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Karolinska Institutet

From Department of Microbiology, Tumor and Cell biology
Karolinska Institutet, Stockholm, Sweden
and
Swedish Institute for Infectious Disease Control

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The red ribbon project, created in New York 1991 by the Visual AIDS Artists Caucus. A symbol to demonstrate compassion for people living with AIDS and their caregivers. An international symbol for AIDS.

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To my family

ABSTRACT

Combinations of antiretroviral drugs against human immunodeficiency virus type 1 (HIV-1) have effectively postponed the progression to acquired immunodeficiency syndrome (AIDS). However, an effective vaccine against HIV-1 would undisputedly be the optimal protective strategy against the virus, especially in resource-poor settings. Because of HIV's unique ability to adapt to environmental pressure, drug-resistant viral strains develop during treatment. In this thesis, we have evaluated vaccine strategies targeting drug-resistant HIV-1. Such a vaccine, together with antiretroviral drugs, would potentially act synergistically against the virus. The drugs would limit viral replication, and the immune pressure specific for resistance mutations would prevent mutant virus from evolving.

Epitopes that commonly mutate during therapy and are restricted to HLA-A0201 were selected as potential vaccine components. Synthetic peptides, representing the epitopes, were evaluated for binding to HLA-A0201 and HLA-A2402 allelic proteins. We found that some of the mutant epitope variants had an enhanced binding capacity over their wild type to HLA-A0201; a few epitopes also cross-bound to the HLA-A2402 protein. Next, we linked the nucleotide sequences of five epitopes, and assessed the immunogenicity of the DNA construct in HLA-A0201 transgenic mice. Contrary to our expectations, the strongest immune response was induced when we immunized mice with the wild type construct. This response was found to cross-react with mutated variants of the epitope. In addition, we explored the possibility to enhance the immune response to mutant peptides by either bridging an HIV-1 protease derived peptide to erythrocytes and use those for vaccination, or by genetically conjugating different epitopes (two of which are presented here) with the B subunit of Cholera toxin (CTB). The expressed fusion proteins were used as immunogens. A weak immune response was measured with the peptide linked to erythrocytes ten weeks after the last immunization. This response was significantly stronger than by giving the peptide alone; despite a 500-fold higher dose of the unconjugated peptide.

Conjugation of the epitope to CTB strongly enhanced the immune response to the epitope. The response was cross-reactive with the wild type epitope, was long-lived and sustained over a four-month period. Interestingly, we observed a correlation of binding capacity of the fusion protein to the natural receptor of CTB, and the adjuvant effect of CTB. The stronger the binding, the better the immune response. We also investigated the potential use of the HIV-1 reverse transcriptase (RT) gene and a multi resistant RT variant. The proteasomal degradation of the proteins was increased by fusing them to ornithine decarboxylase (ODC) or the degradation signal of ODC. After immunization, an inflammatory response was observed in all groups. The RT-specific immune response was relatively weak. The most potent response was detected when RT was fused to the degradation signal of ODC.

In conclusion, we evaluated strategies to target drug-resistant HIV by a vaccine. By using epitopes harbouring drug-resistance mutations as vaccines components, we have consistently detected epitope-specific immune responses that were cross-reactive to wild type sequences. Similar observations were found using wild type epitopes as immunogens. However, the homologous epitope responses were always stronger than, or as strong as, the heterologous epitope responses. This suggests that mutated epitopes representing drug-induced changes are desirable when targeting drug-resistant HIV.

SAMMANFATTNING FÖR LEKMÄN

Arbetet i denna avhandling är fokuserat på de för humant immunbrist virus (HIV) essentiella enzymerna omvänt transkriptas (RT, reverse transcriptase) och viralt protease (PR). RT omvandlar HIVs arvsmassa till DNA som därefter av viralt integrase införlivas i cellens kromosomer. PR katalyserar den mognadsprocess där klyvningar av HIVs äggviteämnen, proteiner, leder till att nya infektiösa viruspartiklar bildas. Dagens antiretrovirala behandling, som länge varit fokuserad på dessa enzymproteiner, har hos vissa individer selekterat HIV som är mer eller mindre resistent mot existerande läkemedel. Det har visat sig att de förändringar inom RT och PR, som leder till en ökad resistens, är snarlika hos flertalet patienter med liknande behandling. Mutationerna ansamlas till kända regioner av proteinerna. Vidare är RT och PR naturligt immunogena, dvs. de ger upphov till ett immunologiskt svar hos patienter. Detta kan utnyttjas inom vaccinforskningen.

Den frågeställning vi arbetat med har varit Hur kan vi rikta patientens immunsvar mot läkemedelsframkallade mutationer? En sådan vaccinering, tillsammans med nuvarande antiretrovirala läkemedel skulle kunna vara av fördel för patienterna då en kombinerad effekt av läkemedelsbehandling och vaccinering skulle kunna uppnås.

Vi har utvärderat tio epitoper där uppkomsten av mutationer är associerad till läkemedelsresistens. Epitoperna syntetiserades i form av peptider, både som den vanligaste förekommande vildtypen och varianter av denna. I ett första skede jämfördes bindning av vildtypspeptider med sina muterade varianter till transplantationskomplexet klass I (MHC) av typ A0201 och vissa av peptiderna också till A2402. Vi fann att vissa av de muterade epitopvarianterna hade en stark bindning till MHC proteinet. Detta skulle kunna innebära en förbättrad förmåga hos de muterade epitoperna att stimulera ett immunologiskt svar. För att undersöka detta kopplade vi samman fem bindande epitoper med en T-hjälparepitop. Dessa DNA vacciner utvärderades i transgena möss som uttrycker mänskliga transplantationskomplex av typ A0201. Vi fann att vissa epitoper var starkt immunstimulerande. Våra resultat tyder också på att immunsvaret blir starkast genom genetisk vaccinering med vildtypsvarianten. Detta var inte förväntat eftersom muterade varianter ofta hade den bästa bindningen till transplantationskomplexet. Vidare fann vi korsreaktivitet mellan vildtyp och mutationstyp. Det starkaste svaret var alltid riktat mot den peptid som användes vid immunisering.

I ett nästa steg undersökte vi tillvägagångssätt för att förbättra den immunostimulerade förmågan hos epitoperna. Detta undersöktes genom att kemiskt koppla samman peptiderna till erytrocyter, röda blodkroppar, eller genetiskt till en receptorbindande enhet av koleratoxin (CTB). De röda blodkropparna eller fusionsproteinerna användes sedan för immunisering av human MHC-transgena möss. Vi förväntade oss en förbättrad presentation till T lymfocyter av peptider som kopplats till röda blodkroppar än av peptider som användes okopplade. Ett svagt immunsvar kunde utläsas hos möss immuniserade med peptider kopplade till de röda blodkropparna. Detta svar var starkare än i möss som fått en 500 gånger högre dos av den okopplade peptiden. Immunisering med CTB fusionproteinerna inducerade ett starkt immunsvar. Den förbättrade förmågan hos fusionsproteinerna att ge ett immunologiskt svar, var korrelerad till hur de band den naturliga cellreceptorn för CTB. För att utnyttja koppling av peptid till CTB krävdes att CTB proteinet kunde bibehålla sin biologiska konformation.

Parallellt med ovanstående studier har vi undersökt skillnader i nedbrytning mellan vildtyps- och läkemedelsresistenta varianter av HIVs enzym RT. Vi avsåg att rikta den intracellulära nedbrytningen av RT till proteasomen; en process som leder till immunologisk presentation. Detta gjordes genom att sammanlänka RT med ornitindekarboxylas (ODC, ett snabbdegraderande kroppseget protein), eller med degraderingssignalen från ODC (ODCsig). I ett sista skede, undersökte vi om en sådan modulering medförde en förbättrad immunostimulerande förmåga hos RT. Vi fann att mutationer associerade till läkemedelsresistens medför en snabbare nedbrytning av RT. Denna nedbrytning medförde ett ökat beroende av proteasomen. Genom fusion av RT till ODC eller ODCsig kunde vi öka nedbrytningstakten. Efter immunisering av möss med DNA vacciner kodande vildtyps, eller modifierat RT fann vi ett inflammatoriskt svar hos alla grupper, men enbart ett relativt svagt RT-specifikt immunsvar. Det starkaste RT-specifika svaret återfanns i gruppen av möss som var immuniserade med RT-ODCsig.

Sammanfattningsvis har vi utvecklat strategier för att vaccinera emot läkemedelsresistent HIV. Ett sådant vaccin skulle teoretiskt kunna utgöra ett komplement till existerande läkemedelsbehandling. Genom att använda peptider med läkemedelsinducerade mutationer kunde vi inducera ett starkt immunsvar mot mutationerna. Vi kunde också påvisa ett korsreagerande svar mot vildtypsvarianten av peptiden. Detta tyder på att immunisering med varianter innehållande kända mutationer kan inducera immunsvar som potentiellt skulle kunna känna igen HIV varianter med läkemedelsresistens.

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II. Boberg A, Sjöstrand D, Rollman E, Hinkula J, Zuber B, Wahren B. Immunological cross-reactivity against a drug mutated HIV-1 protease epitope after DNA multi-CTL epitope construct immunization. Vaccine 2006 May 22;24(21):4527-30.

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IV. Boberg A, Gaunitz S, Brave A, Wahren B, Carlin N. Enhancement of epitope-specific cellular immune responses by immunization with HIV-1 peptides genetically conjugated to the B-subunit of recombinant cholera toxin. Vaccine 2008 Sep 19;26(40):5079-82.

V. Boberg A, Johansson A, Bråve A, Hinkula J, Wahren B, Carlin N. The pentameric structure of the Cholera Toxin B-subunit is important for the immunogenicity of linked HIV peptides. Submitted

VI. Starodubova ES, Boberg A, Kashuba EV, Wahren B, Karpov V, Isaguliants M. HIV-1 reverse transcriptase targeted for proteasomal degradation as a prototype vaccine against drug-resistant HIV-1. Vaccine 2006 May 22;24(21):4541-7.

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CONTENTS

LIST OF ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
APOBEC3G	Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G
ARV	AIDS-associated retrovirus
AZT	Azidothymidine
BCR	B cell receptor
CD	Cluster of differentiation
CTB	Cholera toxin B-subunit
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DNA	Deoxyribonucleic acid
ER	Endoplasmic reticulum
GM1	Monosialoganglioside 1
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HTLV	Human T lymphotropic virus
IDAV	Immunodeficiency associated virus
IFA	Incomplete Freunds adjuvant
IFN	Interferon
Ii	Invariant chain
IL	Interleukin
IN	Integrase
LAV	Lymphoadenopathy associated virus
LTR	Long terminal repeat
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
NK	Natural killer
NNRTI	Non-nucleoside/nucleotide reverse transcriptase inhibitor
NRTI	Nucleoside/nucleotide reverse transcriptase inhibitor
ODC	Ornithine decarboxylase
PI	Protease inhibitor
PR	Protease; refers to HIV protease
RANTES	Regulated on activation normal T cell expression and secretion
RNA	Ribonucleic acid
RT	Reverse transcriptase
SHIV	Simian/Human immunodeficiency virus
SIV	Simian immunodeficiency virus
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TRIM5α	Tripartite interaction motif 5α
β_2m	Beta-2-microglobulin

1 INTRODUCTION

The aim of vaccination is to educate the cells of the immune system to recognize and respond to a particular microorganism. This is carried out by pre-exposing immune cells to the pathogen or fragments of the pathogen. Upon re-exposure to the pathogen, the immune response in vaccinated individuals reacts faster and more vigorously to the microorganism, than in unvaccinated individuals. The immune response thus prevents or restricts the spread of the infection, and limits the symptoms of a disease.

The era of vaccinology begun in 1796 when Edward Jenner inoculated a young boy with extracts from a cowpox lesion. Later, after recovery from the mild infection that followed, the boy was exposed to the human pathogenic smallpox virus but remained healthy. Thus, cowpox virus, closely related to smallpox virus, stimulated protective immunity to the human pathogen. Almost 100 years later, the next vaccines became available. These were developed by Louis Pasteur and were based on killed or attenuated microbes. The vaccines targeted bacterial (anthrax or cholera) and viral (rabies) infections. With improved techniques and more knowledge of infectious diseases, more vaccines were developed. The use of vaccines has markedly decreased the morbidity and mortality from infectious diseases [1, 2]. The conventional vaccines used in humans today are based on live attenuated microbes, inactivated microbes or inactivated purified subunits of a microbe, Table 1 [3, 4]. Subunit based vaccines can be classified into toxoids (bacterial), polysaccharides (bacterial), recombinant proteins (bacterial and viral) or synthetic peptides based (experimental only) vaccines. Moreover, purified pathogen-specific antibodies can be administered and prevent disease soon after exposure of a microbe, before the pathogen reaches its target tissue. These passively administered antibody-based vaccines give a short-lived protection, and are usually given to vulnerable populations only (infants and immunosuppressed individuals that both have a limited immune response). Newer vaccine strategies use genetic material from microbes as vaccine components, and are referred to as genetic or DNA vaccines. The vaccine protein is produced in the body of the vaccinated individual by the host cell machinery. This strategy seems promising, and four DNA vaccines are licensed for animal use.

Table 1. Some vaccines for human use

Vaccine component	Targeted pathogen
Live attenuated pathogen	Measles virus, Mumps virus, Rubella virus, Varicella-Zoster virus, Polio virus (Sabin strain), *Salmonella typhi*
Inactivated/Killed pathogen	Polio virus (Salk strain), Hepatitis A virus, Rabies virus, *Bordetella pertussis*
Subunit or Protein from a pathogen	Hepatitis B virus (surface protein), Human papilloma virus (protein that self-assembles into virus like particles), Influenza virus (surface protein), *Corneybacterium diphtheriae* (toxoid), *Clostridium tetani* (toxoid), *Haemophilus influenzae* (polysaccharide-protein conjugate), *Streptococcus pneumoniae* (polysaccharide-protein conjugate)
Antibodies specific to a pathogen	Rabies virus, Hepatitis B virus, Respiratory syncytial virus

1.1 IMMUNOLOGY

The defence mechanisms against a pathogen can schematically be divided into the innate and the adaptive immune systems [3]. Many naturally existing barriers are

included in the innate immune defence, e.g. skin, mucous and mucous surfaces, and acidic environments. Cells of the innate immune system, e.g. natural killer (NK) cells, macrophages and dendritic cells (DC), act non-specifically on microorganisms and respond to the invader within hours after exposure. These three cell types are especially important, serving as links to the adaptive immune system. They provide chemokines and cytokines required for activation, maturation and differentiation of the lymphocytes of the adaptive immune system. Moreover, cells of the innate immune system have mechanisms to directly kill the pathogen. This prevents growth and spread of the microorganism [3].

The adaptive immune system is divided into humoral and cell-mediated immune responses. B lymphocytes are the key players of the humoral response. These cells express surface-bound antibodies that serve as the B cell receptor (BCR). Upon activation of B cells, rearrangement of the BCR genes takes place, leading to a class switch and to the production and secretion of antibodies. The secreted antibodies can then bind to, and neutralize the invading microorganism. The cell-mediated immune response includes the T lymphocytes. This lymphocyte population is broadly classified as CD4 expressing helper, and CD8 expressing cytotoxic T cells. Their helper and cytotoxic properties are acquired following activation and differentiation. Induction of the adaptive immune response depends on the presentation of microbial epitopes to T lymphocyte receptors (TCR), and the additional co-stimulation of the T lymphocyte by an antigen presenting cell. Macrophages, DCs, and B cells are professional antigen presenting cells and possess the ability to process and present epitopes of pathogen derived proteins to naïve T lymphocytes of both $CD4^+$ and $CD8^+$ phenotypes. The professional antigen presenting cells also express the additional co-stimulatory molecules required for naïve T cell activation and differentiation to effector cells. The adaptive immune response is highly antigen-specific. Introducing a few amino acid substitutions in an epitope may prevent the induction of the response, or reduce the strength of the response to that particular epitope. The adaptive immune response develops within 1-2 weeks after the initial exposure to the pathogen. Some of the activated B and T lymphocytes may differentiate into long-living memory cell populations. These memory cells are then responsible for the more rapid and vigorous immune response upon re-exposure to the pathogen.

