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A VIROLOGIST'S GUIDE TO HIDE AND SEEK: EVASION OF INNATE IMMUNITY BY PRIMATE LENTIVIRUSES

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A Virologist's Guide to Hide and Seek: Evasion of Innate Immunity by Primate Lentiviruses

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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**Und jedem Anfang wohnt ein Zauber inne,
Der uns beschützt und der uns hilft, zu leben.**

Herman Hesse (aus Stufen)

*A magic dwells in each beginning
protecting us, telling us how to live.*

SUMMARY

HIV is the cause of a chronic, incurable infection in 37 million people worldwide in 2014. This thesis investigates how the immune system detects HIV and how in turn HIV avoids detection by the immune system. The understanding of the viral evasion mechanisms that prevent immune detection (“Hide and Seek”) is important to successfully develop future vaccines and cure strategies for HIV.

Primate lentiviruses belong to the retrovirus family and include the human immunodeficiency viruses (HIV-1 and HIV-2) and simian immunodeficiency viruses (SIV). HIV infects cells of the immune system, including subsets of T cells and dendritic cells (DC). Upon cell entry, the detection of the virus by cellular pattern recognition receptors triggers an intracellular cascade of innate antiviral defense mechanisms. In DCs, these mechanisms include the secretion of interferon α and the induction of cellular restriction factors, among these members of the APOBEC3 family that inhibit viral replication. As demonstrated in **Paper III**, low doses of interferon α protected DCs from HIV-1 infection and limited viral spread from DCs to T cells by inducing an increase in APOBEC3G, F and A expression.

DCs are professional antigen presenting cells that present antigen to cells of the innate and adaptive immune system. Invariant natural killer T cells (iNKT) cells are innate T cells that recognize endogenous and exogenous lipid antigens presented by CD1d. Activated iNKT cells regulate the immune response by producing cytokines that recruit and activate innate and adaptive immune cells. Previous studies have shown that the HIV-1 accessory proteins Vpu and Nef interfere with CD1d cell surface expression in infected DCs, thus inhibiting the effective activation of iNKT cells. The results of **Paper II** demonstrated that infected DCs respond to HIV-1 infection by increasing CD1d surface levels and enhanced presentation of the endogenous lipid GlcCer. This enabled iNKT cell activation by HIV-infected DCs. However, HIV-1 counteracts iNKT cell activation by reducing CD1d cell surface expression using the HIV-1 proteins Nef and Vpu. In **Paper I**, efforts to elucidate the mechanism of CD1d antagonism by Vpu identified a highly conserved C-terminal APW motif in HIV-1 group M subtype B Vpu proteins that was necessary for CD1d downregulation. Moreover, we identified this immune evasion mechanism to be a conserved function of diverse HIV-1 and related SIV Vpus. These findings emphasize the role of CD1d-mediated immunity in the antiviral defense against HIV-1 and support the need for further studies investigating the therapeutic potential of Vpu inhibition in the future.

Previous studies found that innate cellular immune responses are altered in chronic HIV-1 infection. Our results in **Paper IV** from an occupational cohort in Guinea-Bissau suggest that this is a general phenomenon of chronic HIV infection as NK and iNKT cells were partly lost and the remaining populations displayed elevated activation levels in chronic HIV-1, HIV-2, and dual infections.

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LIST OF ABBREVIATIONS

α-GalCer	α -GalactosylCeramide
AIDS	Acquired ImmunoDeficiency Syndrome
AP	Adaptor Protein
APOBEC3	Apolipoprotein B messenger RNA-editing Enzyme Catalytic polypeptide-like 3
ART	AntiRetroviral Therapy
CD	Cluster of Differentiation
CRF	Circulating Recombinant Form
DC	Dendritic Cell
EBV	Epstein-Barr Virus
ER	Endoplasmic Reticulum
GIT	Gastro-Intestinal Tract
GlcCer	GlucosylCeramide
HIV	Human Immunodeficiency Virus
IFN	Interferon
iNKT	Invariant Natural Killer T cell
IL	Interleukin
IRF	Interferon Regulatory Factor
ISG	Interferon-Stimulated Gene
MDDC	Monocyte-Derived Dendritic Cell
MFG	Mit Freundlichen Grüßen
MHC	Major Histocompatibility Complex
Nef	Negative Factor
NK	Natural Killer cell
PAMP	Pathogen-Associated Molecular Pattern
PRR	Pathogen-Recognition Receptor
SIV	Simian Immunodeficiency Virus
TGN	Trans-Golgi Network
TLR	Toll-Like Receptor
TMD	TransMembrane Domain
Vpu	Viral Protein U

INTRODUCTION

1 PRIMATE LENTIVIRUSES

Viruses are very small infectious agents that require living cells in order to replicate and cause diseases. HIV is the virus that causes AIDS in humans. The immune system fights HIV, but fails to remove the virus completely. HIV infection is a chronic, incurable disease that affects 37 million people worldwide in 2014.

Retroviruses are enveloped viruses that use reverse transcription to produce DNA from their viral RNA genome. The genus of *Lentiviruses* (Latin: *lenti* meaning slow) belongs to the family of *retroviridae* and infects various mammalian species including primates. Nonhuman primates (monkeys and apes) and humans are the natural hosts of primate lentiviruses, which consist of SIV, HIV-1 and HIV-2 [1].

1.1 ORIGIN AND EVOLUTION OF SIV, HIV-1 AND HIV-2

African nonhuman primates are the natural hosts of simian immunodeficiency virus (SIV) of which more than 40 species-specific strains are known [2,3]. Among these are the SIV strains of the Central Chimpanzee (*Pan troglodytes troglodytes*; SIVcpzPtt), the Western lowland gorilla (*Gorilla gorilla*; SIVgor) and the sooty mangabey (*Cercocebus atys*; SIVsmm). It is thought that these SIV strains crossed the species barrier from nonhuman primate to humans successfully multiple times resulting in 4 HIV-1 groups and 2 endemic HIV-2 groups [2,4]. How the original SIV infections of humans occurred is unknown but might have various sources such as bites and the handling and consumption of undercooked bush meat [4].

In natural hosts SIV usually causes a chronic yet nonpathogenic infection [5]. However, there are recent examples of AIDS-like symptoms in SIV-infected chimpanzees [6,7]. In non-natural hosts such as the rhesus macaque, SIV causes a pathogenic disease, comparable to AIDS in humans, which therefore serves as an animal model for HIV infection [8-10].

1.1.1 HIV-1

HIV-1 was first described in 1983 as the retrovirus causing the acquired immunodeficiency syndrome (AIDS) in humans [11-15]. Since, it has become clear that HIV-1 virus strains are diverse and can be categorized into groups and subtypes based on phylogenetic analysis. Today, HIV-1 is classified into four groups: M (for main), N (for non-M, non-O), O (for outlier), and P [16,17]. HIV-1 group M is the cause of the global HIV pandemic and is further subdivided into 9 subtypes A, B, C, D, F, G, H, J, K and numerous circulating recombinant forms (CRFs) [16,18].

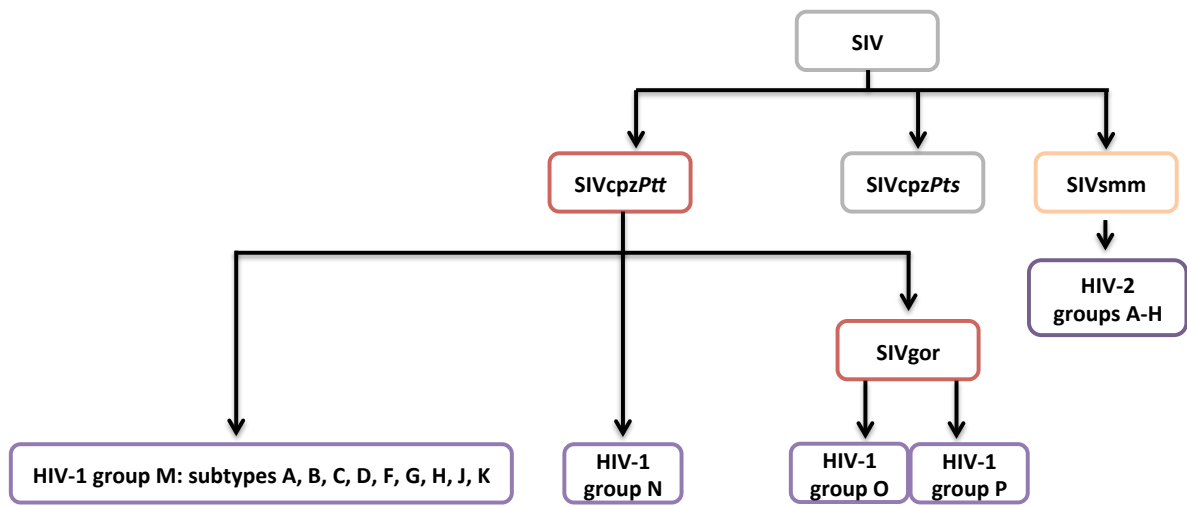
Subtypes A, B and C are the most prevalent subtypes globally. Subtype A is most common in Eastern Europe and Central Asia, whereas subtype B is the most prevalent subtype in North

America, Brazil and Western Europe. Subtype C infects most individuals worldwide and is most prevalent in India, South and East Africa [19] (**Figure 1**).

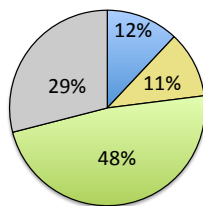
Whereas the pandemic group M has spread worldwide, groups N, O and P are geographically restricted mainly to West-Central Africa. Group O caused a local epidemic in Cameroon and is responsible for 1-2% of all HIV-1 infections [17,20-23], while groups N and P have been detected in a few individuals only [21,24,25]. Based on sequence similarities SIVcpzPtt was identified as the immediate source of HIV-1 groups M and N [26,27], and also as the likely precursor of SIVgor [22]. Groups O and P in turn are most closely related to SIVgor [17,22].

1.1.2 HIV-2

Shortly after the discovery of HIV-1, a closely related retrovirus named HIV-2 was found to also cause AIDS [28]. Interestingly, the closest SIV relative of HIV-2 is not SIVcpzPtt but the SIV strain infecting sooty mangabeys (SIVsmm) [29] (**Figure 1**). Unlike HIV-1, HIV-2 is geographically confined to West Africa and classified into 8 groups (A-H) of which A and B are endemic while the remaining groups have been isolated from a few individuals only [30]. Guinea-Bissau is one of the countries with the highest prevalence of HIV-2, 7.4% in 1996. However, until 2006 the HIV-2 prevalence declined to 4.4% while the prevalence of HIV-1 increased from 0% to 4.6%. Dual infection with HIV, defined as HIV-1 and HIV-2 infection in the same individual, had a prevalence of 0.5% in 2006. Thus HIV-1 is replacing HIV-2 as the major source of HIV infection in young adults [31]. This might be due to HIV-2 being less transmissible compared to HIV-1 [32,33]. HIV-1 and HIV-2 are closely related with up to 60% sequence identity on the amino acid level [34] and while being able to cause a similar AIDS phenotype, only a minority of HIV-2 infected individuals eventually progress to AIDS (reviewed in [35]). This striking difference in disease progression may be the consequence of differences in immunopathogenesis caused by HIV-1 and HIV-2. Therefore HIV-2 has been suggested to be an attenuated model of HIV infection [35]. Furthermore, a study by Esbjörnsson et al. [36] concluded that a preceding HIV-2 infection was able to slow down HIV-1 disease progression in dually infected patients. Studies of HIV-2 infection may provide valuable information for the development of vaccines and treatments aiming to cure HIV-1.



global distribution



■ A ■ B ■ C ■ other subtypes and CRFs

Figure 1: Overview of primate lentiviruses. The simian immunodeficiency virus (SIV) is found in more than 40 African nonhuman primate species. The SIV strains of the Central Chimpanzee (SIVcpzPtt), the Western lowland gorilla (SIVgor) and the sooty mangabey (SIVsmm) have crossed the species barrier from nonhuman primate to humans resulting in 4 HIV-1 groups (M, N, O, P) and 8 HIV-2 groups (A-H). HIV-1 group M is the cause of the global HIV pandemic and comprises 9 subtypes A-D, F-H, J and K. Subtypes A, B and C are the most prevalent subtypes globally. Subtype A is most common in Eastern Europe and Central Asia and accounts for 12% of cases globally, whereas subtype B is the most prevalent subtype in North America, Brazil and Western Europe accounting for 11%. Subtype C infects most individuals worldwide, 48%, and is most prevalent in India, South and East Africa. Other subtypes and CRFs (circulating recombinant forms) account for the remaining infections. Subtype distribution as of 2007. Figure is based on information from [19,37].

