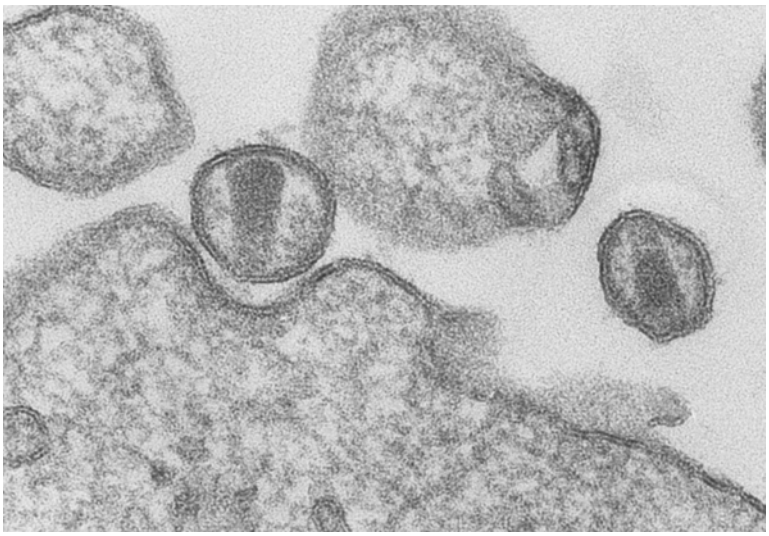
ENHANCEMENT OF HIV-1 DNA IMMUNOGENS

ANNE KJERRSTRÖM ZUBER



Stockholm 2002

Karolinska Institutet

**FROM MICROBIOLOGY AND TUMORBIOLOGY CENTER,
KAROLINSKA INSTITUTET AND THE SWEDISH INSTITUTE FOR
INFECTIOUS DISEASE CONTROL, STOCKHOLM, SWEDEN**

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Front cover: Electron micrograph of HIV-1 particles, kindly provided by Kjell-Olov Hedlund, Swedish Institute for Infectious Disease Control.

Anne Kjerrström Zuber: Enhancement of HIV-1 DNA immunogens. Karolinska Institutet, Stockholm, 2002.

Published and printed by Repro Print AB, Stockholm, Sweden

ISBN 91-7349-304-X

ABSTRACT

Human immunodeficiency virus type 1 eludes control by the immune response through a high degree of variability and immune escape mechanisms. Induction of a broad specific immune response is important to clear virus-infected cells. DNA vaccination is a relatively new approach that induces both humoral and cellular immune responses in vaccinated hosts. The aim of this thesis was to enhance immune responses to different HIV-1 proteins using different DNA vaccine regimens.

The three regulatory genes *tat*, *rev* and *nef* of HIV-1 have been of particular interest in vaccine design. A strong cytotoxic T-lymphocyte response against these three proteins correlates to long-term non-progression of disease. The protein expression from regulatory genes was characterized from patient and laboratory strain viruses. The laboratory strain derived genes resulted in the most efficient protein expression and were used for further studies. We examined single versus combined genes and found that individual responses to each protein were strongest after single gene administration. Immune responses to several targets were induced when the three genes were used together, which is important when developing an effective HIV-1 vaccine. The strongest responses were seen to the Nef protein. However, these responses decreased when co-immunizing with the *tat* and *rev* genes, as was the case with responses to Rev. Different combinations of plasmids, different injection sites and different doses might however overcome these drawbacks.

Several immunization strategies, using DNA, recombinant modified vaccinia Ankara (MVA) vectors, protein mixed with CpG oligodeoxyribonucleotides (ODN), and a novel adjuvant, were evaluated. Different prime-boost regimes were used to enhance Nef-specific immune responses. The combination of *nef* DNA and MVA*nef* resulted in partial resistance from challenge with HIV-1/MuLV infected cells. The combination of recombinant Nef protein mixed with CpG ODN with or without a booster immunization with MVA*nef* also cleared HIV/MuLV infected cells. A broad response to Nef after HIV-1/MuLV challenge was apparent in the groups of mice that had received the recombinant Nef protein mixed with CpG ODN. To develop these findings, another HIV-1 gene, the reverse transcriptase (RT) gene, was used. *RT* gene priming followed by RT protein mixed with CpG ODN booster was used in primates. Again, strong cellular responses were induced by *RT* DNA followed by RT protein mixed with CpG ODN.

A combination of regulatory and structural genes might give a beneficial broad immune response. The compound imiquimod activates the Toll like receptor 7 and is used in clinic for treating genital warts. Imiquimod was evaluated as an adjuvant with the *nef*, *p37* (*p17* and *p24* genes) and *RT* genes of HIV-1, and was shown to potentiate cellular immune responses capable of clearing HIV-1/MuLV infected cells.

In conclusion, we were able to induce strong immune responses to all antigens tested using DNA vaccination. The responses were increased by using either adjuvants in combination with the DNA, by boosting with protein mixed with CpG ODN or by boosting with a recombinant modified vaccinia Ankara vector. The strongest cellular responses related to partial protection from challenge with HIV-1/MuLV infected cells.

Keywords: HIV-1, DNA vaccination, regulatory genes, adjuvants, experimental HIV-1 model

ISBN 91-7349-304-X

LIST OF PUBLICATIONS

This thesis is based on the following original papers and manuscripts, which will be referred to in the text by their Roman numerals:

I. **Anne Kjerrström** and Britta Wahren

Expression of HIV regulatory DNA vaccine constructs

Biogenic Amines, 15: 93–112 (1999)

II. **Anne Kjerrström**, Jorma Hinkula, Gunnel Engström, Vladimir Ovod, Kai Krohn, Reinhold Benthin and Britta Wahren

Interactions of Single and Combined Human Immunodeficiency Virus Type 1 (HIV-1) DNA Vaccines

Virology, 284: 46–61 (2001)

III. Sandra A. Calarota, **Anne Kjerrström**, Khalid B. Islam and Britta Wahren

Gene Combination Raises Broad Human Immunodeficiency Virus-Specific Cytotoxicity

Human Gene Therapy, 12: 1623–1637 (2001)

IV. **Anne Kjerrström Zuber**, Bartek Zuber, Gerd Sutter, Birgit Kohleisen, Sandra A. Calarota, Malin Fredriksson, Reinhold Benthin, Heather L. Davis, Jorma Hinkula, Volker Erfle and Britta Wahren

Clearance of human immunodeficiency virus type 1 after immunization with the Nef protein

Submitted

V. Bartek Zuber Barbro Mäkitalo, **Anne Kjerrström Zuber** and Britta Wahren

A novel potent strategy for induction of immunity to HIV-1 reverse transcriptase in primates

AIDS, 16: 1839–1840 (2002)

VI. **Anne Kjerrström Zuber**, Bartek Zuber, Karl Ljungberg, Malin Fredriksson, Reinhold Benthin, Maria G. Isaguliants, Eric Sandström, Jorma Hinkula and Britta Wahren

Topical administration of imiquimod is a potent adjuvant for HIV-1 DNA vaccination

Manuscript

This work was supported by the Foundation for Knowledge and Competence Development, SBL Vaccines (formerly SBL Vaccin AB), the Swedish Medical Research Council, the Swedish Society for Medical Research, the Swedish Agency for Research Cooperation (SAREC) and the European Community

CONTENTS

LIST OF ABBREVIATIONS

AIDS	acquired immunodeficiency syndrome
ADCC	antibody dependent cell-mediated cytotoxicity
APC	antigen presenting cell
CAT	chloramphenicol acetyltransferase
CD	cluster of differentiation
CTL/CTLp	cytotoxic T lymphocyte/cytotoxic T lymphocyte precursor
DC	dendritic cell
dsDNA	double stranded deoxyribonucleic acid
Env	envelope
Gag	group specific antigen
GM-CSF	granulocyte macrophage-colony stimulating factor
gp	glycoprotein
HIV-1	human immunodeficiency virus type 1
HLA	human leukocyte antigen
IFN	interferon
Ig	immunoglobulin
IL	interleukin
kb	kilobase
kD	kilodalton
LTNP	long-term non-progressors
LTR	long terminal repeat
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
NK cell	natural killer cell
Nef	negative factor
ODN	oligodeoxyribonucleotides
PBMC	peripheral blood mononuclear cells
Pol	polymerase
polyA	polyadenylation
Rev	regulator of virion expression
RRE	Rev responsive element
RT	reverse transcriptase
SIV	simian immunodeficiency virus
TAR	Tat-responsive element
Tat	transactivator
TLR	Toll-like receptor
TNF	tumor necrosis factor

1 INTRODUCTION

In the history of man, one of the most successful types of medical intervention is vaccination. Smallpox and soon polio have virtually been eradicated by preventive vaccination. Therapeutic vaccination has not yet been widely used against established chronic infections caused by viruses. This is partly because viruses that establish chronic infections have developed mechanisms for evading the host immune system. One of the most well-defined mechanisms for viruses to escape immune prevention is the down-modulation of antigen processing and presentation to T cells. The human immunodeficiency virus type 1 (HIV-1) eludes the immune system by mutational escape, by integration of its genome in the host genome, by downregulation of the major histocompatibility complex (MHC) class I molecules, and by upregulation of the Fas ligand on the surface of infected cells (186). A strong cellular and humoral response will likely be needed to control HIV-1 infection. Over 40 million people were infected by HIV-1 at the end of 2001 (241). Although 20 years have passed since the virus was first discovered by Barré-Sinoussi and colleagues in 1983 (17), confirmed by Popovic and colleagues in 1984 (190), no successful cure or vaccine is yet available.

1.1 THE HUMAN IMMUNODEFICIENCY VIRUS

There are two types of HIV, type 1 and type 2. Both types are causative agents of acquired immunodeficiency syndrome (AIDS), although infection with HIV-1 is worldwide while HIV-2 infection is not. HIV-2 seems to be less pathogenic than HIV-1 and causes a disease that develops more slowly (202, 254). HIV-1 and HIV-2 belongs to the Lentivirus genus of the *Retr viridae*. Like all viruses, HIV replicates within the living cells of the host. HIV-1 contains nine open reading frames (figure 1) encoding 15 proteins. It is unique in that the single stranded RNA genome of approximately 9.2 kilobases (kb) is diploid within the virion.

1.1.1 THE HIV-1 VIRION

The mature virion of HIV-1 is an icosahedral sphere with a diameter of approximately 110 nanometers (figure 1). The outer envelope, which is formed from the host cell membrane, is a lipid bilayer that contains host cell proteins and 72 spikes of the viral envelope glycoproteins (gp): gp120 and gp41. Inside the bilayer, the nucleocapsid protein encapsulates two copies of the genomic RNA.

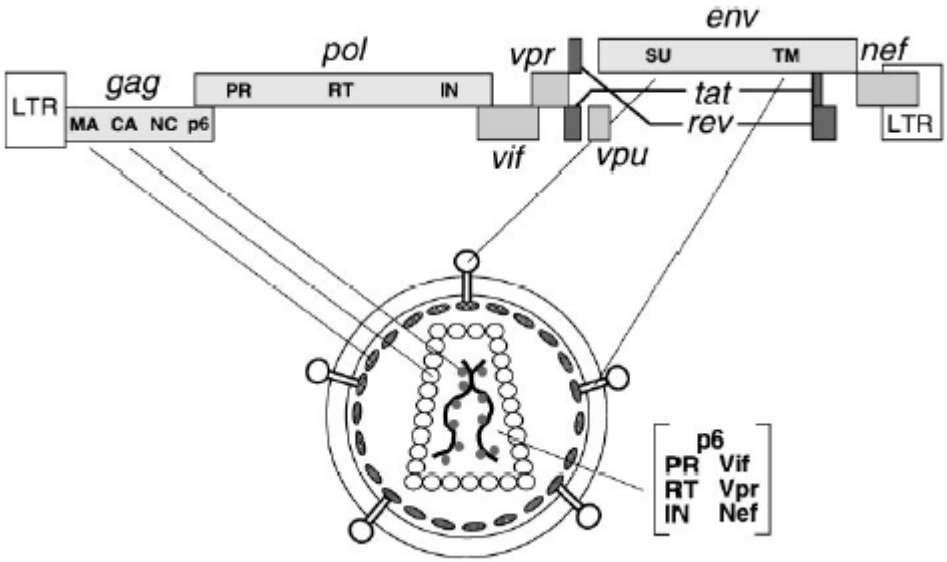

Figure 1. Schematic drawing of the HIV-1 genome and virion.

The long terminal repeats (LTRs) flank the viral genome that encodes 15 proteins. The *gag* gene encodes structural proteins that build the virion: the capsid (CA, p24) protein, the matrix (MA, p17) protein, the nucleocapsid (NC, p7) protein and a small p6 protein. The *p l* gene encodes the protease (PR), reverse transcriptase (RT) and integrase (IN) enzymes. The *env* gene encodes the surface (SU, gp120) and transmembrane (TM, gp41) glycoproteins. In addition, HIV-1 encodes six accessory proteins: Tat (transactivator), Rev (regulator of virion expression), Nef (negative factor), viral infectivity factor (Vif), viral protein R (Vpr) and the viral protein U (Vpu). The virions are approximately 110 nm in diameter. Adapted from (74).

The matrix protein (p17) is located between the nucleocapsid and the virion membrane. The capsid protein (p24) forms the capsid shell surrounding the nucleocapsid. Several other viral proteins can be found inside the virion: the integrase responsible for integration of viral genome into the host genome, the reverse transcriptase (RT) that converts the RNA genome to a DNA molecule, as well as several other accessory proteins (reviewed in (75)).

1.1.2 PATHOGENESIS OF HIV-1

HIV-1 spreads by ways of its host's behavior, crossing sexually from man to woman, woman to man and man to man, crossing vertically from mother to child and horizontally through needles (251). The pathogenesis of HIV-1 is dependent on several immune abnormalities. Altered cytokine expression, decreased cytotoxicity, decreased humoral and proliferative response to antigens and mitogens, decreased MHC class II expression, decreased monocyte chemotaxis, depletion of CD4+ cells, impaired delayed type hypersensitivity reactions, and polyclonal B cell activation can be seen after HIV-1 infection. The continued generation of new antigenic variants eventually destroys the immune system. Over 100 opportunistic infections by viruses, bacteria,

fungi and protozoa have been associated with AIDS (reviewed in (251)). HIV-1 infects the cells of the immune system, mainly by binding to the receptor CD4 (cluster of differentiation). T helper lymphocytes, macrophages and dendritic cells (DC) are the main cells infected by HIV-1. The macrophages are important reservoirs for viruses, especially the microglia in the brain. Induction of innate immune responses within the central and peripheral nervous system is largely mediated by microglia, and influences the development of primary HIV-related neurological disease (reviewed in (193)). There are two main HIV-1 phenotypes; the rapid high (syncytia inducing) isolates using the CXCR4 (CXC chemokine receptor 4) coreceptor and the slow low (non-syncytia inducing) isolates using the CCR5 (CC chemokine receptor 5) coreceptor. In addition, dual-tropic isolates capable of using both CCR5 and CXCR4 are found. The type of coreceptor used directs the choice of cell tropism; the CCR5 is mainly expressed on macrophages and CXCR4 on T-helper cells (22).

