

TARGETING HIV-1 ENTRY AND REVERSE TRANSCRIPTION BY VACCINATION

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STOCKHOLM 2002

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AND REVERSE TRANSCRIPTION
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STOCKHOLM 2002

To my family

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ABSTRACT

Human immunodeficiency virus type 1 (HIV-1) is a complex retrovirus, which uses the CD4 receptor and chemokine receptors to infect its target cells. The chemokine receptor CCR5 is essential for primary HIV-1 infection. The hallmark of retroviruses is the enzyme reverse transcriptase (RT), which transcribes the virus genome from RNA to DNA. RT is a major target for HIV drugs but antiviral treatment often selects for drug resistant virus variants. RT lacks proofreading, which accounts for a great deal of the high variability of HIV-1 genomes. The aim of this thesis was to evaluate the RT gene and the CCR5 gene as immunogens and to develop an animal model for HIV-1 drug resistance.

The human CCR5 gene was used together with the adjuvant granulocyte macrophage colony-stimulating factor (GM-CSF) for immunization of mice and monkeys (*cynomolgus* macaques). Immunization with the CCR5 gene induced antibody responses in both mice and monkeys. The macaques were boosted with CCR5 peptides in the rectal mucosa. This potentiated local IgA responses as well as systemic IgA and IgG antibody responses to human and macaque CCR5 peptides. The detected CCR5 antibodies demonstrate that we were able to break tolerance and immunize against the endogenous CCR5 receptor. Interestingly, the CCR5 peptide boost abolished or decreased CCR5 gene-induced antibody responses towards native CCR5. Sera from vaccinated animals blocked infection of peripheral blood mononuclear cells (PBMC) with HIV-1 and with simian immunodeficiency virus sooty mangabey (SIVsm) *in vitro*. Regrettably none of the CCR5-immunized monkeys were protected from SIVsm challenge. In one vaccinated animal the virus surprisingly evolved and started to use the CCR5 receptor more efficiently than before. Thus CCR5 immunization may lead to the evolution of viruses with new properties which may be negative for the patient. This has to be investigated further.

Different immunization strategies were tested in order to induce strong RT specific immune responses. The adjuvant effect of bacterial DNA (CpG-oligodeoxynucleotides, ODN) was evaluated in this context. A prime with the RT gene followed by protein with CpG-ODN boost induced the most potent cellular immune responses in both mice and macaques. After challenge of mice with HIV-1/murine leukemia virus (HIV-1/MuLV), cellular immune responses in splenocytes were equal in the groups: receiving a) DNA followed by protein with CpG-ODN or b) protein with CpG-ODN. In both these groups protection from challenge was observed in some of the vaccinated mice. Consequently the evaluated three-component vaccination strategy induced potent humoral and cellular immune responses to RT and provided partial protection from HIV-1. RT may therefore be an important component of an HIV-1 vaccine.

HIV-1 drug resistance mutations found *in vitro* do not always correlate to the mutations found *in vivo* in treated patients. An *in vivo* model for the study of resistance development in macaques was constructed. The occurrence, type and duration of RT mutations during treatment with the non-nucleoside inhibitor nevirapine were analyzed over time. We found a good correspondence of mutation pattern and kinetics between the monkey model and known data from nevirapine treated patients. Mutation K103N found in the drug treated monkeys was infrequently selected by nevirapine in cell culture. This indicates that the monkey model might be used to detect mutations that are difficult or impossible to predict *in vitro*. This model can also be used to study new vaccines targeted to RT with drug-induced mutations.

In conclusion we were able to induce immune responses to both the CCR5 and the RT gene products. CCR5 specific antibodies induced unpredicted virus evolution to a higher receptor affinity. RT specific immune responses partially protected mice from HIV-1/MuLV infection. A primate model for HIV-1 drug resistance was successfully developed. This model may be used in future studies of vaccines directed to RT with drug-induced mutations.

Key words: HIV-1, DNA vaccination, coreceptor, CCR5, RT, drug resistance, CpG, pseudovirus

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ORIGINAL PAPERS AND MANUSCRIPTS

This thesis is based on the following papers, which will be referred to by their roman numerals.

- I. **Zuber, B.**, Hinkula, J., Vödrös, D., Lundholm, P., Nilsson, C., Mörner, A., Levi, M., Benthin, R., and Wahren, B. (2000). Induction of immune responses and break of tolerance by DNA against the HIV-1 coreceptor CCR5 but no protection from SIVsm challenge. *Virology*, 278(2):400-411.
- II. **Zuber, B.**, Vödrös, D., Mörner, A., Nilsson, C., Walther-Jallow, L., Ten Haaf, P., Heeney, J., and Wahren, B. CCR5 gene immunization may select for SIV that uses CCR5 more efficiently. Manuscript.
- III. **Zuber, B.**, Kjerrström Zuber, A., Mäkitalo, B., Rollman, E., Ljungberg, K., Hinkula, J., Klingström, J., Eriksson, L.E., Mathiesen, I., Isagulants, M.G., Öberg, B., and Wahren, B. Potent and protective immune responses to the HIV-1 RT gene / protein aided by CpG-ODN in a prime-boost regimen. Submitted.
- IV. **Zuber, B.**, Böttiger, D., Benthin, R., Ten Haaf, P., Heeney, J., Wahren, B., and Öberg, B. (2001). An *in vivo* model for HIV resistance development. *AIDS Res Hum Retroviruses*, 17(7):631-635.

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LIST OF ABBREVIATIONS

aa	Amino acid
Ab	Antibody
ADCC	Antibody-dependend cellular cytotoxicity
AIDS	Acquired immunodeficiency syndrome
APC	Antigen presenting cell
bp	Base pair
CCR5	C-C (beta) chemokine receptor 5
CMV	Cytomegalovirus
CpG-ODN	Cytosin-Guanosin dinucleotide (unmethylated)-oligodeoxynucleotide
CTL	Cytotoxic T lymphocyte
CXCR4	C-X-C (alpha) chemokine receptor 4
DC	Dendritic cell
ECL2	Extracellular loop 2
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-Activated Cell Sorter
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HAART	Highly active antiretroviral therapy
HIV-1	Human Immunodeficiency Virus Type One
HIV-2	Human Immunodeficiency Virus Type Two
HIV-1/MuLV	Human Immunodeficiency Virus Type One / Murine Leukemia Virus
HIV-1 R5	HIV-1, which uses the CCR5 receptor
HIV-1 X4	HIV-1, which uses the CXCR4 receptor
HLA	Human Leukocyte Antigen
IgG, IgA, IgM	Immunoglobulin classes G, A, M
IFN	Interferon
IL	Interleukin
MAb	Monoclonal antibody
MHC	Major Histocompatibility Complex
NNRTI	Non-nucleoside RT Inhibitors
NRTI	Nucleoside RT inhibitors
NSI	Non-syncytium inducing
PBMC	Peripheral blood mononuclear cells
pol	Polymerase
RT	Reverse Transcriptase
SHIV	Simian-Human Immunodeficiency Virus
SIV	Simian Immunodeficiency Virus
SIVsm	SIV derived from the sooty mangabey monkey
TNF-	Tumor necrosis factor alpha
wt	Wild type

1 INTRODUCTION TO HIV AND AIDS

In the early 1980s an epidemic of acquired immunodeficiency was observed in the United States. The syndrome was soon linked to groups of people with certain behavioral characteristics, such as homosexuals or injecting drug users. Immunodeficiency often leads to opportunistic infection with microbes, which our immune system usually defeats. They give rise to disease because of deficient cellular immune responses. Reports described an increased incidence of *Pneumocystis Carinii* pneumonia and an aggressive form of Kaposi's sarcoma in previously healthy young homosexual men (CDC, 1981; Gottlieb *et al.*, 1981). Many of the patients had unusually low numbers of CD4+ T-lymphocytes, which explained the immunodeficiency symptoms. The new disease was called acquired immunodeficiency syndrome (AIDS). In 1983 the causative agent of AIDS was identified (Barre-Sinoussi *et al.*, 1983; Gallo *et al.*, 1983). Gallo and coworkers argued that the known human T-cell leukemia virus I (HTLV-I) was the possible cause of the AIDS epidemic, while Montagnier's laboratory described it as a new HTLV. One year later Gallo *et al.* published four papers in *Science* showing that the cause of AIDS was a new virus related to HTLV-1 and HTLV-II and given the name HTLV-III (Gallo *et al.*, 1984; Popovic *et al.*, 1984; Sarngadharan *et al.*, 1984; Schupbach *et al.*, 1984). Later the virus was re-named human immunodeficiency virus type 1 (HIV-1) (Coffin *et al.*, 1986). HIV-1 was identified as a primate lentivirus belonging to the family of retroviruses.

A search for a vaccine was initiated soon after the virus had been identified.

Several species of non-human African primates carry HIV-1 related retroviruses called simian immunodeficiency viruses (SIVs). HIV-1 and the similar but immunologically distinct HIV-2 (Clavel *et al.*, 1986) probably originated in Africa by cross species transmission of SIV from monkeys to humans (Franchini *et al.*, 1987). SIVcpz from chimpanzees probably gave rise to HIV-1 (Huet *et al.*, 1990) while SIVsm from sooty mangabeys probably gave rise to HIV-2 (Hirsch *et al.*, 1989).

More than 60 million people have been infected with the virus since the epidemic began and 22 million have died because of the disease. HIV/AIDS is now the leading cause of death in sub-saharan Africa. Worldwide, it is the fourth biggest killer. At the end of 2001 the World Health Organization (WHO) estimated that 40 million people globally were living with HIV.

Infectious diseases can be controlled effectively and even eradicated if a vaccine is available. The virus is transmitted via sexual contacts, blood products and from mother to child. According to WHO, a vaccine is required to complement and enhance the effectiveness of existing preventive strategies to control the HIV/AIDS pandemic, especially in developing countries.

2 HIV-1 PATHOGENESIS

The evolution of HIV-1 infection can be divided into three stages. The first stage is acute HIV-1 infection corresponding to primary infection with HIV-1 (Feinberg and Greene, 1992). Acute HIV-1 infection may be asymptomatic or manifest as a mononucleosis-like illness. During the acute stage the plasma viral load usually peaks and then gradually declines to a steady state level (Fig. 1). The second stage is chronic, representing a period of clinical but not necessarily virological latency. It has been demonstrated that there is continuous production of virus during this stage that can be controlled by the immune system (Ho *et al.*, 1995). After the asymptomatic stage, the immune system gradually starts to lose the battle with the virus. The number of CD4⁺ cells declines and the viral load increases (Fig. 1). Finally there is a crisis stage where immunodeficiency is manifest by the development of opportunistic infections and other pathological conditions. At that stage the immunocapacity of the host is poor (Wahren *et al.*, 1986).

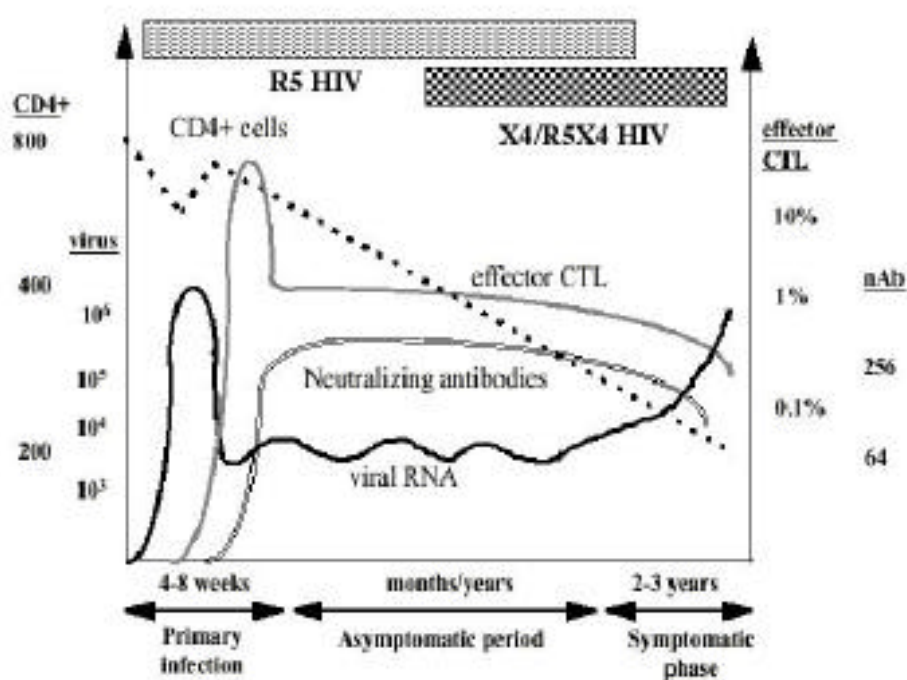


Figure 1. The HIV-1 infection. Neutralizing antibody titer (nAb) (Moore *et al.*, 1994) and percent effector CTL in PBMC (Calarota *et al.*, 1998; Moss *et al.*, 1995; Pantaleo *et al.*, 1994). CD4⁺ T cell count (x10⁶/ml) and virus load as RNA copies/ml blood (Ho *et al.*, 1995). Predominant viral tropism shown as lines on top of the graph: R5; CCR5 (macrophage-tropic) and X4; CXCR4 (T-cell-tropic) virus isolates. There is a peak of virus, which stabilizes to a steady state level when the virus is controlled by CTL.

3 MOLECULAR BIOLOGY OF HIV

3.1 THE VIRAL GENOME

The HIV-1 and SIV genomes both encode precursor polypeptides and also contain several additional open reading frames (Fig. 2). The *gag* gene encodes the precursor for virion capsid proteins, the *pol* gene encodes the precursor for three important enzymes (protease, reverse transcriptase with RNase H, and integrase), and the *env* gene encodes the precursor of envelope glycoprotein. Two overlapping exons encode the genes for transcriptional transactivator (*tat*) and regulator of viral expression (*rev*); these small nonvirion proteins are essential for viral replication. A number of HIV-1 and SIV genes are dispensable for viral replication in tissue culture cells. These genes, also called “accessory” genes encoded by HIV-1, are *vif*, *vpr*, *vpu* and *nef*.

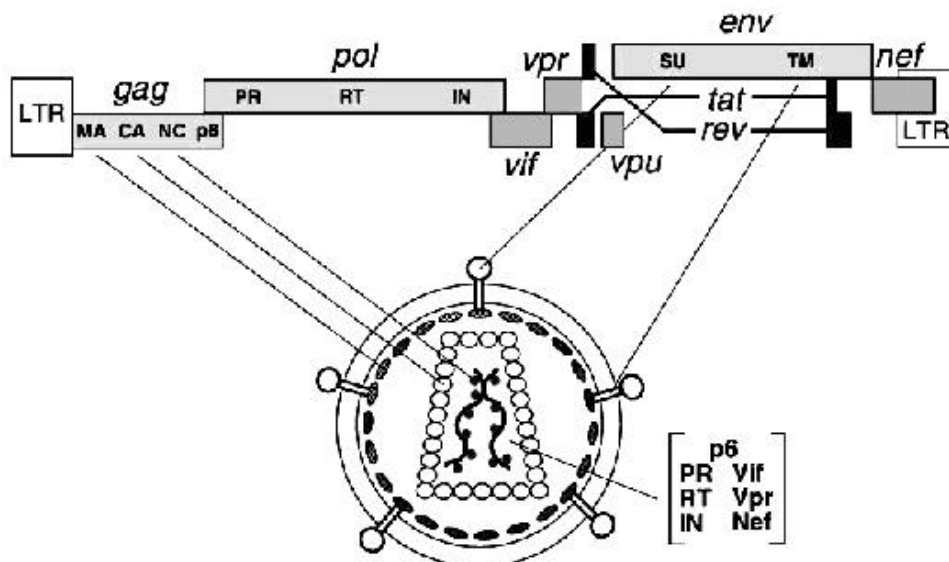


Figure 2. The viral genome and virion structure. Long terminal repeat (LTR) promoters flank the three major genes shared by all retroviruses: *gag*, *pol* and *env*. Precursor polypeptides Gag-pol (Pr160), Gag (pr55) and Env (gp160) are enzymatically processed to yield mature virion proteins. Gag generates capsid (CA) p24, matrix (MA) p17, nucleocapsid (NC) p7, and the p6 protein which binds Vpr. Polymerase (*pol*) of the Gag-pol precursor is processed to form protease (PR) p10, reverse transcriptase (RT) p66/p51, ribonuclease H (RNase), and integrase (IN) p32. Envelope (ENV) gp160 is cleaved to form gp120 (SU) surface and gp41 (TM) transmembrane envelope. Adapted from (Frankel and Young, 1998).

3.2 THE REPLICATION CYCLE

HIV-1 is a complex retrovirus belonging to the lentivirus genus of the Retroviridae family (Fields, Knipe, and Howley, 1996). The virus contains two identical copies of a positive single stranded RNA, of approximately 9.2 kb. The RNA is associated with structural proteins and enzymes such as reverse transcriptase (RT). The virus enters by pH independent fusion and uncoats. RT transcribes the viral RNA into double stranded DNA, which is transported into the cell nucleus (Frankel and Young, 1998). In the nucleus the proviral DNA is stably integrated into the host cell genome by the viral enzyme integrase. The virus can remain dormant in this stage for a very long time before it is activated, for instance by viral transactivators such as tat and by cellular transcription factors such as NF B (Frankel and Young, 1998). After activation the virus is transcribed, replicates, assembles and eventually buds from the host cell membrane (Fig. 3).

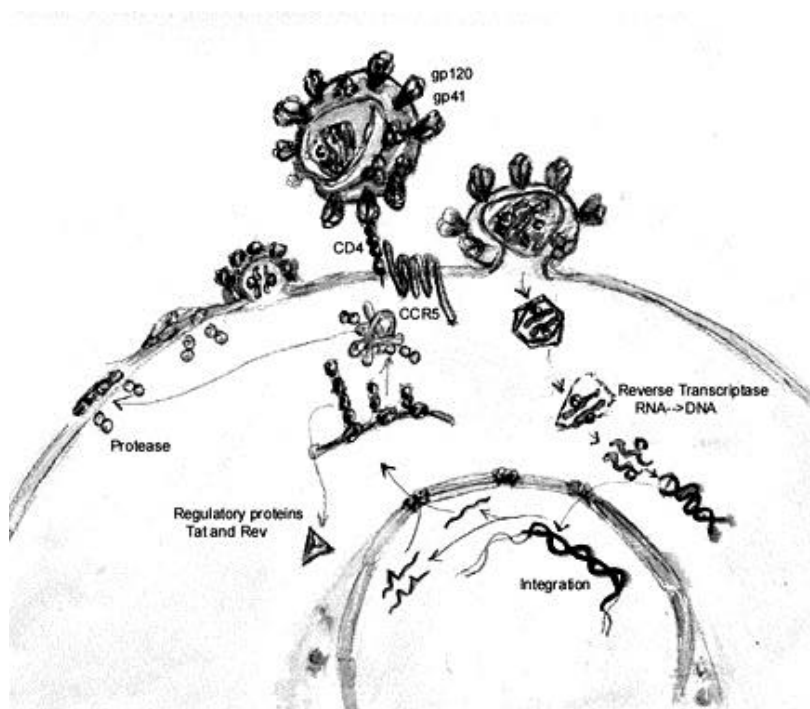


Figure 3. The virus life cycle. When the proviral DNA is activated, transcription starts. Three classes of mRNA are produced: the 9 kb, 4.5 kb partially spliced and the 2 kb multiply spliced transcripts. The 9 kb and 4.5 kb RNA classes are dependent on the viral Rev protein for transport to the cytoplasm. The multiply spliced transcript moves into the cytoplasm and is translated into the early viral proteins Tat, Rev and Nef. Rev then shuttles back into the nucleus and brings the 9 kb and 4.5 kb transcripts to the cytoplasm where they are translated to structural proteins and some accessory proteins. Tat shuttles to the nucleus and enhances transcription of the viral genome.

3.3 HIV-1 ENTRY

3.3.1 HIV-1 gp120 binds to the CD4 receptor

HIV-1 uses the outer surface glycoprotein gp120 for attachment to its main host cell receptor CD4 (McDougal *et al.*, 1986) (Fig. 4). The CD4 receptor plays a vital role for the interactions of cells in the immune system. The receptor is present on cells such as macrophages, CD4 positive T lymphocytes, dendritic cells and some hematopoietic cells in the nervous system. The depletion of CD4⁺ T-cells is the major cause of AIDS.

Gp120 is made from a viral precursor protein called gp160; this glycoprotein is cleaved by a cellular protease to form the mature envelope proteins gp120 and gp41. On the viral surface, a trimer of gp120 is noncovalently linked to three gp41 molecules.

CD4 binds in a recessed pocket on gp120, making extensive contacts over 800 Å² of the gp120 surface (Kwong *et al.*, 1998). Two cavities are evident in the gp120-CD4 interface. A shallow cavity is filled with water molecules, and a deep cavity extends roughly 10 Å into the interior of gp120. The opening of this deep cavity is occupied by phenylalanine-43 of CD4, which has been shown by mutagenic analysis to be critical for gp120 binding.

After binding of gp120 to CD4, conformational changes of the viral envelope gp120 are initiated. These changes contribute to the formation or exposure of the binding site for chemokine receptors (Wyatt and Sodroski, 1998). The interaction of the gp120-CD4 complex with the appropriate chemokine receptor triggers additional conformational changes in the envelope glycoprotein complex. Proposedly as an effect of these changes, the hydrophobic N-terminal part of Gp41 (the fusion peptide) is inserted in the target cell membrane and fusion between virus and host cell takes place (Fig. 4).

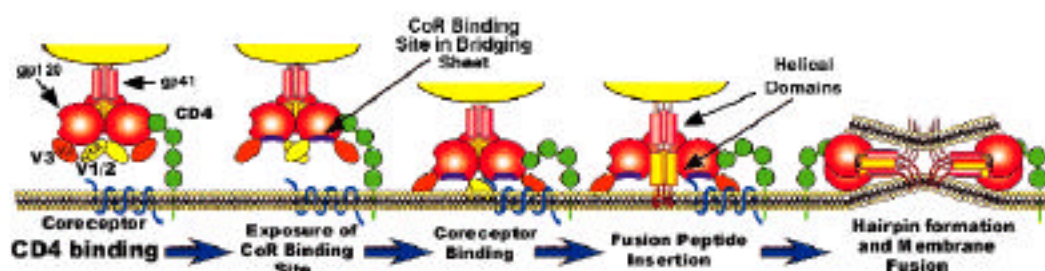


Figure 4. HIV-1 attachment and fusion process. Gp120 binding to CD4 triggers conformational changes in the gp120 protein. These changes expose the coreceptor binding site (CoR) located in the bridging sheet and the base of the V1/V2 stem-loop structure. The interaction of coreceptor with the CoR triggers additional conformational changes which induce the gp41 mediated fusion process. Adapted from (Doms and Trono, 2000).

