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Concepts in

DNA IMMUNIZATION

overcoming viral diversity and enhancing plasmid immunogenicity

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Front cover and picture: Electron micrograph of $CD4^+$ T-lymphocytes infected with HIV (blue), kindly provided by Lennart Nilsson, Karolinska Institutet, Stockholm, Sweden.

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To my family,

ABSTRACT

On April 23, 1984, the prominent scientist Robert Gallo held a historical press conference at the Department of Health and Human Services, Washington D.C., USA. He announced that his laboratory at the National Institutes of Health had over the last months isolated a retrovirus named Human T-cell Leukemia Virus type III (HTLV-III). The virus came from 48 patients in the homosexual community in San Francisco. The city had just been hit by the mysterious epidemic of acquired immunodeficiency syndrome (AIDS). HTLV-III was later renamed human immunodeficiency virus (HIV). At the same meeting, Gallo further explained that his laboratory was able to grow large quantities of the virus in cell cultures and as a consequence it was stated that "*we believe that the new process will enable us to develop a vaccine to prevent AIDS in the future…we hope to have such a vaccine ready for testing in approximately two years*."

This thesis is printed on the very same day, twenty years later and the now mature field of HIV/AIDS vaccine development has still not discovered what exactly mediates protection against HIV infection, and are still far away from a clinically useful vaccine. Why is this? What makes HIV so special when other virus diseases, like polio, can be recognized and eliminated by the immune system, and where vaccination works very well?

The focal points of this thesis are two major problems in modern vaccine development. Many viruses exist in multiple subtypes or serotypes, a phenomenon that has serious implications for the choice of vaccine target. It is especially critical when trying to vaccinate against HIV of which the surface structure (gp120) presents immense antigenic variability. Moreover, modern genetic vaccines are based on smaller units of the virus or consisting of multiple genes (combination genetic vaccine) are weak immunogens. We have in multiple ways tried to increase the potency of such immunogens.

More specifically, we have shown that removal of inhibitory elements in a DNA immunogen is of importance for efficient induction of immunity. Further, antibody responses to a DNA immunogen can be substantially enhanced if the genetic immunogen is coupled to a carrier, in our case the polyomavirus VP1 capsid. In combination with generally immunoactivating agents, for instance recombinant granulocyte macrophage colony stimulating factor (GM-CSF), the HIV envelope genes from multiple subtypes (A, B and C) can change the envelope DNA immunogen into a potent entity that induces high titers of broadly reactive antibodies as well as cellular responses. We also show that immunization with proteins followed by DNA immunogens, a strategy tentatively called "reverse prime-boost immunization" induces strong immunity. These findings will be further validated in human clinical trials within the near future. Last but not least, we have developed an HIV murine challenge model based on pseudotyped viral particles; combining the HIV genome and the murine leukemia virus (HIV/MuLV) envelope. This model resembles human acute primary HIV infection. Protection in this model can be ascribed to cellular immunity, in the complete absence of antibodies. Using this model, we have shown that prime-boost immunization induces better protection against subtype homologous HIV challenge, than against heterologous exposure.

The immunization strategies covered in this thesis describe the biological problems that face vaccine development in general and HIV vaccinology in particular. The problems and concepts illustrate why the statement by several scientists in the 1980s has proven to be somewhat premature.

Key words: HIV, DNA-vaccine, gp160, GM-CSF, HPV-16 L1, VLP, MuLV, prime-boost

LIST OF PUBLICATIONS

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1 THE AIM OF THIS THESIS

The aim of this thesis work has been to develop immunogens and immunization strategies against primarily Human Immunodeficiency Virus (HIV), but also Human Papilloma Virus (HPV) infection. Efficient protection against viral infections involves virus-neutralizing antibodies but also induction of $CD8^+$ cytotoxic T lymphocytes. Our strategies have therefore been based on genetic immunization, a recently discovered vaccination principle known to induce antibodies and to confer especially good cellular immunity.

Our studies centred on the following questions:

How to

- increase the protein expression of DNA immunogens (paper I),
- facilitate the uptake of DNA plasmids *in vivo* (paper II),
- enhance the host immune system during DNA immunization (papers I and IV),
- broaden immunity in order to mediate protection against multiple viral subtypes (papers IV and V),

  and

- combine DNA immunogens with other vaccine components/strategies (paper VI).

The above concepts have been validated in mammalian expression systems *in vitro* and in murine immunization/protection models *in vivo* (paper III).

2 MICROORGANISMS CAUSING HUMAN INFECTION

The world is teeming with microbial life. From the deepest marine ecological systems to the top of extreme alpine environments there are prokaryotic microorganisms[1] which fall into two classes of organisms.

2.1 BACTERIA AND VIRUSES

All organisms contain information about their construction. The genetic material consists of deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) that is converted into messenger RNA molecules (mRNA), used as templates for the synthesis of life's main building blocks, proteins. The typical bacterium is a small (~0.1 mm) free-living cell with a DNA genome protected by a two-layer plasma membrane and a sturdy cellular wall. Bacteria are not directly dependent on the replication apparatus of other living organisms and can reproduce by dividing (binary fission) under optimal nutrient and temperature conditions. Viruses are tiny (~0.0001mm) intracellular obligate parasites that contain DNA or RNA genomes protected by a protein/lipid envelope. A virus is a true parasite as its existence is completely dependent on the replication machinery of other organisms. It has been said analogously that viruses are nothing but "small escaping pieces of genetic material".

Most microorganisms play a crucial role in the balance of our ecosystems by synthesizing and degrading biological material. In the human body there are about ten times as many bacteria as human cells. In a sense, we can be said to be a walking mass of prokaryotes supported by a matrix of human cells! The majority of these human bacteria are found in the gut, where they are generally beneficial for our existence. Still, we are liable to fall sick from bacterial food poisoning (*Salmonella* bacteria) or, even worse, develop gastric cancer (*Helicobacter* bacteria) [1]. Why is exposure to some microorganisms relatively harmless, like the Influenza virus that causes the common cold, whereas other similar encounters are directly life threatening, like the Corona virus that causes a severe acute respiratory syndrome (SARS) [2]?

One explanation lies in the existence of microbial virulence factors. The *Helicobacter pylori* bacteria carry genes that produce cytotoxins and enzymes (mucinase) that destroy the mucosal epithelia and cause cancer [1]. The site of exposure is also important. A case in point is the serious bowl inflammation that is caused by the “friendly” *Escherichia coli* bacteria, which are normally present in the duodenum but may accidentally invade the peritoneum. If the organ invaded at the site of infection carries optimal receptors for the microorganism, then infection can take place. Finally, the manifestation of disease is dependent on the host's ability to respond to the organisms. Influenza infections are self-limiting in an immune competent young person, whereas it kills large numbers of old immuno-compromised people each winter [3]. This thesis discusses the development of vaccines, that is, strategies for aiding the host immune system to respond appropriately to a given organism. We have focused on interventions that will help the immune system to attack a DNA virus; Human Papilloma Virus (HPV), or an RNA virus; Human Immunodeficiency Virus (HIV).

[1] There are also multicellular microbes such as fungi and parasites but they will not be considered here.

2.2 HUMAN PAPILLOMA VIRUS (HPV)

2.2.1 Introduction to Papillomaviruses

Almost a century ago, the Italian physician Ciuffo demonstrated that a filtered suspension of human warts transfers papillomas to a healthy recipient [4]. The etiologic agent that causes cutaneous warts in rabbits was recognized in 1933 and later named cottontail rabbit papillomavirus [5]. Attempts to characterize the agent were thwarted by the impossibility of growing papillomaviruses (PV) in cell cultures. In the 1980s, scientists started cloning the PV genome into bacteria and slowly learned more about its biological mechanisms. PVs are small DNA viruses that induce skin lesions (warts or papillomas) in many different vertebrate species. Within medicine, most attention has been devoted to certain types of human papilloma viruses (HPV) that are known to induce cellular transformation in a number of organs, most commonly in the female cervix, sometimes causing cervical cancer [6].

2.2.2 HPV - structure and viral life cycle

HPV is a small non-enveloped DNA virus that replicates in the nucleus of epithelial cells. The viral particles are approximately 55 nm in size and the viral double stranded DNA (dsDNA) genome (~8000bp) encodes early regulatory proteins (E1-8) and two late structural proteins (L1 and L2) [7]. The viral genes are located on one strand of the dsDNA and serve as a template for transcription. The early regulatory gene products E6 and E7 proteins are known oncogenes. The major capsid protein (L1) is about 55 kDa in size and together with the L2 minor capsid protein it forms pentameric structures that selectively encapsulate PV DNA, creating an icosahedral structure consisting of 72 capsomers.

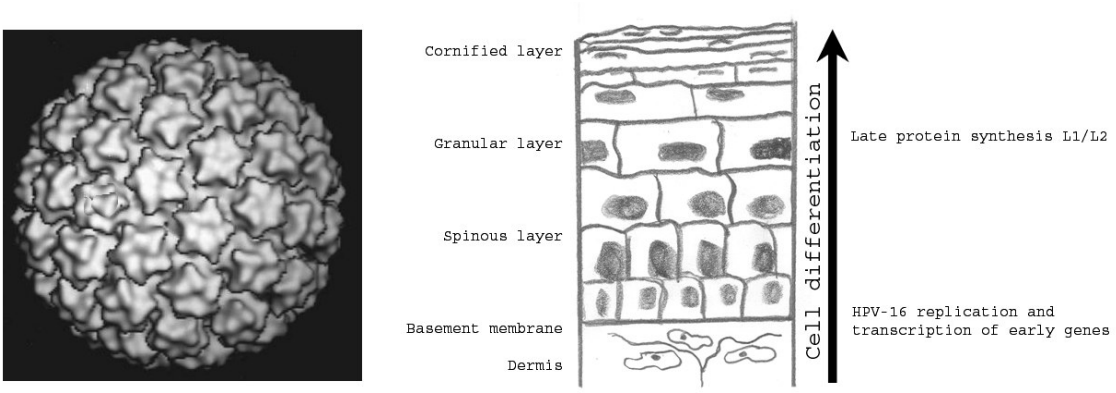


Figure 1. *Human Papilloma Virus.* Cryoelectron microscopy of a papilloma virus particle (left). The image shows the virion consisting of 72 L1/L2 pentamers. A schematic figure of stratified epithelium (right). HPV replicates in the proliferating cells of the basement membrane. The late viral gene products are not synthesized until the migrating cells have reached the upper cell layers. Modified from Baker et al., 1991 [8] and Schwartz 2000 [9].

The HPV life cycle is closely linked with the differentiation of the cervical epithelium. Figure 1. PVs preferably bind to the a6ß4 integrin complex present on many different cell types [10]. They are well conserved in mammalians. Integrin-independent entry has also been described and recently heparin and glycosaminoglycans has been demonstrated to mediate entry [11]. The first steps in the replication cycle, such as cell entry and transport to the nucleus, have been poorly charachterized [12]. The basal cell is the only cell in the squamous epithelium

that is capable of dividing and the only cell layer that allows for HPV replication together with syntheses of the early regulatory proteins [13]. Production of the later proteins and virion assembly are carefully regulated and occur only in the terminally differentiated keratinocyte. Conversely, the major capsid protein (L1) is exclusively found in the superficial cells of a skin wart [14]. Little is known about the mechanism involved in the release of the HPV particles.

2.2.3 HPV - extreme gene regulation

The HPV transcription machinery is highly complex. Multiple promoters are involved in the production of early and late transcripts. In addition, the HPV genome contains multiple cis regulatory elements and host transcriptional factors that *in trans* can modulate viral gene expression. The L1 and L2 genes are expressed from one of the HPV late promotors in terminally differentiated cells, with synthesis of one common L1/L1 transcript. The L1/L2 mRNA is alternatively spliced and early discoveries revealed regulatory elements in the 3´coding region of the viral genome [9, 15]. Recently, the research group headed by Prof. Stefan Schwartz at Uppsala University, Sweden, discovered multiple independent negative *cis*-acting elements located in the 5' end of the HPV L1 gene [16]. When these elements were removed or replaced, high levels of L1 protein could be transiently produced *in vitro*. A few host factors have been shown to interact with these negative elements [17]. In collaboration with the group in Uppsala, we have evaluated the capacity of such expression-modified L1 DNA constructs to induce anti-L1 immunity when delivered as genetic immmunogens *in vivo* (paper I).

2.3 HUMAN IMMUNODEFICIENCY VIRUS (HIV)

2.3.1 Where does HIV come from?

Clinical manifestations of immunodeficiency in people living on the west coast of the United States in the early 1980s were the first signs of what was then an unknown human disease [18, 19]. Rather than emerging randomly throughout the community, the clinical signs were initially found in the risk groups for blood-borne disease (homosexuals, hemophiliacs and intraveneous drug-users), suggesting a common environmental exposure or common routes of infectious disease transmission. In early 1983, the French scientist Dr. Barré-Sinoussi at the Pasteur Institute in Paris, France, isolated a retrovirus from a patient with lymphoadenopathy [20]. US scientists confirmed the French finding and identified the virus, which was initially named lymphoadenopathy-associated virus (LAV) or Human T-cell leukemia Virus type III (HTLV-III). It was renamed human immunodeficiency virus (HIV) in the light of evidence that it really was the causative agent of acquired immunodeficiency syndrome (AIDS) [21]. Since then, the HIV epidemic has spread to millions of people throughout the world. It is clear that the virus originates from more than one cross-species transmission (zoonosis) between monkey species and humans, but when and exactly where this occurred is still unknown [22, 23]. The retrovirus pathogen is believed to have existed in macaques and chimpanzees for thousands of years and to have emerged in humans in the late 1950s as a consequence of globalization and general population growth [24, 25]. It is important to distinguish between HIV type 1 (HIV-1), which has caused the present pandemic, and the significantly less transmissible HIV type 2 (HIV-2), which is mostly found in western parts of Africa and in India. HIV-2 is evolutionarily more closely related to the

simian immunodeficiency virus (SIV) and is probably a more recent cross-species transmission [26, 27].

