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INVARIANT NKT CELLS AND CD1d IN HIV-1 INFECTION

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Till Ossian

“Du min förtvivlan och min kraft,
du tog allt eget liv jag haft,
och därför att du krävde allt
gav du tillbaka tusenfalt."

Karin Boye

ABSTRACT

The invariant natural killer T (iNKT) cells are a subset of T lymphocytes that share characteristics of both innate and adaptive immunity. These cells are defined as T cells restricted by the lipid antigen presenting molecule CD1d and express an invariant T cell receptor (TCR) as well as classical natural killer (NK) cell markers. Upon stimulation iNKT cells rapidly produce large amounts of cytokines and chemokines and activate various other cell types including NK cells and dendritic cells (DCs). iNKT cells have been implicated in the protection against infectious pathogens, including viruses, bacteria and parasites. In addition they are involved in the immune responses against tumors and in regulating autoimmune and inflammatory diseases.

Several viruses have been shown to evade human iNKT cell responses by down-regulating CD1d expression in infected cells. In this thesis the effect of HIV-1 infection on CD1d expression in DCs and the ability of DCs to activate iNKT cells were investigated. We found that HIV-1 interferes with CD1d expression and that this effect was mediated by the HIV-1 accessory protein Vpu. Down-regulation of CD1d contributed to inhibition of iNKT cell activation, as indicated by lower IFN-γ production and less centrosome polarization in iNKT cells in contact with infected DCs. The mechanism behind CD1d down-regulation was dependent on inhibition of CD1d recycling, and interaction between CD1d and Vpu in early endosomal compartments. Vpu down-regulates different host-proteins involved in innate immunity. Interestingly, down-regulation of CD1d and CD4 was found to occur by distinct mechanisms. In addition to Vpu-mediated interference with CD1d expression and iNKT cell activation, HIV-1 infection leads to depletion and functional impairment of iNKT cells in infected individuals. Collectively, these findings suggest that the Vpu protein of HIV-1 may play a significant role in evasion from CD1d-specific immune responses, and moreover support the notion that iNKT cells play an important role in the host defence against HIV-1.

In addition to recognition of exogenous lipid antigens presented by CD1d, iNKT cells can be activated by cytokine or surface receptor stimulation with or without presentation of endogenous lipids. In this thesis, we demonstrate NKG2D-dependent iNKT cell activation. Specifically, the CD4-negative iNKT cell subset was able to degranulate and kill target cells upon NKG2D triggering in a TCR-independent manner. Moreover, NKG2D-engagement co-stimulated TCR-mediated iNKT cell activation in response to endogenous CD1d ligands. These alternative pathways for iNKT cell activation may be particularly important in settings where target cells express no or low levels of CD1d, such as transformed or virus infected cells. In summary, the work presented in this thesis expands our knowledge about how iNKT cells respond to target cells and how viruses work to counteract such recognition.

LIST OF PUBLICATIONS

This thesis is based on three publications and one manuscript. The individual papers are referred to by roman numerals.

I. Markus Moll*, **Sofia K. Andersson***, Anna Smed-Sörensen and Johan K. Sandberg. Inhibition of lipid antigen presentation in dendritic cells by HIV-1 Vpu interference with CD1d recycling from endosomal compartments. *Blood 2010 Sep 16; 116(11): 1876-84. * Shared first authors*

II. **Sofia K. Andersson**, Susanna M. Bächle, Mirko Kroll, Johan K. Sandberg and Markus Moll. HIV-1 Vpu-mediated down-regulation of CD1d and CD4 follow distinct mechanistic pathways. *Manuscript*.

III. **Sofia K. Andersson**, Dominic Paquin-Proulx, Mirko Kroll, Johan K. Sandberg and Markus Moll. Measurement of iNKT cell responses at the single cell-level against rare HIV-1 infected dendritic cells in a mixed culture. *Journal of Leukocyte Biology*. *In Press.*

IV. Carlotta Kuylenstierna, Niklas K. Björkström, **Sofia K. Andersson**, Peter Sahlström, Lidija Bosnjak, Dominic Paquin-Proulx, Karl-Johan Malmberg, Hans-Gustaf Ljunggren, Markus Moll and Johan K. Sandberg. NKG2D performs two functions in invariant NKT cells: Direct TCR-independent activation of NK-like cytolysis and co-stimulation of activation by CD1d. *European Journal of Immunology 2011 Jul;41(7):1913-23.*

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LIST OF ABBREVIATIONS

AP	Adaptor Protein
AIDS	Acquired Immunodeficiency Syndrome
APC	Antigen Presenting Cell
APOBEC3G	Apolipoprotein B mRNA-editing Enzyme Catalytic Polypeptide-like 3G
B_2M	β_2-Microglobulin
BST2	Bone Marrow Stromal Antigen 2
CCR5	C-C chemokine Receptor type 5
CD	Cluster of Differentiation
CTL	Cytotoxic T Lymphocyte
CXCR4	C-X-C Chemokine Receptor type 4
DC	Dendritic Cell
DNA	Deoxyribonucleic Acid
DP	Double Positive
EBV	Epstein-Barr Virus
eGFP	Enhanced Green Fluorescent Protein
ER	Endoplasmatic Reticulum
FACS	Fluorescence-Activated Cell Sorting
FASL	Fas Ligand
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
Gp120	Envelope Glycoprotein 120
HCMV	Human Cytomegalovirus
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HPV	Human Papillomavirus
HSV-1	Herpes Simplex Virus type 1
IAV	Influenza A Virus
IFN	Interferon
Ii	Invariant Chain
IL	Interleukin
iNKT	Invariant Natural Killer T
IRF3	Interferon Regulatory Factor 3

KSHV	Kaposi´s Sarcoma-associated Herpes Virus
MAPK	Mitogen-activated Protein Kinase
MCMV	Mouse Cytomegalovirus
MHC	Major Histocompatibility Complex
MS	Multiple Sclerosis
MTOC	Microtubule Organizing Center
Nef	Negative factor
NK	Natural Killer
NKG2D	Natural-Killer Group 2 member D
NTB-A	NK-T-B Antigen
PD-1	Programmed Cell Death 1
PIM4	Phospatidylinositolmannoside 4
PVR	Poliovirus Receptor
RNA	Ribonucleic Acid
RT	Reverse Transcriptase
SAP	SLAM-Associated Protein
SIV	Simian Immunodeficiency Virus
SLAM	Signalling Lymphocytic- Activation Molecule
T1D	Type 1 Diabetes
TCR	T-cell Receptor
TGN	Trans Golgi Network
T_H	T helper
TLR	Toll-Like Receptor
TNF	Tumor Necrosis Factor
Vif	Virulence factor
Vpr	Viral Protein R
Vpu	Viral Protein U
VSV	Vesicular Stomatitis Virus
VZV	Varizella Zoster Virus
α-GalCer	Alpha-Galactosylceramide
β-GlcCer	Beta-D-Glucopyranosylceramide
β-TrCP	Beta-Transducin repeat-Containing Protein

1 INTRODUCTION

Antigen presentation is a highly regulated immune process where dendritic cells, macrophages and other cells types capture and present antigenic material to T cells. Studies have established that the immune system has a unique ability to respond to antigens of both endogenous and foreign origin. Before the discovery of the lipid antigen presenting CD1 proteins it was believed that all antigens were derived from proteins and presented by major histocompatibility complex (MHC) class I and II molecules to peptide-specific T cells. Today it is clear that not only peptides but also lipids, glycolipids and lipopetides can be presented as antigens and activate lipid-specific T and natural killer T (NKT) cells. The peptide and lipid antigen presentation pathways function probably cooperatively to defend the host from pathogens illustrating the complexity of the immune system. A wide range of endogenous and microbial lipids have already been identified as antigenic and the field of lipid-specific immunity in health and disease is growing. CD1d and CD1d-restricted invariant NKT cells (iNKT) have been implicated in various diseases such as viral and bacterial infections, autoimmunity and cancers. Interestingly, a recent study identified microbial vitamin B9 metabolites as a novel third class of antigens. Vitamin B9 metabolites are presented by the MHC-class I-like molecule MR1 and activate mucosal-associated invariant T (MAIT) cells (1). It cannot be excluded that future studies will identify so far unknown antigen presentation pathways and novel classes of antigens.

The overall aim of this thesis was to study the potential impact of human immunodeficiency virus type 1 (HIV-1) infection of dendritic cells on CD1d expression and iNKT cell activation. Specifically, the interaction of the HIV-1 accessory gene *vpu* with CD1d was studied, and a new microscopy based methodology to study iNKT cell activation was developed. An additional aim was to study iNKT cell activation via natural killer (NK) cell receptor triggering. This thesis starts with an introduction into the field followed by presentation and discussion of our main findings and the four papers (**papers I-VI**) that this thesis is based upon.

2 THE CD1 SYSTEM

In 1979 the first CD1 protein was described as the thymocyte antigen HTA1 (later called CD1a) (2). Today, five CD1 genes located on chromosome 1 that encode five different CD1 proteins (CD1a-CD1e) have been identified in humans. The molecular structure of CD1 proteins is similar to that of MHC class I proteins including a transmembrane heavy chain with three α-domains non-covalently attached to β_2-microglobulin (β_2m) (3-5). The luminal part of the CD1 protein is followed by a transmembrane segment and a short cytoplasmic tail. On the basis of their sequence, CD1 proteins are classified into three different groups; group 1 comprises CD1a, CD1b, and CD1c; group 2 CD1d and group 3 CD1e (6, 7). While group 1 and 2 CD1 molecules function as antigen presenting molecules, CD1e is not expressed on the cell surface and is believed to be involved in lipid antigen processing and loading (4, 8). Even though CD1 proteins were described more than 30 years ago (2) the actual role of CD1 proteins and their function in lipid-antigen presentation to T cells were only discovered much later (9, 10).

Similar to MHC I proteins, the α1 and α2 domains of the CD1 molecules form the antigen-binding region. These domains are linked to the α3 domain, which is attached to the transmembrane segment followed by a relatively short cytoplasmic tail (3). Unlike MHC class I proteins, CD1 proteins bind alkyl chains in their hydrophobic pockets. MHC I proteins are highly polymorphic and each molecule specifically binds peptides of a particular length and motif. In contrast, there are only four CD1 proteins that present lipid antigens and each CD1 member is specialized to interact with a range of lipid ligand variants. Compared to MHC class I molecules, the grooves of CD1 molecules are deeper and consist of up to four hydrophobic pockets: A', C,' F' and T' (Figure 1). The anchoring of the alkyl chain occurs in the deeply located A' pocket and the hydrophilic head group of the lipid is protruding out from the groove and is thereby available for direct contact with the T cell receptor (TCR). The antigen binding grooves of the different CD1 molecules differ in their volumes as well as in the number of pockets (two or four), which determines their ability to bind different types and sizes of lipid antigens (11). In CD1b, which has four pockets, a long so called A'T'F' super channel is formed enabling CD1b to harbour extremely long alkyl chains (12).

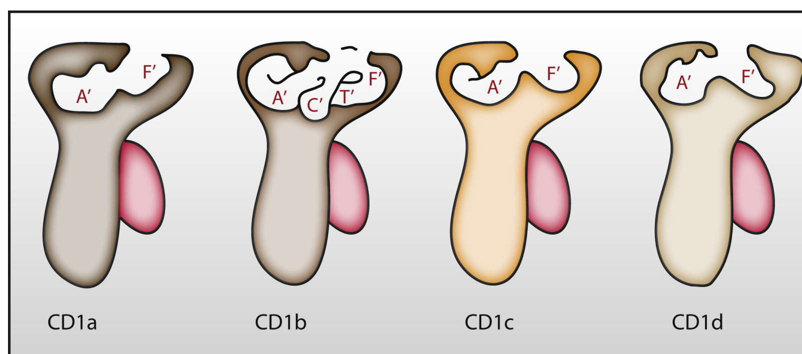

***Figure 1**: Antigen-binding pockets of human CD1 molecules* *(14).*

So far, all mammals studied have been shown to express one or several CD1 proteins. While humans express all isoforms, group 1 genes have been evolutionarily lost in rodents (13).