3.3.2 Chemokine receptors and their ligands

The HIV-1 coreceptors belong to the chemokine receptor family. Chemokine receptors have a central role in host defence and they have been exploited by several intracellular pathogens for cell entry. These proteins are seven-transmembrane spanning, G-protein coupled receptors that normally bind to soluble ligands called chemokines. The chemokines are a group of chemotactic cytokines with molecular weights in the range of 8-10 kDa, they can direct leukocytes to migrate to sites of inflammation (Horuk, 2001). The chemokines have been subdivided into four subfamilies on the basis of the position of either one or two cysteine residues located near the N-terminus of the protein: CC, CXC, C and CXXXC (Teran, 2000). The chemokine receptors are divided into groups depending on which ligands they bind; some receptors bind to an array of different chemokines, while others are very selective in terms of ligand binding. The receptors are widely distributed on hematopoietic and other cells. The extracellular portions of the receptors are those involved in interactions with the ligands, and in the case of HIV-1 infection interactions with the viral gp120 (Doranz *et al.*, 1997; Rucker *et al.*, 1996). The extracellular domains are also the most variable between different related chemokine receptors and between receptors in different species. The transmembrane sequences are conserved and the intracellular domains are involved in cell signaling. Upon receptor-ligand interaction, the signal is transmitted through G-protein coupled interactions, resulting in alterations in cell function such as activation, motion, and migration, usually along a chemokine concentration gradient (Baggiolini, Dewald, and Moser, 1994).

3.3.3 Chemokine receptors and HIV-1 phenotypes

In addition to the CD4 receptor, HIV and SIV viruses need to interact with coreceptors in order to enter the host cells. The chemokine receptors used by HIV-1 are primarily CCR5 (Choe *et al.*, 1996; Deng *et al.*, 1996) and CXCR4 (Berger, Murphy, and Farber, 1999; Lu *et al.*, 1997), while HIV-2 and SIV can use a broader array of receptors (Mörner *et al.*, 1999; Vödrös *et al.*, 2001).

The choice of coreceptor is largely determined by variable parts of the gp120 protein. The V3 loop and to a lesser degree the V1/V2 region are the most important regions for coreceptor choice (Doms and Trono, 2000). Recently a conserved coreceptor binding region was described. The crystal structure of gp120 complexed with CD4 reveals the presence of an extraordinarily well conserved region in the bridging sheet, a portion of gp120 that lies between the base of the V3 and V1/2 regions, and is involved in coreceptor binding (Kwong *et al.*, 1998; Rizzuto *et al.*, 1998).

Before the discovery of the chemokine receptors which act as coreceptors in HIV infection, three overlapping classification systems were used to describe the phenotype of HIV isolates. These systems described how HIV replicated in different celltypes. The first system was based on virus growth kinetics in cellculture; the viruses were defined as slow/low (SL) or rapid/high (RH) depending on their growth in PBMC (Åsjö *et al.*, 1986). A second system categorized viruses as syncytium inducing (SI) or non-syncytium-inducing (NSI) on the basis of whether they induced syncytia in MT-2 cells which express the chemokine receptor CXCR4 but not CCR5. NSI viruses however also form syncytia with cells that express CCR5, which makes the NSI/SI terminology a bit confusing. In a third system primary isolates were

defined as Macrophage (M)-tropic or T-cell-line (T)-tropic. This system was not optimal since T-tropic viruses sometimes infect macrophages which express low amounts of CXCR4 and all primary isolates replicate in activated primary CD4+ T-lymphocytes. After the discovery of HIV coreceptors, a new classification system was suggested (Berger *et al.*, 1998). In this system the virus isolates are classified according to their coreceptor usage, CCR5-using viruses are called R5, CXCR4-using viruses are called X4 and viruses using both receptors are called R5X4. R5 viruses are slow/low, NSI, M-tropic viruses (CCR5 is expressed by macrophages). X4 viruses are rapid/high, SI and many T-tropic primary isolates are R5X4.

Primary sexual HIV-1 infections are normally caused by M-tropic viruses (Berger, Murphy, and Farber, 1999; Cornelissen *et al.*, 1995; Scarlatti *et al.*, 1997; Veenstra *et al.*, 1995). Many chemokines have been found to block infection by the different types of HIV-1 viruses. The chemokines RANTES, MIP-1 α and MIP-1 β , which bind to CCR5 (Cocchi *et al.*, 1995), and CCR2b and eotaxin, which bind to CCR3, have been shown to inhibit R5 viruses. Another chemokine called SDF-1 which is a CXCR4 ligand inhibits X4 viruses.

SIV infection can be mediated by CCR5, but SIV can also infect CD4+ cells that lack CCR5 indicating that they can use other receptors. Two such receptors called Bonzo and BOB were identified (Deng *et al.*, 1997); BOB and Bonzo are also frequently used by HIV-2. It was also demonstrated that SIV can use CXCR4 for entry (Owen *et al.*, 2000).

3.3.4 CCR5 is important for primary HIV-1 infection

A mutated allele of the CCR5 gene has been identified in highly HIV-1 exposed, seronegative individuals. The mutated CCR5 gene has a 32 bp deletion (Liu *et al.*, 1996; Samson *et al.*, 1996) which causes a frameshift and premature termination of translation. The protein encoded by the mutant allele lacks the last three transmembrane segments of the receptor and is not expressed on the cell surface. Therefore cells from people who are homozygous for this allele are protected against infection by M-tropic HIV-1 viruses. An enrichment of the homozygous 32 bp CCR5 deletion has been observed in highly HIV-1 exposed persons who remained uninfected (Huang *et al.*, 1996). The 32 bp deletion in the CCR5 gene does however not give 100% protection from HIV-1 infection, rare HIV-infected persons homozygous for the 32 bp CCR5 deletion have been reported (Michael *et al.*, 1998; O'Brien *et al.*, 1997; Theodorou *et al.*, 1997). Viral sequences isolated from these persons indicate that they were infected by SI virus. Since SI viruses use the CXCR4 receptor for attachment and infection this may be an infection route against which the mutation does not protect. Non SI viruses can also use other receptors such as CCR3 and CCR2b as entry cofactors. Even though the CCR5 $\Delta 32/\Delta 32$ genotype does not confer 100% protection from HIV-1 infection it seems to play an important role for protection from mucosal transfer in highly HIV-1 exposed persons. Also the CCR5 $\Delta 32$ /wt genotype has a certain protective effect against HIV-1 disease progression (Bratt *et al.*, 1998).

3.3.5 Target cells in primary infection

The target cells for SIV during vaginal transmission include large numbers of Langerhans cells (LC), dendritic cells (DC), T lymphocytes and macrophages. Lymphocytes and dendritic cells migrate very rapidly to draining lymph nodes, where the virus can spread to CD4⁺ T cells, which in turn disperse the virus systemically. The blocking of productive infection of DC did not prevent these cells from transmitting virus to T cells (Blauvelt *et al.*, 1997). This phenomenon can be explained by the discovery of a receptor called DC-SIGN, which is expressed on DC (Geijtenbeek *et al.*, 2000). DC-SIGN binds gp120 and may transfer HIV to CD4⁺ cells.

In humans and macaques, CXCR4 is expressed primarily on naive (CD45RA⁺CD62L⁺) lymphocytes, whereas the expression of CCR5 is limited primarily to memory (CD45RO⁺ CD62L⁻, CD26⁺) lymphocytes. The memory lymphocytes which express CCR5 are mostly activated, terminally differentiated effector cells that preferentially induce T helper 1 (Th1)-type (cell-mediated) cytokine responses. The majority of CD4⁺ cells in the peripheral blood, spleen and lymph nodes express very little CCR5. In contrast, CD4⁺ cells in the intestine, vagina and rectum express high levels of CCR5. This may explain why primary infection is mediated by viruses which use the CCR5 receptor for entry (Veazey, Marx, and Lackner, 2001). Although the intestinal CD4⁺ cells also express CXCR4, this receptor might be blocked by its ligand SDF-1, which is expressed by endothelial cells in the intestine. SIV usually uses CCR5 for infection and in primary SIV infection a major loss of the CD4⁺ T-cell population is observed in the intestinal tract before any changes can be seen in the peripheral blood.

SIV-HIV chimeric viruses (SHIVs), which exclusively use either CCR5 or CXCR4 for infection have been designed. When monkeys were infected with these viruses, CD4⁺ cells in the peripheral blood were specifically depleted by the CXCR4 virus while CD4⁺ cells in the intestine were depleted by the CCR5 virus (Harouse *et al.*, 1999). These results indicate that CD4⁺ CCR5⁺ cells at the mucosal surfaces are major targets of entry for SIV and HIV and that immune responses induced at these surfaces may be key to a preventive vaccine. It has been shown that memory T cells are more sensitive than naive T cells to cytopathic effects induced by HIV-1 (Chun *et al.*, 1997). Recently it was demonstrated that HIV preferentially infects CD4⁺ T-cells with a memory for HIV, which may explain the loss of HIV-specific CD4⁺ T-cell responses during the course of HIV infection (Douek *et al.*, 2002).

Several studies have indicated that individuals in the early stages of HIV-1 infection typically often have R5 viruses, while those progressing to full-blown AIDS with advanced immune deficiency have predominately R5X4 or X4 (Connor *et al.*, 1997; Penn *et al.*, 1999). The switch between the viral types is mediated by the very high mutation rate of the virus. Certain regions of the envelope protein gp120 are hypervariable and over time the regions of gp120 interacting with CCR5 (Cocchi *et al.*, 1996) are mutated to a higher affinity for CXCR4.

3.3.6 CCR5 specific immune responses

Antibodies directed against the N-terminus and the second extracellular loop of CCR5 can neutralize HIV-1 infection of cells *in vitro*; monoclonal antibodies directed against the second extracellular loop seem to be more effective in neutralization assays than antibodies recognizing the N-terminus (Olson *et al.*, 1999). The most potent neutralizing Mab identified so far, 2D7, recognizes a conformational epitope in the first part of the second extracellular loop, which seems to carry a good HIV-blocking epitope (Lee *et al.*, 1999).

The CCR5 receptor has been reported to act as an alloantigen in individuals homozygous for the 32 bp CCR5 deletion; serum from these individuals inhibited infection of peripheral blood lymphocytes by R5, but not X4 viruses (Ditzel *et al.*, 1998). Recently it was demonstrated that CCR5-reactive antibodies in seronegative partners of HIV-1-seropositive individuals down-modulate surface CCR5 *in vivo* and neutralize the infectivity of R5 strains of HIV-1 *in vitro* (Lopalco *et al.*, 2000).

It has also been suggested that induction of CCR5 inhibitory antibodies is important in preventing SIV infection in macaques (Lehner *et al.*, 1999). In this paper, antibodies to CCR5 were elicited by immunization with inactivated SIV grown in human CD4⁺ T cells. Immunized monkeys were completely protected against i.v. challenge with SIV grown on human CD4⁺ T cell lines. Sera from immunized monkeys inhibited SIV replication in the absence of significant neutralizing antibodies to SIV. The authors suggest that the CCR5 might have been incorporated in SIV budding from the human CD4⁺ T cells and that the incorporated CCR5 acted as an immunogen. In the case of HIV, incorporation of CCR5 and CD4 in the virus membrane is selected against (Frank *et al.*, 1996; Lalloos *et al.*, 1999). Budding SIV and HIV also incorporates MHC class I and II and other cell membrane molecules. The SIV inhibitory effect in the immunized monkeys might have been partially dependent on antibodies directed to these molecules.

3.4 HIV-1 REVERSE TRANSCRIPTASE

After viral entry into the cell, the viral RNA genome is reversely transcribed by RT into duplex DNA, which integrates in the cellular genome. RT has three distinct activities: RNA- and DNA-dependent DNA polymerase activities and RNase H activity. The transcription process starts from the 3' end of a tRNA₃^{Lys} primer annealed to the primer binding site near the 5' end of the genomic RNA (Frankel and Young, 1998). RNase H degrades the RNA moiety of RNA/DNA hybrids, uncovering the template for viral DNA synthesis. RNase H also appears to generate oligoribonucleotide primers for the reverse transcription process. RT and RNase H are necessary for virus replication. Both enzymes are processed in two steps from the Gag-Pol polyprotein by protease during virion assembly. First p66 is cleaved and forms a homodimer; one p66 subunit in the homodimer is then cleaved to yield a heterodimer composed of p66 and p51. Both the homo and the heterodimers display RT and RNase H activities, whereas p51 alone is not active. The structure of the RT domain in the heterodimer resembles a right hand; the subdomains are called fingers, palm, and thumb (Fig. 5 A).

3.4.1 The active site of RT and nucleic acid interactions

The polymerase active site of HIV-1 RT, which contains three conserved aspartic acid residues (Asp110, Asp185, and Asp186), is located in the p66 palm and lies at the base of the DNA-binding cleft (Fig.5 B). Asp185 and Asp186 form part of the YMDD motif of HIV-1 RT, corresponding to the more general YXDD motif, which is highly conserved in retroviral RTs (Johnson *et al.*, 1986) and also in cellular polymerases. Substitution of these three aspartate residues with other amino acids (including glutamate) dramatically impairs catalytic activity. The dsDNA is bound in a cleft formed by the fingers, palm, and thumb subdomains of p66. The connecting subdomains of both p66 and p51 form the floor of the DNA-binding cleft. The interactions between the dsDNA and protein involve primarily the sugar-phosphate backbone of the nucleic acid and structural elements of the fingers, palm, thumb and RNase H of p66, and are not sequencespecific (Ding *et al.*, 1998). The three catalytically essential amino acid residues (Asp110, Asp185, and Asp186) are located close to the 3' terminus of the primer strand. A hydrogen bond between the 3'-OH of the primer terminus and the side-chain of Asp185 suggests that the carboxylate of Asp185 could act as a general base in initiating the nucleophilic attack during polymerization (Ding *et al.*, 1998).

3.4.2 Treatment and drug resistance

Almost all HIV-1 drugs on the market today are directed at HIV-1 RT or protease. Because of the virus variability, large population size and rapid replication virus escape mutants soon emerge if monotherapy is used. At present, different combinations of drugs targeting both RT and protease are used, yet virus escape mutants are still found.

The RT inhibitory drugs can be divided into nucleoside or nucleotide RT inhibitors (NRTI), non-nucleoside RT inhibitors (NNRTI) and pyrophosphate analogues. The nucleoside RT inhibitors have to be phosphorylated by cellular kinases to become active. After proper phosphorylation they compete with RTs dNTP substrate for incorporation into the growing template/primer. The lack of a 3'OH prevents further elongation of the primer with other dNTPs, resulting in chain termination. The nucleotide inhibitors represented by acyclic nucleoside phosphonates like PMPA also act like chain terminators, but they require less activation by cellular kinases. The pyrophosphate analogue foscarnet (PFA) competes in a noncompetitive way with respect to the natural substrates or RTI. The NNRTIs bind allosterically to a hydrophobic pocket close to the active site and lock RT in a non functional conformation. The NNRTIs do not have to be metabolized in order to inhibit RT and the inhibition is generally noncompetetive with respect to substrate. The NNRTI binding pocket is not present in HIV-2 or SIV RT, this is why NNRTIs only inhibit HIV-1. The mutations induced by NNRTIs usually cluster around the hydrophobic pocket to which the drug binds (Fig.5 C). When the aminoacids close to the drug mutate, the optimal fit is lost, the drug loses effect and the virus becomes resistant.

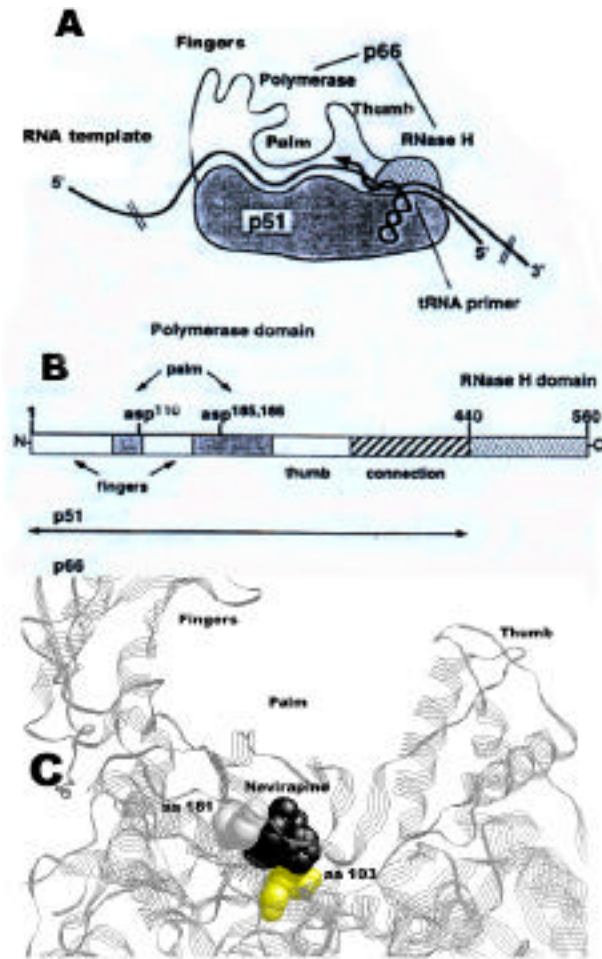


Fig. 5

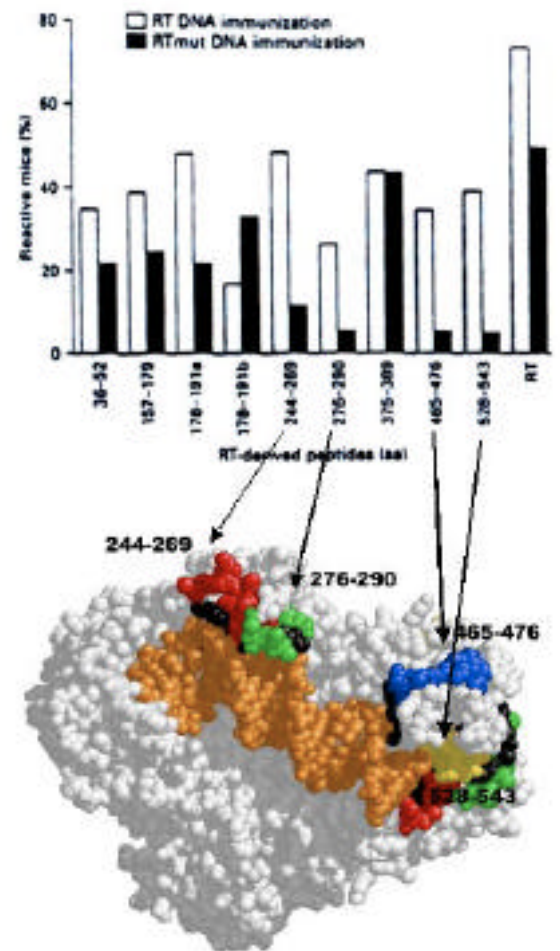


Fig.6

Figure 5. HIV-1 RT structure and activity converted from (Ding *et al.*, 1998). The RT heterodimer is built up of p66 and p51 (A). The polymerase domain contains three amino acids (Asp110, Asp185 and Asp186) essential for catalysis of the primer elongation process (B). The drug nevirapine is in close contact with aminoacids 181 and 103, which both mutate during treatment with nevirapine (C).

Figure 6. The YMLL mutation destroys certain Ab epitopes in RT . The antibody responses in mice immunized with RT or mutated RT containing the YMLL mutation were measured. RT (white) binding to DNA (grey) is shown. RTm (YMLL) immunized mice reacted weakly to indicated epitopes (244-269, 276-290, 465-476 and 528-543), which all lie in parts of RT that interact with the primer-template complex. RT amino acid residues interacting with DNA are shown in black (Ding *et al.*, 1998).

3.4.3 RT specific immune responses

HIV-1 specific CTL are considered the most efficient virus specific immune responses (Koup *et al.*, 1994; Ogg *et al.*, 1998; Schmitz *et al.*, 1999). RT is one of the most frequent targets for CTL recognition; 80% of HIV-1 infected individuals have RT specific CTL (Haas *et al.*, 1998). Virus mutations can decrease immunogenicity by interfering with the intracellular processing or with the HLA binding of viral peptides, resulting in a lack of CTL recognition. New HIV-1 variants that do not disturb such processes can in contrast be more immunogenic for specific new CTL clones. The RT sequence is very highly conserved among different HIV-1 isolates. DNA vaccination with the RT gene can give both antibody and cellular responses in rabbits (Isagulants *et al.*, 2000a) and in mice (Isagulants *et al.*, 2000b). The pol gene which contains RT has also been shown to induce RT specific immune responses in mice (Casimiro, 2002; Huang, Kong, and Nabel, 2001) and monkeys (Casimiro, 2002).

Immunization with enzymatically inactive RT induced different antibody response profiles to RT peptides than did immunization with wild type RT (Isagulants *et al.*, 2000b). Interestingly the antibody responses differed in regions of RT, which interact, with the primer-template complex (Fig. 6). The low reactivity to these peptides may be a consequence of disturbed primer-template interactions with RT because of the D185L, D186L mutations. The destruction of the hydrogen bond between D185 and the primer may distort the contact with the primer thereby preventing the first step in the formation of the active RT complex with primer, template hybrid duplexes.

RT specific drug treatment of HIV-1 induces a number of escape mutations in the RT enzyme. In a recent paper it was demonstrated that mutations induced by mono- or bitherapy with NRTI, M41L, L74V, M184V, and T215Y/F, did not impair CTL recognition of the RT sites of drug-induced mutations in treated patients (Samri *et al.*, 2000). The RT M184V mutation within an HLA-A2 restricted HIV-1 CTL epitope (RT 179-187) induces drug escape against the NRTI lamivudine (3TC) and abolishes recognition by CTL in HIV-1 infected patients. It has been shown that an HIV-1 patient receiving lamivudine treatment could generate RT specific CTL that targeted lamivudine-resistant viruses (Schmitt *et al.*, 2000). Epitopes with drug-escape mutations represent attractive new targets for HIV-1 specific vaccines. More studies are necessary to examine whether the induction of CTL against drug-escape variants can help delay or prevent the emergence of drug-resistant HIV-1 strains.

Although RT is an intracellular enzyme, antibodies to RT can have HIV-1 inhibitory effects. Antibodies to certain epitopes of RT inhibit its enzymatic activity by interfering with the interaction between RT and the template-primer or by inhibiting the RNase H activity (Restle *et al.*, 1992; Örvell *et al.*, 1991). A high prevalence of these antibodies was associated with asymptomatic infection and an inability to isolate virus from the sera of HIV-1 positive individuals (Laurence, Saunders, and Kulkosky, 1987; Sano *et al.*, 1987).