2.3.2 HIV - molecular structure

HIV is a lentivirus belonging to the family of *retroviridae* [28]. The ball-shaped virion is about 110 nm in diameter and consists of structural proteins together with two single-stranded RNA (ssRNA) molecules, 9.2 kB in size. The virion also contains enzymes and structural proteins as well as cellular membrane proteins. Figure 2. Long terminal repeats (LTR) flank the viral genome and constitute enhancer and promoter regions involved in the careful regulation of viral transcription and replication (Marcello, Lusic et al. 2004). The HIV genes can be divided into four groups according to biological function. The envelope (*env*) gp160 gene encodes a membrane protein (gp120) and a transmembrane protein (gp41) [28]. The group antigen (*gag*) gene encode proteins that make up the inner structure (p24), the matrix (p17) and two RNA stabilizing nucleocapsid structures (p6 and p7) [29]. The polymerase (*pol*) genes encode proteins with enzymatic activity, some of which are unique for HIV. The Protease (PR) gene product is transcribed as a gag-pol precursor protein (p55) with activated protease catalyzing the autologous cleavage of the gag precursor protein. The Reverse Transcriptase (RT) is located in the virion close to the RNA genome and is responsible for converting RNA into DNA prior to integration of HIV DNA into the host genome (provirus) [30]. The reverse transcription has an extremely high error frequency (~ 3 $x10^{-5}$ mismatches per replication cycle), leading to large numbers of non-functional particles and an incredibly great viral diversity [31]. The fourth group of genes (*tat, rev, nef, vif, vpr, vpu*) encodes regulatory proteins that act together with cellular molecules to initiate and regulate viral transcription. In this thesis we describe vaccine strategies targeting mostly the virus envelope (papers IV, V and VI) but also the virus gag protein (paper II) and the small regulatory proteins Nef and Tat (paper VI).

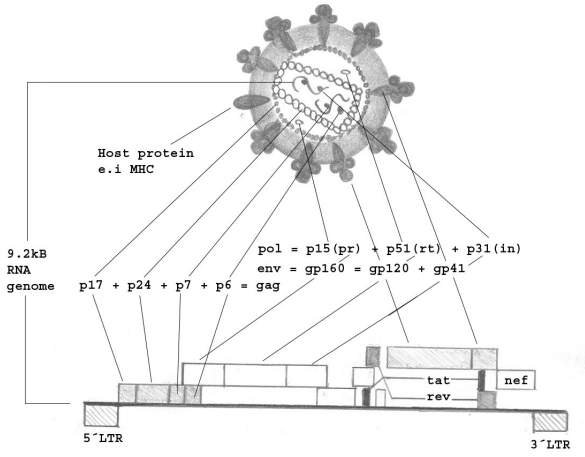


Figure 2. *A schematic drawing of the HIV-1 virion (top) and genome (bottom).* The long terminal repeats (LTRs) flank the viral genome that encodes 15 proteins. The *gag* gene encodes structural proteins involved in the virion architecture. The matrix protein (p17) stabilizes the viral membrane, the core protein (p24) protects the two RNA genomes and the p6 and p7 are also found within the core. The *pol* gene encodes three unique viral enzymes; the protease (p15, pr), the reverse transcriptase (p51, rt) and the integrase (p31, in). The *env* gene encodes the gp160 envelope polyprotein. Trimeric gp120 is assembled together with the transmembrane gp41 molecule. The virus also encodes six regulatory proteins (rev, nef, tat, vpu, vif, and vpr).

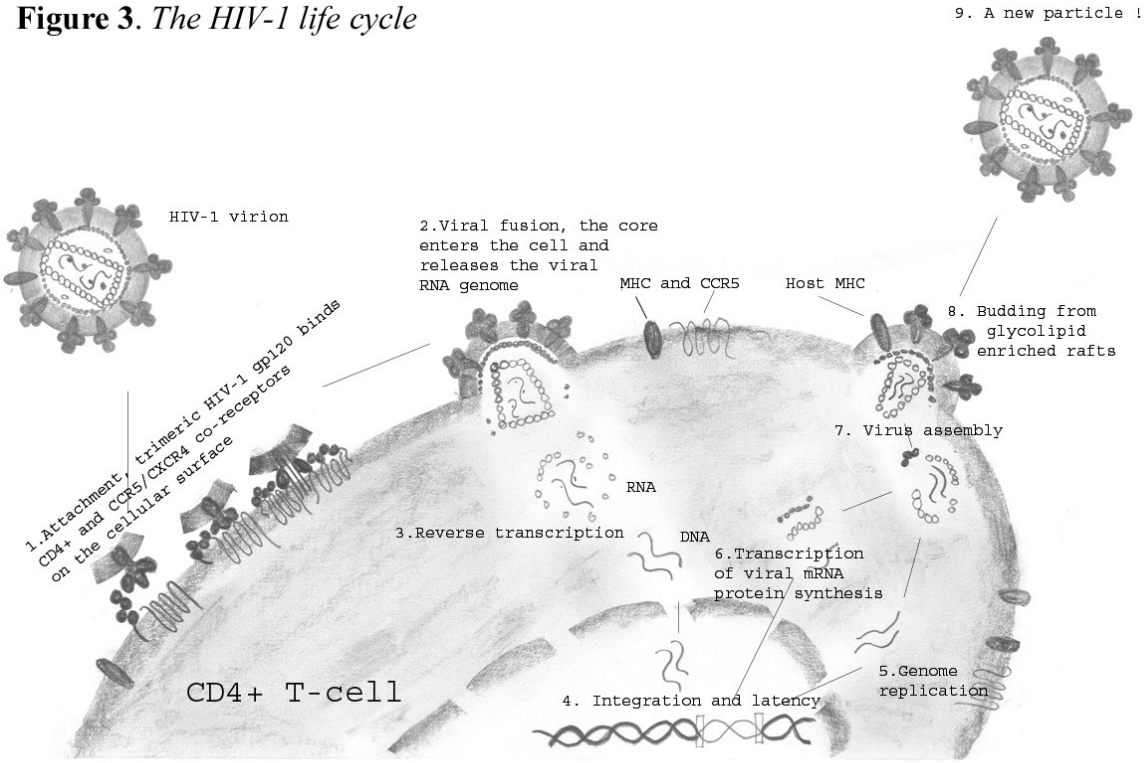
2.3.3 HIV - replication cycle

The HIV-1 life cycle is initiated when the extracellular envelope spikes (gp120) attach to CD4 molecules on cells such as T-helper cells, monocytes, macrophages, dendritic cells or brain microglia cells [32]. Figure 3. The first loop of the CD4 molecule has been mapped to

interact with conformational epitopes primarily in the constant regions 3 and 4 (C3 and C4) of the envelope protein [33]. The gp120 molecule has five extremely variable regions (V1-V5). The V3 loop is known to be the principal neutralization inducing domain, attracting most immunological responses *in vivo* [34, 35]. One of the immunogens is the rather conserved Gly317-Pro318-Gly319 (GPG) motif found on the very tip, the crown region of the V3 loop [36]. The gp120-CD4 interaction leads to a conformational change in the virion surface and exposure of the transmembrane protein gp41 which triggers a pH-independent fusion of the viral and host membranes [32, 37]. In addition to the CD4-gp120 binding, the virus needs a co-receptor interaction to successfully enter a cell. The family of HIV co-receptors is growing continuously and as of now consists of ten members, of which the best known are CCR5, CXCR4, CCR3, CCR2b, BOB and Bonzo [38, 39]. All the molecules that participate in the reaction can be seen as one "receptor complex", as it is believed that multiple gp41, gp120, CD4 and cytokine co-receptor (CCR) oligomers are involved. In general, HIV-1 is either macrophage tropic, using preferentially CCR5 receptors (R5 viruses), or shows T-cell tropism by preferring CXCR4 usage (X4 viruses) [40, 41]. Only the core particle of the virion is transmitted into the host cytoplasm, where it undergoes partial uncoating [42]. The exact architecture of the fusion site and the process of membrane fusion have not yet been fully characterized; some claim that the interaction is highly specific, as in influenza virus haemagglutinin mediated cellular entry, whereas others believe that receptor independent mechanisms, such as entry via apoptotic bodies, may also be involved [43-45].

Reverse Transcriptase (RT) mediates conversion of the ssRNA genome into an RNA-DNA hybrid. The host DNA polymerase then catalyses the syntheses of a DNA-DNA copy, called a provirion. The provirion is transported to the nucleus, where it is integrated into the host genome by HIV integrase [46]. At this stage of infection the processes may be silent for a considerable time, until cellular (NFkB) and/or viral (Tat, Rev) activators trigger transcription from the LTR, a process catalyzed by the host RNA-polymerase II [47, 48].

Figure 3. *The HIV-1 life cycle*



After having passed a number of transcriptional/translational checkpoints, high levels of viral proteins are synthesized. The structural gp160 polyprotein is cleaved by a host protease furin and trimeric structures of gp120-gp41 heterodimers are formed at the cell surface [49, 50]. Presence of correctly folded envelope proteins is essential for the virus infection capacity [51]. These molecules are therefore also targets for vaccination. In this thesis we have modified the processing pathway of the gp160 polyprotein in an attempt to achieve as good envelope protein folding as possible (paper IV). Recent crystallographic and topographic studies also reveal highly complex post-translational envelope modifications, with carbohydrate glycosylation accounting for approximately 50% of the molecular mass [52-54]. The structural proteins and two full-length RNA copies are assembled into new particles close to the cellular membrane. During budding, the HIV-1 particles obtain virally derived gp120 surface proteins, together with various host-derived molecules such as major histocompatibility complexes (MHC) [38, 55]. The budding of virions occurs from glycolipid enriched membrane areas called rafts. The high lipid content of such rafts increases the flexibility of the membrane, facilitating the release of viral particles [56].

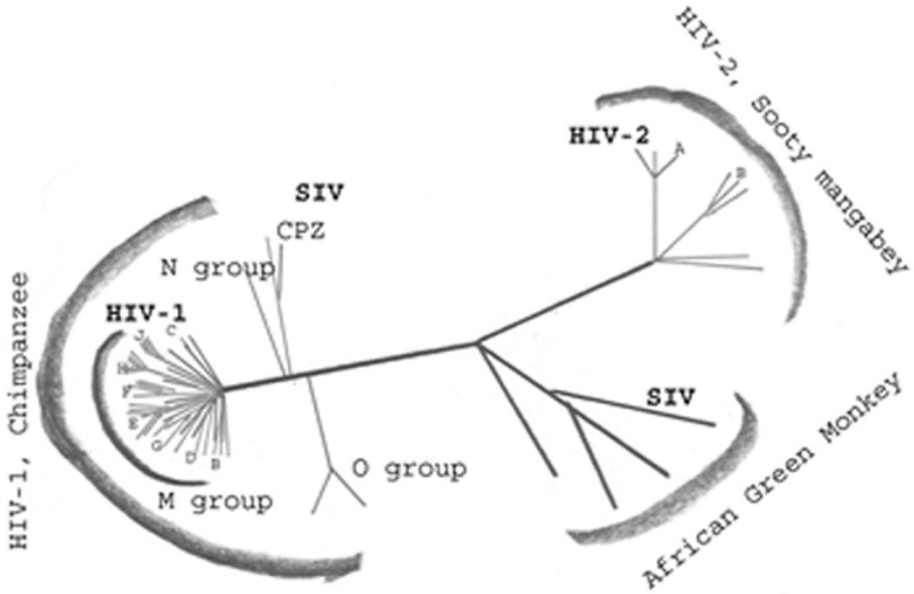


Figure 4. *The phylogentic tree of primate lentiviruses.* HIV-1 was introduced into humans after a transmission (zoonosis) from the chimpanzee, whereas HIV-2 originates from the primate named Sooty mangabey. The African Green Monkey is the natural host of closely related retroviruses. Data from the M group is extensive and the branches depicted here contain thousands of isolates. This basic tree is created by *pol* gene alignments. Modified from Leitner, 2004 [57].

2.3.4 HIV variation - driven by mutation and selection

The highly error-prone HIV RT creates continuous genetic variation throughout each viral life cycle [29]. Far from all viral particles succeed in becoming viable virions capable of initiating a new infection. The host immune system exerts tremendous selective pressure on the highly heterogeneous virus population [58, 59]. Extracellular antibodies mediate extensive pressure on exposed gp120 molecules, explaining the fact that the Env protein is highly variable [60, 61], whereas the structural Gag protein is less variable and the Pol protein with a limited allowance for mutations is more stable. Nucleotide sequence data from the gp120 and gag genes constitute the database for genotypic classification. HIV-1 genotypes can be divided into three families, designated M (main), N (non M, non O) and O (outlier). Figure 4. More than 90% of all HIV-1 isolates belong to the M family, which is further divided into 11 subtypes or clades (designated A-K) based on evolutionary distances [62]. The group O and N viruses are relatively rare and have so far not been very well characterized [63, 64]. Within group M, subtype A is mostly found in central Africa, subtype B is the major transmitting agent in Europe and North America, while Africa and India are mostly infected with subtype C. In Thailand, subtype A was the dominant virus until the mid 1990s, when subtype E started to appear [65]. Today, we know that many of the infected Thais are carrying the circulating recombinant form A_E (CRF A_E) [66]. Recombinants are now spreading rapidly throughout southeast Asia [67, 68]. Similarly, some central African countries may have over 50% of recombinant strains, mainly chimeras between subtypes A, C and D [69]. The global subtype prevalence changes continuously and depends more on migration of infected persons than on the mutational rate of the different subtypes [63].

Phenotypic classification of HIV-1 is classically based on parameters like cell tropism and ability to induce syncytia in the target cell [70]. The R5 viruses use the CCR5 receptor for cellular entry and are non-syncytium inducing (NSI) viruses that have a macrophage cell tropism and slow replication. The X4 viruses use the CXCR4 receptor for cellular entry and are syncytium inducing (SI) viruses that preferably infect T-cells and replicate more rapidly. These viruses can be detected in leukemia T-cell line-2 (MT-2) assays. Dual-tropic viruses (R5X4), capable of using both receptors also exist. Positional mapping has revealed that minor changes in the envelope V3 loop sequence alter the gp120 net charge, leading to dramatic effects on cellular tropism [71, 72].

3 IMMUNITY AND PATHOGENESIS

3.1 THE IMMUNE SYSTEM - AN INTRODUCTION

The immune system has evolved to defend us against infections and to remove damaged cells. Under certain conditions the same system may induce allergic reactions and autoimmune disorders, such as multiple sclerosis, rheumatoid arthritis and diabetes. Controlling this double-edged sword is difficult. This introduction presents an overview of the main components involved in this delicate balance. The dynamics of the system will also be described (reviewed in [73])

The innate immune system consist of soluble and cellular factors that can be activated without previous exposure to the bacteria/virus and do not change after exposure. Adaptive immunity consists of soluble components and cells that need to be educated to recognize a specific antigen[2]; and once this has been achieved, the adaptive immune response has mechanisms for creating immunological memory.

