

THE HUMORAL IMMUNE RESPONSE TO HIV AND ITS EFFECT ON VIRUS REPLICATION AND EVOLUTION

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RESEARCH SUMMARY

This thesis focuses on two different mechanisms of the humoral immune defence against HIV-1 in humans and its effect in virus replication and evolution, neutralisation and antibody-dependent cellular cytotoxicity (ADCC). Development of HIV-specific antibodies in an infected individual happens shortly after infection, neutralising antibodies appear later but are thought to play an important role in disease containment. However, the role of antibodies that mediate effector functions (complement and ADCC) remains largely unclear. The thesis presented here, is structured in two parts that deal with different mechanisms of antibody responses. The first part reflects work on establishing a novel assay system to evaluate ADCC activity and its role in HIV infection. The second part analyses the effect of neutralising antibodies in viral replication and most notably evolution and development of resistant viral strains to the neutralising antibody response.

The development of an *in vitro* assay that allows to measure in a sensitive and standardised way, ADCC mediated by HIV-specific antibodies is discussed in part 1. Since development of ADCC-mediating antibodies in patients occurs very early in infection together with the emergence of HIV specific cytotoxic T lymphocyte response, we aimed to develop a method that will allow us to investigate the influence of ADCC on disease progression. Monitoring changes in ADCC-antibody titres during the evolution of disease, would allow deriving a better understanding of the timing of these antibody responses in comparison to neutralising antibodies and estimating the respective impact of these mechanisms. Measuring ADCC in a system that most closely resembles *in vivo* conditions and allows assessment of activity against autologous patient virus, has not been developed previously and has proven challenging. The current work presents a thorough analysis of parameters in the development of the assay and succeeds in presenting an optimised strategy that allows measuring ADCC against primary HIV infected cells by flow cytometry.

The second part of this work is based on a previous study conducted in our lab where we assessed the ability to induce immune selection *in vivo* to evaluate the impact of the neutralizing antibodies 2G12, 2F5 and 4E10 against HIV-1 *in vivo*. During passive immunisation all patients harbouring 2G12 sensitive strains developed escape mutants to this antibody while no relevant changes in the sensitivity to the

monoclonal antibodies (MAb) 2F5 and 4E10 occurred. These results were confirmed by sequence analysis of the membrane proximal external region (MPER) where the epitopes of these two MAb are located.

We performed a detailed sequence analysis of the 2G12 resistance pattern of viral strains from 13 patients that emerged *in vivo* following immunisation, demonstrating that resistance is dependent on five N-linked glycosylation sites that form the antibody's epitope. Additional detailed clonal analysis of the MPER of virus derived from three patients confirmed that *in vivo* no changes in the epitopes of 2F5 and 4E10 occurred. To investigate if this disparity in escape mutant generation reflects that 2F5 and 4E10 antibodies were ineffective *in vivo* or if alternatively, patient virus strains were coherently refractory to changes in the relevant regions of the MPER, we also performed *in vitro* escape mutant studies. To this end primary viruses were derived from four patients before the passive immunisation trial and subjected to immune selection with the three antibodies *in vitro*. We found that escape to 2G12 occurs also rapidly *in vitro*. Nevertheless, although resistance conferring mutations also were strictly found in the 2G12 core epitope, they differed in most cases from the resistance mutations found *in vivo*. Additionally, in contrast to the *in vivo* situation, 2F5 and 4E10 escapes can emerge *in vitro* although less frequently than 2G12 escapes. 4E10 resistant mutants were the most difficult to generate and frequently resulted in abortive infection or in viral mutants with impaired replicative capacities. Sequence analysis of the MPER generated *in vitro* mutants revealed changes in the respective epitopes of the antibodies and confirmed phenotypic resistance. In conclusion viruses preserved sensitivity to 2F5 and 4E10 *in vivo* despite the ability of the isolates to tolerate mutations in the epitope of these antibodies *in vitro*, however gain of resistance may be difficult to attain and can result in loss of infectivity and replication capacity.

ZUSAMMENFASSUNG

Die vorliegende Dissertation behandelt verschiedene Mechanismen der humoralen Immunantwort gegen das Humane Immundefizienz-Virus vom Typ 1 (HIV-1) und deren Auswirkungen auf die Replikation und Evolution des Virus, die Neutralisation und die durch Antikörper vermittelte zelluläre Zytotoxizität (ADCC). Die vorliegende Arbeit befasst sich mit den unterschiedlichen Mechanismen der Antikörperantwort und ist zweigeteilt. Der erste Teil behandelt Versuche zur Etablierung eines neuen Assay-Systems, um die ADCC-Aktivität und ihre Rolle in der HIV Infektion zu evaluieren. Der zweite Teil analysiert den Effekt von neutralisierenden Antikörpern auf die Virusreplikation und vor allem auf die Evolution und Entwicklung von Virusstämmen, die gegen diese Antikörperantworten resistent sind.

Begonnen wurde mit der Entwicklung eines *in vitro* Assay-Systems, welches auf sensitive und standardisierte Weise, die durch HIV-spezifische Antikörper ausgelöste ADCC bestimmt. Auf der Tatsache beruhend, dass ADCC verursachende Antikörper zusammen mit den zytotoxischen T Lymphozyten in Patienten früh nach der Infektion entstehen, setzten wir uns das Ziel, eine Methode zu entwickeln, in der man die ADCC während des Krankheitsverlaufes verfolgen kann. Dadurch könnte die Entwicklung und den Einfluss dieses Mechanismus auf die HIV-Krankheit besser verstanden werden und schliesslich mit der neutralisierenden Antikörperantwort verglichen werden. Dies würde zu besseren Kenntnisse über die Entstehung und der Bedeutung beider Antikörperantworten führen. Ein System, in der man die ADCC-Antwort direkt aus dem autologen Patientenvirus messen kann und das gleichzeitig am ehesten den *in vivo* Konditionen entspricht, wurde bis jetzt noch nicht entwickelt und hat sich als sehr anspruchsvoll gezeigt. Diese Arbeit legt eine detaillierte Analyse der benötigten Parameter für die Entwicklung eines solchen Assay-Systems dar. Es zeigt eine optimierte Strategie, in der man mittels Durchflusszytometrie die ADCC direkt auf primär infizierten Zellen messen kann.

Der zweite Teil der Arbeit beruht auf einer passiven Immunisierungsstudie mit 14 HIV-Patienten die in unserer Gruppe durchgeführt wurde. In dieser Studie wurde *in vivo* die Wirksamkeit von drei neutralisierenden Antikörpern (2G12, 2F5 und 4E10), HIV zu unterdrücken, getestet. Alle Patienten, die vor der Immunisierung 2G12

sensitive Stämme aufwiesen, entwickelten Resistenzen gegen diesen Antikörper. Im Gegensatz dazu sind keine Sensitivitätsveränderungen bei den anderen zwei Antikörpern aufgetreten. Dies wurde durch Sequenzierung der externen proximalen Membranregion (MPER), in der die Epitope beider Antikörper liegen, bestätigt.

In dieser Arbeit wurde von 13 Patienten, HIV-Stämme aus verschiedenen Zeitpunkten der Immunisierungsstudie isoliert und die Region in der 2G12 bindet, sequenziert. Dank einer detaillierten Analyse dieser Region in den auftretenden Stämmen konnte ein Muster der 2G12-Resistenz erstellt werden. Ausserdem konnten wir nachweisen, dass die 2G12-Resistenz von den fünf bekannten N-gebundenen Glykosilierungsstellen abhängig ist. Bei drei Patienten wurde durch zusätzliche klonale Analyse der MPER-Region bestätigt, dass *in vivo* keine Resistenzveränderungen in den Epitopen von 2F5 und 4E10 vorgekommen sind. Als nächstes wurde untersucht, ob die MPER-Antikörper *in vivo* wirkungslos waren oder ob Viren in dieser Region keine Mutationen ertragen. Erreicht wurde dies, indem HIV-Stämme, die von vier der Versuchspatienten vor der Studie isoliert wurden, *in vitro* einem Selektionsdruck durch einen der drei Antikörper ausgesetzt wurden. Wie *in vivo* entstehen *in vitro* schnell Resistenzmutationen gegen 2G12. Auch in den *in vitro* Mutationen hat sich die 2G12 Resistenz auf das Epitop beschränkt, trotzdem waren das Resistenzmuster meistens anders als die *in vivo* aufgetretenen. Im Gegensatz zu den *in vivo* Erkenntnissen, sind bei den *in vitro* Experimenten auch Viren entstanden, die Mutationen aufwiesen die zu einer Resistenz gegen 2F5 und 4E10 führen. Diese Mutationen traten aber seltener als 2G12 Resistenzmutationen auf und konnten zu Replikationsdefiziente Viren führen. Sequenzanalysen haben die phenotypischen Resistenzen bestätigt und gezeigt, dass die Mutationen an den respektiven Epitope der MPER-Antikörpern aufgetreten sind. Obwohl Viren Veränderungen im MPER erlauben sind *in vivo* keine 2F5 oder 4E10 Mutationen entstanden. Allerdings könnte das Erlangen von Resistenz schwierig sein, da dies zu verminderten Replikationskapazitäten oder zu Verlust von Infektiosität führen könnte.

INTRODUCTION

1. The HIV/AIDS epidemics

Over the 25 years since it was first diagnosed, human immunodeficiency virus (HIV), the agent causing acquired immune deficiency syndrome (AIDS), has become one of the most significant global health problems. With an estimated of 39.5 million infected people, 4.3 million of new infections and nearly three million of deaths in 2006 (UNAIDS 2006) (Figure 1) the realisation of an effective preventive vaccine is one of the most urgent goals for world health.



Figure 1. Global view of adult HIV infection in 2006. Adapted from UNAIDS, 2006.

Where antiretroviral therapy is available, it has led to a dramatic reduction in AIDS-related morbidity and mortality. However, despite recent improved access to antiretroviral treatment and care in many regions of the world, the AIDS epidemic is still expanding. Moreover the inability of the actual treatment to completely eliminate the virus from the body and the fact that resistance to those agents develops readily in a number of patients, necessitates lifelong treatment of infected individuals and the continuous development of novel drugs. Great efforts have also been put towards development of protective and therapeutic vaccines. Various different types of vaccines are currently under development, (reviewed in (Haynes *et al.* 2005b;

Heeney *et al.* 2006; McMichael 2006)), aiming to elicit either CD8 T cell-mediated responses against HIV, or broadly neutralising antibodies, or both.

Successful development of vaccine will thus require the identification of protective and broadly active immune responses against HIV-1.

2. Virion structure and genomic organisation

HIV is a lentivirus, which belongs to the family of retroviruses. Lentiviruses are capable of long-term latent infection of cells, of cytopathic effects, and cause slowly progressing but fatal diseases in humans and animals. Two closely related HIV types have been identified, HIV-1 and HIV-2, the latter being more closely related to the simian immunodeficiency virus (SIV) than HIV-1 with whom it only shares 40% of homology (Chakrabarti *et al.* 1987; Franchini *et al.* 1987). HIV-1 is genetically very diverse, its phylogenetic tree is divided into three main branches, the M (main), the N (new) and the O (outlier), group M viruses being the most widespread and causing up to 99% global infections. This group is divided into nine different subtypes or clades (A through K). Classing was originally based on short sequences within the envelope glycoprotein but more recently on full length sequences (McCutchan 2000; Robertson *et al.* 2000; Binley *et al.* 2004). Clade B is the most prominent class in Europe, America and Australia (Kuiken *et al.* 2000). Clade C viruses are dominant in Southern Africa, China and India and are responsible for most of the infections worldwide (McCutchan 2000). Clades A and D are common in Central and Eastern Asia. Nevertheless it is difficult to class every virus in a specific clade as recombination between viruses from different clades has taken place, creating interclade recombinants, such as the circulating recombinant form (CRF)01, whose envelope belongs to clade E while other genome regions stem from clade A ((Heyndrickx *et al.* 2000; Robertson *et al.* 2000), reviewed in (McCutchan 2006))

The mature virion as shown in Figure 2 has a diameter of around 100nm and is enveloped by a host cell derived lipid bilayer. Within lies a matrix (MA) shell composed of p17, which contains the two RNA copies. These are stabilised as a ribonucleoprotein complex with the nucleocapsid protein, p7, and surrounded by a capsid core made of the protein p24. In addition some non-structural viral proteins

(such as the reverse transcriptase) and cellular proteins are also incorporated in the virion.

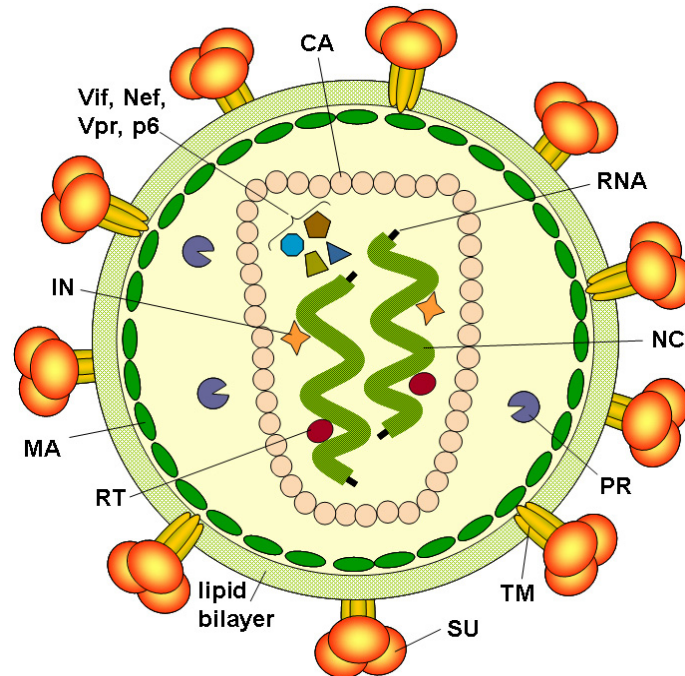


Figure 2. Structure of the mature HIV-1 virion and its components. Abbreviations: capsid (CA), integrase (IN), matrix (MA), nucleocapsid (NC), reverse transcriptase (RT), protease (PR), surface unit (SU), transmembrane unit (TM).

The HIV genome is composed of two positive copies of single stranded RNA that encode nine open reading frames of a total size of ~ 9kb. See Figure 3 and Table 1 for an overview.

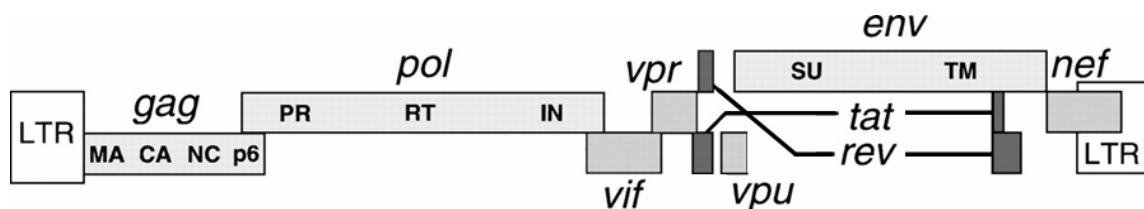


Figure 3. Genome organisation of HIV-1. The proviral DNA is flanked by LTRs which control the expression of the viral genes. The relative positions of genes that encode the structural and enzymatic proteins (Gag, Pol and Env), the regulatory proteins (Tat and Rev), and the accessory proteins (Vif, Vpu, Vpr and Nef) are indicated. Adapted from Frankel and Young, 1998.

Gene	mRNA splicing	Encoded protein(s)
<i>Regulatory genes</i>		
<i>tat</i>	multiple	Tat (16kDa)
<i>rev</i>	multiple	Rev (19kDa)
<i>Accessory genes</i>		
<i>vif</i>	single	Vif (23kDa)
<i>vpr</i>	single	Vpr (14kDa)
<i>vpu</i>	single	Vpu (15-20kDa)
<i>nef</i>	multiple	Nef (25-27kDa)
<i>Structural and enzymatic genes</i>		
<i>gag</i>	unspliced	Pr55^{gag} : MA (p17), CA (p24), NC (p7), p6
<i>pol</i>	unspliced	Pr160^{gag-pol} : PR (p10), RT (p61/p52), IN (p31)
<i>env</i>	single	gp160 : gp120, gp41

Table 1. Overview of genes encoded by the HIV-1 genome. Adapted from Ramezani and Hawley, 2002.

2.1. The structural and enzymatic genes

Gag (group-specific antigen), *pol* (polymerase) and *env* (envelope) code for polyproteins that are cleaved into individual proteins and are common to all retroviruses.

The four *Gag* proteins, matrix (MA; p17), capsid (CA; p24), nucleocapsid (NC; p7) and p6 together with the two *Env* proteins, gp120 (surface subunit; SU) and gp41 (transmembrane subunit; TM) are structural proteins. As mentioned above the matrix is located underneath the viral membrane. The capsid forms the structural core protecting the nucleoprotein complex which is stabilised by p7, and p6 is involved in budding and release of the HIV particles from the infected cell, (reviewed in (Freed 1998; Turner *et al.* 1999; Ramezani *et al.* 2002)). *Env* is the most variable gene of the HIV genome, it possesses 35% sequence diversity between clades, 20% of difference within clades and up to 10% sequence diversity within an infected person (Kuiken *et al.* 1996; Shankarappa *et al.* 1999). The *Env* proteins, gp120 and gp41 spikes are anchored in the bilayer membrane surrounding the viral core and mediate

viral entry to the host cell. Functional envelope spikes are formed by trimers of non-covalently linked gp120 and gp41. Further details to the structure and the functions of the Env glycoproteins will be given later in this thesis.

The three Pol proteins, protease (PR), reverse transcriptase (RT) and integrase are essential for the HIV life cycle and are encapsidated into the viral core. The protease cleaves the HIV polyproteins into their individual functional units. Viral RNA is transcribed into double-stranded DNA by the RT and is then integrated into the host's genome by the virus-encoded integrase, (reviewed in (Freed 1998) (Turner *et al.* 1999)).

2.2. The regulatory genes

Tat (transactivator) and *rev* (regulator of the viral gene expression) are regulatory genes and the first to be expressed after infection and integration in the host genome. *Tat* encodes a nuclear protein that binds to the viral RNA enhancing transcription initiation up to 1000 fold, acting principally to promote the elongation phase of HIV transcription (Ruben *et al.* 1989). Rev participates in the sequence-specific transport of unspliced and incompletely spliced viral mRNAs from the nucleus to the cytoplasm, (reviewed in (Freed 1998; Turner *et al.* 1999)).

2.3. The accessory genes

The four genes *nef* (negative factor), *vif* (virion infectivity factor), *vpu* (viral protein U) and *vpr* (viral protein R) are also known as accessory genes. Nef has several functions in the viral life cycle. By interfering with cellular factors it downmodulates CD4 surface expression as well as the expression of certain MHC class I molecules. It also can disturb T cell activation and stimulates HIV infectivity ((Kim *et al.* 1989), reviewed in (Greene *et al.* 2002; Anderson *et al.* 2003)). Vif is required for HIV replication by inducing degradation of the cellular cytidine deaminase APOBEC3G which otherwise inhibits viral replication. Vpu indirectly assists in assembly of the virion. Vpr is involved in the formation of the pre-integration complex (PIC) and mediates the nuclear import of this complex, (reviewed in (Freed 1998; Turner *et al.* 1999)). Nef, Vif and Vpr are packaged into the virus particles.

3. Virus-cell interactions

3.1. HIV-1 replication cycle

The replication cycle of an HIV virion (Figure 4) starts with docking to the viral receptors and entry into a target cell, followed by reverse transcription, transport and integration of the proviral DNA into the host's genome. Subsequently the genetic information is transcribed and the mRNA translated. The different viral proteins and genomic RNA are thought to assemble at the cellular membrane. This process is followed by budding, release and final maturation of the newly synthesised virion, (reviewed in (Turner *et al.* 1999; Freed 2001; Nielsen *et al.* 2005)).

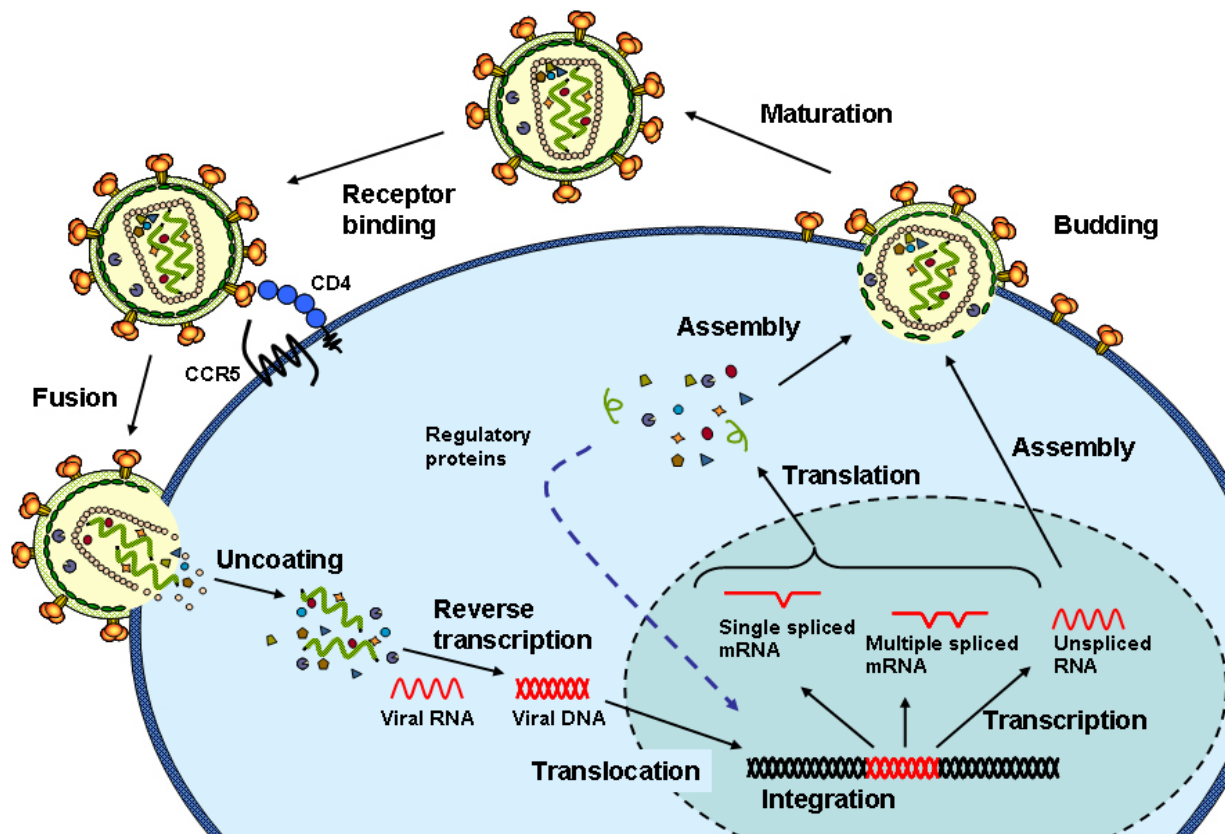


Figure 4. Schematic representation of the HIV-1 life cycle. A mature virion binds to its receptors on the target cell, followed by cell fusion, release of viral contents into the cytoplasm of the target cells, reverse transcription and translocation of the viral cDNA into the nucleus and integration of the viral genetic material into the host's genome. Subsequent transcription and translation of viral proteins leads to assembly and budding of the immature virion. Finally viral maturation occurs after release from the target cell.

3.1.1. Virus entry

Three different steps can be discerned in the processes that lead to virus entry into a target cell, Figure 5. The first step in viral infection is defined by the attachment of virions to the cell surface of permissive cells. This first interaction is mediated through the binding of the viral envelope protein gp120 to its principal receptor, the CD4 molecule, which is expressed on helper T cells, macrophages and dendritic cells. Binding is followed by conformational changes leading to exposure of the co-receptor binding thereby enabling interactions with the secondary receptor. HIV co-receptors are members of the family of seven-transmembrane chemokines receptors, the main co-receptors used by HIV-1 are CCR5 and CXCR4. CCR5 is the main receptor utilised by viruses after transmission and during early stages of infection. Viruses that only use this receptor are called R5-tropic viruses. This preference in co-receptor usage changes in about 50% of the patients during disease. First viruses that use both CCR5 and CXCR4 (dual tropic viruses) as co-receptors emerge. Later in disease the virus population switches to exclusive use of CXCR4 (X4-tropic viruses). This change in co-receptor usage is associated with accelerated loss of CD4 T cells and faster disease progression. However R5 viruses with high fitness may also be responsible of this (Quakkelaar *et al.* 2006).

Binding to the co-receptor in turn induces conformational changes initiating the third step in infection, the fusion process. Thereby cell and virus membrane are brought into close proximity. The N-terminal section of viral envelope protein gp41 gets exposed and forms a fusion peptide. This change in conformation weakens the interaction between gp120 and gp41. The N-terminal fusion peptide is inserted into the cell membrane and forms a pre-hairpin structure, ((Chan *et al.* 1997; Weissenhorn *et al.* 1997) reviewed in (Doms *et al.* 2000)). This structure is hardly accessible due to the proximity of both membranes and it only exists for some minutes. In its extended conformation gp41 displays two heptad repeat (HR) regions (N-and C-terminal) consisting of two antiparallel α -helices connected by an extended loop, separated by a sequence forming a small conserved disulfide loop. The succeeding formation of the stable six-helix bundle structure leads to membrane fusion. The six-helix bundle consists of a symmetrical trimer of heterodimers composed of one N-terminal and one C-terminal HR (HR1), where the three N-terminal HR (HR2) helices form a central parallel three-helix coiled coil that is

surrounded by three antiparallel arranged C-terminal HR helices. (Chan *et al.* 1997; Tan *et al.* 1997; Weissenhorn *et al.* 1997).

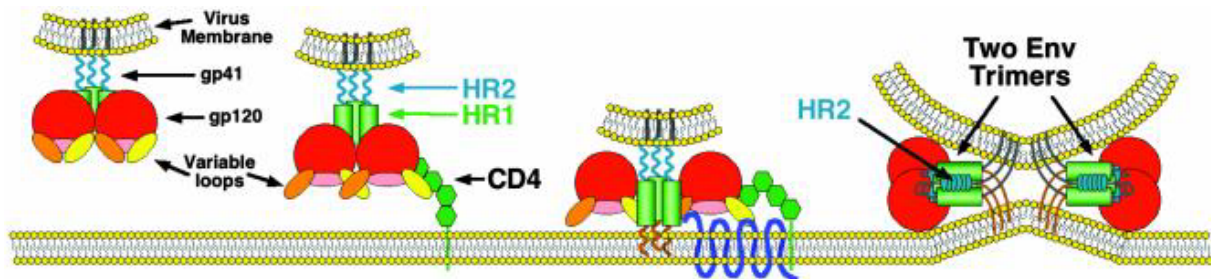


Figure 5. A model for HIV entry. Binding to CD4 is mediated by the Env gp120 subunit. After CD4 binding, gp120 binds to a seven-transmembrane domain co-receptor. Co-receptor binding ultimately results in formation of a six-helix bundle in which the helical HR2 domains in each gp41 subunit fold back and pack into grooves on the outside of the triple-stranded HR1 domains, bringing the fusion peptide and transmembrane domain of gp41 (and their associated membranes) into close proximity. Figure adapted from Moore and Doms, 2003.

It is likely that several Env trimers need to undergo this conformational change in order to form a fusion pore, (reviewed in (Moore *et al.* 2003)). It is currently not known whether gp120 remains associated during the fusion process or dissociates from gp41, (reviewed in (Moore *et al.* 2003)). Membrane fusion results in release of the viral core into the cytoplasm of the host cell (Wyatt *et al.* 1998a).

3.1.2. Reverse transcription and proviral integration

After the viral core enters the cytoplasm the capsid is disassembled progressively, a mechanism referred to as uncoating. Thereby a complex that contains the virus RNA genome and other viral (RT, Vif, Vpr, Nef, and integrase) and cellular proteins packaged in the virion are released. The viral RNA is then copied into double-stranded cDNA, a process mediated by the viral reverse transcriptase (RT) which forms a complex with a cellular tRNA₃^{lys} and the viral genome (Cen *et al.* 2001). The cellular tRNA₃^{lys} acts as primer to initiate synthesis of the negative DNA strand (Goff 1990; Das *et al.* 1994). After synthesis of the negative DNA strand the RNase H activity of the RT degrades the viral RNA template strand, initiating subsequently the synthesis of the second strand of proviral DNA (Panganiban *et al.* 1988). During this process the RT incorporates mutations into the virus. The *in vitro* mutation rate for

HIV-1 is of 4×10^{-5} mutations per target base pair per replication cycle, which predicts that about one mutation occurs in each three new genomes produced (Mansky *et al.* 1995; Mansky 1996), and is thus responsible for the fast evolution rate of HIV. The cDNA forms a pre-integration complex (PIC) together with several viral (MA, IN, RT and Vpr) and cellular proteins (Farmer *et al.* 1991; Burins *et al.* 1993). This complex in a next step translocates to the nucleus by connecting to microtubules and entering through the nuclear pores. This process is only partially understood but is probably mediated through the interaction of IN, MA and Vpr with the cellular import pathway (Trono 1995; Henklein *et al.* 2000; Sherman *et al.* 2001). Inside the nucleus integration of the proviral DNA occurs in a non sequence specific manner but preferentially is found at transcriptionally active regions of the host DNA (Schroder *et al.* 2002; Mitchell *et al.* 2004). The integrase first cleaves the viral DNA and the chromosomal DNA, followed by ligation of both sequences (Bushman *et al.* 1991).

3.1.3. Viral gene expression

Transcription of the integrated viral cDNA uses the cellular machinery by binding to the viral 5' long terminal repeat (LTR) flanking the provirus which contains the promoter and transcriptional enhancer elements. Initially transcription only produces short multiple spliced RNAs encoding Tat, Rev and Nef that are then transported out of the nucleus for translation. As Tat accumulates, the interaction of Tat with the transactivation response element (TAR) at the stem-loop of the nascent RNA enhances the efficiency of transcription elongation of the other HIV-1 genes (Keen *et al.* 1996). Unspliced and partially spliced RNA, which encode for structural proteins and genomic RNA, are generated by action of Rev, which upon translation give rise to progeny virus production.

3.1.4. Virus assembly, release and maturation

HIV assembly occurs either at the plasma membrane or on late endosomes. HIV-1 virus assembly starts with the association between the Gag precursor polyprotein Pr55 and the Gag-Pol precursor polyprotein Pr160, and leads to the formation of immature virion core (Sandefur *et al.* 2000). Both polyproteins are later cleaved into their final proteins during virus maturation. The complex formed by Pr55 and Pr160

next migrates to the cellular or endosomal membranes where the viral envelope trimer spikes, composed of gp120 and gp41, are accumulated. Gag proteins recruit components of the ESCRT cellular machinery through AIP-1, which directly binds to Gag or, indirectly, through TSG-101, a component of an ESCRT family, to facilitate and complete the assembly reactions (Freed 2003) (Pornillos *et al.* 2002). The double strand full-size genomic RNA, cellular tRNA primer and viral and cellular compounds associate with the budding structure (Tritel *et al.* 2001). Virions are then released from the cell surface as immature, non-infectious particles. Budding triggers the activation of the protease cleaving the polyproteins, thus generating mature, infectious virus particles (Freed 1998; Gottlinger 2001).

3.2. Natural course of infection

HIV infection and transmission occurs through exposure to body fluids (blood, mucus) containing the virus. Transmission occurs most commonly via sexual contact (Peterman *et al.* 1985; Friedland *et al.* 1987), but also through blood transfusions (Curran 1985; Fauci *et al.* 1985; Peterman *et al.* 1985), needles shared with infected persons (Ginzburg 1984; Rhame 1984) or transplantation of infected tissue (Curran 1985; Fauci *et al.* 1985; Peterman *et al.* 1985). HIV may enter the host through open wounds or sores, or mucous membranes. In the case of mother to child transmission, the transmission can happen perinatally, during delivering, or post partum, through breast feeding for instance (Dinsmoor 1989; Scarlatti 2004; Luzuriaga *et al.* 2006).

The clinical course of HIV-1 infection in humans in absence of treatment is characterised by three different disease stages (Figure 6). The first stage immediately after infection comprises an initial high peak of viremia and is referred to as acute phase of infection. At this point HIV-1 has emerged from the site of entry to the lymphoid organs, and has established viral reservoirs throughout the body (Pierson *et al.* 2000). Numbers of CD4 T cells, the primary target of HIV-1, decrease fast at this initial stage (Cooper *et al.* 1985; Clark *et al.* 1991; Daar *et al.* 1991; Tindall *et al.* 1991). The immune system consequently is stimulated and reacts against the viral boost by expansion of HIV-1 specific cytotoxic (CD8) T cells (CTL) and production of high levels of HIV specific antibodies. This response correlates with the decrease of viral load in the plasma however it is not able to completely eradicate the

virus (reviewed in (Goulder *et al.* 2004)). During the following chronic phase of infection plasma viremia attains a stable level known as the viral set-point. CD4 cell numbers typically have recovered to some extent from the initial drop. The set-point of continuous replication is maintained during a long period ranging from 5 to 15 years and is thought to be obtained by the efficacy of the immune response, host and viral genetic factors. In that time the patient is asymptomatic but CD4 counts gradually decline (Hazenbergh *et al.* 2000; McCune 2001). The viral set-point differs in every infected individual, usually higher set-point levels correlate with faster CD4 loss and faster progression to AIDS (Genesca *et al.* 1990; Schnittman *et al.* 1990; Simmonds *et al.* 1990). The symptomatic infection phase, also known as AIDS stage is the last phase of the disease and is characterised by plasma CD4 numbers below 200/ml, (reviewed in (Pinching 1988)). This leads to a collapse of the immune system rendering patients prone to opportunistic infections and/or tumours, eventually causing death.

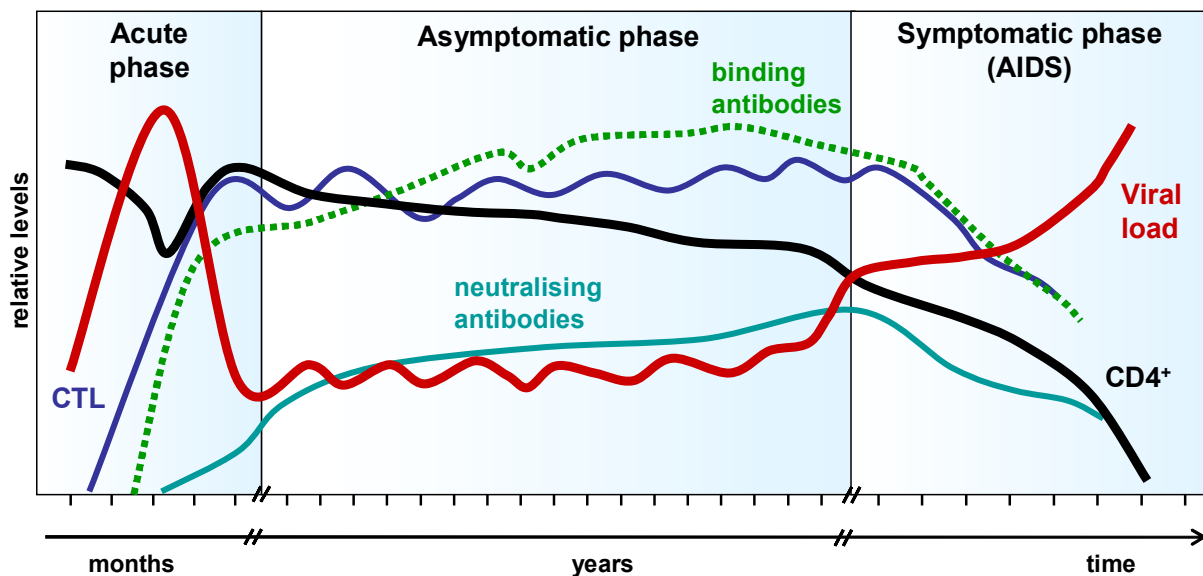


Figure 6. Natural course of HIV infection divided into its three infection phases. In addition to the viral load and the CD4 T cell numbers, specific immune response against HIV, such as the CTL response and the emergence of binding and neutralising antibodies are depicted.

3.3. Immune responses against HIV-1

The human defence against infections is composed of three levels: surface barriers, innate defences and adaptive responses. The immune system is a complex network

of cells, organs and tissues that are equipped to efficiently detect and eliminate the invading pathogen. Through the adaptive or acquired immune response the human body gains the ability to specifically recognise an infectious agent. It is mainly on these acquired responses against HIV that this chapter will focus on.

Upon infection with a pathogen the adaptive immune system responds with both B-cell (humoral) and T-cell (cell-mediated) response mechanisms. Humoral- and cell-mediated immune responses both depend on the action of a subset of T lymphocytes, the CD4 T cells. CD4 T cells have no pathogenic or cytotoxic activity but they recognise foreign particles bound to host class II MHC molecules. Upon recognition CD4 T cells get activated resulting in proliferation and secretion of cytokines, which in turn will enhance CTL and humoral immune responses. However CD4 T cells are the principal target of HIV. The infection results in continuous decay of CD4 T cell numbers as well as impaired functions of the remaining cells. In summary this leads to a dramatic impairment of the adaptive immune responses.