1.1.3 THE REPLICATION CYCLE OF HIV-1

Upon entry into the host at mucosal sites, DCs bind HIV-1 through DC-SIGN receptors and carry the virus from mucosal sites to lymph nodes, where subsequent infection and activation of lymphocytes occur before dissemination through the body (83). The released virions infect host cells expressing the CD4 receptor and one or several of the chemokine co-receptors, CXCR4 or CCR5, which the HIV-1 uses for entry. Entries by binding to the galactocyl ceramide receptor, the Fc receptor or via M-cells have also been shown. The gp120 binds to the CD4 receptor and the gp41 undergoes a conformational change that allows fusion of the virus and cell membrane, thereby allowing the virus core to enter the cell (figure 2 and reviewed in (47)). The core is uncoated and exposes the nucleocapsid complex that contains the RT, the p17 matrix protein, the integrase, the viral protein R and the viral RNA. This complex is transported to the nucleus and the genomic RNA is reversed transcribed into a duplex DNA by the RT. The integrase catalyzes the integration of the viral DNA into the host chromosome and the DNA is repaired (reviewed in (75)).

The HIV-1 promoter is located in the 5´ long terminal repeat (LTR) and contains binding sites for various cellular transcription factors (122). These transcription factors allow transcription initiation from the provirus and their abundance determines when the provirus is quiescent or actively replicating. However, the transcription complexes are inefficient at elongation and need the viral transactivator protein (Tat) to enhance the elongation step in transcription by the transcribing RNA polymerase II. A set of

spliced and genomic-length viral messenger RNA (mRNA) is transported to the cytoplasm with the aid of the viral protein regulator of virion expression (Rev). The viral mRNA is translated using host cell ribosomes and the produced group specific-antigen (Gag) and Gag- polymerase (Pol) polyproteins are localized to the cell membrane. The envelope (Env) polyprotein is associated with the cellular CD4 receptor in the endoplasmic reticulum and the viral protein U degrades the CD4, thereby enabling the Env polyprotein to be transported to the cell surface. The core particle is assembled from the Gag and Gag-Pol polyproteins, the viral infectivity factor, the viral protein R, Nef (negative factor) and two copies of subgenomic RNA.

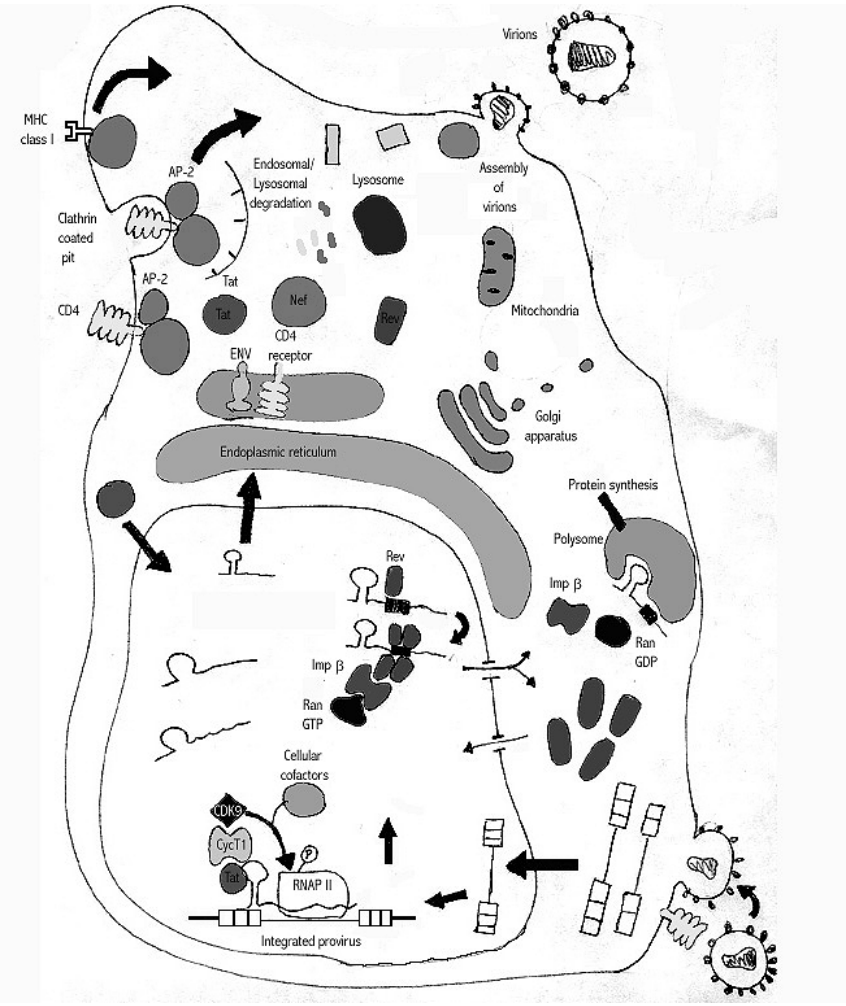

Figure 2. Model of the two phases of the viral life cycle.

Interactions between the viral glycoprotein with the cellular receptors lead to fusion of the cellular and viral membranes. After uncoating of the capsid, the reverse transcriptase transcribes the genome into a DNA molecule, which is transported into the nucleus for subsequent integration. The cellular RNA polymerase II (RNAP II) starts the late phase of the life cycle by transcribing the proviral DNA for subsequent translation in the cytoplasm. New particles form at the cellular membrane and bud from the surface. Subsequent proteolytic trimming by the viral protease included in the virion yields the mature, infectious virions. MHC = major histocompatibility complex, Impβ = importin β, Cyc T1 = cyclin T1, CDK 9 = cyclin dependent kinase 9, Ran GTP/GDP = Ran guanosine triphosphatase/diphosphatase, AP 2= adaptor protein 2

The immature virion now begins to bud from the cell surface. The virus buds coated with the gp120 and gp41, receiving a host-derived lipid bilayer. After budding, the virion undergoes a morphologic change, which involves proteolytic processing of the Gag and Gag-Pol polyproteins by the viral protease (reviewed in (75)).

The gene expression of HIV-1 can be divided into two temporal phases, an early, regulatory phase and a late, structural phase. All the mature viral mRNAs contain 5´ ends that are posttranscriptionally capped with 7-methylguanosine by cellular enzymes and 3´ ends that have a polyadenylation (polyA) signal similar to eukaryotic mRNAs. The genome is transcribed into three classes of mRNA: the 9.2 kb genomic class, the 4.5 kb partially spliced class and the 2 kb multiply spliced transcripts (175, 225, 226). The 4.5 kb and the unspliced mRNAs coding for structural and accessory proteins, contain an RNA element designated the Rev responsive element (RRE) in the *env* region (72). The multiply spliced mRNA transcripts encode the three early proteins Tat, Rev and Nef that appear shortly after infection (72, 187).

1.1.4 THE TAT PROTEIN

The Tat protein is a 14 - 16 kilodalton (kD) transactivating positive regulator that increases the processitivity of RNA polymerase II activity from the viral LTR by a hundredfold (84). The virus is under tremendous pressure to replicate rapidly since the half-life of infected cells is very short, less than 2 days (250), making Tat an essential protein for viral replication. Tat binds to a 55-nucleotide hairpin structure called transactivating response element (TAR) that is located at the 5`end of all viral transcripts to enhance transcription. Tat-TAR independent expression may however occur (84).

The Tat protein contains a nuclear localization signal that allows Tat to be transported into the nucleus directly after production. Tat associates with cyclin T1 (26, 81, 249), one subunit of the cellular positive acting transcription elongation factor that elongates many cellular genes**.** One of the other subunits is the cyclin dependent kinase 9 that hyperphosphorylates the RNA polymerase II C-terminal domain (26, 104, 183, 249) resulting in efficient elongation of transcription. Several other cellular Tat co-factors have been identified (53, 128, 180, 183, 256, 267). The function of Tat is regulated by two acetylation events performed by two Tat-associated histone acetyltransferases (21, 109, 163) that control binding to RNA polymerase II and dissociation of Tat from TAR (132). Viruses with defective Tat do not replicate efficiently (reviewed in (75)).

Tat can be found in at least two forms. One form is the full-length 16 (kD) Tat protein consisting of 86 amino acids encoded by two exons. The other form is a 72 amino acid, 14 kD protein expressed from the first Tat exon. The second exon of Tat has been implicated to repress the transcription of the MHC class I gene (110) and overlaps with the *env* gene, displaying more variation than the first exon.

Tat protein is actively secreted by HIV-1 infected cells (69). Secreted Tat renders uninfected cells more susceptible to productive viral infection by upregulating chemokine receptors (84). Tat acts as a chemotactic factor for monocytes that express a vascular endothelial growth factor receptor (5). Tat also differentially induces chemokine receptors CXCR4, CCR5 and CCR3 expression in peripheral blood mononuclear cells, correlating with Tat-enhanced infectivity (3, 4, 113). The activity of Tat leads to expression of the Fas ligand, leading to cellular apoptosis (147). Tat has also been found to upregulate expression of cytokines like tumor necrosis factor (TNF), interleukin (IL) -2, IL-4, and IL-6 (27, 34, 182, 195, 198). Thus, the release and secondary uptake of Tat can augment viral activation in latently infected cells.

Two major domains of immunogenicity have been found in Tat. Monoclonal antibodies raised against the N-terminal sequence (amino acids 2–19) completely inhibit Tat transactivation *in vitr* (60, 61, 197) and delay HIV-1 replication in peripheral blood mononuclear cells (PBMC) (266). The second major immunogenic region is found in the basic domain of Tat at amino acids 44-62 (84). In addition, anti-Tat antibodies correlate inversely with disease progression (201). In HIV-1 infected individuals, cytotoxic T lymphocyte (CTL) precursors (CTLp) against Tat correlate inversely with rapid disease progression to AIDS (77, 242, 252) and a high frequency of CTLs against both Tat and Rev can be found in HIV-1 infected individuals (2). Recent reports indicate that Tat specific CTLs induced after primary simian immunodeficiency virus (SIV) infection in rhesus macaques are able to control primary infection, but that rapid CTL escape occurs, and correlates with increase in viral load (7).

Several factors make Tat an attractive target for vaccine design: the early expression, the critical role in the virus life-cycle, and the correlation of anti-Tat immune response with non-progression in infected individuals. Tat has been proven safe, immunogenic and effective in mice, macaques and humans (reviewed in (70)).

1.1.5 THE REV PROTEIN

The Rev protein is a 13 - 19 kD basic, nucleocytoplasmic phosphoprotein of 116 amino acids that regulates the gene expression at a post-transcriptional level. Rev allows transport from the nucleus to the cytoplasm of the late unspliced and partially spliced mRNA classes containing the RRE sequence as well as cis-acting inhibitory sequences that affect the stability, transport and translation of the mRNAs (125, 189). In the absence of Rev, structural proteins are generally not made, but some transcripts containing RRE can escape to the cytoplasm. These transcripts are generally not translated due to the lack of Rev. Rev also mediates binding of ribosomes to the viral transcripts (160). The RRE is present in the *env* region of the genome and allows multimerization of four Rev monomers through protein-protein and protein-RNA interactions (244).

The nuclear localization signal directs Rev to the nucleus directly after synthesis in the cytoplasm. An arginine-rich region in Rev mediates the RRE binding, resulting in multimerization of Rev and complete masking of a nuclear localization signal (72). Rev also contains a highly conserved leucine-rich nuclear export signal that allows Rev to shuttle back to the cytoplasm. Mutations in this region have been associated with attenuated Rev function and asymptomatic infection (111). Several cellular cofactors to Rev have been identified (25, 28, 71, 73, 76, 100, 170, 234). The nuclear export signal of Rev interacts with a nucleoporin-like protein called human Rev interacting protein (earlier known as the Rev activation domain-binding protein) that is located at the nuclear pore (76). This interaction occurs through binding to exportin 1 (earlier designated CRM1), a member of the importin-β (karyopherin-β) superfamily of shuttling nuclear transport receptors (179). The nuclear export signal of Rev interacts with exportin 1 and the Ran guanosine triphosphatase (an essential nuclear transport factor). This interaction targets the Rev bound RRE-containing mRNA for nuclear export through the nuclear pore complexes (76, 176). Nuclear export is directed by a gradient of Ran guanosine diphosphatase and triphosphatase in the cytoplasm and nucleus, respectively. In the cytoplasm, hydrolysis of guanosine triphosphatase triggers the dissociation of importin β from Rev (176). It has been shown *in vitr* that the eukaryotic translation initiation factor 5A binds to the activation domain of Rev in the cytoplasm. Rev thus targets the mRNA to the polysomes for protein synthesis, by interacting with the ribosomal protein L5 (218). The nuclear localization signal of Rev is exposed and Rev is translocated back to the

nucleus. Rev has also been found to interact and possibly destabilize the microtubules in HIV-1 infected cells (68, 248).

CTL and CTLp against Rev correlate inversely with rapid disease progression to AIDS (242). CTLs against Rev have been found in one symptom-free individual who had been infected for 12 years. On the other hand, CTL against Rev can lead to a rapid selection of escape mutants (243).

The crucial functions of Rev make it an attractive target for vaccine design. The early expression, the correlation of anti-Rev immune response with non-progression in infected individuals (242, 243), and that it has been proven safe, immunogenic and effective in mice, macaques and humans (35, 106, 181) contribute to making a vaccine against Rev desirable.

1.1.6 THE NEF PROTEIN

The Nef protein is a 27–35 kD myristoylated membrane-associated protein of 206 amino acids. The Nef protein is dispensable for virus replication *in vitr* in CD4 + T cells and macrophage cell lines. However, Kestler *et al*. (131) have shown that an intact SIV *nef* gene was essential for maintenance of high viral load and progression to AIDS in adult rhesus macaques. Similarly, humans infected with HIV with a deleted *nef* are long-term non-progressors who maintain low viral loads (59, 162). Nef mutants have been shown to exhibit decreased rates of viral DNA synthesis following infection (91).

Multiple activities have been ascribed to Nef *in vitr* : (a) downregulation of CD4 and MHC class I molecules from the cell surface (144, 161); (b) increase of viral infectivity; (c) disruption of the signal transduction pathways in T cells (reviewed in (120). MHC class I is only partially downregulated (70%) from the surface of infected cells, human leukocyte antigen (HLA)-A and –B are downregulated while HLA-C and –E are still present on the surface (224). The downregulation may alter the immune recognition by CTL (48); however, HIV-1 infected cells are still susceptible to natural killer (NK) cell lysis (114).