4 PRIMATE MODELS OF HIV-1 INFECTION

The primate models of AIDS can be used to study pathogenesis, transmission and immune responses to infection. These models are also useful in the study of new vaccines and drugs. The HIV-1/Chimpanzee, SIV or HIV-2/macaque and SHIV/macaque models are the most widely used.

In the wild, infections by primate lentiviruses occur only among African monkeys and apes. Different simian lentiviruses are found in sooty mangabey (SIVsm), african green monkey (SIVagm), mandrill monkey (SIVmnd), sykes monkey (SIVsyk) and chimpanzees (SIVcpz), respectively (Joag, 2000). The primate lentiviruses have little or no pathogenicity in their natural hosts and disease occurs only after transmission to another species. SIV was first isolated in immunodeficient captive Asian macaques (Daniel *et al.*, 1985).

SIVsm is the progenitor of HIV-2 and SIVmac. HIV-2 and SIVsm infections of macaques resemble SIVmac infections in most respects. The cynomolgus macaque / HIV-2 model was developed by Biberfeld (Putkonen *et al.*, 1989) and coworkers for HIV vaccine studies.

Macaques from South East Asia infected with certain strains of SIV, such as SIVmac and SIVsm, develop a fatal immunosuppressive disease with almost identical clinical and pathological findings to AIDS in humans. Infection is associated with increased viral load, a decrease in CD4+ T-cells and the eventual development of AIDS-like symptoms.

Rhesus (*Macaca mulatta*), Cynomolgus (*Macaca fascicularis*), and pig-tailed macaques (*Macaca nemestrina*) have been used in the majority of studies. There are a number of pathogenic and non-pathogenic viral strains that can be used in therapeutic and challenge studies.

Recently it was demonstrated that the chimpanzee virus SIVcpz is the probable progenitor of HIV-1 (Gao *et al.*, 1999). In the light of this finding it is not surprising that HIV-1 can easily be transmitted to common chimpanzees (*Pan troglodytes*). HIV/SIVcpz infected chimpanzees do not develop significant CD4+T-cell loss or AIDS, which limits the utility of this model in studying pathogenesis. Also chimpanzees are very expensive and endangered, countering their use in experiments requiring large numbers of animals.

Unlike apes, many monkey species are not endangered and breed well in captivity but attempts to infect monkeys with HIV-1 have not been successful.

Chimeric simian human immunodeficiency viruses (Fig. 7) have been created to better mimic HIV-1 disease in macaques. SHIV chimeras created by inserting the HIV-1 *env*, *rev*, *tat* and *vpu* genes were shown to readily infect macaques, though the infected animals failed to develop disease. Highly virulent SHIV strains have been developed through animal passage. More restricted chimeric viruses, in which only a single gene or a portion thereof is exchanged, have been constructed to investigate the function of a particular gene, or to test antiretroviral drugs. One example is SHIV-RT, in which the RT gene of SIV has been replaced by that of HIV-1. Unlike SIVmac, SHIV-RT can be inhibited by NNRTI (Balzarini, De Clercq, and Uberla, 1997). The SHIV-RT model could also potentially be used to investigate the effect of a vaccine directed to HIV-1 RT.

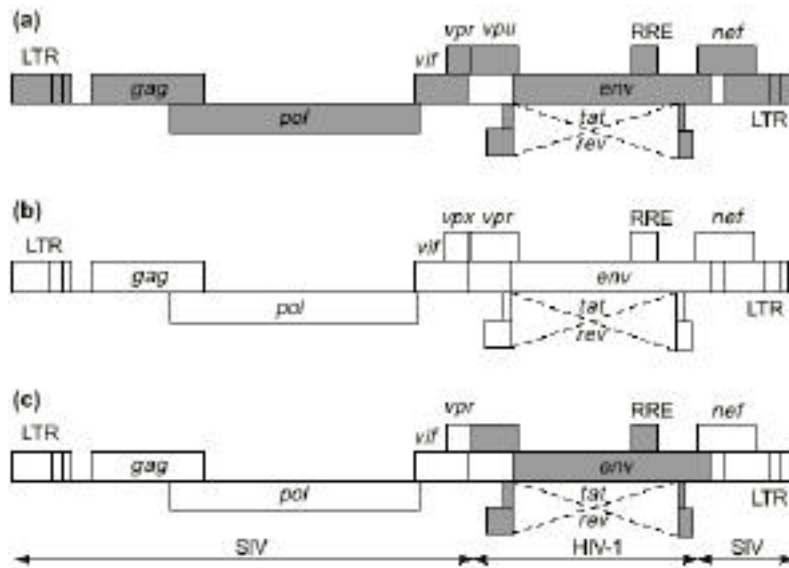


Figure 7. The genomic arrangement of HIV-1 (A), SIV (B) and SHIV (C). Adapted from (Nath, Schumann, and Boyer, 2000).

5 THE HOST IMMUNE RESPONSES TO HIV-1 INFECTION

5.1 INTRODUCTION TO IMMUNE RESPONSES INDUCED BY VIRUS INFECTIONS

5.1.1 Innate and specific responses

The immune responses to viral infections can be divided into non antigen specific (innate) and antigen specific (adaptive) responses (Fig. 8). The innate response does not change after repeated exposure to a virus, while the adaptive response improves with each successive encounter with the same virus. To enter its host, the virus has to pass barriers such as skin and mucous membranes. It also encounters physiological barriers such as temperature, pH, oxygen tension and various soluble factors such as interferons. The virus may also encounter cells involved in the innate response. Leukocytes important for the innate response are the phagocytic cells, such as the monocytes, macrophages and polymorphonuclear neutrophils. These cells can internalize and kill free virions with the help of antibodies which recognize and bind to the virions. The phagocytic cells are more important for antibacterial immune responses than for antiviral responses.

Natural Killer (NK) cells kill tumor cells and virus infected cells lacking inhibitory cell surface receptors. NK cells can also kill by antibody-dependent cell-mediated cytotoxicity (ADCC) through virus specific antibodies, which recognize virus proteins on the surface of a infected cell. The latter mechanism is a combination of innate and adaptive responses.

Once an infection is established, specific immune responses are most important for clearance of the virus. The adaptive anti-virus immune response is mediated by lymphocytes and antibodies. Two key features of the adaptive system are memory and specificity. The cells specifically recognize individual pathogens, inside or outside host cells. There are two types of lymphocytes: T-lymphocytes (T-cells) and B-lymphocytes (B-cells). T-cells recognize antigen presented by antigen presenting cells (APC). The APCs include dendritic cells (DC), macrophages and B cells. The antigen presenting molecules on the APC surface are membrane glycoproteins called the major histocompatibility complex (MHC) molecules classes I and II. In general, the responses to the virion are B-cell mediated and those acting on infected cells are T-cell mediated.

B-cells produce antibodies to all viral proteins, some of which may cause viral enhancement, as well as neutralization. Virus neutralization prevents the attachment of virus to the host cell. Neutralization is mediated by IgG, IgA or IgM antibodies. Only antibody directed to viral components responsible for attachment are neutralizing, but antibodies directed to viral molecules transferred between cells may also be important for control of the infection, which makes it important to induce the right kind of immune response. T-helper lymphocytes interact with B-cells and help

them to divide, differentiate and make antibodies; they also stimulate cytotoxic T-lymphocytes (CTL). Virus infected cells are detected by CTL, which recognize viral antigens in association with MHC class I. CTL lyse virally modified cells and limit disease by eliminating the production of infectious progeny; they can also produce soluble virus-inhibiting factors.

In most acute viral infections, specific CTL activity arises within 3-4 days after infection, peaks after 7-10 days and then declines but persists. Within 7-10 days of primary infection most virions are eliminated by the CTL.

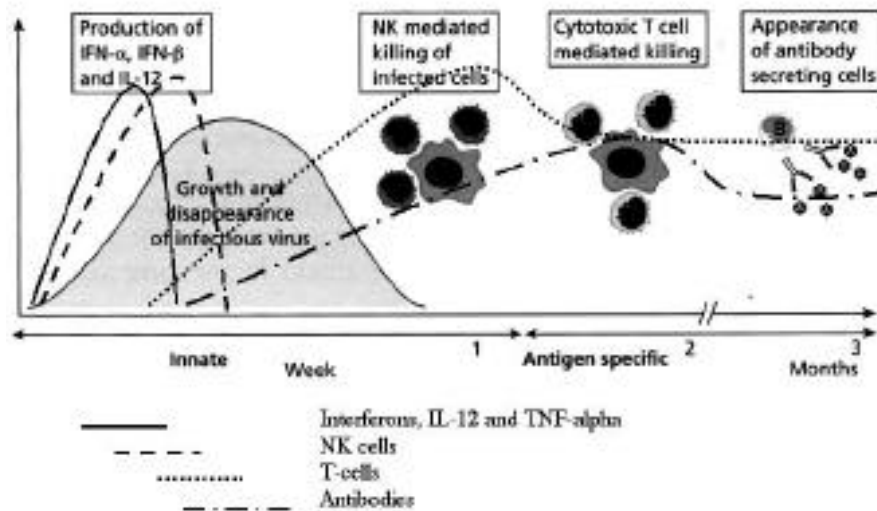


Figure 8. The immune responses to an acute viral infection. Modified from (Janeway *et al.*, 1999). Viral infections of cells induces the production of interferon- α and interferon- β . The interferons induce resistance to viral replication in all cells, increase MHC I expression and antigen presentation in all cells and activate NK cells to kill virus-infected cells. Another cytokine produced early in many infections is IL-12 which together with the interferons increase NK cell activity. NK cells control the virus replication but do not eliminate the virus. Virus elimination is accomplished when specific CD8+ T cells are produced, these cells as well as B cells are stimulated by CD4+ T helper cells. The peak antibody production usually comes at 2-3 weeks and then persists for life time.

5.1.1.1 The generation of MHC-I binding peptides

A large protein complex called the proteasome degrades proteins in the cytosol of cells into peptides, reviewed in (Rock and Goldberg, 1999). These peptides are 4-20 residues long; approximately 10% of the generated peptides are 8-10 residues, which is the right size to fit into the groove of the MHC-I protein. Peptides are degraded by the proteasome and transported by the TAP protein into the endoplasmic reticulum. In the endoplasmic reticulum, peptides associate with and stabilize MHC-I, which is transported to the golgi and presented on the cell surface. Cells targeted for fast destruction are attached to the ubiquitin protein by specific enzymes.

Polyubiquitination of lysin residues targets proteins for fast proteasome degradation. The stability of a protein is largely determined by its N-terminal protein (N-end rule) (Bachmair, Finley, and Varshavsky, 1986). Proteins with large, bulky or charged amino termini are rapidly ubiquitinated and degraded. The N-end and other degradation rules can be used to target ubiquitin-minigene fusion constructs for degradation and more efficient MHC-I peptide presentation. The proteasome complex has many components and several proteolytic activities. Proteins are preferentially cleaved after large hydrophobic, basic or acidic residues. Mammalian proteasomes have also been reported to cleave after branched-chain amino acids and small neutral residues. IFN- γ induces a change in the proteasome complex; certain components are replaced by others. This generates a new complex referred to as the immunoproteasome (Gaczynska, Rock, and Goldberg, 1993), which preferentially cleaves after hydrophobic and basic amino acids and has reduced cleavage after acidic residues. The majority of peptides presented by MHC-I are of this type (Rammensee, Falk, and Rotzschke, 1993). In general, the proteasome generates the C-termini of peptides, while the N-termini are generated by cytosolic amino or endo peptidases. Several studies have shown that changes in the C-terminal-flanking region affect the generation of the MHC-I binding peptide (Bergmann *et al.*, 1996; Livingston *et al.*, 2001; Mo *et al.*, 2000; Shastri, Serwold, and Gonzalez, 1995). This knowledge can be used to design minigenes with N and C-terminal residues optimal for generation of the correct MHC-I peptide (Livingston *et al.*, 2001).

5.1.2 Antigen presentation and CTL escape

The MHC is the most polymorphic gene cluster in the human genome, having a large number of alleles. MHC is called the human leukocyte antigen (HLA) in humans and H-2 in the mouse. MHC class I is expressed on all nucleated cells in mammals. The peptide MHC I complex is recognized by the CD8⁺ subset of T-cells. MHC class II molecules are constitutively expressed on APC and specialized in presenting exogenous peptides. The MHC-II/peptide complex is recognized by CD4⁺ T-cells.

A given MHC molecule can bind numerous different peptides and some peptides can bind to several MHC molecules. Each type of class I MHC molecule (e.g., K, D and L in mice and A, B and C in humans) binds a unique set of peptides. Each allelic form of a class I molecule also binds a distinct set of peptides. The different allelic forms are represented in different frequencies in distinct human populations. For example, HLA-A2 is the most frequent allele in the world's population (Fernandez-Vina *et al.*, 1992). The peptide-binding cleft of class I MHC molecules is closed at both ends, thus limiting the size of the peptides that can fit in the cleft. Binding studies have shown that nonameric peptides bind to MHC I with the highest affinity. The strongest peptide-MHC I interactions involve the first two and the last two amino acid residues of the peptide while the middle part carries the sequence interacting with T-cell receptors which confers immunological specificity. This means that each MHC allele recognizes a special pattern of aa residues in a peptide. The knowledge about which patterns different MHC I alleles recognize can be used to predict potential CTL epitopes in viral proteins. CTLs exert an evolutionary pressure on infecting viruses. If the CTL is directed to a vital viral protein, the virus may quickly mutate in the CTL epitope so that it is no longer recognized by CTL. This phenomenon has been

demonstrated in vivo in HIV-1 infected humans and in SIV infected monkeys (Allen *et al.*, 2000; Phillips *et al.*, 1991; Sewell *et al.*, 2000).

5.2 HUMORAL IMMUNE RESPONSES TO HIV

The strongest antibody responses are induced against the Env (gp120 and gp41) and Gag (p24 and p17) proteins. Most antibodies generated during natural HIV-1 infection bind but do not neutralize virus well and are probably of limited efficacy in controlling the virus. The antibody recognition of an antigenic HIV-1 epitope is disturbed by several mechanisms, such as variation in the amino acid sequence, alteration of conformation and masking by other protein domains and carbohydrate groups (Wyatt and Sodroski, 1998). Neutralizing antibodies may be a component of the initial control of HIV-1 replication. However, as HIV-1 variants emerge over time, new variants are frequently not neutralized by autologous sera (Albert *et al.*, 1990). During infection, disassembled envelope glycoproteins elicit most of the antibodies directed to these viral components (Parren, Burton, and Sattentau, 1997). This results in antibodies which are unable to recognize the functional envelope glycoprotein complex. Specific antiviral antibodies are detected early in the infection but most of these antibodies lack the ability to inhibit virus infection (Moog *et al.*, 1997). The infection is already established when the neutralizing antibodies emerge (Wyatt and Sodroski, 1998). HIV-1 specific antibodies have also been shown to enhance infection under certain conditions. Antibody dependent enhancement of infection has been shown to be mediated by Fc receptors (Matsuda *et al.*, 1989) and complement (Robinson, Montefiori, and Mitchell, 1988).

Antibody mediated protection against HIV-1 and SHIV infection has been achieved in primate models (Baba *et al.*, 2000; Emini *et al.*, 1992; Shibata *et al.*, 1999) but protection required much more potent antibodies than those that have been induced by vaccination of monkeys or humans (Moore and Burton, 1999).

5.3 CELL-MEDIATED IMMUNE RESPONSES TO HIV

CD8⁺ cytotoxic T lymphocytes (CTLs) appear to be important in containing the spread of HIV-1 in infected individuals (Letvin, 1998). The replication of HIV-1 in CD4⁺ T-cells can be inhibited by autologous CD8⁺ CTLs (Walker *et al.*, 1986), through mechanisms which probably involve both lysis and the release of chemokines (i.e. MIP-1 α , MIP-1 β and RANTES) and cytokines. The viral containment in early HIV-1 infection coincides in time with the emergence of a virus specific CTL response (Pantaleo *et al.*, 1994). In chronically HIV-1 infected individuals, a high-frequency of CTL is correlated with the maintenance of low viral load and stable clinical status (Ogg *et al.*, 1998). Generally, HIV-1 specific CTL response is maintained throughout the asymptomatic stage but often declines with disease progression (Autran, Hadida, and Haas, 1996). Despite vigorous CTL responses, virus is not eradicated from infected persons. This implies that the T-cells (T-helper and CTLs) of HIV-1 infected persons may be dysfunctional in some way (Brander and Walker, 1999). Although CTL escape has been observed, this is probably not the major mechanism. In an interesting study on rapidly progressing HIV-1 infection, the patient was shown to have strong CTL responses against a

specific epitope. Despite this, his disease progressed. The patients' CTLs to a specific epitope in the envelope could be expanded to large numbers *in vitro* but did not expand *in vivo*. The patient lacked virus specific T helper cells which may have been needed to activate the CTLs (Hay *et al.*, 1999).

Natural killer cells, along with neutrophils, monocytes and macrophages, can also exert an indirect cellular cytotoxicity using complement or antibodies as mediators. In complement dependent cell-mediated cytotoxicity, membrane deposited C3b on the surface of an HIV infected cell binds to C3b receptors on the effector cell. Antibody-dependent cell-mediated cytotoxicity (ADCC) (Rowland-Jones, Tan, and McMichael, 1997), involves aggregation of antigen-specific IgG molecules, on the surface of an infected cell, which in turn induces binding of the Fc part of the IgG molecules to specific Fc receptors (CD16) on the leucocyte responsible for the ADCC mediated lysis. In both cases the binding activates the release of lytic mediators from the effector cells which lyse the target cells. The lysis is dependent on complement or antibodies for direction but not much is known about the induction of the effector cells needed for lysis. The importance of ADCC in defense against infection with bacteria or viruses has not yet been fully established (Janeway *et al.*, 2001).

6 VACCINES

6.1 INTRODUCTION TO VACCINES

The first vaccine can be attributed to Edward Jenner, who inoculated an 8-year-old boy with vaccinia in 1796, which protected the boy from subsequent challenge with virulent smallpox. Since then, substantial knowledge in virology, immunology and molecular biology has been gained. This knowledge is now being applied to make more efficient vaccines with less side effects. The first generation of viral vaccines was based on attenuated live virus or killed whole virions. Then immunogenic parts of viruses were isolated and subunit vaccines based on viral proteins or peptides were made. Viral vectors are now being developed to deliver genes of other viruses. DNA plasmids containing viral genes can also be delivered and induce immune responses. The plasmids can be replicating or non-replicating. This thesis focuses on non-replicating DNA vaccines.

6.1.1 Immunization using self proteins

Vaccines can be used to break tolerance to self proteins. Anti self responses can be achieved using fusion proteins between an immunogenic protein and a self antigen. For example, anti-fertility vaccines have been developed in which human chorionic gonadotropin has been coupled to tetanus or diphtheria toxoid; these vaccines were effective in human trials (Talwar *et al.*, 1997). Also different cytokines such as GM-CSF can be administered simultaneously with the self antigen to break tolerance (Samanci *et al.*, 1998).

One approach to inhibiting viral cell entry is to induce autoantibodies to viral receptor proteins. In one study chimpanzees were immunized with recombinant soluble human

CD4 receptor. The chimpanzees developed anti-self CD4 antibody responses with anti HIV-1 activity (Watanabe *et al.*, 1992), similar results were obtained in mice (Cassatt *et al.*, 1991) and macaques (Watanabe *et al.*, 1991). DNA based immunization has also been shown to induce anti-CD4 antibodies directed primarily to native epitopes (Attanasio, Pehler, and Scinicariello, 1997).

Some viruses also incorporate host cell proteins in the envelope during the budding process, such molecules can also be targeted. HIV-1 is known to incorporate HLA antigens in the envelope. Allogeneic anti-class I MHC antibodies have been elicited by plasmid DNA immunogens in mice transgenic for various HLA alleles (Dela Cruz *et al.*, 1999). Break of tolerance to macaque MHC by DNA immunization with macaque MHC genes has also been demonstrated (Dela Cruz, MacDonald, and Barber, 2000).

Autoimmunization can also be used to induce anti-cancer immune responses. Immune recognition of human cancers is commonly directed against differentiation antigens. Immune responses to these antigens are difficult to induce because of immune tolerance. It has been demonstrated that tolerance to the mouse melanosome glycoprotein 75 (gp75) can be broken by immunization with DNA encoding an evolutionarily conserved, xenogeneic gp75 protein that is highly homologous to the mouse protein. Thus, immunization with homologous DNA may lead to immunity that cross-reacts with the self protein, breaking tolerance to the self protein (Weber *et al.*, 1998).

A fusion design strategy has also been exploited to break tolerance against cancer antigens. It is known that vaccination with idiotypic protein protects from B-cell lymphoma. DNA vaccines containing single chain Fv derived from tumor induce low anti idiotypic responses. It was shown that fusion of the gene-encoding fragment C of tetanus toxin to single-chain Fv substantially promoted the anti idiotypic response (King *et al.*, 1998).

T-cell receptor (TCR) based immunotherapy with DNA vaccines has also been used with some success to inhibit organ specific autoimmune disease inducing T cells (Matsumoto, 2000). Immunotherapy targeting disease-associated TCR using DNA vaccines significantly reduced the histological severity, and completely suppressed the inflammation in some animals (Matsumoto, Jee, and Sugisaki, 2000).

Recently it was demonstrated that administration of naked DNA encoding tumor necrosis factor alpha (TNF-alpha) results in the generation of immunological memory to its gene product. The immune response to TNF-alpha was shown to prevent chronic adjuvant-induced poly arthritis (Wildbaum, Youssef, and Karin, 2000).

6.2 HIV-1 VACCINES

The search for an HIV-1 vaccine began almost directly after the discovery of the virus. In 1984 the discoverers of the virus predicted that a vaccine would be available within 3-10 years. Today, 18 years later, we are still far from a functional vaccine,

although progress has been made. The initial attempts focused on the envelope protein of the virus: gp160 (fusion of gp120 and gp41) or gp120. It was envisioned that a recombinant gp120 or gp160 could be used as a vaccine. In a phase II study with recombinant gp160 no clinical benefit of therapeutic immunization could be demonstrated, despite strong T helper cell responses (Goebel *et al.*, 1999). In a larger trial vaccination with gp160 led to a transient clinical benefit as demonstrated by an improved survival after two years of immunization. At three years the effect was not significant (Sandström and Wahren, 1999).

One of the gp120 vaccine candidates is now in phase III trials but given the problems with induction of broadly neutralizing antibodies hopes are low (Cohen, 2002). The gp120 is very variable and highly glycosylated. Furthermore, parts of gp120 which are good targets for antibodies are hidden from the immune response.