3.3.1. Humoral immune responses

Since viral proteins are recognised by the immune system as foreign (non-self) antibodies against many viral proteins are generated upon infection. Natural HIV infection generally elicits an early and persisting antibody response specific against the structural proteins of HIV (Gag and Env proteins) (Belec *et al.* 1995; Moore *et al.* 1995; Sattentau *et al.* 1995; Binley *et al.* 1997). However only a small fraction of these antibodies have direct neutralising activity, meaning that these antibodies are able to interfere with the virus life cycle through binding with relative high affinity and/or avidity to exposed sites on the virus surface (Dimmock 1993; Roost *et al.* 1995; Burton 2002; Zolla-Pazner 2004). The interaction of neutralising antibodies to the virion leads to inhibition of virus attachment to the target cell or blocking of the entry/fusion process. Theoretically neutralising antibodies can bind to free virus as well as to cell-associated virus and infected cells however it is thought that the most prominent activity of neutralising antibodies is the clearing of free virus particles (Burton 2002). In HIV-1 infection neutralising activity appears to be weak in the first weeks and of narrow specificity (Poignard *et al.* 1996). In general, neutralising antibodies at high titres only appear two to three months after infection (Safrit *et al.*

1994; Wei *et al.* 2003) (Richman *et al.* 2003) and are normally directed against specific epitopes on the envelope proteins gp120 and gp41. Nonetheless the overall effect of neutralising antibodies *in vivo* is not clear as the virus rapidly acquires resistance to the autologous antibody response, necessitating that the immune response has to adapt to continuously evolving viral escape mutants (Richman *et al.* 2003; Wei *et al.* 2003; Derdeyn *et al.* 2004). The protective role and the effectiveness in virus containment of neutralising antibodies *in vivo* will be discussed in detail later in Part 2 of this work. As mentioned above, neutralising antibodies represent only a fraction of the elicited antibodies during HIV infection. The remaining antibodies either recognise structures on the Env spikes that are irrelevant for the entry process or bind to non-functional spikes such as precursor forms or monomeric structures (Fouts *et al.* 1997; Parren *et al.* 1997; Herrera *et al.* 2003). These HIV-specific but non-neutralising antibodies may also contribute in clearing HIV *in vivo* by inducing effector functions such as complement dependent lysis, antibody dependent cellular-mediated cytotoxicity (ADCC) or phagocytosis.

The complement system is a component of the innate immune defence and its role as effector mechanism in the humoral immune system is significant (Walport 2001; Blue *et al.* 2004; Carroll 2004). Either directly, or through activation by virion bound antibodies, complement has been found effective in lysing various enveloped viruses, resulting in fragmentation of the outer membrane and disintegration of the nucleocapsid (Blue *et al.* 2004). In addition to lysis, the complement system has opsonising, phagocytosis inducing, chemoattractant and immune stimulatory functions, which shape the immune response to pathogens (Davies *et al.* 1994; Walport 2001; Stager *et al.* 2003; Blue *et al.* 2004; Carroll 2004). Complement-mediated lysis of HIV-1 virions is predominantly regulated by antibody activation of the classical pathway of complement lysis (Blue *et al.* 2004). Thereby virions are coated with antibodies and complement fragments, which are recognised by a member of the complement system. The activation cascade triggered by this initial interaction leads to the formation of the membrane attack complex on the virion surface. This complex disrupts the viral membrane by creating a pore leading to disintegration of the particle (Walport 2001; Blue *et al.* 2004; Carroll 2004).

In phagocytosis, an opsonised cell or virus is taken up by phagocytic cells via Fc-receptors or complement bound at their surface, internalised and destroyed in an

actin-dependent manner (Brown 1991; Rabinovitch 1995). Mostly responsible for this function are mononuclear phagocytes (macrophages and monocytes) and polymorphonuclear granulocytes (neutrophils), but also dendritic cells or fibroblasts are able to phagocytose (Rabinovitch 1995).

ADCC functions as potent immune effector mechanism in a wide variety of infectious diseases by combining components of the innate and the acquired immune system (Lowell *et al.* 1979; Nathan *et al.* 1980; Hashimoto *et al.* 1983; Sinclair *et al.* 1988b; Kohl 1991a). In ADCC, killer cells recognise pathogenic antigens expressed on infected cells via their Fc receptor. This binding will subsequently trigger killing of the infected cells by release of cytotoxic granules (perforin, granzymes), cytokines (TNF), chemokines, proteases, nitric oxide, reactive oxygen radicals or Fas/FasL interactions (Kagi *et al.* 1994; Berke 1995; Griffiths 1995; Trinchieri 1995; Cifone *et al.* 2001; Russell *et al.* 2002). This mechanism will be discussed in detail in Part 1 of this work. The exact implications of each of these mechanisms in containing virus replication at acute or chronic stages of HIV infection are however not yet clearly understood.

3.3.2. Cellular immune responses

Typically HIV-1 infection is characterised by an initial phase of high-level viremia (the acute phase), followed by a longer period of persistent lower to medium level virus replication (the chronic phase). The cytotoxic T lymphocyte (CTL) response, mediated by CD8 T cells, is developed after this first burst of viral replication and augments steadily with the viral load. The initial appearance of CTL responses during the acute phase coincides with a decrease in viral supporting the implication of CTL in disease control (reviewed in (Goulder *et al.* 2004), (Kiepiela *et al.* 2007)). CTL response is restricted to recognition of viral peptides presented by human leukocyte antigen (HLA) class I proteins or major histocompatibility type I proteins on the surface of infected cells. MHC class I proteins bind foreign peptides of a length of 9-11 amino acids that are synthesised within the cell itself, the CD8 T cells will then respond to the foreign antigen starting a CTL response. Different HLA alleles will have different specificities for viral peptides and therefore also for different CTL epitopes. According to this, some HLA have been associated with better disease

outcome, such as HLA-B27 and HLA-B57 (Kaslow *et al.* 1996) or on the contrary found to confer increased susceptibility to HIV infection (HLA-B35) (Carrington *et al.* 1999).

As much as 10% of circulating CD8 T cells are involved in these first response against HIV (Kuroda *et al.* 1999; Wilson *et al.* 2000). After infection there is a massive expansion of naïve CD8 T cells that is however restricted to a few clones ((Pantaleo *et al.* 1994; Kiepiela *et al.* 2007), reviewed in (Goulder *et al.* 2004)).

In the chronic phase of infection, some of the expanded CTL persist at high frequencies, it is possible that 1-2% of total CD8 T cells in a human are specific for an HIV epitope (Altman *et al.* 1996; Ogg *et al.* 1998). This high number of HIV-specific CD8 T cells is dependent on continued antigen stimulation as the number of specific T cells declines upon antiretroviral therapy when viral load falls (Kuroda *et al.* 1999). Without therapy there is a constant turnover and proliferation of HIV-specific CD8 T cells and the high number of specific cells will persist into late infection and up to development of AIDS. These antigen-stimulated cells can be divided into two groups, a large group of terminally differentiated cells that are likely to die and a smaller group of long-term memory cells from where the terminally differentiated cells probably expand (Pantaleo *et al.* 1990; Carmichael *et al.* 1993; Dunbar *et al.* 1998; Goulder *et al.* 2000).

CTL responses may decrease HIV-infection by either producing both cytokines and chemokines or by direct killing through secretion of perforins and granzymes. The cytokines produced include interferon- γ (IFN- γ), which inhibits viral replication and tumour-necrosis factor- α (TNF- α), which can upregulate HIV replication. CTL also produce CC chemokines MIP-1 α , MIP-1 β and RANTES, which suppress replication by competition for, or downregulation of the co-receptor CCR5. Additionally CD8 T cells secrete a yet undefined factor, the CD8 T cell antiviral factor (CAF) that inhibits HIV transcription (Mackewicz *et al.* 1994; Levy 2003).

HIV is nevertheless able to overcome CTL response in the same manner it evades antibody neutralisation by mutating rapidly in critical sites within or near sequences that encode CTL epitopes resulting in either defective antigen processing, loss of HLA binding or escape to CTL recognition (Moore *et al.* 2002; Draenert *et al.* 2004). Another escape route is the downregulation of HLA type I expression in HIV infected

cells through Nef (Collins *et al.* 1998). Nef is able to re-route newly synthesised HLA molecules containing a specific sequence in their cytoplasmic tail, to clathrin-coated pits for endosomal degradation (Le Gall *et al.* 1998; Blagoveshchenskaya *et al.* 2002). As HLA-A and HLA-B have this motif they are downregulated whereas HLA-C and HLA-E do not possess this motif and consequently their expression levels do not change upon infection (Cohen *et al.* 1999b; Ward *et al.* 2004).

In conclusion the immune response against HIV depends on a complex network which is functionally impaired. The CD4 T cell response is known to be diminished and is damaged already early in acute infection. The role of neutralising antibodies *in vivo* is also uncertain as virus always seems to escape and to be a step ahead in evolution. The positive effects of non-neutralising antibodies in engaging effector-mediated responses have not been established yet *in vivo*. Additionally although the CD8 T cell response is strong in primary infection its role during chronic infection is also unclear as the effects of the CTL response are suboptimal due to continuous evolving virus escape variants. An optimal strategy for controlling disease would likely require the stimulation of both humoral and cellular immune responses early in infection, before the immune system is severely damaged.

PART 1

4. Natural Killer cells, an introduction

Human natural killer (NK) cells are a bone marrow-derived subset of lymphocytes important in innate immune responses against several tumours and intracellular pathogens such as virus, fungi, bacteria and parasites as they recognise and lyse transformed and infected cells (Trinchieri 1989; Whiteside *et al.* 1989; Lanier 2001). Unlike cytotoxic T-cells (CTL), NK cells do not require prior activation for functionality. Innate responses induced by NK cells occur within hours, therefore providing strong defence activities before the humoral immune response is sufficiently developed. NK cells comprise around 10% to 15% of peripheral blood lymphocytes and are also found in the liver, peritoneum and placenta.

NK cells can potentially inhibit virus infected cells through several different mechanisms. The first mechanism is spontaneous cytotoxicity or natural cytotoxic activity, a mechanism that does not need priming. Nevertheless this process is strongly regulated by the balance between signalling through inhibitory NK receptors (iNKR) and natural cytotoxic receptors (NCR) on NK cells (Lanier 1998) (Sun 2003; Chiesa *et al.* 2005). These killer cells are also able to destroy target cells indirectly by inducing apoptosis of those cells. Interleukin-2 (IL-2) activated NK cells, also known as lymphokine-activated killer (LAK) cells express on their surface a Fas (CD95) ligand that will elicit in CD95⁺ target cells a cascade of events leading to cell death upon binding (Vujanovic *et al.* 1996). As NK cells express TRAIL on their surface the same process may be induced in TRAIL receptor⁺ cells (Cretney *et al.* 2002; Takeda *et al.* 2002). Moreover NK cells are also able of production and secretion of cytokines such as interferon γ (IFN γ), tumour-necrosis factor α (TNF- α) and granulocyte/macrophage colony-stimulating factor (GM-CSF) and chemokines such as the CC-chemokine ligands 3 (CCL3 or MIP1 α), 4 (CCL4 or MIP1 β) and 5 (CCL5 or RANTES) (Trinchieri 1989; Biron *et al.* 1999) that suppress replication of many viruses. Another mechanism of NK mediated antiviral activity is dependent on antibody recognition of an infected cell. In antibody-dependent cell cytotoxicity (ADCC), an antibody coated target cell is crosslinked to CD16 on NK cells via the Fc

portion of the antibody triggering lysis through secretion of cytotoxic granules. These three different effector mechanisms are summarised in Figure 7.

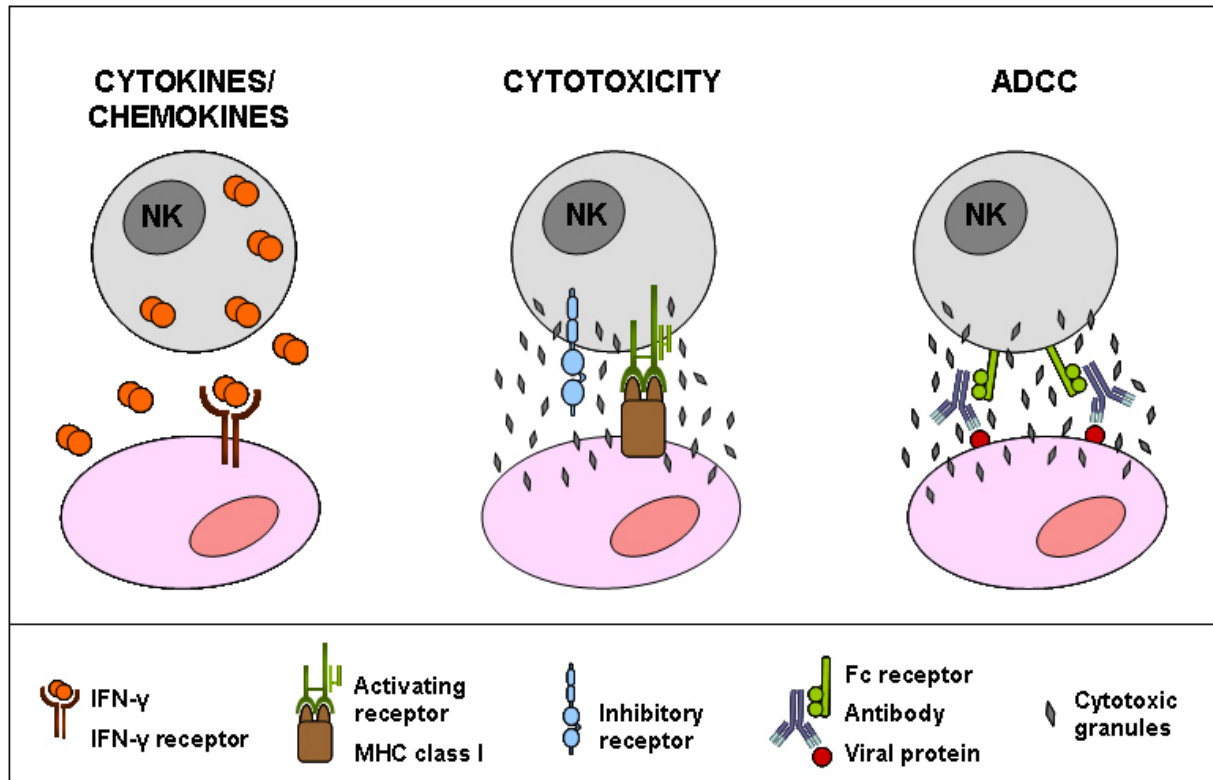


Figure 7. Responses induced by natural killer cells. a) Secretion of cytokines and chemokines, b) natural cytotoxicity, and c) ADCC.

Natural killer cells are identified phenotypically by the surface expression of CD56 and CD16 (FcγRIII), a low affinity IgG receptor specific for the Fc portion of IgG1 and IgG3 subclasses, and the lack of CD3. However at least two different NK subsets are known based on the expression levels of CD16 and CD56 (Nagler *et al.* 1989; Cooper *et al.* 2001; Lanier 2001). The CD16^{hi}CD56^{low} subset constitutes the biggest subset corresponding to 90% of the peripheral-blood NK cells; this subset produces low level of IFN γ , and is mainly responsible for both the cytotoxic activity and the antibody-dependent cell-mediated cytotoxicity. The CD16^{low/-}CD56^{hi} subset is principally responsible for cytokine and chemokine production and possesses low cytotoxicity.

4.1. Natural killing, the missing-self hypothesis

Natural killer cells were first identified by their ability to lyse cells without previous sensitisation (Herberman *et al.* 1986; Karre *et al.* 1986; Lanier *et al.* 1986). Initially Karre *et al.* showed in 1986 (Karre *et al.* 1986) that NK cells were able to discriminate between potential target cells by the amount of MHC class I expressed on the cell surface. According to the missing-self hypothesis, cell lysis via secretion of granzymes and perforins would be induced in those cells lacking or having downregulated the expression of MHC class I molecules on their surface, for instance missing self-recognition. It is now known that the mechanism involved in this recognition is far more complex and is modulated by the balance of interactions between activating and inhibitory receptors on NK cells with ligands available on the target cells. The response induced by NK cells includes beside the cytotoxic activity also secretion of cytokines and chemokines. A revisited version of the missing-self hypothesis supports the idea that NK lysis of a target cell will be induced if MHC class I molecules are low or absent but also if the target cell overexpresses ligands for activating NK receptors (Chiesa *et al.* 2005; Hamerman *et al.* 2005; Lanier 2005), as summarised in Figure 8. Experimental models have shown that even when the MHC class I molecule binds to its inhibitory receptor on NK cells, the target cell can be lysed, if a sufficient strong stimulation of activating NK receptors exists (Cerwenka *et al.* 2001; Diefenbach *et al.* 2001). On the other hand, lack of response to a potential target can also be due to the absence of activation signals as can be seen in the cases of human erythrocytes (no MHC expression) or neural tissues (low MHC expression), where both cells are safe of NK cytotoxicity. The fact that NK cells do not lyse human blood cells even though they do not express MHC class I molecules, may be explained by a deficiency of ligands on erythrocytes capable to engage NK activating receptors or also by expression of other molecules on blood cells that act as ligands for inhibitory NK receptors different to the expected MHC receptors, reviewed in (Lanier 2005).

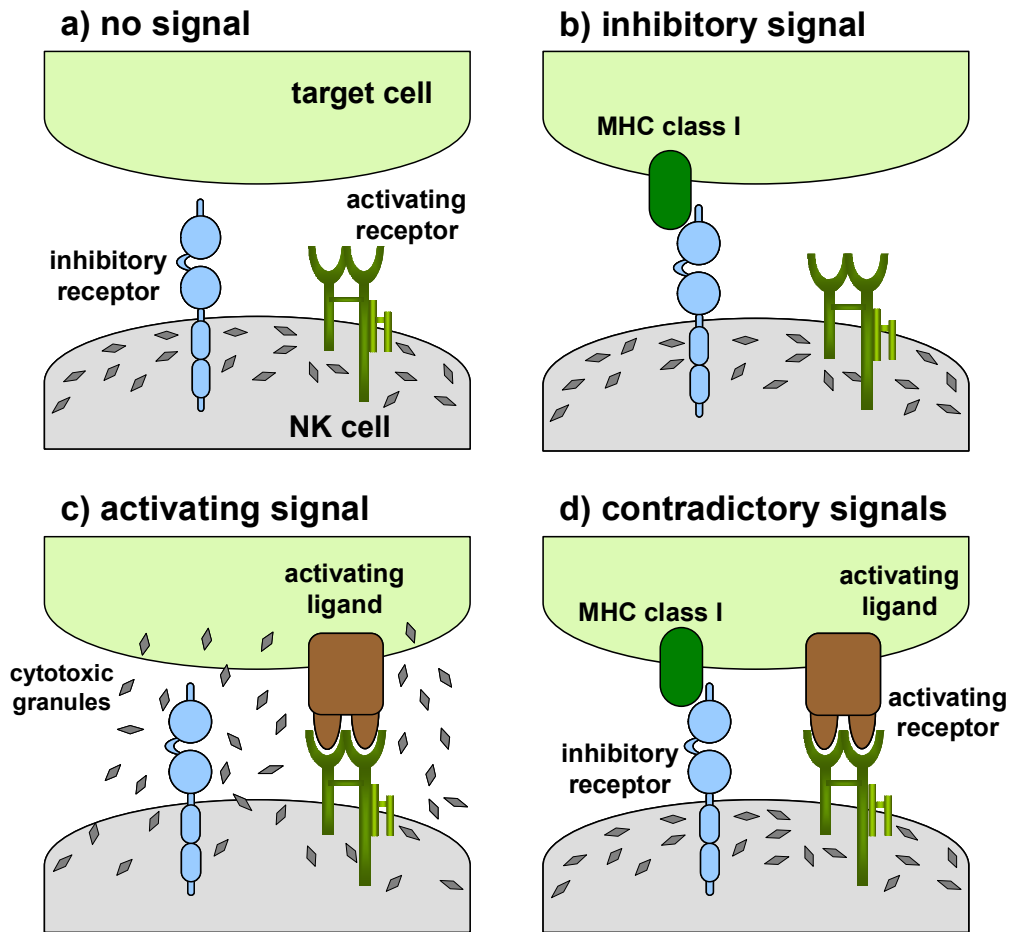


Figure 8. Encounter of an NK cell with a potential target and outcome options. a) no response, since neither activating nor inhibitory receptors are engaged, b) inhibitory receptors recognising MHC class I suppress NK cell responses, c) NK response is released by the binding of an activating ligand to the activating receptor, d) quality and strength of the opposing signals will determine the final response.

Human NK cells express an array of different inhibitory receptors that bind directly to MHC class I molecules. Structurally these receptors are members of two distinct superfamilies: killer cell immunoglobulin-like receptor (KIR) family or C-type lectin-like receptors (CTLR) such as CD94/NGK2A and -B heterodimers. All of these inhibitory receptors are characterised by a cytoplasmic tail containing the consensus sequence I/V/L/SxYxxL/V referred as an immunoreceptor tyrosine-based inhibitory motif (ITIM). Upon ligand binding, the ITIM domain is phosphorylated, possibly by a src-family kinase, with subsequent recruitment of different phosphatases (SHP-1, -2 or SHIP) depending on the receptor engaged. Recruitment of these phosphatases results in decreased phosphorylation of intracellular signalling, inhibiting or diminishing NK cell

effector functions (release of cytotoxic granules or secretion of chemokines and cytokines (Burshtyn *et al.* 1996; Vivier *et al.* 2004).

Activating NK receptors can be classified into two groups, those that bind to MHC class I molecules and those who do not. MHC binding receptors include members of families that also encode inhibitory NK receptors such as KIR or CD94/NKG2. The non MHC receptors include the lectin-like NKG2D (Vivier *et al.* 2002), NKR-P1A, NKR-P1C receptors and the Ig-like NKp30, NKp44 and NKp46 receptors, a third group of stimulating receptor molecules presumably act as co-activators rather than directly activating NK cells. These include the receptors CD16, CD28, CD40L, 2B4, LFA1/DNAM-1 and Lag-3 (reviewed in (Diefenbach *et al.* 2001) (Lanier 2001) (Vivier *et al.* 2004)). All stimulatory molecules lack the ITIM motif in their cytoplasmic domain, they have instead charged residues in their transmembrane domains that are necessary for association with adapter signalling proteins that possess an immunoreceptor tyrosine-based activation motif (ITAM) defined by the sequence, D/ExxYxxL/Ix(6-8)YxxL/I. The first step in the stimulatory signalling includes the binding of one adapter protein to the charged transmembrane domain of the stimulating receptor and subsequent tyrosine phosphorylation of the ITAM domain. Upon phosphorylation the tyrosine kinases Syk and ZAP70 are recruited initiating signalling events including Ca^{2+} influx, degranulation, and transcription of cytokine and chemokine genes.

In summary, unlike in B- or T-cells, effector functions triggered by NK cells are not dominated by a single signalling pathway but by the interaction of several opposing pathways elicited by inhibitory and stimulating receptors.

4.2. Antibody-dependent cell-mediated cytotoxicity (ADCC)

Antibody-dependent cell-mediated cytotoxicity (ADCC) is a powerful immune effector mechanism that requires components of the innate and the acquired immune system. In this mechanism, antigen-specific antibodies direct immune effector cells to the lysis of antigen-expressing target cells. It has been found that antibody dependent lysis is an important defence against bacteria (Lowell *et al.* 1979; Smith *et al.* 1980; Tagliabue *et al.* 1983), parasites (Mauel 1978; Albright *et al.* 1994; Bouharoun-Tayoun *et al.* 1995) and tumours (Nathan *et al.* 1980; Bright *et al.* 1994). In viral

infections, the significance of ADCC is not clearly elucidated, but it is nevertheless considered an important mechanism in control of herpes simplex virus (HSV) (Kohl *et al.* 1989; Kohl *et al.* 1990; Kohl *et al.* 2000), human T cell leukaemia virus type I (HTLV-I) and –II (HTLV-II) (Sinclair *et al.* 1988a) and Epstein-Barr virus (EBV) (Chan *et al.* 1979; Patel *et al.* 1982). A protective role of ADCC has been established in an *in vivo* mouse model against HSV (Kohl 1991b), and prevalence of high ADCC-antibody titres in sera of HSV and EBV infected patients have been found to correlate with slower disease progression (Chan *et al.* 1979; Kohl *et al.* 1989).

Functional ADCC requires three components a) the expression of the target antigen on the surface of the infected or cancer cells, b) the presence of antibodies specific for the target antigen having IgG or IgA isotype, and c) effector cells bearing the Fc gamma or alpha receptor (FcγR, FcαR), Figure 9. The specific antibodies recognise a target cell, subsequently binding to the surface-expressed antigens, the effector cells then interact with the Fc portion of the antibody via the Fc receptor. This crosslinking will engage the release of cytotoxic granules leading to lysis of the target cell.

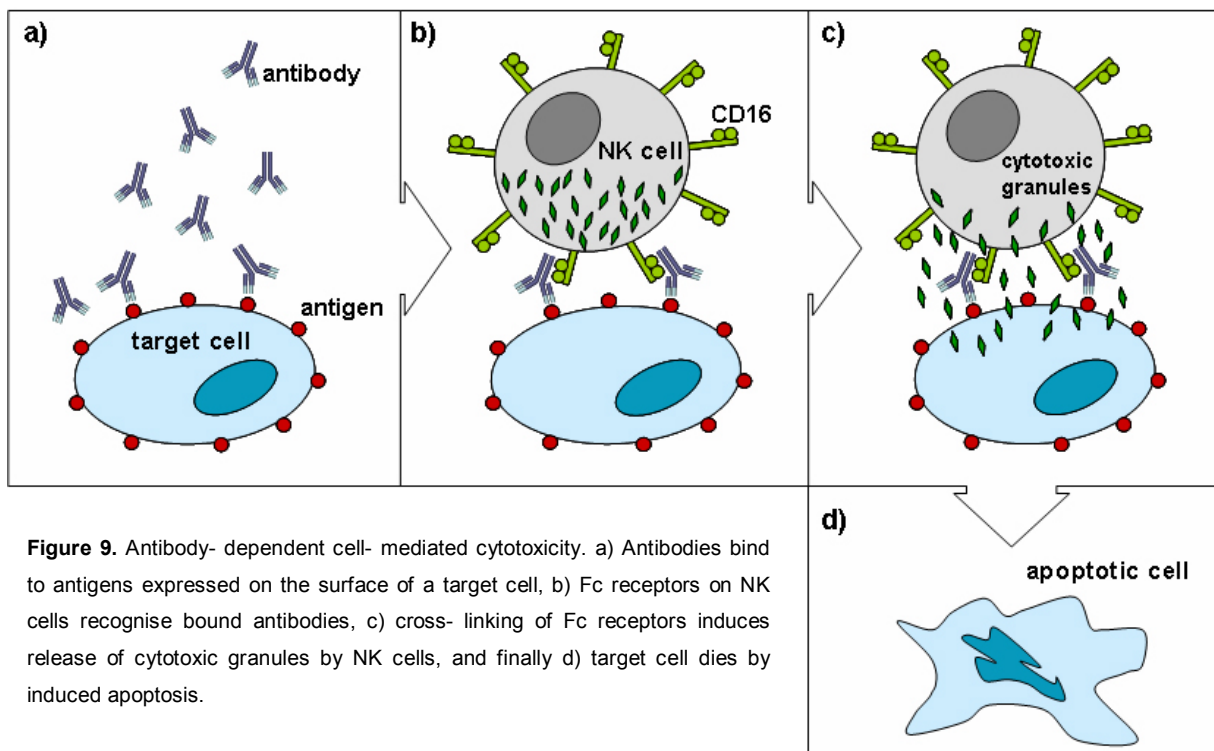


Figure 9. Antibody- dependent cell- mediated cytotoxicity. a) Antibodies bind to antigens expressed on the surface of a target cell, b) Fc receptors on NK cells recognise bound antibodies, c) cross- linking of Fc receptors induces release of cytotoxic granules by NK cells, and finally d) target cell dies by induced apoptosis.

NK cells are the major effector cells mediating ADCC but it can also be triggered by $\gamma\delta$ T cells (Braakman *et al.* 1992), neutrophils (Petty *et al.* 1989; Stockmeyer *et al.* 2003; Otten *et al.* 2005) and macrophages (Young *et al.* 1990; Boyle 2004). On their surface NK cells bear surface Fc γ receptors of type II (Fc γ RIIc; CD32) and type III (Fc γ RIIIA; CD16) (Morel *et al.* 1999; Metes *et al.* 2001; Ernst *et al.* 2002). NK cell mediated lysis occurs by secretion of cytotoxic granules as well as cytokines and chemokines. Macrophages release NO, reactive oxygen radicals and several proteases and phagocytose the antibody-coated target cells, they may also kill through Fas/FasL interactions (Kagi *et al.* 1994; Boyle *et al.* 2001; Boyle *et al.* 2002; Boyle 2004). Neutrophils mediate ADCC via Fc α RI (CD89) but the mechanism of killing is not completely unravelled and may include caspase release (Petty *et al.* 1989; Stockmeyer *et al.* 2003; Otten *et al.* 2005).

ADCC mediated by NK cells is initiated by crosslinking of the low-affinity Fc γ RIIIA (CD16) with the Fc region of the antibody-coated target cell. The Fc γ RIIIA on human cells is a multimeric complex consisting of CD16, the ligand binding α subunit and the non-covalently associated homodimers or heterodimers of ζ and γ subunits (Anderson *et al.* 1989; Lanier *et al.* 1989; Orloff *et al.* 1990; Kurosaki *et al.* 1991). Although none of the subunits possess intrinsic kinase activity signal transduction is mediated by ITAM motifs in the ζ and γ subunits. Whether or not the signalling pathway used in ADCC is the same as those initiated by activating receptors in natural cytotoxicity is not clear. The fact that ITAM motifs can bind different intracellular signalling molecules including ZAP-70, Syk, phospholipase C- γ 1 (PLC- γ 1) and phosphatidylinositol 3-kinase (PI3K) (Exley *et al.* 1994; Isakov *et al.* 1995; Johnson *et al.* 1995; Osman *et al.* 1996) suggests that different pathways may be triggered by the different ITAM regions (Leibson 1997). In summary, antibodies mediate specificity of NK cell activity that otherwise is not antigen specific.

4.3. Role of NK cells in HIV-1 infection

NK cells could potentially inhibit HIV infection by cytolytic mechanisms but also by non-cytolytic mechanisms, via chemokine and cytokine secretion. Nevertheless the protective contribution of NK cells during the different stages of HIV-1 infection is not clearly elucidated.

4.3.1. Cytokines and chemokines

It has been demonstrated *in vitro* that NK cells of HIV-infected individuals are able to produce CC-chemokines that inhibit viral replication (Fehniger *et al.* 1998; Oliva *et al.* 1998). The produced chemokines (CCL3, CCL4 and CCL5) are ligands of CCR5, the entry co-receptor of R5 tropic viruses, therefore these chemokines will probably block virus entry by competitive inhibition of CCR5 and downregulation of receptors (Oliva *et al.* 1998; Kottlil 2003). Since NK cells also produce numerous cytokines it is probable that secretion of TNF, IFN γ and GM-CSF, helps to prevent infection through recruitment of other effector cells, such as neutrophils, macrophages and eosinophils.

4.3.2. Natural cytotoxicity

HIV downregulates MHC class I expression of infected cells *in vitro* probably to escape recognition by CD8 cytolytic T cells (Scheppler *et al.* 1989; Collins *et al.* 1998), however because of the downregulation this infected cells become potential targets for NK mediated cytotoxic activity. Responsible for this downregulation are at least three viral encoded proteins, Tat, Nef and Vpu (Kamp *et al.* 2000). Consistent with MHC class I downregulation some studies have demonstrated that NK cells were able to lyse infected cells either via direct cytotoxicity or through ADCC (Sirianni *et al.* 1988; Bandyopadhyay *et al.* 1990; Fortis *et al.* 1999). However in studies where HIV-1 primary T-cell blasts with downregulated MHC-I expression levels were used as target cells, it has been shown that autologous NK cells were unable to lyse those cells. However the same NK cells were indeed able of cytotoxic activity against K562, the control cell line used (Bonaparte *et al.* 2003; Tasca *et al.* 2003; Bonaparte *et al.* 2004). The discrepant results may be explained by the fact that HIV selectively downregulates the expression of HLA-A and HLA-B on the cell surface while preserving expression of HLA-C and HLA-E (Cohen *et al.* 1999a; Blagoveshchenskaya *et al.* 2002; Ward *et al.* 2004). HLA-A, -B and -C interact with members of the KIR family, whereas HLA-E binds to the CD94/NKG2 family, all members of the inhibitory NK receptors (Moretta *et al.* 1996; Leibson 1997; Braud *et al.* 1998; Cohen *et al.* 1999a). In summary the HIV Nef protein is able to retain stable expression of HLA-C and HLA-E to inhibit NK cytotoxicity whereas it downmodulates

the expression of HLA-A and HLA-B, molecules that also trigger CTL activity. Consequently it is probable that HIV not only evades CTL killing by downregulating MHC I molecules on the surface of infected cells, but also evades NK cytolytic activity by modulating the expression of receptors that balance stimulatory and inhibitory signals for NK killing. Nevertheless it also has to be considered that not all NK cells possess the same inhibitory receptors on their surface as well as the level of downregulation of HLA in HIV-infected cells may also diverge. To what extent HIV may evade NK killing is therefore very likely variable.

4.3.3. ADCC

Early studies attempted to elucidate the significance of ADCC in controlling HIV infection. A study in 1987 showed that gp120-coated CD4 cells could be lysed by PBMC when patient sera was added (Lyerly *et al.* 1987). Others also showed the presence of NK cells that were armed with HIV-specific antibodies in infected patients, these NK cells were also able to lyse gp120-coated CD4 cells (Tyler *et al.* 1989a; Tyler *et al.* 1989b; Tanneau *et al.* 1990). Rook *et al.* (Rook *et al.* 1987) demonstrated, also in 1987, a positive correlation between functional ADCC activity and slower disease progression. This finding was confirmed by several groups, who found that patients having AIDS had lower ADCC titres than HIV-1 patients in the asymptomatic phase and that the levels of ADCC antibodies positively correlated with better health of infected individuals (Ljunggren *et al.* 1987; Sawyer *et al.* 1990; Ahmad *et al.* 1994a; Baum *et al.* 1996). Furthermore although higher levels of ADCC titres in HIV-infected mothers or their newborns were not associated with lower perinatal transmission, these titres correlated with better clinical stages of the infected children (Ljunggren *et al.* 1990; Broliden *et al.* 1993; Jenkins *et al.* 1993). These different studies demonstrating the presence of ADCC-mediating antibodies in sera from HIV-patients are supported by *in vivo* studies in the macaque model (Belo *et al.* 1991; Ferrari *et al.* 1993; Ohkawa *et al.* 1994; Broliden *et al.* 1996). SIV infected chimpanzees display persistent levels of ADCC, which appear to control initial plasma viremia and correlate with delay in disease progression. In human infection ADCC mediating antibodies are developed early in infection, they emerge at about the same time as CTL functions are detectable (Connick *et al.* 1996) and can be detected during all disease stages (Koup *et al.* 1991). In addition several epitopes on

gp120 and gp41 have been identified to bind ADCC specific antibodies (Evans *et al.* 1989; Koup *et al.* 1989; Nixon *et al.* 1992). The first antibody described to specifically mediate ADCC activities was the monoclonal antibody 15e (Koup *et al.* 1991) but also MAb 2G12 was found to activate ADCC (Trkola *et al.* 1996; Parren *et al.* 1997). However, as mentioned before ADCC mediating antibodies could also mediate lysis of uninfected CD4 T cells that have bound gp120 on their surface, contributing to the depletion of this cells in infected patients and consequently to the disease progression (Lyerly *et al.* 1987; Ahmad *et al.* 1994b; Hober *et al.* 1995).

4.3.4. NK dysfunction

HIV triggers several phenotypic and functional irregularities in NK cells either through direct infection, binding on the surface of NK cells or indirectly by the general effect that viremia has on the immune system. Although all NK cells express CCR5 and CXCR4, the HIV co-receptors, only a small subset also expresses CD4, the primary receptor of HIV and is thus susceptible to infection. Viral DNA in NK cells of patients receiving ART showed that NK cells could be persistently infected, contributing possibly to the latent HIV reservoir (Valentin *et al.* 2002; Valentin *et al.* 2003). Furthermore, CCR5 levels on NK cells of viremic patients are increased, HIV interaction with the chemokine co-receptor might induce intracellular signalling that could result in deregulated activation (Kottlilil *et al.* 2004).

In infected NK cells the expression of activating receptors of natural cytotoxicity is reduced, similar to the MHC class-I downregulation in CD8 T cells, while the expression of inhibitory receptors is upregulated resulting in reduced natural cytotoxicity (Mavilio *et al.* 2003; Kottlilil *et al.* 2004). All three activating receptors on NK cells (NKp30, NKp44 and NKp46) were shown to be expressed at lower levels in HIV-viremic individuals than in HIV-negative controls (De Maria *et al.* 2000; Mavilio *et al.* 2003). Stable expression of NKG2D, another activating receptor, together with several activating co-receptors may explain residual cytotoxicity (Sirianni *et al.* 1994; Mavilio *et al.* 2003; Kottlilil *et al.* 2004).

The observed defect in NK function can also be explained in part by phenotypic changes and distribution of NK subsets during HIV-infection. The proportion of cytolytic CD16^{hi}CD56^{low} NK-cell subset diminishes whereas the number of CD16^{hi/-}

CD56⁺NK cells increases. This subset expresses higher levels of iNKR and lower levels of activating receptors and it secretes lower amounts of cytokines and chemokines than normal CD16^{hi}CD56^{low} NK (Sondergaard *et al.* 1999; Mavilio *et al.* 2003; Mavilio *et al.* 2005).

