IMPROVING THE IMMUNOGENICITY OF HIV-1 DNA VACCINES

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ABSTRACT

Nearly 30 years have passed since the start of the global HIV epidemic and we are still unable to control the spread of the virus. HIV predominately infects cells crucial for the function of the immune system, integrates into the host genome and demonstrates a huge genetic variability, and the use of therapeutic antiretroviral drugs can restrain but not clear the infection. Therefore a protective vaccine is considered the best approach to counteract HIV. The failure of HIV vaccine candidates based on classical vaccine strategies have paved the way for novel vaccine modalities, such as genetic vaccines. These vaccines have induced broad and robust immune responses in animals but need to be optimized to ultimately induce protection against HIV infection. Furthermore, no definite correlates of protection against HIV infection are yet identified and this severely complicates the vaccine development. Nevertheless, the licensure of several DNA vaccines for veterinary use and the induction of protection against SIV infection/disease in non-human primate models raise hope for the possibility to induce protection against HIV-1/AIDS by DNA vaccination also in humans. This thesis describes both the effect of optimizing the gene insert and the use of adjuvants to augment the immune responses after immunization with HIV-1 plasmids.

A HIV-1 protease gene was genetically optimized by changing the amino acid composition. By altering the enzymatic active site, rendering the protein inactive, it was possible to greatly increase the *in vitro* protein expression and significantly increase the immunogenicity of the gene in various mouse strains, including mice transgenic for the human HLA-A0201 molecule. We thus identified a rather simple strategy to drastically increase the immunogenicity of HIV-1 protease and induce strong immune responses against wild type protease as well as against protease carrying drug resistance mutations. The optimized protease construct will be integrated into the clinically evaluated multigene vaccine, HIVIS, and initial results have shown that the immunogenicity of the protease construct, as well as the immunogenicity of the other constructs in the vaccine, is not negatively affected by the addition of the new plasmid. Also, by administering our multigene vaccine formulated in a lipid-based adjuvant intranasally to young mice we could increase both the systemic cellular and humoral immune responses. In addition, antibody responses could be detected at mucosal sites distant from the intranasal mucosa, demonstrating the ability of DNA vaccines to induce broad immune responses in different compartments of the body. The induction of these mucosal anti-HIV antibodies presents a possible means to prevent infection at the mucosal surface where the majority of HIV transmissions take place. These and other ways to augment the induction of strong immune responses by DNA vaccination will hopefully provide clues on how to construct and deliver the next generation of potent DNA vaccines against HIV as well as against other infectious diseases and cancers.

LIST OF PAPERS

I. **Hallengärd D**, Hallermalm K, Boberg A, Petersson S, Roos AK, Wahren B and Bråve A. Increased expression and immunogenicity of HIV-1 protease following inactivation of the enzymatic activity. *Manuscript.*

II. Bråve A, **Hallengärd D**, Schröder U, Blomberg P, Wahren B and Hinkula J. Intranasal immunization of young mice with a multigene HIV-1 vaccine in combination with the N3 adjuvant induces mucosal and systemic immune responses. *Vaccine 2008, Sep 19;26(40): 5075-8.*

ADDITIONAL PUBLICATIONS NOT INCLUDED IN THE THESIS

I. Bråve A, Johansson U, **Hallengärd D**, Heidari S, Gullberg H, Wahren B, Hinkula J, Spetz AL. Induction of HIV-1-specific cellular and humoral immune responses following immunization with HIV-DNA adjuvanted with activated apoptotic lymphocytes. *Vaccine 2009, Dec 28. Ahead of print, PMID: 20044053.*

II. Bråve A, **Hallengärd D**, Gudmundsdotter L, Stout R, Walters R, Wahren B and Hallermalm K. Late Administration of Plasmid DNA by Intradermal Electroporation Efficiently Boosts DNA-primed T and B cell Responses to Carcinoembryonic Antigen. *Vaccine 2009, Jun 8;27(28): 3692-6.*

III. Bråve A, **Hallengärd D**, Malm M, Blazevic V, Wahren B, Rollman E and Krohn K. Strong humoral and cellular anti-HIV immune responses can be generated with DNA vaccines in mice by combining optimal deliveries with potent vector concepts. *Vaccine 2009, Jan 7;27(2): 184-6.*

IV. Gudmundsdotter L and **Hallengärd D**, Challenges of global vaccine development. *Expert Rev Vaccines 2008, Feb;7(1):21-3. Review*

LIST OF ABBREVATIONS

Ab	Antibody
ADCC	Antibody-dependent cellular cytotoxicity
AIDS	Acquired immunodeficiency syndrome
APOBEC3G	Apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3G
CD	Cluster of differentiation
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DNA	Deoxyribonucleic acid
ELISpot	Enzyme-linked immunosorbent spot
Env	Envelope
EP	Electroporation
ER	Endoplasmatic reticulum
Fc	Fragment crystallizable
Gag	Group-specific antigen
GALT	Gut associated lymphoid tissue
GM-CSF	Granulocyte macrophage colony-stimulating factor
Gp	Glycoprotein
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
IFN	Interferon
IL	Interleukin
IN	Integrase
LTR	Long terminal repeat
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
Nef	Negative regulatory factor
NK	Natural killer
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside reverse transcriptase inhibitor
PAMP	Pathogen associated molecular patterns
RANTES	Regulated on activation normal T cell expressed and secreted
Rev	Regulation of viral expression
RNA	Ribonucleic acid
PR	Protease
PRR	Pattern recognition receptors
RT	Reverse transcriptase
SIV	Simian immunodeficiency virus
Tat	Transactivator for transcription
TCR	T cell receptor
Th	T helper
TLR	Toll like receptor
TRIM5a	Tripartite motif protein 5a
Vif	Viral infectivity factor
VLP	Virus-like particle
Vpr	Viral protein R
Vpu	Viral protein

CONTENTS

1 THE HUMAN IMMUNODEFICIENCY VIRUS

In the beginning of the 1980's numerous young men in the US, mainly homosexual and intravenous drug users, fell ill from opportunistic infections and Kaposi's Sarcoma, a rare form of cancer. The symptoms demonstrated clear evidence that the patients suffered from immune suppression and these clustered symptoms were named acquired immunodeficiency syndrome (AIDS) (1). The causative agent of AIDS was identified in 1983 by a French research group led by Luc Montagnier (2), a finding that was awarded the Nobel Prize in medicine 2008. Soon after, an American research group lead by Robert Gallo also published their findings of a novel virus isolated in AIDS patients (3, 4). Both the French and the American groups noted that the virus infected T lymphocytes, why they named it lymphoadenopathy-associated virus (LAV) and human T-lymphotropic virus type III (HTLV III, due to the resemblance with HTLV I), respectively. It was later shown that the two groups had in fact isolated the same virus, and the virus was renamed human immunodeficiency virus (HIV) in 1986 (5).

Today, almost thirty years after the start of the global HIV/AIDS epidemic, we are still unable to control the spread of the virus and during 2008 approximately 2,7 million people became infected with HIV (www.unaids.org). There are two types of HIV, type 1 (HIV-1) that was first isolated and type 2 (HIV-2) that was discovered in 1986 (6). The more pathogenic HIV-1 is spread worldwide and is responsible for the vast majority of cases of AIDS, whereas the less pathogenic HIV-2 is mostly found in the western parts of Africa (7). HIV is thought to originate from the simian immunodeficiency virus (SIV) prevalent in African non-human primates and the passage to humans is thought to have taken place during handling of infected blood in the beginning of the 20th century (8-11). HIV-1 (from now on referred to as HIV) is divided into groups; major (M), outlier (O) and non-M non-O (N), and the M group is further divided into subtypes A-K.

