

Thesis for doctoral degree (Ph.D.)
2009

HIV-1 IMMUNE RESPONSES INDUCED BY NATURAL INFECTION OR IMMUNISATION



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“We must accept finite disappointment, but we must never lose infinite hope.”
Martin Luther King Jr.

ABSTRACT

The development of an effective HIV-1 vaccine must be considered as one of today's greatest biomedical goals and challenges. The nature of the HIV-1 virus, characterised by its tropism for the CD4+ T cells of the immune system and its integration into the host genome, together with the vast viral variability, allows the HIV-1 virus to establish a latent infection and evade the host defence mechanisms.

In our endeavour to develop a genetic HIV-vaccine that elicits broad and robust immune responses to several virus variants, we have demonstrated major cross-clade Gag responses in patients infected with different subtypes of HIV-1. This indicates that genetic vaccines encoding Gag from a few strains may act to induce immunity to broad ranges of HIV-1 clades. We also found that HIV-1 infection results in release of intracellular perforin into the serum of HIV infected individuals and in SIV/SHIV infected monkeys. As perforin secretion/production has been shown to be associated with control of HIV-1 infection, the aberrant perforin release may be a result of a yet unknown viral immune escape mechanism.

Individuals enrolled in two therapeutic and one prophylactic HIV-1 vaccine trial were analysed for their vaccine-specific immune responses. Analyses of the therapeutic vaccine samples revealed that: 1) Multiple injections with recombinant HIV-1 glycoprotein 160 induces an increased central memory T cell population, which can be associated with increased antigen-specific cellular immune responses in treatment naïve HIV-1 infected individuals. 2) Topical DNA immunisation, combined with repeated treatment interruptions in patients on successful antiretroviral therapy, induced novel HIV-1 specific cellular immune responses. Such treatment did permit extended drug-free periods for all participants. Moreover, the viral load set points were significantly lower after the treatment protocol than before initial onset of any antiretroviral treatment. However, the decreased viral load set points were not related to the HIV-1 immunisation.

In the prophylactic study, HIV-1 DNA followed by a recombinant vaccinia virus vector boost induced strong and broad HIV-1 specific cellular immune responses. In healthy individuals, we found that previous immunity to vaccinia only moderately reduced the HIV-specific immune responses when vaccinia was used as a vector for HIV genes. This finding suggests that vaccinia-based immunogens can be used despite the presence of pre-existing immunity to this vector.

Immunisation of HIV-1 infected individuals is difficult, as HIV-1 infection leads to a dysfunctional immune system. Despite efforts to induce new and improve existing immune responses by therapeutic vaccination, no clinical trial has yet been able to show long-term sustained clinical effects of immunisation. The modest and transient effects observed by us with currently available DNA vaccines highlight the need to develop immunisation strategies which combine immunisation with novel antiviral therapies.

SAMMANFATTNING PÅ SVENSKA

Över 33 miljoner människor världen över lever med Humant immunbrist virus typ 1 (HIV-1) idag. HIV-1 kännetecknas av att viruset infekterar immunförsvarets T-celler och därefter integrerar sin egen arvsmassa i värdcellens DNA. Viruset är mycket variabelt vilket gör att det kan undgå kroppens försvarsmekanismer och dessutom upprättar det en latent infektion. Effektiva läkemedel för att behandla HIV-infektion finns, men man har ännu inte lyckats ta fram något vaccin mot viruset, och något annat sätt att bota infektionen finns inte heller. Utvecklingen av ett effektivt vaccin mot HIV-1 måste därför betraktas som en av de största medicinska utmaningarna i vår tid.

I vårt arbete med att utveckla ett genetiskt HIV-vaccin som kan stimulera ett immunsvär mot flera varianter av HIV-1, har vi visat att det finns en betydande immunologisk korsreaktivitet i de cellulära immunsvaren mot olika subtyper av HIV-1. Detta tyder på att genetiska vacciner som består av delar av virus från några få stammar av HIV-1 skulle kunna användas för att stimulera immunsvär mot många subtyper av HIV-virus. Vi har också upptäckt att HIV-1-infektionen leder till utsöndring av perforin i serum hos HIV-infekterade individer. Samma observation gjordes även i blodprover från apor infekterade med ett närbesläktat virus, SIV. Proteinet perforin utsöndras aktivt från immunförsvarsceller när virus infekterar oss och proteinet har till uppgift att döda virusinfekterade celler. En förhöjd perforinutsöndring kan antingen tyda på ökad avdödning av virusinfekterade celler eller möjligen på en ännu okänd mekanism som orsakar perforinutsöndring vid virusinfektion.

Antiviral behandling av HIV-infektion är livslång och för att behandlingen ska fungera effektivt måste läkemedel intas dagligen. Om immunförsvaret mot HIV-1 skulle kunna förstärkas genom specifik immunisering i samband med/under framgångsrik HIV-medicinering skulle behandlingsuppehåll kunna göras och risken för biverkningar och resistensutveckling skulle därmed minska. Vi har analyserat prover från två vaccinstudier där HIV-1 infekterade individer immuniserats med två olika typer av HIV-vaccin. Vi fann att immunisering med höljeproteinet från HIV-1 gav upphov till en ökad mängd minnes-T-celler hos de vaccinerade individerna. Detta kan ha bidragit till positiva effekter hos dessa patienter då andra studier har visat att förekomsten av HIV-specifika minnes-T-celler korrelerar med en bättre kontroll av HIV-infektionen. I den andra studien såg vi att immunisering med ett DNA-vaccin mot HIV-1 i kombination med upprepade avbrott i antiviralbehandlingen, gav upphov till nya HIV-specifika T-cellsvar. Virusnivåerna hos patienterna i studien var lägre efter immuniseringarna och behandlingsavbrotten än de var innan de första gången började behandlas. Vi kunde dock inte direkt relatera dessa resultat till immuniseringen, då placebogruppen hade en likvärdig minskning i virusnivåer jämfört med vaccingruppen.

I en tredje studie immuniserades friska (HIV-1 negativa) individer. Denna studie visade att vaccinering med ett DNA-vaccin mot HIV-1, följt av en dos med rekombinant vaccinia virus innehållande HIV-gener, inducerade ett starkt HIV-1-specifikt immunsvär. Vi fann att individer som tidigare blivit vaccinerade med vacciniavirus,

vilket användes som smittkoppsvaccin under 1900-talet, hade ett något sänkt HIV-specifikt immunsvår jämfört med de individer som inte vaccinerats mot smittkoppor. Resultaten visar att vacciner baserade på vacciniaviruset kan användas trots förekomst av befintliga immunsvår mot vektorn.

Vaccinering HIV-1 är svårt då infektionen genast leder till ett dysfunktionellt immunsystem och då viruset lägger vilande i kroppens egna celler, gömt från immunförsvaret. Trots att många försök gjorts, har ingen klinisk prövning ännu kunnat visa någon långsiktig och hållbar effekt av immunisering mot HIV-1. De blygsamma effekter som har observerats i den här avhandlingen belyser behovet av att utveckla nya immuniseringsstrategier som kombinerar vaccination med antiviral behandling.

LIST OF PUBLICATIONS

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

- I. Gudmundsdotter L, Bernasconi D, Hejdeman B, Sandström E, Alaeus A, Lidman K, Ensoli B, Wahren B and Buttò S. *Cross-clade immune responses to Gag p24 in patients infected with different HIV-1 subtypes and correlation with HLA class I and II alleles*. *Vaccine*. 2008 Sep 19;26(40):5182-7
- II. Klingström J, Gudmundsdotter L, Zuber B, Hinkula J, Mörner A, Wahren B and Rollman E. *Elevated levels of serum perforin in chronic HIV-1 and acute SIV/SHIV infection*. *AIDS* 2006 Jan 2;20(1):125-7
- III. Gudmundsdotter L, Boström AC, Burton C, Rosignoli G, Sandström E, Hejdeman B, Wahren B, Imami N and Gotch F. *Long-term increase of CD4(+) central memory cells in HIV-1-infected individuals by therapeutic HIV-1 rgp160 immunization*. *Vaccine*. 2008 Sep 19;26(40):5107-10
- IV. Gudmundsdotter L, Wahren B, Hallermalm K, Rosignoli G, Edbäck U, Boberg A, Petz I, Ekroth S, Bernasconi D, Butto S, Gaines H, Imami N, Gotch F, Lori F, Lisiewicz J, Sandström E and Hejdeman B. *Therapeutic DNA vaccination against HIV-1 during highly active antiretroviral therapy followed by repeated treatment interruptions*. Manuscript
- V. Gudmundsdotter L, Nilsson C, Brave A, Hejdeman B, Earl P, Moss B, Robb M, Cox J, Michael N, Marovich M, Biberfeld G, Sandström E and Wahren B. *Recombinant Modified Vaccinia Ankara (MVA) effectively boosts DNA-primed HIV-specific immune responses in humans despite pre-existing vaccinia immunity*. Submitted

CONTENTS

1	Aims	13
2	The Human Immunodeficiency Virus	14
2.1	HIV-1 Structure and Replication	14
2.2	Course of HIV-1 infection.....	16
2.3	Genetic variability and Implications for Vaccine Design.....	18
3	Non-human primate models in HIV-1 Vaccine development	22
4	Immunology.....	24
4.1	Innate immunity.....	24
4.2	Adaptive immunity	25
4.2.1	Cellular immune response	25
4.2.2	Signalling through the T cell receptor.....	26
4.2.3	HLA and immune responses	27
4.2.4	Differentiation of memory T cell subsets	27
4.2.5	B cells and induction of humoral immune responses	28
4.2.6	Immune correlates of protection against HIV-1	29
4.2.7	Host genetic factors and control of HIV- infection	30
4.2.8	The role of perforin for cytotoxicity	30
4.2.9	Perforin and HIV-1 infection	31
5	Current HIV-1 Treatment.....	33
5.1	Prolonged treatment free periods.....	34
5.1.1	Pre- and post-exposure prophylaxis	34
5.2	Prevention of HIV-1 transmission	34
6	Immunisation.....	36
6.1	Antigen presentation following immunisation.....	36
6.2	Therapeutic HIV-1 immunisation.....	37
6.3	Live attenuated vaccines	38
6.4	Inactivated vaccines.....	38
6.5	Subunit vaccines	39
6.5.1	Recombinant proteins	39
6.5.2	Peptides	41
6.5.3	Dendritic cell based vaccines	42
6.6	DNA immunisation.....	42
6.7	Enhancing the potency of DNA immunisation	43
6.7.1	Adjuvants	43
6.7.2	Delivery methods and formulation.....	44
6.8	Viral vectors	49
6.8.1	Modified Vaccinia Virus Ankara.....	49
6.8.2	Adenovirus vectors	51
6.9	Prime-boost concept in prophylactic vaccine trials.....	51
7	Implications of viral vector immunity.....	53
7.1	Antivector immunity reduces insert specific immune responses ..	53
7.1.1	Safety concerns antivector immunity.....	54
8	Concluding remarks.....	56
9	Acknowledgements	58
10	References	60

LIST OF ABBREVIATIONS

ADCC	Antibody Dependent Cytotoxicity
AIDS	Acquired Immune Deficiency Syndrome
APC	Antigen Presenting Cell
cART	Combination Anti-Retroviral Therapy
CD	Cluster of Differentiation
CMV	Cytomegalovirus
CRF	Circulating recombinant form
CTL	Cytotoxic T Lymphocyte
CTLA-4	Cytotoxic T Lymphocyte Antigen 4
DC	Dendritic Cell
DNA	Deoxyribonucleic Acid
EC	Elite controllers of HIV-1 infection
ELISA	Enzyme-Linked Immuno Sorbent Assay
ELISpot	Enzyme-Linked Immunospot Assay
Env	Envelope
ESN	Exposed seronegatives
FEC	Flu/EBV/CMV peptide pool
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
i.d.	intradermal
Ig	Immunoglobulin
i.m.	intramuscular
IL	Interleukin
LTR	Long terminal repeats
LTNP	Long-term nonprogressor
mAb	Monoclonal Antibody
MHC	Major Histocompatibility Complex
MVA	Modified Vaccinia virus Ankara
Nef	Negative factor (in HIV)
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PD-1	Programmed Death-1
PD-L	Programmed Death- Ligand
PFU	Plaque forming unit
Rev	Regulator in virion expression (in HIV)
PHA	Phytohaemagglutinin
RNA	Ribonucleic Acid
RT	Reverse Transcriptase
SHIV	Simian-Human Immunodeficiency Virus
SIV	Simian Immunodeficiency Virus

Tat	Transactivator of transcription
Th	T helper cell
T _{CM}	Central Memory T cell
TCR	T Cell Receptor
T _{EM}	Effector Memory T cell
T _{EMRA}	Terminally Differentiated Effector Memory T cell
TLR	Toll-Like receptor

1 AIMS

The specific aims of my thesis were:

- To investigate cross-clade immune responses in HIV-1 seropositive individuals infected with different clades of HIV-1 and to investigate the association of HIV-specific immune responses to the patient-specific expression of HLA class I and II alleles.
- To investigate T cell memory differentiation in HIV-1 infected individuals immunised with multiple doses of recombinant HIV-1 glycoprotein 160.
- To evaluate the clinical and immunological effects of topical DNA plasmid immunisations in combination with repeated treatment interruptions, in HIV-1 seropositive individuals.
- To evaluate the impact of antivector immunity on the HIV-specific immune responses in healthy HIV-1 seronegative volunteers immunised with HIV-1 DNA followed by a recombinant vaccinia virus vector encoding HIV-1 proteins.

2 THE HUMAN IMMUNODEFICIENCY VIRUS

The Human immunodeficiency virus type 1 (HIV-1) was isolated by French scientists led by Dr Luc Montagnier in 1983 (19). The same year, another group of researchers led by Robert Gallo, at the American National Cancer Institute, developed cell lines which were permanently and productively infected with the virus (108). It was rapidly established that a new virus causing immunodeficiency was spread among certain risk groups (177, 206, 257). The virus was established as the causative agent for acquired immune deficiency syndrome (AIDS) (226, 260). But the transmission of HIV-1 from the proposed reservoir, the common chimpanzee (*Pan troglodytes troglodytes*), probably occurred during the first part of the twentieth century (1915-41) (163). Retrospective studies of stored serum samples have revealed that the first documented HIV-1 seropositive sample was collected from a man in former Zaire in 1959 (237).

Since its discovery over 25 years ago, more than 60 million people have been infected with HIV-1, and more than 20 million have died from AIDS related disease. There are several explanations as to why HIV-1/AIDS has spread to become a world wide pandemic. The onset of AIDS is caused by a slow degeneration of the immune system as a consequence of HIV-1 infection. Thus the infection may have a silent period for many years before onset of serious symptoms. During this asymptomatic period the host is highly infectious and the virus can be spread through the exchange of body fluids. In general, the signs of primary HIV infection are non-specific, resembling influenza like symptoms. Therefore the infected individual does not know that he or she carries the HIV virus. The most common route of transmission is by sexual contact, and sexually transmitted diseases are difficult to control, even when treatment is available. HIV/AIDS disease is emotionally and politically charged and disease denial has frequently prevented rational countermeasures in many countries. For example, South African political leaders have until recently disputed the causal relationship between the HIV infection and AIDS (60). This oblivium has contributed to the critical situation in sub-Saharan Africa, where more than 25 million people live with HIV today. HIV-1 also targets people of economically productive ages which have serious economic, political and demographic consequences. These factors make it difficult to combat the infection in the developing world, despite the introduction of highly efficient antiretroviral drugs. Thus, a vaccine against HIV-1 would indisputably be the most efficient means to halt the epidemic.

2.1 HIV-1 STRUCTURE AND REPLICATION

HIV-1 belongs to the Lentivirus genus of the *Retroviridae* family. Retroviruses are small enveloped viruses that contain two copies of positive single stranded RNA molecules. HIV-1 is a spherical virus with a diameter of approximately 110 nm. The virus particle contains a conical nucleocapsid that surrounds the viral nucleic acid, as well as enzymes required for early replication events. The nucleocapsid is enclosed by the matrix which in turn is surrounded by the envelope, consisting mainly of a host cell lipid bilayer membrane, including the viral glycoprotein gp41. Trimeric gp41 is non-covalently linked to the trimeric gp120. Gp120 is the viral protein that initiates the

infection by binding to the CD4 receptors present on T cells, macrophages, dendritic cells (DC) and microglia cells in the central nervous system.