An early breakthrough came when monkeys were immunized with whole inactivated SIV virus preparations produced in human cells. This immunization regimen completely protected monkeys from infection with SIV (Desrosiers *et al.*, 1989; Murphey-Corb *et al.*, 1989). The main reason for this was reactivity against human HLA proteins carried by the virus. When SIV is grown in human cells, the budding viruses incorporate human cell components into their envelopes. James Stott and coworkers could show that the protective effect of the whole killed SIV vaccine was caused by immune responses towards components of human cells incorporated into the SIV envelope (Stott, 1991). The concept of human proteins as a HIV vaccine was suggested and backed by animal data (Arthur *et al.*, 1992; Arthur *et al.*, 1995; Shearer, Clerici, and Dalgleish, 1993). Although this concept was very promising, it was pursued only by a few scientists. "It's too simplistic", said a frustrated Gene Shearer, years later (Cohen, 2001). Another promising vaccine approach was demonstrated by Ronald Desrosiers and coworkers. They successfully protected monkeys from SIV infection by using attenuated SIV strains with a deleted nef gene (Daniel *et al.*, 1992). This approach can probably never be used in humans because of the risk of revertants (Ruprecht, 1999).

Later vaccine attempts focused on virus vectors which could be used to deliver HIV-1 genes. Often different pox viruses like vaccinia or modified vaccinia ankara were used. Recently adenovirus vectors have been tried with good results (Shiver *et al.*, 2002). These approaches induce both antibody responses and cellular immune responses. The HIV-1 proteins are produced inside cells in a native conformation and virus vectors often activate the immune response and act as adjuvants.

The latest vaccine approaches combine different regimens in prime-boost immunization schemes. DNA vaccine prime followed by virus vector boost has been shown to induce very potent immune responses in primates (Amara *et al.*, 2001; Robinson *et al.*, 1999). Human trials using the promising prime-boost approach have been initiated (Cohen, 2002; Hanke and McMichael, 2000).

6.3 DNA VACCINES

Wolff *et al.* were the first to show that non-replicating DNA expression vectors can be taken up by muscle cells when injected into mice intramuscularly (i.m.) without the use of a delivery vehicle (Wolff *et al.*, 1990). When mice were injected with plasmids encoding human growth hormone by the particle bombardment method (Tang, DeVit, and Johnston, 1992), specific antibodies were elicited, demonstrating that DNA immunization can induce a humoral response. The influenza hemagglutinin (HA) was shown to confer protection from influenza challenge in chicken and mice. Efficient protection was achieved using only 0.4 µg of DNA coated on gold beads and delivered by the gene gun approach (Fynan *et al.*, 1993). In another study mice were immunized intramuscularly with plasmids encoding the influenza nucleoprotein; this resulted in protection from a subsequent challenge with a heterologous influenza strain. Both an nucleoprotein-specific cytotoxic T-lymphocyte response and high-titer Ab were detected (Ulmer *et al.*, 1993). DNA vaccines have since been reported to generate immune responses against various antigens, and also protective immunity in several diseases (Liljeqvist and Ståhl, 1999). DNA vaccines encoding a multitude of antigens from viruses, bacteria, parasites, fungi, allergens and tumors have been used successfully to induce immune responses in animal species ranging from mice to humans (Liu and Ulmer, 2000).

6.3.1 Construction of DNA vaccines

A DNA vaccine consists of a bacterial plasmid into which a foreign gene of interest is cloned. It is important to engineer the plasmid for optimal expression in eukaryotic cells. The plasmid usually contains an origin of replication that allows growth in bacteria, and may have an antibiotic resistance gene that allows plasmid selection during bacterial culture (Wahren and Brytting, 1997).

A strong promoter is needed for optimal expression in eukaryotic cells. For this, virally derived promoters, such as those from cytomegalovirus (CMV) or simian virus 40, provide the most optimal gene expression. Stabilization of mRNA transcripts can be achieved by incorporation of polyadenylation sequences (Poly-A) (Gurunathan, Klinman, and Seder, 2000). Translation initiation can also be optimized by incorporation of a translation initiation region (An *et al.*, 2000).

6.3.2 Advantages of DNA vaccines

Immunization with DNA vaccines offers several advantages. Unlike most conventional inactivated vaccines, DNA vaccines can induce both cell-mediated and antibody-based immune responses. Immunization with plasmid DNA encoding allergens may provide a novel type of immunotherapy for allergic diseases by shifting the immune response from a T-helper type two (Th2) to a T-helper type one (Th1) response (Raz and Spiegelberg, 1999). DNA vaccines are easy to manipulate and combination vaccines are readily made because all the components can be purified by the same procedure. This method of producing the protein antigens of interest directly in host cells can provide an appropriate tertiary structure for the induction of conformationally specific antibodies, and also facilitate the induction of cellular immune responses. The same vector can also be used for subsequent immunization

since no immune response is elicited against the vector itself, as in the case of vaccinia and other live virus vectors.

6.3.3 Mechanism of immune induction by DNA vaccines

DNA vaccines are potent inducers of T-cell responses, but for priming antibody responses they are less strong than immunization with recombinant protein in adjuvant. This has been shown in mice, monkeys and humans. One explanation may be that very low amounts of antigen are produced within cells of the plasmid inoculated host. Based on estimates from reporter gene studies, only picogram to nanogram amounts of protein are expressed (Manthorpe *et al.*, 1993; Wolff *et al.*, 1990). Such protein amounts would not be sufficient to induce a strong immune response if they were given as a subunit vaccine. In contrast, it has been shown that very small amounts of antigen in the context of an antigen presenting cell is sufficient to induce T-cell responses (Fields, Shimizu, and Mule, 1998). Two factors are especially important for priming of T-cell responses by DNA vaccines: the type of cells involved and the potential effects of immunostimulatory motifs within bacterial plasmid DNA. After intramuscular injection of plasmids, most of the gene product is produced by non-antigen presenting cells such as myocytes and cells of the skin. These cells do not directly prime T-cell responses; for this, professional bone marrow derived antigen presenting cells are required (Corr *et al.*, 1996; Fu *et al.*, 1997; Iwasaki *et al.*, 1997). Antigen presenting cells can either be directly transfected by the DNA vaccine or they can take up antigens synthesized by another transfected cell (i.e., cross-priming) (Fig. 9). Evidence now exists for both production of antigen by APCs (Akbari *et al.*, 1999) and cross-priming after DNA vaccination (Ulmer *et al.*, 1996).

DNA vaccines are efficient at inducing broad-based immune responses in small animal models (Donnelly *et al.*, 1997; Hinkula *et al.*, 1997). However, the immune responses induced in primates are generally of lower magnitude or more variable than those induced in small animals. Also, immunization of primates requires much larger amounts of DNA to induce efficient immune responses. Several immunizations are usually required, involving boosting with recombinant proteins or viral vectors (Putkonen *et al.*, 1998). Data from DNA vaccine phase I human trials have been published for infected and non infected individuals. In these studies cellular immune responses and weak antibody responses have been observed (Boyer *et al.*, 1999; Boyer *et al.*, 2000; Calarota *et al.*, 1998; Calarota *et al.*, 1999; MacGregor *et al.*, 1998; Tacket *et al.*, 1999; Wang *et al.*, 1998).

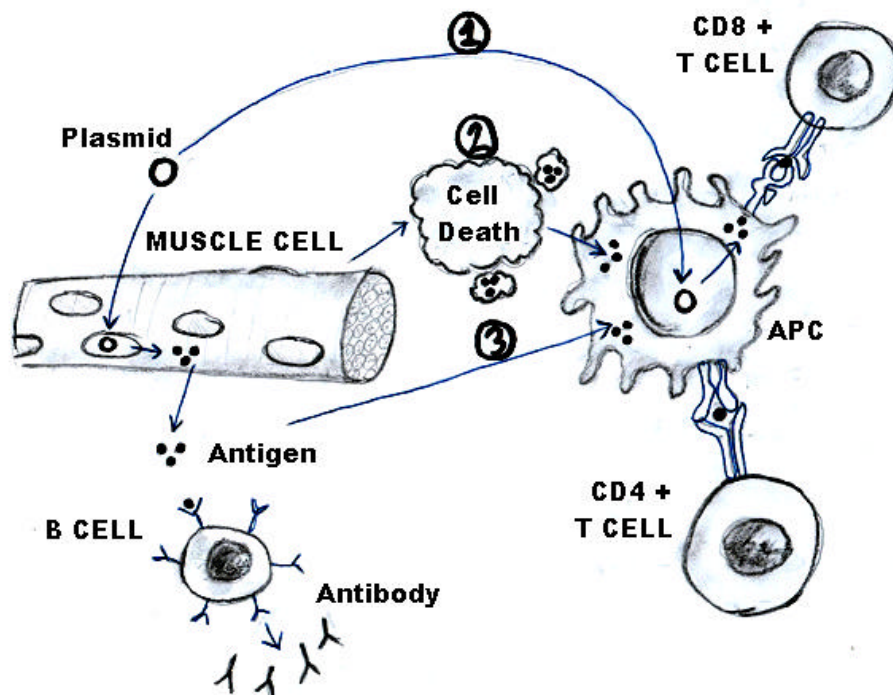


Figure 9. Mechanism of immune activation by DNA vaccines. The injected plasmid is taken up by myocytes or APCs (1). The cells which have taken up the plasmids start to transcribe the gene of interest and the protein is produced. The cells expressing protein may die by either necrosis or apoptosis (2). The apoptotic bodies containing expressed protein can be taken up by APCs, processed and presented on MHC II or MHC I molecules. Protein can also be secreted by myocytes and taken up by APC (3). The myocytes cannot stimulate T-cells because they lack important co-stimulatory molecules such as B7 which interact with CD28 on T-cells and produce a co-stimulatory signal (Linsley and Ledbetter, 1993).

6.3.4 Immunostimulatory properties of bacterial DNA

Unmethylated CpG dinucleotides are more frequent in the genomes of bacteria than in vertebrates. Bacterial CpG containing DNA is immunostimulatory in vertebrates; They are danger signals recognized by a pattern recognition receptor (PRR). The pattern recognition receptors trigger innate immunity by recognition of pathogen specific molecular structures such as lipopolysaccharide, which is a component of bacterial cell walls. In general, CpG DNA can directly activate B cells, dendritic cells and to a lesser degree monocytes/macrophages, while NK cells and T cells are indirectly stimulated; overall, a Th1 environment is created (Fig. 10). CpG ODN drive the differentiation of CTL and IFN- secreting T-cells, activate APCs to present antigen more efficiently and potentiate antibody responses (Krieg, 2002). The CpG effect is mediated by the toll-like receptor 9 (TLR-9) which is a pattern recognition receptor (Hemmi *et al.*, 2000), the signal pathway triggered by CpG and TLR-9 ends in NFkB expression and immune activation.

Murine B cells are directly stimulated to proliferate and secrete immunoglobulin *in vitro* and *in vivo* by CpG in bacterial DNA or in synthetic oligodeoxynucleotides (ODN). The optimal motif for stimulation of murine B cells is an unmethylated CpG dinucleotide flanked by two 5' purines (PuPu) and two 3' pyrimidines (PyPy) (GACGTT) (Krieg *et al.*, 1995). CpG DNA is a stronger Th1-like adjuvant for inducing B-cell and T-cell responses than for instance the golden standard complete Freund's adjuvant (CFA).

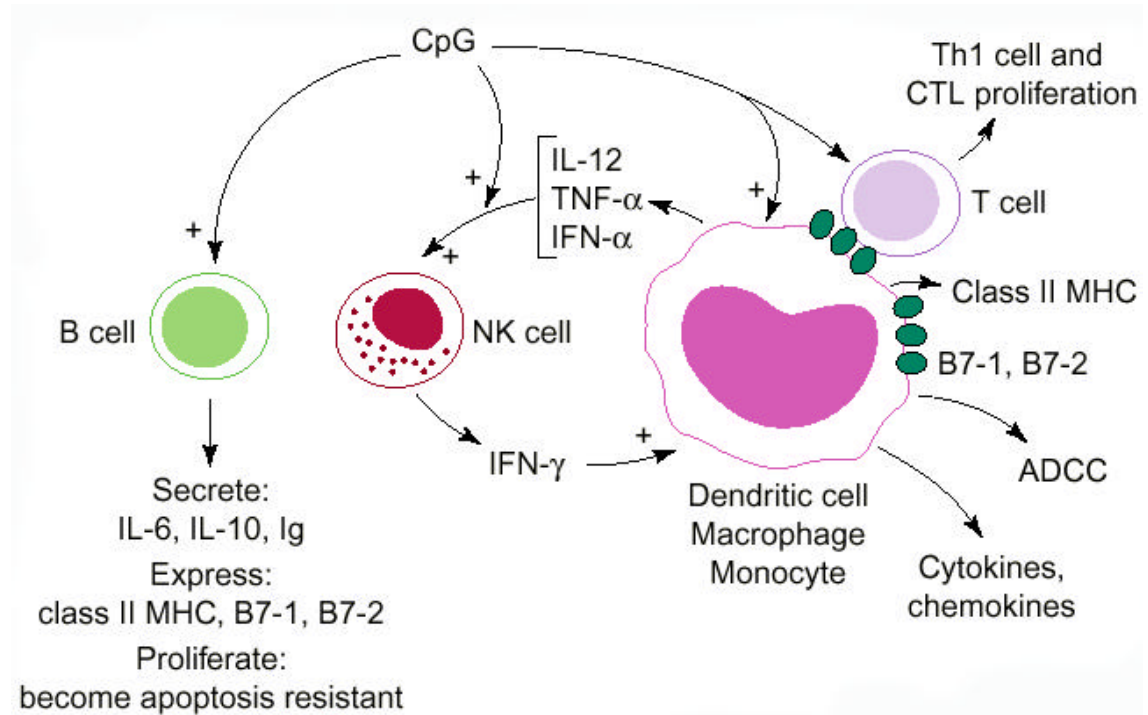


Figure 10. Mechanism of immune activation by CpG-ODN. Adapted from (Krieg and Wagner, 2000).

The optimal CpG motif recognized by human and primate cells is different from the mouse motif (Hartmann *et al.*, 2000) and the TLR-9 protein seems to determine the species specificity of the CpG motifs (Bauer *et al.*, 2001). After screening of more than 250 ODNs with phosphothioate backbones, which makes them more stable, the optimal sequence for stimulation of human B cells and NK cells was described to be GTCGTT (called K or B, Table I). ODNs containing this sequence have later been shown to increase the seroconversion rate and antibody response of orangutans, chimpanzees, cynomolgus macaques and aotus monkeys immunized with hepatitis B vaccine and malaria proteins (Davis *et al.*, 2000; Hartmann *et al.*, 2000; Jones *et al.*, 2001). A control ODN lacking CpG did not affect antibody responses induced by vaccination (Jones *et al.*, 2001). Cellular IFN- immune responses to proteins have also been enhanced in primates (Verthelyi *et al.*, 2002; Zuber *et al.*, 2002).

A novel CpG motif (called D or A, Table I) that stimulates human and monkey cells was recently discovered (Krug *et al.*, 2001; Verthelyi *et al.*, 2001). These ODNs have a mixed phosphodiester/phosphorothioate backbone, express a single self-complementary purine/pyrimidine/CpG/purine/pyrimidine motif, and are capped by a 3' poly G tail.

These two classes were shown to have distinct immunostimulatory properties on human cells *in vitro*; (Table 1) K/B ODN stimulate B cells to proliferate and secrete IgM, plasmacytoid dendritic cell precursors to mature and secrete IL-8, and monocytes to produce IL-6. In comparison, D/A ODN trigger plasmacytoid dendritic cells to produce large amounts of IFN- α , directly or indirectly trigger natural killer cells to secrete interferon- α , and myeloid dendritic cells to mature. In one *in vivo* study, D/A ODN boosted antibody responses to ovalbumin more potently than did K/B ODN (Verthelyi *et al.*, 2002); this seems contradictory to the *in vitro* data, which show that K/B is the optimal motif for B cell stimulation. In the same study, D/A and K/B ODN induced similar IFN- responses in cells from monkeys immunized with a heat-killed *Leishmania* vaccine and restimulated with *Leishmania* protein. In conclusion, more *in vivo* studies in primates are needed to evaluate the different effects induced by CpG motifs.

Table.I Nucleotide motifs that enhance immunogenicity

Cell type	¹ ODN K/B <u>TCGT</u><u>CGT</u>TTTTGT<u>CGT</u>TTTTGT<u>CGT</u>T	² ODN D/A <u>GGTGCAT</u><u>CGATGCAG</u>GGGGG
B	+++ Activation; Upregulated CD69,CD25,CD86 Proliferation Secretion; IgM, IL6	+
Plasmo- cytoid dendritic cell (PDC)	Secretion; TNF- (Krug <i>et al.</i> , 2001)	Secretion; TNF- , IFN- (Krug <i>et al.</i> , 2001)
Mono cyte	Activation; Upregulated CD69,CD25 Proliferation Secretion; IL-6 Maturation into macrophages (Klinman <i>et al.</i> , 2002)	Maturation into DC (Klinman <i>et al.</i> , 2002)
NK	+ Activation; Upregulated CD69, CD25	+++ Activation; Upregulated CD69, CD25 Secretion; IFN- (Verthelyi <i>et al.</i> , 2001) Lysis (Krug <i>et al.</i> , 2001)
T (CD4+)		Secretion; IFN- (Krug <i>et al.</i> , 2001)

¹PS backbone, the optimal mouse motif is Pu/Pu CG Py/Py Example, ODN 1826:

TCCATGACCGTTCCTGACCGTT

The optimal primate motif is GTCCGTT, example, ODN 2006:

TCGTCGTTTTTGTCGTTTTTGTCGTT.

²PO/PS mixed backbone

Single hexameric Pu/Py CG Pu/Py motif flanked by selfcomplementary bases that form a stem-loop structure capped at the 3' end by a poly G tail (Verthelyi *et al.*, 2001)

Example, ODN D19 (Verthelyi *et al.*, 2002)

GGTGCATCGATGCAGGGGGG

Pu= A, G, Py= T, C, PS = phosphothioate, PO = phosphodiester

6.3.5 How can DNA vaccines be improved?

There are many ways to improve the efficacy of DNA vaccines.

The vector can be optimized to express more protein, using optimal promoters, enhancers, poly(A) signals and translation initiation signals. The foreign genes expressed can also be humanized to enhance expression (Andre *et al.*, 1998).

Uptake of DNA and delivery to the cytoplasm is a barrier to efficient transfection of cells *in vivo*. DNA can be coated on gold particles and delivered epidermally by a gene gun to enhance delivery to APC (Porgador *et al.*, 1998). Uptake can be enhanced by *in vivo* electroporation, which has been shown to substantially increase DNA delivery and DNA vaccine potency (Widera *et al.*, 2000). Adsorption of DNA onto cationic microparticles may also result in enhanced delivery of DNA to APC (Singh *et al.*, 2000).

The micro-environment in which the antigen is presented to the immune system can be changed to enhance and direct the immune response (Gurunathan, Klinman, and Seder, 2000). Cytokines are soluble molecules that generally act locally on immune cells. For example, interleukin-2 (IL-2) causes the activation and proliferation of T-lymphocytes. It is the major autocrine growth factor for T lymphocytes and is an important determinant of the magnitude of T cell-dependent immune responses. Recently, control of viremia and prevention of clinical AIDS in rhesus monkeys by cytokine-augmented DNA vaccination were demonstrated (Barouch *et al.*, 2000). In this study, an IL2/Ig fusion protein was used to enhance DNA mediated immune responses.

The cytokine IL-12 can be used to activate the Th1 subset of CD4⁺ T cells and to repress the Th2 subset. Co-delivery of cytokines or cytokine genes can also be used to recruit and mature dendritic cells. This can be achieved using GM-CSF (Conry *et al.*, 1996; Samanci *et al.*, 1998).

7 AIMS OF THE THESIS

The general objective was to induce strong immune responses towards the HIV-1 coreceptor CCR5 and to the HIV-1 enzyme RT using gene vaccination. Furthermore we wanted to study the protective capacity of these vaccine components. We also wanted to make a primate model for HIV resistance development, which could be used to investigate a vaccine towards RT containing drug induced mutations.

Specific aims:

- To study the immune responses induced by CCR5 immunization of mice and macaques (I).
- To evaluate the protective potential of a CCR5 vaccine in a macaque SIV challenge model (I, II).
- To study if CCR5 specific antibodies induce virus evolution in SIV challenged, CCR5 immunized macaques (II).
- To study the humoral and cellular immune responses to HIV-1 RT induced by a potent vaccine regimen in mice and monkeys (III).
- To study the protective capacity of RT in a HIV-1/MuLV model (III).
- To develop a monkey model for the study of HIV drug induced mutations (IV).

8 RESULTS AND DISCUSSION

8.1 CHARACTERIZATION OF IMMUNE RESPONSES INDUCED BY VACCINATION WITH THE HUMAN CCR5 GENE / PEPTIDES (I, II)

8.1.1 Introduction

Currently most HIV-1 drugs focus on the inhibition of different steps in the viral life cycle, by targeting important viral enzymes. An alternative or complementary approach is to target the receptors and coreceptors which the virus uses for infection. Chemokine analogues which block the CCR5 receptor have been developed. A derivate of RANTES called AOP-RANTES, did not induce chemotaxis but was a very strong antagonist of CCR5 function in monocytes (Simmons *et al.*, 1997). It potently inhibited infection of diverse cell types (including macrophages and lymphocytes) by NSI M-tropic HIV-1 strains.

A small molecule nonpeptide inhibitor of CCR5 called TAK-779 which has potent anti HIV-1 activity has been discovered (Baba *et al.*, 1999), another drug called AMD-3100 selectively blocks CXCR4 and has potent anti HIV-1 activity (De Clercq *et al.*, 1994). Alternatively CCR5 could be blocked by antibodies (Olson *et al.*, 1999; Trkola and Olson, 2001; Wu L *et al.*, 1997a; Wu L *et al.*, 1997b).

We evaluated immunization with the human CCR5 gene in mice and monkeys. Several immune enhancing approaches were studied. Gene deliveries by different routes were compared and the effects of immunostimulatory cytokines were evaluated.

A CCR5-tetanusepitope fusion gene was constructed to induce stronger CCR5 specific responses. We studied both cellular and humoral immune responses and evaluated the ability of induced CCR5 specific antibodies to neutralize HIV-1 and SIV. Furthermore we studied if tolerance of macaques to endogenous CCR5 could be broken by immunization with the human CCR5 gene (98% homology). Protection from SIV challenge was studied in monkeys.

8.1.2 Immune responses induced by the human CCR5 gene in mice

A gene was constructed in which a promiscuous-T-helper cell epitope from the tetanus toxin was fused in frame with the human CCR5 gene. This tetanus epitope has earlier been shown to enhance immune responses when fused to other proteins (Chin *et al.*, 1994; Kumar *et al.*, 1992; Panina-Bordignon *et al.*, 1989). The mice were first immunized with the tet-CCR5 construct by the intramuscular (i.m) route together with the murine GM-CSF gene on a separate plasmid, used as an immune enhancer. This protocol did not induce CCR5 specific humoral or cellular responses.