1.1 STRUCTURE AND REPLICATION

HIV is a Lentivirus belonging to the *Retroviridae* family. The virus is spherical with a diameter of approximately 100 nm. The bi-layered lipid envelope, containing the envelope glycoprotein (gp) spikes, is derived from the host cell during budding (Fig. 1). Inside the envelope a protective cone-shaped capsid surrounds the genome consisting of two identical 9,2 kBp single stranded RNA molecules with positive polarity. The genome encodes three major polyproteins; Group-specific antigen [Gag], Envelope [Env] and the enzymatic proteins [Pol]) as well as several regulatory and accessory proteins.
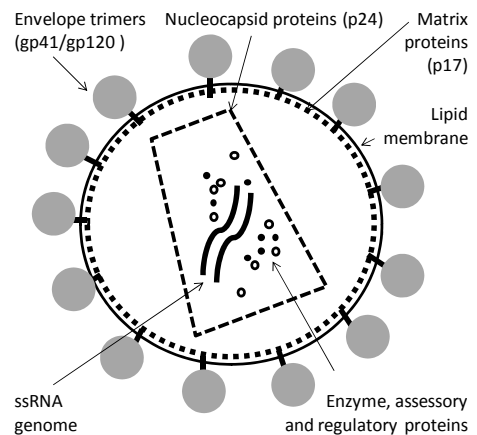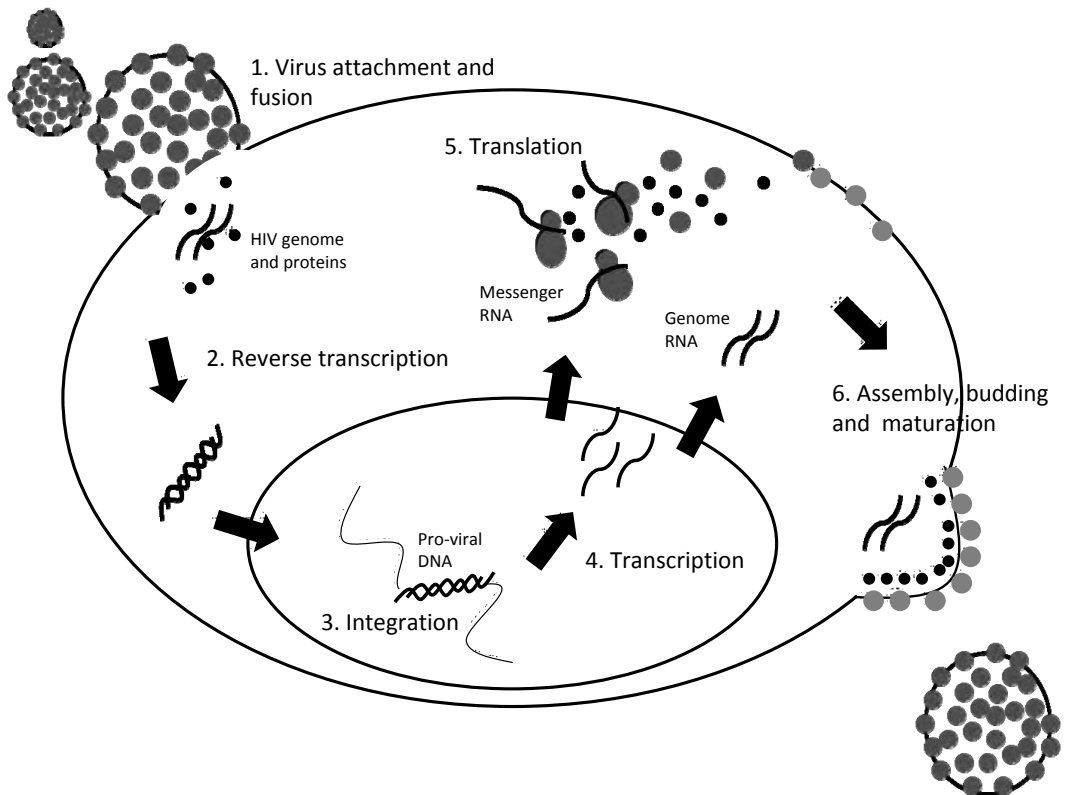

Figure 1. The HIV virion

1. Virus attachment and fusion

5. Translation

HIV genome and proteins

Messenger RNA

Genome RNA

2. Reverse transcription

6. Assembly, budding and maturation

Pro-viral DNA

4. Transcription

3. Integration

The viral life cycle begins when the extracellular viral gp120 binds to CD4 molecules present predominantly on T lymphocytes, macrophages, dendritic cells (DCs) and brain microglia (12) (Fig. 2). In addition to the CD4 molecule on the host cell, the virus requires either of the co-receptors CCR5 or CXCR4 for entry. After binding, gp120 undergoes conformational changes allowing for the transmembrane part of the envelope spike, gp41, to insert its hydrophobic terminus into the host cell membrane, which enables fusion of the viral and host cellular membrane. The nucleocapsid is then released into the cytoplasm and undergoes uncoating during which the genomic RNA strands, enzymes and additional molecules required for the initiation of translation are released. The reverse transcriptase (RT), which is the trademark of retroviruses, reversely transcribes the single stranded RNA genome into a complementary strand of DNA (12). The template RNA is then degraded by the Ribonuclease H domain of RT and a complementary DNA strand is synthesized, creating a double stranded DNA HIV genome, called the provirus. During reverse transcription, long terminal repeats (LTRs) are added to both the 5' and 3' end of the DNA and the LTRs are crucial for facilitating the subsequent transcription of the viral genome. Another viral enzyme, Integrase (IN), then form a pre-integration complex with the double stranded DNA and other viral and cellular proteins and enters the nucleus where the HIV genome is inserted into the host genome (12). Once the viral genome is integrated, the infected cell can become latent (13), which makes it very difficult to clear the viral infection.

When the integrated pro-viral DNA is being transcribed by the host RNA polymerase II, either to produce novel viral genomes or viral messenger RNA (mRNA), the regulatory proteins are the first ones to be translated and these, amongst other things, facilitates the expression of the late structural viral proteins. The transactivator for transcription (Tat) protein forms complex with several cellular proteins and binds the LTRs of the viral genome and thereby enhances transcription of viral RNA (14, 15). The regulator of viral expression (Rev) protein increases the expression of the viral Gag, Env and

Pol poly-proteins as it binds Rev-responsible elements present in the viral RNA and thereby facilitates the export of unspliced viral mRNA from the nucleus (14, 15). The negative regulatory factor (Nef) protein accelerates the endocytosis and subsequent degradation of CD4 and major histocompatibility complex (MHC) class I molecules so that the cell evades recognition by the immune system (14, 15). Besides the regulatory proteins, three accessory proteins; viral infectivity factor (Vif), viral protein R (Vpr) and viral protein U (Vpu) are expressed from the viral genome. Vif counteract the antiretroviral effect of apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3G (APOBEC3G), which is a protein that inhibits retroviral infection by hypermutating the negative RNA strand during reverse transcription resulting in deamination of the pro-viral DNA (16, 17). The Vpr constitutes a part of the pre-integration complex and the Vpu enhances the release of virions from the cell surface (16, 17).

The Env gp160 precursor protein is expressed and glycosylated in the endoplasmatic reticulum (ER) and subsequently cleaved by the cellular protease furin into gp120 and gp41 in the Golgi apparatus and transported to the cell surface. There, trimers of transmembrane gp41 protein associate to trimers of the extracellular gp120 protein. Simultaneously, two copies of the viral genome and p55 Gag and p160 Gag-Pol poly-proteins are assembled at the cell membrane, associates with the glycoproteins and a new particle subsequently buds from the cellular membrane. After budding of the immature virions, the viral Protease (PR), which is auto-cleaved from the Pol precursor protein, cleaves the Gag and Gag-Pol poly-proteins into: p17 (matrix protein), p24 (capsid protein), p7, p6 (nucleocapsid proteins) and the viral PR, RT and IN enzymes (12). This last step of the replication completes the life cycle and the mature virion is now ready to infect new CD4+ cells.

1.2 COURSE OF INFECTION

HIV is transmitted by sexual contact, transfer of infected blood and from mother to child during pregnancy, birth or breastfeeding. Infection through heterosexual intercourse, which accounts for the majority of infections, is estimated to occur in as low as one in 100 sexual contacts (18). However, the likelihood of being infected increases with the viral load of the infected partner, the state of the mucosal surfaces and the type of sexual activity (19, 20). The initial symptoms of an HIV infection during the primary infection are either absent or manifests as "flu-like" symptoms that includes fatigue, headache, lymph node swelling and fever. The symptoms arise when the immune system reacts in response to HIV infecting leukocytes and the virus starts to multiply. The initial infection results in a massive loss of CD4+ lymphocytes, predominately CD4+ memory T cells in the gut associated lymphoid tissue (GALT) (21, 22) (Fig. 3). Despite the loss of CD4+ T helper (Th) cells, which normally coordinate the adaptive immune system, the onset of the adaptive immune responses decreases the viral load, which after the initial viral peak, settles at a level that is referred to as the viral set-point. This set-point is often a good predictor of the outcome of the infection (19, 20) and the higher viral set-point the more rapid disease progression. The acute phase is followed by a clinical latency phase that often lasts for several years. Here, the CD4+ T cell count steadily decreases as the viremia gradually increases. When the CD4+ T cell count has dropped below 200 copies/μL blood and/or when opportunistic infections occur, the clinical phase starts and the patient progresses to AIDS (23).
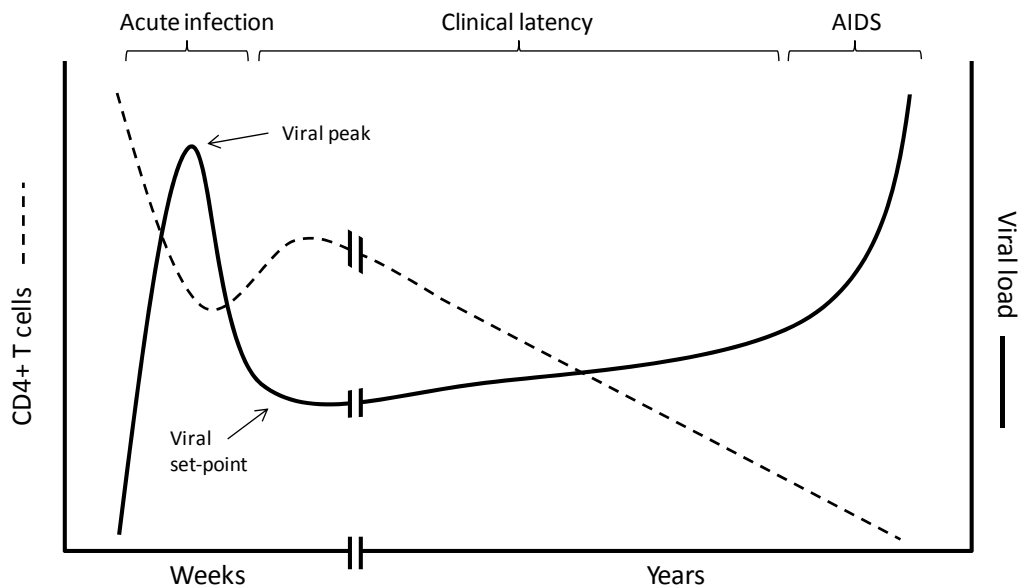
Figure 3. The course of untreated HIV infection