The *in vivo* functionality of ADCC during HIV viremia is still uncertain. While some groups have found that ADCC remains functional during infection (Katz *et al.* 1988), others reported that ADCC effector cells were functionally compromised (Weinhold *et al.* 1988; Ljunggren *et al.* 1989; Tyler *et al.* 1990).

In summary, although NK cells may contribute *in vivo* to disease control, their functions can also be severely compromised during infection. It has also to be taken in consideration that the total number of NK cells in peripheral blood diminishes in infected patients and as a result the impact of NK mediated actions may be reduced. Therefore, high titres of ADCC mediating antibodies do not guarantee that ADCC-mediated killing occurs.

5. Project outline

There is evidence from studies in long term non-progressing patients and in animal experiments that ADCC positively influences HIV containment, however its exact role in protection remains unclear. Since ADCC mediating antibodies appear very early during infection, we wanted to investigate the importance of this mechanism during the acute and chronic infection. For that reason we aimed to assess activity of ADCC-mediating antibodies in HIV patients during different stages of infection, in the acute phase and in the chronic phase as well as in patients following structured treatment interruption (STI). We also aimed to compare ADCC-mediating antibodies to neutralising antibodies and monitor possible overlaps. The long term aim is to be able to assess neutralising titres to ADCC antibody titres side by side to define the impact of these mechanisms in viral control.

The reason why until now the role of ADCC in suppressing HIV-1 is not clearly defined is due to the lack of appropriate assay systems that allow to measure ADCC activity of patients sera against autologous virus. To date the role of ADCC has only partially been investigated, since most assay systems measure reactivity against lab adapted HIV strains and not the patients' own isolates (Ljunggren *et al.* 1987; Lyerly

et al. 1987; Koup *et al.* 1991; Ahmad *et al.* 1994b; Alsmadi *et al.* 1997; Loubeau *et al.* 1997; Forthal *et al.* 2001; Bonaparte *et al.* 2003) or with target cells that were coated either with recombinant gp120 proteins (Weinhold *et al.* 1988; Parker *et al.* 1995; Nag *et al.* 2004; Gupta *et al.* 2005; Kantakamalakul *et al.* 2006) or with whole virus via centrifugation (Ojo-Amaize *et al.* 1989b; Ziegner *et al.* 1999) to obtain surface expression of viral antigens. However, neither attaching of monomeric gp120 nor centrifugation of virus particles to the cell surface will result in proper presentation of antigens. In both cases no autologous envelope proteins were available and so the *in vivo* activity of the ADCC-mediating sera cannot be clearly elucidated. The majority of studies performed measured ADCC by using whole PBMC (Ljunggren *et al.* 1989; Ojo-Amaize *et al.* 1989a; Murayama *et al.* 1990; Ziegner *et al.* 1992; Ahmad *et al.* 1995; Forthal *et al.* 2001; Gomez-Roman *et al.* 2006) or monocyte and macrophages depleted PBMC, since these cells, through their FcγR, have ADCC activity (Alsmadi *et al.* 1997; Ziegner *et al.* 1999; Nag *et al.* 2004; Kantakamalakul *et al.* 2006; Kim *et al.* 2006) as effector cells. We decided to use purified NK cells to measure ADCC activity, since measured lysis can so directly be attributed to the effector cells and their input be better controlled.

We aim to gain insight in the overall impact and significance of ADCC by measuring ADCC mediated by sera against cells infected with the autologous virus isolate at different time points using purified NK cells as effectors. In summary, ADCC depends on three variables: a) infected cells that express viral antigens on their surface, b) presence of ADCC eliciting antibodies that recognise the expressed particles and c) appropriate effector cells. Care has to be taken to define conditions *in vitro* that mimic such a complex *in vivo* situation. The aim of the project was to develop an assay that would permit measurement of NK cytotoxicity as well as autologous ADCC activity in a fluorometric based manner. Such an assay would help to clarify the role of ADCC in HIV infection since changes in ADCC-mediating antibody levels against cell infected with autologous virus would be measured and compared longitudinally to the autologous neutralisation activity in the same patients.

6. Assay development

As mentioned before three factors are necessary for efficient ADCC activity *in vitro*. A principal factor in the assay is the need of a target cell line that can easily be infected by CCR5 using viral isolates but at the same time is resistant to natural NK lysis. The CEM.NKR cells were produced by subcloning of the human T lymphoblastoid CEM cell line with PBMC, the surviving cells (CEM.NKR cells) had diminished susceptibility to NK lysis (8.4% of lysis by PBMC compared to 20.4% of parental CEM cells) but had still similar sensitivity as the parental cell line to ADCC activity (Howell *et al.* 1985). Such a cell line permits to discern between both antibody-specific and unspecific NK lysis. The CEM.NKR cell line was then transduced to stably express the HIV co-receptor CCR5 by Trkola *et al.* (Trkola *et al.* 1999) resulting in CEM.NKR CCR5 cells that can easily be infected by R5 using HIV-isolates.

To assess natural killer (NK) activity and antibody-dependent cellular cytotoxicity (ADCC), human natural killer cells were used as effector cells. K562 cells, which are highly susceptible to natural killer cell activity (Jondal *et al.* 1975; Ortaldo *et al.* 1977), were used as target cells for the NK activity assays. For the ADCC assay we initially used the above described NK resistant cell line, CEM.NKR CCR5 cells (Trkola *et al.* 1999), infected with different HIV-1 isolates.

We developed an assay to measure ADCC lysis based on a previously published protocol on fluorometric assessment of T lymphocyte mediated lysis (Sheehy *et al.* 2001). Traditionally ⁵¹chromium release assay, are used to measure ADCC. Thereby the target cells are loaded with ⁵¹Cr, which they release after the cell membrane is perforated by cytotoxic granules. Although widely used, this method bears several disadvantages. It depends on the use of radioactivity label, from spontaneous leakage of ⁵¹Cr, influence of ⁵¹Cr upon the effector cell population, difficulties in labeling of several cell types and low sensitivity (Slezak *et al.* 1989; Volkmann *et al.* 1989; Heo *et al.* 1990; Kruger-Krasagakes *et al.* 1992; Papadopoulos *et al.* 1994). We aimed to use a novel method, which is based on dual staining with two fluorescent markers PKH-26 and CFSE (5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester). Using dual label allows effective differentiation of effector from target cells. The PKH-26 dye associates irreversibly with membrane lipids. In contrast CFSE, an uncharged fluorescein derivate, is transported through the cell membrane,

and is cleaved in the cytoplasm by intracellular esterases. The resulting charged CFSE derivate is retained in the cell and is instantaneously lost upon membrane damage. PKH-26 stays associated with the membrane even in the apoptotic cells and can therefore be used to differentiate target from effector cells. The quantification of target cell lysis is based on measuring the decrease in CFSE stained cells.

6.1. Materials and methods

Cell lines

- K562: (ATCC nr. CCL-243™) is an immortalised cell-line derived from a chronic myelogenous leukaemia patient in terminal blast crisis.
- CEM.NKR CCR5 (CNC): CEM.NKR the surviving cells produced by subcloning of the human T lymphoblastoid CEM cell line with PBMC, (CEM.NKR cells) possess diminished susceptibility to NK lysis. They were transduced with a retroviral vector to express human CCR5 (Trkola *et al.* 1999).
- CEM 5.25.EGFP.Luc.M7 (CEM 5.25): The cell is a genetically engineered clone of CEMx174 that expresses multiple entry receptors (CD4, CXCR4, and GPR15/Bob) and is transduced to express CCR5. It also possesses Tat-responsive reporter genes for luciferase and green fluorescent protein (GFP) (Brandt *et al.* 2002).
- HELA CD4: these HELA cells (human epithelial carcinoma cells) stably express CD4 through infection with a CD4 expressing retrovirus, pSFF-CD4 (Kabat *et al.* 1994).
- HELA CD4 CCR5: HELA CD4 were transduced with virus produced from the retroviral expression vector pSFF-CCR5 to express CCR5 (Platt *et al.* 1998).
- HELA JR-FL: express the HIV-1 JRFL Env proteins (Progenics Pharmaceutical, Inc).
- AA2 CD4 CCR5: is a CD 4-positive lymphoblastoid B cell line, previously shown to be especially permissive for HIV replication (Chaffee *et al.* 1988). AA2 CD4

clones expressing CCR5 were isolated via FACS sorting by C. Gordon and A. Trkola.

- Ghost-3 hi CCR5/CXCR4 (Ghost): are derived from the human osteosarcoma cell line, HOS, and have been engineered to stably express CD4 and the co-receptors CCR5 and CXCR4. The indicator cell line carries the HIV-2 long terminal repeat-driven green fluorescence protein (GFP) gene, which becomes activated upon infection with HIV or SIV (Morner *et al.* 1999).

Culture Buffers

- Normal culture medium: RPMI 1640, 10% heat inactivated Fetal Calf Serum (FCS) and 1% Penicillin-Streptomycin (P/S) (Biowhittaker)
- NK-stimulation medium: RPMI 1640 media supplemented with 10 units (U) Interleukin-2 (IL-2),
- Infection medium: RPMI 1640 media supplemented with 10% heat inactivated FCS, 1% P/S and 10µg/ml Polybrene (Pb)
- Selection buffer for CEM 5.25 cells: this cell line is carried in RPMI 1640, 10% heat inactivated FCS and 1% P/S, supplemented with 400µg/ml of geneticin (G418; Gibco-BRL).
- Medium for adherent cell lines: Dulbecco's Modified Eagle Medium (DMEM), 10% heat inactivated FCS and 1% P/S
- Ghost-cell medium: Cells were maintained in DMEM containing 10% FCS, P/S, hygromycin (100ug/ml), and puromycin (1ug/ml).

Stains and antibodies

- CFSE: 5-(and -6-) carboxyfluorescein diacetate succinimidyl ester (Molecular Probes, Eugene, Oregon) is a cell permeant, fluorescein-based tracer. Approximate fluorescence excitation and emission maxima, are 492nm and 517nm respectively, FL-1.

- PKH-26: is a red fluorochrome that incorporates into the membrane associating to membrane lipids (Sigma, St. Louis, MO), its excitation maxima is at 551nm and emission maxima at 567nm, FL-2.
- DiD: ,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine, 4-chlorobenzene sulfonate salt is a carbocyanine that is incorporated into cell membranes (Molecular Probes, Eugene, Oregon). Excitation and emission maxima are 644nm and 665nm respectively, FL-4.
- PI: is a fluorescent vital dye that stains DNA, it is not permeable to intact cell membrane however in cells where membrane integrity is destroyed, the dye is able to penetrate and integrate into the nucleus (BD Biosciences), FL2.
- All antibodies and isotype controls used were from Caltag Laboratories, excepting KC57 antibody, provided by Beckman Coulter (Miami, Florida).

Blood donations

Written informed consent was obtained from the HIV-1 negative and the HIV-1 positive blood donors according to the guidelines of the local ethics committee.

6.1.1. Generation of effector cells

Peripheral blood monocyte cells (PBMC) were isolated from buffy-coats of healthy blood donors by ficoll-hypaque centrifugation. NK cells were then purified from PBMC using magnetic beads of a negative isolation kit from Dynal Biotech that were coated with antibodies against CD3, CD14, CD36, CDw123 and HLA class II DR/DP, only, therefore only NK cells would not bind to the beads and in consequence pass through the magnetic column. Purified NK cells were then cultured not longer than two days in normal medium or alternatively up to seven days in NK-stimulation media.

6.1.2. Handling of target cells

6.1.2.a. Dual cell staining

Target cells (K562 or CEM.NKR CCR5) (1×10^6 – 3×10^6 cells) were first stained with PKH-26 at a final concentration of 2.5×10^{-6} M (Sigma, St. Louis, MO). Staining was performed as recommended by Sigma. Cell pellets were first washed twice with PBS. The pellet is then resuspended in diluent C, provided in the staining kit, as well as the dye. Both solutions are mixed together and incubated five minutes at room temperature (RT). The staining reaction is stopped by adding an equal volume of heat inactivated FCS. Cells were then washed with PBS and centrifuged. The pellet resuspended in PBS and 5-(and –6-) carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, Oregon) was added at a final concentration of 0.3×10^{-6} M. Cells were then incubated five minutes at RT and the reaction again stopped with an equal volume of heat inactivated FCS. Finally the cells were washed twice with PBS and resuspended in NK culture medium. If infected target cells were used, the NK culture medium was additionally supplemented with 10 µg/ml Pb.

6.1.2.b. Infection of target cells

CEM.NKR CCR5 cells used for infection experiments with R5 HIV-1 isolates were cultured in infection medium and passaged 1:3 the day before infection or addition as feeder cells to continuously infected cultures. Optimal conditions for maintaining continuous cell infection were achieved by splitting the culture 1:5 twice a week and add 2×10^5 cells/ml non-infected cells at each passage. Infectivity was controlled by production of p24, measured in cell supernatant by p24 ELISA or by assessing intracellular p24 by FACS.

6.1.2.c. Intracellular p24 stain

For successful ADCC to occur, target cells have to efficiently express viral antigens on their surface in order to be recognised by ADCC-mediating antibodies. In order to quantify ADCC activity over a meaningful range, the target cell population has to be highly infected with HIV-1. The percent number of infected target cells was estimated by staining intracellular p24. Cells were first fixed and permeabilised with the BD

Cytofix/Cytoperm™ solution (BD Biosciences) according to the manufacturer's instruction and then stained with an anti-p24 antibody, KC57 PE. The KC57 antibody identifies the 55, 39, 33 and 24kDa proteins of the core antigens of HIV-1. As the signal of that staining was not very high (Figure 10a), and the population of KC57 positive cells can not be clearly separated from the control cells, with only 26.2% positives, the signal was amplified using the enzymatic amplification staining (EAS) kit from FlowAmp Systems Ltd (Figure 10b). Using this method a significant increase of intracellular p24 detection was achieved (26.2% to 88.4%).

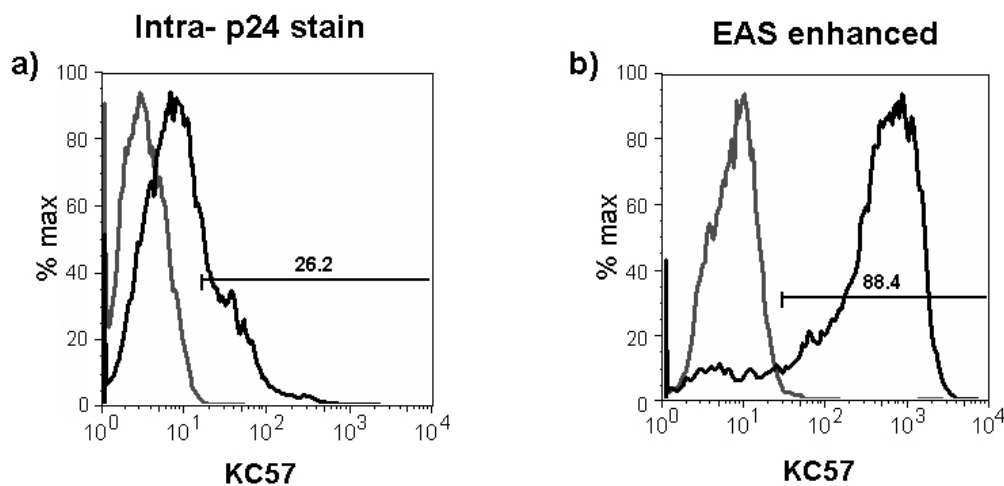


Figure 10. HIV-infection levels of CEM.NKR CCR5 cells. Grey line: isotype control, black line KC57 stain. Gate refers to the KC57 staining a) KC57 stain without EAS b) enhanced expression levels of KC57 after EAS.

This method was used routinely to measure percentage of infected target cells in the cell culture.

7. Results

7.1. Characterisation of natural killer cells

Natural killer cells were analysed in order to evaluate donor variability and influences of *in vitro* culturing to determine the optimal handling procedures. In particular, cell viability in the population, changes in cytotoxic activity, and expression levels of several CD surface markers were monitored.

7.1.1. Viability

Viability of NK cells was assessed using propidium iodide (PI) (BD Biosciences). The cell population is washed in PBS, before 1ul of PI at a concentration of 50ug/ml is added, after five minutes incubation the sample can directly be measured by flow cytometry.

PI is a fluorescent vital dye that stains DNA, it is not permeable to intact cell membrane. However in cells at late apoptotic stages or already dead where membrane integrity is destroyed, the dye is able to penetrate and integrate into the nucleus. Viable cells are therefore defined as the amount of PI negative cells in a given population.

As described we found that non-stimulated NK populations die rapidly. Viable cells drop to 75% at day 2 post isolation and to 70% at day 3 post isolation (Figure 11). In contrast when the cell population was stimulated with low IL-2 concentrations, viability was maintained for longer periods. With this stimulation at day 7 after isolation still 65% of the cell population is alive.

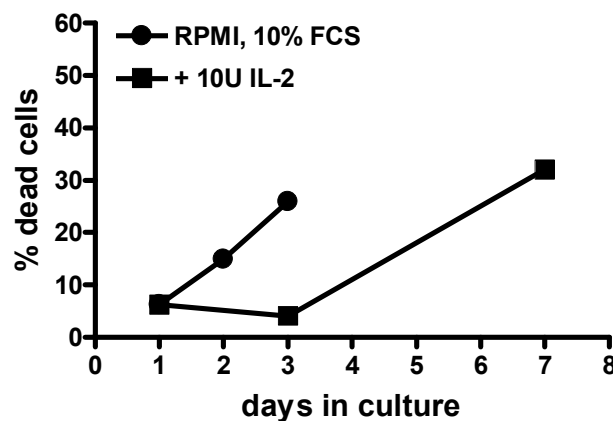


Figure 11. Assessment of natural killer cell viability with a propidium iodide stain. Isolated NK cells were maintained in culture either normal RPMI, 10% FCS medium or stimulated with 10U IL-2.

Therefore, assays executed with unstimulated NK populations were performed at day 1 or 2 post isolation. Stimulated cells were used within 7 days post isolation. Assays presented here used both, unstimulated NK cells and stimulated cells at days 6 or 7 post isolation.

7.1.2. Expression levels of different CD markers

Adequate expression levels of CD16 (FcγRIII) on NK cells are crucial to define ADCC effector activity since ADCC is mediated through CD16 bearing NK. The CD56 molecule represents the lineage marker characteristic for NK cells. The CD16^{hi}CD56^{low} subset produces low levels of IFNγ, and has been defined to be mainly responsible for both the cytotoxic and ADCC activity of NK cells, whereas the CD16^{low/-}CD56^{hi} subset is principally responsible of cytokine and chemokine production and possesses low cytotoxicity. Given that the CD16^{hi}CD56^{low} subset represent 90% of the NK population in blood, and the remaining 10% are mostly composed of the CD16^{low/-}CD56^{hi} subset, it is essential to verify that the CD16 expression level and the compositions of subsets do not vary during stimulation, since in consequence diminished CD16 levels in NK cells results in lower cytotoxicity and ADCC levels. We therefore assessed CD16 and CD56 levels in the isolated NK cell population. Purity of the populations was controlled with an anti-CD3 stain, as CD3 is solely expressed on lymphocytes but not on NK. Additionally an anti-CD69 stain was used to define the activation state of the cells.

In the isolated NK cell population, mainly three different subsets can be identified. The most predominant subset at day 1 post isolation (Figure 12a), was with 90.9%, the CD16^{hi}CD56^{low} subset as expected, the other two subsets, CD16^{low/-}CD56^{low} and CD16^{low/-}CD56^{hi}, only represented around 4% each. During stimulation the composition of the subsets changed, although not strikingly (Figure 12c), since both CD16^{low/-}CD56^{low} and CD16^{low/-}CD56^{hi} subsets increased their percentage to 13.8% and 7.08% respectively and the CD16^{hi}CD56^{low} subset decreased from 90.9% to 76.4%.

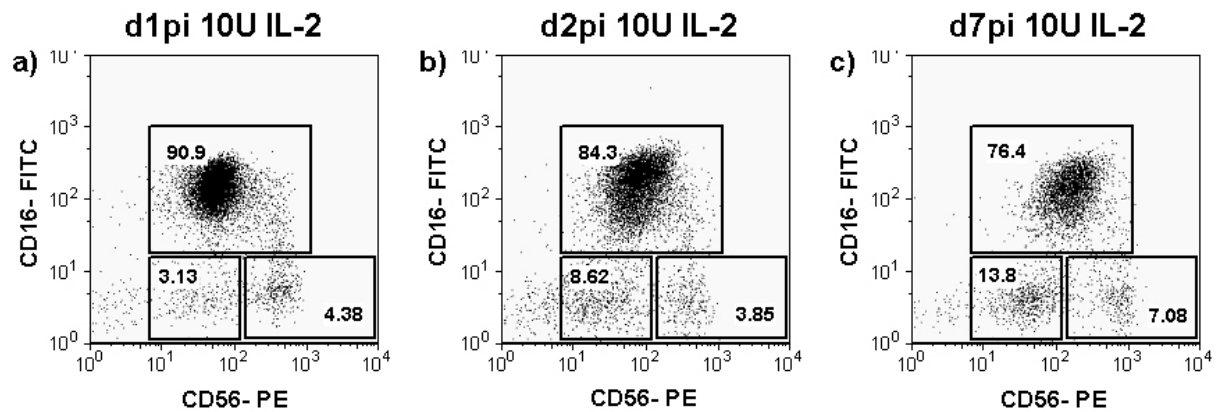


Figure 12. Subset of natural killer cells and their evolution during IL-2 stimulation. NK cells were stimulated with 10U IL-2 directly after isolation. Cells were double stained with anti-CD56 and anti-CD16 antibodies and analysed by flow cytometry.

Staining with anti-CD3 antibodies revealed that the contaminating CD3 positive cells in the NK culture after isolation was small making up only 1.05% of the population. However this cell population increased during culturing, with 13.07% at day 2 already and 9.83% at day 7, Figure 13. Whether or not the increase of CD3 positive cells is real, remains uncertain as this would require a massive expansion of the initial population within one to two days, which is unlikely. A more plausible explanation is that this result is at least to some extent due to false positive staining as activated cells (Figure 14) may have an increased background. Based on the day 1 data, we conclude that the percentage of CD3 positive cells is relatively small, and should consequently not interfere with measurements of NK activity.

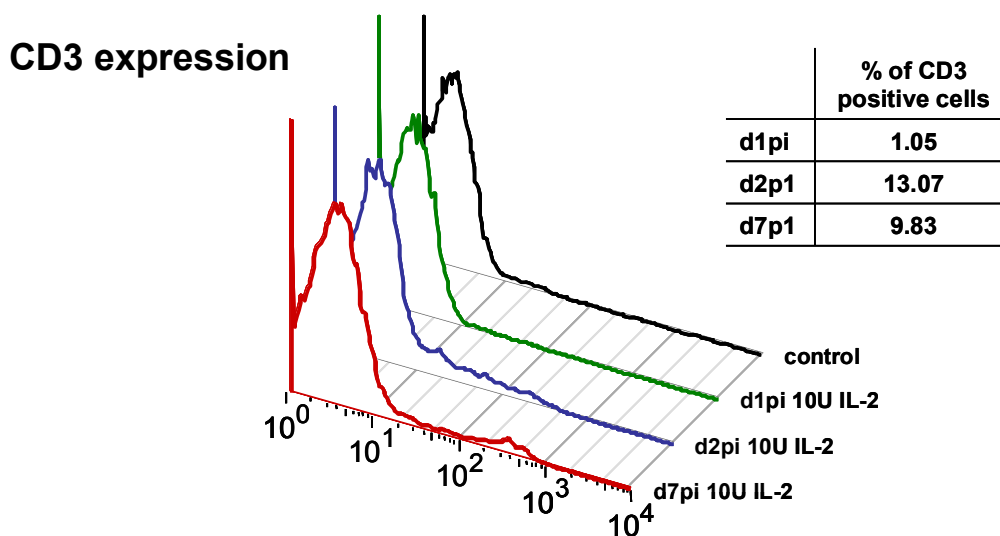


Figure 13. CD3 expression levels of isolated NK population after different culturing times and its isotype control.

The human CD69 differentiation antigen is one of the earliest cell surface molecules expressed after activation of T and B lymphocytes and other haematopoietic derived cells (Testi *et al.* 1994; Sancho *et al.* 2005). In NK cells CD69 is rapidly expressed after activation (Borrego *et al.* 1993; Gerosa *et al.* 1993; Borrego *et al.* 1999). We looked for CD69 expression of our NK populations. Already at day 1 post isolation and culturing in NK-stimulation media, 40.4% of NK cells express CD69, CD69 expression level reaches 68.21% at day 2 and diminishes to 56.72% of NK cells expressing CD69 (Figure 14).

CD69 expression

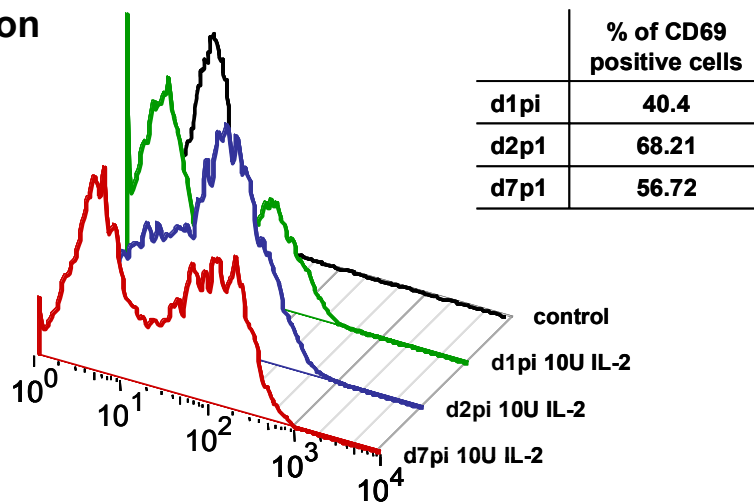


Figure 14. CD69 expression levels of isolated NK population after different culturing times and its isotype control.

7.1.3. Killing assay

Double stained (PKH-26/CFSE) K562 cells were plated in 96 V-shaped well plates at a concentration of 1×10^5 cells/well. K562 stained cells were plated either alone, for controls, or mixed together with natural killer cells (effector to target ratio of 1:1) for 5h incubation at 37°. Each of the conditions was tested in duplicates or triplicates. Cells were fixed with 1% formaldehyde and measured for fluorescence on a FACSCalibur (Becton Dickinson, Mountain View, CA).

As mentioned before, the assay is based on detection of lysed cells as being the PKH-26 positive population that has lost CFSE staining. After 5 hours of co-culture we expect to distinguish three different populations. PKH-26/CFSE double positive cells, representing the intact cells, PKH-26 positive, CFSE low or negative cells,

indicating the lysed target cells and since the effector population has no staining they will appear as double negative cell population in the FACS readout. The experimental evaluation revealed that intensity of PKH-26 also diminishes in killed cells. Thus the cell membrane is severely damaged by released cytotoxic granules so that both dyes are released. That not only CFSE as expected but also PKH-26 stain was lost posed a problem to the differentiation of target and effector cells population by flow cytometry. To overcome this, a time point 0, was introduced in the assay. Parallel to the five hour co-incubations of target and effector cells, double stained target cells were incubated for five hours separately and mixed together with NK cells just prior fixation to set time point 0. This initial ratio of effector and target cells permits to calculate percent killing by defining the reduction of dual stained target cells after five hours of incubation with effector cells. Figure 15 illustrates the shape of time point 0 dot plot, the expected as well as the measured dot plots after 5 hours co-culture.

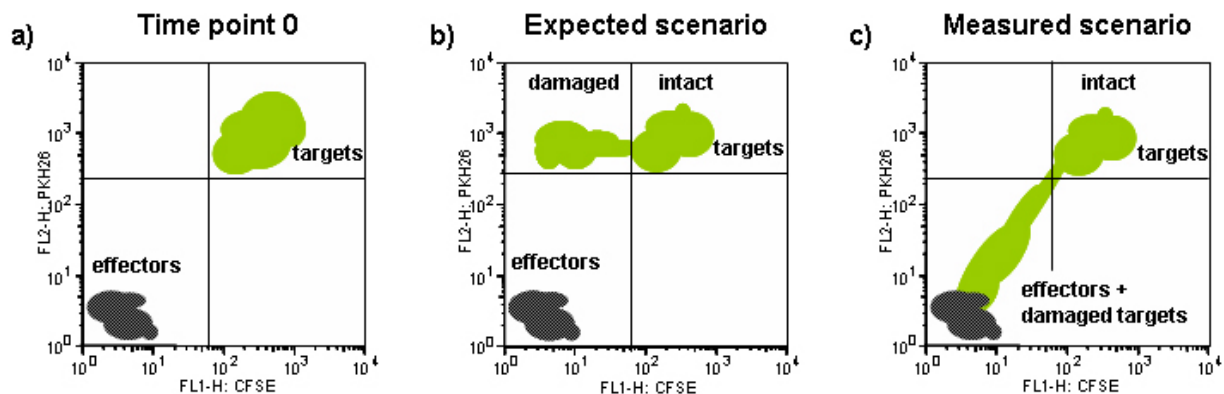


Figure 15. Interpretation of FACS data after cytotoxicity assay. a) Time point: % of target cells and effector cells in the assay. b) Expected scenario: % killing measured as % cells in the upper left quadrant, with diminished CFSE concentration compared to intact cells in the upper right quadrant. CFSE levels in intact cells can be controlled measuring intensity of dual stained target cells cultured alone. c) Measured scenario after 5 h co-culture: % killing could not be defined since lysed target cells can not be discerned from effector cells. Target cells not only decrease CFSE but also PKH-26 concentrations. Final method: % intact target cells at time point 0 are compared to % intact target cells after 5 hour incubation with effector cells in c). Percent killing is calculated as the % decrease of intact cells after 5 hours.

Flow cytometry dot plot of dual-stained target cell incubated at the same conditions as the effector-target co-cultures, is used to set the gate of living target cells, in which the cell membrane is still intact. The amount of killing is consequently calculated as the percentage decrease of intact cells after five hour co-incubation.

Percent of NK activity is calculated as the decrease of intact double positive stained cells between the time point zero hours and five hours incubation.

$$\% \text{ killing} = 100\% - \frac{(\% \text{ intact cells after 5h}) * 100}{(\% \text{ intact cells at 0h})}$$

7.1.4. Cytotoxicity levels

To verify the activity of NK cell preparations we assessed their activity in lysing the NK sensitive target cells K562. Figure 16 depicts a typical experiment. Based on the presented raw data, we calculated that 54.55% of K562 were subjected to NK killing in this experiment.

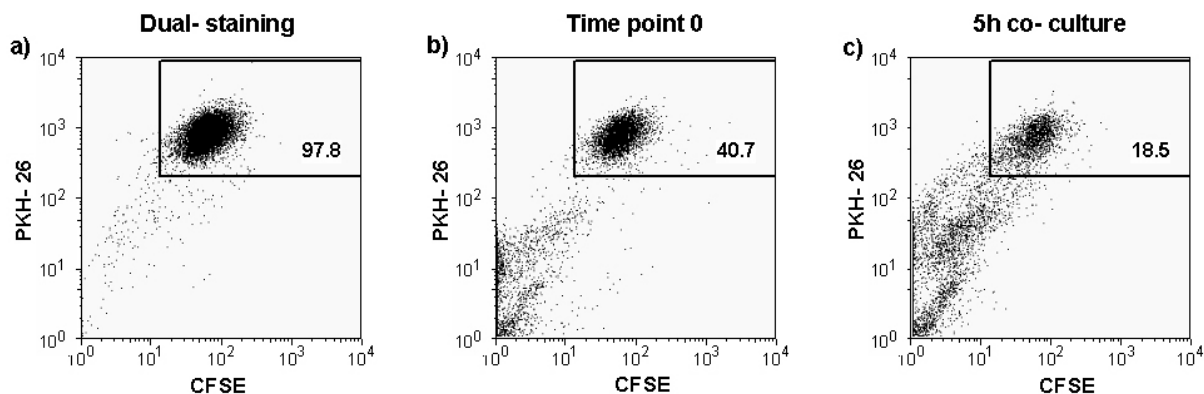


Figure 16. FACS dot plot analysis of natural killer cytotoxicity against K562 dual-stained cells. a) dual-stained K562 cells, the gate represents K562 cells with intact cell membrane, b) NK cells and K562 at time point 0, c) NK cells and K562 cells after 5 hours co-culture.

In a next step we controlled the variability of killing capacity of NK cells derived from different donors after varied duration of IL-2 stimulation. NK cells were isolated either directly from healthy donors (will be abbreviated as fresh) or from buffy-coats (abbreviated as buffy) obtained through the blood bank. A major logistic difference of these two NK donor pools is that blood for the buffy-coats are collected in the morning or the day before by the blood bank and thus are only available for process in our lab in the afternoon, whereas blood contributions of healthy donors in our clinic

could be directly processed. Killing assays were performed with NK cells at different cultivation and stimulation (with 10U IL-2) levels.

NK cell preparations used:

- Non-stimulated NK cells at days 1 and 2 post isolation
- Stimulated NK cells at days 1, 2 and 7 post isolation

Figure 17 exemplifies the various degrees of killing activity we observed between different donors as well as at different stimulation durations. In general median killing levels after seven days of IL-2 stimulation are similar for both buffy-coat and fresh-derived NK cells. However as can be seen by comparing killing activity on day one and day two post isolation, NK derived from buffy-coats, appear less active immediately after isolation. It appears nevertheless, that these cells recover in culture and killing activity increased at day 2. Stimulation with IL-2 raised killing activity levels from different donors as can be seen on Figure 17b. Notably low killing levels tend to increase during stimulation, whereas those NK that already have high killing activity at day two, normally do not improve but may instead even slightly diminish. In summary, natural killer cell activity after seven days of stimulation reach up to 55% killing at a 1:1 ratio of effector to target cells. Especially the differences of killing capacities at day 7 is still high and ranges between 18% and 55% in buffy derived NK cells and at comparable levels in fresh-derived NK cells (26%-53%).

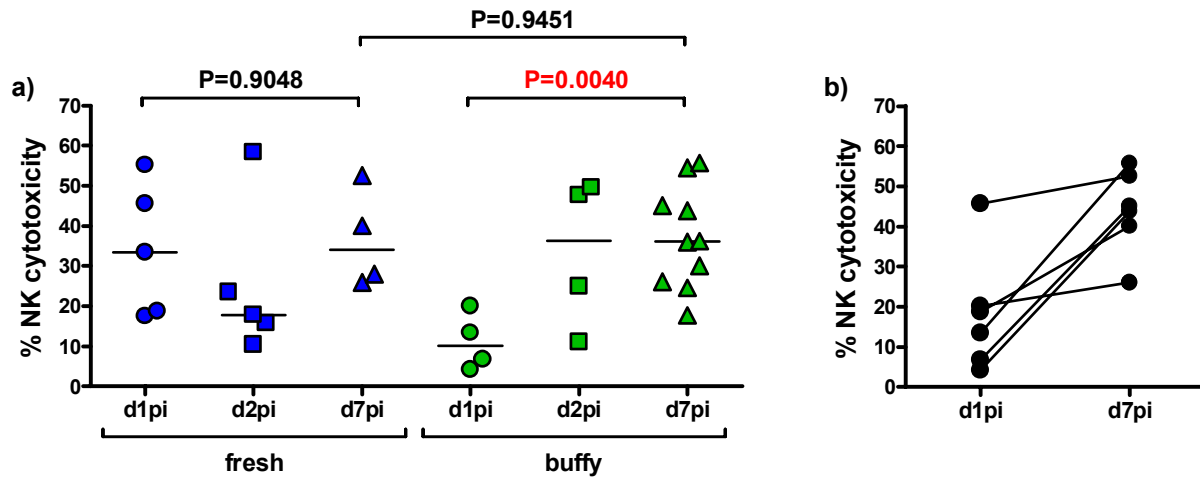


Figure 17. Variability in cytotoxic activity of natural killer cells against K562 target cells. A) Variability between fresh and buffy NK donors and between different stimulation durations with 10U IL-2. The median value is drawn, statistical analysis were done using a Mann-Whitney paired T-test. b) Variability in killing capacity of NK cells after stimulation.

7.2. Measurement of ADCC activity against HIV infected cells

Double stained HIV infected CEM.NKR CCR5 cells were dispensed in 96 V-shaped well plates at a concentration of 1×10^5 cells/well in duplicates or triplicates. NK cells were mixed together at effector: target ratios of 1:1. For ADCC activity assays, ADCC-mediating antibodies at concentrations up to 25ug/ml were first incubated for 30 minutes with the infected cells in order to pre-coat the cells with the antibody. Binding of NK cells to the antibody-coated cells will then be facilitated. After five hours incubation at 37°C, cells were fixed with 1% formaldehyde. Time point 0h was set as in the NK activity assay. Assay was set in duplicates or triplicates. Cells were then measured for fluorescence on a FACSCalibur. Percent of ADCC lysis is estimated as the difference in amount killing in presence and absence of a given antibody.

$$\% \text{ ADCC} = \% \text{ killing in presence of antibodies} - \underbrace{\% \text{ killing in absence of antibodies}}_{\text{NK cytotoxicity}}$$

Killing is calculated from the measured FACS raw data as described above.

$$\% \text{ killing} = 100\% - \frac{(\% \text{ intact cells after 5h}) * 100}{(\% \text{ intact cells at 0h})}$$

7.2.1. Killing of CEM.NKR CCR5 cells

An essential feature of the ADCC assay is the requirement that target cells are highly resistant to natural cytotoxicity. This should be the case of the selected cell line, the CEM.NKR CCR5 cells as this cells were described to have an increased NK resistance. Nonetheless, in our assay, unspecific killing directly through NK cytotoxicity was very high (Figure 17), which in turn made it more difficult to differentiate between NK cytotoxicity and ADCC in our assays.