1.1.1 Antigen presentation

Antigen presentation via major histocompatibility complexes (MHC) of class I (MHC-I) or II (MHC-II) to T lymphocytes is important for the humoral immune response and a prerequisite for the cell-mediated immune responses. Antigens are presented via MHC-I proteins to TCRs of CD8 expressing T cells, whereas antigen presentation to CD4 expressing T lymphocytes occurs via MHC-II molecules. The MHC molecules are also referred to as human leukocyte antigen (HLA) in humans and H-2 in mice.

The MHC-I molecule is expressed by all nucleated cells and consists of a heavy α-chain to which β_2-microglobulin (β_2m) protein is non-covalently attached. The genes of the heavy chain are encoded by three different loci in humans (A, B or C) that are expressed from both maternal and parental chromosomes. This leads to a maximal possibility to express six different variants of MHC class I molecules, if the individual is heterozygotic for the MHC-I genes. In an outbred population, multiple variants or alternative forms of each gene (allele) are represented in a locus, making this chromosomal region highly polymorphic among humans. The MHC-I proteins are translated in the membrane of rough endoplasmic reticulum as membrane-bound proteins. Chaperon proteins associate with the MHC-I molecule, and stabilize the

partially synthesized MHC complex. The peptide binding groove of the MHC protein is closed at both ends, which limits the length of the epitope permitted to bind. The preferred length of a peptide with good MHC-I binding is 8-11 amino acids, with preferentially basic or hydrophobic amino acid residues at the C-terminus [5]. The interactions between specific amino acid residues of a peptide with specific sites of the binding groove of an MHC-I molecule (the so-called anchor positions) determine how well the peptide will bind to a specific MHC-I protein. Common anchor positions are positions two and nine of the binding groove, but they differ between different MHC-I allelic proteins [6]. Preferred anchor position residues have been used to group MHC alleles into supertypes. Members of a supertype share similar preferential amino acid residues at their anchor positions [7]. Using the approach of supertypes, almost all known HLA-A and HLA-B alleles have been grouped into supertypes A1, A2, A3, A24, A26, B7, B8, B27, B39, B44, B58, and B62 [8, 9]. Thus, each MHC-I molecule can theoretically bind many different peptides as long as they have the preferred anchor position residues. Thereby, the MHC-I allelic proteins expressed by an individual determine the repertoire of peptides that the individual will present to immune cells.

MHC class II molecules are constitutively expressed on professional antigen presenting cells, and expression may be induced on endothelial and epithelial cells upon infection or during inflammatory conditions. The MHC-II genes are encoded by three loci (HLA-DP, HLA-DQ and HLA-DR). Each molecule consists of two chains (α- and β-chains) that are synthesised and assembled in the rough endoplasmic reticulum. The codominant expression of the alleles results in a maximal possibility to express 12 different MHC-II molecules by an individual. The peptide binding groove is open at the ends and the preferred length of the presented peptide is 12-18 amino acids [3, 10].

1.1.1.1 Endogenous antigens loaded on MHC class I molecules

The principle degradation of endogenous proteins is accomplished by the proteasome. The proteasome is a multicatalytic cylinder-like protein complex that is found both in the cytosol and in the nucleus of eukaryotic cells. The protein complex is composed of four homoheptameric rings ($\alpha_7\beta_7\beta_7\alpha_7$), where each α or β subunit is distinct from the other. The individual order of each subunit of a ring is conserved between proteasomes. The catalytic activity is associated with three of the β-proteins (β_1, β_2, and β_5) of each heptameric β-ring of the proteasome complex. These three subunits which possess trypsin-like (β_2), chymotrypsin-like (β_5) and peptidylglutamyl peptide hydrolytic, also called caspase-like activity (β_1), cleave proteins after basic, hydrophobic or acidic amino acids, respectively. The proteasome has also been reported to exhibit two additional proteolytic activities that cleave after small neutral amino acids (SNAP), or branched amino acid residues (BrAAP) [11]. However, these activities has only been reported on model peptides and following modification of the proteasome [12]. The α-rings surround the central opening trough which the proteins enter, and the narrow passage restricts the proteins to be in an unfolded conformation [12-15]. In addition to the $\alpha_7\beta_7\beta_7\alpha_7$-core, regulatory complexes (e.g. PA28 and PA700) may associate with each α-ring of the proteasome [16]. Binding of these regulatory complexes dramatically enhance peptide hydrolysis of ubiquitinated proteins by the proteasome. Endogenous synthesised proteins are poly-ubiquitinated and thereby targeted for degradation by the proteasome when they become misfolded, abnormal or improperly assembled (Figure 1, page 5) [3, 17-21]. The proteasome cuts the proteins into shorter peptides (normally 3-20 amino acid residues long) in an energy dependent manner. A fraction of the newly made peptides escapes further degradation and are transported into the ER through the transporter associated with antigen processing (TAP). Under

the influence of interferon-γ (IFN-γ) the three catalytic subunits of each β-ring of the proteasome are replaced with LMP2 (β_1), LMP7 (β_5) and MECL-1 (β_2) proteins, forming the so-called immunoproteasome. The replacement of these subunits makes the catalytically active pocket more constricted and hydrophobic, and consequently favours the chymotrypsin-like, and trypsin-like catalytic activities. As a result, more peptides with basic or hydrophobic C-termini are generated [3, 13, 15, 22].

The two proteins TAP I and TAP II constitutes the TAP complex, which forms a channel through the ER membrane. The complex preferentially binds peptide with basic or hydrophobic C-terminus. The length of the peptide may be upto 40 amino acid residue long, but peptides of a length of 8-13 amino acid residues are preferred [14]. The binding of peptides to the TAP complex is energy-independent, whereas the translocation of peptides across the ER membrane requires energy in the form of ATP hydrolysis. Cytosolic or ER-associated peptidases trim the N-terminal of the peptide to fit the peptide binding groove of the MHC-I protein [23].

As the last steps of the endogenous antigen presentation pathway, the β_2m and the translocated peptide associates with the MHC-I protein and replaces the chaperon molecules that stabilized the heavy α-chain of the MHC-I molecule following its synthesis. The final trimeric peptide/MHC-I/β_2m complex is then transported through the golgi-apparatus to the cell surface where it is recognized by the T cell receptor of CD8 expressing T cells (Figure 1) [3].

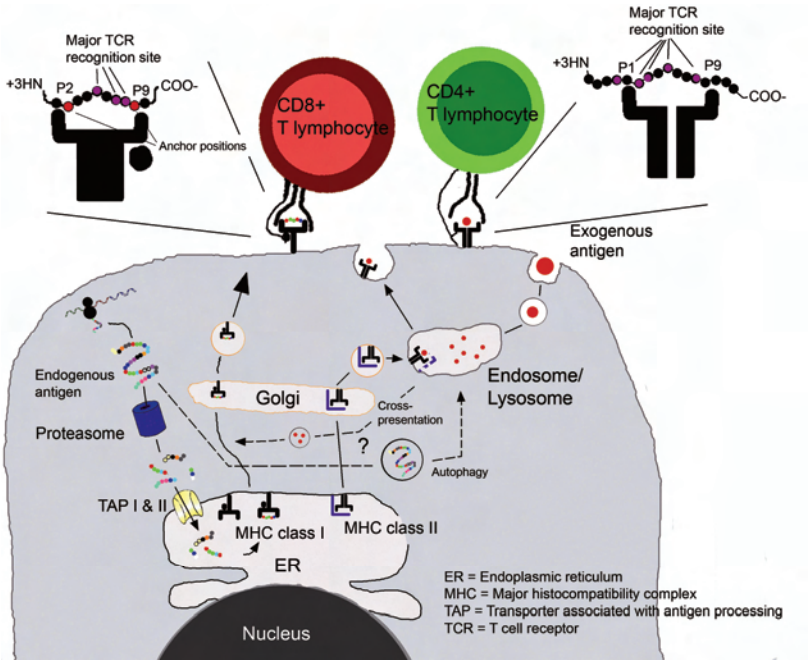

Figure 1. Antigen presentation (*Section 1.1.1*).

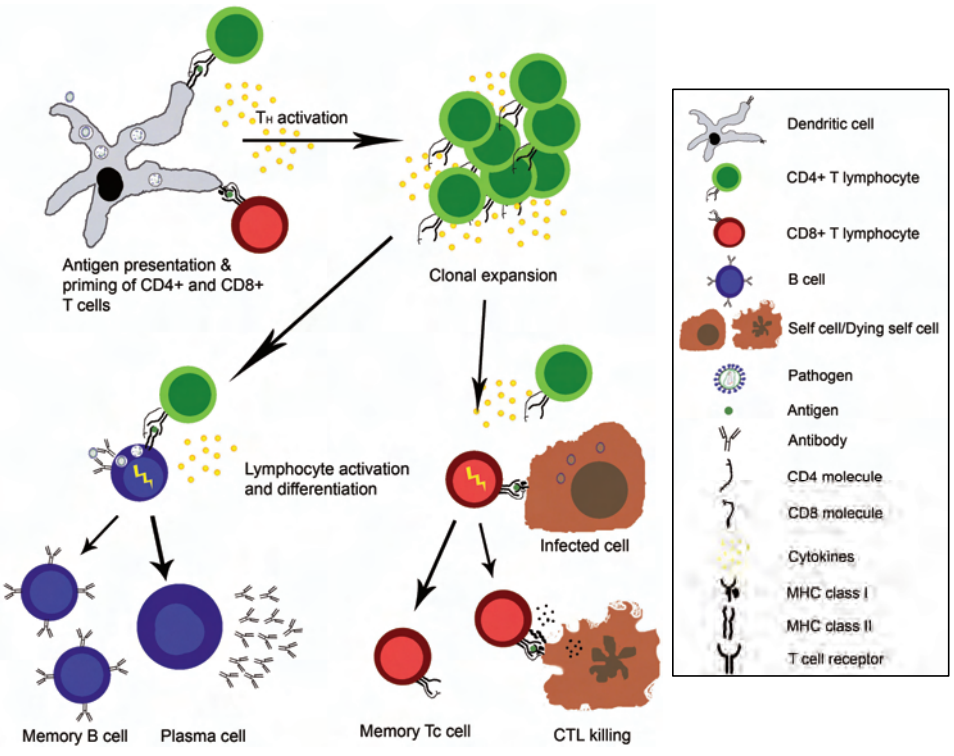

Figure 2. Induction of an immune response (*Section 1.1.2*).

1.1.1.2 Exogenous antigens loaded on MHC class II molecules

Exogenous antigens are taken up by cells either through phagocytosis (e.g. cells or pathogens), or through endocytosis (e.g. fluid or suspended proteins). Following the uptake of the different antigens, the formed phagosome or early endosomes, respectively, are transported inwards to the interior of the cell, and eventually ends up in the lysosome. The environment of the lysosome is acidic, and processing of the delivered antigen is accomplished though the activity of endopeptidases, exopeptidases and reductases (Figure 1, page 5) [24].

During the ER-associated synthesis and assembly of the MHC-II molecule, the chaperone molecule, the invariant (Ii) chain, interacts with the lumenal part of the MHC class II protein and assures correct folding. The Ii protein contains a signalling motif that targets the MHC-Ii complex to the lysosome, either directly through the trans-golgi network, or via the plasma membrane through the endosomal route following its internalization [25]. The acidic environment and the peptidases of the lysosome make the invariant chain to become gradually processed, leaving only a small fraction attached to the binding groove of the MHC-II protein. This so-called class II-associated invariant chain peptide (CLIP) prevents antigenic peptides to be loaded pre-maturely onto the MHC class II molecule. CLIP may become replaced with lysosomally degraded antigenic peptides that have a higher affinity to the MHC-II binding groove. This peptide exchange is aided by the chaperon molecule DM. The final antigenic peptide/MHC-II complex is then transported to the cell surface where it is recognized by the T cell receptor of CD4 expressing T cells (Figure 1, page 5) [25-29].

1.1.1.3 Cross-talk between the processing pathways

As has been previously described, endogenous antigens are normally degraded by the proteasome and are presented on MHC-I proteins to the CD8 expressing T cells, whereas exogenous antigens end up in the lysosome, associate with the MHC-II molecules and become presented to the CD4 expressing T cells. However, as more knowledge of antigen presentation is obtained, the situation becomes more complex. There is evidence that professional antigen presenting cells (especially dendritic cells) can present exogenous antigens on MHC class I molecules, a process known as cross-presentation (Figure 1, page 5) [19, 30, 31]. This process is believed to be important in stimulating immune responses to tumour antigens or to pathogens that do not directly infect the dendritic cell, but where the DC phagocytise apoptotic bodies of an infected dying cell. How the exogenous antigen reaches the MHC-I molecule is not exactly known. One model suggests that a protein translocon, associated with the endoplasmic reticulum, ends up in the endosome through membrane fusion of the ER and the endosome [19]. Other models point at the possibility of endosomal leakage, or disruption of the endosomal membrane. In all these models, the exogenous antigen accesses the cytoplasm. Once there, the exogenously derived antigens may be targeted to the proteasome for degradation. The generated peptides may then be transported through the TAP complex and associate with the MHC-I proteins in the ER. The final MHC complex may then be transported to the cell surface, and presented. An additional model suggests that the MHC-I molecules are internalized and targeted to the endolysosomal compartment [19, 30, 31]. In the lysosome exogenous derived antigens may associate with MHC-I proteins, which then are re-cycled to the cell surface for presentation.

Professional antigen presenting cells also possess the ability to load endogenous antigens on MHC class II molecules by a process called autophagy (Figure 1, page 5) [19, 32, 33]. The autophagy is involved in maintaining cell homeostasis. It has been described to remove damaged intracellular organelles, protein aggregates and to provide nutrients during starvation of the cell. The content of the autophagosomes is transported to the endolysosomal pathway for degradation. Once in the lysosome, the degradation product may be loaded on the MHC-II molecules. It is thus believed that endogenous proteins are internalized into autophagosomes as they are accumulated. Following lysosomal degradation of the endogenous proteins, their peptides may be loaded on the MHC-II molecules. These complexes may then be transported to the cell surface [19, 32, 33].

1.1.2 Induction of an immune response

1.1.2.1 Sensing of pathogens in the periphery

Immature dendritic cells act as sentinels for invading microbes and migrate through blood, peripheral tissues and the lymphoid system when no inflammatory or immune responses are ongoing. These cells constitutively express MHC-I and -II molecules, as well as co-stimulatory molecules for lymphocyte activation. In the periphery the cells take up and process antigens, but presentation of the antigens is inefficient. Upon the appearance of a pathogen-derived antigen, danger-associated signalling molecules are triggered e.g. via Toll-like receptors [34-36]. The dendritic cells become activated and start to mature. As a result of the maturation, the phagocytic activity of the dendritic cell is reduced and the antigen presenting property is enhanced. The activation also leads to up-regulation of co-stimulatory molecules (e.g. CD80, CD86 and CD40), induces the production of cytokines, and changes the expression of chemokine receptors (e.g. CCR5 is down regulated and the CCR7 is up regulated) by the dendritic cell [20, 37]. The enhanced expression of CCR7 on the activated cells enables them to travel to the draining lymph nodes via the afferent lymph vessels into the T-cell rich areas of the organ.

The Toll-like receptors belong to the so-called pattern recognition receptors, and recognise the pathogen-associated molecular patterns. To date, 11 different human Toll-like receptors have been identified, which recognize distinct molecular patterns of different pathogens (e.g. bacterial lipopolysaccharides (endotoxins), lipopeptides, flagellin, unmethylated CpG DNA motifs, and viral single or double stranded RNA) [35, 38, 39]. A major field of vaccine research concerns finding strategies for Toll-like receptor stimulation by vaccine components [40, 41]. Such strategies may increase the potency of a vaccine to elicit an immune response.