The main effect of secreted Nef would be to activate T cells to allow the virus to establish a secondary pool of infected cells large enough to escape the primary CTL immune response (172). Approximately 70 molecules of Nef are incorporated per virion and are subsequently cleaved by the protease to generate a soluble C terminal fragment with an unknown function (91). Nef contains a consensus SH3 domain

binding sequence that mediates binding to several tyrosine kinases, thereby regulating their activities (20, 89, 171). Nef also associates with a serine/threonine kinase (217) that might play a role in how Nef interferes with both endocytosis and T cell signaling (54).

The depletion of T cells that follows HIV-1 infection is a result of a high degree of apoptosis by the CD4+ and CD8+ T cells. The majority of this apoptosis is mediated by Fas-Fas ligand interactions. Nef upregulates Fas ligand expression in, and might contribute to the loss of, CD4+ lymphocytes. Crosslinking of CD4 by HIV Env in the presence of the Tat protein can induce Fas ligand expression and apoptosis of uninfected cells as well (186).

Nef is produced in abundance and the majority (2/3) of HIV positive patients develop antibodies to Nef (56, 143), while CTLs can be found in approximately 50% of infected individuals (46, 55, 137). Two regions are dominant immunogenic sites in HIV infected individuals, amino acids 45–69 and 176–206 for both B and T cells (10, 11, 86, 211). Some Gambian women, who remain HIV-1 seronegative despite repeated exposure, have high levels of Nef specific CTL that is considered to contribute to resistance of infection (209), making Nef an attractive target for vaccine design.

1.1.7 THE MATRIX PROTEIN (P17) AND THE CAPSID PROTEIN (P24)

The p17 matrix protein is the N-terminal component of the Gag polyprotein and is important to target Gag and Gag-Pol precursor polyproteins to the plasma membrane prior to viral assembly. In the mature particle, the p17 lines the inner surface of the virion membrane. The p17 also aids incorporation of the Env glycoproteins into the viral particles. The array of threefold symmetric holes located between matrix trimers appears to be large enough to accommodate the long cytoplasmic tails of full-length Env. The p17 also facilitates infection of non-dividing cell types, principally macrophages (reviewed in (75)).

The p24 protein capsid, produced from the second open reading frame of Gag, is important for assembly of the virion. The p24 participates in viral uncoating through its association with a cellular chaperone, cyclophilin A. Inhibition of cyclophilin A results in a post-entry block of infection (31). The major homology region in p24 is a 20 amino acid sequence that is highly conserved within all retroviral Gag proteins and essential for particle assembly (reviewed in (75)).

Antibodies directed against p17 and p24 are induced in HIV-1 infection. Most antibodies fail to neutralize the viruses. Gag-specific CD8 and CD4 T cell responses negatively correlate to viral load in infected individuals, with p17 and p24 displaying many epitopes for CD8 T cells and p24 epitopes for CD4 T cells (124, 178, 208). Including both p27 and p24 will likely enhance the efficacy of an HIV-1 vaccine candidate.

1.1.8 THE REVERSE TRANSCRIPTASE

The RT was the first target of antiviral drugs in clinical use (205). Before integration, the RNA must be reversed transcribed into a dsDNA molecule. RT catalyzes both RNA-dependent and DNA-dependent DNA polymerization reactions and contains an RNase H domain that cleaves the RNA portion of RNA-DNA hybrids generated during the reaction. RT initiates from the 3´ end of the $tRNA_3^{Lys}$ primer annealed to the primer-binding site near the 5´ end of the genomic RNA. The tRNA is incorporated into virions during assembly and is often extended by several nucleotides inside the particle. Reverse transcription involves two DNA strand transfer reactions that are catalyzed by RT and are important for priming the synthesis of both minus and plus strands. RT consists of a heterodimer subunit containing 560 amino acids (p66) and a 440 amino acid subunit (p51), are both derived from the Pol polyprotein. Each subunit contains a polymerase domain composed of four subdomains, called fingers, palm, thumb and connection, and p66 also contains an additional RNase H domain. The active site of the p66 polymerase consists of three amino acids in a catalytic triad, which is conserved in many polymerases (reviewed in (75)).

More than 40 different peptides containing RT-specific CTL epitopes have been identified. The most conserved epitopes are located within the “fingers” and “palm” subdomains of the enzyme. Approximately 50% of seropositive individuals develop CTL against RT. RT is also likely to stimulate cross-clade immune responses together with p24 (reviewed in (168)), making RT an attractive target for vaccination.

1.2 THE IMMUNE SYSTEM

The immune system provides protection from a wide range of pathogens. For protection, the host must be able to recognize and destroy a variety of pathogens. Vertebrates have two lines of defense against pathogens. One component of immunity is the innate immune response that fights pathogens from the moment of first contact, and offers protection against pathogens without prior exposure. The other component is the acquired, specific immune system. It is mediated by lymphocytes that have

evolved to express an enormous array of recombinant receptors recognizing any pathogen that the host might ever encounter. The reactive lymphocyte clone specific for the particular antigen is expanded through clonal proliferation. The adaptive immune system takes days or weeks to develop. It is dependent on the innate immune system, through the granulocytes, macrophages, neutrophils, eosinophils, and basophils, which are dedicated to the ingestion and destruction of microorganisms.

1.2.1 THE INNATE IMMUNE RESPONSE

The innate immunity is the unspecific defense system that the pathogens first encounter in the host. Macrophages engulf and destroy microorganisms upon first contact, and secrete cytokines that influence both the innate and the adaptive immune responses. The macrophages are distributed throughout the body, especially in the liver, spleen and lymph nodes. The DC is a specialized relative of the macrophages, and professionally presents antigens to the lymphoid cells to stimulate the adaptive immunity. Neutrophils patrol the blood to detect pathogens, but rapidly attach to the walls of small blood vessels and leave the circulation by extravasation when the body is infected. The NK cells mediate another type of nonspecific effect by killing tumor cells and virus infected cells lacking inhibitory cell surface receptors. The NK cells can also destroy antibody-coated target cells by antibody dependent cell-mediated cytotoxicity (ADCC). This is triggered when antibodies bound to the cell interact with Fc receptors on the NK cells. The *in viv* importance of ADCC in defense against virus infected cells has not yet been fully established (119).

Recognition of the pathogen involves pattern recognition receptors. In mammalians, these receptors are called Toll like receptors (TLRs) named after the Toll receptor in Drosophila with which they are homologous. The TLR family consists of phylogenetically conserved transmembrane proteins (167) that recognize pathogen derived ligands and direct subsequent cell activation via the Toll/IL-1R signal pathway. Today ten human TLRs have been found that protect against microorganisms (reviewed in (134)). For instance, TLR 2 and 4 are responsible for immune responses to peptidoglycan and lipopolysaccharide antigens respectively, while TLR 9 is capable of recognizing bacterial DNA (99).

1.2.2 THE ADAPTIVE IMMUNE RESPONSE

The innate response is followed by the adaptive (acquired) immunity that specifically recognizes and eliminates foreign microorganisms and molecules. It displays specificity, diversity, memory and self/non-self recognition. The cells of the

phagocytic system are intimately involved in activation of the specific system and several factors are produced by the innate system that attract the cells of the specific immune system. The specific system can be subdivided into humoral and cellular immune responses. The humoral immune system involves B cells that express cell-surface immunoglobulin molecules as receptors for antigens. Upon activation, they secrete the immunoglobulins as soluble antibodies that provide defense against pathogens in the extracellular spaces of the body. Effector T cells generated in response to antigen are responsible for cellular immunity. The effector T cells are the CTLs and T helper cells

Activated CD4+ T cells have a critical role in promoting B cell survival and antibody production through CD40L/CD40 interactions. Through IL-2 secretion and/or through CD40L/CD40 costimulation, the activated CD4+ T cells provide helper function to CD8+ T cells. CD4+ T cells secrete many cytokines that have profound immunoregulatory effects. The T helper cells can be subdivided into two main types: the T helper 1 cells produce interferon-γ (IFN-γ), whereas the T helper 2 cells produce IL-4, IL-5, and IL-13. The cytokine milieu present at the time of initial T cell priming appears to be the most important. T cells require costimulatory molecules for full stimulation. Stimulation occurs through interactions of intracellular adhesion molecules or lymphocyte function associated antigens and the B7 molecules, B7-1 (CD80) and B7-2 (CD86) (214). If B7 engages CD28, they activate T cell responses, whereas if they bind CTLA-4, they inhibit T cell responses (112).

T cells have receptors that recognize peptide fragments of intracellular pathogens transported to the cell surface by the MHC molecules. Two classes of MHC molecules bind peptides from different intracellular compartments to present them to the effector T cells. MHC class I molecules collect peptides derived from proteins synthesized in the cytosol, like viral proteins, while MHC class II molecules bind peptides derived from pathogens that have been internalized by phagocytic cells and B cells. CTLs kill virus-infected cells by recognizing viral peptides in the context of MHC class I molecule.

Besides the cytolytic activity of CD8+ T cells specific for HIV-1 infected target cells, these cells secrete cellular soluble antiviral factors that can suppress HIV-1 replication (159). Other factors that inhibit HIV-1 are the natural ligands to the chemokine receptors. The ultimate goal of an HIV-1 vaccine is to develop long-lived

immunological protection, to enhance memory responses that either completely prevent reinfection or greatly reduce the severity of disease.

1.3 DNA VACCINATION

DNA vaccination is a therapeutic and prophylactic strategy in which nucleic acids are introduced into human cells in order to evoke both humoral and cellular immune responses to an encoded antigen. DNA vaccines are considered to be of particular interest against organisms such as HIV-1, where a strong cell-mediated immunity as well as a humoral response seem to be required (66). Nucleic acids are not subject to neutralizing antibodies that can hamper the efficacy of vaccines based on recombinant viral vectors. In 1990, Wolff *et. al.* (255) showed that intramuscular injection of bacterial plasmid DNA resulted in a picogram-sized expression of the encoded reporter gene that could be detected for several months after injection. The elicitation of humoral responses to DNA vaccines in animals was demonstrated in 1992. Using a biolistic system to propel DNA-coated gold microprojectiles directly into skin in living animals, Tang *et al.*, showed that an antibody response could be elicited to the human growth hormone protein by injecting the corresponding gene (237). The priming of cellular responses and protection against challenge were shown the following year, along with different immunization routes (80, 206, 240, 246). Today, several viruses, bacteria and parasites along with certain cancer forms have been targeted using DNA vaccination (reviewed in (66)). Different animal species have been used, ranging from mice to humans (29, 30, 36, 37, 158, 247). The list of DNA vaccines to viruses where protection or partial protection against challenge with infectious viruses is increasing. Partial and complete protective immunity have been induced against many different viruses (Table 1) using DNA alone with different administration techniques (reviewed in (204)).

Table 1. Examples of viruses where DNA vaccination alone has induced protection or partial protection against virus challenge.

Viridae	*Virus*	*Animal models*	*Protection*	*References*
Arena	Lymphocytic choriomeningitis virus	Mice	P/P/P	(145, 265)
Hepadna	Hepatitis B virus	Chimpanzees	P	(194)
	Duck hepatitis B virus	Ducks	PP	(239)
	Woodchuck hepatitis virus	Woodchucks	P	(155, 229)
Herpes	Herpes Simplex virus type 1	Mice	P	(40)
	Herpes Simplex virus type 2	Mice	PP	(82)
	Murine cytomegalovirus	Mice	PP/PP	(87, 173)
	Channel catfish herpes virus	Channel catfish	PP	(177)
Papova	Human papilloma virus type 16	Mice	P/PP	(207, 227)
	Canine oral papillomavirus	Beagle dogs	P	(233)
	Cottontail rabbit papillomavirus	Rabbits	P/PP	(65, 95)
Reo	Rotavirus	Mice	P/PP	(105, 261)
Flavi	Japanese encephalitis virus	Mice	PP/P/PP	(44, 129, 149)
	Russian spring-summer encephalitis virus	Mice, Rhesus macaques	P/P	(220, 221)
	Central European encephalitis virus	Mice, Rhesus macaques	P/P	(220, 221)
	Hepatitis C	Mice	PP	(230)
	Murray Valley virus	Mice	P	(49)
	Tick-borne encephalitis virus	Mice	P	(1)
	Dengue type 1	Monkeys	PP/PP	(136, 199)
	Dengue type 2	Mice	PP	(192)
	West Nile virus	Mice, Horses	P	(57)
	St. Louis encephalitis virus	Mice	PP	(184)
Filo	Ebolavirus	Mice	PP	(245)
Paramyxo	Measles	Cotton rats, Rhesus monkeys	PP/P	(188, 219)
	Respiratory syncytial virus	Mice	PP	(148)
Rhabdo	Rabies virus	Mice, Cynomolgus monkeys	P/P	(154, 258)
Bunya	La Crosse virus	Mice	P	(223)
	Hantavirus (Seoul virus)	Hamster	P	(108, 126)
	Hantavirus (Puumala)	Bank voles	PP	(33)
Orthomyxo	Influenza	Chicken, Mice	PP/ P/P	(45, 153, 206, 240)
Retro	HIV-1	Mice, rhesus monkey	PP/P	(146, 152)
	SIV	Rhesus	P	(67)
Picorna	Foot-and-mouth disease virus	Swine	PP	(19)
	Coxsackievirus B3	Mice	PP	(102, 103)
Parvo	Canine parvoviruses	Dogs	P	(121)
Poxviridae	Vaccinia virus	Mice	P	(107)

PP=partial protection
P=protection

1.3.1 THE VECTOR

The plasmid DNA encodes an origin of replication for propagation in a prokaryotic host and an antibiotic resistance gene (usually the ampicillin or the neomycin/kanamycin resistance gene) to enable selective growth conditions. The plasmid also contains a strong promoter / enhancer element and a mRNA transcript termination / polyA signal for expression of the encoded antigen in an eukaryotic cell (66, 151). Another important feature of the vector backbone is the CpG motif, described below. The DNA is administered to the host by one of various routes: intramuscular, intraperitoneal, intranasal, intravaginal, subcutaneous, oral, or intradermal (reviewed in (215)). Vaccinologists have tried to increase the uptake by adding cationic lipids capable of binding to the DNA and facilitating transport across the cellular and possibly nuclear membranes. Another way of increasing uptake is by blasting the DNA into the cell with helium gas using a gene-gun (237). Electroporation, where an electric field is applied across cell membranes to create a large transmembrane potential that allows DNA to enter, is another method of increasing the transfection efficiency of intramuscularly injected plasmid DNA. The transfected fibers in rat increased from 1% to over 10% after electropulsing (164). The cells take up the DNA and express the antigen that is presented to the immune system of the host. The expressed protein will have the same native conformation, glycosylation, and other post-translational modifications that occur during natural infection of the host cell. The responses induced by a single DNA vaccination can last up to 1 year after immunization, as shown by Thomson *et al.* (238).

