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Natural Killer Cells and Antibody-mediated Effects in Human Immunodeficiency Virus Infection

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The figure on the front shows an NK cell, antibodies and HIV-1 virions and was made by Annika Wiklund.
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ABSTRACT

The human immunodeficiency virus (HIV-1) effectively avoids immunologic eradication. Certain characteristics of the virus contribute to this, including the specific targeting of the body's own defense system, the ability to hide in the host cell genome and the vast viral variability. Natural killer (NK) cells are important effectors in resistance to virus infections and there are indications that NK cells have a role also in HIV-1 infection. HIV-1 specific antibodies can cause neutralization of free virions, but also contribute to killing of infected cells through interaction with effector cells, such as NK cells. In this thesis the involvement of NK cells in acute and chronic HIV-1 infection has been studied and novel treatment modalities based on NK cells or antibodies have been examined.

In our mouse model for acute HIV-1 infection, we show that NK cells have a role, since more infectious virus could be retrieved from NK cell depleted mice than normal mice after inoculation of spleen cells infected with a HIV-1/MuLV pseudovirus (**paper III**). In addition, frequency of NK cells at the site of injection of infected cells were increased and the NK cells had a more mature phenotype. These studies are interesting in relation to previous reports on an enhanced NK cell activity in acute HIV-1 infection in humans and highlight the importance of considering activation of NK cells in future treatment modalities targeted to HIV-1.

One possible therapeutic strategy is activation of NK cells by blocking inhibitory NK cell receptors. The KIR binding antibody, 1-7F9, induced activation of NK cells from HIV-1 infected patients and healthy individuals, and the effect could be associated to KIR genotype of the blood donor (**paper I**). However, we could not detect increased NK cell recognition of HIV-1 infected compared uninfected autologous T cells. This lack of HIV-1 specific effect may be due to the *in vitro* system used.

Phenotypic and functional differences in NK cells from patients that spontaneously control their disease were compared to NK cells from patients with viremia or healthy individuals, but no striking differences were found (**paper I and II**). NK cells can specifically kill antibody coated infected target cells in a process called antibody dependent cellular cytotoxicity (ADCC). The antibodies elicited in patients differed in ability to induce Env specific ADCC (**paper II**). Qualitative differences in HIV-specific ADCC antibody responses may thus contribute to control of disease.

For induction of efficient antibody mediated killing, monoclonal antibodies may be chemically coupled to cytotoxic drugs. This treatment modality was examined in our model for acute HIV-1 infection and was found to contribute to eradication of HIV-1/MuLV infected cells in the mouse (**paper IV**).

We conclude, based on our studies in the HIV-1/MuLV mouse model, that NK cell activity in acute HIV-1 infection may contribute to early control of infected cells and that HIV-1 envelope specific antibodies conjugated to drugs may efficiently resolve virus infected cells. In addition the possible differences in ADCC inducing antibodies in controller patients deserve further investigation, as do KIR blockade for the treatment of HIV-1 infection.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Humant immunbrist virus (HIV) orsakar en av de största epidemierna i världen idag. Viruset attackerar främst de celler i vår kropp som skyddar oss mot infektioner och detta tillsammans med HIVs förmåga att snabbt förändra sig gör att vårt immunsystem inte klarar av att utplåna viruset. Den behandling som finns idag hindrar HIV från att föröka sig, men eftersom viruset kan gömma sig i kroppens celler i latent form kan behandlingen inte helt utplåna det. Det saknas därför ett botemedel mot HIV och detta är en av de större utmaningarna inom den biomedicinska forskningen idag.

I den här avhandlingen har vi studerat en typ av immuncell som tillhör det medfödda immunförsvaret och som kallas natural killer (NK) cell. Det är känt sedan tidigare att denna cell snabbt och effektivt kan döda virusinfekterade celler. Vi har använt en musmodell för att undersöka om NK-celler är viktiga för att skydda mot infektion med HIV och funnit att möss som saknar NK-celler har ett sämre skydd. Det visade sig också att frekvensen mogna NK-celler ökade där de HIV infekterade cellerna fanns, vilket är intressant eftersom det är troligt att en mer mogen NK-cell är bättre på att döda infekterade celler.

Vi har även studerat NK-celler från HIV-infekterade patienter. Vissa patienter klarar av att kontrollera sina virusnivåer utan behandling och är därmed intressanta att studera för att ta reda på vilka faktorer som bidrar till detta skydd. Vi undersökte om dessa patienter har NK-celler som skiljer sig från NK-cellerna hos patienter med höga virusnivåer och friska individer, men vi fann inga stora skillnader som skulle kunna förklara det fördelaktiga sjukdomsförloppet. NK-celler kan också känna igen infekterade celler specifikt genom att binda till antikroppar, det vill säga kroppsegna proteiner som kan angripa HIV infekterade celler. Detta antikropps-orsakade dödande av infekterade celler kallas för ADCC (antibody dependent cellular cytotoxicity). Vi fann att patienter som kan kontrollera sina virusnivåer har antikroppar med något annorlunda ADCC förmåga än patienter med höga virusnivåer och vi spekulerar därför i om detta kan vara en bidragande orsak till ett långsammare sjukdomsförlopp.

Antikroppar kan även användas experimentellt för att påverka NK-cellernas funktion, genom att blockera vissa proteiner som uttrycks på NK-cellytan. Vi har studerat blockering av NK-cell hämmande proteiner och undersökt om det på så vis är möjligt att öka NK-cellernas förmåga att reagera mot HIV-infekterade celler. Vi fann att NK-cellerna reagerade starkare när den blockerande antikroppen tillsattes till cellkulturerna, men denna aktivitet var lika stor mot oinfekterade celler som mot HIV-infekterade celler.

Vi har också studerat effekten av att kemiskt koppla ett cellgift till en antikropp som specifikt binder till HIV-infekterade celler. Detta ökade antikroppens förmåga att döda HIV-infekterade celler i cellkultur och även i vår musmodell för HIV.

Sammanfattningsvis har vi funnit att NK-celler utövar ett skydd i den tidiga fasen av HIV-infektion i möss och att patienter som kontrollerar sina virusnivåer kan ha antikroppar med en bättre förmåga att orsaka NK-cells dödande av infekterade celler. Vi har även visat att antikroppar kopplade till toxiska läkemedel är en effektiv strategi för att utplåna HIV-infekterade celler.

LIST OF PUBLICATIONS

I. Susanne Johansson, Bo Hejdeman, Jorma Hinkula, Maria H Johansson, François Romagné, Britta Wahren, Nicolai R Wagtmann, Klas Kärre, Louise Berg.
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Clinical Immunology 2009 Oct 30 (Epub ahead of print)

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Manuscript

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Role of NK cells in local intraperitoneal HIV-1/MuLV infection.
Manuscript

IV. Susanne Johansson, David M. Goldenberg, Gary L. Griffiths, Britta Wahren, Jorma Hinkula.
Elimination of HIV-1 infection by treatment with a doxorubicin-conjugated anti-envelope antibody.
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LIST OF ABBREVIATIONS

ADCC	Antibody dependent cellular cytotoxicity
AIDS	Acquired human immunodeficiency syndrome
AML	Acute myelogenous leukemia
APC	Antigen presenting cell
APOBEC	Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like
ART	Antiretroviral treatment
CCR	Chemokine receptor
CD	Cluster of differentiation
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DNAM	DNAX accessory molecule 1
Env	Envelope
EU	Exposed uninfected
Fc	Fragment crystalline
HCMV	Human Cytomegalovirus
HIV	Human immunodeficiency virus
HIVIG	HIV immunoglobulin
HLA	Human leukocyte antigen
ICAM	Intercellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motifs
KIR	Killer immunoglobulin-like receptor
KLRG	Killer cell lectin-like receptor G
LFA	Leukocyte functional antigen
LIR	Leukocyte immunoglobulin-like receptor
LTNP	Long-term non-progressor
mAb	Monoclonal antibody
MCMV	Murine Cytomegalovirus
MHC	Major Histocompatibility complex
MIC	MHC class I chain related protein
MIP	Macrophage inflammatory protein
MuLV	Murine leukemia virus
Nef	Negative factor
NK	Natural killer
NCR	Natural cytotoxicity receptors
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PRR	Pattern recognition receptors
PVR	Polio virus receptor
RANTES	Regulated on activation normal T cell expression and secretion

SHIV	Simian-human immunodeficiency virus
SIV	Simian immunodeficiency virus
TCR	T cell receptor
TGF	Tumor growth factor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis inducing ligand
ULBP	UL16-binding protein

1 AIMS OF THE THESIS

The overall aim with my studies has been to investigate different aspects of NK cells and antibodies in HIV-1 infection. The specific aims were the following:

- To investigate if HIV-1 controller patients differ from patients with viremia regarding their NK cell phenotype and function (**paper I and II**).

- To investigate if ADCC-inducing antibodies from HIV-1 controller patients differ from antibodies from patients with viremia (**paper II**).

- To delineate the role of NK cells in a mouse model for acute HIV-1 infection (**paper III**)

- To evaluate if an antibody blocking inhibitory KIR receptors could increase the NK cell activity in response to HIV-1 infected cells (**paper I**)

- To evaluate the therapeutic effect of a HIV-1 envelope specific antibody conjugated to a cytotoxic drug in a mouse model for HIV-1 infection (**paper IV**)

2 THE IMMUNE SYSTEM

The immune system is our defense against intruding organisms, like viruses and bacteria. For simplicity, the immune system can be divided into two parts, the innate and the adaptive. The innate immune system contributes to the activation of the adaptive immune system and controls the infection during the first days to weeks it takes for the adaptive immune system to become active and effective. Cells belonging to either part of the system have specific functions but they interact through soluble molecules and cell-to-cell contacts in a complex manner.

2.1 INNATE IMMUNE RESPONSES

The innate immune system is the rapid first defense against intruders. Physical barriers, like skin, mucosa and tears prevent pathogens from entering the body. If the pathogen passes through these barriers it will come across various cells and molecules with ability to eliminate the intruder. The cells of the innate immune system have germline encoded receptors that recognize certain pathogen structures, not found on self molecules. These pattern recognition receptors (PRR), including for example Toll-like receptors (TLR), recognize the pathogen-associated molecular patterns (PAMPs) found on for example bacteria, fungi, protozoa and viruses [1]. PAMPs found on viruses include for example double and single stranded RNA bound by TLR3 and TLR7/8 respectively and CpG motifs in viral DNA recognized by TLR9 [2]. Signaling through TLRs trigger immediate inflammatory and antiviral responses, and influences subsequent activation of the adaptive immune response. DCs can capture pathogens and transport the foreign material to lymphoid organs where it is presented to cells of the adaptive immune system. DCs are induced to express co-stimulatory molecules by activation through TLRs and this increases their ability to induce better activation of the T cells of the adaptive immune system. Cells presenting antigens to the adaptive immune cells in presence of co-stimulatory molecules are called professional antigen presenting cells (APCs) and include B cells and macrophages in addition to DCs.

Plasma proteins belonging to the complement system circulate in the blood and bind to pathogens or tumor cells and induce killing either directly or by recruiting phagocytic cells. Phagocytes like macrophages, monocytes and neutrophils can engulf pathogens and kill them. These cells also secrete molecules, like cytokines and chemokines. Cytokines induce responses and affect the behavior of cells with the cytokine receptor, while chemokines are a class of cytokines that activates cells and stimulates migration towards the chemokine source. An inflammatory response is induced by the release of these factors and this will attract more cells and effector molecules and also prevent the pathogen from spreading. Natural killer (NK) cells are lymphocytes, which can recognize and kill infected cells but also secrete cytokines and chemokines with ability to influence the overall immune response to a pathogen [3]. They are regulated by a fine balance between stimulation of activating and inhibitory receptors [4]. NK cells will be introduced in more detail in section 3.

2.2 ADAPTIVE IMMUNE RESPONSES

The adaptive immune system provides a specific response to individual pathogens. It can develop memory, leading to a fast and specific response to reappearing antigens. The adaptive immune system consists of two kinds of lymphocytes, B cells and T cells.

T cells are responsible for the cellular response and also aid in the humoral response produced by B cells. Both of these cell types have antigen receptors that are produced by site-specific somatic DNA recombination, which leads to an almost unlimited repertoire of receptors and thus recognition of most antigens.

2.2.1 Major histocompatibility complex molecules

The proteins encoded by the major histocompatibility gene complex have a central role in the adaptive, but also in the innate immune response. There are two kinds of major histocompatibility complex (MHC) molecules on the cells in our body, MHC class I and MHC class II. MHC class I consists of a class I α chain and a non-covalently linked, non-polymorphic β chain called β2-microglobulin. MHC class II consists of two chains, the class II α chain and the class II β chain. MHC class I molecules are expressed on all nucleated cells in the body and present cytoplasmic peptides, which are 8 to 10 amino acids long and derived from for example virus proteins. MHC class II molecules are highly expressed on professional APCs and present longer peptide fragments of exogenous antigens that have been taken up from the extracellular environment.

In humans, the MHC molecules are termed human leukocyte antigens (HLA). There are three different genetic loci encoding the MHC class I α chain, HLA-A, HLA-B and HLA-C. These genes are highly polymorphic, leading to a wide variety of alleles. Both alleles at each locus of an individual are transcribed and translated. Hence each individual can express up to six different MHC class I molecules. In mice the MHC class I molecules are H-2K, H-2D and H-2L. The human MHC class II molecules are named HLA-DP, HLA-DQ and HLA-DR. They can exist in even more variants than the MHC class I molecules, since the class II α and β chains are both polymorphic. Humans also express the non-classical MHC class Ib molecules HLA-E, HLA-G and HLA-F, which display a rather limited polymorphism [5]. Qa-1^b is a MHC class Ib molecule present in mice.

2.2.2 T cells

T cells are produced in the bone marrow but mature in the thymus. They circulate between the blood and peripheral lymphoid organs, searching their specific antigen. On encountering it in a lymph node or in the spleen, they are induced to proliferate and differentiate into effector cells and memory cells. The T cell receptor (TCR) on T cells specifically recognizes peptides bound to MHC molecules. Activation of a naïve T cell requires that co-stimulatory molecules are present at the same time as the TCR recognizes its antigen. Co-stimulatory molecules are expressed on professional APCs and include B7 molecules that bind to the activating receptor CD28 on T cells [6].

Cytotoxic T lymphocytes (CTLs), which are $CD8^+$, can recognize antigenic peptides bound to MHC class I and this leads to killing of the target cell. CTLs kill cells by release of cytotoxic effector molecules, like perforin and granzymes [7], and/or the expression of the death receptors, such as Fas ligand (FasL), which can induce apoptosis in the target cell. CTLs also release cytokines like interferon-γ (IFN-γ) and tumor necrosis factor (TNF) that for example enhance antigen presentation and mediate anti-viral effects [8].

T helper lymphocytes, which are $CD4^+$, recognize antigens bound by MHC class II molecules. Upon activation, these cells secrete cytokines, which help to activate other parts of the immune system, like macrophages, CTLs and B cells [8]. There are several

different subsets of $CD4^+$ T cells, including T helper 1 (Th1), Th2, Th17 and regulatory T cells. Th1 cells help controlling intracellular pathogens by secreting, for example IFN-γ and interleukin-2 (IL-2) that activate CTLs and macrophages. They can also activate B-cells and induce their switch towards production of opsonizing antibody isotypes which is important in clearing of extracellular pathogens. Th2 cells produce cytokines like IL-4 and IL-10 and activate B cells, inducing switching to neutralizing antibody isotypes. The IL-17 producing Th17 cells have a role in the host defense against certain extracellular pathogens [9]. Regulatory T cells are able to suppress effector T cell proliferation and cytokine production either through secretion of anti-inflammatory cytokines such as IL-10 and tumor growth factor β (TGF-β) or direct cell-to-cell contact [10].

NKT cells are a subset of T cells with both T and NK markers. Most express an invariant TCR molecule and NK-receptors such as CD161. These cells become activated upon TCR mediated recognition of glycolipids presented by the non-classical MHC class I molecule CD1d, which is nonpolymorphic. A key feature for this subset of T cells is the immediate production of large quantities of cytokines like IFN-γ and IL-4 after antigen stimulation [11].

2.2.3 B cells

B cells develop in the bone marrow and mature in secondary lymphoid organs like the spleen, lymph nodes and mucosa-associated tissue. B cells express a B cell receptor (BCR) that specifically recognizes antigens, which can for example be viral or bacterial proteins. Each B cell expresses BCRs of one specificity. Different variable gene segments are combined by a somatic recombination process, which results in the high number of variable BCRs expressed by the B cells in one individual. The BCRs have antigen binding parts, called complementarity determining regions (CDRs) that are the most variable parts of the molecule, in addition to a constant fragment crystalline (Fc) part. When a B cell recognizes its antigen and receive co-stimulatory signals from T-cells, it will proliferate undergo "switching" and differentiate into plasma cells or memory cells (see further below). Plasma cells produce large amounts of secreted BCRs, called antibodies. Memory cells stay in the body for a long time and quickly respond if the antigen reappears. During stimulation by antigen and repeated rounds of proliferation, B-cells may go through a process called somatic hypermutation. This is based on stochastic mutations in the CDRs [12], followed by selection through competition for antigen If this process is successful it may result in BCRs with a higher affinity for the antigen – a process named affinity maturation. These B cells will then proliferate and can differentiate into plasma cells or memory cells.

During B cell maturation and proliferation the Fc part expressed by the B cell may be exchanged for another Fc part at the gene level, the so called "switch" [12]. The cytokine milieu affects this process, called class switching, and the Fc part of the immunoglobulin determines the characteristics of the antibody. There are five classes of antibodies or immunoglobulins (Ig) with different functions. These are called IgM, IgG, IgA, IgE and IgD. IgG is the most abundant antibody in the blood and can be divided into four subclasses: IgG1 to IgG4. IgAs are the main antibodies at mucosal surfaces. Upon binding of an antibody to a pathogen or a cell, different immunological pathways are induced, depending on the antibody class. Antibodies of the IgG and IgA class can neutralize pathogens by preventing them from binding to their receptor on host cells [13]. Antibodies may also induce activation of the complement cascade, a property of mainly IgM, IgG3 and IgG1. By binding of the antibody Fc part to Fc

receptors the antibody-coated antigen can be opsonized, i.e. antibodies can promote phagocytosis of for example pathogens [14]. Antibody binding to Fc receptors can also result in killing of infected cells by NK-cells, macrophages or eosinophils through a mechanism called antibody-dependent cellular cytotoxicity (ADCC). IgG1 and IgG3 are the main antibody subclasses involved in ADCC mediated by NK cells. The type of effector mechanism induced by a certain antibody may also be affected by the glycosylation profile of the antibody, which affects the binding to Fc-receptors [15].

3 NATURAL KILLER CELLS

3.1 EARLY CHARACTERIZATION OF NK CELLS AND "MISSING SELF" RECOGNITION

Natural killer (NK) cells were defined more than 30 years ago as non T , non B- large granular lymphocytes capable of killing tumor cells *in vitro* without prior sensitization [16, 17]. This "natural" killing contrasted to the priming required for T cell responses. *In vivo* evidence for NK cell resistance to tumor growth in mice was soon to follow, as was evidence on a role for NK cells in rejection of bone marrow transplants [18]. NK cells also seemed to be involved in response to infections [19]. This concept was later strengthened in humans lacking NK cells or with defective NK cell activity, who are more susceptible to certain infections and may be at increased risk of malignancies [20, 21].

The molecular basis for NK cell recognition of these various target cells was however poorly understood. A first clue to the complex specificity of NK cells came when Klas Kärre proposed the "missing self" hypothesis [22-24], based on inhibition of NK cell activity by self MHC class I molecules on a potential target cell. This was later shown to be due to interaction between inhibitory receptors on NK cells and self MHC class I molecules on the target cell. Down-regulation of MHC class I molecules on tumor cells [22, 24] or infected cells [25, 26] can result in increased NK cell sensitivity *(Figure 1b)*. Lack of one recipient self MHC molecule on bone marrow transplants is another area where "missing self" recognition is important [27]. However NK cells do not attack all cells lacking MHC class I molecules equally efficiently [28], which is explained by the presence of various ligands on target cells binding to activating receptors on NK cells [4]. Interaction between activating receptors and their ligands on target cells is necessary for an NK cell to be able to kill a target cell. These activating ligands can be upregulated upon transformation or infection of cells or by other stress stimuli acting on cells [29-31] *(Figure 1c)*. Additionally, viral proteins expressed on infected cell surfaces also bind activating receptors [32-35]. Thus, a potential target cell can avoid NK cell killing either through active inhibition, via MHC class I molecules, or through absence of activating ligands [4]. "Natural" killing by NK cells is thus not "non-specific" as previously thought, rather is it influenced by complex interactions with a multitude of receptors, as well as various cytokines present upon infection [36].

3.2 NK CELL DEVELOPMENT AND EDUCATION

Human NK cells derive from $CD34^+$ hematopoietic progenitor cells present in the bone marrow. Interestingly, recent data indicate that further maturation to NK cells can take place not only in the bone marrow but also in secondary lymphoid organs [37, 38], possibly in close contact with DCs and APCs expressing membrane-bound IL-15, needed for NK cell development [3]. In mice, bone marrow has been considered the primary site of NK cell maturation, although emerging data indicate, similarly as in humans, that NK cell precursors and immature NK cells that give rise to mature NK cells are present in other organs as well, including lymph nodes, liver, spleen and thymus [39]. Thus it is possible that multiple sites support NK cell differentiation.

