predominant C-type lectin in MuLC (see **Figure 3.3** for MDLC and **Figure 3.2** for MuLC). Expression patterns of these lectins in MDLC and MuLC is particularly important for our research, as DC-SIGN and Langerin could have opposite functions in respect to HIV-1 infection (Lee et al. 2001; de Witte et al. 2007). Accordingly, attachment of virions to Langerin results in their degradation in Birbeck granules (Kawamura et al. 2000; de Witte et al. 2007). In contrast, DC-SIGN is known to support entry of HIV-1 into Dendritic Cells and to further enhance DC-mediated virus transmission to $CD4^+$ T cells (Lee et al. 2001). Therefore, direct comparison of wild type HIV-1 infection of MuLC and MDLC might have conflicting outcomes. Additionally, functional studies of MuLC might be misleading because of the partial maturation and impaired responsiveness of these cells to interferon stimulation (see **Figure 3.8**).

As SAMHD-1 induces an efficient post-entry block to HIV-1 replication in myeloid cells, cells were treated with VSV-G-pseudotyped Vpx-expressing SIV-derived lentivector. Effective SIV3-Vpx-mediated SAMHD-1 degradation was recorded for MuLC, MDLC and MDDC (see **Figure 4.1**) as soon as 3-4 hours post exposure to SIV3-Vpx addition. Rapid and consistent disappearance of SAMHD-1 between donors in SIV3-Vpx treated cells confirmed the feasibility of this method aimed at investigating HIV-1 restriction in LC in the presence or absence of SAMHD-1 expression. Furthermore, the stable effect of SIV3-Vpx ensured the absence of SAMHD-1 in the cells during the whole duration of virus infection experiments (3-4 days). Despite complete down-regulation of SAMHD-1 in the model cells, susceptibility of MuLC and MDLC to HIV-1 infection remained negligible in contrast to robust infection observed in MDDC following SIV3-Vpx-mediated SAMHD-1 degradation. The apparent increase of Gag-$p24^{+}$ MDDC observed in the experiments validated previous findings describing a predominant viral restrictive function of this cellular factor in Dendritic Cells (Berger et al. 2011a; Hrecka et al. 2011; Laguette et al. 2011). However, infection results obtained from LC models suggested the presence of a SAMHD-1-independent HIV-1 restriction in these cells. A closer analysis of infection results in MuLC revealed a reproducibly low MFI of p24-gag positive MuLC population barely distinguishable from the main population suggesting a very limited viral production in this cell line even when considering cells productively infected (see **Figure 4.3**). In contrast, the p24 positive populations from productively HIV-1 infected MDDC and MDLC were clearly separated (see **Figure 4.2**) and significantly affected in the presence of AZT (see **Figure 4.7**). The p24-gag readout of HIV-1 infection does not distinguish between virus capture and productive infection. Thus gag^{+} MuLC population might represent weakly effective replicating HIV-1 but

also langerin-captured particles destined for lysosomal degradation. The slight increase in gag^+ MuLC during SIV3-Vpx co-infection, compared to the infection in the presence of SAMHD-1, indicates productive infection of the virus rather than capture, however both processes could be taking place in MuLC. Although the capture of HIV-1 in MuLC by Langerin would significantly decrease the percentage of gag positive cells, similar infection susceptibility of these cells compared to MDLC could results from partial maturation of MuLC derived in the presence of TNF-α. In fact, I observed that TNF-α signalling can favour virus infection in cells (see **Figure 4.15**). Nevertheless, to eliminate the possibility of C-type lectin involvement in HIV-1 entry in LC models, VSV-G envelope pseudotyped GFP expressing HIV-1 virus (VSV-G HIV-GFP) (Naldini et al. 1996; Dull et al. 1998) was used in this work instead of wild type HIV-1. The use of VSV-G HIV-GFP brings numerous benefits into the system including the easy readout of productively infected cells by a measurement of GFP expression, which excludes the potential background of captured particles such as in the case of p24-gag staining. However, during VSV-G HIV-GFP stock production, GFP mRNA or a translated protein itself can be packaged into lentiviral particles, and be consequently detected in target cells. This process called a retroviral pseudotransduction may lead to the expression of foreign proteins, without delivering integrating proviral DNA (Haas et al. 2000; Galla et al. 2004; Nash and Lever 2004). Thus, some levels of GFP detected in cells transduced with VSV-G HIV-GFP cells may represent pseudotyped molecules rather than a result of productive lentiviral infection. However, GFP protein expression in both MDLC and MDDC was sensitive to AZT suggesting the productive infection is the main source of GFP in these cells. Although, susceptibility of cells to pseudotransduction could introduce some low variation in the total amount of GFP+ cells between MDDC and MDLC, the effect of

SAMHD-1 degradation is mostly attributed to release of productive infection restriction in MDDC, which does not have the same effect in MDLC.