Epidermal gene gun immunization is known to induce good antibody responses. Therefore this route was evaluated as a way of enhancing the immune responses in mice. Gene gun immunization with a 50-fold lower DNA dose than was used in i.m

immunization induced good cellular and humoral responses. Using peptides, the strongest antibody response was mapped to the N-terminus of CCR5 while moderately strong responses were detected to the 2:nd extracellular loop.

Monoclonal antibodies (MAbs) have been used to study the different parts of the CCR5 receptor and their importance in HIV-1 interactions. It was shown by Lee *et al.* (Lee *et al.*, 1999) that N-terminal mAbs blocked gp120-CCR5 binding more effectively than mAbs directed to extracellular loop 2 (ECL2). Surprisingly, ECL2 mAbs were more potent inhibitors of viral infection than N-terminal mAbs. These results suggest that the N-terminal domain of CCR5 is more important for gp120 binding while the extracellular loops are more important for inducing conformational changes in Env that lead to membrane fusion and virus infection.

Sera from CCR5 immunized mice did not neutralize R5 HIV-1 infection of human PBMC despite the CCR5 specific binding antibodies. One explanation for this might be that the strongest antibody response was directed to the wrong part of CCR5 i.e. the N-terminus instead of the ECL2 binding site. The antibody response may also have been too weak.

8.1.3 Break of tolerance by heterologous gene immunization in monkeys

Antibody reactivity against both human and macaque CCR5 peptides was found after immunization of monkeys with the human CCR5 gene together with human GM-CSF protein (I). Similar results were obtained in a recent study in which macaques were immunized with baculovirus-produced human CCR5 protein or macaque CCR5 peptides (Lehner *et al.*, 2001). Human and cynomolgus macaque CCR5 are 98% identical. They differ by eight amino acids of which four are located in the N-terminus and the ECL2. The ECL2 of human and macaque CCR5 differ in amino acid 171, which is crucial for recognition of CCR5 by the strongly neutralizing MAb 2D7. These differences may be important for the macaque to recognize human CCR5 as foreign and to produce antibodies to both human and macaque CCR5. The GM-CSF may also have contributed to the breakage of tolerance. The immunization of humans with the macaque CCR5 gene may be a feasible way to break tolerance to human CCR5.

8.1.4 Immune responses induced by the human CCR5 gene in monkeys

Induction of mucosal CCR5 specific antibody responses may be beneficial in protecting from primary HIV-1 infection. To induce mucosal and systemic immune responses, we immunized macaques with the human CCR5 gene in the rectal mucosa. After two immunizations with the CCR5 gene the monkeys were immunized with the tet-CCR5 gene followed by two immunizations with human CCR5 peptides. This was done to further enhance CCR5 specific antibody responses. The genes and peptides were administered together with human GM-CSF protein to stimulate immune responses.

The monkeys developed both humoral IgG, IgA and cellular CCR5 specific responses after DNA immunization. The antibody responses towards CCR5 peptides were equally strong against the N-terminus and ECL2 after three DNA immunizations. After two peptide boosts, the response against the N-terminus was 25 times higher than the ECL2 directed response in monkey U9 and 5 times higher in monkey B189. The antibody responses towards conformational epitopes were mapped on native CCR5 expressed on the cell surface of CEM cells. Two monoclonal antibodies recognizing the N-terminus and ECL2 of native CCR5 were used in a competition assay with sera from the CCR5 immunized monkeys. Inhibition of MAb binding was evaluated using FACS. After three DNA immunizations monkey U9 recognized ECL2, while B189 recognized the N-terminus (Fig.11, B, D, E). Very interestingly the peptide boost destroyed the recognition of conformational epitopes, while it enhanced recognition of linear epitopes (Fig. 11). After CCR5 peptide boost monkey U9 still recognized the native ECL2 although the recognition was much weaker (Fig.11 B). Recognition of the native N-terminus was totally abrogated by the peptide boost (Fig. 11 D).

To conclude, DNA immunization with the CCR5 gene induced antibody responses towards native CCR5 epitopes. These responses were significantly reduced by CCR5 peptide boosting.

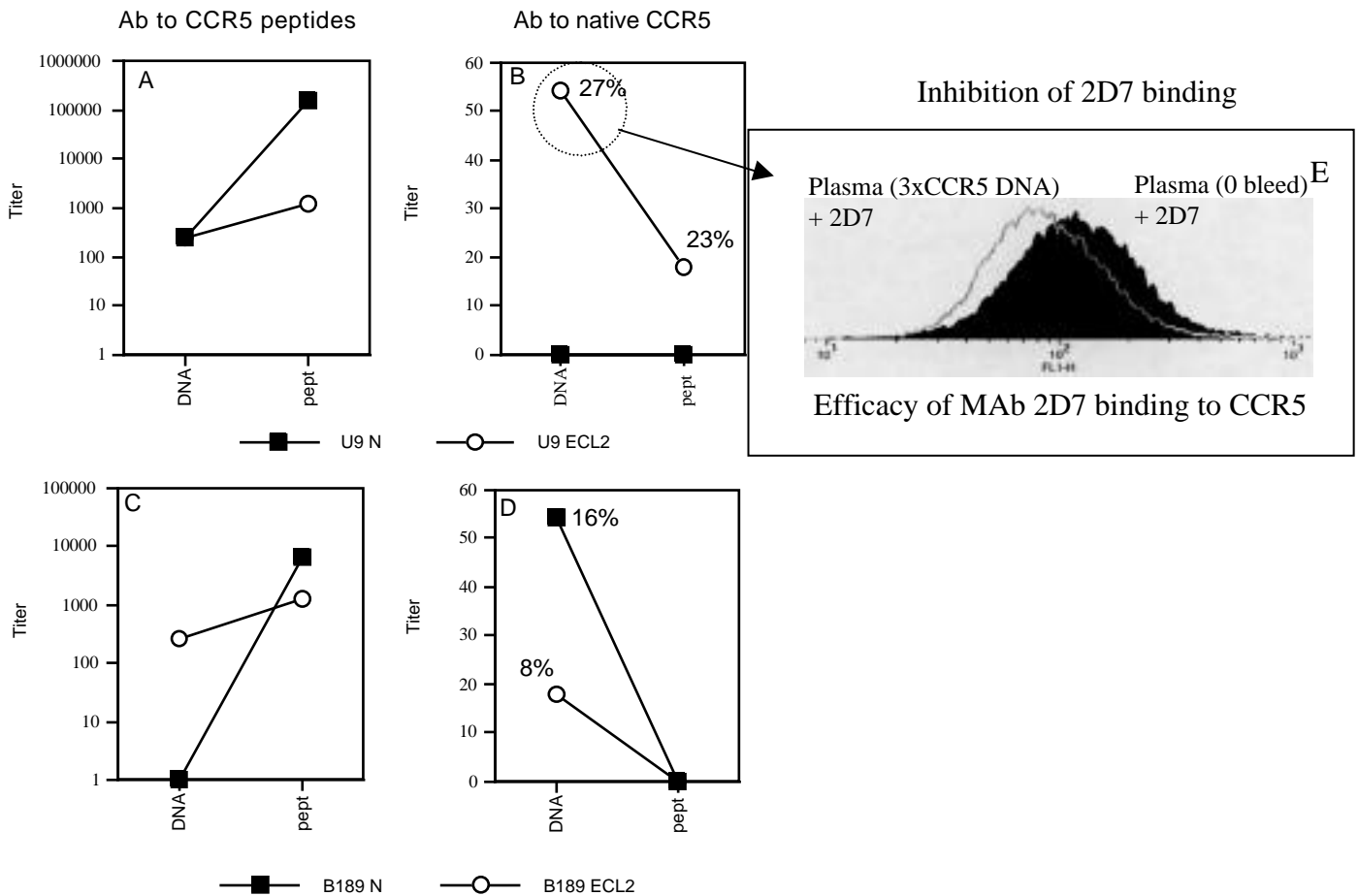


Figure 11. Antibody responses to CCR5 in macaques. The responses were measured after three immunizations with the human CCR5 gene (day 162, DNA) and after two boosts with CCR5 peptides (day 260, pept). IgG responses to peptides representing the N-terminus (N1c) and 2:nd extracellular loop (X2.1c) of macaque CCR5 were measured by ELISA (A, C). Antibody responses to the N-terminus (N) and 2:nd extracellular loop of native human CCR5 expressed on the surface of CEM cells were measured using FACS (B, D). Antibody responses in macaque sera were measured indirectly using competition with two CCR5 specific monoclonal antibodies; 2D7 (recognizing a conformational epitope in the 2:nd extracellular loop) and 3A9 (recognizing the N-terminus of native CCR5). The results are shown as the highest serum dilution which induced a difference in % inhibition of Mab binding as compared to % inhibition induced by control serum (before immunization). The percent inhibition of Mab binding is also shown (B, D). An example of the FACS data is shown (E).

Sera from the immunized monkeys neutralized several primary R5 HIV-1 isolates and SIVsm but not an HIV-1 X4 strain. This indicated that the neutralizing capacity was CCR5 specific. Sera from monkey U9 could block infection by two primary HIV-1 R5 strains while sera from B189 only blocked infection by one strain (I). This may reflect the fact that sera from monkey U9 contained Ab directed to the native ECL2 of CCR5 while sera from B189 did not.

We also found that CCR5 gene immunization increased the RANTES levels in the plasma of monkey U9. Increased levels of the CCR5 ligand RANTES may act in synergy with CCR5 specific antibodies to block virus binding to CCR5 expressing cells.

The monkeys were challenged intrarectally with SIVsm one month after the last immunization. Both immunized and control monkeys became infected after challenge; neither protection nor enhancement was found. A 3.4 log decrease of virus load at virus setpoint was observed in the monkey B189. The small number of animals in the study makes it difficult to draw any conclusions from the observed decrease in virus setpoint. It is tempting to speculate that the CCR5 peptide boost may have abrogated potentially protective immune response induced by the CCR5 gene vaccination.

8.1.5 Virus evolution after CCR5 immunization of monkeys

It has been demonstrated that SIVsm uses CCR5 very efficiently but also facilitates the coreceptors BOB and Bonzo for entry (Vödrös *et al.*, 2001). Sequential virus isolates from monkey U9 used the CCR5 receptor much more efficiently than did virus isolated from monkey B189 (II). Virus from monkey U9 was also more CCR5 dependent than virus from B189 (II).

Other studies using CCR5 specific antibodies (Aarons *et al.*, 2001) or small molecule inhibitors (Trkola *et al.*, 2002), have shown that HIV-1 does not switch coreceptor when subjected to CCR5 blockers but rather starts to use CCR5 more efficiently or in a different manner. In conclusion, CCR5 specific antibody responses together with enhanced RANTES levels induced virus evolution and a change in coreceptor usage.

8.2 CHARACTERIZATION OF IMMUNE RESPONSES INDUCED BY VACCINATION WITH THE RT GENE / PROTEIN (III)

8.2.1 A new prime boost strategy induces strong immune responses to HIV-1 RT

We wanted to induce a strong, RT specific cellular and humoral immune response. In order to do so, we evaluated several prime boost immunization strategies in mice and monkeys. We also investigated protection from HIV-1/MuLV challenge in mice.

The RT gene was used for priming, and the animals were boosted with RT protein with or without CpG-ODN. Cellular immune responses were evaluated by measuring IFN- γ and IL-2 secretion from RT peptide stimulated splenocytes or blood derived

PBMCs. The strongest IFN- responses in PBMC from mouse and macaque blood were induced by DNA prime, followed by RT protein with CpG-ODN.

The immunized mice were challenged intraperitoneally with syngeneic splenocytes infected with HIV-1/MuLV (Fig. 12). The animals were sacrificed and cells from the intraperitoneal cavity were cocultured with HIV-1 permissive cells. Protection was defined as inability to rescue HIV-1 from the intraperitoneal cells. Mice immunized with the RT gene followed by the RT protein with CpG-ODN and mice receiving RT protein with CpG-ODN without the RT gene prime were partially protected from the HIV-1/MuLV challenge. The strongest IFN- and IL-2 cellular immune responses after challenge were also found in these two mouse groups. Protective effect from HIV-1/MuLV challenge was also observed in an earlier study in which mice were immunized with the RT gene (Isaguliantz *et al.*, 2000b). In conclusion, the gene prime followed by protein with CpG-ODN boost immunization approach induced potent humoral and cellular immune responses in both mice and macaques. This immunization regimen also partially protected mice from HIV-1/MuLV challenge.

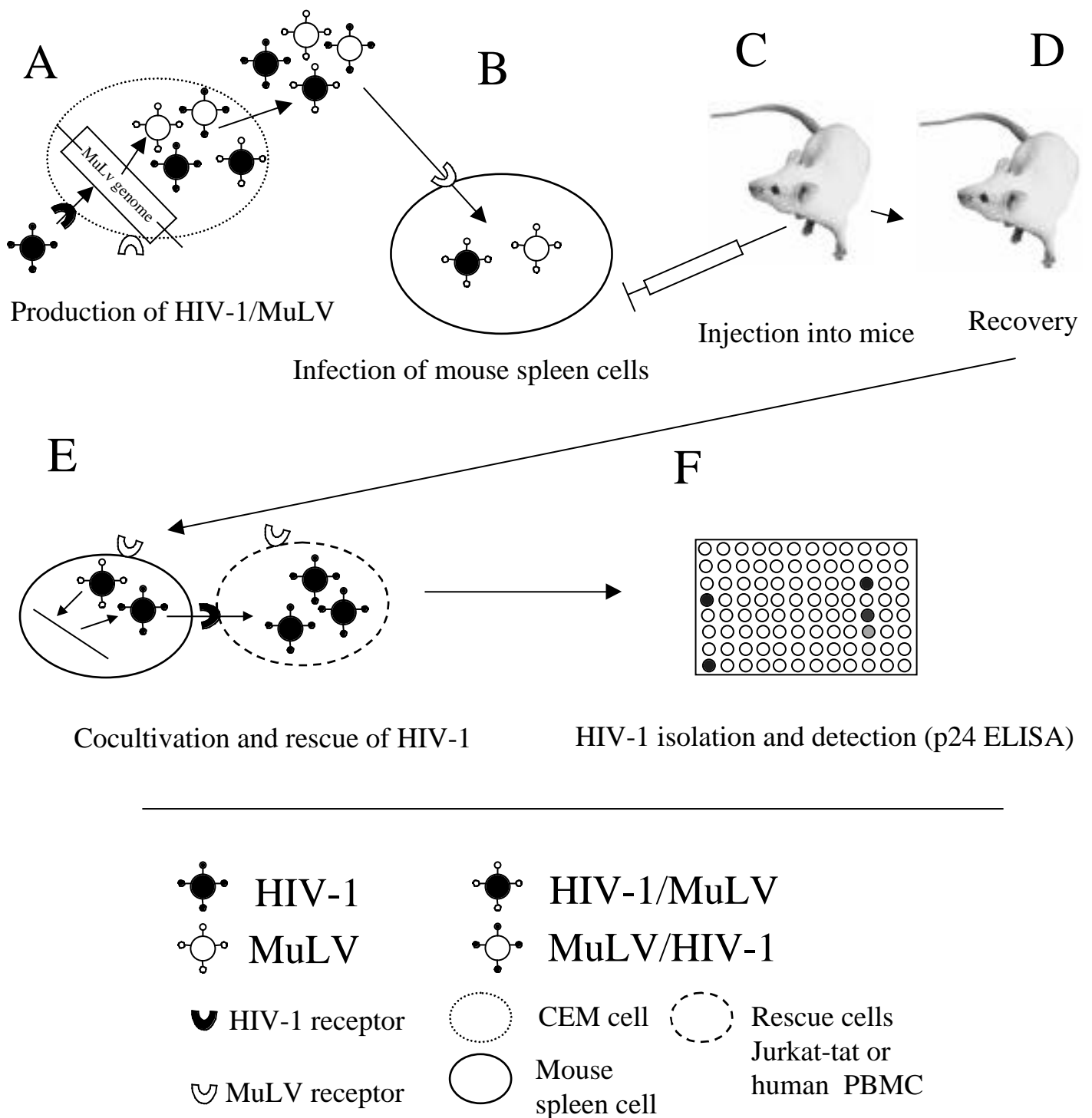


Figure 12. A model for HIV-1/MuLV challenge of immunized mice. Human T-cell line (CEM-B), permissive for HIV-1. This cell contains a integrated MuLV genome, which can be induced to produce MuLV (A). Infection of primary mouse spleen cells with HIV-1/MuLV (B). Syngeneic HIV-1/MuLV infected spleen cells are injected intraperitoneally (ip) into mice (C). Cells are recovered from the peritoneal cavity 10 days after challenge (D). Recovered mouse spleen cells from ascites were cocultivated for 21 days with HIV-1 permissive human jurkat-tat or PBMC (E). ELISA was performed every third day on supernatants from the cocultivated cell cultures (F).

8.3 AN *IN VIVO* MODEL FOR HIV-1 RESISTANCE DEVELOPMENT (IV)

HIV-1 resistance development was studied in a monkey model. This model can be used in future studies on vaccination against drug-induced RT mutants and to study the development of resistance to novel HIV-1 drugs.

NNRTIs are very specific for HIV-1 RT and do not inhibit HIV-2 or SIV RT. Therefore a hybrid virus (RT-SHIV) of simian immunodeficiency virus (SIV) with its RT exchanged for HIV-1 RT was used. The NNRTI nevirapine was chosen as a model drug because nevirapine monotherapy of HIV-1 patients induces a specific set of mutations in RT within a few weeks.

Cynomolgus monkeys infected with RT-SHIV were treated with varying doses of nevirapine. Viral load and resistance development were monitored. Effects of treatment interruption on resistance development were also studied.

8.3.1 Low dose drug treatment

Chronically SHIV-RT infected monkeys were treated with a low dose of nevirapine to give the virus the best opportunity to accumulate mutations. Treatment interruptions were also used to facilitate resistance development. The usual human oral dose regimen for nevirapine is 200 mg given daily for 14 days and then 200 mg administered twice daily. These doses correspond to 3 and 6 mg/kg (for a 70 kg person) respectively. Monkeys were treated with 3 mg/kg given s.c. for 75 days in total, interrupted by rest periods. This treatment did not induce any mutations in the RT gene. The dose was probably too low to inhibit the viral replication, since no effect was seen on the viral load in plasma.

The same monkeys were then treated with a higher nevirapine dose of 9 mg/kg for 10 days without interruptions. After this treatment regimen, the nevirapine specific mutation K103N (Fig.5 C) was found in one of three monkeys. This mutation has been observed in nevirapine treated patients (Richman *et al.*, 1994). The K103N mutation was not selected by nevirapine treatment of RT-SHIV (Balzarini *et al.*, 1995) or HIV-1 (Dolores *et al.*, 2002) in cell culture, but has been observed infrequently by others (Loemba *et al.*, 2002). This indicates that the monkey model might be used to detect mutations that are difficult or impossible to predict *in vitro*.

The biotransformation pathway of nevirapine in female cynomolgus monkeys is identical with the pathways observed in humans. Although qualitatively the metabolism of nevirapine is similar, significant differences in the rate of elimination of nevirapine have been noted between cynomolgus macaques and humans. The half-life of nevirapine is 1.5 h in macaques compared to a mean of 20 h in humans and the systemic clearance rate is about 30-fold higher in macaques. These differences may explain why the viral load was unaffected by the 3mg/kg dose in macaques.

8.3.2 High dose drug treatment

A new group of monkeys was treated with the higher initial nevirapine dose of 9 mg/kg. This dose had an effect on the viral load in 1/3 monkeys. After 11 days of treatment a low amount of the Y181C (Fig.5 C) mutant was detected in 2/3 monkeys by using direct sequencing of the whole viral population. The Y181C mutation is the one most commonly found in HIV-1 patients receiving nevirapine monotherapy. The monkeys were rested for 23 days and then treated with a still higher nevirapine dose of 18 mg/kg. After this increased dose the nevirapine-specific resistance mutation Y181C was identified in virus from all treated monkeys and the combined Y181C/K103N mutation was found in monkey 83 (Fig. 5 C).

During the 18 mg/kg treatment, virus load decreased 1.3 and 0.6 log, respectively, in monkeys 82 and 83, while the virus load in 81 remained unchanged. 14 days after the start of therapy, virus loads in monkeys 82 and 83 had increased despite continued treatment, suggesting that nevirapine-resistant virus had emerged.

These observations correspond well to studies made in human patients receiving nevirapine monotherapy. In one study a consistent maximum decrease of 2 log₁₀ HIV-1 RNA copies per ml of plasma (range: 1.96 to 2.43) from baseline after 2 weeks was observed in all monotherapy subjects. After 14 days of therapy, HIV-1 RNA levels began to increase and aa Y181C mutant virus was detected. No reversion of mutants was observed during the 23-day rest period of our macaques, which also correlates with data from nevirapine treated HIV-1 patients.

In conclusion, a moderately high nevirapine dose induced the same pattern of mutations in RT in the chronically SHIV-RT infected macaque as observed in the RT of HIV-1 patients given nevirapine monotherapy. The need for a higher nevirapine dose to affect viral load in the infected monkeys could be explained by the differences in nevirapine metabolism between macaques and patients.

The K103N mutation was not detected in nevirapine treated SHIV-RT *in vitro* (Balzarini *et al.*, 1995). This indicates that the *in vivo* macaque model in which the K103N mutation was induced may give a more true reflection of the real mutation patterns. This may be used for characterization of novel drugs.

9 GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

In this thesis two genes (CCR5 and RT) were examined as vaccine targets for the development of a HIV-1 vaccine. The genes were evaluated for their immunogenicity and protective capacity. New immunization protocols were developed to enhance immune responses towards these genes. Furthermore, an animal model was developed to study the evolution of RT drug-induced mutations *in vivo*.

First we studied whether the human CCR5 gene could be used for immunization and whether the generated immune responses could protect from challenge. This thesis has demonstrated that immunization with the human CCR5 gene gives CCR5 specific humoral and cellular responses in mice and monkeys. Antibodies to human CCR5 were induced and break of tolerance to endogenous CCR5 was achieved in macaques by immunization with the human CCR5 gene together with the human GM-CSF protein. The CCR5 gene immunization was followed by CCR5 peptide injection in an attempt to increase CCR5 specific antibody responses. Responses towards CCR5 peptides were enhanced by CCR5 peptide immunization but antibody responses towards native CCR5 were abolished or decreased. Sera from immunized monkeys inhibited HIV-1 and SIV infection of cells *in vitro*. Although the monkeys had high titers of CCR5 peptide specific antibodies at the time of challenge with SIV, all monkeys became infected. It is possible that a better effect of the vaccine would have been achieved if more antibodies were directed to native CCR5. Recent results from Lehner indicate that immunization with CCR5 together with virus components may elicit clearance or a decrease in viral load in macaques (Lehner, 2002; Lehner and Shearer, 2002).