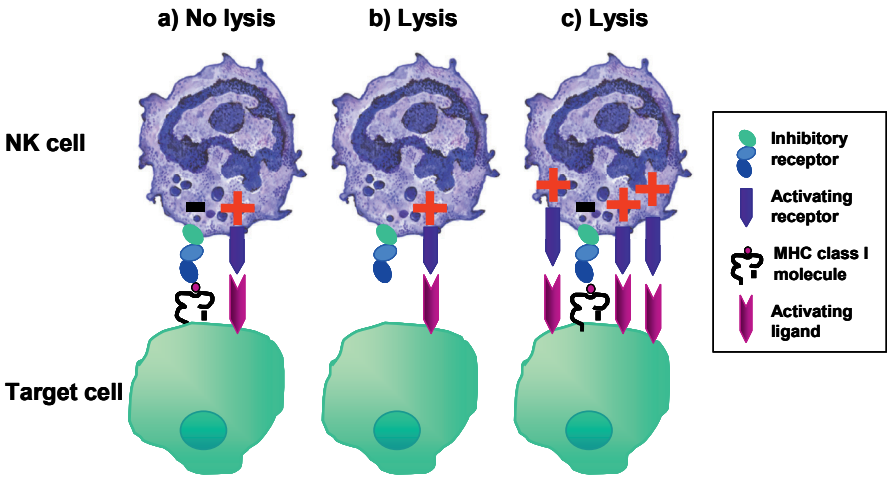

Figure 1: NK cell recognition of target cells. a) NK cell interaction with normal cell expressing MHC class I molecules does not result in lysis. b) An NK cell recognising for example an infected cell, which has downregulated the expression of MHC class I molecules, kills the target cell due to "missing-self" recognition. c) An infected target cell expressing many activating ligands in addition to normal levels of MHC class I molecules will also be killed by the NK cell.

To prevent NK cells from killing autologous cells, NK cells are educated to become unresponsive or tolerant to the "self" phenotype. This process is believed to be dependent on recognition of MHC class I molecules by the inhibitory NK cell receptors. Usually each NK cell expresses a selection of the inhibitory receptor genes present in an individual [40]. The acquisition of these receptors is believed to be a stochastic event and it results in an NK cell population with diverse MHC class I specificities [41]. MHC class I expression in the host influence the NK cell repertoire to some extent. NK cells lacking inhibitory receptors for self MHC class I molecules are functionally impaired [42-44]. This finding has led to the proposal of two opposing models to explain how NK cell activity is influenced by the interaction of MHC class I molecules and inhibitory receptors for self MHC during development. The "disarming model" states that persistent activation of an initially responsive NK cell in the absence of inhibitory signals leads to an unresponsive state [43]. This is similar to activation induced anergy in T and B cells. The "licensing model" on the other hand suggests that NK cells become activated or "licensed" when they receive signals through their inhibitory receptors [44]. Irrespective of model it seems that the number and type of MHC class I alleles recognized by an NK cell during education can influence the responsiveness of that particular cell in a quantitative manner. Thus, according to the recently proposed "rheostat model" education is a quantitative tuning of NK cell activity along a continuous scale [45].

3.3 NK CELL PHENOTYPE

Human NK cells are defined phenotypically as cells expressing an isoform of the neural cell-adhesion molecule (NCAM), CD56, but lacking expression of CD3, to distinguish them from T cells expressing CD56 [46] *(Figure 2)*. Mice lack CD56 and instead NK cells are defined depending on expression of NK1.1 (NKR-P1C) in C57BL/6 mice, and CD49b (bound by the DX5 mAb), expressed in a wide range of mouse strains [47], together with lack of CD3 expression *(Figure 2)*. Recently, a new phenotypic definition of NK cells as NKp46^{+}CD3^{-} cells was proposed, since this population includes the vast majority of NK cells in humans and mice, permitting comparisons between species [48].

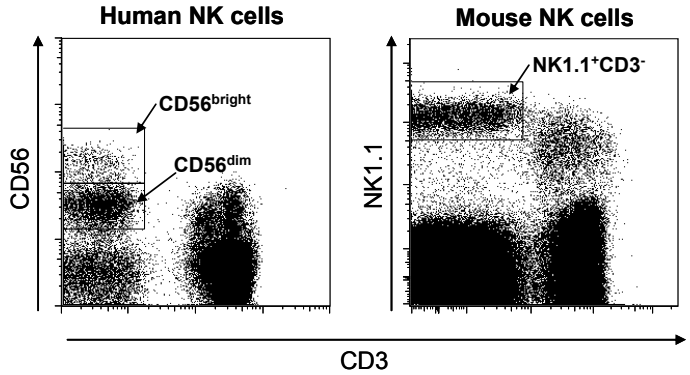

Figure 2: Gating strategies used to define NK cells in humans and mice. Human CD56^{+}CD3^{-} NK cells from blood and C57BL/6 mouse NK1.1^{+}CD3^{-} NK cells from spleen. FACS plots show events within the lymphocyte gate.

3.3.1 Human NK cell subsets

In human peripheral blood from healthy individuals about 5-20% of all lymphocytes are NK cells. Based on the level of expression of CD56, human NK cells can be divided into two subsets with distinct functional capabilities [49]. CD56^{bright} NK cells have a high density of CD56 on the surface and these cells primarily produce cytokines in response to stimulation. CD56^{dim} NK cells, on the other hand, have low levels of CD56 and are naturally more cytotoxic than the CD56^{bright} NK cells [36, 50]. This is also reflected by expression of the Fc-receptor, CD16, involved in ADCC, on CD56^{dim} cells [49]. The majority of NK cells (approximately 90%) in blood of healthy individuals belong to the CD56^{dim} subset, while the minority is CD56^{bright} *(Figure 2)*. The ratio between the subsets differs in various diseases [51, 52]. In human immunodeficiency virus (HIV) infected patients a hyporesponsive CD56^{-}CD16^{+} NK cell subset has also been identified [53, 54]. In secondary lymphoid organs, such as lymph nodes and tonsils CD56^{bright} NK cells are the dominant subset [55]. Recent data suggest that CD56^{bright} NK cells mature into CD56^{dim} NK cells and that the later have shorter telomere length [56, 57]. In addition to differences in CD56 and CD16 expression, there are other major phenotypic differences between the NK cell subsets for example in expression of inhibitory receptors, where most CD56^{dim} NK cells

preferentially express killer immunoglobulin-like receptors (KIRs) whereas $CD56^{bright}$ NK cells mainly express CD94/NKG2A heterodimer [36, 58]. Chemokine receptors are also differently expressed on the NK cell subsets, since $CD56^{bright}$ cells express CCR7, important in homing to secondary lymph nodes and CXCR3, while $CD56^{dim}$ cells express CXCR1 and CX_3CR1 [59]. Further, $CD56^{bright}$ NK cells express higher levels of certain cytokine receptors, including preferential expression of the high-affinity IL-2Rαβγ, explaining the strong activation induced by IL-2 on this subset [36].

The possibility that NK cells develop under various influences in different tissues in can be one explanation for the different NK cell subsets found in certain organs. One distinct NK cell subset is found in the uterus during pregnancy, where NK cells dominate among the decidual lymphocytes. These have mainly a $CD56^{bright}$ phenotype, but in contrast to other NK cells of this subset they express KIRs. These NK cells are not cytotoxic despite the expression of several activating receptors, although they secrete high amounts of cytokines and angiogenic factors important in vascular remodeling during pregnancy [60]. Another recently described NK cell subset is the IL-22 producing NK cells that are present at mucosal sites and may help constrain inflammation and protect mucosal sites [61].

3.3.2 Mouse NK cell subsets

Recently it was realized that mouse NK cells similarly to human NK cells can be divided into subsets [62, 63]. Based on the expression of the TNF receptor CD27 and the integrin Mac-1 (CD11b), four differently mature subsets with distinct functions have been identified [62, 64]. Starting from the most immature $Mac\text{-}1^{low}CD27^{low}$ (double negative, DN) and going through $Mac\text{-}1^{low}CD27^{high}$ ($Mac\text{-}1^{low}$) and $Mac\text{-}1^{high}CD27^{high}$ (double positive, DP) and ending with the most mature subset, $Mac\text{-}1^{high}CD27^{low}$ ($CD27^{low}$). The tissue distribution of these NK cell subsets is quite distinct, with mainly $Mac\text{-}1^{low}$ and DP NK cells in lymphoid organs, such as bone marrow and lymph nodes and mostly DP and $CD27^{low}$ NK cells in spleen, blood and liver [62]. The most immature subsets (DN and $Mac\text{-}1^{low}$) have the highest capacity for homeostatic expansion [62-64]. DP cells produce high amounts of IFN-γ upon stimulation, an ability which is less pronounced in the $CD27^{low}$ subset [62]. However, the $CD27^{low}$ subset has the highest expression of genes involved in NK cell mediated cytotoxicity and secretory pathways [64]. A higher frequency of NK cells of the $CD27^{low}$ subset also expresses inhibitory Ly49 receptors, indicating a possible need for inhibition of cytotoxic activities [62, 64]. Thus, although expression of CD27 and CD56 in humans does not completely overlap [65, 66], there are similarities in function and distribution between mouse subsets based on Mac-1 and CD27 and human CD56 subsets. Chemokine receptors expressed on mouse NK cells further reflect this similarity since $Mac\text{-}1^{low}$ and DP NK cells express CXCR3 and $CD27^{low}$ express CX_3CR1 similarly to $CD56^{bright}$ and $CD56^{dim}$ human NK cells respectively [67]. In human and mouse, L-selectin, CD62L, is used by the more immature NK cell subsets for homing and recruitment to lymph nodes, [62, 68], whereas egress from lymph nodes involves the sphingosine 1-phosphate (S1P) receptor, $S1P_5$, expressed on the most mature NK cell subsets [69].

3.4 NK CELL EFFECTOR FUNCTIONS

3.4.1 Cytotoxicity

NK cells can kill target cells by a variety of mechanisms, with the directed release of cytotoxic granules (also known as secretory lysosomes) considered to be the most important. The release of cytotoxic granules induce apoptosis in the target cell, since the granules contain the membrane-disrupting proteins perforin and granulysin as well as granzymes, belonging to the serine protease family [70]. The exact mechanism whereby perforin contributes to granzyme delivery is debated. One model suggests that perforin is needed for release of granzymes from endosomal compartments, into the cytoplasm of the target cell, while another model proposes that it allows entrance of granzymes through the plasma membrane [7]. The granzymes induce apoptosis of the target cell through distinct pathways. Granzyme B can for example induce direct activation of cellular caspases or promote mitochondrial permeabilization [7]. Granules also contain cell membrane bound proteins, such as lysosomal-membrane-associated glycoprotein 1 (LAMP-1, also known as CD107a) that are exposed on the cell-surface upon degranulation [70, 71]. Proteins belonging to this family are highly glycosylated and have been proposed to protect the effector cell membrane from the lytic enzymes released upon degranulation [72]. Thus, CD107a can be used as a marker for activated NK cells that have degranulated [71, 73, 74]. Death-receptor-mediated apoptosis is another mechanism by which NK cells can kill target cells. NK cells express Fas ligand (FasL) and TNF-related apoptosis inducing ligand (TRAIL) upon activation [75, 76]. When these are bound by their receptors, death domain containing Fas and TRAIL receptor, expressed on target cells, apoptosis is induced

3.4.2 Release of cytokines and chemokines

NK cells are not only “killers”, they are also potent producers of cytokines, such as IFN-γ, TNF, granulocyte macrophage-colony stimulating factor (GM-CSF), IL-5, IL-10 and IL-13 [50, 77]. Immature NK cells can produce IL-13 and IL-5, whereas mature NK cells mainly produce IFN-γ [78]. NK cell-produced IFN-γ contributes to antiviral responses, stimulates macrophages and other antigen presenting cells and contributes to differentiation of T cells [77, 79, 80]. NK cells secrete CC-chemokines like macrophage inflammatory protein (MIP)-1α, MIP-1β and RANTES, which have chemoattractant and pro-inflammatory functions [77, 81]. The CC-chemokines are important in HIV-1 infection, since they are natural ligands for the co-receptors commonly used by the HIV-1 virus [82].

3.5 REGULATION OF NK CELL ACTIVITY

The type of effector function generated by an NK cell depends on the activation or inhibitory signals received. These signals can either be transmitted through soluble factors that bind cytokine and chemokine receptors on the NK cell or through direct interaction between the NK cell and the potential target cell.

3.5.1 Soluble factors

NK cells develop in the bone marrow in the presence of the crucial cytokine IL-15 [83, 84]. Several cytokines regulate NK cell activity, such as IL-2, IL-12, IL-15, IL-18, IL-21, IL-10, TGF-β and IFN-α and-β [77, 85]. These cytokines can have multiple effects on NK cells, including induction of proliferation, maturation, cytotoxicity and cytokine

production as well as inhibition of the same. Certain combinations of these cytokines induce distinct effects. During viral infections type I interferons IFN-α and -β are produced by infected cells and TLR stimulated plasmacytoid DCs [86]. IFN-α and -β stimulate NK cell cytotoxic activity and induce expression of TRAIL on NK cells [77, 86-88]. Type I interferons also activate DCs, monocytes and macrophages to produce IL-15, which in addition to its role in NK cell development and differentiation seems to promote NK cell survival, proliferation and cytotoxicity [87, 89]. IL-2 shares the medium-affinity IL-2Rβγ with IL-15 and promotes similar effects on NK cells. In addition this cytokine induces expression of KIRs and CD16 on $CD56^{bright}$ NK cells [89]. TLR stimulated or IFN-γ stimulated monocytes produce IL-12, which in turn induces IFN-γ production by NK cells [87, 90, 91]. There is also evidence that NK cells express TLRs that directly sense the presence of viral particles and activates the cell [92, 93]. Various chemokines affect NK cell migration and whether a specific NK cell will be affected depends on the chemokine receptors expressed which varies depending on the NK cell subset, as discussed previously (section3.3).

3.5.2 NK cell receptors

At the site of interaction between the NK cell and its potential target cell a local and transient immunological synapse is formed to which inhibitory and activating receptors are recruited. The net outcome of inhibitory versus activating signals transduced at the site determines the fate of the target cell [94]. Inhibitory receptors have a cytoplasmic tail with immunoreceptor tyrosine-based inhibitory motifs (ITIM), which are phosphorylated upon ligand binding. This results in recruitment of phosphatases like the tyrosine phosphatases SHP-1 and SHP-2 and the lipid phosphatase SHIP-1, which suppress NK cell responses by dephosphorylating molecules of importance in signal transduction from activating NK cell receptors [95]. Several activating receptors interact through charged transmembrane amino acids with transmembrane-anchored adaptor proteins that contain immunoreceptor tyrosine-based activation motifs (ITAM). These are constitutively expressed by NK cells and include FcεRI-γ, CD3-ζ and DAP12, which forms dimers linked by disulfide bonds. Upon crosslinking of an activating receptor ITAMs are phosphorylated by protein tyrosine kinases, which in turn recruit the tyrosine kinases Syk and ZAP70. This initiates a cascade of events, which in the absence of inhibitory signals results in cytoskeleton reorganizations needed for release of cytotoxic granules and transcription of cytokine and chemokine genes [96]. Other activating receptors signal through other adaptor proteins or signaling motifs in their own cytoplasmic tails, inducing diverse signaling cascades. There are a multitude of activating and inhibitory receptors expressed by NK cells and the receptors most relevant to the thesis are described below. Other important receptors not mentioned in the text can be found in *table 1*.

3.5.2.1 Killer immunoglobulin-like receptors (KIR)

Two structurally different NK receptor systems with analogous function are the KIR and the Ly49 receptors, in humans and mice, respectively. These highly variable receptors recognise the polymorphic MHC class I molecules and have evolved independently. The human genome possesses several KIR genes but only one non-functional Ly49 pseudogene. Mice on the other hand lack KIR genes but have a varying number of Ly49 receptors depending on the mouse strain [4]. Also other species have diversified KIR or Ly49 genes. Primates and cattle use KIR genes, whereas rodents and horses use Ly49 genes.

KIR genes and proteins

KIR proteins are expressed on NK cells and subsets of T cells and belong to the Ig superfamily. The nomenclature is based on structure and KIR proteins can either have two (KIR2D) or three (KIR3D) extracellular Ig-like domains [97, 98] *(Figure 3)*. The cytoplasmic tail varies in length, a property connected to functional activity, since inhibitory KIRs have long (L) cytoplasmic tails with ITIM motifs and activating KIRs have short (S) cytoplasmic tails which can associate with the ITAM-containing DAP12 adaptor protein. To date about 14 KIR genes and 2 pseudogenes have been described in the KIR gene cluster on chromosome 19 in humans. The number of genes varies greatly between individuals and there are only three commonly shared framework genes present in all individuals (KIR2DL4, KIR3DL2 and KIR3DL3). Since the KIR genes are highly homologous it is likely that the variability is due to gene duplications or non-allelic homologous recombinations during evolution [99]. Based on the gene content two kinds of haplotypes have been defined, where B haplotypes have more activating KIRs compared to A haplotypes. Individuals with a KIR haplotype B have one or more of the KIR genes; 2DS1, 2DS2, 2DS3, 2DS5, 3DS1 and 2DL5 and individuals with haplotype A lack all of these and can instead possess inhibitory KIRs including 2DL1, 2DL3 and 3DL1 as well as the activating 2DS4 [100] (http://www.ebi.ac.uk/ipd/kir/). An extensive allelic variation in several genes adds to the diversity and influences the amount of KIR protein expressed on each NK cell [101-103]. In addition, some allelic variants do not generate functional proteins expressed at the cell surface [102]. It is believed that at the population level, this highly diverse expression would be important for giving a broad NK cell response against different pathogens. The KIR proteins are also expressed in a variegated manner on NK cells, i.e. a certain KIR is expressed on some NK cell clones but not on others [40, 97, 98]. However, once an NK cell clone has started to express a certain KIR gene during development the expression is stable and does not appear to be affected by cytokines [103, 104]. Recently it was realized that KIR gene transcription is controlled by epigenetic mechanisms, like methylation, and by the presence of a bidirectional promoter able to stochastically switch direction of transcription during development of the NK cell, thus determining whether the NK cell will express the KIR gene or not [105, 106]. This results in a diverse repertoire of KIR expressing NK cell clones that can recognize almost every MHC class I molecule [40].

KIR ligands

The inhibitory KIRs are known to bind MHC class I molecules *(Figure 3)*. KIR2DL1 and the alleles KIR2DL2/3 recognize HLA-C molecules that can be divided into two groups called C1 and C2 that are distinguished by the amino acids at positions 77 and 80 in the α1 domain. All alleles fall into either of these two groups, where KIR2DL1 binds HLA-C2 and KIR2DL2/3 bind HLA-C1. This specificity in binding appears to be determined by amino acid 44 in the D1 domain of the KIR molecules [107]. However the binding specificity might not be as strict as previously thought since recent data suggest that KIR2DL2/3 bind to some HLA-C2 alleles as well, a property mainly found in KIR2DL2 which has a higher affinity for HLA-C [108, 109]. KIR3DL1 interacts mainly with HLA-A and HLA-B allotypes with the Bw4 epitope [110, 111], differing from the Bw6 epitope at position 79-83 in the α1 helix [112]. HLA-Bw4 allotypes with isoleucin at position 80 (Bw4Ile80) have been suggested to bind better to KIR3DL1 than those with threonine at the same position (Bw4Thr80), although this is not true for all KIR3DL1 alleles [110, 113]. Peptides bound to HLA has in some instances been shown to affect the interaction with KIR molecules [114, 115]. Although KIR molecules do not seem to distinguish self from non-self peptides, KIR3DL2 was shown to bind certain HLA-A molecules only in presence of specific Epstein-Barr virus

(EBV) peptides [116]. The unique framework gene KIR2DL4 with features typical of both inhibitory and activating receptors binds the non-classical MHC class I molecule HLA-G expressed on fetal trophoblasts, possibly indicating a role for KIR2DL4 during pregnancy [117].

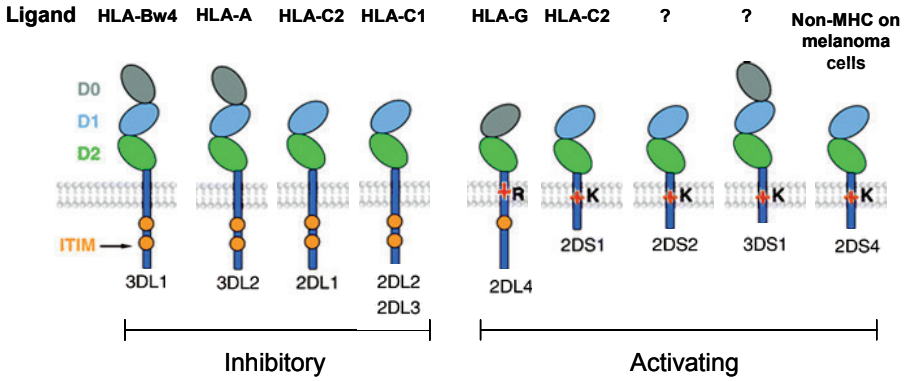

Figure 3: Schematic representation of killer immunoglobulin-like receptors (KIR) and their ligands. Inhibitory and activating KIR receptors with known ligands are included in the figure, in addition to KIR2DS2 and KIR3DS1 for which the ligands are still unknown. Extracellular immunoglobulin-like domains D0, D1 and D2 are depicted in gray, blue and green respectively. Inhibitory KIRs and 2DL4 have ITIMs in their cytoplasmic tail. Activating KIRs possess a basic amino acid in the transmembrane domain that can interact with the ITAM-containing DAP12 adaptor protein. *Figure modified from Bashirova et.al. Annu Rev Genomics Hum Genet. 2006, 7, 277-300*

Less is known about the ligands for activating KIRs, but since they are similar in the extracellular sequence to inhibitory KIRs, it is believed that they bind to the same ligand as their corresponding inhibitory receptor *(Figure 3).* Although KIR2DS1 binds weakly to HLA-C2 no conclusive evidence for binding of KIR2DS2 to HLA-C1 or KIR3DS1 to HLA-Bw4 has been established [109, 118-120]. It is possible that activating KIR receptors recognize MHC class I molecules loaded with disease-specific or stress-induced peptides or bind non-class I MHC ligands. The later seems to be the case for KIR2DS4 that upon recognition of a ligand on melanoma cells induces killing of the tumor cells [121]. However since a substantial proportion of humans lack most activating KIR genes (individuals homozygous for haplotype A) these genes are probably not absolutely required for NK cell function. Disease association studies have on the other hand indicated a role for both inhibitory and activating KIR receptors and there respective ligands in infectious diseases, autoimmunity, cancer and reproduction [122]. This will be discussed in more detail in section 5.1.3.3 regarding KIR associations to HIV-1 progression.