1.3 PREVENTION, TREATMENT AND DRUG RESISTANCE

Even though massive information campaigns have been carried out in order to increase the knowledge about HIV and AIDS, the virus is still rapidly spreading in large parts of the world. The simplest way to decrease the sexual spread of HIV is by preventive measures such as reducing the number of sexual partners and using condoms. However, these precautions might be hard to implement in cultural and educational settings where women have little or no control and where religious believes overrides logic and scientific reasoning (24). Transmission of HIV via infected blood is, in some countries, prevented by offering intravenous drug users to replace their used needles. However, this strategy is highly debated (25, 26).

Fortunately there are several efficient anti-HIV drugs available that targets different stages of the viral life cycle. Initially, monotherapy with azidotymidin (AZT) was attempted but the therapy failed due to the rapid mutation rate of the virus resulting in virus replication despite the presence of the drug. Later, in 1996, the "highly active antiretroviral therapy "(HAART), consisting of combinations of drugs acting by different mechanisms, was introduced (27, 28). Since then, this therapy has been successfully used to suppress viral replication, delay the development of AIDS and substantially increase the quality of life for HIV-infected individuals.

Antiretroviral drugs are divided into different classes according to function. The RT inhibitors are divided into nucleoside and non-nucleoside RT inhibitors (NRTI and NNRTI, respectively) where the NNRTIs directly affect the enzymatic activity by binding to the viral enzyme, and the NRTIs act indirectly as they disrupt the chain elongation during transcription by taking the place of the natural nucleoside (29). The PR inhibitors act by binding in the active site of the protein and thereby prevent proper enzymatic function (30). According to the Swedish reference group for antiviral therapy, the first line of anti-HIV therapy should include two NRTI and either a NNRTI or a PI and treatment should be initiated when the CD4+ T cell count has dropped below 350 copies/μL of blood (www.smittskyddsinstitutet.se/rav). In addition to these classical targets for HIV-inhibition, novel

antiretroviral drugs affecting other parts of the viral life cycle have been licensed over the last years. These includes drugs targeting integration by IN (31), viral/host cell fusion (32, 33) and co-receptor binding (34). However, so far no antiretroviral drugs are able to clear infection and the access to HAART is limited in low-income countries, why an effective prophylactic vaccine is urgently needed.

Since RT lacks proof-reading mechanism the enzyme frequently induces mutations during the reverse transcription and approximately three nucleotide substitutions are introduced per 10^5 incorporated nucleotides during each round of replication (35). In addition, recombination between the two strands of viral RNA by RT promote further genetic diversity (36). By combining the high mutation and recombination rate with a rapid viral turnover and an extremely efficient production of new virions (37, 38), HIV easily escapes recognition by both HIV inhibitors and the adaptive immune system. The induction of drug resistance mutations is, however, usually prevented when complying with the HAART regimen.

2 HIV ANIMAL MODELS

HIV only replicates efficiently in humans and chimpanzees as several species-specific host factors restrict the replication (39, 40). Hence the initial studies of pathogenesis and the efficacy of vaccine candidates were performed in chimpanzees (41, 42). This model is however rarely used today due to ethical aspects, huge costs and the fact that chimpanzees seldom develop AIDS. Instead SIV infection of Asian macaque monkeys is frequently used as a model system for HIV infection. Depending on the pathogenesis of the strain of SIV used, the infected macaques develop a disease that mimics that of HIV infection in humans, although the infection often progresses more rapidly than in humans (43). In order to be able to evaluate HIV vaccine candidates in macaques, chimeras of HIV and SIV, called SHIV, are used to challenge vaccinated monkeys. (44). In addition, small animals such as mice are frequently used in HIV research, and the mice are often genetically modified to express various human proteins (45). Nevertheless, the optimal means to study the complex interaction between HIV and the immune system is via clinical studies.

3 IMMUNOLOGY IN HIV INFECTION

3.1 INNATE IMMUNITY

The innate immune system is our first line of defense against invading pathogens. This arm of the immune system consists of external barriers such as skin and mucus membranes as well as various leucocytes and proteins. Upon infection, the innate immune system acts by various mechanisms, for instance by recognizing conserved microbial structures known as pathogen associated molecular patterns (PAMPs). These structures can for example be DNA, RNA or endotoxins and they are recognized by the pathogen recognition receptors (PRRs) located on and within cells of the innate immune system. This interaction initiates a cascade of antiviral events including the production of molecules with a direct antiviral activity and cytokines and chemokines that direct both the innate and the adaptive immune system (46, 47).

For HIV there are a number of different components of the innate immune system that affects the outcome of the infection. For example, natural killer (NK) cells, DCs and macrophages produce chemokines such as regulated on activation normal T cell expressed and secreted (RANTES) and macrophage inflammatory protein (MIP) type 1 α and β, which are the natural ligands for the CCR5 molecule that is used as a co-receptor by HIV. These molecules thus prevent CCR5-tropic HIV from infecting cells and interestingly, these chemokines have been shown to be upregulated in exposed but uninfected individuals (48-50). Other anti-HIV proteins produced by the innate immune system are the intracellular APOBEC3G and tripartite motif protein 5a (TRIM5a) molecules. As previously

described, APOBEC3G deaminates, and thereby impedes, the pro-viral DNA (51-53) and TRIM5a binds to the viral capsid and prevents viral uncoating (54, 55). In addition, antigen presentation and signaling by the innate immune system is crucial for the development of functional HIV-specific adaptive immunity (56).

3.2 ADAPTIVE IMMUNITY

The adaptive immune system acts in a more specific manner than the innate system and is dependent on antigen-specific memory. It is however slower than the innate system and requires days or weeks to develop. The system is divided into a cellular and a humoral arm that is, simplified, coordinated by the CD4+ Th type 1 or 2 (Th1 or Th2) cells, respectively. Additional subsets of CD4+ T cells include the regulatory T cell population that restrain/control the activity of lymphocytes and thereby prevent the induction of auto reactivity (57), and the more recently discovered Th17 cells that has been shown to play an important role in combating specific pathogens, especially at mucosal compartments and in inducing tissue inflammation and auto-immunity (58). The fact that the CD4+ T cells are the main target cells for HIV has severe consequences for the induction of HIV-specific immunity, as the infection prevents both arms of the adaptive immune system to function properly. Furthermore HIV escapes the immune system by down-regulating MHC molecules from the host cell membrane (59), and by constantly changing the amino acid composition the immunogenic epitopes, resulting in reduced recognition of virus by the immune cells (60, 61).

3.2.1 Humoral immune responses

The main component of the humoral immune system is the B cells that express membrane-bound antibodies (Abs). The Abs bind and promotes engulfment of antigens, which are subsequently processed, associated with MHC class II molecules and presented on the B cell membrane for the T cell receptor (TCR) on Th cells. If the proper co-stimulatory signal is present on the B cell, the Th cell secretes cytokines that allows the B cell to differentiate into an Ab-secreting plasma cell and also stimulates the B cells to switch Ab isotype, from IgM or IgD to IgG, IgA and IgE.

Vaccine-induced neutralizing (n) Abs is the known correlate of protection for most microbial vaccines developed to date, partly since this is relatively easy to measure with classical laboratory methods. In order to achieve sterile protection with a HIV vaccine, it should be able to induce broadly nAbs. Despite the difficulties to induce such nabs against HIV there are some nabs that has been isolated from HIV-infected individuals. These monoclonal nAbs have been shown to prevent SHIV infection in macaques when administered post challenge (62) and more recently passive immunization with nAbs have been shown delay the rebound after treatment interruption of antiretroviral therapy in HIV infected individuals (63). These Abs does, however, most often have unusual characteristics, which makes it difficult to induce similar Abs by immunization (64).