In addition to easy readout method in VSV-G HIV-GFP infection studies, VSV-G envelope bypasses the requirement for entry receptors (CD4) and co-receptor (CCR5) for lentivirus fusion and prevents binding of lentivirus to C-type lectins. Entry receptors of VSV-G are poorly characterised but recent reports underline the role of plasma membrane fatty acids in VSV-G envelope attachment to the cell (Finkelshtein et al. 2013). The origin of MDLC and MDDC from the same monocytic pool would predict comparable content of these molecules in cell membranes and therefore an equal efficiency of VSV-G binding. In addition to the above features, VSV-G HIV-GFP retains all the initial HIV-1 infection steps, including reverse transcription and integration, making it a subject to post-entry viral restriction factors. Although, usage of VSV-G pseudotyped particles overcomes entry restrictions associated with wild type HIV-1 gp120, VSV-G HIV-GFP also largely bypass cytoplasm as it fuses with endosomal membrane for entry. Upon binding low density lipoproteins (LDLR) on the cell surface, VSV-G HIV-GFP is endocytosed and fuses with endosomal membrane only after pH change imposed by lysosomal fusion (Sun et al. 2005; Finkelshtein et al. 2013). This different route of entry compared to HIV-1 localise VSV-G HIV-GFP close to the nucleus and possibly prevents interaction of lentivirus with cytoplasmic sensing mechanism or restriction factors. On the other hand, it could also impair binding of viral core to CPSF6 or Nup proteins, which were described to be essential for early infection steps of HIV-1 (Price et al. 2012; Rasaiyaah et al. 2013). Therefore, the exact consequence of endosomal entry route of VSV-G HIV-GFP on its host protein interaction is largely unknown. However, accordingly to this study, SAMHD-1 remains the main restriction to VSV-G HIV-GFP infection in MDDC, leading to

assertive assumption that efficiency of early infection steps is not affected in these and related cells, such as MDLC. The use of VSV-G HIV-GFP concomitantly with the down-regulation of SAMHD-1 expression confirmed the refractory phenotype of MDLC and MuLC to lentivirus infection in these cells. MDLC susceptibility to infection remained unchanged compared to HIV-1 experiments, possibly confirming little involvement of Langerin in HIV-1 infection blockage. In contrast, VSV-G pseudotyped HIV-1 (+SIV3-Vpx) successfully bypassed Langerin restriction in MuLC resulting in increased lentiviral infection of these cells, as compared to HIV-1. Although the GFP signal in MuLC theoretically indicates only successful infection VSV-G HIV-GFP, AZT pre-treatment of lentiviral transduced cells did not result in lower percentage of GFP^+ MuLC. The defect of AZT can be excluded from the possible explanation of this result because VSV-G HIV-GFP infection performed in parallel in MDDC and MDLC was almost completely abolished in these cells in the presence of AZT (see **Figure 4.7**). Being a tumour-derived cell line MuLC could have a higher concentration of cytoplasmic nucleotides compared to blood-derived MDLC. In such case, high availability of these components for viral reverse-transcription might have not been fully altered by supplementation with AZT, consequently leading to low infection of the lentivirus. Possibly, observed GFP levels in MuLC may be a result of pseudotransduction that would be insensitive to AZT treatment. In that case, increase of GFP detection in MuLC upon SAMHD-1 degradation could result from inhibition of RNase activity of SAMHD-1 thus increased stability of pseudotransduced GFP mRNA. However, this would not explain the evident difference in GFP expression levels between MuLC and MDDC transduced with equal VSV-G HIV-GFP dose in the absence of SIV3-Vpx pre-treatment (see **Figure 4.6**). If pseudotransduction contributes to GFP levels in MuLC, the same background

detection should be theoretically recorded for MDDC. Whether partial activation of MuLC could influence the expression of GFP mRNA was not investigated in this study, however, infection of MuLC with VSV-G HIV-GFP cannot be excluded from the above data. Nevertheless, limited understanding of HIV-1/VSV-G HIV-GFP infection in MuLC influenced the choice not to use this cell model for further experiments in this study, including the fact that MuLC poorly mimic other immunological functions from LC. Instead, the investigation of VSV-G HIV-GFP restriction in LC models was performed in MDLC in comparison to MDDC. Although $CD141^+$ DC delivered in the presence of Vitamin D_3 were recently considered as appropriate dermal DC model (MacDonald et al. 2002; Jongbloed et al. 2010; Chu et al. 2012), MDDC have been also extrapolated as a model mimicking dermal DC subsets. These cells were therefore used in this work due to their good characterization in the literature, growing conditions like those in MDLC medium (except for TGF-β) and comparable to $CD141^+$ DC in their susceptibility to HIV-1 infection (see **Figure 4.4**). Together the results showed that restriction to HIV-1/VSV-G HIV-GFP infection in model LC cells is not eliminated after SAMHD-1 degradation, therefore suggesting some other major cellular mechanisms of viral restriction in these cells.