3.5.2.2 Ly49 receptors

Ly49 receptors belong to the C-type lectin-like family and are expressed on NK cells and subsets of T cells. Ly49 receptors are either inhibitory or activating. The inhibitory

Ly49s encode an intracellular ITIM and interact with certain MHC class I molecules, resulting in inhibition of NK cell cytotoxicity [4]. Activating Ly49 receptors associate with the ITAM-containing DAP12 and in C57BL/6 mice two such receptors exist, Ly49D and Ly49H [4]. Ly49D recognize the MHC class I molecule H-2D^{d}. Ly49H on the other hand binds the "MHC-like" murine Cytomegalovirus (MCMV) encoded protein m157, which activates NK cell functions and contributes to control of MCMV infection [32, 35].

3.5.2.3 NKG2A/NKG2C

Another MHC class I binding receptor family are the heterodimers CD94/NKG2, expressed in mice and humans and belonging to the C-type lectin-like receptor family [123]. As for KIR and Ly49 these are either inhibitory or activating receptors. The inhibitory NKG2A contains intracellular ITIMs and the CD94/NKG2A complex is expressed on most normal NK cells. The ligands for the CD94/NKG2 receptors are the non-classical MHC class Ib molecules, HLA-E in humans [124, 125] and Qa-1^{b} in mice [126]. The most abundant peptides bound to the peptide groove of HLA-E are leader sequence peptides from other MHC class I molecules. It was demonstrated that the inhibitory receptor binds with a higher affinity than the activating receptor and that peptides bound to HLA-E can affect the binding [127]. As an example, HLA-E molecules that have bound stress induced peptides derived from heat shock protein 60 (hsp60) do not bind to the inhibitory CD94/NKG2A, and this loss of inhibition could lead to NK cell elimination of these stressed cells in a peptide-dependent manner [128].

An activating counterpart for the inhibitory CD94/NKG2A heterodimer is the CD94/NKG2C complex. NKG2C associates with the adaptor protein DAP12, which induces activation signals in the cell [129]. As stated above, activating and inhibitory CD94/NKG2 bind the same MHC class I ligands albeit with different affinity. Few resting blood NK cells express CD94/NKG2C, although the expression can be regulated by the cytokine environment and might be affected by ongoing infections [4, 130].

3.5.2.4 NKR-P1

The NK1.1 protein, bound by the PK136 antibody [131] is used to define NK cells in C57BL/6 mice. This protein belongs to the NKR-P1 gene family, which encode proteins of the C-type lectin-like family. The ligand for NK1.1 (NKR-P1C) is not known but upon antibody cross-linking NK1.1 associates with an ITAM-containing adapter protein, resulting in NK cell activation [132]. In humans only one receptor of this family is present, CD161 (NKR-P1A), expressed by most NK cells since immature NK cells express it before they acquire CD56 and CD16 [133]. It is also present on subsets of T cells. CD161 has been suggested to be inhibitory upon binding of its ligand, lectin-like transcript 1 (LLT1), which leads to inhibited cytotoxicity and IFN-γ production by NK cells [134, 135]. However, in certain instances it seems to have an activating function as seen in T cells when CD3 and CD161 are ligated simultaneously [134].

	Receptor	Species	Cellular ligand	Viral ligand
Inhibition	**KIR***	Human	MHC class I alleles	
	Ly49	Mouse	MHC class I alleles	CMV m157 ($Ly49I^{129}$)
	CD94/NKG2A	Human/Mouse	HLA-E/$Qa\text{-}1^{b}$	
	LIR-1 (ILT2,CD85j)	Human	HLA-A, B, C, E, F, G	CMV UL18
	CD161 (NKR-P1A)	Human	LLT1	
	NKR-P1B/D	Mouse	Clr-b	
	2B4	Mouse	CD48	
	KLRG-1	Human/Mouse	Cadherins	
	CEACAM1 (CD66a)	Human/Mouse	CD66	
Activation	**KIR***	Human	MHC class I alleles?	
	Ly49D **Ly49H**	Mouse Mouse	$H\text{-}2D^{d}$	 CMV m157
	CD94/NKG2C/E	Human/Mouse	HLA-E/$Qa\text{-}1^{b}$	
	CD16	Human/Mouse	IgG Fc-part	
	NKp46	Human/Mouse	Heparan sulfate	Hemagglutinin
	NKp30	Human	Heparan sulfate, BAT3	CMV pp65
	NKp44	Human	Heparan sulfate	Hemagglutinin, HIV-1 gp41 induced ligand
	NKG2D	Human Mouse	MICA, MICB, ULBP1-4 Rae1, H60, MULT1	
	DNAM-1 (CD226)	Human/Mouse	PVR (CD155), Nectin-2 (CD112)	
	NK1.1 (NKR-P1C)	Mouse (C57BL/6)	Unknown	
	CD69	Human/Mouse	Unknown	
Adhesion/ Costimulation	**LFA-1 (CD11a/CD18)**	Human/Mouse	ICAM-1-5	
	Mac-1 (CD11b/CD18)	Human/Mouse	ICAM-1, iC3b, Fibrinogen	
	2B4 (CD244)	Human	CD48	
	CD27	Human/Mouse	CD70	

Table 1: Some NK cell receptors expressed by human and mouse NK cells and their respective ligands. *More information regarding inhibitory and activating KIRs, see figure 3

3.5.2.5 LIR-1

Leukocyte Ig-like receptor (LIR) -1 (ILT2, CD85j) belongs to the Ig superfamily and has four extracellular Ig-like domains and four intracellular ITIM motifs [136]. LIR-1 is expressed on a varying number of NK cells in different human individuals, on some T cells and all B cells and monocytes. Ligands for LIR-1 are all HLA class I molecules, including HLA-A, -B, -C, -E, -F, and -G [137]. Interestingly, LIR-1 expression has been implicated in human CMV (HCMV) disease [138]. LIR-1 binds an HCMV encoded MHC class I-like molecule, UL18, with a much higher affinity than for MHC

class I molecules [136, 137]. This probably contributes to inhibition of LIR-1$^+$ NK cell activity in response to HCMV infected cells, despite downregulation of MHC class I on the infected cells [139].

3.5.2.6 KLRG1

Inhibitory NK cell receptors also bind non-MHC class I ligands. The inhibitory killer cell lectin-like receptor G1 (KLRG1) possesses an ITIM and binds to members of the cadherin family [140, 141]. KLRG1 is present on subsets of NK cells and T cells in both man and mice. The most mature subset of NK cells in naïve mice express KLRG1 and upon MCMV infection a transient expansion of this NK cell subset was detected [142, 143].

3.5.2.7 CD16

The first activating NK cell receptor to be characterized was the Fc-receptor, FcγRIIIa (CD16), which binds the Fc part of IgG antibodies [144, 145]. When IgG molecules have recognized a specific antigen on for example an infected cell or tumor cell, the NK cell is able to bind the target cells, which results in the lethal hit called antibody dependent cellular cytotoxicity (ADCC). CD16 associates with homo- and heterodimers of ITAM-containing CD3-ζ and FcεRI-γ in humans and with FcεRI-γ homodimers in mice [146-148]. Upon CD16 stimulation these adaptor proteins transduce signals that in addition to ADCC result in cytokine production and release, proliferation and apoptosis of activated NK cells [149, 150]. In contrast to most activating receptors that need co-activation via other activating receptors in order to induce NK cell activation, CD16 stimulation is sufficient for NK cell degranulation [151]. However, a more effective target cell killing was induced when another activating receptor and CD16 were engaged simultaneously by their respective ligands [151]. CD16 have different affinities for IgG subclasses, with the highest affinity for IgG1 and IgG3 in humans and IgG1, IgG2a and IgG2b in mice [14, 152, 153]. Polymorphisms in the human CD16 gene have been reported [152]. A polymorphism at amino acid 158 influences binding efficacy of IgG, since NK cells from individuals homozygous for Valine (V) at this position bind IgG better than NK cells from individuals with at least one allele with Phenyalanin (F) [153, 154]. Patients with lymphomas treated with the monoclonal antibody Rituximab (IgG1 anti-CD20) had a better prognosis if they were homozygous for V at position 158, indicating an ADCC mechanism in killing of cancer cells *in vivo* [155, 156]. Another polymorphism in CD16 has been associated with severe herpes virus infections, although *in vitro* ADCC function in these patients was normal [21].

3.5.2.8 NCRs

The natural cytotoxicity receptors (NCRs), including NKp30, NKp44 and NKp46 belong to the immunoglobulin superfamily and are selectively expressed by NK cells [157-159]. NKp30 and NK46 are expressed by all NK cells, while NKp44 is only expressed by activated NK cells, as seen after culture in presence of IL-2. The NCRs do not share structural homology but they all associate with membrane-anchored adaptor proteins with ITAM motifs. The ligands for the NCRs are not well characterized but seem to exist on tumor cells and virally infected cells. NKp46 and NKp44 bind to hemagglutinin of Influenza and Parainfluenza virus [34, 160] and mice lacking NKp46 (Ncr1) were more susceptible to influenza induced death [33]. NKp46, NKp30 and NKp44 bind heparan sulfate proteoglycans expressed on tumor cells [161, 162]. NKp30 mediates NK cell killing of immature DCs [163] and binds the nuclear factor

HLA-B-associated transcript 3 (BAT3) released by tumor cells and DCs [164]. NKp30 can also be bound by a HCMV tegument protein called pp65, which leads to decreased surface expression of NKp30 [165]. An unknown ligand for NKp44 is suggested to be induced on $CD4^+$ T cells in HIV-1 infected patients [166].

3.5.2.9 NKG2D

NKG2D is an activating receptor expressed on all human and mouse NK cells and a fraction of T cells. The expression can be manipulated by different cytokines [4]. NKG2D forms homodimers and is not structurally related to the other NKG2 receptors. Ligands for NKG2D are structurally related to MHC class I and human ligands include MHC class I chain related protein A (MICA), MICB and UL16-binding protein 1-4 (ULBP1-4) [167-169] and mouse ligands are Rae1, H60 and MULT1 [170-172]. These ligands are often upregulated on stressed cells, i.e. during infection or transformation [173]. NKG2D signals via its associated adaptor protein DAP10 [174], which instead of an ITAM contain a phosphatidylinositol-3 kinase (PI3K) binding motif, resulting in activation of other signaling pathways. Mouse NKG2D exists in two isoforms, differing in length of the cytoplasmic domain. The short isoform (NKG2D-S), not present in humans, interacts with either DAP10 or DAP12, whereas the long isoform (NKG2D-L) only interacts with DAP10 [4]. The importance of NKG2D ligands in infections can be exemplified by presence of several viral proteins in human and mouse CMV that interfere with these ligands and inhibit cell surface expression, protecting the cells from killing through NKG2D activated NK cells [168, 175]. A similar mechanism seem to be present also in HIV-1 infection [176].

3.5.2.10 DNAM-1

The leukocyte adhesion molecule CD226 (DNAX accessory molecule 1 (DNAM-1)) is an activating receptor expressed on most NK cells, T cells and monocytes [177]. It belongs to the Ig superfamily and the ligands for this receptor are Poliovirus receptor (PVR, CD155) and Nectin-2 (CD112) [178]. These are highly expressed in many tumor cell lines and DNAM-1 mediated NK cell activation is involved in killing of tumor cells as well as DCs [179, 180]. DNAM-1 association with the adhesion molecule leukocyte functional antigen 1 (LFA-1) in the NK cell might be required for activation of cytotoxicity [181]. The expression of the ligand PVR was shown to be downregulated by a viral gene product in cells infected with HCMV, probably contributing to a decreased killing of CMV-infected cells by NK cells [182].

3.5.2.11 Integrins

NK cells express integrins such as leukocyte functional antigen LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18), which bind intercellular adhesion molecule (ICAM) -1 on target cells. LFA-1 and ICAM-1 interaction contributes to the initial contact and adhesion to the target cell as well as to granule polarization in the NK cell [151]. This interaction is important for efficient ADCC through the CD16 receptor and also in induction of natural cytotoxicity. LFA-1 is also involved in NK cell extravasation [67].

3.6 NK CELL INTERACTION WITH OTHER IMMUNE CELLS

Immune cells interact in complex networks with each other and this is an area where new knowledge is constantly unraveled. Here I will only touch on this briefly. Interestingly, DCs which are required for activation of NK cells are also affected by the NK cells in many ways [183, 184]. Immature DCs can be killed by NK cells, while

mature DCs are protected and this has been shown to be due to upregulation of MHC class I molecules on the surface of mature DCs [185, 186]. Killing of immature DC may involve interaction with NKp30 and DNAM-1 and killing through TRAIL [163, 179, 180, 187]. NK cells activate immature DCs by releasing the cytokines IFN-γ and TNF in addition to cell-contact dependent signals [188]. This leads to production of IL-12 by DCs, further stimulating NK cells, but the cytokines also indirectly activate T cell responses. Another indirect contribution to the adaptive immunity is the antigens from NK cell lysed target cells provided to DCs, which are presented to T cells. NK cells are also involved in direct priming of Th1 T cells, by contributing with the initial source of IFN-γ [80]. Induction of isotype class switching in B cells is another area where NK cells might be involved .[183]. In addition, NK cells do not only activate immune responses they can also terminate them, which could be of importance in preventing immunopathology [183].

3.7 NK CELLS IN VIRAL INFECTIONS

NK cells contribute to control of certain viral infections, as has been mentioned previously. The majority of data concerns Herpesvirus infections and mainly CMV infection, both in humans and mice. However, NK cells play a role also in other viral infections, for example in infections with Poxviruses, Influenza virus, Hepatitis viruses and HIV-1 [189, 190]. From this research an understanding on how NK cells are activated through virally induced cytokines [86-88, 90, 91] or upregulation of ligands for activating NK cell receptors on infected cell surfaces [32-35] has been gained. NK cells have also been shown to be recruited [191, 192] and to proliferate [193, 194] at the site of infection. This virally induced NK cell activation results in release of cytokines and direct killing of infected cells [88, 90, 195]. Recently, in an adoptive transfer model, specific NK cells expanded during MCMV infection, were shown to respond more vigorously to stimulation than naïve NK cells. This capacity remained up to two months after the infection [196], which indicates that NK cells might have a memory-like function, similarly to the adaptive T and B cells. In two other corroborating reports NK cells stimulated with cytokines or haptens have also had a prolonged memory-like response [197, 198].

In humans, *in vivo* data on the role of NK cells in infection comes from rare immunodeficiencies affecting NK cell numbers or function [21, 199]. The importance of NK cells *in vivo* in infected mice has often been studied by depletion of NK cells with antibodies specific for NK cell markers, such as NK1.1, or by using transgenic mice lacking certain genes important in NK cell function. A problem however has been the lack of specific NK cell depletion since some T cells share most NK cell markers and have similar effector functions as NK cells do. Mice depleted of NKp46 are increasingly used for studies on NK cell functions [48]. In addition, it was recently reported that deletion of a transcription factor acting downstream of the IL-15 receptor results in a specific lack of NK cells [200]. These transgenic mice could be an important tool in future research on many areas concerning NK cell function and development.

Despite the sometimes vigorous NK cell response to infections it is often not enough for complete control of a viral infection. One reason for this could be the immune evasion mechanisms evolved by many viruses [190]. To avoid NK cell activation due to recognition by inhibitory NK cell receptors MHC class I molecules important for NK cell recognition are kept on the cell surface, while others are selectively downregulated

to avoid T cell recognition [201]. Some viruses also express viral homologues of MHC class I which bind inhibitory receptors, as exemplified by the HCMV UL18 protein, which bind LIR-1 [136, 137]. To avoid recognition by activating receptors cell surface expression of activating ligands [168, 175, 182] or receptors [165] are inhibited by certain viral proteins. In HCMV infection certain NKG2D and DNAM-1 ligands are affected by the UL16 and UL141 proteins respectively and NK cell expression of NKp30 is as previously mentioned decreased by binding of the HCMV pp65. Several of these escape mechanisms are used by HIV-1 to avoid NK cell recognition and this is an exciting field of research which will be covered in detail in section 5.2.

4 HUMAN IMMUNODEFICIENCY VIRUS INFECTION

In 1981 USA clinicians identified an immunodeficiency syndrome that was affecting previously healthy individuals that now succumbed to rare infections [202, 203]. The main risk groups were men who had sex with men, intravenous drug users and hemophiliacs. Early on it was suspected that a virus would be the causative agent and in 1983 the French scientists Luc Montagnier and Francoise Barré-Sinoussi with co-workers managed to isolate a previously unknown retrovirus from a patient with symptoms of acquired immunodeficiency syndrome (AIDS) [204]. Knowledge on how to grow the virus in cell culture and how to easily detect it in human specimens was soon to follow [205]. Although the virus was named differently from the beginning the name human immunodeficiency virus (HIV) was subsequently agreed on. Since then the HIV epidemic has escalated to a pandemic with over 60 million people infected of whom 33 million are currently living with the infection. Globally there are about 2.7 million newly infected each year and 2 million people dying from AIDS-related illnesses [206]. Despite this a pleasant news is that the spread of the disease is decreasing, a trend seen over the last ten years [206]. The leading cause of death among HIV-infected world-wide is co-infection with Mycobacterium tuberculosis, which approximately on third of the world's population is infected with. The problem with HIV-1 infections is greatest in Sub-Saharan Africa and Asia, where the health problem is compounded by economic and infrastructural effects.

4.1 THE ORIGIN OF HIV

HIV is a *Lentivirus* belonging to the *Retroviridae* family. Like its relatives within the retrovirus family, the viruses causes a slow chronic disease targeted to hematopoietic cells [207]. There are two different human immunodeficiency viruses: HIV-1, which is the main cause of the AIDS epidemic in the world, and HIV-2, which is a less pathogenic virus spread mostly in West African countries. The HIV viruses resemble viruses present in African non-human primates. It is likely that HIV-1 originates from the chimpanzee (*Pan troglodytes troglodytes*) simian immunodeficiency virus (SIV_{CPZ}) [208] and that HIV-2 originates from the sooty mangabey SIV (SIV_{SM}) [209]. It is believed that humans acquired these viruses through cross-species transmission and it is estimated that the first infection with HIV-1 took place in the early 20^{th} century [210]. The oldest evidence for HIV-1 infection was identified in a retrospective study on stored plasma samples and dates back to 1959 [211]. There are three different groups of HIV-1: M (major), O (outlier) and N (non-M, non-O). Approximately 95% of all HIV-1 isolates belongs to group M, which can be further divided into eleven clades or subtypes (A-K) [207]. Circulating recombinant forms of these subtypes are also present to varying extents in the world.

4.2 HIV-1 INFECTION AND PATHOGENESIS

HIV-1 is spread by sexual contacts, contaminated blood, or from mother to child during pregnancy, birth or breastfeeding. The majority of infections in the world occur through heterosexual contacts, this despite that the risk of infection per sexual act is estimated to be below 1% [212]. Factors that are known to influence the risk of transmission are genital infections and lesions, type of sexual activity and viral load in the infected partner.

HIV-1 infection through the genital mucosa results in binding to DCs or direct infection of DCs or Langerhans cells, which is another APC present in the epithelium [213]. DCs express a molecule called DC-SIGN which traps the HIV-1 virus in the genital mucosa and allows it to be transported to lymphoid tissues [214]. Infected APCs also disseminates to draining lymph nodes where active $CD4^+$ T cells, the prime target for HIV-1, are infected. In the case of presence of other genital infections or inflammation the risk of viral transmission increases. Co-infections can cause ulcerations, which leads to breaching of the epithelium and influx of $CD4^+$ T cells to the site of viral entry [213].

In the acute phase, occurring within a few weeks of HIV-1 transmission, the viral load is high *(Figure 4)* and flu-like symptoms appear in most individuals due to immune hyperactivation. During this phase the majority of the gastrointestinal tract $CD4^+$ T cells are lost [215] and the gastrointestinal tract mucosal barrier is injured. After the acute phase, the level of virus in plasma decreases to a stable level, called the viral set point. The viral set point is predictive of the disease course, since a high viral set point correlates with a more rapid disease progression [216]. Following the acute phase of the infection, comes the latent phase that may last for years. During this phase the $CD4^+$ T cell count decline slowly, probably due to viral replication and the chronic systemic immune activation. Chronically infected patients have elevated levels of LPS in the blood, which might contribute to the chronic immune activation. The elevated LPS levels is thought to result from microbial translocation over the injured mucosal barrier of the gastrointestinal tract [217].

When the $CD4^+$ T cell count falls below 200 cells/µl of blood, opportunistic infections usually appear and this defines the AIDS stage of the infection. Examples of opportunistic infections that are common for AIDS patients include Pneumocystis carinii, Mycobacterium avium and viral infections that cause AIDS defining malignancies like Kaposi's sarcoma and various lymphomas. In the late stage of the disease, the viral load increases sharply and the $CD4^+$ T cell counts decrease rapidly resulting ultimately in immune incompetence and death due to opportunistic infections.

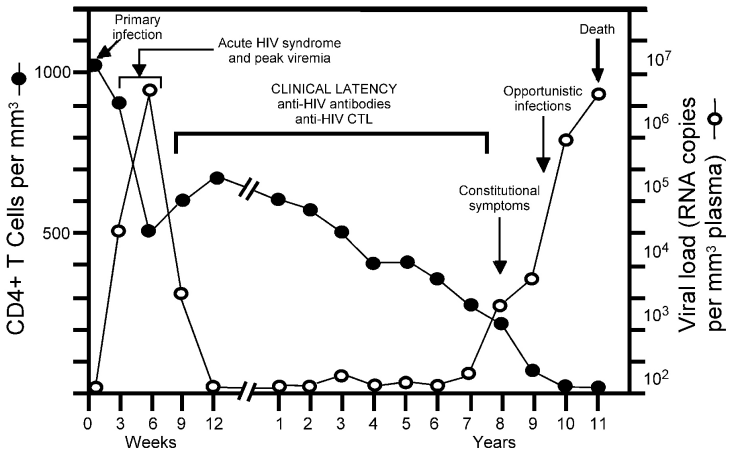

Figure 4: The clinical course of HIV-1 infection

4.3 VIRAL STRUCTURE AND REPLICATION

4.3.1 The replication cycle of HIV-1

HIV-1 is an enveloped virus with a protein core that encloses two single-stranded genomic RNAs and various regulatory proteins important for the replication cycle *(Figure 5a)*. The infection starts when the HIV-1 envelope protein gp120 binds to a CD4 molecule on a target cell *(Figure 5b)*. CD4 is mainly expressed on T helper cells, macrophages, monocytes, DCs and brain microglia. When gp120 binds its receptor conformational changes are induced that result in exposure of the co-receptor binding-site on gp120. The most important co-receptors for HIV-1 are the chemokine receptors CCR5 and CXCR4 [218]. The interaction with the co-receptor leads to an additional conformational change in the viral transmembrane envelope protein gp41, which is linked non-covalently to the gp120 protein. The gp41 protein then mediates fusion between the viral and cellular membranes [219].