Infection begins when gp120 binds to the CD4 molecule on the target cell and undergoes subsequent conformational changes that enable the protein to bind to one of the co-receptors, mainly CCR5 or CXCR4. Subsequently, gp41-mediated fusion of the viral- and cellular membranes enables the viral capsid and genome to enter the host cell. Like all other retroviruses HIV-1 has an enzyme, reverse transcriptase (RT), which is responsible for converting ssRNA into dsDNA. Inside the host cell, the viral genome is released and the viral RNA is transcribed to DNA by RT. The RT has a less exact proof-reading mechanisms than cellular polymerases and generates more than 3 substitutions per 10^5 incorporated nucleotides (205). The introduction of mutations during transcription is one of the major mechanisms for viral diversity which in turn allows for escape from the immune system as well as from antiviral drugs.

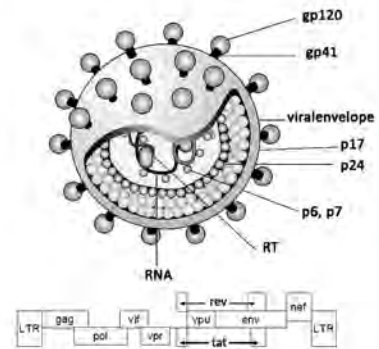


Figure 1: HIV-1 genome and virion structure.

After reverse transcription, the retroviral genome is contained in a pre-integration complex that subsequently is integrated into the host cell DNA. The pre-integration complex essentially consists of newly synthesized viral DNA, nucleocapsid protein, structural protein p6, accessory protein Vpr (described below) and viral integrase. The viral integrase facilitates integration of the viral DNA into the host cell genome forming a pro-virus. Once integrated the viral DNA remains permanently associated with the host genetic material. The integrated DNA can either remain in a latent stage (and be passed on by cell division) or be activated, transcribed into viral mRNA by cellular RNA polymerase and form new infectious particles.

Viral transcription begins with synthesis of a complete RNA copy of pro-viral DNA. The viral protein Tat (transactivating), initiates and stabilizes the elongation of viral mRNA transcripts, as it binds to tat responsive regions (TAR) in the long terminal repeats (LTRs) of the pro-viral DNA. Tat also assists in HIV-1 replication and infection; playing a key role in neurological damage (160), where it negatively affects the protein synthesis in neurons (241). The mRNA can be translated into viral proteins or be incorporated in new viral particles as genomes. The initial transcripts are spliced into mRNAs encoding the early regulatory proteins, Tat, Nef and Rev. The Tat protein made early in the infection accelerates the rate of viral RNA transcription throughout the course of infection (298, 305). The Nef (negative regulatory factor) protein can down regulate CD4 as well as MHC class I and class II molecules and thereby the virus may escape CD8+ T cell recognition (153, 316). Nef also facilitates CD4+ T cell apoptosis through membrane permeabilisation (167). Both Tat and Nef are expressed by infected cells and can be released into the extra-cellular environment and exert actions on bystander cells. The Rev (regulation of viral expression) protein binds to

Rev responsive elements and permits un-spliced mRNA to exit the nucleus into the cytoplasm. It thus enables translation of the structural proteins Env and Gag together with the viral enzymes (307).

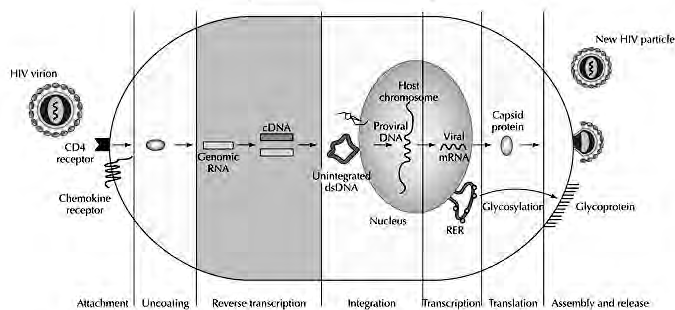


Figure 2. HIV-1 life cycle (images.md)

The final step of the HIV-1 replication cycle, assembly of new virions, begins at the plasma membrane. The envelope protein (gp160) is glycosylated and processed in the endoplasmic reticulum and the Golgi complex, where it is cleaved by cellular protease (furin) into the surface proteins gp41 and gp120. These are transported to the plasma membrane of the host cell where the transmembrane-spanning gp41 anchors the gp120 on the outside of the membrane of the infected cell. Two HIV genomic RNA molecules associates with the inner surface of the plasma membrane along with the Gag (p55) and Gag-Pol poly-protein (p160) as the virion begins to bud from the host cell. Maturation of the virus occurs during and after budding from the host cell. During this process the Gag-Pol poly-protein is cleaved by an HIV-1 encoded protease to produce the viral internal proteins p7, p9, p17, p24 and viral enzymes protease, integrase and reverse transcriptase. The various structural components are subsequently assembled to produce a mature infectious HIV virion.

Three accessory proteins Vif, Vpr and Vpu are packaged into the viral particle. The Vif (viral infectivity factor) protein is important for cell to cell transmission of virus. Moreover, it is crucial for proviral DNA synthesis and is also involved in packaging of the core proteins (122). Vif counteracts the antiviral activity of apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G (APOBEC3G), a cell protein that contributes to resistance to retroviral infection, by deamination of the proviral negative DNA strand (61). The Vpr (viral protein r) protein plays an important role in regulating import of the HIV-1 pre-integration complex into the cell nucleus, and is required for viral replication in non-dividing cells such as macrophages. Vpr also induces cell cycle arrest and apoptosis in proliferating cells, which contributes to immune dysfunction (122). The Vpu (viral protein U) enhances virion release by counteracting the cellular protein tetherin that causes retention of viral particles on the cell surface (239).

2.2 COURSE OF HIV-1 INFECTION

The most common route of HIV-1 transmission is heterosexual intercourse. The genital (or rectal) mucosa is often the portal of virus entry. Transmission can also

occur by transfer of contaminated blood through needle stick injuries or the sharing of needles between intravenous drug users or from mother to child during pregnancy, birth or breast feeding. HIV-1 gains entry to the host in a variety of ways depending on the integrity of the mucosal surface and the frequency and activation state of locally susceptible cells. The risk of acquiring HIV-1 through heterosexual intercourse is however rather low. By studying discordant couples and continuously testing the non infected partner for their HIV status, it has been calculated that infection will take place approximately once in every 100 heterosexual contacts (125). But if the integrity of the host epithelial surface is altered due to abrasions or sores, HIV-1 may have direct access to primary target cells. Hence, the risk of obtaining HIV-1 depends on the type of sexual activity and the presence of genital infections. The risk is also dependent on the viral load of the infected partner, the higher viral load the greater risk of infection (219, 337). It was previously believed that an infected person always remains infectious, but with effective cART for an extended time it has been suggested that such persons do not sexually transmit virus, this is however debated (169).

HIV-1 infects CD4⁺ cells and primary transmission of HIV is mainly caused by CCR5 tropic virus strains that use the CCR5 as a co-receptor for entry. In the case of an intact mucosal epithelial layer, antigen presenting cells (APC) in the mucosa, dendritic cells and Langerhans cells (LC) trap HIV-1 (74, 156). This is induced by binding of the gp120 subunit to a C-type lectin, such as DC-SIGN. This binding is however not sufficient to trigger fusion of the virus and the cellular membrane (114). Rather the virus becomes internalized into endosomes in the DC, and is carried to the draining lymph nodes, where subsequent transmission of virus to T cells occurs. In addition, DCs can also be directly infected as many of them express both CD4 and CCR5. The acute phase of HIV-1 infection is characterized by a massive drop in peripheral blood CD4⁺ T cell numbers and high plasma viral load (129, 209, 289). This aggressive replication is especially severe in gut mucosal associated lymphoid tissue, where almost 80% of memory CD4⁺ T cells are depleted within the first few days of infection (42). HIV-1 preferentially infects activated CD4⁺ T cells, leading to destruction of many activated HIV-1-specific CD4⁺ T cells including uninfected bystander CD4⁺ T cells (83). The depletion of memory T cells is devastating for the hosts' immune system since the majority of memory CD4⁺ T cells are found in lymphoid tissues in the gut. Within a few weeks of initial HIV-1 infection, most infected persons experience symptoms that resemble influenza or mononucleosis, with fever, headache, sore throat, diarrhoea, muscle ache and swollen lymph glands. The development of an HIV-1-specific immune response, including activation of CD8⁺ T cells and development of anti-HIV antibodies, occurs within a few weeks after primary infection (325). In most cases, this results in suppression of viral replication and an increase in the number of CD4⁺ T cells. The viral load set point, defined as the stable level of virus in the blood after the initial peak viremia, serves as a good indicator of the rate of disease progression (94). The higher the set point is the more rapid progression to disease. The latent, and in most cases asymptomatic phase, of HIV-1 infection may last for several years.

Figure 3 illustrates the T cell and viral dynamics, and immune activation during HIV-1 infection (adapted from (128)). The massive loss of memory cells in the gastrointestinal mucosa, occurring in the acute phase of infection, together with the already compromised integrity of the mucosal barrier, favours the translocation of

microbial products into the systemic circulation (41). Failure to clear bacterial antigens effectively results in both opportunistic infections and chronic immune activation of both CD4+ and CD8+ T cells because immune responses are constantly triggered. The excessive immune activation later leads to T cell exhaustion and apoptosis, eventually tipping the balance between T cell turnover and increased viral replication, such that the immune reconstitution cannot keep pace with the viral infection. T cell exhaustion is probably the main reason for ineffective control of viral replication during HIV-1 infection. This further weakens the potency and effectiveness of host adaptive immune responses, resulting in failure to control the viral replication and accelerates the disease progression to AIDS.

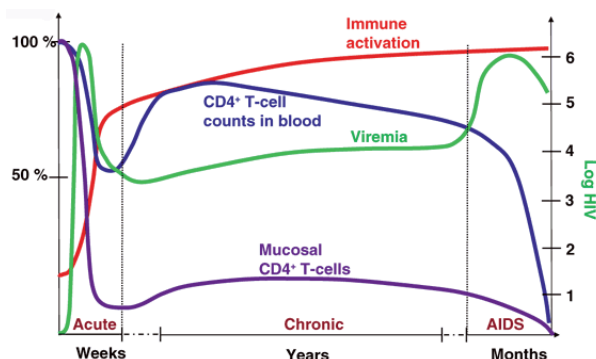


Figure 3. The dynamics of plasma HIV-1 viral load, peripheral blood and mucosal CD4+ T cell counts and immune activation, in the timeline of HIV-1 disease progression (adapted from (128)).

The onset of AIDS usually occur when CD4+ T cell blood counts reach below 200 cells/mm³ and is associated with the emergence of opportunistic infections (218). During late stage infection, as the proportion of CCR5+ CD4+ T cells declines, there is usually a shift to CXCR4 tropism and predominant targeting of CD4+ T cells (30). This in turn can lead to a more rapid progression of infection (as reviewed in (254)).

2.3 GENETIC VARIABILITY AND IMPLICATIONS FOR VACCINE DESIGN

One of the major characteristics of HIV-1 is the vast genetic variability. As described in 1.1, the introduction of mutations during transcription is one of the major mechanisms for viral diversity. Together with a very high rate of viral production (approximately 10¹¹ particles/day), the incorporation of mutations gives rise to a number of virus particles (quasi-species) with different properties that allow for escape from the immune system and antiviral drugs.

HIV-1 variants can be divided into genetic groups based on nucleotide sequences, subtypes (clades) and circulating recombinant forms (CRFs). Three HIV-1 groups have been identified so far: M (main), O (outlier) and N (non-M non-O). Group O and N viruses occur at low frequencies mainly in regions of Central Africa. Group M viruses are spread world wide and are divided into nine subtypes: A, B, C, D, F, G, H, J, and K (314). Co-infection of patients with distinct subtypes can give rise to circulating recombinant forms. The subtypes and CRFs are present to varying extents in different geographical regions in the world.

The diversity of the virus and the constant appearance of novel recombinant viruses represent a significant challenge to HIV-1 vaccine design. It is important to point out

that the genotype (i.e. subtype) of HIV-1 is not the same as the immunotype. No evidence exists to support the idea that immune responses directed against HIV-1 from a certain subtype would confer protection against all other strains of virus within the same subtype. This can be illustrated by the fact that super-infections can occur with two genetically distinct viruses from the same subtype (5). For an HIV-1 vaccine, the envelope glycoproteins are the only viral proteins that are present on the virus surface and thus possible to target with directly neutralising antibodies. They are also the most variable parts of the virus and amino acid sequences of Env can differ up to 20% within a particular clade and over 35% between clades (315).

To evaluate the importance of cellular immune responses to HIV-1 which may target more conserved internal genes, we analysed env and gag cross-clade (subtype non-specific) immune responses in 60 HIV-1 patients infected with different subtypes of HIV-1. These included 7 patients with subtype A infection, 21 with subtype B infection, 19 with subtype C infection, 7 with subtype D infection, 1 with subtype G infection, 4 with subtype CRF_01AE infection and 1 with subtype CRF_02AG infection. The cellular immune response was analysed by IFN- γ ELISpot assay using Env and Gag peptide pools from subtype A, B, C. The results from this study have been published in (130) and in paper I.

The analysis revealed that the T-cell response in this cohort of chronically HIV-infected individuals was higher to gag than env. The overall reactivity to the Env was low and no major cross-clade reactivity was detected. In contrast, we detected a strong cross-reactivity between Gag p24 A and B peptide pools. In total 47 out of 60 (78%) patients responded by interferon gamma secretion to peptides of Gag p24 subtypes A or B and cross-reactivity occurred in 87% of the Gag responders (Figure 4). The results indicate a surprisingly high cross reactivity within the Gag p24 region, considering that we studies cellular immune responses in patients infected with several different subtypes of HIV-1.

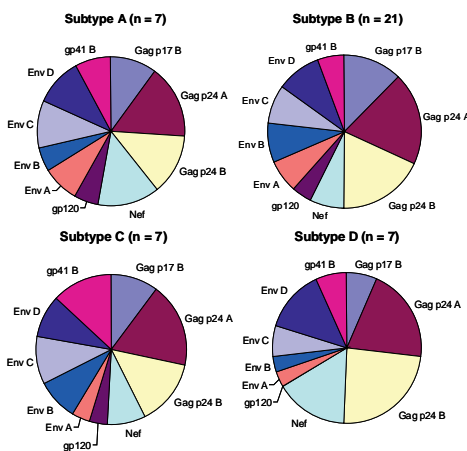


Figure 4: Relative contribution of response to HIV-1 proteins from different subtypes. Each pie chart shows responses from patients infected with a certain subtype as the mean response of the individual protein subunits compared to the total magnitude of response. (gp120 and Nef peptides are derived from subtype B)

Recognition of immunodominant peptides within the Gag p24 region of subtypes A, B and C was assessed, by a peptide matrix approach (2, 130). This revealed a broad cross reactivity between the three different subtypes (Figure 5 and paper I). The most important finding was that lymphocytes from different patients cross-reacted to peptides representing different subtypes and that the reactivity was distributed along all the p24 protein. Peptides to which the majority of patients cross-reacted were located in the region between peptides 8 and 15 corresponding to amino acid 29-78 and peptides 19 and 35 corresponding to amino acid 84-177 (Paper I). All patients reacted to at least one peptide and the individuals with the broadest response reacted with up to six different peptides.

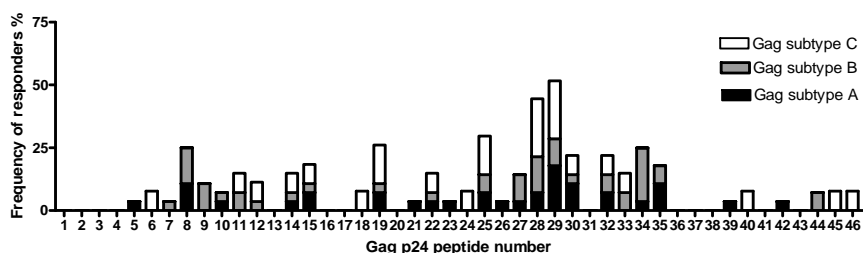


Figure 5: Identification of individual reactive HIV-1 Gag p24 peptides. The frequency of patients responding to the specific peptides from Gag A, B and C peptide pools is shown on the y-axis.

In line with our findings, other studies have demonstrated that CD8+ T cell cross-reactivity differs according to the degrees of conservation in the genes. The most conserved protein induces the highest frequency of cross-clade responses: Pol>Gag>Env (66, 112). This indicates that genes representing conserved proteins (for example Gag and Pol) of a few circulating strains may act to induce cellular immune response to those proteins for a broad range of the HIV-1 viruses circulating world wide (118, 171, 190, 270, 293).