3.1.1 Innate immunity

Physical barriers like skin, cilia, mucosal membranes and the placenta block microorganisms from direct contact with the host. Physiological barriers such as temperature, salinity, acidity and oxygen tension also assist in upholding this first line of defence. Upon entering the body, the invading microorganism triggers the complement system, which then "tags" the intruders with complement factors, making them efficiently recognized by cells such as granulocytes. Actively engulfing phagocytotic cells like monocytes, macrophages and neutrophiles specialize in killing extracellular bacteria. Virus-infected cells secrete soluble factors such as interferon-a and ß, thereby signaling to non-infected cells that an invasion is in progress. The most sophisticated weapon in the antiviral innate defence is the natural killer (NK) cell, which recognizes and kills infected cells on the basis of missing surface receptors. The NK cell can also be guided to the infected target cell with the help of immunoglobulins, a mechanism known as antibody-dependent cell-mediated cytotoxicity (ADCC).

3.1.2 Adaptive immunity

The majority of microbes are cleared by the natural innate immune response, mostly without us even noticing. If an infection is becoming established, the defense is gradually taken over by the adaptive immunity, which consists of the humoral immune response mediated by antibodies that are produced by B-lymphocytes and cellular immunity (T-lymphocytes). T-cells are capable of killing the infected cell and of initiating/terminating the immune cascade. Figure 5. Adaptive immunity is characterized by specificity and memory, features that have to be acquired through lymphocyte training[3].

[2] Can be bacteria/virus components but also allergens, metals and other factors that the host regards as non-self.

[3] Immunological tolerance, positive and negative selection of B and T cells, not further described here.

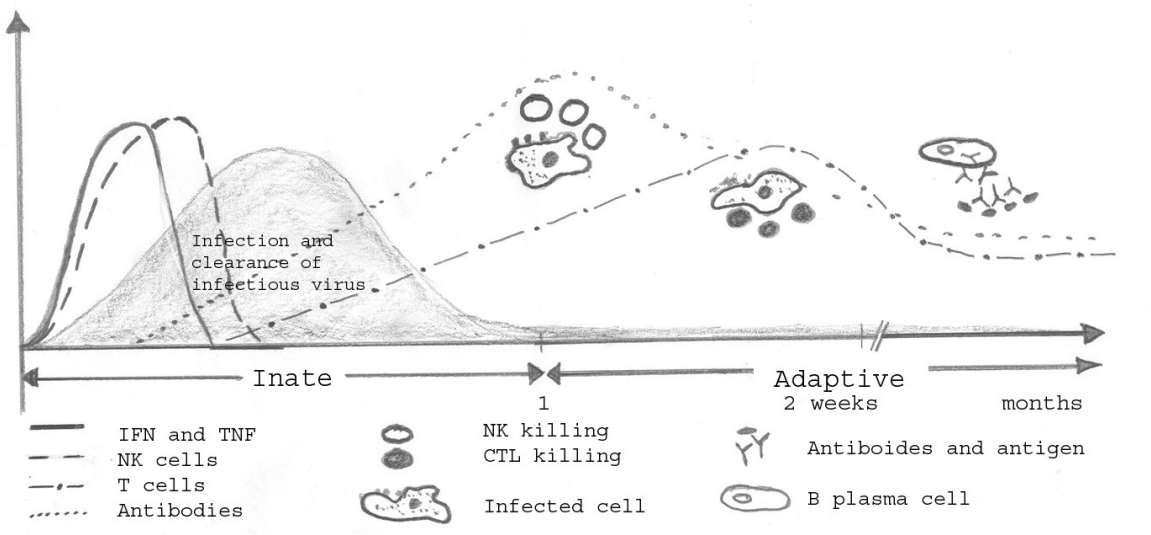


Figure 5. *Innate and adaptive immune responses.* Interferon-α and –β are produced rapidly in response to infection and alert the immune system. The natural killer cells effectively recognise virus-infected cells, which have down regulated their MHC molecules. Gradually the adaptive immunity builds up but it is not until after a couple of weeks that cytotoxic T lymphocytes are functionally active. Antibody induction after primary infection is relatively slow, with specific immunoglobulins appearing after two weeks. Modified from Janeway et al., 1999 [74].

3.1.2.1 Antigen presentation leads to humoral and cellular immunity

Antigen presenting cells (APCs), such as monocytes, macrophages, dendritic cells and B-cells, are the detectives of the immune system. Their role is to constantly screen the body for non-self antigens. Just as a house is continuously restored, so do our cellular components undergo a constant renovation. During this ongoing metabolism, APCs present parts of the degraded building blocks (peptides) on a family of cellular surface molecules called Major Histocompatibility Complex (MHC) class I molecules. Circulating trained $CD8^+$ T-lymphocytes can distinguish between self and foreign peptides with the help of their T-cell receptor (TcR) and will start to proliferate and multiply if they encounter a foreign peptide in the pretext of co-stimulatory molecules and correct cytokine signals. The expanding $CD8^+$ T lymphocyte population gradually matures into cytotoxic T-cells (CTLs) that circulate in the body and efficiently kill infected cells, identified through recognition of the specific foreign peptide. This is the endogenous pathway of antigen presentation, specially evolved to induce cellular immunity.

Alternatively, the APC engulf extracellular molecules, degrade them and present parts of these structures (peptides) on a cell surface receptor called MHC class II. Peptides bound by MHC class II receptors are recognized by $CD4^+$ T-cells, which then start to produce cytokines that in turn stimulate resting B cells in the presence of antigen to produce antibodies. This is the exogenous pathway of antigen presentation that is used to trigger humoral (antibody) immunity to defend against extracellular threats such as bacteria. Antibodies (Ab) are immunoglobulin (Ig) proteins with precise specificity for a given antigen. The most common human antibody classes are IgG, IgA and IgM. IgG is the main Ig class in the blood, whereas IgA is also present at mucosal sites. The IgG class of antibodies can be further divided into subclasses such as IgG1, IgG2 etcetera. Subclass Ig-ratios have been shown to correlate with the type of immunity induced (Feltquate, Heaney et al. 1997).

The mode of APC presentation roughly determines the nature of the induced immunity. The more precise control is conducted by the $CD4^+$ helper T-cells, which after MHC class II to peptide recognition can fine tune the system and stimulate the activation of $CD8^+$ T-cells through T helper type 1 (Th1) cells, specialized in producing cytokines like interferon-γ (IFN-γ), tumor necrosis factor ß (TNF-ß) and interleukin-2 (IL-2). Alternatively, the helper type 2 (Th2) $CD4^+$ T-cell can produce interleukins 4, 5 and 10 to stimulate cell types like B cells, mast cells and eosinophils needed in the war against extracellular organisms. The $CD4^+$ T-cell is absolutely crucial for the induction of a correct response. The network of cytokine signaling is complex but it is worth mentioning the Granulocyte-macrophage colony stimulating factor (GM-CSF), which is secreted from APCs and T-cells upon activation. GM-CSF stimulates both the B and the T-cell compartment to proliferate and differentiate [75]. Interleukin 12 (IL-12) is also of major importance in T-cell activation and stimulates IFN-γ production.

3.2 HPV - CAUSING BENIGN WARTS OR CANCER

3.2.1 HPV types 1 and 4 - the common skin warts

More than one hundred different HPVs have been isolated and the existence of even more HPV genotypes has been suggested on the basis of partial sequence data [76]. Cutaneus infection with HPV types 1 and 4 generate benign skin warts, the mechanisms underlying the cytopathic changes are not fully known. Some of the early HPV proteins can directly stimulate abnormal cell growth but are generally expressed at very low levels in infected basal cells of the skin (reviewed in [12]). Viral gene expression may possibly stimulate the release of cytokines that in turn stimulate growth of the basal cells. Warts can be transmitted by person-to-person contact and readily spread to an individual´s uninfected areas. Skin warts are normally self-limiting and the lesions heal after months or years.

3.2.2 HPV type 16 - the major etiological agent of cervical cancer

Genital HPV is a sexually transmitted infection (STI) and is therefore mostly contracted by young sexually active people. The sexual route of transmission is confirmed by a direct correlation between the number of sexual partners and the occurrence of cervical lesions [77]. Both females and males can be infected with genital HPV, but genital (penile) malignancies in the male are relatively unusual [12]. HPV DNA in cervical cancer tissue was first detected in the early 1980s [78]. Since then, the World Health Organization (WHO) continuously collects data and reports that almost all (99.8%) cervical cancers can be explained by HPV infection [79]. Today, there are estimates of 450.000 cases of cervical cancer each year, causing 200.000 annual deaths [80]. Genital HPVs can be classified according to disease severity, with low-risk types like HPV-6 and 11 causing benign condylomas and high-risk types like HPV-16, 18, 31 and 45 inducing cervical cancer. HPV-16 alone is responsible for more than 50% of the cancers induced by the high-risk types [79]. Still, it should be kept in mind that only approximately 1% of all high-risk HPV infections lead to the development of malignant cervical cancer. Many years may pass between the time of HPV exposure and the occurrence of tumors. The first clinical signs of transformation is mild dysplasia. If the HPV infection persists it may lead to malignancy [81]. The progression towards cervical cancer is thought to be due to the direct action of the E6 and E7 proteins, which interact with cellular

suppressor proteins p53 and pRb respectively [82]. Cell transformation has also been induced after viral DNA integration in the vicinity of cellular regulatory genes [83].

3.2.3 Genital HPV immunity and tumor escape

Since HPV infection does not result in cell lysis, immune responses to infection are relatively weak. The high frequency of spontaneous resolution of early stage genital lesions is most likely due to cell-mediated immune responses [84]. The virus has developed various strategies for hiding within the progressing tumor. Firstly, the oncogenic proteins E6 and E7 are expressed at relatively low levels. Viral interference with host inflammation, cytokine production and induction of E6/E7 tolerance also takes place [85]. In addition, advanced tumors show decreased MHC class I presentation, limiting the CTL mediated tumor killing [86]. HIV positive women are more likely to acquire genital HPV and to develop high-grade dysplasia, additionally demonstrating the role of cellular immunity in the control of HPV infection [87]. The exact role of antibodies in natural immunity is not known. The anti-viral humoral immunity is possibly weakened as an effect of the immune escape. Still, neutralizing anti-L1 antibodies do play an important role in vaccine-induced immunity, as discussed in section 4.3.

3.2.4 Prophylaxis and therapy in cervical cancer

As with other STIs, the risk of acquiring genital lesions due to HPV infection can be reduced by having fewer sexual partners and to at least some extent by using condoms [88]. First-world nations have set up rigorous Papanicolaou (Pap) screening programs that test for abnormal cells and HPV DNA types in cervical smears [89]. Although associated with high costs, such screening programs have been successful in decreasing the incidence of cervical cancer. Pap smear positive women are normally topically treated with podophyllotoxin or the immune stimulatory cream imiquimod (see section 4.2.3.2) [90, 91]. Interferon-α treatment has also been validated [92]. In patients with high-grade tumors, parts of the cervix are surgically removed. As 80% of global cervical cancer cases occur in the third world, with a rising incidence as a consequence of the HIV epidemic, other more effective and less expensive medical interventions, such as HPV vaccines, are needed.

3.3 HIV - LEADING TO CHRONIC INFECTION AND IMMUNODEFICIENCY

3.3.1 The HIV pandemic - the result of a virus, sexual behavior and mobility

The number of people currently living with HIV is estimated to total 40 million and so far the epidemic has caused more than 20 million deaths [66]. It has been estimated that 14,000 people become infected every day and that 95% of these events occur in developing countries, predominantly on the African continent [63]. The common ways of spreading the HIV virus and infected cells include sexual intercourse, intravenous drug-use and vertical mother-to-child transmission. In a historic perspective, global population growth and increased mobility have led to a growing number of personal interactions [93]. The spread of HIV is also fuelled by the never-ending conflicts in sub-Saharan Africa [94]. While some HIV scientists as well as the South African leader Thabo Mbeki have recently questioned whether HIV is in fact the causative agent of AIDS [95], the evidence supporting such a relationship is overwhelming (reviewed in [96]).

3.3.2 HIV infection and pathogenesis

HIV transmission after heterosexual encounters is relatively low (1 to 100 - 1000) [29]. This is likely to be a consequence of physical factors such as the skin, mucosal layers, pH and temperature as well as the innate immune system. Other parameters that affect the risk of transmission are low donor virus load and virus phenotype [97]. During primary infection there are few clinical symptoms but a minority of the patients experience general symptoms like fever, skin rash, headache and enlarged lymph nodes [98]. This makes HIV infection almost impossible to distinguish from an "everyday" infection. Mucosal cell layers with underlying Langerhans cells, macrophages and $CD4^+$ cells are among the first to be infected after initial exposure. Infected cells are then transported to local lymph nodes, where they infect other cells, followed by systemic spread via the lymphatic and circulatory systems to lymphatic tissue throughout the body. After some 8-12 weeks the patient starts to produce anti-HIV specific antibodies (seroconversion) and moves into the second, asymptomatic stage of infection, during which HIV-1 particles are often undetectable in blood [99]. The period of latency - a sort of "status quo" between the defender and the aggressor - can last from months up to many years and is distinguished by slow increase in virus and a slow or no decline in $CD4^+$ T cell counts [100]. The continued loss of $CD4^+$ T cells during infection is partly due to viral cell lysis together with HIV-dependent induction of apoptosis [101-103]. Host cell mediated lysis of HIV infected autologous $CD4^+$ T cells also takes place. During this time the virus spreads throughout the hematopoetic system and usually also infects the nervous system [104, 105]. Early asymptotic disease is often predominated by NSI viruses. Later, a phenotypic switch to viral SI phenotype may cause disease progression towards immune suppression. Without anti-viral treatment (see 3.3.4), HIV-1 infection typically leads to AIDS, which weakens the patient through opportunistic infections, neoplasia, autoimmunity and/or manifestations of neurological dysfunction [106, 107]. This stage ultimately leads to the complete collapse of the immune system.