2.1 CD1 EXPRESSION

The different CD1 proteins differ in their expression profile. While the expression of group I CD1 proteins is generally restricted to professional antigen presenting cells (APCs) (15), CD1d is more broadly expressed and found both on professional APCs, B cells, monocytes as well as some non-hematopoetic cells (16-19). CD1d is also expressed on thymocytes (20), which is important for iNKT cell development and will be discussed later in this thesis. Additionally, CD1d is expressed on Kupffer cells and endothelial cells in the liver (21), up-regulated on hepatocytes in hepatitis C infection (22) and on microglial cells upon inflammation (23). Unlike group I CD1 proteins, CD1d is expressed in a wide number of tissues. Studies have demonstrated CD1d expression in the intestine, liver, kidney, skin, breast and the reproductive organs (24). In the skin, CD1d is expressed in low levels on many different cell types such as epithelial cells. CD1d is also expressed on intestinal epithelial cells, which have been demonstrated to present antigens to iNKT cells (25). Despite some evidence that CD1d expression can be regulated by inflammatory signals (26), surface levels of mouse and human CD1d seem to be stable under normal conditions (19). Monocytes, that express low levels of CD1d, keep their expression levels during differentiation into dendritic cells (DCs), and in contrast to CD1a, CD1b and CD1c, addition of GM-CSF and IL-4 does not upregulate CD1d expression (19, 27). Furthermore, recent work has shown that CD1d expression in monocyte-derived DCs is regulated by FcR mediated signals (28). Although DCs express only low levels of CD1d, they are the most effective cells in activating iNKT cells and are able to stimulate iNKT cells to produce different cytokines and induce their cytolytic response (19).

2.2 CD1 TRAFFICKING

CD1d is synthesized into the ER and glycosylated by N-linked oligosaccharides, which enables association of the ER-chaperones calnexin and calreticulin that regulate glycoprotein folding, and with the thiol oxidoreductase eRp57 that facilitates disulfide bond formations (29). The non-covalent binding to β_2m also occurs in this compartment (6, 30, 31). Similar to MHC class I binding of peptide and β_2m and MHC class II interaction with the invariant chain (Ii), β_2m binding and loading of endogenous lipid contribute to stabilization of CD1 molecules (32, 33). However, a minor fraction of CD1d seems to have the ability to leave the ER without β_2m and can be expressed at the surface as functional antigen presenting proteins (34, 35). The endogenous lipids loaded into the CD1 pocket already in the ER are not clearly defined, but phosphatidylinositol has been suggested as one possible candidate (32). Following assembly in the ER, CD1 molecules traffic through the secretory pathway, via the Golgi complex, to the plasma membrane (6). Whether the CD1 proteins, loaded with endogenous lipid, stimulate autoreactive T cells at this stage is not clear. Although cell surface trafficking is the main route for CD1 proteins, some studies have shown that there are alternative trafficking pathways for CD1d (36-38). These pathways are

dependent on the interaction with MHC II-Ii complexes that are maintained in the endosomal system as well as on the plasma membrane (36). Why a fraction of CD1d interacts with the Ii is not completely understood but it has been suggested that the Ii protects the groove from antigen binding until the molecule reaches the endosomes and exposes CD1d to different sets of antigens (37).

In order to be able to sample the cell for foreign antigens, the CD1 proteins internalize from the plasma membrane and recycle between the plasma membrane and endosmal/lysosomal compartments (Figure 2). To broadly survey the endocytic system, the different CD1 isoforms internalize into different endocytic compartments where various types of endogenous and, if the cell is infected, exogenous lipid antigens are available for loading into CD1 molecules (39). Internalization of CD1b, c and d is dependent on a conserved tyrosine-based motif (YXXØ; where Y is a tyrosine, X is any amino acid and Ø is an amino acid with a bulky hydrophobic group) encoded in the cytoplasmic tail. This motif mediates binding to adaptor proteins (APs) that sort cargo proteins into clathrin coated pits (40). CD1c and CD1d only interact with AP-2, which directs them to late endosomes (41). In contrast, mouse CD1d and human CD1b that require endosomal acidification for efficient lipid binding, bind to AP-2 and AP-3 mediating transport to lysosomes (42, 43). Despite the fact that CD1a does not express a tyrosin-based motif, it is internalized into the endocytic system by a clathrin independent pathway similar to that of MHC class I and is mainly found in recycling endosomes (41, 44, 45). Surface presentation of antigens loaded into CD1 molecules in the endosomal compartments requires recycling of the CD1 proteins to the cell surface. While internalization is well studied, the molecular mechanisms regulating the recycling pathways need further investigation.

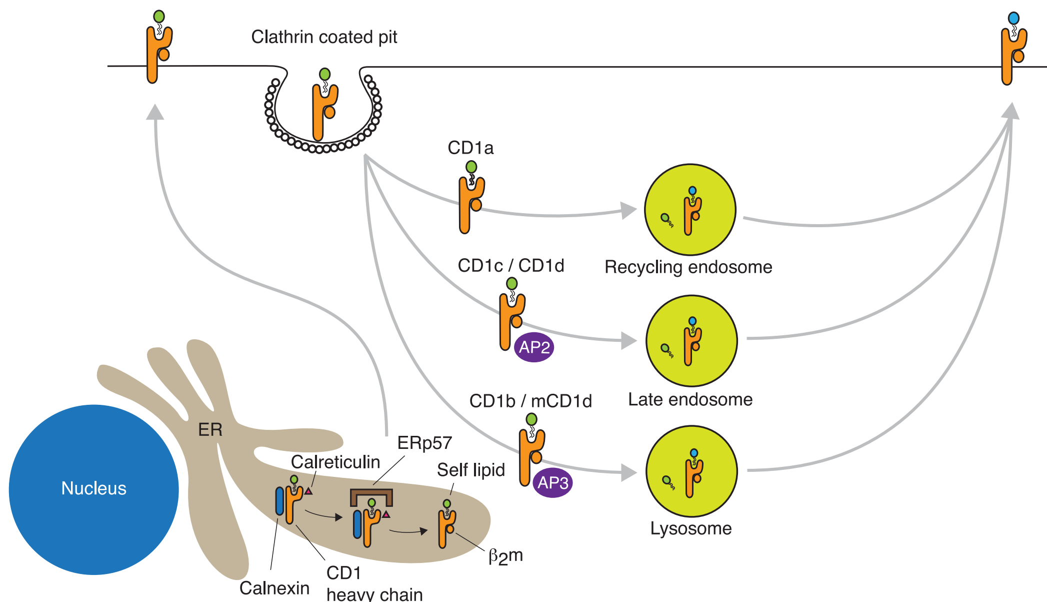

Figure 2: CD1 trafficking in APCs. *CD1 proteins traffic from the ER via the Golgi complex to the plasma membrane and internalize into different endosomal compartments depending on trafficking signals in the cytoplasmic tails of the CD1 proteins. From the endosome the proteins recycle back to the cell surface and present their antigenic content to CD1-restricted T and NKT cells.*

2.3 CD1 LIGANDS

As there are probably distinct glycolipid antigens in the different endosomal compartments, the wide distribution of CD1 molecules throughout the endosomal system may contribute to the presentation of a wide range of endogenous and exogenous lipid antigens. The mechanisms involved in lipid antigen loading into CD1 molecules are incompletely defined. However, lipid transfer proteins (LTP) extract lipids from membranes and have been shown to directly interact with CD1 proteins, suggesting their involvement in lipid loading (46, 47). Another important factor for lipid trimming and binding into the CD1 pocket is the endosomal/lysosomal pH (48). While the lipid binding specificity of the different CD1 isoforms is somewhat overlapping (49) some lipids bind exclusively to one isoform.

A variety of both endogenous lipids synthesized within the cell and foreign derived lipids from extracellular origin have been identified as CD1 ligands. Glycosphingolipids (GSL) and phospholipids (PL) are two major groups of antigenic self-lipids that have been identified as binding to the CD1 grooves, but only some of them have the capacity to stimulate T cells (3). All CD1 proteins are able to bind microbial lipids. CD1a binds and presents lipid antigens from *Mycobacterium tuberculosis* to activate CD1a-restricted T cells (50). Lipids derived from the mycobacterial cell wall are presented by CD1b and include many different lipid types such as lipoarabinomannan (LAM) (51), phosphatidylinositol mannosides (PIM) (8) and mycolic acids (10) and are able to stimulate CD1b-restricted T cells. CD1c molecules bind mycobacterial lipids from both *M. avium* and *M. tuberculosis* (52). Glycosphingolipid antigens derived from the cell wall of gram-negative *Sphingomonas* bacteria were the first well-defined microbial antigens shown to bind to CD1d and to stimulate mouse and human iNKT cells (53, 54). Another gram-negative bacteria, *Borrelia burgdorferi* that is causing Lyme disease (55), as well as the gram-positive bacteria *Streptococcus pneumoniae* (56), contain CD1d-presented lipid antigens that can activate iNKT cells.

3 iNKT CELLS

iNKT cells are a subset of T lymphocytes with an important role in regulating innate and adaptive immunity. iNKT cells can rapidly produce large amounts of cytokines and chemokines upon activation and activate NK cells, T cells, B cells and DCs and are therefore important in the protection against pathogens, including viruses, bacteria and parasites (57, 58).

3.1 DEFINITION

Whereas T cells restricted by CD1a-, b and c display a diverse T-cell receptor repertoire, iNKT cells are defined as CD1d-restricted T cells expressing an invariant TCR as well as classical NK cell markers. The majority of these cells express an invariant Vα24-Jα18 TCR preferentially paired with Vβ11 in humans and Vβ8, Vβ7 or Vβ2 in mice, and due to this limited variability are called invariant NKT/iNKT cells (59). iNKT cells were discovered already in 1987 when researchers found a unique T cell subset in mice with a CD4-CD8+ phenotype and a restricted Vβ repertoire (60, 61). Later on, iNKT cells were given their name, based on the shared characteristics with NK cells (62) and it was not until 1994 that human iNKT cell subsets where discovered (63, 64). In contrast to conventional T cells that recognize peptide antigens presented by MHC class I or II molecules, iNKT cells become activated by lipids bound to CD1d. The model CD1d antigen α-galactosylceramide (α-GalCer) stimulates iNKT cells, also referred to as "classical" or "type I NKT cells" (65, 66). Other subsets of NKT cells exist, called "non-invariant" or "type II NKT cells" (67) (Table 1). The subset that expresses a diverse TCR repertoire and responds to lipids different to those recognized by iNKT cells will not be extensively discussed in this thesis and when discussing NKT cells, iNKT cells will be implied unless otherwise stated.