However, as intra-subtype CD8+ T cells responses are usually stronger and more frequently detected than inter-subtype responses, it is reasonable to assume that obtaining protection by immunisation will be substantially harder with increasing divergence between vaccine antigens and circulating virus. HIV-1 vaccines that are based on immunogens from only one subtype would probably restrict the use of the vaccine to the geographical regions where the strains are predominant. Ideally, the immunogens should induce responses which are broadly reactive against multiple virus clades. It is generally agreed that the control of the HIV-1 pandemic requires the development of vaccines that efficiently protect against several genetic forms of HIV-1 (315).

There are a number of proposed strategies to tackle HIV-1 variability. Vaccines could contain a cocktail of immunogens derived from several different subtypes of HIV-1. Or they could include the most common circulating strains prevalent in a certain geographic area for which the vaccine is intended. Efforts have been made to

generate antigens based on ancestral or consensus sequences since these might have the potential for broader cross-clade responses (97, 210, 338). Immunogens can also be based on conserved regions from the virus which are found in many clades. There is however a concern that too conserved regions does not necessarily contain epitopes that are naturally recognised by the immune system.

3 NON-HUMAN PRIMATE MODELS IN HIV-1 VACCINE DEVELOPMENT

No ideal model exists that can mimic the pathogenesis of HIV-1 infection and AIDS in the human body. Nonetheless, data from animal models provide conceptual insights into immune responses elicited by investigational vaccines. Initially, chimpanzees were used as a model for studying HIV-1 infection and disease since they can be infected with HIV-1. However, chimpanzees are hardly ever used today due to ethical concerns and the huge expense of working with them. Chimpanzees in captivity mount a significantly different immune response against HIV-1 compared to humans and rarely develop AIDS (17). However, recent data suggests that SIV-infected chimpanzees in the wild have a 15-fold higher risk of death than virus-free chimpanzees, implying that SIV-infection poses nearly as great a risk to chimpanzees as HIV-1 does to humans (64).

Monkeys within the genus *Macaca*, including Rhesus, Cynomolgus and Pig-tailed macaques are currently used for evaluating vaccine candidates. As macaques cannot be infected with HIV-1, various strains of simian immunodeficiency virus (SIV) are used. SIVs are endemic to a variety of African non-human primates and the human HIV-1 is derived from the SIV variant that naturally infect chimpanzees. SIVs are non-pathogenic in the natural host, African green monkeys, but become pathogenic when transferred into a new host such as a rhesus or cynomolgus macaques (235). There are a limited number of SIV strains used for AIDS studies in macaques for example, SIVmac251 is an uncloned biological isolate comprised of many related but distinct quasispecies of virus, often described as a 'swarm', whereas SIVmac239 is a single clone derived from a SIVmac251- infected macaque. Both SIVmac251 and SIVmac239 are CCR5-tropic viruses that are broadly used since they mimic HIV-1 disease progression in humans (reviewed in (231)). Only a few vaccine candidates have demonstrated any efficacy in non-human primate challenge models using pathogenic SIV isolates (336). Several vaccine regimens including recombinant vaccinia virus vectors and canarypox virus (ALVAC) encoding SIV proteins have only induced modest levels of control over SIVmac251 replication (145, 244).

Genetically-engineered chimeric viruses consisting of SIV proteins and an HIV-1 envelope called SHIV viruses have been developed for evaluation of HIV vaccines in monkeys (179). These viruses are particularly useful when testing the efficacy of vaccines containing HIV-1 Env, aiming to elicit neutralising antibodies. Some SHIV viruses are highly pathogenic and cause a more rapid disease progression as compared to what is observed in humans. For instance, SHIV89.6P is a CXCR4 tropic virus that causes rapid loss of CD4+ T cells and immunodeficiency in macaques. Vaccinia virus vectors have been used alone or after a DNA prime to vaccinate macaques that were subsequently challenged with SHIV89.6P (204). Amara et al. used a DNA prime followed by a Modified Vaccinia virus Ankara (MVA) boost regimen to control SHIV89.6P replication, as manifested by viral load reduction and preservation of peripheral CD4+ T cells (8). The SHIV89.6P virus has been used extensively in vaccine research, but is not considered to be optimal model since it is relatively easy

to obtain protection from this virus (222, 329). A potential shortcoming of non-human primate research is that SIV and HIV-1, although similar, are different viruses, so that advances made with SIV need to be verified using HIV-1. There are important differences between SIV infected macaques and HIV-infected humans (151, 318), including virulence, pathology, genetics, protein function, infection and host response (as reviewed in (17)). While some researchers claim that the SIV/SHIV model is not adequate for evaluating HIV vaccine research (146, 317) there are others claiming that it is (231, 329). However, the failure of the STEP trial (described in chapter 5.8.2.1) was predicted by a preclinical testing of a similar regimen in SIVmac239-challenged macaques (336).

The ethical aspects, as well as the extensive cost of performing experiments in non human primates have led to the development of other animal models. These include feline immunodeficiency virus (FIV) (174), transgenic mice that contain the human co-receptors allowing viral entry (43), severe combined immune deficiency (SCID) mice reconstituted with human immune system cells or tissues (77) and mice infectable with pseudotypes of HIV-1 (32).

4 IMMUNOLOGY

4.1 INNATE IMMUNITY

The first line of defence against infection is mediated by the innate immune system. This system includes external barriers such as the skin and the mucosal surfaces, but also biologically synthesised products and cells that act in a non pathogen-specific manner against invading microbes. Cells of the innate immune system are responsible for a localized host response that acts within hours of infection. These cells also capture and transport foreign material from the peripheral surfaces of the body to the lymphoid organs and there subsequently present the foreign material to the adaptive immune system (288, 322).

Although historically the innate immune system is termed non-specific, it recognises certain pathogen-specific patterns (e.g. proteins, DNA or RNA sequences, and sugar motifs) via the different pathogen recognition receptors (PRRs) like Toll like receptors (TLRs) and the mannose receptors. The TLRs, which are the most common PRRs are expressed on various cell types and cellular compartments, and the most common response upon TLR activation is the expression of different cytokines (interferons) that in turn can help to activate the adaptive immune response (144, 216). The innate immune responses are more or less a requirement for induction of adaptive immunity. The aim of immunisation is to induce long-lived adaptive immune responses against the pathogen of interest. However, these responses are induced by signals from the innate immune system and thus the vaccine needs to stimulate innate immune responses. Traditionally, the stimulation of a strong vaccine-specific immune response is achieved by using either attenuated or killed pathogens, where viral components stimulate the innate immune responses via TLR 3, 7/8, 9, and RIG-I and MDA-5 (168). This strong initial response, caused by abundance of microbial material stimulating the immune system, is often translated into long-lived immunity against the pathogen of interest. However, with the introduction of purified vaccine antigens (e.g. recombinant proteins, peptides or genetic vaccines) it is necessary to stimulate the innate immune responses by adding adjuvants, which are often immunostimulatory compounds and will be further discussed in the Vaccine section of this thesis.

The cells of the innate immune system can directly contribute to defence against HIV-1 by secretion of antiviral soluble factors. For instance, increased levels of IFN- γ , tumour necrosis factor (TNF) and β -chemokines such as RANTES, MIP-1 α and MIP-1 β have been suggested to contribute to the resistance against HIV-1 infection in individuals who are continuously exposed to HIV-1 but remain uninfected (228, 291). The beneficial effect of the β -chemokines may be explained by their ability to bind the CCR5 receptor on cells and thereby inhibit the binding and entry of the virus (63, 283). Other anti-HIV components include proteins involved in the complement system and a variety of cytokines such as type-1 interferons. Plasmacytoid dendritic cells are the primary producer of type 1 IFN- α (98) that counter acts HIV-1 replication, by indirect inhibition of viral replication and activation of NK- and T cells (227). An intracellular protein, tripartite motif protein 5 α (TRIM-5 α) has been attributed to restrict HIV-1 replication in old world monkeys. By binding to the viral capsid, TRIM-5 α interferes

with the uncoating process, therefore preventing successful reverse transcription (306). However, HIV-1 can infect both NK cells and DCs and thereby interfere with their antiviral activities (53, 132).

4.2 ADAPTIVE IMMUNITY

The adaptive immune response is highly specific and is generally effective in combating infections. In contrast to the innate immune system, the development of an adaptive response is relatively slow. It usually takes several days or weeks to develop following encounter with a “new” pathogen. The two main arms of the adaptive immune response are the cellular and humoral arms, mediated by T and B cells, respectively. Both cell types originate from the same haematopoietic stem cells in the bone marrow. While the T cells mature in the thymus, the B cells mature in the bone marrow. During maturation, both cell types undergo extensive clonal selection during which the immune response is shaped to combat the invading pathogens.

4.2.1 Cellular immune response

The adaptive cellular immune response includes two cell types, the CD4⁺ T helper cells and the cytotoxic CD8⁺ T cells. CD4⁺ T cells play a central role in directing and regulating many processes of the adaptive immune response. They recognise peptide antigens, derived from extracellular pathogens, which are presented by human leukocyte antigen (HLA) class II molecules on professional APCs. There are several different subsets of CD4⁺ T cells, including T-helper (Th) 1, Th2, Th17 and regulatory T cells. One important function of the various T helper cells is to provide support for the other immune cells (25, 232). Importantly, T helper cells secrete cytokines and express co-stimulatory molecules to promote differentiation and maturation of B cells as discussed below. Th1 mediated immune responses have been attributed to control intracellular pathogens, whereas Th2 cells help in clearing extra cellular pathogens. The recently discovered Th17 cells have an important role in host defence against certain extracellular pathogens and can cause tissue inflammation and in some cases autoimmunity (102). The Th17 cells are preferentially lost in the gastrointestinal tract during acute HIV-1 infection (40). Regulatory T cells are essential for maintaining peripheral tolerance, preventing autoimmune diseases and limiting chronic inflammatory diseases (342).

The CD8⁺ T cells or cytotoxic T lymphocytes (CTL) are the main effectors of the cellular immune system, with the objective of eliminating infected cells. Killing of infected target cells can be mediated through a number of effector functions (284). CD8⁺ T cells recognize peptide antigens derived from intracellular pathogens presented on HLA class I molecules of the infected cell. Cytotoxic T cells have the potential to recognize and kill any infected cells that express HLA class I molecules. One way for the CTL to induce apoptosis is by granule-mediated cytotoxicity. This mechanism requires the interaction between the MHC class I molecule and the T cell receptor that results in release of cytotoxic granules from the CTL. The release of pro-apoptotic enzymes, including perforin and granzymes from the granules, induces apoptosis in the target cell (344). Induction of apoptosis in target cells can also be mediated by the CD8⁺ T cells by the engaging the Fas (CD95) molecules on the target cells. Furthermore, cytotoxic T cells can also secrete high amounts of the antiviral cytokine

IFN- γ , which can block viral replication and induce up-regulation of MHC molecules on the surface of infected cells, resulting in more efficient antigen presentation. IFN- γ can also activate macrophages, NK cells and promote isotype switching in B cells. As mentioned above, the control of virus replication during the acute phase of HIV-infection has been largely attributed to the effector functions of CD8+ T cells (6, 149).

4.2.2 Signalling through the T cell receptor

Activation of naïve T cells requires two signals, the first through the T cell receptor (TCR) and the second through co-stimulatory molecules such as CD28, CTLA-4 and PD-1. The TCR molecules recognize peptides presented in the context of MHC molecules on antigen presenting cells. The TCR and its associated CD3 and co-receptor molecules, either CD4 or CD8, form an immunological synapse together with the peptide-MHC complex (Fig 6). When a T cell recognizes a peptide-MHC complex, a signalling cascade is started and this process initiates activation of the cell.

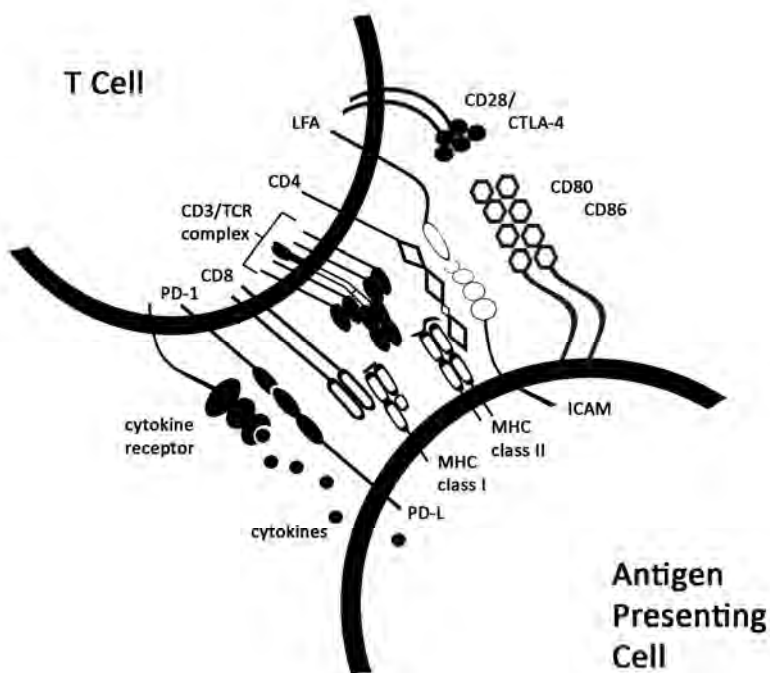


Figure 6. T cell recognition of an antigen. In the case of a CD8+ T cells, the TCR binds to a MHC class I molecule, in the case of a CD4+ T cells the TCR binds to a MHC class II molecule. This binding may be further stabilized by binding of lymphocyte function-associated antigen-1 (LFA-1) on the T cell and intracellular adhesion molecules (ICAMs) on the APC. In the presence of correct co-stimulatory molecules such as CD28, signal transduction through the TCR is modulated by the CD3 molecule. Ligation of PD-1 with PD-L, induces a intracellular inhibitory signalling pathway that inhibits TCR signalling.

Activation of naïve T cells is referred to as priming of a response while activation of a pathogen-specific memory T cells is a recall response. The type of immune response elicited by the activation, depends on a variety of factors including which cell type that presented the antigen and when and where the antigen presentation takes place (208). Without co-stimulation, TCR and CD3 binding to the peptide-MHC alone are not sufficient to activate a naïve T cell. Instead, anergy (failure to respond to antigen) or apoptosis may be induced if TCR binding to the peptide-MHC complex occurs in the absence of co-stimulatory signals (175). Such co-stimulatory signalling includes interaction between CD80 and CD86 on the antigen presenting cell with the CD28 on the T cell. CD28 is involved in the positive regulation of T cell responses. In contrast, CTLA-4 and PD-1 on the T cell are negative regulators.

4.2.3 HLA and immune responses

During chronic HIV-1 infection the magnitude of CD8+ effector T cell responses to viral antigens is determined by the combination of certain HLA types of the individual and the immunodominant epitopes that the antigen presenting cells display on their surface. HLA genes are highly polymorphic and encode for cell surface molecules that are responsible for antigen presentation to T cells. There is a high diversity within the genes encoding for HLA class I (A, B and C) and class II (DR, DQ and DP) alleles. One individual can express up to 6 different class I and class II molecules. The repertoire of expressed HLA-molecules depends on whether the genetic disposition for a certain allele is homozygous or heterozygous. The repertoire of diversity can be illustrated by the HLA class I and II typing performed on the 60 patients (paper I). The analysis distinguished 16, 18 and 17 class I HLA-A, -B, or -C alleles respectively (paper I). Several studies have described associations of particular HLA alleles with different outcomes of HIV-infection, discussed below, and a large number of viral epitopes have been described to bind to certain HLA class I molecules (24). Therefore the majority of cellular responses detected in the cohort had already been described to be associated with the HLA class I alleles expressed by the patients (paper I). However, HLA class II association with peptide responses revealed eight peptides harbouring epitopes not previously associated with those particular HLA class II alleles (paper I). Results from this kind of analysis deepen the understanding of immunogenicity of HIV-1 immunogens in individuals with different genetic backgrounds, which is important for HIV-vaccine design.