3.3.3 Can natural immunity protect against HIV?

3.3.3.1 The Host - innate and adaptive anti-HIV immunity

As with most infections, physical barriers make up the first line of defense against HIV infection. Natural resistance is also aided by circulating hematopoietic cells. Neutrofils, macrophages and NK cells [108] are all parts of the innate non-specific early repertoire against HIV. Later, the adaptive immune system, with its antigen-presenting cells and cytotoxic $CD8^+$ T cells, attacks the HIV-infected target cells through MHC class I restricted recognition and actively kills them by inducing apoptosis via cytotoxic granula [109]. Immunity to HIV-1 also involves the mechanism of ADCC (antibody-dependent cell-mediated cytotoxicity) where the Fc-heavy chains of anti-HIV-antibodies bind to the NK CD16-receptor, activating NK cells. This in turn leads to release of granules and ultimately to ADCC mediated cell lysis [110-112]. In addition to these classical immune responses, $CD8^+$ T cells can secrete a soluble anti-HIV protein called $CD8^+$ Associated Factor (CAF) after encountering infected cells [113]. Other soluble viral suppressors are β-chemokines such as Macrophage Inflammatory Protein-1α (MIP-1α), RANTES and Interleukin 16 (IL-16) [114].

High titers of antibodies against HIV are directed towards the gag antigens (p24 and p17), the transmembrane part of the envelope antigen (gp41) but mostly towards the major surface antigen gp120 [99]. Most of the anti-gp120-antibodies are directed towards the variable loops

(V1-V5) [115]. Antibodies to HIV gp120 are effective in neutralizing virus in primary infection. In chronic infection they have less effect because the V1-V5 rapidly escapes away from the antibody pressure. Passive transfer of monoclonal/polyclonal anti-gp120-antibodies between primates has been shown to give protection against experimental challenge [116, 117], demonstrating that antibodies can play a prophylactic role. However, there are always two sides to the coin and under some conditions anti-envelope antibodies can increase the uptake of HIV *in vitro* and under certain conditions also *in vivo* [118, 119]. The overall impairment of the $CD4^+$ T helper cell function cripples the overall potency of the anti-HIV immune response [120]. Still, 20-30% of HIV patients can remain asymptomatic for 7 and sometimes up to 20 years after infection. These long-term non-progressors (LTNP) are believed to possess favorable combinations of innate and adaptive immune system factors, involving NK cell and $CD8^+$ T-cell functions, leading to prolonged viral control (Wahren and Liu, in press) [121, 122].

3.3.3.2 The Virus - advanced regulation interferes with immunity

HIV has evolved a broad range of strategies for avoiding the host defence. It has developed unique mechanisms to avoid complement-mediated lysis in which the virus, instead of being killed, uses the complement factor C3 to enhance its infectivity through opsonisation [123]. Similarly, not only does HIV infect the dendritic cell (DC) and impair its vital antigen-presenting role; it also hitchhikes on the DC from the site of infection to the regional lymph node, bound to a receptor called DC-SIGN [124]. The fact that HIV mainly infects $CD4^+$ T-cells seriously affects the host's possibility of mountin a proper response. One of the most important viral counter-measures is the generation of large sequence heterogeneity by the error-prone RT, leading to both cellular and humoral immune escape [125]. Virus escape occurs when CTL responses fail to kill an infected cell because the virus has mutated vital amino acids in MHC class I restricted epitopes [126, 127]. There are also other specific mechanisms, such as down-regulation of host MHC or CD4 molecules mediated by the Nef protein [128]. The HIV Vif protein has recently been shown to actively interfere with late stages of the viral life cycle though interactions with the cellular APOBEC3G protein [129]. The small HIV Vpu protein also assists in the strategy of concealment, by down-regulating host CD4 molecules [130]. For antibodies, the viral variation creates a huge problem for efficient neutralization *in vitro* [131] and *in vivo* [132]. Viral shedding of gp120 monomers creates decoy structures that absorb a large proportion of the neutralizing antibodies away from the infectious virus [28]. *In vivo*, the virions in blood are covered with IgG, thus protecting the virus particles from the more efficient virus-neutralizing antibodies (Östlund and Wahren, personal communication). Also extreme glycosylation of the gp120 surface antigen creates a shield against incoming antibodies [133].

3.3.3.3 Cytokine co-receptors - the delta CCR5 story

An intriguing finding, made in 1996, was that resistance to HIV is mediated by the 32 base pair deletion in the CCR5 receptor (CCR5Δ32) [32, 134, 135]. As this truncated version of the HIV co-receptor is not expressed on the cell surface, entry of CCR5 using NSI virus is not feasible. Still, CCR5Δ32 cells/humans can be infected with SI viruses, since they use the CXCR4 co-receptor. The CCR5Δ32 allele is mostly found in the Caucasian population and is absent in African and Japanese populations. Individuals who are CCR5Δ32 heterozygous (10%) show delayed disease progression [136] and homozygous individuals (1%) appear to be highly resistant [135, 137]. The frequency of the homozygous CCR5Δ32 genotype is high

in uninfected people at risk and close to null in an infected population, demonstrating the protective CCR5Δ32 effect against HIV-1 infection [133].

3.3.3.4 HLA and other host genes - the Nairobi story

Genetic susceptibility has been an intense issue in HIV epidemiology. The documented findings include correlations between Human Leukocyte Antigen (HLA) haplotype and the progression of AIDS [138]. A cohort of Nairobi prostitutes who seemed to be resistant to HIV infection were found to have certain uncommon variants of HLA.A2 and HLA.DR genotypes [139, 140]. High levels of HIV-1 specific IgA and local CTL reactivity were found in cervical specimens from the temporarily protected women [141-143]. This illustrates that although short-lived, local mucosal immunity is likely to play an important role in protection against HIV-1 infection. The same Nairobi cases left prostitution for some time but unfortunately became HIV-infected after returning to exposure.

3.3.4 Prevention and treatment of HIV/AIDS

The most straightforward approach to effectively countering the HIV epidemic is of course to sustain from sexual intercourse. More realistically, preventive health measures like well-managed HIV/AIDS monitoring, education and needle/condom distribution have proven effective in slowing the spread of HIV [144, 145].

A large number of polyanionic compounds have been described to block HIV by interfering with the charged gp120 V3 loop [31]. These compounds have been used clinically to prevent HIV-1 infection, as vaginal microbiocides [146]. Nucleoside analogues, such as nucleoside reverse transcriptase inhibitors (NRTI), mimic natural nucleosides but lack the OH-group needed for nucleic acid elongation. There are also non-nucleoside RT inhibitors (NNRTI) that inhibit the RT function by targeting a non-substrate site of the RT [31]. The integrase and protease are needed to generate mature HIV particles and have therefore also been explored as drug targets [147, 148]. During a short time frame of entry, the ectodomain of gp41 becomes blockable with a 26-residue peptide called T-20, now licensed for therapy [149]. Further, a number of new viral co-receptor antagonists have been shown to prevent HIV-1 entry blocking the CCR5 or CXCR4 receptors. Such compounds are now approaching clinical use [150-152].

Monotherapy with the first NRTI, called AZT (3-´azido-3´-deoxythymidine), was for many years the only HIV therapy available. Today the recommended therapy is a combination of two to three RT-inhibitors and often one protease inhibitor in what is referred to as ART (Anti Retroviral Therapy). It should be noted that while ART can interfere with ongoing viremia, it can never clear the infection. Large-scale anti-retroviral treatment can only be successful if managed properly, controlling for viral resistance together with reduced risk behavior [153]. Mother-to-child transmission via breast milk or the placenta is an important infectious route that can be limited by the use of anti-retroviral treatment [154-156]. Tremendous efforts are being made to provide such treatment in sub-Saharan Africa and the rest of the third world [157]. The huge problem of anti-viral resistance [158, 159] and the complications associated with large-scale anti-viral treatment in the third world lends weight to other HIV/AIDS strategies; such as vaccines.

4 VACCINATION

4.1 THE CONTINUOUS REVOLUTION IN VACCINOLOGY

Vaccination can be divided into passive and active varieties. In passive vaccination, antigen-specific antibodies/immune cells are transferred to a person to mediate brief protection from exposure. For example, delivering antibodies against Rabies can therapeutically clear an infected person from this lethal virus [160]. Adoptive transfer of antigen-specific immune cells to HIV infected patients has also been shown to restore immune function [161].

Active vaccination strategies, when the vaccinated person develops his/her own immunity, are for obvious reasons much more applicable, especially for large-scale prophylaxis. Active vaccination can in principle be described as inoculation of a complete or partial infectious agent (vaccine) into a subject (vaccinee), leading to induction of specific immunity that will protect against later exposure to the specific agent (memory). The first recorded attempts at active induction of immunity are from Turkey and China in the Middle Ages, when dried crusts from smallpox (Variola) pustules gave protection from infection (reviewed in [162]). More recent proofs of the concept were provided by Dr. Edward Jenner in 1796 and Dr. Louis Pasteur a hundred years later in 1885 [163]. Jenner observed that milkmaids constantly exposed to cowpox were later protected against smallpox [164]. Jenner also proved his hypothesis by inoculating a small boy with cowpox and later deliberately infecting the child with smallpox, showing that his prophylactic treatment conferred protection. This was one of the first examples of active vaccination with a live virus. Pasteur observed that when he infected chicken with a cholera extract derived from old birds with a chronic infection, onset of disease was followed by recovery and immunity to a subsequent infection. This is an example of vaccination with a live attenuated virus. The other strategy for decreasing the inherent infectivity/pathogenicity of an infectious agent is to make inactivated vaccines with the virus functionally weakened by chemical or heat treatment. Koch, von Behring, Calmette, Guérin and Lister made further important discoveries in the 19th and early 20th century by pioneering the development of attenuated live and inactivated vaccines such as Cholera, Diphtheria and Tuberculosis [165]. This early period can be seen as the first revolution in vaccinology.

After World War II, dramatic breakthroughs occurred in the fields of cell culture, biochemistry and immunology. This started the second revolution in vaccinology with cell cultured vaccines such as Polio, Measles, Mumps and Rubella [162]. The third revolution in vaccinology, starting in the early 1970s, is genetic engineering; where subunit vaccines are produced in animal, plant, bacterial or yeast cells. The production of recombinant Hepatitis B surface antigen (HbsAg) in yeast and human cells resulted in the first recombinant vaccine, which was released for clinical use in the early 1980s [166]. The past two decades have seen incredible advances in the fields of immunology and molecular biology. The fourth, most recent revolution in vaccinology is recombinant vector delivery and immunization with genes from an infectious agent in the form of DNA or RNA. The vaccinated person will make his/her own vaccine antigen *in vivo*. This last approach is called genetic immunization.

4.2 GENETIC IMMUNIZATION - THE MOST RECENT STRATEGY

The breakthrough for DNA immunization came in 1990 when Wolff et al. demonstrated gene transfer *in vivo* after performing intra muscular (i.m) injections with naked DNA plasmids [167]. Since then, DNA vaccine strategies have been successfully applied in aquaculture [168], veterinary medicine [169] and with ongoing clinical trials in human medicine; validating candidate vaccines against Tuberculosis, Malaria, HIV, Hepatitis B, Influenza, Ebola, Rotavirus and Rabies. DNA vaccination is referred to as direct immunization with the gene of interest located on a suitable eukaryotic expression plasmid. After delivery, the DNA is taken up by host cells and travels to the nucleus, where it is expressed using the host machinery. The construct lacks factors needed for complete eukaryotic replication, something that leads to *in vivo* degradation within days to weeks. The DNA immunogen passes through the host endogenous transcription, translation and post-translational machinery, generally resulting in a protein with the correct three-dimensional conformation, phosphorylation and glycolysation. DNA immunizations therefore most often result in high quality antigen presentation, preferentially inducing Th1 cellular immunity [170]. Several proteins also result in MHC class II presentation. A primary targeted cell can somehow transfer the antigen to another APC (cross-presentation), believed to result in MHC class I dependent presentation [171]. Figure 6. The more exact nature of the induced immune response depends on the fine design of the DNA construct, the route and method of administration, the immunization timing and the use of co-delivery of immunomodulatory cytokines/chemokines.

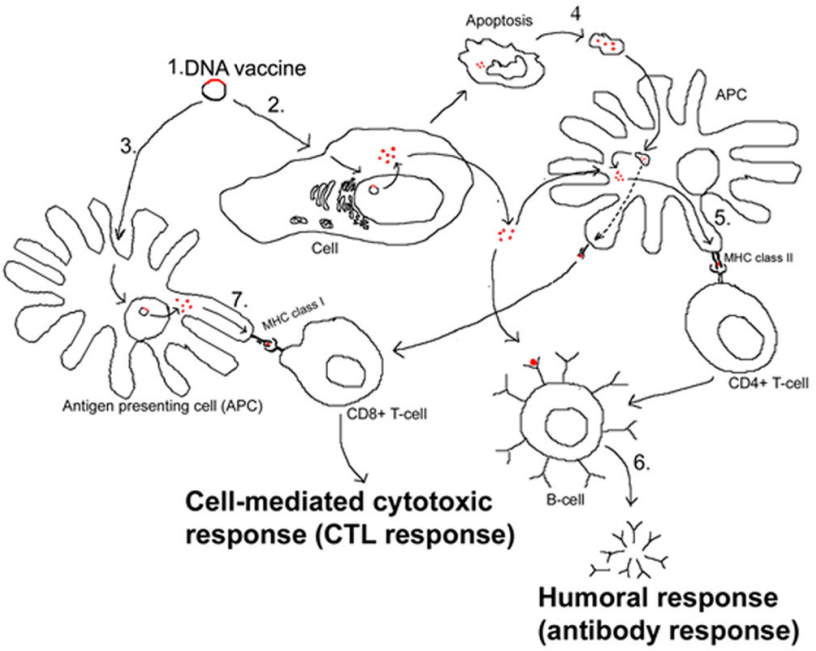


Figure 6. *How a DNA vaccine induces adaptive immunity.* The DNA vaccine plasmid (1) is taken up by somatic cells (2) or by antigen presenting cells (3). The gene product may be secreted by the cells and/or the antigen expression can lead to programmed cell death (apoptosis) (4). The secreted product can be taken up and presented on the surface of B-cells or presented directly to already primed B-cells. The APCs will degrade the product and express the peptides on the major histocompatibility complex class II molecules (MHC II) (5). Together with $CD4^+$ T-cell help, a humoral response can be induced (6). MHC class I presentation (7) leads to induction of the $CD8^+$ T-cell response which can lead to a cytotoxic response. Figure kindly provided by A. Boberg.