1.3.2 MECHANISM OF IMMUNE ACTIVATION

DNA vaccines are thought to prime and cross-prime professional bone marrow derived APCs (DCs) to present the encoded antigen. Three routes are believed to prime APCs (figure 3) (51, 63, 118). First, APCs can be directly transfected with the DNA vaccine (191). Secondly, APCs can be cross-primed by ingesting soluble antigens that have been secreted or released by transfected cells. The internalized antigen will be processed intracellularly by APCs for presentations by MHC class I molecules and priming of CTL responses. Secreted or exogenous proteins also undergo endocytosis or phagocytosis to enter the MHC class II pathway of antigen processing to stimulate CD4+ T cells. Thirdly, APCs can take up cells that have been injured or killed as a result of the vaccine or its function. The death can be either necrotic or apoptotic, but

only cells harboring a high copy number of the DNA vaccine will die as a consequence of the transfection (reviewed (151, 203)).

The primed APCs will be activated and upregulate chemokine receptors and adhesion molecules that will enable them to migrate to lymphatic organs where they activate the immune responses. In the lymph nodes, the antigen derived from the DNA vaccine will be processed for both MHC class I and class II association for presentation to both CTLs and T helper cells. The cell death induced by transfection of host cells is a signal for activation of APCs (165).

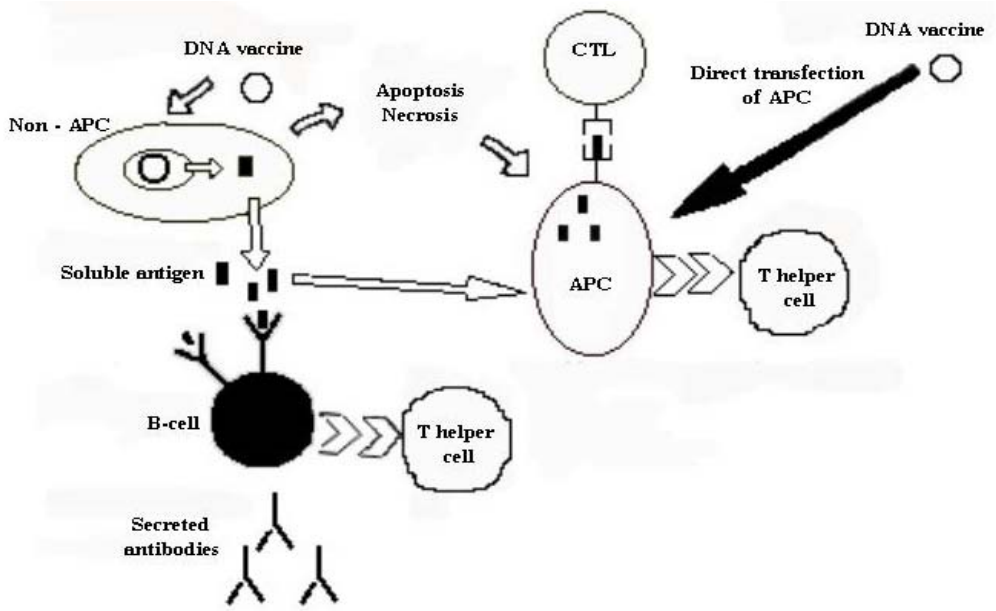

Figure 3. Sketch of immune activation by DNA vaccines.

Antigen presenting cells (APCs) or non-APC cells take up the injected plasmid and the encoded antigen is produced and secreted. The APCs can be dendritic cells (DC), monocytes or macrophages. The non-APCs transfected by the plasmid will eventually die and the apoptotic or necrotic cells are digested by APCs. The APCs will process the antigen for presentation on MHC class I and II molecules and subsequent activation of T helper cells and cytotoxic T lymphocytes (CTLs). Secreted antigen can activate B cells interacting with T helper cells to produce antibodies. APCs can also take up the secreted antigen for presentation on major histocompatibility complex (MHC) class II molecules.

DNA vaccination induces antibodies of the immunoglobulin M (IgM), IgG and IgA types. The predominant IgG subclass generated by DNA vaccination is IgG2a, except after gene-gun when the predominant subclass is IgG1. DNA vaccination using the influenza nucleoprotein gene induced strong antibody responses in mice, non-human primates and humans that peaked 4-12 weeks after a single DNA injection. The responses increased in a dose dependent manner with either single or multiple injections of DNA by various routes of immunization. Antibody responses can be long-lived; significant levels can be present up to 1.5 years post-vaccination (reviewed in (92)). The amount of CD4+ T cells, as measured by proliferation remained elevated for

at least 40 weeks post-immunization. Raz *et al.* (200) showed that CTL responses could be observed for more than 68 weeks after intradermal injection of DNA encoding the influenza nucleoprotein gene. The length of the induced responses is likely to be dependent on the expressed antigen, the vector used, the type of cells primed, and the route and dose of immunization.

There has been some concern that the DNA plasmid may integrate into the host genome, thereby possibly activating oncogenes or inactivating tumor suppressor genes. To date there has been no evidence that plasmids integrate but neither has this possibility been eliminated (reviewed in (92)).

1.3.3 THE IMMUNOSTIMULATORY CPG MOTIFS

Certain bacterial non-methylated, palindromic DNA sequences containing a CpG dinucleotide motif in a particular base context were shown to activate B cell proliferation, induce immunoglobulin secretion, activate T cells, NK cells and DC *in vitr* and *in viv* (reviewed in (138)). Krieg *et al.* (140) formulated a hypothesis for pattern recognition of bacterial or synthetic DNA. They discovered that CpG dinucleotides with selective flanking bases were important and that DNA motifs displaying a 5´-Pu-Pu-CpG-Pyr-Pyr-3´ base sequence were biologically active in eukaryotic cells. Addition of these repeats to a non-coding regions enhanced the immune response to the encoded antigen as measured by IFN-γ and IL-4 secretion, IFN-α, -β and IFN-γ activation and by NK and CTL activity in mouse splenocytes (216). The immunostimulation of these motifs seem to be species specific in activation, the optimal motif for humans being "GTC GTT" and for mice ”GAC GTT” (18). The immune stimulatory effects were further enhanced using phosphorothioate CpG oligodeoxyribonucleotides (ODN), that are nuclease resistant, instead of phosphodiester motifs (140). Phosphorothioate CpG motifs were also shown to have chemotactic effects on primary macrophages that were independent of the CpG motif and were not seen with phosphodiester CpG ODNs (15).

Bacterial DNA has the expected frequency of CpG dinucleotides (1:16), while mammalian DNA exhibits CpG suppression. Nearly all DNA viruses and retroviruses appear to have reduced their genomic content of CpG dinucleotides, adenoviruses being the exception (127). DNA from adenovirus of serotype 12 is immune stimulatory, while DNA from serotype 2 is non-stimulatory and can even inhibit activation by bacterial DNA (139). Thus, depending on the CpG motif, prokaryotic DNA can either stimulate or neutralize immune responses. The inclusion of these

neutralizing CpGs in gene therapy can serve to inhibit unwanted immunostimulatory effects.

It was shown that TLR 9 expression in human immune cells correlated with responsiveness to bacterial CpG DNA to induce proliferation of splenocytes, inflammatory cytokine production (TNF, IL-6 and IL-12) from macrophages and maturation of DC (shown by upregulation of CD40, CD80, CD86 and MHC class II molecules). The extracellular region of TLR 9 contains a DNA-binding motif described to occur in a family of methylated CpG-DNA binding proteins, MBP-1-4 (78, 101). The role of TLR 9 was shown by expressing TLR 9 in normally non-responsive cells and by studying TLR $9^{-/-}$ knockout mice (99). Endosomal acidification is a requirement for CpG-ODN signaling (figure 4) (157, 263).

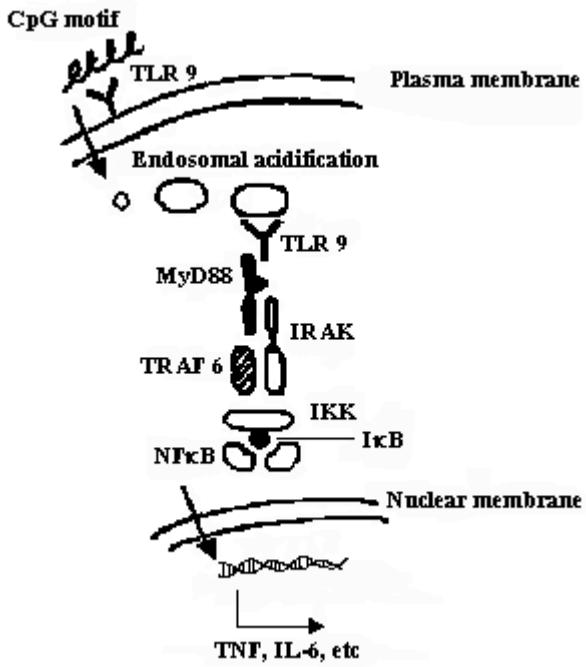

Figure 4. Model of NF-κB signaling in dendritic cells (DC) by CpG DNA.

DNA binding proteins associated with the cell membrane binds to the CpG motifs in a non-sequence specific manner. The motifs are translocated to early endosomes. The linkage between the CpG motif and the myeloid differentiation 88 adaptor (MyD88) activation is believed to be an intracellular Toll like receptor (TLR) 9 molecule. MyD88 acts downstream of the TLR9 to activate the interleukin 1 receptor-activated kinase (IRAK) (130). IRAK then recruits an adaptor, TRAF 6 (tumor-necrosis factor receptor-associated factor 6) which is activated by IRAK (99, 115) and in turn activates IKK (Iκ kinase complex). IκK phosphorylates the inhibitor of NFκB, IκB, targeting it for degradation and releasing the active transcription factor NFκB for translocation into the nucleus and subsequent transactivation of cytokine genes and the TNF genes (reviewed in (24)).

There are two classes of CpG ODNs. Certain CpG ODNs can induce high amounts of IFN-α and β in PBMC, while TNF seems to be upregulated by most CpG ODNs. The

ability to induce IFN-α correlates with their ability to stimulate NK cell lytic activity. IFN-γ production is further dependent on IFN-α. CpG ODN 2006 is a primate CpG ODN that has been shown poor at inducing IFN-α but activates B cells and maturation of plasmacytoid DC. CpG ODN 2216 is another primate CpG ODN, but instead activates NK cells and promotes IFN-γ production of activated CD4+ T cells (142).

CpG DNA directly activates monocytes, macrophages and dendritic cells *in vitr* to upregulate their expression of co-stimulatory molecules and to secrete a variety of cytokines such as IL-12 (figure 5) (42, 94, 135). The IL-12 stimulates NK cells to secrete IFN-γ and increase their lytic activity (135). It generates reactive oxygen species that are detectable within 5 minutes and appear to be a secondary signal, rather than a by-product. Reactive oxygen species is one of the signals reported to activate

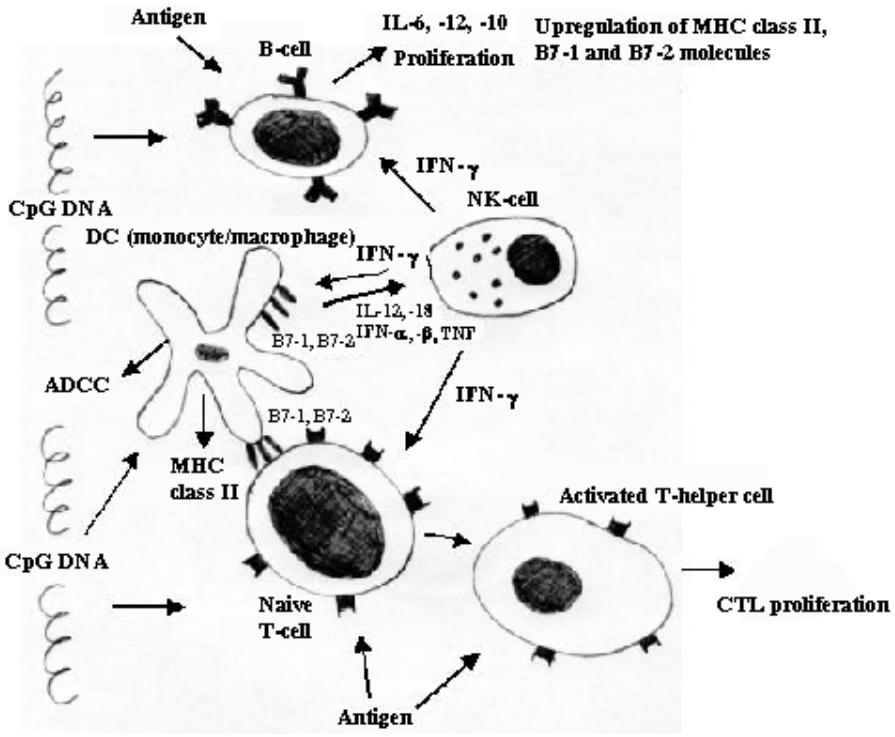

Figure 5. **Model of cellular activation following exposure to CpG DNA.**
CpG directly activates dendritic cells (DC, also macrophages and monocytes) to express increased levels of costimulatory molecules, and increase antigen presentation and cross priming. T helper cell type 1 cytokines such as interleukin-12 (IL), tumor necrosis factor (TNF) and IFN-α (interferon-α) are released and induce natural killer (NK) cells to release IFN-γ. Monocytes can be stimulated to engage in antigen dependent cellular cytotoxicity (ADCC). B-cells produce T helper cell type 2 cytokines such as IL-6 an IL-10 and upregulate their costimulatory molecules as well as major histocompatibility complex (MHC) class II molecules. They become activated to proliferate and increased release of immunoglobulins. All T helper cell type 1 cytokines activate naive T-cells if an antigen is present and activates cytotoxic T lymphocyte (CTL) cells.

NFkB via stress kinases. Protein tyrosine kinases, protein kinase A, and protein kinase C have no roles in CpG mediated leukocyte activation (262). Activation of T cells and NK cells appears to require additional signals, like T cell receptor ligation. B cell activation can be direct and/or require additional signals, however B cell receptor crosslinking is needed for proliferation (reviewed in (150)).

There has been some concern that CpG motifs can induce autoimmunity by enhancing the production of anti-dsDNA autoantibodies in normal mice and accelerate the development of autoimmune disease in lupus-prone animals. Shortly after vaccination, the numbers of IgG anti DNA spot-forming cells increased two to threefold, but the increase was only transient (reviewed in (92)).

1.3.4 VIRAL VECTORS

The use of recombinant viral vectors to booster priming immune responses with DNA has been effective for control of HIV-1 (8). One point to consider is that the viral vectors must be safe to use in immunocompromised individuals, besides having the capacity to carry large foreign genes over multiple passages. Several families of viruses have been used as booster immunizations in combination with DNA vaccinations.