4.2.4 Differentiation of memory T cell subsets

Naïve T cells undergo many complex steps in the process of becoming mature antigen-specific cells with either effector or memory functions. It is not clear whether these cells follow a linear path of development. The maturation process is influenced by interaction with many other cells and products of the immune system, and by the precise location of the cell and nature of the pathogen that the cells respond to. Several models have been proposed to describe the different stages of effector and memory T cells in humans. The model proposed by Sallusto and Lanzavecchia has placed the emphasis on the lymphoid tissue homing profile of CD8+ T cells, where CCR7 a chemokine receptor that controls homing to secondary lymphoid organs, is used to define the different stages of maturation (282). On the other hand, Appay et

al. instead used varying expression levels of the co-stimulatory molecules CD27 and CD28 to divide CD8+ T cells into early, intermediate and late effector cells (12).

On the basis of surface expression of CD45RA and CCR7 as well as their relevant function, Lanzavecchia et al., proposed four stages of differentiated T cell subsets: naive CD45RA⁺ CCR7⁺ T cells, central memory CD45RA⁻ CCR7⁺ T cells (T_{CM}), effector memory CD45RA⁻ CCR7⁻ T cells (T_{EM}) and terminally differentiated effector CD45RA⁺ CCR7⁻ T cells (T_{EMRA}) (59, 281). The T_{CM} cells home to T cell areas of secondary lymphoid organs, where they readily differentiate into proliferating effector cells. They are therefore suggested to play a critical role in effector cell renewal. The T_{EMRA} subset, which is believed to be differentiated from the T_{EM} subpopulation, carries the largest amount of perforin, but possesses the least proliferative potential (113). In HIV-infection T_{CM} are more frequent in the CD4+ T cell population while T_{EM} are more frequent in the CD8+ T cell population. To further distinguish the function of the different subsets of memory type T cells, a study revealed that T_{CM} are the major source of IL-2-secreting CD4+ T cells, whereas the CD4+ T_{EM} cells mainly secrete IFN- γ (135). However, during progression of HIV-1 infection, a large number of HIV-1-specific CD4+ T cells lose the capacity to produce IL-2, thus the response is skewed towards a virus-specific IFN- γ - secreting memory T cell population. As described earlier, HIV-1-specific CD4+ T cells have also been found to be major targets for HIV-1 infection (135, 136). Characteristics of CD8+ T_{EM} are that these cells can rapidly mount an effector response as they carry substantial amounts of perforin. This allows them to exhibit lytic activity immediately they recognise virus-infected cells.

4.2.5 B cells and induction of humoral immune responses

B cells are professional antigen presenting cells that can evolve into antibody producing plasma B cells. The induction of a humoral immune response requires an antigen (soluble or membrane bound) that is recognised by membrane-bound immunoglobulin (Ig) on the surface. The antigen is taken up by the B cell and processed into peptides, which are displayed on the cell surface with HLA class II molecules. The TCR and the CD4 molecule on the T helper cell recognise the foreign epitope and subsequently provide co-stimulatory signals and IL-4. In this manner the T helper cells stimulate the maturation of the naïve B cells into fully differentiated antibody secreting plasma B cells. They also stimulate isotype antibody switching from IgM or IgD to IgG, IgA and IgE. Antibodies can facilitate protective immunity by inducing antibody dependent cytotoxicity (ADCC) or by direct neutralisation of the virus. The protective effects of neutralising antibodies were demonstrated early on in experiments where chimpanzees were shown to resist HIV-1 infection when they received monoclonal antibodies prior to challenge (90).

HIV-1 is unable to infect B cells because of the lack of CD4 expression on the B-cell surface. However, HIV-infection is associated with several B-cell abnormalities such as B cell hyper-activation due to unspecific stimuli of microbial products binding to TLRs, or bystander activation and phenotypic alterations. The mechanisms by which HIV-1 impairs humoral immunity may either be direct virus induced B cell defects, or polyclonal B cell activation (reviewed in (224)).

4.2.6 Immune correlates of protection against HIV-1

It has been suggested that CD8+ T cells play important roles in clearance or control of a number of viral infections (343) including influenza (214), cytomegalovirus (234), measles (225), herpes simplex virus (158), hepatitis C virus (295) and Epstein-Barr virus (52). Studies of individuals exposed to HIV but uninfected and seronegative (ESN) individuals, who are capable of controlling HIV-1, and experiments in non-human primates, provide evidence that it is possible to, at least partially, control HIV-1 infection. ESN do not seroconvert despite multiple exposures to HIV-1 (349). Extensive analyses have been performed on these individuals in order to identify the immunological and/or genetic mechanisms that might explain this phenomenon. Hirbod et al. performed a prospective study of a large group of high-risk female sex workers in Kenya, and found genital HIV-specific neutralizing secreted IgA and HIV-specific proliferative responses. They correlated well with maintained seronegativity (143). Previous studies have also suggested that cytotoxic HIV-specific T cells responses in blood and the cervix play a role for protection of ESN individuals (154, 275). The ESN are often sex-workers that constantly expose themselves to HIV-infection through intercourse. Interestingly, for a proportion of these individuals a pause in multiple exposures can result in late seroconversion despite earlier cell mediated immunity (155). This suggests that the memory of the cell-mediated immune response (either local or systemic) may not be sufficient for protection and that short lived cellular responses or innate immunity is important for these individuals. The protective capacity might need to be maintained by multiple exposures to either the HIV-1 virus or to other components, such as HLA molecules (304).

Further evidence supporting the idea that containment of infection is possible comes from the small percentage (< 2%) of HIV-1 infected individuals who are capable of controlling of HIV-1 infection in the absence of antiretroviral therapy. Two types of HIV-1 seropositive individuals capable of controlling HIV-1 have been defined. Long-term non-progressors (LTNP) are characterized by a stable CD4+ T cell count and variable but low viral loads (46) whereas elite controllers (EC) are characterized by stable CD4+ T cell counts and low viral loads (<50 copies /ml plasma) (75). Partial control of HIV-1 infection has also been seen in individuals infected with a defective virus (172). Most HIV-1 controllers appear to be infected with fully functional virus, suggesting that host factors account for this virus control. Both ECs and LTNP appear to have cell mediated immune responses targeting the HIV-1 protein Gag (278, 330). It has been proposed that viral load in chronic HIV-1 infection is inversely correlated to the level of the Gag-specific cellular responses, while having Env-specific responses is associated with higher viremia (159). Moreover, EC and LTNP have a higher HIV-specific CTL activity that that seen in progressors. For example, Migueles et al showed that the cytotoxic granules of CD8+ T cells isolated from ECs contained higher levels of functional granzyme B and perforin than the granules from patients with progressing disease (221). HIV-specific immune responses in LTNP also exhibit broad specificity and polyfunctionality (e.g. the ability to produce and secrete several cytokines upon specific stimulation), whereas narrow and monofunctional (mainly IFN- γ) responses are seen in progressors (26, 139). The precise mechanism of action behind these findings is largely unknown.

The role of antibodies in HIV-1 protection is illustrated by the fact that antibodies to conserved epitopes of the gp120 or gp41 proteins can also protect against HIV-1 infection in non-human primates (90). Recent results demonstrate that the memory B cell compartment, in some HIV-controllers, is comprised of a diverse neutralising IgG-repertoire targeting many different epitopes on gp120 (290).

4.2.7 Host genetic factors and control of HIV- infection

Several studies describe associations of particular HLA alleles with different outcomes of HIV-infection. For example, HLA B*57 is able to present a wide array of HIV-1 peptides for T cell recognition, and expression of this molecule is associated with a more benign HIV-infection (7, 148). As reviewed in (55), there are independent studies involving different cohorts of patients which show strong correlations between HLA B*57 and B*27 molecules and delayed disease progression, whereas the HLA B*35 is associated with a more rapid disease progression. HLA B*57 and B*27 molecules have been shown to be over-represented in elite controllers, although less than 50% of the elite controllers show the presence of HLA B*57 and even less (20%) express HLA B*27 (248). Thus not the HLA alleles alone but rather the specific epitopes they target may be responsible for viral control. Furthermore, recently published data suggest a dual mechanism for durable control of HIV replication in HLA-B*57+ individuals, consisting of loss of viral fitness as a result of CTL escape mutations, together with strong CD8+ T cell immune responses to the arising variant epitopes (223).

A few individuals originating from the northern parts of Europe have a 32 bp deletion in the gene encoding the HIV-1 co-receptor CCR5. Individuals homozygous for the $\Delta 32$ allelic variant of the CCR5 protein display a strong protection against sexual transmission of HIV-1 and they may have a delayed progression of HIV-disease (189, 283).

4.2.8 The role of perforin for cytotoxicity

As mentioned above, cytotoxic T cells and NK cells are the main effector cells in the immune response against viruses. These cells release the contents of cytotoxic granules into the immunological synapse formed between the infected target cells and the effector cell, which trigger apoptosis (121, 303). The cytotoxic granules contain perforin and serine proteases called granzymes (180). Perforin was originally thought to insert itself into the target cell's plasma membrane, forming a pore and thereby inducing cell death or facilitating the influx of granzymes. Although purified perforin is capable of lysing cells when added in high concentration to cell cultures, this mechanism does not explain the ability of CD8+ T cells and NK cells to induce apoptosis in target cells. The original pore-forming model was challenged when it was discovered that granzymes can be endocytosed alone without perforin, and that apoptosis can be triggered when perforin is added to washed cells that have endocytosed granzyme in the absence of perforin (255). So far, the synergistic mechanism between perforin and granzymes is not fully understood. One theory suggests that perforin and granzymes interact at the target cell membrane and are rapidly internalised into endosomes that are subsequently disrupted by perforin, causing the granzymes to be released into the cytosol (103). A hybrid theory has

emerged suggesting that perforin permeabilises the plasma membrane and triggers Ca^{2+} influx from the extracellular fluid into the cells, triggering a wound healing mechanism. This mechanism includes the mobilisation of intracellular vesicles, like endosomes and lysosomes, which donate their membranes in order to reseal the damaged plasma membrane. This somehow facilitates the uptake of granzymes and perforin into endosomes, followed by a perforin mediated release of granzymes into the cytosol (255). In order to find correlates of immune protection in the setting of HIV disease, attempts have been made to link the ability of CTLs to degranulate (as measured by the expression of CD107a) to disease progression. While some studies claim that the expression of CD107a together with a panel of cytokines (MIP1 β , TNF α , IL-2 and IFN γ) correlates with better outcome of disease (27), recent studies indicate that the content of the granules (e.g. granzymes and perforin) is more important (134, 221).

4.2.9 Perforin and HIV-1 infection

HIV-specific CTL responses are detectable shortly after the acute phase of HIV-1 infection. However the immune responses cannot control viral replication and prevent development of chronic immune suppression in the majority of infected individuals. Some studies have suggested that this lack of control is due to impaired functional activity of cytotoxic T lymphocytes and NK cells. The impaired functional reactivity has recently been attributed to decreased intracellular levels of perforin and granzyme B (10, 101, 258). We have observed a significant increase in levels of serum perforin in HIV-1 infected individuals as compared to uninfected controls, suggesting that HIV-1 infection contributes to the release of perforin in serum. Interestingly, untreated HIV-1-infected individuals had a higher level of perforin than patients on treatment (Figure 7 and (paper II)). This implies that effective treatment, to a large extent, can reverse the excess of serum perforin in HIV-1-infected individuals.

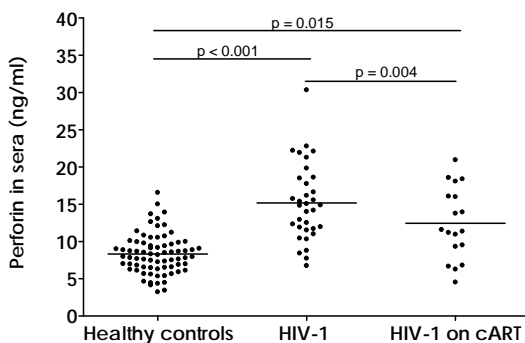


Figure 7. Levels of extracellular perforin in sera from healthy donors, HIV-1 infected untreated patients and HIV-1 infected patients on cART.

We also found that the levels of perforin in serum increase significantly during the acute phase of HIV-infection, as shown in figure 8A. Furthermore, analysis of perforin in serum during the acute phase of SIV or SHIV infection in cynomolgus macaques revealed a markedly elevated level of perforin, coinciding with the plasma virus peak (Figure 8B and C and paper II).

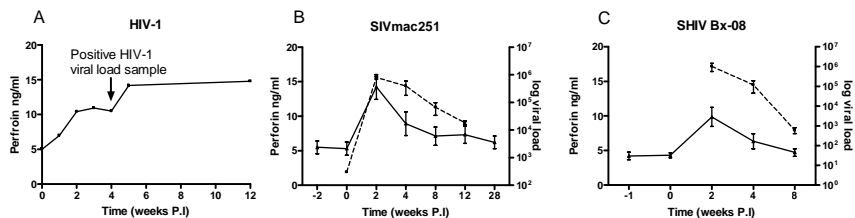


Figure 8. A) Levels of extracellular perforin in sera from an HIV-1 infected individual during seroconversion serum perforin in a human seroconversion panel, B) Levels of extracellular perforin in sera from SIV infected monkeys C) Levels of extracellular perforin in sera from SHIV infected monkeys. Filled lines indicate perforin (ng/ml), striped lines indicate viral load (copies/ml).

In chronically infected HIV-1 patients undergoing repeated structured treatment interruption (STI) we detected an increase in serum perforin after each STI cycle (figure 9A). This increase was also observed in perforin release from unstimulated PBMC as measured by ELISpot (figure 9A and B). A correlation between viral load and the level of secreted perforin from unstimulated PBMC was thus observed in this cohort (Spearman, $r = 0.78$, $p = 0.036$) unpublished data.

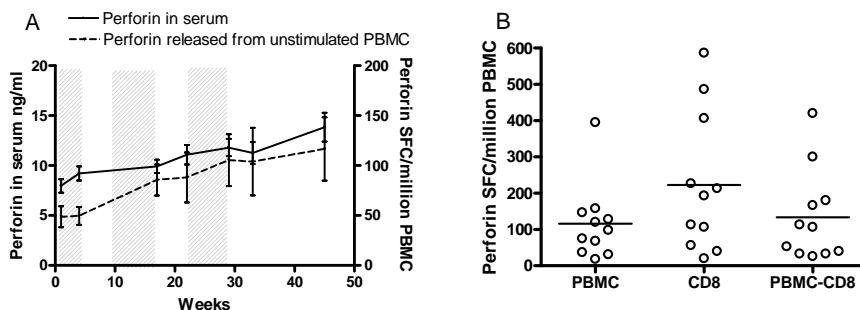


Figure 9. A) Levels of extracellular perforin in sera and levels of perforin released from unstimulated PBMC from HIV-1 infected individuals during repeated treatment interruptions. B) Levels of perforin released from unstimulated PBMC, CD8+ T cells and CD8+ T cell depleted PBMC.

It is conceivable that the increased levels of free perforin mirror the decreased intracellular levels of perforin observed in CD8+ T cells and NK cells during HIV-1 infection and that these cells are the main source of the extracellular perforin. The combination of reduced intracellular perforin levels and increased serum levels shows that HIV-infection induces aberrant perforin secretion. As perforin secretion/production has been shown to be associated with control of HIV-1 infection, the aberrant perforin release may be a result of a yet unknown viral immune escape mechanism. However, such observations have also been made with acute viral infections such as Hanta viruses (162) suggesting that an increased serum level of perforin may be a direct reflection of increased CTL killing due to virus infection.

5 CURRENT HIV-1 TREATMENT

Today, the treatment of HIV-1 infection primarily relies on combination antiretroviral therapy (cART) which normally consists of at least three different drugs targeting different steps in the viral replication cycle. Typically, the first line treatment includes two nucleoside analogue reverse transcriptase inhibitors (NRTIs) plus either a non-nucleoside reverse transcriptase inhibitor (NNRTI) or a protease inhibitor, targeting reverse transcription and viral protease activity. NRTIs inhibit viral transcription by incorporating nucleoside analogues lacking 3'-hydroxyl group on the deoxyribose moiety. As a result viral DNA synthesis is stopped. NNRTIs are not incorporated into the viral DNA and stop viral transcription by inhibiting the movement of protein domains of reverse transcriptase, that are needed to carry out the process of DNA synthesis. New drugs are constantly being developed with the aim of improving potency and minimising the toxic effect of the drugs. During recent years, two novel drugs that inhibit fusion of the virus to the target cell have been released: T20 which is a gp41-binding peptide that disturbs the binding of the virus to the target cell (58), and Maraviroc -a co-receptor antagonist that binds to CCR5, thus preventing an interaction with gp120 and the receptor (79). Integrase inhibitors are a novel class of antiretroviral drug designed to block the action of integrase, the viral enzyme that inserts the viral genome into the DNA of the host cell. The first integrase inhibitor was licensed in 2007 (300). Recently, the viral protein Vif has become highly interesting as a novel target for HIV therapy since recent results demonstrate that it is possible to inhibit HIV-1 replication by antagonising the Vif-APOBEC3G complex (238).