Compared to the ^{51}Cr assay, the FACS readout appears to be more sensitive and CEM.NKR CCR5 cells do not appear to be as resistant to natural cytotoxicity through NK cells as previously described (Howell *et al.* 1985). Measured NK killing reached levels higher than 50% in some cases and ranged typically between 16.93% and 57.73% at day 7 post isolation. In fact these values are very similar to the killing activity observed against the indicator cell line K562 that ranged from 18% to 55% (Figure 18a). In addition, similar to killing with K562 no differences in cytotoxicity levels between freshly isolated and buffy-coat isolated NK cells are apparent, Figure 18a. As well, killing levels increase during stimulation and the increase is higher if the killing capacity was low after isolation (Figure 18b).

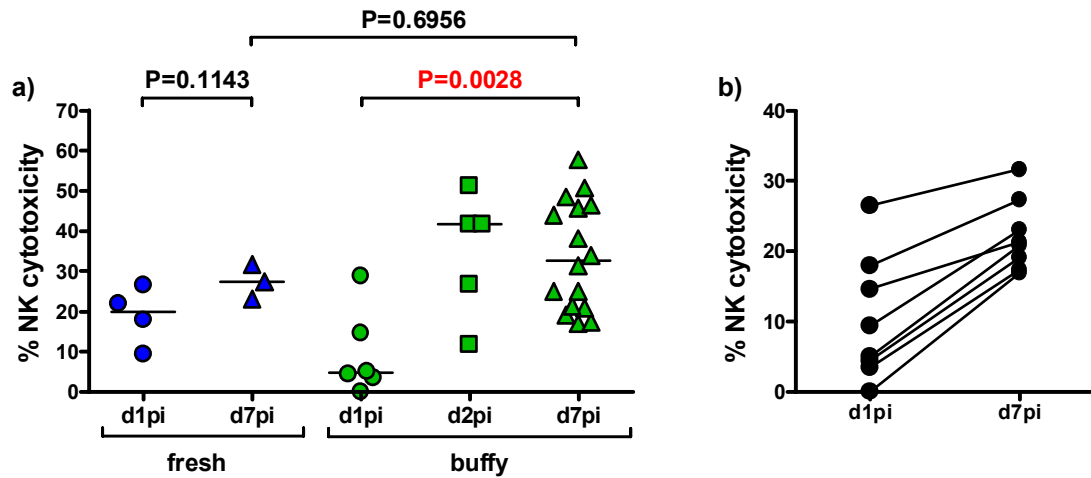


Figure 18. Variability in cytotoxic activity of natural killer cells against CEM.NKR CCR5. a) Variability between fresh and buffy NK donors and between no and 7 days of stimulation with 10U IL-2. The median value of each data set is drawn, statistical analysis were done using a Mann-Whitney paired T-test. b) Changes in killing capacity of NK cells after stimulation.

In view of the fact that NK cytotoxicity levels were so high, we expected that assessment of ADCC activity would be difficult since the differences in killing levels probably might not be high enough to make clear differentiations. We nevertheless probed ADCC activity using this system. ADCC assays in which the CEM.NKR CCR5 cells were infected with an HIV isolate (SB123 or SP121) and cross linking between effector and targets were performed either with MAb 2G12, a neutralising antibody that also mediates ADCC, and MAb 2F5, a neutralising antibody whose ADCC activity has not yet been defined.

Figure 19 depicts a representative experiment with NK lysis activity against the infected target cells of 34.26%. Notably, no increase of killing activity in presence of the monoclonal antibodies 2G12 and 2F5 was seen, (Figure 19c and d).

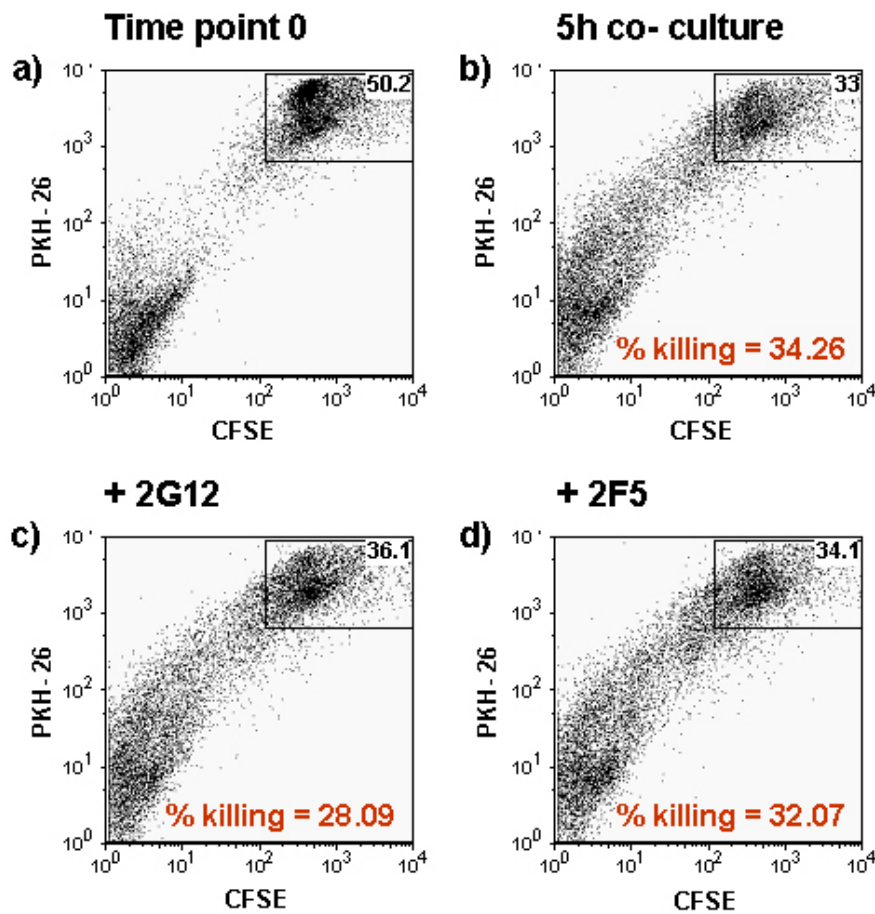


Figure 19. Example of ADCC assay, CEM.NKR CCR5 cells were infected at 66% with SB123 virus, 2G12 and 2F5 were used to mediate ADCC at a concentration of 25ug/ml and pre-incubated with the infected cells for 30min. before killing was started. a-d) Example of a FACS dot plot. Killing of target cells was evaluated by the decrease of target cells after co-culture compared to the time point 0.

Whether this is due to a lack of ADCC activity against this virus or reflects the inadequacy of the assay system remained to be determined. Clearly, conditions with a lower natural killing activity against the target cells were required to address this.

We next investigated following variations to define if activity of NK cells is different depending on source and stimulation and how this influences the outcome of the assay.

Tested conditions:

- Fresh NK cells at day 1 (non-stimulated) and days 2 and 7 post isolation (stimulated).

- Buffy coat-derived NK cells at day 1 (non-stimulated) and days 2 and 7 post isolation (stimulated).

Figure 20 is a representative example of the obtained results and illustrates the limitations of the assay. Although natural cytotoxicity, co-culture without antibody, was around 20%, ADCC activity of 2G12 and 2F5 was not measurable. The above described NK preparations were tested on target cells infected with two different patient-derived viral isolates (SP121 and SB123). Infection rates of the cells used was normally higher than 50%, so that theoretically enough cells could be bound by antibodies and killed subsequently through the interaction with CD16. Figure 20 shows the outcome of this experiment using NK cells at day 7 post isolation. ADCC activity of monoclonal antibodies was evident only against SP121 infected cells and only with NK cells derived from buffy coat but not in any other condition. However the difference is not very marked and it's not noticeable in any of the other settings, regardless of NK stimulation level or source, or infecting virus isolate.

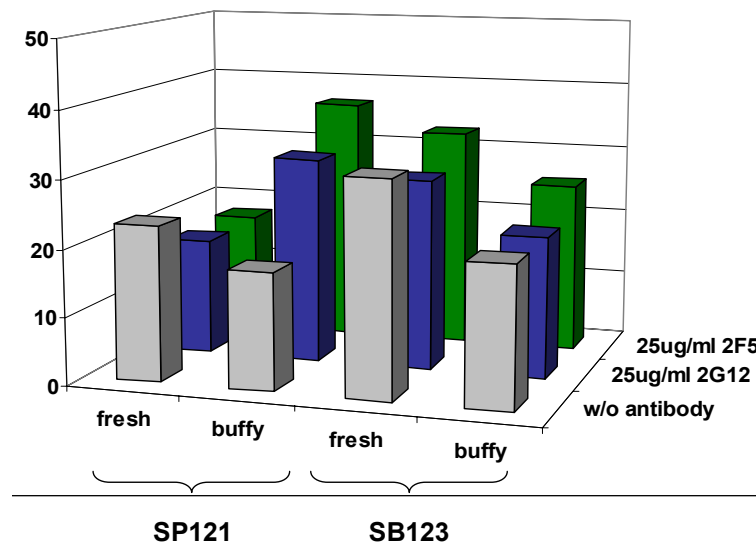


Figure 20. Example of an ADCC assay. CEM.NKR CCR5 cells were either infected with SP121 or SB123 HIV, killing at day 7 post isolation either with buffy or fresh derived NK cells is shown. Antibodies were co-cultured with the cells 30min before effector cells were added to ensure good binding to the target cells.

Why SP121 infected cells were more susceptible to ADCC-mediated by NK cells isolated from buffy coats, is not clear. Several reasons can account for the fact that

we could not measure ADCC activity. Either the antibodies used did not link effector to target cells efficiently, or not enough viral epitopes were expressed on the surface to be recognised by the antibodies. Alternatively, cytotoxicity levels achieved already the maximum killing level of the NK cells so that these cells may be exhausted and therefore not able to kill more cells at the given ratio. Clearly decreasing NK killing appears to be crucial and we attempted to improve the assay in this respect in the following described experiments.

7.2.2. Effects of HLA-G on ADCC assay system

Since reduction of natural cytotoxicity is a prerequisite in order to make measurements of ADCC-activity conceivable, we opted to transfect the target cell lines with an inhibitory ligand to NK cells to render them more resistant to NK killing, without interfering with antibody-specific killing through ADCC. HLA-G was used as inhibitory ligand since it has been previously shown by several groups that HLA-G bears the ability upon transfection to protect NK sensitive cells such as K562 from NK lysis (Rouas-Freiss *et al.* 1997) (Khalil-Daher *et al.* 1999).

7.2.2.a. HLA-G, an introduction

MHC encoded class I molecules are divided into two groups based on their polymorphism and levels of expression. In humans, these two families are the class Ia (classical) molecules HLA-A, HLA-B and HLA-C and the class Ib (non-classical) molecules, principally HLA-E, HLA-F and HLA-G. Classic MHC molecules are polymorphic and widely expressed proteins that bind a diverse set of peptides derived from the cytosol, directing T cells to kill virus or bacteria infected cells (Shawar *et al.* 1994). In contrast, non-classical MHC molecules are less abundant, tissue-specific and non or low polymorphic but have a higher frequency of alternative splicing (Shawar *et al.* 1994) (Le Bouteiller 1994). HLA-G is a non-classical class I protein encoded within the MHC region at chromosome 6p21. HLA-G, is unlike classic MHC-I molecules, is characterised by low polymorphism and restricted tissue distribution, mostly expressed only in placental trophoblasts, thymic epithelial cells (Le Bouteiller *et al.* 1999), and macrophages (Yang *et al.* 1996). All three non-

classical molecules have conserved residues in a putative CD8-binding domain (Gao *et al.* 1997).

HLA-G consists of eight exons, seven introns and a 3'-untranslated region (Figure 21). Furthermore as a result of alternative splicing, HLA-G is transcribed and translated into seven different soluble and membrane-bound isoforms (HLA-G1 to -G7) (Ishitani *et al.* 1992) where HLA-G1 to -G4 are membrane-bound (Carosella *et al.* 1999) (Moreau *et al.* 2002) and HLA-G5 to -G7 are soluble isoforms, created by retention of intron 4 that contains a premature stop codon, preventing the translation of both transmembrane and cytoplasmic domains (Fujii *et al.* 1994) (Paul *et al.* 2000) (Carosella *et al.* 2000).

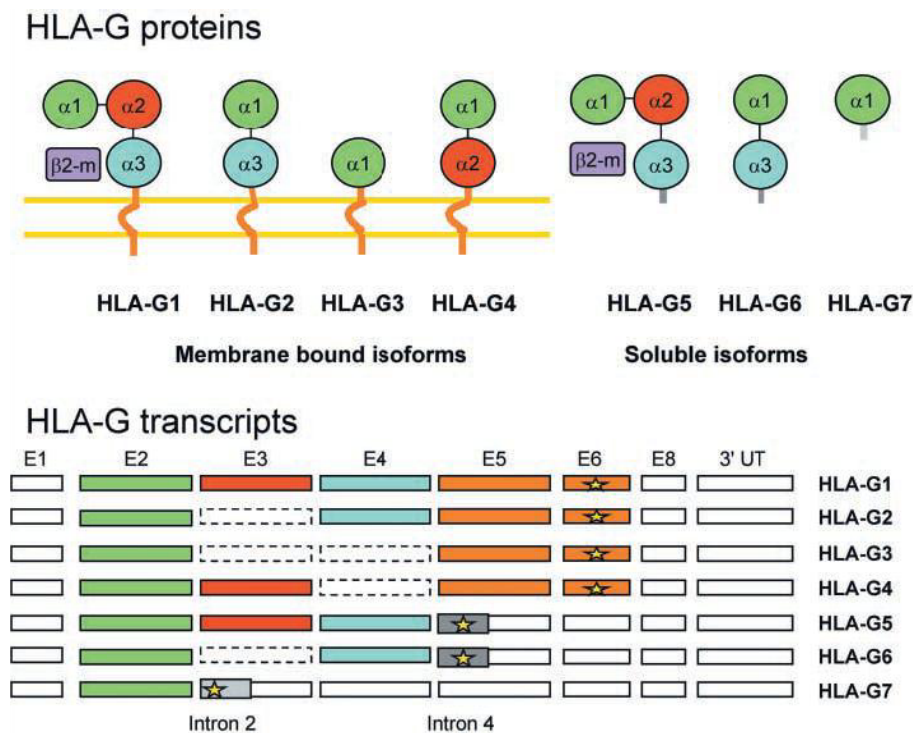


Figure 21. Alternatively spliced HLA-G mRNA and HLA-G protein isoforms. Exon 1 (E1) encodes the leader peptide, exon 2 to 4 (E2-E4) encode the $\alpha 1$ to $\alpha 3$ extracellular domains, exon 5 (E5) encodes the transmembrane region, and exon 6 (E6) encodes the reduced cytoplasmic domain of the HLA-G protein. Stars represent stop codons. Adapted from Moreau *et al.* 2002

HLA-G1 is the full-length isoform and its structure is similar to that of classical MHC class I molecules as it possesses three extracellular domains ($\alpha 1$ - $\alpha 3$) and a heavy chain non-covalently associated with β -2 microglobulin.

The role of HLA-G in immune responses is not clearly elucidated but as it is mainly present in placental tissue. It is thought that HLA-G may present viral peptides to the maternal immune system, triggering lysis of infected foetal cells or could play a role in regulating CD8 T cells, eliminating alloreactive T cells (Zavazava 1998). This hypothesis are supported by studies were HLA-G expressing cells were found to bind to the $\alpha\alpha$ CD8 homodimer (Sanders *et al.* 1991) (Shiroishi *et al.* 2003) and also by the *in vitro* ability of HLA-G expressing cells to induce apoptosis of CD8 T cells via the Fas/FasL pathway (Contini *et al.* 2003) (Fournel *et al.* 2000) (Solier *et al.* 2002).

Furthermore, HLA-G has been implicated in protecting otherwise susceptible target cells from natural lysis by polyclonal NK cells (Chumbley *et al.* 1994) and NK clones (Deniz *et al.* 1994). However, in subsequent studies it has been found that expression of the CD94/NKG2A (-B) complex on NK cells is decisive for protection, clones expressing KIR receptors had normal lysis levels (Perez-Villar *et al.* 1997). NK clones that expressed both inhibitory receptors, and KIR members had restored cytotoxicity against HLA-G transfectants when the CD94/NKG2 receptor was blocked with an anti-CD94 antibodies but not when the KIR receptors where blocked with anti-KIR antibodies respectively.

7.2.2.b. Methods of HLA-G transfection

HLA-G Vector Construction

The plasmid containing the HLA-G gene was generously provided by the group of E. Carosella (Rouas-Freiss *et al.* 1997). Briefly, the 1.5kb HLA-G1 full-length cDNA *EcoRI* fragment was ligated into a *HindIII*-digested pRc/RSV vector blunted and dephosphorylated by calf intestinal phosphatase (GIBCO/BRL). The correct orientation of this construct was confirmed by restriction digest analysis and subsequent sequencing.

HLA-G Transfectant Cell Lines

The vector containing HLA-G1 was transfected into K562 cells and CEM.NKR CCR5 with the effectene transfection reagent (Qiagen). After two days of culture, the cells were selected with 1mg/ml geneticin (G418; GibcoBRL). Transfectant cells were

monitored by flow cytometry analysis to confirm the expression of HLA-G. Binding of an anti-HLA-G antibody [mouse monoclonal MEM-G/11 (Abcam)] was detected by a biotin goat anti-mouse IgG and labelled with streptavidin-FITC.

Both K562 cells and CEM.NKR CCR5 cells stably expressed HLA-G at high levels. Of each cell line the two with the highest expressing clones were selected and probed in killing assays. HLA-G expression in the K562 derived clones, K562 HLA-G-7 and -8 was of 17% and 61.7% HLA-G positive cells respectively Figure 22a, and in the CEM.NKR CCR5 derived clones, CEM.NKR CCR5 HLA-G-2 (will be abbreviated as CNC-HLA-G-2) and -2p (CNC-HLA-G 2p) of 75.7% and 35.1%, Figure 22b.

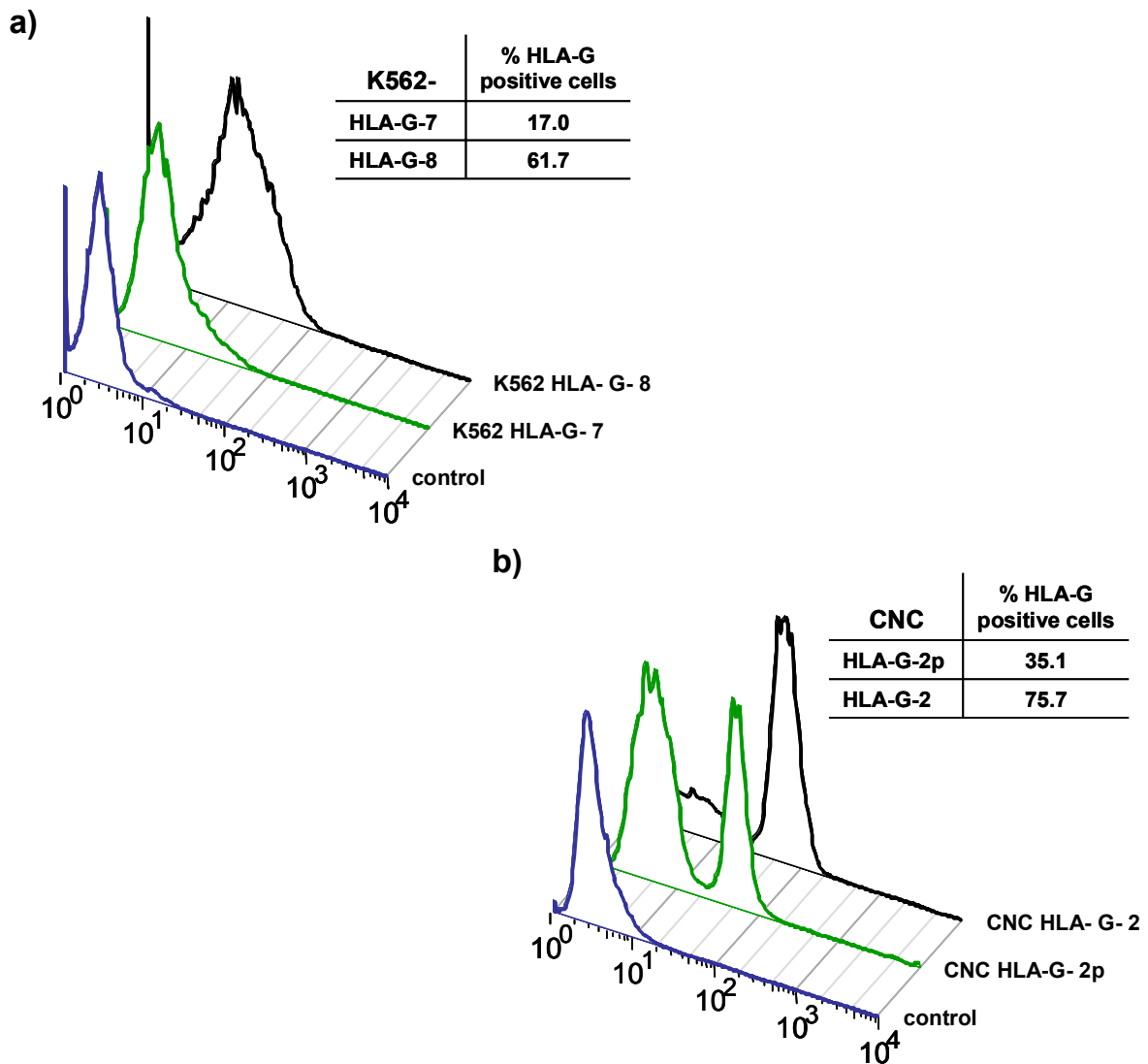


Figure 22. HLA-G expression levels in CEM.NKR CCR5 transfected cells, HLA-G-2 and HLA-G-2p a) and in K562 transfected cells HLA-G-7 and HLA-G-8 b).

7.2.2.c. Killing of HLA-G transfected cells

Despite the successful expression of HLA-G into K562 cells, NK killing sensitivity of K562-HLA-G-7 and -8 cells did not show any differences compared to the untransfected K562 cell line. The percentage of dead cells in all three cell populations was always comparable regardless of the HLA-G expression levels. Figure 23 shows a representative dot plot of a NK killing assay testing the K562 transfectants. In this figure NK cytotoxicity was in the same range than the wild type K562 cells and the HLA-G clones, HLA-G expression does not seem to influence killing levels.

Time point 0

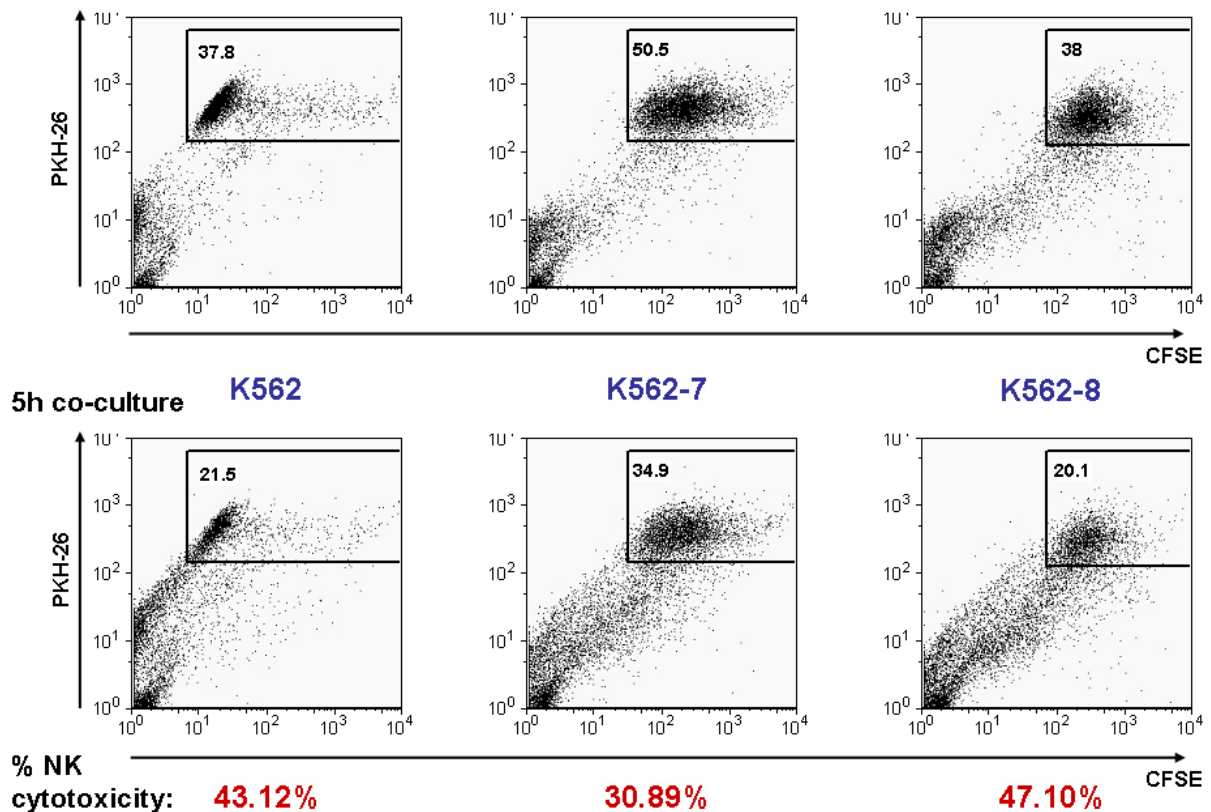


Figure 23. Natural cytotoxicity of K562 cells and K562-HLA-G transfected cell lines, K562-7 and -8. HLA-G expression levels were of 16.3% and 23% in K562-7 and -8 respectively.

A similar scenario was observed when we tested the CEM.NKR CCR5 HLA-G transfectants for sensitivity to NK cytotoxicity. No consistent differences in killing sensitivities were obvious, HLA-G transfectants were more or less equal sensitive to

NK cytotoxicity compared to the parental cell line, as depicted in Figure 24. The differences between sensitivity to NK cytotoxicity of the parental cell line and the HLA-G-2 or HLA-G-2p transfectant clone were not significant.

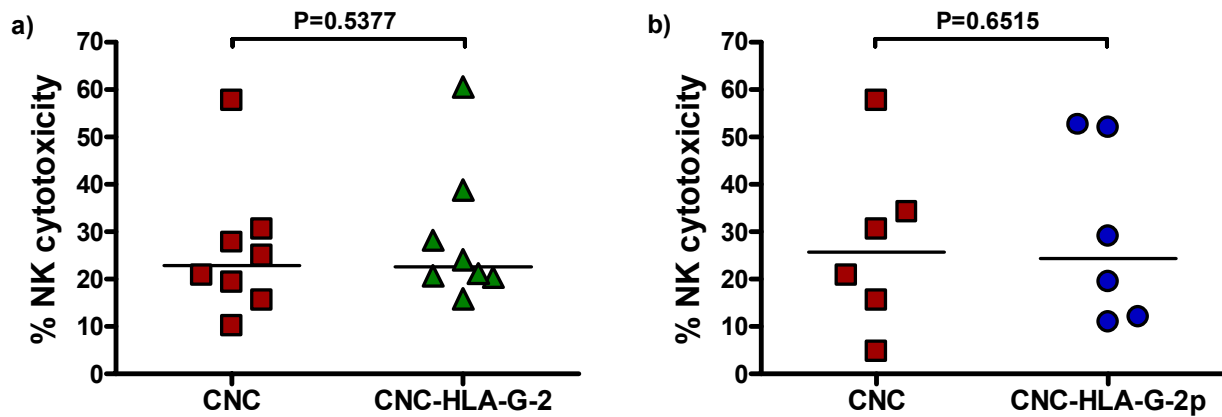


Figure 24. Sensitivity to natural cytotoxicity of HLA-G transfectants, CNC-HLA-G-2 (a) and CNC-HLA-G-2p (b) compared to the parental cell line, CEM.NKR CCR5. The median value is drawn, statistical significance was analysed with a paired T-test, in a) P-value= 0.5377 and $R^2= 0.0568$ and in panel b) P-value= 0.6515 and $R^2= 0.04404$.

ADCC in HLA-G transfectants

The β -2 microglobulin is a component of the MHC class I molecules and is therefore present in almost every cell type, including lymphocytes. ADCC assays were performed in which antibodies do not cross-link target to effector cells via surface expressed viral particles but instead by binding to β -2 microglobulin. The Fc part of these antibodies binds to CD16 on NK cells and thus activates the ADCC killing mechanism. With this setup maximal ADCC activity can be produced, as all target cells express β -2 microglobulin in large numbers and should thus be susceptible to ADCC. This provides an important control for the ADCC assay system. ADCC-mediated killing is calculated as the difference in killing in presence and absence of anti- β -2 microglobulin antibody.

$$\% \text{ ADCC} = \% \text{ killing in presence of antibodies} - \underbrace{\% \text{ killing in absence of antibodies}}_{\text{NK cytotoxicity}}$$

Where killing is calculated as:

$$\% \text{ killing} = 100\% - \frac{(\% \text{ intact cells after 5h}) * 100}{(\% \text{ intact cells at 0h})}$$

As depicted in Figure 25, addition of anti-β-2 microglobulin antibody enhanced killing, thus ADCC occurred and could be dissected from NK cytotoxicity. In the assays performed (Figure 25a) β-2 microglobulin-mediated ADCC levels ranged from 13-33% in parental CEM.NKR CCR5 cells, 15-45% in the HLA-G-2 clone and 27-55% in the HLA-G-2p clone. Enhancement of ADCC levels in the HLA-G transfectants is probably due to the fact that since β-2 microglobulin is a component of MHC-class I molecules; it is therefore more abundant in HLA-G expressing cells. In addition Figure 25b illustrates that higher ADCC levels do not correlate with high NK cytotoxicity levels, indicating that these two mechanisms are independent from each other.

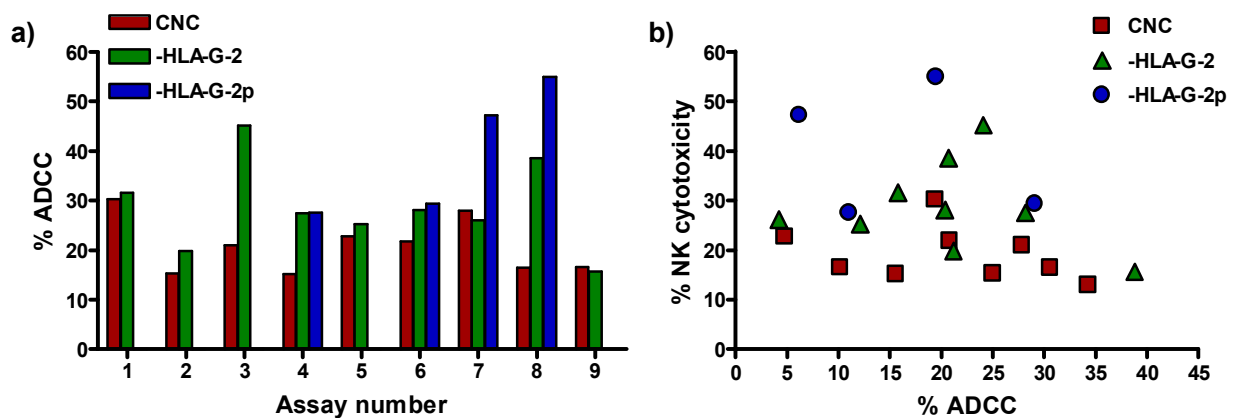


Figure 25. β-2 microglobulin-mediated ADCC levels in the parental cell line CEM.NKR CCR5 (CNC) and in the HLA-G transfectants, and compared to cytotoxicity levels in the same cell lines. a) ADCC levels in the parental and the HLA-g transfectated cell lines. b) No correlation between % cytotoxicity and % ADCC is apparent.

7.2.3. Improving assay conditions

Since in our assay, natural cytotoxicity of fresh and activated NK cells against the NK resistant cell line CEM.NKR CCR5 was too high to allow sensitive ADCC measurement and transfection of target cell lines with HLA-G failed to increase

resistance against natural killing, several parameters were tested to diminish natural lysis. We probed following parameters:

- Duration of co-incubation time
- Effector to target ratios
- Alternate cell lines
- Subcloning of target cells to improve resistance

7.2.3.a. Evaluating duration of incubation time

We performed time courses where we reduced incubation time from 5 hours co-culture to 2 hours to probe if shorter NK-target cell exposure would limit direct NK killing effects. However as depicted in Figure 26, these attempts did not succeed in lowering NK cytotoxicity levels below 10%. Notably though for CEM.NKR CCR5 cells a time dependent reduction was observed.

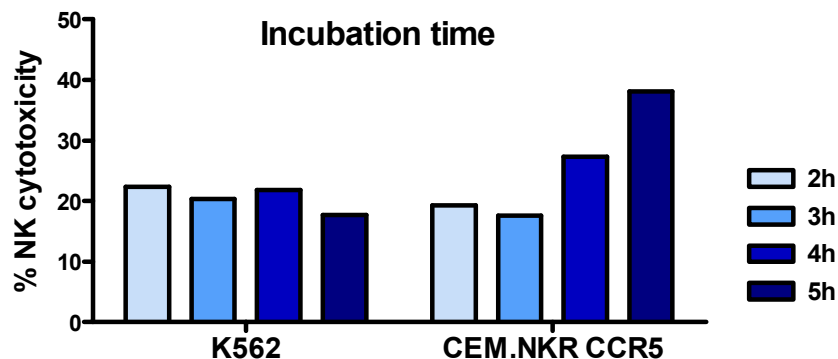


Figure 26. Time course of NK killing activity. Target cells were co-incubated with effector cells at different periods of time, ranging from 2 hours up to 5 hours at a 1:1 effector to target ratio.

7.2.3.b. Decreasing effector to target ratios

Reducing of the number of effector cells in the co-culture resulted in diminished unspecific killing as expected (Figure 27a), however difference between unspecific, NK cytotoxicity and specific, ADCC, killing was not increased since levels of β -2 microglobulin-mediated ADCC also decreased with reduction of effector to target ratios Figure 27a and b respectively.

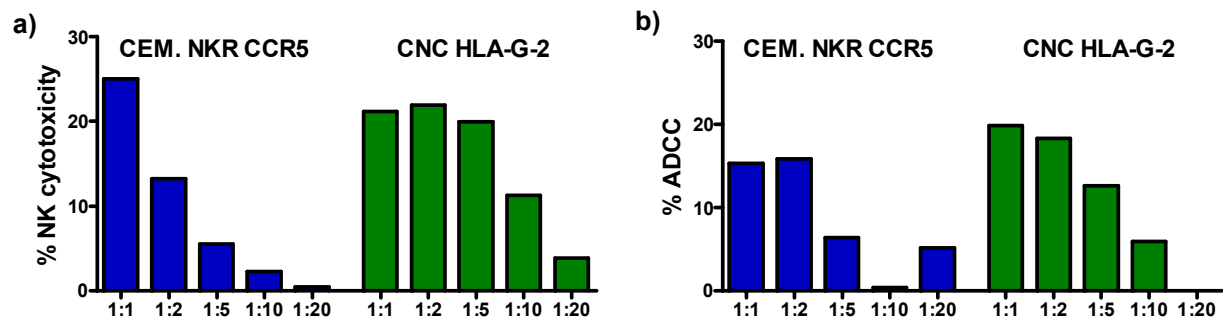


Figure 27. Killing assays with decreasing effector cell numbers. a) Target cells were incubated at different ratios with effector cells, starting at a 1:1 ratio of effector to target cells and decreased until a 1:20 ratio. b) In parallel, β -2 microglobulin-mediated ADCC assays were performed at the same ratios, depicted are the ADCC levels where NK cytotoxicity is already subtracted.

7.2.3.c. Probing alternate cell lines for NK sensitivity

Since reduction of killing time and lower effector numbers in the co-cultures did not help to reduce unspecific killing, other cell lines were also tested for their sensitivity to natural cytotoxicity. Figure 28 depicts cell lines tested for their sensitivity to NK lysis. With the exception of HELA cell, all cell lines probed had too high initial sensitivity for NK cytotoxicity. In addition an adequate cell line should readily be infected with HIV. So HELA CD4 cells, although relative resistant to NK cells, are not suitable target as they do not express CCR5. Unfortunately the HELA CD4 CCR5 cell line, which expresses CCR5, had again higher sensitivity to cytotoxicity with killing levels around 16% (Figure 28).

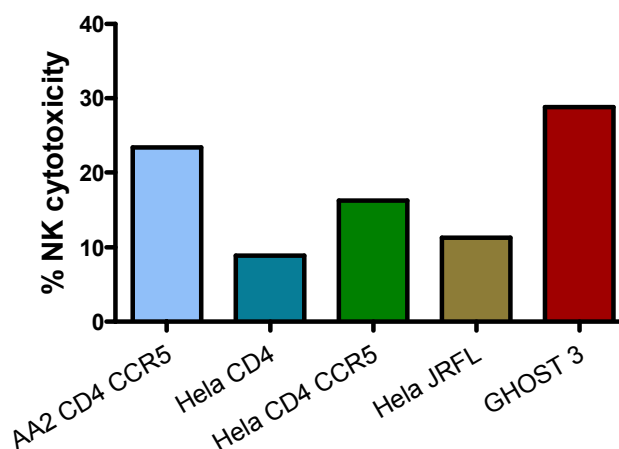


Figure 28. Testing different cell lines for their sensitivity to NK lysis. Assay was performed at a 1:1 effector to target ratio and a co-culture time of 5h.