1.3.4.1 Poxviruses

Poxviruses are large DNA viruses carrying a genome of 130-300 kb with a cytoplasmic lifecycle. They can carry multiple large, foreign genes, which are stably expressed from the recombinant genomes (235). The most commonly used today are recombinant vectors based on the attenuated Modified Vaccinia Ankara (MVA) virus, canary poxvirus and the fowlpox virus (reviewed in (222)).

1.3.4.2 Adenoviruses

These viruses are non-enveloped, and have a linear dsDNA genome of 36-38 kb DNA. These vectors are commonly used for gene therapy. They replicate within mucosal surfaces and can be administered both orally and intranasally. To circumvent the possibility of pre-existing strong humoral responses to different adenovirus subtypes, other species-specific strains have been used lately (reviewed in (222))]. A recent paper by Shiver *et al.* (228) compared prime boost regimens in monkeys using SIV *gag* DNA as prime and either MVA–SIV *gag* or adenovirus 5-SIV *gag* as boost. The adenovirus 5 vector used was replication-incompetent. After challenge with a pathogenic HIV-SIV hybrid virus, animals immunized with the adenovirus vector showed the lowest levels of virus levels in plasma.

1.3.4.3 Alphaviruses

The alphaviruses are single stranded, positive sense RNA viruses that infect many types of animal cells. The vectors are based on the alpha viruses Venezuelan equine encephalitis virus, Sindbis virus or Semliki Forest virus and can be generated to encode an antigen of interest, but do not produce viral structural proteins. The vectors use the viral RNA dependent RNA polymerase to amplify multiple copies of mRNA encoding the gene of interest within the cytoplasm of an infected cell. Mice immunized with a recombinant Semliki Forest vector against influenza were protected against lethal influenza challenge (23). Certain alphaviruses have tropism for DC, making this approach particularly interesting. In addition, DNA vectors with a gene cassette containing an alphavirus replicon and a gene of interest can be used. This results in a dsRNA intermediate that activates interferons, in higher levels of antigen expression, and cross-priming through induction of apoptosis (reviewed in (64)).

1.3.5 ADJUVANTS AND COEXPRESSION OF CYTOKINES

There are several ways to enhance the immune responses induced by a DNA vaccine. One is by co-immunizing with genes expressing one or several cytokine genes. For instance, intramuscular administration of an IL-15 expression vector together with an HIV-1 DNA vaccine enhanced the antigen specific CTL activity compared to using the HIV-1 DNA alone (133). Using HIV-1 *env* DNA and an IL-15 plasmid enhanced cellular responses compared to DNA alone (259). Many more cytokines have been evaluated for their adjuvanticity in combination with DNA vaccines (reviewed in (92)). Protein vaccines usually contain aluminum hydroxide (Alum) as an adjuvant that induces a cytokine milieu that may interfere with CTL induction. Alum is the only adjuvant that is approved by 1998 for use in humans (reviewed in (141)).

2 AIMS OF THE STUDY

The main objectives of this study were to construct, evaluate and enhance DNA vaccines against HIV-1 *in vitr* for expression and *in viv* for immunogenicity.

The specific aims for the different papers and manuscripts were:

- To study the expression of the three regulatory proteins of HIV-1 (Paper I).
- To study the immunogenicity of single and combined regulatory and structural genes of HIV-1 as DNA immunogens in mice and in humans (Papers II &III).
- To evaluate the capacity of different prime boost regimens to induce strong immune responses to HIV-1 (Papers IV, V).
- To evaluate the compound imiquimod as a new adjuvant with DNA immunogens (Paper VI).

The methods used in this thesis are described in detail in the attached articles.

3 RESULTS AND DISCUSSION

3.1 EXPRESSION OF HIV REGULATORY DNA VACCINE CONSTRUCTS (PAPER I)

There is a vast variation of different HIV isolates and clades that display a high degree of genomic variability. On average, one nucleotide substitution is introduced in each replication cycle, resulting in sequence variability due to the millions of new viruses generated every day (reviewed in (185)). HIV-1 isolates from African patients differ by more than 20 % in nucleotide sequence from North American and European isolates, primarily in the structural genes (6). The differences between different clones from one virus isolate (210) or between sequential isolates from one patient are more moderate (93). The *tat, rev* and *nef* genes of HIV-1 are of particular interest as vaccine targets since they are expressed early and are immunogenic *in viv* .

Functional studies of primary *nef* isolates from long-term non-progressors (LTNP) have shown that they may carry isolates with defective Nef functions, for instance with an impaired ability to downregulate MHC class I molecules (74). Another study showed that there was an important variation in Nef, but that the functionally important domains were more conserved, for instance the myristoylation signal was almost totally conserved among different subtypes (123). Similar findings were apparent in the Rev function, with LTNP showing 2–4 fold reduced Rev activity (111). The *tat* gene seems more conserved than the *rev* and *nef* genes. A study by Yamada *et al.* (260) showed mutation frequencies in *nef* and *rev* genes in seven LTNP at 30.6% and 36.7% respectively, while no variation was seen in the *tat* gene. The mutations in *rev* were only seen in the second exon, while the first exon was totally conserved. This is consistent with a study showing conservation of the immunodominant B cell epitopes of Tat among distantly related subtypes (85).

We investigated the biological function and protein expression capacity of different *tat*, *rev* and *nef* genes. We constructed several Tat, Rev and Nef expression plasmids carrying the *tat* and *rev* genes from the HIV-1 virus HXB2 and the *nef* gene from the HIV-1 LAI virus. Further, different polyA signals and vector backbones were investigated for efficient protein expression as characterized by measurement of the biological function of the individual proteins.

Sequence analysis was performed on an amino acid level to investigate variations. Four out of five patient isolates had identical amino acid sequences with the *tat* gene derived from the laboratory strain virus HXB2. The fifth sequence had a variation present in the nuclear localization signal of Tat; at amino acid number 50 (K50E). A Tat based biological activity assay was used to quantify the amount of Tat dependent chloramphenicol acetyltransferase (CAT) protein that was expressed using the different *tat* genes. As expected, the *tat* gene with a mutated nuclear localization signal was found less efficient in Tat dependent CAT production. A less basic nuclear localization signal would result in less efficient transport of the Tat protein from the cytoplasm to the nucleus, where the Tat transactivates the transcription of the *CAT* gene. The *tat* gene derived from HXB2 was more efficient in inducing Tat dependent CAT expression than the patient derived genes, even with identical amino acid sequences. We hypothesize that this might be due to variation in codon usage between laboratory strain derived *tat* and patient derived *tat.*

We compared the ampicillin resistance marker with the kanamycin resistance marker in identical vector backbones in plasmids encoding the *tat* gene. The promoter used was the immediate early promoter of the human cytomegalovirus and the polyA signal was the human papilloma virus type 16 polyA. The vector carrying the ampicillin resistance gene (HCMV*tat*) was more efficient in producing CAT than the vector encoding the kanamycin resistance gene (pKCMV*tat*). The pKCMV*tat* resulted in 20 % less efficient CAT production than the HCMV*tat* vector. This was hypothesized to be caused by less efficient uptake in the cells or nuclei when using the pKCMV*tat* construct.

We also constructed five plasmids encoding the *rev* gene from the above patients and performed sequence analyses. One amino acid alteration was located at amino acid 61 (G61S) compared to the laboratory strain sequence. The position of the variant is located between the nuclear localization signal and the leucine-rich effector domain of Rev. The amino acid is commonly found in several different B isolates.

All five clones from the five patients were identical at the amino acid level. A Rev assay was used to quantify the amount of Rev dependent p24 production. Two different vectors were compared in the Rev assay. The first vector HCMV*rev* uses the human cytomegalovirus promoter and the rat preproinsulin II polyA signal. The ampicillin resistance gene is used as the antibiotic marker gene and the vector backbone derives from pfX3. The second vector, pKCMV*rev*, uses the same vector

backbone as HCMV*tat* and pKCMV*tat* (pUC8). The promoter, polyA signal and antibiotic resistance gene are identical to the sequences in pKCMV*tat.* The patient-derived clones are all expressed from the vector using the kanamycin resistance gene.

The p24 production based on Rev from the pKCMV*rev* was 50% higher than production with the HCMV*rev* construct. This indicates that the pUC8 backbone with the human papilloma virus type 16 polyA produces more protein than the pfX3 backbone with the rat preproinsulin II polyA. The p24 production from the patient-derived *rev* clones was similar to p24 produced using the HCMV*rev* vector.

Nef expressing vectors were constructed using the *nef* gene from one LTNP patient. Three patient-derived *nef* clones were constructed. Two different vectors were investigated for efficient Nef expression from the laboratory strain derived *nef* gene. The HCMV*nef* uses the human cytomegalovirus promoter, the HIV-1 LTR as the polyA signal and the ampicillin resistance gene. The pKCMV*nef* uses the same promoter, polyA signal and antibiotic resistance marker as pKCMV*tat* and pKCMV*rev*.

Sequence analysis showed that all three patient-derived *nef* clones were identical in their nucleotide sequence but that this sequence differed from the laboratory strain derived *nef* at both the nucleotide and the amino acid levels. The patient-derived clones all carried a frame-shift mutation at the 3´- end, resulting in a prolonged protein. Immunofluorescence confirmed the expression of Nef. Immunogenicity studies in mice were performed using the HCMV*nef* plasmid and the patient-derived clones. Both groups induced similar Nef-specific cellular and humoral immune responses as measured with lymphoproliferative assays, IgG synthesis *in vitr* and Nef specific ELISA. T cell and B cell epitope mapping were performed and the different vectors induced similar reactivity patterns.

In conclusion, we have examined the protein expression capacity and biological activity from the HIV-1 regulatory genes in different vector backbones as well as from different viral isolates. The patient-derived *tat* genes generally directed lower CAT expression than the HXB2 *tat* gene. For further studies, we used the HXB2 *tat* gene. Some concern has been raised about the safety of using a functional *tat* gene. However, both biologically active Tat protein and wild type *tat* DNA have been shown to be safe in mice, guinea pigs, monkeys and humans (35, 37, 70).

The patient-derived *rev* genes resulted in expression similar to the laboratory strain derived *rev* gene. Only one mutated *nef* gene could be used in comparison with the laboratory strain derived gene and no quantitative assays for Nef expression were available at that time. Thus, the only conclusions we can draw from this part of the experiment are that, using the different genes, we found similar immune reactivities and similar protein localization in the cell. Further, by comparing different vector backbones and polyA signals, we found that pUC8 vector backbones and the human papilloma virus type 16 polyA signal gave high expression levels of all three genes. The use of attenuated genes (structural and accessory genes) as effective immunogens have been shown previously (14). However, one study showed a poor correlation between protein expression and induced immunity (264). This indicates that with some antigens, protein expression might not always be the best marker for evaluating the efficacy of a DNA vaccine.

3.2 Interactions of Single and Combined Human Immunodeficiency Virus Type 1 (HIV-1) DNA Vaccines (Paper II)

DNA immunization permits selection of single genes and evaluation of possible antagonistic or synergistic effects between the encoded components. Previously, some papers have reported on the use of combined genes or multivalent DNA vaccines (32, 39, 90, 106, 166). None of them reported on interference between the chosen antigens. Co-immunization of mice with genes from the measles virus (39) resulted in an antibody subclass switch from an IgG2a type response elicited by the nucleoprotein gene alone or to an IgG1 response when combined with a hemagglutinin gene. Several groups have evaluated different vaccine concepts against the three early proteins of HIV-1 (35, 37, 196). The *nef* DNA and Nef protein have also been used for vaccination purposes (174, 213). Studies using DNA vaccines encoding *tat*, *rev* and *nef* have also been done in humans (35, 37). By inducing immune responses to Tat, Rev and Nef proteins, it should be feasible to limit primary and chronic infection.

The protein expression capacity *in vitr* and immunogenicity *in viv* of three individual plasmids encoding the *tat*, *rev* and *nef* genes of HIV-1 were examined as single immunogens and as a combined multivalent vaccine. The vectors used carried the kanamycin resistance marker, the human cytomegalovirus promoter and the

human papilloma virus type 16 polyA signal, all described in paper I. All genes were derived from laboratory strain adapted virus isolates.

The protein expression capacity for the *tat* and *rev* encoding plasmids was characterized by assays based on the biological function of the respective protein. No interference of Tat expression, measured as the transcriptional activity of Tat, was apparent when the *tat* encoding plasmid was transfected in combination with the *rev* and *nef* encoding plasmids.

The Rev protein expression capacity was quantified, based on the activity of Rev to transport the viral mRNA transcripts (encoding the p24 of HIV-1 in our experiments) from the cell nucleus to the cytoplasm. No decline in p24 protein production was seen when the *tat* and *nef* encoding plasmids were co-transfected together with the *rev* encoding plasmid, as compared to transfections with the *rev* encoding plasmid alone or with non-coding plasmids.

A capture sandwich ELISA specific for the Nef protein was developed to quantitatively measure Nef protein expression from *nef* encoding plasmids. The amount was measured directly rather than using an assay for biological activity. A decrease (ranging from 36–239%) in Nef protein expression was apparent when the *nef* encoding plasmid was co-transfected with the plasmids encoding the *tat* and *rev* genes as compared to transfection experiments using the *nef* encoding plasmid alone.

Immunogenicity was evaluated *in viv* by immunizing C57BL/6 mice with single plasmid immunogens or with a combination of the *tat*, *rev* and *nef* genes. The intramuscular route was used for immunization since we have previously shown that such immunization induces immune responses to the Tat, Rev and Nef proteins (106). In that study, a combination of the three regulatory genes and three structural genes: p24, p17 and gp160, was used in several different strains of mice. Immune responses were induced to all included antigens, as measured by lymphoproliferative assays and antibody responses. Several modes of delivery were also attempted in that study.

The DNA immunizations either with single immunogens or with the combination of all three genes were not able to induce significant IgG seroconversion to any of the three HIV-1 antigens. Very low antigen titers were seen in a few animals in each group after the last immunization. The low induction of antibody responses has also been reported when using *env* DNA vaccines (43).