Antiretroviral treatment slows down progression to AIDS by reducing the viral load. Thus, cART has revolutionised the care of HIV-1 infected individuals, where it has been available, converting a terminal disease into a chronic manageable infection (245). When cART was introduced in 1996 the initiation of treatment was recommended at HIV-1 diagnosis, according to the principle "hit early and hit hard" (45). However, the limitations of cART following long term use (adherence, side effects and drug resistance) resulted in the recommendations for cART initiation on the basis of CD4+ T cell count in peripheral blood (346). Instead of recommending cART at HIV diagnosis, ART was indicated when CD4+ counts fell below 200 cells/mm³, which usually occurs later during infection. However, the findings of the recent Strategies for Management of Antiretroviral Therapy (SMART) study (described below) suggest that cART should be initiated when CD4+ T cell counts drop below 350 cells/mm³ (253).

Antiretroviral treatment is life-long since non of the available drugs are capable of eradicating the reservoir of latent virus within memory CD4+ T cells (62, 95). It is believed that HIV-1 latency is generally established when an activated CD4+ T cell becomes infected by HIV-1 and undergoes transition to a memory phenotype that prevents it from being killed by the virus or components of the immune system. Because memory cells are long-lived, the virus can persist within the cell for decades, before receiving a stimulatory signal that activates virus production. It is therefore estimated that under current antiretroviral regimens, complete depletion of this viral reservoir would take more than 60 years (95), even if there were no further viral replication. It is not clear whether this is due to poor antiretroviral drug penetration

into some compartments of virus replication, like the central nervous system or because of replication that occurs even under 'optimal' drug concentrations. Drug therapy cannot eradicate infection and the treatment is therefore life-long.

5.1 PROLONGED TREATMENT FREE PERIODS

Problems associated with antiretroviral therapy have led to investigation of treatment-sparing strategies, such as structured treatment interruptions (STI). It was proposed that STI might provide the benefits of antiretroviral therapy while minimizing the time on cART, thus decreasing the problems associated with long-term treatment. However, the SMART study has prompted new considerations regarding the safety of STI. The SMART study was a large multi-centre trial that enrolled 5472 patients in order to determine the safety of cART-free periods, where cART initiation was based primarily on CD4+ T cell counts (88). Patients were divided into two arms, one arm in which patient were maintained on continuous cART and one arm in which the patients were subjected to intermittent cART. The patients in the arm including STIs discontinued cART when the patient had a single CD4+ T cell count above 350 cells/mm³ and cART was then reinstated as soon as the CD4+ T cell count dropped below 250 cells/mm³. This resulted in patients going on and off treatment at varying intervals. The study was stopped when the patients in the intermittent cART arm were found to have more adverse events and higher morbidity than patients on continuous treatment. The results from the study have led to the overall conclusion that multiple treatment interruption is unsafe. In contrast, other smaller treatment interruption studies have shown that prolonged STIs are generally safe (9, 203). These studies also suggested that the rate of CD4+ T cell decline is related to CD4+ T cell nadir which is the lowest CD4+ T cell count reported before cART start. Patients with higher pre-cART CD4+ T cell counts have the potential for longer STIs compared with patients with low pre-cART CD4+ T cell counts (236).

5.1.1 Pre- and post-exposure prophylaxis

Pre-exposure prophylaxis (PrEP) is the long-term use of a prophylactic treatment for a disease prior to the exposure. In the setting of HIV-1, it usually consists of administration of one or several antiretroviral drugs to individuals at risk of infection (16). This is thought to prevent infection or to lower the initial peak viral load. PrEP has been shown to be effective in humanized mice models (76) and the effect on humans is currently under investigation. However, there are some concerns that PrEP might encourage risk behaviour, possibly increasing rather than decreasing the risk of HIV-infection, for both the individual and the population at large. In addition to this, there are concerns that PrEP might select for the transmission of drug resistant strains of HIV-1. Post exposure prophylaxis (PEP) relates to antiretroviral therapy administered shortly (within 72 hrs) after occupational or sexual exposure, to prevent establishment of infection (16).

5.2 PREVENTION OF HIV-1 TRANSMISSION

Although successful cART reduces transmission of HIV-1, by lowering the viral load and thus the risk of transmission, other methods to prevent transmission are required. The single most effective way to prevent the transmission of HIV-1 during sexual

contact is to use a condom (265). Recent clinical trials have demonstrated that male circumcision also can reduce female-to-male transmission of HIV-1 by approximately 50%–60% (18, 124). The beneficial effects of male circumcision may be explained by the high density of cells susceptible for HIV infection in the foreskin (211). Circumcision also reduces the risk of acquiring other genital infections associated with increased risk of HIV-1 infection (340). Other interventions, like microbicides, are designed to prevent infection through the genital mucosa. However, first-generation microbicides such as nonoxynol-9 and cellulose sulphate have failed to show protection in clinical trials (261, 269). These early microbicides instead seemed to increase the risk of infection, most probably through irritation of the mucosa (1). The environment in the vaginal mucosa is very delicate and disruption of this milieu by addition of a microbicide with detergent properties can ruin the natural barrier which in turns leads to enhanced infection. More promising results have been obtained with new microbicide gel formulations like BufferGel and PRO2000, based on an acidic buffered and a sulfonated polymer, respectively. Recent data indicate that PRO2000 was at least 30% more effective in the study in preventing HIV, while BufferGel did not show a protective effect (279). BufferGel's primary action is to lower vaginal pH, while PRO2000 is designed to interfere with HIV's interactions with its target cells (312).

It has also been shown that behavioural prevention methods (sexual abstinence, delayed sexual debut, reduction of sexual partners, and clean needle use for drug users) can significantly reduce the risk of HIV-I infection, when they are used in combination (87, 327).

6 IMMUNISATION

Immunisation is the process whereby a person is made immune or resistant to an infectious disease, by the administration of a vaccine. Along with improved hygiene, immunisation is considered to be the most cost-effective method of prevent infectious diseases and can be divided in two main categories: passive and active immunisation. In passive humoral immunisation, purified antibodies or antibody containing sera are administered to an individual and this can provide temporary protection against the pathogen. Passive immunisation occurs naturally in the case of transfer of maternal antibodies to a foetus or a baby via the placenta or breast feeding, respectively. Prevention of rabies infection is an example where both passive and active immunisation is required to elicit protective immunity if an individual has been exposed to the virus. Passive immunisation with neutralising antibodies has been performed in HIV-1 infected individuals and chimpanzees. Transfusion of neutralising antibodies 2G12, 2F5 and 4E10 resulted in a delay of HIV-1 rebound in acutely infected humans after cessation of antiretroviral therapy; however only a marginal effect was seen in chronically infected individuals (321). Passive transfer of cell-mediated immunity, has been used in humans to treat some types of cancer as well as HIV-1 as reviewed in (176). One study revealed that transfer of autologous gene-modified T cells, expressing a HIV-entry inhibitor peptide, resulted in a significant increase of CD4+ T cell numbers in HIV-infected patients with advanced immunodeficiency (331).

The principle of active immunisation is to present a foreign antigen to the immune system thereby subsequently inducing an antigen-specific immune response. This allows the body's own immune system to protect or fight against subsequent infection or disease. Vaccines used for active immunisation can be prophylactic (e.g. prevent a future infection by a pathogen), or therapeutic (e.g. cure or partially restore immune responses in a host with an existing chronic disease like HIV-1 or cancer).

6.1 ANTIGEN PRESENTATION FOLLOWING IMMUNISATION

The aim of active immunisation is to induce long-lived adaptive immune responses against the pathogen or tumour-antigen of interest. A short summary of the basic mechanisms for antigen presentation following an immunisation is given below.

The majority of vaccines are administered to somatic cells in the skin or muscle, typically via needle and syringe. There are however some exceptions, like the polio and cholera vaccines that are delivered orally. The most common cells directly targeted by the immunisation injection are muscle cells, keratinocytes, monocytes and antigen presenting cells in the skin. Live vaccines enter the cell using virus specific receptors on the host cell surface. In the case of DNA vaccines, the uptake of plasmids into cells is not fully understood. It has been suggested to occur through membrane disruption, transient pore formation in the cellular membrane and/or a more active process including receptor mediated endocytosis (48, 105, 287).

Using the host cellular machinery, the genes in live vaccines or carried by plasmids are transcribed and translated to produce endogenous antigen. Protein production following immunisation with live or genetic vaccines mimic protein production which

occurs during natural infection, as the cell provides endogenous post-translational modifications, including glycosylation, to the vaccine antigen. Endogenously synthesised proteins are poly-ubiquitinated and thereby targeted for degradation by the proteasome. The proteasome cuts the protein into shorter peptides (normally 3-20 amino acid residues long). A fraction of these peptides are then translocated via the transporter of antigenic peptides (TAP) complex to the ER where they associate with HLA class I molecules. As described previously HLA class I presentation of antigens are associated with CD8+ T cell responses. HLA class I presentation can also be elicited by cross-presentation of cell-associated exogenous antigens.

Exogenous antigens are taken up by cells either through phagocytosis or through endocytosis. The exogenous antigens taken up by professional APCs can either be in form of inactivated or protein subunit vaccines or antigens produced and released from cells transfected with live or plasmid based vaccines. Upon phagocytosis or endocytosis, the phagosomes or early endosomes are transported to the inner parts of the cell and end up in the lysosomes where the antigen is degraded into smaller peptides. The peptides are then loaded onto HLA class II molecules in the lysosome, and the final peptide/MHC complex is then transported to the cell surface where it is recognised by the T cell receptor of CD4+ T cells.

In addition to these two pathways, antigens that are produced by somatic cells can be transferred to APCs and enter the HLA class I pathway by a mechanism called cross-priming or cross-presentation. This is a mechanism that can be utilised for the induction of immune responses by a genetic vaccine (80, 104).

6.2 THERAPEUTIC HIV-1 IMMUNISATION

In contrast to preventative vaccination, where specific immunity is induced to prevent viral or bacterial infection, therapeutic vaccination is intended to mobilize a patient's immune response in order to control an already established infection. The concept of augmenting the HIV-specific immuneresponse by therapeutic immunisation was first proposed in 1987 (280). Although successful cART suppresses viral load efficiently and helps to restore the immune response against opportunistic infections it does not fully eradicate the reservoirs of HIV-1 (28, 96, 215), suggesting that a life long intake of drugs will be necessary. Immune responses to HIV-1 are not restored by cART and in fact usually decline over time during treatment (256). cART is also associated with both severe side effects and development of drug resistance if the patient is not fully compliant. It would thus be beneficial to extend treatment options of HIV-1-infected patients with a therapeutic vaccine that can modulate immune responses against the virus, which in turn could control HIV immune deficiency and thereby reduce the need of cART.

The main goal of therapeutic immunisation during the chronic stage of HIV-1 infection is to induce HIV-specific immune responses and restore immunological competence and reverse the anergy-like situation of the T cells. This in turn may reduce viral load and also permit extended periods without the use of antiviral drugs (14, 81, 328). The rationale behind therapeutic immunisation is based on the assumption that the natural history of disease can be modified by enhancing existing or generating new anti HIV-1

immune responses. Immunisation with an exogenous antigen may either boost an existing immune response or induce a qualitatively different immune response from that induced by natural infection. These modified responses are hypothesised to result in better viral control and therefore a slower disease progression (246).

During the past two decades several research groups have evaluated the potential role of therapeutic vaccination as an alternative treatment strategy. The goal has been to increase virus-specific immune responses that are critical in the suppression and control of HIV replication. A range of vaccine candidates, developed primarily as prophylactic HIV vaccines, have been tested in non-human primates and have entered early human therapeutic trials. These include live attenuated vaccines, whole inactivated virus, subunit vaccines (protein and peptides), DNA vaccines, dendritic cell based vaccines and viral vectors.

6.3 LIVE ATTENUATED VACCINES

Vaccines consisting of live, attenuated virus, such as yellow fever, measles and rubella have been cultured under conditions that disable their virulence. This type of vaccine can also be based on closely related, but less dangerous, organisms to induce an immune response. One example of this is the smallpox vaccine that is based on vaccinia virus (cow pox virus), a poxvirus that induces a much more benign infection as compared to the variola virus that causes smallpox. Live attenuated vaccines are produced by growing the virus in tissue culture that will, in the absence of immune pressure, select for less virulent strains either by mutagenesis or loss of virulence factors. As there is a small risk of virulence even if the virus in the vaccine is weakened, attenuated vaccines cannot be used in immunocompromised individuals including HIV-1 infected patients. As the attenuated pathogens in vaccines replicate to a small extent they mimic natural viral infection and provoke both T cell and B cell responses. This results in induction of more durable immune responses as compared to immunisation with inactivated vaccines, and booster doses are required less often.

Initially, this vaccine strategy seemed promising against HIV-1, as macaques immunized/infected with *nef*-deleted SIV were protected from challenge with pathogenic SIV (70). However, the initial enthusiasm was dampened when the *nef*-deleted SIV reverted back to its pathogenic form and caused AIDS in vaccinated macaques (341). *Nef*-deleted HIV-1 has also been shown to cause immunodeficiency in humans, as nine individuals in Australia were infected with a *nef*-deleted HIV-1 virus through a blood transfusion. These individuals had an initial delay in disease progression, most probably associated with low virus replication due to the lack of Nef (172), but eventually progressed in their disease (173).

6.4 INACTIVATED VACCINES

An inactivated vaccine contains virus particles that are inactivated or killed with chemical treatment or heat. The virus particles are rendered unable to replicate, but the virus capsid proteins are intact enough to evoke an immune response. Many vaccines, like rabies, influenza and hepatitis A, are based on inactivated virus. This is

usually a safe and effective way to induce humoral immunity, when the vaccines are manufactured correctly, but improper inactivation can result in intact and infectious particles. The major advantages of inactivated vaccines are that they induce strong humoral immune responses and lack the capacity to replicate and can thus be used safely in immunocompromised hosts. However, they are often poor inducers of T cell mediated immune responses and immunological B cell memory, hence several repeated immunisations (booster doses) are required to achieve long-lasting high antibody levels.

Inactivated HIV-1 vaccines have been extensively evaluated in therapeutic immunisation settings. The Remune immunogen (Immune Response Corp., CA, USA), is composed of purified inactivated virions encoding HIV-1 clade A envelope and clade G gag, stripped of surface gp120 and emulsified in incomplete Freund's adjuvant. In early studies, administration of Remune resulted in stimulation of both CD4+ and CD8+ T-cells, slowing of disease progression, and increased body weight. However, interim analysis of a phase III clinical study of Remune in HIV-1 participants showed no changes in CD4+ and CD8+ T-cell counts and viral loads (324). Further studies where Remune was administered together with IL-2 failed to sustain or induce HIV-1 specific immune responses (137). Aldrithiol-2 (AT-2) is a mild oxidizing reagent, used to eliminate the infectivity of HIV-1 while maintaining its structure and ability to be processed for presentation to T cells. Immunisation with AT-2 inactivated SIV particles has been studied in animal models. However, this immunisation strategy had minor effect on viral load and did not induce protective immunity in macaques, despite induction of SIV specific immune responses (181, 335). Nevertheless, this inactivation technique produce excellent antigens for *in vitro* screening of HIV-1-specific responses in HIV-1 seropositives and vaccinees (277).

6.5 SUBUNIT VACCINES

6.5.1 Recombinant proteins

In the case of subunit vaccines a viral protein is introduced into the vaccine recipient. The hepatitis B vaccine was the first viral subunit vaccine on the market and contains the viral envelope protein, the hepatitis B surface antigen. A human papilloma virus (HPV) vaccine has recently been registered for human use. This vaccine is based on proteins from papilloma viruses which form virus-like particles (VLPs). Virus like particles consist of viral protein(s) derived from the structural proteins of a pathogen. In some cases these proteins are embedded within a lipid bilayer. These particles resemble the virus from which they were derived but lack viral genomes and non-structural proteins, and are therefore not infectious.