7.2.3.d. Subcloning of target cells

We next subcloned target cell with the aim to isolate cell clones that are more resistant to NK cytotoxicity than the average bulk population. This was done by long term co-culture of target cells with NK cells. If successful, surviving target cells should be less sensitive to NK lysis. This was done in analogy to CEM.NKR cells, which were initially identified by co-culture of CEM cells with PBMC.

We therefore subcloned CEM.NKR CCR5 cells by co-culturing these target cells together with NK cells at equal ratios, by seeding per well of a 96-well plate five target cells and five effector cells. The surviving cells after a two to three-week period of co-culturing were mixed together and tested in our assay. Since NK cells die in prolonged culturing we can be sure that the cell mixture only contains CEM.NKR CCR5 cells. Figure 29 shows the killing level of the subcloned CEM.NKR CCR5 cells, mainly after the second round of subcloning, cytotoxicity was reduced from 36% to 14%.

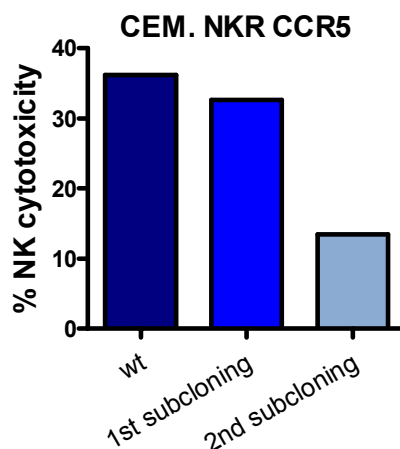


Figure 29. NK cytotoxicity levels in CEM.NKR CCR5 cells at the first and second round of subcloning. Assay was performed at a 1:1 effector to target ratio and a co-culture time of 5h.

7.3. ADCC readout by FACS: Protocol II

An alternate ADCC assay was adapted from Gupta *et al.* (Gupta *et al.* 2005), it is also based on dual stains measured by flow cytometry. Thereby killing is assessed by recording the percent propidium iodide positive cells after co-incubation of effector and target cells. To differentiate target from effector cells we stained with a

carbocyanine dye, in contrast to the PKH stain used by Gupta *et al.* At this end target cells (K562 or CEM.NKR CCR5) (1×10^6 - 3×10^6 cells per tube) were first stained with DiD (,1' -dioctadecyl -3, 3, 3', 3' -tetramethylindodicarbocyanine, 4 -chlorobenzene sulfonate salt), a carbocyanine that is incorporated into cell membranes (Molecular Probes, Eugene, Oregon). Staining was done by incubating cells for five minutes at room temperature with 0.3uM final concentration of the dye. The staining reaction was then stopped by adding an equal volume of heat inactivated FCS. Cells were then washed with PBS and resuspended at 0.5×10^6 cells /ml.

2.5×10^4 target cells were incubated with NK cells at a ratio of effector to target of 1:1 or 5:1 in a final volume of 200ul for 90 min. at 37°C. As control, target cells and NK cells were incubated separately and mixed only prior to FACS analysis, the time point 0 as in the CFSE /PKH-26 assay. In the final 15 minutes of the reaction propidium iodide (50ug/ml) was added to the effector-target cell cultures, this dye incorporates into apoptotic cells and stains the DNA. Cells are then directly transferred to FACS tubes and directly analysed without fixation. 1×10^4 cells were then analysed by FACS. Figure 30 exemplifies a typical dot plot measured.

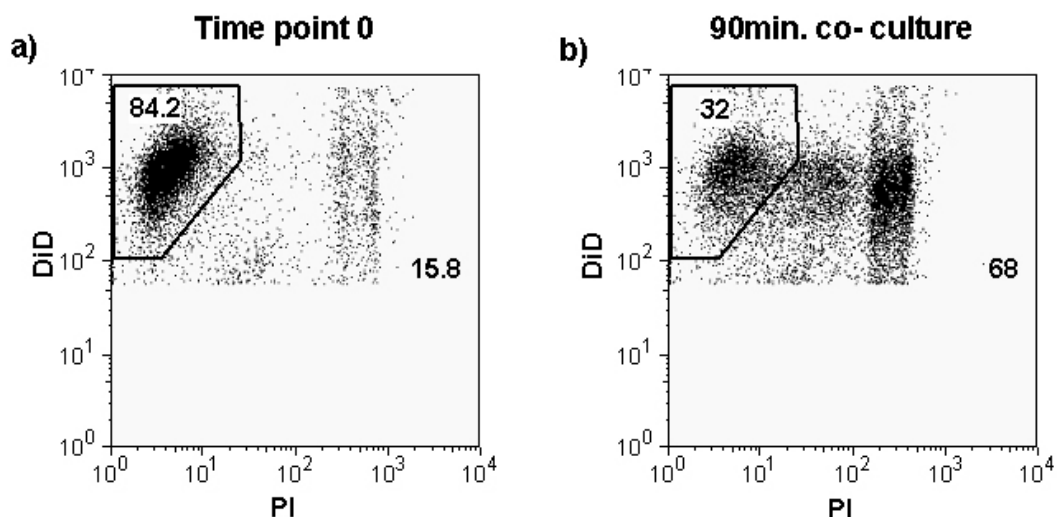


Figure 30. Example of quantification of the killing assay with a DiD/PI dual stain. Dot plots show only target cells, gated as DiD positive cells. a) Time point 0, intact cells (DiD positive) and dead (PI positive) target cells, b) after 90 minutes of co-culture with effector cells, decrease of DiD positive cells.

Analysis of the FACS dot plot, Figure 30, is similar as in the first assay. DiD positive cells (intact target cells) are gated, killing is determined as the decrease of DiD cells by comparing time point 0 and 90 min. co-culture.

$$\% \text{ killing} = 100\% - \frac{(\% \text{ intact cells after 90'}) * 100}{(\% \text{ intact cells at 0h})}$$

Or, alternatively as the increase of PI positive cells, dead cells. When ADCC is evaluated, natural lysis activity is subtracted of the total killing measured as in the PKH-26/CFSE stain.

$$\% \text{ ADCC} = \% \text{ killing in presence of antibodies} - \underbrace{\% \text{ killing in absence of antibodies}}_{\text{NK cytotoxicity}}$$

7.3.1. Evaluation of the alternate protocol

We first determined the cytotoxic sensitivities of K562 and CEM.NKR CCR5 to natural killer cells in this new assay. In addition we also tested the sensitivity of CEM 5.25 (CEM 5.25.EGFP.Luc.M7) cells. This CEM derivate was engineered to express CCR5 and in addition green fluorescent protein (GFP) and luciferase under the control of HIV-1 long terminal repeat. This permits to rapidly quantify HIV infection by flow cytometry. ADCC could thus be easily evaluated by gating first on the GFP positive cells (HIV-infected) and then monitoring the changes of DiD and PI positive populations.

Similar as in the previous killing assay also in the new format sensitivities varied a lot when NK cells were used 1 day after isolation. There NK killing activity as measured against K562, ranged between 15 and 70% at an effector to target ratio of 5:1 (Figure 31). An advantage of the alternate protocol, likely due to shorter assay duration (90 min. versus 2-5 hours) is that natural killing activity is markedly lower against CEM.NKR CCR5 cells. In CEM.NKR CCR5 cells at the same effector to target ratio, 5:1, natural cytotoxicity only attained 10% in this alternate protocol, which renders them again suitable for ADCC assays (Figure 31). CEM 5.25 cells were more sensitive to cytotoxicity, since although they arise from the same parental cell line as

the CEM.NKR CCR5, they were not selected for NK resistance, killing rates range from 2% to 25% for these cells (Figure 31).

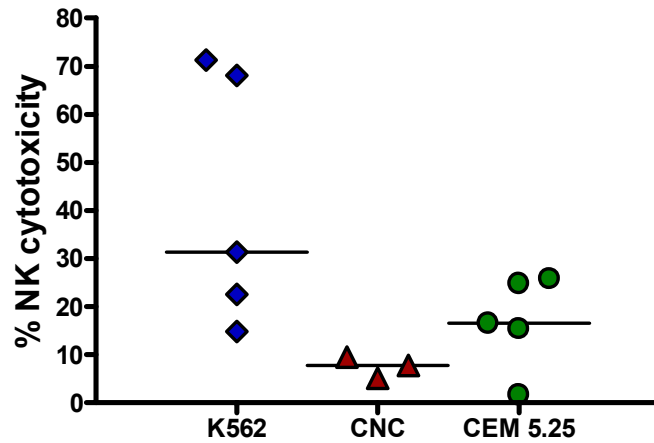


Figure 31. Variability in killing sensitivities of different cell lines through fresh isolated NK cells 1 day post isolation at a effector to target ratio of 5:1. The median of each data set is drawn.

Figure 32 depicts a comparison of cytotoxicity levels reached by the two different protocols used. In the first protocol target cells are previously double stained with CFSE and PKH-26, then co-cultured for 5 hours at an effector to target cell ratio of 1:1. In the second protocol target cells are previously stained with DiD, co-cultured at an effector to target ratio of 1:1 and 5:1 and stained with PI after 90 minutes. In the figure only 5:1 ratios are depicted.

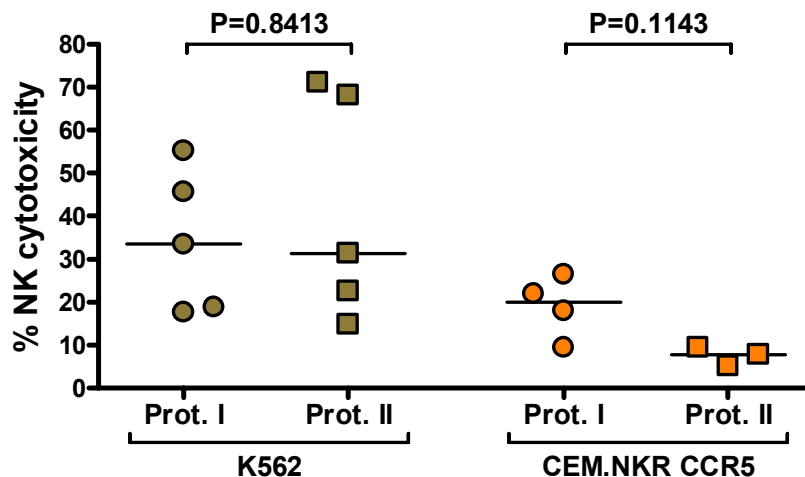


Figure 32. Comparison of NK cytotoxicity levels measured in Protocols I and II with two different target cells, K562 and CEM.NKR CCR5 cells. Median values are drawn and P-values were calculated with a Mann-Whitney test.

The killing levels of K562 cells in both protocols are very similar, the median in both cases is around 32%. The difference of reached cytotoxicity levels in CEM.NKR CCR5 cells in both protocols is more pronounced. Although it is statistically not significant median lysis value attained in the first protocol is 20% and in the second below 8%. Since background killing (cytotoxicity) is smaller in the second protocol (DiD/PI staining, 90 minutes co-culture) and the median in this killings does not reach 10% this protocol is clearly more suitable than the first one to measure specific killing.

We next performed ADCC assays with CEM 5.25 cells infected with JRFL (Figure 33). Fresh isolated NK cells at day 1 post isolation were used as effector cells and the assay was set up at two different effector to target ratios, 1:1 and 5:1. Gating was first done on GFP positive cells corresponding to the infected target cells so that ADCC can directly be measured comparing killing in infected cells without antibody and with the ADCC mediating antibody, in this case 2G12. ADCC was calculated as described before, as the difference of killing in presence and absence of the antibody.

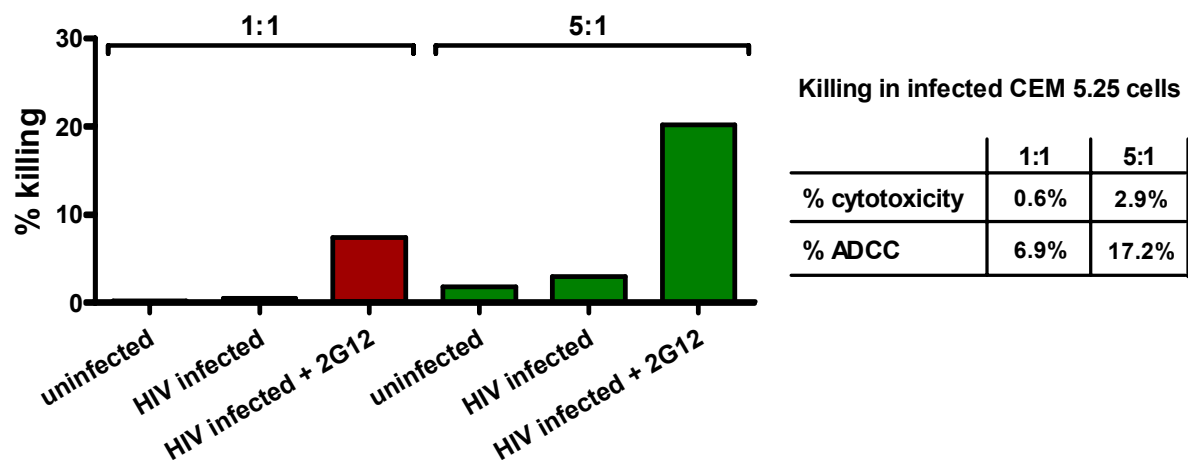


Figure 33. Killing of HIV infected CEM 5.25 cells at two different effector to target ratios, 1:1 and 5:1. Infected cells were gated for GFP expression and killing was evaluated directly in this population. 2G12 antibody was added at a concentration of 25ug/ml.

These results were quite promising, since NK cytotoxicity was low. Even at a target to effector ratio of 5:1 direct NK lysis was below 3% in the 5:1 ratio and specific killing reached 17.2% at the same ratio.

8. Conclusions

Assessing activity of ADCC-mediating antibodies in HIV patients during different stages of infection would help to gain insight in the overall impact and significance of ADCC in HIV suppression. This could be achieved by measuring ADCC activity mediated by antibodies elicited in HIV infected patients against cells infected with the autologous virus isolate at different time points using purified NK cells as effectors and comparing the activity longitudinally to the autologous neutralisation activity in the same patients. The aim of the project was therefore to develop an assay that would permit measurement of NK cytotoxicity as well as autologous ADCC activity in a fluorometry based assay.

Developing a robust assay system, that allows to measure ADCC activity against the autologous virus turned out to be relatively complex. Following criteria had to be fulfilled:

- Readily infectable target cells with patient derived viruses. Since we aimed to use autologous virus for our assays the chosen target cells need to be infectable with primary, R5 and X4 viruses. Therefore we were restricted to cell lines expressing CD4 and both co-receptors. The CEM.NKR CCR5 cells accomplish these criteria and can be infected by both R5 and X4 viruses.
- No radioactivity. We chose fluorometric readout to avoid use of radioactivity as handling of infectious radioactive waste bears several logistic problems. We adapted successfully a flow cytometry based read out for the ADCC assay, which allowed measuring NK cytotoxicity as well as ADCC. It is based on double stain of target cells with CFSE and PKH-26 to differentiate them from effector cells and to differentiate lysed from intact target cells.
- Sensitive. Since we aimed to measure differences in content of ADCC-mediating antibodies in sera of HIV-patients at different time points of infection the measured killing range need to be broad.
- Target cell resistance to NK cytotoxicity. This is ultimately important as the assay is unable to differentiate between natural cytotoxicity and antibody mediated killing (ADCC).

Since ADCC requires the interaction of three players, target cell, effector cells, and the antibody linking those two cell populations, each of these factors has to be precisely analysed. NK cells were isolated from PBMC and used as effector cells. Stimulation of these cells with low IL-2 concentrations was necessary in seven day cultures to avoid cell death. During this stimulation time no changes in expression of CD16, the main receptor responsible of ADCC, were apparent (Figure 12). In addition cytotoxicity levels, as measured in K562 cells, increased significantly after stimulation when using buffy-coat derived NK cells were used (Figure 16). The difference was not so striking if NK cells were directly isolated after blood donation, as these cells were active already shortly after isolation (Figure 16). One factor that has to be considered is the variability in killing ability of NK cells from different donors. The assays performed demonstrate a great range in killing activity, depending on the donor and the stimulation state. Additionally killing capacity may vary in the same donor depending on the activation state and the amount of inhibitory and activating receptors expressed on the NK cells at the time of blood donation and the following isolation and culture. We were however able to minimise influences of activation state to some extent by stimulation with low IL-2 concentrations (10U) for seven days (Figure 16). Nonetheless, donor variability has to be considered as it is likely that differences in ADCC antibody titre will be better discernable when the killing ability of the used NK population is high.

Initially experiments focused on CEM.NKR CCR5 cells as target cells, as the parental cell line CEM.NKR is insensitive to natural cytotoxicity and therefore widely used as target cell population in HIV ADCC assay. Nevertheless in the first protocol (CFSE/PKH-26 stain of target cells), these cells were highly sensitive to NK lysis, reaching cytotoxicity levels similar to those reached in K562 cells, which are known to be very sensitive to cytotoxicity. This could be due to the different read out (flow cytometry/⁵¹Cr release assay) or killing insensitivity was lost during cloning of these cells to express CCR5.

Transfection of HLA-G into CEM.NKR CCR5 cells to reintroduce resistance to NK cells, did not result in expected resistance to NK cytotoxicity (Figure 24), although expression levels were high, 75.2% in CNC-HLA-G-2p cells (Figure 22b).

Improving assay conditions by modifying incubation time of targets and effectors did not decrease cytotoxicity in CEM.NKR CCR5 to levels below 10% (Figure 26). Reduction in effector to target ratio resulted in diminished unspecific killing, but at the same time decrease of ADCC activity and was thus not a suitable approach (Figure 27). We identified no other cell line that could be infected by R5 and X4 primary viruses that had cytotoxicity levels below 10% in this assay (Figure 28). However, selection of CEM.NKR CCR5 cells resistant to NK cells in prolonged co-cultures resulted in clones where killing sensitivity was reduced from 36% to 14% (Figure 29).

The alternate ADCC protocol, that was adapted subsequently, is based on 90 minutes co-incubation of effector and target cells, targets are stained with DiD and lysed cells are detected with PI. Cytotoxicity levels measured comparing both protocols were similar for K562 cells, however CEM.NKR CCR5 cells had considerably lower cytotoxicity levels in this protocol (median <8%) as shown in Figure 32. CEM 5.25 had slightly higher sensitivity to NK cells in this assay with a median level of 16% (Figure 31). CEM 5.25 cells are effective target cells for ADCC as these cells express GFP upon infection, therefore infected cells can be gated directly for GFP expression. This allows ADCC to be calculated more precisely compared to cells without reporter gene. Infection rate of cells without reporter gene has to be measured in a separate experiment and then killing of infected cells estimated mathematically. Therefore the evaluation of ADCC on target cells that can be directly gated for infected cells has a much higher precision. In future experiments it should therefore be considered to subclone this cell line in order to select a clone with higher resistance to natural killing as it was done for CEM.NKR CCR5 (Figure 29). In addition we performed ADCC assays with JRFL infected CEM 5.25 cells. ADCC activity mediated by 2G12 was measurable in these cells even though the used NK cells had low killing ability (Figure 33).

The second protocol has some advantages over the first protocol:

- i) Incubation times are reduced from 5 hours to 90 minutes.
- ii) Natural killing in target cells CEM.NKR CCR5 cells was reduced dramatically to background levels necessary for ADCC measurement.
- iii) Target cells are easily stained with DiD, this fluorochrome has a very high intensity and is measured on the FL-4 channel of the FACS, in contrast

CFSE (FL-1) tends to bleed into FL-2, and puts constraints on compensation.

- iv) DiD (FL-4) and PI (FL-2) stains allows direct gating of HIV-infected cells through expression of GFP in FL-1. Since CFSE is also measured in FL-1, GFP expressing cells cannot be discerned, and percent HIV-infection has to be measured in a separate experiment by intracellular p24.

Why cytotoxicity levels in CEM.NKR CCR5 are so drastically diminished remains unclear. It seems improbable that this is solely due to the shorter incubation time since the killing time course in the first protocol only reduced cytotoxicity to 20% when incubated 2 hours (Figure 26). To what extent the different stains are responsible for that difference in readout, yet needs to be clarified. It could be due because of a more precise staining in the second protocol, which directly stains lysed cells but also because of the CFSE/PKH-26 stain that could leak spontaneously. Despite the promising results, several parameters of the second protocol yet need to be fine tuned. Equally effects of NK stimulation should also be investigated in more detail.

In conclusion, I succeeded in developing an assay system to measure ADCC activity in autologous systems of patient viruses, infected cells, and patient sera. An assay was setup to measure ADCC activity *in vitro* based on dual stain of the target cell line and evaluation by flow cytometry. The latest results are quite promising and proof that we succeeded in deriving a standardised system, where plasma of different patients may be evaluated for the presence of ADCC-mediating antibodies.

PART 2

9. The HIV-1 envelope glycoproteins

HIV-1 is surrounded by an envelope derived from the host-cell lipid bilayer that contains cellular membrane proteins such as major histocompatibility antigens, adhesion factors, actin and ubiquitin (Arthur *et al.* 1992). Imbedded in this outer membrane are viral envelope proteins forming spikes that promote virus entry into target cells. This envelope spikes are composed of the surface glycoprotein gp120 and the transmembrane glycoprotein gp41 that are non-covalently associated as trimers of heterodimers (Schawaller *et al.* 1989; Earl *et al.* 1990; Weiss *et al.* 1990; Thomas *et al.* 1991; Pinter *et al.* 1995).

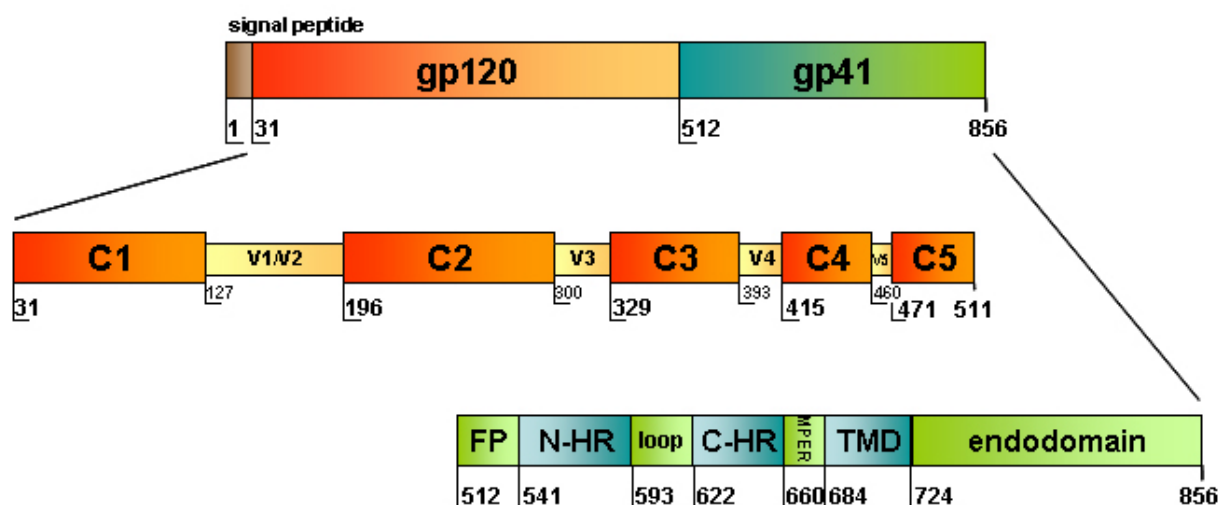


Figure 34. Schematic representation of the *env* gene. Positions correspond to HXB2 numbering, length relations were maintained. Abbreviations in gp120, C: constant region; V: variable region. In gp41, FP: fusion peptide; N-HR: N-terminal heptad repeat; C-HR: C-terminal heptad repeat; MPER: membrane proximal external region; TMD: transmembrane domain.

The envelope proteins are synthesised as gp160 polypeptide precursors and are co-translationally glycosylated by cellular enzymes in the lumen of the rough endoplasmic reticulum (Dewar *et al.* 1989). Glycosylation involves the attachment of asparagine-linked high-mannose type oligosaccharides to the protein backbone, reviewed in (Geyer *et al.* 1988; Hunter *et al.* 1990). This glycosylation is apparently

required to transport the gp160 precursor from the endoplasmatic reticulum to the Golgi apparatus (Dewar *et al.* 1989; Willey *et al.* 1991). Subsequently, accessible glycans on the precursor are trimmed and modified by various cellular enzymes during transport through the Golgi (Leonard *et al.* 1990). The resulting glycoprotein is then cleaved into gp120 and gp41 in the trans-Golgi network (Willey *et al.* 1988; Earl *et al.* 1991; Hallenberger *et al.* 1992).

During the production of progeny virus, infected cells express the envelope trimers on their surface. They are anchored in the membrane through gp41 and upon viral assembly incorporated with the cellular membrane into the budding virus.

Recently a model of the Env spike of SIV, the simian immunodeficiency virus closely related to HIV was generated by Zhu *et al.* (Zhu *et al.* 2006), through aligning and averaging 6175 individual SIV spike tomograms from 122 virions (Figure 35). According to the model derived by Zhu *et al.* the Env spike subunit contains three lobes (main, lateral and proximal), formed by gp120.

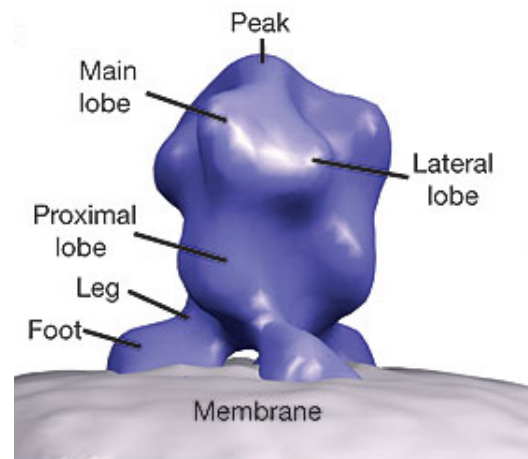


Figure 35. Side view of the trimeric SIV Env spike as obtained from tomograms. Zhu *et al.*, 2006.

Gp41 originates from each proximal lobe and forms a short leg that angles outward reaching the viral membrane and continues parallel to it forming a foot-like structure. From these estimates, the spike has a height of 13.7nm and a diameter of 10.5nm. As the *env* sequences from SIV and HIV are very similar (SIVmac 32H and HIV-1 HXB2 have 35% identity and over 70% similarity) (Chen *et al.* 2005a), it is assumed that HIV spikes will also have a similar structure.

These results are however contradicted by Zanetti *et al.* (Zanetti *et al.* 2006) who by performing very similar studies found no evidence for the proposed lobes-leg conformation. Instead Zanetti *et al.* suggest that gp41 trimers form a stem, without outstanding legs, on top of which gp120 is positioned much like a mushroom head on a stem (Figure 36).

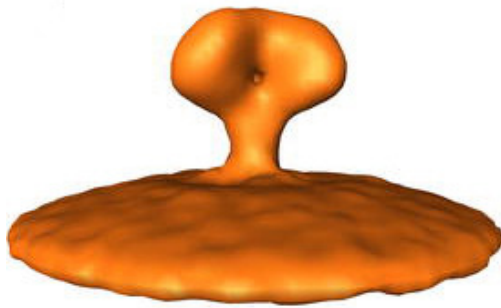


Figure 36. Reconstruction of the Env spike by Zanetti *et al.*, 2006.

More studies are needed to define which conformation model is correct. Studies in which the structure of the trimer will be analysed not only in its native conformation but also after binding to the receptor, co-receptor or during membrane fusion will give more insight into the definitive structure of the native functional envelope spike and the accessibility of each epitope to antigen recognition.

Functional Env trimer spikes are responsible for infection. There is, however, increasing evidence that the majority of spikes on HIV are non functional. These non-functional forms may be a) uncleaved gp160 precursor, b) gp41 stumps, c) alternative binding forms of gp120 to gp41 or d) gp41-gp120 monomers (Moore *et al.* 2006). However not only the organisation of the spike plays a role in productive infection but also the number of spikes on the HIV-1 virion and their orientation on it may have an influence. A recent study suggests that normal HIV possesses 14 ± 7 spikes on its surface and that these tended to cluster (Zhu *et al.* 2006). In contrast a previous study favoured the hypothesis that spikes are anchored in an ordered manner in holes in the matrix network underneath (Forster *et al.* 2000). New infections occur first through binding of the virus to the target cells and takes place by the interaction of gp120 with CD4 followed by binding to the co-receptor protein, usually CCR5 or CXCR4 leading to conformational changes that expose gp41 and initiate viral fusion and subsequent entry into the target cell.

Since all neutralising antibodies are directed against the viral envelope, a precise knowledge of the structure of the exposed protein, their conformational changes and escape mechanisms are prerequisite for developing effective vaccines that elicit neutralising antibody response.

9.1. Gp120

Gp120 is the surface expressed protein of the virus envelope and is responsible for the first interactions between the virus and the target cell by binding to the entry receptor CD4 and a co-receptor. Based on comparative analysis of different primate immunodeficiency viruses, five constant (C1-C5) and five variable (V1-V5) regions were identified in gp120, Figure 34. The conserved regions fold into a core containing elements essential for CD4 and chemokine receptor binding. The first four variable regions are exposed on the surface via disulfide bonds as large loops. Both variable and constant regions are heavily glycosylated, with carbohydrates accounting for up to 40-50% of the molecular weight of gp120. Together the variability and the glycosylation of gp120 surface modulate the immunogenicity and antigenicity levels of HIV-1.

Based on crystal structures of an HIV-1 gp120, the gp120 core is formed by a) the inner domain, b) the outer domain, and c) the bridging sheet (Figure 37) (Kwong *et al.* 1998).

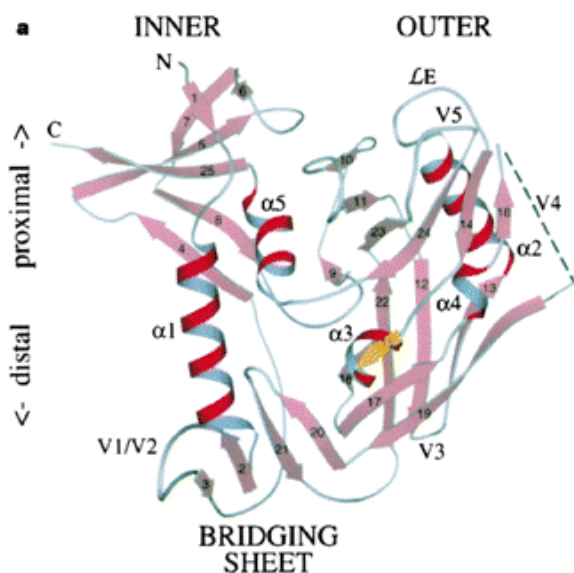


Figure 37. Structure of core gp120 in a ribbon diagram. In this view, the left portion of core gp120 represents the inner domain, the right portion the outer domain, and the 4-stranded sheet at the bottom left of gp120 as the bridging sheet. The viral membrane would be oriented above, the target membrane below, and the C-terminal tail of CD4 would be coming out of the page. α -Helices are depicted in red and β -strands in salmon, except for strand β 15 (yellow). Adapted from Kwong *et al.* 1998.

The inner domain is formed mainly by the C1 and C5 regions and is largely devoided of glycans. This domain is proposed to be responsible for interaction with gp41 as it is only accessible to antibodies in the monomeric conformation. The outer domain is heavily glycosylated and thought to be exposed at the surface of the trimer. Both

domains are linked together by the bridging sheet, which also serves as stem for the projecting V1/V2 loop (Kwong *et al.* 1998).

In a recent paper Zhu *et al.* (Zhu *et al.* 2006) fitted an unliganded SIV gp120 core (Chen *et al.* 2005b) into their unliganded SIV spike cryoEM density map showing the possible trimeric structure of gp120, Figure 38. The optimum fit positioned the core gp120 structures into the main lobes, the CD4 binding site on the periphery of the spikes and aligned the truncated V1/V2 and V3 loops towards the proximal and lateral lobes respectively.

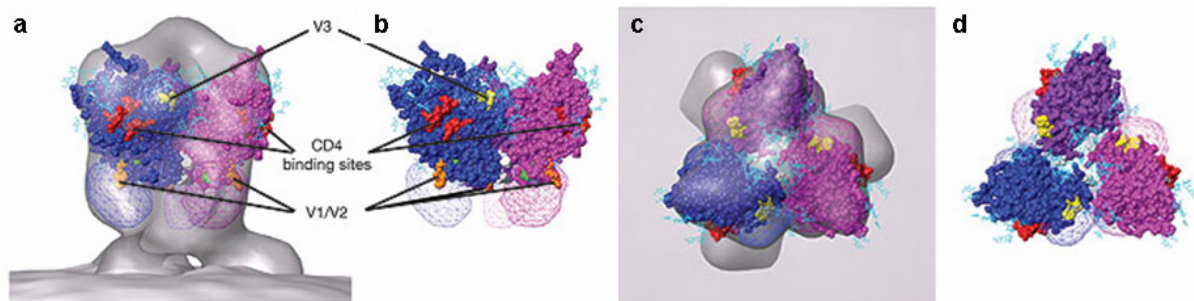


Figure 38. Fitting of unliganded gp120 SIV into the Env spike tomogram structure. Side (a, b) and top (c, d) views. gp120 subunits (blue, magenta, mauve), CD4 binding sites (red), V3 loop stems (yellow), V1/V2 loop stems (orange), N and C termini (green), C2 loop stems (white), proposed V3 and V1/V2 loop sites (mesh-enclosed volumes over stems). Adapted from Zhu *et al.*, 2006.

Here again the results obtained by Zanetti *et al.* are somewhat in contradiction. Their fittings resulted in two different options for the positioning of the different structures. In general glycans are all positioned on the surface of gp120 and the V4 and V5 variable regions are on the top surface. The CD4 binding site would be exposed on the outer edge so that interaction with CD4 would be possible, the V1V2 loop points outwards and the C1 and C5 regions would be near gp41 for interaction. From the first fitting option they proposed a model for receptor engagement. Since in their fitting the V3 loop and the co-receptor binding site are occluded they propose that gp120 changes its structure after CD4 engagement leading to rotation and separation of the gp120 proteins exposing the V3 loop therefore permitting co-receptor binding (Figure 39).

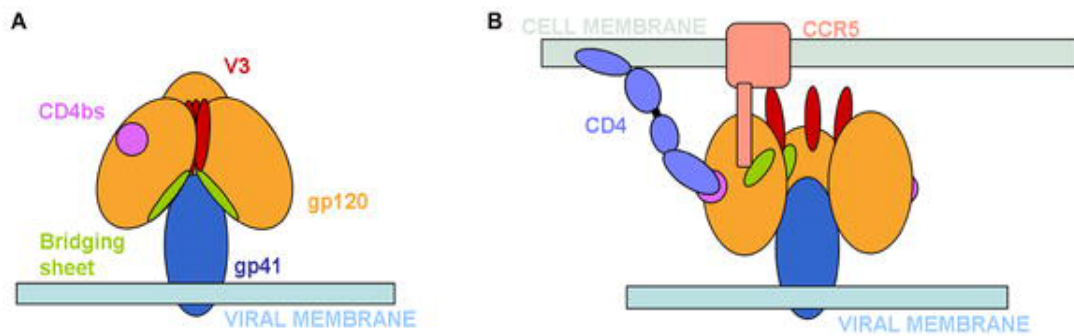


Figure 39. Model for HIV/SIV receptor engagement, based on the first proposed fitting. A) Viral Env spike in its unbound conformation. B) Conformational changes after binding of gp120 to CD4 on the cell membrane. Adapted from Zanetti *et al.*, 2006.

9.1.1. The receptor binding sites on gp120

9.1.1.a. The CD4-binding site

The first step in viral infection process is the high affinity binding of HIV gp120 to CD4 on the surface of target cells (Dalglish *et al.* 1984; Klatzmann *et al.* 1984; McDougal *et al.* 1986). The CD4 binding domain lies on a highly conserved cavity on gp120 formed by the interface between the inner domain, the bridging sheet, and the outer domain and is responsible for this crucial first interaction (Kwong *et al.* 1998; Wyatt *et al.* 1998a). Most of the CD4 interaction lies however on the outer domain. The ectodomain of CD4 consists of four immunoglobulin-like regions (D1-D4) and binding to gp120 occurs mainly through D1, the outermost domain (Ryu *et al.* 1990; Truneh *et al.* 1991). The surface of interaction between both molecules is quite large and covers 740Å on CD4 and 800Å on gp120 (Kwong *et al.* 1998).

9.1.1.b. The co-receptor binding site

Although binding of HIV to CD4 is essential, it is not sufficient for infection as an additional fusion co-receptor is needed ((Maddon *et al.* 1986; Broder *et al.* 1995), reviewed in (Moore *et al.* 1997)). Beside the most common co-receptors CCR5 and CXCR4, other members of this family have been identified as potential co-receptors such as CCR2b, CCR3, CCR8, APJ and others (de Roda Husman *et al.* 1998; van Rij *et al.* 2002). Upon CD4 binding, conformational changes on gp120 are known to expose the co-receptor binding site that involve highly conserved residues (Rizzuto *et*

al. 1998; Wyatt *et al.* 1998b). This conserved region has been mapped by site-directed mutagenesis to residues in the bridging sheet, including the V1/V2 stem and C4 regions, and the V3 loop (Rizzuto *et al.* 1998; Pierson *et al.* 2003). Efficient co-receptor binding depends on the presence of the V3 loop on gp120 (Wu *et al.* 1996), whose sequence also influences the specific co-receptor used (Choe *et al.* 1996; Hoffman *et al.* 2002). Of note, recently the V3 loop has been shown to mimic a beta-hairpin structures of chemokine, supporting its role in chemokine receptor interaction (Sharon *et al.* 2003). While the V3 loop clearly plays a role in co-receptor usage, conserved regions in the V3 stem and in the bridging sheet have been determined to be necessary for binding to either CCR5 or CXCR4 (reviewed in (Moore *et al.* 1997)).