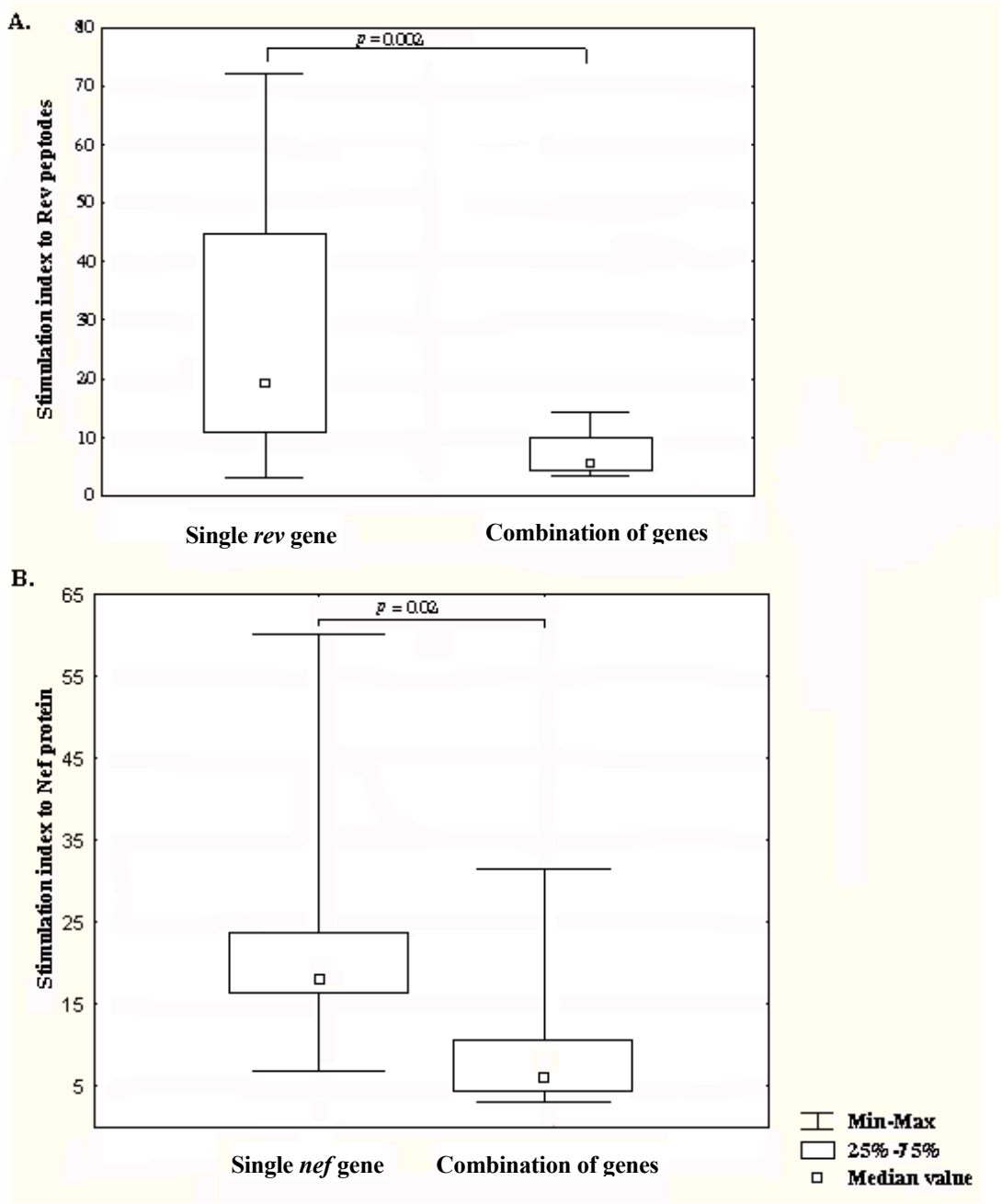

Figure 6. T-cell reactivity.

T-cell reactivity to Rev after DNA immunization **(A)** and to Nef after DNA immunization **(B)**. The magnitude of T-cell proliferative stimulation indices (SI) is shown from mice immunized with the single *rev* or *nef* genes or with the combination of all three *tat*, *rev* and *nef* encoding plasmids. All SI values above 3 were considered positive. The combination of all three plasmids results in a reduced proliferative capacity to Rev and Nef antigens compared to when single plasmid immunogens are used.

The IgG synthesis *in vitr* is considered a more sensitive assay to measure antibody responses, since splenocytes are stimulated with the respective antigen. In this assay, 100% of both the single-immunogen immunized mice and the mice receiving all three immunogens responded to Tat protein or the Tat representative peptide antigens. No statistical difference in the magnitude of responses was seen between the two groups.

The IgG production *in vitr* after stimulation with either Rev protein or the Rev peptide antigens was decreased in the mice immunized with all three genes as compared to those that received the *rev* encoding plasmid as a single immunogen. A statistical difference was seen between these two groups, with a stronger magnitude of response in the mice that received the *rev* encoding plasmid alone. No statistical difference between the two groups was found in response to either Nef protein or Nef peptide antigens.

The cellular responses to the respective antigen were measured using a T cell proliferative assay (106). We used 200,000 cells per well and stimulated these with antigens for 5 days. Different variations of this assay are commonly used, with varied cell numbers and duration of stimulation. The Tat T cell proliferative responses were not influenced by co-expression of the Rev and Nef proteins. The magnitude of cellular proliferation after stimulation with Rev (figure 6A) and Nef (figure 6B) antigens was lower (Mann-Whitney U – test, $p = 0.002$ and $p = 0.02$ respectively), in mice immunized with all three genes than in mice receiving only one of them.

In conclusion, the protein expression capacity and immunogenicity induced by a combination of DNA vaccines seems to be influenced by the choice of a combination of plasmids. The gene expression and immunogenicity from the *tat* gene were not influenced by the combination with *rev* and *nef* genes. However, cellular responses against the Rev and Nef protein were decreased when a combination of *tat*, *rev* and *nef* encoding plasmids was administered. The decrease in cellular responses might be caused by the immunomodulatory effects caused by the Tat and Nef proteins. For vaccination against HIV-1, where a broad specific cellular response is needed, one might have to design specific combinations of genes that have been documented not to interfere with each other. Two possibilities are to deliver certain genes as single DNA immunogens and to combine structural genes with regulatory genes (13). Immune responses can also be optimized by varying the interval between immunizations (79).

3.3 A GENE COMBINATION RAISES BROAD HUMAN HIV-SPECIFIC CYTOTOXICITY (PAPER III)

DNA vaccination is a powerful tool for both prophylactic and therapeutic vaccination for human diseases. DNA immunization has been shown to be safe and can induce immune responses in humans (29, 30, 36, 37, 158, 247), eliciting both cellular and humoral responses. Cellular immune responses, especially CD8+ cytotoxic T

lymphocytes, have been reported to be important for control of HIV replication both in primary disease and in LTNP (242). A strong CTL response can be detected early after infection in humans, with up to 1-5% of the pool of CD8+ T cells specific for the virus (178). The HIV-1 specific CD8+ T cells are thought to be capable of repressing the plasma viremia that occurs within a few weeks of infection, it ultimately fail to control the infection. HIV can hide from CTLs in at least two compartments: the glial cell of the central nervous system, an organ where cell-mediated responses are typically restricted, and the resting T lymphocyte (185).

We have studied the efficacy of a combination of DNA plasmids encoding the *tat, rev* and *nef* genes in inducing MHC class I-restricted CTL in asymptomatic HIV-1 infected patients compared to the use of each immunogen separately. The cytolytic activity was measured using autologous B target cells expressing several HIV-1 viral peptides caused by infection with an HIV-1/Murine leukemia pseudovirus (MuLV, described in paper IV). Target cells expressing the respective antigen were also used, created by infection using recombinant vaccinia viruses (12).

CTL assays based on peptides may not account for the possibility that mutations in and around epitopes may interfere with endogenous processing. Targets may normally be lysed when the peptide is placed on the surface of the cell experimentally, but the peptide might fail to reach the cell surface *in viv* altogether. Assays with virus-infected cells or gene-transfected cells, as targets should be more sensitive to mutations altering peptide processing (reviewed in (88)). We used target cells infected with an HIV-1/MuLV pseudovirus or recombinant vaccinia viruses to attain an endogenous processing of HIV-1 antigens.

Nine patients selected for having no or low antibody titers or T-helper cell reactivity to HIV-1 Tat, Rev or Nef antigens were previously immunized. They received one single gene constructs encoding the *tat, rev* or *nef* gene (each of the plasmids was used in 3 patients) (35, 37). Four of the patients started highly active antiretroviral treatment after the second or third dose of single gene immunization. After 13 – 18 months, eight patients (one patient who previously received the *rev* gene moved to another city and was lost for follow up) continued immunization. They were given three doses of a combination of the *tat, rev* and *nef* genes at days 0, 60 and 180. Six non-DNA immunized HIV-1 positive patients were used as controls; three of them were receiving highly active antiretroviral treatment.

Two different administration routes were used. Immunization at day 0 was performed by intramuscular administration, at day 60 by mucosal intraoral jet injection and at day 180 by intramuscular injection (four patients) and by mucosal intraoral jet injection (four patients) (156).

CTL precursor frequencies and CTL responses against autologous target cells infected with either HIV-1/MuLV or with a recombinant vaccinia expressing a single gene product were evaluated before and after DNA immunizations.

Three patients naive to highly active antiretroviral treatment developed CTLp to HIV-1/MuLV infected targets after the combination of genes. Three out of four patients on highly active antiretroviral treatment had developed detectable CTLp after the single DNA immunization and before the combined DNA immunization. As expected, the immune response was irregular over time as is common in HIV-1 infection (38), but overall the CTLp to targets infected by HIV-1/MuLV were moderately higher after the combined immunization than after single gene immunization. CTLp frequencies to target cells expressing one HIV-1 protein increased in all patients after the single DNA immunization. In the patients naive to antiviral treatment, the responses were transient and undetectable before immunization with the combined genes. These four patients retained detectable CTLp frequencies to the corresponding antigen after the combined DNA immunization. All four patients receiving highly active antiretroviral treatment had detectable CTLp frequencies before the first combined DNA immunization. In two of these patients, CTLp to Nef, Rev and Tat increased following the DNA immunization.

After single DNA immunization, 4 patients showed positive CTL responses (specific lysis > 10%) to HIV-1/MuLV infected targets. Following immunization with the combination of genes, the CTL responses to the HIV-1/MuLV specific target cells increased in all patients but one.

CTL responses against *nef*, *rev* and *tat* infected target cells were induced against the respective antigen after single gene immunization in all except one patient. This patient had received the *rev* gene as single immunogen. The *rev* gene is the poorest of the three plasmids in inducing immune responses in immunized hosts. The Nef responses following co-immunization with the *rev* and *tat* genes were decreased compared to levels after single *nef* gene immunization (figure 7), and new responses to Rev and Tat were induced. Also, the Rev CTL responses were decreased in the one patient who responded to single *rev* gene immunization, when the *rev* gene were coimmunized with the *nef* and *tat* genes as compared to levels after single *rev* gene immunization. New responses to Nef and Tat were induced and in the Rev non-responder, new Rev responses were detectable after the combined immunization. In the patients previously immunized with the *tat* gene, low Tat specific and no Nef specific responses were detected after immunization with the combination of genes. New CTL responses to Rev were, however, induced in two of these patients.

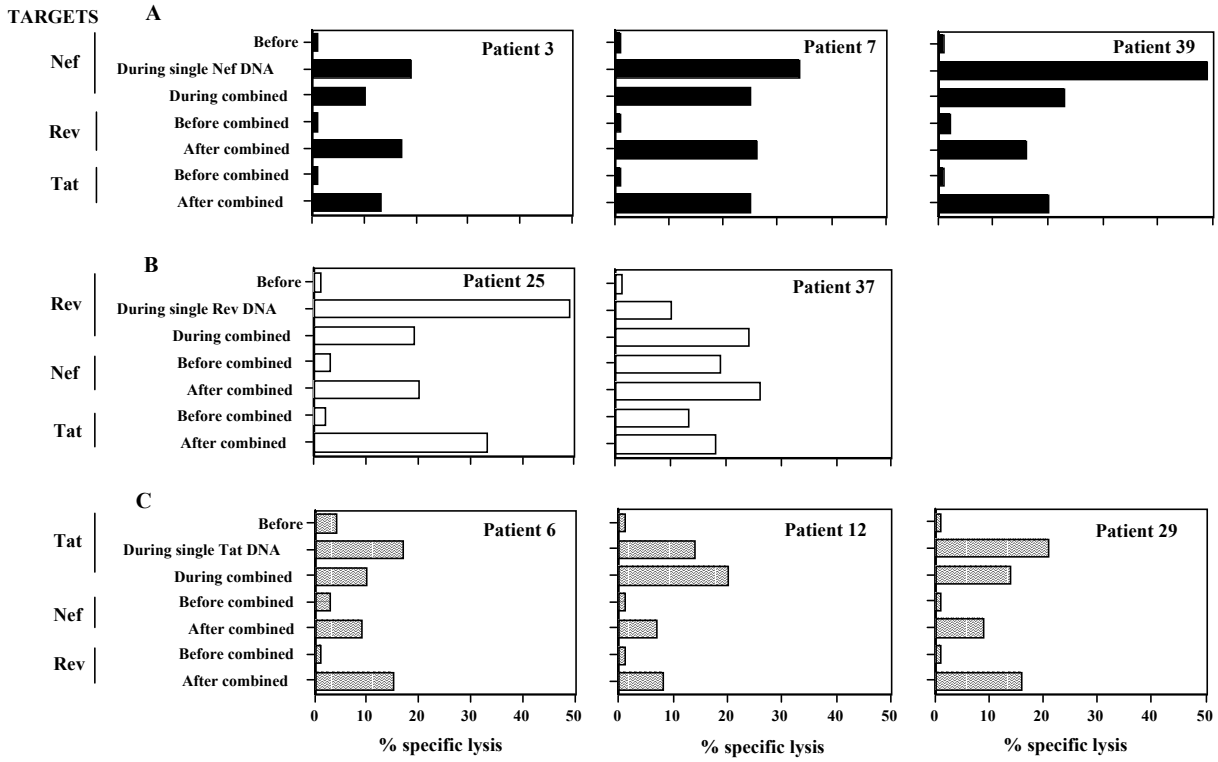

Figure 7. Cytotoxic T lymphocyte activity after DNA immunization.

The highest cytotoxic T lymphocyte (CTL) activity of peripheral blood mononuclear cells (PBMC) against HIV-1/MuLV infected targets in eight DNA immunized asymptomatic HIV-1 infected patients, before DNA immunization, during single DNA immunization and during combined DNA immunization. The results are shown at effector to target ratios of 6:1 for patients 3, 7 and 39 and 50:1 for the remaining five patients. CTL activities to Nef targets are decreased after combined DNA immunization compared to after single DNA immunization with the *nef* gene.

The results from this study indicate that the combination of genes induces target cell lysis consisting of an HIV specific and a bystander effect. The basic lytic activity against control target cells also increased after the combined plasmid immunization. This might be due to the presence of human CpG motifs in the bacterial plasmid backbone of the DNA vaccines, previously shown to contribute to unspecific immune responses in human peripheral cells (140). Some CTLp responses were also seen against HIV-1/MuLV in the non-DNA immunized HIV-1 positive patients induced by the natural HIV-1 infection. Compared with most acute viral infections, HIV is unusual in that circulating CTL effector cells and CTLp are present in the peripheral blood of many patients. The most frequently recognized proteins are Gag, Pol, Env and Nef but responses to Tat, Rev and Vif proteins have also been detected but at a lower frequency (reviewed in (38)).