Subunit vaccines containing HIV-1 proteins have also been studied. The first phase III clinical trial of an HIV-1 vaccine was conducted by Vaxgen Inc. using a candidate HIV-1 monomeric gp120 subunit vaccine. The vaccine consisted of two recombinant gp120 subunits derived from two different subtype B isolates MN and GNE8 (AIDSVAX B/B) or from the subtype B MN strain and subtype E A244 stain (AIDSVAX B/E). The proteins were delivered in alum adjuvant and the healthy volunteers were immunised seven times with 300 µg of protein (100). The hope was that the vaccine would

induce antibodies against gp120 that would subsequently bind, neutralise and clear the virus before the infection was established. Upon completion of the trial in 2003, it was clear that the vaccine neither prevented HIV-1 infection nor delayed the disease progression. The results from the trial indicate that monomeric gp120 is a suboptimal vaccine component for eliciting broadly neutralising antibodies. It also weakened the hypothesis that antibodies against monomeric envelope glycoproteins would bind and neutralise HIV before infection was established.

Therapeutic vaccination with HIV-1 proteins was initially tested in the pre-cART era but failed to induce any significant clinical benefits. Recombinant baculovirus-expressed envelope protein, rgp160 (VaxSyn MicroGeneSys, Meriden, CT) and HIV-1 gag (p17/p24) expressed in a yeast vector as self-assembled virus like particles were not found to have any significant beneficial effect, in terms of disease progression, CD4+ T cell count or viral load despite boosting HIV-1-specific immunity (28, 116, 157, 165, 182, 264, 339). These results may reflect both poor immunogenicity of the vaccines and antigenic overload due to uncontrolled HIV-1 replication.

A large Scandinavian study performed in 1992, revealed that multiple intramuscular injections of rgp160 significantly enhanced HIV-specific proliferative responses and improved short-time survival in vaccinated HIV-1 patients (286). In paper III, we analysed cells from patients in this study in order to evaluate the effect of multiple rgp160 immunisations on the differentiation of T cell subsets. The differentiation of naïve T cells into effector and memory subsets represents one of the most fundamental elements of T cell mediated immunity. HIV-specific polyfunctional CD8+ central memory cells have been associated with superior control of HIV (6, 27). Frozen cells samples from 12 individuals (6 vaccine recipients and 6 placebos) were selected from this study. They had been immunized intramuscularly during one year (months 0, 1, 2, 3, 4, 6, 9, 12) with eight doses of 160 µg of rgp160 (VaxSyn, MicroGeneSys, Meriden, CT) or placebo (alum adjuvant alone). Thereafter the patients received vaccinations every three months up to three years from the initiation of the study (286).

We observed a significant increase in the number of central memory T_{CM} (CD4⁺CD45RA⁻CCR7⁺) cells in rgp160 vaccinated individuals (Figure 10A and paper III). The increase in the CD4+ memory cell population was consistent with increased HIV-1 specific CD4+ proliferative responses (Figure 10B and paper III). No increase in central memory population or proliferative responses was observed in the placebo group, where the number of central memory cells stayed constant throughout the monitoring period (paper III). These results indicate that rgp160 administration significantly enhanced the induction of an effector memory T cell subset. In the original study there were significantly fewer deaths among the vaccine recipients than among the placebo-group patients at 2 years but not at the end of the study at 3 years (286). This transient improvement may be due to functional immune activation as shown by the increased central memory population. Although the administration of rgp160 did not have an effect on viral load it induced memory virus-specific responses, presumably thus enabling the HIV-1 infected individual to better regulate the disease and improve survival.

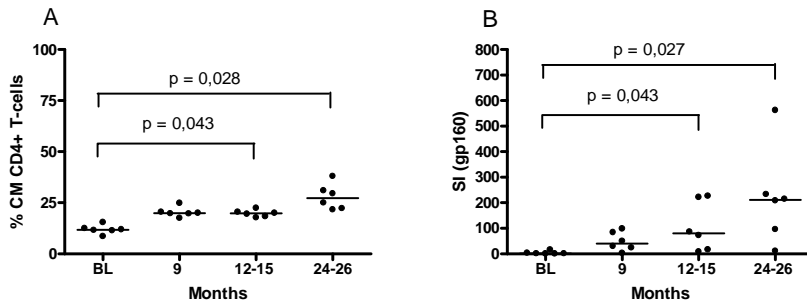


Figure 10. A) Frequency of central memory CD4+ T-cells (%) and B) HIV-specific proliferative responses (SI = stimulation index) in patients are immunised with rgp160.

An absence of cytotoxic T-cells displaying the activation marker CD38 has recently been suggested to correlate with control of viremia. This has been observed in a small number of drug naïve HIV-infected individuals who have persistently undetectable viral load (278). In our study (paper III), an up-regulation of the immune activation markers HLA-DR and CD38, compared to levels in healthy individuals was seen on both CD4+ and CD8+ T cells in all patients (29), throughout the monitoring period (paper III). This reflects the immune activation induced by uncontrolled HIV-1 virus replication. Further enhanced expression of the activation marker CD38 was observed over time in the group vaccinated with rgp160, but not the placebo group. This is likely due to further antigenic stimulation from the rgp160 vaccine. This may be one of the explanations as to why this vaccine did not induce control of the HIV infection.

Other HIV proteins, such as the regulatory protein Tat have been tested in clinical trials more recently and have been shown to induce immune responses but have so far failed to show any beneficial effect on viral load or CD4+ T cell counts (91, 191).

6.5.2 Peptides

A number of synthetic peptides designed to induce immune responses to dominant CTL epitopes or HIV neutralizing determinants have been tested in humans. Synthetic peptides designed to contain combinations of T-helper, CTL and neutralising antibody epitopes from the V3 loop from several HIV-1 strains were tested in eight HIV-infected patients (21). This vaccine did not reduce viral load or increase CD4+ T-cell count despite inducing an increase in neutralizing antibody titres and lymphoproliferative responses. However, a recent phase I study analysing the immunogenicity of a multiepitope peptide vaccine containing both CTL and T helper cell epitopes resulted in failure. Only six of 80 volunteers who received vaccine developed low and transient HIV-specific responses (299).

In a further study, a peptide-based vaccine candidate consisting of highly conserved regions of HIV-1 p24 recognised by long-term non-progressors was designed to induce cellular immunity (274). When examined in several trials in HIV-infected volunteers in the absence or presence of cART, Gag-specific responses were observed in vaccinees. Those that responded to the vaccine had significantly reduced viral loads and better preserved CD4+ T cell counts as compared to low or non-responders (13, 164, 242). Data indicated that a significantly slower decline in CD4+ T-cells was noted for the

peptide immunised patients. The median time of treatment interruption achieved for all the patients that participated in the Vacc-4x phase II clinical trial was 17 months as compared to 4 months for the control group (199).

Peptide vaccines have also been linked to lipids in an attempt to enhance their uptake through cell membranes. There have been a number of promising results, at least in terms of immunogenicity of the lipopeptides, either delivered on their own (107, 294), or in combination with other vaccine candidates (178).

6.5.3 Dendritic cell based vaccines

A promising strategy in the SIV model is to use autologous dendritic cells pulsed with chemically inactivated virus *in vitro*. Administration of dendritic cells loaded with AT-2 inactivated SIV particles has led to an increase in T-cell responses, and reduced viral loads in monkeys infected with SIV (196). Vaccination of 18 chronically HIV-1 infected treatment naïve-patients with their own dendritic cells loaded with their own AT-2 inactivated HIV led to a 5 fold decrease of plasma viral load after one year. This decrease in viral load correlated with the percent increase of CD4+ T cells in the patients' blood, secreting IL-2 and IFN- γ when cultured with HIV-1 antigen (195). To confirm this finding a trial with 100 HIV-1 infected individuals is planned (11). However, this type of autologous vaccine is very cumbersome to manufacture and is not suitable for individuals in developing countries.

6.6 DNA IMMUNISATION

Genetic immunisation is a novel approach to vaccination. DNA or RNA encoding a specific antigen is transferred to a host cell, either as plasmids or carried in a recombinant viral or bacterial vector. The antigen is expressed by the host cell and can be subsequently presented to the host's immune system, thereby eliciting an immune response. DNA vaccines display several features that make them interesting for immunisation; they are easy to modify, they induce both humoral and cellular immunity and they do not induce immunity towards the vector itself (166). Furthermore, the use of DNA vaccines does not have the safety concerns associated with live vaccines (276).

The DNA based vaccine approach was first proposed in the early 1990s. Several independent research groups reported induction of immune responses after immunisation with genes encoding influenza proteins, human growth hormone, various tumour antigens and proteins from HIV-1 (106, 310, 326, 332). In the two most important studies, DNA constructs encoding Influenza antigens were shown to induce protective responses against influenza challenge in mice and chickens (106, 326). Since the initial findings, DNA vaccines against various pathogens and tumour antigens have been evaluated in animal models. In the past years, four DNA vaccine products have been licensed for animal use. One against West Nile virus in horses (72), one against infectious haematopoietic necrosis virus in salmon (111), one for treatment of melanoma in dogs (23), and one for growth hormone releasing hormone used to prevent foetal loss in swine (313).

The first human study, using plasmid DNA constructs encoding Rev, Nef and Tat demonstrated a significantly enhanced cellular immune response in treatment naïve HIV-1 infected individuals (49), and later in individuals who were receiving antiretroviral therapy (50). This vaccine also induced local mucosal cellular and humoral responses when administered to HIV-positive patients by intra-oral jet-injections (198). Weiner and colleagues performed an early study with a DNA plasmid construct encoding Env and Rev genes in untreated HIV-positive patients and demonstrated safety but marginal cellular and humoral immune responses (200, 201). The group developed an additional DNA construct encoding Gag and Pol, which was immunogenic and demonstrated protective effect in experimental animals (36). When the HIV-1 DNA vaccine encoding Env/Rev and Gag/Pol was administered to HIV-infected humans, a difference in viral detection events in vaccinated compared to control patients was observed. The frequency of "viral blips" (transient elevations of HIV RNA) (140) were significantly fewer in vaccine recipients as compared to placebo controls (202). These results indicate that DNA immunisation of cART treated HIV-positive subjects with vaccines designed to stimulate CD8+ T cell responses may have a therapeutic effect and may decrease the frequency of transient viremia.

6.7 ENHANCING THE POTENCY OF DNA IMMUNISATION

The principal issue regarding the future of DNA vaccines concerns improving their immunogenicity in larger animals and in humans. DNA vaccines have induced significantly weaker immune responses in non-human primates and humans compared to mice. Naked DNA also seems less immunogenic as compared to recombinant viral vectors or recombinant protein for induction of antibody responses. However, a variety of methods are being developed to increase the immunogenicity of DNA vaccines. Efforts to increase antigen expression and vaccine immunogenicity by different delivery adjuvants and methods will be discussed below.

6.7.1 Adjuvants

Adjuvants are substances that enhance the immunogenicity of an immunogen and they have been used for decades to improve the immune response to vaccine antigens. By adding adjuvants to vaccine formulations one aims to enhance and prolong the vaccine-specific immune response. By using the proper adjuvant it is possible to modify the nature of the immune response (e.g. with respect to immunoglobulin classes and induction of cytotoxic or helper T cell responses) and to reduce the amount of antigen needed for a successful immunisation, and also reduce the number of booster immunisations needed. Adjuvants can be classified according to their source, mechanism of action, or physical and chemical properties.

So far the only approved adjuvants for human use are aluminium hydroxide or phosphates (alum); alum in combination with monophosphoryl lipid A (MPL) (AS04 from GSK); and an oil-in-water squalene emulsion (MF59 from Novartis). The classical proposed mechanism by which it was believed that alum provides its adjuvant effect is by causing the formation of an antigen depot at the inoculation site resulting in slow release of the antigen. However, as reviewed in (141), more recent findings indicate that immune stimulation also occurs through stimulation of uric acid production,

inflammation and recruitment of antigen-presenting cells, uptake of antigen to DCs and subsequent DC maturation. MPL is a non-toxic derivate from a lipopolysaccharide component of the cell wall of bacteria and is well described to stimulate the innate immune responses by acting through TLR4 (4). The mechanism of action of adjuvant emulsions is to promote slow antigen release and to protect antigen from rapid elimination (243). Another important mechanism for some emulsion adjuvants including MF59 is the recruitment of immune cells to the site of injection and the promotion of uptake of vaccine by APCs (86).

In general, the mode of action of adsorbents and particulate adjuvants involves delivery of the antigen to APCs, whereas the microbial, synthetic and endogenous adjuvants act by direct stimulation or modulation of the immune system (252). Bacterial components are often potent immune activators although commonly associated with toxicity, for example, bacterial DNA with immunostimulatory CpG motifs is a potent cellular adjuvant. CpGs are unmethylated cytosine-guanine motifs found in bacterial DNA, including plasmids, but absent in mammalian DNA. CpG DNA is recognized by TLR9 and induces a milieu that favours activation of Th1-dominated immune responses. This is mainly due to activation of dendritic cells and subpopulations, thereby inducing an intense interferon and IL-12 response (142).

The stimulatory effect of human endogenous cytokines, on both innate and adaptive immune responses, makes them interesting as adjuvants. Recombinant cytokines have been evaluated as adjuvants. However, the major limitations in their use are their short half-life in vivo. Thus, cytokine-encoding plasmid DNA has been evaluated as an alternative, avoiding the toxicities associated with the administration of systemic protein. As reviewed in (51) the addition of cytokines including IL-2, IL-12, IL-15 and IL-18 to SIV DNA constructs have augmented protective immune responses in macaques. The cytokine granulocyte macrophage colony-stimulating factor (GM-CSF) has been shown to enhance the primary immune response by activating and recruiting antigen presenting cells to the site of inoculation (183). Both recombinant GM-CSF and plasmids encoding GM-CSF have been used to enhance cellular and humoral immune responses in mice and non-human primate models (38, 267, 348). Although GM-CSF seems to enhance immune responses in animals, two recent phase I trials in man failed to demonstrate the beneficial effect of human GM-CSF. When the cytokine was delivered in conjunction with a HIV-1 peptide based vaccine it did not enhance immune responses (299) and when delivered together with a HIV-1 DNA vaccine the human GM-CSF proved to have a negative effect on both cellular and humoral immune responses (285).

6.7.2 Delivery methods and formulation

The route of vaccine delivery as well as the devices used to deliver the DNA is important for the induction of immune responses. The optimal protocol varies with the antigen/adjuvant used. While DNA vaccines delivered by needle and syringe have induced low to moderate antigen expression, novel application approaches have been able to enhance their potency. Forcing the DNA into the target cells can be accomplished by the use of special devices, such as the gene gun that was the first device used for the delivery of the DNA vaccines (310). The gene gun was originally

developed for gene transfer into plant cells and enables DNA coated gold particles to be transferred into the cells by a helium-propelled acceleration. Epidermal immunisation by gene gun targets both epithelial cells and Langerhans cells, resulting in a presentation to CD4+ and CD8+ T cells (20, 152). In addition to the gene gun, several different techniques like the Biojector, *in vivo* electroporation and different topical administration methods have been developed for delivering DNA.

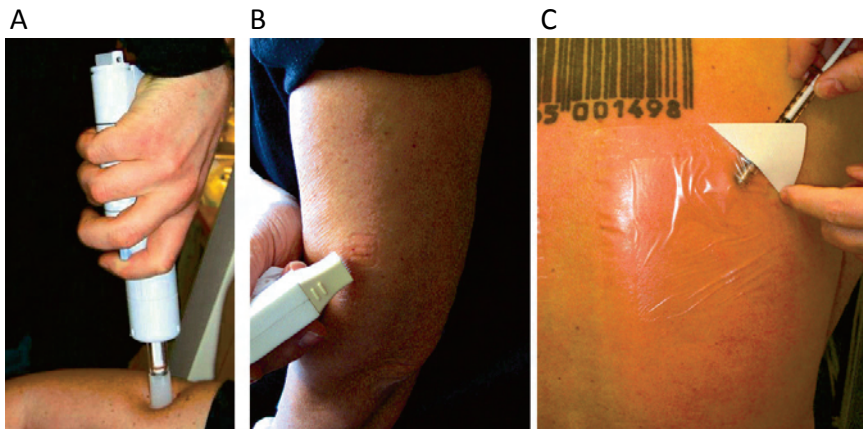


Figure 11. Different administration techniques for DNA immunisation A) Biojector, B) Electroporation and C) topical administration using DermaPrep.