9.2. Gp41

Gp41 is the transmembrane subunit of the envelope spike and promotes fusion of the viral and cellular membranes. It is activated by the conformational changes on gp120 that are induced upon binding to CD4 and the co-receptor. Gp41 consists of a N-terminal external domain, a hydrophobic membrane-spanning domain and a C-terminal intracytoplasmic segment (Hunter *et al.* 1990; Pancino *et al.* 1994), Figure 34.

The intracytoplasmic tail (ICT) or endodomain has a length of 150 amino acids, which is thought to interact with the matrix underneath at least during assembly (Murakami *et al.* 2000a). Its presence is essential for viral replication in primary PBMC as demonstrated *in vitro* (Murakami *et al.* 2000b). Furthermore mutations in the ICT attenuate SIV infection in macaques *in vivo* (Shacklett *et al.* 2000). The intracytoplasmic tail contains two structurally conserved amphipathic α -helical domains, lentivirus lytic peptides 1 and 2 (LLP-1 and LLP2) at the C-terminus (Miller *et al.* 1993). Mutations in this domains result in inhibition of Env incorporation (LaBranche *et al.* 1995; Berlioz-Torrent *et al.* 1999) and viral infectivity (Chakrabarti *et al.* 1989; Johnston *et al.* 1993; Ritter *et al.* 1993) as well as inhibition of cell-cell fusogenicity (Ritter *et al.* 1993; Spies *et al.* 1994) and lower Env antigenicity *in vitro* (Kalia *et al.* 2003; Kalia *et al.* 2005).

The N-terminal ectodomain contains a hydrophobic fusion peptide at the N-terminus followed by the N-terminal heptad repeat (N-HR) region and the C-terminal heptad

repeat (C-HR) region and finally the membrane proximal external region (MPER), defined roughly as residues 660 to 683 (HXB2 numbering) of gp160 (Ofek *et al.* 2004; Zwick 2005), a highly hydrophobic region because of its sequence rich in tryptophan (Trp). In the native trimer conformation the fusion peptide appears to be buried near the C-terminal transmembrane domain, it is exposed after CD4 and co-receptor engagement, (see section 3.1.1. Virus entry, for details). Currently there are no crystal structures of the full length gp41 in any form, bound or unbound to gp120 available. However X-ray structures of the N-HR and C-HR at the postfusion structures that form leucine zippers as well as of the MPER gave some insight of the gp41 structure. Crystal structures of the MPER regions will be discussed in more detail in chapters 10.1.2. and 10.1.3 together with the antibodies 2F5 and 4E10, that bind in this region.

Gp41 appears to be mostly occluded from antibody recognition by gp120, only the region close to the viral membrane, the MPER is accessible to some extent to antibody neutralisation as evidenced by the three neutralising antibodies, 2F5, 4E10 and Z13 (Zwick *et al.* 2001). However after receptor engagement, during formation of the pre-hairpin structure, gp41 is still protected from antibody binding by the closeness of both membranes. In contrast, the more stable postfusion six-helix bundle is highly immunogenic, the immunodominant domain between amino acid residues 586 and 620 (Ratner *et al.* 1985). Notably peptides corresponding to the different HR regions can inhibit HIV entry into the cells.

10. Neutralising antibodies

Antibody-based immunity, particularly virus neutralising antibodies, are an important defence mechanism in many different human viral diseases ((Groothuis *et al.* 1995; Robbins *et al.* 1995; Lee *et al.* 1997; Hilleman 2001) and reviewed in (Keller *et al.* 2000)). The role of neutralising antibodies in HIV infection is still not completely unravelled but it is generally agreed that protection vaccines will need to induce both cellular and humoral immunity to HIV. In HIV infection the antibody response is strong and usually directed against the structural proteins of the virus (Belec *et al.* 1995; Moore *et al.* 1995; Sattentau *et al.* 1995; Binley *et al.* 1997). However the minority of antibodies elicited to HIV-1 proteins have direct antiviral, neutralising

effects. Neutralising antibodies bind to the free virus and interfere with viral replication and infectivity through different mechanisms: a) inhibiting receptor binding, b) interfering with the fusion process, or later on c) preventing virus uncoating or budding. However the latter has not yet been described for antibodies to HIV-1. For neutralisation to happen antibodies need to have relative high affinity and/or avidity to epitopes that are exposed on outer structures of the virus. The HIV envelope possesses several exposed sites that may be recognised by neutralising antibodies: the CD4 binding site (Ho *et al.* 1991; Barbas *et al.* 1992; Karwowska *et al.* 1992), the carbohydrate cluster at the outer face of gp120 (Trkola *et al.* 1996; Scanlan *et al.* 2002), the V2 loop (Gorny *et al.* 1994; Scanlan *et al.* 2002), the V3 loop (Scott *et al.* 1990; Gorny *et al.* 1992; Gorny *et al.* 2002), the C4 induced site (Thali *et al.* 1993; Moulard *et al.* 2002; Xiang *et al.* 2002), and the MPER on gp41 (Muster *et al.* 1993; Trkola *et al.* 1995; Zwick *et al.* 2001). Although the antibody response is vigorous the majority of elicited antibodies are non-neutralising. In addition most neutralising antibodies only have relatively weak actions. They do not have the ability to potently cross-neutralise (or broadly neutralise) primary viruses and only have strain-specific effects. To date only four different human monoclonal antibodies have been described that are capable to broadly neutralise primary viruses from divergent clades (Burton *et al.* 2004; Zolla-Pazner 2004). These antibodies target highly conserved structures on gp120 and gp41. Among the broadly neutralising antibodies, IgG1b12 (Burton *et al.* 1994; Roben *et al.* 1994) and 2G12 (Trkola *et al.* 1996) target structures in gp120, whereas the other two, 2F5 and 4E10 cross-react with neighbouring regions at the MPER of gp41 (Muster *et al.* 1993; Buchacher *et al.* 1994; Stiegler *et al.* 2001; Zwick *et al.* 2001).

A reason for the relatively weak neutralising response against HIV is that the virus has evolved a variety of defence mechanisms to evade a robust antibody response. This mechanism include oligomeric exclusion, conformational masking, epitope variation, glycan cloaking, and steric interference at the cell-virus interface (Kwong *et al.* 1998; Wyatt *et al.* 1998a; Wyatt *et al.* 1998b; Kwong *et al.* 2002; Labrijn *et al.* 2003; Burton *et al.* 2004; Zolla-Pazner 2004). Numerous studies have shown that although neutralising antibodies are present in the plasma of patients, they often are not able to neutralise the current autologous virus but only the preceding virus strain,

indicating that HIV is able to rapidly escape to antibody pressure (Moore *et al.* 1994; Moog *et al.* 1997; Richman *et al.* 2003; Wei *et al.* 2003; Deeks *et al.* 2006).

10.1. Broadly neutralising antibodies and HIV's escape routes

This chapter discusses the mechanisms of action of three broadly neutralising antibodies, 2G12, 2F5 and 4E10 against HIV, which we used for our continuing studies.

10.1.1. 2G12

HIV gp120 contains approximately 25 sites for asparagine (N)-linked glycosylation that form a complex branched carbohydrate network accounting for approximately 50% of the molecular mass of the glycoprotein. This glycan shield is supposed to protect the virus by hiding conserved viral structures. Removal of specific glycan sites increases dramatically sensitivity to neutralising antibodies (Kolchinsky *et al.* 2001). However one antibody, 2G12, is able to recognise specifically glycans on gp120, more precisely $\alpha 1 \rightarrow 2$ -linked mannose residues on the distal ends of oligomannose sugars located on the carbohydrate-covered silent face at the outer face of gp120 (Trkola *et al.* 1996; Sanders *et al.* 2002; Scanlan *et al.* 2002)

Crystal structure analysis of 2G12 reveals that this antibody binds to the carbohydrate cluster in an unusual way in which the antigen-binding fragments (Fab) form a interlocking domain-swapped Fab dimer (Calarese *et al.* 2003). Thus, the variable heavy chains (V_H) from each Fab arm have exchanged positions in order to interact with the variable light chains (V_L) of the other Fab arm. The resulting conformation preserves the conventional antigen binding sites formed by the V_L/V_H interface but has an additional interface between the two V_H domains permitting 2G12 to bind to multiple glycans on the surface of gp120 (Figure 40).

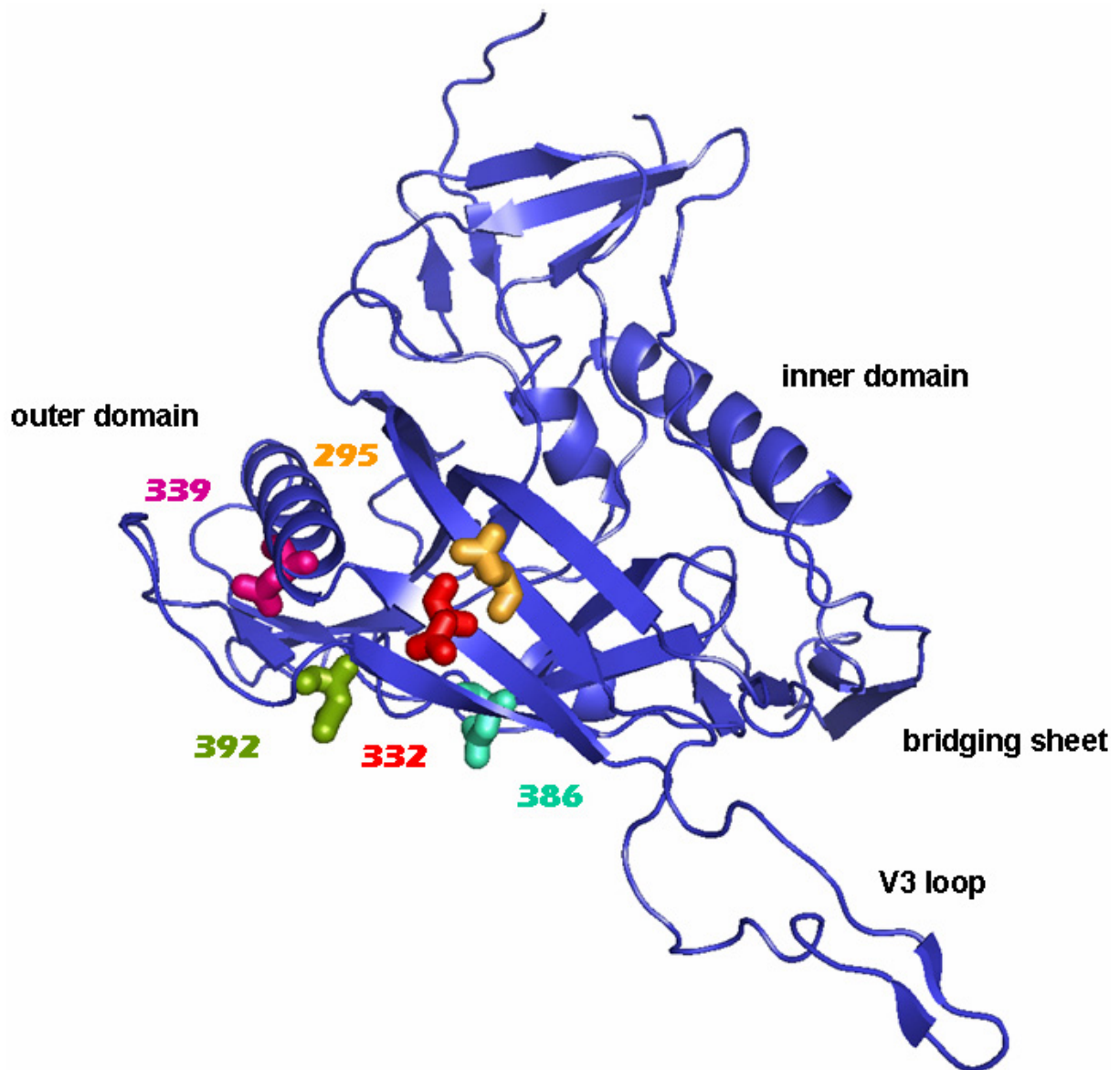


Figure 40. Structure of monomeric gp120 with the 2G12 relevant N-glycosylation sites in colour in context of the crystal structure of the protein. The structural coordinates of 2B4C (Huang *et al.* 2005) were obtained from the PDB database and the model was created using PyMOL software.

2G12 binding depends on presence of specific N-linked glycosylation sites at positions 295, 332, 339, 386 and 392 of gp120, according to numbering of the HXB2 reference strain as extensive alanine mutation analysis suggest (Scanlan *et al.* 2002). By binding to a cluster of carbohydrates and not only to one N-linked moiety, 2G12 selectively binds HIV Env and not random glycosylated proteins of the host. Notably resistance to 2G12 recognition is achieved by a single mutation in one amino acid that causes loss of one N-glycosylation site (Scanlan *et al.* 2002).

In vitro, 2G12 has shown to inhibit primary and T-cell line-adapted clade B virus strains as well as clade A members (Trkola *et al.* 1996; Scanlan *et al.* 2002). However, 2G12 is ineffective or only rarely effective against clade C, D, and AE viruses, likely due to the absence of one or more specific glycan sites required for efficient 2G12 binding to these strains (Binley *et al.* 2004; Chen *et al.* 2005b).

10.1.2. 2F5

The human monoclonal antibody, 2F5 has been shown to possess broadly neutralising activity against diverse HIV-1 isolates (Buchacher *et al.* 1994; Trkola *et al.* 1995). Like 2G12 and 4E10 it was first isolated from immortalised B cells of an HIV infected donor in a screen for anti-HIV antibodies. 2F5 recognises an epitope, composing the sequence ELDKWA at position 662 to 667 in the MPER of gp41 (Muster *et al.* 1993), this sequence is the core of a relatively conserved 16-amino acid sequence. As the affinity of 2F5 for the MPER is increased in the presence of lipids it has been suggested that the proximity of the viral membrane increases 2F5 binding (Sanchez-Martinez *et al.* 2006a; Sanchez-Martinez *et al.* 2006b). X-ray crystal structure of the MPER were determined by binding of 2F5 to a 7-mer peptide (Pai 2000) and a 17-mer peptide (Ofek *et al.* 2004). This analysis revealed that the initial portion of the MPER has an extended conformation with a distinct Type I β turn at the core of the peptide epitope (DKW) and another Type I β turn at the end of the epitope from residue 666 to 668. These overlapping turns reverse directional changes so that overall the peptide remains essentially in an extended conformation (Ofek *et al.* 2004) (Figure 41).

Mainly D664, K665 and W666 account for over 40% of the surface area that is buried by 2F5 and for almost 50% of the hydrogen bonds between 2F5 and gp41, this core epitope has been shown to be essential for 2F5 binding (Muster *et al.* 1993; Conley *et al.* 1994) (Purtscher *et al.* 1996; Zwick 2005), even though they are not sufficient for recognition by themselves. Consequently mutations in the sequence of the core epitope lead to decrease neutralisation sensitivity (Zwick 2005).

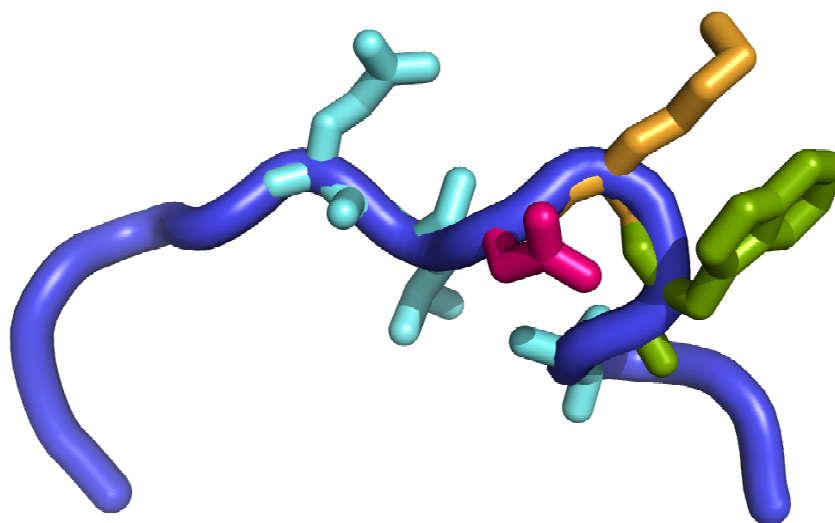


Figure 41. Crystal structure of the 17-mer 2F5 epitope on gp41 as determined bound to 2F5 in context of the crystal structure of the protein. The side chains of the epitope ELDKWA are coloured in aquamarine and the amino acids forming the core epitope DKW coloured in pink, yellow and olive green respectively. The structural coordinates of 1TJI (Ofek *et al.* 2004) were obtained from the PDB database and the model was created using PyMOL software.

10.1.3. 4E10

The region immediately carboxy-terminal to the 2F5 epitope is recognised by 4E10, the human monoclonal neutralising antibody that until now has the broadest ability to neutralise a large range of virus strains from all different subtypes (Zwick *et al.* 2001; Binley *et al.* 2004). 4E10 is able to recognise an epitope containing the sequence NWF(D/N)IT at residues 671 through 676 a Trp-rich region in the MPER. In addition 4E10 is able to neutralise viruses different from the consensus clade B sequence in which the core epitope differs at positions one (N), four (D) and six (T) (Zwick *et al.* 2001). Two crystal structures of 4E10 complexed with a peptide comprising either 13 residues from 668 to 680 (Cardoso *et al.* 2005) or a 19-mer from residue 665 through 683 (Schibli *et al.* 2001) showed that the 4E10-bound Trp-rich peptide adopts a helical conformation with a characteristic 'loosening' of the helix within and N-terminal to the residues W672 and F673, which are deeply buried in a paratope of 4E10, additionally the Trp residues form a 'collar' around the helix axis (Schibli *et al.* 2001; Cardoso *et al.* 2005) (Figure 42). Alanine substitutions showed that only mutations in

W672, F673 and W680 were able to confer neutralisation resistance (Zwick *et al.* 2004).

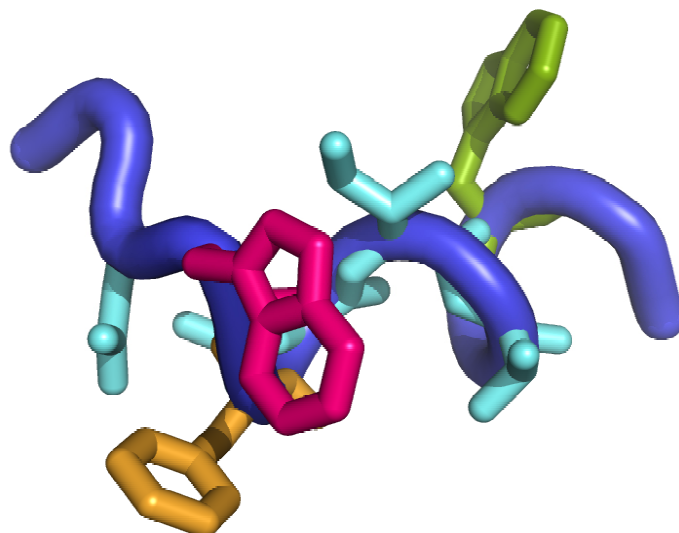


Figure 42. Crystal structure of the 4E10 epitope. Side chains of the NWFDIT are coloured in aquamarine, Trp672 is coloured in pink, Phe673 in yellow and Trp680 in olive green forming the core epitope of 4E10 in context of the crystal structure of the protein. The structural coordinates of 1TZG (Cardoso *et al.* 2005) were obtained from the PDB database and the model was created using PyMOL software.

11. Passive immunisation

An *in vivo* passive immunisation study to determine the protective role of neutralising antibodies was performed in our group (Trkola *et al.* 2005). The study was performed with eight chronically and six acutely HIV-1-infected individuals in whom plasma viremia was suppressed to undetectable levels by ART. Patients were selected from 58 individuals pre-screened for the sensitivity of the viral isolates to the neutralising antibodies 2G12, 2F5 and 4E10 *in vitro*. The fourteen selected HIV-infected patients were treated with a cocktail of three monoclonal antibodies, 2G12, 2F5 and 4E10 and the potential of the MAb to suppress viral rebound was evaluated. The six acutely and eight chronically infected individuals, who all carried virus highly sensitive to these neutralising antibodies, received 13 doses of an antibody cocktail, consisting of 1.3g 2F5, 1g 4E10 and 1g 2G12. MAb were administered over an 11-week period starting one day before antiretroviral therapy (ART) was stopped (Figure 43). Throughout this period and the following 12 weeks of wash out phase, the patients

were monitored for viral rebound kinetics and emergence of antibody-resistant virus variants. The results obtained in this study gave the background for the design of this second project of my PhD thesis.

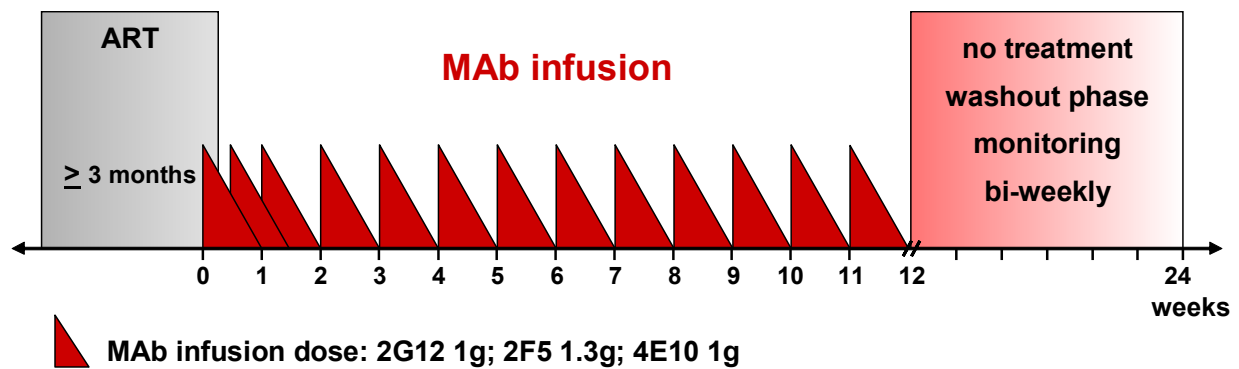


Figure 43. Design of the passive immunisation study.

In the eight chronic HIV-infected patients following immunisation viral rebound was compared to their rebound in previous structured treatment-interruption trials (Fagard *et al.* 2003a; Fagard *et al.* 2003b). In six patients viral rebound kinetic was similar to the rebound observed during STI without antibody infusion. The other two patients showed either delayed rebound or no rebound until week 18 of the study during the wash out phase. The evaluation of the influence of immunisation in the acute group was more complex than in the chronic group since no direct comparison with previous STI can be done. The rebound of the acutely infected individuals following immunisation was therefore compared with a control group of 12 acute HIV-infected patients who interrupted ART without receiving antibody infusions. Viral rebound in the acute patients occurred slightly later, by week 5 the earliest, than in the chronic patients where some rebounded already after 2-3 weeks. However in this group, three patients controlled viremia for prolonged time, compared to the control group, viral load being detectable the earliest by week 10 of the study and one patient although it virus could be measured by week 6, levels remained low up to week 9.

In summary two chronically and four acutely HIV-infected individuals showed delay in viral rebound whereas in the other eight passive immunisation did not show any effect (Figure 44).

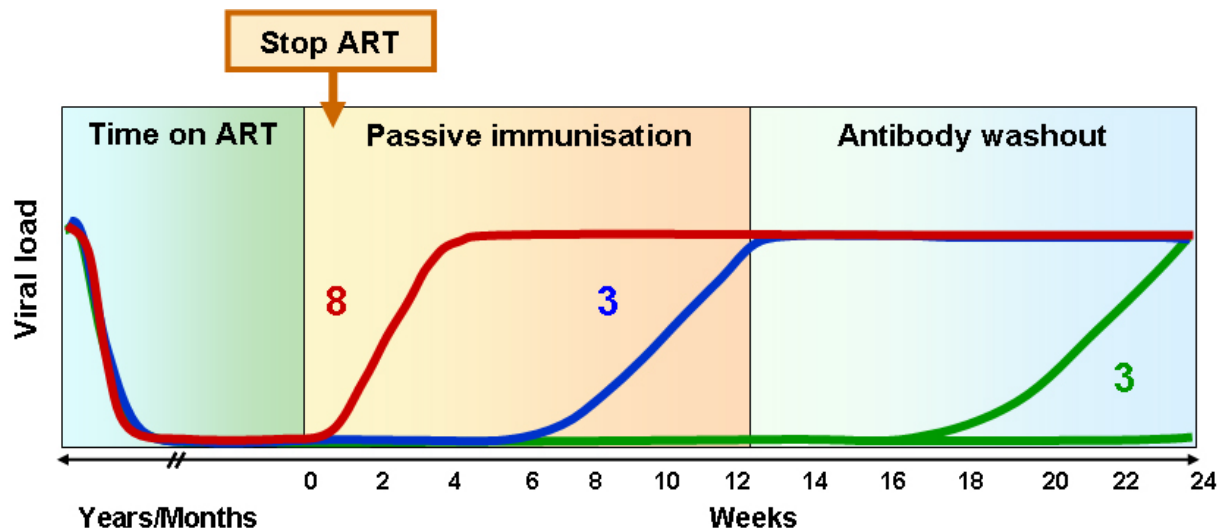


Figure 44. Schematic illustration of the outcome of the passive immunisation study. No delay in viral rebound of 8 patients (red), delay of viral rebound in 3 patients (blue), and viral suppression in 3 patients (green). Changes in viral load before, during and after immunisation are schematically depicted.

Surprisingly, most of the effect of antibody treatment could be attributed to a single monoclonal antibody, 2G12. Patients who experienced a delay in viral rebound had higher effective plasma concentrations of 2G12 (based on *in vitro* potency against pre-ART virus) compared to those who did not control their virus. In addition during passive immunisation all patients that harboured 2G12 sensitive virus strains developed escape mutants to this antibody, while no relevant changes in the sensitivity to 2F5 and 4E10 occurred. Emergence of 2G12 resistant variants was strongly associated with failure to respond to antibody treatment. 2G12 showed also longer plasma half-life than the MPER antibodies, which permitted much higher concentrations of this monoclonal antibody to be achieved *in vivo* compared to 2F5 and 4E10. This raises the possibility that only 2G12 reached plasma concentrations that are effective *in vivo* and that the concentrations achieved for 2F5 and 4E10 were below a crucial threshold needed to control the virus or to exert a selective pressure.

To investigate if this disparity in escape mutant generation reflects that 2F5 and 4E10 antibodies were ineffective *in vivo* or if alternatively, patient virus strains were coherently refractory to changes in the relevant regions of the MPER, we performed *in vitro* escape mutant studies. To this end primary viruses were derived from 3 patients before the passive immunisation trial and subjected to immune selection with

the same three antibodies *in vitro*. Culture conditions were used to mimic *in vivo* conditions in as such that cell-cell transmission of virus is favoured and antibody levels were kept at high doses while still allowing residual viral replication. In addition sequence analysis was done of the carbohydrate-dependent core epitope of 2G12 and the epitopes for 2F5 and 4E10 of all patients that followed passive immunisation.

12. *In vivo* and *in vitro* escape to neutralizing antibodies 2G12, 2F5 and 4E10

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12.1. Abstract

Recently, passive immunization of HIV infected individuals with MAbs 2G12, 2F5 and 4E10 provided evidence of the *in vivo* activity of 2G12 but raised concerns on the function of the two membrane proximal external region (MPER) specific MAbs (51). In the light of MPER targeting vaccines under development we performed an in-depth analysis of the emergence of resistance mutations to these three MAbs to further elucidate their activity. Clonal analysis of the MPER region of plasma virus samples derived during antibody treatment confirmed that *in vivo* no changes in this region had occurred. Sequence analysis of the 2G12 epitope relevant N-glycosylation sites of viruses derived from 13 patients during the trial supported the phenotypic evaluation, demonstrating that mutations in these sites are associated with resistance. *In vitro* selection experiments with isolates of four of these individuals corroborated the *in vivo* finding that virus strains rapidly escape 2G12 pressure. Notably, *in vitro* resistance mutations differed in most cases from those found *in vivo*. Importantly, *in vitro* selection with 2F5 and 4E10 demonstrated that resistance to these MAbs can be difficult to achieve and can lead to selection of variants with impaired infectivity. This remarkable vulnerability of the virus to interference within

MPER calls for a further evaluation of the safety and the efficacy of MPER targeting therapeutic and vaccination strategies.

12.2. Introduction

Neutralizing antibodies are considered a key component of protective vaccines against HIV-1 but despite tremendous efforts vaccination approaches tested so far have failed to induce broad neutralization activity (4, 5, 14, 18, 20, 34). Challenges for vaccine design have proven manifold. Most notably, the high genetic variability of the HIV envelope proteins (27), their high degree of glycosylation (58) and their complex conformation as membrane embedded trimer of two subunits (15, 59, 60, 62) represent considerable obstacles in vaccine development. The most promising leads in vaccine design remain the few potent and broadly neutralizing antibodies known to date, foremost the four intensively characterized antibodies IgG1b12, 2G12, 2F5 and 4E10 (2, 5-7, 38, 45, 51, 54, 65). Epitope characteristics, modes of action and potency as well as biochemical properties and structures of these antibodies have been unraveled and provide the basis for the design of vaccines aiming to elicit like responses. Both antibodies recognizing gp120, IgG1b12, which binds to a distinct epitope overlapping the CD4 binding site (CD4BS) (6, 45) and 2G12, which recognizes an unique mannose-dependent epitope within gp120 (49, 54), target fairly complex, non-linear epitopes and are in general more potent within subtype B than against other subtypes of HIV (1, 54). Binding sites of 2F5 and 4E10, however, lie within a well defined region of the membrane proximal external region (MPER) of gp41 (2, 38, 51, 65). MPER based vaccine approaches have gained particular interest due to the high conservation of this region and its role in the fusion process (36, 48, 63). In a comprehensive screening against 90 HIV isolates of diverse subtypes, 4E10 was found to be active against 100% of the viruses, 2F5 still neutralized 67% whereas IgG1b12 and 2G12 neutralized only 50% and 41% of the isolates in this survey, respectively (1). Nevertheless, thus far attempts to elicit 2F5- and 4E10-like responses have failed to yield potent neutralization activity (10, 11, 16, 22, 26, 28, 29, 32, 37, 61, 63). Recent biophysical analysis of the MPER revealed that this region adopts a helical conformation, which may be required for proper recognition by neutralizing antibodies and likely needs to be considered in vaccine

development (8, 9, 41, 50). Based on these findings efforts to redefine immunogen design are underway and raise hopes that eliciting neutralizing MPER reactive antibodies may be possible (63). However, whether or not MPER specific antibodies will be able to effectively control infection *in vivo* will not be known for years. To investigate the *in vivo* activity of these antibodies to pre-assess their potential in vaccine mediated protection, we performed a clinical trial of passive immunization study with a cocktail of the MAbs 2G12, 2F5 and 4E10, which was designed to mimic conditions of therapeutic vaccination (52). The study provided formal proof that neutralizing antibodies are active *in vivo* as a delay of viral rebound occurred in several trial participants. Strikingly, in all patients who initially harbored 2G12 sensitive virus strains resistance against 2G12 but not to the MPER antibodies developed and loss of sensitivity to 2G12 coincided with viral rebound in plasma (52). In sum, from all 14 patients enrolled in this passive immunization trial, 14 isolates before immunization and 40 isolates during the trial were assessed for the sensitivity to the three MAbs. None of the strains revealed phenotypic changes in sensitivity to the MPER MAbs upon *in vitro* exposure to the antibodies (52). The fact that viral rebound was associated with resistance to 2G12 but neither of the MPER antibodies, raised questions whether the latter antibodies had been active *in vivo*. In view of the MPER and carbohydrate targeting vaccines under development these findings necessitated an in-depth analysis of the underlying events.

12.3. Materials and methods

Clinical specimen

Patient plasma and isolates utilized in this study were derived during a recently conducted passive immunization trial as described (52). Written informed consent was obtained from all individuals according to the guidelines of the ethics committee of the University Hospital Zurich.

Stimulated primary CD8-depleted PBMC

Buffy coats obtained from three healthy blood donors were depleted of CD8⁺ T cells using Rosette Sep cocktail (StemCell Technologies Inc.) and PBMC isolated by Ficoll-Hypaque centrifugation. Cells were adjusted to 4x10⁶ per ml in culture medium

(RPMI 1640, 10% FCS, 10 U/ml IL-2, glutamine and antibiotics), divided into three parts and stimulated with either 5 ug/ml phytohemagglutinin (PHA), 0.5 ug/ml PHA or anti-CD3 MAb OKT3 as described (46). After 72 h, cells from all three stimulations were combined (referred to as 3-way stimulated PBMC) and used as source of stimulated CD4⁺ T cells for infection and virus isolation experiments.

Virus stock preparation

Autologous virus was isolated from patient PBMC by co-culturing patient CD4⁺ T cells with stimulated CD8-depleted PBMC as described (52). Virus was isolated in absence of the neutralizing antibodies to ensure that escape from antibody treatment occurred *in vivo* and not *in vitro*. Only early passage virus was used for further studies (passage 1-3). The 50% tissue culture infectious dose (TCID₅₀) and coreceptor usage of the obtained virus stocks were determined as described (46).

Generation of *in vitro* escape mutants

Three-way stimulated primary CD8-depleted PBMC (1x10⁶/ml) were infected with 1000 TCID₅₀ of the respective HIV-1 isolates in a total volume of 6 ml. Cultures were maintained either in medium alone or in presence of either 2G12, 2F5 or 4E10 or a cocktail of all three antibodies. Initial MAb concentrations for the selection experiments were adjusted to the 70% inhibitory dose of the MAbs against the respective virus. Viral growth was monitored weekly by measuring p24 antigen levels. Continuous viral replication was maintained by passaging weekly one tenth of the infected culture (supernatant and cells) to freshly stimulated PBMC. During this passage also the according adjustments in antibody concentration were made. Testing for sensitivity changes to determine required antibody concentration changes is in this regime not possible as the readout of the inhibition assay is only available with a 7 day delay. Growth kinetics were thus compared based on p24 antigen determination. By comparing viral antigen production of a specific Ab selection culture to the growth in the same culture in preceding weeks and the untreated control culture of the respective isolate, it was then decided whether or not increases or if necessary decreases Ab concentration were necessary in order to keep a notable selection pressure while still allowing sufficient viral production. The readout based on p24 antigen production provides only rough estimates on the effect of the antibodies as viral replication in these long term cultures can vary substantially from

week to week due to differences in PBMC donor infectability. The selected viral cultures were therefore continuously assessed in parallel for the sensitivity to the MAbs. Virus supernatant and cells were collected weekly and stored at -80°C . Virus supernatants were then used to characterize escape variants in neutralization assays, for sequencing and as source for virus stock generation.

Neutralization assay

Neutralization activity of MAbs against sequential virus isolates was evaluated on CD8-depleted PBMC as described (53). Briefly, virus inoculum (100 TCID_{50}) was incubated with serial dilutions of antibodies for 1h at 37°C . Then stimulated PBMC were infected with aliquots of this preincubation mixture. The total infection volume was 200 μl . Cultures were incubated in 96 well culture plates and assayed for p24 antigen at multiple time points between days 4 and 14, depending on the viral growth kinetic. The antibody concentrations ($\mu\text{g/ml}$) causing 50%, 70% and 90% reduction in p24 antigen production were determined by linear regression analysis. If the appropriate degree of inhibition was not achieved at the highest or lowest drug concentration, a value of $>$ or $<$ was recorded.

Viral cDNA synthesis and amplification

Viral RNA was extracted using an automated extraction robot (MagNA Pure instrument, Roche) according to the manufacturer's instructions. cDNA synthesis of the full length envelope was done using SuperScriptTM III Reverse Transcriptase (Invitrogen) with primer envM (17), envN (17), PR5 (5'-AGCTGGATCCGCTCTCGAGATACTGCTCCCACCC-3') or PR10 (5'-CGAGCTGGATCCTTTTGACCACTTGCCACCCATCTTATAGC-3') depending on the virus isolate. Subsequent amplification was performed in 50 μl reactions [94°C 2 min, $5\times$ (94°C 10 sec, 58°C 30 sec, 68°C 180 sec), $45\times$ (94°C 10 sec, 60°C 20 sec, 68°C 150 sec), 68°C 10 min] with Platinum[®] Taq DNA Polymerase High Fidelity (Invitrogen). The primer pair env1A (31) and PR5 was used for the majority of isolates. In those cases where PR5 did not match the isolates' sequence and failed to amplify *env*, the alternative primer sets env1A/envM, env1A/envN or env1A/PR10 were used. PCR amplicons were loaded on a 1% agarose gel and purified using the QIAquick Gel extraction Kit (Qiagen, Basel, Switzerland) before sequencing and

cloning was performed. Clones were sequenced after high purity plasmid miniprep (QIAprep 8 Turbo Miniprep Kit, Qiagen, Basel, Switzerland).