	Type I/iNKT	**Type II/non-iNKT**
CD1d dependent	Yes	Yes
α-GalCer reactive	Yes	No (but sulfatide reactive)
Subsets	CD4+, CD8+, DN (humans) CD4+, DN (mice)	CD4+, DN (mice)
TCR α-chain	Vα24-Jα18 (humans) Vα14-Jα18 (mice)	Diverse, but some Vα3.2-Jα9, Vα8 (mice)
TCR β-chain	Vβ11 (humans) Vβ8.2, 7, 2 (mice)	Diverse, but some Vβ8.2 (mice)

***Table 1**: **Classification of NKT cells**. Adapted from (67).*

iNKT cells have been well characterized in both mice and humans. Human iNKT cells have been reported in peripheral blood, cord blood and liver as well as in the bone

marrow. In mice, iNKT cells can be detected at all sites where conventional T cells can be found. The proportions of iNKT cells vary between different tissues. In mice iNKT cells are 0.5% of the blood T cells, 3% of the spleen T cells and up to 50% of the T cells in the liver (68). In humans, iNKT cells are less frequent in all locations, but highly variable between different individuals, ranging from less than 0.001% to over 3% (68). Interestingly, twin studies have suggested that iNKT cells numbers in humans may be genetically determined. This was indicated by studies in type I diabetes patients where there was no difference in the iNKT cell compartment between healthy or diseased twins (69), supporting the hypothesis that the variability between iNKT cell numbers in humans is most probably influenced by genetic factors. However, recent studies have also uncovered differences in peripheral blood iNKT cell percentages between different geographic locations (unpublished observation), supporting the notion that environmental factors also may influence iNKT cell levels in the periphery.

3.2 iNKT CELL DEVELOPMENT

Both mouse and human iNKT cells develop in the thymus (70, 71) and derive from the same progenitor as conventional T cells but separate from them at the double positive (DP) thymocyte stage in the thymic cortex (72, 73). In contrast to MHC class I restricted conventional T cells, iNKT cell precursors are positively selected by glycolipids bound to CD1d, expressed on DP cortical thymocytes (73, 74, reviewed in 75), and both CD1d and endogenous endosomally derived antigens are necessary for the selection (76). Important for the development is generation of secondary signals that are generated by the interaction between two DP T cells leading to engagement of receptor members of the signalling lymphocytic-activation molecule (SLAM) family that in turn leads to downstream engagement of the SLAM-associated protein (SAP) and SRC kinase Fyn (77-79). After positive selection, iNKT cells expand in the thymus. However, most of the iNKT cells that leave the thymus are immature and complete their maturation process in the periphery (80), where they acquire different NK cell markers (70). In contrast to conventional T cells, where ongoing interaction with their ligand is necessary, CD1d is not required in the iNKT maturation process. However, co-stimulatory molecules and signals (70, 81), the transcription factor PLZF (81) as well as IL-15 (82, 83) are critically involved. In humans, IL-7 has been shown to regulate iNKT cell development where high doses promote proliferation of iNKT progenitors and low doses support survival and drive maturation of these cells (71). There are some major differences between iNKT cell development in mice and humans. First, the intra-thymic precursors of iNKT cells can be readily identified in the adult mouse but in humans only in the early fetal thymus. Second, the mouse iNKT progenitors can be both CD4+ and CD4-. The CD4- iNKT cells subset can be found in human adult peripheral blood, but it is only the CD4+ subset that is detected in the human fetal thymus (71). The pattern of CD4 and CD8 molecules on iNKT cells during their development is poorly understood. The fact that CD4- iNKT cells appear later than CD4+ iNKT cells (84, 85) suggests that CD4- iNKT cells derive from the CD4+iNKT cell subset (71).

3.3 MODES OF iNKT CELL ACTIVATION

iNKT cells are described as innate immune effector cells that have the ability to within minutes to hours respond with cytokine production, proliferation and cytolysis. Due to their fast response to antigen they are believed to bridge the gap between innate and adaptive immunity. The fast response to stimuli contributes to their importance in many different diseases including cancer, autoimmunity, infection and allergy (59). iNKT cells can become activated during infections as well as inflammatory and autoimmune processes. The first discovered iNKT ligand α-GalCer came from the marine sponge *Agelas mauritianus*, collected in the Okinawan sea. This lipid was first identified as having anti-tumor activities (86), and later on led to the identification of iNKT cells (65). α-GalCer is today broadly used in immunoassays as a very potent iNKT cell antigen, contributing to a CD1d-TCR affinity that is one of the highest ever recorded (87). In addition to α-GalCer, iNKT cells are activated by a number of exogenous glycolipids. These lipids, that can directly stimulate iNKT cells to produce cytokines, include lipids found in the cell wall of the gram-negative bacteria *Sphingomonas* (53, 54, 88, 89), *Borrelia burgdorferi* as well as phospatidylinositolmannoside (PIM4) from the mycobacterial cell wall (55, 90) (Figure 3).

iNKT cells are not only activated by foreign lipids. Recently, β-GlcCer was shown to be an endogenous antigen for iNKT cells (91). Importantly, this antigen, which is the precursor of most glycosphingolipids in the eukaryotic cell, is present in the thymus as well as in peripheral lymphoid tissues where iNKT cells are present, suggesting that it plays a physiological role. β-GlcCer accumulated in APCs after stimulation with LPS or bacteria, and iNKT cell autoreactivity decreased after pharmacological inhibition of β-GlcCer production (91) indicating its role in infection and regulation of iNKT cell activity. Some microbes, such as *Salmonella*, do not produce lipid antigens that can activate iNKT cells. In this case iNKT cell activation is dependent on APC activation by toll-like receptor (TLR) signalling and IL-12 release by the APC that, together with presentation of endogenous ligand enhances the ability of the DC to stimulate iNKT cells (54, 92). Additionally, a role for IL-18 in activation of iNKT cells has been shown in *M. tuberculosis* infection as well as in atopic eczema (93, 94). Even a role of IL-12 and IL-18 in iNKT activation without TCR engagement has been demonstrated (95) (Figure 3).

Another possible way to trigger iNKT cells is via NK receptor cross-linking. Human iNKT express various NK cell receptors such as CD161, NKG2D, CD94 and DNAM-1 (96). Activation of mouse iNKT via NK1.1 (97) and human iNKT via the activating receptor NKG2D (96) has been demonstrated and will be discussed in further detail in the results and discussion section.

3.4 iNKT CELL FUNCTIONS

The ability to rapidly produce large amounts of cytokines and chemokines upon activation is an important characteristic of iNKT cells (98-100) (Figure 3). iNKT cells contain cytokine transcripts and can therefore upon stimulation respond very fast, and

in turn activate and regulate several other immune cells including DCs, NK cells, T and B cells (101-103). Most of the studies assessing iNKT cell cytokine profiles are limited and focus on interferon-γ (IFN-γ) and interleukin-4 (IL-4) and sometimes tumor necrosis factor (TNF) (104), but some studies also include GM-CSF, IL-2, IL-5, IL-6, IL-10 and IL-13 as well as the chemokines RANTES, Eotaxin, MIP-1α and MIP-1β (105, 106). Recently characterized subsets of mouse iNKT cells are the NK1.1 - and ROR-γt dependent IL-17 producing iNKT subset (107, 108) as well as iNKT cells that produce IL-21 and IL-22 (109, 110).

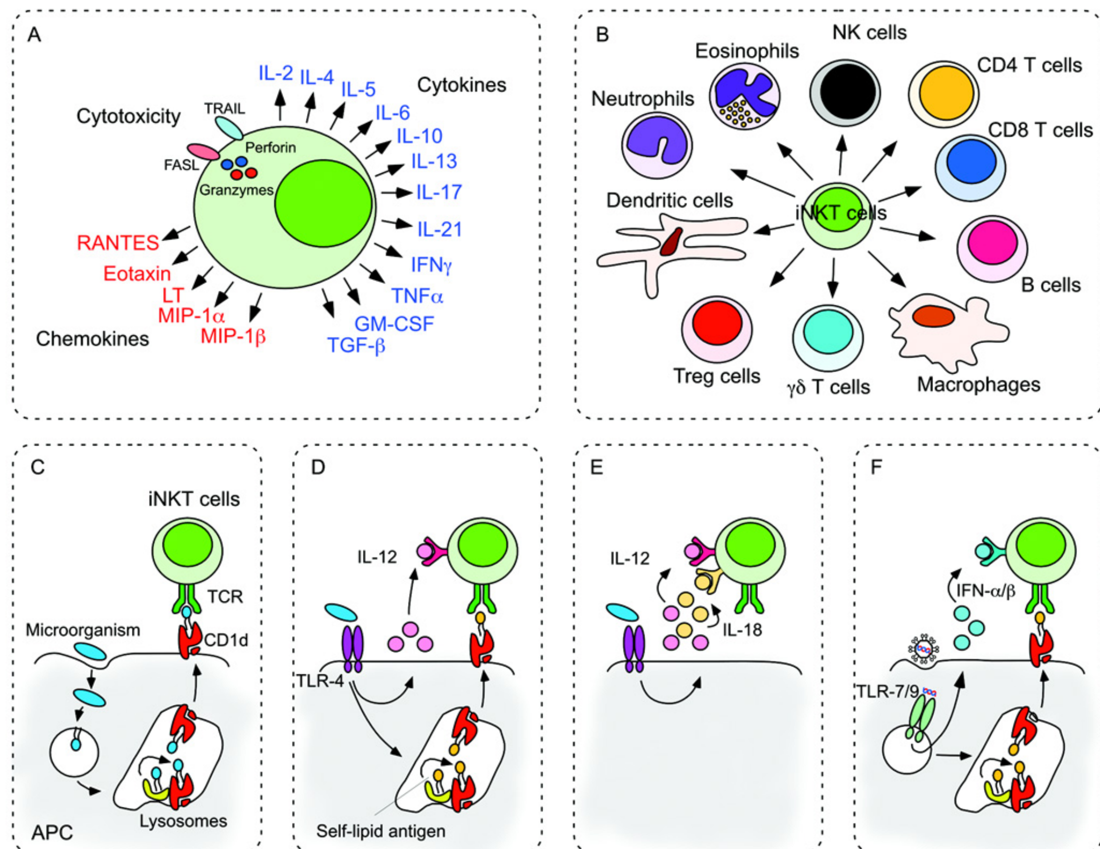

Figure 3. Activation of iNKT cells and their functions. *A. Activated iNKT cells release various cytokines and chemokines and exert a potent killing activity. B. Activated iNKT cells have an influence on many other cell types. C- F. Different pathways of iNKT cell activation including CD1d-dependent activation by foreign antigen (C), TLR4-dependent stimulation of DCs leading to endogenous antigen presentation and IL-12 stimulation (D), activation through IL-12 and IL-18 (E) and TLR7/9 dependent stimulation of DCs leading to endogenous antigen presentation and IFN-γ stimulation (105).*

iNKT cells are divided into different subsets depending on their expression of CD4 and their ability to produce either T_H1 and/or T_H2 cytokines upon activation. In mice, iNKT cells are either CD4-CD8- double negative (DN) or CD4+. In humans, CD4+, DN, and CD8+ iNKT cell subsets all exist (111). While the CD4+ subset expresses relatively high levels of CD62L and may exhibit a lymph-node homing phenotype, CD4- iNKT cells express low levels of CD62L and high levels of CD11a and may therefore be more of a tissue-infiltrating subset (112). All these subsets do respond to the same

ligands, but with different outcomes. When stimulated with α-GalCer, human CD4+ iNKT cells produce both T_H1 and T_H2 cytokines while the CD4- iNKT cell subset produces mainly T_H1 cytokines and has an effector profile (113, 114). Not only expression of CD4 but also CD28 and the programmed death 1 (PD-1) receptor can have an influence on the cytokine polarization of iNKT cells (115). Compared to the present knowledge about iNKT cytokine production, less is known about the mechanisms of cytotoxicity. iNKT cells produce both perforin and granzyme (116) (Figure 3), and there is evidence demonstrating a correlation between cytotoxicity and CD1d expression levels on the target cells and with the potency of the iNKT cell antigen (117). α-GalCer (116, 118, 119) and NK cell receptor triggering (96) can also generate iNKT cell cytolytic responses. Additionally, iNKT cells are able to kill target cells through FasL (Fas ligand)-mediated pathways (120). Since FasL is mainly expressed by CD4+ iNKT cells this way of killing might be preferentially used by this subset (121).