1.1.2.2 Interaction of T lymphocytes with antigens and the priming of an immune response

Once in the lymph node, the activated dendritic cells present epitopes of the foreign antigen on MHC-I and MHC-II molecules, to the $CD8^+$ and $CD4^+$ T lymphocytes, respectively. The $CD4^+$ T cells are of special importance for eliciting and coordinating the adaptive immune response. The interaction between the MHC complexes on the dendritic cell and the TCR/CD4 or TCR/CD8 on the T lymphocytes generates the first stimulatory signal. This signal is antigen-specific, but is not sufficient to activate the T cell. Binding of CD80/CD86, on the dendritic cell to CD28, on the T cell generates a second co-stimulatory signal that activates the T cell, which starts to differentiate. The co-stimulatory signal also results in the expression of CD40L on the T cell. Interaction between CD40 and CD40L enhances the dendritic cell function to activate T lymphocytes, including priming of $CD8^+$ T cells [42, 43]. Overall, this interaction

triggers the clonal expansion of the microbe-specific $CD4^+$ and $CD8^+$ T lymphocytes ensuring that sufficient numbers of immune cells recognize the pathogen. In addition, cytokines provided by the dendritic cell (sometimes referred to as signal 3) result in a T helper (T_H) phenotype of type 1 or 2 [42]. T_H1 cells provide the optimal cytokine environment (IFN-γ, interleukin-12 (IL-12), and tumour necrosis factor-α (TNF-α)) for $CD8^+$ T cell activation, which in turn causes this subset of lymphocytes to become fully activated and differentiate into cytotoxic T lymphocytes (CTL; Figure 2, page 5). The activated CTLs may then leave the lymph node and travel back to the site of infection and kill the infected cells [3]. T_H2 cells produce cytokines (IL-4 and IL-10) that are important for B cell activation and maturation. The interaction between the T_H2 cell and the B cell creates an optimal environment for B cells, e.g. provision of the co-stimulatory signal via CD80/CD86-CD28 interaction, resulting in the B cell activation and differentiation (Figure 2, page 5) [3]. This T_H2-B cell interaction is, however, not a strict requirement for B cell activation. T_H-independent B cell activation may occur by cross-binding of multiple BCRs by an antigen. This cross-binding may result in a sufficient signal to activate B cells.

1.1.2.3 MHC – TCR interactions

The interplay between the peptide/MHC complexes and the TCR is relatively weak [44] and brief [45, 46]. Additional interactions between adhesion molecules are needed for continued signalling through the TCR complex and T cell activation [47]. It has been found that serial triggering of multiple TCRs is needed to reach the threshold for T cell activation [48]. These numerous engagements are thought to be performed by a limited number of MHC complexes [48]. This can be achieved by the formation of immunological synapses, where peptide/MHC complexes, TCRs, co-stimulatory receptors and adhesion molecules are brought in close proximity to one another [49, 50]. Following the first two signalling events and T cell activation, antigen-specific TCRs and CD4 or CD8 molecules are down regulated, suggesting a regulatory event of T cell activation [51, 52]. To prime and activate naïve T cells higher amounts of antigen are required than to reactivate the effector or memory cells [53-57]. The naïve T cells are also more dependent on co-stimulatory molecules for their activation. The duration of the signal influences the outcome of the MHC complex-TCR interaction. Some studies suggest that a short exposure to a high amount of antigen is sufficient for activation of T cells, whereas others suggest the need for a longer interaction before activation occurs [58-61]. Again, there is a discrepancy between naïve cells and effector or memory cells in the demand for signal duration. Too strong or too long signalling to effector or memory cells, makes them undergo activation-induced cell death [58, 62].

2 HUMAN IMMUNODEFICIENCY VIRUS

Early in the summer of 1981, the Centers for Disease Control and prevention in the United States, described cohorts of gay men suffering from the Kaposi's sarcoma or Pneumocystis carinii infection [63, 64]. These are diseases usually affecting immunosuppressed patients exclusively. Common to all affected patients was the absence of, or the significant reduction of $CD4^+$ T lymphocytes. The disease was defined as acquired immunodeficiency syndrome (AIDS). The causative agent, a retrovirus, was identified in 1983 by French scientists Francoise Barrè-Sinoussi and Luc Montagnier [65] (awarded with the Nobel Prize in Medicine and Physiology 2008). Initially as different research groups isolated the virus, it was denoted differently; lymphoadenopathy associated virus (LAV), Immunodeficiency associated virus (IDAV), human T lymphotropic virus III (HTLV-III), and AIDS-associated retrovirus (ARV) [65-68]. Later, the viruses were found to be the same. From 1985 it is known as Human Immunodeficiency Virus type 1 (HIV-1) [69-71]. From being an infection reported in a marginalized population as in 1981, HIV-1 infection has spread to become pandemic. Over 60 million persons have been infected, of whom approximately 30 million are currently living with the infection [72]. The annual infections reached almost 3 million during 2007, and more than 2 million died due to opportunistic infections related to AIDS [72].

2.1 ORIGIN AND CLASSIFICATION

HIV is grouped into one of two types, designated HIV-1 and HIV-2. Epidemiological studies have shown that HIV-1 and HIV-2 were transmitted from two different African nonhuman primate species to humans. Phylogeny analysis of sequences from early human samples in which HIV-DNA has been detected, suggests that these zoonotic events took place in the beginning of (1908), or early (1931) of the 20^{th} century [73, 74]. The different HIV types originate from different simian immunodeficiency viruses (SIVs). HIV-1 is related to chimpanzee SIV (SIV_{CPZ}) [75, 76], while HIV-2 is related to sooty mangabey SIV (SIV_{SM}) [77].

HIV-1 infections are spread globally, while HIV-2 infections are mainly observed in the Western Africa and India. HIV-1 is branched into three groups: Main (M), Outlier (O) and Non-M-non-O (N). The M-group is further divided into subtypes, also referred to as clades, A to K. The dominant subtype in the Western world and Australia is B, whereas subtype C is commonly found in southern Africa and parts of Asia. The diversity is, however, becoming more complex with recombination of genes between the subtypes. These complex virus variants are called circulating recombinant forms (CRFs) [78]. Today, most subtypes are found in highly HIV-1 infected areas.

2.2 STRUCTURE

HIV belongs to the Retroviridae family, and is found under the Lentivirus genus. It is a spherical, enveloped virus, with a diameter of approximately 110 μm. The virion contains two copies of a positive-sense, single-stranded ribonucleic acid (RNA) genome. The genome, of approximately 10 Kb, contains the three major genes common for all retroviruses: group-specific antigen (*gag*), polymerase (*pol*) and envelope (*env*) (Figure 3, page 13). In addition, HIV carries regulatory genes: transactivator of viral transcription *(tat)*, regulator of RNA transport (*rev*); and accessory genes: viral infectivity factor (*vif)*, viral protein R (*vpr)*, negative factor (*nef)* and viral protein U (*vpu;* or viral protein X, *vpx*, for HIV-2). This makes the genetic structure of HIV unique among retroviruses. HIV uses three overlapping open reading frames to

maximize the use of the genome. Long terminal repeats (LTRs) flank the structural genes. The 5' LTR encodes a promoter, the transcriptional regulatory elements and the specific binding site for Tat protein. Tat is necessary for efficient transcription of the HIV-1 genome. Both LTRs are needed for the incorporation of the viral genome into the host cell chromosomes, a process catalyzed by the viral enzyme integrase (IN) [79, 80]. Table 2 summarises important functions of HIV proteins.

The membrane-bound spikes, protruding from the viral envelope, are built up by trimers of the viral gp120 protein, non-covalently associated with trimers of the transmembrane viral gp41 protein (Figure 3, page 13) [79, 81]. The capsid and the nucleocapsid proteins are associated with the genome and protect the RNA strands from degradation. The matrix protein is found between the envelope and the capsid. Packed inside the nucleocapsid are the viral enzymes reverse transcriptase (RT), protease (PR) and integrase, all encoded by the *pol* gene [80].

Table 2. Important properties of HIV-1 proteins

Gene	Gene product	Function
gag (group-specific antigen)	p17 (Matrix)	N-terminal part of *gag*. Involved in targeting the Gag and Gag-pol poly-proteins to the plasma membranes. Important structural protein of the virus.
	p24 (Capsid)	Forms the core of the viral particle.
	p7 (Nucleocapsid)	Binds to the psi-signal (packaging signal) on the full-length genomic RNA, and targets the viral genome into new virions. Serves to protect the viral genome from degradation.
	p6	Essential for efficient budding and release of new virus particles. Important for incorporation of Vpr into new virions.
pol (polymerase)	Protease (PR)	Catalytic enzyme, responsible for cleavage of viral precursor proteins and maturation of the virus particle. This makes the newly formed virus infectious.
	Reverse transcriptase (RT)	Reversely transcribes the viral RNA genome into a double-stranded DNA (provirus). Possesses RNase H activity that degrades RNA in the RNA/DNA hybrid formed during the reverse transcription.
	Integrase (IN)	Catalyses the integration of the provirus into the host cell chromosome. A latent infection is thereby established.
vif (viral infectivity factor)	Vif	Involved in HIV infectivity. The Vif protein enhances degradation of the endogenous deaminase APOBEC3G. APOBEC3G would otherwise induce hypermutations that impair the viral DNA.
vpr (viral protein R)	Vpr	Involved in nuclear localization of viral genomes and enzymes. Important for infection of non-dividing cells by enhancing nuclear uptake of viral DNA. Can induce G2 cell cycle arrest.
tat (transactivator of viral transcription)	Tat	Increases the transcription of integrated HIV genome by stabilizing the host RNA polymerase II complex. Promotes apoptosis in infected cells and in bystander cells.
rev (regulator of RNA transport)	Rev	Involved in the transport of unspliced viral mRNA from the nucleus to the cytoplasm, thereby enabling production of structural proteins.
vpu (viral protein U)	Vpu	Interacts with CD4 molecules in the endoplasmic reticulum. This causes degradation of surface expressed CD4 molecules. May also interfere with MHC class I expression on the plasma membrane, and with virion

Table 2. Important properties of HIV-1 proteins

Gene	Gene product	Function
		release.
env (envelope)	gp120 (Surface protein)	One of the two proteins in the viral spikes protruding from the viral envelope. Involved in binding to host cell CD4 and co-receptors on target cell surface.
	gp41 (Transmembrane protein)	Constitutes the transmembrane part of the viral spike. Involved in the fusion of viral envelope with host cell plasma membrane.
nef (negative factor)	Nef	Involved in down regulation of CD4 and MHC class I molecules on host cell surface. Involved in enhancement of viral replication and infection. Prevents apoptosis in infected cells and promotes apoptosis in bystander cells.

2.3 REPLICATION CYCLE

The initial step of HIV infection involves binding of the gp120-trimeric spike to the primary target cell receptor, the CD4 antigen (Figure 4. Step 1, page 13). Binding to CD4 induces a change of conformation of the gp120 spike. The conformational change exposes the co-receptor site of gp120, and enables gp120 to interact with co-receptors the chemokine receptor CCR5 or CXCR4 [82]. Binding to the co-receptor brings the virion in close proximity to the cellular plasma membrane, permitting a part of gp41 to penetrate the plasma membrane.The virion then fuses with the cell (Figure 4. Step 2, page 13).

Once in the cytoplasm, the uncoating of the capsid starts. The reverse transcription of the viral genome occurs simultaneously with the transport of the genome to the nucleus (Figure 4. Steps 3 and 4, page 13). This transcription is catalysed by the viral enzyme reverse transcriptase and involves the conversion of the single stranded RNA genome into a double stranded DNA copy (the so-called provirus). RT is error-prone in its catalytic activity, and lacks the ability to exactly proofread the progeny DNA strands, as they are synthesised [83-86]. Moreover, the enzyme jumps between the two viral RNA genome copies during reverse transcription. As a result, mutations are introduced into the provirus. The provirus is transported into the nucleus and integration into the host cell chromosome is catalyzed by the viral enzyme integrase (Figure 4. Step 5, page 13).

Once integrated, the transcription of HIV genes is carried out by the cellular RNA polymerase II transcription machinery [87]. Initially, only transcription and translation of Tat, Rev and Nef occurs. The messenger RNAs of these proteins are spliced multiple times before being transported to the cytoplasm for translation. The Tat and Rev proteins are transported back to the nucleus, where Tat binds to specific motifs in the 5' LTR of the provirus. This enhances the transcription of the viral genome by stabilizing the binding of the host RNA II polymerase complex to the integrated provirus. Rev has the function to bind to unspliced HIV transcripts, and to transport them to the cytoplasm for translation. Thus, the efficient transcription and translation of structural proteins and enzymes can start only upon accumulation of Tat and Rev proteins (Figure 4. Step 6, page 13) [88]. When the HIV structural gene transcripts enter the cytoplasm, they are translated to proteins that may be post-translationally modified. The transport of the proteins to the cellular plasma membrane begins as they are synthesised. The structural and enzymatic transcripts (gag, gag-pol, and env) are translated into precursor proteins that need to be processed to become functional. A ribosomal frameshift (of -1

nt), enables translation of the Gag-pol poly-protein. The cleavage of the Env precursor protein occurs with the help of host cell enzymes. Then the final gp120 and gp41 proteins are formed (Figure 3). Gag-, and Gag-pol precursor proteins are cleaved by the viral protease. The process starts with the auto-catalytic release of PR from the Gag-pol precursor, followed by continuous separation of the individual proteins. The cleavage of the Gag generates matrix protein (p17), capsid protein (p24), and small nucleocapsid proteins (p6, p7; Figure 3). Cleavage of the Gag-pol protein gives more matrix, capsid, and nucleocapsid proteins and functional protease, reverse transcriptase and integrase (Figure 3) [80]. This proteolysis begins during assembly, and continues after release of new virions from the cell (Figure 4. Steps 7-9). The new HIV particles are released by budding from the plasma membrane (Figure 4. Step 8).

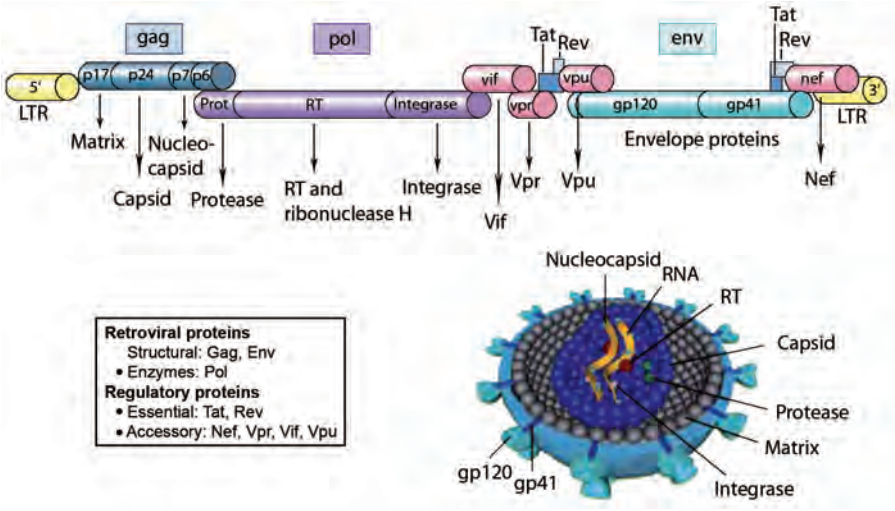

Figure 3. HIV genome and particle. Modified from Klinger P.P *et. al.* Expert Rev Anti Infect Ther 3: 61-79 with permission of Expert Reviews Ltd (*Section 2.2*).