Sequence analysis

Bi-directional sequencing was performed by dye terminator cycle sequencing (ABI Prism BigDye Version 2.0, Applied Biosystems, Rotkreuz, CH) using an automated capillary sequencer (ABI 3100). Full length sequencing of 10 overlapping regions spanning from *rev* through *nef* genes was performed using specific primers including 5allspl (5'-AAGAAGCGGAGACAGCGACGAAGA-3'), MF178 (5'-ATGGTAGAACAGATGCATGAGGATATAAT-3'), V3Fin2 (24), MF181 (5'-TGCAGAATAAAACAATTTATAAACATGTGGC-3'), MF159 (47), PR10, MF 182 (5'-TGTATTAAGCTTGTGTAATTGTTAATTTCTCT-3') or MF155 (52), MF169 (5'-TGATGGGAGGGGCATACATTG-3'), MF180 (5'-TGAGTTGATACTACTGGCCTAATTCCATGTG-3'), and MF179 (5'-CACATGGCTTTAGGCTTTGATCCCAT-3').

Precipitation with ethanol/sodium acetate was used for purification of the extension products. Where specified, sequencing was confined to the C2-V4 region of gp120 or the MPER spanning region in gp41, respectively. The primers V3Fin2 (24) and MF169 were used to sequence the C2-V4 region. MPER sequencing gp41 was done using primers MF158 (52) /MF155 (52).

Sequence heterogeneity was detected in some instances as a consequence of direct sequencing of bulk PCR products derived from samples containing mixed virus populations. Only the major variants represented in bulk sequences, i.e. those exhibiting the strongest signal in the chromatograms, are depicted except where mixed populations are indicated. Mixed populations in bulk sequences were identified by visual examination of chromatograms for data sets depicted in Figures 1, 4 and Supplement Figure 1. Where specified, clonal analysis was performed.

Clonal analysis of the MPER region in gp41

Extraction of viral RNA from patient plasma from week 12 samples of patients NAB01, NAB02 and NAB08, cDNA synthesis and hot start PCR was performed in duplicates as previously described (47, 52). Alternatively, MPER cDNA synthesis was performed using primers MF155 and MF158 and amplification for one cycle (50°C 30

min, 95°C 15 min) and 50 cycles (95°C 10sec, 55°C 15 sec and 72°C 120 sec) followed by 72°C for 7 min. Cloning (pCR 4 TOPO® TA or pcDNA™3.1/V5-His TOPO® TA, Invitrogen, Groningen, Netherlands), amplification (HotStarTaq Master Mix, Qiagen, Basel, Switzerland) and sequencing (BigDye, Applied Biosystems, Rotkreuz, Switzerland) of individual clones was performed as described (24) using the primer pair MF155/MF158 and otherwise identical conditions. This procedure has previously been demonstrated to result in low error rates (24).

Nucleotide sequence accession numbers

The reported clonal envelope sequences will be deposited in the GenBank database (<http://www.ncbi.nlm.nih.gov/Genbank/index.html>). The assigned accession codes will be added here.

Full length-envelope cloning and generation of envelope pseudotyped HIV particles

Full length envelope genes amplified for sequencing were also used for cloning of envelope genes into expression vectors. To this end the PCR amplicons were introduced in the Expression Kit (Invitrogen, Groningen, Netherlands) followed by transformation into One Shot TOP10 or STBL3 competent bacteria (Invitrogen, Groningen, Netherlands). Since ligation of inserts using AT-cloning can occur bi-directionally, the orientation of the envelope inserts in derived clones was verified by PCR using two different primer pairs PR12 (5'-GGCTAACTAGAGAACCCACTGCTTA -3') and PR13 (5'-ACTACTTACTGCTTTGATAGAGAACTTGA -3') and primer pair PR14 (5'-GCCCCGAAGGAATCGAAGAAGAAGGTGG -3') and PR15 (5'-AGGCTTACCTTCGAACCGCGGGCCCTCTA -3') spanning small regions at both ends of the cloning site resulting in amplification of different sized products depending on the orientation of the insert. PCR was performed using the HotStarTaq Master Mix (Qiagen, Basel, Switzerland) in 22 ul reactions [94°C 15 min, 33× (94°C 10 sec, 55°C 15 sec, 72°C 60 sec), 72°C 7 min]. Subsequent gel electrophoresis of PCR amplicons allowed to select clones which carry correctly oriented envelope inserts. Pseudotyped HIV reporter viruses were generated as described (31, 56). Briefly, 293T cells were transfected in 24 well plates with 6 ul of the Effectene Transfection Reagent (Qiagen, Basel, Switzerland), 3 ul of the plasmid carrying the

reporter gene expressing virus backbone, pNLluc-AM ((43) ; kind gift by A. Marozsan and J.P. Moore) and 2 ul of the purified respective *env* carrying plasmid (QIAprep 8 Turbo Miniprep Kit, Qiagen, Basel, Switzerland). Viral supernatants were harvested 2 days post transfection and screened for infectivity. To this end TZM-bl cells (National Institutes of Health AIDS Research and Reference Reagent Program; (13, 42, 55)) were infected with viral supernatants in Dulbecco's Modified Eagle Medium (DMEM), 10% heat inactivated FCS and 1% Penicillin-Streptomycin (BioWhittaker) containing 10 ug/ml DEAE Dextran (Amersham Biosciences). Luciferase activity was measured three days after infection as described (23, 31). Functional envelope clones were used to produce large scale virus stocks for further analysis in infection assays and for sequence verification.

Neutralization assays with Env-pseudotyped reporter gene viruses

Large scale virus stocks of Env-pseudotyped virus were prepared by transfecting 10 cm dishes seeded with 293-T cells with 15 ug of the backbone plasmid (pNLluc-AM) and 5 ug of the functional envelope clone and 40 ug of PEI (linear, 25 kDa, Polysciences, Inc.). Virus stocks were titrated as described (23). Neutralization activity of MAbs against pseudotyped virus carrying the patient derived and *in vitro* selected envelope genes was evaluated on TZM-bl cells essentially as described (35). Briefly 200 TCID₅₀ of the virus were preincubated with serial dilutions of the antibody. The antibody concentration causing 50% (IC₅₀), 70% (IC₇₀) and 90% (IC₉₀) reduction in luciferase reporter gene production was determined by regression analysis.

Infection experiments using replication competent TN6 NL constructs

The TN6 NL vector, a replication competent viral backbone engineered from strain NL4-3 (40) was kindly provided by M. Dittmar. The NL4-3 envelope was deleted from the TN6 NL vector by digestion with the restriction enzyme BstEII and NcoI (New England Biolabs). *Env* depleted vectors were purified through separation on agarose gel. The NAB01 envelope genes encoded in pcDNA3.1 vectors were amplified by a primer pair introducing a BstE II-restriction site (PR20: 5'-AATTGTGGGTCACCGTCTATTATGGGGTACCTGT-3') at the 5' end, and a NcoI-restriction site (PR21: 5'-GACTGCGTGCCATGGCTTATAGCAAAGCTC-3') at the 3' end of the *env* using identical PCR conditions as described for *env* amplification

above. *Env* PCR product were digested with BstEII and NcoI, purified, and ligated into the linearized TN6 NL vectors using DNA T4 ligase (New England Biolabs,) according to the manufacturers instructions. Virus stocks were generated by transfecting 293-T cells with the various TN6 constructs. P24 antigen content of stocks and 50% tissue culture infectious doses of the stocks on 3-way stimulated PBMC were determined to evaluate the infectivity of the various virus mutants.

12.4. Results

Characteristics of *in vivo* escape from 2G12

Resistance to 2G12 has been commonly described to result from mutations at one or several of the N-linked glycosylation sites composing the antibody's epitope. Five N-linked glycosylation sites have been defined to form the canonical sites of the 2G12 epitope and are located at aa positions 295, 332, 339, 386 and 392 within gp120 of the reference strain HXB2 (49). Of note, a recent study suggested that escape from 2G12 may also occur without involvement of these five sites (39). Since 2G12 is considered as a prototypic antibody to model vaccines that elicit anti-carbohydrate responses blocking HIV, we performed an in-depth analysis of the 2G12 escape mutants evolved in our *in vivo* study to derive further insight into the composition of the antibody's epitope and *in vivo* escape pathways. To this end we sequenced for each patient envelope (*env*) genes spanning C2-V4 regions from isolates derived before passive immunization (pre-isolates) and directly from plasma at relevant time points during the trial when viral rebound and phenotypic resistance had been observed (Figure 1). By comparing 2G12 sensitive pre-treatment sequences with those of the matching escape variants we analyzed whether resistance conferring mutations mapped to those glycosylation sites previously defined as the 2G12 epitope, or whether alternate glycosylation sites or amino acids may also be involved in forming the epitope and were preferentially changed during *in vivo* escape. Pre-treatment sequences were derived from the cultured virus isolates used as patient relevant reference strains throughout the study (52). In this analysis 13 of the 14 patients enrolled in the clinical trial were included. Samples from patient NAB14 were not available for this analysis as this patient suppressed viremia throughout the entire observation period (24 months). Sequences of the *in vivo* evolved escape variants of

the remaining 13 patients were derived directly from bulk viral RNA isolated from plasma. This strategy allowed us to determine the dominant mutations in the viral quasispecies which evolved during the *in vivo* treatment with the MAbs excluding potential influences of *in vitro* culturing (Figure 1). In the majority of patients escape mutants emerged that had sequence changes in at least one of the five previously defined glycosylation sites composing the 2G12 epitope. Notably different were the results obtained initially for bulk sequences from patient NAB05, which despite phenotypic resistance to 2G12 portrayed no apparent change in the canonical sites of the epitope in this analysis (data not shown). To verify whether indeed resistance in this case was conferred by mutations in other regions as previously suggested (39), we performed a clonal analysis of the envelope genes derived from viral RNA in the plasma of this patient (Figure 1). Data obtained in this survey revealed that multiple different mutations within the 2G12 epitope had occurred. The latter could not be detected in a bulk analysis as none of them by itself was forming a majority amongst viral quasispecies. However, in sum these mutant sequences formed the majority, clearly outnumbering wild type sequences and thus were in concordance with the observed phenotypic resistance profile (Figure 1 and data not shown). Of note, virus from patient NAB09 was already at the pretreatment time point relatively resistant to 2G12 (IC₉₀ > 25 ug/ml; IC₇₀ = 8.71 ug/ml). Although sensitivity to 2G12 did not further decrease in the phenotypic assay we nevertheless observed new mutations in the 2G12 epitope suggesting that the antibody even in this case exerted some selection pressure. Several patients lacked one of the 2G12 canonical sites already before treatment: Two patients (NAB02, NAB10) lacked the glycosylation site at position 332, three individuals (NAB01, NAB05, NAB13) the site at position 339 and patient NAB07 the glycan at site 386. Interestingly, isolates of these patients were phenotypically sensitive against 2G12 in the PBMC-based neutralization assay. Thus, depending on the viral strain 2G12 reactivity appears not to require always all five glycosylation sites (Figure 1). Nevertheless, higher antibody concentrations were needed to achieve inhibition of several of these isolates supporting the notion that these sites are relevant for 2G12 binding and activity (Figure 1 and (52)). Reactivity in at least some of these cases may have been preserved through introduction of adjacent glycosylation sites at positions 330 and 334 (NAB02), 334 (NAB10) and 344 (NAB13). Upon *in vivo* exposure to 2G12 we observed different patterns of escape

mutations amongst individuals: Patients NAB01, NAB03, NAB10 and NAB13 lost only one glycosylation site, patients NAB04, NAB05, NAB08, NAB11 and NAB12 two sites, patients NAB06, NAB07 and NAB09 three and patient NAB02 four sites at different time points. Mutation at sites 295, 332 and 339 occurred each in four individuals, whereas mutations at sites 386 and 392 were detected in 7 and 8 individuals, respectively (Figures 1 and Supplement Figure 1). As expected the number of 2G12 core epitope glycosylation sites were significantly decreased after passive immunization ($P=0.0002$, Wilcoxon, signed rank test two-tailed) whereas no influence on other N-linked glycosylation sites within aa 264 and 415 was apparent ($P=1.00$ Wilcoxon, signed rank test two-tailed).

Characteristics of viral evolution during *in vivo* treatment with 2F5 and 4E10

We previously reported that in contrast to the effects seen for 2G12 upon prolonged *in vivo* exposure, we failed to detect escape from the MPER reactive antibodies 2F5 and 4E10 (52). Neither changes in phenotypic sensitivity to the MAbs nor mutations in the core epitopes of 2F5 and 4E10 were detected. To exclude that mutations had been lost during culturing of virus isolates, we had previously sequenced both the derived cultured isolates which emerged during the trial and viral RNA derived directly from plasma (52). Both analyses provided identical results and gave no evidence for mutational changes (52). The epitopes of 2F5 and 4E10 are prime candidates for the development of vaccines due to the exceptional broad reactivity of these MAbs against divergent viral strains. It was thus of particular interest to define why escape from these antibodies had not occurred *in vivo* and whether this reflects an overall incapacity of the antibodies to function under *in vivo* conditions. In a first step to address this, we performed an in-depth clonal analysis of the MPER region of three representative patient isolates to define whether escape mutants had developed at low frequency. In total we analyzed 150 clonal sequences of virus from patients NAB01 (51 clones), NAB02 (37 clones) and NAB08 (62 clones). Sequences were derived directly from viral RNA isolated from patient plasma at the peak of antibody exposure (week 12) when assumingly the strongest selection pressure was induced. Within the core epitope of 2F5 no amino acid mutation was detected amongst clones analyzed for patients NAB01 and NAB08. One clone of patient NAB02 had a synonymous substitution in the epitope at position 664D. Within the 4E10 epitope patients NAB01 and NAB02 had non synonymous substitutions in three

and one clone respectively (Table 1). Substitution of S to N at position 671 and of D to G at position 674 that occurred in clones of NAB01 are known not to interfere with 4E10 activity (1, 47, 64). The substitution I675M detected in two clones is highly uncommon. Only 8 of currently 187'347 entries listed in the Los Alamos HIV sequence database (<http://www.hiv.lanl.gov/content/hiv-db/mainpage.html>) contain the respective residue. Importantly, we found that introduction of this mutation into the wild type *env* sequence of virus NAB01 does not affect infectivity nor sensitivity to 4E10 (P. Rusert and A. Trkola data not shown). The deletion spanning the 4E10 epitope observed in one clone of patient NAB02 has not been described and likely represents a defective viral sequence. Taken together, this clonal analysis confirmed our previous observations that no escape mutations in the epitopes of 2F5 and 4E10 occurred *in vivo* despite prolonged exposure to high concentrations of the MAbs.

***In vitro* escape from 2G12, 2F5 and 4E10**

The central question thus remained to define why escape solely to 2G12 and not to the MPER MAbs had occurred *in vivo*. Our previous studies suggested that effective doses of MPER MAbs reached by passive immunization may have been too low to reach *in vivo* efficacy (52). While emergence of escape mutations is evidence that a MAb induced selection pressure, we can nevertheless not defer with certainty that absence of escape proves the MAbs' inactivity. Clearly, the MPER Abs by themselves had had no effect in our *in vivo* study as otherwise lower viremia should have been observed also after escape from 2G12. However, it has to be considered that the three MAbs can act in synergy (30, 33, 66). While the impact of the MPER MAbs by themselves must have been too low for *in vivo* efficacy, it cannot be ruled out, that as long as all three antibodies were present and active, 2F5 and 4E10 might in combination increased the 2G12 selection pressure. Once resistance to 2G12 was established, the pressure of the MPER MAbs alone may have been too low to affect selection. If this scenario is correct, it would imply that 2G12 resistance can be obtained more easily without loss in fitness, whereas MPER mutations are selected against. Nevertheless, naturally occurring and *in vitro* or *in vivo* emerged phenotypically resistant virus isolates to both MPER MAbs have been described and appear at least in the case of 2F5 not to be uncommon (3, 33, 44, 51, 57, 65).

To determine if restrictions for HIV in generating escapes to MPER antibodies exist, we performed *in vitro* escape selection experiments using viral isolates from four patients, NAB01, NAB02, NAB03 and NAB08, derived before the passive immunization study. These four isolates were chosen amongst the 13 patient isolates due to their high sensitivity to all three MAbs at pre-screening which allowed escape selections at comparable antibody concentrations. By exposing these virus isolates *in vitro* to the three MAbs separately, we investigated if isolates from our patient cohort were particularly refractory to changes within MPER.

All four selected patients had experienced a rebound of viremia during the trial that coincided with emergence of 2G12 resistant viral strains (52). Virus resistant against 2G12 evolved rapidly in patients NAB01 and NAB02 (after eight and four weeks, respectively). NAB03 rebounded only when passive immunization had stopped and antibody levels had declined. NAB08, a patient who initiated ART treatment during acute infection, showed delayed viral rebound compared to a control group of untreated acutely HIV-1 infected patients. Importantly, sensitivity loss to 2G12 was associated with loss of viremia control in all these patients.

To explore the escape patterns to the three antibodies *in vitro*, we cultivated pre-immunization virus isolates on stimulated PBMC in presence of the respective antibody. Antibody concentrations were adjusted in order to attain high selection pressure while still allowing residual viral replication (for a detailed description see materials and methods). To closely mimic *in vivo* conditions both supernatant and infected cells were passaged weekly onto fresh stimulated PBMC allowing free virus infection as well as cell-cell transmission to occur. Viral replication was monitored weekly by assessing p24 content in cultures and antibody concentrations were increased according to the extent of viral replication to guarantee a sufficient selection pressure. Figure 2 depicts a representative profile of the selection experiments performed with each individual antibody against isolate NAB01. After a series of long term selection experiments with all four isolates, we performed additional experiments for NAB01 and NAB03 to obtain information on the reproducibility of our observations (Figure 3). Viral supernatant was collected weekly and assayed periodically for sensitivity against all three MAbs. Resistance increased gradually under elevated antibody pressure in those cases where successful selection of escape variants occurred (Figures 2 and 3). Irrespective of the isolate

used, we observed a relative rapid escape from the 2G12 antibody in all 9 selection experiments. Timing of the phenotypic detection of escape varied to some extent between patient isolates but also in repeat experiments with the same isolates and ranged from 3 weeks to 11 weeks. This is not unexpected, as viral replication in these experiments depends on PBMC infectivity which fluctuates from donor to donor.

In respect to 2G12, the *in vitro* experiments confirmed our *in vivo* observation: While the MAb is capable of exerting pressure on viral replication, the virus nevertheless fairly easily evades 2G12. However, contrasting the *in vivo* situation, escape virus against both MPER antibodies could be generated *in vitro*. Viruses from all four patients developed resistance against 2F5 during immune selection albeit escape selection was not successful in all attempts (Figure 3). The fastest resistance evolved after 4 weeks of culture (NAB02) and the slowest after 8 weeks (NAB01). In comparison, 4E10 escapes were even more difficult to induce. No 4E10 escapes could be generated in virus cultures derived from patients NAB03 and NAB08. Virus of patient NAB01 developed 4E10 resistance in all three selection experiments after 9 to 16 weeks of culture and 4E19 resistance of virus NAB02 developed only after 17 weeks. We noted that, compared to 2G12, infected cultures were highly sensitive to increases in 4E10 concentration and to a lesser extent also to 2F5. Elevation of MPER antibody levels repeatedly resulted in abortive infection (Figure 3). The latter confirmed that at least *in vitro* the antibodies are highly effective and that at the same time resistance is not easily achievable for the virus. Notably, we did not succeed in generating stable fully resistant virus populations to 4E10 in these experiments. While the *in vitro* emerged virus strains clearly had lost substantial sensitivity to 4E10, they remained partially sensitive to the MAb (IC₉₀ < 25 ug/ml) (Figure 3).

Genotypic analysis of *in vitro* escape variants

We next sought to confirm the introduction of resistance conferring mutations in the generated viral variants by genotypic analysis. The *in vitro* selected virus cultures are expected to contain a mixture of wild type subspecies that will progress to a population with increasing content of escape variants as antibody pressure is elevated. Sequencing of bulk virus cultures has therefore limitations in defining escape mutants at early stages of the mutant selection as only the most frequent

population is represented in this analysis. Since clonal analysis at all time points in our extensive mutant selection would not have been feasible we chose a two geared approach: We initially performed bulk sequencing of virus cultures at all time points where phenotypic resistance was detected, by analyzing the respective epitope regions (C2-V4 for 2G12; aa 620-720 spanning MPER for 2F5 and 4E10). Selected time points of interest were analyzed more thoroughly by single clone sequence analysis of the full length gp160 gene. In all selection experiments untreated controls were cultured in parallel to provide reference viral strains that were subjected to identical culturing conditions. This allowed us to specifically discriminate between changes introduced upon long term *in vitro* culturing and those induced directly by MAb pressure.

Genotypic analysis of *in vitro* selected 2G12 escape variants

In total we analyzed the 2G12 epitope of nine viral cultures with phenotypic resistance to the MAb and the matching control cultures. While *in vivo* selected 2G12 resistant strains harbored multiple amino acid substitutions that were not associated with potential N-glycosylation sites, the pattern observed *in vitro* was markedly different (Figures 1 and 4 and data not shown). Mutations that did not affect glycosylation sites were only found occasionally. The majority of substitutions occurred in N-glycosylation relevant sites. As expected mock controls displayed no sequence changes within the 2G12 relevant N-glycosylation sites with the exception of the NAB02 mock culture strain where a glycosylation site at position 332 was reintroduced. Accordingly, all control culture isolates remained fully sensitive to 2G12 (Figure 4).

Interestingly, 2G12 selection of virus NAB01 gave rise to the same single mutation (T394I) which resulted in loss of the glycosylation site at position 392 in all three long term selection experiments performed with this virus. These escape mutants thus differed from those *in vivo* where solely mutants who lost the N-glycan at position 386 had evolved (Figure 1). Phenotypic resistance against 2G12 was detected for the NAB02 derived virus isolate already at week 6. However, at this early time point of the selection wild type virus was apparently still present as detected by bulk sequencing (Figure 4). Further clonal analysis revealed that resistant species with a mutation in T341I (loss of N-glycosylation site at position 339) had emerged. The

same mutation also occurred *in vivo* although there it was not the dominant variant as this patient developed multiple different escape mutants over time (Figure 1). *In vitro* cultivation of all four selection experiments performed with NAB03 yielded virus with phenotypic resistance to 2G12. Three escapes had lost the glycosylation site at position 339 (N339K), whereas *in vivo* again a different mutation had evolved (N386D). Clonal analysis of NAB03 *in vitro* selection experiments revealed that while loss of site 339 was predominant, mutations of N-glycosylation sites at position 295 and 332 also occurred. Clones of the escape virus derived of NAB08 after 5 weeks of selection harbored a mutation at aa 339 which affects the N-glycosylation. Interestingly, sequence analysis of the same long term culture at week 11 revealed that over time a different mutant had become dominant which lacked the glycosylation site in position 392 (T394I). Of note, both sites had been lost during *in vivo* escape.

Genotypic analysis of the MPER upon *in vitro* exposure to 2F5 and 4E10

In view of the fact that during *in vivo* treatment with the MPER antibodies no loss in neutralization sensitivity nor mutations in the antibody epitopes were detected, it was of particular interest to analyze the *in vitro* generated escape mutants to 2F5 and 4E10 phenotypically and genotypically (Figures 2, 3 and 5).

In one of three experiments with virus derived from patient NAB01 we succeeded in generating a 2F5 escape variant at week 8 of culture which harbored the mutation D664N within the core epitope of 2F5. All three attempts to generate 4E10 escapes of NAB01 resulted in evolution of viral variants with mutations within the 4E10 epitope. Two selection series gave rise to mutation F673L whereas the third selected strain had introduced a change to W672G (Figure 5). However, phenotypically these virus isolates had gained only a relative modest decrease in sensitivity to 4E10 (Fig. 3), which could be caused by a mixed population of wild type and escape mutants in these viral strains. We thus cloned full length viral envelopes from these cultures and performed inhibition experiments using Env-pseudotyped virions and TZM-bl cells (Figure 6). Both, *env* mutants carrying W672G and F673L were fully resistant to 4E10 in this analysis.

Virus from patient NAB02 developed a 2F5 (K665T) and a 4E10 (F673V) escape mutation after culturing for 4 and 17 weeks with the respective antibody (Figures 3

and 5). Only one of the 4 attempts to generate a 2F5 escape of virus from patient NAB03 was successful. Bulk sequencing at week 7 revealed that the majority of quasispecies carried mutation K665E. However, clonal analysis detected quasispecies carrying a K665N mutation which became prevalent by week 12 of culture. All four attempts to generate 4E10 escapes of NAB03 virus failed and resulted in abortive infection at relatively early time points (Figure 3). Virus of NAB08 also introduced a mutation at position D664N which appeared after 5 weeks of culture with 2F5. However in the same series 4E10 escape selection of NAB08 was unsuccessful. To more closely mimic the *in vivo* experiment we also performed selection experiments using the three MAbs in combination (Fig. 2). Briefly, we found that triple selection experiments followed very much the profile seen for the MPER MAb selection, in as much that frequent abortive infection was observed (data not shown). By applying relatively low antibody pressure, we nevertheless succeeded in maintaining three triple selection cultures derived from isolate NAB01 (Figure 2 and data not shown). Resistance was evaluated phenotypically by inhibition assays and resistance conferring mutations located by sequencing of the respective epitopes (data not shown). Two triple selected strains developed resistance to both MAbs 2F5 and 4E10 (mutations D664N and F673L, respectively), while the third isolate only developed resistance to 2F5 (D664N). However, likely as a result of the lower 2G12 concentration used in the combination, no resistance to 2G12 developed (data not shown).

In summary all tested isolates were capable of tolerating mutations in the MPER region. Importantly, the observed resistance conferring mutations were all located at the canonical sites of the respective antibody epitopes defined in previous studies (12, 38, 44, 64, 65).

Infectivity and stability of *in vitro* selected escape mutant viruses

Our observations during the *in vitro* selection of MAb escape mutants, strongly suggested that 2G12 escape mutations are tolerated well while 4E10 and to some extent also 2F5 resistance conferring mutations appear to pose constraints on viral replication. To address this more directly we compared the functionality and infectivity of *env* genes from patient NAB01 derived during selection *in vivo* and *in vitro* (Figure 7). To exclude influences on viral infectivity outside *env* we inserted the respective

envelope genes of the mock control virus and the respective MAb escapes in the TN6 vector, a replication competent viral backbone engineered from strain NL4-3 (40). Infectivity was determined by measuring the 50% tissue culture infectious doses (TCID) of the viruses on stimulated PBMC. Figure 7 depicts TCID₅₀ normalized to p24 content of the respective stock (TCID₅₀/pg p24). Infectivity of the NAB01 mock control at week 11 of culture was highest in this analysis. 2G12 and 2F5 escape variants displayed lower but still relatively high infectivity. In contrast to this, 4E10 resistant virus had a markedly decreased infectivity substantiating the evidence that the resistance conferring mutations may potentially interfere with viral fitness.

To further investigate the phenotypic features of MPER-MAb and 2G12 resistant viruses we probed to what extent these mutations restrict viral replication by analyzing the stability of the mutations in selected clones from patient NAB01 during long term culturing in absence of antibody pressure (Fig. 8). Cultures were propagated in analogy to the escape selection on PBMC with weekly passaging onto fresh donor cells. Viral supernatants were periodically analyzed for their sensitivity to the three MAbs to assess if and how rapidly resistance conferring mutations are reverted to wild type sequences. As anticipated, 2G12 escape virus showed no apparent changes in replication pattern in the long term culture and remained fully resistant to the MAb until the termination of the experiment at week 16. Considering the observed impairment in replication of the MPER resistant clones and the comparative difficulties to select these mutants, we hypothesized that a rapid reversion to wild type sequences upon removal of antibody pressure could occur. Surprisingly, the 2F5 escape mutant replicated for several weeks in absence of the antibody without regaining sensitivity. Only by week 11 sensitivity to 2F5 begun to moderately increase (Figure 8). However, compared to the wild type strain the mutants retained reduced sensitivity to the MAb. These data were supported by clonal analysis of virus collected at the beginning of the long term culturing and at weeks 11, 13 and 16 which revealed that the majority of clones still harbored the resistance conferring mutation D664N (Figure 9 left panel). Notably though, at week 11 one clone carrying the 2F5 sensitive wild type sequence was detected while different minor variants were detected at weeks 13 and 16.

In contrast to 2F5, upon phenotypic evaluation of 4E10 escape isolates during long term culture a gradually increased sensitivity to 4E10 from week 7 onwards was

detected. Surprisingly, clonal analysis revealed that this gain in neutralization sensitivity was not linked to a reappearance of quasispecies carrying the wild type 4E10 epitope (Fig. 9). Solely at one time point (week 13) 2 of the 36 tested clones had mutated to the wild type residue W672. Which changes were introduced that rendered the 4E10 escape viruses more sensitive to neutralization upon long term culture will require further analysis. Similar to the 2F5 escape viruses, other fluctuations in the epitope were apparent but none of these mutants increased in frequency. In sum, these analyses suggest that in absence of competition with fitter viral strains and selection pressure, MPER mutants are relatively stable.

12.5. Discussion

We recently conducted a passive immunization study to evaluate the *in vivo* potency of the MPER MAb 2F5 and 4E10 together with the carbohydrate specific MAb 2G12. The outcome of this trial was remarkable: The cocktail of these three neutralizing antibodies was capable of suppressing or delaying viremia rebound in several individuals. However, activity was lost as soon as 2G12 resistant virus emerged. The fact that no resistance to the MPER MAb occurred raised concerns that these MAb were not active *in vivo*.

To elucidate the mechanisms behind the selection and resistance patterns *in vivo* and to gain insights into potential effects of MPER vaccines, we set out in the current study to investigate the escape pathways of patient isolates to the three antibodies both *in vivo* and *in vitro*.

In sum, our analysis confirmed that 2G12 activity depends on the presence of glycosylation sites at positions 295, 332, 339, 386 and 392 as previously determined (49). However, the relevance of the individual sites appears to vary depending on the viral strain and in the context of some viral *envs*, 2G12 remains active also when one of the sites is lacking. It may be possible that adjacent N-glycans that were introduced in some cases, can compensate for the loss of the 2G12 core epitope sites (Figure 1). Most frequently we observed amongst the *in vivo* emerged escape variants aa changes in positions 386 and 392 (7 of 12 and 8 of 13 patients respectively). *In vivo* emerged 2G12 escape variants, included not only mutations in the epitope core sites but also in other potentially glycosylated and non-glycosylated

amino acids positions. Whether or not these latter changes compensate for 2G12 escape mutations, support resistance to 2G12 or reflect selection processes of the autologous immune responses cannot be dissected with certainty. Our current analysis nevertheless allows to defer that *in vivo* 2G12 escape requires mutations in one or more of the 5 canonical sites. Ensuing introduction of novel glycosylation sites may occur to secure the viral glycan shield. Thus, carbohydrate targeting vaccines could have the potential to be effective if they succeed in recognizing a variety of epitope compositions. When we induced escape from 2G12 *in vitro* with pre-study isolates of four patients that had participated in the passive immunization trial, we found different patterns of escapes: mutations were almost exclusively restricted to the N- glycosylation sites and the mutations within the 2G12 core epitope differed from those found *in vivo* in three of the patients. Escape was in general rapid confirming that the virus quite easily can evade the MAb pressure.

Our detailed analysis of *in vivo* and *in vitro* escape pathways of 2F5 and 4E10 however, gave strikingly different results. To verify our previous observations that no resistance conferring mutations had occurred *in vivo*, we performed a detailed clonal analysis of the MPER region in plasma viral RNA derived from three patients at week 12 of antibody treatment which is the latest time point under high antibody pressure in the study. The results confirmed our previous analyses: amongst 150 tested clones none with a resistance mutation to 2F5 or 4E10 was detected.

We conducted *in vitro* selection experiments with the MPER MAbs to verify whether or not these isolates from study participants can tolerate mutations in MPER needed to gain resistance to the MAbs. The outcome of these *in vitro* selection experiments was notable different from those against 2G12 as frequent abortion of infection occurred when antibody levels were increased. The latter observations confirm that the MPER antibodies are highly active against these isolates and that escape from the MAbs is difficult to achieve. We nevertheless, succeeded in generating 2F5 resistant viruses of all four, and 4E10 resistant strains of two of the four tested isolates. Phenotypic and genotypic analyses demonstrated that resistance was in all cases associated with changes in the known core epitopes of the MAbs.

The often slow evasion process against the MPER MAbs and the observed fragility of the emerging viruses prompted us to investigate whether the MPER mutations

required for resistance to 2F5 and 4E10 may to some extent impair viral replication. Indeed viral infectivity of the tested 4E10 escape was markedly reduced. Surprisingly though, long term culture in absence of antibody pressure did not lead to rapid reversion of the epitope to wild type sequence for any of the three MABs. Nevertheless, variation in both MPER epitope regions occurred suggesting that a certain drift was induced.

Several reasons for a potential inactivity of the MPER antibodies in our previous trial have been considered: As MPER antibody concentrations in plasma were significantly lower than those of 2G12 during passive immunization, the required concentration for *in vivo* efficacy may not have been reached (25, 52). Lower MPER antibody concentrations were the consequence of considerably shorter elimination half lives, the cause of which remains unknown (25). Of note, no endogenous antibody response to either MAb developed (25, 52). Tissue distribution was not measured directly, but pharmacokinetic analyses deferred no differences in distribution half lives, central and steady state distribution volumes suggesting that generally tissue distribution may not be distinctively different. However, whether or not tissue distribution to the relevant sites of infection is comparable remains unknown (25). Concerns that the activity of 4E10 and 2F5 was blocked *in vivo* due to cross reactivity with host antigens such as phospholipids (19), could thus far not be corroborated as in *ex vivo* studies all three passively administered antibodies maintained their neutralization activity in plasma (Trkola et al., Manuscript in preparation). Which, if any of these effects could account for inactivity *in vivo* will require further investigation. Despite the fact that the MPER MABs lacked *in vivo* activity by themselves, as no effect on viral load upon escape to 2G12 nor escape mutant selection to the MPER MABs was seen, it cannot be ruled out with certainty that the MPER antibodies were active *in vivo*. (52). While difficult to assess, it is possible that *in vivo* activity of the antibody cocktail depended on synergistic effect of all three MABs. Synergy, as previously described for these MABs (30, 33, 66) in combination with 2G12 could potentially have led to an augmenting the selection pressure induced by the latter. We have recently investigated these synergy effects in detail and found evidence that the antibodies acted in combination (Trkola et al., Manuscript in preparation).

Here we provide further information on the nature of the escape evolution of the three MAbs. Whereas escape from 2G12 is also rapid *in vitro*, resistance selection to MPER antibodies is notably difficult and can result in abortive infection or - as in the case of the analyzed 4E10 resistant variant – in virus strains with low infectivity. Both, high threshold for resistance evolution and impaired infectivity of emerged escapes would be highly desirable features of vaccine elicited immunity and highlight once more the potential of MPER targeting immunogens or drugs. A further detailed survey of the potential but also the suggested risks (21) of MPER specific strategies for therapy and prevention is therefore pivotal.

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12.7. Figure legends

Figure 1. Changes in potential glycosylation sites within C2-V4 induced during *in vivo* treatment with 2G12. Different time points derived from plasma bulk cultures and single clones from 13 patients (NAB01-NAB13) following passive immunization where sequenced and changes are summarized. The 5 glycosylation sites defining the 2G12 epitope are shaded in dark grey. Non-2G12 related potential glycosylation sites are shaded in light grey. X denotes sequence mutations in the glycosylation site that lead to loss of that specific site. Glycosylation sites created through mutations during passive immunization are indicated by dashed boxes. Presence of mixed populations is indicated by boxes with diagonal divisions.

Sensitivity to 2G12 (IC90) of available replication competent viruses derived during passive immunization study was measured in a PBMC based assay (52). * Indicates that isolate was sensitive to 2G12 at IC 70. ** IC90 level measured against isolate derived at week 24.

Figure 2. Representative profile of *in vitro* selection experiments performed with MAbs 2G12, 2F5 and 4E10. Individual panels depict evolution of the resistant viral variants of isolate NAB01 for each of the three MAbs (2G12, 2F5, 4E10) tested and all three MAbs in combination. Grey lines and symbols (grey axis) signify p24 antigen production in cultures. Shaded areas represent the antibody level present in the culture during selection (orange for 2G12, magenta for 2F5 and blue for 4E10). The same colors are used to illustrate neutralization sensitivities (IC50 clear, IC70 dashed and IC90 filled bars) of the emerged viruses against the respective antibody at the indicated time points.

Figure 3. Overview of *in vitro* selection experiments against 2G12, 2F5 and 4E10. Selection experiments were conducted one to four times (indicated by Latin numbers) with patient isolates from NAB01, NAB02, NAB03 and NAB08. Neutralization sensitivity (IC50 clear, IC70 dashed and IC90 filled bars) attained during each selection series is depicted. Genotypic resistance was further characterized by sequencing (Figures 6 and 7). When selection resulted in abortive infection timing of the termination of an experiment is indicated. Roman letters (I-IV) signify individual selection experiments.

Figure 4. Overview of sequence changes within the C2-V4 region of *in vitro* 2G12 selected isolates. The C2-V4 region of the 2G12 selected isolates derived from NAB01, NAB02, NAB03 and NAB08 are shown together with the respective mock isolates and the sequence changes found *in vivo* during passive immunization. 2G12 relevant glycosylation sites are shaded in dark grey. Non-2G12 related potential glycosylation sites are shaded in light grey. X denotes sequence mutations in the glycosylation site that lead to loss of that specific site. Presence of mixed populations is indicated by boxes with diagonal divisions.