After fusion the viral capsid is released into the cytoplasm of the cell. The viral RNA genome is then reversibly transcribed by the viral reverse transcriptase (RT) into double-stranded DNA. During this process, mutations are frequently introduced since the reverse transcriptase lacks proof-reading mechanisms. These mutations are important for the viral diversity and can result in escape from the immune system and from antiretroviral drugs. The preintegration complex, consisting of viral DNA and integrase, is directed to the nucleus by the accessory protein viral protein r (Vpr) [220]. Linear double-stranded DNA is then integrated into the host genome, catalyzed by the viral enzyme integrase. The site of integration is not chosen randomly, rather it targets specifically transcriptionally active genes [221].

The virus can establish a latent infection if the infected cell returns to a resting state when viral DNA has been integrated in the genome. This transition from an active to a resting state can be exemplified by activated $CD4^+$ T cells that develop into memory cells. Virus is produced by transcriptionally active cells, as cellular transcription factors are necessary for transcription to be initiated from the HIV-1 long terminal repeat (LTR) flanking the HIV-1 DNA genome. In resting cells, these transcription factors are not expressed and this together with the formation of a heterochromatin structure at the site of integration is believed to contribute to lack of proviral transcription [222]. The heterochromatin structure results in reduced capacity of binding of transcriptional factors due to sterical hindrance [222]. The latent phase can persist for years, throughout the life-span of the infected cell, but is reverted upon activation of the cell.

In activated cells, the viral DNA is transcribed into RNA and this step is greatly enhanced by the transactivating viral protein Tat. RNA is then transported to the cytoplasm, aided by the viral regulator of RNA transport (Rev) protein [220], where translation into polyproteins takes place. The polyproteins are formed from the structural genes: group-specific antigen (Gag), polymerase (Pol) and envelope (Env). Three different polyproteins are formed: Gag, Gag-Pol and Env [223]. Gag directs the assembly of viral proteins and RNA beneath the cell membrane. These proteins include Gag and Gag-Pol polyproteins and accessory proteins like Vpr, viral infectivity factor (Vif) and negative factor (Nef). The Env gene is translated into the fusion protein gp160 in the ER. Cleavage into the envelope proteins gp120 and gp41 takes place in the Golgi apparatus before the proteins are transported to the cell surface. The envelope

proteins form trimers consisting of three transmembrane gp41 molecules in complex with three gp120 molecules [219]. These are incorporated in the budding virion by binding to the matrix proteins, which line the inner surface of the virion membrane.

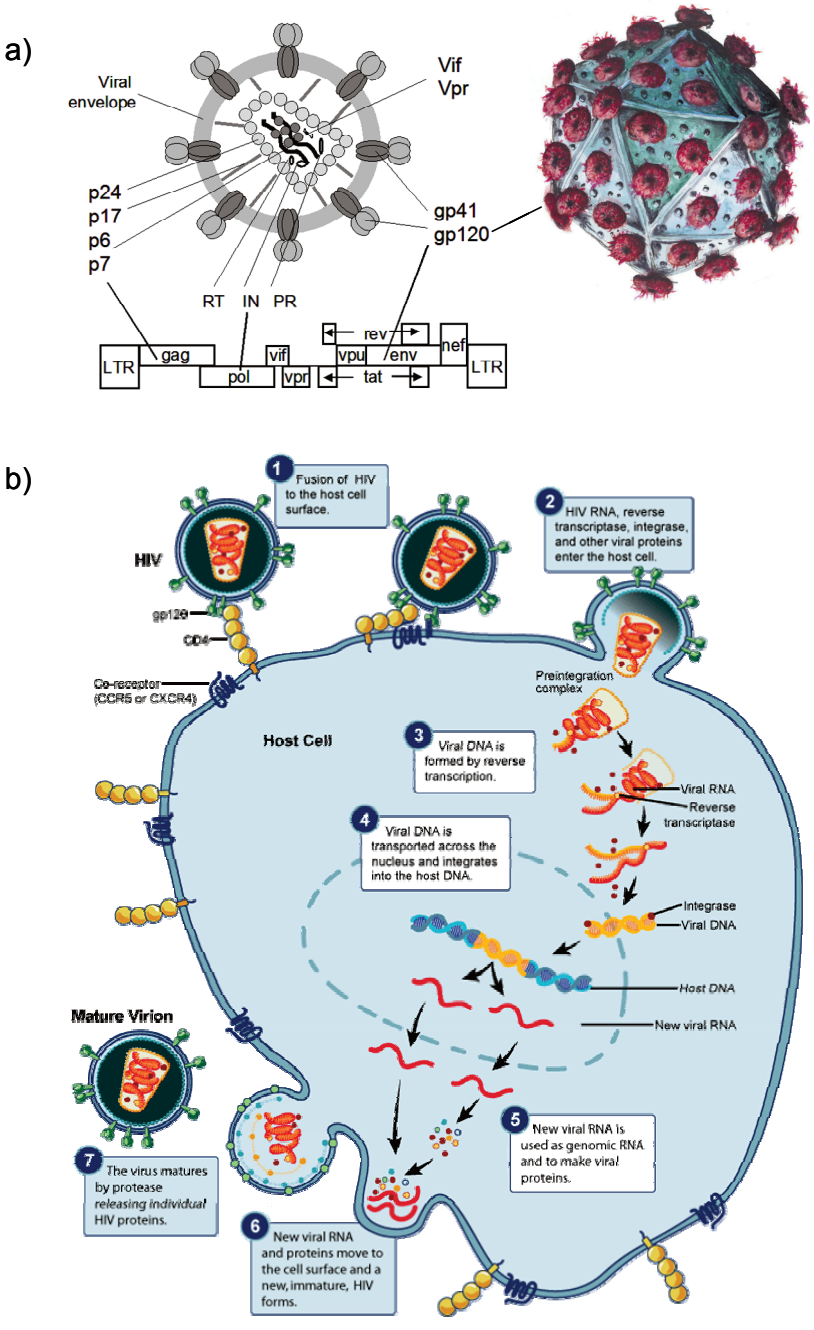

Figure 5: a) The viral structure and genome. b) The viral replication cycle of HIV-1.
Courtesy: a) Annika Wiklund b) National Institute of Allergy and Infectious Diseases

The Vpu and Nef proteins are involved in degradation of CD4 molecules, in order to avoid interaction between the newly synthesized Env proteins, enabling the viral surface proteins to be transported to the surface and new virions to be released from the cell [223]. During assembly and during budding from the cell surface the Gag and Gag-Pol polyproteins are cleaved by protease. Cleavage of Gag results in formation of p17 matrix, p24 capsid, nucleocapsid (NC or p7) and p6 proteins. Pol encodes the enzymes viral protease, reverse transcriptase and integrase. This cleavage leads to structural reorganizations essential for viral infectivity [207, 220, 223]. HIV-1 can also infect neighboring cells through directed release at cell to cell contacts where a virological synapse is formed with a preferential accumulation of Gag and Env proteins [224].

4.3.2 The envelope protein structure

The gp120 molecule is divided into five conserved (C1-C5) and five variable (V1-V5) segments. The inner domain of the gp120 protein in its trimeric conformation is mainly formed by the C1 and C5 regions [225]. This part of gp120 is in contact with the transmembrane gp41 molecule. The gp120 molecule also has a heavily glycosylated outer domain and in between the inner and outer domain lies a bridging sheet. The interface between the inner domain, bridging sheet and outer domain forms the binding site for CD4. As mentioned, when CD4 is bound conformational changes in gp120 results in exposure of regions with co-receptor binding properties. One of these regions is the V3 loop, which is partly shielded by the variable loops V1 and V2. V3 is likely to protrude from the core and interact with the co-receptor upon CD4 binding [226]. This extended form of the V3 loop could be an explanation to the immune dominant responses to this region. CCR5 is the primary co-receptor during mucosal transmission but later in the course of infection, when the immune pressure is lower, the virus often changes the co-receptor usage to CXCR4. This change in co-receptor usage is believed to be due to mutations in the V3 loop [227].

4.4 PREVENTION AND TREATMENT

4.4.1 Antiretroviral treatment

Since the introduction of antiretroviral treatment (ART) in the mid 1990´s, the death rate in AIDS has declined sharply in the Western world. Antiretroviral treatment now consists of a combination of drugs targeting different parts of the viral life cycle. The most commonly used drugs target the reverse transcriptase and the protease. Drugs that inhibit the fusion of HIV-1 with the target cell, by binding to gp41 and preventing the conformational change needed for fusion, are also used. Novel drugs include integrase inhibitors that block integration of HIV-1 DNA into the host genome and CCR5 antagonists that interfere with the binding of HIV-1 to the target cell [228]. Efficient ART treatment leads to a rapid decrease in the plasma virus levels down below the limit of detection (<50 copies per ml), because infection of new susceptible cells is almost completely stopped. ART treatment also results in increased $CD4^+$ T cell counts.

Despite the positive effects of ART, the virus is not eradicated and HIV-1 is still a life-long disease without any cure as of today. This is due to latently infected resting memory $CD4^+$ cells that persist despite long-lasting ART treatment [229]. The virus also hides in compartments like the central nervous system with poor antiretroviral drug penetration. Upon therapy interruption there is a rapid onset of virus replication and production of new viruses. An interesting field of research is the development of

interventions to specifically antagonize the mechanisms of latency [230]. This is meant to induce proviral replication in latently infected cells in presence of effective ART, something that might result in a reduction of the pool of latent virus.

A major drawback of the antiretroviral drugs is that they are toxic to a varying degree and can cause numerous side effects, such as gastrointestinal disturbances and metabolic rearrangements, and this can lead to problems with medical compliance since the treatment needs to be continued indefinitely. Although the availability of ART in low and middle income countries has increased substantially in the last five years [231], the high costs and difficulties in distribution of ART are problematic for combating the HIV-1 epidemic. Due to the high mutation rate in HIV-1 combined with the problems of adherence, resistance to drugs is also becoming a problem in HIV-1 treatment [228]. New ways of treating HIV-1 are therefore needed and much attention has been given to different ways of activating the immune response against HIV-infected cells.

4.4.2 Preventive measures

The most effective strategy for preventing sexual HIV-1 transmission as of today is education on HIV infection and transmission, and promotion of condom use. Male condoms, if consistently and correctly used, decreases the risk of infection with 95%, although problems with non-coherent usage decreases the estimated protective effect substantially [232]. Another preventive measure is male circumcision, which in three large randomized trials performed in Africa reduced the risk of male infection with about 60% [233]. The high concentration of cells susceptible for HIV-1 in the foreskin might explain this beneficial effect [234]. A major problem is that condom use and circumcision both are controlled by males and as females globally account for 60% of all HIV-1 infections there is a need for female-targeted preventive measures. Intravaginal application of microbicides, which are substances that reduce the infectivity of a microbe, could serve such a purpose. The action of a microbicide can be to disrupt the pathogen membrane, mainly through the use of detergents, or to lower the pH of the intravaginal tract to reduce infectivity. Microbicides can also be specifically targeted to the HIV-1 virus or host cell proteins bound by the virus [235]. New types of microbicides are constantly developed and tested in the search for a successful agent. A good preventive measure is lowering of the viral load in infected patients by ART treatment, which reduces the risk of transmitting the virus. Importantly, ART treatment of HIV-1 infected mothers is an effective countermeasure for mother-to-child transmission which together with terminated breastfeeding have decreased the risk of transmission to only 1% in high income countries [236]. The best preventive strategy would be a protective HIV-1 vaccine. It is believed that such a vaccine should induce specific neutralizing antibodies and/or T cell responses, and different vaccine modalities inducing this kind of responses are constantly being explored (see section 6.2.3)

4.5 ANIMAL MODELS IN HIV RESEARCH

There is no proper animal model for HIV-induced AIDS; and the closest model has been the chimpanzee, which can be infected with HIV-1, but rarely develop AIDS [237]. Rhesus and cynomolgus macaques can be infected with SIV or a genetically engineered variant of the virus, called SHIV, which is carrying some of the genes from HIV-1. Depending on which virus is used SIV and SHIV can cause AIDS in macaques within a couple of months from infection [238, 239]. Macaques are used for studying HIV pathogenesis and evaluation of potential vaccines and immunotherapeutics. Sooty

mangabey monkeys are naturally infected with SIV but do not develop AIDS and are therefore an interesting model for studying natural resistance to disease [240].

For ethical and economic reasons, primates cannot be used as a first line model for testing new immunotherapeutics. Instead, small animal models, such as mice, are preferable. HIV-1 replication in rodent cells is restricted due to species differences in cellular host factors like Cyclin T1 [241] and APOBEC3G (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G) [242]. Mouse cells also lack the receptors for HIV-1 entry. To overcome these species barriers models of various kinds have been developed for HIV-1 infection in mice. These include sever combined immunodeficiency (SCID) mice with human xenografts, mice irradiated and repopulated with human cells and transgenic mice [243]. In the SCID mouse models human peripheral blood mononuclear cells (PBMCs) or tissues from thymus and liver, permissive for HIV replication, are engrafted into the mice. In some models such as the SCID-hu (Thy/Liv) mice $CD4^+$ T cells are depleted resembling the infection in humans [244]. In newer SCID-models, where human hematopoietic stem cells are transplanted, functional human immune systems with primary humoral and cellular responses to HIV-1 have been achieved, although for a limited period of time [245, 246].

Instead of modulating the mice, it is possible to instead construct pseudoviruses that express HIV-1 antigens and have the ability to infect murine cells. This can be done by constructing HIV-1 particles that bind other receptors than CD4 and CCR5/CXCR4. The sodium phosphate channel receptor Ram-1 is expressed on cells of many species and is used by amphotropic retroviruses for entry [247-249]. By exchanging the HIV-1 envelope with the envelope protein from a virus with tropism for this receptor HIV-1 can enter murine cells [250]. Exchange of viral envelopes can be achieved naturally by infecting a cell with two viruses, a process referred to as pseudotyping. One model for this is the HIV-1/MuLV challenge model where pseudovirions of HIV-1 and murine leukemia virus (MuLV) are produced (described below) [251, 252]. Another model based on the same principle and recently developed by Potash et.al. takes the advantage of genetical engineering to exchange the coding region for the HIV-1 gp120 envelope with the MuLV envelope gp80 in a plasmid encoding the virus [253]. This virus is able to spread to multiple organs in infected mice and induce immune responses to HIV-1.

4.5.1 The HIV-1/MuLV mouse challenge model

We have chosen to use the HIV-1/MuLV challenge model as a model system for HIV-1 infection. Pseudoviruses are produced by *in vitro* HIV-1 infection of a cell line that carries an integrated murine leukemia virus (MuLV) genome *(Figure 6)*. The cell line will theoretically produce four different viral variants, with HIV-1 or MuLV genomes each contained in virions surrounded by HIV-1 or MuLV envelopes. These pseudoviruses are harvested and subsequently used for infecting activated murine splenocytes, which can be infected with the viral particles with a MuLV envelope (MuLV/MuLV or HIV-1/MuLV). The syngeneic HIV-1/MuLV infected splenocytes are then injected intraperitoneally (i.p.) into mice. After ten days, cells from the peritoneal cavity are harvested from the mice and the peritoneal cells are co-cultured *in vitro* with HIV-1 susceptible cells, such as Jurkat T cells or activated human PBMCs. The amount of viral replication is measured by production of HIV-1 p24. This is used as an indirect measurement of amount of infected cells present in the peritoneal cavity of the mice [252].

It is unclear if HIV-1 actively replicates in the transplanted murine cells and the virus is thereby unlikely to spread to new cells *in vivo,* unless helper MuLV is present. The model thus mostly resembles the antigen exposure during the acute phase of HIV-1 infection in humans. Injection of infected cells induces HIV-1 specific cellular and humoral immune responses probably contributing to the complete clearance of the infected cells 4-6 weeks after challenge [252]. Unspecific stimulation with Toll-like receptor adjuvants, like CpG-oligonucleotides (ODNs), seem to induce a level of protection against the HIV-infected cell challenge [243]. This indicates that also innate immune mechanisms can contribute to clearing of the infected cells. The role for NK cells in the HIV-1/MuLV challenge model was investigated in **paper III.** Several vaccine evaluation studies have been performed with this model and have shown that vaccinated mice can be protected against challenge with infected cells [243, 252, 254]. The model is also suitable for investigating effects of other immunotherapeutic strategies as discussed in relation to a drug-conjugated anti HIV-1 envelope antibody in **paper IV**.

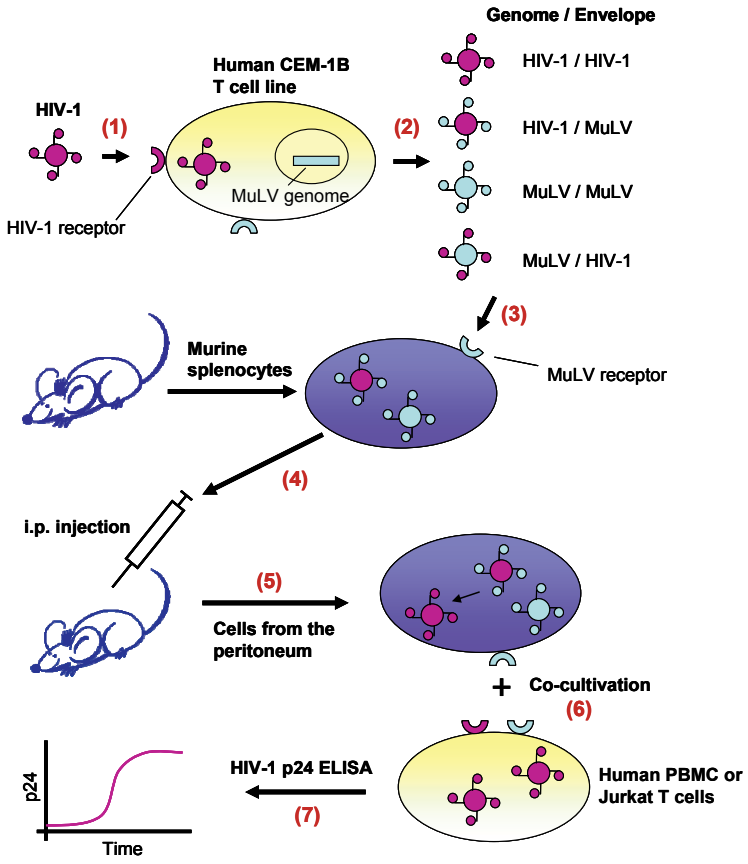

Figure 6: The HIV-1/MuLV challenge model. (1) A human T cell line with a proviral MuLV is infected with a HIV-1 isolate. **(2)** Four types of particles are produced with HIV-1 and/or MuLV genome/envelope. **(3)** These are used to infect primary murine splenocytes *in vitro*. **(4)** The infected cells are injected intraperitoneally into syngeneic mice. **(5)** One to ten days later peritoneal cells are harvested and **(6)** co-cultivated with a human T cell line or activated PBMCs. **(7)** Supernatant is retrieved and HIV-1 p24 is quantified.

5 THE HOST RESPONSE AGAINST HIV-1 INFECTION

5.1 CORRELATES OF PROTECTION

There are no definite correlates of protection against HIV-1 infection or control of viral replication, but there are many factors identified that are suggested to be important. Some HIV-1 infected patients are able to control their infection without treatment. These individuals have been grouped into three different categories. *Long-term non-progressors* (LTNPs) are patients that have been infected for a long time with stable CD4 counts and often low viral loads [255]. *Controller patients* are patients that are able to suppress the viral load for an extended period of time and *elite controller patients* are patients that maintain a viral load of below 50 HIV-1 RNA copies/ml for long periods (at least one year) [256]. In **paper I and II** we use the designation controller patients, although some of these patients might also fit into the LTNP or elite controller groups. Another interesting group of individuals is the exposed uninfected (EU) that seem to have some kind of protection against infection, as they remain uninfected despite repeated exposures to the virus [257]. These individuals have been found among commercial sex workers, discordant couples and intravenous drug users [258-260].

Extensive studies have been performed on these groups of patients and the exposed individuals to try to identify parameters that correlate with a slower disease progression or protection from infection. Although there are some studies suggesting that patients that control their infection are infected with a defective virus, the majority of results indicate that most controller patients are infected with replication-competent viruses, indicating that there is some protective mechanism in these patients acting to control the virus [261]. It is plausible to believe that genetic differences and host immune responses may contribute to the outcome of the infection.

5.1.1 Innate immune responses to HIV-1 infection

The innate immune response is a possible contributor to protection from HIV-1 infection. Early events in the acute HIV-1 infection appear to have an effect on disease progression, and it is therefore likely that features of the innate immune response play a role in the establishment of a state of control of the virus. HIV-1 can activate the pDCs to produce type I interferons by binding of single stranded RNA or CpG DNA motifs to TLR7/8 and TLR9 respectively. Certain polymorphisms in the TLR9 gene have been associated with rapid disease progression [262]. In addition, pDCs are present in increased numbers in controller patients compared to uninfected individuals and patients with a progressive disease [263]. NK cells and NKT cells from EUs have an increased activity as compared to uninfected individuals, indicating a role in protection from infection [260, 264]. Other innate immune mechanisms include intracellular antiviral proteins such as APOBEC3G, which hypermutate the viral RNA leading to lethal mutations in the viral genome [242]. HIV-1 counteracts APOBEC3G via the viral protein Vif, which targets APOBEC3G for degradation. However, the protein may be upregulated in EUs and an increased APOBEC3G mRNA level has been shown to correlate with lower viral load [265].