5.2. Primary Skin Isolated Epidermal LC and Dermal DC Confirm Restriction of HIV-1 in LC

In order to validate the results obtained in cell lines and primary cells I set up conditions to obtain primary skin isolated dermal DC (dDC) and epidermal LC (eLC). Challenge of dermis and epidermis walkout populations with VSV-G HIV-GFP, with or without SIV3-Vpx pre-treatment, demonstrated similar infection profiles to those

observed in MDDC and MDLC respectively. Accordingly, epidermal Langerhans cells were weakly permissive to VSV-G HIV-GFP in contrast to higher proportion of GFP positive dermal DC (see **Figure 4.9**). However, skin walkout cells comprise mixed population of immune cells and tissue cells, which all could be susceptible to VSV-G HIV-GFP entry. To address the possibility of lentivirus entry into other targets than myeloid cells, epidermal and dermal walkout population have been analysed by considering GFP expression related to specific eLC or dDC cell surface markers (**Figure 4.10 and Figure 4.11**). Results showed a predominant localization of GFP into LC and DC populations of epidermis and dermis respectively, suggesting a lack of significant infection of other skin cells. Nevertheless, a limited VSV-G HIV-GFP entry into other cells than DC and LC remains probable and our GFP readout method cannot rule out a non-productive infection, therefore possibly underestimating viral skin targets. The pseudotyping of lentivectors with other amphotropic or pantropic envelopes (Hemagglutinin from Influenza virus, MLV envelope, etc) could have helped in answering which was, however, beyond the scope of our study. As LCs appear in lower numbers in the epidermis, compared to DC population in the dermis, the corresponding virus/cell ratio might be different and the results obtained in this work could be skewed due to reduced entry of VSV-G HIV-GFP into LC. Similarly, viral-like particles carrying Vpx could have reached the other epidermis walkout cells than LC, leading to incomplete degradation of SAMHD-1 in these cells. In both cases, epidermal LC infection would appear lower compared to dermal DC. Additional staining of stimulated cells with p24-gag antibody could identify cells targeted by lentivirus; however, this readout was not used in this work. Increase of the initial VSV-G HIV-GFP input in epidermal infections could also partially resolve the underestimate of infection in eLC occurring due to their low numbers in walkout

population. In such case, the amount of particles reaching eLC would equalise to this entering dDC, thus allowing better comparison of these cells types. Although skin walkout cells infection challenge insinuate lower susceptibility of epidermal LC than dermal DC to HIV-1-like vector infection, the experiment protocol could be modified to address the above concerns. Nevertheless my results strongly suggest that *ex vivo* eLC, in contrast to dDC, remain strongly refractory to HIV-1 infection even upon SIV3-Vpx-mediated SAMHD-1 degradation.

5.3. Matured MDLC are More Permissive to VSV-G HIV-GFP Infection

The data obtained from this work (see Chapter 4) suggest that MuLC and MDLC are refractory to HIV-1/VSV-G HIV-GFP infection due to downstream action of TGF-β signalling. However, partial maturation of MuLC triggered by TNF-α can possibly lift the restriction imposed during differentiation resulting in slightly higher expression of GFP in these cells, compared to MDLC. The effect of TGF-β on cells depends on the cumulative impact of the microenvironments factors, such as cytokines, activation stimuli and inflammation. For instance TGF-β has a weaker effect on activated T cells possibly due to down-regulation of TGFβRII expression on these cells (Cottrez and Groux 2001). Indeed, I have also reported a significant, yet limited, effect of TGF-β on VSV-G HIV-GFP infection in differentiated MDDC (see **Figure 4.20**). Similarly, restrictive effect of TGF-β exerted on MuLC could be partially eliminated by TNFα-mediated maturation of these cells resulting in increased infection. Eventually the origin of MuLC from immortalized cell line might also promote GFP expression in these cells possibly due to the presence of high levels of factors required for cell

division and virus infection. However, this statement is only a speculation, which requires verification.

However, in agreement with the role of TNF-α on induction of GFP expression in MuLC treatment of differentiated MDLC with this cytokine or TLR-2 agonist increased VSV-G HIV-GFP infection of these cells (see **Figure 4.10**). TLR2 and TNF-α signalling pathways converge towards the activation of the master transcription factor NF-κβ that regulates expression of genes involved in multiple immune processes like cytokine production, inflammation or pyroptosis (Nabel and Baltimore 1987). However, NF-κβ is also known to bind several viral promoters including the Cytomegalovirus (CMV) promoter (Roux et al. 2000; Gangwani et al. 2013) and the long terminal repeat sequences flanking HIV-1 genome (Legrand-Poels et al. 1990; Kretzschmar et al. 1992). In fact, the HIV-1 encoded Tat protein mediates binding of NF-κβ to its LTR (Dandekar et al. 2004) supporting expression of integrated HIV-1 DNA. The phosphorylation of NF-κβ p62 at serine 536 in parallel with the degradation of its negative regulator IκBα are potential indicators of the activation of this signalling pathway. Indeed phosphorylated IκBα (p-IκBα) becomes ubiquitinated and is degraded in the proteasome, resulting in the release and phosphorylation of NF-κβ p62 and activation of gene expression. In agreement, phosphorylation of NF-κβ p62 and IκBα were observed in TNF-α stimulated MDLC (see **Figure 4.16**) suggesting activation of the cells. The results correlated with the increased GFP expression in transduced cells, suggesting the involvement of this signalling pathway in enhancement of VSV-G HIV-GFP infection of MDLC and MDDC. As EF1α promoter upstream of GFP in VSV-G HIV-GFP lentivector is not known to be directly bound by NF-κβ, related GFP expression increase could be a result of general cell activation. Thus, addition of TNF-α to MDLC culture would

activate the cells and consequently alleviate the virus restriction operating in these cells. In vivo, TNF-α induced NF-κβ signalling promotes activation of chronically infected T cells (Duh et al. 1989; Aukrust et al. 1994), but at the same time it was demonstrated to inhibit HIV-1 infection of peripheral blood mononuclear cells and alveolar macrophages (Herbein et al. 1996; Lane et al. 1999). However, elevated levels of TNF-α are detected in HIV-1 positive patients and these correlate with increased viral replication and systemic depletion of CD4+ T cells (Dezube et al. 1997; Valdez and Lederman 1997). Thus, *in vivo* TNF-α plays an notable role in HIV-1 progression and pathogenesis.