HIV possesses several features that complicate the induction of nAbs. The first, and perhaps most important, trait of HIV is the vast genetic diversity that due to pressure from the immune system results in a constant change in appearance of the gp120/gp41 envelope spike (60). Moreover, the spike is heavily glycosylated and this can sterically hinder Abs from binding the protein (65, 66). The existence of non-functional spikes, misleading the Ab responses, has also been suggested to constitute an obstacle for the induction of nAbs (67, 68).

In addition to the sterical hindrance of the virus-host cell contact mediated by nAbs, Abs can activate the complement system and promote cell killing by Ab-dependent cellular cytotoxicity (ADCC). This occurs as cells of the immune system interacts with the Fc-part of a bound Ab, and the importance of

the Fc-receptor mediated activity was demonstrated in an experiment where removal of the Fc part of a broadly HIV-nAb resulted in reduced protection from challenge in the SHIV/macaque model (69).

3.2.2 Cellular immune responses

The CD8^{+} Cytotoxic T Lymphocytes (CTLs) are the main cells of the cellular adaptive immune system. These cells recognize antigens that are presented on MHC class I molecules at the surface of an infected cell. Subsequent to encountering the MHC/peptide complex, the CTL develop into a antigen-specific memory CTL, effector CTL or both (70). The effector CTLs can mount strong immune responses that can, together with HIV-specific Abs, contribute to controlling viral replication during the different stages of the infection (Fig. 3). One of the main modes of cytotoxic killing by CTLs is by the release of granules containing granzymes and perforin. Perforin is traditionally thought to create pores in the cell membrane of the infected cell, allowing for an influx of granzymes into the infected cell that initiates a cascade of cellular events eventually leading to apoptosis. More recently the role of perforin in the uptake of granzymes into cells has been questioned as perforin and granzymes have been observed to be co-endocytosed into cells, and that perforin perturbs the endosomal membrane rather than the cell membrane to release the endosomal contents and induce cell death (71). Also, the Fas-Fas ligand interaction between infected cells and e.g. NK cells or CTLs, respectively, is an important route to induce apoptosis of infected cells (72). Furthermore, the secretions of several cyto- and chemokines have prominent impact on the HIV infection. For example, the production of IFN-γ can, in addition to activating an array of cells of the immune system, also directly inhibit HIV replication (73).

In the case of HIV it is believed that the CTLs are unable to prevent infection. However, there are several observations indicating the importance of the cellular immune responses for controlling the HIV infection. Rhesus monkeys were shown unable to control the primary SIV infection when an anti-CD8 monoclonal Ab was administered prior to challenge (74). Additionally, if the Ab was administered during chronic infection, the virus levels increased for as long as the Ab was in circulation (75). There are also examples of individuals that are not infected with HIV despite repeated exposures during unprotected sexual intercourse. The now famous cohort of sex workers in Nairobi is one example of individuals that demonstrate HIV-specific CTLs but lack Ab responses (76). Another unique group is the elite controllers that are infected with HIV but capable of suppressing the virus and able to retain their viral loads at undetectable levels (77). In these cohorts there is an overrepresentation of individuals expressing the HLA-B57 and -B27 alleles, which are both associated with a better outcome of the HIV infection (78, 79). Nevertheless, this does not solely explain why these individuals succeed in controlling HIV infection, but it does imply the importance of CTLs in controlling HIV infection. Further evidence for the positive effects of CTLs on the clinical outcome of HIV infection are the correlation between CTL responses to HIV Gag epitopes and low viremia, and the importance of polyfunctional CTL responses (80, 81).

4 HIV VACCINES

The goal of immunization is to educate the immune system to recognize and thereby prevent infection by microbes. The first well documented example of vaccination was performed by Edward Jenner in 1796 when he observed protection against smallpox after inoculation with the closely related cowpox virus. Today smallpox is eradicated thanks to extensive vaccination campaigns, and several other infectious diseases can be very efficiently prevented by vaccination.

A prophylactic HIV vaccine that can confer protection against infection is most likely the most efficient way to end the HIV pandemic. Several vaccine strategies have been evaluated in the search for an effective HIV vaccine but, so far, with limited success. It has therefore been argued that a

vaccine inducing partial protection, i.e. is able to decrease the viral load in an infected person and by this reduce the further spread to uninfected individuals, is adequate (82). For already infected individuals a therapeutic vaccine could strengthen the immune system and help to suppress the infection (83).

4.1 LIVE ATTENUATED VACCINES

Most classical viral vaccines, such as the measles, mumps, rubella, polio and yellow fever vaccines, consists of live attenuated viruses that still have parts of their replicative capacity intact but without causing disease. The vaccine-microbe has typically gone through several passages through cell cultures to lose its pathogenic elements. Another strategy is to use a closely related but benign virus, such as the cowpox virus that was used as a vaccine against smallpox. As these vaccines actually cause an infection they are able to stimulate many parts of the immune system and by this induce a broad both humoral and cellular immune response. However, one negative characteristic of this vaccine modality is that the replication competent vaccines can in some cases cause disease and usually cannot be administered to immune-suppressed individuals.

To examine this vaccine strategy against HIV, *nef*-deleted mutants of SIV were assessed for their ability to protect macaques from intravenous challenge with pathogenic SIV. The vaccinated monkeys, as opposed to control monkeys, were protected against infection (84). However, as a result of the high mutation rate of HIV, the virus might revert to a pathogenic form, resulting in disease (85). A similar observation was made in individuals that were accidently infected with a *nef*-deleted HIV via blood transfusion. Similar to what was observed in the non-human primates, the initial control of disease progression was followed by progression to AIDS as the virus reverted to a more pathogenic form (86, 87). Hence this vaccine modality is considered too dangerous to be used against HIV.

4.2 INACTIVATED VACCINES

The inactivated viral vaccines are unable to replicate and can thus be used in immunocompromised individuals. Today several vaccines, including those against Polio, Infuenza and Hepatitis A are based on this type of vaccine. However, the inability to replicate also results in induction of a skewed immune response resulting in a strong humoral response rather than a broad both cellular and humoral immune response. Against HIV, one example of an inactivated vaccine is the Remune vaccine candidate consisting of an inactivated HIV particle devoid of the gp120 protein and formulated in incomplete Freunds adjuvant (88). When Remune initially was administered to HIV infected individuals, a significant decrease in viral load, a tendency of increased CD4+ T cell count and an increase in the HIV-specific immune response were observed. However, another clinical trial in HIV infected on treatment was stopped because no difference was found between patients receiving the vaccine and those who did not (89).

4.3 RECOMBINANT SUBUNIT VACCINES

As an alternative to using the whole virion as a vaccine one can use proteins, often the surface proteins, from a microbe. These vaccines mainly induce humoral responses and potentially nAbs and this vaccine modality is today used successfully against for instance Hepatitis B and Human papilloma virus. For HIV, most subunit vaccine candidates have been based on the envelope spike as this is the only protein displayed on the viral particle and thus the sole viral component that is accessible to nAbs. For example, repeated vaccination of infected individuals with a recombinant (r) gp160 antigen decreased mortality in AIDS during a 2-year period, but after initiation of HAART this advantage was lost (90). However, the vaccine candidate that has received most attention is the AIDSVAX rgp120

vaccine developed by VaxGen (91). Initial phase I and II trials showed promising results with regard to safety and immunogenicity and two phase III trials in uninfected volunteers were conducted. The first trial enrolled 5000 participants, mainly men who have sex with men, and was conducted predominantly in North America using a subtype B rgp120. The second trial was performed in Thailand with about 2500 intravenous drug users using a subtype B/E rgp120 vaccine to better mimic the HIV subtype present in the country. Although promising results in the initial immunogenicity studies, there were no significant difference in the number of newly infected between the participants receiving the vaccine and the ones receiving placebo (adjuvant only) (www.clinicaltrials.gov). The AIDSVAX rgp120 subtype B/E vaccine has however successfully been used for boosting a viral vector vaccine in a phase III efficacy trial in Thailand (further described in the virally vectored vaccines section) (92). In addition to vaccines based on rgp120, there are several examples of recombinant subunit vaccines based on other HIV proteins, for example recombinant Tat (93). Also virus-like particles (VLPs), consisting of self-assembled Gag and Env proteins that forms particles resembling HIV but lacking the genome, enzymes and regulatory proteins, are used (94).

4.4 PEPTIDE VACCINES

In a peptide vaccine, short stretches of amino acids (normally between 8 to 30 aa) representing strong either Th or CTL epitopes are used as immunogens. This vaccine technology possesses advantages such as easy production and the possibility to rapidly alter the amino acid composition. Peptide based vaccines are however normally less immunogenic than proteins and typically require the use of strong adjuvants or linkage to carrier or adjuvant proteins (95, 96) to induce an immune response. Recently a peptide vaccine termed HIV-LIPO-5, containing five long peptides from HIV Gag, Pol and Nef coupled to a lipid tail, was reported to induce sustained both CD4 and CD8 T cell responses in a phase II placebo-controlled trial (97). Interestingly, the low dose group receiving only 50µg/peptide generated as strong responses as did the high dose group receiving 500µg/peptide, why this dose will be used in further trials examining the efficacy of this vaccine.