5.1.1.1 NK cell responses to HIV-1 infection

The potential importance of NK cell activity in protection from primary infection was first shown in a study of exposed uninfected Vietnamese intravenous drug users. PBMCs from these EUs had a significantly increased cytotoxic activity against the NK cell sensitive cell line K562 and an increased proportion of NK cells from these patients produced the cytokines IFN-γ and TNF-α and the CC-chemokines MIP-1α, MIP-1β and RANTES than both intravascular drug users who became HIV-1 infected, or uninfected controls [260]. They also had increased frequency of $CD69^+$ NK cells *ex vivo* and an increased frequency NK cells degranulating *ex vivo* without *in vitro* addition of stimulants [266]. The correlation between a high cytokine production by NK cells and protection from infection has also been shown in a cohort of sexually exposed EUs [264].

In acute HIV-1 infection, before onset of the adaptive immune response, the frequency and absolute number of NK cells in the blood are increased [267, 268]. There is a preferential expansion of $CD56^{dim}CD16^+$ NK cells [268, 269] and NK cell activity is elevated and parallels the decrease of viral load from peak viremia to the viral set point [267]. This indicates that NK cells may contribute to the early immune response to HIV-1 infection. Similarly, in SIV infected rhesus macaques NK cell cytotoxicity and CD69 expression on NK cells is elevated early after infection [270]. When investigating the effects of NK cells in control during infection, SIV infected macaques that control their viral load for an extended time were shown to have increased production of IFN-γ as compared to viremic macaques [271]. NK cell cytotoxicity and chemokine production is elevated in humans infected with the less pathogenic HIV-2 virus as compared to HIV-1 infected patients with similar CD4 counts [272]. This suggests that an enhanced NK cell activity may be involved in control of HIV-2 infections. Studies on human HIV-1 controller patients have not given any clear correlates of control regarding NK cells. Controller and viremic patients have similar frequencies of NK cells and this frequency is often lower than for healthy individuals *(Figure 11)* (**paper I** and [268, 273, 274]). In addition, controller patients do not seem to have a stronger NK cell activity as compared to viremic patients and healthy controls (**paper I** and [274]).

NK cells can mediate control of infection *in vitro* through several mechanisms *(Figure 7).* Direct cytotoxic killing of HIV-1 infected tumor cell lines have been reported [275], while presence of NK cell cytotoxicity against autologous HIV-1 infected $CD4^+$ T cells have been less clear. Bonaparte et.al showed that NK cells from healthy individuals are unable to kill autologous $CD4^+$ T cells infected with HIV-1 [25, 276], whereas Tomescu et.al reported an increased NK cell cytotoxicity against autologus $CD4^+$ T cells infected with a primary HIV-1 strain [277]. NK cells from viremic patients also had an increased NK cell cytotoxicity against autologous $CD4^+$ T cells stimulated to express endogenous virus [278]. NK cells can inhibit viral replication in autologous $CD4^+$ T cells in a cell-contact dependent manner *in vitro* [279]. However they can also suppress viral infection *in vitro* by production of CC-chemokines, which bind to the CCR5 co-receptor and prevent the virus from entering the cell, by competing for or down-regulating CCR5 [82, 235, 280]. Another potent NK cell mediated mechanism for killing of HIV-1 infected cells is ADCC in presence of HIV-1 specific antibodies (**paper II** and [281]).

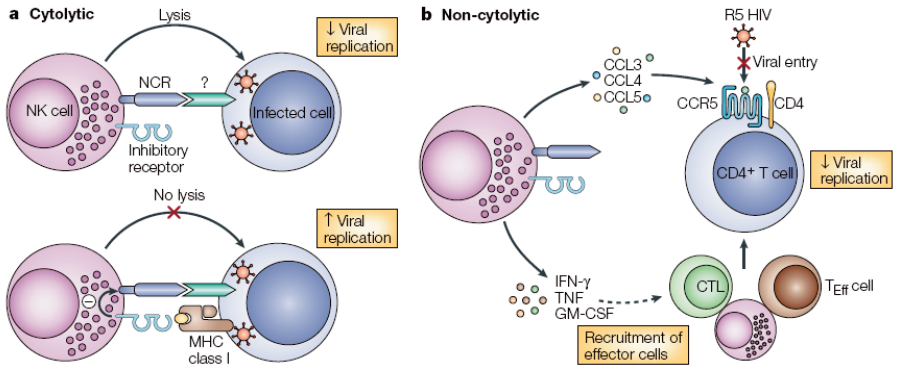

Figure 7: Possible effects exerted by NK cells on HIV-1 infection. a) NK cells that recognise activating ligands on the infected cell in the absence of MHC class I molecules may kill the infected cell. b) NK cell secreted chemokines and cytokines block viral entry or activate other immune effector cells to respond to the virus infection.CCL3 = MIP-1α, CCL4 = MIP-1β, CCL5 = RANTES. *Reprinted by permission from Macmillan Publishers Ltd: Nature reviews immunology, Fauci et.al. 2005 Nov;5 (11):835-43, copyright 2005.*

5.1.2 Adaptive immune responses to HIV-1 infection

The adaptive immune response reacts specifically to the HIV-1 antigens and it is well established that the mounted response is able to suppress the viral replication. However, ultimately in the vast majority of cases, the virus will win this struggle.

5.1.2.1 Anti-HIV-1 antibodies

In non-human primate experiments it has been shown that antibodies are important for protection from HIV-1 and SHIV infections [282-285]. This was proven by passive immunizations of non-human primates with neutralizing monoclonal antibodies in different infection models [282-285]. Although in most of these trials the passive immunizations did not induce complete protection, antibody treatment before challenge was able to prolong the time until infection. Interestingly, antibodies delivered after SIV challenge of macaques also led to control of disease [286], indicating that antibodies may exert effects during the acute infection. This was corroborated by a study where B cells were depleted in macaques before SIV challenge, resulting in lower antibody responses, higher viral loads and a more rapid progression [287]. In addition to neutralization, antibodies can also exert protective effects via other mechanisms including ADCC or complement mediated lysis. Recently Hessell et.al. investigated the importance of the Fc-part of the antibody molecule in SHIV challenged macaques and their results indicate that it is not merely the neutralizing ability of antibodies that is important but also the ability to bind to Fc-receptors [288, 289]. The potential effect of the antibody Fc-part was further confirmed in neonatal macaques that was protected from oral SIV challenge by infusion of sera without neutralizing ability but with known antibody-dependent cell-mediated virus inhibition activity [290, 291]. Passive immunization has also been carried out in HIV-1 infected patients and the results show that neutralizing antibodies can transiently affect the viral load, but that the

rapid development of virus with resistance against the transferred antibodies limits the therapeutic use of such a treatment [292].

In infected humans, HIV-1 triggers production of high levels of antibodies against the envelope proteins gp120 and gp41, but most of these antibodies either cannot bind the virion or binds to epitopes that are not important for neutralization of the virus. The reason for this may be that the majority of antibodies produced have been triggered by shedded free envelope proteins and viral debris from lysed cells [293]. The HIV-1 virus can escape antibody recognition, indicating that antibody-mediated mechanisms can contribute to immunologic control of viral replication [225, 294]. Both high antigenic variation at immunogenic regions and structural features of the envelope protein contribute to the resistance against antibody binding. The highly variable loops of gp120 can cover and sterically hinder antibodies from reaching parts that are essential for entry of virus. The trimeric envelope complex also makes it possible for potential epitopes to hide inside the trimer and only become exposed when gp120 binds to CD4 and changes conformation. Another mechanism for masking of essential epitopes is the extensive glycosylation of the envelope protein, with glycans making up 50% of the protein mass [295]. It is also possible that HIV-1 specific antibodies enhance infection of cells by mediating binding of antibody-bound virus particles to Fc-receptors or complement receptors on cells. This may result in uptake of HIV-1 via phagocytosis and subsequent infection of the phagocyting cell or dissemination to other cells [13]. However, none of the human or animal studies where high amounts of antibodies have been injected *in vivo* have given any evidence of antibody-driven enhancement [292].

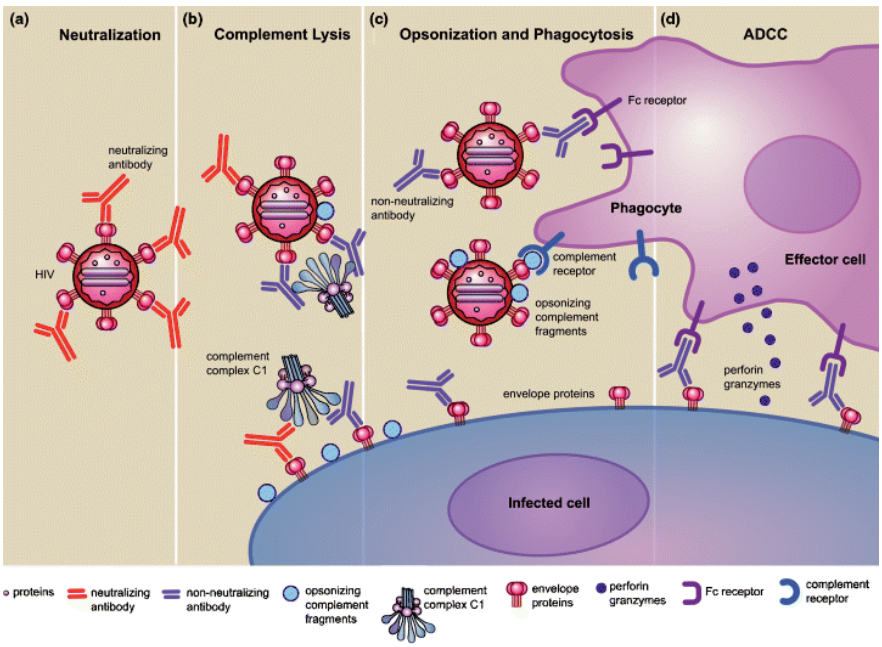

Figure 8: Antibody mediated mechanisms for combating HIV-1. a) Neutralization of free virus by antibodies, b) complement-mediated lysis of free virus and infected cells triggered by antibodies, (c) opsonization of virus particles by antibodies and phagocytosis of virus particles via Fc- or complement-receptors, (d) ADCC against infected cells. *Reprinted by permission from Wiley InterScience: Journal of Internal Medicine, Huber and Trkola, 2007 Jul 262, (1), 5-12, copyright 2007.*

Neutralizing antibodies

Neutralizing antibodies against HIV-1 *(Figure 8a)* have been suggested to appear after the viral load has declined in the acute phase of HIV-1 infection, which would indicate that they are not important for this first control of viral replication after infection [296-298]. However neutralizing antibodies to autologous virus can develop within weeks of seroconversion and drive the evolution of viral escape mutations in the envelope protein [299], indicating that neutralizing antibodies exert a certain level of selective pressure. Most neutralizing antibodies appearing early during infection are highly specific for the autologous virus, while cross-reactive antibodies that can neutralize heterologous viral isolates may develop later over time as the infection progresses [296, 297, 299]. Strong broadly neutralizing antibodies have been described in some LTNPs [297, 300], however most elite controller patients have low levels of neutralizing antibodies suggesting that an effective control of the virus can be achieved without these antibodies [301-303]. An effective neutralizing antibody can inhibit infection by preventing receptor or co-receptor interactions or preventing virus-membrane fusion [13, 225, 304]. Thus, most neutralizing antibodies are directed to the CD4-bindig site, to the CD4-induced binding epitopes involved in binding to co-receptors and to the V3 loop in gp120. They can also be directed to the membrane proximal external region of gp41. HIV-1 specific mucosal IgA antibodies have been correlated with possible immune protection. These antibodies are present in many EUs and some may neutralize HIV-1 [257, 258, 305].

ADCC inducing antibodies

HIV-1 specific ADCC responses are readily detected in most HIV-1 infected patients *(Figure 8d)*. ADCC inducing antibodies can hinder cell to cell spread of the HIV-1 virus, since infected cells are targeted by the effector cells [306]. ADCC inducing antibodies are present early in infection before the initial drop in viral load, implying an important function in the acute infection [307-309]. These antibodies also have a broad cross-reactivity against the envelope protein, evident already in the acute infection [308, 310]. ADCC-mediating antibodies have been described to influence disease progression in HIV-1 infected patients; strong ADCC responses associate with slower disease progression and lower viral loads (paper II and [302, 311-313]). In addition to antibodies in plasma, HIV-1 specific ADCC mediating antibodies are also present in vaginal fluid where antibody titers correlate negatively with genital viral load [314]. However, contradicting reports exist where no correlation between disease progression and ADCC antibodies were observed [315, 316].

Other antibody mediated effector mechanisms

Another critical effector mechanism exerted by HIV-1 specific antibodies is activation of the complement system which results in lysis of virions and infected cells *(Figure 8b)*. Complement lysis activity correlates inversely with viral load in the acute phase of the infection and could thus be involved in the early immune-mediated control of the virus [317]. Phagocytosis and opsonization of antibody coated virions are also important mechanisms in clearing virus particles [13] *(Figure 8c)*.

5.1.2.2 T cell responses to HIV-1 infection

HIV-1 infected patients develop strong virus-specific CTL responses against HIV-1 proteins [318]. In primary infection, HIV-1 specific CTL responses correlate to the decrease in viral load after the acute phase of the disease [298, 319]. Depletion of $CD8^+$ cells in monkeys points to the importance of these cells in control of SIV viremia [320].

The appearance of mutations in CTL epitopes of viral proteins, which probably is due to a selective pressure by the CTLs on the virus to change its amino acid sequence, strengthens the importance of the cytotoxic cellular responses [321]. Recently the quality of the CTL response has been appreciated and shown to correlate with disease progression. A polyfunctional response has been defined as CTLs producing several cytokines, often including IFN-γ, MIP-1β, IL-2, TNF-α and being positive for the degranulation marker CD107a. HIV-1 controller patients have a more polyfunctional CTL response [322], in addition to a response preferentially directed to the more conserved Gag protein as compared to the Env protein [303, 323, 324]. Some HIV-1 infected controller patients have $CD8^+$ T cells that can control HIV-1 infection in autologous $CD4^+$ T cells *ex vivo* and this activity is correlated with HIV-1 Gag specific CTL responses [325, 326]. A high percentage of EUs have HIV-1 specific CTL responses, although this response decline over time after the last exposure and does only seem to be protective during the period of time when the person is frequently exposed to antigen [257, 259, 327].

The lymphoproliferative response to HIV-1 antigens is inversely correlated to HIV-1 viral load [328, 329]. Also in $CD4^+$ T cells is co-production of IL-2 and IFN-γ beneficial since it correlates to control of viremia [303, 324, 330]. HIV-1 specific $CD4^+$ T cell responses occur in EUs [257, 331] and these $CD4^+$ T cells proliferate and produce high amounts of IL-2 in addition to CC-chemokines [332].

5.1.3 Genetic factors associated with disease progression and protection.

There are several gene polymorphisms that have been associated to slower disease progression [265], however only co-receptors, HLA and KIR, which are the most relevant to the thesis, will be described here.

5.1.3.1 CCR5

One factor that can contribute to protection from infection and control of disease is the genotype of the chemokine receptor CCR5 [333]. Strikingly, it has been shown that individuals homozygous for a mutation (CCR5Δ32) resulting in a defective form of CCR5, are almost completely protected from infection of CCR5-tropic HIV-1. Heterozygotes have lower expression of functional CCR5 on cell surfaces and have often a delayed disease progression. The presence of this genotype is highest in the Caucasian population, especially in people in northern Europe, and it is almost absent in black Africans [265, 334]. Several other genetic variants of CCR5 and other co-receptors for HIV-1 that may influence disease progression have been described [265]. There are also genetic variants of the co-receptor ligands, most importantly the CC-chemokines RANTES and MIP-1α that may contribute to protection from infection or a slower rate of disease progression [265].

5.1.3.2 HLA

Several HLA types associate with different outcomes of HIV-1 infection. Homozygosis for HLA alleles in general and presence of certain HLA alleles, such as HLA-B35 and Cw4, have been shown to associate with a more rapid disease progression [335]. Presence of the HLA-B57 allele, on the other hand, is associated with slower progression to AIDS and is more prevalent in controller patients, as is the HLA-B27 allele and homozygosis for the HLA class I Bw4 epitope [336, 337]. Recent studies estimate that the frequency of protective HLA alleles is about 60% in patients that can

control their viremia [303, 324]. It is possible that the HLA associated effects on disease progression are linked to differences in presentation of antigen for T cells [338] and this strengthens the fact that cytotoxic T cell responses are important for control of the infection. Another explanation can be that different HLA alleles are recognized differently by KIR molecules expressed on NK cells as will be discussed in the next section [339]. A single nucleotide polymorphism in the HLA-C promoter was associated with lower viral loads in a whole genome association study [340]. This polymorphism results in a higher expression of the HLA-C protein per cell which might have an effect on NK cell recognition, but also on T cell recognition since HIV-1 infected cells preferentially downregulate HLA-A and -B, but keep the expression of HLA-C [201]. However, a subsequent study in another cohort of patients pointed out that a linkage disequilibrium between the HLA-C promoter polymorphism and presence of the protective HLA-B57 allele may explain the positive effect on viral load [341].

5.1.3.3 KIR

The importance of KIR recognition in HIV-1 progression is supported by studies which show that KIR genes alone, as well as interactions between different KIRs and HLA ligands, can influence the outcome of HIV-1 infection [339]. Not all studies agree on which KIRs or KIR/HLA combinations that associate with a better outcome in HIV-1 infection, but KIR3DL1/DS1 and HLA-Bw4 alone or in combination have received particular attention. In the first study by Martin et.al., the combination of genes for the activating KIR3DS1 and HLA-Bw4Ile80 associated with delayed progression to AIDS [342], subsequently suggested to be due to both a modest lowering of viral load and protection against opportunistic infections [343]. In early HIV-1 infection presence of KIR3DS1 has been associated with higher CD4 counts, and presence of HLA-Bw4Ile80 with lower viral load, when investigated separately [344]. However, Gaudieri et.al. found that possession of both KIR3DS1 and HLA-Bw4Ile80 associated with faster disease progression, but also a slower decline in $CD4^+$ T cells [345]. This report also suggested a detrimental effect of possessing KIR2DL2/DS2, which was associated with a faster $CD4^+$ T cell decline [345].

When possible immune mediated mechanisms behind the protective effects of possessing both KIR3DS1 and HLA-Bw4Ile80 were investigated, NK cells from individuals with this genetic combination were found to inhibit viral replication *in vitro* to a greater extent [279]. In addition, in presence of HIV-1 infected autologous $CD4^+$ T cells, $KIR3DS1^+$ NK cells from HLA-$Bw4^+$ individuals degranulated more than NK cells from HLA-$Bw6^+$ individuals [279]. This indicates that these molecules might interact, although no such interaction have been found despite attempts to show binding of KIR3DS1 with Bw4Ile80 tetramers in complex with HIV-1 derived epitopes [118]. In acute HIV-1 infection $KIR3DS1^+$ NK cells expand in presence of HLA-Bw4Ile80 [346], indicating that a potential interaction between KIR3DS1 and HLA-Bw4Ile80 results in a proliferation signal to the NK cell. Interestingly, patients with an early HIV-1 infection that possess KIR3DS1 also have more active NK cells *ex vivo* [347].

The inhibitory counterpart of KIR3DS1, KIR3DL1, has also been implicated in lowering the rate of progression to AIDS. Different KIR3DL1 alleles are expressed to different levels and some, like $KIR3DL1^*004$, are not expressed on the cell surface [102]. Patients possessing the HLA-Bw4 epitope and $KIR3DL1^*004$ have a slower disease progression, as do patients with HLA-Bw4Ile80 and KIR3DL1 alleles expressed to a high degree [348]. The later association may be explained by stronger

education signals during NK cell development when the highly expressed inhibitory receptor and its ligand are simultaneously present [45]. These NK cells would be expected to have a higher activation potential and be more responsive to the lack of inhibition when interacting with HIV-1 infected cells that have down-regulated HLA-B.

Exposed uninfected individuals have also been investigated with regards to their KIR genes. KIR3DS1 homozygotes were more prevalent in the EU group as compared to primary HIV-1 infected patients [349] and the KIR3DS1 transcript levels are reported to be increased in some EUs [266]. In addition, the frequency of EUs with high expressing KIR3DL1 alleles plus the HLA-B57 allele is increased as compared to the frequency in infected patients [350]. An earlier study indicated that lack of cognate HLA ligand for KIRs were more prevalent among EUs, which was suggested to contribute to a lower threshold for NK cell activation in these individuals [351].

5.2 PATHOGENIC INFLUENCE BY THE HIV-1 VIRUS

The pathogenesis in HIV-1 infection can be due both to the destruction of immune cells and to the chronic immune activation induced. The selective destruction of $CD4^+$ T helper cells, important for activation of other immune cells, may for example result in functionally impaired HIV-1 specific $CD8^+$ T cells [328]. The memory $CD4^+$ T cells in the gut are preferentially targeted by the virus and this is devastating for the hosts' immune system since the majority of memory $CD4^+$ T cells are found in the lymphoid tissues of the gut. The chronic immune activation correlates with disease in HIV-1 infected patients. Interestingly, a group of HIV-1 infected patients with high viral loads but no disease progression were shown to have low immune activation [352]. The importance of immune activation is further stressed when comparing HIV-1 infected patients and patients infected with the HIV-2 virus. HIV-2 infected patients have reduced levels of immune activation and a less pathogenic disease process [353]. Also different primate species infected with SIV have either non-pathogenic or pathogenic infections, which may be due to the differences in immune activation [240]. The immune activation may affect the cells of the immune system in several ways and it causes many of the defects found in NK cells [354], NKT cells [355], DCs [356], B cells [357] and T cells [358] in HIV-1 infected patients. Since this thesis is focused on NK cells I will discuss phenotypic and functional defects found in these cells in more detail below.