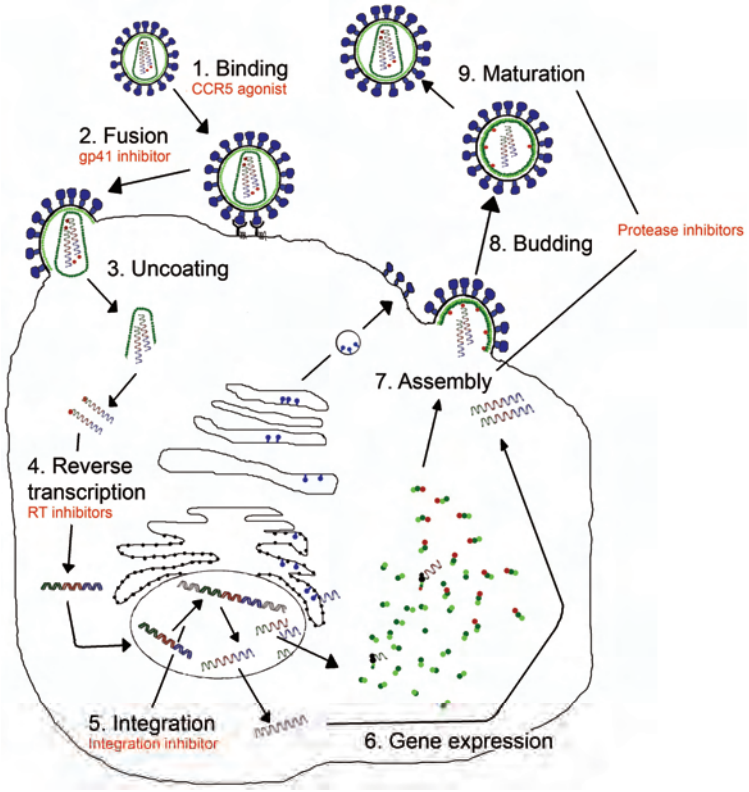

Figure 4. Replication of HIV (*Section 2.3*). Red text indicates antiretroviral drug interference (*Section 2.5*).

2.4 HIV INFECTION

2.4.1 Transmission

HIV is transmitted via unprotected sex (oral, vaginal, or anal), by contaminated blood (sharing needles among intravenous drug users, blood transfusion, or organ transplantation), and vertically from mother to child (congenital, at delivery, or during breastfeeding). It is estimated that sexual transmission of HIV stands for 90% of HIV infections. Heterosexual transmission is the major route of transmission and accounts for 60-70% [89].

2.4.2 Target cells

As mentioned, HIV uses the CD4 antigen as its primary receptor for binding to the target cell, and one of the chemokine receptors CCR5 or CXCR4 as co-receptor to enable fusion of the virion with the susceptible cell. Susceptible cells are $CD4^+$ T lymphocytes, dendritic cells, macrophages and microglia cells of the brain [87]. These cell types concurrently express the CD4-receptor and the CCR5 or CXCR4 co-receptor. HIV may enter the body through lesions in the mucous, by transcytosis via epithelial cells or by direct infection of susceptible cells when being sexually transmitted [90]. Susceptible cells may be dendritic cells interlaying the epithelia, or $CD4^+$ T cells, macrophages and DCs being located in the underlying tissue. Moreover, HIV can bind to C-type lectin receptors, e.g. DC-SIGN, Langerin, and mannose receptor, expressed on the surface of dendritic cells [91, 92]. This binding enables HIV to be disseminated to $CD4^+$ T cells without infecting the dendritic cell. HIV infection of DCs results in TLR signalling and activation of the dendritic cell. The activated DC then migrates to the draining lymph node, where it presents epitopes of HIV to naïve CD4 and CD8 expressing T lymphocytes. The presentation to T cells and the subsequent co-stimulation activate the lymphocytes, leading to their clonal expansion. However, the antigen presentation by the dendritic cells to $CD4^+$ T cells, also brings surface bound HIV to its primary target cells. Primary contact between HIV and $CD4^+$ T lymphocytes is followed by massive infection of this cell type and high viral replication. During this period, HIV replicates to high titers in the blood, and disseminates to lymphatic tissues, and other organs [93]. The activation of T-helper cells aids the $CD8^+$ T cells to differentiate into cytotoxic T lymphocytes that can kill the HIV infected cells [94, 95]. This, together with the cell death induced by the infection, causes the dramatic loss of $CD4^+$ T cells observed following primary HIV infection [96, 97].

2.4.3 Symptoms

Symptom-wise, the acute phase of the infection normally proceeds subclinically, or there are mild to moderate flu-like symptoms [93].

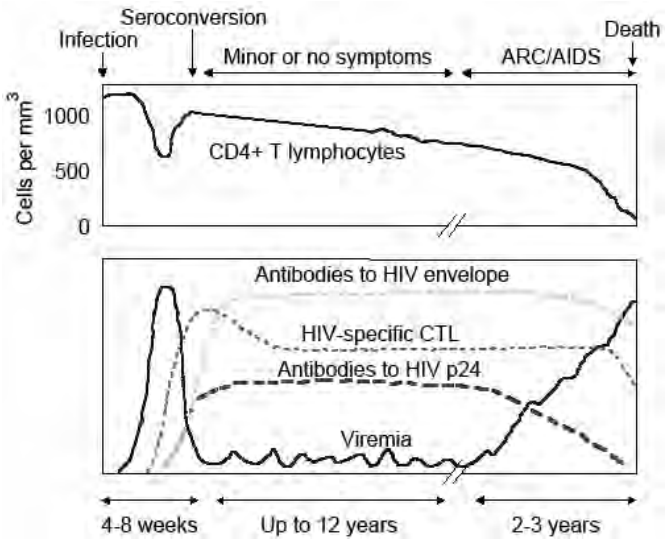

Figure 5. Features of HIV infection. Image adapted from Images.MD.

This primary stage of HIV infection proceeds for 2-3 months, and is associated with an initially high plasma viral load that subsequently declines to a steady viral load set point (Figure 5). Then follows a chronic stage, when the immune system is able to reduce HIV replication [98]. Untreated, this phase can last from a few years to up to 10-15 years [99, 100]. Despite limited viral replication, a continued loss of the $CD4^+$ T cells occurs, which eventually ends up in the symptomatic phase. During the symptomatic stage, the $CD4^+$ T cell number is generally decreased to below 200 cells/mL of blood. When the $CD4^+$ T cells drop below 200 cells/mL of blood, opportunistic infections and malignancies frequently occur. At this stage, the patient has developed AIDS. The opportunistic infections are also the ultimate cause of patient death.

2.5 TREATMENT

In 1986, the American Food and Drug Administration approved the first anti-HIV drug. The drug, azidothymidine, AZT (or zidovudine, ZDV), was until 1991 the only drug available for HIV treatment. From 1991, new drugs became available, and combination therapies, with more than one drug were initiated. Since 1996, therapy regimens include combinations of 3-4 drugs, from at least two drug classes [101]. This regimen is termed highly active antiretroviral therapy (HAART). The introduction of HAART decreased the morbidity and mortality from HIV-associated infections, and slowed the progression to AIDS, thereby making HIV a persistent chronic disease [102].The drugs interfere with specific steps in the replication cycle of HIV, and are grouped into different classes on the basis of viral molecule or viral process that is affected by the drug (Figure 4, page 13, red text). Today, the antiretroviral drugs are grouped into six classes; nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), non-NRTIs (NNRTIs), protease inhibitors (PIs), fusion inhibitors, integrase inhibitors and CCR5 inhibitors.

An important effect of the therapy is the suppression of HIV replication to undetectable levels (<50 viral RNA copies/mL of blood), enabling the restoration of the immunocompetence of the patient. This makes the patient capable of coping with many

common infections. A beneficial consequence of the effective suppression of viral replication by HAART is a decreased risk of HIV transmission, since high HIV viral load is correlated with an increased risk of disseminating HIV [103, 104]. Although the antiretroviral drugs are the most important intervention in the battle against HIV, HAART will never clear the infected cells. A reservoir of infected cells remain dormant [105]. Moreover, some viral replication still occurs during treatment, which enables viral evolution [106, 107].

The patients' $CD4^+$ T cell-count decides when therapy should be initiated in asymptomatic patients. The Swedish reference group for antiviral therapy (RAV) and the Swedish medical products agency (MPA) have currently revised the previous recommendations from 2007 for HIV therapy initiation (www.rav.nu). The main change in the new recommendations is that therapy should start when the patient has a $CD4^+$ T cell-count < 350 T cells/mL of blood. Previously, the recommendation was that therapy should be considered when the CD4 cell-count was < 350, and therapy should be started when the T cell-count was < 200 T cells/mL of blood. This number is normally around 1000-1500 cells/mL. Treatment should be initiated when AIDS-like symptoms appear, as recommended before.

2.5.1 Antiretroviral drugs

NRTIs are pro-drugs and act as nucleoside triphosphate analogues. They need to be phosphorylated by host cell kinases to become functional. The drugs are competing with the natural nucleoside triphosphates to be incorporated into the elongating viral DNA strand. NRTIs act as chain terminators, and incorporation of the analogue in the growing DNA chain stops the reverse transcription [108, 109]. NNRTIs do not bind to the active site of RT. Instead, the drugs bind to a hydrophobic pocket proximal to the active site of the enzyme. The interaction inactivates RT, by inducing a conformational change of the protein [108, 109].

PIs are small molecules that compete with HIV poly-proteins for binding to the active site of viral protease. The binding of the drug inhibits the proteolytic cleavage of the Gag and Gag-pol precursor proteins. Then no infectious particles are formed. [110, 111].

One fusion inhibitor is licensed for human use. There are two glycine-rich heptad repeat domains (HR1 and HR2) N-terminally of gp41. These two regions normally associate to form a six-helix bundle that is thought to draw the viral and cellular membranes together. This enables the fusion of the two membranes [112]. The drug mimics the HR2 domain and associates with the HR1 region of gp41. This association inhibits the bundle formation, and prevents fusion of the viral membrane with the cell membrane [112, 113].

Integrase inhibitors are drug classes to be approved for HIV therapy. There is one drug on the market that blocks the integration step of the viral provirus into the host chromosome. The drug binds to the host DNA binding site of IN and prevents the establishment of a latent infection [114].

The latest drug class belongs to the chemokine inhibitors. There is one drug licensed in this class and the drug does not bind HIV proteins. Instead, the drug inhibits infection by binding to the CCR5 co-receptor, and prevents the fusion of the virion and the cell membrane [115]. The drug specifically targets CCR5-using HIV strains and will

consequently not be effective against CXCR4-using viruses. A list of the antiretroviral drugs approved for HIV therapy in Sweden is given in Table 3.

Table 3. Drugs approved for HIV treatment in Sweden 2009

Name(s)[A]	Drug class	Key mutations associated with specific drugs [B]
Zidovudine, AZT, ZDV (Retrovir)	NRTI	M**41**L, D**67**N, K**70**R, L**210**W, T**215**YF, K**219**QE
Stavudin, d4T (Zerit)	NRTI	M**41**L, D**67**N, K**70**R, L**210**W, T**215**YF, K**219**QE
Lamivudin, 3TC (Epivir)	NRTI	K**65**R, M**184**VI
Abacavir, ABC (Ziagen)	NRTI	K**65**R, L**74**V, Y**115**F, M**184**V
Didanosin, ddI (Videx)	NRTI	K**65**R, L**74**V
Emitricitabin, FTC (Emtriva)	NRTI	K**65**R, M**184**V/I
Tenofovir, TDF (Viread)	NRTI	K**65**R, K**70**E
Nevirapin, NVP (Viramune)	NNRTI	L**100**I, K**103**N, V**106**AM, V**108**I, Y**181**CI, Y**188**CLH, G**190**A
Efavirenz, EFV (Stocrin)	NNRTI	L**100**I, K**103**N, V**106**M, V**108**I, Y**181**CI, Y**188**L, G**190**SA, P**225**H
Etravirin, ETR (Intelence)	NNRTI	V90I, A98G, L100I, K101EP, V106I, V179DFT, Y181CIV, G190SA
Ritonavir, RTV (Norvir) [C]	PI	L10FIRV, K20MR, V32I, L33F, M36I, M46IL, I54VL, A71VT, V77I, V**82**AFTS, I**84**V, L90M
Indinavir, IDV (Crixivan)	PI	L10IRV, K20MR, L24I, V32I, M36I, M**46**IL, I54V, A71VT, G73SA, L76V, V77I, V**82**AFT, I**84**V, L90M
Saquinavir, SQV (Fortovase, Invirase)	PI	L10IRV, L24I, G**48**V, I54VL, I62V, A71VT, G73S, V77I, V82AFTS, I84V, L**90**M
Nelfinavir, NFV (Viracept)	PI	L10FI, D**30**N, M36I, M46IL, A71VT, V77I, V82AFTS, I84V, N88DS, L**90**M
Atazanavir, ATV (Reyataz)	PI	L10IFVC, G16E, K20RMITV, L24I, V32I, L33IFV, E34Q, M36ILV, M46IL, G48V, I**50**L, F53LY, F53LY, I54LVMTA, D60E, I62V, I64LMV, A71VITL, G73CSTA, V82ATFI, I**84**V, I85V, N**88**S, L90M, I93L/M
Darunavir, DRV (Prezista)	PI	V11I, V32I, L33F, I47V, I**50**V, I**54**M/L, G73S, L**76**V, I**84**V, L89V
Fosamprenavir, fAPV (Telzir)	PI	L10FIRV, V32I, M46IL, I47V, I**50**V, I54LVM, G73S, L76V, V82AFST, I**84**V, L90M
Lopinavir, LPV (Kaletra)	PI	L10FIRV, K20MR, L24I, V**32**I, L33F, M46IL, I**47**VA, I50V, F53L, I54VLAMTS, L63P, A71VT, G73S, L76V, V**82**AFTS, I84V, L90M
Tipranavir, TPV (Aptivus)	PI	L10V, I13V, K20MR, L**33**F, E35G, M36I, K43T, M46L, I47V, I54AMV, Q58E, H69K, T74P, V**82**LT, N83D, I**84**V, L90M
Enfuvirtid, T-20 (Fuzeon)	Fusion inhibitor	G36DS, I37V, V38AME, Q39R, Q40H, N42T, N43D
Raltegravir, RAL (Isentress)	Integrase inhibitor	Q**148**HKR, N**155**H

Table 3. Drugs approved for HIV treatment in Sweden 2009

Name(s)[A]	Drug class	Key mutations associated with specific drugs [B]
Maraviroc, MVC (Celsentri)	CCR5-inhibitor	-
Combination pills		
AZT/ZDV + 3TC + Abacavir (Trizivir)	3x NRTIs	
Abacavir + 3TC (Kivexa)	2x NRTIs	
AZT + 3TC (Combivir)	2x NRTIs	
Lopinavir + Ritonavir (Kaletra)	2x PIs	
TDF + FTC (Truvada)	2x NRTIs	
TDF + FTC + EFV	2x NRTIs + 1x NNRTI	

[A] Generic name, acronym and sales name
[B] Information obtained from [116]. Major mutations are in bold letters.
[C] Information obtained from [117].
NRTI= nucleoside/nucleotide reverse transcriptase inhibitor; NNRTI= non- nucleoside/nucleotide reverse transcriptase inhibitor; PI= protease inhibitor

2.5.2 Drug-resistance

As mentioned in *Section 2.3*, HIV RT is error-prone during the reverse transcription and lacks the ability to correctly proofread the progeny DNA strands [83-86]. It is estimated that $3x10^{-5}$ errors occur per base pair per replication cycle [118]. During the reverse transcription, two or three recombination events also occur between the two viral RNA copies [119]. Together with the extremely efficient production of new viral particles (deriving from 10^7-10^8 infected cells) and rapid viral turnover (approximately 2 days) [98, 120], many mutations are introduced into the viral population. This results in a natural polymorphism within the HIV proteins, and makes the virus able to adapt to changes in the microenvironment. Usually patients show resistance patterns which are specific for treatment regimen. However, not all changes in the viral genome conferring resistance to the antiretroviral drugs are the result of drug treatment. Drug-resistance mutations can be observed in treatment-naïve patients, infected with wild type virus [121-124]. Such naturally resistant viral variants may underlie the limited viral replication seen in patients during effective HAART, and result in the viral evolution to a drug-resistant phenotype. The known mutations conferring drug-resistance are summarized in [116].