3.5 iNKT CELLS IN DISEASE

iNKT cells have been implicated in immune responses against microbes, tumors and in regulating autoimmune and inflammatory diseases (57, 99). Tools such as the model antigen α-GalCer, CD1d tetramers to reliably identify and quantify iNKT cells (122, 123), and iNKT cell deficient mice (65, 76, 124) have been instrumental in gaining insight into the role of iNKT cells in infectious and non-infectious diseases.

3.5.1 iNKT cells in viral infections

Both human and mouse studies have increased our knowledge about the role of iNKT cells in viral infection. Despite that viruses do not express glycolipids, iNKT cells are involved in viral infections and anti-viral responses. By using iNKT cell deficient mice, there has been progress in understanding the role iNKT cells might play in viral diseases though some results are conflicting. In a herpes simplex virus type 1 (HSV-1) skin model, CD1d -/- mice were more susceptible to infection and similar results were seen in Jα18 -/- mice (125). However, another study did not see any involvement of iNKT cells in HSV-1 infection (126) but that could be due to that different viral strains where used. Additionally, CD1d/iNKT cell deficient mice infected with respiratory syncytical virus or Influenza A virus (IAV) showed a worse disease than wild-type mice (127, 128). In IAV infection, the absence of iNKT cells resulted in higher number of myeloid suppressor cells that inhibited IAV-specific immune responses and resulted in increased virus titer and mortality. The IAV-specific immune responses were restored after adoptive transfer of iNKT cells (128). iNKT cells have also been studied in murine cytomegalovirus (MCMV) infection, which is widely used as an experimental model for human CMV (HCMV). In MCMV infection, DCs are activated via TLR9 to produce IL-12 that in turn stimulates the iNKT cell IFN-γ production. Interestingly, the involvement of CD1d in iNKT cell activation in this context seemed to be minor (129).
In humans, only a few studies show a correlation between iNKT cell deficiency and infectious disease. iNKT cells have been suggested to participate in the host defence

against varicella zoster virus (VZV). Two independent case reports described two cases of uncommon responses against the live attenuated varicella vaccine, which is considered as a safe vaccine (130, 131). These two patients, that got severe, life-threatening symptoms from the vaccine, were iNKT cell deficient and in one patient this was combined with deficient expression of CD1d. In addition, children with mutations in the gene encoding the signalling protein SAP that is required for iNKT development and therefore lack iNKT cells, suffered from more Epstein-Barr virus (EBV) infections (132). This suggests a potential role of iNKT cells in the immune response against VZV and EBV.

In HIV-1 infected humans iNKT cell numbers are reduced or depleted (112, 133, 134). This has also been demonstrated in SIV infected macaques (135). Due to the fact that CD4+ iNKT cells express both the primary receptor and the co-receptor for HIV-1 entry, the CD4+ subset is preferentially depleted (112). Compared to conventional T cells, R5-tropic HIV-1 viruses replicate better in iNKT cells and this might contribute to their rapid loss. In some HIV-1 infected individuals the iNKT cell numbers stay normal but most of these iNKT cells are functionally impaired. In these individuals, both CD4- and CD4+ subsets have reduced capacity to produce cytokines (136-138) and have an up-regulated PD-1 receptor expression (137), in line with an exhausted phenotype. Highly active antiretroviral therapy (HAART) may contribute to the recovery of predominantly CD4- iNKT cells (139), although this could be explained by their redistribution from earlier tissue sequestration. In simian immunodeficiency virus (SIV)-infected Asian macaques, a monkey species that is susceptible to SIV infection and therefore is used as an HIV-1 infection model, a reduction in iNKT cell-activation induced cytokine production has been observed. These monkeys show a decline in CD4+ iNKT cell numbers and an association between loss of CD4+ iNKT cells and increased immune activation. In contrast, the function and number of iNKT cells from Soothy mangabeys, that are natural hosts of SIV and AIDS-resistant, remain intact (140). The difference in iNKT cell function and activation in non-natural and natural hosts could be one factor contributing to the differential susceptibility to AIDS. Similar to HIV-1, some studies report lower levels of iNKT cells in Hepatitis C virus (HCV) infected individuals compared to healthy controls (141, 142). This has been debated though, since some studies show no change in the iNKT compartment in HCV infection (143, 144).

3.5.2 Viral interference with CD1d expression

Viruses have evolved mechanisms to escape from iNKT cell responses by down-regulating CD1d from the cell surface. In mice, vaccinia virus, lymphocytic choriomeningitis virus and vesicular stomititis virus (VSV) where all reported to down-regulate CD1d from the surface of infected cells (145, 146) by mechanisms that at least for some of them involved changes in the intracellular trafficking of CD1d.

In humans, a number of otherwise unrelated viruses have been identified to evade iNKT cell responses by inhibiting CD1d expression. Although these viruses share this immune evasion strategy the molecular mechanisms resulting in CD1d down-

regulation differ (reviewed in 58, 106). HIV-1 interferes with CD1d surface expression by mechanisms that involve at least three viral genes. Both the accessory genes *negative factor* (*nef*) and *viral protein u* (*vpu*) contribute to this action, that involves inhibition of CD1d internalization and retaining CD1d in the TGN (147, 148) and inhibition of CD1d recycling back to the cell surface (149) as described in detail in the results and discussion section of this thesis. Another HIV-1 protein, the envelope glycoprotein gp120, has also been described to interfere with CD1d surface expression (150). Not only HIV-1, but also HSV-1 (151, 152), Kaposi Sarcoma- associated herpes virus (KSHV) (153) and human papillomavirus (HPV) (154) interfere with CD1d expression. The mechanisms these viruses use are often complex. HSV-1 glycoprotein B interacts with CD1d already in the ER and together with the viral kinase US3 accumulates CD1d in the TGN (151, 152). This results in lower CD1d surface expression levels and contributes to a decrease in iNKT activation. However, one study reports that HSV infection of keratinocytes does not induce CD1d down-regulation but inhibited iNKT cell cytokine production through a cell-to-cell contact dependent mechanism (155). Similar to HIV-1 Nef, the KSHV protein MIR2 enhances CD1d endocytosis thereby reducing CD1d-mediated iNKT cell activation (153). The HPV E5 protein targets CD1d *in vitro* and *in vivo* by both interacting with calnexin to inhibit CD1d assembly and directing CD1d for proteosomal degradation (154). Despite extensive research on viral mechanisms to escape from iNKT cell recognition, no virally encoded ligands that bind any of the CD1 proteins have been discovered so far. However, CD1c has the ability to present a lipopeptide with a peptide sequence similar to HIV-1 Nef (156) and this supports the hypothesis that lipidated viral proteins may function as CD1 ligands.

3.5.3 iNKT cells in bacterial and parasitic infections

Studies based on iNKT/CD1d deficient mouse models have supported the importance of iNKT cells in the immune defence against many bacterial infections. Several bacterial glycolipid ligands have been found to activate iNKT cells (53, 54). iNKT cells play a role in *Sphingonomas* infections, as well as in the tickborn infections *Ehrlichia muris* and *Rickettsia,* with increased mortality in iNKT cell deficient mice (53, 54). CD1d -/- mice infected with *Borrelia burgdorferi,* the causative agent of Lyme disease, developed a worse disease profile than control mice (157). In *Salmonella* infection, the bacteria activate DCs to produce IL-12 via LPS mediated TLR4 stimulation and the IL-12 together with presentation of endogenous lipid antigen activates iNKT cells (54, 92). In *Salmonella* infection, LPS is also able to induce iNKT cell activation without the need for CD1d, but instead through exposure to IL-12 and IL-18 only (158).

Also parasites have been studied in iNKT/CD1d deficient mouse models. *Leishmania donovani* surface antigens bind to CD1d and are recognized by iNKT cells. Infection in CD1d -/- mice leads to significantly higher parasite levels in liver and spleen, and a defective anti-parasite response compared to wild-type mice (159). Similar to *L. donovani* infection, *L. major* infection in iNKT cell deficient mice leads to an increase in parasite numbers as well as a decrease in NK cell activity and IFN-γ production by iNKT cells (160, 161).

3.5.4 iNKT cells in inflammatory and autoimmune diseases

iNKT cells have been implicated in several autoimmune diseases in mouse and human. One of them is asthma, a disease characterized by a T_H2 immune response, inflammation and airway hyper-reactivity (162). In experimental models, iNKT cells seem to play a role in the development of disease, since iNKT cell deficient mice show a defect in the development of airway hyper-reactivity and airway inflammation (163). In patients suffering from asthma iNKT cells numbers are increased (164, 165) and since iNKT cells can become activated by several environmental substances such as dust particles they might play an important role in airway hyper-reactivity and inflammation (166-168). Similar to asthma, the relationship between iNKT cells and type 1 diabetes (T1D) is controversial. T1D is caused by T cell-mediated destruction of insulin-producing pancreatic beta cells and both CD4+ and CD8+ T cells are involved in the pathogenesis of T1D (169). In diabetic mice and humans it has been reported that iNKT cell frequencies are lower than in healthy controls and cytokine production is decreased or altered (170-172). Other studies have disagreed with these findings, and report normal iNKT cell numbers in T1D patients (69, 173). The conflicting results could be explained by genetic and environmental factors as well as the age of the patients and the stage of the disease. In multiple sclerosis (MS) patients iNKT cell numbers are decreased and the cytokine production is defective (174, 175). The reason for the iNKT cell deficiency is not known and moreover it is unknown if this defect promotes the development of MS or is a consequence of the disease.

3.5.5 iNKT cells in cancer

iNKT cells can have a protective role against tumours either by recognizing tumour cells directly, or through an indirect mechanism by activating other cells, like NK cells, that can kill the cancer cells. Studies have shown that some leukaemia cells that express CD1d are sensitive to lysis by human iNKT cells (176). In mice, iNKT cells have a protective role against tumour growth and this effect is dependent on CD1d (177). iNKT cells can also have an effect against CD1d- tumours by killing of tumour-associated CD1d+ macrophages that stimulate tumour growth (178). The role for the activating receptor NKG2D in iNKT cell recognition of tumour cells will be discussed in the results and discussion part.

Studies in mice suggest that iNKT cells have a crucial role in tumor immune-surveillance. A protective role has been demonstrated in different tumor models including metylcholanthrene-induced fibrosarcoma (179), p53 deficiency (180) and prostate cancer (181) as observed using Jα18 -/- mice, CD1d -/- mice and wild-type mice. IFN-γ production is important for at least some of the anti-tumor effects, as it stimulates NK cell activation (182). In humans, iNKT cells numbers are decreased and function is impaired in patients suffering from cancers such as melanoma, colon, lung and breast cancer. Similar to the studies in mice, relatively high levels of iNKT cells may be associated with a good cancer prognosis (183-187). The CD4- subset of iNKT cells seems to have the main role in tumor surveillance in mice and there are indications that this subset has a similar role in humans (102, 188).