At the time of SIV challenge the two CCR5 immunized monkeys had similar antibody titers towards extracellular parts of CCR5 in a peptide ELISA. Sera from monkey U9 but not from monkey B189 reacted with native CCR5 on the cell surface. Sera from monkey U9 also had a broader neutralization capacity after DNA vaccination (I). This indicated a qualitative difference in the antibody responses.

We also found that CCR5 gene immunization increased RANTES levels in plasma of monkey U9. This finding suggests that CCR5 gene immunization may induce CCR5 blockage by two mechanisms: 1) by inducing CCR5 specific antibodies, and 2) by increasing the amount of the natural CCR5 ligand RANTES.

Virus was isolated from the CCR5 immunized and SIV challenged monkeys over time after infection. Coreceptor usage of these viruses was studied using indicator cell lines expressing different coreceptors used by SIV and HIV. Interestingly, virus isolated from monkey U9 used CCR5 much more efficiently than did virus from monkey B189 or control monkeys. This indicates that antibody responses towards CCR5 may select for viruses which use CCR5 in a novel way. No coreceptor switch was observed as a cause of CCR5 specific antibody pressure. It has recently been shown by others that both a CCR5 specific antibody (Aarons *et al.*, 2001) and a small molecule inhibitor of CCR5 (Trkola *et al.*, 2002) selected for resistant HIV-1 virus,

which used CCR5 more efficiently; no coreceptor switch was observed in these studies either.

The env gene from virus of the CCR5-immunized animals was partially sequenced. Especially the V3 loop was sequenced with several overlapping primers since it has been shown to be important in coreceptor interactions. No changes were found in the V3 loop. In other studies where CCR5 specific antibody or drug pressure was applied *in vitro* to HIV-1 isolates, changes were not found in the V3 loop, but in other parts of the env gene (Aarons *et al.*, 2001; Trkola *et al.*, 2002). In future studies it would be very relevant to clone and fully sequence the envelope genes from viruses isolated from the CCR5 immunized monkeys. The cloned envelopes could also be used to pseudotype viruses and to study the env-receptor binding in more detail (Hoffman *et al.*, 2000).

In conclusion, the human CCR5 gene together with GM-CSF was successfully used for immunization of mice and macaques. CCR5 gene immunization induced antibodies directed to native CCR5, while this response was reduced by CCR5 peptide boosting. CCR5 immunization did not protect macaques from SIV challenge. Virus isolated from one CCR5 immunized monkey started to use CCR5 more efficiently, probably because of antibody responses specific for conformational epitopes on native CCR5. As we argue in Science (Fig. 13) this phenomenon has to be investigated further, before anti CCR5 agents are introduced in humans.

In the second part of this thesis we studied and tried to enhance immune responses induced by immunization with the RT gene. The RT gene alone induced weak cellular IFN- responses in mice and macaques as measured by ELISPOT with overlapping peptides covering the whole RT protein. To enhance immune responses, a DNA prime protein boost approach was evaluated. This immunization scheme was further potentiated using immunostimulatory bacterial DNA (CpG-ODN). The most potent cellular immune responses in PBMC from mouse and macaque blood, were induced by the RT gene followed by RT protein with CpG-ODN. This immunization regimen also induced the most potent antibody responses in monkeys and a good antibody response in mice.

DNA followed by protein with CpG-ODN as well as protein with CpG alone conferred a certain degree of resistance to challenge with HIV-1/MuLV. Cellular immune responses were also evaluated in splenocytes recovered from the immunized mice after challenge. Both IFN- and IL-2 responses were strong in the two mouse groups with partial protection from challenge.

In future studies the optimized RT based immunization regimen could be tested in larger groups of macaques. These monkeys could be challenged with a SHIV-RT virus to evaluate protective capacity of the RT gene / protein. Furthermore the potential dangers of immunization with an active RT should be evaluated in these monkeys.

In the next step it would be interesting to evaluate immune responses towards RT containing drug-induced mutations. A gene vaccine based on RT CTL epitopes

containing drug-induced mutations could in theory have synergistic effects with RT specific HIV drugs. We have designed synthetic genes containing RT CTL epitopes with drug induced mutations (Fig. 14). Evaluation of these gene constructs is ongoing.

In the last study we wanted to develop a primate model for HIV-1 drug resistance, which could be used to study vaccines against resistant virus. The model may also facilitate studies of mutation patterns induced by HIV-1 drugs *in vivo*. A macaque model for the study of HIV-1 drug resistance development was successfully created. Nevirapine was used as a model drug to study the development of resistance in seven macaques chronically infected by RT-SHIV. Different parameters were compared between the monkey model and published results on nevirapine monotherapy of HIV-1 patients. Overall the parameters were very similar between humans and macaques. Resistance to nevirapine was observed when viral load started to increase during ongoing nevirapine treatment of the monkeys. The same key mutations in the RT gene were induced by nevirapine treatment of monkeys and humans (Y181C and K103N).

It would be interesting to use this model for evaluation of vaccines targeted to resistant HIV-1. This would facilitate identification of macaque specific CTL epitopes which overlap with HIV-1 drug induced mutations.

HIV coreceptor immunization - unexpected risks?

23 September
2002



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In their Letter "Alternative HIV vaccine strategies" (23 August, p. 1276), Thomas Lehner and Gene Shearer argue in favor of a vaccine strategy directed toward self components. The authors suggest prevention of HIV infection by targeting the viral coreceptor CCR5. A vaccine strategy to avoid escape from virus-specific cytotoxicity or antibody responses is valid and very important. We investigated the ability of CCR5 immunization to delay or prevent HIV-1 infection of macaques (1). Surprisingly, however, the induction of high titers of CCR5 specific antibodies appeared to select for new virus variants in vivo. Virus isolated from a macaque after immunization by the CCR5 gene and challenge by simian immunodeficiency virus (SIV) used the CCR5 receptor much more efficiently, indicating a higher affinity of the newly selected virus variants to the coreceptor (2). Usually, the CCR5 usage of SIV is very stable over time (3). These in vivo data were confirmed by in vitro experiments. Moore and co-workers used a CCR5-specific small molecular inhibitor to select virus in cell culture. The selected virus acquired over 20,000-fold resistance to the inhibitor without change in coreceptor usage (4). Instead of using an alternative receptor, the selected virus required lower levels of CCR5 for entry and probably used the drug-bound form of the receptor. In a similar experiment, a CCR5 specific monoclonal antibody was used to select resistant virus in cell culture. The adapted virus became approximately 100-fold less sensitive to the antibody, but it did not change coreceptor usage (5).

Thus, it has been demonstrated in vitro and in vivo that inhibition of CCR5 may select for viruses that use the receptor in a novel and more effective way. The expected virus variation--drift to usage of the coreceptor CXCR4--was demonstrated after treatment of HIV-1- infected hu-PBL-SCID mice with an analog of the natural chemokine ligand RANTES (6). The impact of CCR5-induced virus evolution on pathogenesis and disease evolution has to be thoroughly investigated before a CCR5-based vaccine is introduced into humans.

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1. B. Zuber et al., Virology 278, 400 (2000).
2. B. Zuber et al., CCR5 gene immunization lowers viral setpoint and may select for SIV that uses CCR5 more efficiently, HIV-1 protection and control by vaccination, Keystone, Colorado (2002).
3. D. Vödrös et al., Virology 291, 12 (2001).
4. A. Trkola et al., Proc. Natl. Acad. Sci. U.S.A. 99, 395 (2002).
5. E. J. Aarons et al., Virology 287, 382 (2001).
6. D. E. Mosier et al., J. Virol. 73, 3544 (1999).

Figure 13. Potential risks in receptor immunization.

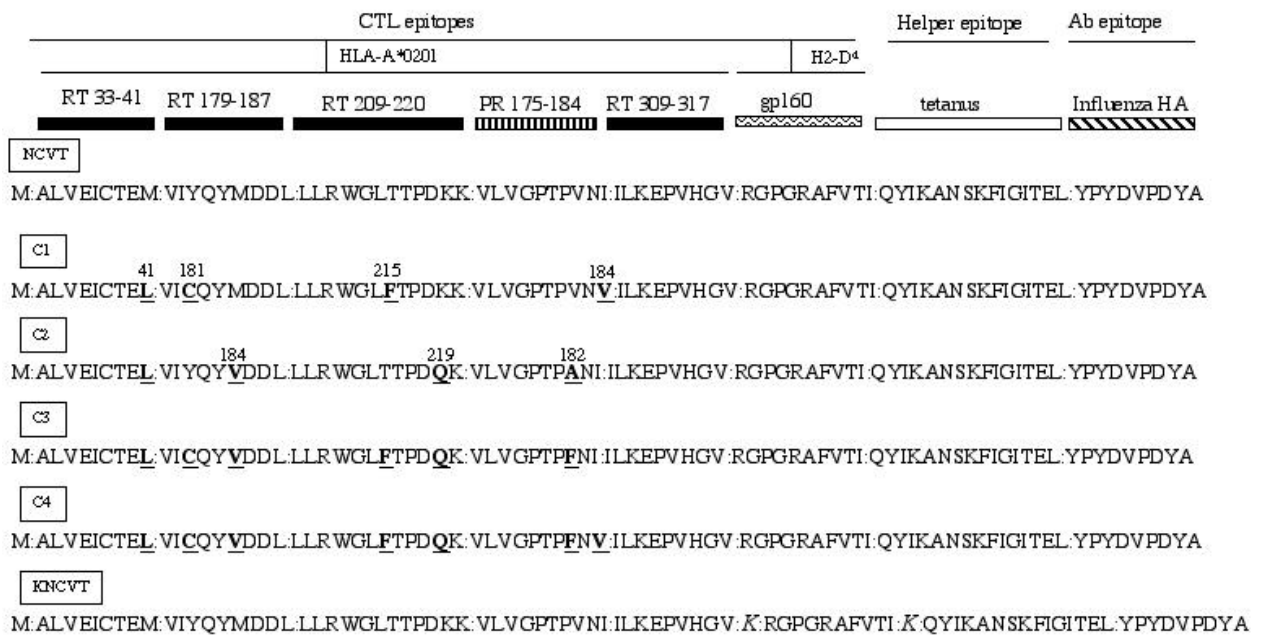


Figure 14. Minigene constructs. KNCVT contains wild type RT and protease epitopes. Constructs C1-C4 contain epitopes with drug induced mutations (bold letters).

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11 REFERENCES

- Aarons, E. J., Beddows, S., Willingham, T., Wu, L., and Koup, R. A. (2001). Adaptation to blockade of human immunodeficiency virus type 1 entry imposed by the anti-ccr5 monoclonal antibody 2d7. *Virology* **287**(2), 382-90.
- Akbari, O., Panjwani, N., Garcia, S., Tascon, R., Lowrie, D., and Stockinger, B. (1999). DNA vaccination: transfection and activation of dendritic cells as key events for immunity. *J Exp Med* **189**(1), 169-78.
- Albert, J., Abrahamsson, B., Nagy, K., Aurelius, E., Gaines, H., Nyström, G., and Fenyő, E. M. (1990). Rapid development of isolate-specific neutralizing antibodies after primary HIV-1 infection and consequent emergence of virus variants which resist neutralization by autologous sera. *Aids* **4**(2), 107-12.
- Allen, T. M., O'Connor, D. H., Jing, P., Dzuris, J. L., Mothe, B. R., Vogel, T. U., Dunphy, E., Liebl, M. E., Emerson, C., Wilson, N., Kunstman, K. J., Wang, X., Allison, D. B., Hughes, A. L., Desrosiers, R. C., Altman, J. D., Wolinsky, S. M., Sette, A., and Watkins, D. I. (2000). Tat-specific cytotoxic T lymphocytes select for SIV escape variants during resolution of primary viraemia. *Nature* **407**(6802), 386-90.
- Amara, R. R., Villinger, F., Altman, J. D., Lydy, S. L., O'Neil, S. P., Staprans, S. I., Montefiori, D. C., Xu, Y., Herndon, J. G., Wyatt, L. S., Candido, M. A., Kozyr, N. L., Earl, P. L., Smith, J. M., Ma, H. L., Grimm, B. D., Hulsey, M. L., Miller, J., McClure, H. M., McNicholl, J. M., Moss, B., and Robinson, H. L. (2001). Control of a mucosal challenge and prevention of AIDS by a multiprotein DNA/MVA vaccine. *Science* **292**(5514), 69-74.
- An, L. L., Rodriguez, F., Harkins, S., Zhang, J., and Whitton, J. L. (2000). Quantitative and qualitative analyses of the immune responses induced by a multivalent minigene DNA vaccine. *Vaccine* **18**(20), 2132-41.
- Andre, S., Seed, B., Eberle, J., Schraut, W., Bultmann, A., and Haas, J. (1998). Increased immune response elicited by DNA vaccination with a synthetic gp120 sequence with optimized codon usage. *J Virol* **72**(2), 1497-503.
- Arthur, L. O., Bess, J. W., Jr., Sowder, R. C., 2nd, Benveniste, R. E., Mann, D. L., Chermann, J. C., and Henderson, L. E. (1992). Cellular proteins bound to immunodeficiency viruses: implications for pathogenesis and vaccines. *Science* **258**(5090), 1935-8.
- Arthur, L. O., Bess, J. W., Jr., Urban, R. G., Strominger, J. L., Morton, W. R., Mann, D. L., Henderson, L. E., and Benveniste, R. E. (1995). Macaques immunized with HLA-DR are protected from challenge with simian immunodeficiency virus. *J Virol* **69**(5), 3117-24.
- Attanasio, R., Pehler, K., and Scinicariello, F. (1997). DNA-based immunization induces anti-CD4 antibodies directed primarily to native epitopes. *FEMS Immunol Med Microbiol* **17**(4), 207-15.

- Autran, B., Hadida, F., and Haas, G. (1996). Evolution and plasticity of CTL responses against HIV. *Curr Opin Immunol* **8**(4), 546-53.
- Baba, M., Nishimura, O., Kanzaki, N., Okamoto, M., Sawada, H., Iizawa, Y., Shiraishi, M., Aramaki, Y., Okonogi, K., Ogawa, Y., Meguro, K., and Fujino, M. (1999). A small-molecule, nonpeptide CCR5 antagonist with highly potent and selective anti-HIV-1 activity. *Proc Natl Acad Sci U S A* **96**(10), 5698-703.
- Baba, T. W., Liska, V., Hofmann-Lehmann, R., Vlasak, J., Xu, W., Ayehunie, S., Cavacini, L. A., Posner, M. R., Katinger, H., Stiegler, G., Bernacky, B. J., Rizvi, T. A., Schmidt, R., Hill, L. R., Keeling, M. E., Lu, Y., Wright, J. E., Chou, T. C., and Ruprecht, R. M. (2000). Human neutralizing monoclonal antibodies of the IgG1 subtype protect against mucosal simian-human immunodeficiency virus infection. *Nat Med* **6**(2), 200-6.
- Bachmair, A., Finley, D., and Varshavsky, A. (1986). In vivo half-life of a protein is a function of its amino-terminal residue. *Science* **234**(4773), 179-86.
- Baggiolini, M., Dewald, B., and Moser, B. (1994). Interleukin-8 and related chemotactic cytokines--CXC and CC chemokines. *Adv Immunol* **55**, 97-179.
- Balzarini, J., De Clercq, E., and Uberla, K. (1997). SIV/HIV-1 hybrid virus expressing the reverse transcriptase gene of HIV-1 remains sensitive to HIV-1-specific reverse transcriptase inhibitors after passage in rhesus macaques. *J Acquir Immune Defic Syndr Hum Retrovirol* **15**(1), 1-4.
- Balzarini, J., Weeger, M., Camarasa, M. J., De Clercq, E., and Uberla, K. (1995). Sensitivity/resistance profile of a simian immunodeficiency virus containing the reverse transcriptase gene of human immunodeficiency virus type 1 (HIV-1) toward the HIV-1-specific non-nucleoside reverse transcriptase inhibitors. *Biochem Biophys Res Commun* **211**(3), 850-6.
- Barouch, D. H., Santra, S., Schmitz, J. E., Kuroda, M. J., Fu, T. M., Wagner, W., Bilska, M., Craiu, A., Zheng, X. X., Krivulka, G. R., Beaudry, K., Lifton, M. A., Nickerson, C. E., Trigona, W. L., Punt, K., Freed, D. C., Guan, L., Dubey, S., Casimiro, D., Simon, A., Davies, M. E., Chastain, M., Strom, T. B., Gelman, R. S., Montefiori, D. C., and Lewis, M. G. (2000). Control of viremia and prevention of clinical AIDS in rhesus monkeys by cytokine-augmented DNA vaccination. *Science* **290**(5491), 486-92.
- Barre-Sinoussi, F., Chermann, J. C., Rey, F., Nugeyre, M. T., Chamaret, S., Gruest, J., Dauguet, C., Axler-Blin, C., Vezinet-Brun, F., Rouzioux, C., Rozenbaum, W., and Montagnier, L. (1983). Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science* **220**(4599), 868-71.
- Bauer, S., Kirschning, C. J., Hacker, H., Redecke, V., Hausmann, S., Akira, S., Wagner, H., and Lipford, G. B. (2001). Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. *Proc Natl Acad Sci U S A* **98**(16), 9237-42.
- Berger, E. A., Doms, R. W., Fenyo, E. M., Korber, B. T., Littman, D. R., Moore, J. P., Sattentau, Q. J., Schuitemaker, H., Sodroski, J., and Weiss, R. A. (1998). A new classification for HIV-1. *Nature* **391**(6664), 240.

- Berger, E. A., Murphy, P. M., and Farber, J. M. (1999). Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease. *Annu Rev Immunol* **17**, 657-700.
- Bergmann, C. C., Yao, Q., Ho, C. K., and Buckwold, S. L. (1996). Flanking residues alter antigenicity and immunogenicity of multi-unit CTL epitopes. *J Immunol* **157**(8), 3242-9.
- Blauvelt, A., Asada, H., Saville, M. W., Klaus-Kovtun, V., Altman, D. J., Yarchoan, R., and Katz, S. I. (1997). Productive infection of dendritic cells by HIV-1 and their ability to capture virus are mediated through separate pathways. *J Clin Invest* **100**(8), 2043-53.
- Boyer, J. D., Chattergoon, M. A., Ugen, K. E., Shah, A., Bennett, M., Cohen, A., Nyland, S., Lacy, K. E., Bagarazzi, M. L., Higgins, T. J., Baine, Y., Ciccarelli, R. B., Ginsberg, R. S., MacGregor, R. R., and Weiner, D. B. (1999). Enhancement of cellular immune response in HIV-1 seropositive individuals: A DNA-based trial. *Clin Immunol* **90**(1), 100-7.
- Boyer, J. D., Cohen, A. D., Vogt, S., Schumann, K., Nath, B., Ahn, L., Lacy, K., Bagarazzi, M. L., Higgins, T. J., Baine, Y., Ciccarelli, R. B., Ginsberg, R. S., MacGregor, R. R., and Weiner, D. B. (2000). Vaccination of seronegative volunteers with a human immunodeficiency virus type 1 env/rev DNA vaccine induces antigen-specific proliferation and lymphocyte production of beta-chemokines. *J Infect Dis* **181**(2), 476-83.
- Brander, C., and Walker, B. D. (1999). T lymphocyte responses in HIV-1 infection: implications for vaccine development. *Curr Opin Immunol* **11**(4), 451-9.
- Bratt, G., Leandersson, A. C., Albert, J., Sandstrom, E., and Wahren, B. (1998). MT-2 tropism and CCR-5 genotype strongly influence disease progression in HIV-1-infected individuals. *Aids* **12**(7), 729-36.
- Calarota, S., Bratt, G., Nordlund, S., Hinkula, J., Leandersson, A. C., Sandstrom, E., and Wahren, B. (1998). Cellular cytotoxic response induced by DNA vaccination in HIV-1- infected patients. *Lancet* **351**(9112), 1320-5.
- Calarota, S. A., Leandersson, A. C., Bratt, G., Hinkula, J., Klinman, D. M., Weinhold, K. J., Sandstrom, E., and Wahren, B. (1999). Immune responses in asymptomatic HIV-1-infected patients after HIV-DNA immunization followed by highly active antiretroviral treatment. *J Immunol* **163**(4), 2330-8.
- Casimiro, D. (2002). Vaccine-Induced Immune Responses in Rodents and Nonhuman Primates by use of a Humanized Human Immunodeficiency Virus Type 1 pol gene. *Journal of Virology* **76**(1).
- Cassatt, D. R., Sweet, R. W., Arthos, J. A., and Truneh, A. (1991). Immunization with soluble murine CD4 induces an anti-self antibody response without causing impairment of immune function. *J Immunol* **147**(5), 1470-6.
- CDC. (1981). Kaposi sarcoma and Pneumocystis Carinii pneumonia among homosexual men. In "Morbidity and Mortality Weekly Report", Vol. 30, pp. 305-308, New York and California.