Due to HIVs' ability to mutate, the prescribed therapy requires a high compliance. During suboptimal therapy, drug-resistant HIV readily emerges [125, 126]. The first generation of antiretroviral therapy was associated with a complicated dosing schedule (including many pills that needed to be taken at regular hours with food-intake restrictions) and drug toxicity. Physicians needed to adjust the treatment to fit to the daily routine of the patients to increase their adherence to the prescribed therapy [101, 127, 128]. Today, the use of combination pills where two or three drugs are combined has eased the pill burden and the drugs are also less toxic. However, suboptimal HAART, and the suboptimal compliance to prescribed therapy, lead to break-through in viral replication and permit mutations to emerge and persist. As long as the therapy remains suboptimal these viral variants can replicate faster than the wild type virus. Over time, these resistant variants may constitute the major detectable phenotype, limiting the effectiveness of currently used drugs [113, 129]. Drug-resistance mutations are classified as primary or secondary. Primary mutations are associated with resistance

to a particular drug, and decreased viral susceptibility to that drug. These mutations are often associated with a loss of fitness of the mutated viral strain. The virus may replicate slower than wild type virus. Secondary mutations refer to changes in the genome that compensate for the loss of fitness introduced by the primary mutations [111]. Thus, secondary mutations restore viral replication capacity.

Most primary mutations involved in drug-resistance, have been introduced in the reverse transcriptase and protease genes. Crystallographic studies of RT have shown that the conformation of the protein resembles a right hand; with a thumb-, palm-, and finger-like sub-domains [130]. Resistance to NRTIs involves an increased discrimination of the drugs by RT, thus, favouring incorporation of the natural nucleoside triphosphates. This occurs by either a higher restriction of incorporation of normal nucleoside triphosphates, or by an enhanced excision (depolymerisation reaction) of the chain terminating drugs, allowing the DNA polymerisation to proceed [109, 130-132]. NNRTI resistance occurs when mutations arise in the pocket where the drug binds [109, 133]. This allows RT to preserve the activity, and enables reverse transcription to proceed.

HIV protease is a homodimer where the substrate binding site is located within the active site, formed by the two subunits. Drug-resistance involves the replacement of amino acids responsible for the interactions between the drug and the active site of the protease, consequently reducing the binding affinity of the drug to the viral protease [111, 129, 134]. Then, the maturations of Gag and Gag-pol precursor proteins proceed.

Fusion inhibitor resistance involves one or more mutations within the codons 36 to 45 of gp41, and is located within the heptad repeat 1 domain. The mutations reduce the binding of the drug to HR1 and thus permit bundle formation and membrane fusion [112, 113].

The HIV integrase protein consists of three functional domains, a catalytic core domain surrounded by the N- and C-terminal domains involved in coordination of zinc molecules and host DNA binding, respectively. Mutations that confer resistance to integrase inhibitors are located within, or near the target DNA binding site of IN and limits access of the drug to the active site [113, 114].

The knowledge of CCR5 inhibitor resistance mutations is relatively limited. Known resistance mutations are, however, located within the V3 loop of HIV gp120. The V3 loop is responsible for the interaction of gp120 with the co-receptor, and resistance involves phenotypical changes in the co-receptor sequence [113, 135].

2.5.3 Other preventive measures

The most effective way of limiting the sexual transmission of HIV, is a complete abstain from sex. This is however unlikely, therefore information campaigns and education programs on HIV infection and transmission are effective ways to reduce the spread of HIV. Moreover, promoting the practice of safer sex by the use of male or female condoms may limit HIV transmission even further. The male condom has been estimated to reduce HIV transmission by approximately 80% [136]. Disruption of the condom is a reason why the effectiveness does not reach a 100%. It has also been evidenced from three large clinical trials conducted in Africa that male circumcision reduces the rate of female-to-male transmission of HIV [137-139]. The removal of the Langerhans cells that are frequent in the foreskin is considered to be an important factor for this effect [140, 141].

As women account for half of the HIV infected individuals, and since condom use may be refused by the male partner, a prevention controlled by women is desirable. The development of an effective microbicide against HIV is therefore as important as HIV vaccine development. Microbicides are substances, the purpose of which is to reduce infectivity of a microbe. The first generation of microbicides was based on pH modulators, detergents or polyanionic gels. One of the first microbicide to be tested in efficacy clinical trial was nonoxynol-9. This substance showed disruption of HIV membranes *in vitro* [142], but failed to reduce HIV transmission in a clinical trial [143, 144]. Moreover, a trend to an increased susceptibility to HIV was observed in the nonoxynol-9 treated group. Induction of an inflammatory response by nonoxynol-9 at the mucosal surfaces was considered to be a reason for the negative effect. The inflammatory response would activate and bring more target cells to the site of infection [145, 146]. Newer generations of microbicides include combination of antiretroviral drugs formulated in gel or silicone rings [147]. This approach of using a combination of drugs in microbicides seems more promising than the first generation of the microbicide formulations. Based on the positive effect of HAART, a great hope is put on these newer microbicide strategies. Especially promising is the silicone ring, where a constant release of the drugs for a sustained period of time has been achieved. By using a silicone ring, a weekly or monthly exchange of the ring may only be required to still provide good protection against HIV infections. Antiretroviral drugs in microbicide formulations are currently evaluated in early clinical trials [147, 148].

2.6 IMMUNE RESPONSES AGAINST HIV

2.6.1 Innate immune responses

To establish an infection, HIV first has to overcome the innate immune response. The interaction of the glycosylated gp120 and mannose receptors on dendritic cells and macrophages stimulate production of type I interferons (IFN-α and IFN-β) [149]. IFN-α brings the exposed as well as adjacent cells to an anti-viral stage [150-152]. An additional effect of IFN-α, is an enhanced production of the tripartite interaction motif 5α (TRIM5α). This antiretroviral protein affects the early (entry) and late stages (expression and release) of the HIV life cycle [153-156]. The endogenous deaminase APOBEC3G (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G), acts to inhibit the viral replication cycle by hypermutating the negative strands of the viral RNA genome. This results in introduction of lethal mutations into the viral genome [157-163]. Moreover, NK cells secrete soluble β-chemokines such as RANTES (regulated on activation normal T cell expressed and secreted), MIP-1 α and MIP-1 β (macrophage inflammatory proteins), and cytokines such as IFN-γ, and TNF-α that are thought to contribute to resistance against the viral infection [164-167]. NK cells also express Fc-receptors that recognize and bind to the Fc-part of antibodies bound to infected cells. The interaction triggers the release of granzyme and perforin from intracellular granules of the NK cells. The release of these proteins leads to the killing of the infected cell, a mechanism known as antibody-dependent cellular cytotoxicity [168-174].

2.6.2 Adaptive immune responses

Strong proliferative T-helper and cytotoxic T lymphocyte responses are clinically associated with reduction of viral load (Figure 5, page 15) [175-182]. These responses develop during acute HIV infection [175-179]. The strength of the CTL response declines as the disease progresses [183]. There is evidence from SIV infected monkeys that prove the important role of $CD8^+$ T cells in controlling SIV infection [184-187]. Depleting rhesus macaques of the $CD8^+$ T cells prior to challenge, resulted in a lack of

control of the viral replication that followed [185, 187]. In addition, a rebound of viremia was observed when chronically SIV infected monkeys were treated with anti-CD8 antibody [187]. Immune responses to Gag antigens develop quickly, and may be detected as soon as 6 hours following HIV infection [188]. Responses to Env- and Pol-proteins develop later [176]. The CTL response acts by lysis of HIV infected cells performed by the release of perforin and granzyme B, or by apoptosis through Fas-FasL interactions [176, 189].

Later, patients develop high titers of anti-HIV antibodies (Figure 5, page 15) [190]. The majority of the antibodies reacts with Env (gp41 and gp120) or Gag (p17 and p24) antigens. Neutralizing antibodies to Env can bind free virus and prevent the attachment of the HIV gp120 protein to the CD4 antigen. This will limit the number of cells being infected. Sterilizing immunity has been obtained in monkeys after passive immunization with high doses of anti-Env antibodies prior to challenge with simian/human immunodeficiency virus 89.6P (SHIV89.6P) [191-194]. However, induction of broadly neutralizing antibodies in the natural HIV infection is rare, and most of the antibodies produced during the infection will only bind to the viral particle, but not prevent the infection to be established. There are also studies showing that such binding antibodies may enhance the infectivity of HIV [195-198].

In addition to the primary immune responses, the HIV infection triggers memory B and T cell responses. Long-living memory cells are important for containing HIV infection. Induction of central memory CTL is associated with lower viral RNA levels in the blood, and a slower decline of $CD4^+$ T helper cells [199-203].

Despite the capacity of the immune responses to control HIV replication in the natural viral infection the responses are developed too late, when the infection is already established. Moreover, as HIV replication may be slow following the infection, a complete depletion of HIV infected cells by CTLs is unlikely to occur. However, by inducing potent humoral and cell-mediated immune responses by a vaccine prior to infection, HIV infection may be prevented or the transmission of HIV may be limited.

2.6.3 Immune responses against drug-resistant virus

The combination therapy used today may select for the drug-resistant HIV variants [116]. These variants may then replicate to high titers and become the major viral phenotype in the patient. Patients in which drug-resistant HIV can be detected have been found to elicit an immune response to such drug-resistant variants [204, 205]. This may be an effect of increased load of the resistant virus, manifested as an increase in the dose of immunogens harbouring the mutated epitopes, which induce immune response of new specificities. Some of the drug-escape mutations, seen in resistant HIV have been found to coincide with T cell epitopes [206, 207]. This will potentially cause escape from both drug and immune pressures [205, 206]. However, the mutations may also result in new CTL epitopes that potentially may elicit a new immune response. The CTL epitopes may emerge by the introduction of new proteasomal cleavage sites in the HIV derived protein. Moreover, the introduction of drug-escape mutation may end up in presentation of an epitope by other MHC-I allelic proteins expressed by the patient [208]. This may induce new immune responses to the drug-resistant virus. In addition, the immune response against wild type virus may be cross-reactive, i.e. capable of recognizing both the wild type and drug-resistant virus [209, 210]. These findings [204-213] show that even when drug-resistant virus emerge, the patient can still elicit an immune response to those variants, either by a cross-reactive immune recognition by existing HIV-specific cells, or by the induction of new specific immune responses to

the drug-resistant HIV variants. By restoring the immunocompetence using antiretroviral drug treatment the immune responses can be further improved [211, 214]. Taken together, these findings form the fundament of the concept of vaccination against drug-resistant HIV [211, 215-218]. By combining therapy and vaccination against known drug-escape variants, the aim is to elicit strong immune responses that would suppress the resistant HIV from replicating. Such strategies may have an impact on the spread of drug-resistant HIV [219].

2.6.4 Escape mechanisms by HIV

HIV has evolved mechanisms to specifically interfere with host immune systems. The HIV Vif protein interacts with the APOBEC3G protein enhancing the proteasomal degradation of APOBEC3G. Thus, Vif prevents APOBEC3G from introducing lethal hypermutations into the viral genome [158, 220]. HIV Tat modifies the catalytic subunit of the immunoproteasome, and thus affects the generation and presentation of MHC-I-binding CTL epitopes [221]. Tat has also been found to interfere with apoptosis by down-regulating the cellular bcl-2 protein, and up-regulating the expression of the cellular bax protein [222]. These two cellular proteins normally inhibit (bcl-2) or induce (bax) apoptosis. Thus, the expression of Tat promotes apoptosis in infected cells. In addition, the viral protein Nef can selectively down regulate HLA class I (HLA-A and HLA-B) and CD4 proteins, without affecting expression of inhibitory NK cell ligands (HLA-C and HLA-E), which are important for cellular escape from NK killing [223, 224]. Consequently, these mechanisms protect virus-infected cells from being eliminated by the immune system. Moreover, although robust and strong immune responses are elicited during HIV infection, studies have shown that HIV-specific $CD8^+$ T lymphocytes are impaired in maturation and differentiation. This results in a reduced expression of perforin in response to HIV-specific antigens as compared to good perforin expression of cytomegalovirus specific $CD8^+$ T-cells [225-228]. This results in a less efficient killing of HIV infected cells.

Furthermore, the ability of HIV to establish a latent infection that may be non-productive for years [81], makes it difficult for the immune response to eradicate all infected cells. Thus, a reservoir of HIV infected cells that can maintain the infection is preserved. The reservoirs may be located at immunologically privileged sites e.g. in microglia cells of the central nerve system. Next, the preference in infecting $CD4^+$ T lymphocytes makes the infection devastating to the host. The infection and depletion of CD4 cells, will eventually result in susceptibility to opportunistic infections. Further, the error-prone replication [118], and rapid evolution of HIV [120, 229], generate swarms of slightly different virus variants. A few virions may adapt and escape immune responses [230-234] or antiretroviral drugs [109]. Moreover, the rapid variation in the hypervariable loops of gp120 may decrease the binding of anti-HIV antibodies to the viral surface protein [233, 234]. Together, these escape mechanisms may result in a burst of viral replication and a spread of the infection.

3 HIV VACCINES

3.1 NATURAL RESISTANCE

There are individuals that show a decreased HIV-1 susceptibility and that remain HIV uninfected despite being constantly exposed to the virus (i.e. neither HIV RNA or proviral DNA, nor antibodies against HIV can be detected in their blood). The underlying reason for this natural resistance remains to be determined, but both innate immune responses (e.g. increased chemokine production at mucosal sites), and adaptive immune responses (both T-helper and CTL responses as well as secretory IgA production), have been suggested to play a role [235-241]. In addition, there are genetic factors for resistance to HIV infection. Individuals with a 32 amino acid deletion in the CCR5 gene have been found to be resistant to HIV-1 strains using that co-receptor for entry [242]. Also, an HIV infected leukaemia patient that received a bone marrow transplant from a CCR5-deficient donor was recently described [243]. 20 months following the transplantation, HIV was still not detectable. Moreover, slower disease progression has been associated with expression of certain HLA alleles, such as HLA-B27 and HLA-B57 [244-246].

Some HIV-1 infected individuals show differences in their ability to control their infection without treatment. Studies of the so-called long-term non progressors (with viremia below 5000 RNA copies/mL), or elite controllers (no detectable viremia), have shown that they possess strong T-helper proliferative and cytotoxic T lymphocyte responses to HIV antigens, and that disease progression was associated with decline of these responses [247-250].

Together, these results show that virus-controlling immune responses to HIV infection can be achieved. However, the lack of correlation with protection against HIV infection makes it difficult to develop vaccines since the properties of the immune responses are multiple and not always the same.

3.2 VACCINE APPROACHES

Ideally, an HIV vaccine should induce sterilizing immunity against HIV infection. This can only be achieved by inducing neutralizing antibodies that prevent HIV to bind to and infect its target cells. As been previously mentioned, evidence that such neutralizing antibodies work comes from experiments with passive immunization of macaques with broadly neutralizing antibodies prior to intravenous SHIV-89.6PD [192], or vaginal SHIV-162P4 [251] challenge. Sterilizing immunity was achieved in half of the passively immunized animals that received a combination of three broadly neutralizing antibodies [192], or in all monkeys receiving a high dose of one broadly neutralizing antibody [251]. In addition, a reduction in plasma viremia was observed in some immunized monkeys. One other study showed good protection against homologous HIV-2 or SIV challenges of monkeys passively immunized with pooled sera from HIV-2 or SIV infected animals, respectively [252]. Inducing such antibodies by immunization is, however, hard. The extensive glycosylation, the exposure of antigenic sites following conformational changes of gp120 and hypervariability of structural loops of gp120 are some of the obstacles that need to be overcome before a vaccine inducing broadly neutralizing antibodies could be developed [253]. Also, these antibodies need to be present at the port of entry, for instance in the genital mucosa.

A suboptimal HIV vaccine may reduce the viral peak following the primary infection and lower the viral load set point. As this set point is a predictive marker for disease

progression [254], such a vaccine may improve the clinical prognosis. Moreover, since HIV viral load has been correlated with an increase risk of HIV transmission [103, 104], a suboptimal vaccine, reducing viral load, may have an impact on limiting the spread of HIV. As was discussed previously, the decline in viral load has been associated with the development of $CD8^+$ T cell responses against HIV in humans [175, 177-179], and against SHIV and SIV in monkeys [184-187]. These evidences suggest that a vaccine that induces potent $CD8^+$ T cell responses could limit the infection.

Over the years, many vaccine approaches and candidates have undergone preclinical trials in different animal models [255, 256]. When evaluated in clinical trials, they have appeared to be safe, well tolerated and immunogenic.