CTLs to the Tat, Rev and Nef proteins would target the virus-infected cells before there is a substantial release of virus. There is an immunodominant region in the central portion of the Nef protein (amino acid 73-144) that is recognized by most of the patients producing a CTL to Nef in association with several HLA class I molecules (52). Mutations occurring in the dominant epitopes of the Nef proteins have been shown to allow the virus to evade recognition by specific CTLs in SIV infected monkeys (reviewed (185)). The mutations resulted in reduced efficacy of CTL-mediated killing of cells expressing such peptides. CTL escape variants have also been documented in HIV infected individuals. Anti-Tat CTLs are able to control early virus replication after primary infection in the SIV model and exert a selective immune pressure on the virus, leading to the appearance of slow replication and less pathogenic escape mutants (7). Targeting conserved regions of the regulatory proteins essential for the function of the respective proteins, possibly with mini gene constructs, might be one way to avoid CTL escape.

Responses induced in mice correlated to the responses seen after immunization of humans. This indicates that mice can be used to screen for optimal combinations of plasmids to avoid decreases in responses when immunizing with several genes.

3.4 CLEARANCE OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 AFTER IMMUNIZATION WITH THE NEF PROTEIN (PAPER IV)

DNA vaccinations have been shown to induce broad cellular and humoral responses in small animal models against HIV-1 (66). Immune responses induced in primates and in man are generally lower and more varied, than those induced in small animals.

Several options for boosting these responses are available using different viral vectors such as poxvirus (8), different adjuvants (16) and recombinant proteins for enhancing immune responses.

We evaluated the efficacy of different Nef immunization schedules for their ability to induce strong HIV-1 Nef specific responses using *nef* DNA or recombinant Nef protein (table 2). To evaluate the Nef responses we used an experimental HIV-1/MuLV challenge model for mice (figure 8) (12, 117, 152, 231, 232). HIV-1 is used to superinfect CEM-1B cells that are stably transfected with the whole genome of MuLV, an amphotropic murine retrovirus. The supernatant is collected and contains a mixture of the different viruses. MuLV, HIV-1, particles with the MuLV envelopes containing the HIV-1 genome and vice versa and mosaic virus particles with HIV-1/MuLV envelopes with either HIV-1 or MuLV genome are particles that may be found in the supernatant. The virus suspension is then used to infect primary splenocytes from naive mice. Only particles carrying the MuLV envelope will be able to infect these murine splenocytes. Presence of HIV-1 in the infected cells is assayed by an HIV-1 specific p24-capture ELISA (62). The splenocytes are subsequently transplanted intraperitoneally in immunized and control mice. This model is believed to mimic the situation when an HIV-1 infected cell enters the host and meets the primary immune response. HIV-1 proteins will be presented on MHC class I and class II molecules on the surface of infected cells and enable recognition and subsequent killing of virus infected cells. Mature virions may be produced from the transplanted cells, but these virions will not be able to cause a productive infection *in viv* . Ten to 14 days after transplantation, the mice are sacrificed and the peritoneal cells are collected for co-culturing with HIV-1 permissive PBMC or Jurkat cells. Supernatants are collected every third day and assayed for the presence of HIV-1 p24 representing the kinetics of the infectious HIV-1 production. Using this challenge model, in 40 out of 41 control mice, viable HIV-1 has been recovered (unpublished data).

Table 2: Immunization schedule and experimental results.

Prime;Boost (number of mice)	Prime (weeks 0, 4)	Boost (week 12)	Clearance of HIV-1 (%)	IFNγ SFC per million cells after Nef stimulation
Nef protein (5)	50 μg Nef	-	20	6±14
Nef protein/ODN (10)	50 μg Nef / 50 μg ODN	-	70	74±104
Nef protein/ODN; MVA*nef* (6)	50 μg Nef/ 50 μg ODN	MVA*nef*[2]	33	21±99
ODN ; MVA*nef* (6)	50 μg ODN	MVA*nef*[2]	0	54±75
nef DNA/MVA*nef* (6)	-	100 μg pKCMV*nef* mixed with MVA*nef*[2]	0	5±118
nef DNA ; MVA*nef* (5)	100 μg pKCMVnef	MVAnef[2]	0	ND[3]
nef DNA ; MVA (6)	100 μg pKCMVnef	MVA[2]	0	36±82
Saline (6)	Saline	Saline	17	4±35

[1]Mice were challenged at week 16 and sacrificed 14 days later.

[2]Both MVA constructs were used at a plaque forming unit of 10^7.

[3]Not performed due to lack of cells

Cellular responses to Nef were assessed by lymphoproliferative assays, measurement of IL-2 production and by IFN-γ production. High specific lymphoproliferation was seen to Nef protein and to a Nef specific peptide after immunization with Nef protein mixed with CpG ODN 1826 motifs (58) followed by boosting with MVA*nef* (9, 236). The group receiving CpG ODN and MVA*nef* without Nef protein and *nef* DNA mixed with MVA*nef* proliferated after stimulation with Nef protein or a Nef specific peptide. Nef specific IFN-γ secretion was induced in all groups of immunized mice except the saline control group and the group that received Nef protein alone. IL-2 secretion was seen in cells from all groups except the saline immunized mice. Humoral responses were measured using a Nef specific ELISA. Weak responses were seen in the group that received Nef protein and CpG ODN and in the group that received *nef* DNA mixed with MVA*nef*.

It has previously been shown that CpG ODN 1826 is effective in increasing antibody titers in combination with protein. The effect can be further enhanced by using CpG ODN in combination with another adjuvant, Alum (Al_2O_3) (58). Addition of CpG ODN 1826 to plasmid DNA however, results in an CpG ODN dose-dependent reduction in gene expression from the plasmid, possibly because of competitive interference at binding sites on the surface of target cells.

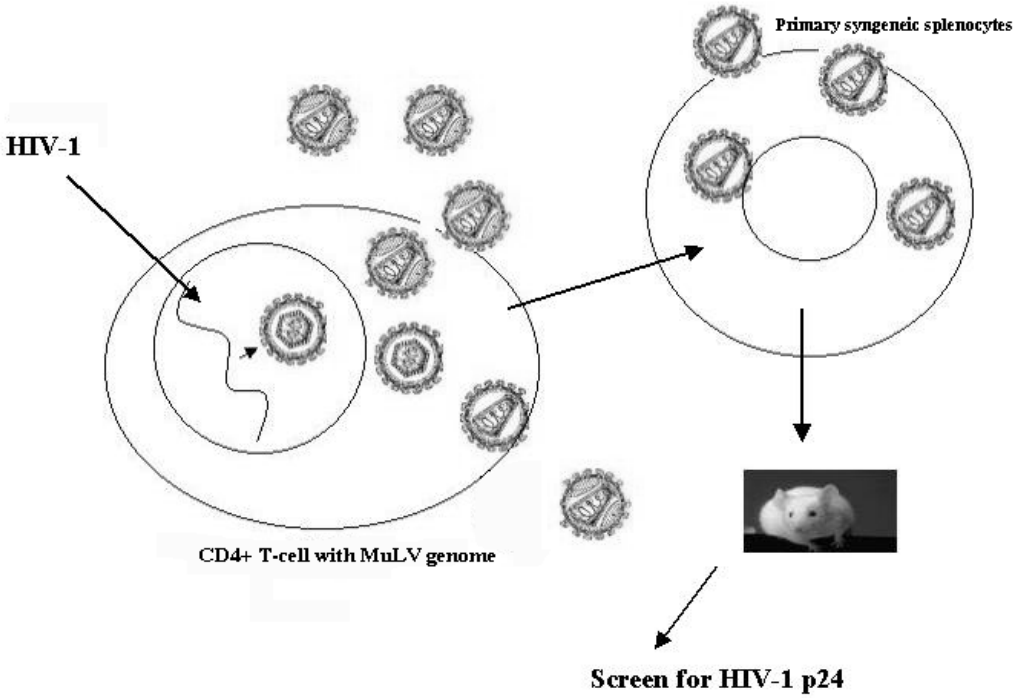

Figure 8. Scheme of the HIV-1/MuLV challenge model.

HIV-1 is used to superinfect CD4+ T-cells (CEM 1B cell lines) that are stably transfected with the whole genome of murine leukemia virus (MuLV). A mixture of different virus particles will be harvested from the cell cultures. The virus suspension is then used to infect primary splenocytes from naive mice. Only particles carrying the MuLV envelope will be able to infect these murine splenocytes. The splenocytes are transplanted intraperitoneally into immunized and control mice. Ten to 14 days after transplantation, the mice are sacrificed and the peritoneal cells are collected for coculturing with HIV-1 permissive cells. Supernatants are then assayed for presence of infectious virus.

Following immunization, we challenged the mice with the HIV-1/MuLV infected splenocytes. The groups that had received Nef protein with CpG ODN with or without MVA*nef* as boost displayed the lowest p24 values (figure 9). A statistical difference in p24 values at day 21 was seen between the Nef protein CpG ODN group and the saline or Nef protein group. Seven out of ten mice in the Nef protein CpG ODN group cleared the HIV-1 infected cells.

Nef specific IFN-γ secretion appeared to be the variable that appeared to be most related to viral clearance. All protected animals had more than 50 IFN-γ specific spot-forming cells per million spleen cells. Only low antibody responses were induced in our study, indicating that in our model, cellular responses seemed to be most important. This argues for that our model mimics the situation when the primary infection occurs through virus-infected cells. In addition, Nef specific immune responses were able to clear virus infected cells on their own. By combining several

antigens, it should be feasible to induce a broader and more potent defense against HIV-1. The combination of an HIV-1 protein with the potent CpG ODN was more efficient in inducing strong responses in our study than using *nef* DNA and boosting with MVA or MVA*nef*.

A study by Amara *et al.* (8) showed the efficacy of priming with a multicomponent DNA immunogen and boosting with a recombinant MVA vector for inducing responses capable of containing a highly pathogenic immunodeficiency virus challenge. We used only one gene in our study, which may explain why priming with DNA and boosting with MVA*nef* was not so successful here. Other recombinant vectors, such as recombinant Semliki Forest virus expressing HIV-1 genes have previously been shown to induce HIV-1 Tat and Rev specific immune responses in monkeys (181).

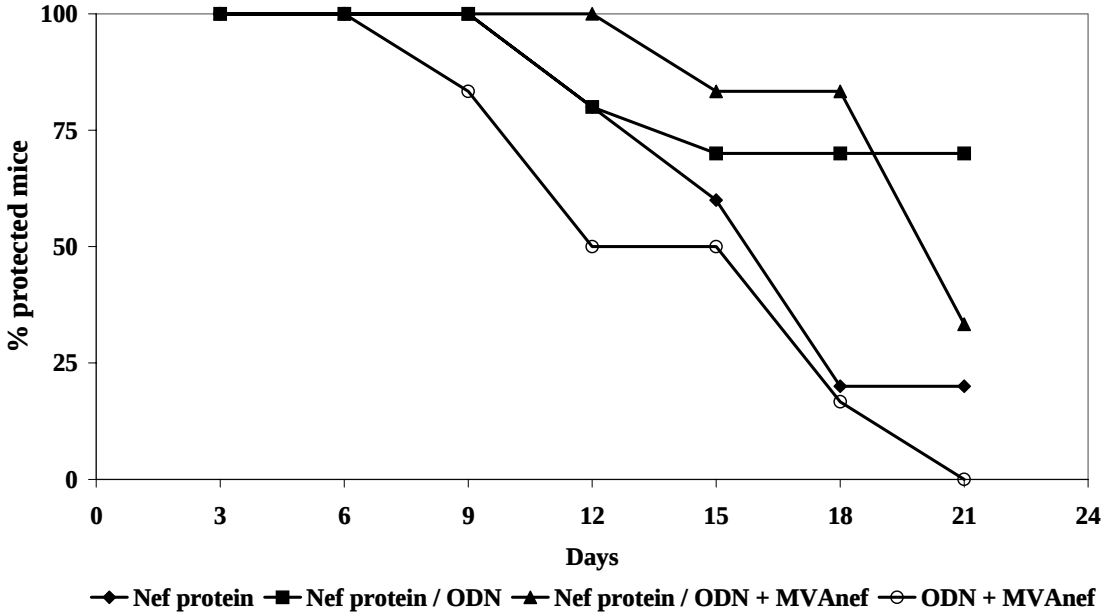

Figure 9. Clearance of viable HIV-1 replication after Nef immunizations.

Peritoneal cells from mice challenged with HIV-1/MuLV were co-cultured with HIV-1 permissive cells for 21 days. Protection against virus replication is shown over time as the percentage of mice that were able to clear viable HIV-1 replication. The groups that were immunized with recombinant Nef protein mixed with CpG oligodeoxyribonucleic acid (ODN) with or without MVA*nef* as a booster immunization, were able to partly clear HIV-1 infected cells. Protection against viable virus replication is shown over time as percentage of mice that were able to clear viable HIV-1 replication.

3.5 A NOVEL POTENT STRATEGY FOR INDUCTION OF IMMUNITY TO HIV-1 REVERSE TRANSCRIPTASE IN PRIMATES (PAPER V)

The HIV-1 RT gene or the *p 1* gene product has previously been shown to induce humoral and cellular immune responses in mice (117), rabbits (116) and primates

(41). Strong CTL epitopes are found within this viral enzyme, which is targeted by several HIV-1 drugs by non-nucleoside or nucleoside analogues (168). Drug-induced mutations are frequent, and some of them have been associated with failure of CTL to recognize the RT as a target, while others seem to increase the frequency of recognition. Antibodies to RT have also been shown to inhibit the enzymatic activity of RT and have been associated with asymptomatic infection.

In paper IV, we showed that immune responses induced by Nef protein mixed with CpG ODN targeting the Nef protein of HIV were efficient in inducing strong cellular immune responses and cleared HIV-1 infected cells. To develop this strategy and to induce strong RT specific immune responses, combinations of RT DNA and RT protein were evaluated in monkeys.

CpG ODN has previously been shown to induce strong cellular responses in macaques (96). In addition, CpG ODN induced activation of B cells and NK cells with 10 fold increases in antibody titers in monkeys when they were administered as an adjuvant to hepatitis B antigen (97). Another study showed that a primate specific CpG ODN (ODN 2006) improved protection against *Leishmania maj r* in monkeys when coadministered with a heat-inactivated *Leishmania* vaccine (253).

We used the RT protein mixed with or without CpG ODN 2006 and compared to a group of monkeys that had been primed three times with the *RT* DNA. However, as we were only able to use 4 monkeys, these results are very preliminary.