4.5 GENETIC VACCINES

The last two decades a lot of focus of HIV vaccine development has been on genetic vaccines, either in the form of DNA plasmids or recombinant viral vectors. The expressed vaccine-antigens are being presented by the immune system in the same way as for intracellular microbes and can therefore, depending on the type of antigen being expressed, induce a balanced both cellular and humoral immune response. Today, several clinical HIV vaccine trials are based on this vaccine modality.

4.5.1 Plasmid DNA vaccines

That DNA plasmids could be used as vaccine vectors was discovered in the early 90's and the first proof that genes could induce protection from infection was demonstrated against influenza virus. It was shown that the genes could induce strong immune responses capable of protecting mice from challenge with pathogenic influenza virus (98, 99). This finding resulted in interest in this safe and seemingly effective non-live vaccine approach that has been proven to induce both cellular and humoral immune responses without being hampered by the anti-vector immunity observed for the attenuated vaccine vectors. Soon after the initial findings, the first DNA vaccine encoding the HIV gp160 antigen were constructed and shown to induce strong and balanced immune responses in mice (100).

In the late 90's Wahren and colleagues performed the first clinical trial examining the safety, tolerability and immunogenicity of a DNA vaccine. The trial enrolled nine asymptomatic and untreated HIV-infected who were administered plasmids encoding HIV Rev, Nef and Tat. The vaccine

was safe and tolerable, and induced cellular immune responses to all three antigens (101). Soon after, Weiner et al conducted a study in asymptomatic HIV-infected volunteers that examined the immunogenicity of a DNA vaccine encoding HIV Env and Rev. Although safe and tolerable, no significant difference in the number of CD4+ T cells, plasma viral load or strength of immune responses were observed (102). Later, using DNA vaccines encoding more HIV antigens, Weiner and colleges observed increased CTL responses and a moderate effect on the viremia of HIV-infected individuals on HAART as the presence of viral blips (transient elevations of HIV RNA levels) were lower in the vaccine group than for the placebo group (103). Since then DNA vaccines encoding antigens from numerous pathogens as well as from tumors have been explored and there are now several DNA vaccines licensed for veterinary use (104-107). Additionally, the induction of protection against SIV and SHIV infection (108-110) in non-human primates raises hope for an effective plasmid-based vaccine in humans.

In an attempt to target the vast genetic variability of HIV, we have designed a HIV DNA vaccine candidate termed HIVIS that represents several HIV genes of different subtypes (111). This multigene/multiclade vaccine encodes; Env (subtypes A, B and C), Gag (subtype A and B) and Rev and an inactivated RT (subtype B), and has proven to induce strong immune responses both in animals and humans (112, 113). This vaccine will be further discussed in the following sections.

4.5.1.1 Features of the vaccine plasmid

To function as an expression vector the plasmid is required to contain some basic elements including: 1) an origin of replication (ORI) that allows for the replication of the plasmid in bacteria; 2) a selection marker; 3) a strong eukaryotic promoter to initiate transcription of the encoded protein; and 4) a polyadenylation (poly-A) signal in the 3' end of the gene to stabilize the mRNA (Fig. 4). Additional modifications including the addition of a Kozak sequence just upstream of the translational start of the gene enhance translation and hence increases expression (114).
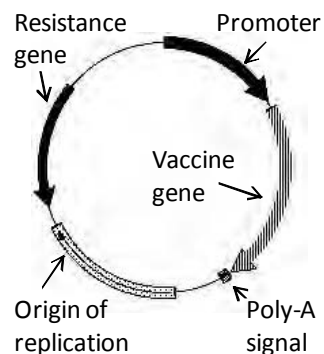

Figure 4. The plasmid DNA vector

4.5.1.2 Protein expression and induction of an immune response

Plasmid DNA vaccines can be injected (intradermally or intramuscularly) or administered via the mucosal surfaces (orally, intranasaly, intravaginally or intrarectally) and transfect cells via a so far unknown mode. Inside the cell, the host cell machinery transports the plasmid to the nucleus and transcribes the gene into mRNA, which is then translated into protein in the cytoplasm. The endogenously produced antigen can then be degraded by the proteasome into shorter peptides, typically 8-10 amino acids long, that can be associated with the MHC class I molecules in the endoplasmatic reticulum (ER) and transported through the golgi network to the cell membrane (Fig. 5). The complex is then presented to the TCR on the CD8+ T cells that, in the presence of the proper co-stimulatory signals, induce an antigen-specific cellular immune response.
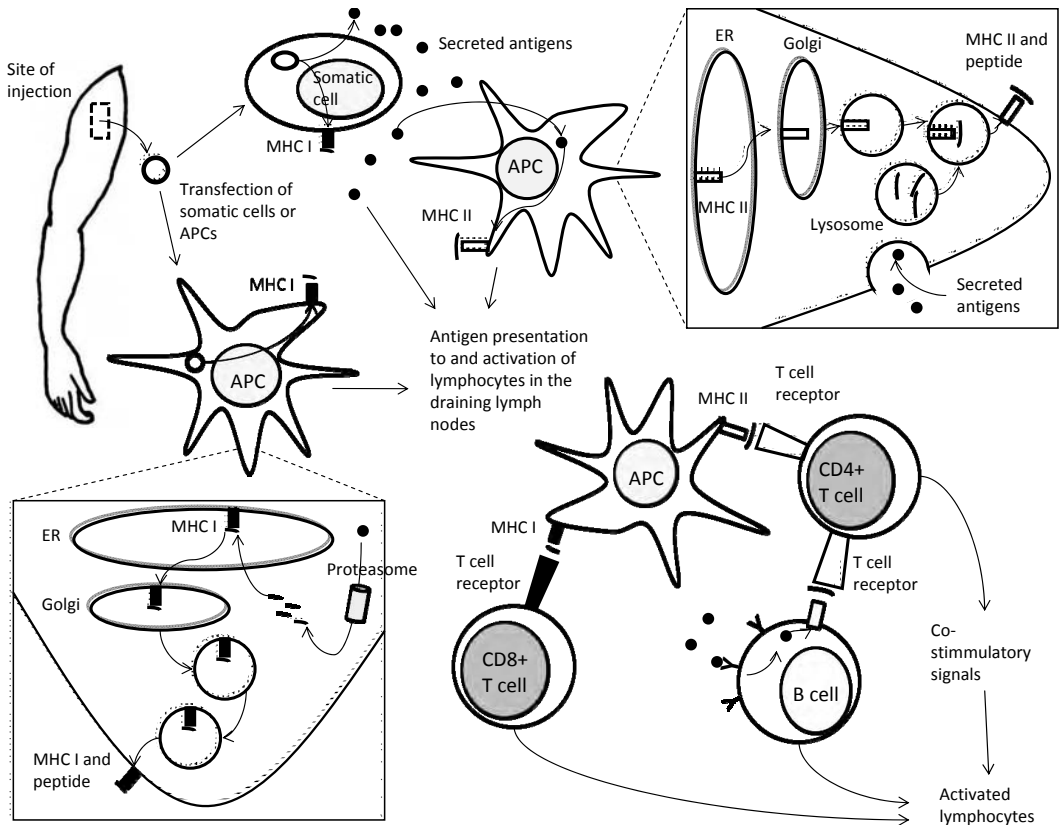
Figure 5. Immune response after plasmid DNA vaccination

Moreover, if a secretion signal is present in the expressed protein, it can be transported to the cell surface and either be bound to the membrane or secreted and can then stimulate humoral responses. The secreted protein then binds to Abs on B cells or is endocytosed by other APCs. Subsequent degrading and association of the peptides with the MHC class II molecules takes place in the lysosome. The resulting peptide/MHC complex is then transported to the cell surface and presented to the TCR on the CD4+ T cells, which in turn stimulates the production of Abs by B cells (Fig. 5).

Antigen presentation by the different parts of the immune system is, however, not a static process. This is highlighted by the occurrence of cross-presentation and autophagy. Cross presentation is the event when exogenous antigens are degraded and presented via the MHC class I pathway. This way of priming the immune system has been suggested to play a major role in the induction immune responses after DNA vaccination (115) as well as after immunization with certain viral vectors (116). Autophagy is the process when endogenously produced antigens are degraded by the lysosome and presented via the MHC class II pathway. Either way, the APCs travels to the draining lymph nodes where they present the antigen to the lymphocytes and stimulate the induction an antigen-specific immune response.

4.5.1.3 Means of increasing the immunogenicity of DNA vaccines

DNA vaccines have, despite inducing strong immune responses in small animal models, not yet met the success that it was expected to do in non-human primates and humans. Therefore, there are many strategies being employed in order to try to enhance the potency of naked DNA vaccines. As

the level of expression of the vaccine antigen most often correlate with immunogenicity (117-119) it is important to optimize the antigen expression. Besides using a potent/highly efficient plasmid vector, a simple and now days standard routine to increase the expression is to codon-optimize the gene by replacing the wild-type codons with the codons most frequently used in humans (120). Examples of other strategies employed to increase the immunogenicity to DNA vaccines is the removal of inhibitory sequences that is often present in microbial genes and results in reduced level of transcription (121, 122). It is also possible to genetically link the antigen to a secretory signal that allows for an increased antigen excretion and an increased Ab response (123, 124). In addition, various ways to deliver the plasmids and the use of potent adjuvants can be explored to tilt the immune response in a desired direction (i.e. Th1 or Th2) or augment the immunogenicity and thereby reduce the number of immunizations and the amount of antigen needed.