As discussed before (see Introduction Section 1.4.3) apart from Vpx, SIV3-Vpx particles used in this study deliver also Vpr proteins for which multiple conflicting functions regarding NF-κβ were described (Ayyavoo et al. 1997; Majumder et al. 2005; Varin et al. 2005; Hoshino et al. 2010). A study by Roux P (2000) demonstrated that Vpr uses NF-κβ signalling cascade in macrophages and T cells to induce IL-8 and to promote transcriptional activation of viral promoter such as LTR of HIV-1. However, recent study suggest that Vpr blocks TNF-α induced NF-κβ signalling in macrophages (Kogan et al. 2013). Thus, the TNF-α-mediated NF-κβ signalling in cells observed in this study could have been restricted by the presence of Vpr protein. This could explain why Pam3CSK and TNF-α induced maturation of MDLC were insufficient to fully release viral restriction in MDLC.

Interestingly Pam3CSK supplementation to MDDC brought the opposite effect to this observed in MDLC and resulted in a decreased percentage of GFP positive MDDC population. Although, both MDDC and MDLC respond to Pam3CSK by release of TNF-α/IL-10 cytokines (see **Figure 3.12 and 3.13**) production of other pro-inflammatory cytokines might differ in these cells. As Langerhans cells are constantly

exposed to bacterial components they induce mostly tolerogenic responses. This is in contrast to Dendritic Cells that induce strong pro-inflammatory response when stimulated with TLR agonists of bacterial origin, which could possibly also decrease susceptibility of these cells to other infections, for example HIV-1. However, the change in VSV-G HIV-GFP expression in Pam3CSK treated MDDC was not significant compared to non-stimulated MDDC, possibly suggesting a lack of strong immune response to this TLR1/2 agonist.

In contrast to Pam3CSK and TNF-α, poly I:C stimulation of MDLC and MDDC almost completely blocked VSV-G HIV-GFP infection in these cells possibly due to the establishment of a potent antiviral state in the cell. Activation of TLR-3 signalling was reported to be associated with induction of type-I interferon (IFN-I) response in the cells. Consequently, IFN from both, paracrine and endocrine source induces expression of interferon-stimulated genes (ISG) among which some of them are known to be potent antiviral. Thus, as expected 6 hours stimulation of MDDC and MDLC with poly dA:dT resulted in accumulation of IFN-β mRNA (see **Figure 4.13**) possibly involved in the late restriction of VSV-G HIV-GFP transduction of these cells. In contrast, IFN-β response was not detected in SAMHD-1 depleted MDLC infected with VSV-G HIV-GFP until 24 hours post infection. This effect was even more delayed in case of MDDC, where IFN-β mRNA was recorded only at 48 hours post infection. Interestingly, induction of IFN-β mRNA timely correlated with increase of MX2 mRNA in MDLC and MDDC, suggesting that this restriction factor has limited function at early time points of VSV-G HIV-GFP infection. In addition to PCR studies, blocking antibodies to IFN receptor or disruption to IFN signalling cascade could be employed in this research to identify the exact involvement of interferon in MDLC infection control. Restoration of GFP expression in such treated

MDLC would allow identification of an early role of interferon in VSV-G HIV-GFP restriction in these cells.

A robust reverse-transcription of VSV-G HIV-GFP in MDLC in the absence of SAMHD-1 could be a target for cytoplasmic DNA sensors, which consequently inhibit GFP expression. In fact high levels of the early products of HIV-1 infection of SAMHD-1 depleted cells could be detected in MDDC by cyclic-GMP-AMP (cGAMP) synthase (cGAS), resulting in induction of interferon response and cells maturation (Manel et al. 2010; Manel and Littman 2011; Gao et al. 2013). However, accordingly to other studies, intracellular sensing of HIV-1 cDNA in MDDC is ineffective as viral capsid shields it from detection by cGAS and other DNA sensors, even in the presence of Vpx (Manel et al. 2010; Lahaye et al. 2013; Rasaiyaah et al. 2013). Instead, it was suggested that a newly synthesised gag protein of HIV-1 would be required to fully activate infected DC (Luban et al. 1993; Manel et al. 2010). As a single round replication construct, VSV-G HIV-GFP does not encode *gag* gene, which excludes sensing of this gene product in stimulated MDDC or MDLC. Additionally, entry of VSV-G HIV-GFP to cell cytoplasm from endosomal compartments could possibly limit the exposure of lentiviral genome to sensing molecules. On the other hand, bypassing of the cytoplasm may limit binding of CPSF6 and other cellular proteins to VSV-G HIV-GFP capsid thus affect shielding of viral nucleic acids. A successful infection of VSV-G HIV-GFP in SIV3-Vpx-treated MDDC confirms the lack of lentivirus restriction imposed by DNA/RNA sensing, which however cannot exclude viral detection by a cell.