4 HIV-1

HIV-1 is a member of the genus *Lentivirus*, which is a part of the *Retroviridae* family, and is the causative agent of the acquired immune deficiency syndrome (AIDS). AIDS was discovered in homosexual American men in 1981 as a disease characterized by severe immune deficiency, opportunistic infections and rare cancers (189). Two years later, HIV-1 was discovered as the virus causing AIDS (190-192) and it became quickly clear that this virus not only affected homosexual men but also other groups in society, and that the virus was able to spread through sexual contact, via blood or blood products as well as through mother-to-child transmission (193). Since then, more than 60 million people have been infected with HIV-1 and approximately 34 million individuals are living with the infection today (194). A few years later, a similar virus, HIV-2, was detected in West African patients suffering from AIDS (195). HIV-1 and HIV-2 bear 30-60% homology at the genetic level and they share many biological and virological features. While HIV-1 has spread globally, HIV-2 is mainly found in West Africa (196). HIV-1 is more pathogenic than HIV-2, the transmission rate is higher and it accounts for most of the HIV infections in the world (196, 197).

HIV-1 and HIV-2 originate from cross-species transmission of simian immunodeficiency virus (SIV) from African primates to humans in West and Central Africa sometime between 1853 and the early 1900s, most probably when people were hunting monkeys for bushmeat or kept them as pets (198-200). The closest relatives to HIV-1 are the SIVs from chimpanzees (SIVcpz) and gorillas (SIVgor), while HIV-2 is most closely related to the SIV from sooty mangabeys (SIVsmm) (201-203). Since the discovery of HIV-1, the virus has been classified into different groups and subtypes, with their differences mainly located in the envelope genes (197). It is believed that several independent transmissions of SIV to humans have led to the generation of the different HIV-1 groups. HIV-1 is today divided into group M (main), N (non-M, non-O), O (outlier) and P. Group M, the oldest HIV-1 group, is further categorized into nine subtypes and numerous circulating recombinant forms (CRF) (204), and is due to its high replication and transmission fitness responsible for the global AIDS epidemic (205). Group M subtype C viruses are the most prevalent HIVs today (206). Due to the lack of proof-reading activity in its reverse transcriptase (RT) and the consequently high error-rate during reverse transcription, the genetic variation between the existing HIV-1 groups and subtypes ranges usually between 17% and 35% (207, 208). Moreover, there is a huge genetic variability within each infected individual. The high mutation (209, 210) and replication rate of HIV-1 in combination with the selection pressure by the host immune system contribute to a genetically diverse viral population that can differ up to 10% in each infected individual (207). Interestingly, it is believed that heterosexual transmission may be mediated by a single virus particle and thus represents a genetic bottleneck (211). The intact genital tract mucosal barrier, if it is not disturbed by infections or hormonal contraceptives, sharply limits the number of variants transmitted during the transmission event (212).

4.1 THE LIFE CYCLE OF HIV-1

The life cycle of HIV-1 can be divided into two phases: the early phase that includes binding, entry and integration, and the late phase that begins with expression of viral genes and ends with the release of progeny virus (Table 2). In order to enter a cell, HIV-1 uses receptor-mediated fusion at the plasma membrane. The envelope glycoprotein gp120 (Env) that is present on the surface of viral particles and joined together with gp41, binds the primary receptor CD4. This leads to conformational changes in CD4 and gp120 (213). The interaction between gp120 and CD4 exposes coreceptor-binding sites that, depending on the viral tropism determined by the V3 loop of gp120, are engaged with the coreceptors CCR5 or CXCR4 (214). This leads to another conformational change and dissociation of gp120 and gp41 (215). HIV-1 is also able to bind C-type mannose binding lectins, such as DC-SIGN expressed on DCs, but this type of binding does not directly lead to productive infection (216). After dissociation of gp120 and gp41, gp41 is incorporated into the target cell membrane, allowing fusion of the viral envelope with the cell membrane and subsequently release of the viral core into the cytoplasm (217). Once in the cytoplasm, the viral RNA is transcribed into DNA by the virus-packaged RT (218) and a provirus is formed by integration of the viral DNA into the host cell genome. The integrated provirus can either undergo transcription and translation to become an infectious virion or remain in a latent state (219). In the cytoplasm, mRNA serves as template for translation of the various viral proteins and all components are then directed to the site of assembly (220). Gag is the protein that drives the assembly that occurs at the plasma membrane (221). At this site, HIV-1 incorporates the proteins as well as two copies of its RNA genome into the virion that buds from the cell membrane (reviewed in 222)

Gene	Protein	Function
Gag	Matrix/p17	Structural protein
	Capsid/p24	Structural protein
	Nucleocapsid/p7	Protects viral RNA
	P6	Viral assembly
Pol	Reverse transcriptase/p66, p51	Reverse transcription
	Protease/p10	Processing of viral proteins after translation
	Integrase/p32	Integration of viral DNA into host DNA
Env	Envelope surface protein/gp120	Binding of receptors on target cells
	Envelope transmembrane protein/pg41	Penetration of cell membrane
Rev	Rev/p19	Regulates viral mRNA processing
Tat	Tat/p14	Transcription factor
Vif	Vif/p23	Antagonizing the restriction factor APOBEC3G
Vpr	Vpr/p15	Transportation of viral genome to nucleus
Vpu	Vpu/p16	CD4 down-regulation, promotion of viral particle release, CD1d down-regulation
Nef	Nef/p27	CD4 down-regulation, protection from CTL and NK cell recognition

Table 2: The HIV-1 genes and the protein functions *(149, 223-225).*

4.2 HIV-1 INFECTION

Most HIV-1 transmissions occur via sexual intercourse where the virions interact with epithelial cells in the genital or intestinal tract and traverse the epithelium by infecting CD4+ cells, through endocytosis or penetration through gaps between the cells (226). Once the epithelial barrier is crossed, HIV-1 is ready to infect DCs and CD4+ T cells for permanent establishment of the infection (227). Sexual intercourse often leads to microabrasions in the mucosal surface that facilitates viral entry and allows the virions access to DCs, T cells and macrophages that are present in the basal epithelium and underlying stroma (228). During the first days of infection, innate responses to viral exposure can inhibit or at least limit viral replication. However, these responses can also contribute to an inflammatory milieu in the mucosa that will likely attract CD4+ T cells, the main target cells of HIV-1, triggering viral replication and spread. Pre-existing inflammation within the genital mucosa due to other sexually transmitted diseases is common. Therefore, there is often a local accumulation of DCs and activated CD4+ T cells at the site of transmission supporting viral replication and establishment of infection (229, 230). Once infection is established, HIV-1 is able to spread rapidly and eliminate the majority of CCR5+CD4+ T cells, mainly the cells in the gastro intestinal (GI) tract, within a few weeks (231).

DCs are important for establishment of infection and efficient spread of the virus. Due to their ability to bind and internalize the virus and transfer it to T-cell dense parts of lymph nodes, HIV-1 abuses them as carriers for establishment of infection (232). HIV-1 can be stored as integrated provirus following infection of the DC, in multivesicular bodies following endocytosis, or as surface bound virions (228). In addition to infecting the cell via CD4 binding, HIV-1 is also able to bind C-type lectins such as DC-SIGN expressed on DCs. This type of binding allows the virus to use the DC as a Trojan horse for spread (216). Before DCs carrying HIV-1 reach lymph nodes and form infectious synapses with CD4+ T cells, which facilitates viral passage from DC to T cells, no HIV-1 RNA is detectable in the blood (228). At this stage, replication and plasma viremia are rapidly increasing and the virus is able to spread to other lymphoid tissues. A particular target site is the gut-associated lymphoid tissue (GALT) where the vast majority of the CD4+ T cells are lost after direct infection or through activation induced apoptosis (233, 234). During the first weeks after transmission, the virus blood levels reach a peak level and the CD4+ T cell numbers are often very low. The ensuing adaptive immune response controls the viremia and a viral load set-point level is established. As the infection proceeds to the chronic phase, the chronic immune activation is a central characteristic. Persistent immune activation involves increased T cell activation and turnover, immune exhaustion, B cell activation and elevated levels of pro-inflammatory cytokines and chemokines. Other characteristics are microbial product translocation across mucosal barriers into the circulation, loss of T regulatory cells (T_{regs}) and mycobacterial co-infection. Immune activation is thought to support HIV replication, since it replicates well in activated CD4+ T cells (235).

4.3 HIV-1 ACCESSORY GENES

In addition to Gag, Pol and Env that are contained in all retroviral genomes, HIV-1 has six additional genes. Two of them encode essential regulatory proteins (Tat and Rev) and the remaining four encode so-called accessory proteins (virion infectivity factor (Vif), viral protein R (Vpr), Vpu and Nef) that are not absolutely required for viral replication. The HIV-1 accessory proteins interact with numerous cellular factors and thereby allow the virus to replicate and spread in the presence of strong innate and adaptive immune responses.

4.3.1 Vif and Vpr

Vif is a 23 kDa protein that targets the anti-viral factor APOBEC3G by linking it to a cullin 5-based E3 ubiquitin ligase complex that induces poly-ubiquitination and subsequent proteosomal degradation (236, 237). If Vif is not present, APOBEC3G is incorporated into the virions, inhibits viral DNA synthesis and induces G-to-A hypermutations to inactivate the virus (238). The 14 kDa Vpr protein has been reported to have many activities such as arresting the cell-cycle in the G2 phase, inducing cell death and activating proviral transcription (239, 240). Although Vpr is not required for viral replication it is conserved among all primate lentiviruses and is therefore suggested to have an important role in SIV and HIV infection.

4.3.2 Nef

Nef is a 27 kDa protein produced early during the viral replication cycle. Nef is essential for maintenance of high viral loads and disease progression in HIV-1 infected humans and SIV infected monkeys (241, 242). Nef has multiple activities and interferes with cellular trafficking and surface expression of proteins, antigen presentation and signal transduction. The best-characterized targets of Nef are MHC class I and CD4, but Nef also down-regulates a number of other cell surface proteins important in host immune function. Selective internalization of the MHC class I alleles human leukocyte antigen (HLA) -A and -B but not HLA-C and –E induced by a cooperative binding of Nef, the cytoplasmic domain of the respective MHC class I molecules and the adaptor protein AP-1 inhibits lysis of infected cells by cytotoxic T lymphocytes (CTLs) and NK cells (243, 244). Down-regulation of the HIV-1 entry receptors CD4, CCR5 and CXCR4 contributes to protection against super-infection of cells, which may otherwise lead to death of the infected cells and overall lower production of new virus (225, 245, 246). Impairment of antigen presentation and the subsequent activation of T cells is an important immune evasion mechanism. In addition to MHC-I down-regulation, that will inhibit peptide antigen presentation, Nef impairs T cell activation also by down-modulating the costimulatory molecules CD28, CD80 and CD86 (247, 248). Moreover, Nef also impairs lipid antigen presentation by interfering with the surface expression of CD1d. Mechanistically, Nef induces both retention of CD1d in the TGN and an increase in the internalization of CD1d from the cell surface (147, 148).

4.3.3 Vpu

Vpu is a 16 kDa integral membrane protein that is produced during the late stage of the viral life cycle and has an important role in viral virulence (249). Vpu consists of two major domains (Figure 4): a transmembrane domain and a cytoplasmic domain than contains two serine residues that are constitutively phosphorylated by casein kinase II (250, 251). Vpu exist in HIV-1 and SIVcpz, gsn, mon, mus, gor, den, but not in other SIV strains or HIV-2 (252, 253). The importance of Vpu for viral spread and pathogenesis has been demonstrated in many studies, for example in monkeys infected with SIV-HIV chimeras lacking expression of the *vpu* gene or with mutated *Vpu* sequences. Monkeys infected with these types of mutant viruses had lower virus levels as compared to monkeys infected with viruses with functional Vpu expression. In addition, animals infected with Vpu-deficient virus had little or no loss of CD4+ T lymphocytes, demonstrating the importance of Vpu in viral release and immune evasion (254-256).