4.5.1.3.1 Technical devices
The first delivery device employed to increase the efficiency of DNA vaccines was the Gene gun. Here, the vaccine plasmids are coated on gold particles and shot into the skin. This has been shown to drastically increase the uptake of plasmids into the cell, resulting in an increased protein expression and immune response (98) and even protective Ab titers to Hepatitis B after vaccination with a DNA vaccine encoding the Hepatitis B surface antigen (125). Although efficient, only small amounts of DNA can be delivered using this method and the use of gold particles limits the relevance of this technology. Other delivery methods that increase the immunogenicity of plasmid vaccines are the needle-free Syrijet (126) or Biojector (112, 113) devices that propel the vaccine into the skin in a stream of liquid. Even a conventional tattoo apparatus that repeatedly penetrates the skin and thereby deliver the vaccine can be used (127, 128).

Currently the most promising approach for delivering plasmid vaccines is *in vivo* electroporation (EP). This method have since long been used in *in vitro* settings to transfect both pro- and eukaryotic cells, but is now employed to increase the uptake of plasmids after immunization (129, 130). EP can be applied either intradermally or intramuscularly, as determined by the length of the needles and electrodes used, and can either both deliver the vaccine and electroporate (TriGrid EP device, www.ichorms.com and www.inovio.com) or solely electroporate after vaccine injection (DermaVax EP device, www.cytopulse.com). The mechanisms by which EP increases the plasmid uptake is thought to be via the transient formation of pores in the cell membrane, which increases the influx of plasmids into cells (131), and by the tissue damage caused by the electric pulses that can recruit leukocytes to the site of injection (132) and promote local inflammation. EP has been shown to increase the immunogenicity of numerous vaccine antigens and is currently employed in several clinical trials (www.clinicaltrials.gov) both against infectious disease and cancer.

The first clinical trial evaluating the safety and immunogenicity of a HIV DNA vaccine candidate delivered by EP (ADVAX) is currently ongoing and according to the preliminary data presented at the AIDS vaccine meeting 2009, the use of EP increased the magnitude of the immune responses as well as increased the breadth of responses (97). We have also experienced the advantage of using EP in mice (Bråve and Hallengärd, unpublished data and (133)), why we, in the beginning of 2010, will initiate a phase I clinical trial to evaluate the HIVIS vaccine using this method in healthy volunteers.

4.5.1.3.2 Adjuvants
Vaccination using attenuated or inactivated pathogens typically induces strong innate immune reactivity due to the presence of microbial material that binds and activates various receptors associated with the innate immune system. This potent activation of the innate immune system helps the subsequent development of a strong and long-lasting adaptive immune response. The microbial structures that interacts with the various receptors on immune cells are often absent when using pure recombinant subunit and peptide vaccines, why these vaccines might need the use of adjuvants, i.e. a substance that augment the vaccine-specific immune responses, to at least partially

mimic the complex natural course of infection. DNA vaccines, however, carry bacteria-derived unmethylated cystidine-phosphate-guanosine (CpG) motifs in the plasmid backbone that can stimulate the innate immune system by signaling via the toll-like receptor (TLR) 9. In addition, cytoplasmic molecules such as DNA-dependent activator of IFN regulatory factors (DAI) functions as a DNA sensor and activates the innate immune system upon interaction with DNA (134). Still, other co-delivered adjuvants may be crucial to induce more potent immune responses with a DNA vaccine. The most classical adjuvant is alum, which is typically used to augment the humoral response to subunit vaccines such as the Tetanus, Diphtheria and Hepatitis A vaccines. The classical hypothesis is that alum acts via the formation of a depot from which the vaccine is slowly released and by the introduction of inflammatory responses by APCs. More recently, alum has been shown to act via the induction of ureic acid that recruits and activates DCs (135). However, for DNA vaccines alum is rarely used and other types of adjuvants may be better suited (136). Examples of novel adjuvants that are used in conjunction with DNA vaccines are immune stimulating TLR agonists and cytokines and formulations that can protect and/or increase the transfection efficacy of the plasmid vaccine as well as the efficacy of the plasmid DNA vaccine, resulting in strong immune responses.

TLRs belong to the PRRs and are present on or within a wide range of innate immune cells. Subsequent to the interaction of microbial material and immune receptors on APCs, a signaling cascade is initiated that ultimately results in the activation of transcription factors such as NF-κB. This results in the production of cytokines and chemokines and upregulation of co-stimulatory molecules on the APC. Signaling via TLRs can thus stimulate the adaptive immune system by producing cytokines and cause migration of cells to the site of infection (or immunization). Examples of TLR-agonists are double-stranded RNA, lipopolysacharide (LPS) on the surface of the gram-negative bacteria, bacterial flagellin and viral single-stranded RNA. These PAMPs are recognized by the TLR3, TLR4, TLR5 and TLR7, respectively (137, 138). Hence, in addition to the built-in adjuvant effect generated by CpG and DNA, one can further augment the vaccine-specific immune response by stimulating with other viral or bacterial derived components. Examples of such adjuvants used with DNA vaccines are monophosphoryl lipid A (MPL), a non-toxic form of LPS, and imiquimod which is normally used to treat genital warts. These adjuvants efficiently stimulate macrophages and DCs to engulf and present the vaccine antigen and typically induce a Th1 type of immune response (139, 140).

Cytokines (and chemokines) are small molecules secreted by cells of the immune system and act as signals to direct, coordinate and activate the immune response. When cytokines are used for adjuvants for DNA vaccines they are usually encoded by plasmids and co-delivered with the vaccine. Different cytokines can be used to direct the immune response in a certain direction (e.g. direct against Th1 or Th2 responses). Examples of classical Th1 cytokines are interleukin (IL) 2, IL-12 and IL-15, whereas cytokines usually inducing Th2 responses are IL-4, IL-7 and granulocyte macrophage colony-stimulating factor (GM-CSF) (reviewed in (141)). We have used rGM-CSF to augment the immunogenicity of our DNA vaccine, and in mice rGM-CSF delivered prior to vaccination was shown to be crucial for the induction of strong both cellular and humoral responses (113). The effect of GM-CSF observed in the mice was, however, not observed in a clinical trial where a multigene vaccine was delivered with or without recombinant human GM-CSF. Instead the participants receiving the adjuvant displayed lower magnitude of immune reactivity than the volunteers that received only the vaccine (112). Also, the individuals receiving GM-CSF displayed some adverse events, stressing the possible negative features of using cytokines for this purpose.

The main function of many adjuvants, in particular for DNA vaccines, is to create a depot effect, protect the vaccine from degradation and enhance the entry of plasmids into the target cells. Examples of such adjuvants are lipid emulsions and chemical adjuvants. Emulsion adjuvants are two-phased systems, either oil-in-water or water-in-oil, that are typically used to augment humoral responses against recombinant protein-based vaccines. Freunds complete or incomplete water-in-oil

adjuvants, containing or lacking bacteria (Mycobacteria) derived material, respectively (142), and the licensed Novartis MF59 oil-in-water emulsion that activates APCs (143, 144), are two common emulsion adjuvants. The chemical adjuvants include liposomes, polymers and nano- and microparticles, which typically protects the DNA plasmids from degradation and facilitate the transport of DNA across the cell membrane by neutralizing the negative charge of the vaccine plasmid (145). In paper II we examined the adjuvant properties of the cationic lipid N3 adjuvant from Eurocine AB. This and other chemical adjuvants are suitable to deliver DNA vaccines across mucosal surfaces aiming to induce mucosal immunity at the major sites for HIV entry (146). In a previous study, mice immunized in with N3 formulated with plasmids encoding HIV gp160 and Rev and boosted with a gp41-derived peptide formulated in the L3 adjuvant (Eurocine AB) was shown to induce both mucosal and systemic cellular and humoral responses as well as lowering the amount of DNA needed (147).

4.5.2 Virally vectored vaccines

The use of viral vectors in vaccine research have raised the question of pre-existing immunity as infections of some of the parental viruses are either prevalent in the target population (e.g. Adenovirus of subtype 5) or vaccinated against using attenuated or inactivated virus as vaccines (e.g. measles) (148). Nevertheless, viral vectors are commonly used and two families of viruses are frequently employed in the HIV vaccine research: Poxvirus and Adenovirus.