5.2.1 Effects of HIV-1 on the infected cell

The HIV-1 virus can induce changes in the infected cells, which make them escape killing by NK cells and CTLs. One such mechanism is the downregulation of cell surface expressed MHC class I molecules on HIV-1 infected cells via regulatory HIV-1 proteins like Nef and Vpu [359, 360]. The Nef dependent downregulation is specific for HLA-A and HLA-B, which are recognized by CTLs, but the expression of HLA-C and HLA-E, which are mainly recognized by inhibitory receptors on NK cells, is spared [201]. The end result is that HIV-1 infected cells can avoid killing by both CTLs and NK cells. This is reflected by studies showing that NK cell cytotoxicity of autologous $CD4^+$ cells infected with HIV-1 depends on the ability of the virus to modulate MHC class I expression [25, 277]. Furthermore blocking the interaction between MHC class I molecules and inhibitory NK cell receptors increases NK cell killing of HIV-1 infected target cells [25, 277, 278, 281]. Another potential benefit of keeping HLA-C on the

infected cell surface is the selective incorporation of HLA-C into the HIV-1 envelope which increases the infectivity of HIV-1 [361].

HIV-1 infection also affects ligands for activating NK cell receptors. NKG2D may be involved in control of HIV-1 infection since recent reports show that blocking of NKG2D on NK cells decreases the killing of HIV-1 infected autologous T cells [278, 362]. NKG2D interacts with the ULBP-1, -2 and -3 ligands that are upregulated on *in vitro* infected T cells [176, 278, 362]. It has been suggested that the HIV-1 protein Vpr induces expression of the NKG2D ligands [363], while the viral protein Nef might downregulate their expression [176]. It is thus possible that while infection per se induces the expression of NKG2D ligands, which are of importance in NK cell recognition of HIV-1 infected cells, the virus has evolved a strategy to dampen this effect. In addition, the expression of ligands for the co-receptors NTB-A and 2B4 on NK cells are also downregulated on HIV-1 infected cells [362].

Expression of ligands for the activating NCRs (NKp30, NKp44 and NKp46) are not induced on HIV-1 infected cells [278, 362], but a ligand for NKp44 is expressed on uninfected $CD4^+$ T cells from HIV-1 infected patients and on uninfected cells in infected *in vitro* cultures [166, 362, 364]. A peptide from HIV-1 gp41 induces the expression of the unknown NKp44 ligand, which results in increased sensitivity of uninfected $CD4^+$ T cells to NK cell lysis [166, 364]. Nef mediates intracellular retention of the NKp44 ligand in infected cells, thereby avoiding NK cell recognition of these cells [364]. When macaques were immunized with the gp41 peptide and challenged with SHIV, a decreased frequency of NKp44 ligand expressing $CD4^+$ T cells and increased CD4 counts were found [365]. This indicates that NK cells may be involved in the pathogenic destruction of uninfected $CD4^+$ T cells.

5.2.2 Effects of HIV-1 disease on NK cells

HIV-1 infection can affect NK cells directly or indirectly. A direct effect is the possible infection of NK cells. NK cells express the HIV-1 co-receptors CCR5 and CXCR4 and a subset of NK cells also express the CD4 receptor, which is needed for HIV-1 infection. These NK cells were shown to be infected even in patients on ART, indicating that NK cells could be a latent reservoir for HIV-1 production [366]. Direct infection of NK cells may lead to some of the dysfunctions seen in the NK cell repertoire *(Figure 9)*. The general chronic immune activation seen in HIV-1 infection also affects the NK cells, partly due to a decrease in the release of cytokines of importance for NK cell activation and proliferation, like IL-2, IL-12, IL-15 and IL-21 [354]. The increased concentrations of the immunosuppressive cytokines IL-10 and TGF-β in the blood of HIV-1 infected patients may also contribute.

5.2.2.1 Phenotypic differences in the NK cell compartment

Although it is hard to determine whether the effects on NK cells are direct or indirect, many studies have reported defects detected in the NK cell repertoire after HIV-1 infection *(Figure 9)*. The number of NK cells in blood decreases in HIV-1 infected patients (**paper I** and [54]), but this may be restored after ART treatment [367, 368]. Similarly, frequency of NK cells in colonic lamina propria is decreased in viremic patients [369]. NK cell subset proportions are different in HIV-1 infected patients compared to healthy individuals. They have a decreased proportion of cytolytic $CD56^{dim}CD3^-$ NK cells [53, 54, 370] and an increased frequency of $CD56^-CD16^+$ NK cells [53, 54, 370, 371]. The later subset is functionally defective in cytotoxicity and

production of cytokines [372], but may have a different functional profile as compared to "normal" NK cells since they produce increased levels of MIP-1β [53].

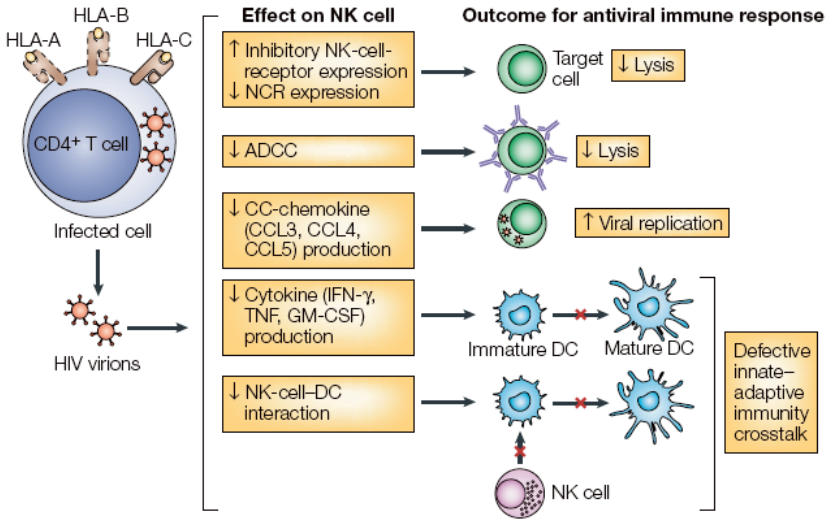

Figure 9: HIV-1 viremia effects on NK cell phenotype and function. This has consequences for the antiviral immune response elicited. *Reprinted by permission from Macmillan Publishers Ltd: Nature reviews immunology, Fauci et.al. 2005 Nov;5 (11):835-43, copyright 2005.*

Several reports have investigated the expression of receptors on NK cells from viremic HIV-1 infected patients. Generally, the expression of the inhibitory receptors, KIR and LIR-1, increases (**paper I** and [274, 371, 373]) and the expression of the activating receptors, NKp30 and NKp46, decreases on NK cells from patients (**paper I** and [53, 371, 374]). This could probably make the NK cells unresponsive to infected cells and might be a reason for the decreased function seen in NK cells from HIV-1 positive patients. However, the C-type lectin like receptors NKG2A (inhibitory) and NKG2C (activating) behave just the other way around (**paper I** and [375, 376]). This has been suggested to be due to a concomitant CMV infection in HIV-1 infected patients since HIV-1 negative individuals with CMV infection have a similar pattern of NKG2A and NKG2C expression [130, 377]. The CD161 receptor which may have an inhibitory function [134, 135] is also downregulated on NK cells from HIV-1 infected patients (**paper I** and [53, 367, 368, 370]). These abnormalities in the NK receptor repertoire is in most cases restored in patients with a low viral load due to ART treatment (**paper I** and [371]).

5.2.2.2 Differences in NK cell function

Cytotoxicity against tumor target cell lines is decreased in HIV-1 infected patients with a high viral load [278, 368, 371], but can be restored in patients with an undetectable viral load receiving ART, who have a cytotoxic capacity similar to healthy individuals [368, 371]. This decrease in cytolytic function may be due to presence of the non-cytotoxic $CD56^{-}CD16^{+}$ cells in the NK cell pool. NK cell degranulation ability on the

other hand is not compromised in HIV-1 infected patients, their NK cells either degranulate more vigorously or to the same degree as NK cells from healthy individuals (**paper I** and [53, 266, 268, 367]). The NK cell ability to mediate ADCC may be decreased in HIV-1 infected patients [378, 379]. Possible factors that might contribute are defects in expression of the ζ chain associated with CD16 [380] and the recently reported increased expression of matrix metalloproteases (MMPs) that removes CD16 from the cell surface following NK cell activation [379].

The production of cytokines like IFN-γ and TNF by NK cells is affected by the infection *(Figure 9)*. When NK cells from patients with a high viral load and from ART treated patients with a low viral load are stimulated with different combinations of IL-2, IL-12, IL-15 and IL-18, a decreased production of IFN-γ has been observed compared to healthy controls [368, 381, 382]. In other studies, however, where NK cells were stimulated with the NK cell sensitive K562 cell line, which lacks MHC class I expression, an increased production of IFN-γ was seen in patients with a high viral load compared to successfully treated patients and healthy controls [268, 367]. It is possible that the increased expression of inhibitory MHC class I specific receptors on NK cells from viremic HIV-1 infected patients make these cells more responsive to target cells lacking MHC class I. Secretion of CC-chemokines from NK cells has been shown to suppress HIV-1 infection *in vitro* and both the amount of suppression and the levels of MIP-1α and RANTES correlate to HIV-1 viral load [82, 383]. Thus, in patients with a high viral load, the secretion of CC-chemokines and the suppression of HIV-1 infection by these soluble factors, are defective [383].

NK cell interaction with other immune cells is also affected by the HIV-1 infection *(Figure 9)*. Killing of immature DCs by NK cells is defective in chronic HIV-1 infection [384] and this is mainly due to the presence of the dysfunctional $CD56^{-}CD16^{+}$ NK cell subset and their impaired NKp30 and TRAIL expression/secretion and function [179]. Interestingly, a recent study showed that NK cells can inhibit HIV-1 replication in monocyte-derived DCs by interaction between LIR-1 and an unknown non-MHC class I ligand expressed during HIV-1 infection [385]. This interaction seems to be inhibited by the downmodulation of NK cell expression of LIR-1 upon interaction with the infected cells.

6 IMMUNOTHERAPIES BASED ON NK CELLS AND ANTIBODIES

6.1 NK CELL BASED IMMUNOTHERAPIES

6.1.1 Stimulation of NK cell activity

The cytokine IL-2 can be used to stimulate lymphocytes *in vitro* to obtain lymphokine-activated killer (LAK) cells, which include NK cells. Infusion of LAK cells into cancer patients have been performed in several clinical trials but the clinical response rate have only been about 20% [386]. Cytokines involved in NK cell differentiation and activation have also been infused into cancer patients, including IL-2, IL-12, IL-18, IL-21 and IFN-α with varying effects on NK cell activity and clinical response [386]. NK cells may also be stimulated by different vaccine modalities, which have mainly been studied in relation to DC-based vaccines [386, 387]. The role for NK cells in these vaccine-mediated immune responses is believed to be due to the immunomodulatory function of NK cells [387].

6.1.2 "Missing self" reactivity of NK cells in hematopoietic stem cell transplantations

NK cells may be important effector cells in hematopoietic stem cell transplantations to patients with leukemia. In a retrospective study Ruggeri et.al. found that relapses in stem cell transplanted acute myeloid leukemia (AML) patients were eliminated when there was a KIR-ligand mismatch between the transplanted cells in the graft and host cells in the recipient [388]. This was interpreted as a "missing self" reaction mediated by donor-derived NK cells expressing KIR molecules that did not recognize MHC class I molecules in the host. Interestingly, it seems as tumor cells are specifically targeted by these alloreactive NK cells, since only graft-versus-leukemia (GVL) effects and not increased graft versus host disease (GVHD) was seen. The preferential killing of leukemic cells is probably due to an increased expression of activating ligands on the tumor cells making them sensitive to NK cell killing. NK cell killing of host DCs [185, 186] may contribute to the lack of GVHD since it prevents DCs of the recipient from presenting antigens to donor T cells [388, 389]. The protective effect of KIR-ligand mismatch was reported in transplantations between related donors and recipients (haploidentical transplantation), but has not been as clearly documented in transplantations from unrelated donors [390]. The reason for controversies in this field may be the many discrepancies between the studies regarding for example T cell depletion of the graft, stem cell source and dose, preconditioning treatment and GVHD prophylaxis as well as type of malignancy and disease stage of the patient [391].

Adoptive transfer of haploidentical NK cells have also been performed in the clinical setting. Miller et.al. injected pure NK cells along with IL-2 in AML patients that had been treated with immunosuppressive medication, which resulted in production of endogenous IL-15 and subsequent expansion of donor NK cells [392]. Complete remission was achieved in five of nineteen AML patients with poor prognosis. Three of the four patients that had received cells from a KIR-ligand mismatched donor were among the five that responded to the therapy.

6.1.3 Blocking of inhibitory receptors to induce "missing self" reactions

"Missing self" reactivity can also be achieved by blocking the interaction between inhibitory NK cell receptors and MHC class I molecules. This enhanced the anti-tumor response in a mouse system when inhibitory Ly49 receptors were blocked using monoclonal antibodies [393]. A similar approach for blocking of KIR receptors is presently evaluated by the company Innate Pharma in a phase I clinical trial in patients with relapsed AML and multiple myeloma. The antibody used is called 1-7F9 and is a fully-human IgG4 antibody [394]. It induces NK cell mediated lysis of primary AML cells *in vitro* and protects immunodeficient mice injected with human NK cells and autologous primary AML blasts from disease [394]. This antibody has been used by us in **paper I** to delineate its potential as an immunotherapeutic for use in HIV-1 infected patients, with the aim to increase NK cell recognition of virus infected cells. Antibodies can also be used to specifically target the infected cells as is further discussed in the next section.

6.2 IMMUNOTHERAPIES BASED ON HIV-1 SPECIFIC ANTIBODIES

6.2.1 Passive immunization

Passive immunization with antibodies is a well-known treatment modality. Treatment with sera from humans or animals that have previously encountered a specific antigen has long been used as prophylaxis and treatment after potential exposure to, for example, Hepatitis and Rabies [395]. In recent decades the ability to produce monoclonal antibodies (mAb), specific for particular antigens [396], have resulted in new group of drugs emerging. These have many possible applications including treatment of various tumors and infectious diseases [395, 397]. The first to be used for human treatment were mouse mAbs, but many of them had a low efficacy *in vivo*, mainly because they were immunogenic in humans, had a short half-life and did not kill target cells efficiently since they did not bind well to human complement or Fc-receptors inducing ADCC. This has now been circumvented by production of human or humanized mAbs [397]. Several mAbs are approved for clinical use against cancer including for example Rituximab in Non-Hodgkin lymphoma targeting CD20 on B cells [398]. The main mechanism behind the effect of unconjugated mAbs in cancer therapy has been proposed to be ADCC, although other mechanisms are also plausible [397]. The importance of the Fc-receptor CD16 expressed on NK cells has been substantiated by the associations between clinical effect of mAb treatment and presence of polymorphisms in CD16 affecting binding of the antibody [153, 155, 156].

Despite the potential for antibody induced protection against HIV-1 infection, which has been shown in non-human primate models [282-286] human trials have so far been disappointing [292]. Due to the high mutation rate in HIV-1, a cocktail of several antibodies binding to different parts of the envelope may be preferable to antibodies to a single epitope, similar to the strategy used during highly active antiretroviral therapy. Neonatal rhesus macaques orally challenged with SHIV and then treated with three different unconjugated neutralizing antibodies were protected from infection [284]. When the same antibodies were used in HIV-1 infected patients undergoing ART interruption, two out of eight chronically infected patients were able to retain the viremia for a longer time than during previous treatment interruptions. The study concluded that neutralizing antibodies can have an effect on persistent HIV-1 infection,

but that a high concentration of antibodies to multiple conserved epitopes is required. Unfortunately, the immune pressure elicited by the antibodies induced a rapid escape in most patients [292].

6.2.2 Conjugated antibodies

One plausible way to specifically and effectively kill HIV-1 infected cells is by using immunoconjugates. This is successfully used in cancer therapies and several immunoconjugates are approved for clinical use [399]. Antibodies or soluble proteins targeted to HIV-1 infected cells and conjugated to toxins [400-403], radioisotopes [404] or cytotoxic drugs (**paper IV**) have been investigated both *in vitro* and *in vivo* for their potential use against HIV-1 infection. Despite promising preclinical results, the failure of the first clinical trial with an immunotoxin consisting of a soluble CD4 protein conjugated to a variant of the toxin *Pseudomonas* exotoxin (PE) [400, 401], hampered the field. This toxin was immunogenic and the short half life of the CD4 fusion protein probably contributed to the lack of efficacy. The treatment was also hepatotoxic which may be due to binding of the immunotoxin to free gp120 and subsequent clearance of immune complexes in the liver. The highly glycosylated gp120 protein can likely pose as a substrate for hepatic receptors, leading to killing of hepatocytes when levels of free gp120 are high [405]. Lessons learnt from this study were that a non-immunogenic conjugate with a longer half life should be tested in patients with low viral loads, i.e. in combination with ART treatment.

Another goal with immunotoxins could be to target latently infected cells in ART treated patients in order to eradicate these reservoirs [406]. Specific targeting of the $CD45RO^{+}$ memory $CD4^{+}$ T cells, one of the main reservoirs for latent viruses, with a CD45RO specific antibody conjugated to a ricin A chain is one approach with promising preclinical results [402]. The *in vitro* treatment of PBMCs from successfully ART treated patients with this immunotoxin resulted in decreased numbers of viral copies and infected cells [402]. Another approach for reducing the number of latently infected cells is to reactivate latently infected cells in the presence of ART. This can be achieved by immune activation therapy [407, 408] or by a more selective induction of viral expression [230, 409]. Reagents tested with partial success include IL-2, IL-7, prostratin and histone deacetylase inhibitors [407-409]. This type of therapy could be combined with immunoconjugates that specifically kill the reactivated and HIV-1 antigen expressing cells [407] and ART that inhibit reinfection of new cells. The problem with this kind of treatment is the difficulty in getting a complete eradication of the latent reservoir which will be crucial for cure of the disease [406].

6.2.3 Active immunization

Instead of passive immunization, antibody production can be induced by immunizing with antigen from the pathogen of interest. Vaccines based on the HIV-1 envelope protein have been tested in humans, but the first phase III efficacy trial with a recombinant gp120 protein did not protect immunized persons from infection, despite induction of specific antibodies [410]. It is widely believed that for an antibody response to be protective the antibodies need to be broadly neutralizing, i.e. able to neutralize a wide range of subtypes and strains of HIV-1 [225]. So far, immunization with monomeric gp120 has not been able to evoke this kind of response [410]. A proof-of-concept trial, called the STEP trial, investigated the protective effect of a recombinant adenovirus vaccine encoding the HIV-1 genes Gag, Pol and Nef [411]. This trial was terminated due to futility and, disturbingly, a higher frequency of

individuals in the vaccine group became infected as compared to the placebo group. The primary goal with this vaccine was to induce specific T cell responses able to blunt the initial viremia and lower the viral set point [411]. Since the vaccine did not include gene sequences encoding the HIV-1 envelope protein no neutralizing antibodies were induced.

Results from the third HIV-1 vaccine efficacy trial, performed in Thailand, were recently released [412]. This prime boost vaccine regimen contains two vaccine modalities. A recombinant canarypox virus encoding HIV-1 Env, Gag and protease was used to prime the immune response, which was then boosted with a gp120 protein. An increased protection from infection in individuals receiving the vaccine was reported [412]. Over 16 000 participants were enrolled, half of them receiving the vaccine, and 51 in the vaccine group got infected with HIV-1 as compared to 74 in the placebo group [412]. Despite the total low number of infections and the marginal statistical significance, this study is the first to show that protective immunity can be achieved by vaccination and that has increased the hopes for the development of a future protective vaccine. The type of immune response that might have contributed to this possible protective effect is now being investigated. The vaccine has previously been reported to induce binding, but not broadly neutralizing, antibodies [412, 413]. Since only a few percent elicited potent T cell responses [412] it is believed that the binding antibodies might have contributed to protection [413]. In the absence of directly neutralizing antibodies, ADCC is one potential effector mechanism that might be responsible for the protective effects of the antibodies.

Many new types of HIV-1 vaccines that elicit either strong T cell responses or neutralizing antibodies are in the pipeline and the potential for both prophylactic and therapeutic vaccination is investigated. One strategy for induction of T cell responses, with promising clinical results, is to prime with a DNA vector encoding viral genes and then boost with a viral vector [414]. To achieve better antibody responses, oligomeric HIV-1 envelope proteins, more closely mimicking the native form of the protein, are being developed. In contrast to the earlier monomeric envelope proteins these oligomers are expected to elicit more broadly neutralizing antibodies as the aim is to target epitopes present in the functional viral envelop spike [225].

7 RESULTS & DISCUSSION

In this thesis I have investigated different aspects of the immune system that may contribute to the control of HIV-1 infection *(Figure 10)*. The discussion is divided into three parts starting with possible roles for NK cells and ADCC in natural control of HIV-1 disease progression, i.e. in the rare HIV-1 infected patients that spontaneously control their viremia. In the second part different factors affecting NK cell reactivity, including HIV-1 virus infection of target cells, a KIR blocking antibody and the KIR genotype are discussed. In the last part, results on the effect of NK cells and a drug-conjugated antibody in our *in vivo* HIV-1/MuLV mouse model are presented.