4.2.1 The DNA vaccine architecture - ways to modify protein expression

The basic structure of a DNA immunogen in the form of an artificial DNA plasmid is shown in Figure 7. The most commonly used transcriptional starting point used in a naked DNA immunogen is the Cytomegalovirus (CMV) early gene promotor [172]. The downstream antigen encoding gene/cDNA sequence has been isolated from an infectious agent or made synthetically [173]. In strategies targeting autoimmunity and cancer, the DNA antigen often encodes an autologous gene. To achieve optimal processing in the eukaryotic cell, a suitable polyadenosine (polyA) signal follows the open reading frame (ORF). The DNA immunogen needs to be propagated in a prokaryotic system and therefore typically encodes an antibiotic resistance gene for selection.

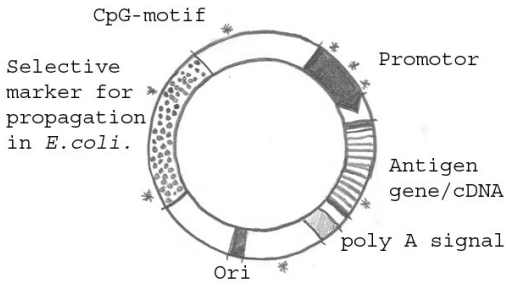


Figure 7. *The DNA vaccine architecture*. The cytomegalovirus (CMV) promoter is the most commonly used transcription initiator of the foreign or endogenous DNA vaccine antigen. An eukaryotic polyA signal is needed for efficient processing of microbial antigen. The vector needs a selective marker and an inmitation site for propagation in *E.coli*. Multiple immunostimulatory CpG motifs (*) may also affect the immuno-activating properties of the DNA immunogen.

Modifying the intracellular processing of the antigen has been extensively explored, using plasmids that drive the antigen towards either the proteosome through linkage to ubiquitination signals resulting in enhanced MHC class I presentation or via the endoplasmatic reticulum (ER) for facilitated MHC II processing [172]. Other important plasmid properties are size and presence of immunostimulatory DNA sequences in the plasmid backbone (see Figure 7 and section 4.2.3.2).

Genes from prokaryotic organisms have evolved sophisticated regulatory mechanisms that sometimes are incompatible with that of the mammalian expression system. Transcriptional regulation of the prokaryotic genome is mediated in two principle ways. Elements such as HIV-1 Tat protein can bind their own transcriptional region TAR *in trans*, leading to transcriptional modification of this specific gene/gene region [174]. Alternatively, the regulatory/coding mRNA can regulate itself *in cis* by the formation of inhibitory secondary structures such as hairpin loops [175]. In the work with the HPV-16 L1 gene (paper I) we made a DNA construct that circumvents the transcriptional inhibitory features of HPV RNA by introducing a number of point mutations into the coding gene. The engineering of DNA immunogens also allows for modification of post-translational cleavage patterns of the encoded protein. This has been performed by deliberately inactivating the recognition site for the host protease cleavage of the HIV-1 gp160 DNA immunogen (paper IV). One aspect of genome evolution concerns codon usage, by which is meant that different organisms prefer different tRNA anti-codons [176]. In many DNA vaccine systems it has been shown that immunization with synthetic genes using codons preferred by eukaryotic organisms (codon optimization) leads to increased levels of expression and consequently enhanced immunity compared to immunization with wild type DNA [177-179]. In the last work of this thesis (paper VI) we perform experiments both with a wild type HIV-1 gp160, nef and tat DNA plasmids and with synthetic codon optimized versions.

4.2.2 Routes of immunization and gene delivery vectors

A variety of routes are available for immunizations with naked DNA [180]. Epidermal gene gun administration using DNA-coated gold particles requires 100-1000 fold less DNA than intramuscular injections for a similar response [181]. Gene gun immunization leads to a high frequency of direct transfection of APCs (Langerhang´s cells), which in turn are able of produce enough antigen to trigger an antibody response. The relatively small number of APCs present in muscular tissue is believed to result in production of relatively small amounts of antigen and the induced immunity by this route is preferentially cellular [182-184]. In this thesis we have mostly utilized the intramuscular route of immunization. In addition, the work on HPV16 L1 DNA (paper I) and the HIV-1 DNA prime-boost inoculations with gp120, Nef and Tat antigens (paper VI) were given by gene gun immunization. Characterization of the murine experimental challenge model (paper III) involved a mixture of immunization routes. The main drawback with naked DNA immunization is that the induced immunity tends to be weak and relatively short-lived becuse the antigen expression is limited. Developments in genetic engineering have made it possible to clone the antigen-encoding gene into a live vector such as adeno virus, modified vaccinia virus (MVA), Semliki Forest virus (SFV) [185], rhabdovirus [186] or even salmonella, listeria and shigella bacteria [187-189]. A major problem in the field of vector mediated vaccine delivery is that of pre-existing immunity, resulting in vector neutralization and impaired gene transfer efficiency. This problem can partially be overcome by combining different vaccine strategies like DNA, protein and live vaccine vectors [190]. Also such heterologous prime-boost immunization strategies have proven to successfully enhance the vaccine induced immunity [191]. We have continued to explore the field of heterologous prime-boost immunization using HIV-1 gp160 DNA and protein antigens (paper V). We have also investigated "reverse prime-boost immunization"; priming with HIV-1 gp120, nef and tat genes followed by boosting with HIV-1 protein (paper VI).

4.2.3 Adjuvants in genetic immunization

4.2.3.1 Toxoids, classical adjuvants and novel carriers

One of the main problems in DNA vaccinology is delivery of the DNA plasmids *in vivo* so as to efficiently activate APCs and the adaptive immune system (Alpar 2002). An adjuvant can be described as a substance that amplifies the vaccine-induced immune response. For oral administration of DNA immunogens, the heat labile enterotoxin (*Eschischera coli*) or various versions of cholera toxin (*Vibrio cholerae*) have been well studied but generally lead to problems with toxicity [192]. Various aluminum salts have been used as adjuvants in protein immunization, and have also been shown to assist in enhancing antibody induction by DNA immunogens [193] [194, 195]. Binding naked DNA onto cationic carrier molecules like poly lactide-co-glycolide (PLG) [196], liposome encapsulation [197], oil-in-water micro-emulsions [198], virosomes [199] and synthetic nanorods [200] are some of the DNA carrier principles that have been explored. We aimed to validate the carrier potential of the recombinant murine polyomavirus VP1 capsid conjugated to an HIV-1 DNA plasmid (paper II). The result revealed enhanced humoral immunity and detectable cellular immunity. The increased antibody response was most likely an effect of two different mechanisms. Firstly, increased DNA uptake (carrier effect) mediated by the VP1 pseudocapsids that bind multiple cell-types *in vivo* through the promiscuous sialic acid receptor [201]. Secondly, we also

observed strong immune activation by the VP1 molecule itself. It is therefore likely that the VP1 in this case also works as an adjuvant, enhancing general immunity.

4.2.3.2 Toll receptor ligands - imiquimod and CpG-ODN

In the evolution of eukaryots, the capacity to respond appropriately to invading microorganisms has been continuously selected for [91]. Nature has evolved specific receptor-ligand signaling systems that by a specific recognition trigger dendritic cells (DCs), leading to efficient immune activation [202]. One such system consists of the mammalian Toll-like receptor (TLR) family that has eleven known receptor members, each recognizing a specific parasite structure [203]. Antigen stimulation together with TLR activation triggers various signal pathways that lead to induction of interferons, cytokines, chemokines, costimulatory molecules and T/B cell activation [119]. Different TLR-ligand interactions lead to different patterns of immune triggering. One of the most studied inflammatory TLR signaling interactions is the TLR-4 interaction with bacterial lipopolysaccharide (LPS) [204]. The natural ligand of TLR-7 is unknown but can be triggered by synthetic immune modifiers like loxoribine, bropirimine and imiquimod [205]. We recently showed that epidermal DNA vaccination is enhanced after pre-treating the skin of mice with imiquimod [206]. This strategy was used as part of this thesis to enhance the epidermal delivery of HPV-16 L1 DNA (paper I). The most interesting TLR interaction for DNA vaccination purposes is probably that of TLR-9, known to specifically bind hypomethylated cysteine guanosine (CpG) bacterial DNA motifs. Such hypomethylated bacterial nucleotide sequences efficiently distinguish between self and foreign [207]. Synthetic CpG- oligodeoxynucleotides (CpG-ODNs) have been shown by many research groups to stimulate inflammation, infection and immunity [208-210]. We have continued the early work by Deml and colleagues [211] on enhancing the immunogenicity of a recombinant HIV-1 gp160 protein using CpG-ODN motifs as adjuvants (paper V). Immunization with HIV-1 rgp160 together with CpG-ODNs results in a tilting of the immune system towards cellular (Th1) immunity compared to immunization with rgp160 alone, which mostly induced antibody production.

4.2.3.3 Cytokines - IL-12 and GM-CSF

In order to improve DNA vaccination, plasmids or live vectors that express cytokines/chemokines have been used as genetic adjuvants [212]. The cytokine adjuvant, unlike the Toll receptor ligands, does not mediate triggering of danger signals but rather works by stimulating specific cells of the immune system [213]. Many cytokines/chemokines have been used as adjuvants in DNA immunization [214]. GM-CSF attracts APC and activates T-cells. The GM-CSF gene is known to enhance immunity induced by HIV-1 gp160 DNA [215]. In comparison, we show that co-delivery of recombinant GM-CSF (rGM-CSF) together with the HIV-1 gp160 encoding DNA plasmid leads to induction of high titers of binding antibodies, whereas gp160 DNA immunization alone does not (papers V-IV). Intramuscular immunization with the structural HPV16 L1 gene was performed with pegylated rGM-CSF, also resulting in antibody induction (paper I). Interleukin 12 (IL-12) is a key cytokine in stimulating cellular immunity, especially $CD8^+$ T-cells (se section 3.222). Co-delivery of IL-12 encoding plasmids together with DNA immunogens strongly enhances cellular immunity, as previously shown by us and others [216, 217]. Immunization with multiple HIV-1 genes together with an IL-12 expressing plasmid (paper III) resulted in Th1 biased immunity, with protection against experimental HIV-1/MuLV challenge (se section 5.2).

4.3 HPV VACCINE DEVELOPMENT

4.3.1 What mediates protection against HPV infection?

The chronic nature of HPV infection and the lethality of tumors induced by some types make a good setting for developing a prophylactic vaccine. Injections with inactivated PV resulted in protection against experimental challenge in a rabbit model [218]. This early experiment demonstrated that systemic immunization could induce protective immunity. Recently it has been shown that vaccine induced anti-L1 antibodies are associated with protection in humans [219]. The importance of anti-L1 cellular immunity has also been demonstrated, as L1 immunized mice that are completely protected against experimental challenge loose the viral control after removal of the $CD8^+$ T-cells *in vivo* [220]. Cellular immunity against the E6 gene product has also been shown to be important in the control of human natural infection [221]. Most likely both cellular and humoral responses are needed to protect against HPV infection and obstruct the potential development of cervical tumors. Due to difficulties in propagating the virus *in vitro* and the presence of viral oncogenes, the strategy of prophylactic inoculation of healthy young women with an attenuated virus-based vaccine had to be abandoned. Instead, the field of prophylactic HPV vaccines rapidly focused on the development of subunit immunogens.

4.3.2 Prophylactic HPV vaccines - virus like particles almost in the clinic

The L1 protein can spontaneously assemble *in vitro* into virus-like particles (VLPs). Bacculovirus infected insect cells or yeast expression systems (*Saccharomyces cervisiae*) are most commonly used to make HPV L1 VLPs [222]. Multiple pre-clinical [223-225] and clinical [219, 226, 227] immunization studies have shown that the HPV VLPs induce neutralizing anti-L1 antibodies and that immunization leads to protection. The human serum IgG induced by VLPs are 40 times higher than after natural infection [226, 227]. The cellular immunity induced by the VLP immunogen is limited. Large phase III trials with VLPs are ongoing and it has been predicted that a licensed prophylactic product will be on the market within 5 years [228]. Approaches to develop E6 and E7 based therapeutic immunization strategies, which can aid a patient already infected with HPV, have also been sought (reviewed in [229]).

4.3.3 HPV vaccines for the third world - the role for DNA vaccines

Unfortunately, VLPs are not stable at room temperature. Therefore VLP immunogens are less suitable for large-scale field use in the third world. Recently, expression of the L1 protein in *E.coli* resulted in production of L1 pentameric capsomers that somewhat surprisingly can induce neutralizing antibodies [230, 231]. This kind of immunogen is much more stable and could therefore be advantageous for third world use. An alternative approach is genetic immunogens that can easily be freeze-dried, facilitating storage and transportation under rough conditions. Already in 1996, Donnelly and colleagues showed that a rabbit PV L1 DNA immunogen could induce L1 specific antibodies and mediate protection in the rabbit model (see section 5.1) [232]. Utilizing the HPV L1 gene as a DNA immunogen was for many years problematic since L1 gene transcription is limited in cells other than differentiated keratinocytes (see section 2.2.2). Constructing a completely synthetic HPV-16 L1 gene resulted in high expressions of L1 protein and showed induction of L1 specific antibodies [178]. A number of inhibitory sequence elements were recently discovered in a

detailed analysis of the L1 gene regulation [16]. We compared the immunogenicity of two modified L1 constructs with that of a wild type L1 DNA plasmid and showed significant enhancement of both humoral and cellular anti-L1 immunity in mice (paper I). The levels of induced L1 antibodies were in the same range as with fully codon optimized L1 DNA [178] or after intra nasal/oral VLP immunization [233, 234]. DNA immunization alone or combined with protein L1 immunogens will most likely be the strategy of choice for third world prophylactic vaccine trials [228].

4.3.4 Do HPV serotypes matter?

Many hundred different HPV genotypes exist and sera from these individuals recognize and neutralize viruses in a highly type-specific manner [222, 235]. In other words, HPV genotypes appear to be distinct serotypes [84]. Clinical data demonstrate that infection with one genotype does not protect against infections with another type [236]. Detailed analyses of L1 proteins originating from different genotypes reveal that the generally conserved protein has substantial antigenic variation within its neutralizing antibody epitopes. This could explain genotype-specific neutralization [224]. In conclusion, at least in theory a prophylactic HPV vaccine has to contain L1 antigens of at least HPV-16, 18, 31 and 45 in order to confer efficient protection against the most common cancer-causing agents. This has still to be demonstrated in large-scale vaccine trials and the relevance of reported cellular cross-reactivity needs to be determined in the field [226].