1.2 HIV INFECTION AND AIDS

Global summary of the AIDS epidemic | 2014

Number of people living with HIV in 2014	Total	36.9 million	[34.3 million – 41.4 million]
	Adults	34.3 million	[31.8 million – 38.5 million]
	Women	17.4 million	[16.1 million – 20.0 million]
	Children (<15 years)	2.6 million	[2.4 million – 2.8 million]
People newly infected with HIV in 2014	Total	2.0 million	[1.9 million – 2.2 million]
	Adults	1.8 million	[1.7 million – 2.0 million]
	Children (<15 years)	220 000	[190 000 – 260 000]
AIDS deaths in 2014	Total	1.2 million	[980 000 – 1.6 million]
	Adults	1.0 million	[890 000 – 1.3 million]
	Children (<15 years)	150 000	[140 000 – 170 000]

WHO – HIV department | July 21, 2015



According to the World Health Organization's (WHO) report of 2014 (**Figure 2**), approximately 37 million people are living with an HIV infection worldwide and a global infection rate of 2 million people per year keeps the epidemic active [38], causing not only individual hardship but also having a significant socioeconomic impact in endemic countries [39].

Figure 2: The AIDS epidemic as of 2014 (WHO) [38].

Both HIV-1 and HIV-2 cause AIDS and impressive achievements in understanding the HIV life cycle and pathogenesis as well as in developing successful drugs to inhibit viral replication (antiretroviral therapy, ART) have been made. However, there is still no cure for HIV infection or AIDS [40], despite one successfully cured case after hematopoietic stem cell transplantation [41].

The general course of infection and progression to AIDS is similar for both HIV-1 and HIV-2, referred to here as 'HIV' (**Figure 3**). Primary infection with HIV starts by the transmission of the virus from one human to the other through direct contact with body fluids. HIV infects cells using the cell surface molecule CD4 and the co-receptors CCR5 and CXCR4 [42] expressed on subsets of immune cells such as T cells, dendritic cells, macrophages and NKT cells, the function of which will be discussed below in chapter 2.

The **acute phase** is characterized by an early and pronounced loss of CD4+ T cell in the peripheral blood and the gastro-intestinal tract (GIT) in addition to high viral replication. At the peak in viral load the immune system starts to partly control viral replication and viral load decreases until it reaches a relatively stable level during the chronic phase. The level of this so-called 'viral set point' can vary significantly between individuals (reviewed in [43]). Despite the absence of disease symptoms during the **asymptomatic chronic phase**, the functionality of the immune system of HIV-infected individuals is undermined by the continued loss of CD4+ T cells [44], ultimately resulting in the progression to AIDS. **AIDS** is characterized by the inability to suppress viral replication, to mount successful immune responses leading to a raise in HIV viral load, the outbreak of opportunistic bacterial, yeast, and viral infections as well as specific types of cancer [45]. The CD4+ T cell count is the primary clinical parameter measured when assessing disease progression [46,47]. Progression from the chronic phase to AIDS is commonly defined in adults as a peripheral blood CD4+ T cell count ≤ 200 cells/ μL (healthy range 500-1000 cells/ μL in Ethiopian adults) or CD4+ T cell percentage $\leq 14\%$ (healthy average 40%) [48,49].

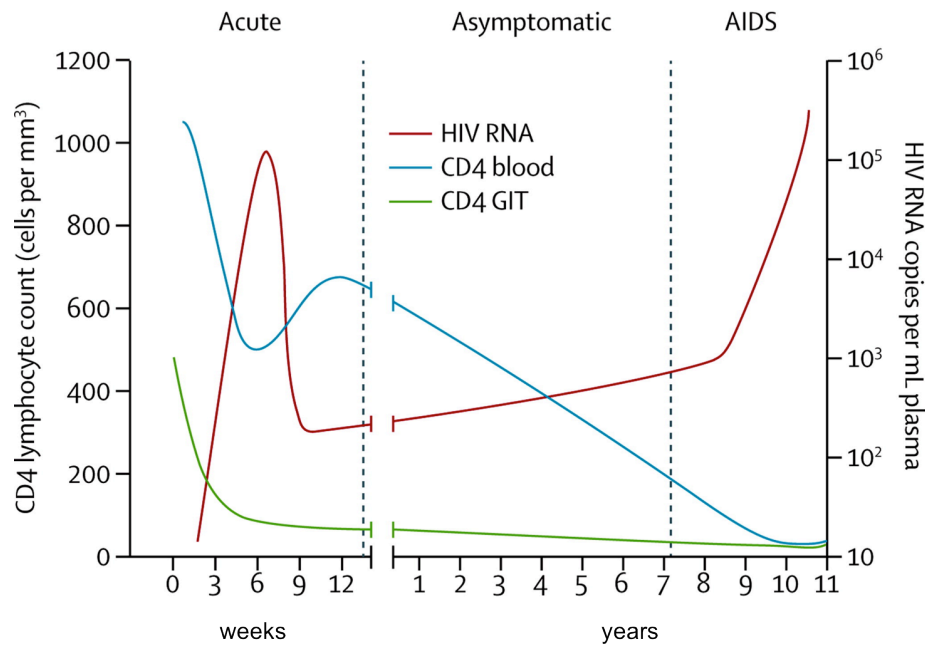


Figure 3: Progression of untreated HIV-1 infection from acute infection to AIDS.

The acute phase of HIV-1 infection is characterized by a pronounced loss of CD4+ T cells in the blood and the gastro-intestinal tract (GIT) accompanied by increasing viral replication during the first weeks of primary infection. As the immune system combats the virus, the viral load decreases and reaches a stable level, the so-called viral set point, during the asymptomatic chronic phase. Despite the absence of disease symptoms, the functionality of the immune system is undermined by the continued loss of CD4+ T cells that ultimately results in the progression to AIDS. The onset of AIDS is commonly defined as CD4+ T cell count ≤ 200 cells/ μ L in the peripheral blood and characterized by the inability to control opportunistic infections and to suppress viral replication. This leads to a fast rise in HIV viral load, the outbreak of opportunistic bacterial, yeast, and viral infections as well as specific types of cancer. The CD4+ T cell count is the primary clinical parameter measured when assessing disease stages and progression. Figure from [50] and reproduced with permission.

There are several interesting differences between HIV-1 and HIV-2 infection, despite the similarities in disease progression [51]. Most HIV-1 infected untreated individuals eventually progress to AIDS, whereas only 15-20% of the HIV-2 infected individuals mirror HIV-1 disease progression. The majority of HIV-2 infected untreated individuals maintains very low or undetectable viral loads and remains in the asymptomatic chronic phase for life [52]. Furthermore, in HIV-2 infection the transmission rate is lower [32,53], the mortality rate is lower [54] and the viral set point is almost 30-fold lower [54,55] compared to HIV-1 infection. However, CD4+ T cell count and plasma viral load are independent predictors of disease progression and mortality in both HIV-1 and HIV-2 [56].

2 INNATE IMMUNITY AGAINST HIV

The immune system is the defense of the body against infections. Innate immunity refers to the arm of the immune system that acts against pathogens quickly and in a general manner. It requires no knowledge of the specific attributes of the threat. After entry, innate immunity is the first barrier that HIV has to overcome in order to establish an infection in the body.

Antiviral immune responses at the sites of virus exposure dictate subsequent protective immune responses, virus control, and immunopathology. After HIV enters a cell, HIV-specific pathogen-associated molecular patterns (PAMPs) activate an intracellular cascade of innate antiviral defense mechanisms. These include type 1 interferons that restrict viral replication and spread. The infected state of this cell is signaled to its direct environment by the secretion of cytokines and chemokines, which in turn recruit and activate cells of the innate immune system (reviewed in [57]). Innate immune cells comprise cells such as monocytes, dendritic cells (DC), macrophages, neutrophils, granulocytes, natural killer cells (NK) and natural killer T cells (NKT). Their task is to recognize pathogens and to respond quickly to infections. After sensing a viral infection, DCs function as antigen-presenting cells and release proinflammatory cytokines. This in turn activates effector cells such as NK and NKT cells. Activated NK and NKT cells regulate the immune response by producing cytokines that recruit and activate innate and the adaptive immune cells [58].

2.1 TYPE 1 INTERFERON

Type 1 interferons (IFNs), particularly IFN α and IFN β , are key modulators of the human immune response. The detection of viral PAMPs leads to a signaling cascade activating a specific set of transcription factors called interferon regulatory factors (IRFs) and the NF- κ B transcription factor which induces the expression of type 1 IFNs, proinflammatory cytokines and chemokines [59]. These affect both the infected cell and adjacent cells. After binding of type 1 IFNs to the IFN receptor on the cell surface, a signaling pathway initiates the expression of interferon-stimulated genes (ISG). This results in an **antiviral state** within the infected cell and adjacent cells aimed to limit viral production and spread [60,61] (**Figure 4**).

The release of type 1 IFN marks the beginning of the immune response to a viral infection and signals systemically the presence of the infection leading to the activation and recruitment of innate and adaptive immune cells [62] (see **Figure 7** in chapter 2.6).

Several studies indicate the importance of IFN α in HIV infection [63]. Elevated levels of IFN α are found in the peripheral blood of HIV-infected patients [64,65] and HIV-1 replication is blocked by IFN α *in vitro* [66]. The presence of HIV-1 elicits IFN α production in human DCs *in vitro*, probably through toll-like receptor (TLR) 7 and/or TLR9 stimulation [67]. Recently, a study in SIV-infected Rhesus macaques showed that blocking IFN α with antibodies resulted in a reduced expression of cellular restriction factors, described below, whereas administration of IFN α prevented systemic infection [68].

2.2 CELLULAR RESTRICTION FACTORS

After cell entry the single-stranded RNA genome of HIV is reverse-transcribed to viral cDNA, a process that produces viral RNA and DNA intermediates which represent viral PAMPs that are recognized by cellular pathogen-recognition receptors (PRR) [69], for example TLRs [70]. The detection of PAMPs triggers a signaling cascade ending in the activation of the transcription factors NF- κ B and IRF3 to induce gene expression of cellular restriction factors, type 1 IFNs and proinflammatory cytokines (**Figure 4**).

The term restriction factor identifies host proteins that limit viral replication and spread. Human restriction factors important for the anti-HIV response are TRIM5 α , SAMHD1, the apolipoprotein B messenger RNA (mRNA)-editing enzyme catalytic polypeptide-like 3 (APOBEC3), and tetherin. TRIM5 α is a post-entry inhibitor, while SAMHD1 and the APOBEC3 family interfere with the reverse transcription process, and tetherin inhibits the release of virions from infected cells (reviewed in [71,72], see **Figure 4**).

One anti-HIV mechanism elicited by IFN α is the induction of the human APOBEC3 family. APOBEC3 interferes with HIV replication by introducing mutations into the viral genome. This leads to substitutions and stop codons that result in the production of non-functional viral proteins [73]. The APOBEC3 family has 7 members (A-H) of which at least APOBEC3A (A3A), A3G and A3F are expressed in DCs to counteract HIV within the cell [73-75].

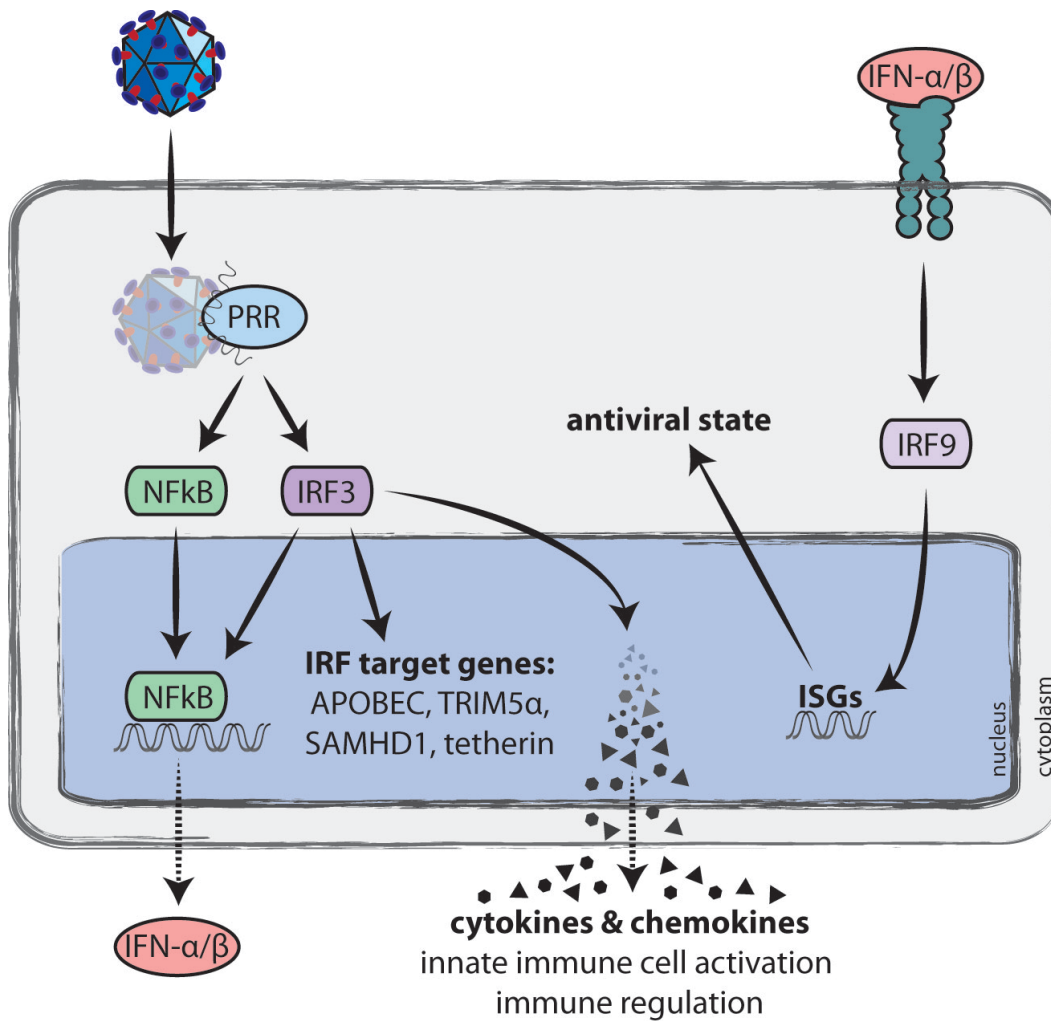


Figure 4: Induction of the antiviral state and innate immune activation after virus infection.