The cellular immune responses were evaluated by RT specific IFN-γ secretion by stimulating PBMC with a library of 15-mer peptides covering the amino acids 151-282 of the RT protein as measured by the ELIspot assay. The strongest IFN-γ responses in PBMC from macaque blood were induced by the *RT* gene prime followed by RT protein with CpG ODN boost. The DNA prime is likely to induce RT specific memory cells that are expanded by the RT protein CpG boost, arguing for the necessity of both the prime and boost in this experimental setting. The CpG ODN component was also an essential complement to the RT protein. Low responses were seen to the RT protein compared to responses to the RT peptide pool, arguing for a CD8+ cellular response rather than a CD4+ response.

In conclusion, the gene prime followed by a boost of protein mixed with CpG-ODN induced potent humoral and cellular immune responses in immunized macaques.

3.6 TOPICAL ADMINISTRATION OF IMIQUIMOD ENHANCES CELLULAR IMMUNE RESPONSES INDUCED BY HIV-1 DNA VACCINATION (PAPER VI)

DNA vaccination alone is capable of inducing memory responses to the encoded antigens but is poorer at expanding these responses *in viv* . To potentiate the DNA vaccine, several investigators have used different cytokine and costimulatory genes (92). Other agents, such as GM-CSF, recruit and facilitate the maturation of DC at the site of immunization and stimulate both cellular and humoral responses (50, 212, 257).

Imiquimod has been shown to stimulate primarily IFN-α secretion and IL-12 when used for topical treatment of human papilloma virus induced genital warts (169). Imiquimod stimulation functions through TLR 7 signaling (98) and subsequent activation of NFκB activation, in a manner similar to CpG ODN, which signals through the TLR 9 pathway. Results from the use of CpG ODN with DNA vaccines have been contradictory, indicating both enhancement and decreases of immune responses.

We aimed to evaluate an imidazoquinoline compound called imiquimod as a possible adjuvant for DNA vaccination using three HIV-1 genes in mice. We used the *nef*, *gag* and a mutated version of the *RT* gene to induce responses to both an early regulatory gene and two structural genes in order to induce a broad specific response. The *nef* gene was administered four times by gene gun immunization and the *gag* and *RTmut* genes were administered intramuscularly with the third and fourth *nef* immunizations. The efficacy of imiquimod was related to that of recombinant GM-CSF, which was used as a positive control. The responses were evaluated using an experimental HIV-1/MuLV challenge in mice.

IFN-γ secretion following immunizations was evaluated by stimulating pools of the peripheral blood lymphocytes from all animals in each group. IFN-γ secretion following DNA vaccination in the groups receiving either imiquimod or GM-CSF was consistent and comparable between the two groups when the cells were stimulated by the three antigens. Comparable Nef specific IL-2 secretion was also seen in the two groups. Both IFN-γ and IL-2 responses were measured using cytokine specific ELIspot assays with peptide antigens.

Immune responses after the HIV-1/MuLV challenge were measured using antigen specific proliferative assays, and by IFN-γ, IL-2 and IL-4 secretion. Broader IFN-γ and IL-2 specific responses were seen after challenge in the group that had received imiquimod as an adjuvant compared to responses in the group that received GM-CSF as adjuvant. The group that had received GM-CSF as adjuvant induced a broader IL-4 secretion to both Gag and RT antigens, while the imiquimod group only responded with IL-4 to Gag peptides. The group that was immunized by DNA alone failed to stimulate IL-4 production.

As with the IL-4 responses, the humoral response was broader in the group that received GM-CSF as compared to the group that received imiquimod. Responses were measured with antigen specific ELISA on serum from individual mice before, during and after immunization as well as after challenge.

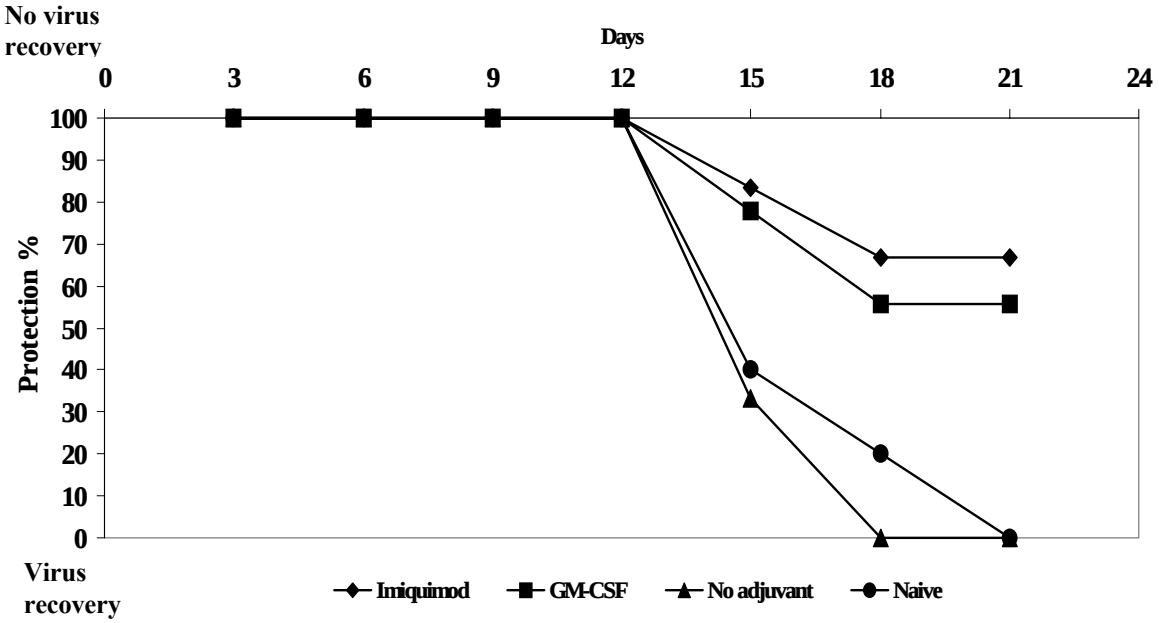

Figure 10 Clearance of viable HIV-1 replication

Clearance of viable HIV-1 replication after *nef, p37* and *RTmut* gene immunizations with or without GM-CSF (granulocyte macrophage-colony stimulating factor) or imiquimod as adjuvant. Peritoneal cells from mice challenged with HIV-1/MuLV, was co-cultured with HIV-1 permissive cells for 21 days. Protection against viable virus replication is shown over time as the percentage of mice that were able to clear HIV-1 infected cells. The groups that received imiquimod or GM-CSF, as adjuvants to DNA immunizations were able to partly clear HIV-1 infected cells.

Following challenge, a virus recovery assay as described earlier was performed on a mixture of cells from the intraperitoneal cavity and HIV-1 permissive Jurkat cells. Supernatants were collected over a period of 21 days. After 21 days of co-culturing, virus could be recovered in samples from all individual mice in the naive control

group and in the DNA immunized group that did not receive adjuvant (figure 10). In the mice that received imiquimod as an adjuvant to DNA immunization, 67% cleared the HIV-1 infected cells. In the GM-CSF immunized mice, 56% cleared the viable HIV-1.

In conclusion, the immune modulator imiquimod seems to be a potent adjuvant for cellular immune responses with DNA vaccination, comparable to the effect of GM-CSF. GM-CSF induces both humoral and cellular immune responses while imiquimod seems to be better at enhancing cellular immune responses. Imiquimod has the advantages of being easy to administer and of already being used in the clinic for the treatment of genital warts.

4 GENERAL CONCLUSIONS

In this thesis, DNA vaccines directed against regulatory and structural genes of HIV-1 were evaluated for their immunogenicity and different vaccination schedules were evaluated for enhancing the induced immune responses.

The first aim was to evaluate the efficacy of different regulatory gene isolates for expression or biological activity compared to laboratory strain derived genes. Laboratory strain derived genes expressed higher levels of protein than patient-derived genes. The assays used for Tat and Rev expression studies are indirect and measure biological activity rather than direct protein expression. By developing assays to directly measure protein expression of the Tat and Rev protein, it might be possible to detect such quantitative differences. The *nef* genes were evaluated for their immunogenicity and similar patterns of immune responses were seen between the laboratory and primary isolates. We decided that for further studies, we would use the laboratory strain derived genes since the Tat and Rev proteins expressed from those genes were more efficient in their respective biological activities.

The second aim was to evaluate combination immunizations using three plasmids encoding the respective regulatory gene of HIV-1 in mice and humans. The advantage of using multivalent vaccines rather than single gene immunogens is the broader immune response obtained. For immunization against HIV-1, it is very likely that single gene immunization will not induce protective immunity and it will be necessary to combine regulatory, enzymatic and structural genes, as was shown in a macaque model (8). When using several plasmids in a multivalent vaccine, it will be very important to consider the synergistic or antagonistic effects of combining the particular genes. In mice, both Rev- and Nef-specific immune responses decreased in magnitude when the genes were combined with the *tat* gene. This antagonistic effect might be overcome by adjusting the dose of the respective gene or by separating the immunizations and even the sites of immunization. The combination of structural and regulatory genes will result in a broad specific immune response, targeting different time-points in the viral life cycle. However, combining different genes as components in a multivalent vaccine is likely to result in different synergistic or antagonistic effects, depending on which genes are combined and from which pathogen the genes are derived.

When evaluating the effect of combining the three regulatory genes in humans, we observed an increase in immunological memory and an increase in the basic CTL level. This may be due to additional bystander stimulation induced by the CpG motifs present in the plasmid backbone (138). The effect of these CpG motifs is most likely additative, since more DNA resulted in higher specific reactivity as well as higher background reactivity.

When the plasmids were combined in a vaccine, we observed that the cytotoxic activity was more efficient against targets presenting more than one specific peptide. It appears that the combination of plasmids can induce CTL responses to at least two of the individual components in those patients. By adjusting the dose of a specific plasmid in a mixture, it should be possible to induce CTL responses against all components of the mixture.

The third aim was to evaluate different immunization regimens to enhance HIV-1 specific responses in mice. The Nef protein was studied more intensely since the best responses were seen to this protein. To enhance the immune responses induced with the *nef* gene alone, different prime-boost regimens were evaluated for their capability to induce Nef-specific humoral and cellular responses. The immune responses were evaluated using an HIV-1/MuLV challenge model, believed to mimic the primary infection of cell-bound HIV-1 (232). The combination of *nef* DNA and MVA*nef* conferred a certain degree of resistance from challenge with HIV-1/MuLV, as did the combination of recombinant Nef protein mixed with CpG ODN with or without a booster immunization with MVA*nef*. A broader and more efficient response to Nef after challenge with the HIV-1/MuLV infected cells, was apparent in the groups of mice that had received the Nef protein mixed with CpG ODN, compared to mice that received *nef* DNA followed by MVA*nef*. In future studies, the dose of the *nef* gene to optimize responses could be tested as well as different vector systems, such as Semliki Forest virus based vectors.

To develop these findings, RT specific responses induced by priming with *RT* DNA and boosting with either RT protein alone or mixed with CpG ODN or nothing were evaluated in cynomolgus macaques. The most potent cellular responses were induced by the immunization with the RT DNA followed by RT protein mixed with CpG ODN. These studies were performed in a small number of animals and should be evaluated in larger groups of macaques to see eventual significant differences between the groups.

The last aim of this thesis was to evaluate if the compound imiquimod could enhance cellular immune responses induced by HIV-1 DNA immunogens. Again, the responses induced were evaluated using the experimental HIV-1/MuLV model system. Three genes were used to evaluate the effect of imiquimod: *nef, p37* (*p17* and *p24* genes) and a mutated version of the *RT* gene. As expected, GM-CSF potentiated both humoral and cellular responses to the vaccines, while imiquimod potentiated the induced cellular immune responses. In this study, the *nef* gene was administered by gene gun immunization to enhance humoral responses to Nef. However, only low Nef responses could be detected and only in the group was potentiated with the GM-CSF protein.

In future studies, it would be interesting to combine priming immunizations using regulatory and structural DNA immunogens potentiated by imiquimod and/or GM-CSF with booster immunizations using recombinant proteins mixed with CpG ODN or recombinant viral vectors. Further studies could be made to evaluate different immunization routes such as intranasal, intramuscular and intradermal to evaluate the responses for protection against infection.

5 ACKNOWLEDGEMENTS

I wish to express my deepest gratitude to everyone involved in the work with this thesis, in particular:

Britta Wahren, the greatest of all supervisors, for scientific discussions, support and all your time for me during these years together.

Jorma Hinkula, "helt otroligt", the greatest co-supervisor, for always being there early in the mornings, at weekends and whenever I needed help with mice experiments, for all your time, knowledge and support. ***Nils Carlin*** and ***Stefan Schwartz***, my "company" and "early-days" co-supervisors for sharing your knowledge and time.

My boys: ***Erik Rollman***, ***Karl Ljungberg***, ***Dan Sjöstrand***, ***Jonas Klingström*** and ***Lars Lund*** for making life inside and outside the lab fun and enjoyable.

The co-authors of my papers not already mentioned: ***Gunnel Engström*** for being there since my very first day at the department, ***Reinhold Benthin*** for all your invaluable help with my mice experiments during workdays, weekends and holidays, ***Sandra Calarota, Malin Fredriksson*** and ***Marja Isaguliants*** for great friendship and fun times, ***Eric Sandström, Khalid B. Islam, Barbro Mäkitalo***, ***Vladimir Ovod, Kai Krohn, Volker Erfle, Gerd Sutter, Birgit Kohleisen*** and ***Heather Davis*** for good collaboration.

All friends in the Wahren group and outside: ***Soo Aleman***, ***Andreas Bråve, Shirin Heidari, Anna Linda Hultström, Kicki Brus Sjölander, Joacim Elmén, Dominique Buteux, Lars Eriksson***, ***Andreas Mörner, Peter Lundholm***, ***Mikael Levi***, ***Lotta Leandersson***, ***Åsa Björndal***, ***Cristina de Carvalho***, ***Lisen Arnheim, Angelo De Milito, Karin Wilbe***, ***Lottie Schloss***, ***Eleonor Brandin***, ***Malin Enbom, Jonas Hardestam, Camilla Kolmskog (now Mittelholzer), Margareta Benthin, Åke Lundkvist*** and ***Elisabeth Gustafsson***.

All other friends at ***MTC*** and the ***Department of Virology*** at SMI.

The people behind the Ph.D. program in biotechnology with an industrial focus (FFB), ***Agneta Mode***, ***Pia Hartzell*** and ***John Skår***, for support during these years.

My ***family-in-law.***

My two grandmothers ***Britta*** and ***Stina***

My parents ***Lars*** and ***Gunilla***, my sister ***Jessica***, my brother-in-law ***Andreas*** and the newest member of the Kjerrström family, my lovely nephew ***Eskil***, for love, support and always believing in me.

Bartek, words are not enough.

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