4.4 HIV VACCINE DEVELOPMENT

4.4.1 HIV and the vaccine saga[4]

Over 35% of the populations in Zimbabwe and Botswana are infected with HIV and the overall prevalence in sub-Saharan Africa is almost 20% [66]. The life expectancy of a child born today in these areas is about 40 years, compared to 70 years if he/she was born before the advent of the HIV/AIDS epidemic [66]. For almost 20 years there has been a search for a vaccine against HIV, without any substantial success [18]. Why has the development of a vaccine against HIV-1 not succeeded?

The first pre-clinical HIV vaccine studies were done in the mid 1980s with inactivated HIV that resulted in induction of sterilizing immunity [237-239]. Due to the risk of reversion and/or transmission to immuno-compromised humans, the use of live attenuated HIV for vaccination has not been regarded as a realistic alternative. Later it also turned out that some of the protective immunity induced by inactivated HIV was due to anti-host reactivity as host antigens were acquired from the cell culture systems used for growing the virus[240]. Instead, the focus turned to subunit vaccines. Early on, recombinant or patient-isolated envelope gp120 proteins were used to induce antibodies that theoretically should mediate protection. This was not the case in experimental challenges in chimpanzee [241]. The early recombinant gp120 molecules were monomeric gp120, a feature that does not create the correct antigen conformation for induction of neutralizing antibodies *in vivo* [51]. Secondly, heavy glycosylation of the gp120 molecule creates a "protective umbrella", hiding the gp120

[4] Saga = “A long, continued story that is action-packed, but not especially romantic, and that is historical and legendary or both.”, analogy by Smith et al.,2003.

from incoming neutralizing antibodies, an unknown phenomenon at the time of the first rgp160 immunization experiments [242-244]. Third, the high mutation frequency easily leads to virus variation and escape. Recent large-scale human trials with similar protein antigens confirm that the envelope antigen needs to be presented in a correct conformation in order to induce antibodies that can mediate protection [214, 245, 246].

The small regulatory HIV-1 proteins Rev, Nef and Tat have been shown to be potent targets for cytotoxic T-cells and numerous vaccine strategies have aimed at inducing immunity against these [247, 248]. Substantial Rev, Nef and Tat mediated protection has also been demonstrated in experimental challenge models [39, 249]. The most potent of all HIV antigens in eliciting cytotoxicity is the nef and gag (p24) antigens. Ongoing trials with the gag protein show promising induction of cellular immunity in primates [250] and humans [251]. The HIV RT has also been targeted [252, 253]. HIV integrase is probably less suitable as an immunogen since it has been shown to create antibodies that cross-react with endogenous serum proteins [254]. The CCR5 phenomenon (see section 3.3.3.4) has also been explored in efforts to break tolerance to the host CCR5 receptor, the aim being to create antibodies to this HIV-1 binding receptor and thereby hinder viral entry [255]. Immunization strategies for interfering with host receptors have also been explored by Lehner and colleagues, demonstrating the principle of allo immunization [256].

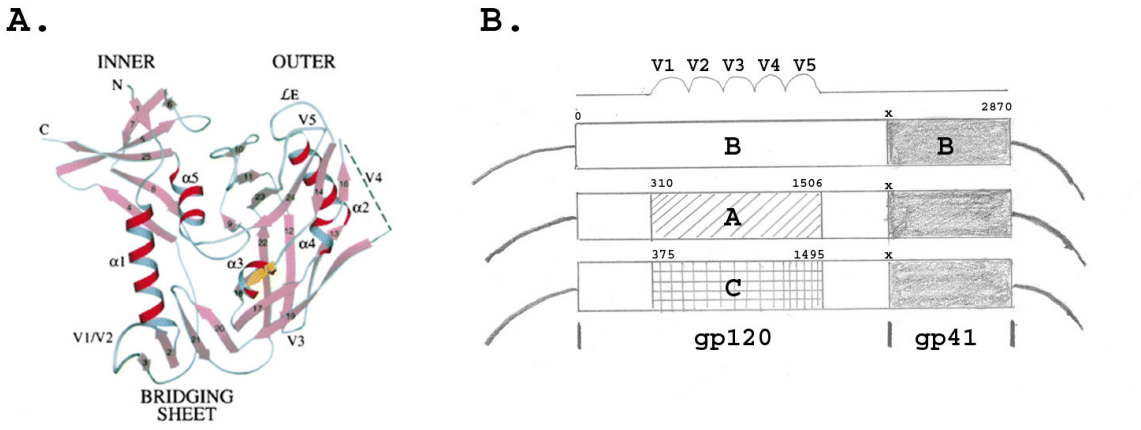


Figure 8. *HIV-1 envelope glycoprotein 160 (gp160).* **A.** A model of the HIV-1 gp120 envelope protein based on crystallographic data (picture adapted from Kwong et al., 1998). The five variable loops (V1-V5) are highly diverse and located on different regions of the molecule. **B.** The gp160 DNA immunogens derived from subtype A, B and C viruses are used as DNA immunogens in papers IV and V. The gp160 B/A and B/C plasmids are chimeric constructs made from the basic backbone encoding HIV-1 subtype B LAI gp160. X indicates the mutations R511S and A512M destroying the furin cleavage site.

4.4.2 HIV DNA vaccines - bringing new hope to the envelope immunogen

Processing the HIV-1 envelope gp120/gp160 antigen through the endogenous MHC class I pathway leads to appropriate protein folding and translational modifications. A convenient way of doing this *in vivo* is DNA immunization. Experiments in our laboratory show that inoculations with a mixture of HIV-1 genes, among them gp160 encoding plasmids (Figure 8), are able to induce envelope specific humoral and cellular immunity and complete protection against experimental challenge (paper III). In early 2000 we initiated immunization studies with our gp160 DNA plasmid alone and were successful in detecting cellular immunity and envelope-specific neutralizing antibodies (paper IV). We also show that mice immunized with gp160 DNA partially clear an experimental challenge with homologous HIV. In the follow-up study, we decided to deliver the gp160 DNA immunogen together with recombinant GM-CSF. This resulted in antibody enhancement, increasing anti-envelope antibody titers from <100 in DNA inoculated mice to 10^4 in DNA + GMCSF immunized animals. This effect was already evident after a single inoculation (paper V). We also continued the DNA priming with recombinant protein boosting, leading to further antibody production.

The gp160 gene encodes AT rich inhibitory sequences that inhibit export of nuclear mRNA transcripts. Binding of HIV Rev to the Rev Responsible Element (RRE) of the gp120 mRNA do turn on the nuclear transmembrane transport of envelope transcripts [257]. Co-administration of our envelope gp160 DNA plasmid together with a Rev encoding plasmid can lead to nuclear transport of gp160 mRNA *in vivo* (papers III-VI). Another strategy to circumvent the dilemma of AT-rich inhibitory elements in the gp120 is to adapt the gp120 genetic code to the codons used by humans [258] (paper VI and section 4.2.1 above).

4.4.3 Do HIV subtypes matter?

A typical untreated HIV-patient carries a cocktail of $5x10^5$ viral particles per ml blood, where millions of variants of the envelope antigen are present at any one time. Complete turnover of this viral quasi-species population occurs in a matter of days, generating an incredible intra-patient virus evolution, unknown to any other virus [57, 259]. It becomes obvious that anti-HIV-1 interventions are literally dealing with a range of dynamic "enemies", not just a single static agent [260]. The diversity displayed by HIV is further complicated by inter-subtype recombination. This diversity leads to an unusual viral property, to replicate despite immune responses.

There is evidence that cellular and antibody cross-reactivities are sufficiently large to cope with the dilemma of variation, described in both clinical and experimental studies [261-263]. On the other hand, cytotoxic T-cells are strictly epitope-restricted and encountering a slightly different antigen/virus unquestionably leads to CTL immune escape [264, 265]. Super-infection, where infection with one subtype of the virus is followed by infection with a second subtype, does not confer protection [266]. This strengthens the importance of subtypes in active immunization.

We and others believe that the development of multi-subtype HIV-1 vaccines is necessary in order to achieve broad antiviral immunity [241, 267, 268]. Here we show that immunization with envelope genes originating from subtype A, B and C viruses resulted in broadly neutralizing antibodies and overall humoral immune enhancement (papers IV and V). Peptide mapping showed that mixing subtypes A, B and C DNA immunogens lead to induction of

V3, C4, V4 and V5 specific antibodies with overall recognition of 73% of overlapping envelope peptides. This can be compared to subtype B DNA immunization, where only 29% of the peptides were recognized.

Usually HIV immunogens are classified according to antigen phenotype/genotype. Over the last couple of years, work on identifying envelope "immunotypes" has deepened our knowledge of what defines anti-envelope antibody immunity [115, 269, 270]. This is likely to be the future path to a rational understanding of how broad immunity to the HIV envelope can be created and how the problem of viral diversity should be overcome.

4.4.4 What is the aim of an HIV vaccine ?

An efficient vaccine is normally not considered for large-scale field trials unless it has been shown to be safe and to confer a high rate of protection (95% for Hepatitis B vaccine) [162]. In the case of HIV there is an ongoing discussion as to what level of protection a vaccine will need to achieve in order to stop the ongoing epidemic. It is ethically highly controversial whether the efficacy criterion should differ between the western world and Africa. From a purely scientific point of view, it can be argued that an HIV vaccine could be based on the phenomena of “heard immunity”. This means that if a given number of people in a population respond to a putative vaccine and are immune or show reduced viremia/viral transmission [271], the unprotected individuals will also benefit from an overall decrease in the spread of the pathogen. Since the transmission frequency is low, even a moderately efficient vaccine is likely to have an effect in the field.

5 ANIMAL MODELS IN VACCINE DEVELOPMENT

5.1 HPV VACCINE MODELS - COWS, RABBITS, DOGS, MICE AND HUMANS

Both humans, non-human primates, cattle, rabbits, horses, dogs, sheep, elk, deer, birds (!) and mice have been shown to carry various papillomaviruses. Humans can be infected with HPV [12]. Because HPV infection is so species-specific, no animal model has been developed. Instead, HPV researchers have been relying on vaccination of the natural host and then adapting the findings to the human system.

Cattle develop cancer of the upper gastrointestinal tract and the bladder after infection with bovine papilloma virus types 1 and 2 [272]. The bovine model has been used for many years for basic virology/infection studies. Bovine PVs are also the only exceptions to the rule of species-specificity since it has been shown that these viruses can infect horses, donkeys and mules [273]. The domestic rabbit was one of the first species in which the connection between PV infection and wart formation was demonstrated (Shope RE 1933). Since rabbits develop papillomas very readily after exposure to the cottontail rabbit PV, this model has been very powerful in studying the natural induction of warts and progression to malignancy [272]. The same model was also used to validate one of the first protective effects seen with a PV DNA immunogen [232]. The most recent model for HPV infection is the beagle dog model that can be infected with canine oral papilloma virus. As the name suggests, this virus infects the oro-pharynx of dogs and causes papillomas that can spread to other parts of the body, where they can induce squamous cell carcinomas [274]. Canine PV has been used to study vaccine mediated protection [218]. A number of murine tumor challenge models also exist [231, 275].

5.2 HIV VACCINE MODELS - MANY SPECIES, BUT NO ONE LIKE HUMANS

5.2.1 Primates and SIV/SHIV

After breaking the primate-human species barrier, HIV has displayed a rapid adaptability, evolving highly remarkable mechanisms in sustaining the viral life cycle within its new host. Experimental inoculation back into the chimpanzee (HIV-1) or sooty mangabey (HIV-2) demonstrates this close host-virus co-evolution, since the human viruses have reduced pathogenicity/infectiousness in their former hosts. In the mid 1980s the chimpanzee was seen as the only real model for HIV-1 infection. Later it came clear that the chimpanzee rarely develops AIDS and there are fundamental differences in anti-HIV immunity [276, 277]. As a result of this, outrageous animal costs and rising ethical concerns, the idea of using chimpanzees in pre-clinical vaccine development was gradually abandoned. Instead, attention was drawn to the simian/simian-human immunodeficiency virus (SIV/SHIV) models of rhesus or cynomologus macaques [278, 279]. The SHIV virus is a genetically engineered version of SIV in which various HIV genes are introduced. One of the major differences between SHIV induced AIDS in the macaque and HIV/AIDS in the human is the rate of development towards immunodeficiency. Infection with the most commonly used SHIV strain (SHIV89.6P) lead to development of primate-AIDS within a couple of months, whereas it normally take years to develop the human disease [280]. This makes this SHIV model an acute progressive infection model, compared to the chronic slow disease

progression in human HIV/AIDS [281]. Factors like Fut-2, TRIM5α, Lv-1, Ref-1 and Cyclophilin A have been discovered to be important primate-to-human species barriers [282-285].

5.2.2 Cats

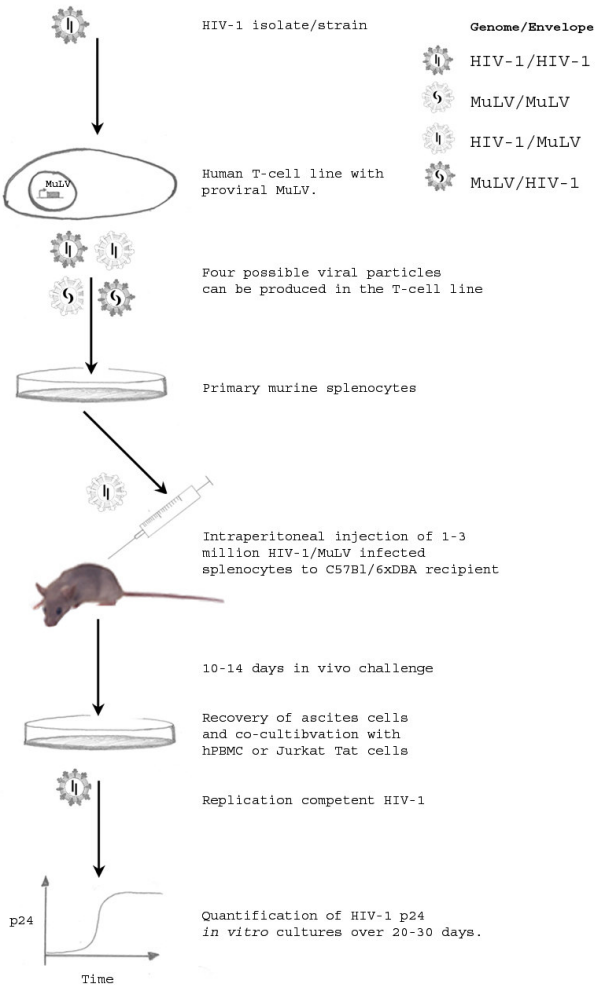
Feline immunodeficiency virus (FIV) is a natural infection of domestic cats with a worldwide prevalence of 1-28% [286]. FIV infection in cats leads to AIDS-like symptoms and has therefore been explored both for veterinary and human vaccine purposes. The FIV model has mostly been used to mimic HIV-related dementia in humans, since the feline virus is highly neurotropic [287]. Recently, a commercially available FIV vaccine composed of inactivated virus of two subtypes was released to veterinary clinics [288, 289].