- Chin, L. T., Hinkula, J., Levi, M., Ohlin, M., Wahren, B., and Borrebaeck, C. A. (1994). Site-directed primary in vitro immunization: production of HIV-1 neutralizing human monoclonal antibodies from lymphocytes obtained from seronegative donors. *Immunology* **81**(3), 428-34.
- Choe, H., Farzan, M., Sun, Y., Sullivan, N., Rollins, B., Ponath, P. D., Wu, L., Mackay, C. R., LaRosa, G., Newman, W., Gerard, N., Gerard, C., and Sodroski, J. (1996). The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell* **85**(7), 1135-48.
- Chun, T. W., Chadwick, K., Margolick, J., and Siliciano, R. F. (1997). Differential susceptibility of naive and memory CD4+ T cells to the cytopathic effects of infection with human immunodeficiency virus type 1 strain LAI. *J Virol* **71**(6), 4436-44.
- Clavel, F., Guetard, D., Brun-Vezinet, F., Chamaret, S., Rey, M. A., Santos-Ferreira, M. O., Laurent, A. G., Dauguet, C., Katlama, C., Rouzioux, C., and et al. (1986). Isolation of a new human retrovirus from West African patients with AIDS. *Science* **233**(4761), 343-6.
- Cocchi, F., DeVico, A. L., Garzino-Demo, A., Arya, S. K., Gallo, R. C., and Lusso, P. (1995). Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells. *Science* **270**(5243), 1811-5.
- Cocchi, F., DeVico, A. L., Garzino-Demo, A., Cara, A., Gallo, R. C., and Lusso, P. (1996). The V3 domain of the HIV-1 gp120 envelope glycoprotein is critical for chemokine-mediated blockade of infection. *Nat Med* **2**(11), 1244-7.
- Coffin, J., Haase, A., Levy, J. A., Montagnier, L., Oroszlan, S., Teich, N., Temin, H., Toyoshima, K., Varmus, H., Vogt, P., and et al. (1986). Human immunodeficiency viruses. *Science* **232**(4751), 697.
- Cohen, J. (2001). "Shots in the dark." Norton & Company, New York.
- Cohen, J. (2002). Vaccines. Monkey puzzles. *Science* **296**(5577), 2325-6.
- Connor, R. I., Sheridan, K. E., Ceradini, D., Choe, S., and Landau, N. R. (1997). Change in coreceptor use correlates with disease progression in HIV-1--infected individuals. *J Exp Med* **185**(4), 621-8.
- Conry, R. M., Widera, G., LoBuglio, A. F., Fuller, J. T., Moore, S. E., Barlow, D. L., Turner, J., Yang, N. S., and Curiel, D. T. (1996). Selected strategies to augment polynucleotide immunization. *Gene Ther* **3**(1), 67-74.
- Cornelissen, M., Mulder-Kampinga, G., Veenstra, J., Zorgdrager, F., Kuiken, C., Hartman, S., Dekker, J., van der Hoek, L., Sol, C., Coutinho, R., and et al. (1995). Syncytium-inducing (SI) phenotype suppression at seroconversion after intramuscular inoculation of a non-syncytium-inducing/SI phenotypically mixed human immunodeficiency virus population. *J Virol* **69**(3), 1810-8.
- Corr, M., Lee, D. J., Carson, D. A., and Tighe, H. (1996). Gene vaccination with naked plasmid DNA: mechanism of CTL priming. *J Exp Med* **184**(4), 1555-60.

- Daniel, M. D., Kirchhoff, F., Czajak, S. C., Sehgal, P. K., and Desrosiers, R. C. (1992). Protective effects of a live attenuated SIV vaccine with a deletion in the nef gene. *Science* **258**(5090), 1938-41.
- Daniel, M. D., Letvin, N. L., King, N. W., Kannagi, M., Sehgal, P. K., Hunt, R. D., Kanki, P. J., Essex, M., and Desrosiers, R. C. (1985). Isolation of T-cell tropic HTLV-III-like retrovirus from macaques. *Science* **228**(4704), 1201-4.
- Davis, H. L., Suparto, II, Weeratna, R. R., Jumintarto, Iskandriati, D. D., Chamzah, S. S., Ma'ruf, A. A., Nente, C. C., Pawitri, D. D., Krieg, A. M., Heriyanto, Smits, W., and Sajuthi, D. D. (2000). CpG DNA overcomes hyporesponsiveness to hepatitis B vaccine in orangutans. *Vaccine* **18**(18), 1920-4.
- De Clercq, E., Yamamoto, N., Pauwels, R., Balzarini, J., Witvrouw, M., De Vreese, K., Debyser, Z., Rosenwirth, B., Peichl, P., Datema, R., and et al. (1994). Highly potent and selective inhibition of human immunodeficiency virus by the bicyclam derivative JM3100. *Antimicrob Agents Chemother* **38**(4), 668-74.
- Dela Cruz, C. S., Chamberlain, J. W., MacDonald, K. S., and Barber, B. H. (1999). Xenogeneic and allogeneic anti-MHC immune responses induced by plasmid DNA immunization. *Vaccine* **17**(20-21), 2479-92.
- Dela Cruz, C. S., MacDonald, K. S., and Barber, B. H. (2000). Anti-major histocompatibility complex antibody responses in macaques via intradermal DNA immunizations. *Vaccine* **18**(27), 3152-65.
- Deng, H., Liu, R., Ellmeier, W., Choe, S., Unutmaz, D., Burkhart, M., Di Marzio, P., Marmon, S., Sutton, R. E., Hill, C. M., Davis, C. B., Peiper, S. C., Schall, T. J., Littman, D. R., and Landau, N. R. (1996). Identification of a major co-receptor for primary isolates of HIV-1. *Nature* **381**(6584), 661-6.
- Deng, H. K., Unutmaz, D., KewalRamani, V. N., and Littman, D. R. (1997). Expression cloning of new receptors used by simian and human immunodeficiency viruses. *Nature* **388**(6639), 296-300.
- Desrosiers, R. C., Wyand, M. S., Kodama, T., Ringler, D. J., Arthur, L. O., Sehgal, P. K., Letvin, N. L., King, N. W., and Daniel, M. D. (1989). Vaccine protection against simian immunodeficiency virus infection. *Proc Natl Acad Sci U S A* **86**(16), 6353-7.
- Ding, J., Das, K., Hsiou, Y., Sarafianos, S. G., Clark, A. D., Jr., Jacobo-Molina, A., Tantillo, C., Hughes, S. H., and Arnold, E. (1998). Structure and functional implications of the polymerase active site region in a complex of HIV-1 RT with a double-stranded DNA template- primer and an antibody Fab fragment at 2.8 Å resolution. *J Mol Biol* **284**(4), 1095-111.
- Ditzel, H. J., Rosenkilde, M. M., Garred, P., Wang, M., Koefoed, K., Pedersen, C., Burton, D. R., and Schwartz, T. W. (1998). The CCR5 receptor acts as an alloantigen in CCR5Delta32 homozygous individuals: identification of chemokine and HIV-1-blocking human antibodies. *Proc Natl Acad Sci U S A* **95**(9), 5241-5.

- Dolores, M., Casado, C., Yuste, E., Olivares, I., and Lopez-Galindez, C. (2002). In vitro analysis of human immunodeficiency virus type 1 resistance to nevirapine and fitness determination of resistant variants. *Journal of General Virology* **83**, 93-101.
- Doms, R. W., and Trono, D. (2000). The plasma membrane as a combat zone in the HIV battlefield. *Genes Dev* **14**(21), 2677-88.
- Donnelly, J. J., Ulmer, J. B., Shiver, J. W., and Liu, M. A. (1997). DNA vaccines. *Annu Rev Immunol* **15**, 617-48.
- Doranz, B. J., Lu, Z. H., Rucker, J., Zhang, T. Y., Sharron, M., Cen, Y. H., Wang, Z. X., Guo, H. H., Du, J. G., Accavitti, M. A., Doms, R. W., and Peiper, S. C. (1997). Two distinct CCR5 domains can mediate coreceptor usage by human immunodeficiency virus type 1. *J Virol* **71**(9), 6305-14.
- Douek, D. C., Brenchley, J. M., Betts, M. R., Ambrozak, D. R., Hill, B. J., Okamoto, Y., Casazza, J. P., Kuruppu, J., Kunstman, K., Wolinsky, S., Grossman, Z., Dybul, M., Oxenius, A., Price, D. A., Connors, M., and Koup, R. A. (2002). HIV preferentially infects HIV-specific CD4⁺ T cells. *Nature* **417**(6884), 95-8.
- Emini, E. A., Schleif, W. A., Nunberg, J. H., Conley, A. J., Eda, Y., Tokiyoshi, S., Putney, S. D., Matsushita, S., Cobb, K. E., Jett, C. M., and et al. (1992). Prevention of HIV-1 infection in chimpanzees by gp120 V3 domain- specific monoclonal antibody. *Nature* **355**(6362), 728-30.
- Feinberg, M., and Greene, W. (1992). Molecular insights into human immunodeficiency type one pathogenesis. *Curr Opin Immunol* **4**(4), 466-474.
- Fernandez-Vina, M. A., Falco, M., Sun, Y., and Stastny, P. (1992). DNA typing for HLA class I alleles: I. Subsets of HLA-A2 and of -A28. *Hum Immunol* **33**(3), 163-73.
- Fields, B., Knipe, D., and Howley, P. (1996). "Fields Virology." Lippincott-Raven.
- Fields, R. C., Shimizu, K., and Mule, J. J. (1998). Murine dendritic cells pulsed with whole tumor lysates mediate potent antitumor immune responses in vitro and in vivo. *Proc Natl Acad Sci U S A* **95**(16), 9482-7.
- Franchini, G., Gurgo, C., Guo, H. G., Gallo, R. C., Collalti, E., Fargnoli, K. A., Hall, L. F., Wong-Staal, F., and Reitz, M. S., Jr. (1987). Sequence of simian immunodeficiency virus and its relationship to the human immunodeficiency viruses. *Nature* **328**(6130), 539-43.
- Frank, I., Stoiber, H., Godar, S., Stockinger, H., Steindl, F., Katinger, H. W., and Dierich, M. P. (1996). Acquisition of host cell-surface-derived molecules by HIV-1. *Aids* **10**(14), 1611-20.
- Frankel, A. D., and Young, J. A. (1998). HIV-1: fifteen proteins and an RNA. *Annu Rev Biochem* **67**, 1-25.
- Fu, T. M., Ulmer, J. B., Caulfield, M. J., Deck, R. R., Friedman, A., Wang, S., Liu, X., Donnelly, J. J., and Liu, M. A. (1997). Priming of cytotoxic T lymphocytes by DNA vaccines: requirement for professional antigen presenting cells and evidence for antigen transfer from myocytes. *Mol Med* **3**(6), 362-71.

- Fynan, E. F., Webster, R. G., Fuller, D. H., Haynes, J. R., Santoro, J. C., and Robinson, H. L. (1993). DNA vaccines: protective immunizations by parenteral, mucosal, and gene-gun inoculations. *Proc Natl Acad Sci U S A* **90**(24), 11478-82.
- Gaczynska, M., Rock, K. L., and Goldberg, A. L. (1993). Gamma-interferon and expression of MHC genes regulate peptide hydrolysis by proteasomes. *Nature* **365**(6443), 264-7.
- Gallo, R. C., Salahuddin, S. Z., Popovic, M., Shearer, G. M., Kaplan, M., Haynes, B. F., Palker, T. J., Redfield, R., Oleske, J., Safai, B., and et al. (1984). Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. *Science* **224**(4648), 500-3.
- Gallo, R. C., Sarin, P. S., Gelmann, E. P., Robert-Guroff, M., Richardson, E., Kalyanaraman, V. S., Mann, D., Sidhu, G. D., Stahl, R. E., Zolla-Pazner, S., Leibowitch, J., and Popovic, M. (1983). Isolation of human T-cell leukemia virus in acquired immune deficiency syndrome (AIDS). *Science* **220**(4599), 865-7.
- Gao, F., Bailes, E., Robertson, D. L., Chen, Y., Rodenburg, C. M., Michael, S. F., Cummins, L. B., Arthur, L. O., Peeters, M., Shaw, G. M., Sharp, P. M., and Hahn, B. H. (1999). Origin of HIV-1 in the chimpanzee *Pan troglodytes*. *Nature* **397**(6718), 436-41.
- Geijtenbeek, T. B., Kwon, D. S., Torensma, R., van Vliet, S. J., van Duijnhoven, G. C., Middel, J., Cornelissen, I. L., Nottet, H. S., KewalRamani, V. N., Littman, D. R., Figdor, C. G., and van Kooyk, Y. (2000). DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. *Cell* **100**(5), 587-97.
- Goebel, F. D., Mannhalter, J. W., Belshe, R. B., Eibl, M. M., Grob, P. J., de Gruttola, V., Griffiths, P. D., Erfle, V., Kunschak, M., and Engl, W. (1999). Recombinant gp160 as a therapeutic vaccine for HIV-infection: results of a large randomized, controlled trial. European Multinational IMMUNO AIDS Vaccine Study Group. *Aids* **13**(12), 1461-8.
- Gottlieb, M. S., Schroff, R., Schanker, H. M., Weisman, J. D., Fan, P. T., Wolf, R. A., and Saxon, A. (1981). Pneumocystis carinii pneumonia and mucosal candidiasis in previously healthy homosexual men: evidence of a new acquired cellular immunodeficiency. *N Engl J Med* **305**(24), 1425-31.
- Gurunathan, S., Klinman, D. M., and Seder, R. A. (2000). DNA vaccines: immunology, application, and optimization. *Annu Rev Immunol* **18**, 927-74.
- Haas, G., Samri, A., Gomard, E., Hosmalin, A., Duntze, J., Bouley, J. M., Ihlenfeldt, H. G., Katlama, C., and Autran, B. (1998). Cytotoxic T-cell responses to HIV-1 reverse transcriptase, integrase and protease. *Aids* **12**(12), 1427-36.
- Hanke, T., and McMichael, A. J. (2000). Design and construction of an experimental HIV-1 vaccine for a year-2000 clinical trial in Kenya. *Nat Med* **6**(9), 951-5.
- Harouse, J. M., Gettie, A., Tan, R. C., Blanchard, J., and Cheng-Mayer, C. (1999). Distinct pathogenic sequela in rhesus macaques infected with CCR5 or CXCR4 utilizing SHIVs. *Science* **284**(5415), 816-9.
- Hartmann, G., Weeratna, R. D., Ballas, Z. K., Payette, P., Blackwell, S., Suparto, I., Rasmussen, W. L., Waldschmidt, M., Sajuthi, D., Purcell, R. H., Davis, H. L., and

- Krieg, A. M. (2000). Delineation of a CpG phosphorothioate oligodeoxynucleotide for activating primate immune responses in vitro and in vivo. *J Immunol* **164**(3), 1617-24.
- Hay, C. M., Ruhl, D. J., Basgoz, N. O., Wilson, C. C., Billingsley, J. M., DePasquale, M. P., D'Aquila, R. T., Wolinsky, S. M., Crawford, J. M., Montefiori, D. C., and Walker, B. D. (1999). Lack of viral escape and defective in vivo activation of human immunodeficiency virus type 1-specific cytotoxic T lymphocytes in rapidly progressive infection. *J Virol* **73**(7), 5509-19.
- Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K., and Akira, S. (2000). A Toll-like receptor recognizes bacterial DNA. *Nature* **408**(December 7), 740-745.
- Hinkula, J., Svanholm, C., Schwartz, S., Lundholm, P., Brytting, M., Engstrom, G., Benthin, R., Glaser, H., Sutter, G., Kohleisen, B., Erfle, V., Okuda, K., Wigzell, H., and Wahren, B. (1997). Recognition of prominent viral epitopes induced by immunization with human immunodeficiency virus type 1 regulatory genes. *J Virol* **71**(7), 5528-39.
- Hirsch, V. M., Olmsted, R. A., Murphey-Corb, M., Purcell, R. H., and Johnson, P. R. (1989). An African primate lentivirus (SIVsm) closely related to HIV-2. *Nature* **339**(6223), 389-92.
- Ho, D. D., Neumann, A. U., Perelson, A. S., Chen, W., Leonard, J. M., and Markowitz, M. (1995). Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. *Nature* **373**(6510), 123-6.
- Hoffman, T. L., Canziani, G., Jia, L., Rucker, J., and Doms, R. W. (2000). A biosensor assay for studying ligand-membrane receptor interactions: binding of antibodies and HIV-1 Env to chemokine receptors. *Proc Natl Acad Sci U S A* **97**(21), 11215-20.
- Horuk, R. (2001). Chemokine receptors. *Cytokine Growth Factor Rev* **12**(4), 313-35.
- Huang, Y., Kong, W. P., and Nabel, G. J. (2001). Human immunodeficiency virus type 1-specific immunity after genetic immunization is enhanced by modification of Gag and Pol expression. *J Virol* **75**(10), 4947-51.
- Huang, Y., Paxton, W. A., Wolinsky, S. M., Neumann, A. U., Zhang, L., He, T., Kang, S., Ceradini, D., Jin, Z., Yazdanbakhsh, K., Kunstman, K., Erickson, D., Dragon, E., Landau, N. R., Phair, J., Ho, D. D., and Koup, R. A. (1996). The role of a mutant CCR5 allele in HIV-1 transmission and disease progression. *Nat Med* **2**(11), 1240-3.
- Huet, T., Cheynier, R., Meyerhans, A., Roelants, G., and Wain-Hobson, S. (1990). Genetic organization of a chimpanzee lentivirus related to HIV-1. *Nature* **345**(6273), 356-9.
- Isagulians, M. G., Gudima, S. O., Ivanova, O. V., Levi, M., Hinkula, J., Garaev, M. M., Kochetkov, S. N., and Wahren, B. (2000a). Immunogenic properties of reverse transcriptase of HIV type 1 assessed by DNA and protein immunization of rabbits. *AIDS Res Hum Retroviruses* **16**(13), 1269-80.

- Isaguliants, M. G., Petrakova, N. N., Zuber, B., Pokrovskaya, K., Gizatullin, R., Kostyuk, D. A., Kjerrstrom, A., Winberg, G., Kochetkov, S. N., Hinkula, J., and Wahren, B. (2000b). DNA-encoding enzymatically active HIV-1 reverse transcriptase, but not the inactive mutant, confers resistance to experimental HIV-1 challenge. *Intervirology* **43**(4-6), 288-93.
- Iwasaki, A., Torres, C. A., Ohashi, P. S., Robinson, H. L., and Barber, B. H. (1997). The dominant role of bone marrow-derived cells in CTL induction following plasmid DNA immunization at different sites. *J Immunol* **159**(1), 11-4.
- Janeway, A., Travers, P., Walport, M., and Capra, J. (1999). "Immuno biology, The immune system in health and disease." 4:th ed. Elsevier Science, London, UK.
- Janeway, A., Travers, P., Walport, M., and Shlomchik, M. (2001). "Immuno biology, The immune system in health and disease." 5:th ed. Elsevier Science, London, UK.
- Joag, S. V. (2000). Primate models of AIDS. *Microbes Infect* **2**(2), 223-9.
- Johnson, M. S., McClure, M. A., Feng, D. F., Gray, J., and Doolittle, R. F. (1986). Computer analysis of retroviral pol genes: assignment of enzymatic functions to specific sequences and homologies with nonviral enzymes. *Proc Natl Acad Sci U S A* **83**(20), 7648-52.
- Jones, T. R., Narum, D. L., Gozalo, A. S., Aguiar, J., Fuhrmann, S. R., Liang, H., Haynes, J. D., Moch, J. K., Lucas, C., Luu, T., Magill, A. J., Hoffman, S. L., and Sim, B. K. (2001). Protection of Aotus monkeys by Plasmodium falciparum EBA-175 region II DNA prime-protein boost immunization regimen. *J Infect Dis* **183**(2), 303-312.
- King, C. A., Spellerberg, M. B., Zhu, D., Rice, J., Sahota, S. S., Thompson, A. R., Hamblin, T. J., Radl, J., and Stevenson, F. K. (1998). DNA vaccines with single-chain Fv fused to fragment C of tetanus toxin induce protective immunity against lymphoma and myeloma. *Nat Med* **4**(11), 1281-6.
- Klinman, D. M., Takeshita, F., Gursel, I., Leifer, C., Ishii, K. J., Verthelyi, D., and Gursel, M. (2002). CpG DNA: recognition by and activation of monocytes. *Microbes Infect* **4**(9), 897-901.
- Koup, R. A., Safrit, J. T., Cao, Y., Andrews, C. A., McLeod, G., Borkowsky, W., Farthing, C., and Ho, D. D. (1994). Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J Virol* **68**(7), 4650-5.
- Krieg, A. M. (2002). CpG motifs in bacterial DNA and their immune effects. *Annu Rev Immunol* **20**, 709-60.
- Krieg, A. M., and Wagner, H. (2000). Causing a commotion in the blood: immunotherapy progresses from bacteria to bacterial DNA. *Immunol Today* **21**(10), 521-6.
- Krieg, A. M., Yi, A. K., Matson, S., Waldschmidt, T. J., Bishop, G. A., Teasdale, R., Koretzky, G. A., and Klinman, D. M. (1995). CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* **374**(6522), 546-9.

- Krug, A., Rothenfusser, S., Hornung, V., Jahrsdorfer, B., Blackwell, S., Ballas, Z. K., Endres, S., Krieg, A. M., and Hartmann, G. (2001). Identification of CpG oligonucleotide sequences with high induction of IFN-alpha/beta in plasmacytoid dendritic cells. *Eur J Immunol* **31**(7), 2154-63.
- Kumar, A., Arora, R., Kaur, P., Chauhan, V. S., and Sharma, P. (1992). "Universal" T helper cell determinants enhance immunogenicity of a *Plasmodium falciparum* merozoite surface antigen peptide. *J Immunol* **148**(5), 1499-505.
- Kwong, P. D., Wyatt, R., Robinson, J., Sweet, R. W., Sodroski, J., and Hendrickson, W. A. (1998). Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. *Nature* **393**(6686), 648-59.
- Lallos, L. B., Laal, S., Hoxie, J. A., Zolla-Pazner, S., and Bandres, J. C. (1999). Exclusion of HIV coreceptors CXCR4, CCR5, and CCR3 from the HIV envelope. *AIDS Res Hum Retroviruses* **15**(10), 895-7.
- Laurence, J., Saunders, A., and Kulkosky, J. (1987). Characterization and clinical association of antibody inhibitory to HIV reverse transcriptase activity. *Science* **235**(4795), 1501-4.
- Lee, B., Sharron, M., Blanpain, C., Doranz, B. J., Vakili, J., Setoh, P., Berg, E., Liu, G., Guy, H. R., Durell, S. R., Parmentier, M., Chang, C. N., Price, K., Tsang, M., and Doms, R. W. (1999). Epitope mapping of CCR5 reveals multiple conformational states and distinct but overlapping structures involved in chemokine and coreceptor function. *J Biol Chem* **274**(14), 9617-26.
- Lehner, T. (2002). The role of CCR5 chemokine ligands and antibodies to CCR5 coreceptors in preventing HIV infection. *Trends Immunol* **23**(7), 347-51.
- Lehner, T., Doyle, C., Wang, Y., Babaahmady, K., Whittall, T., Tao, L., Bergmeier, L., and Kelly, C. (2001). Immunogenicity of the Extracellular Domains of C-C Chemokine Receptor 5 and the In Vitro Effects on Simian Immunodeficiency Virus or HIV Infectivity. *J Immunol* **166**(12), 7446-55.
- Lehner, T., and Shearer, G. (2002). Alternative HIV vaccine strategies. *Science* **297**(August 23), 1276-1277.
- Lehner, T., Wang, Y., Doyle, C., Tao, L., Bergmeier, L. A., Mitchell, E., Bogers, W. M., Heeney, J., and Kelly, C. G. (1999). Induction of inhibitory antibodies to the CCR5 chemokine receptor and their complementary role in preventing SIV infection in macaques. *Eur J Immunol* **29**(8), 2427-35.
- Letvin, N. L. (1998). Progress in the development of an HIV-1 vaccine. *Science* **280**(5371), 1875-80.
- Liljeqvist, S., and Ståhl, S. (1999). Production of recombinant subunit vaccines: protein immunogens, live delivery systems and nucleic acid vaccines. *J Biotechnol* **73**(1), 1-33.
- Linsley, P. S., and Ledbetter, J. A. (1993). The role of the CD28 receptor during T cell responses to antigen. *Annu Rev Immunol* **11**, 191-212.
- Liu, M. A., and Ulmer, J. B. (2000). Gene-based vaccines. *Mol Ther* **1**(6), 497-500.