Despite the huge success in eradicating smallpox, the safety profile of Vaccinia virus was questioned as the virus could cause severe disease in immunocompromised individuals (149). In order to attenuate the virus further, it was extensively passaged in calves, donkeys and chicken fibroblasts to remove elements associated with disease in humans (150). As this was done in Ankara, Turkey, the new improved vaccine was named Modified Vaccinia Virus Ankara (MVA) and due to the well established safety profile of MVA, this virus is suitable for use as a vector for genetic vaccines. A rMVA encoding HIV Env (subtype E), Gag and a PR and RT fusion protein (subtype A) does however only induce relatively weak immune responses, and mainly against the Env component when administered as a single modality in human (97). However, when the vector is used to boost DNA-primed responses, it is highly efficient and augments both Gag- and Env-specific responses (112) why MVA as well as the related NYVAC virus (151) have been used extensively in prime-boost settings.

Another safe vector that is based on a Poxvirus is the canarypox virus ALVAC. ALVAC-HIV (Sanofi Pasteur), encoding HIV Env (subtype B/E), Gag and PR (subtype B), have successfully been evaluated for safety and immunogenicity in humans to prime responses prior to boosting with the AIDSVAX rgp120 vaccine (subtypes B/E) (described in the recombinant subunit vaccines section) (152). A large placebo-controlled efficacy trial conducted in Thailand, termed Thai trial, enrolled 16 000 uninfected individuals and was completed in October 2009. Encouragingly, this is the first HIV vaccine candidate that has shown any significant protection (31,2% vaccine efficacy, p=0,04) against infection (92). Numerous immunologic assays analyzing both humoral and cellular immune reactivity have been performed. However, as of yet, no correlates of protection have been identified why more recently developed immunological assays will be employed to hopefully identify such. Furthermore, no difference in viral load was seen between the participants receiving vaccine or placebo why single-genome amplification of breakthrough infections could gain insights regarding the features of these viruses (92).

Replication deficient Adenovirus has been extensively used as vaccine vectors and the most commonly used vectors are based on Adenovirus of serotype 5 (Ad5). A vaccine candidate developed by Merck encoding HIV Gag, Pol and Nef of subtype B was shown to be safe and immunogenic in a phase I trial (153) and a placebo-controlled phase IIB study was performed to test the efficacy of the vaccine. The study, termed STEP, enrolled 3000 uninfected individuals at high risk of HIV-infection.

The trial was however discontinued due to futility (154, 155). The interim analysis showed that there were a higher HIV-incidence in the vaccine group as compared to the placebo group and there were no effect of the vaccine on the viral load in the infected individuals. Subsequent multivariate analysis identified that the group of volunteers with the greatest risk of acquiring HIV was uncircumcised men with pre-existing Ad5 immunity (154). However, further studies have reduced the impact of the pre-existing Ad5 immunity on acquiring infection (156). These results left the field cautious and reluctant to initiate new trials with Ad5 and emphasis has now been put into bringing vectors based on other serotypes of adenoviruses with lower seroprevalence in to the clinic (reviewed in (157)). The failure of the STEP trial was naturally a huge drawback for the HIV vaccine research at the time but the more recent Thai trail have again raised the hopes for genetic vaccines and emphasizes the importance of prime-boost vaccination strategies.

4.5.2.1 Heterologous prime-boost immunization

The combination of more than one vaccine modality has been shown to strengthen and broaden the immune response to several HIV vaccine candidates and today most clinical trials based on genetic HIV vaccines utilize this strategy (www.clinicaltrials.gov). In our clinical trials in Stockholm and Tanzania the HIVIS vaccine have successfully been combined with a MVA vector encoding similar but not identical HIV antigens as boost (112, 158). The main advantage of this strategy is the combination of the capacity of DNA to prime a highly specific, but usually rater weak, response and the capacity of the viral vector, with its inherited immune stimulating components, to efficiently boost the responses. By priming the immune system, the recombinant vaccine antigen, encoded both by the plasmid and by the viral vector, gets an immunological advantage over the other viral antigens and this allows for a very efficient expansion of the vaccine antigen-specific immune response. By this strategy it is possible to circumvent the effects of the vector-specific immune responses that in most cases prevent repeated injections of a recombinant viral vector.

5 AIMS

The aim of this thesis was to increase the immunogenicity of HIV-1 DNA vaccines with the specific objectives being:

I.   To construct and optimize DNA vaccines based on HIV-1 protease (Paper I).
II. To evaluate the impact of the route of immunization and the effect of adjuvants on the immune responses induced by a HIV-1 DNA vaccine (Paper II).

6 RESULTS AND DISCUSSION

6.1 INCREASED EXPRESSION AND IMMUNOGENICITY OF HIV-1 PR FOLLOWING INACTIVATION OF THE ENZYMATIC ACTIVITY (PAPER I)

As an attempt to address the issue of the vast genetic variability of HIV we have included several HIV genes of different subtypes in our clinically evaluated HIV vaccine (111, 112). To further broaden the immune response we aim to include still more HIV genes, including the viral PR gene. The PR enzyme is crucial in the late stage of the viral life cycle and is relatively conserved between viral strains (159), why PR could be suitable to use as a vaccine antigen. Also, by introducing mutations that are commonly induced during treatment with antiretroviral drugs, it might be possible to raise immune responses against drug resistant PR (160) or prevent the induction of drug resistance. Therefore different variants of a subtype B HIV-1 PR gene were constructed and cloned into the pKCMV expression vector. The genes were codon-optimized (co) in order to enhance expression and mutations conferring inactivation of the enzymatic activity of the PR (D25N) and mutations commonly induced in the protein during treatment with PR inhibitors (V84F/I84V) were introduced. The inactivating mutation was introduced for safety reasons, but also in order to investigate if the enzymatic activity affects expression and immunogenicity of the protein. Such a phenomenon has been observed for plasmid-encoded RT, which is less expressed and thus less immunogenic when a mutation that inactivates the enzymatic activity is introduced in the gene (161).
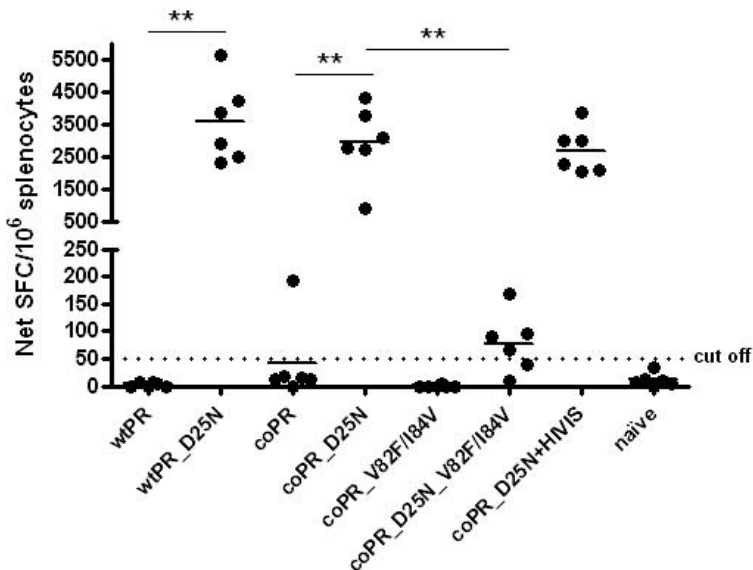

*Figure 6. PR-specific IFN-γ responses as measured by ELISpot on splenocytes from BALB/c mice immunized with the different PR-encoding vaccine plasmids. Important significant differences are marked ** (p<0.01)*

By inactivating PR, both *in vitro* expression and immunogenicity of the protein in BALB/c (Fig. 6) and HLA-A0201 transgenic C57Bl/6 mice were significantly increased. This finding is the opposite of what has been observed for plasmid-encoded RT and raises the question of how the activity affects the

expression and immunogenicity of the enzymes. For PR, several mechanisms are possible. One explanation could be that the active protein undergoes auto-proteolysis. Rosé and colleagues examined this phenomenon by designing a protein where locations for predicted self-digestion was altered, and a mutation in amino acid position 7 (Q7K) was shown to block the self-cleavage without hampering the enzymatic activity (162). However, when we introduced this mutation in the active PR gene, neither *in vitro* expression nor *in vivo* immunogenicity were affected. Another explanation for the increased expression of the inactivated PR could be that active PR causes cytotoxic effects as it can induce apoptosis by cleaving several cellular proteins (163). We have, however, not observed any difference in pathogenic effects caused by active or inactive PR *in vitro*. We thus conclude that it seems unlikely that autolysis or cytotoxicity can explain the low immunogenicity induced by the active protein.

The strong immunogenicity of inactivated PR was retained also when the gene was delivered as a part of our multigene HIV vaccine indicating that the presence of an additional immunogen did not, in this case, hamper the vaccine-responses to PR (Fig. 6). Neither did the presence of PR decrease the immune response to the other vaccine-antigens. However, the drug resistance mutations negatively affected the immunogenicity, and we could show that this was due to the fact that the immunodominant H2D^d (BALB/c) and the HLA-A0201 epitopes in PR are located in the region where these mutations occur. Encouragingly, immunizing with wild type PR generated both wild type- and drug resistance-specific reactivity.