Only in the absence of SAMHD-1 does VSV-G HIV-GFP trigger maturation of MDDC and MDLC. If a high virus infection was required for this phenotype CD86 increase recorded in MDLC would strongly suggest the successful reverse

transcription of VSV-G HIV-GFP in these cells. Consequently sensing of infection products by MDLC would impose the restrictive phenotype observed in these cells that efficiently stalls GFP expression. However, the measurement of IFN-β mRNA in VSV-G HIV-GFP (+SIV3-Vpx) transduced MDLC at 5 hours post infection did not show any changes compared to non-infected cells. Perhaps this was performed too early in the infection to detect cellular activation. In contrast 48 hours after transduction the increase of expression of some maturation markers (CD86, HLA-DR) at the surface of MDLC could be observed, suggesting that infection could modulate the immune response of these cells depending on the context. Investigation of interferon response in MDLC at later stages of infection should be performed to clearly establish the role of interferon in VSV-G HIV-GFP inhibition in these cells. The blockade of IFN-mediated signalling by IFN receptor antagonists (antagonizing Abs) could be used to assess such role for example. If interferon-inducible restriction factors mediate the observed inhibition of VSV-G HIV-GFP infection of MDLC, measurement of these proteins levels after few days infection could also provide a clear indication. Unfortunately, this experiment has not been performed in this work however, available data shows no induction of MX2 or BST-2/Tetherin in SIV3-Vpx-treated MDDC or MDLC (see **Figure 4.12**) suggesting that down-regulation of SAMHD-1 itself does not induce Interferon responses in the MDDC/MDLC. Additionally, it is possible that Interferon independent mechanisms of VSV-G HIV-GFP restriction operate in LC. This is particularly valid in the light of VSV-G HIV-GFP infection inhibition in MuLC that I demonstrated to be irresponsive to IFN-α stimulation.

Regardless of the mechanism of virus sensing in LC, IFN-α pre-treatment of MDLC further restricts GFP expression in infected cells (see **Figure 4.12**). A range of

restriction factors are unregulated in these cells including MX2, APOBEC3F/G, RIG-I and BST-2/Tetherin (see **Figure 4.12**). MX2 (also called MXB) is a member of the IFN-inducible GTPase superfamily closely related to MX1, which was recently described to act as an IFN-dependent inhibitor of HIV-1 infection of macrophages (Goujon et al. 2013; Kane et al. 2013). MX2 acts at early stages of HIV-1 replication seemingly targeting the HIV-1 uncoating process (Kane et al. 2013; Buffone et al. 2015). For the first time expression of this restriction factor could be demonstrated in MDLC but, as expected, exclusively after IFN-α stimulation (see **Figure 4.12**). As MX2 affects early infection steps of the virus, the induction of MX2 in interferon treated MDLC could potentially explain LC restriction to VSV-G HIV-GFP. However this is unlikely as at early time points of VSV-G HIV-GFP infection (up to 24 hours) MX2 mRNA is absent in MDLC (see **Figure 4.13**), suggesting that MX-2 role in VSV-G HIV-GFP inhibition is limited unless cells are pre-treated with interferon. Similarly, as a single replication construct, VSV-G HIV-GFP is not susceptible to the action of APOBEC3G/F that is constrained to secondary infection (Mangeat et al. 2003; Newman et al. 2005; Malim 2009). However, it was showed that APOBEC3G could limit HIV-1 infection in monocyte-derived DC during primary infection (Pion et al. 2006). Down regulation of APOBEC3G would be necessary to fully exclude it from the list of restrictions operating in MDLC. Whether function of APOBEC3G could be modulated by TGF-β remains to be determined.

Interestingly the results suggest that HIV-1 infection in type I interferon stimulated MDLC could be also restricted by increased expression of surface and intracellular Langerin in these cells (see **Figure 3.9**). Langerin plays an important function in pathogen capture therefore an increased expression of this CLR mediated could be an additional innate mechanism operating during infection. Although the exact signalling

triggering Langerin expression during interferon treatment has not been identified it could be expected to involve common signalling components within the type-I interferon signalling cascade. Such mechanism, if verified, would further emphasize the role of Langerin in viral and possibly bacterial infections. Additionally, if levels of Langerin on MDLC could represent an indirect measure of interferon signalling in the cells increased expression of this CLR in MDLC transduced with SIV3-Vpx and VSV-G HIV-GFP would again suggest an antiviral response in these cells. The mechanism of Interferon-mediated Langerin increase is not understood, hence not verified in freshly isolated epidermal Langerhans cells.