6.7.2.1 Biojector

The Biojector 2000 is CO₂-propelled device used to deliver DNA (either intramuscularly or intradermally) to target cells by a thin stream of liquid (figure 11A). The device disperses the antigen to a large area of muscle or the dermis, the latter has a high frequency of antigen presenting cells. Delivery by Biojector has been shown to enhance cellular and humoral immune responses against various infectious diseases including HIV-1, herpes simplex, and dengue in mice (38, 220, 263). Although several animal experiments have demonstrated that DNA delivery by biojector might be superior to vaccine delivery using needle and syringe (3, 123), a recent experiment in non-human primates showed no difference in immune responses induced by a plasmid vaccine delivered by needle or jet injection (262). We have used the Biojector for delivering DNA intradermally and intramuscularly in two clinical trials (285). The Biojector has also been used for delivering malarial genes in clinical trials, with good immunological responses (333).

6.7.2.2 Electroporation

Electroporation is so far the most impressive delivery method for DNA vaccines. This technique has been used for two decades to improve delivery of chemotherapy drugs intratumourly and in basic research to aid in the transfer of DNA into cells *in vitro* (figure 11B) (119, 240). By electroporation an electric pulse transiently destabilizes the cellular plasma membranes, facilitating the entry of foreign molecules into the cells (272). Although the exact mechanism of entry of macromolecules into the cells is still unclear, entry of small molecules such as anticancer drugs seems to occur by simple diffusion after the pulse (92). Larger molecules such as plasmid DNA are thought to

enter through a multistep mechanism involving the interaction of the DNA molecule with the destabilized membrane during the pulse and then its passage across the membrane (92). Studies in mice and non-human primates have shown that electroporation is a potent technique for inducing vaccine-specific immune responses, inducing a 28-fold higher immune response as compared to conventional plasmid injection (297). Intramuscular electroporation of macaques with SIV gag + IL-15 plasmids, led to an expansion of antigen-specific CD4+ and CD8+ T cells of both central and effector memory phenotype (273). Importantly for HIV vaccines, electroporation has been shown to increase the breadth of immune responses after intramuscular immunisation with a multigene vaccine (197). This study also showed an enhanced humoral immune response with 2-3 log higher Env –specific serum antibodies. So far, only one clinical trial has started in which electroporation is used together with HIV-1 immunogens and is performed by D. Ho at the Aaron Diamond AIDS Research Centre in New York. In this trial, a multi antigen, clade C DNA vaccine is delivered with the Ichor TriGrid device (www.clinicaltrials.gov). There are, however, an abundance of pre-clinical studies testing different electroporation devices together with HIV-1 DNA constructs, and there will most likely be several clinical trials starting in the near future.

6.7.2.3 *DermaPrep*

The DermaPrep vaccine delivery method is a novel approach developed to enhance the uptake of plasmid DNA by antigen presenting cells in the skin (figure 11C) (185, 193). To apply the formulation using the DermaPrep procedure, the skin is first exfoliated to remove the outer stratum corneum, which is a layer of the skin that is able to regenerate rapidly. The DNA plasmids are formulated in a transfectant formulation containing polyethylenimine mannose (PEIm) and glucose solution. A patch is then applied onto the exfoliated skin and the formulation of DNA with PEImannose is delivered under the patch (185, 193).

PEI is able to condense DNA into cationic particles (figure 12) able to bind to syndecan surface receptors and enter the cell via endocytosis (185, 193). Due to its property of acting as a proton sponge, it triggers endosomal swelling and rupture leading to efficient DNA release in the cytoplasm (35). Upon appropriate formulation (5% glucose), the generated PEI/DNA complexes are small, stable and homogenous in size

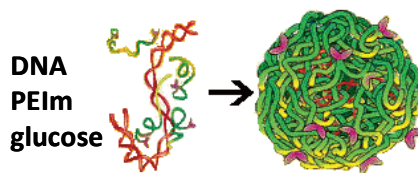


Figure 12. The DNA is formulated with polyethylenimine-mannose (PEIm), and glucose solution to form cationic particles (Adapted from (193)).

(50-80 nm), facilitating diffusion and tissue penetration as any nanoparticle of similar size (33, 120, 350). PEI also protects the DNA from degradation and promotes nuclear transport (44). Formulation of plasmid DNA into nanoparticles may enhance plasmid uptake and gene expression significantly as well as immune responses (reviewed in (126)). In this case, the chemical coupling of mannose to PEI enhances targeting to cells expressing mannose receptors such as dendritic cells.

The exfoliation or disruption of the stratum corneum is required because it triggers Langerhans cells to migrate from the epidermis to the stratum corneum. The exfoliation step is necessary for the penetration of the DNA into the epidermis and to Langerhans cells. Langerhans cells take up and process epicutaneous antigens and migrate to the lymphoid organs. The transfer of antigens to the lymph nodes is crucial for the induction of an immune response.

Topical DermaPrep immunisation of rhesus macaques with plasmids encoding SHIV proteins induced both CD8+ and CD4+ vaccine-specific T cell responses (186). Furthermore, this immunisation regimen induced responses that controlled viral load during repeated interruptions of anti-retroviral chemotherapy in chronically SIVmac251-infected macaques (186). Macaques were immunized during ART treatment which included the cytostatic drug Hydroxyurea (HU) that limits the expansion of CD4+ T cells and works synergistically with NRTIs to lower HIV viral load (109, 192). It was suggested that the immunomodulating properties of HU might prevent excess immune activation and thus favour induction of virus-specific immune responses also in humans (192, 194).

6.7.2.4 Therapeutic immunisation in humans using DermaPrep

We evaluated a HIV-1 DNA vaccine administered with the DermaPrep application method in 12 HIV-infected patients treated with cART (paper IV). The HIV-1 DNA vaccine encodes envelope gp160 of HIV-1 subtypes A, B and C; rev B; Gag A and B and reverse transcriptase (RT) B and has proven to be immunogenic in healthy individuals (285). The patients were randomly assigned to receive 0.4 mg of HIV DNA plasmids using DermaPrep, 0.4 mg of HIV DNA plasmids using DermaPrep with a daily addition of 500 mg of hydroxyurea or placebo (i.e. PEI without DNA using Dermaprep). Six immunisations were performed during three cycles of 7 weeks of cART followed by four weeks of treatment interruption (figure 13). After the last cycle of cART the patients were on long-term treatment interruption until CD4+ T cell counts dropped below 350 cells/mm³ on two occasions.

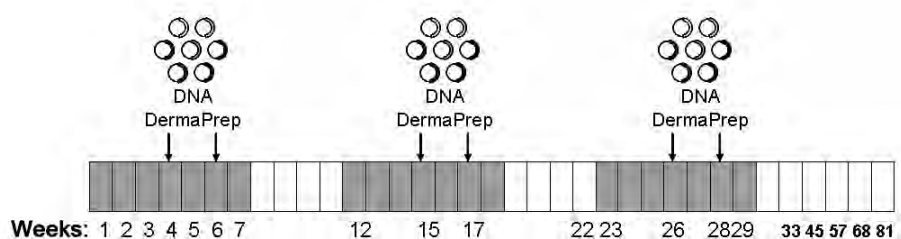


Figure 13. Immunisation schedule of a HIV-1 DNA vaccine trial using repeated treatment interruptions, grey boxes indicate time on cART and white boxes indicate time on STI. Arrows indicate topical HIV-1 DNA immunisation.

After six HIV-1 DNA immunisations were completed, we found a modest vaccine specific increase in IFN- γ responses, suggesting that this delivery technology can be used to induce antigen specific responses in humans (paper IV). The mean net gain of accumulated HIV-specific IFN- γ responses (defined as the mean HIV-specific IFN- γ

SFC/million PBMC detected at visit 10 with the baseline responses subtracted) was significantly higher in the vaccine recipients, as compared to the placebo group (Figure 14 and paper IV).

Furthermore, epitope mapping with vaccine specific peptides revealed that 7 out of 8 patients receiving vaccine had an enhanced immune response to one or more vaccine-specific peptides by visit 10 (after 6 HIV DNA immunisations and 2 STIs) as compared to baseline (table I). In contrast, only one individual in the placebo group revealed an immune response to a new epitope. This suggests that the HIV-1 DNA vaccine strengthens or induces de novo responses to vaccine-specific epitopes in already infected individuals.

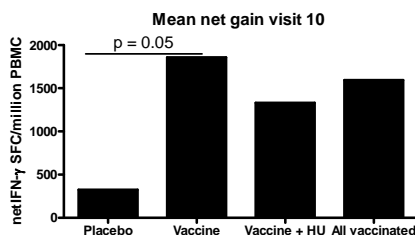


Figure 14: Mean net gain of HIV-specific IFN-γ responses detected at visit 10 with the baseline responses subtracted.

Table I. HIV-specific epitope reactivity at baseline, visit 7 and visit 10 and association with the patients HLA class I alleles

Immunization Arm	Study #	Peptide sequence	Visit/ Peptide reactivity			The patients HLA class I restricting allele
			1	7	10	
Placebo	9013	ALGPAATLEEMMTAC				A*0201
	9013	MFSALSEGATPQDLN				A*0201
	9013	VGGHQAAQM LKETI				A*0301
Vaccine	9003	QMVHQAI SPRTLNAW				B*5701
	9003	EKA FSPEVIPMFSAL				A*6601
	9009	KKKYK LKHIVWASRE				A*2402
	9009	SKVSQ NYPI MQNAQG				A*0201
	9011	WEIKRLRPGGKKKYK				A*0301
	9012	WEIKRLRPGGKKKYK				A*0301
	9012	LETSEGCRQILGQLQ				⊘
Vaccine + HU	9001	EEQNSK KKKAAQAAA				⊘
	9001	QMVHQAI SPRTLNAW				B*0702
	9007	PRGSDIAGTTSTLQE				⊘
	9014	EWDRVHPVHAGPIAP				B*35
	9014	HPVHAGPIAPGQMRE				B*35
	9014	IPVGEIYKRWILGL				B*0801

Bold letters in the peptide sequence indicate the epitope binding to the proposed HLA-1 molecule. Peptide reactivity; white area = non reactive, light grey area = 55-500 SFC/million PBMC, dark grey area = 500-1000 SFC/million PBMC, black area = >1000 SFC/million PBMC, ⊘ = epitopes not previously associated with the patients particular HLA class I alleles.

The vaccine-induced immunogenicity was not associated with decreased viral replication following the last treatment interruption (paper IV). In the whole group, however, the viral load set point, determined after the final treatment interruption was significantly lower, 0.5 log viral load, in all patients (including placebos) compared to the viral load set point before the first the initiation of cART (paper IV). A transient decrease in viral load levels has been observed in many studies evaluating STIs (34,

184). We conclude that our vaccination approach did not induce any direct clinical benefits. The lack of effect can be attributed to a number of parameters including the immune status of the vaccinated host, the low amount of genetic material used and the repeated STIs. During time off treatment, viral rebound occurs with concomitant activation and turnover of CD4+ T-helper populations that are generally in low numbers during effective ART mediated viral suppression (292). The increased immune activation and the increased infection of CD4+ T cells may destroy the potential benefit of the immunisation. Our conclusion is, that repeated STIs should not be combined with immunisation.

6.8 VIRAL VECTORS

Recombinant viral vectors have emerged as a potentially useful vaccine modalities in genetic vaccine technology. Viruses have evolved specialized molecular mechanisms to deliver their genomes inside the host cells and to produce viral proteins using the host cell machinery. Therefore, various viruses have been explored as delivery vehicles for foreign genes, with the aim to induced potent and long lasting immune responses in the vaccinated host (reviewed in (39)). In HIV-1 vaccine research, the most commonly used viral vectors are replication incompetent Adenovirus 5 (Ad5) (47) and various attenuated poxviruses including avipox viruses canarypox virus (ALVAC) (320) and Fowlpox (89), Modified Vaccinia virus Ankara (MVA) (285) and New York Vaccinia virus (NYVAC) (133), the latter two derived from vaccinia virus.

6.8.1 Modified Vaccinia Virus Ankara

Poxviruses have played an important role in the field of vaccinology ever since Edward Jenner demonstrated in 1798 that it was possible to protect humans against smallpox by inoculation of the related cowpox virus. Poxviruses are the largest virus known to induce disease in humans. They are double-stranded DNA viruses with a genome size of 150-300 kb. Vaccinia virus is the prototypical poxvirus and has been administered to more than 1 billion people during the successful smallpox eradication program. The large size of the vaccinia genome and the stability of recombinant vectors allow large amounts of foreign genetic material to be contained in a single vaccinia virus vector. Another major advantage is that proteins encoded by vaccinia virus tend to be more immunogenic than genes or proteins delivered without a vector, most likely secondary to the inflammatory response triggered against highly immunogenic vaccinia proteins. In contrast to other DNA viruses like Adenovirus, poxviruses replicate in the cytoplasm of the host cell as they encode and carry proteins necessary for replication outside the nucleus.

Despite the success of using vaccinia virus during the smallpox eradication campaign, there were concerns regarding safety. While inoculation of the vaccine produced pustular lesions in the majority of vaccinees, immunocompromised individuals could in rare cases experience severe neurological side effects (170). This prompted the development of a safer smallpox vaccine. This process started with the development of vaccinia virus Ankara by sequential passages of vaccinia virus in calves and donkeys in the vaccine institute in Ankara, Turkey. Several hundreds of additional passages of vaccinia virus Ankara in chicken embryo fibroblasts resulted in the deletion of genes encoding immunomodulatory functions and host range determinants, and thus

Modified vaccinia virus Ankara was generated (217, 233, 308, 309). This replication-deficient virus has a substantially improved safety profile as compared to the original vaccinia virus (217, 302). MVA has an excellent safety record as a smallpox vaccine, for which it was used during the late phase of the smallpox eradication campaign. Moreover, MVA is safe in immunocompromised animals and humans and might therefore be applicable as a vector even in HIV-infected persons (31, 67). While rMVA carrying HIV-1 genes is highly immunogenic in mice (37), it loses some of its potency in non-human primates (56) and humans (251). Thus, different prime-boost combinations with plasmid DNA or subunit vaccines are presently under investigation in clinical trials (85, 133, 268, 285).

As a consequence of smallpox immunisations carried out worldwide until the mid-1970s (65, 131), anti-vaccinia virus immunity is present in the older population, but not in younger individuals. The numbers of vaccinia-naïve individuals will continue to increase, enabling wider use of poxviruses as vaccine vectors. MVA has been further processed to carry genes from other pathogens like malaria, tuberculosis and HIV-1. However, the increasing use of poxvirus as vectors these pathogens might in the long run restrict the use of these vectors, due to the potential drawbacks of vector specific immunity.

6.8.1.1 Poxviral vectors in therapeutic HIV-1 vaccine trials

The potential of poxviral vectors as therapeutic HIV-1 vaccines has been evaluated in a number of studies. The most widely explored poxvirus vectors for therapeutic HIV vaccination are based on the canarypox virus, the recombinant ALVAC vectors. Several generations of recombinant canarypox virus vaccines have been tested in HIV-1-seronegative and HIV-1-infected individuals, where they have been proven safe. The immune responses, however, have been variable (22, 323). A study evaluating a canarypox vaccine (vCP-1433) plus a lipopeptide vaccine and subcutaneous IL-2 in cART treated patients, followed by analytical therapy interruption revealed a significant delay in viral rebound. A delay in time to restart cART was also observed in vaccine recipients, although this translated into a median increase in time off therapy of three weeks (178). Another recombinant canarypox vaccine ALVAC-vcP1452 was used in a prime-boost combination with recombinant gp160 and was found to be immunogenic in patients who had initiated cART within three months of seroconversion (150). However, no effect on viral rebound was apparent in vaccine recipients who subsequently stopped cART in a rollover study (207). Moreover, a recent study revealed that administration of the ALVAC-vcP1452 vaccine alone was associated with a shorter time to resume therapy and higher viral load rebound, despite significant induction of immune responses (15).

In two phase-I trials, a recombinant MVA expressing the HIV-1 nef protein was shown to be safe and immunogenic in cART treated HIV-1-infected subjects (68, 138). This vaccine induced CD4+ T cell responses and lowered viral load set-point as compared to pre-cART viral load levels in some patients after cART withdrawal. A recent trial evaluated HIV-1 recombinant MVA and Fowlpox vectors in young HIV-1 infected individuals on cART treatment. The vaccine induced increased frequencies of HIV-1

specific CD4+ T cell proliferation responses and increased the breadth (i.e. number of recognised epitopes) of CD8+ T cell responses (127).