Taken together, our data demonstrates an efficient strategy to increase the immunogenicity of HIV-1 PR and mount strong immune responses against wild type as well as drug resistant PR, and thus immunogenicity to most HIV strains. Another vaccine candidate that contains PR is the ALVAC-HIV vaccine that is a part of the vaccine used in the Thai trial. However, no PR-specific responses have been reported from this trial (92) nor from other trials where the ALVAC-HIV (164) or other vaccines (165, 166) encoding PR is included. The lack of information regarding the immunogenicity of PR in different vaccine constructs might be due to the limited immune responses, but this is not clear. However, our finding that the immunogenicity of various types of plasmid-encoded PR could be significantly increased by inactivating the enzymatic activity can easily be applied to most likely all PR-encoding vaccines. The immunogenicity of PR in combination with the HIVIS plasmids will be assessed in future clinical trials and further studies are needed to reveal the mechanism linking the enzymatic activity to the immunogenicity of the protein.

6.2 INTRANASAL IMMUNIZATION OF YOUNG MICE WITH A MULTIGENE HIV-1 VACCINE IN COMBINATION WITH THE N3 ADJUVANT INDUCES MUCOSAL AND SYSTEMIC IMMUNE RESPONSES. (PAPER II)

The evaluation of mucosal responses as well as the development of vaccines aiming to induce mucosal immunity is generally not prioritized in HIV vaccine research. As it is well known that mucosal delivery of immunogens can raise antigen-specific responses even at distant mucosal compartments, we focused on intranasal vaccine delivery with the intention to raise responses in vaginal and rectal mucosa (147). Furthermore, as the amount of vaccine that can be delivered in the nasal cavity is limited, especially in children, the use of a potent adjuvant is most likely required in order to induce strong antigen-specific immune responses with a plasmid DNA vaccine. In this project the clinically evaluated multigene HIVIS vaccine was formulated in the lipid adjuvant, N3, and administered intranasally to very young C57Bl/6 mice (two weeks at the time of first immunization). The mice were immunized three times and both humoral and cellular vaccine-specific reactivity were assessed (Fig. 7).
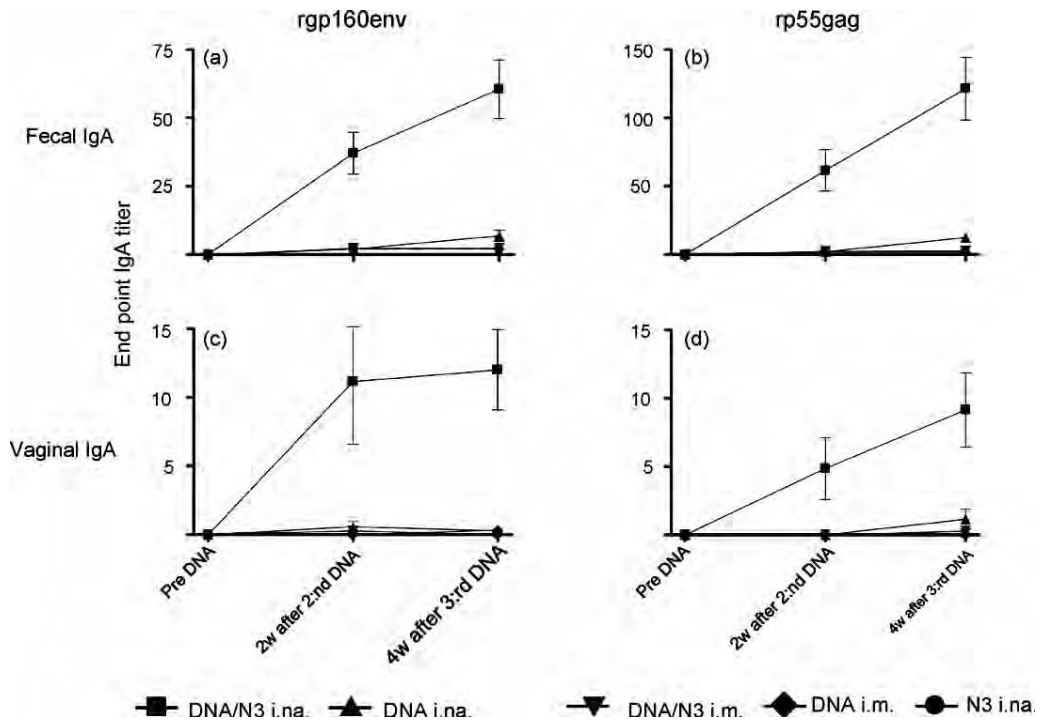
Figure 7. HIV-1 rgp160env- or rp55gag-specific IgA in (a and b) fecal pellets and (c and d) vaginal washings. Graph shows mean values of all animals in each group and error bars represent the standard deviations.

The intranasal delivery of the vaccine induced higher immune responses using less DNA than intramuscular immunization. Also, intranasal vaccination induced both humoral and cellular systemic responses, and IgA-responses could be detected at mucosal sites (vaginal and rectal) distant from the intranasal mucosa (Fig. 7). Furthermore, the mucosal responses were only induced when the vaccine was co-administered with the N3 adjuvant. As there have been reports of side effects such as Bell's palsy or damaged olfactory nerves and/or nasal epithelium after intranasal immunization it is highly important to carefully study local effects after immunization (167-169). The good safety profile of N3 has been shown in other experiments (170) and also in our experiment the adjuvant was well tolerated by the very young mice.

This study demonstrates the ability of DNA vaccine to induce broad immune responses in different compartments of the body, and the data is in agreement with other mucosal immunization schedules using DNA vaccines and adjuvants (171, 172). Furthermore, and perhaps more importantly, the induction of mucosal anti-HIV IgA presents a possible means to prevent infection at the mucosal surface where the majority of HIV infections take place (146). Therefore, inducing such mucosal responses is one of the major goals of HIV vaccine development. It is, however, traditionally difficult to obtain mucosal responses with plasmid DNA vaccines, partly because we lack knowledge regarding how to design the envelope antigen to induce nAbs, but also because the magnitude of the immune response is too low and it is difficult to induce a response that is maintained over time. The potency can be increased by the use of adjuvants but it still remains to determine how to immunize in order to obtain mucosal IgA that remain at a high level. Another, not trivial, feature of intranasal delivery is that it does not require any sharps and therefore the risk of transmission of infectious diseases is reduced.

7 CONCLUDING REMARKS

A vaccine that is able to induce protection from HIV infection might be the most realistic hope for ending the global HIV/AIDS epidemic. However, the virus possess several features including the unusually high mutation rate that complicates vaccine development, why HIV vaccine candidates evaluated to date have only induced, at best, modest protective responses in humans. The relatively novel vaccine modality based on plasmid DNA has so far not been an exception. These vaccines have the potential to induce, in addition to antibody responses, strong cellular immune responses, which has proven important for suppressing HIV replication (75, 81, 173, 174). However, the immuno-genicity induced by plasmid vaccines has so far been limited in humans and thus needs to be enhanced. The advantage of using prime-boost immunization strategies have been shown by the success of the Thai trial, where two seemingly low-immunogenic vaccines induced protection against infection when administered in a prime-boost protocol (92). Today, novel DNA vaccine delivery technologies such as *in vivo* electroporation might be able to induce similarly strong responses as seen when boosting plasmid DNA-primed responses with a viral vector or a recombinant protein. Still, the immunogenicity of DNA can be further enhanced by other strategies of optimization. This thesis highlights the effects on the induction of immune responses by genetically modifying the HIV protease gene and the effects of using the N3 lipid-based adjuvant.

The importance of protease in the viral life cycle and the possibility to induce immune responses to drug resistant strains of HIV makes protease a potential vaccine target. Our finding that the immunogenicity of wild type protease, as well as drug resistant variants, could be significantly increased by inactivating the enzymatic activity can easily be applied to protease-based vaccines. Additionally, by formulating the HIVIS vaccine plasmid-cocktail in the lipid-based N3 adjuvant and by using intranasal delivery, we increased the systemic as well as mucosal immune responses. This finding can most likely be extended also to other plasmid vaccine constructs.

In addition to the need for increasing the immunogenicity of HIV DNA vaccines, the development of vaccines against HIV is severely complicated by the lack of known correlates of protection from infection and disease. This means that it is not known which antigens to include, or what the optimal vaccine modality is, in order to induce potent neutralizing antibody responses or to induce a strong cytotoxic response able to eradicate infected cells. To increase the possibility of including the correct HIV-antigen we have decided to target many of the viral antigens from various subtypes of the virus with our multigene/multiclade DNA vaccine (112). Other ways to approach this issue are to construct vaccines expressing consensus (175) or mosaic (176, 177) antigens or sequences representing multiple variants of the HIV antigens. During the last two decades massive efforts have been put into developing a HIV vaccine and the recent positive results from the ALVAC-HIV/AIDSVAX vaccine trial, have provided much needed hope to the field of HIV vaccine research. Although there is now, for the first time, indications that it might be possible to develop a vaccine against HIV, there is most likely a long way to go before a broadly protective vaccine can be distributed to the world.

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