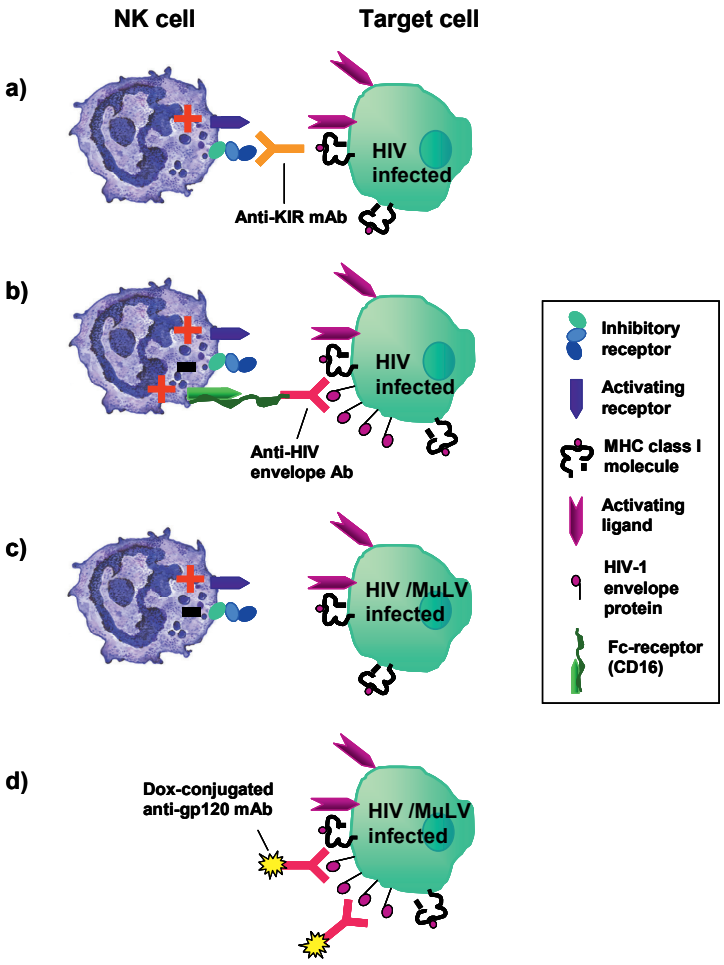

Figure 10: Schematic representation of the NK cell and antibody effector mechanisms investigated in the thesis. a) Antibody-mediated blocking of inhibitory KIRs **(paper I)** b) ADCC of HIV-1 infected cells **(paper II)** c) NK cell effect on HIV-1 **(paper I)** and HIV-1/MuLV infected cells **(paper III)** d) Effect of doxorubicin-conjugated anti-gp120 mAb on HIV-1 or HIV-1/MuLV infected cells **(paper IV).**

7.1 CHARACTERISTICS OF HIV-1 CONTROLLER PATIENTS

7.1.1 NK cell phenotype in controller patients does not seem to influence the disease (paper I).

To investigate whether NK cell phenotype and function differ in HIV-1 infected controller patients as compared to viremic patients and healthy individuals we recruited six HIV-1 controller patients, fourteen viremics and a number of healthy individuals. The controller patients had a viral load below 500 HIV-1 RNA copies/ml blood, and had been infected for at least eight years without receiving treatment (table 1in **paper I**). For the phenotypic investigations a group of ten patients on ART was also included. Controller patients, ART treated patients and viremic patients had a similar frequency of $CD56^{+}CD3^{-}$ NK cells, which was lower than the frequency in healthy individuals *(Figure 11a)* (**paper I and II**) and this corroborates previous studies [268, 273, 274]. There were no differences in NK cell subsets between any of the groups, in line with some [274] but not other reports [268, 273].

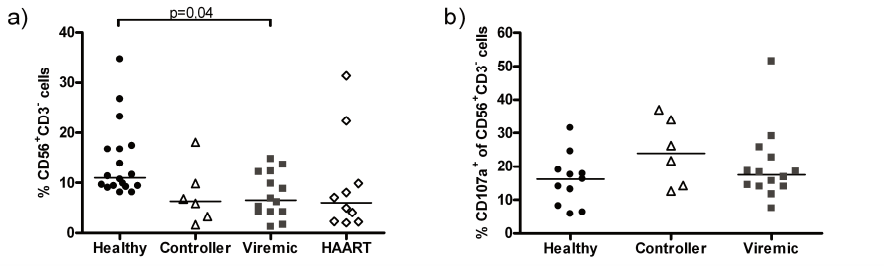

Figure 11: Frequency of NK cells in blood and degranulation in response to target cells. a) Frequency of $CD56^{+}CD3^{-}$ NK cells in blood and b) frequency of NK cells degranulating in response to the MHC class I deficient K562 tumor cell line. The horizontal line represents the median in each group. P-values in the figure are derived by the Kruskal-Wallis post-hoc test.

Expression of a panel of NK cell receptors was investigated and we found a similar effect in viremic and ART treated patients as previously reported (Table 1, Figure 1 and 2 in **paper I**) [354, 371, 376]. Regarding controller patients they had an NK cell receptor phenotype most similar to the phenotype in viremic patients [274]. Possible exceptions include frequency of KIR^{+} and $NKG2A^{+}$ NK cells, where the controller patients NK cells were more similar to NK cells from healthy individuals as compared to viremic patients (Figure 1 and 2A in **paper I**). Since frequency of NK cells expressing KIR2DL2/L3/S2 (positive correlation) and KIR2DL1/S1 (negative correlation) correlated with viral load it is likely that the deviant KIR expression in viremic patients is due to the viremia [267]. Interestingly when comparing the two patient groups with low viremia, there was a trend towards a higher frequency of $NKG2A^{+}$ NK cells in controller patients as compared to ART treated patients. Since it is known that CMV infection results in decreased NK cell expression of NKG2A [130, 375] one possible explanation is that differences in CMV infection contribute to the NKG2A expression. Although the frequencies of CMV seropositive individuals were

similar in all groups, the lower CMV antibody titers in controller patients as compared to ART treated patients (5000 (910-25000) and 21000 (4500-28000) respectively, p=0,06 when using the Mann-Whitney U-test) may reflect a lower CMV viral load or more infrequent periods of reactivation in controller patients, possibly affecting NKG2A expression. Thus the controller status is probably not affected by the NK cell phenotype. A more likely explanation is that the NK cell phenotype abnormalities in active HIV-1 infection are due to viremia or constant immune activation.

7.1.2 NK cell activity in controller and viremic patients is similar to the activity in healthy individuals (paper I and II).

The activity of NK cells from the controller patients was investigated by staining NK cells with an antibody to CD107a, which is a membrane-bound protein present in secretory lysosomes and which is exposed upon degranulation of NK cells. PBMC effector cells were purified from blood and then incubated in IL-2 over night, before the effector cells were mixed with various target cells. NK cells, defined as $CD56^{+}CD3^{-}$ from controller patients, viremic patients and healthy individuals degranulated to similar levels in response to the tumor cell lines lacking MHC class I (K562 and 721.221) *(Figure 11b),* tumor cell lines expressing HLA-C (721.Cw3 and 721.Cw4), uninfected and HIV-1_{IIIB} infected Jurkat T cells or autologous T cells (**paper I**). Infected Jurkat T cells coated with HIV-1 specific antibodies also induced similar NK cell degranulation in the various groups (Figure 4 in **paper II**). Thus, NK cells from our patient cohorts have similar degranulation abilities as NK cells from healthy individuals regardless of the target cell lines used. When staining for intracellular IFN-γ, only low frequencies of positive NK cells were detected (Figure 4 in **paper II**) and no differences between the controller patients viremic patients and healthy individuals were detected for this marker either. Since the activity of NK cells from controller patients did not differ from viremic patients or healthy individuals, our controller patients do not seem to have NK cells with a generally increased activity that could explain the control of the disease.

Similarly to us Alter et.al. previously showed that HIV-1 infected patients with a non-progressive disease had similar degranulation ability as healthy individuals [268, 379]. Viremic patients have been reported to have an increased [53, 268, 367] or similar [266] frequency of NK cells degranulating in response to K562 cells as compared to healthy individuals, although a decreased response to antibody coated target cells was also reported [379]. The higher CD4 counts in our viremic patients compared to previous studies where degranulation has been investigated probably reflects a better clinical status, which may contribute to the more normal NK cell function in our viremic patients.

7.1.3 ADCC mediating antibodies in controller patients may differ from antibodies in viremic patients (paper II).

HIV-specific antibodies may induce killing of infected cells through ADCC involving NK cells or other Fc-receptor bearing cells. This is a potent mechanism for induction of NK cell responses and antibody ADCC activity has been correlated to a better disease progression [302, 311-313]. We therefore compared the ability of controller patients' and viremic patients' antibodies ability to induce an ADCC response. Plasma from the HIV-1 infected patients contained antibodies that induced killing of target cells coated with HIV-1 gp140, a soluble oligomeric envelope protein [415]. Increased plasma mediated killing correlated with lower viral loads in the viremic patients (Figure 2 in

paper II) substantiating previous findings [308, 416]. The ability of plasma antibodies to induce NK cell degranulation and IFN-γ secretion was also investigated [415, 417]. Interestingly, antibodies from controller patients induced higher NK cell secretion of IFN-γ compared to antibodies from viremic patients (Figure 1 in **paper II**) which is in line with the recently reported increase in killing of target cells via ADCC in elite controller patients compared to viremic patients [302]. It is thus possible that ADCC inducing antibodies are involved in control of disease or viremia. Another possible interpretation is that a lower viral load and less progressive disease could contribute to the ability to produce antibodies with better ADCC function. The detrimental immune activation in HIV-1 viremic patients contributes to polyclonal activation and functional exhaustion of the B cell pool and may result in antibodies with lower specificity [357]. To further delineate this, investigations of the ADCC mediating ability of antibodies from controller and HAART treated patients with similar low viral loads are desirable.

Further, the antibody specificity for the V3 loop in the HIV-1 gp140 protein was investigated in our cohort by incubating plasma with either wt gp140 or a V3 truncated version of gp140 (gp140ΔV3) [415]. Whereas plasma from all viremic patients and some of the controller patients lost their ability to induce NK cell degranulation in presence of gp140ΔV3, two of the controller patients induced a similar response to the wt and ΔV3 gp140 protein (Figure 1 in **paper II**). This indicates that these two controller patients may have ADCC inducing antibodies to other parts of the gp140 protein than the V3 loop. However, one should keep in mind that only one viral isolate (AD8) has been investigated and since some antibodies to the V3 loop may be strain specific it is possible that using an envelope protein from another viral isolate might have given different results. The two controller patients that mediated V3-independent ADCC participated in a vaccination study with gp160 protein more than seven years ago [418] and it may be that this vaccine has induced antibodies directed to other parts of the envelope protein than the naturally induced antibody epitope specificities present in HIV-1 infected patients. Nevertheless, it is possible that antibodies to other parts of gp140 than to the immunodominant V3 loop may be more beneficial and contribute to control of disease. As an example an immunogenic epitope in the C5 region of gp120 that induced functional ADCC antibodies was earlier identified in a long-term non-progressive patient [419].

Antibody IgG1 titers to the ΔV3 gp140 protein correlated positively to the antibody induced NK cell degranulation (Figure 1 in **paper II**). Interestingly this was not seen for IgG1 antibody titers to wt gp140 protein. This may be interpreted as antibodies binding to V3-independent regions of the envelope protein being more important for mediating ADCC. Further studies are warranted to delineate the epitopes in the HIV-1 envelope protein that may contribute to ADCC responses and possibly to control of the disease. This is important for future developments of HIV-1 specific vaccines and other immunotherapies based on ADCC.

7.1.4 What makes a controller patient control HIV-1? (paper I and II)

The reasons why certain HIV-1 infected patients control viremia without ART treatment may be several and although it is not possible to know the exact reason why some patients can control their viremia, I will here summarize the possible correlates to control that we found in our six controller patients. Mutations in the CCR5 gene is known to influence disease progression [333] and one of our controller patients was heterozygous for the CCR5Δ32 mutation. Interestingly, five of the viremic patients

with similar viremia and CD4 counts as the other viremic patients were also heterozygous for the CCR5Δ32 mutation, indicating that this might not be sufficient for control over the virus. The relatively high frequency of CCR5Δ32 heterozygous individuals in our cohort (7/31) may be due to the high prevalence of this polymorphism in Scandinavia [334]. Other genetic factors that correlate with disease progression are polymorphisms in the HLA and KIR genes [335, 337, 339]. Presence of the KIR3DS1 gene together with a HLA-Bw4Ile80 allele correlates with slower disease progression [342] and this combination was found in two of our controller patients, but only one of the viremic patients. Certain immune responses also seem to confer control over the disease, as discussed in section 5.1 [261]. Two of our controller patients had ADCC mediating antibodies directed to V3-independent parts of the gp140 protein whereas viremic patients had a more V3 specific ADCC activity. Thus from our investigations of patients that control their viremia a potential explanation to this control has been found for five out of the six patients. However, there may be many other factors that have contributed to their controller status as well, which were not investigated by us, including for example viral fitness, specific HLA alleles and T cell responses [261, 322, 326, 336, 337].

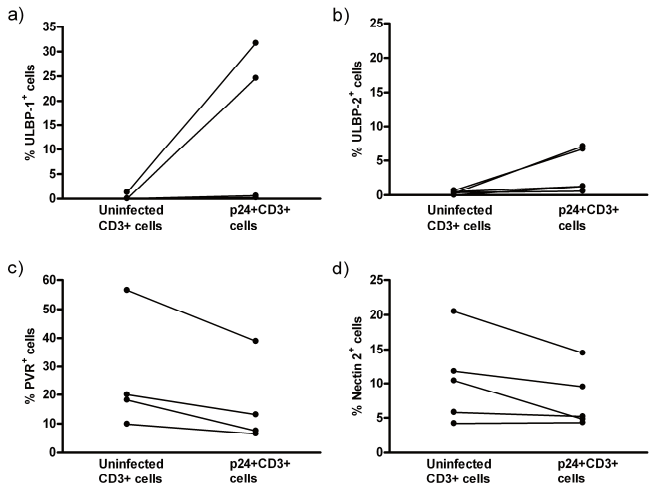

Figure 12: Ligands for NK cell receptors expressed on PHA and IL-2 stimulated uninfected $CD3^+$ cells and HIV-1IIIB infected $p24^+CD3^+$ cells seven days after infection. Frequency of cells positive for a) ULBP-1, b) ULBP-2, c) PVR and d) Nectin-2. Frequency of cells positive for control IgG have been subtracted from ULBP-1, -2, PVR and Nectin 2 values. Lines connect results from one experiment.

7.2 FACTORS AFFECTING NK CELL ACTIVITY

7.2.1 Similar NK cell activity to autologous HIV-1 infected cells and uninfected cells (paper I).

There are conflicting data regarding the ability of NK cells to recognize and kill autologous HIV-1 infected T cells *in vitro*. While some reports state that there is no difference in the level of NK cell mediated killing of HIV-1 infected versus uninfected cells [25, 276], others have found such a difference [277, 278]. We aimed at comparing the ability of NK cells from patients and healthy individuals to recognize HIV-1 infected autologous T cells, as opposed to allogeneic T cells or immortalized cell lines studied in some of the previous reports [420, 421]. $CD4^+$ T cells were purified from blood and stimulated with IL-2 and PHA before infection with the viral isolate HIV-1_{IIIB}. These infected cells or uninfected control cells were incubated for four hours with IL-2 stimulated PBMCs derived from the same individual and NK cell degranulation was analyzed. We did not detect any difference in NK cell degranulation in response to uninfected and HIV-1_{IIIB} infected autologous T cells, neither were there any difference in activity of NK cells from HIV-1^+ patients and healthy individuals (Figure 3 in **paper I**). Similar results were obtained with uninfected and HIV-1_{IIIB} infected Jurkat T cells.

Previous studies that have detected a difference in NK cell response to infected autologous cells have used primary isolates to infect cells from healthy individuals [277] or endogenously virally infected cells from viremic patients [278]. A difference compared to our study is the decreased expression of MHC class I molecules on these virally infected cells, which was not detected by us. This is probably due to the use of the lab adapted strain HIV-1_{IIIB} [277]. We had anticipated that viral effects on ligands for activating NK cell receptors would increase NK cell recognition of HIV-1 infected cells [176, 278, 362]. When investigating this, an increased expression of the NKG2D ligands ULBP-1 and -2 was found upon *in vitro* infection in some but not all individuals investigated *(Figure 12)*. The expression of the DNAM-1 ligands, Nectin-2 and PVR, on the other hand was, if anything slightly decreased on HIV-1_{IIIB} infected cells as compared to uninfected cells *(Figure 12)*. Thus, we conclude that NK cells cannot distinguish HIV-1_{IIIB} infected from uninfected autologous T cells due to a similar level of expression of inhibitory and activating NK cell ligands. One important factor is the *in vitro* activation of T cells necessary for efficient HIV-1 *in vitro* infection. We observed that the activation protocol used induced the expression of the NK receptor ligands ICAM-1, PVR and MHC class I *(Figure 13)*. Future studies should include infection with primary viral strains.

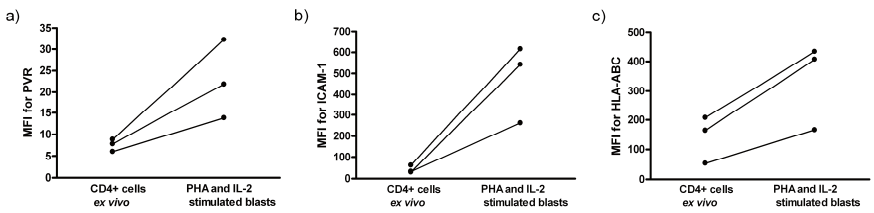

Figure 13: Ligands for NK cell receptors expressed on CD4+ cells ex vivo and on blasted cells after PHA and IL-2 stimulation. Mean fluorescent intensity (MFI) for a) PVR, b) ICAM-1 and c) HLA-ABC.

7.2.2 A KIR-binding antibody increases NK cell activity in HIV-1 infected patients (paper I).

The reported downregulation of HLA-A and -B but not HLA-C on HIV-1 infected cells [25, 201] and the increased expression of KIRs on NK cells from viremic patients (**paper I** and [371]) indicate that the interaction between HLA-C and KIRs may be important in preventing NK cells from killing HIV-1 infected cells. This is supported by the increased NK cell mediated killing of HIV-1 infected autologous T cells when blocking inhibitory receptors on NK cells, depleting NK cells with inhibitory receptors or blocking MHC class I molecules on target cells [25, 278]. The KIR specific 1-7F9 antibody binds all KIRs that use HLA-C as a ligand, including KIR2DL1/S1 and KIR2DL2/L3/S2, and is therefore expected to induce "missing self" reactivity in the whole human population (see section 6.1) [394]. We aimed at evaluating the possible future use of this antibody as a treatment for HIV-1 infected patients and investigated the effects of 1-7F9 *in vitro*.

PBMC from HIV-1 infected patients and healthy individuals were incubated with the 1-7F9 antibody and then added to autologous HIV-1 infected or uninfected T cells. This resulted in increased NK cell degranulation, although to a similar extent in presence of uninfected and HIV-1 infected autologous T cells (Figure 4 in **paper I**). One explanation for the failure of NK cells to distinguish infected and noninfected cells could be the IL-2 activation of the effector cells in PBMC, which could result in disrupted self-tolerance mechanisms [422]. In addition, NK cells were induced to degranulate by 1-7F9 in the absence of added target cells, which may also be explained by the IL-2 stimulation of PBMCs since it is possible that this could induce expression of activating ligands on potential target cells in the PBMC culture [423]. In the mouse system, cytokine stimulation of NK cells and blocking of inhibitory Ly49 receptors *in vitro* resulted in killing of autologous T cell blasts [424]. However *in vivo* blocking did not result in autoreactivity on normal autologous spleen cells, but a specific effect on leukemic cells [424, 425]. Thus, although our *in vitro* study did not show any HIV-1 specific effect of the 1-7F9 antibody, it can not be excluded that the use of 1-7F9 *in vivo* in HIV-1 infected patients could have an advantageous effect. The need for cytokine stimulation of both effector and target cells *in vitro* may conceal preferential activity towards virus infected cells.

7.2.3 The KIR genotype influences the NK cell response to a KIR-binding antibody (paper I).

While there was no difference in effect of the 1-7F9 antibody on NK cells from HIV-1 infected patients and healthy individuals, there was a heterogeneous response of NK cells from different individuals to the 1-7F9 treatment. Some showed a strong effect of the antibody and others showed no effect at all, and this pattern could be seen with all target cells used. We therefore correlated the effect of 1-7F9 on NK cell degranulation with frequency of KIR expressing NK cells and with KIR genotype. In healthy individuals and viremic patients there was a positive correlation between frequency of NK cells expressing the 1-7F9 bound KIRs and effect of the antibody on NK cell degranulation (table 3 in **paper I**). This indicated that treatment with 1-7F9 might have a strong effect on NK cells from viremic patients that have an increased expression of KIRs. However in the controller group this correlation was reversed, meaning that frequency of NK cells expressing 1-7F9 binding KIRs can not fully explain the heterogeneous effect of 1-7F9 treatment. We therefore explored how the KIR genotype might have influenced the effect (Figure 5 in **paper I**). Presence of at least one

KIR2DL2 allele resulted in stronger NK cell degranulation, as did presence of the activating KIR2DS2; these two KIRs are in linkage disequilibrium. Individuals with at least one of the activating KIRs bound by 1-7F9 (KIR2DS1 and KIR2DS2) also had a significantly increased NK cell activity in presence of 1-7F9. The KIR B haplotype is characterized by the presence of more activating KIR genes compared to the KIR A haplotype and NK cells from individuals possessing the B haplotype responded more to 1-7F9 treatment. Thus presence of activating KIRs seems to influence the effect of the 1-7F9 antibody.

There are several potential explanations for the possible importance of activating KIRs on the 1-7F9 effect on NK cells. Although 1-7F9 is an IgG4 antibody with low affinity for Fc-receptors, it may bind the Fc receptor FcγRI (CD64) on monocytes [394]. Presence of these cells in the PBMC cultures might induce cross-linking and subsequent activation of NK cells via activating KIRs bound by the 1-7F9 antibody. Blocking with sera could be used to reduce this kind of activity and this should be included in future experiments. It is also possible that activating KIRs could infer a higher responsiveness on the NK cell during the education process, similarly to what have been described for inhibitory receptors. However a recent publication stated that the activating KIR2DS1 tunes down the NK cell responsiveness in presence of its ligand HLA-C2 [426]. Nevertheless, NK cells with the activating KIR3DS1 gene seem to have an increased activity [347, 427], which will be discussed in more detail below (section 7.2.4). This gene is present in the KIR B haplotype and a trend towards increased NK cell activity in presence of 1-7F9 in individuals with the KIR3DS1 gene was found (data not shown).