The activities of Vpu that have been most extensively investigated are CD4 degradation and enhancement of virus particle release by antagonizing the host cell restriction factor Tetherin. Together with Env, Vpu reduces CD4 levels at the cell surface, a mechanism that is dependent on phosphorylation of Ser52 and Ser56 in the Vpu protein (257). Vpu physically interacts with the cytoplasmic tail of CD4 in the ER (258, 259) and with the SCF-E3 ubiqutin ligase complex subunit β-TrCP. This interaction leads to ubiquitination of CD4, export from the ER and subsequent proteosomal degradation. Interestingly, Vpu itself is not degraded in this process (260). Importantly, Vpu-mediated degradation of CD4 destabilizes CD4–Env complexes in the ER, allowing Env to mature and to be incorporated into progeny virions (261).

In addition to its effect on CD4, it has been reported that Vpu promotes viral release from HIV-1 infected cells (262, 263). In 2008, two groups independently reported identification of the interferon-inducible host restriction factor BST2/Tetherin (CD317) as the protein involved in viral release restriction and antagonized by Vpu (264, 265). The mechanisms underlying this function are not yet completely resolved, but the suggested molecular pathways include blocking of the traffic of newly synthesized Tetherin to the cell surface (266, 267), inhibition of recycling of Tetherin (268) or increasing the internalization of Tetherin (269). In addition to these three potential mechanisms, Tetherin has been suggested to undergo Vpu-mediated proteososmal (270) or lysosomal degradation (269, 271), but intracellular sequestration has also been suggested (272, 273).

Besides its important role in down-regulation of CD4 and Tetherin, Vpu interferes with the cellular expression of a number of other proteins. Interestingly, recent studies have demonstrated Vpu interference with NK cell activation (274). One study demonstrated that Vpu down-regulates the co-activating NK-T-B antigen (NTB-A) protein, a member of the SLAM receptor family (275, 276). Down-regulation of NTB-A from HIV-1 infected T cells inhibited lysis of infected cells by NK cells. Mechanistically, this does not involve proteosomal degradation but altered intracellular trafficking by an

interaction with the Vpu transmembrane region (275). Vpu does also, together with Nef, down-regulate the DNAM-1 ligand Poliovirus receptor (PVR), probably to inhibit NK cell mediated lysis of infected cells (277). Other Vpu-interacting proteins include the transcription factor interferon regulatory factor (IRF) 3 (278, 279), MHC class I (280), the calcium-modulating cyclophilin ligand (CAML) (281) as well as MHC class II Invariant chain/CD74 (282). Furthermore, as shown in this thesis, Vpu interferes with CD1d surface expression and activation of CD1d-resticted iNKT cells (**Paper I,** 149). This will be discussed in detail in the results and discussion section.

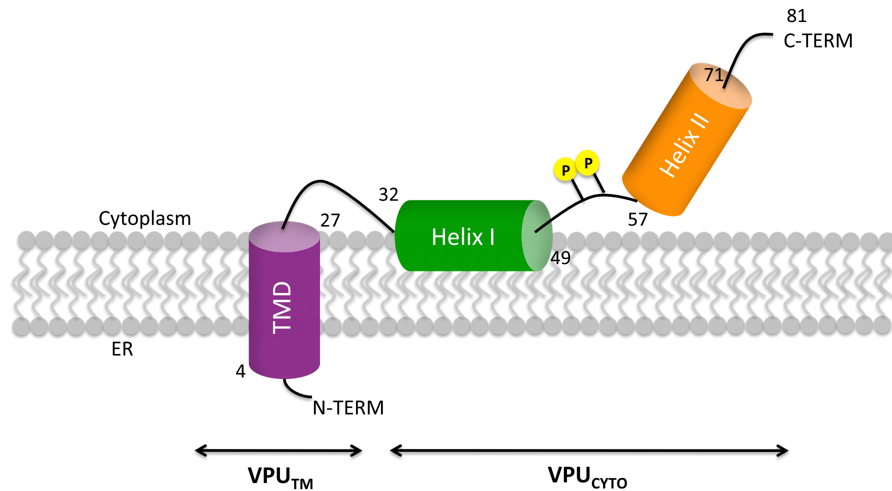

***Figure 4**: **The HIV-1 Vpu protein.** Schematic representation of HIV-1 Vpu with its transmembrane domain (TM) and cytoplasmic domain consisting of two α-helixes and a flexible loop containing two phosphorylation sites.*

5 AIMS OF THIS THESIS

Paper I: To study the effect of DC HIV-1 infection on CD1d expression and iNKT cell activation.

Paper II: To study molecular and structural requirements for HIV-1 Vpu-mediated inhibition of CD1d.

Paper III: To develop methodology to study iNKT cell activation by rare HIV-1 infected DCs in a mixed culture.

Paper IV: To study the role of NKG2D in iNKT cell activation.

6 RESULTS AND DISCUSSION

6.1 HIV-1 VPU INTERFERENCE WITH CD1d

To establish chronic infection it is essential for HIV-1 to evade innate and adaptive immune responses early after transmission. One important strategy is to avoid elimination of infected cells by T cells and NK cells by interfering with the functional expression of HLA-A and B (283), MHC II (284) and CD1d (147-149). Besides its structural proteins, HIV-1 expresses two regulatory and four accessory proteins. The latter are not absolutely required for viral replication but are known to interact with cellular proteins such as retroviral restriction factors to facilitate viral spread and pathogenesis. The HIV-1 accessory proteins Nef and Vpu are known for their ability to interfere with different host proteins and their important contribution to viral immune evasion.

6.1.1 Down-regulation of CD1d by HIV-1 Vpu

HIV-1 does not only interfere with peptide antigen presentation by down-regulating MHC proteins, but also with the presentation of lipid antigens. This has been shown by several groups that have demonstrated Nef-mediated inhibition of CD1d and CD1a surface expression (147, 148, 285) and CD1d down-regulation mediated by HIV-1 gp120 (150). In **paper nr I** in this thesis, we identify CD1d down-regulation as a novel function of HIV-1 Vpu. Initially, we demonstrated HIV-1 mediated inhibition of CD1d surface expression in productively infected DCs (Figure 1, **paper I**). This activity was dependent on HIV-1 Vpu, which we could demonstrate in both Vpu-EGFP transfected CD1d-expressing 293T cells (Figure 2A-C, **paper I**) and DCs infected with a Vpu-deficient virus mutant (Figure 5). DCs infected with the Vpu-deficient virus had a partially reduced capacity to down-regulate CD1d as compared to DCs infected with the parental virus. A similar partial effect was seen with a Nef-deficient HIV-1 mutant confirming and extending previously published results demonstrating Nef-dependent CD1d down-regulation in cell lines (147, 148). When DCs were infected with a Nef- and Vpu double-defective virus down-regulation was completely abolished demonstrating that HIV-1 mediated CD1d inhibition requires the activity of both Nef and Vpu (Figure 5). Interestingly, the co-operative activity of Vpu and Nef has not only been demonstrated in the context of CD1d but also in down-regulation of the NK cell activating ligand PVR (277) where maximum PVR reduction is dependent on the activity of both proteins.

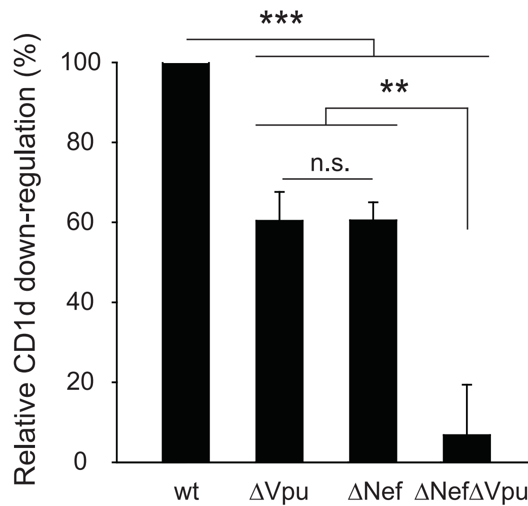

Figure 5*: Down-regulation of CD1d is mediated by HIV-1 Vpu and Nef**. *CD1d down-regulation 4 days after infection in human monocyte-derived DCs infected with HIV-1 81Awt or mutants lacking the expression of Vpu (ΔVpu), Nef (ΔNef) or both (ΔNefΔVpu). CD1d down-regulation was set as 100%. **P<.01;P<.001. Adapted from* ***paper I***.

6.1.2 Mechanisms of Vpu-mediated CD1d down-regulation

As described earlier, one of the mechanisms Vpu utilizes to inhibit host protein expression is to subject these proteins to proteosomal or lysosomal degradation. CD4, Tetherin, and the interferon regulatory factor IRF3 are down-regulated by Vpu-mediated proteasomal or lysosomal degradation (223, 279, 286). In addition, Vpu has been described to down-regulate proteins, including NTB-A and PVR, with so far poorly defined mechanisms that do not involve enhanced degradation (Table 3).

6.1.2.1 Differences between Vpu-mediated down-regulation of CD1d and CD4

In **papers nr I** and **II**, we studied the mechanisms underlying Vpu-mediated down-regulation of CD1d. Surface protein expression can be modulated by accelerating the internalization rate and this mechanisms has been demonstrated for Nef-mediated down-regulation of MHC class I and CD1d (147, 243, 244). Moreover, also the Karposi-sarcoma associated herpes virus interferes with CD1d endocytosis (153). **In paper nr I** we compared the rate of CD1d internalization in Vpu+ and Vpu- 293T cells and found that CD1d endocytosis rates were similar indicating that Vpu employs a different mechanism to down-regulate CD1d (Figure 4B, **paper I**). Interestingly, we could demonstrate a decreased rate of CD1d recycling in cells that expressed Vpu suggesting that Vpu interacts with CD1d in the endosomal system and thereby inhibits its recycling (Figure 4C and D, **paper I**). Inhibition of recycling has also been demonstrated in HSV-1 infection, where the virus interferes with CD1d by redistributing endocytosed CD1d to the lysosome (152). We hypothesize that while Nef increases the rate of CD1d internalization into endosomal compartments (147), Vpu traps CD1d molecules after internalization and inhibits their recycling back to the cell surface (Figure 6). Co-localization studies using confocal microscopy demonstrated a close interaction of Vpu and CD1d in early endosomal compartments (Figure 5A and B, **paper I**). Since co-localization does not give a clear answer to whether the proteins

physically interact or not, we performed co-immunoprecipitation assays. The results from these assays demonstrated interaction between CD1d and Vpu (Figure 5C, **paper I**). Further investigations are needed to study if there is a direct interaction between the proteins or if a third protein is involved.