Figure 5. Overview of sequence changes in isolates selected for resistance to 2F5 and 4E10. The epitope of the selected isolates derived from NAB01, NAB02, NAB03 and NAB08 are shown together with the respective pre isolate and the mock

isolates. Intact epitopes for 2F5 and 4E10 are shaded in magenta or blue respectively, mutations in the epitopes conferring resistance are colored in red. Aa corresponds to amino acid numbering in HXB2 reference strain.

Figure 6. Neutralization sensitivities of 4E10 escape mutants. Neutralization sensitivities of Env-pseudotyped viruses expressing gp160 derived from 4E10 escape virus strains from patients NAB01 and NAB02 were measured on TZM-bl cells. Inhibitory concentrations (IC₅₀ clear, IC₇₀ dashed and IC₉₀ filled bars) of wild type (pre) and 4E10 escape mutants are depicted. The respective mutation of the envelope clones in the core epitopes is indicated.

Figure 7. Infectivity of neutralization escape viruses. The influence of 2G12, 2F5 and 4E10 resistance conferring mutations on the infectivity of the envelope of the virus NAB01 was studied. *Env* genes of the mock control (black), 2G12 escape (NAB01 2G12 II wk10, gray), 2F5 escape (NAB01 2F5 I wk8, dashed) and 4E10 escape (NAB01 4E10 II wk11, white) of isolate NAB01 were introduced in the replication competent NL4-3 backbone TN6-NL and compared for infectivity on 3-way stimulated PBMC. Infectivity (TCID₅₀ per pg p24) of the viral stocks is displayed. Means of two independent experiments each performed on pools of three independent donor PBMC are shown.

Figure 8. Stability of escape mutations in absence of antibody pressure. NAB01 isolates selected to escape 2G12 (NAB01 2G12 II wk10) (left), 2F5 (NAB01 2F5 I wk8) (middle) and 4E10 (NAB01 4E10 II wk11) (right panel) were subjected to long term *in vitro* culture in the absence of antibody pressure. Inhibitory doses of the respective antibodies against virus strains derived during these long term cultures are depicted (IC₅₀ clear, IC₇₀ dashed and IC₉₀ filled bars).

Figure 9. Clonal analysis of the MPER region after long term culture. MPER regions of 2F5 (NAB01 2F5 I wk8) and 4E10 resistant (NAB01 4E10 II wk11) virus strains obtained after long term culturing in absence of Ab pressure. Sequences of the respective antibody epitopes were analyzed to detect whether reversion to the respective wild type sequences (ELDKWA or SWFDIT for the epitopes of 2F5 and 4E10 respectively) occurs.

Table 1: Clonal analysis of MPER in plasma virus during *in vivo* treatment with MAb

4E10 epitope													
aa	668	669	670	671	672	673	674	675	676	677	678	679	680
NAB01													
Pre-Isolate	S	L	W	S	W	F	D	I	T	Q	W	L	W
week 12- clone 05	G	M
week 12- clone 13	M
week 12- clone 27	.	.	.	N
NAB02													
Pre-Isolate	S	L	W	N	W	F	D	I	T	K	W	L	W
week 12- clone 35	x	x	x	x	x	x	.	.

Sequence changes in MPER retrieved from the analysis of 51, 37 and 62 clones derived from NAB01, NAB02 and NAB08 respectively at wk12 of the *in vivo* passive immunization study.

aa: amino acid numbering in the HXB2 reference strain.

X corresponds to amino acid deletion in the sequence.

12.8. Figures

Figure 1.

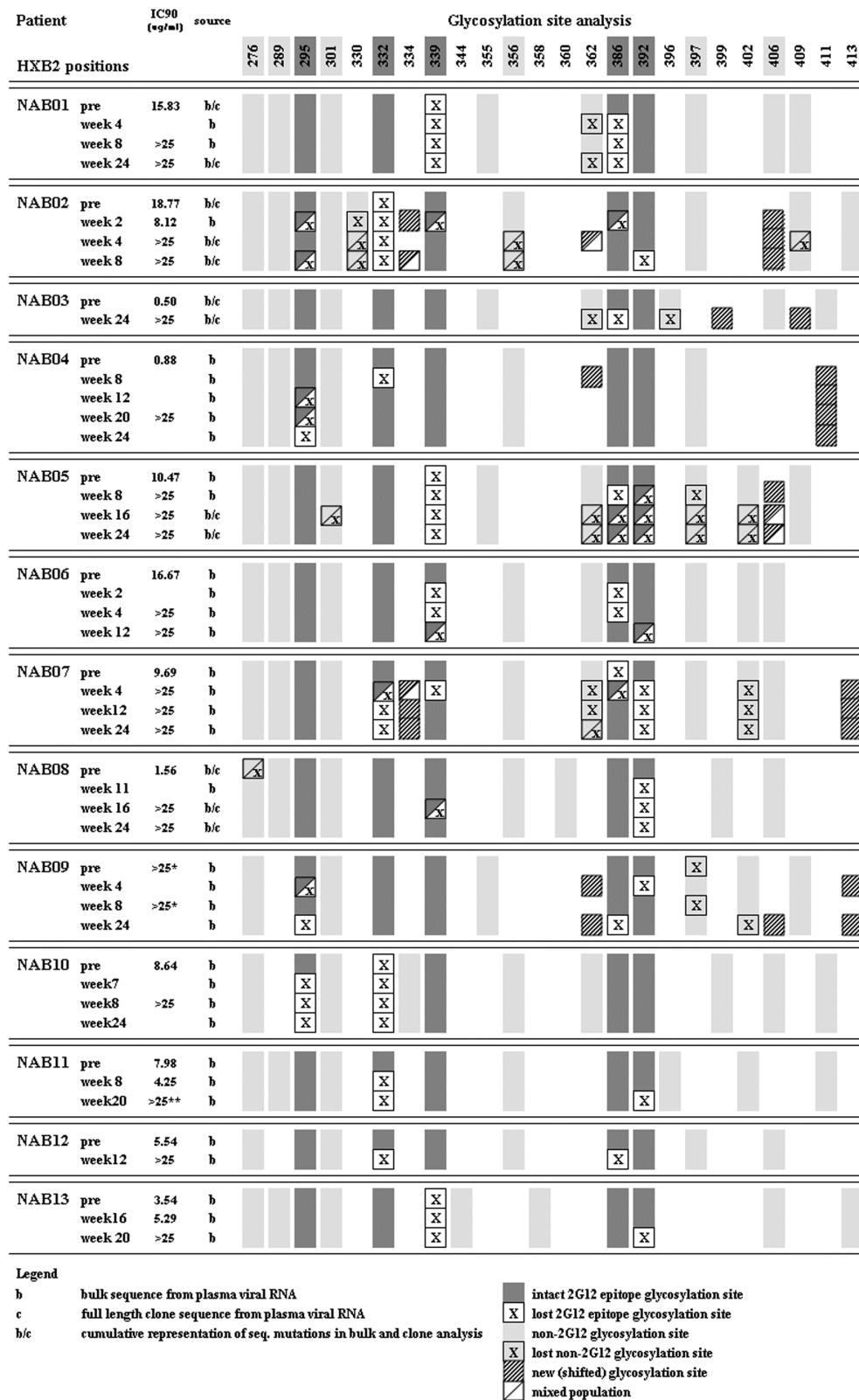


Figure 2.

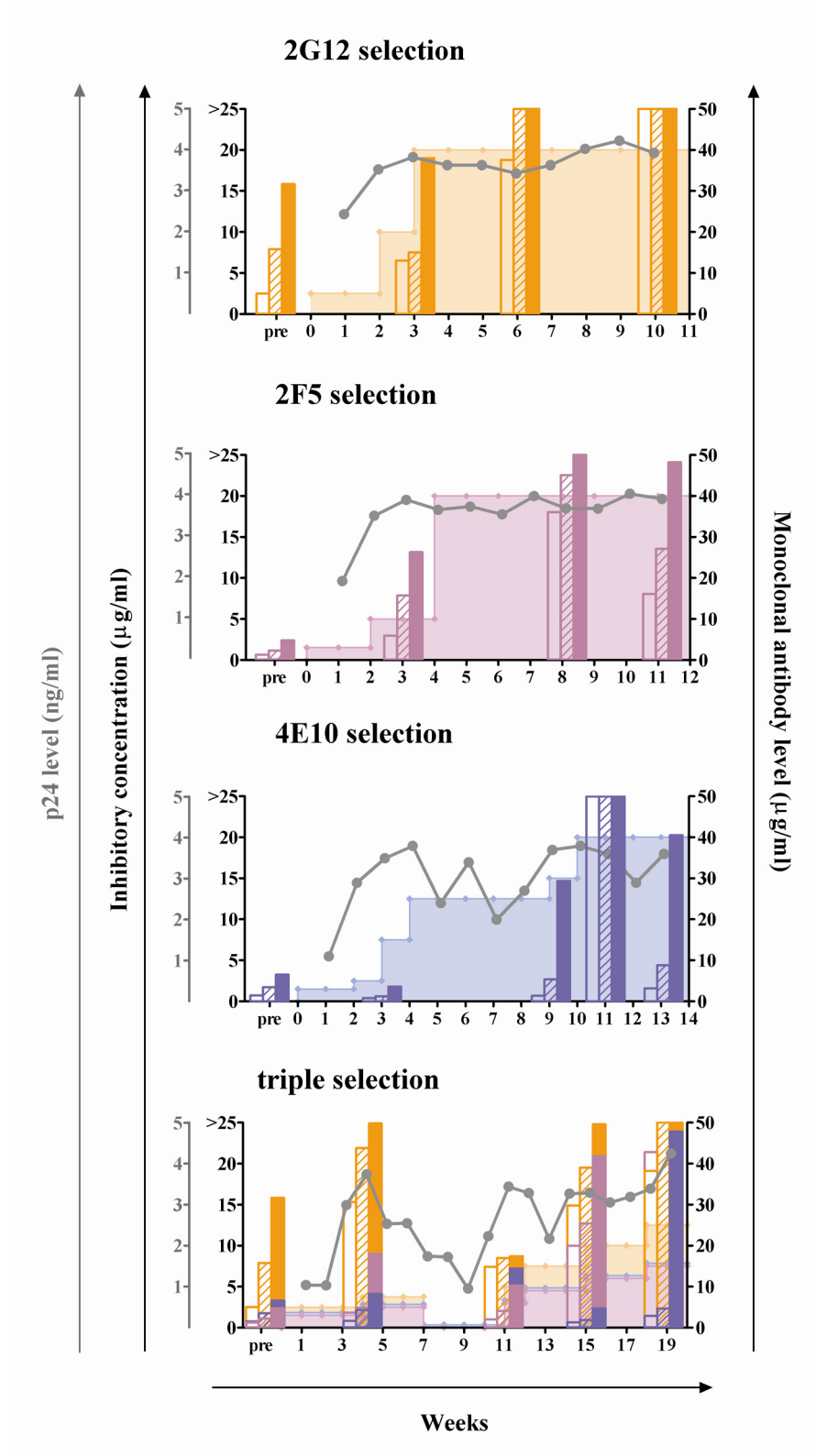


Figure 3.

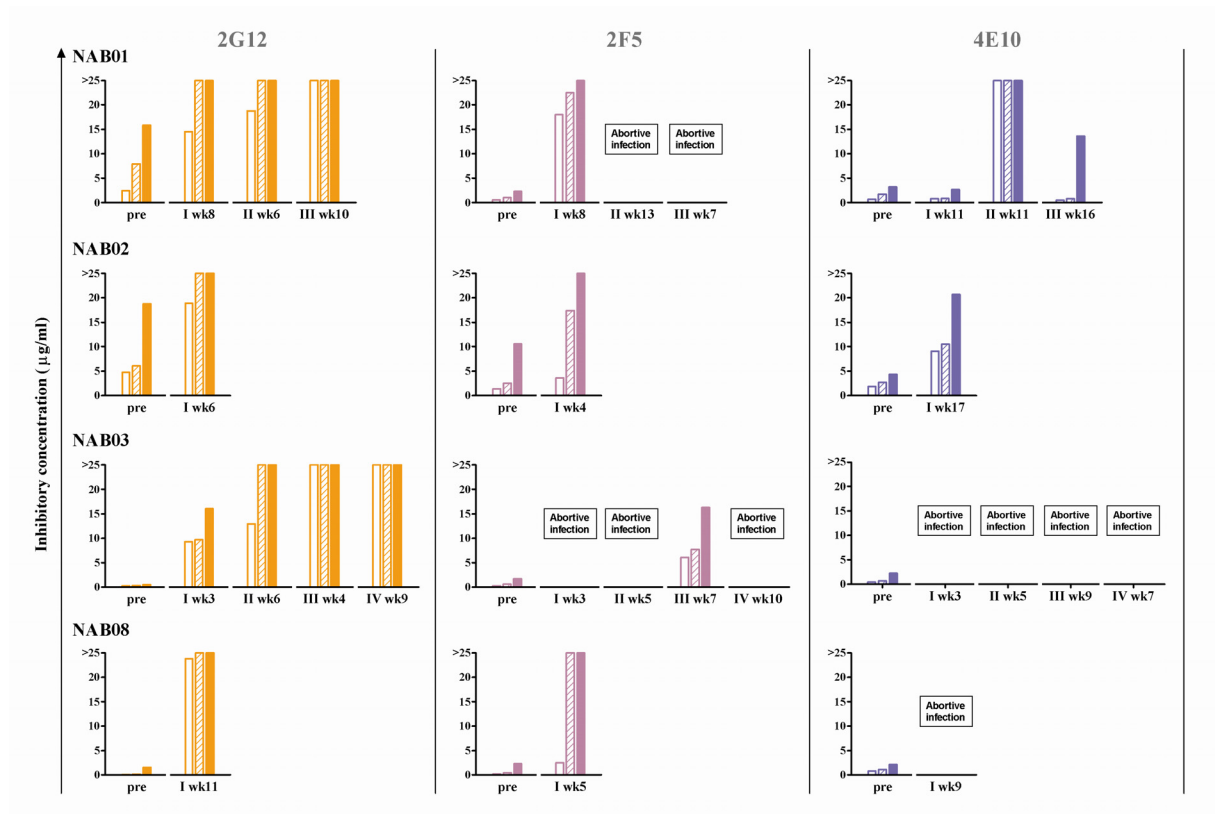


Figure 4.

Patient				assay	weeks in culture	source	Glycosylation site analysis																				
HXB2	reference strain						276	289	295	301	330	332	334	339	355	356	360	362	386	392	397	399	401	402	406	409	411
NAB01	<i>in vivo</i>	pre isolate*			bp									X													
					cp								X														
					cp								X														
	<i>in vitro</i>	mock	wk11	b									X														
				2G12	I	wk16	b							X						X							
			II	wk6	b								X							X							
				wk10	b								X							X							
				c									X							X							
				c									X							X							
		III	wk10	b				X					X							X							
				c									X							X							
		III	wk15	c									X								X						
				b									X							X							
NAB02	<i>in vivo</i>	pre isolate*			bp							X															
					cp							X															
	<i>in vitro</i>	mock	wk11	b																							
				2G12	I	wk6	b					X															
				c							X																
NAB03	<i>in vivo</i>	pre isolate*			bp																						
					cp															X							
	<i>in vitro</i>	mock	wk11	b																							
				2G12	I	wk3	b						X														
			II	wk6	b							X															
				c								X							X								
				c															X								
				c															X								
		III	wk4	b								X															
				c																							
		IV	wk5	b									X														
				c										X													
NAB05	<i>in vivo</i>	pre isolate*			bp																						
					cp	X																					
	<i>in vitro</i>	mock	wk13	b	X																						
				2G12	I	wk5	b	X											X								
				c	X							X															
			wk11	b	X														X								
				c	X														X								
				c	X														X								

Legend

- * indicates the viral sequence used as reference for the respective virus isolate
- bp bulk sequence C2-V4, plasma viral RNA
- cp full length clone sequence from plasma viral RNA
- b bulk sequence C2-V4, cultured viral RNA
- c full length clone sequence from cultured viral RNA

- intact 2G12 epitope glycosylation site
- X lost 2G12 epitope glycosylation site
- non-2G12 glycosylation site
- X lost non-2G12 glycosylation site
- mixed population

Figure 5.

Patient		assay		weeks in culture	source	2F5 Epitope										4E10 Epitope									
						aa	661	662	663	664	665	666	667	668	669	670	671	672	673	674	675	676	677	678	679
HXB2	reference strain					L	E	L	D	K	W	A	S	L	W	N	W	F	N	I	T	N	W	L	
NAB01	in vivo	pre*		bp	L	E	L	D	K	W	A	S	L	W	S	W	F	D	I	T	Q	W	L		
			cp		
			cp		
	in vitro	mock	wk11	b		
				in vitro	2F5	I	wk8	b	.	.	.	N	
								c	.	.	.	N		
	c	.	.					.	N						
	in vitro	4E10	I	wk11	b	L		
					c	L					
					II	wk11	b	G					
		III	wk16	c	G							
				b	L							
		NAB02	in vivo	pre*		bp	L	E	L	D	K	W	A	S	L	W	N	W	F	D	I	T	K	W	L
cp					
in vitro	mock		wk11	b		
				in vitro	2F5	I	wk4	b	.	.	.	T	
								c	.	.	.	T			
in vitro	4E10	I	wk17					b	V		
				c	V							
NAB03	in vivo	pre*		bp	L	E	L	D	K	W	A	S	L	W	N	W	F	D	I	T	K	W	L		
			cp		
	in vitro	mock	wk11	b		
				in vitro	2F5	III	wk7	b	.	.	.	E		
								c	.	.	.	N				
	c	.	.					.	N							
	wk12	b	.	.	.	N						
		wk16	b	.	.	.	N					
			c	.	.	.	N						
NAB08	in vivo	pre*		bp	L	E	L	D	K	W	A	S	L	W	N	W	F	N	I	T	N	W	L		
			cp		
	in vitro	mock	wk13	b		
				in vitro	2F5	I	wk5	b	.	.	.	N		
								c	.	.	.	N				
	c							

Legend

- * indicates the viral sequence used as reference for the respective virus isolate
- bp bulk sequence C2-V4, plasma viral RNA
- cp full length clone sequence from plasma viral RNA
- b bulk sequence C2-V4, cultured viral RNA
- c full length clone sequence from cultured viral RNA

Figure 6.

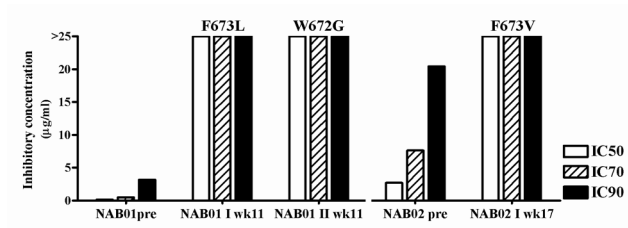


Figure 7.

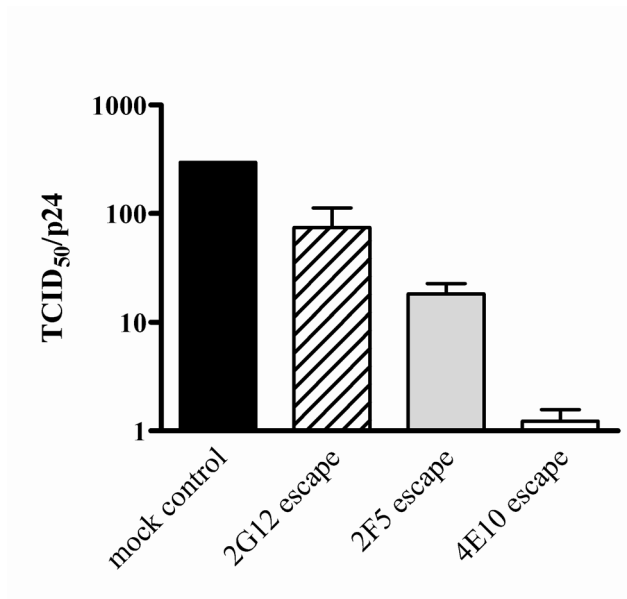


Figure 8.

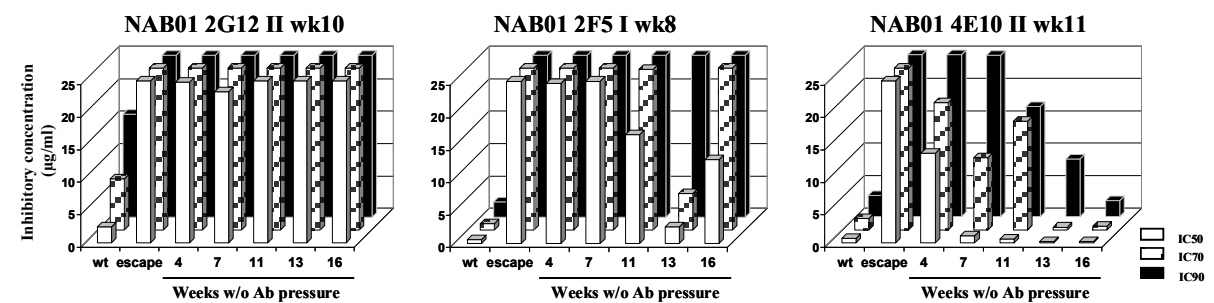
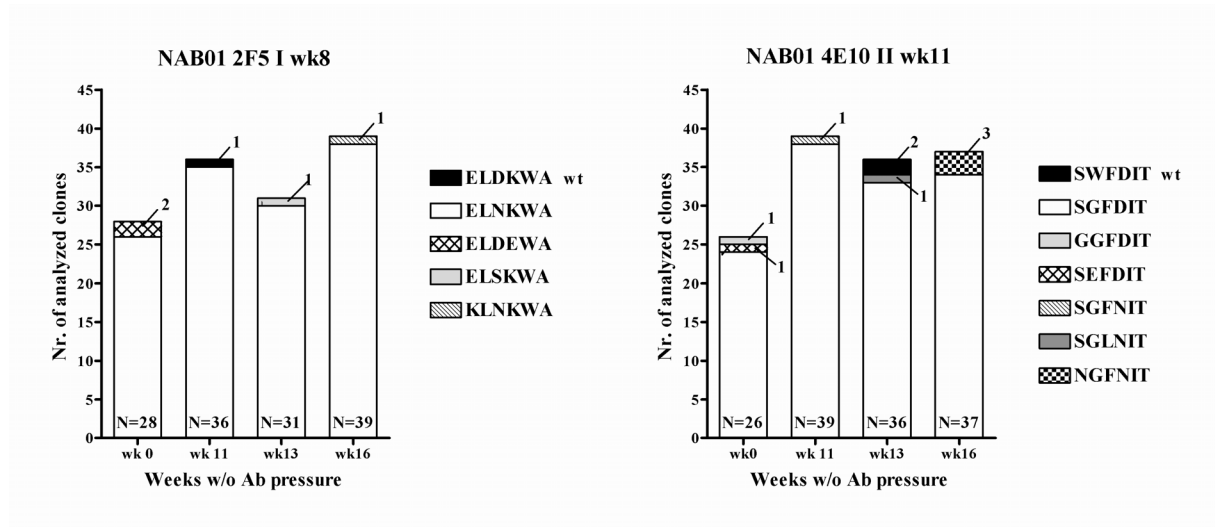


Figure 9.



Supplement Figure 1.

		295			332			339			386			392		
HXB2		N	C	T	N	I	S	N	N	T	N	S	T	N	S	T
RNA source																
NAB01 pre	b/c	N	C	T	N	I	S	E	N	T	N	T	T	N	S	T
wk4	b	N	C	T	N	I	S	E	N	T	H	T	T	N	S	T
wk8	b	N	C	T	N	I	S	E	N	T	D	T	T	N	S	T
wk24	b/c	N	C	T	N	I	S	E	N	T	D	T	T	N	S	T
NAB02 pre	b/c	N	C	T	T	L	S	N	D	T	N	T	T	N	S	T
wk2	b	N/H	C	T	N	I	N	N	N	A	Y	T	S	N	S	T
wk4	b/c	N	C	T	T	L	N	N	D	T	N	T	S	N	S	T
wk8	b/c	N/I	T/C	T/C	N	I	N	N	N	T	N	T	S	N	S	T
NAB03 pre	b/c	N	C	T	N	I	S	N	K	T	N	S	T	N	S	T
wk24	b/c	N	C	T	N	I	S	N	K	T	D	S	T	N	S	T
NAB04 pre	b	N	C	T	N	I	S	N	N	T	N	T	T	N	S	T
wk8	b	N	C	T	K	I	S	N	N	T	N	T	T	N	S	T
wk12	b	N	C	T/I	N	I	S	N	N	T	N	T	T	N	S	T
wk20	b	N/D	C	T/I	N	I	S	N	N	T	N	T	T	N	S	T
wk24	b	D	C	T	N	I	S	N	N	T	N	T	T	N	S	T
NAB05 pre	b	N	C	T	N	I	S	E	N	T	N	T	T	N	S	T
wk8	b	N	C	T	N	I	S	E/G	N	T	N	T	A	N/D	S	T
wk16	b/c	N	C	T	N	I	S	E/G	N	T	N/D	T	T/A	N/K	S/G	T/A
wk24	b/c	N	C	T	N	I	S	E/G	N	T	N/D	T	T/A	N/K	S/G	T/A
NAB06 pre	b	N	C	T	N	L	S	N	N	T	N	T	T	N	S	T
wk2	b	N	C	T	N	L	S	E	N	T	D	T	T	N	S	T
wk4	b	N	C	T	N	L	S	E	N	T	D	T	T	N	S	T
wk12	b	N	C	T	N	L	S	N/I	N/D	T	N	T	T	N/D	S	T
NAB07 pre	b	N	C	T	N	L	S	N	N	T	S	T	T	N	S	T
wk4	b	N	C	T	N	L	S/N	Y	N	T	N/S	T	T	S	S	T
wk12	b	N	C	T	N	L	N	N	N	T	N	T	T	S	S	T
wk24	b	N	C	T	N	L	N	N	N	T	N	T	T	S	S	T
NAB08 pre	b/c	N	C	T	N	L	S	N	D	T	N	V	T	N	S	T
wk11	b	N	C	T	N	L	S	N	D	T	N	V	T	S	S	T
wk16	b/c	N	C	T	N	L	S	N/S	D	T	N	V	T	S	S	T
wk24	b/c	N	C	T	N	L	S	N	D	T	N	V	T	S	S	T
NAB09 pre	b	N	C	T	N	I	S	N	S	T	N	T	S	N	S	T
wk4	b	N/D	C	T/I	N	I	S	N	S	T	N	T	S	S	G	T
wk8	b	N	C	T	N	I	S	N	S	T	N	T	S	N	S	T
wk24	b	N	C	I	N	I	S	N	S	T	S	T	S	N	S	T
NAB10 pre	b	N	C	T	T	L	N	N	N	T	N	T	T	N	S	T
wk7	b	T	C	T	T	L	N	N	N	T	N	T	T	N	S	T
wk8	b	T	C	T	T	L	N	N	N	T	N	T	T	N	S	T
wk24	b	T	C	T/I	T	L	N	N	N	T	N	T	T	N	S	T
NAB11 pre	b	N	C	T	N	I	S	N	K	T	N	T	S	N	G	T
wk8	b	N	C	T	N	I	N	N	K	T	N	T	S	N	G	T
wk20	b	N	C	T	N	I	N	N	K	T	N	T	S	S	G	T
NAB12 pre	b	N	C	T	N	I	S	N	T	T	N	T	S	N	S	T
wk12	b	N	C	T	H	I	S	N	T	T	N	T	L	N	S	T
NAB13 pre	b	N	C	T	N	I	S	M	N	M	N	T	S	N	T	S
wk16	b	N	C	T	N	I	S	M	N	M	N	T	S	N	T	S
wk20	b	N	C	T	N	I	S	M	N	M	N	T	S	D	T	S

Legend

- b bulk sequence from plasma viral RNA
- c full length clone sequence from plasma viral RNA
- b/c cumulative representation of seq. mutations in bulk and clonal analysis
- X/Y mixed populations
- intact glycosylation site
- lost glycosylation site
- mix of lost and intact glycosylation site

Supplement Figure 1. Genotypic analysis of in vivo changes within the 2G12 core epitope.

Changes in the 5 glycosylation sites defining the 2G12 epitope of viruses derived from all 13 patients at different time points during passive immunization. Grey shaded areas represent intact glycosylation sites and red shaded lost glycosylation sites. X/Y corresponds to possible sequence combinations leading either to intact or lost glycosylation site due to mixed populations detected at the analyzed time point. Aa corresponds to amino acid numbering in HXB2 reference strain.

DISCUSSION

To date antiretroviral therapy (ART), where available, succeeds in dramatically reducing mortality and morbidity and significantly lowers rates of mother-to-child transmission. However, although initiation of ART suppresses viral loads to undetectable levels for several years in most individuals (Gulick *et al.* 2006), it is not able to definitely clear the virus. HIV research of almost 25 years has revealed that it is indispensable to understand not only the mechanisms involved in infection defence but also the mechanisms used by the virus to evade those defences. It is therefore important to understand which immune responses are most efficient against the virus and in which way the immune response has to be boosted to protect, treat or even eventually eliminate the virus. On the other hand it is also essential to identify how the virus functions, how it enters the body, how it is transmitted to different sites of replication and to the various target cells, which features of the host are beneficial or detrimental for viral life cycle, and mostly the means by which the virus is able to rapidly evade the host immune responses and how these ways can be blocked.

Initial efforts to develop antibodies for therapeutic use focused on defining antibodies with neutralising capacity. Neutralising antibodies are therefore considered to be an important component of vaccine-induced immunity and thus have also been prime candidates for the development as therapeutics. Therapeutic antibodies should fulfil the following criteria: they have to be safe, potent and broadly active against divergent HIV strains. However it has been demonstrated that most of the neutralising activity elicited to HIV-1 *in vivo* is strain and subtype specific. So far, only a handful of monoclonal antibodies have been isolated that have broad cross-neutralising activity, IgG1b12 (Burton *et al.* 1994), 2G12 (Trkola *et al.* 1996) and the antibodies 2F5 and 4E10 (Muster *et al.* 1993; Stiegler *et al.* 2001; Zwick *et al.* 2001). However it is not clear if vaccine-induced antibodies solely should have neutralising activity or whether they should also activate cell-mediated defence mechanisms. Besides neutralising free viruses, antibodies could have significant impact on virus elimination by inducing phagocytosis, complement-dependent lysis of opsonised viral particles, or antibody-dependent cellular cytotoxicity (ADCC). This first part of this thesis focuses on the contribution of ADCC to disease suppression. ADCC-mediating antibodies could eliminate HIV-1 infected cells and thereby reduce production of

progeny *in vivo*. Although antibodies which mediate ADCC are present in HIV infected individuals, functionality of natural killer cells, the principal effector cells, could be impaired upon disease progression. Evidence has accumulated that ADCC mediating antibodies may nevertheless function *in vivo*, as they were described to positively influence disease progression (Broliden *et al.* 1993; Baum *et al.* 1996; Ahmad *et al.* 2001; Forthal *et al.* 2001).

The current work presents a detailed analysis of parameters in the development of an assay to measure ADCC against primary HIV infected cells and the challenges encountered. One of the biggest problems research faces is mimicking *in vitro* as closely as possible processes that occur *in vivo*. Consequently the more complex, and the more variables a mechanism has, the more challenging it is to set up an appropriate assay *in vitro* that fulfils all criteria. In the case of an ADCC assay three factors need to be controlled: infected target cells, activity of effector cells and antibodies that by binding to target cells trigger lysis by effector cells. Different aspects have to be controlled, such as the level of infection of target cells. Too high infection would result in high percentage of apoptotic cells, low infection levels would have too low levels of viral antigen expression on the cell surface to be detected by plasma antibodies. The differences in killing activity between the different NK donors have also to be taken in consideration, as differences can be rather large. The most challenging problem encountered was the differentiation between natural killing activity and antibody-mediated ADCC. Conditions were defined which maintained natural cytotoxicity low enough to not interfere or misinterpret the ADCC response measured. The final proposed assay is promising, and is probably the most adequate system for longitudinal assessment of ADCC-mediating antibodies in plasma of patients. However some fine tuning experiments have to be considered to standardise the system. In this respect CEM.NKR CCR5 cells and CEM 5.25 cells have to be evaluated further. CEM.NKR CCR5 cells are effective target cells because of their high resistance to direct NK killing. CEM 5.25 cells on the other hand have the advantage that due to expression of the reporter gene GFP upon infection, infected cells can directly be identified by FACS. However CEM 5.25 cells are more susceptible to cytotoxicity. Further engineering of these cells should be considered to improve their features. So could the CEM.NKR CCR5 be engineered to express GFP

upon HIV infection, and CEM 5.25 could be subcloned in order to select a less sensitive clone to natural killing.

Development of a safe and effective HIV-1 vaccine is one of the highest priorities in HIV research. Over the last two decades significant efforts have been made towards inducing potent humoral and cellular immune responses by vaccination, however antibodies and CTL responses alone are likely not sufficient for inducing sterilizing immunity or long-term control of viral replication. Therefore, it is generally believed that both humoral and cellular responses will be needed for an effective HIV vaccine. Early pre-clinical studies established a correlation between a strong CTL activity and reduced viral load. However, recent data obtained in the rhesus macaque model suggest that cellular response focused on a single epitope is not sufficient for effective control of viral replication (Barouch *et al.* 2002b) (Barouch *et al.* 2002a; Barouch *et al.* 2003; Vogel *et al.* 2003). In addition HIV is able to overcome CTL response not only by mutating rapidly which results in either defective antigen processing, loss of HLA binding or escape to CTL recognition (Moore *et al.* 2002; Draenert *et al.* 2004) but also by downregulating HLA type I expression in HIV infected cells through Nef (Collins *et al.* 1998). Presence of neutralising antibodies prior to infection has been shown to provide sterilising immunity in non human primates (Mascola *et al.* 1999; Shibata *et al.* 1999; Mascola *et al.* 2000; Parren *et al.* 2001). Sterilising immunity against HIV in humans may therefore depend on presence of pre-existing neutralising antibodies and great efforts are been made to create vaccines that are able to induce high titres of neutralising antibodies in humans. However until now either Env immunogen derived vaccines (Ferrantelli *et al.* 2004; Burton *et al.* 2005) nor immunisation with gp120 (Haynes *et al.* 2005b), nor with inactivated virus (Levine *et al.* 1996; Gilbert *et al.* 2005) were able to induce neutralising antibodies effectively.

We conducted a passive immunisation study (Trkola *et al.* 2005) with eight chronically and six acutely HIV-1-infected individuals pre-screened for high-sensitivity to 2G12, 2F5 and 4E10 in whom plasma viremia was suppressed to undetectable levels by ART. ART was stopped one day after starting an 11-week course of treatment with the triple combination of monoclonal antibodies. This study provides the first direct proof of the effects of neutralising antibodies in HIV-infected patients. However, despite the sensitivity of patient isolates to the infused antibodies,

only 6 of 14 patients had delayed or no viral rebound in absence of antiretroviral therapy. In addition all of the patients harbouring 2G12 sensitive strains developed resistance against this MAb during immunisation or the following wash out phase, although rebound viruses remained highly sensitive to MPER MAb. Analyses of sequence changes in these patients confirmed that 2G12 resistance is dependent of the five known N-glycosylation sites that compose the 2G12 core epitope (Scanlan *et al.* 2002). In addition to mutations in the 2G12 epitope *in vivo* variants also displayed a considerable amount of mutations in other potential N-linked glycosylation sites as well as in other amino acid positions. What the implications of these mutations are regarding their function in either compensating the loss of 2G12 linked glycans to maintain the immunogenic defence set up by the gp120 glycan shield or if the additional mutations are created to support 2G12 resistance counter weighting fitness or infectivity costs or if for instance these mutations appeared due to autologous immune responses can not be discerned clearly.

To confirm the *in vivo* results and to elucidate the role of MPER MAb we generated *in vitro* escape variants against the three antibodies used in passive immunisation. In the case of 2G12 selections the results were similar to the *in vivo* situation. Mutations leading to 2G12 resistance were always situated in the core epitope of this antibody. However the mutation pattern in the epitope of three of the four patients analysed differed. As well non glycosylation linked mutations were much rarer than in the *in vivo* situation.

Detailed analysis of the MPER region of 150 clones derived from three patients that followed immunisation resulted in rare mutations at the epitopes of 2F5 and 4E10 none of them resulting in loss of sensitivity to the given MAb. On the contrary *in vitro* we were able to generate mutations against the MPER MAb. Notably, evolution of resistance was difficult and frequently resulted in abortive infections underlining the importance of this region for viral fitness and infectivity. Generated MPER resistance like the 2G12 resistance was found to be dependent on the previously described core epitopes (Muster *et al.* 1993; Buchacher *et al.* 1994; Stiegler *et al.* 2001; Zwick *et al.* 2001). Since the frequency of abortive infections suggested that MPER antibodies have an may select for viral strains with low replication capacity, we probed the infectivity levels of MPER and 2G12 resistance mutants and compared these to infectivity level of the mock culture. As expected the mock culture had the highest

infectivity followed by the 2G12 mutant and the 2F5 mutant (5 and 9 fold lower infectivity respectively). The 4E10 mutant had the lowest infectivity having a 164 fold lower infectivity than the mock culture.

In summary the virus from the four patients analysed *in vitro* where able to support mutations in the epitopes of 2F5 and 4E10 although these mutations resulted in lower infectivity. Which factors accounted for the inactivity of MPER MAb *in vivo* still have to be elucidated and require further investigation. But it can not be ruled out that the MPER MAb where active *in vivo* despite the lack of escape selection.

Altogether, my studies provide a detailed insight into the escape pattern to 2G12 but also to 2F5 and 4E10 and are thus of use for further design and development of vaccines that target these regions.

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