Due to the linkage disequilibrium between the KIR2DL2 and KIR2DS2 genes another possibility is that the inhibitory KIR2DL2 receptor is responsible for the increased effect of 1-7F9 in individuals that also posses the KIR2DS2 gene. The increased affinity of HLA-C for KIR2DL2 as compared to the allele KIR2DL3 [109] might contribute since a high affinity interaction during education between an inhibitory receptor and its MHC class I molecule can result in an increased functionality of that NK cell [45]. When this interaction is blocked by 1-7F9 an increased NK cell activity might be anticipated. To further delineate the effect of 1-7F9 in individuals possessing certain KIR genotypes the activity of NK cells with different combinations of KIRs could be investigated with NK cell clones or the newly described antibody combinations for specific KIR molecules [426].

7.2.4 NK cell phenotype and genotype influence NK cell mediated ADCC to HIV-1 infected cells (paper II).

The NK cell mediated ADCC activity in presence of HIV-1_{IIIB} infected Jurkat T cells and pooled IgG from HIV-1 infected patients (HIVIG) did, as mentioned above, not differ between HIV-1 infected patients and healthy individuals. However there was a heterogeneous response to this stimulation between individuals within the groups. We investigated whether expression of NK cell receptors or presence of certain receptor genotypes could contribute to this. An increased frequency of NK cells expressing the activation marker CD69 *ex vivo* correlated with a higher proportion of NK cells degranulating in response to HIVIG coated HIV-1 infected Jurkat T cells (Figure 4d in **paper II**). CD69 is usually seen as a marker for *in vivo* activation but it might also be functional in the *in vitro* interaction between NK cells, infected cells and antibodies. One of the proposed functions for CD69 is as a stimulatory receptor [428], which may

explain the effect found by us, although several other suggestions on CD69 function are equally possible [429, 430].

The Fc receptor CD16, involved in ADCC, possesses polymorphisms that influence the binding of IgG antibodies [153, 154]. We genotyped all individuals included in the study for the polymorphism at position 158, but this did not explain the heterogeneous NK cell ADCC activity among donors (Figure 4e in **paper II**).

7.2.4.1 KIR3DL1/S1 and HLA-B genotype influence NK cell ADCC function.

Another genetic variable is the presence of different KIR genes, which could potentially influence NK cell ADCC activity [431]. We investigated the association of KIR3DL1/S1 and HLA-B alleles in the recorded ADCC results, since these genes have been associated with the rate of HIV-1 disease progression [339, 342, 345]. When grouping individuals according to presence or not of the HLA-B Bw4Ile80 epitope, which is a high affinity ligand for KIR3DL1 [110, 111], an interesting pattern associated with possession of KIR3DL1 or KIR3DS1 was found *(Figure 14)*. Although the number of individuals with the HLA-B Bw4Ile80 allele was small, there was a trend towards increased NK cell ADCC activity in individuals with the activating KIR3DS1 allele as compared to individuals only possessing the inhibitory KIR3DL1 allele *(Figure 14a)*. Intriguingly individuals lacking this HLA-B epitope and instead possessing the HLA-B Bw4Thr80 or HLA-B Bw6 epitopes had a contrasting pattern with increased NK cell ADCC activity in individuals homozygous for the KIR3DL1 allele as compared to individuals homozygous for the KIR3DS1 allele *(Figure 14b)*. Individuals lacking the HLA-B Bw4Ile80 epitope still can possess HLA-B Bw4 and HLA-A Bw4 epitopes binding KIR3DL1 [111, 115, 432]. However ADCC induced activity did not differ between KIR3DL1/S1 genotypes when comparing individuals with the HLA-Bw4 or Bw6 epitopes (data not shown), indicating that only presence of the HLA-B Bw4Ile80 influence the ADCC activity in our cohort.

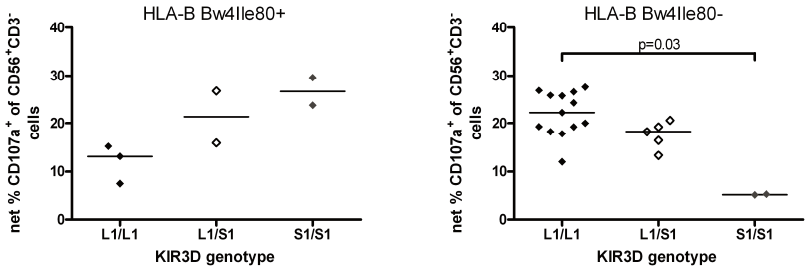

Figure 14: Influence of KIR3DL1/S1 and HLA-B genotype on NK cell mediated ADCC activity. Frequency of NK cells degranulating in presence of HIV infected Jurkat T cells and HIVIG (250 µg/ml) in individuals with different genotypes. Individuals possessing (a) or lacking (b) the HLA-B Bw4Ile80 epitope grouped into KIR3DL1 homozygous (L1/L1), KIR3DS1 homozygous (S1/S1) or KIR3DL1/S1 heterozygous (L1/S1). The horizontal line represents the median in each group. P-values in the figure are derived by the Kruskal-Wallis post-hoc test.

The simplest explanation for the results in *Figure 14a* would be that an interaction between KIR3DL1 and HLA-B Bw4Ile80 during the effector target interaction influenced NK cell ADCC activity. However, genotyping of the Jurkat T cells, used as target cells, indicated that these cells completely lack HLA molecules with the Bw4 epitope, which argues against that KIR binding to HLA in the *in vitro* setup contributes to the effects recorded. Rather a difference in activation state of NK cells from these individuals, possibly induced during education, might be the reason. The results are especially interesting since they are difficult to explain using the current dogma on NK cell education, which states that NK cells lacking inhibitory receptors for self MHC are rendered hyporesponsive [42-44]. In addition, as mentioned previously, a recent publication suggested that activating KIRs in presence of their ligand could reduce the responsiveness of the NK cell [426]. One possibility is that linkage disequilibrium with other inhibitory receptors and HLA molecules contributes to the increased activity of NK cells from individuals possessing the KIR3DS1 allele together with HLA-B Bw4Ile80.

Previous reports analysing NK cell degranulation in the unstimulated state or in response to stimulation through CD16 found an increased activity of $KIR3DS1^+$ NK cells as compared to $KIR3DS1^-$ NK cells [427, 433]. In presence of HIV-1 infected autologous $CD4^+$ T cells $KIR3DS1^+$ NK cells from $HLA\text{-}Bw4^+$ individuals degranulated to a higher degree as compared to NK cells from $HLA\text{-}Bw6^+$ individuals, whereas $KIR3DL1^+$ NK cells did not degranulate at all [279]. A similar effect might be operating in ADCC activation and could have resulted in the increased degranulation in KIR3DS1 positive individuals that possesses the HLA-B Bw4Ile80 epitope compared to individuals lacking this HLA epitope. A difference in frequency and level of expression of KIR3DL1 and KIR3DS1 in individuals with different HLA alleles [346, 427, 433], which could contribute to frequency of NK cells activated is one possible explanation.

From these results it seems as KIR genotype in conjunction with certain HLA alleles can influence NK cell ADCC activity. However, the low number of individuals positive for HLA-B Bw4Ile80 and/or KIR3DS1 in our study needs to be increased before any firm conclusions can be made. We also plan to extend the study and investigate differential responses in NK cell subpopulations expressing KIR3DS1 compared to KIR3DL1.

7.3 NK CELL AND ANTIBODY-MEDIATED EFFECTS IN THE *IN VIVO* HIV-1/MULV MOUSE MODEL

7.3.1 NK cells are involved in the early control of HIV-1/MuLV infected cells *in vivo* (paper III)

There are implications on a role for NK cells in protection from infection [260, 264, 266] and during the acute HIV-1 infection [267] in humans. To further investigate the effects of NK cells in an *in vivo* model for acute HIV-1 infection, we turned to the HIV-1/MuLV challenge model in mice. HIV-1/MuLV infected syngeneic cells were injected intraperitoneally into the mouse and at different timepoints thereafter the peritoneal cells were harvested and presence of NK cells and infected cells investigated *(Figure 6)*. An increased frequency of NK cells but not T cells was detected in the peritoneal fluid already at day one after injection of infected cells (Figure 1 in **paper III**). No

effect on the frequency of NK cells in the spleen was seen, indicating a specific expansion or recruitment of NK cells at the site of injection. The maturation stages of NK cells are known to vary in various tissues and in response to infection [62, 63, 143]. We therefore analyzed the maturation stages of NK cells in the peritoneum by staining for the markers CD27 and Mac-1 (see section 3.3.2) [62, 64]. Injection of HIV-1/MuLV infected cells resulted in an increased proportion of more mature NK cells of the $CD27^{low}$ subset, while the less mature subsets, double negative (DN) and $Mac\text{-}1^{low}$, decreased (Figure 2 in **paper III**). It thus seems that there is a shift towards higher frequency of NK cells of a more mature phenotype at the site of injection of HIV-1/MuLV infected cells.

The early increase in frequency of NK cells in the peritoneum upon injection of a number of different virus types was previously reported by Daniels et.al. [193]. An increased proportion of the more mature Mac-1 positive NK cells in spleen and liver after systemic MCMV infection has also been reported [63, 143]. The increased proportion of mature NK cells upon injection of infected cells may be due to recruitment, proliferation or maturation of NK cells in the peritoneum and possible mechanisms for this are discussed below.

Recruitment of NK cells to the peritoneum could be due to induction of chemokines that bind the chemokine receptors expressed by NK cells. One of these is the CCR5 receptor that binds MIP-1α which is involved in recruitment of NK cells to the liver upon MCMV infection [192]. MCMV infection induces production of type I interferons which are involved in recruiting MIP-1α producing macrophages and this in turn results in recruitment of NK cells [191, 192]. In addition, NK cell recruitment to vaginal HSV-2 infection was regulated via regulatory T cells and production of the CCR5-binding chemokine RANTES [434]. Different NK cell subsets express different chemokine receptors [67], which may explain a possible preferential recruitment of $CD27^{low}$ NK cells to the site of infection. These cells express the sphingosine 1-phosphate (S1P) receptor $S1P_5$, that contributes to increased NK cell migration [69]. Also, integrins such as Mac-1 could be involved in differential recruitment [67].

NK cells proliferate in response to multiple cytokines which are induced by infection, exemplified by the increased NK cell turnover in liver upon MCMV infection [194]. The proliferation in MCMV infection is occurring in two phases. The first phase (day 1-2) is unspecific and involves all NK cell subsets and the later phase (day 4-6) is specific for mature NK cells expressing the activating receptor Ly49H [63, 194]. The $Ly49H^{+}$ cells are stimulated by the MCMV protein m157 that binds the receptor [32, 35]. NK cell interaction with MCMV infected cells also leads to an accelerated phenotypic NK cell maturation [143]. Thus phenotypic maturation during unspecific proliferation of the NK cells may result in the early increased frequency of $CD27^{low}$ NK cells detected by us in the HIV-1/MuLV model. However, further studies are warranted to determine the responsible mechanism.

To investigate whether NK cells could have any effect on the infected cells, mice were depleted of NK cells with an NK1.1 specific antibody before challenge with HIV-1/MuLV infected cells. The effect on the infected cells was measured by an HIV-1 p24 ELISA on *in vitro* cultured peritoneal cells [252]. Depletion of $NK1.1^{+}$ cells in mice resulted in an increased number of HIV-1 p24 positive cell cultures from these mice (Figure 4 in **paper III**). This indicates that $NK1.1^{+}$ cells are involved in the early control of HIV-1/MuLV infected cells in the peritoneal cavity. A likely explanation is an NK cell mediated killing of the infected cells. In addition to NK cells, $NK1.1^{+}$ T

cells are also depleted with the antibody treatment used. However CTL responses are not affected by anti-NK1.1 treatment [435]. When CD16 expressing cells (potentially NK cells, T cells and monocytes) were depleted in rhesus macaques infected intravenously with SIV, efficient depletion of $CD16^+$ cells lasted for a week and in this early phase after infection there was a trend towards increased viral load in the CD16 depleted macaques [436]. This effect was however lost in the later phase of the infection. The route of infection might have influenced the response since a mucosal route of infection might be more relevant for determining the contribution of NK cells in the early host defence. Thus, further investigations are needed to determine if NK cells are also important for early control of infection in non-human primates and patients.

We investigated whether the HIV-1/MuLV infected cells differently expressed MHC class I molecules and a ligand for NKG2D as compared to uninfected cells. The ligands were chosen based on reported differences on HIV-1 infected human cells [359, 360, 363]. Only slight differences in MHC class I expression and no effect on the NKG2D ligand Rae-1 was found (data not shown). This is in accord with our ligand stainings on $HIV\text{-}1_{IIIB}$ infected human T cells and may be explained by the use of the same HIV-1 viral strain in the mouse system as in the above mentioned human studies (**paper I and II**). Other ligands than the ones mentioned are probably involved in the NK cell recognition of HIV-1/MuLV infected cells. This recognition may be directed to the HIV-1 virus or to the MuLV virus since the pseudovirus used consists of both these viruses. In the future we plan to investigate the effect of MuLV versus HIV-1 on NK cell recruitment and activation in the model.

In conclusion this study shows that $NK1.1^+$ cells are involved in the control of HIV-1/MuLV infected cells *in vivo* and that these infected cells stimulate a response resulting in an increased frequency of NK cells with a more mature phenotype in the peritoneum.

7.3.1.1 Pros and cons with the HIV-1/MuLV mouse model.

There are some characteristics of the HIV-1/MuLV mouse model that deserves attention when interpreting data from these studies. The high doses of the HIV-1/MuLV virus supernatant needed for infection of mouse cells results in a high frequency of dead or dying splenocytes in the infected cell cultures. When these are injected into the mouse it may induce immune reactivities that induce the recruitment, expansion or activation of NK cells, induced by apoptotic cells rather than in a virus-specific manner. However, HIV-1 is a lytic virus which induces cell death, and therefore the injection of cells which are induced to die by virus may be relevant. The infection of cells with an uncharacterized virus consisting of a mixture of HIV-1 and MuLV may also affect the interpretation of these data. We have not yet investigated the role of MuLV itself in the data we have obtained. It is possible that the increased NK cell numbers we find and the mature phenotype of these cells result from MuLV as well as from HIV-1. Nonetheless, it is interesting to note that virus infected cells can affect NK cells in the peritoneum in this model.

The data of NK cell control of virus infection in the HIV-1/MuLV model depends on retrieval of infected cells from the peritoneum, and thus that infected cells do not migrate from this site. There is no evidence for migration of cells or for the transport of infected cells to other organs [252]. However, if this kind of migration occurs it would probably not be affected by depletion of NK cells. In most mouse strains injection of

HIV-1/MuLV infected cells results in early clearance of the injected cells. Only a few mouse strains, including the HLA-A2 transgenic mouse used by us permit, persistence of the virus infected cells [252]. It is possible that this is due to some kind of immune defect or genetic difference that affects the host response to infected cells, which may involve NK cells. This should also be kept in mind when interpreting data from this model.

Despite these considerations, studies with this model may contribute with important information regarding the *in vivo* effects of different immune cells and treatment modalities directed to HIV-1 infected cells during acute infection. This small scale model allows a great number of variables to be combined at limited space and time when choosing among several anti-retroviral strategies or drugs and combinations thereof. It may therefore be used as an *in vivo* advisor before embarking on larger animal models or clinical studies.

7.3.2 A drug-conjugated anti-envelope antibody is effective against HIV-1/MuLV infected cells *in vivo* (paper IV).

Despite the many unconjugated and conjugated mAbs used in human cancer therapy [397, 399] and the promising effects of passive immunizations in non-human primates [282-286], no antibody treatment has as yet had any long term effect in HIV-1 infected patients [292]. We investigated whether a HIV-1 specific antibody conjugated to the cytotoxic drug doxorubicin, used in treatment of cancer patients, is effective against HIV-1 infected cells *in vitro* and *in vivo* in the HIV-1/MuLV mouse model. In contrast to previous immunoconjugates with toxins, doxorubicin is not immunogenic. Conjugation of doxorubicin to cancer antigen specific mAbs is effective in mouse models for leukemia [437], but in a phase II clinical trial for treatment of metastatic breast carcinoma there was only a small number of partial regressions [438]. It is possible that this kind of therapy is most effective in minimal disease settings, exemplified by ART treated HIV-1 infected patients, and that the effect of the conjugate depends on the antigen targeted. We conjugated doxorubicin to a mouse mAb, specific for HIV-1 gp120, called P4/D10 [439]. This antibody binds to the V3 loop and mediates both neutralization and ADCC *in vitro* [439]. It was proven safe in a phase I clinical trial for late-stage HIV-1 infected patients [440].

The neutralizing capacity of the conjugated antibody was tested by incubating it with the virus isolate HIV-1_{IIIB} before adding the virus to Jurkat T cell cultures. The p24 production in the cell cultures was measured by ELISA and the percent inhibition compared to Jurkat T cells mixed with only HIV-1_{IIIB} was calculated. Doxorubicin-conjugated P4/D10 neutralized HIV-1_{IIIB} with an efficacy similar to that of the unconjugated P4/D10 mAb and purified IgG from HIV-1 infected patients (HIVIG) (Figure 1a, **paper IV**). The doxorubicin conjugated P4/D10 was also able to eradicate virus infection in already infected cell cultures and this ability was greater than that of unconjugated P4/D10 mAb, free doxorubicin or irrelevant doxorubicin-conjugated mAb (Figure 1b, **paper IV**).

The possible *in vivo* effect of the doxorubicin-P4/D10 mAb on HIV-1 infected cells was investigated in the HIV-1/MuLV challenge model [252]. The conjugated or unconjugated antibodies were injected intraperitoneally at the same time as the mice were challenged with HIV-1_{IIIB}/MuLV infected cells. Mice were completely protected from challenge when treated with doxorubicin-P4/D10 mAb, since no cell cultures from these mice became positive for HIV-1 p24 (Figure 2 **paper II**). Unconjugated

P4/D10 mAb, free doxorubicin or irrelevant doxorubicin-conjugated antibodies were not protective. Thus doxorubicin-P4/D10 was significantly better than all the controls in inhibiting HIV-1_{IIIB}/MuLV replication *in vivo*, indicating that the effect of an anti-HIV-1 antibody could be significantly enhanced by coupling it to doxorubicin.

The effect of doxorubicin on HIV-1 infected cells could be due to induction of apoptosis in actively proliferating cells since it binds to topoisomerase II and hinders resealing of DNA breaks [441]. Repression of topoisomerase II by RNA antisense resulted in impaired HIV-1 replication *in vitro* [442], suggesting that doxorubicin may inhibit HIV-1 replication as well. Doxorubicin induced tumor cell death leads to an effective $CD8^+$ T cell anti-tumor immune response [443]. DC uptake of apoptotic cells killed by doxorubicin was more effective than the DC uptake of cells killed by other cytostatics [443]. It is thus possible that doxorubicin-conjugated mAbs could induce an effective HIV-1 specific immune response at the same time as it specifically kills HIV-1 infected cells.

For future clinical development of this drug-conjugated mAb concept, a cocktail of antibodies targeting conserved regions of the HIV-1 envelope should be used to avoid viral escape mutations [225, 284, 292]. On top of this it may be an equally important strategy to couple different cytotoxic drugs to the antibodies to be able to reduce the dose of each single drug and thus decrease risks of toxicity (very much the same way as is today done with antiretroviral drugs by obtaining synergistic antiviral effects with reduced toxicity). Immunogenicity of mouse antibodies is problematic and human or humanized antibodies are preferable [397]. ART treated patients would be the target group to avoid problems associated with high viral load [405]. This could increase the chances of success by simultaneously introducing both agents that kill infected cells and ART, which inhibits replication. If it were possible to induce reactivation and drive out HIV-1 from latently infected cells in a safe way [230], simultaneous treatment with immunoconjugates and ART could potentially decrease the latent reservoirs [405, 407]. However, the feasibility to cure latent HIV-1 infection with this kind of treatment is questionable and needs further investigation [406].

8 CONCLUDING REMARKS

Despite the tremendous research performed to understand the HIV-1 virus and how to combat it, no cure or preventive measure for the disease exists today. During the last years the interest for the role of NK cells in HIV-1 infection has increased substantially, much thanks to the elucidation of the associations of certain KIR and HLA genes with a slower disease progression [339]. In addition, the increased activity of NK cells from individuals that avoid HIV-1 infection despite repeated exposures to the virus [260] justifies the increased interest in this field of research. In this thesis, potentially effective treatment modalities based on NK cells or antibodies have been examined in addition to studies on the involvement of NK cells in acute and chronic HIV-1 infection.

Our results concerning the role for NK cells in the acute HIV-1/MuLV model stresses the importance of considering NK cells as a component in protection from infection. Further knowledge on the mechanisms for this NK cell effect on infected cells are needed, as is understanding on what the triggers for NK cell activity in this model are. This type of information could contribute to development of treatments or vaccines for which an increased NK cell response may be advantageous.

The interesting associations between KIR and HLA genotype and NK cell ADCC activity need to be extended and the phenotype of the NK cells responsible for the effect discerned. The possible contribution of certain KIR genes to the NK cell activity deserves further attention since it may affect the individuals NK cell response to HIV-1 infection.

The possible differences in specificity of ADCC inducing antibodies in controller patients may indicate that the epitopes targeted by this type of antibodies contributes to a slower disease progression. Further studies with increased numbers of controller patients are needed to confirm or refute our tentative conclusions and to in more detail investigate the epitopes on oligomeric envelope proteins targeted by ADCC inducing antibodies. Such epitopes may be good candidates for induction of protective antibodies with active immunization strategies. This knowledge may also be used when choosing which free or conjugated antibodies to use in future passive immunization trials.

The potent effect of a drug-conjugated anti HIV-1 envelope antibody in the HIV-1/MuLV mouse model deserves further attention and the concept should be developed to include conjugated-antibodies targeting different epitopes on the HIV-1 envelope protein.

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