3.2.1 Live attenuated vaccines

Several vaccines used in childhood are based on live attenuated virus. These vaccines can potentially elicit both strong humoral and cellular immune responses by only a few vaccinations. For example, the highly effective trivalent measles, mumps and rubella virus vaccine included in the childhood immunization program is administered twice. It gives an almost lifelong immunity against these viruses. This suggests an attenuated HIV as a vaccine option. A unique cohort of patients (The Sydney blood bank cohort) was accidentally infected by blood transfusion with a *nef*-deficient, attenuated HIV-1 strain. Despite their infection, these patients maintained stable CD4 counts indicating that the *nef*-defective HIV strain was less pathogenic. However, in a long-term follow-up, some of the patients showed signs of immune damage [257, 258]. Experiments with live attenuated SIV vaccines, where Nef and/or Vpr deletions were introduced, were shown to protect monkeys against challenge with SIV or SHIV strains. In the follow-up, some of the animals showed, however, signs of disease or had developed AIDS [259-261]. These studies demonstrate the risk of reversion of attenuated HIV strain to become pathogenic. Thus, the use of attenuated HIV vaccines is not applicable in humans for safety reasons.

3.2.2 Recombinant vaccines

Two of the existing recombinant protein-based vaccines are those against Hepatitis B and Human papilloma virus. The vaccines induce cellular, but mainly humoral immune responses that prevent the viruses from infecting the host. This approach was extensively evaluated for HIV vaccines. In an early therapeutic vaccine trial in asymptomatic non-treated HIV-1 infected patients, the potential to use recombinant gp120 protein of HIV-1 was investigated. An increase in $CD4^+$ T cell count, and a two year improved survival was demonstrated for the vaccinated group, as compared to the placebo group [262]. Long-term follow-up also revealed an increase in the central memory $CD4^+$ T cell population and an increased expression of the immune activation markers HLA-DR and CD38 in the vaccinated group [263]. Other studies using gp120 protein as immunogen showed potential to induce neutralizing antibodies, mainly effective against laboratory adapted HIV strains [264, 265]. One of these gp120-based vaccines was evaluated in an efficacy clinical trial. However, in the field trial the vaccine failed both in preventing HIV infection and in reducing the viral load in infected individuals [264, 266-268].

3.2.3 Genetic vaccines

Over the last decades, the use of genetic material as vaccines has been the focus of many research groups, including ours. DNA vaccines were early shown to induce protective immunity against lethal challenge with influenza in both mice and chickens [269, 270]. Since then, DNA vaccination has been found to induce strong humoral and

cell-mediated immune responses that resulted in protection against other infectious diseases and cancer in different animal models [271, 272]. Despite these promising preclinical results, no genetic vaccine is licensed for human use due to the limited success in inducing strong immune responses in humans [273]. There are, however, four DNA vaccines licensed for veterinarian use [272].

More advanced vaccine strategies use genetic vaccines in heterologous prime-boost settings. In such a setting, one vaccine component is used to prime a specific immune response and a second vaccine is used to boost the response. Using DNA vaccines to prime an immune response prior to a viral vector boost, has been found to enhance the potential of DNA vaccines in preclinical and clinical settings [274-279]. Commonly used viral vectors for heterologous prime-boost vaccine strategies are adenovirus type 5 and attenuated poxvirus, fowlpox, and canarypox virus vectors. The use of vaccinia derivatives, such as modified vaccinia Ankara and NYVAC, is also common [280]. These vectors are genetically related to cowpox. The strategy of using viral vector as vaccine component was tested in a proof-of-concept trial in high risk populations. The trial evaluated the concept of eliciting cell-mediated immune responses to prevent HIV infection. The set up of the trial included three vaccinations of healthy HIV seronegative volunteers with replication-defective recombinant adenovirus 5 vectors each expressing HIV-1 *gag*, *pol* or *nef* genes [253, 281, 282]. The trial was stopped by the data safety and monitoring board due to futility. The vaccine did not induce protective immune responses against HIV infection. It did not either reduce the viral set point in infected individuals. More so, a trend to an increased risk of HIV infection was observed in vaccinated individuals with high pre-existing adenovirus 5 immunity [253, 281-283].

3.2.4 Peptide-based vaccines

The advantages of the peptide-based vaccines are the ease in production of high amounts of pure synthetic peptides, and the ability to quickly change the amino acid composition of a peptide. However, peptides are generally less immunogenic than proteins. To circumvent this problem, early studies evaluated the use of carrier-proteins or strong adjuvants. Peptides were mixed or linked to T-helper epitopes to enhance the immunogenicity of the peptides. The peptide-specific immune responses were however limited, following immunization with such peptide-based vaccines [284, 285]. On the other hand, it was possible to induce neutralizing antibodies against laboratory HIV-1 strains [286-289], and to protect mice from infection with recombinant vaccinia expressing HIV-1 gp160 [290] after immunization with peptides mixed with more potent adjuvants (Cholera toxin or Freund's complete/incomplete adjuvant).

Other approaches that have been evaluated to increase the immunogenicity of the peptide-based vaccines include the improvement of the peptide binding to MHC molecules [215, 291], or the enhancement of the peptide delivery to immune cells either by pulsing dendritic cells [292, 293], or red blood cells with peptide cocktails [213]. It was found that enhanced MHC binding of a peptide correlated with better immunogenicity of the peptide. Immunization of monkeys with dendritic cells pulsed with a cocktail of peptides, induced vaccine-specific immune responses that controlled a pathogenic SHIV89.6P challenge [292, 293]. Moreover, by using red blood cells pulsed with peptides containing drug-resistance mutations, it was possible to elicit peptide-specific immune responses in SHIV infected macaques. Altogether, these studies suggest that optimizing the MHC binding of a peptide and by combining an effective peptide delivery method with a potent adjuvant, it might be possible to develop a peptide-based vaccine for human use.

Additional problems that need to be addressed when designing peptide-based vaccines is the polymorphic nature of the MHC class I allelic proteins among humans, and the preference for specific amino acid residues at anchor positions requested for proper peptide binding to a specific MHC molecule. To overcome the limitations of a peptide-based vaccine due to MHC restriction, several individual or overlapping epitopes in peptide-vaccine mixtures have been used [294, 295]. Using such peptide mixtures for immunization of mice it was possible to elicit peptide-specific immune responses in mice with different MHC haplotypes. Moreover, using longer lipopeptides for vaccination, peptide-specific immune responses has been elicited in HIV infected patients expressing different MHC allelic proteins [296]. Thus, by using overlapping peptide cocktails or longer peptides representing epitopes from HIV as vaccines, it may be possible to develop a peptide-based vaccine that induces vaccine-specific immune responses in individuals expressing different MHC proteins.

In the goal to find a universal HIV vaccine it will, however, be unlikely that a peptide-based vaccine would be given alone. Such a vaccine will most likely be included in a heterologous prime-boost vaccine setting, where the potency of peptides to induce, or enhance CTL responses to specific epitopes is considered. One setting where such a vaccine would be beneficial is the targeting of drug-resistant HIV. Combining the knowledge on MHC diversity between different ethnical populations [8, 297], with HIV vaccine development, it may be possible to develop peptide mixtures specific for different geographic regions. For example, a peptide-based vaccine to be used in Caucasians may include epitopes restricted for binding to MHC-A1, A2, B7 and B44 supertypes, whereas a vaccine including peptides restricted to A2, A3, A24 and B7 supertypes may be more efficient in eliciting vaccine-specific immune responses in some black populations [8].

4 AIMS

The overall aim of the thesis has been to evaluate vaccine strategies targeting drug-resistant HIV-1. The specific objectives were:

- To explore differences in antigen presentation of the wild type and drug-resistant HIV epitope variants (Paper I; Manuscript)
- To evaluate the ability to target drug-resistant HIV-1 by minimal epitope-based DNA vaccines (Paper II).
- To evaluate different peptide delivery strategies to improve vaccine-induced immune response (Papers III-V).
- To compare how the wild type and drug-resistant HIV-1 reverse transcriptases are degraded by the proteasome and to investigate the possibility to promote such degradation by modifications of the proteins (Papers VI and VII).
- To assess if targeting the proteasome makes HIV-1 reverse transcriptase a better immunogen than the wild type, when delivered by DNA vaccination (Paper VII).

5 RESULTS & DISCUSSION

5.1 SELECTION OF RT AND PROTEASE PEPTIDES

Most of the mutations that occur during antiretroviral drug treatment are located in the protease and reverse transcriptase regions of the HIV *pol* gene. A strong immune response against such mutated epitopes may be beneficial for a patient. To trigger such a response, we have chosen epitopes from regions in RT and PR that harbour common drug-resistance mutations [116] and evaluated them as vaccine components. We focused on CTL-epitopes, as $CD8^+$ T lymphocyte responses have been shown to be important for containing HIV infection. The described drug-resistance mutations of RT and PR ([298]; http://resdb.lanl.gov/Resist_DB) were incorporated into short peptides, including hydrophobic amino acid residues at the second and/or ninth position. In evaluation of the concept of targeting drug-resistant HIV by a vaccine, we have to select a certain HLA allele. We have selected HLA-A0201 restricted epitopes since this allele is frequent in the Caucasian population [8, 297], and we had access to a HLA-A0201 transgenic murine model [299, 300], in which our vaccine approach could be evaluated. The epitopes chosen are summarized in Table 4.

Binding of peptides to HLA-A0201 molecules was evaluated *in vitro*, using a peptide stabilization assay [301-303]. The human T lymphocyte cell line T2, defective in the *tap* genes, was incubated with exogenous peptide and peptide binding was measured as stabilization of HLA-A0201 molecules on the cell surface. The ability of a peptide to bind the HLA protein was correlated with the up-regulation of surface expressed HLA molecules. Using this method, we evaluated ten different HIV-1 CTL epitopes and their drug-resistant mutants for binding to HLA-A0201 (Table 4). A negative mock-peptide control [304], $Nef_{158\text{-}166}$ KGENNCLLH (KH9), was included in all experiments, and verified by our method as non-binding. The peptide-specific mean fluorescence intensity (MFI) was divided by the MFI of unexposed or mock treated cells. The ratio indicate how well the peptide binds to HLA-A0201 complexes [305]. The well-characterized HLA-A2 restricted peptide SLYNTVATL (SL9) from HIV p17 was used as an assay control. Based on the ratio between MFI_{SL9} and MFI_{KH9} at the highest peptide concentration, we ranked the epitopes to be: strongly (a ratio of $\geq$1.6), intermediately (a ratio >1.1 and $\leq$1.5) or non- (a ratio <1.1) associating to HLA-A0201 molecules.

Three of the ten epitopes (two of which were mutated) demonstrated strong binding capacity to HLA-A0201 (Table 4, bold numbers). In four other epitopes, some mutated variants showed intermediate or strong binding, whereas the wild type peptide did not bind the HLA-A0201 molecule. The other three regions did not bind to HLA-A0201 molecules in our assay. Interestingly, all four of the drug-resistant variants of the HIV-1 protease derived epitope ($PR_{75\text{-}84}$) had an increased binding and may therefore enhance the immunogenicity of the epitope (Table 4, Paper III).

Table 4. *In vitro* peptide binding to HLA-A0201 assessed by the stabilization assay.

			Relative ratio of peptide binding[A] Peptide concentration (μM)				
Sequence	**Epitope**	**Mutation**	**100**	**10**	**1**	**0.1**	**HLA restriction of epitope [B]**
ALVEICTEM	RT_{33-41}	-	**2.5**	**2.0**	1.4	1.1	A2, A0201, A3
ALVEICTE**L**		M41L	**2.6**	**2.3**	**1.6**	1.2	A2, A0201, A3
NTPVFAIKK**V**	RT_{57-66}	K66V	1.0	0.9	0.9	0.9	A3 supertype, A68, A6801, A11, B8
N**L**PVFAIKKV		T58L	1.0	0.8	0.9	1.0	A3 supertype, A68, A6801, A11, B8
NTPVF**V**IKK**V**		A62V / K66V	1.0	1.0	1.0	1.0	A3 supertype, A68, A6801, A11, B8
NTPVFAIK**RV**		K65R / K66V	0.9	0.9	1.0	0.9	A3 supertype, A68, A6801, A11, B8
GIPHPAGLKK	RT_{93-102}	-	1.0	1.0	1.0	0.9	A11, A3, A0301
GIPHPAGL**E**K		K101E	0.9	0.9	0.9	0.9	A11, A3, A0301
GIPHPAGLK**V**		K102V	1.0	1.0	1.0	0.9	A11, A3, A0301
PAGLKKKKSV	RT_{97-106}	-	1.0	0.9	0.9	0.9	
PAG**I**KKKKSV		L100I	1.0	0.9	0.9	0.9	
PAGL**E**KKKSV		K101E	0.9	0.9	0.9	0.9	
PAGL**I**KKKSV		K101I	0.9	0.9	0.9	0.9	
PAGLKK**E**KSV		K103E	0.9	0.9	0.9	0.9	
PAGLKK**Q**KSV		K103Q	0.9	0.9	0.9	0.9	
VIYQYMDDL	$RT_{179-187}$	-	1.1	1.0	0.9	1.0	A2, A0201, A0202
IYQYMDDLYV	$RT_{180-189}$	-	1.3	1.1	1.0	1.0	A0201
VI**C**QYMDDL		Y181C	1.1	1.1	1.1	1.1	A2, A0201, A0202
VIYQY**V**DDL		M184V	1.5	1.2	1.0	1.0	A2, A0201, A0202
VI**C**QY**V**DDL		Y181C / M184V	1.1	1.0	1.0	1.0	A2, A0201, A0202
IYQY**V**DDLYV		M184V	1.4	1.1	1.0	1.0	A0201
IYQYMDDL**C**V		Y188C	1.4	1.1	1.0	1.0	A0201
LLRWGLTTPDKK	$RT_{209-220}$	-	1.1	1.1	1.0	1.1	A2, A0201
LLRWGLTTP	$RT_{209-217}$	-	1.0	0.9	0.9	1.0	Part of A2, A0201
GLTTPDKK	$RT_{213-220}$	-	0.1	0.9	0.9	ND	
LLRWGL**F**TPDKK		T215F	1.0	1.0	1.0	1.0	A2, A0201
LLRWGLTTPD**Q**K		K219Q	1.0	1.0	1.0	1.1	A2, A0201
LLRWGL**F**TPD**Q**K		T215F / K219Q	1.0	1.0	1.0	1.0	A2, A0201
LLRWGL**Y**TPV		T215Y	1.3	1.0	0.9	0.9	A2, A0201
GL**Y**TPDKK**V**		T215Y / H221V	1.2	1.0	0.9	0.9	
ILKEPVHGV	$RT_{309-317}$	-	**2.8**	**2.3**	1.5	1.1	A2 supertype, A2, A0201, A0202, A0205
VTLWQRPLV	PR_{3-11}	-	0.9	1.0	0.9	ND	A74, A28 supertype, A28, A2, A19, A7401, A6802
VTLWQRP**R**V		L10R	1.1	1.0	0.9	ND	A74, A28 supertype, A28, A2, A19, A7401, A6802
VTLWQRP**V**V		L10V	1.0	1.0	0.9	ND	A74, A28 supertype, A28, A2, A19, A7401, A6802
VTIKIGGQLK	PR_{11-20}	-	1.0	1.0	1.0	0.9	A3 supertype
VTIKIGGQL**M**		K20M	0.9	0.9	1.0	0.9	A3 supertype
VTIKIGGQL**R**		K20R	0.9	0.9	1.0	0.9	A3 supertype
IGGQLKEAL	PR_{15-23}	-	1.0	0.9	0.9	ND	
IGGQL**R**EAL		K20R	0.9	09	0.9	ND	-
KMIGGIGGF	PR_{45-53}	-	1.0	1.1	1.0	ND	A2 supertype
KMIGG**V**GGF		I50V	0.9	0.9	0.9	ND	A2 supertype
KMI**V**GIGGF		G48V	1.0	0.9	0.9	ND	A2 supertype
K**I**IGGIGGF		M46I	1.1	1.1	0.9	ND	A2 supertype

Table 4. *In vitro* peptide binding to HLA-A0201 assessed by the stabilization assay.