Following HIV-1 entry into the cell, the virus uncoats and viral RNA is reverse-transcribed into cDNA producing viral RNA and DNA intermediates that are detected by cytoplasmic pathogen recognition receptors (PRR). This leads to a signaling cascade ending in the activation of the transcription factors IRF3 and NF-κB, which induce target gene expression including HIV restriction factors (APOBEC, TRIM5α, SAMHD1 and tetherin), type 1 interferon (IFN) and proinflammatory cytokines and chemokines. IFN signals back into the infected cell and bystander cells through binding to the IFN-α/β receptor and induces via IRF9 the expression of interferon-stimulated genes (ISGs). ISGs create an antiviral state that inhibits different stages of the viral life cycle. For example, viral replication is hindered by a general or virus-specific blockage of protein synthesis. Figure based on information from [57,61,72,76].

2.3 ANTIGEN-PRESENTING CELLS: DENDRITIC CELLS (DC)

As professional antigen-presenting cells with the unique capacity to activate naive T cells, DCs have a key function in the immune system. DCs induce and regulate innate and adaptive immune responses by antigen-presentation, expression of co-stimulatory molecules, and cytokine release [77,78]. DCs are present at sites of antigen encounter such as the skin, mucosal surfaces, lymph nodes and the blood where they capture, process and present exogenous antigen to T cells via the major histocompatibility complex (MHC) and CD1 antigen-presentation systems (reviewed in [58,79]).

In mammals, DCs can be broadly subgrouped into ‘conventional’ or myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) [80]. mDCs are very efficient antigen presenters and are characterized by high MHC class II levels and expression of CD1 molecules. In contrast to mDCs, pDCs are less efficient in antigen-presentation and express lower levels of MHC class II, but upon viral infection pDCs are potent producers of IFN α [80], vitally contributing to the antiviral immune response. DCs are essential to initiate innate and adaptive immune responses, but also play a central role in disease progression during HIV infection [78,81]. Tissue-resident mucosal DCs are among the first cells that encounter HIV after mucosal transmission *in vivo* [82]. Here HIV can bind to and/or infect DCs [83,84] and be subsequently transported with the DC to lymph nodes rich in HIV target cells, thus facilitating the establishment of the infection in T cells. Infected DCs assist infection of CD4⁺ T cells by passing on membrane-bound viral particles and newly produced virus during the formation of an immunological synapse [85-87]. HIV-1 infection leads to impaired activity and early loss of DCs in the peripheral blood further hampering the immune system [88-90].

2.4 CD1D-RESTRICTED ANTIGEN PRESENTATION

After processing by a DC, peptide antigens are loaded on antigen-presenting molecules on the cell surface belonging to the highly polymorphic MHC family. MHC class I and II molecules present the peptide antigen to CD8⁺ and CD4⁺ T cells, respectively [91]. Not only peptides but also lipids can serve as T cell activating antigens, presented by MHC-like molecules belonging to the CD1 family (reviewed in [92]). Unlike the MHC, the CD1 family displays very limited allelic polymorphism [93] and is found in all studied mammals [94] - potentially conserved even throughout jawed vertebrates as evidence for CD1 and CD1-mediated immunity was recently found in cold-blooded vertebrates [95,96]. CD1 molecules are glycosylated transmembrane proteins expressed on the cell surface that share structural and domain homology with MHC molecules. The extracellular part is made up of the α 1-3 domains of which α 1 and α 2 form the antigen-binding groove. All CD1 molecules commonly form a heterodimer with β 2-microglobulin (β 2m) that non-covalently associates with the α 3 domain (reviewed in [97]).

In humans the CD1 family comprises of 5 members: CD1a, CD1b, CD1c, CD1d and CD1e. CD1a-c expression is mainly restricted to professional antigen-presenting cells while CD1d is widely expressed including by HIV-1 target cells such as macrophages, monocytes, DCs and activated T cells [98], but also on epithelial cells at sites of HIV entry such as the mucosa of the gastro-intestinal and the female genital tract [99,100]. Notably, mice encode only CD1d [101,102]. CD1e is only expressed intracellularly and assists lipid antigen processing and presentation by the other CD1 molecules [103].

Depending on their biochemical properties, lipids travel through different intracellular compartments [104]. During synthesis, CD1a-d molecules are loaded with endogenous lipids and then transported via the secretory pathway to the cell surface. Next, they get internalized to patrol various endo-lysosomal compartments where exchange of self-lipids with foreign lipid antigen can occur. Upon return to the cell surface the antigen is presented to CD1-restricted T cells. The recycling of CD1 molecules through intracellular compartments plays an important role for the antigen presentation process (reviewed in [105,106]).

Human and mouse CD1d contain a tyrosine-based sorting signal that mediates recycling into the endocytic pathway by binding to adaptor protein 2 (AP2) [107] and passing through early and late endosomes [108] (**Figure 5**). In contrast to mouse CD1d that additionally binds to adaptor protein 3 (AP3) and localizes to lysosomes [109], human CD1d lacks interaction with AP3 and is only partially localized in lysosomes [110]. Recycling of CD1d into endocytic compartments is important for antigen presentation as was shown for both mouse [111] and human CD1d [112,113].

CD1d-mediated immunity started to gain attention when a tumor screen revealed a glycosphingolipid, α -galactosylceramide (α -GalCer), that had anti-metastatic activity [114]. After the identification of α -GalCer as a model lipid antigen presented by both mouse and human CD1d [115,116] it has been a vital tool to study CD1d-restricted immune responses in health and disease.

Despite the fact that no viral lipid antigen has been described so far [117], several viruses have developed different strategies to evade CD1d-mediated antigen presentation and recognition, emphasizing the role of CD1d-mediated immunity in the antiviral defense. The presentation of self-lipids and the cytokine-mediated activation of iNKT cells provide potential pathways of indirect recognition of virus infections [118] (see chapter 2.5, iNKT activation modes 1+2).

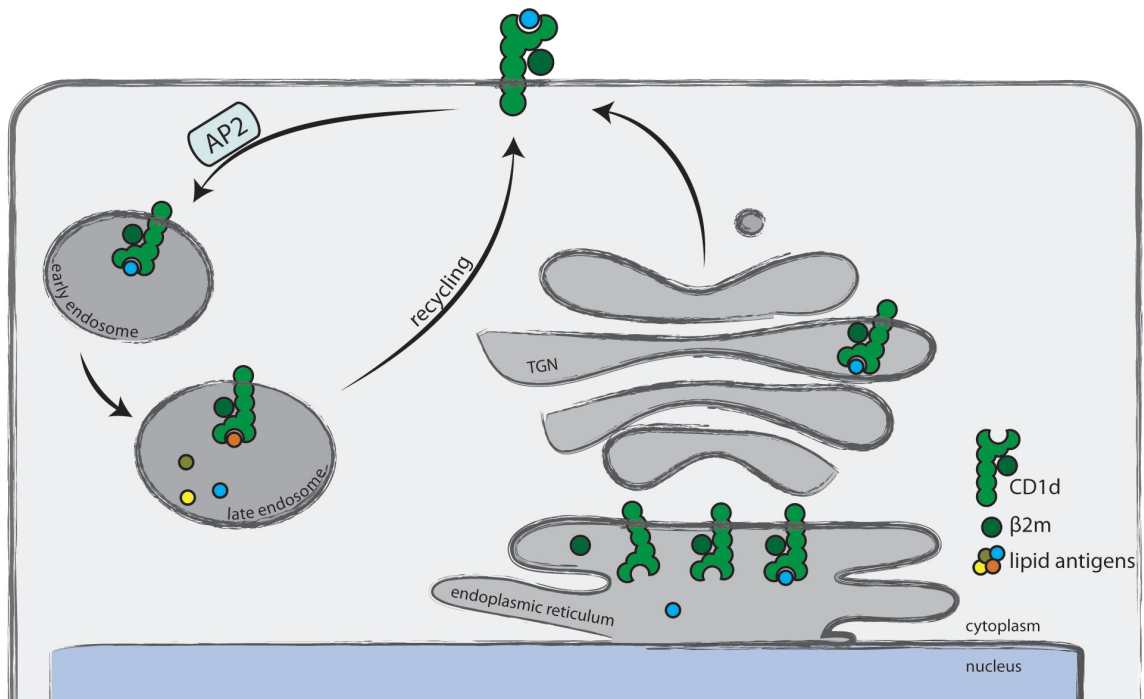


Figure 5: Human CD1d synthesis and recycling. CD1d is synthesized in the endoplasmic reticulum where it forms a heterodimer with beta2microglobulin ($\beta 2m$) and is loaded with a self-lipid. After glycosylation in the Golgi, CD1d gets transported via the secretory pathway through the trans-Golgi network (TGN) to the cell surface. CD1d is internalized from the cell surface by binding to adaptor protein 2 (AP2) and enters the endocytic pathway. While passing through early and late endosomes CD1d encounters new foreign and self-lipid antigens. After antigen exchange CD1d recycles back to the cell surface where it presents the antigen to CD1d-restricted T cells [107,108].

2.5 NATURAL KILLER T (NKT) CELLS

The main effector cells of CD1d-mediated immunity are a subset of T cells called natural killer T (NKT) cells. As suggested by the name, NKT cells have characteristics of both natural killer (NK) and T cells, such as CD161 and a T cell receptor (TCR), respectively.

CD1d-restricted NKT cells are divided in 2 groups based on differences in their α/β TCR: type I or invariant NKT (iNKT) cells and type II or diverse NKT cells. Whereas iNKT cells are identified by their semi-invariant α/β TCR containing a V α 24J α 18 rearrangement paired with V β 11 in humans (V α 14J α 18 combined with V β 2, 7 or 8.2 chains in mice), type II NKT cells employ a diverse TCR repertoire. In stark contrast to iNKT cells, type II NKT cells do not recognize the model lipid antigen α -GalCer (reviewed in [119,120]). This thesis focuses on iNKT cells. In the human peripheral blood iNKT cell levels range from undetectable to 1% of all lymphocytes with an average level of 0.1-0.2% [121,122]. Some evidence suggests that iNKT cell numbers in the blood might be gender dependent [121,123].

Upon activation iNKT cells are potent and rapid producers of a large variety of both pro- and anti-inflammatory cytokines such as IFN γ and TNF α (reviewed in [124]), indicating an immunoregulatory role of iNKT cells in human health and disease [125]. This role of iNKT cells can be explained by the fact that they function as coordinators between innate and adaptive immunity by activating DCs [126,127], B cells [128,129] and natural killer (NK) cells [130,131].

Subpopulations of iNKT cells can be distinguished based on their CD4 and CD8 expression. The human iNKT cell compartment consists of CD4+, CD4-CD8- double negative (DN) and CD8+ subsets [125,132]. The cytokine profiles of these iNKT cell subsets overlap partly (i.e. all iNKT cells produce IFN- γ) but differ in the production of individual cytokines, extent and duration of the particular cytokine expression, and in cytolytic activity [132,133]. The iNKT subset size might depend on whether the analyzed total iNKT cells were derived from fresh blood or from cultures expanded *in vitro* [132] and thus the reported subset numbers in peripheral blood vary, however at least 20% of circulating iNKT cells in healthy donors are CD4+ [132,134,135]. iNKT cell populations with a larger CD4+ iNKT cell subset compared to peripheral blood were demonstrated for the gut mucosa [136], a potential entry site for HIV. It is well established that the CD4+ iNKT cell subset can get infected with HIV-1 [134,135] and is lost during HIV-1 infection [136-138]. This was also shown in a primate model of SIV infection [139]. Despite not expressing the HIV receptor, the functionality of the remaining iNKT cell population is impaired and displays an exhausted phenotype [138,140].

iNKT cells recognize lipid antigen presented by CD1d (**Figure 6**). These lipid antigens can be self-derived (endogenous, **activation mode 1**) or pathogen-derived (exogenous, **activation mode 3**), recently reviewed in [141] and [142]. Recognition of foreign lipid antigen was described for certain bacteria, such as *Mycobacterium* [143], *Streptococcus* [144] and *Borrelia* [145].