Thus, the above data provide an interesting observation regarding the SAMHD-1-independent inhibition of HIV-1 infection in TGF-β derived MDLC. Although no interferon release could be observed in MDLC at initial stages of VSV-G HIV-GFP infection, such response cannot be excluded to take part at later time-points. Additionally, Infection related maturation of MDLC and up-regulation of Langerin could correlate with the involvement of sensing mechanism in these cells that blocks viral propagation. However, such mechanism has not been confirmed in this work.

5.4. TGF-β Induces an Anti-retroviral State in MDLC

The fact that MDLC and MDDC derived from the same monocytes show such distinguished profile of HIV-1 infection is intriguing. Available data suggest that the action of TGF-β present exclusively in MDLC differentiating medium imposes cells restrictive phenotype. TGF-β is known for its strong immunosuppressive activity on various cell types (Borkowski et al. 1996; Letterio and Roberts 1998; Kobie et al. 2003; Li et al. 2006a). For instance, TGF-β treated DC fail to up-regulate MHCII and

other co-stimulatory molecules when activated with pathogens (Geissmann et al. 1999). This immunosuppressive activity of TGF-β also affects the responses of Langerhans cells to bacterial components. Limited TLR repertoire and tolerogenic activity of LC prevents pro-inflammatory responses to commensal bacteria and sustains body mucosal homeostasis. Correspondingly, TGF-β activity also modulates the permissiveness of MDLC to HIV-1 infection as demonstrated in this work. Accordingly, soon after the first supplementation of TGF-β differentiating monocytes become more restrictive to VSV-G HIV-GFP infection, compared to monocytes grown in the absence of this cytokine. This TGF-β mediated effect amplifies during cell culture and reaches its greatest restriction upon full differentiation (see **Figure 4.18**). However, prior to establishment of the restrictive phenotype, differentiating MDLC show a variable susceptibility to infection, which corresponds to TGF-β supplementation. In fact at day 3 of differentiation, 2 days since last addition of TGF-β to the cells medium, VSV-G HIV-GFP susceptibility of SAMHD-1 depleted cells increases substantially in comparison to an infection challenge performed a day after TGF-β supplementation. Perhaps at this time, restriction in MDLC is not yet fully developed and it depends purely on the recent TGF-β signalling. Alternatively, during differentiation TGF-β induced restriction can operates on different levels, including brief decrease of dNTP levels in the cells or general, transient suppression of the cell activity. In contrast, fully developed cells may acquire a new permanent mechanism that leads to inhibition of HIV-1 infection in these cells. Such mechanism could include down-regulation of viral dependency factors or up-regulation of restriction factors levels or activity mediated by TGF-β. A systematic analysis (microarrays, proteomics…) of MDLC and MDDC could potentially indicate candidate genes that differ between these cells and could be responsible for viral restriction in MDLC.

Further testing of each candidate would have to follow in order to identify the exact mechanism of HIV-1 restriction imposed by TGF-β.

TGF-β signals via SMAD2 and SMAD3, which form a complex in the cytoplasm. Activation of this complex and consequent translocation of SMAD2/3 to the nucleus is achieved by phosphorylation of both SMAD2 and SMAD3 at Ser465/467 and Ser423/425 respectively. The pharmacological component LY2109761 is a small molecule inhibitor of the TGF-β receptor type 1/type II kinase activity and inhibits, therefore, the phosphorylation and activation of downstream effectors of the TGF-β-mediated signalling pathway such as SMAD2 and SMAD3. The effectiveness of LY2109761 in the inhibition of SMAD2 activation was analyzed in differentiating MDLC and was subsequently shown to prevent the establishment of the viral restriction in these cells. As a result, the level of VSV-G HIV-GFP infection in MDLC increased, particularly when SAMHD-1 expression was previously down modulated (see **Figure 4.19**). Therefore the infection results obtained in LY2109761 treated MDLC showed that these cells were behaving like MDDC thus confirming that TGF-β signalling is crucial for induction of non-permissive state in MDLC.

Although the exact mechanism of viral inhibition in MDLC could not be fully characterised during this study, attempts to identify the stage of HIV-1 infection affected in these cells indicated a possible block at the viral reverse transcription step. Quantitative PCR of VSV-G HIV-GFP (+/-SIV3-Vpx) transduced cells showed lower accumulation of *gfp* DNA in MDLC compared to MDDC (see **Figure 4.21**), reflecting an infection pattern observed in these cells (see **Figure 4.5**). Perhaps a block to virus propagation is elicited only at later stages of virus reverse transcription as accumulation of some *gfp* DNA products could be observed in the cells. In that case

sensing of reverse transcription products could be a plausible explanation. However, synthesis of viral DNA from its RNA genome could be also restricted independently of sensing by a pre-existing restriction. As a result, *gfp* DNA detected in MDLC might originate from a restriction escape. Perhaps the arising transcript would be incomplete additionally leading to inhibition of further VSV-G HIV-GFP infection steps including integration or transcription. The fact that some particles could avoid restriction would eliminate the possibility that MDLC are completely depleted in a viral replication dependency factor.