6.8.2 Adenovirus vectors

Adenoviruses are stable non-enveloped viruses with a double stranded DNA genome of approximately 38 kb. As adenoviruses replicate in the nucleus of a broad range of cells, they are popular as vaccine vectors. The majority of adenoviruses do not integrate their viral genome into the host chromosomes; however there are some concerns about integration (301). The recombinant vectors are made by inserting the foreign genetic material directly into the adenovirus genome, or by depletion of the early genes E1 or E1 and/or E3 that enables stable insertion of larger gene segments (7-10kb) (71). The deletion of the early genes renders the vector replication incompetent, which is a benefit with regard to safety. As the most frequently used Adenovirus vectors are based on serotype 5 and this serotype is highly prevalent in the human population (247), there may be a problem with anti-vector immunity. In addition, Adenovirus vectors are highly immunogenic and induce strong immune responses against the vector itself. As rAd5 vectors containing HIV-1 genes were pre-clinically shown to elicit high levels of HIV-specific CTL responses (56), this vaccine platform entered a phase IIb vaccine trial.

6.8.2.1 The STEP trial

A phase IIb proof-of-concept trial known as STEP was conducted by the pharmaceutical company Merck together with the HIV Vaccine Trials network (HVTN). Persons at high risk of acquiring HIV-1 were immunized three times with a trivalent recombinant Ad5 vaccine encoding HIV-1 Gag, Nef, and Pol. However, the trial was suspended based on interim data that concluded that the vaccine could not be shown to prevent HIV-1 infection or reduce the amount of virus in those who became infected (47). Despite the presence of vaccine-induced HIV-specific CD8+ T cell responses, the vaccine failed to reduce viral load in participants who acquired HIV-1 infection (213). Disturbingly, the post-hoc analysis of the data revealed that uncircumcised men with pre-existing neutralizing antibody responses against Ad5 receiving the vaccine were at higher risk for HIV-1 infection following immunisation than the volunteers in the group that received placebo (47). The influence of vector immunity and its association with higher HIV-1 infection is not fully understood, this is further discussed below in 6.1.1. Efforts to develop new effective adenovirus vectors based on rare serotypes like Ad26 and Ad35 are currently ongoing. These constructs show protective effect in SIV challenged monkeys when used in combination with Ad5 (188).

Due to the results from the STEP trial and the issues regarding safety of Ad5 vectors, several phase I/II trials planned to use DNA prime followed by Ad5 booster doses, have been stopped (259).

6.9 PRIME-BOOST CONCEPT IN PROPHYLACTIC VACCINE TRIALS

Heterologous prime-boost protocols, defined as sequential immunisation using different vaccine modalities, have been shown to greatly enhance both antibody and T

cell responses against a variety of pathogens and tumour antigens. This approach is a potent way to focus and strengthen antigen-specific immune responses. By priming with DNA the immune response is focused on the desired antigen, and by boosting once or twice with antigens in a heterologous vector like MVA or adenovirus or with recombinant protein, the initial response can be amplified (229). The importance of DNA as a priming agent has been described by several groups (212, 266). Currently, the only ongoing Phase III HIV-1 vaccine trial is with the Sanofi Pasteur live recombinant ALVAC-HIV prime and VaxGen gp120 B/E (AIDSVAX B/E) boost. This study is anticipated to end in 2010.

Our group uses a HIV DNA prime HIV MVA boost strategy where the DNA contains HIV-1 Env subtypes A, B, C; Gag subtypes A and B; Rev, RT subtype B (271). The MVA is produced by Walter Reed Army Institute, MD, USA and contains Env subtype E and a Gag/Pol fusion protein of subtype A (285). Thus, this is a heterologous prime-boost schedule both in terms of vectors and the subtypes of the genes. This strategy is currently under evaluation in two clinical trials in Stockholm, Sweden and in Dar Es Salaam, Tanzania, respectively. The frequency of responders after the first, (out of two) MVA booster doses was 97% in the Swedish volunteers (285). Geovax has used a similar DNA prime MVA boost strategy where the DNA contains several HIV-1 antigens. DNA and MVA constructs contain identical genes encoding Gag, Pol and Env from HIV-1 clade B. After the second MVA injection a balanced cellular response between Gag and Env was detected as well as an Env antibody response frequency of 88% (117). A DNA prime MVA boost strategy has also been studied by McMichael and colleagues (251). Their DNA vaccine encoded a string of T cell epitopes from HIV-1 and a fragment encoding a subtype A HIV-1 Gag; this was followed by an MVA boost that encoded the same antigens. However, like other epitope vaccines delivered by different DNA approaches, only low level T cell responses to the individual epitopes were induced in healthy individuals. When delivering this vaccine, to HIV-1 infected individuals, a significant boosting of existing Gag-specific CD8+ and CD4+ T-cell responses was observed. These responses seemed to have no effect on viral load (82).

The EuroVacc consortium have used a DNA prime-NYVAC boost strategy in which the vaccine constructs encode HIV-1 subtype C Env, Gag, Pol and Nef. This vaccine induced Env-specific T cell responses in almost all vaccine recipients; in contrast the T cell responses against Gag, Pol, and Nef were generally low and transient (133).

A DNA prime followed by protein boost schedule has also been studied in humans. Novartis is currently performing a trial where they use a HIV DNA prime followed by a trimeric gp140 envelope protein boost. Lu et al. uses a DNA vaccine containing one subtype C *gag* gene and 5 *env* genes (subtypes A, C, & E + two from clade B), plus a rgp120 protein boost that contains the same antigens. Both studies have shown strong induction of anti-HIV-1 antibodies as well as CD4+ T cell responses, but only a limited neutralising antibody response (166, 334).

7 IMPLICATIONS OF VIRAL VECTOR IMMUNITY

A potential drawback with viral vectors is vector specific-immunity. An immune response to the vector component may hamper the induction of an effective immune response against the foreign antigen delivered by the vector (345). Anti-vector antibodies may reduce the actual vaccine dose by early neutralisation of the inoculum, thus preventing efficient delivery of the foreign gene. The immune responses to viral vectors can either be induced by closely related naturally occurring virus infections or by previous immunisation with a vaccine based on the same (or closely related) viral vector.

7.1 ANTIVECTOR IMMUNITY REDUCES INSERT SPECIFIC IMMUNE RESPONSES

The effect of pre-existing immunity against vaccinia has been studied predominantly in the context of neutralizing humoral immunity (69, 99, 249). However, pre-existing Ad5-specific neutralising antibodies have been reported to reduce cellular immune responses of rAd5 vector based vaccines in clinical trials (57, 259). We have evaluated the impact of pre-existing immunity to MVA in individuals receiving a HIV-1 DNA MVA vaccine (paper V). This study included, forty healthy HIV-1 negative Swedish volunteers, that were immunized three times with HIV-1 DNA plasmids (described in chapter 5.7.2.4) intradermally (id) or intramuscularly (im), with or without adjuvant GM-CSF. The volunteers were block re-randomized at month 9 to receive a single boost of either 10^7 plaque-forming units (PFU) id or 10^8 PFU im of recombinant HIV-1 MVA, which expresses subtype E gp150 Env and Gag/Pol from Thai isolates CM235 and CM240. This immunisation strategy resulted in a 97% HIV-specific cellular response frequency as described in (285). We found that pre-existing immunity to vaccinia did not affect the frequency of vaccinees with a positive cellular response to HIV peptide pools. However, pre-existing humoral immunity to vaccinia decreased the magnitude of cellular (figure 15 A and B) and humoral HIV-specific responses (paper V).

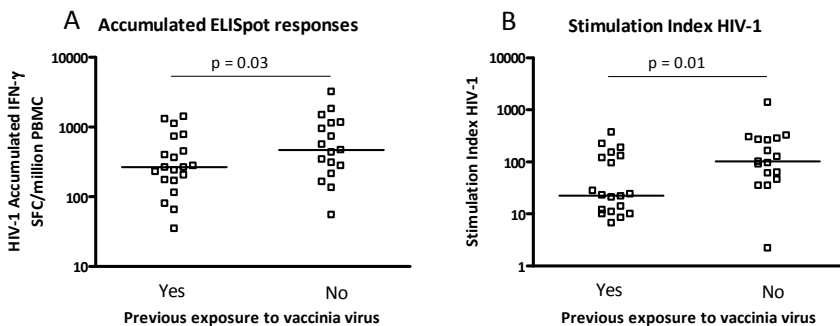


Figure 15. HIV-specific cellular immune response after HIV-1 MVA boost, related to vaccinia vaccination status. Short horizontal bars indicate medians. A) Distribution of accumulated HIV-1 IFN- γ ELISpot responses. B) Distribution of HIV-1 antigen-specific T-cell proliferative responses.

The reduced HIV-specific immune responses may be due to: 1) Anti-vector antibodies that reduce the vaccine dose through early neutralization of the inoculum, thus preventing the delivery of the recombinant gene. 2) Antibody-dependent cellular cytotoxicity that may eliminate antigen-producing cells and subsequent induction of immune response. 3) Competition between vector-specific and HIV-specific antigen presentation, where the more immunodominant vector components may be displayed to a higher extent.

We also observed that even when 30 years had passed since smallpox immunisation, several volunteers still maintained clearly detectable levels of vaccinia-neutralizing antibodies in serum (paper V). A previous study of the persistence of immunity following smallpox vaccination showed that vaccinia-specific antibodies may persist up to 75 years after vaccination, whereas T-cell responses decline, with a half-life of 8-15 years [22]. In our study (paper V), the higher dose of 10^8 PFU HIV-1 MVA induced significantly higher serological vaccinia-specific responses than a lower dose of 10^7 PFU. After the HIV-1 MVA boost, the anti-vaccinia antibodies persisted at high levels for several months in individuals displaying pre-existing vaccinia responses prior to the immunisation. In vaccinia-naïve individuals, on the other hand, the primary anti-vaccinia antibody responses induced by HIV-1 MVA were transient. After twelve weeks, the titers of neutralizing antibodies had dropped to baseline levels in a majority of the vaccines (paper V).

Our results suggest that vaccinia-based antigens can be used efficiently to induce immunity to the vectored HIV-1 antigens, even in individuals with pre-existing vaccinia immunity. As the magnitude of the HIV-1 specific immune responses was somewhat dampened by vector immunity and vector specific immune responses in vaccinia naïve individuals were transient, it may be beneficial to lengthen the interval between sequential administrations of MVA, to elicit the best conceivable HIV-specific immune responses.

Heterologous prime-boost regimens are one way to avoid the potential impact of pre-existing immunity against the vector (251, 345). By reducing the risk of neutralisation of the vaccine vectors, the use of heterologous prime-boost promotes efficient delivery of the inserted gene and favours expansion of the insert-specific memory population over the vector-specific responses (187, 230).

7.1.1 Safety concerns antivector immunity

In light of the discontinued STEP trial, safety concerns regarding anti-vector immunity have emerged. As mentioned above, a post-hoc analysis of the data revealed that those who entered the trial with existing immunity to Ad5, and were uncircumcised, appeared to have an increased risk of HIV-1 infection if they received the vaccine rather than placebo (47). The reasons behind the increased risk of infection are not yet completely understood (93, 213). One hypothesis is that the vaccine induces activation and expansion of Ad5 specific T cells in Ad5 seropositive individuals. These cells could then serve as targets for HIV-1 infection, as HIV-1 mainly infects activated T cells. However, recent results from M. Betts lab show that there is no correlation between anti Adenovirus antibodies and Ad5 specific CD4+ T cells in the blood of Ad5

seropositive individuals (147). The group has studied individuals included in a precursor study to the STEP trial where the volunteers received the same vaccine as the volunteers in the STEP trial. Furthermore, the MRKAd5/HIV-1 vaccine does not induce higher activation in infected as compared to non-infected persons, as measured by Ki67 expression or up-regulation of HIV-1 co-receptor CCR5 (213). These results suggest that the reason behind the increased risk of infection is not linked to activated Ad5 specific CD4+ T cells in the blood. Another hypothesis is that immunisations with either Ad5 vector alone or with combinations of different Adenovirus vectors may induce a general CD4+ T cell activation and that such cells home to the mucosa (311). As the primary target for HIV-1-specific cells is activated CD4+ T cells, a high number of activated T cells in the mucosa might allow more efficient infection of HIV-1 at mucosal surfaces resulting from immunisation with Adenovirus vectors (296). This could explain the higher incidence of HIV-1 acquisition in the vaccine recipients of the STEP trial.

Pantaleo et al. set up an in-vitro model to look at the effect of Ad5 immune complexes, found in Ad5 seropositive patients. They reported that Ad5 immune complexes together with an Ad5 vector caused enhanced HIV-1 infection in DC-T cell co-cultures(250). These results suggest the Ad5 immune complexes activate a DC-T cell axis that may set up a permissive environment for HIV-1 infection, which could account for the increased acquisition of HIV-1 infection among Ad5 seropositive vaccine recipients in the STEP trial.

8 CONCLUDING REMARKS

The development of effective therapeutic immunisation aimed at improving control of HIV-1 infection remains an important goal for HIV research. Structured treatment interruption can be used to evaluate whether vaccine recipients experience any benefit following cessation of cART. The time to resumption of cART may be based upon pre-defined cycles, plasma viral load/CD4+ T cell measurements or other relevant parameters. As described above, STIs are controversial, and there is a general consensus that STIs might cause harm under certain conditions, (54, 110, 346). However, it is the only endpoint that can prove the true benefit of therapeutic immunisation in HIV-1 infected cART treated patients. If STIs are to be used as an analytical tool in HIV-vaccine development, close monitoring of viral load and CD4+ T cell counts and high standard criteria for resumption of cART is crucial for the safety of the patients. Viral load set point is not predictive of time to treatment in HIV-1 infected cART treated patients on treatment interruption (78), the nadir CD4+ T-cell count, however, appears to be predictive of the length of time that patients take off treatment when re-initiation is defined by predetermined parameters (115). Thus it is crucial for studies using time to re-initiation of cART as a marker for vaccine success to start to randomise patients on the basis of their nadir CD4+ T cell count.

It would be very interesting to explore the possibility of performing HIV-1 vaccine trials in the context of cART in patients in the very early phase of the infection, as we know that the outcome of the infection is set during this period. The results from the QUEST trial, where the effect of therapeutic immunisation with either Remune or ALVAC was evaluated in patients who initiated cART during acute HIV infection, suggested that immunisation in the early phase of infection did not improve the outcome of disease progression (161). However, it is not clear whether the lack of apparent effect of these vaccination strategies on viral rebound indicates poor immunogenicity.

Recent advances in the HIV-1 vaccine field have provided important information for the design of therapeutic antigens. Ideally therapeutic immunisation should stimulate polyfunctional CD4+ and CD8+ T cell responses that can slow down CD4+ T cell decline reduce or eliminate viral reservoirs, and induce an immune status allowing control of infection. Although there has been much focus on designing antigens and enhancing uptake and immunogenicity of vaccines, so far no measures have translated into significant clinical effect. The modest and transient effects observed with currently available vaccines highlight the need to develop novel strategies. As T cell exhaustion may be the main reason for ineffective viral control during HIV-1 infection, it could be beneficial to try to reduce the immune activation, rather than targeting the virus, or to target both in combination approaches. PD-1 has been shown to be highly expressed on HIV-specific CTLs, and it's expression correlates with exhaustion and inability to respond to proper stimuli (73, 319, 347). Thus PD-1 is defined as a potential therapeutic target for restoring the functional capacity of HIV-specific CTLs. Initial experiments of a PD-1 blocking antibody have been completed in monkeys and clinical trials in cancer with diffuse large-B cell lymphoma have begun (as reviewed in (84)). These initial studies will provide a foundation on which decisions to move into human trials in HIV-infected subjects can be based.

Within the scope of this thesis we have observed modest effects using currently available DNA vaccines in combination with treatment interruptions in HIV-1 infected individuals. This emphasises the need to develop novel immunisation strategies which combine immunisation with antiviral therapy. We have found that previous immunity to vaccinia only moderately reduced the HIV-specific immune responses when vaccinia was used as a vector for HIV genes in healthy individuals. Since the recombinant adenovirus used by Merck failed as an HIV-1 vaccine, there are concerns that other viral vectors including vaccinia virus will meet the same fate. However, findings included in this thesis provide evidence that recombinant MVA could effectively boost DNA-primed HIV-specific immune responses, despite pre-existing vaccinia immunity. Over the past several years there has been significant scientific progress in understanding HIV-infection, and how the virus interacts with the human immune system. Despite these advances, HIV-1 is a difficult virus to target and developing a safe and effective vaccine that protects people against infection will involve overcoming several scientific obstacles. On this long, winding and often bumpy road towards an effective HIV-vaccine 'we must accept finite disappointment, but we must never lose infinite hope' (quote by Martin Luther King Jr).

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