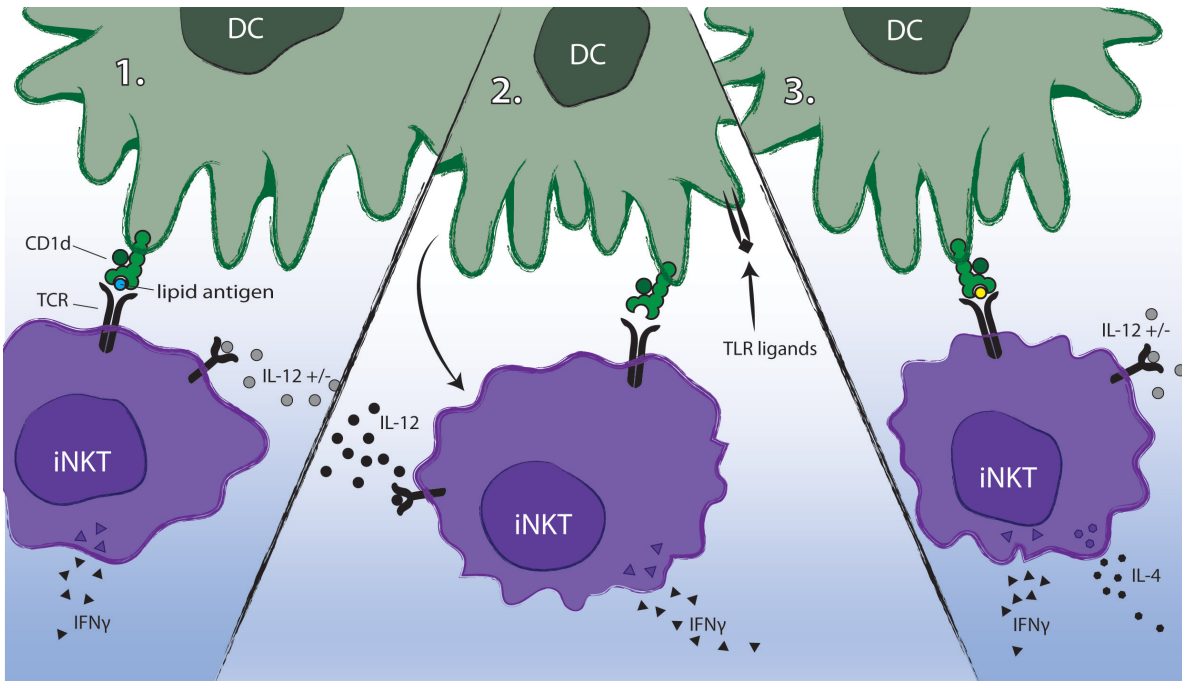


Figure 6: The activation modes of iNKT cells.

iNKT cells are most likely activated by activation modes 1 and 2 in the presence of viral infections.

- 1) Recognition of self-lipid antigen presented by CD1d (in the context of IL-12).
- 2) Activation by IL-12 secreted from DCs after stimulation by TLR ligands.
- 3) Recognition of foreign lipid antigen presented by CD1d (in the context of IL-12).

1) The profile of self-lipids presented by CD1d is potentially altered during viral infection and can activate iNKT cells in the absence of a viral lipid antigen (possibly with the assistance of IL-12). **2)** Even in the complete absence of a lipid antigen, the constitutive expression of high levels of IL-12 receptor enables iNKT cells to respond rapidly to cytokine-mediated stimulation. DCs produce IL-12 in response to infectious agents that possess toll-like receptor (TLR) ligands and allow iNKT cells to overcome their restricted TCR specificity. **3)** The model lipid antigen α -GalCer and some bacterial lipids are able to activate iNKT cells directly without the assistance of IL-12. Figure based on information from [105,117,143,146].

The profile of self-lipids presented by CD1d is altered during viral infection and can therefore activate iNKT cells in the absence of a viral lipid antigen (**activation mode 1**). iNKT cells were shown to be part of the antiviral defense against dengue virus in mice [147] and interestingly changes in the serum lipidome were found in patients with dengue virus infection [148]. Zeissig et al (2012, [149]) showed that during hepatitis B virus infection more lysophospholipids are produced which have been previously shown to activate human iNKT cells [150]. Enveloped viruses, such as HIV, use host membrane lipids to form their envelope when budding from an infected cell. The envelope of HIV-1 is enriched in unusual sphingolipids [151], a lipid class presented by CD1d [123,152]. Additionally, changes in the human lipidome are described in individuals with HIV infection, as a recent study found several classes of plasma lipids to be dysregulated in HIV-1 infected individuals [153].

Thus, alterations in the endogenous lipid profile could make HIV-infected cells a target for CD1d-mediated immunity.

The TLR system enables DCs to detect the presence of viruses without requiring a lipid antigen. Upon ingesting the debris of infected cells or direct infection, human DCs can detect viral double stranded RNA via TLR3, single stranded RNA via TLR7 and TLR8, and single stranded DNA via TLR9 [154,155]. Engagement of TLRs leads to the production of proinflammatory mediators such as cytokines and type I interferon, leading to DC maturation and the initiation of a variety of inflammatory and antiviral responses (reviewed in [154]). Notably, iNKT cells are also activated by cytokines released from TLR-stimulated DCs (**activation mode 2**) in the absence of TCR stimulation. Several studies in mice suggest that activation of TLR7 and TLR9 induces IL12, IL18 or IFN α production in DCs, which in turn is sufficient to induce IFN γ production of iNKT cells [118,156-158]. Zhu et al (2015, [159]) reported that in mice iNKT cells are activated by macrophages infected with enterovirus and that this activation is dependent on TLR3. Less is known in humans, but TLR9 agonists were reported to induce TNF and IFN α secretion from DCs and an activated phenotype in co-cultured iNKT cells [160]. Furthermore, DCs matured by TLR3, TLR4 and TLR7/8 agonists activated human iNKT cells [161].

The antiviral effects mediated by human iNKT cells are demonstrated in patients with CD1d [162] or NKT cell deficiency [163]. In these rare cases, vaccination with varicella zoster virus belonging to the herpesvirus family, caused a life-threatening infection. Further evidence is added by the observation that individuals with mutations causing X-linked lymphoproliferative disease [164,165], lacking iNKT cells among other immune defects, are particularly susceptible to Epstein-Barr virus (EBV) infection, another member of the herpesvirus family [166] (reviewed in [167-169]).

2.6 NATURAL KILLER (NK) CELLS

NK cells are innate effector cells that play an important role in the defense against cancer and viral infections (reviewed in [170,171], respectively). NK cells are present in human peripheral blood (ca. 10-15% of all lymphocytes) and are commonly identified as CD56⁺ CD16⁺ CD3⁻ cells. Two major subpopulations of NK cells are distinguished based on the level of CD56 surface expression: CD56^{dim}, the more abundant subset comprising 90-95% of all human NK cells, and CD56^{bright} cells [172].

NK cells are essential players in the antiviral defense and fulfill multiple functions. They recognize and kill virus-infected cells, and can recruit and activate cells of the innate and adaptive immune system via the production of proinflammatory cytokines (**Figure 7**, reviewed in [171]). NK cells are regulated by DCs and vice versa, enabling NK cells to influence the adaptive immune responses through cytokine secretion and the selective killing of infected DCs [173]. In addition, iNKT cells are able to modulate NK responses [130] and shape NK cell functionality [131].

Similar to iNKT cell deficiencies, NK cell deficient patients are highly susceptible to herpesvirus infections [174,175]. Due to their ability to bridge between innate and adaptive immunity, NK cells play an important role in modulating the immune response to HIV [62,176] and like iNKT cells, NK cells are dysregulated during HIV infection. For example, the interaction between NK cells and DCs as well as NK cell activation, is impaired [177]. Additionally, some studies reported decreased numbers of NK cells and subset dysregulation in the peripheral blood during chronic HIV-1 infection [178,179]. A decrease in NK cells was recently described in a longitudinal study of early HIV-1 infection, with a more rapid NK cell loss in progressing patients [180]. In the human gut mucosa, a major site of HIV-associated pathogenesis, Sips et al. (2012, [181]) found the distribution of mucosal NK cells was altered in HIV-infected individuals, especially in treated patients with poor CD4+ T cell recovery.

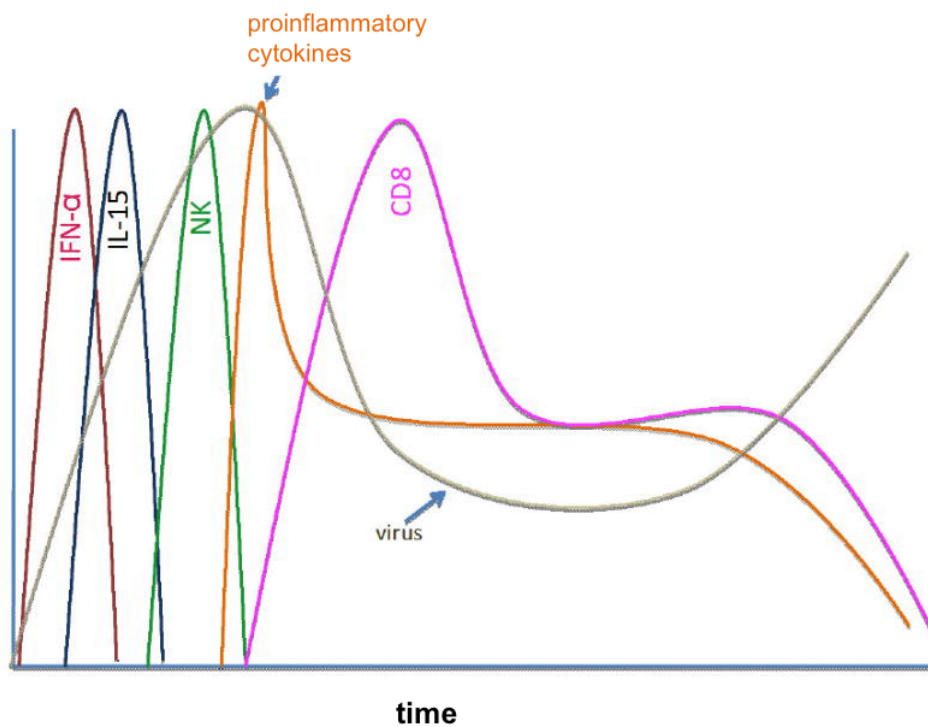


Figure 7: The immune response to viral infections. After viral infection, the first step is the release of type 1 interferons by DCs and followed by the induction of NK cell proliferation via increased levels of IL-15. This contributes to early suppression of viral replication. The subsequent release of proinflammatory cytokines, such as IFN γ , drives the adaptive immune response to elicit virus-specific CD8+ T cells that eliminate or control the virus. In HIV infection CD8+ T cells control viral replication throughout the chronic phase until viral replication escalates during AIDS. Figure reproduced with permission from [62].

3 HIV VERSUS INNATE IMMUNITY

In order to prevent a virus from spreading, virus-infected cells attempt to alert the immune system. However, HIV has developed several ‘immune evasion’ mechanisms to prevent the infected cell from setting off alarms. HIV infection leads to a competition of “Hide and Seek” between the immune system and HIV.

3.1 HIV ACCESSORY GENES

In addition to the structural genes *gag*, *pol* and *env* commonly found in all retroviruses, primate lentiviruses encode so called accessory genes (**Figure 8**). The accessory genes differ between HIV-1 and HIV-2: HIV-1 encodes viral infectivity factor (Vif), viral protein R (Vpr), viral protein U (Vpu) and negative factor (Nef), whereas HIV-2 lacks Vpu but encodes viral protein X (Vpx) [71]. The accessory proteins received their name due to the fact that they are dispensable for viral replication in some cell types *in vitro* [182]. However the name may be misleading, as accessory proteins are necessary for successful viral replication *in vivo* and efficient virus spread in the primate host. Research investigating the function of HIV accessory genes revealed their previously unknown human targets, namely the cellular restriction factors introduced in chapter 2.2 (reviewed in [183]). An overview of selected functions of HIV-1 accessory proteins is given in **Figure 9**. In addition, accessory proteins target host proteins that are important for the immune response, for example MHC class I, CD1d and CD4 [184] (**Table 1**).

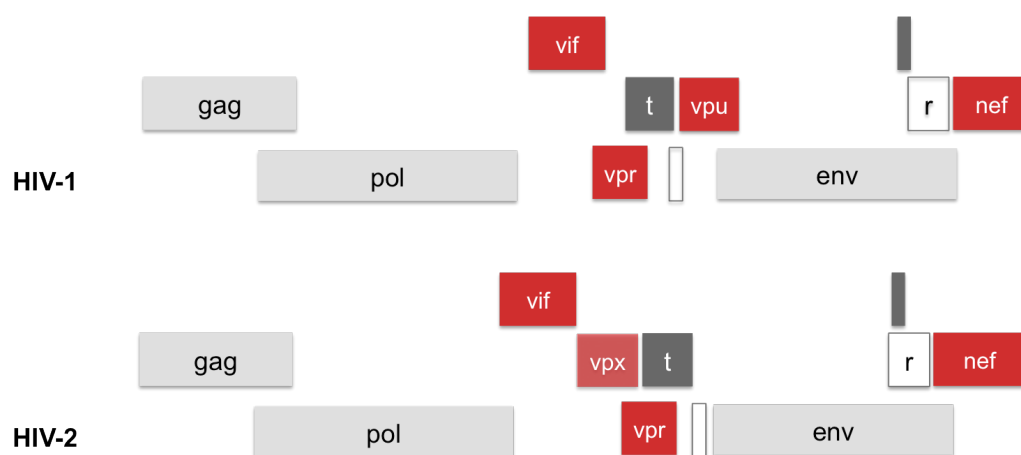


Figure 8: The genomic organization of HIV. Open reading frames of the structural genes *gag*, *pol* and *env* (in grey), the viral regulatory factors *tat* (*t*) and *rev* (*r*) and the accessory genes *vif*, *vpr*, *vpu* or *vpx*, and *nef* (in red) [185].