Based on the data collected in this work I have characterised several aspects of a mechanism involved in HIV-1 restriction in LC. Apparently HIV-1 infection is restrained in MDLC prior to integration, possibly during the reverse transcription process, however several explanation as to the mode of restriction are possible. These are considered as follows:

1) Firstly, SAMHD-1-independent restriction in MDLC could be mediated by expression of a yet unidentified cellular factor in these cells. This restriction could act on dNTP levels similarly to SAMHD-1, or have another function that blocks early steps of HIV-1 reverse transcription. Western blot analysis of APOBEC3 in MDDC and MDLC showed comparable levels of this protein in both cells types excluding it from potential candidates although a difference in APOBEC3G complexes could mediated this effect as shown for other myeloid cells (Stalder et al. 2010). Lack of MX2 detection in non-stimulated MDLC also suggests that TGF-β itself does not trigger expression of this protein. Other anti-retroviral restriction factors operate in cells including TRIM5α and Fv1 but their expression has not been investigated in our experiments. Eventually TGF-β action could focus on the qualitative improvement of

restriction factors (other than SAMHD-1) activity rather than stimulating their amount.

2) Ultimately, instead of acting on restriction factors TGF-β could induce, as evoked already, an effective sensing mechanism in MDLC. In that case detection of VSV-G HIV-GFP early during infection could trigger an antiviral state in the cells resulting in low GFP expression. In line with this hypothesis, maturation of SAMHD-1 depleted MDLC after transduction with VSV-G HIV-GFP was observed (see above 5.3). The same conditions also induced expression of Langerin on MDLC suggesting that Interferon could play a role in inducing a resistance to HIV-1 in MDLC. Although, IFN-β mRNA production in these cells has not been observed, the experiment was performed at very early steps of VSV-G HIV-GFP infection, and possibly more time is required to induce an Interferon response.
3) HIV-1 relies almost entirely on cellular factors and components for its replication. For instance the viral reverse transcription step requires the presence of deoxynucleotides in the cell cytoplasm, and SAMHD-1 mediated depletion of these nucleotide derivatives efficiently blocks virus propagation. Similarly, TGF-β signalling could result in restriction or depletion of proteins or molecules required for HIV-1 replication in MDLC. As mentioned above such dependency factor would be limited but not completely depleted from MDLC as some level of infection is observed in these cells. The knowledge about the nature of these reduced factors would definitely contribute towards development of treatment strategies.
4) Another hypothesis to explain TGF-β induced restriction of VSV-G HIV-GFP in MDLC relies on the biology of these cells. Langerhans cells are long-lived

cells critically contributing to tolerogenic immune responses. Perhaps the activity of cellular factors required for HIV-1 infection in these cells is also reduced, therefore resulting in an unfavourable environment for virus. This situation would mimic the one observed in T lymphocytes for which quiescent T cells are strongly resistant to productive HIV-1 infection (Zack et al. 1990; Zack et al. 1992; Vatakis et al. 2010). Activation of these cells restores the transcription factors activity and promotes expression of latent virus. Similarly, activation of MDLC with TLR signalling or pro-inflammatory cytokines (ex. TNFα) partially relieves VSV-G HIV-GFP restriction in these cells (see **Figure 4.15**). Therefore, inactive state of Langerhans cells could explain their resistance to infection. It is worthwhile to remember that results for this work were acquired in LC models that could differ in some aspects to LC at steady state in mucosal surfaces. Although low infection susceptibility of MDLC was reproduced in epidermal LC interferon and maturation studies were limited to MDLC. Strictly controlled differentiating conditions in MDLC are different to those present in epidermal microenvironment, which could possibly result in slightly different experimental outcomes. For instance, maturation of epidermal LC might result in much higher or lower infection by of VSV-G HIV-GFP in these cells. Therefore, further work on this project should concentrate on deciphering the exact mechanism of HIV-1 inhibition in both MDLC and real epidermal LC.

Regardless of the mechanism, the results presented in this work are unexpected, especially in the light of other studies that describe TGF-β to support HIV-1 propagation. Some reports suggested that TGF-β contributes to apoptosis and depletion of HIV-1 positive lymphocytes (Wang et al. 2001), consequently speeding

up the progression to AIDS. Non-cytotoxic mechanism of TGF-β induction of immunodeficiency was also suggested and proposed to rely on the impaired proliferation ability of antigen stimulated $CD4^+$ T cells (Kekow et al. 1990). Infected astrocytes, macrophages, monocytes, T cells and Dendritic cells, all can produce TGF-β (Wahl and Chen 2005), which acts in paracrine and autocrine manner to promote virus spread. For instance TGF-β increases expression of HIV-1 co-receptor, CXCR4, on monocyte-derived macrophages (Chen et al. 2005), T cells (Wang et al. 2001), monocyte-derived Dendritic cells (Sato et al. 2000) and Langerhans cells (Zoeteweij et al. 1998) hence promoting HIV-1 attachment and entry into cells, while R5-tropic viruses are usually the ones found at infection sites. The importance of this cytokine in HIV-1 infection is also highlighted by the fact that HIV-1 encoded Tat protein induces TGF-β in antigen-stimulated T cells and monocytes (Gibellini et al. 1994; Reinhold et al. 1999). However, the study described above showed the effect of TGF-β on already differentiated or HIV-1^+ cells, which are possibly less susceptible to TGF-β-induced changes. In agreement, I showed that in terms of VSV-G HIV-GFP inhibition TGF-β effect on differentiated MDDC is limited. In contrast, the same monocytes derived in the presence of this immunosuppressive cytokine show the opposite phenotype, clearly demonstrating the role of TGF-β in mediating this effect in MDLC.