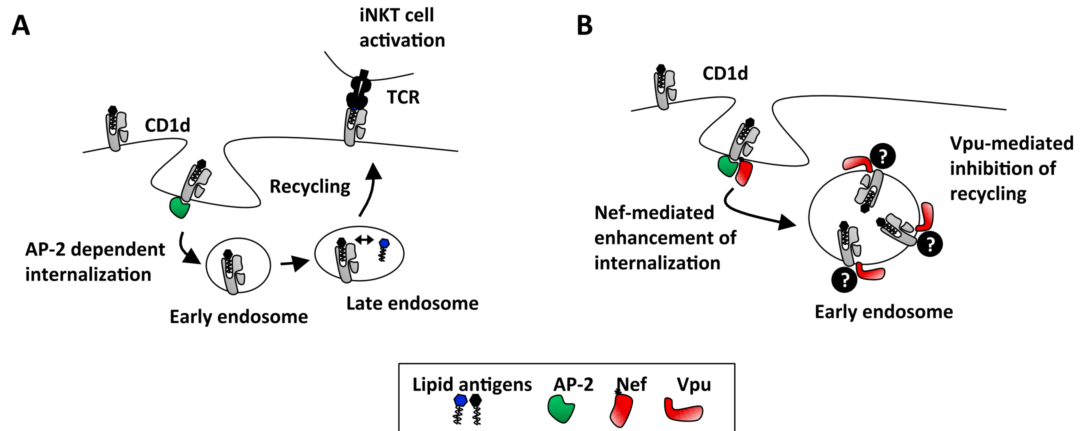

Figure 6: HIV-1 Vpu and Nef interfere with CD1d-mediated antigen presentation. *A. Normal AP-2 dependent internalization and recycling of CD1d and antigen presentation to iNKT cells. B. In HIV-1 infected cells, Nef enhances CD1d internalization and Vpu inhibits CD1d recycling back to the cell surface. Possible co-factors in the Vpu-mediated inhibition of recycling remain to be identified. Adapted from (274).*

In **paper nr II** we investigated further molecular and structural details of Vpu-mediated CD1d down-regulation. Vpu-mediated down-regulation of CD4 requires phosphorylation of Vpu at two serine residues in the cytoplasmic domain (257). Vpu variants mutated at these specific sites are unable to interact with β-TrCP and cannot mediate ubiquitination of target proteins (260). By creating a phosphorylation defective mutant (VpuΔP), we tested if down-regulation of CD1d was dependent on Vpu phosphorylation. Interestingly, down-regulation of CD1d was significantly reduced in cells expressing VpuΔP as compared to wild-type, but CD1d expression levels were still significantly higher as compared to cells transfected with a control plasmid (Figure 2A, **paper II**). This clearly distinguishes CD1d from other Vpu targets (CD4, PVR, IRF3) where interference is strictly dependent on Vpu phosphorylation. Moreover, it is also different from the mechanisms underlying Vpu-mediated NTB-A down-regulation, where phosphorylation of Vpu is not at all involved. To further confirm that proteasomal degradation was not involved in CD1d down-regulation, a mutant form of CD1d that does not contain the ubiquitination site in the cytoplasmic tail (CD1dΔUb) was constructed. This CD1d mutant was down-regulated to the same level as the wild-type CD1d confirming that β-TrCP dependent ubiquitination is not involved in Vpu-mediated CD1d down-regulation (Figure 2B, **paper II**). These results are in line with our results in **paper nr I,** where total amounts of CD4 and CD1d in Vpu transfected cells were investigated. While CD4 total and surface levels were reduced, the levels of CD1d were only reduced on the cell surface (Supplemental figure 1, **paper I**). The fact that CD1d is retained in the cell indicates that CD1d is not rapidly degraded and that the mechanisms underlying Vpu-mediated down-regulation of CD4 and CD1d differ.

As described above, Nef and Vpu seem to cooperate in the CD1d down-regulation process. While Nef increases CD1d internalization, Vpu interacts with CD1d in early endosomes and thereby prevents the molecule from recycling back to the cell surface. However, while Nef uses the same mechanism for down-regulation of both CD1d and CD4, Vpu has developed different mechanisms to decrease CD1d and CD4 surface expression (147, **paper II**).

6.1.2.2 Structural requirements for Vpu interference with CD1d

Our results from **paper nr I** indicated a physical interaction between CD1d and Vpu. In **paper nr II** our aim was to go further with the details of this interaction with the hope to find specific domains or amino acids in the proteins that were involved in the interaction. Vpu has been shown to interact with NTB-A through its transmembrane region and this interaction is required to down-modulate the expression of NTB-A in T cells (275). To test if the transmembrane domain of Vpu was involved in CD1d down-regulation, CD1d expressing 293T cells were transfected with wild-type Vpu, a Vpu mutant lacking the transmembrane domain (VpuΔTMD), or with a Vpu mutant with a randomized transmembrane domain sequence (VpuTMDrd). Clearly, CD1d down-regulation was dependent on the presence of a Vpu transmembrane domain and membrane anchoring (Figure 3, **paper II**). Interestingly, there was a small but significant difference in CD1d down-regulation by wild-type Vpu and VpuTMDrd indicating the possible requirement of particular amino acid residues at certain positions in the transmembrane domain for Vpu-mediated CD1d down-regulation. In fact, for CD4 it was recently shown that the Vpu transmembrane domain contains determinants contributing to CD4 down-regulation (287), and therefore Vpu mutants with specific amino acids substitutions in the transmembrane domain need to be tested for their effect on CD1d. Next, C-terminal deletion mutants of Vpu were created where 5 up to 30 amino acids in the Vpu cytoplasmic domain were deleted (VpuΔ5-Δ30). Down-regulation assays in 293T cells demonstrated a decrease in down-regulation capacity with tail deletions of increasing size. There was a significant decrease in the down-regulation of CD1d by VpuΔ10-Δ30 compared to wild-type Vpu (Figure 4A, **paper II**). These results indicate that sites in the cytoplasmic tail of Vpu, in particular the second α-helix, may be critically involved in CD1d down-regulation. This region of Vpu may interact either directly with CD1d or with a third protein that may link Vpu and CD1d. Introducing larger deletions in the cytoplasmic tail of Vpu could also result in a modified expression pattern in the cell, and this mistrafficking of Vpu could affect the interaction with CD1d. However, as results from CD4 down-regulation assays with the same Vpu deletion mutants confirmed published results this seems unlikely. Here, there was a significant difference in down-regulation of CD4 by wild-type Vpu compared to VpuΔ15-Δ30 (Figure 4B, **paper II**). The valine residue at position 68 and the leucine residue at position 63 in the second α-helical domain of Vpu have been reported to be important for Vpu-mediated reduction of CD4 from the cell-surface (288). These residues were deleted in the mutants VpuΔ15 and VpuΔ20, respectively, explaining the observed pattern.

In addition to sites in the Vpu protein that could be involved in the interaction between Vpu and CD1d, we were also interested in potentially important CD1d sites. Here, we

constructed chimeric molecules consisting of the luminal domain of MIC-A, a molecule that is not affected by Vpu (Figure 5B, **paper II**), and the transmembrane domain or cytoplasmic domain or both domains of CD1d (called MMC, MCM and MCC, respectively) (Figure 5A, **paper II**). By using confocal microscopy, interaction between the chimeric molecules and Vpu in double transfected 293T cells was studied (Figure 5C, **paper II**). In contrast to CD1d, that is localized in endosomal compartments and co-localized with Vpu, MIC-A was localized almost exclusively at the cell surface and did not co-localize with Vpu. The MMC and MCM chimeras demonstrated that the cytoplasmic domain but not the transmembrane domain of CD1d is required for the interaction with Vpu. MCM, which has the CD1d transmembrane domain, was mainly localized on the cell surface. In contrast, the chimera MMC co-localized with Vpu, which can be explained by the introduction of the CD1d cytoplasmic tail to the MIC-A molecule. It is known that the cytoplasmic tail is required for CD1d internalization into the endosomal system (40), and we have shown that efficient Vpu-mediated CD1d down-regulation depends on the YXXØ trafficking motif in the CD1d cytoplasmic tail (Figure 4A, **paper I**). The chimera MCC showed a similar expression pattern as MIC-A, and was mainly expressed on the cell surface and did not overlap with Vpu. This was surprising, since the molecule contains the cytoplasmic tail of CD1d. It is unclear why the CD1d cytoplasmic tail was incapable of mediating the normal distribution in the context of the MCC chimera and further experiments are required to investigate this. In conclusion, our results indicate an important role of the CD1d cytoplasmic domain in the interaction with Vpu. If this is related to a specific amino acid motif that directly interacts with Vpu or a third protein involved, or if mutations in CD1d cytoplasmic tail mainly disturb the trafficking of CD1d is not clear yet and will be an important aspect of our continued studies.

	Down-regulation by VpuΔP	**Involvement of Vpu-**			**Proteosomal or lysosomal degradation**
		TMD	**αhelix I**	**αhelix II**	
CD4	no	(yes)	yes	yes	p
Tetherin	(no)	yes	yes	yes	p , l
CD1d	(yes)	(no)	*n.d.*	(yes)	no deg.
PVR	(no)	yes	*n.d.*	*n.d.*	*n.d.*
NTB-A	yes	yes	*n.d.*	*n.d.*	no deg.
IRF3	no	*n.d.*	*n.d.*	*n.d.*	l

Table 3**: **Vpu targets and mechanisms of interference. *TMD: transmembrane domain, VpuΔP: Vpu lacking its phosphorylation sites, p: proteosomal, l: lysosomal, n.d.: not determined, no deg.: no degradation involved (149, 223, 264, 265, 275, 277-279, 287, 289-291).*

6.2 iNKT CELL ACTIVATION

iNKT and NK cells can become activated early in HIV-1 infection (292, 293) and may contribute to the temporary control of viral replication by producing cytokines and chemokines as well as direct killing of infected cells (105). Therefore, it may be

relevant for the virus to inhibit these effects already at mucosal sites by interfering with the expression of molecules involved in the activation of iNKT and NK cells.

6.2.1 A microscopy-based method to study iNKT cell responses

In paper nr I one aim was to demonstrate the biological relevance of Vpu-mediated CD1d down-regulation, and we therefore wanted to measure the capacity of HIV-1 infected DCs to induce iNKT cell activation. However, detailed studies of this aspect are hampered by the low frequency of DCs productively infected with HIV-1 *in vitro*. Therefore, we developed a microscopy-based method using an eGFP-expressing HIV-1 virus. This method, described in **paper nr III**, enabled detailed studies of iNKT cell activation after conjugate formation with infected or non-infected DCs on a single cell level (Figure 7). The eGFP-expressing HIV-1 mutant was constructed, to easily and without further manipulation be able to identify rare HIV-1 infected cells in a mixed culture. Functional gene expression of this virus was verified in FACS, microscopy and western blot (Figure 1 and 2, **paper III**). DC cultures were infected with 81A-eGFP and co-incubated with iNKT cell lines, and subsequently fixed and stained for markers of activation and microscopically analyzed. Although iNKT cells formed complexes with both infected and uninfected DCs, IFN-γ production was mainly confined to iNKT cells in complex with uninfected DCs (Figure 5B and C, **paper III**). This indicates that CD1d down-regulation in HIV-1 infected DCs results in decreased antigen-presentation and iNKT cell activation. Noteworthy, there was no significant difference in IFN-γ production between iNKT cells in contact with uninfected DCs and unexposed DCs (MOCK) demonstrating that exposure to virus is not sufficient to induce changes in the iNKT cell activation capacity of DCs (Figure 5B, **paper III**).

An advantage of the described methodology is that it can be modified in many different ways including infected/non-infected cultures, different types of read-out and time points of analysis including very early events (synapse formation, vesicle polarization) as well as late events (cytokine production, degranulation) of activation. We have used this method to study both TNF-α and IFN-γ production (**paper III and I**, respectively), centrosome polarization (**paper I and IV**) and polarization of perforin and NKG2D (**paper IV**). Moreover, the proviral construct eGFP-81A could be further manipulated to knockout further genes of interest. With some changes in the protocol, this method should also be possible to use in live cell imaging. In our study, iNKT cell activation was analyzed by counting cytokine expressing cells without help from microscope analysis software. To further improve the quality of analysis, computerized analysis tools would be beneficial.