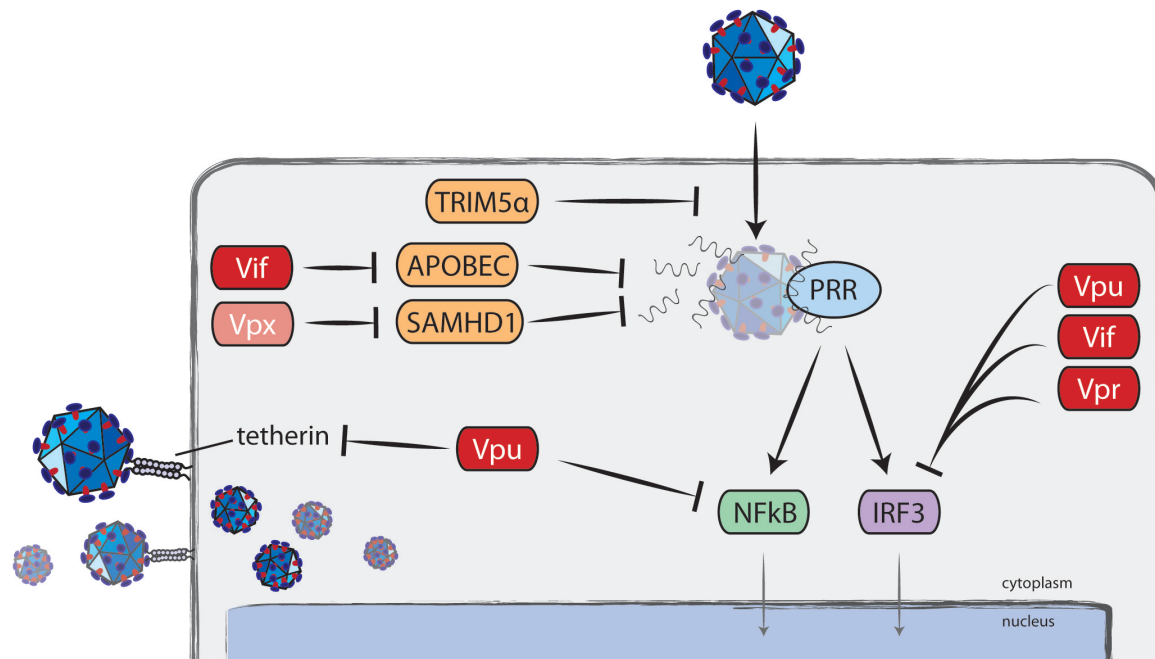


Figure 9: The effect of HIV accessory proteins on cellular restriction factors. The cellular restriction factors TRIM5 α , APOBEC, SAMHD1 and tetherin are expressed constitutively or are induced by type 1 interferons upon viral infection and subsequent recognition by pattern-recognition receptors (PRR). After entering the cell, HIV meets TRIM5 α that binds to the HIV capsid and interferes with the uncoating process. SAMHD1 and APOBEC restrict the HIV replication process. SAMHD1 depletes the pool of cellular dNTPs and decreases reverse transcription of HIV-1 RNA. SAMHD1 is antagonized by HIV-2 Vpx, but not by HIV-1. APOBEC introduces mutations into the viral genome and is counteracted by HIV-1 Vif. Tetherin tethers budding virions to the cell surface and inhibits the release of virions. HIV-1 Vpu binds and targets tetherin to degradation. Additionally, Vpu inhibits activation of NF- κ B and together with Vif and Vpr suppresses the transcription factor IRF3 resulting in the reduced expression of interferon and interferon-stimulated genes. The figure is based on information from [57,72,183,186].

3.1.1 Nef

Nef is expressed early during the viral life cycle and also found in virions [187,188]. Despite the name ‘negative factor’, the functional expression of Nef is required for high viral replication and the development of immunodeficiency. This was demonstrated a decade ago in a primate model [189] and human patients [190] infected with SIV and HIV lacking the *nef* gene, respectively. Meanwhile several functions of Nef have been described which are all related to the manipulation of cellular factors leading to immune evasion (recently reviewed in [184]). One example is the interference of Nef with MHC class I surface expression. MHC class I is a key cell surface receptor that presents peptide antigen to CD8⁺ T cells and initiates immune responses. Nef blocks the transport of newly synthesized MHC class I molecules and increases their endocytosis from the cell surface thereby reducing significantly MHC class I surface expression [184,191]. In addition, Nef inhibits lipid-specific immunity in a similar manner by interfering with CD1d and preventing iNKT cell activation. CD1d and Nef form a complex that leads to accelerated CD1d internalization from the cell surface thus reducing CD1d cell surface levels [192,193].

3.1.2 Vpu

Vpu is a small type I transmembrane protein: the prototypic Vpu of the virus NL4-3 (HIV-1 group M, subtype B) is 81 amino acids long (ca. 16 kDa) [194]. The predicted structure of Vpu contains from the N-terminus a short luminal domain, the transmembrane domain (TMD, an α -helix), and a cytoplasmic domain (CD) made up of two α -helices ($\alpha 1$ and $\alpha 2$). The α -helices of the cytoplasmic domain are linked by a flexible loop structure that contains two highly conserved phosphorylation sites (serines 52 and 56 in NL4-3 Vpu [195]) [196] (**Figure 10**).

Vpu is expressed by HIV-1 and its direct SIV precursors, SIVcpz and SIVgor [197]. HIV-2 and most SIV strains lack Vpu with the exception of a subset of SIVs infecting guenons (*Cercopithecus*) which encode Vpu: SIV of the greater spot-nosed monkey (SIVgsn), the mona monkey (SIVmon), the mustached monkey (SIVmus) and Dent's mona monkey (SIVden) [198-201] and reviewed in [2,202]. The fact that Vpu is missing from most SIV genomes and that Vpu resembles a subdomain of the primate TASK-1 channel led to the speculation that SIV acquired a precursor Vpu by 'molecular piracy' from a guenon early in the evolution of SIV [203].

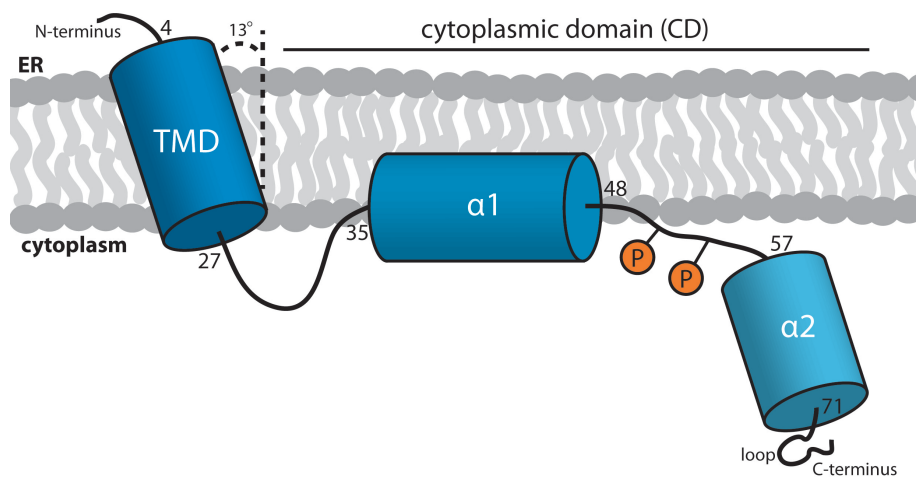


Figure 10: The predicted protein structure of HIV-1 Vpu. The protein consists of the transmembrane domain (TMD) and a cytoplasmic domain (CD). The two α -helices ($\alpha 1$ and $\alpha 2$) of the CD are linked by a flexible region containing two highly conserved phosphorylation sites (P). A potentially functional loop structure close to the C-terminus is indicated [204]. The numbers refer to the amino acid positions in NL4-3 Vpu.

Vpu displays high sequence variability between HIV-1 subtypes and even within subtypes [205-208]. The amino acid sequence logo [209,210] in **Figure 11** demonstrates variable and conserved motifs in subtype B Vpus.

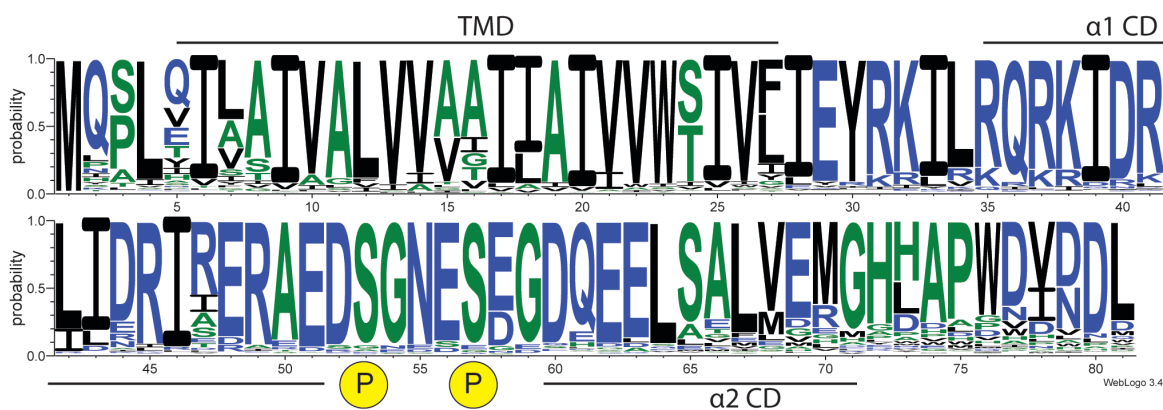


Figure 11: Logo of HIV-1 subtype B Vpus. A graphical representation of the alignment of Vpu amino acid sequences retrieved from the HIV sequence database (www.hiv.lanl.gov, accessed 08082015; [211]). A probability of one represents absolute conservation of this amino acid at the particular position. The color-coding of the amino acids represents hydrophobicity: blue, hydrophilic; green, neutral; black, hydrophobic. The relative positions of the protein domains and the conserved phosphorylation sites are indicated (P).

Evidence for an important role of Vpu in HIV pathogenesis comes from several nonhuman primate studies showing that infection with simian-human immunodeficiency viruses (SHIV) either lacking or containing dysfunctional Vpu causes decreased pathogenicity compared to the wild type virus [8,212-215].

Vpu was first described to be important for virus release [194], an observation later explained by Vpu's ability to interfere with the restriction factor tetherin [216]. In recent years the immune evasion functions of Vpu have grown into an impressive list (a current overview is given in **Table 1**). The fact that Vpu masters several different mechanisms to interfere with the innate immune mechanisms is fascinating [217].

Most of Vpu's cellular targets are cell surface receptors whose expression on the cell surface is an essential part of their function. Examples of these targets are CD4, tetherin, CD1d and the NK cell co-activating receptors PVR and NTB-A. Vpu has developed different mechanisms to interfere with these cellular targets (**Table 1**). Currently two major strategies of Vpu interference are distinguished. The first strategy is to directly interact with the cellular target and to mediate its degradation by the proteasome. The second strategy is to disturb the cellular trafficking of the target and to sequester it in intracellular structures.

The glycoprotein **CD4** plays a central role in the immune system as it binds to MHC class II molecules and mediates cell adhesion and CD4⁺ T cell activation [218,219]. CD4 surface levels are severely reduced in Vpu-expressing cells [220]. Vpu binds to newly synthesized CD4 molecules in the endoplasmic reticulum (ER) causing their ubiquitination and proteosomal degradation (reviewed in [221]). The successful degradation of CD4 involves phosphorylation of Vpu, the interaction CD4 and Vpu mediated by their TMDs [222-224] and the 2nd α -helix of the Vpu cytoplasmic domain [225].

The function of **tetherin** (CD312 or BST2) was discovered when investigating the IFN α induced antiviral activity of human cells [216]. In virus-producing cells tetherin is found on the cell surface where it binds to the virions, retains them on the cell surface and blocks virus release. Tetherin recycling occurs from the cell surface via endosomes to the trans-Golgi Network (TGN) and back to the cell surface [226]. The exact mechanisms and their importance in different cell types are not fully elucidated but Vpu was described to target tetherin protein turnover and vesicular trafficking (recently reviewed in [227,228]). The interaction of the TMDs of Vpu and tetherin [229], phosphorylation of Vpu and a motif in the 2nd α -helix of Vpu are required for tetherin antagonism [230,231].