Therefore, I conclude that TGF-β induces major changes in monocytes biology during differentiation that perhaps cannot be recapitulated in already differentiated cells, such as MDDC. These changes arise via the SMAD signalling cascade and trigger a SAMHD-1 independent restriction in MDLC.

5.3. Summary and Future Directions

Modern advances in antiretroviral therapy extend life expectancy of HIV-1^{+} individuals comparable to this of a healthy individual. However, life-long treatments come with a decreased quality of life, appearance of serious side effects and hefty price tag for the government. Therefore, the attempts to develop preventative measures against HIV-1 transmission are urgently required. Recently the scientific community advised on the implementation of prophylactic approach in form of a free access to Truvada (Heneine and Kashuba 2012; Administration 2013; CDC.com 2014). Accordingly, available data suggests this strategy to limit the number of new HIV-1 transmission and to reduce the costs of life-long treatment of infected patients.

As Langerhans cells are believed to be the first targets for HIV-1 infection during sexual transmission it is important to understand the events taking place at the mucosa. This work adds on to the knowledge of LC-HIV-1 interactions by investigating a post entry blockade event occurring in infected cells. Importantly the potential presence of TGF-β induced HIV-1 restriction in fully differentiated MDLC and fresh epidermal LC is described. TGF-β plays a crucial role in mediating a non-permissive phenotype in MDLC and blocking TGF-β-mediated signalling pathway reverses the effect. Operating at post entry level MDLC restriction appears independent of SAMHD-1 expression and possibly involves sensing of replicating virus. Preliminary data suggest block to HIV-1 infection in MDLC prior to integration, thus making viral DNA a likely target for sensing. TGF-β mediated activation of restriction factors, expression of new antiviral protein, or down-regulation of some molecules required for virus propagation might also be possible explanations for TGF-β action in MDLC. Although a single mechanism cannot be indicated based on available data, it is undisputable that TGF-β mediates the observed

effect. Further investigation into the topic could benefit from the microarrays or proteomics studies looking at expression or down modulation of genes in response to TGF-β treatment. If such genes/proteins are identified, possible modulation of their expression by gene knockdown could provide the answers as to the exact mechanism of TGF-β induced HIV-1 restriction in LC.

Interestingly, I observed that Langerin is up regulated in response to type I interferon stimulation, an observation not published before. Commonly recognised as a potent antimicrobial barrier for the first time Langerin is described to be responsive to warning signals such as type I interferon. It would be interesting to see if other pro-inflammatory cytokines induce similar effect on Langerin levels in these cells. Verification of the above data in skin-isolated cells would also benefit to our understanding of Langerhans cells behaviour in inflammatory condition.

In summary, this work provides new insight into Langerhans cells interactions with HIV-1. For the first time I describe the role of SAMHD-1 in these cells in preventing virus replication. Additionally, I shown that additional, TGF-β inducible restriction to virus infection operates in these cells and its function cannot be overcome by the presence of SIV Vpx or Vpr proteins. Decoding the role of this cellular restriction to HIV-1 infection in MDLC could offer new insights into the development of preventative vaccines or microbicides, which could help, prevent over 2 million of new HIV-1 transmissions happening each year worldwide.

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Appendix 1

Appendix 1. TLR expression profile in cells and their agonists.

Receptor	Ligand (origin)	Agonists used in our study (concentration)	Location in cells	expression in vivo*	monocyte derived models expression*
TLR 1	Lipopeptides (bacteria)	Pam3CSK4 (1µg/ml)	Cell surface	DC: LC:++	MDDC+/- MDLC +
TLR 2	Zymosam (fungi); lipotechoic acid (bacteria)	Peptidoglycan (10µg/ml)	Cell surface	DC LC+	MDDC+/- MDLC+
TLR 3	ds-RNA (viruses)	Poly I:C (2.5µg/ml)	Intracellular compartments	DC LC	MDDC MDLC
TLR 4	Lipopolysaccharide (bacteria)	LPS (1µg/ml)	Cell surface	DC LC-	MDDC+/- MDLC+
TLR 5	Flagellin (bacteria)	Flagellin (1µg/ml)	Cell surface	DC LC+	MDDC++ MDLC++
TLR 6	Diacyl lipopeptides (mycoplasma)	FSL1 (1µg/ml)	Cell surface	DC LC++	MDDC++ MDLC++
TLR 7	Small syntetic compounds; ssRNA (viruses)	Imiquimod (100µg/ml)	Intracellular compartments	DC LC-	MDDC MDLC
TLR 8	ssRNA (viruses)	ssRNA (1µg/ml)	Intracellular compartments	DC LC	MDDC MDLC
TLR 9	Unmethylated CpG oligodeoxynucleotide DNA (bacteria, viruses)	E.coli ssDNA (5µg/ml)	Intracellular compartments	DC LC++	MDDC++ MDLC++
TLR 10	unknown	Not investigated		DC LC	