5.2.3 Mice

Ethics, economy, convenience, genetics and animal preservation are among the arguments for postponing the evaluation of early HIV-1 vaccine candidates in "higher" species and instead screen for protection in small-animal models. Over the years, an ever-growing number of viral factors that limit HIV-1 replication in rodent cells have been identified. One of the first factors that was found to be missing in the rodent system was the viral entry port, consisting of the CD4 and CCR5 receptors [290, 291]. Later it was found that viral replication is mediated by HIV-1 tat in concert with a number of host proteins (reviewed in [292]), and that one of the components in this complicated machinery, the Cyclin T1, was not functional in the rodent system [293]. Other factors that supposedly affect the life cycle of HIV-1 in the murine cells are certain restriction factors [294], the host splicing inhibitor p32 [295] and the vif-mediated inhibition of host APOBEC3G involved in viral assembly [296]. Rat models have also been described [291, 297].

5.2.3.1 Transgenic and SCID mice infected with wild-type HIV

A number of transgenic murine models have been described (reviewed in [298]). Although the most obvious human-to-mouse limiting factors have been introduced into mice, productive HIV-1 replication with a complete viral life cycle has not been obtained *in vivo* [290]. Further, a number of versions of the Severe Combined Immunodeficiency (SCID) model have been developed, in which human T-cells/lymphoid organs are transferred to a mouse that naturally lacks a functional immune system [299]. The virus can effectively replicate in the human xenograft but since the SCID mouse does not have a functional immune system, immunotherapeutics are difficult to study in this model. Instead it is mostly used to evaluate anti-virals. Irradiated mice have also been used as recipients for human T-cell xenografts [300].



5.2.3.2 Inbred mice infected with pseudotyped HIV

Finally there are a few murine challenge models where vaccine-induced HIV-1 immunity can be validated in response to exposure to HIV antigens. Replication but usually no viral spread takes place in such models. In the recombinant vaccinia virus (rVV) model, the HIV-1 envelope gene is cloned into a replication-competent vaccinia virus [301]. The rVV is inoculated into the mice (intravenous, intranasal or intrarectal route), followed by recovery of the poxvirus from the ovaries 6-8 days post challenge [39]. Viral glycoproteins can be exchanged after infection by two or more viruses, often referred to as pseudotyping.

Figure 9. *A murine HIV-1 challenge model.* The CEM-1B T-cell line carries an integrated copy of the MuLV genome. This T-cell line is infected by HIV-1. After infection it produces four virus particle variants. Only the particles with a MuLV envelope can infect the murine splenocytes, since they have the receptor for the MuLV envelope. Mice of the same strain as the splenocytes are inoculated with the infected cells. After 10-14 days peritoneal ascites cells are harvested from the mice and added to new, HIV-susceptible cells in culture. The production of HIV-1 p24, a result of the viral replication, is quantified.

This approach has been used to make mixtures between the HIV genome and envelopes of the vesicular stomatitis virus (VSV) G-protein [302], the baculovirus gp64 protein [303] or the murine leukaemia virus (MuLV) envelope protein [304]. We show that HIV can be delivered into the murine peritoneal cavity by injection of cells infected by pseudotyped HIV (paper III and Figure 9). High levels of viral RNA can be found in the challenged mice, but spread to new cells probably does not take place *in vivo*. An indication of this is, that pseudotyped virions by them selves do not cause infection of the mice. Replication-competent HIV can, be recovered from the infected animals within 14 days. The experimental challenge induces both cellular and humoral anti-HIV immunity. Prophylactic vaccination of the mice with a cocktail of HIV-1 DNA plasmids demonstrates sufficient protection against subsequent challenge. We have also continued the early work with primary HIV-1 isolates by screening approximately 30 non-B subtype isolates, resulting in a subtype A challenge virus (paper VI). This virus was used to perform heterologous challenge after immunization with DNA/protein or apoptotic bodies. We now have a flexible small animal challenge model that can be used to investigate vaccine immunity against desired subtypes and variants of HIV-1.

6 NOVEL RESULTS USING DNA VACCINATION

6.1 A PROPHYLACTIC CERVICAL CANCER VACCINE BASED ON HPV-16 L1 DNA THAT IS SUITABLE FOR THE THIRD WORLD (PAPER I)

Each year about a quarter of a million women die in cervical cancer. Virtually all these malignancies (99.8%) are caused by HPV [79]. Much effort is a made to prevent cervical cancer by HPV vaccination and a commercially developed vaccine based on a virus-like particle (VLP) is in phase III clinical trials [219, 227, 305]. Still, 80% of all cervical cancers occur in the developing countries where the expensive and cold-chain dependent VLP vaccine is unlikely to be used. DNA immunization is a low-cost vaccination strategy that might be useful in the third world. In addition, this strategy leads to a broadening of the cellular immune responses.

We aimed to evaluate the immunogenicity, induced by HPV-16 L1 plasmids where inhibitory RNA elements had been removed [16]. Wild-type or modified HPV L1 plasmids were delivered to C57Bl/6 mice by intramuscular co-injections with pegylated murine granulocyte-macrophage colony stimulating factor. A gene-gun immunization schedule was also performed, pretreating the skin with immiquimod. Indeed, we found that this key modification of the L1 DNA immunogen resulted in improved immunogencity, with induction of high titers of neutralizing antibodies and broad cellular immunity, especially after intramuscular immunization. Figure 10. In comparison, the wild type L1 gene failed to induce comparable humoral immune responses and only weak cellular responses. We also demonstrated that the cellular immunity consists of both $CD4^+$ and $CD8^+$ T cells. A separate peptide mapping experiment revealed T-cell epitopes in multiple regions of the L1 immunogen. The epidermal delivery system did result in antibody production but with limited cellular induction. We explain this as an effect of gene gun immunization, since this technique is known to induce limited cellular responses [306]. Alternatively the weak cellular immunity after epidermal delivery can be explained by the limited capacity of the Langerhans cells to present the very HPV-16 L1 protein antigen [307]. We have also immunized mice intranasally or muscularly with 100 µg of the modified HPV-16 L1 plasmid without adjuvant, which did not result in either humoral or cellular responses (data not shown). Therefore it appears that GM-CSF is necessary for induction of neutralizing L1-antibodies. Topical treatment with imiquimod has been shown to enhance DNA plasmid immunization [206] and most likely also assists the L1 immunogen.

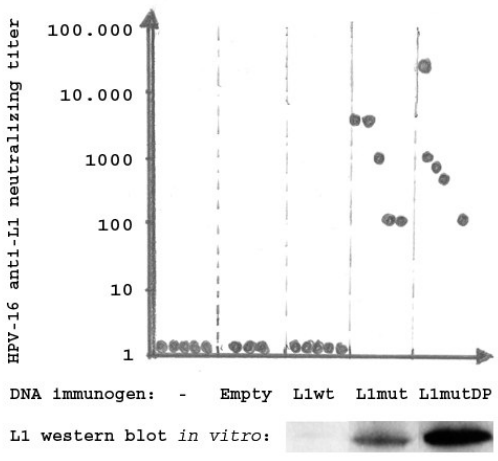


Figure 10. *HPV-16 L1 DNA immunization.* Humoral immunity was induced by modified HPV-16 L1 DNA illustrated here as neutralizing antibody titers in mice given three intramuscular immunizations. Empty = non-coding plasmid, L1wt = HPV-16 L1 wild-type encoding plasmid, L1mut = HPV-16 L1 encoding plasmid with modified 5´-end, L1mutDP = HPV-16 L1 encoding plasmid with modified 5´-end and inactivated late polyA signal (paper I).

It has been shown that VLP L1 immunization in humans induces robust but declining serum IgG titers (Koustky, unpublished data). A comparison of the durability of IgG responses over

time between VLP and DNA immunization would therefore be highly relevant. The research group led by Martin Müller has also showed efficient L1 expression and potent anti-L1 antibody production after immunization with a synthetic L1 gene in which all the codons were optimized for translation in the human [178]. A future experiment comparing the immunogenicity of the two DNA constructs head-to-head would produce useful data for further development of the HPV-16 L1 DNA immunogen. The recent finding that *E.coli* expressed L1 pentamers can induce neutralizing antibodies and mediate protection is highly intriguing [230, 231]. Combining our L1 DNA immunogen with such L1 pentamers would most likely result in synergistic immune enhancement, as seen in other DNA-protein prime-boost systems [191] or by reversed boost-prime as shown by us (paper VI). If proven successful, such a prophylactic prime-boost vaccine strategy based on stable immunogens could be attractive for field usage in the third world.

6.2 INCREASING IGG TITERS BY DELIVERING GENETIC IMMUNOGENS IN COMPLEX WITH POLYOMAVIRUS VP1 CAPSIDS (PAPER II)

First generation DNA immunogens induced specific immunity but were righteously criticized for being weak immnogens as such. This is conceptually understandable in that inoculated naked DNA is highly exposed to nucleases in the interstitial fluid and/or hampered by a low frequency of cellular transfection *in vivo*. The limited numbers of antigen-presenting cells also impair the efficacy of genetic immunization. Many DNA vaccine carriers and adjuvants have therefore been tried involving liposomes, bacterial endotoxins, macroglobulin, chromosomal proteins, mineral adsorbents, polymers and various peptides. Delivering green fluorescent reporter plasmids together with polyomavirus VP1 protein has been shown to mediate a 10-50 fold increase in intracellular DNA copy numbers, as well as an increase of reporter protein expression [201, 308]. We aimed to explore whether polyomavirus VP1 could mediate an adjuvant effect in DNA immunization.

Mice were injected with 20 µg HIV-1 DNA (p37 gag) alone or in conjunction with 2, 20 or 100 µg of polyoma virus VP1 capsids. We found that the animals receiving DNA plus 20 or 100 µg VP1 capsids responded with 10-fold higher anti-HIV p24 titers than the animals immunized with DNA alone. Figure 11. The increase in humoral response observed with DNA combined with the higher VP1 loads could be the effect of several functions: the pseudocapsids, known to bind to sialic acid receptors *in vivo*, constitute a carrier for DNA transfer at this VP1/DNA ratio, and hence increase cellular uptake of the plasmid [201]. In addition, the VP1 may protect the plasmid immunogen and is therefore likely to shield the DNA from degradation *in vivo*. Finally, the VP1 molecule is in itself highly immunogenic and can therefore stimulate general immunity, acting like an adjuvant.

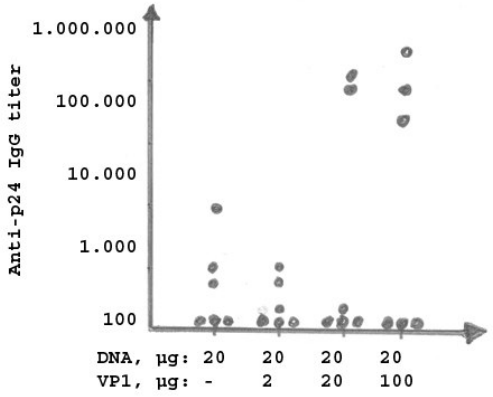


Figure 11. *Genetic immunization with polyomavirus VP1.* Immune responses in C57Bl/6 mice immunized with 20 µg HIV-1 p37 DNA plasmid together with increasing amounts of polyoma virus VP1 capsids. DNA was given at weeks 0, 4 and 7 and the animals were sacrificed 10 days post the final immunization. A significant difference (p=0.025) was observed when comparing responding animals receiving only DNA with those given the higher doses of VP1 protein (20 and 100 µg) together with the DNA

(paper II).

Only half of the animals responded with antibodies when given 20 ug DNA while the other half was unresponsive. This could be explained by the deliberately chosen threshold dose of DNA [306] or sub-optimal *in vivo* stability of the DNA-VP1 complex. No significant difference in cellular immunity, as measured by IFN-γ Elispot, was detected between the groups. A possible clinical dilemma with the use of VP1 carrier immunization is the presence of previous immunity to the carrier, leading to a limited possibility of repeated vaccine boosting. However, general strategies aiming to solve such boosting problems are under way [309]. We conclude that our novel DNA-VP1 immunization strategy can be used for protocols where the aim is to induce high titers of antibodies.

6.3 THE DEVELOPMENT OF A SMALL ANIMAL CHALLENGE MODEL USED FOR PRE-CLINICAL HIV-1 VACCINE EVALUATIONS (PAPER III)

HIV-1 can only infect humans and chimpanzees. Simian/simian-human immunodeficiency viruses (SIV/SHIV) can infect rhesus or cynomologus macaques. Since experimentation in the primate models is expensive and ethically questionable there is great need for a small-animal HIV-1 model to study infection, immunity and vaccine efficacy. This work describes the development of a murine challenge model for HIV-1.