Our group showed previously that Vpu interferes with **CD1d** surface expression on HIV-1 infected DCs [232]. Endocytosis of CD1d is not affected but Vpu and CD1d are found to localize to the same vesicular structures identified as early endosomes by EEA-1 expression. The rate of CD1d recycling is reduced in cells expressing Vpu, which in turn leads to a decreased CD1d cell surface expression. In contrast to the CD4 interference mechanism, Vpu does not induce degradation of CD1d. The exact molecular mechanism of Vpu's interference with CD1d is unknown. In addition to Vpu, Nef decreases CD1d cell surface levels by increasing CD1d internalization and retention of CD1d in the TGN [192,193]. Vpu and Nef employ different mechanisms to interfere with CD1d and it is likely that these mechanisms complement each other. Dendritic cells infected with HIV-1 deficient in Nef and Vpu display no CD1d cell surface downregulation. The biological relevance of reducing CD1d cell surface expression was demonstrated as decreased CD1d levels resulted in impaired iNKT cell activation [232].

Table 1: Overview of Vpu functions and mechanisms modulating cellular targets involved in immune responses. Degradation (Degrad) via the proteasome (P) or the lysosomal (L) pathways; interference with transport pathways: anterograde (A), endocytosis (E), recycling (R), cleaving (Clv). Vpu domains involved: transmembrane domain (TMD), cytoplasmic domain (CD), phosphorylation (P). Evidence for (+) or against (-) interference with the respective pathway or involvement of the particular domain. No experimental evidence in grey. The information depicted in this table is based on current reviews [217,227,228] and the following references: general receptor array: [233]; CD4: [223-225]; tetherin: [226,227,229,230,234-239]; CD1d: [192,193,232]; NTB-A: [240,241]; PVR: [242]; CCR7: [243]; CD62L: [244]; tetraspanins (CD81): [233,245]; IRF3: [246-249]; NF- κ B: [186,247].

Target	Mechanism					Vpu domains				HIV-1		
	Degrad (P/L)		A	E	R	Clv	TMD	CD α 1 α 2		P	Subtype difference	Nef target
CD4	+	-	-	-	-		+		+	+	+	+
tetherin	+	+	+	-	+		+	+	+	+	+	- ¹⁾
CD1d	-	-		-	+		-		+	-	+	+
NTB-A	-	-	+	-	+		+		+	-		-
PVR	-	-	+				+		-	-		+
CCR7	-	-	+	-			+			-		-
CD62L	-	-	+				-			-		+
CD81 ²⁾	+	+	+				-			-		+
IRF3		+ ³⁾				+ ³⁾						
NF- κ B										+	-	+ ⁴⁾

¹⁾ only Nef of Group O targets tetherin; ²⁾ CD81 as example of tetraspanins; ³⁾ conflicting results; ⁴⁾ Nef induces NF- κ B.

The fact that several human viruses have evolved the capacity to interfere with CD1d-mediated immunity emphasizes its importance in the antiviral defense. Kaposi sarcoma-associated herpesvirus (KSHV) [250], herpes simplex virus (HSV) [251,252], human cytomegalovirus (HCMV) [253] and human papillomavirus (HPV) [254] were shown to interfere with CD1d surface expression through different mechanisms (summarized in **Figure 12** and reviewed in [117,169,255]).

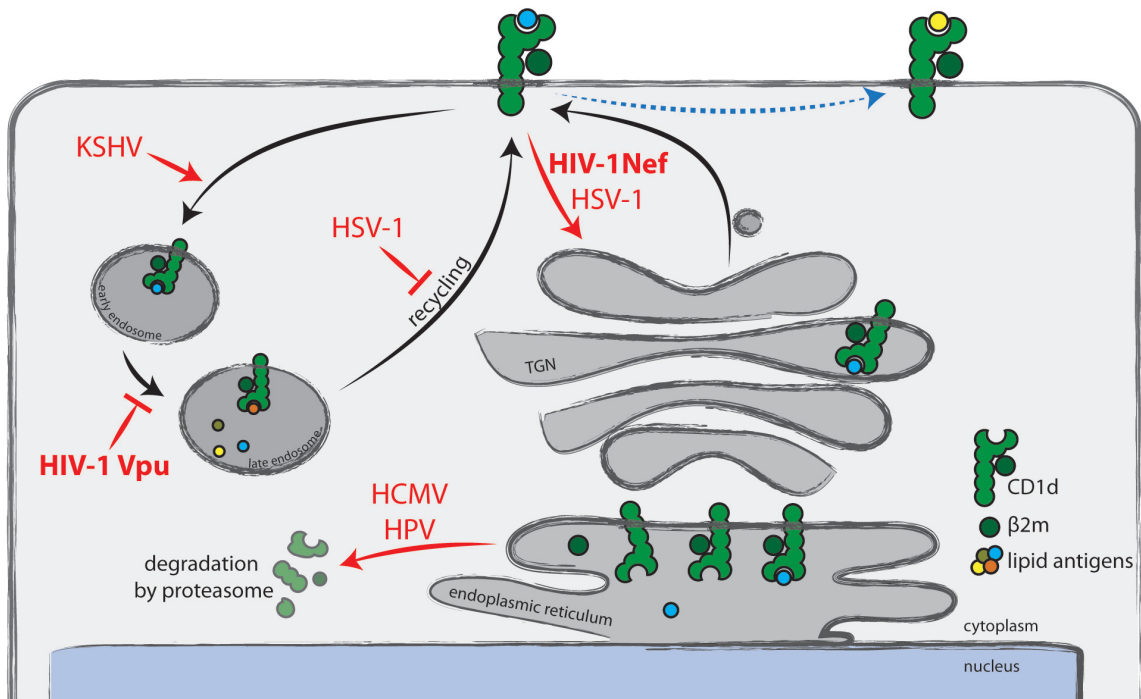


Figure 12: Viral interference with CD1d-mediated lipid antigen presentation.

After synthesis, human CD1d is transported via the secretory pathway to the cell surface. From here CD1d is internalized and patrols early and late endosomes. During the journey through the endocytic pathway lipid antigen exchange can occur and new self-lipids or foreign lipids may be loaded onto CD1d before recycling back to the cell surface. Viruses have developed different mechanisms to interfere with CD1d-mediated antigen presentation by reducing cell surface expression of CD1d. Viruses are shown in red. Kaposi sarcoma-associated herpesvirus (KSHV) increases CD1d endocytosis. HIV-1 Nef accelerates CD1d internalization and sequesters CD1d in the trans-Golgi network (TGN). HIV-1 Vpu interferes with CD1d recycling and is found together with CD1d in early endosomes, whereas herpes simplex virus (HSV-1) traps CD1d in late endosomes. Human cytomegalovirus (HCMV) interacts with CD1d in the ER and, similar to human papillomavirus (HPV), supports CD1d degradation by the proteasome. The dotted blue line indicates possible changes in the self-lipid antigen repertoire presented by CD1d due to viral infections. This figure is based on information from [117,250-254].

AIMS

The aim of this thesis is to explore the role of innate immunity and viral evasion strategies in HIV infection.

PAPER I

To determine the evolutionary conservation as well as mechanistic aspects of CD1d downregulation mediated by primate lentiviral Vpu proteins.

PAPER II

To investigate HIV recognition in dendritic cells and the mechanisms that translate viral recognition into CD1d-mediated activation of iNKT cells.

PAPER III

To study the effect of interferon α on HIV infection in dendritic cells.

PAPER IV

To analyze iNKT and NK cell populations and phenotype in chronic HIV-1, HIV-2, and dual infections in individuals from Guinea-Bissau.

SUMMARY AND CONCLUSIONS

PAPER I

Interference with CD1d is a conserved lentiviral immune evasion strategy.

- Interference with human CD1d is conserved among HIV-1 Vpus and their SIV precursors.
- A highly conserved C-terminal motif in subtype B Vpu is necessary for CD1d downregulation.

PAPER II

HIV-1 infection is sensed in dendritic cells and induces CD1d-mediated antigen presentation and the activation of iNKT cells.

- HIV-infected dendritic cells upregulate CD1d surface levels and the endogenous lipid antigen GlcCer.
- TLR7 stimulation induces CD1d cell surface expression and GlcCer synthesis.
- iNKT cells recognize HIV-1 infected dendritic cells in the absence of exogenous antigen.
- HIV-1 Nef and Vpu inhibit the detection of HIV-infected dendritic cells by iNKT cells.

PAPER III

Low levels of interferon α protect dendritic cells and T cells from HIV-1 infection.

- Interferon α induces APOBEC3 expression in dendritic cells.
- Interferon α restricts HIV-1 replication and transmission from dendritic cells to T cells.

PAPER IV

Alterations in iNKT and NK cells contribute to/are a consequence of the systemic immune activation in chronic HIV.

- Subsets of iNKT and NK cells are reduced in HIV-1 and HIV-2 infections.
- iNKT and NK cell activation are increased in HIV-1 and HIV-2 infections.
- Activation of iNKT and NK cells correlates with markers of disease progression and immune activation.

METHOD DISCUSSION

HEK293T transfection system

The transformed human embryonic kidney cell line (HEK293T, [256]) is commonly used for *in vitro* cell biological assays [257] because of its favorable properties such as fast cell growth, semi-attached phenotype and high rates of transfection. However, it is not clear which cell type or tissue is represented by HEK293T cells [257], thus functional assays need to be interpreted with care. Primary DCs and even MDDCs are notoriously hard to transfect, and upon transfection they often mature, thus the HEK293T cell line is a practical model system allowing the analysis of general aspects of viral interference on a larger scale. HEK293T cells co-transfected with plasmids encoding CD1d and Vpu, respectively, mimic the CD1d cell surface downregulation observed in human MDDCs [232] and were used to assess CD1d downregulation in **Paper I**. If possible, it is important to confirm mechanistic studies in the target cell type, such as DCs, due to cell type specific protein expression profiles and the particular endocytosis system present in antigen-presenting cells [258].

Monocyte-derived DCs (MDDCs)

Primary DCs are rare in the human blood and therefore more abundant monocytes were used to generate MDDCs for *in vitro* cell biological assays in **Papers I-III**. After monocyte isolation from the peripheral blood and differentiation in IL-4 and GM-CSF, MDDCs share similarities with primary DCs [259]. If cultured in the presence of human IgG, MDDCs express CD1d and are able to activate CD1d-restricted iNKT cells [260]. Yet, it is important to keep in mind that there are morphological and phenotypic differences between primary blood DCs and cultured MDDCs and if feasible primary DCs should be used to validate results [261].

HIV infection of MDDCs

Like primary DCs, MDDCs can get productively infected with HIV [83,90,262]. HIV-infected MDDCs were identified in **Papers I-III** by the intracellular detection of the viral capsid protein p24 using flow cytometry. p24 expression requires *de novo* synthesis of viral proteins and thus represents productively infected DCs and not just HIV virions attached to the cell surface [263]. The infection rate *in vitro* is usually low (1-3% in primary DCs [264]) and differs greatly between donors [265]. Therefore, we assessed the activation of iNKT cells by HIV-infected DCs in a mixed culture by microscopy on single cell basis [266].

HIV strains

Due to their high infectivity *in vitro*, the commonly used T cell line-adapted strains HIV-1 NL4-3 and BaL [267] were used in the infection assays for **Papers I-III**. It was shown recently that HIV-1 NL4-3 Vpu is less potent in interfering with cellular restriction factors compared to Vpus from patient isolates [239]. Thus, it is important to assess the immune evasion strategies of HIV not only in prototypic lab strains but also in patient isolates.

iNKT culture and activation

In order to obtain human iNKT cells for functional assays, iNKT cells were isolated from peripheral blood and expanded *ex vivo* in the presence of IL-2 and the lipid antigen α -GalCer. Expanded iNKT cells show a skewed subset distribution compared to freshly isolated cells, with a preferred expansion of CD4+ iNKT cells [132]. This needs to be kept in mind when interpreting iNKT cytokine production and effector functions in response to infected cells. Similar to MDDCs, donor-dependent variations are also observed. In order to overcome these issues, established iNKT cell clones were used in **Papers I and II** [268]. The use of the strongly activating model lipid α -GalCer in functional assays might induce greater iNKT cell responses compared to weakly activating endogenous lipids. Therefore, this assay might underestimate the effect HIV-1 Vpu *in vivo*.

Population and phenotype analyses of iNKT and NK cells using flow cytometry

For **Paper IV**, human iNKT cells were identified using antibodies against CD3 and the iNKT-specific TCR chains (V α 24 and V β 11). NK cells were defined as CD3 negative and CD56 positive cells. Due to technical limitations we omitted the NK cell marker CD16 from our staining panel. It was previously shown that chronic HIV infection increased the number of CD56 negative NK cells [269], a subset excluded from our analysis. The staining panel for the identification and phenotyping of iNKT and NK cells was tested and optimized using blood samples from Swedish donors. Population-specific and transport-derived sample differences may exist. In order to facilitate the logistics, blood samples from the cohort in Guinea-Bissau were preserved using CytoChex tubes (Streck) as this allows transport at room temperature. The preservation process fixes the cells permitting subsequent phenotypical analyses. Cross-sectional cohort studies on patient material are always subjected to limitations and biases. Despite the noted confines, **Paper IV** is an important characterization of unique patient samples to further our scientific and clinical understanding of HIV infections.