			Relative ratio of peptide binding[A] Peptide concentration (μM)				
Sequence	**Epitope**	**Mutation**	**100**	**10**	**1**	**0.1**	**HLA restriction of epitope [B]**
K**L**IGGIGGF		M46L	1.2	1.1	0.9	ND	A2 supertype
KMIGGIGG**V**		F53V	**1.8**	**1.6**	1.1	1.0	A2 supertype
VLVGPTPVNI	PR_{75-84}	-	**2.0**	**1.7**	1.2	1.0	A2 supertype, A2, A0201, A0205
VLVGPTPVN**V**		I84V	**2.6**	**2.2**	**1.5**	1.1	A2 supertype, A2, A0201, A0205
VLVGPTP**A**NI		V82A	**2.3**	**1.8**	1.2	1.1	A2 supertype, A2, A0201, A0205
VLVGPTP**F**NI		V82F	**2.3**	**1.9**	1.4	1.1	A2 supertype, A2, A0201, A0205
VLVGPTP**F**N**V**		V82F / I84V	**2.6**	**2.3**	**1.6**	1.2	A2 supertype, A2, A0201, A0205
RGPGRAFVTI	$Gp160_{311-320}$	-	1.3	1.1	1.1	1.1	A2, A0201
SLYNTVATL	$p17_{77-85}$	-	**2.4**	**1.9**	1.3	1.1	A2. A0201, A0202, A0205, A0214
KGENNCLLH	$Nef_{158-166}$	-	1.0	1.0	1.0	1.0	

[A] Relative ratio of peptide binding: $MFI_{test\ peptide}$ / $MFI_{mock\text{-}peptide}$
A relative ratio of: 0≥ Non-binding <1.1; 1.1≥ Intermediate binding ≤1.5; Strong binding ≥1.6.
Bold numbers are classified as strongly HLA-A0201 associating epitopes (a relative ratio ≥1.6).
[B] From reference [306]. ND=Not done. MFI = mean fluorescence intensity

5.2 DRUG-RESISTANCE MUTATIONS ALTER BINDING, AFFINITY AND OFF-RATE OF PEPTIDES TO HLA ALLELES A0201 AND A2402 (PAPER I)

Basic knowledge of how epitopes from HIV-1 proteins are presented by and to the immune cells is required for understanding the immunological features of HIV infection. Such information can be implemented in the design and development of vaccines targeting HIV. Knowledge on how the introduction of drug-resistance mutations affects the antigen presentation, and how that changes over time, is of importance for the understanding of how the patient's respond to emerging viral variants. In this study we aimed to find how HIV-1 epitopes from HIV RT and PR were presented by HLA class I molecules and how this antigen presentation was affected by the introduction of drug-resistance mutations.

Some of the epitopes (RT_{33-41}, $RT_{179-187}$, PR_{75-84}) evaluated by the stabilization assay, see *Section 5.1* and Table 4, were also selected and evaluated for binding, affinity and off-rate from HLA-A0201 and A2402 using the *iTOPIA*™ assay. *iTOPIA*™ 96-well plates were coated with recombinant HLA-A0201 or HLA-A2402 molecules. To ensure correct folding, the different HLA molecules were co-incubated with placeholder peptides (known to bind the different HLA proteins) as well as an external β_2-microglobulin. Peptide binding was measured by unfolding the coated HLA molecules, releasing the placeholder peptides and β_2m, and then adding the peptide of interest together with new β_2m. The HLA proteins were allowed to re-fold, which only occurs if the added peptide can bind to the HLA molecule. Re-folded HLA molecules were stained with a fluorescently labelled antibody, which recognized the properly folded peptide/HLA/β_2m complex. The relative fluorescence intensity of each of the peptides was used to calculate the percent binding of the test peptide as compared to binding of the specific HLA allelic protein controls. Affinity measurements were performed by peptide titrations and the calculation of the effective dose 50 (ED50). ED50 was defined as the peptide concentration at which 50% of the initial binding was achieved. Off-rate was defined as the time when peptide binding had been reduced two-fold, i.e. 50% of the initial binding. It was defined by adding the peptide to the HLA molecules and measuring the binding at eight different time points. The $t_{1/2}$ (h) was extrapolated from the curves depicting these time points.

Most of the mutations conferring drug-resistance did not affect HLA-A0201 binding of the peptides much, as measured by the *iTOPIA* assay. However, one of the mutations (Y181C of $RT_{179-187}$) did not affect binding to A0201 proteins, but reduced the affinity of the peptide to the HLA molecule by one $\log_{10}$. At the same time, the dissociation of this peptide from the HLA molecule was reduced, i.e. the off-rate was prolonged. Another mutation (V82F/I84V of PR) did not affect binding, or affinity to the HLA-A0201 molecule, but stabilized the peptide/HLA complex as compared to the wild type sequence. We could also detect cross-binding of the HLA-A0201 restricted epitopes to the A2402-allelic protein. This was true for both the wild type and the drug-resistant epitope variants. This may be partly explained by an overlap in preferred amino acid residues in anchor positions between the two HLA supertypes A2 and A24. Introducing the drug-resistance mutations into the epitopes had a more pronounced effect upon binding of the epitopes to HLA-A2402 than to HLA-A0201. In addition, the mutations had multiple effects on affinity; some reduced, other enhanced the affinity to the A2404 molecules by two $\log s_{10}$, some did not affect affinity as compared with the wild type epitope.

Thus in our assay, introduction of mutations conferring drug-resistance does not affect the HLA binding of the epitopes in a consistent way, at least not in case of binding to HLA-A0201 or A2402 proteins. One can only speculate how a prolonged association of a mutated peptide will affect the immunogenicity of that peptide. The observation that a sustained MHC-I/TCR interaction was needed for activating naïve T cells [58], suggests that the longer association of the mutant peptide would result in an enhanced activation of that T cell subset. In line with this observation, the reduced dissociation of the PR_{75-84} mutant epitopes (I84V and V82F/I84V) from HLA-A0201 protein suggests that these variants would be more immunogenic than their wild type prototype. A comparison of the immune response to HIV-1 epitopes in chronically infected untreated or treated viremic patients was made [307]. The authors found a discrepancy in the immune response against SL9 (Gag_{77-85}) and PR_{76-84} between the two groups. In chronically infected but untreated patients, the main immune response was detected against SL9, whereas in the treated viremic patients the immunodominant response was directed against PR_{76-84}. This discrepancy in epitopes to which the dominant immune response was elicited may in part be explained by the occurrence of drug-resistant HIV and an enhanced presentation of epitopes harbouring mutations. The introduction of resistance mutations in the PR derived epitope may result in a prolonged presentation of that particular epitope to naïve T cells and consequently be a reason for the strong immune response observed. This is in line with our findings of a more stable PR_{75-84}/MHC complex when V82F and/or I84V mutations were introduced.

5.3 ENHANCED HLA BINDING DOES NOT NECESSARILY LEAD TO ENHANCED IMMUNOGENICITY (PAPER II)

Based on the knowledge that antiretroviral treatment of HIV-1 infected patients selects for drug-resistant viral variants over time [116], we aimed to design a vaccine candidate with the potential to target drug-resistant as well as the wild type HIV-1.

Epitopes in different concentrations were added to T2 cells, and binding to HLA-A0201 was evaluated, see *Section 5.1* and Table 4. Five of the epitopes (RT_{33-41}, $RT_{179-187}$, $RT_{209-220}$, PR_{75-84}, $RT_{309-317}$ of either wild type or mutant variants) were selected to be evaluated as vaccine components in a minimal-epitope based DNA vaccine. The epitopes were selected harbouring key mutations associated with drug-resistance to NRTIs and PIs [116, 117], being restricted to HLA-A0201 [306], and being naturally

immunogenic in HIV infected patients [204-206, 308, 309]. The corresponding nucleotide sequences of the epitopes were linked together, using the nucleotide sequence of lysine as a linker. The lysine linker was included to potentially enhance the proteasomal cleavage after the epitopes following expression [310]. A T helper epitope from tetanus toxoid (TT_{88-102}) was included in the constructs to potentially increase the immunogenicity of the DNA-encoded product [311]. These DNA constructs, mixed with the granulocyte-macrophage colony stimulating factor, or the individual peptides, in emulsion with incomplete Freunds adjuvant (IFA), were used for immunization of HLA-A0201 transgenic mice. Immune responses elicited by different wild type and drug-resistant epitope variants were assessed by the IFN-γ ELISpot assay.

Table 5. Immune reactivity to HIV-1 derived epitope PR_{75-84} and variants.

Immunogen	PR peptide used for *ex vivo* stimulation				
	$_{75}$VLVGPTPVNI$_{84}$	$_{75}$VLVGPTP**F**N**V**$_{84}$	$_{75}$VLVGPTPVN**V**$_{84}$	$_{75}$VLVGPTP**A**NI$_{84}$	$_{75}$VLVGPTP**F**NI$_{84}$
PR wt ($_{75}$VLVGPTPVNI$_{84}$)	*5[a] (232[b])*	2 (45)	5 (192)	3 (79)	1 (56)
PR d.mut ($_{75}$VLVGPTP**F**N**V**$_{84}$)	1 (34)	*4 (151)*	3 (98)	2 (46)	4 (171)
RT/PR wt DNA	*5 (484)*	4 (250)	5 (483)	5 (303)	3 (197)
RT/PR d.mut DNA	2 (61)	*3 (192)*	1 (56)	2 (68)	3 (146)
Naive	0 (6)	0 (3)	0 (13)	0 (7)	0 (13)

wt: wild type; d.mut: double mutant; PR: protease; RT: reverse transcriptase; values in italic: homologous *ex vivo* stimulation.

a Numbers of responding animals per group (n=5). Cut off is set to >50 SFC/million splenocytes after the response to the irrelevant HIV-1 Nef peptide $_{159}$KGENNCLLH$_{167}$ has been subtracted.

b Net mean SFC per million splenocytes (non-responding animals included).

As shown in Table 4 (page 29), three of the chosen epitopes bound strongly to the HLA-A0201 protein. As commented, the most pronounced effect was detected for the PR_{75-84} epitope where all mutants showed an increased capacity to bind HLA-A0201. Next, we investigated whether increased binding of the mutant peptide correlated with enhanced immunogenicity. When immunizing transgenic mice with the different DNA vaccines, we detected only an immune response against the PR derived epitope. The underlying reason for this is unclear. It might be that the protease derived epitope is immunodominant over the other epitopes encoded within the same construct. Another explanation may be that the PR derived epitope was located in the centre of the expressed product and the peptide better cleaved out by the proteasome following degradation. The most potent immune response against the PR derived peptides was detected in the group that received the wild type construct (Table 5). This response was cross-reactive to all PR epitope-variants. The fact that we detected the strongest immune response when immunizing with the construct encoding wild type epitopes was unexpected, as we thought that the mutant peptides would be better immunogens due to better HLA-A0201 binding.

The relatively weak response detected in the peptide immunized group was unexpected. However, a suboptimal immunization strategy (intramuscularly) may be an explanation. In previous immunization studies using the wild type or mutant peptide in emulsion with IFA, the peptides were found to be highly immunogenic. In those studies the peptides were injected subcutaneously, suggesting that such immunization was more efficient in eliciting peptide-specific immune responses (unpublished data).

Thus, the enhanced binding of the mutant PR peptide did not elicit a stronger immune response than that evoked by the wild type sequence. An important finding was, however, development of cross-reactive immune responses against the wild type and

drug-resistant epitope variants in all groups. This suggests that our immunogen may be used to target both the wild type and mutated epitope variants.

5.4 LINKAGE OF PEPTIDE TO ERYTHROCYTES FOR INCREASING VACCINE DELIVERY DOES NOT COMPENSATE FOR THE LOW AMOUNT OF LOADED ANTIGEN (PAPER III)

The previous finding that we could only elicit a moderate epitope-specific immune response by peptide immunization made us search for a more efficient way to enhance the delivery of peptides. Optimally such approach would be applicable to human use.

The wild type or the doubly mutated variant (V82F/I84V) of the protease derived epitope PR_{75-84} were biotinylated and linked to syngeneic erythrocytes by biotin-streptavidin bridges. The erythrocytes were exposed to a moderate chemical treatment to make them appear as aging, and thus be recognized by phagocytic cells, and become rapidly cleared from the circulation [312, 313]. HLA-A0201 transgenic mice were immunized with the erythrocytes or the individual peptide in IFA, and immune responses were assessed by IFN-γ ELISpot assay.

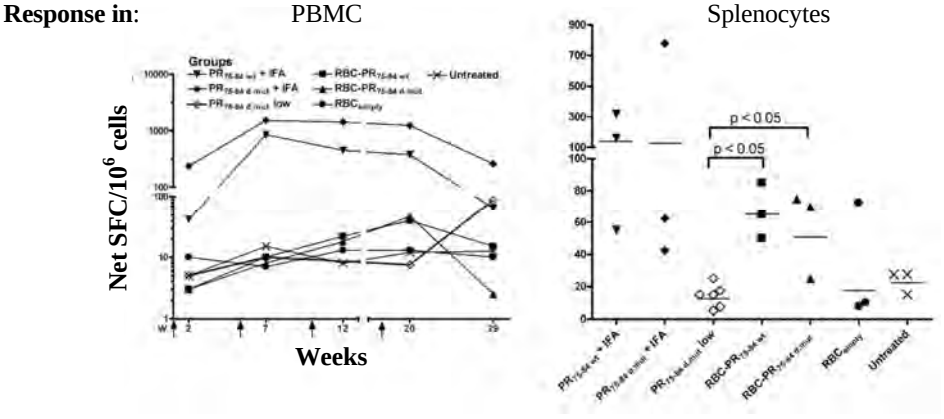

Figure 6. Immune response to the mutated epitope PR_{75-84} V82F/I84V. Arrows indicated time of immunization.

Immunization with the peptide (50 μg) in IFA induced strong immune responses detected 10-12 days after each immunization. This response peaked after two immunizations (Figure 6, PBMC). The response diminished over a ten week period following the last immunization. A weak response could be detected ten weeks after the last immunization in the group that was immunized with the peptide linked to erythrocytes (Figure 6, Splenocytes). This response was significantly stronger than that generated by the peptide alone in a low dose (5μg). The dose of the peptide in solution was 500-fold higher than the dose used for the peptide-erythrocyte immunization. The fact that we could detect a response following peptide-erythrocyte immunization ten weeks after the last injection suggests that this method of delivery may increase the immunogenicity of the peptide better than the peptide alone. This is most likely achieved by improving delivery of the peptide to macrophages, and consequently enhancing the peptide presentation to T cells.

5.5 A STRONG AND CROSS-REACTIVE IMMUNE RESPONSE CAN BE ELICITED UPON IMMUNIZATION WITH FUNCTIONAL PENTAMERIC

FUSION PROTEINS OF CTB AND MUTATED HIV-1 EPITOPES (PAPERS IV AND V)

We also aimed to enhance cellular responses against epitopes harbouring drug-induced mutations by fusing mutant epitopes deriving from HIV-1 reverse transcriptase (Paper IV) or protease (Paper V) to the B subunit of Cholera toxin (CTB). This unit is non-toxic and is responsible for the binding of the cholera toxin to the target cell monosialoganglioside receptor, GM1.

The nucleotide sequences of the epitopes from reverse transcriptase (RT_{33-41}M41L) or protease (PR_{75-84}I84V or V82F/I84V) were genetically linked to the 3' end of the nucleotide sequence of CTB. The expressed fusion proteins were purified by column chromatography, and characterized by SDS-PAGE followed by Comassie staining, Silver staining, or by Western blot analysis. Binding capacity of the fusion proteins to the native receptor of CTB, was measured with affinity column and Biacore techniques. To circumvent difficulties in production of pentameric CTB-PR fusion protein, the sequential five or ten amino acids of HIV-1 PR were added (Paper VII). HLA-A0201 transgenic mice were immunized with the fusion proteins, a mix of rCTB and peptides representing the epitopes, or peptides alone. Immune response to the mutant and the wild type epitope variant was assessed by IFN-γ ELISpot.