Expression of HIV-1 antigens and release of HIV virions have been described in mice expressing human CD4, the CCR5 co-receptor and human cyclin T1 proteins [310]. In the transgenic mouse model described by Mariani et al., a low, but clearly detectable release of infectious HIV virions was seen. The production of intracellular HIV-1 p24 antigen was similar to what has been shown in human cells, but restriction appeared in the release of virions. Our approach is to generate pseudovirions between the HIV-1 genome and the murine leukemia virus envelope (HIV-1/MuLV) [311, 312]. The capacity of numerous different HIV-1/MuLV particles to infect primary spleen cells from C57Bl/6xDBA/2 (C57Bl/6.A2), C57Bl/6, BalbC, FvN/B and DBA/2 mice was evaluated. Subtype B LAI HIV-1/MuLV did efficiently infect primary C57Bl/6.A2 splenocytes, especially $CD4^+$ T cells and macrophages. We show that HIV-1/MuLV particles enter the mouse cell through its highly promiscuous virus envelope bringing with it the encapsulated HIV-1 genome. Despite the fact that our mouse cells lack a functional human cyclin T1 we can detect production of infectious virus. Recently we have shown that transfection of primary mouse spleen cells with human cyclin T1 prior to HIV-1/MuLV infection results in a 20-fold increased HIV-1 p24 antigen release [313]. This would indicate that human cyclin T1 in primary murine cells substantially enhances HIV transcription and expression, but is not an absolute requirement for release of infectious virions [314].

Pseudo HIV-1 infected splenocytes were injected into the peritoneal cavity of C57Bl/6.A2 mice and high levels of HIV-1 RNA ($>10^5$ RNA copies/ml) could be detected in sampled murine peritoneal cells on day 10 after challenge. Proviral HIV-1 DNA could also be detected both in the peritoneal fluids and in the spleen up to 24 days after infection, demonstrating both that HIV-1/MuLV infection has passed the step to reverse transcription and that infected cells can spread to secondary lymphoid organs. In this respect the model resembles human acute primary HIV-1 infection. Most importantly, viable HIV-1 could be recovered from the infected animals within 14 days of inoculation. The experimental HIV-1/MuLV inoculation resulted in broad long-lasting (at least four months) cellular and humoral anti-HIV immune responses.

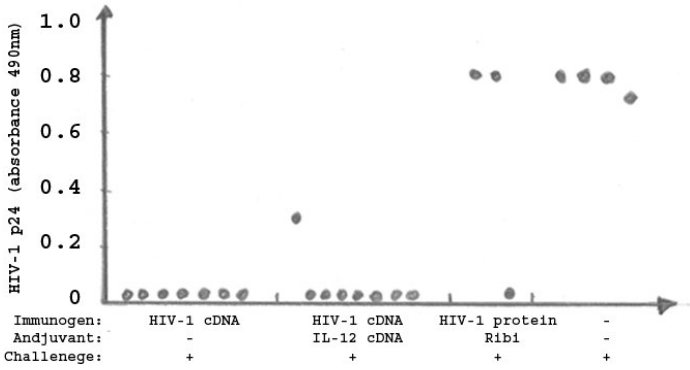


Figure 12. *HIV-1 DNA immunization protects against HIV-1/MuLV challenge.* One intranasal immunization with HIV-1 DNA encoding the gp160, Gag, Nef, Tat and Rev antigens was given to C57Bl/6.A2 mice with or without an IL-12 encoding plasmid. Alternatively, three intramuscular inoculations with a mixture of the corresponding recombinant HIV-1 proteins were delivered in formulation with Ribi. DNA immunization, but not protein immunization, protected against a subsequent HIV-1/MuLV challenge (paper III).

Genetic immunization with an HIV-1 DNA plasmid cocktail (gp160, p24, p17, nef, rev and tat) resulted in full protection from isolation of viable virus in this model. A parallel group of animals were given the same DNA plasmid cocktail together with an IL-12 encoding DNA plasmid, resulting in a strongly biased Th1 response without any detectable antibodies. These animals were still protected against the experimental challenge. Mice immunized with the corresponding HIV-1 proteins produced HIV specific IgG but were not protected after HIV-1 challenge. This demonstrates that cellular immunity is important for protection against experimental HIV-1/MuLV splenocyte challenge. The system has so far been used to validate vaccine-induced immunity in over 400 animals, as part of multiple projects, among them Andäng et al., Spetz et al., papers IV and VI. Future plans for the experimental challenge system involve distinguishing the exact correlates of protection and continuing with the search for and development of new viral isolates that can be fitted as challenges of primary-like HIV into the model (se paper VI).

6.4 STRATEGIES TO ENHANCE BROAD IMMUNITY TO SUBTYPES OF HIV (PAPERS IV AND V)

There is an obvious need for a preventive HIV-1 vaccine, especially in the developing countries. The high HIV-1 mutation rate results in the rapid development of viral variation and consequent circulation of multiple HIV subtypes and recombinants thereof. Several arguments point to the existence of immunological cross-reactivity, but *in vivo* proof of the concept has not been presented.

By using *in vitro* homologous recombination we have been able to create a set of three HIV-1 gp160 DNA immunogens originating from subtype A, B and C viruses. The cloning was designed to exchange the V1-V5 loops from the subtype B backbone construct with that of subtype A and C envelope sequences differing to 30% from the subtype B sequence. In order to construct an immunogen with the capability of protecting against both syncytium inducing (SI) and non syncytium inducing (NSI) HIV-1 isolates, we deliberately choose envelope genes of both kinds, the subtype B LAI strain being SI and subtype A and C isolates being NSI [315]. Recombinant gp160 was produced in eukaryotic cells *in vitro*. All constructs expressed gp160 protein in HeLa cells as detected by immunofluorescence and immuno blot. Co-transfection with Rev-encoding plasmid enhanced the envelope protein transcription approximately ten fold.

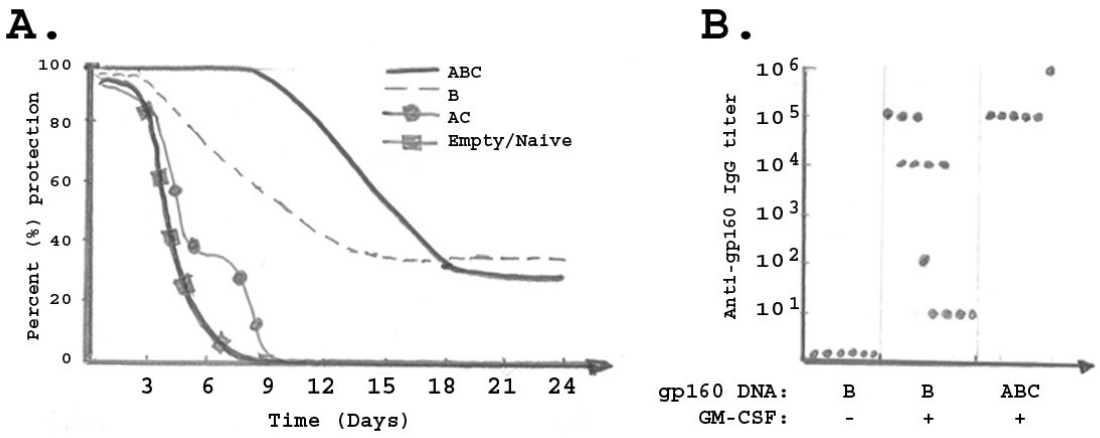
Genetic immunization of C57Bl/6.A2 mice with each gp160 DNA plasmid induced envelope specific cellular immunity and a combination of all three constructs induced the strongest immune response. Only weak anti-envelope antibody production was detected. We then investigated the ability of the multivalent vaccine to protect against challenge with a HIV-1/MuLV pseudotype virus. The subtype B DNA partially protected against experimental challenge with subtype B virus, whereas immunization with subtypes A and C did not result in protection against the heterologous subtype B challenge. Interestingly the animals immunized with a mixture of A, B and C DNA were among the best protected even though the amount of subtype B envelope gene was three-fold less in this group than in the B DNA immunized group. It appears that the envelope specific protective immunity in this experimental model is subtype dependent. Also, for the ABC immunized group there was a significant delay in the onset of p24 production. In these "slow progressors" there are arguably fewer infected cells present in the ascites fluid, which in turn would reflect a more potent immune response against the HIV-1 challenge. These animals thus seemed to be able to control the challenge virus better than non-immunized animals, and may resemble the short-term viral control in certain clinical HIV-1 infections. Also, post challenge anti-envelope IgG was produced in the vaccine primed animals. Still, no direct correlate to protection was observed in this experiment and we were therefore unable to determine if the subtype specific protection was dependent on antibodies or cellular immunity. In conclusion, for the first time we observed immune enhancement when combining three different HIV-1 envelope DNA immunogens.

Still, the humoral immunity induced after gp160 DNA immunization was overall weak and we therefore continued to validate strategies in enhancing the anti-envelope antibody response. Delivery of gp160 DNA together with rGM-CSF resulted in high titers of anti-gp160 specific IgG (mean reciprocal titer $>10^4$). The end-point titers were enhanced further (titers $>10^5$) if a mixture of subtype A, B and C gp160 DNA was delivered together with rGM-CSF. The addition of rGM-CSF to the gp160 DNA immunogen(s) strongly favors antibody production with a Th2 tilted IgG subclass profile and enhanced IL-4 production. Sera from ABC immunized animals were also able to neutralize A, B and C viral isolates, whereas sera from animals immunized solely with subtype B DNA neutralized only subtype B virus. The combined DNA vaccine gave serum antibodies with broad recognition of HIV-1 envelope epitopes as determined by peptide mapping. We believe that the enhancement of antibody production, as well as the broader antibody specificity in the multi-subtype immunized animals, are due to the presentation of multiple epitopes and thereby the stimulation of a broad clonal expansion of B cells. Similarly, broad antibody responses have been reported in the clinic with multi-subtype infected individuals being capable of producing IgG molecules that recognize HIV from several subtypes [316].

As for cellular immunity, enhanced cross-reactive T cell proliferative responses were detected after immunization with a mixture of plasmids encoding subtype A and C proteins in comparison to inoculation with the individual plasmids.

In conclusion, this demonstrates the ability of a DNA vaccine encoding multiple envelope genes to induce the desired antibody response against several HIV-1 subtypes. It also documents the ability of rGM-CSF to enhance the potency of such a vaccine when given simultaneously. This strategy may be useful for making an HIV vaccine more potent and broadly effective against strains of different clades. In the near future this approach will be tested in clinical trials.

Figure 13 *Enhancing the immunogenicity of the HIV-1 gp160 DNA immunogen.* **A.** gp160 DNA immunized



mice were challenged with syngeneic splenocytes infected with subtype B HIV-1/MuLV. Ten days after challenge, cells from the ascites fluid were collected and co-cultured with human PBMCs. Eradication of infected cells by immunized mice was assessed as a reduction of infectious HIV. Animals that received the subtype A, B and C gp160 antigens were protected if compared to controls (p=0.005). Animals immunized with subtype A and C gp160 DNA where not protected against the heterologous subtype B virus (paper IV). **B.** Robust antibody production after gp160 DNA immunization together with recombinant GM-CSF. By mixing gp160 DNA plasmids originating from subtypes A, B and C all recipients acquired very high antibody titers (paper V).

6.5 DNA/PROTEIN PRIME-BOOST IMMUNIZATION AND ITS CAPACITY TO PROTECT AGAINST HETEROLOGOUS HIV-1 CHALLENGE (PAPER IV).

The potency of existing HIV-1 vaccine candidates needs to be increased. Mixed modality prime-boost immunization, where different kinds of immunogens like DNA, protein, peptides or live vectors are combined have been found to broaden and enhance immunity. Still, knowledge within this field of research needs to be expanded further.

In collaboration with colleagues at GlaxoSmithKline, Stevenage, England and Riixenart, Holland we have performed a number of pre-clinical immunization experiments with HIV-1 DNA and protein vaccine candidates. In BalbC and C57Bl/6.A2 mice we compared the immunogenicity of HIV-1 Env, Nef and Tat DNA immunogens given alone, with that of the corresponding recombinant proteins given alone. In an initial experiment, animals received mixed modality immunizations with DNA priming followed by protein boosts and another group was given the same immunogens but in the reverse order. We show that the protein component induced high titers of anti-envelope IgG antibodies, whereas non-codon optimised DNA did not. To our surprise, we are able to show that protein priming followed by DNA boosting resulted in outstanding cellular immunity with remaining high envelope antibody titers. We tentatively call this novel strategy "reverse prime-boost immunization".

In a second experiment, we immunize with codon-optimised DNA and corresponding proteins. The most notable finding was, that codon-optimised DNA given several times, alone gave rise to complete protection in the majority of challenged mice (Figure 14). The protective effect of the vaccine-induced immunity was validated by experimental homologous subtype B HIV-1 challenge or heterologous subtype A challenge. Altogether, the type-specific protection was stronger than the cross-protection to experimental HIV infection. We have highlighted the complex relations of adjuvants, immunogen dose, route of transmission, timing of inoculations, and difficulties in choosing predictive variables. In conclusion, the results suggest that immunization with HIV-1 env, nef and tat DNA can result in a high frequency of protection and that DNA immunogens may be helped by innate immunity. An in-depth study of the observations of "reverse prime-boost immunization" in humans is planned for the near future.

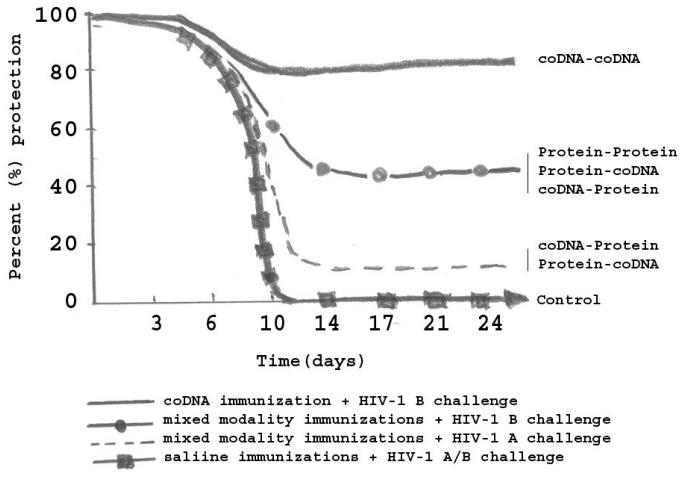


Figure 14. Gene gun immunization using codon-optimized HIV-1 env, nef and tat (coDNA) and prime/boost with recombinant proteins (Env, Nef and Tat). Gene gun immunization three times with a codon optimised env, nef, tat DNA (coDNA) construct resulted in potent protection against subtype B HIV-1 challenge (coDNA – coDNA). Three groups had partial protection; Protein alone (Env, Nef and Tat) or in combination with coDNA. Animals subjected to heterologous HIV subtype A challenge were poorly protected.

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