RESULTS AND DISCUSSION

HIV-1 EVASION OF INNATE IMMUNITY (PAPERS I-III)

Dendritic cells (DCs) are essential in the initiation of innate and adaptive immune responses after HIV infection [78,81]. Tissue-resident DCs express CD1d and are among the first cells to encounter HIV after mucosal transmission *in vivo* [82]. As potent producers of interferon α (IFN α), DCs create an antiviral state intracellularly and systemically [78]. Upon virus detection, DCs activate innate immune cells and initiate the adaptive immune response. HIV-1 in turn employs its accessory proteins Vpu and Nef to realize a variety of evasion strategies enabling viral replication and avoiding detection by the innate immune system [57,183]. Viral evasion of CD1d-mediated invariant natural killer T (iNKT) cell activation is described for several viruses infecting humans [117].

Figures in the papers are referred to as “Fig.” and figures in this thesis as “Figure”.

PAPER I: INVOLVEMENT OF A C-TERMINAL MOTIF IN THE INTERFERENCE OF PRIMATE LENTIVIRAL VPU PROTEINS WITH CD1D-MEDIATED ANTIGEN PRESENTATION

Summary of Results & Discussion

In HIV-1 infection, the accessory protein Vpu is a critical factor mediating evasion of the detection by the innate immune system [217,228,270]. Our previous study showed that Vpus of two HIV-1 group M subtype B strains (NL4-3 and BaL) downregulate CD1d from the surface of productively infected DCs and thereby inhibit their crosstalk with CD1d-restricted iNKT cells [232].

In **Paper I** we investigated whether CD1d inhibition is a conserved Vpu function of primate lentiviruses. In addition we set out to identify the molecular determinants in Vpu involved in interference with CD1d cell surface expression.

We analyzed a set of 63 *vpu* alleles derived from the four HIV-1 groups M, N, O and P and the direct SIV precursors of HIV, namely the SIV strains infecting the central chimpanzee (SIVcpzPtt) and the western lowland gorilla (SIVgor). The HIV-1 *vpu* alleles from group M contained all 9 subtypes. In order to gain an even broader evolutionary picture we also evaluated alleles from more distant Vpu-encoding SIV strains infecting guenons, such as the greater spot-nosed monkey (*Cercopithecus nictitans*, SIVgsn), the mona monkey (*Cercopithecus mona*, SIVmon), and the mustached monkey (*Cercopithecus cephus*, SIVmus).

We found that the ability to downregulate human CD1d from the cell surface was conserved in Vpu proteins from HIV-1 groups M, O and P (Fig. 1a, b). In line with previous results [271], Vpu from rare HIV-1 group N strains showed only weak CD1d downregulation (less

than 20%; Fig. 1a, b). Notably, antagonism of human CD1d was even detected in Vpus from the HIV-1 precursor strains SIVcpzPtt and SIVgor and also in the more distant SIVgsn and SIVmus (Fig. 1b). These results suggest a pre-existing susceptibility of human CD1d to SIVcpzPtt and SIVgor Vpu proteins rather than host-specific adaptation of these Vpus to restrain human CD1d. This is in contrast to tetherin antagonism, which is thought to represent a species barrier due to the fact that Vpu proteins from chimpanzee SIVs are poor antagonists of human tetherin [197]. The conservation of primate lentiviral CD1d antagonism might be explained by the high degree of sequence conservation between human, chimpanzee and gorilla CD1d. In order to assess the biological relevance of the observed CD1d downregulation levels we employed an iNKT cell activation assay. Lower CD1d cell surface levels induced less iNKT cell activation in response to α -GalCer, demonstrating that iNKT cell activation is indeed inversely correlated with CD1d downregulation (Fig. 2). Based on our results we conclude that **interference with CD1d expression and iNKT cell activation is conserved among diverse primate lentiviral Vpu proteins indicating the importance of this immune evasion strategy.**

Traditionally, most HIV-1 strains used in research are derived from group M subtype B, such as the model strains HXB2, NL4-3 and BaL [272]. Subtype B and subtype C differ in their geographic prevalence and while subtype B accounts for most infections in Europe and North America, subtype C represents about 50% of all HIV-1 infections globally [19]. Several differences in disease progression and drug resistance have been reported for HIV-1 group M subtypes [19,273,274]. One study found lower levels of CD4⁺ T cells and iNKT cells in subtype D compared to subtype A [275]. In a European cohort the HIV-1 subtype significantly influenced CD4⁺ T cell decline but had no effect on the viral set point [276]. Therefore, it is important to not only focus research efforts on subtype B but to also include other subtypes, such as subtype C.

Therefore, we analyzed in **Paper I** CD1d downregulation of group M Vpu proteins on a subtype level. Notably, in comparison to subtype B, subtype C Vpu proteins were significantly less efficient CD1d antagonists (Fig. 1d). It was suggested previously that subtype C displays lower replicative fitness compared to subtype B *in vitro* [277,278]. However, subtype C viruses may be more efficient in regard to transmission and infectivity [279,280]. It remains to be investigated if a different accessory protein substitutes Vpu's function and if subtype C viruses cause downregulation of CD1d in infected cells.

The functional difference between subtype B and C Vpus provided a tool to study the molecular requirements for CD1d downregulation. Here, we employed different mutants and chimeric proteins derived from active subtype B and inactive subtype C Vpus and revealed the C-terminal third of Vpu as relevant domain (Fig. 3 and 4). Generally, Vpu sequences are highly variable between HIV-1 subtypes and even within subtypes [205,206,208]. However, our amino acid sequence analysis identified **a highly conserved amino acid APW motif present in subtype B and absent from subtype C Vpus** (Fig. 4b and [206,207]).

Mutational analyses demonstrated that **the C-terminal APW motif in subtype B was necessary for CD1d downregulation**. Interestingly, the APW motif was described as part of a potentially functional secondary structure, a C-terminal hydrophobic tight loop [204] (depicted in chapter 3.1.2, **Figure 10**). The APW motif is absent from subtype C and did not transfer downregulation capacity when introduced into the subtype C sequence (Fig. 5a). The downregulation of subtype B Vpu was decreased but not completely lost after removal of the APW motif (Fig. 5a and d) indicating the involvement of additional Vpu motifs. In order to elucidate whether known Vpu motifs are important for CD1d downregulation, we investigated the EXXXLV motif in the 2nd α -helix of Vpu, which was recently shown to be important for binding to cellular adaptor protein 1 (AP1) [236] and tetherin antagonism [230,231]. However, this motif was not required for CD1d downregulation (Fig. S3). Possible additional domains and motifs required for CD1d downregulation in subtype B and the other CD1d downregulating HIV-1 subtypes, which do not contain the APW motif, remain to be investigated.

Human CD1d contains a tyrosine-based sorting signal that enables internalization into the endocytic pathway mediated by binding to AP2 [107] (see chapter 2.4, **Figure 5**). In addition to AP1, AP2, a central component of clathrin-mediated endocytosis [107], was recently shown to interact with Vpu [236]. Future experiments should aim to analyze whether Vpu targets AP2 in order to interfere with the cellular recycling machinery.

PAPER II: INNATE iNKT CELL RECOGNITION OF HIV-INFECTED DENDRITIC CELLS VIA INDUCED EXPRESSION OF ENDOGENOUS GLYCOLIPID ANTIGEN

Summary of Results & Discussion

To date, no viral lipid antigen has been described [117], still interference with CD1d-restricted lipid antigen presentation is a conserved function of HIV-1 Vpu (Paper I) and a immune evasion strategy developed by several viruses, emphasizing the role of CD1d-mediated immunity in the antiviral defense (chapter 3.1.2, **Figure 12**). As described in Paper I and [232], CD1d cell surface expression in HIV-1 infected DCs is reduced by the accessory proteins Nef and Vpu. A recent study investigating the role of iNKT cells in EBV infections found that, similar to HIV-1, CD1d expression is lost in EBV-infected cells abrogating iNKT activation. However, after synthetic induction of CD1d expression in EBV-infected cells, iNKT cells recognized the infected cells despite the absence of an exogenous lipid antigen and responded by cytokine secretion and cytotoxicity [166]. This led us to investigate whether iNKT cells are able to respond to HIV-infected DCs in the absence of Nef and Vpu.

In **Paper II** we examined how HIV-1 infection is sensed in DCs and if this virus recognition is translated from endogenous antigen presentation on CD1d to immune responses mediated by iNKT cells.

The female genital mucosa is a major portal for HIV transmission and entry [83]. In contrast to other mucosal tissues, the female genital tract lacks organized lymphoid structures and contains disseminated immune cells [281,282]. Therefore we determined the presence of CD1d⁺ DCs and iNKT cells in the female genital tract. We found CD1d⁺ DCs and iNKT cells expressing the HIV receptors CD4 and CCR5 present in the mucosa of human endometrial and cervical samples (Fig. 4 and 5).

The effect of HIV infection on CD1d expression was investigated by infecting MDDCs (from now referred to as DCs) *in vitro*. HIV-1 DHIV-3 (derived from NL4-3) infected DCs had decreased CD1d cell surface expression, which was mediated by the accessory proteins Nef and Vpu, and was lost in DCs infected with a Nef and Vpu deficient virus (Δ nef Δ vpu) (Fig. 1a and b), as expected (Paper I and [232]). Interestingly, in HIV-1 Δ nef Δ vpu-infected DCs CD1d cell surface levels were not at the level of uninfected DCs but were significantly increased (Fig. 1b). TLRs in general, and sensing of genomic HIV RNA by TLR7 and 8 in particular [283], are involved in the innate sensing of HIV [57]. In order to investigate the mechanism for CD1d upregulation in HIV-infected DCs, we used a TLR7 agonist, shown previously to induce CD1d expression [156], to stimulate uninfected DC and detected increased cell surface expression of CD1d (Fig. 1d).

CD1d presents endogenous and exogenous lipids to activate iNKT cells [284,285]. The first reported endogenous lipid capable of activating human iNKT cells was a β -linked glycosphingolipid [286]. Subsequently, lactosylceramide (LacCer, [287]) and glucosylceramide ([288], GlcCer) have been shown to activate iNKT cells.

Enzymes regulating GlcCer and LacCer biosynthesis are glucosylceramide synthase (*Ugcg*) and lactosylceramide synthase (*B4galt6*) [289,290] (**Figure 13**).

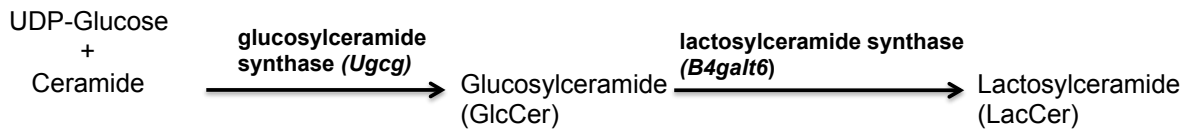


Figure 13: Biosynthesis of Glucosylceramide (GlcCer) based on [291,292].

In order to determine if the elevated CD1d cell surface expression in HIV-infected or TLR7 agonist stimulated DCs is accompanied by changes in the cellular lipid metabolism, we analyzed the expression of the enzymes glucosylceramide synthase and lactosylceramide synthase. Whereas TLR7 stimulation of DCs led to increased expression of glucosylceramide synthase and reduced expression of lactosylceramide synthase (Fig. 2a and b), infecting DCs with HIV-1 wt or HIV-1 Δ nef Δ vpu only decreased lactosylceramide synthase expression (Fig. 2c and d). In any case, these changes favored an increase in GlcCer levels. Importantly, glycolipid extraction and mass spectrometry of sorted DCs confirmed the accumulation of GlcCer in HIV-infected DCs (Fig. 3).

In wild type HIV, Vpu and Nef inhibit iNKT cell activation by downregulating CD1d from the cell surface. Interestingly, HIV-1 Δ nef Δ vpu-infected DCs activated iNKT cells significantly more than uninfected DCs of the same culture (Fig. 4b), indicating that iNKT cells respond to HIV-infected DCs in the absence of exogenous lipid antigens. This activation was CD1d-dependent and reduced by blocking the endogenous GlcCer synthesis (Fig. 4c). Thus, these findings indicate that **in the absence of an exogenous lipid antigen, HIV-infected DCs can connect viral recognition via the observed changes in enzyme expression, GlcCer levels and CD1d cell surface expression to iNKT cell activation.** This mechanism is counteracted by the HIV-1 accessory proteins Vpu and Nef.

PAPER III: IFN- α INDUCES APOBEC3G, F, AND A IN IMMATURE DENDRITIC CELLS AND LIMITS HIV-1 SPREAD TO CD4+ T CELLS

Summary of Results & Discussion

HIV-1 infection triggers the production of proinflammatory cytokines such as TNF and IFN α , which in turn create an antiviral state in the infected cell and in adjacent cells (chapter 2.2, **Figure 4**). The induction of cellular restriction factors is important for the creation of the antiviral state and the limitation of viral spread. The restriction factor family APOBEC3 restricts viral replication and is induced by IFN α [61].