The purified CTB-RT_{33-41}M41L fusion protein, showed to form functional pentamers that were able to bind to GM1. In contrast, the CTB-PR_{75-84}I84V fusion protein remained as a monomer and could not bind to GM1. However, after including five or ten extra amino acid residues to the C-terminal of the epitope, the resulting chimeric protein could form pentamers. We observed a discrepancy in binding capacity to GM1 by different CTB-PR fusion proteins. The fusion protein with a 20 amino acid residue long PR derived region, harbouring the I84V mutation (CTB-PR_{75-94}I84V), bound to GM1 as well as the native recombinant CTB. When the V82F mutation was introduced in the same fusion protein (CTB-PR_{75-94}V82F/I84V), a partial reduction of the GM1-binding was observed (74% binding capacity as compared to rCTB). The fusion protein with the 15 amino acid residue extension C-terminally of CTB (CTB-PR_{75-89}I84V), demonstrated a binding capacity of 93% as compared with rCTB. This PR epitope had only the I84V mutation introduced. Fusion proteins that bound well to GM1, elicited a strong immune response, whereas mixing rCTB with the peptide only stimulated a just detectable response (Figure 7A). Delivering a ten-fold higher concentration of the peptide alone (without adjuvant) was not sufficient to trigger an immune response (Paper IV).

The immune response induced by the fusion protein immunization cross-reacted with the wild type epitope sequence (Figure 7A). Interestingly, we detected a correlation between immunogenicity and the capacity of the chimeras to bind to GM1 (Paper V). Immunizing with the monomeric fusion protein elicited an immune response similar to that induced by the mixture of the peptide with CTB (Figure 7B). Long-term follow-up of immunized animals showed that immune responses persisted for over four months, and this response could readily be boosted with an additional late immunization.

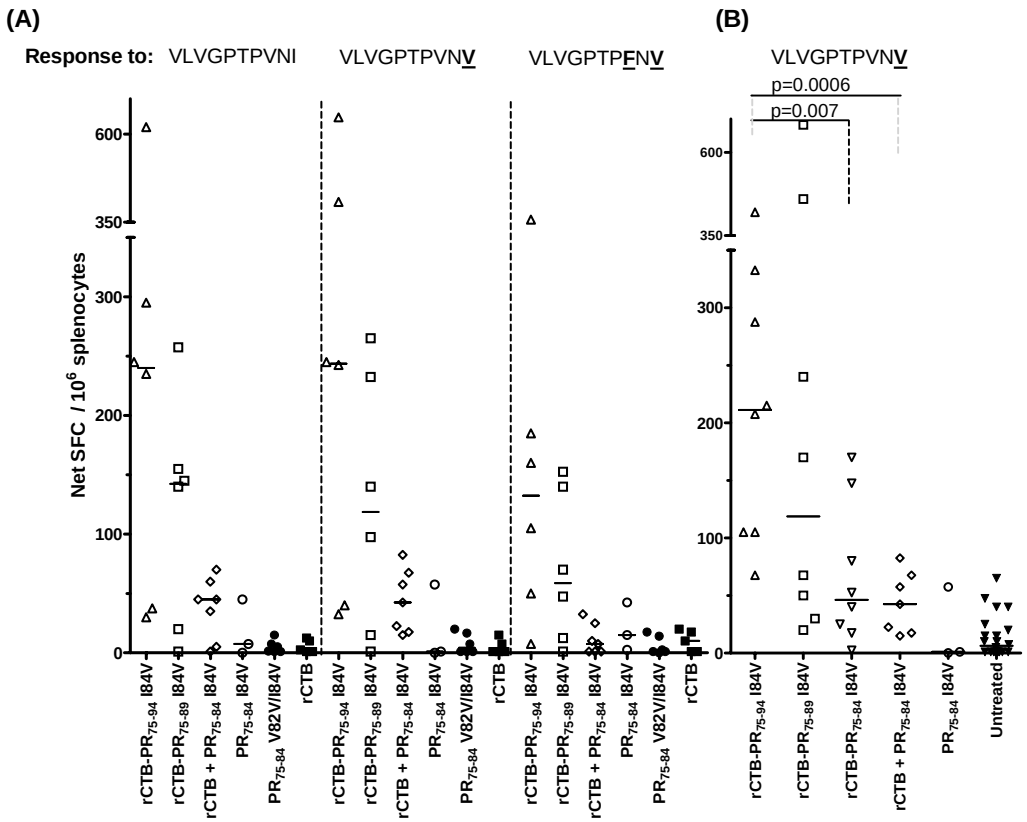

Figure 7. (A and B) Immune responses to PR epitope variants elicited by CTB-PR epitope fusion protein immunization.

Thus, by linking HIV peptides to the B subunit of cholera toxin it is possible to stimulate a strong and long-lasting immune response, significantly stronger than the response evoked by the peptide alone. Our findings also suggest that the binding between the rCTB-pentamer and the natural cellular receptor of CTB, is needed to enhance the immune response. This was evidenced by the adjuvant effect of CTB being related to the capacity of the fusion protein to bind to GM1. Moreover, immunizing with sequences containing a drug-induced mutation triggered a cross-reactive immune response against the wild type epitope in addition to the homologous response. The long-term persistence of the cellular immune response implies that the fusion proteins may be able to induce a peptide-specific memory response.

5.6 DRUG-RESISTANCE MUTATIONS TARGET HIV-1 REVERSE TRANSCRIPTASE TO PROTEASOMAL DEGRADATION (PAPER VI)

Earlier observations showed that drug-resistant variants of HIV-1 reverse transcriptase were more rapidly degraded than the wild type RT [314]. Since all RT genes were expressed under similar conditions, it is likely that the differences in the protein expression were due to the biological characteristics of the proteins. One can speculate

that the introduction of mutations to a protein during drug-treatment may change the properties of that protein, such as folding, or the intracellular processing pathway. Such changes may affect the generation of antigenic peptides and their further presentation. In the present study we investigated the role of the proteasome in the degradation of HIV-1 reverse transcriptase.

Cells (HEK293 and HeLa) were transiently transfected with plasmids encoding a wild type RT (wtRT) or a multi-drug resistant variant of RT (RT1.14). The cells were treated with proteasomal inhibitors MG132 or epoxomicin for 18 hours, or were left untreated. MG132 is a reversible inhibitor of the proteasome that affects chymotrypsin-like activity [315]. However, the inhibitor also affects other proteases like cathepsin and calpain. Epoxomicin on the other hand, inhibits the proteasome specifically and irreversibly, without affecting the activity of other proteases [316].

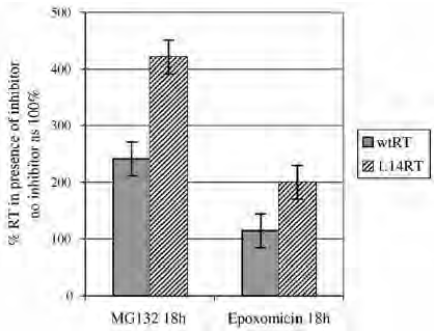

Figure 8. Stabilization of HIV-1 reverse transcriptase variants in the presence of proteasome inhibitors as compared to untreated RT expressing cells, in which expression was taken to be 100%.

Consistent with our previous findings [314], we observed that higher quantities of wtRT than of RT1.14 were detected in the untreated cells (25 fg/cell and 7 fg/cell, respectively). Adding the MG132 inhibitor led to a 2-2.5-fold increase in the amount of accumulated wtRT. Under the same condition, we detected a 4-fold accumulation of RT1.14. Treating the cells with epoxomicin had only a marginal effect on accumulation of wtRT, whereas a two-fold increase was detected for the multi-drug resistant RT-variant (Figure 8).

Stabilization of the wild type HIV-1 reverse transcriptase in the presence of MG132 and the lack of accumulation in the presence of epoxomicin suggest that wtRT is not solely degraded by the proteasome. Other proteases like cathepsins and calpains are likely to be involved in the natural degradation of reverse transcriptase. On the other hand, the considerable effect on the accumulation of the multi-drug resistant variant of RT following epoxomicin treatment, suggests that the introduction of resistance-mutations targets mutant RT protein to be degraded by the proteasome complex. It is conceivable that the mutations introduced by the antiretroviral drugs shift the processing pathway of the protein by increasing the number of misfolded proteins produced during the translation of RT mRNA. This would enhance the poly-ubiquitination of drug-resistant RT, and direct the protein to proteasomal degradation.

Lower expression of multi-drug resistant RT may in part be responsible for less efficient replication of multi-drug resistant HIV strains as compared to wild type strains. The differences in degradation of wtRT and drug-resistant RT may also be translated into their immunogenicity. If the result of the introduction of mutations is an increased poly-ubiquitination of RT, this may enhance the immunogenicity of mutated

RT protein. In that case, the mutated RT would serve as a better vaccine candidate than the wtRT. However, the mutated gene may be less efficiently transcribed or the transcript less efficiently translated, which in both cases would result in a reduced amount of protein produced. Moreover, the mutation may destroy antigenic epitopes by introducing new proteasomal cleavage sites in the protein [317]. Taken together, this would decrease the immunogenicity of mutated RT.

5.7 SPECIFIC PROTEASOMAL DEGRADATION LIMITS THE IMMUNOGENICITY OF RT-ODC FUSION PROTEIN (PAPER VII)

To explore the effect of proteasomal degradation on RT immunogenicity, we modified the protein to target it to the proteasome. Potentially an enhanced antigen presentation of RT epitopes would thereby be achieved. We fused the wild type RT to mouse ornithine decarboxylase (ODC). ODC is a rapidly degrading enzyme involved in the biosynthesis of polyamines. It is degraded through the proteasome without the need of poly-ubiquitination [318, 319].

Fusion protein was constructed by cloning the gene for murine ODC in frame with the wild type RT (RT-ODC; Paper VI). To limit the risk of inducing an autoimmune response to ODC, a variant of RT-ODC was made that contained only a minimal ODC sequence (ODCsig) required for proteasomal degradation of ODC (Paper VII). Expression of the fusion proteins was detected as described (Paper VI). We compared immunogenicity of the parental RT gene and RT-ODC fusion constructs in BALB/c mice. The mice were intramuscularly immunized with the DNA constructs encoding the RT and RT-ODC variants. The RT-specific cellular immune responses were assessed by intracellular cytokine staining, ELISpot, and ELISA; and the humoral response by ELISA (Paper VII).

By the fusion of RT to ODC or ODCsig, we could enhance the degradation of RT-ODC/ODCsig fusion proteins, as compared with the wild type RT (Figure 9A). The half-life of the fusion proteins were 2 (RT-ODCsig) and 5 hours (RT-ODC), respectively, whereas it was 20 hours for the wild type RT protein. We believe that this effect can be partially explained by a more prone proteasomal degradation of the fusion proteins. Adding MG132 (that inhibits both the proteasome and other proteases) to transfected cells yielded a 24-fold increase in the amount of both RT-ODCsig and RT-ODC. However, when adding the proteasomal-specific inhibitor epoxomicin, a discrepancy in the expression of the two proteins was observed. RT-ODCsig content increased 4.5-fold, whereas the content of RT-ODC 9-fold. Our results thus suggest that a more specific targeting to the proteasome is achieved when fusing RT to the entire ODC-protein, than in case of fusion to the degradation signal alone.

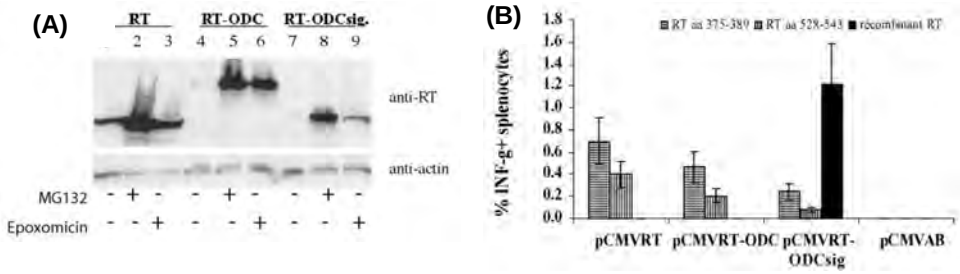

Figure 9. (A) Expression of RT and RT-ODC chimeras treated or non-treated with a proteasomal inhibitor. (B) Percent of total splenocyte population positive for IFN-γ production upon *ex vivo* stimulation by recombinant RT or RT derived peptides.

In BALB/c mice, we measured a humoral response to RT following immunization with either wtRT or RT-ODCsig gene chimeras (Paper VII). The RT-ODC immunization elicited no antibodies to RT. Following *ex vivo* stimulation of splenocytes with RT peptides or RT proteins, we measured the strongest IFN-γ (Figure 9B) and TNF-α production in the groups immunized with wtRT or RT-ODCsig. A tendency for IL-2 production was only measurable in the group immunized with the RT-ODCsig construct. The mice that were injected with RT-ODC elicited only a weak cellular immune response. Some TNF-α production could, however, be detected in that group. The production of IFN-γ, TNF-α and some IL-2 after stimulation with RT antigens in the RT-ODCsig immunized group, suggests that a T_H1-type of a response was triggered. This was further strengthened by the detection of IgG2a antibody production in that group. However, the overall immune response was weak in all groups.

Thus, our results suggest that too efficient targeting to degradation may prevent enough antigens to be accumulated and thereby decrease the immunogenicity of that particular protein. Keeping a balance in accumulation and degradation of a protein is therefore of importance for optimal immunogenicity.

6 CONCLUDING REMARKS

The HIV-1 pandemic is one of the most severe infectious diseases humans have experienced. The HIV virus preferentially infects and destroys T-helper cells that normally orchestrate the adaptive immune system. The virus replicates to high titers in body fluids and causes a persistent infection through integration of the viral genome into the host chromosome. HIV spreads in contaminated blood and other body fluids to new human hosts. Rapid viral replication and high error-rate during transcription enable HIV to adapt to pressures from immune responses and antiretroviral drugs. In order to develop a vaccine, these factors need to be addressed. The effective antiretroviral drug therapy used in developed countries has markedly decreased the mortality in HIV infection, and patients live a relatively normal life. However, in resource-poor countries, therapy is still limited, which accentuates the need for a vaccine against HIV.

Three candidates (of approximately 170 clinical trials conducted) have been brought forward to efficacy trials to investigate the vaccine's potential to prevent infection or to reduce the viral load/set point upon HIV infection. In the first trial, the concept of inducing sterilizing immunity against HIV infection was tested, but failed. The second trial, which assessed the potential of a cell-mediated response by HIV genes in adenovirus vectors, was stopped by the data safety and monitoring board at the first interim analysis due to futility. The third trial, including a canarypox vector prime and protein boost schedule with HIV envelope antigens subtype B and E, is ongoing and is estimated to be completed during summer 2009. The repeated failures of these extended clinical trials and the lack of correlation with protection against HIV infection may suggest that initiating smaller proof-of concept trials instead of these large licensure trials may be more strategic.

My personal view is that an HIV vaccine will not be composed like conventional vaccines, where a few injections are given to obtain lifelong immunity to a particular microorganism. Rather, an HIV vaccine will most likely support the existing antiretroviral drug therapy. The early observations that drug-resistant HIV readily develops when anti-HIV therapy is suboptimal (e.g. too few drugs or poor treatment adherence) imply that, besides targeting as many subtypes of HIV-1 as possible, vaccine development needs to address drug-resistant HIV variants. By including regions that contain both the mutant and the wild type virus sequences in the vaccine mix, both wild type and mutant variants may be targeted.

We have pre-clinical observations that immunization with mutated variants of an epitope induces not only epitope-specific immune response, but also a cross-reactive response to the wild type epitope variant. Thus, including drug-resistant variants as vaccine antigens, besides suppressing the development of drug-resistance, may have an effect on the wild type virus as well. There is a potentially synergistic effect of simultaneous antiretroviral drug therapy and vaccination against drug-resistant HIV; thus, vaccination may improve immune responses to virus variants. If an antiretroviral drug causes the HIV virus to introduce drug-escape mutations, the immune response against such viral variants should evolve and suppress the replication of the virus variants. There will be fewer chances for HIV to escape both these forces. It would

enable the administration of an effective anti-HIV therapy to more patients and consequently limit the risk of HIV shedding and transmission. While such a vaccine may neither prevent nor eradicate the HIV infection, it may reduce the continuous need for newer and different drugs.

7 ACKNOWLEDGEMENTS

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