DCs that reside in the genital mucosa are critical for HIV transmission. Small micro-abrasions that occur during sexual intercourse may allow the virus to directly reach susceptible target cells (294). In the mucosa, virus can productively infect DCs or be internalized into the endocytic compartment of the DCs and pass across the infectious synapse to CD4+ T cells (reviewed in 228). Additionally, the virus can directly infect mucosal CD4+ T cells (295), and possibly mucosal iNKT cells as well. Due to the capacity of iNKT cells to produce large amounts of cytokines, chemokines and

cytolytic responses very early after activation, it may be particularly relevant for HIV-1 to evade iNKT cell responses. It may therefore be beneficial for the virus to down-regulate CD1d in infected DCs at a very early stage of infection, probably already in the mucosa. A role for iNKT cells in HIV-1 infection is supported by the fact that iNKT cell numbers are reduced in HIV-1 infected individuals (112, 133, 134) and those who are left are exhausted and functionally impaired (136-138). Down-regulation of CD1d strengthens the role of iNKT cells in HIV-1 infection as well as the importance for HIV-1 to evade iNKT cell responses.

Viruses do not encode for any lipids, and therefore possible antigen candidates may be unusual self-lipids presented in HIV-1 infected cells due to disturbed lipid metabolism. Self-lipid presentation together with cytokine production by the infected DCs does activate iNKT cells (54, 92) and may be a potential iNKT cell activation pathway in HIV-1 infection. Other lipid antigen candidates could be lipidated peptides. Both CD1a and CD1c have the ability to present lipopeptides (156, 296) supporting the idea that lipidated viral proteins may be a source for CD1-presented antigens. Interestingly, the lipopeptide that has been shown to bind CD1c has an amino acid sequence that is identical to a sequence found in the Nef protein (156).

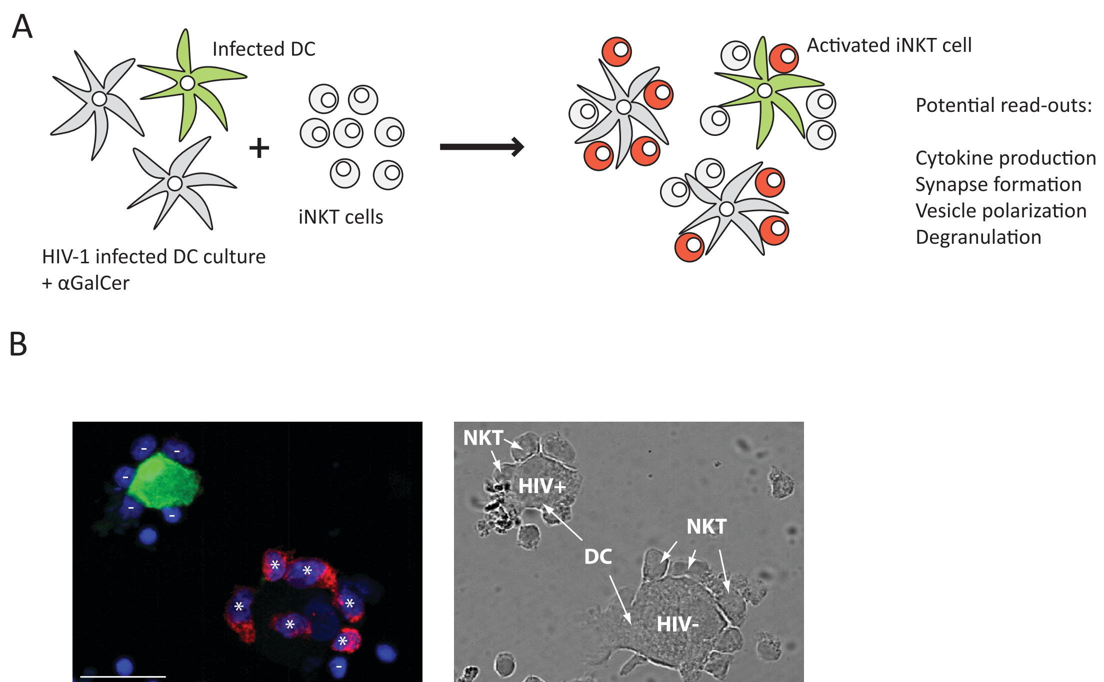

***Figure** 7: **A microscopy-based method to study iNKT cell activation**. A. Schematic representation of the assay. B. Confocal pictures of iNKT cells in contact with HIV-1 infected or uninfected DCs. Minuses indicate IFN-γ negative, asterisks IFN-γ positive iNKT cells in contact with DCs. Red: IFN-γ, Green: HIV-1-eGFP Blue: DAPI. Sale bars, 15 μm. Adapted from **paper nr I and III**.*

6.2.2 NKG2D-mediated activation of iNKT cells and its potential role in viral infection

In addition to recognition of lipid antigen bound to CD1d, iNKT cells can become activated through NK cell receptor triggering (96, 97). This could be an important mechanism to recognize cells that do not express CD1d as for example tumor cells and in cells where CD1d is down-regulated as a consequence of infection. In **paper nr IV** we describe NKG2D-mediated, CD1d-independent iNKT cell activation.

Not much is known about NK receptor expression on iNKT cells. Therefore, we stained iNKT cells for different NK cell receptors and analyzed the expression profile on the CD4- and CD4+ iNKT cell subsets. CD4- iNKT cells expressed the receptors 2B4, NKG2D, CD94 and NKG2A. DNAM-1 and CD2 were expressed on both subsets while NKG2C, NKp30, NKp44, NKp46, KIR2DL1, KIR2DL2/3 as well as KIR3DL1 were undetectable on iNKT cells (Figure 1, **paper IV**). This indicates that CD4- iNKT cells express several NK cell activating receptors, which could be involved in their innate function. Our continued studies focused on the activating receptor NKG2D that is recognizing the ligands MIC-A and B and ULBP1, 2 and 3, all up-regulated upon cellular stress, including infection and transformation (297, 298). NKG2D has been shown to play a role in HIV-1 infection and the NKG2D pathway seems to be important for NK-cell mediated killing of HIV-1 infected cells (299). However, conflicting results exist concerning the effect of HIV-1 infection on the expression of NKG2D ligands. Whereas one study demonstrated up-regulation of ULBP molecules in HIV-1 infected cells (300), HIV-1 Nef was shown to down-regulate ULBP1 and 2 as well as MIC-A, probably to avoid NK cell recognition and killing (299, 301). Moreover, also other viruses interfere with NKG2D and its ligands, indicating the importance of the NKG2D-MIC-A pathway in immune responses against viruses. In acute HSV-1 infection, there is an increased expression of NKG2D on blood NK cells, and in the same study MIC-A was shown to be down-regulated in HSV-1 infected HeLa-cells (302). MCMV and HCMV also block the expression of several NKG2D ligands in infected cells (303). Interestingly, our results demonstrated perforin and granzyme B expression in NKG2D+ iNKT cells suggesting a role of NKG2D in iNKT cell effector activity (Figure 2C, **paper IV**). To study the localization of NKG2D and effector molecules in the contact zone between iNKT and target cells, we employed and modified the methodology described in **paper nr III**. iNKT cells formed complexes with the classical NK-cell target cell line K562, and we observed NKG2D localization close to the immunological synapse indicating a role in iNKT cell responses to target cells (Figure 8). Interestingly, it was mainly the NKG2D+ iNKT cells that were in contact with K562 cells (Figure 3E, **paper IV**). Moreover, polarization of microtubule organizing centres and perforin granules indicated activation of iNKT cells and NKG2D-dependent cytolytic activity towards the K562 target cells (Figure 3G and 4, **paper IV**).

To confirm the involvement of NKG2D in iNKT cell mediated killing, we performed CD107a degranulation and ^{51}Cr release assays. NKG2D triggered degranulation in the CD4- iNKT cell subset towards anti-NKG2D coated target cells (Figure 2D, **paper**

IV). Moreover, we observed killing of anti-NKG2D coated target cells, demonstrating that iNKT cells have the ability to kill in an NKG2D-dependent manner (Figure 5, **paper IV**). Interestingly, we could also demonstrate that NKG2D can act as a co-stimulatory molecule. NKG2D engagement increased the level of TCR-mediated activation, especially when TCR triggering was weak (Figure 6, **paper IV**). This could be an important mechanism in cells that express low levels of CD1d and need additional signals to stimulate an effective iNKT response.

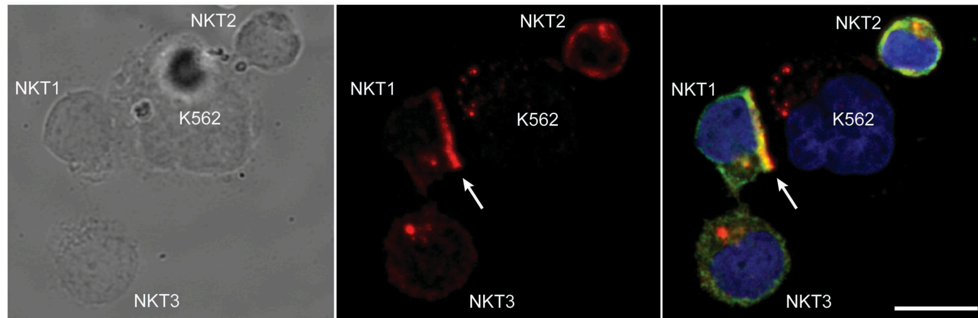

***Figure 8: NKG2D polarization in iNKT cells upon target cell contact**. NKG2D expression in three iNKT cells. The confocal picture shows polarization of NKG2D in NKT1 in contact with a K562 cell. Red: NKG2D, Green: CD3, Blue: DAPI. Scale bars, 10 μm. Adapted from **paper nr IV**.*

7 CONCLUDING REMARKS

The main aim of this thesis was to extend our knowledge about the role of the lipid antigen-presenting molecule CD1d and CD1d-restricted iNKT cells in HIV-1 infection. A number of viruses have evolved evasion strategies to escape from host immune responses. It is well known that HIV-1 evades adaptive immunity by interfering with the functional expression of MHC class I and II proteins. In this thesis, our main findings demonstrate:

- HIV-1 Vpu interference with lipid antigen presentation via down-regulation of CD1d, in infected DCs, as described in **paper nr I**.

- Degradation-independent down-regulation of CD1d by Vpu, a mechanism that is different from that of interference with CD4 and other cellular Vpu targets. This is described in detail in **paper nr II**.

- Impaired activation of iNKT cells in contact with HIV-1 infected DCs, demonstrated by a novel microscopy-based analysis method described in **paper nr III**.

- An NKG2D-dependent and CD1d-independent iNKT cell activation pathway, described in **paper nr VI**.

In conclusion, the findings presented in this thesis indicate that CD1d-restricted immunity may contribute to the host defence against HIV-1, and that the Vpu protein may play a significant role in the evasion from the CD1d-restricted immune response. Since Vpu has been shown to interfere with iNKT and NK cell activation as well as the induction of interferon responses, Vpu is emerging as an important inhibitor of innate immune mechanisms and a significant HIV-1 pathogenicity factor. It is tempting to speculate that compounds antagonizing Vpu could make HIV-1 infected cells accessible to iNKT and NK cell recognition and killing, and thereby contribute to reduce the viral burden. Vpu may therefore be an important target in the development of novel HIV-1 therapeutics.

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