In **Paper III** we investigated the effect of exogenously added IFN α and TNF on HIV-1 infection in MDDCs (from now referred to as DCs).

DCs were exposed to HIV-1 BaL (a model HIV-1 lab strain, group M, subtype B) and treated with different amounts of recombinant IFN α and TNF. The cell surface expression of DC activation markers, CD80 and CD86, was increased by TNF in a dose-dependent manner and by the highest dose of IFN α (Fig. 1). Interestingly, when analyzing the percentage of productively HIV-1 infected DCs the result was the opposite: there was a dose-dependent inhibition of viral replication for IFN α but only the highest dose of TNF successfully blocked virus replication (Fig 2). This demonstrates that low doses of IFN α block viral replication without inducing maturation (as defined by increased activation marker expression and migration) in DCs.

Infected DCs have been shown previously to assist infection of CD4+ T cells either by passing membrane-bound viral particles or newly produced virus to T cells during the close contact when forming an immunological synapse [85-87]. We therefore investigated if transfer of HIV-1 from infected DCs to autologous T cells was altered by the presence of IFN α . In contrast to TNF that had no effect on virus transfer, even low doses of IFN α reduced T cell infection (Fig. 3).

In order to determine the mechanism of viral replication blockage in DCs, we turned to APOBEC3, a restriction factor family known to be expressed and restrict viral replication in DCs [74]. Additionally, one member of the family, APOBEC3G was previously found to be induced by IFN α in T cells [293]. Virus-exposed DCs showed an increased expression of APOBEC3A, 3G and 3F in a dose-dependent manner after treatment with IFN α , but not after treatment with TNF (Fig. 4).

Taken together **this study showed that the lowest dose of IFN α (100 U/mL) induced expression of APOBEC3 and restricted HIV-1 replication in DCs while maintaining an immature DC phenotype. As a consequence, viral transfer from infected DCs to T cells was reduced in the presence of IFN α .**

Future Perspectives: Antiviral responses and viral evasion mechanisms thereof in HIV-1 infection (Papers I-III)

As demonstrated and discussed in papers I-III, local sites of HIV exposure and entry are populated by DCs, which play a central role in initiating and modulating the immune responses. Alarmed DCs release IFN α and other proinflammatory cytokines to activate adaptive or innate immune cells, among the latter iNKT and NK cells, which act immediately by either eliminating HIV-infected cells or recruiting additional immune cells [86]. Indirect evidence for the importance of IFN α in restricting HIV infection comes from the observation that HIV employs Vif, Vpr and potentially Vpu to interfere with its induction in infected cells [294] (recently reviewed in [57]).

Despite the importance of IFN α for the antiviral response, IFN α has also been linked to the chronic immune activation and disease progression in HIV infection. IFN α was found to modulate a hyperproliferative state in CD4⁺ T cells and potentially assist CD4⁺ T cell depletion in chronic HIV [295]. In addition, a recent longitudinal study investigating DC dynamics in chronic HIV infections found that DCs relocate from the peripheral blood to the gastrointestinal mucosa, which correlated to increased IFN α expression and activation of CD8⁺ T cells [296]. This is in line with the finding that systemic IFN α treatment led to increased levels of CD8⁺ T cells in HIV-infected persons [297]. However, as suggested in **Paper III**, low doses of IFN α could have beneficial effects while avoiding the detrimental effects of chronic immune activation. Further evidence for the therapeutic potential of IFN α comes from a recent study showing a more rapid and prolonged production of IFN α in DCs from so-called HIV elite controllers compared to chronically infected patients [298]. HIV elite controllers are individuals who are able to maintain undetectable viral loads despite the absence of antiretroviral therapy. Apart from the amount, timing might be another important aspect determining whether IFN α has a beneficial or detrimental influence in HIV infection. SIV infection in natural hosts, monkeys who are chronically infected by SIV but do not progress to AIDS and remain in an asymptomatic phase, differs from HIV infection in regard to the timing of the IFN α response [299]. In contrast to the chronic immune activation in HIV, in non-pathogenic SIV infection the initial IFN α response resolves after 1-2 months despite sustained viral replication [300]. Future studies are needed to investigate the potential beneficial effect of treatment with low doses of IFN α on innate immune cell responses in early HIV infection.

One promising example of successfully boosting innate immune responses in a viral infection is given by Lim et al. (2014, [301]). The authors show that a combination of type I interferons, TNF and receptor-ligand interactions with DCs could rescue cytotoxic NK cell responses that were otherwise blocked by the dengue virus immune evasion mechanisms. A similar therapeutic strategy of combining IFN α with increased CD1d antigen presentation or indirect activation via cytokines could be hypothesized for iNKT cells during HIV infection. CD1d⁺ DCs and iNKT cells are found in the mucosa of the female genital tract (**Paper II**) where iNKT cells could play a role as effectors and immune regulators [124].

Thus enhancing iNKT cell responses could assist the success of mucosal vaccines or new therapeutics [302,303].

Several viruses including HIV-1 have developed strategies to escape from CD1d-mediated lipid immunity and iNKT cell activation (**Figure 12** and reviewed in [117]). A study in mice showed that treatment with IFN α triggered TLR expression in iNKT cells and subsequent stimulation with TLR ligands resulted in increased and prolonged production of IFN γ [304]. Interestingly, Bego et al. (2015, [305]) revealed that HIV-1 uses a Vpu-mediated mechanism to downregulate surface tetherin in infected T cells to allow viral spread, while using the remaining tetherin to provide a negative signal to DCs resulting in decreased IFN α production. Adding to the HIV Vpu-mediated immune evasion catalogue, we show in **Paper II** that HIV-1 infection in DCs leads to increased CD1d surface expression and the presentation of a specific endogenous lipid enabling recognition by iNKT cells. As demonstrated in **Paper I**, higher CD1d levels lead to a greater percentage of activated iNKT cells. However, HIV-1 employs Vpu to interfere with CD1d-mediated immunity by decreasing CD1d cell surface levels, thus hindering activation of iNKT cells [232]. As this immune evasion strategy is conserved among HIV-1 groups M, O and P Vpus (**Paper I**), it provides therapeutic potential for Vpu inhibition. If Vpu is neutralized, the antiviral state of infected DCs would lead to decreased viral release, increased IFN α secretion, elevated and altered CD1d-mediated antigen presentation and finally detection of infected DCs by iNKTs. This could allow the activated iNKT cells to either eliminate infected cells or to accelerate the immune response by cytokine production. In favor of this approach is the recent finding that immune evasion strategies of the HIV-1 accessory proteins Vif, Nef and Vpu are conserved in acute and chronic HIV-1 infection [306].

Synthetic inhibitors of Vpu are available and have been analyzed for their antiviral potential. A novel small molecule inhibitor of Vpu named BIT225 (Biotron Limited) reduced viral release from HIV-1 infected macrophages [307], which was independent of tetherin antagonism [308]. In addition to Vpu, Nef has been shown to reduce CD1d cell surface levels [192,193] and several small molecule inhibitors of Nef have been described [309,310]. It remains to be investigated if BIT225 or Nef inhibitors can rescue CD1d cell surface expression in infected cells. A current noteworthy approach to find new HIV-1 inhibitors screened the pan-African Natural Product Library, “the largest collection of medicinal plant-derived pure compounds on the African continent”, and detected several molecules with potential to interact with Vpu [311]. More specific analyses need to show if these compounds prove to be useful therapeutic candidates.

PAPER IV: ELEVATED LEVELS OF iNKT AND NK CELL ACTIVATION CORRELATE WITH DISEASE PROGRESSION IN HIV-1 AND HIV-2 INFECTIONS

Summary of Results & Discussion

HIV-1 and HIV-2 are related viruses that can cause AIDS. While HIV-1 has given rise to a pandemic, HIV-2 is mainly restricted to West Africa [30]. The rate of CD4⁺ T cell decline and progression to AIDS is slower in HIV-2 compared to HIV-1 infection. In addition to disease progression, several differences in the course of HIV-1 and HIV-2 infection are known [51]. The reason for this is not fully understood, but in HIV-2 infection a great proportion of infected individuals maintains low or undetectable plasma viral load and with a viral set point at least one log lower than in HIV-1 infection [55,312]. Similar to HIV-1, T cell activation in HIV-2 infection appears to be related to CD4 levels as well as viral load [313-315]. However, HIV-2-infected individuals display lower levels of chronic immune activation assessed by detection of activated T cell populations compared to HIV-1 [316]. The innate and adaptive immune response to HIV-1 has been studied extensively, while much less is known about immune responses in HIV-2 and HIV-1/2 dual (HIV-D) infections [51].

In **Paper IV** we studied the innate cellular immune response, in particular iNKT and NK populations and phenotype, in chronic HIV-1 and HIV-2 and dual infection in a cross-sectional occupational cohort from Guinea-Bissau.

Consistent with previous findings, detecting a selective loss of CD4⁺ iNKT in HIV-1 infected individuals [134,317], we found **a significant reduction of CD4⁺ iNKT cells in HIV-1 and HIV-D infected individuals** (Fig. 1b). There was no loss of CD4⁺ iNKT cells detected when analysing all HIV-2 infected individuals. However, after subgrouping into aviremic and viremic HIV-2 infection, the latter group had a strong decrease in CD4⁺ iNKT cells, similar to that seen in HIV-1 infection (Fig. 1c). While the CD4⁺ iNKT subset can be infected with HIV-1 [318], this has not been investigated for HIV-2 thus far. The NK cell population also changed in chronic HIV infection, with a reduced CD56^{bright} NK cell subset in HIV-1 and HIV-2 infections (Fig. 1b).

We then analyzed phenotypic changes in the innate cellular immune response such as the expression levels of activation markers. In chronic HIV-1 infection, the phenotype of activated iNKT cells is characterized by the elevated expression of the PD-1 exhaustion marker and impaired cytokine production [140]. Here, we detected **elevated activation levels of the iNKT and NK cell subsets in chronic HIV-1 and HIV-2 single as well as HIV-1/HIV-2 dual infections**, with the highest levels in HIV-1 infection (Fig. 2a). Interestingly, viremic HIV-2 infected participants showed increased iNKT and NK cell activation levels, compared to aviremic HIV-2 infection and healthy controls (Fig. 2b). The observed elevated iNKT and NK cell activation levels correlated with CD4 T cell percentages and viral load, both clinical markers of disease progression. Additionally, iNKT, NK and CD4⁺ T cell activation levels correlated with each other.

These results suggest that **alterations in iNKT and NK cells contribute to/are a consequence of the systemic immune activation in chronic HIV infections.**

Future Perspectives: Innate cellular immunity in HIV-1 and HIV-2 infections

The relationships between different immune cells contributing to chronic immune activation in HIV infections are complex and far from elucidated (reviewed in [319]). One way to systematically analyse immune changes during the course of HIV infection is to conduct longitudinal studies preferably from the time of the primary infection. However, recruiting cohorts to study primary infection is challenging, especially in the setting of HIV-2 infection. Additionally, the number of HIV-2 infections is declining and is replaced by HIV-1 as the major source of HIV infection [31]. A recent longitudinal study by Jiao et al (2014, [180]) reported that individuals with a lower CD4⁺ T cell count during the first year of HIV-1 infection also had a more pronounced loss of CD56^{dim} NK cells in the first months of infection. We found a reduction of CD56^{bright} NK cells during both chronic HIV-1 and HIV-2 infections, however, the early dynamics of innate cells in HIV-2 remain to be investigated.

Another way to study differences in the dynamics of the immune response is to compare HIV-1 to less pathogenic infection settings, such as SIV infection in natural hosts and HIV-2 infection in the large proportion of individuals with slow disease progression [312,320]. An interesting example of this approach is an *in vitro* study from Cordeil et al (2013, [321]) that compared the effects of IFN α on viral replication of HIV-1, HIV-2, and SIVmac from the rhesus monkey. Their results indicated that HIV-1 is more resistant to the IFN α -induced antiviral response than HIV-2 and SIVmac.

Several clinical trials evaluating antiretroviral treatment in HIV-2 are ongoing [322], which could provide a possibility to further analyze human innate and adaptive responses in HIV-2, and compare them to the present knowledge of HIV-1 [323].

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“Driving down the road, road tripping. Feel my energy running over me.

So let’s stop a while for a break – life is serving rum & cake.”

© *The Band*

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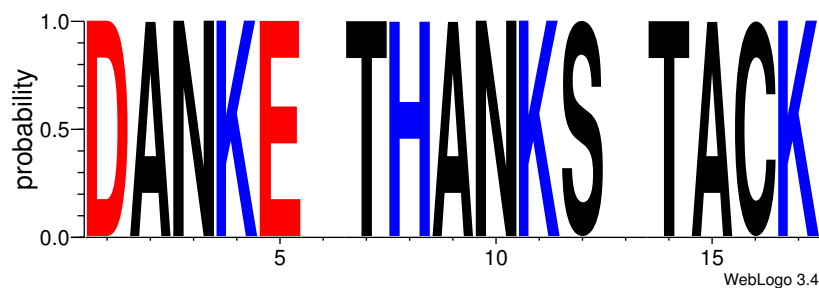
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