- Liu, R., Paxton, W. A., Choe, S., Ceradini, D., Martin, S. R., Horuk, R., MacDonald, M. E., Stuhlmann, H., Koup, R. A., and Landau, N. R. (1996). Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. *Cell* **86**(3), 367-77.
- Livingston, B. D., Newman, M., Crimi, C., McKinney, D., Chesnut, R., and Sette, A. (2001). Optimization of epitope processing enhances immunogenicity of multiepitope DNA vaccines. *Vaccine* **19**(32), 4652-60.
- Loemba, H., Brenner, B., Parniak, M., Ma'ayan, S., Spira, B., Moisi, D., Oliveira, M., Detorio, M., Essex, M., and Wainberg, M. (2002). Polymorphisms of cytotoxic T-lymphocyte (CTL) and T-helper epitopes within reverse transcriptase (RT) of HIV-1 subtype C from Ethiopia and Botswana following selection of antiretroviral drug resistance. *Antiviral Res* **56**(2), 129.
- Lopalco, L., Barassi, C., Pastori, C., Longhi, R., Burastero, S. E., Tambussi, G., Mazzotta, F., Lazzarin, A., Clerici, M., and Siccaldi, A. G. (2000). CCR5-reactive antibodies in seronegative partners of HIV-seropositive individuals down-modulate surface CCR5 in vivo and neutralize the infectivity of R5 strains of HIV-1 In vitro. *J Immunol* **164**(6), 3426-33.
- Lu, Z., Berson, J. F., Chen, Y., Turner, J. D., Zhang, T., Sharron, M., Jenks, M. H., Wang, Z., Kim, J., Rucker, J., Hoxie, J. A., Peiper, S. C., and Doms, R. W. (1997). Evolution of HIV-1 coreceptor usage through interactions with distinct CCR5 and CXCR4 domains. *Proc Natl Acad Sci U S A* **94**(12), 6426-31.
- MacGregor, R. R., Boyer, J. D., Ugen, K. E., Lacy, K. E., Gluckman, S. J., Bagarazzi, M. L., Chattergoon, M. A., Baine, Y., Higgins, T. J., Ciccarelli, R. B., Coney, L. R., Ginsberg, R. S., and Weiner, D. B. (1998). First human trial of a DNA-based vaccine for treatment of human immunodeficiency virus type 1 infection: safety and host response. *J Infect Dis* **178**(1), 92-100.
- Manthorpe, M., Cornefert-Jensen, F., Hartikka, J., Felgner, J., Rundell, A., Margalith, M., and Dwarki, V. (1993). Gene therapy by intramuscular injection of plasmid DNA: studies on firefly luciferase gene expression in mice. *Hum Gene Ther* **4**(4), 419-31.
- Matsuda, S., Gidlund, M., Chiodi, F., Cafaro, A., Nygren, A., Morein, B., Nilsson, K., Fenyo, E. M., and Wigzell, H. (1989). Enhancement of human immunodeficiency virus (HIV) replication in human monocytes by low titres of anti-HIV antibodies in vitro. *Scand J Immunol* **30**(4), 425-34.
- Matsumoto, Y. (2000). Characterization of T cell receptor (TCR) of organ-specific autoimmune disease-inducing T cells and TCR-based immunotherapy with DNA vaccines. *J Neuroimmunol* **110**(1-2), 1-12.
- Matsumoto, Y., Jee, Y., and Sugisaki, M. (2000). Successful TCR-based immunotherapy for autoimmune myocarditis with DNA vaccines after rapid identification of pathogenic TCR. *J Immunol* **164**(4), 2248-54.
- McDougal, J. S., Kennedy, M. S., Sligh, J. M., Cort, S. P., Mawle, A., and Nicholson, J. K. (1986). Binding of HTLV-III/LAV to T4+ T cells by a complex of the 110K viral protein and the T4 molecule. *Science* **231**(4736), 382-5.

- Michael, N. L., Nelson, J. A., KewalRamani, V. N., Chang, G., O'Brien, S. J., Mascola, J. R., Volsky, B., Louder, M., White, G. C., 2nd, Littman, D. R., Swanstrom, R., and O'Brien, T. R. (1998). Exclusive and persistent use of the entry coreceptor CXCR4 by human immunodeficiency virus type 1 from a subject homozygous for CCR5 delta32. *J Virol* **72**(7), 6040-7.
- Mo, A. X., van Lelyveld, S. F., Craiu, A., and Rock, K. L. (2000). Sequences that flank subdominant and cryptic epitopes influence the proteolytic generation of MHC class I-presented peptides. *J Immunol* **164**(8), 4003-10.
- Moog, C., Fleury, H. J., Pellegrin, I., Kirn, A., and Aubertin, A. M. (1997). Autologous and heterologous neutralizing antibody responses following initial seroconversion in human immunodeficiency virus type 1-infected individuals. *J Virol* **71**(5), 3734-41.
- Moore, J. P., and Burton, D. R. (1999). HIV-1 neutralizing antibodies: how full is the bottle? *Nat Med* **5**(2), 142-4.
- Moore, J. P., Cao, Y., Ho, D. D., and Koup, R. A. (1994). Development of the anti-gp120 antibody response during seroconversion to human immunodeficiency virus type 1. *J Virol* **68**(8), 5142-55.
- Moss, P. A., Rowland-Jones, S. L., Frodsham, P. M., McAdam, S., Giangrande, P., McMichael, A. J., and Bell, J. I. (1995). Persistent high frequency of human immunodeficiency virus-specific cytotoxic T cells in peripheral blood of infected donors. *Proc Natl Acad Sci U S A* **92**(13), 5773-7.
- Murphey-Corb, M., Martin, L. N., Davison-Fairburn, B., Montelaro, R. C., Miller, M., West, M., Ohkawa, S., Baskin, G. B., Zhang, J. Y., Putney, S. D., and et al. (1989). A formalin-inactivated whole SIV vaccine confers protection in macaques. *Science* **246**(4935), 1293-7.
- Mörner, A., Achour, A., Norin, M., Thorstensson, R., and Björling, E. (1999). Fine characterization of a V3-region neutralizing epitope in human immunodeficiency virus type 2. *Virus Res* **59**(1), 49-60.
- Nath, B. M., Schumann, K. E., and Boyer, J. D. (2000). The chimpanzee and other non-human-primate models in HIV-1 vaccine research. *Trends Microbiol* **8**(9), 426-31.
- O'Brien, T. R., Winkler, C., Dean, M., Nelson, J. A., Carrington, M., Michael, N. L., and White, G. C., 2nd. (1997). HIV-1 infection in a man homozygous for CCR5 delta 32. *Lancet* **349**(9060), 1219.
- Ogg, G. S., Jin, X., Bonhoeffer, S., Dunbar, P. R., Nowak, M. A., Monard, S., Segal, J. P., Cao, Y., Rowland-Jones, S. L., Cerundolo, V., Hurley, A., Markowitz, M., Ho, D. D., Nixon, D. F., and McMichael, A. J. (1998). Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA. *Science* **279**(5359), 2103-6.
- Olson, W. C., Rabut, G. E., Nagashima, K. A., Tran, D. N., Anselma, D. J., Monard, S. P., Segal, J. P., Thompson, D. A., Kajumo, F., Guo, Y., Moore, J. P., Maddon, P. J., and Dragic, T. (1999). Differential inhibition of human immunodeficiency virus type 1 fusion, gp120 binding, and CC-chemokine activity by monoclonal antibodies to CCR5. *J Virol* **73**(5), 4145-55.

- Owen, S. M., Masciotra, S., Novembre, F., Yee, J., Switzer, W. M., Ostyula, M., and Lal, R. B. (2000). Simian immunodeficiency viruses of diverse origin can use CXCR4 as a coreceptor for entry into human cells. *J Virol* **74**(12), 5702-8.
- Panina-Bordignon, P., Tan, A., Termijtelen, A., Demotz, S., Corradin, G., and Lanzavecchia, A. (1989). Universally immunogenic T cell epitopes: promiscuous binding to human MHC class II and promiscuous recognition by T cells. *Eur J Immunol* **19**(12), 2237-42.
- Pantaleo, G., Demarest, J. F., Soudeyins, H., Graziosi, C., Denis, F., Adelsberger, J. W., Borrow, P., Saag, M. S., Shaw, G. M., Sekaly, R. P., and et al. (1994). Major expansion of CD8⁺ T cells with a predominant V beta usage during the primary immune response to HIV. *Nature* **370**(6489), 463-7.
- Parren, P. W., Burton, D. R., and Sattentau, Q. J. (1997). HIV-1 antibody--debris or virion? *Nat Med* **3**(4), 366-7.
- Penn, M. L., Grivel, J. C., Schramm, B., Goldsmith, M. A., and Margolis, L. (1999). CXCR4 utilization is sufficient to trigger CD4⁺ T cell depletion in HIV-1-infected human lymphoid tissue. *Proc Natl Acad Sci U S A* **96**(2), 663-8.
- Phillips, R. E., Rowland-Jones, S., Nixon, D. F., Gotch, F. M., Edwards, J. P., Ogunlesi, A. O., Elvin, J. G., Rothbard, J. A., Bangham, C. R., Rizza, C. R., and et al. (1991). Human immunodeficiency virus genetic variation that can escape cytotoxic T cell recognition. *Nature* **354**(6353), 453-9.
- Popovic, M., Sarngadharan, M. G., Read, E., and Gallo, R. C. (1984). Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. *Science* **224**(4648), 497-500.
- Porgador, A., Irvine, K. R., Iwasaki, A., Barber, B. H., Restifo, N. P., and Germain, R. N. (1998). Predominant role for directly transfected dendritic cells in antigen presentation to CD8⁺ T cells after gene gun immunization. *J Exp Med* **188**(6), 1075-82.
- Putkonen, P., Böttiger, B., Warstedt, K., Thorstensson, R., Albert, J., and Biberfeld, G. (1989). Experimental infection of cynomolgus monkeys (*Macaca fascicularis*) with HIV-2. *J Acquir Immune Defic Syndr* **2**(4), 366-73.
- Putkonen, P., Quesada-Rolander, M., Leandersson, A. C., Schwartz, S., Thorstensson, R., Okuda, K., Wahren, B., and Hinkula, J. (1998). Immune responses but no protection against SHIV by gene-gun delivery of HIV-1 DNA followed by recombinant subunit protein boosts. *Virology* **250**(2), 293-301.
- Rammensee, H. G., Falk, K., and Rotzschke, O. (1993). Peptides naturally presented by MHC class I molecules. *Annu Rev Immunol* **11**, 213-44.
- Raz, E., and Spiegelberg, H. L. (1999). Deviation of the allergic IgE to an IgG response by gene immunotherapy. *Int Rev Immunol* **18**(3), 271-89.
- Restle, T., Pawlita, M., Sczakiel, G., Muller, B., and Goody, R. S. (1992). Structure-function relationships of HIV-1 reverse transcriptase determined using monoclonal antibodies. *J Biol Chem* **267**(21), 14654-61.

- Richman, D., Havlir, D., Corbeil, J., Looney, D., Ignacio, C., Spector, S., Sullivan, J., Cheesman, S., Barringer, K., Pauletti, D., Shih, C., Myers, M., and Griffin, J. (1994). Nevirapine resistance mutations of human immunodeficiency virus type 1 selected during therapy. *Journal of virology* **68**(3), 1660-1666.
- Rizzuto, C. D., Wyatt, R., Hernandez-Ramos, N., Sun, Y., Kwong, P. D., Hendrickson, W. A., and Sodroski, J. (1998). A conserved HIV gp120 glycoprotein structure involved in chemokine receptor binding. *Science* **280**(5371), 1949-53.
- Robinson, H. L., Montefiori, D. C., Johnson, R. P., Manson, K. H., Kalish, M. L., Lifson, J. D., Rizvi, T. A., Lu, S., Hu, S. L., Mazzara, G. P., Panicali, D. L., Herndon, J. G., Glickman, R., Candido, M. A., Lydy, S. L., Wyand, M. S., and McClure, H. M. (1999). Neutralizing antibody-independent containment of immunodeficiency virus challenges by DNA priming and recombinant pox virus booster immunizations. *Nat Med* **5**(5), 526-34.
- Robinson, W. E., Jr., Montefiori, D. C., and Mitchell, W. M. (1988). Antibody-dependent enhancement of human immunodeficiency virus type 1 infection. *Lancet* **1**(8589), 790-4.
- Rock, K. L., and Goldberg, A. L. (1999). Degradation of cell proteins and the generation of MHC class I- presented peptides. *Annu Rev Immunol* **17**, 739-79.
- Rowland-Jones, S., Tan, R., and McMichael, A. (1997). Role of cellular immunity in protection against HIV infection. *Advances in Immunology* **65**, 277-346.
- Rucker, J., Samson, M., Doranz, B. J., Libert, F., Berson, J. F., Yi, Y., Smyth, R. J., Collman, R. G., Broder, C. C., Vassart, G., Doms, R. W., and Parmentier, M. (1996). Regions in beta-chemokine receptors CCR5 and CCR2b that determine HIV-1 cofactor specificity. *Cell* **87**(3), 437-46.
- Ruprecht, R. M. (1999). Live attenuated AIDS viruses as vaccines: promise or peril? *Immunol Rev* **170**, 135-49.
- Samanci, A., Yi, Q., Fagerberg, J., Strigård, K., Smith, G., Ruden, U., Wahren, B., and Mellstedt, H. (1998). Pharmacological administration of granulocyte/macrophage-colony- stimulating factor is of significant importance for the induction of a strong humoral and cellular response in patients immunized with recombinant carcinoembryonic antigen. *Cancer Immunol Immunother* **47**(3), 131-42.
- Samri, A., Haas, G., Duntze, J., Bouley, J. M., Calvez, V., Katlama, C., and Autran, B. (2000). Immunogenicity of mutations induced by nucleoside reverse transcriptase inhibitors for human immunodeficiency virus type 1-specific cytotoxic T cells. *J Virol* **74**(19), 9306-12.
- Samson, M., Libert, F., Doranz, B. J., Rucker, J., Liesnard, C., Farber, C. M., Saragosti, S., Lapoumeroulie, C., Cognaux, J., Forceille, C., Muylldemans, G., Verhofstede, C., Burtonboy, G., Georges, M., Imai, T., Rana, S., Yi, Y., Smyth, R. J., Collman, R. G., Doms, R. W., Vassart, G., and Parmentier, M. (1996). Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. *Nature* **382**(6593), 722-5.

- Sandström, E., and Wahren, B. (1999). Therapeutic immunisation with recombinant gp160 in HIV-1 infection: a randomised double-blind placebo-controlled trial. Nordic VAC-04 Study Group. *Lancet* **353**(9166), 1735-42.
- Sano, K., Lee, M. H., Morales, F., Nishanian, P., Fahey, J., Detels, R., and Imagawa, D. T. (1987). Antibody that inhibits human immunodeficiency virus reverse transcriptase and association with inability to isolate virus. *J Clin Microbiol* **25**(12), 2415-7.
- Sarnagadharan, M. G., Popovic, M., Bruch, L., Schupbach, J., and Gallo, R. C. (1984). Antibodies reactive with human T-lymphotropic retroviruses (HTLV-III) in the serum of patients with AIDS. *Science* **224**(4648), 506-8.
- Scarlatti, G., Tresoldi, E., Bjorndal, A., Fredriksson, R., Colognesi, C., Deng, H. K., Malnati, M. S., Plebani, A., Siccardi, A. G., Littman, D. R., Fenyo, E. M., and Lusso, P. (1997). In vivo evolution of HIV-1 co-receptor usage and sensitivity to chemokine-mediated suppression. *Nat Med* **3**(11), 1259-65.
- Schmitt, M., Harrer, E., Goldwisch, A., Bauerle, M., Graedner, I., Kalden, J. R., and Harrer, T. (2000). Specific recognition of lamivudine-resistant HIV-1 by cytotoxic T lymphocytes. *Aids* **14**(6), 653-8.
- Schmitz, J. E., Kuroda, M. J., Santra, S., Sasseville, V. G., Simon, M. A., Lifton, M. A., Racz, P., Tenner-Racz, K., Dalesandro, M., Scallan, B. J., Ghrayeb, J., Forman, M. A., Montefiori, D. C., Rieber, E. P., Letvin, N. L., and Reimann, K. A. (1999). Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. *Science* **283**(5403), 857-60.
- Schupbach, J., Popovic, M., Gilden, R. V., Gonda, M. A., Sarnagadharan, M. G., and Gallo, R. C. (1984). Serological analysis of a subgroup of human T-lymphotropic retroviruses (HTLV-III) associated with AIDS. *Science* **224**(4648), 503-5.
- Sewell, A. K., Price, D. A., Oxenius, A., Kelleher, A. D., and Phillips, R. E. (2000). Cytotoxic T lymphocyte responses to human immunodeficiency virus: control and escape. *Stem Cells* **18**(4), 230-44.
- Shastri, N., Serwold, T., and Gonzalez, F. (1995). Presentation of endogenous peptide/MHC class I complexes is profoundly influenced by specific C-terminal flanking residues. *J Immunol* **155**(9), 4339-46.
- Shearer, G. M., Clerici, M., and Dalgleish, A. (1993). Alloimmunization as an AIDS vaccine? *Science* **262**(5131), 161-2.
- Shibata, R., Igarashi, T., Haigwood, N., Buckler-White, A., Ogert, R., Ross, W., Willey, R., Cho, M. W., and Martin, M. A. (1999). Neutralizing antibody directed against the HIV-1 envelope glycoprotein can completely block HIV-1/SIV chimeric virus infections of macaque monkeys. *Nat Med* **5**(2), 204-10.
- Shiver, J. W., Fu, T. M., Chen, L., Casimiro, D. R., Davies, M. E., Evans, R. K., Zhang, Z. Q., Simon, A. J., Trigona, W. L., Dubey, S. A., Huang, L., Harris, V. A., Long, R. S., Liang, X., Handt, L., Schleif, W. A., Zhu, L., Freed, D. C., Persaud, N. V., Guan, L., Punt, K. S., Tang, A., Chen, M., Wilson, K. A., Collins, K. B., Heidecker, G. J., Fernandez, V. R., Perry, H. C., Joyce, J. G., Grimm, K. M., Cook, J. C., Keller, P. M., Kresock, D. S., Mach, H., Troutman, R. D., Isopi, L. A., Williams, D.

- M., Xu, Z., Bohannon, K. E., Volkin, D. B., Montefiori, D. C., Miura, A., Krivulka, G. R., Lifton, M. A., Kuroda, M. J., Schmitz, J. E., Letvin, N. L., Caulfield, M. J., Bett, A. J., Youil, R., Kaslow, D. C., and Emini, E. A. (2002). Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. *Nature* **415**(6869), 331-5.
- Simmons, G., Clapham, P. R., Picard, L., Offord, R. E., Rosenkilde, M. M., Schwartz, T. W., Buser, R., Wells, T. N. C., and Proudfoot, A. E. (1997). Potent inhibition of HIV-1 infectivity in macrophages and lymphocytes by a novel CCR5 antagonist. *Science* **276**(5310), 276-9.
- Singh, M., Briones, M., Ott, G., and O'Hagan, D. (2000). Cationic microparticles: A potent delivery system for DNA vaccines. *Proc Natl Acad Sci U S A* **97**(2), 811-6.
- Stott, E. J. (1991). Anti-cell antibody in macaques. *Nature* **353**(6343), 393.
- Tacket, C. O., Roy, M. J., Widera, G., Swain, W. F., Broome, S., and Edelman, R. (1999). Phase 1 safety and immune response studies of a DNA vaccine encoding hepatitis B surface antigen delivered by a gene delivery device. *Vaccine* **17**(22), 2826-9.
- Talwar, G. P., Singh, O. M., Gupta, S. K., Hasnain, S. E., Pal, R., Majumbar, S. S., Vrati, S., Mukhopadhyay, A., Srinivasan, J., Deshmukh, U., Ganga, S., Mandokhot, A., and Gupta, A. (1997). The HSD-hCG vaccine prevents pregnancy in women: feasibility study of a reversible safe contraceptive vaccine. *Am J Reprod Immunol* **37**(2), 153-60.
- Tang, D. C., DeVit, M., and Johnston, S. A. (1992). Genetic immunization is a simple method for eliciting an immune response. *Nature* **356**(6365), 152-4.
- Teran, L. M. (2000). CCL chemokines and asthma. *Immunol Today* **21**(5), 235-42.
- Theodorou, I., Meyer, L., Magierowska, M., Katlama, C., and Rouzioux, C. (1997). HIV-1 infection in an individual homozygous for CCR5 delta 32. SeroCo Study Group. *Lancet* **349**(9060), 1219-20.
- Trkola, A., Kuhmann, S., Strizki, J., Maxwell, E., Ketas, T., Morgan, T., Pugach, P., Xu, S., Wojcik, L., Tagat, J., Palani, A., Shapiro, S., Clader, J., McCombie, S., Reyes, G., Baroudy, B., and Moore, J. (2002). HIV-1 escape from a small molecule, CCR5-specific entry inhibitor does not involve CXCR4 use. *Proceedings of the national academy of sciences* **99**(1), 395-400.
- Trkola, a., and Olson, C. (2001). Potent, Broad-Spectrum Inhibition of Human Immunodeficiency Virus Type 1 by the CCR5 Monoclonal Antibody PRO 140. *Journal of Virology* **75**(2), 579-588.
- Ulmer, J. B., Deck, R. R., Dewitt, C. M., Donnelly, J. I., and Liu, M. A. (1996). Generation of MHC class I-restricted cytotoxic T lymphocytes by expression of a viral protein in muscle cells: antigen presentation by non-muscle cells. *Immunology* **89**(1), 59-67.
- Ulmer, J. B., Donnelly, J. J., Parker, S. E., Rhodes, G. H., Felgner, P. L., Dwarki, V. J., Gromkowski, S. H., Deck, R. R., DeWitt, C. M., Friedman, A., and et al. (1993). Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science* **259**(5102), 1745-9.

- Veazey, R. S., Marx, P. A., and Lackner, A. A. (2001). The mucosal immune system: primary target for HIV infection and AIDS. *Trends Immunol* **22**(11), 626-33.
- Veenstra, J., Schuurman, R., Cornelissen, M., van't Wout, A. B., Boucher, C. A., Schuitemaker, H., Goudsmit, J., and Coutinho, R. A. (1995). Transmission of zidovudine-resistant human immunodeficiency virus type 1 variants following deliberate injection of blood from a patient with AIDS: characteristics and natural history of the virus. *Clin Infect Dis* **21**(3), 556-60.
- Verthelyi, D., Ishii, K. J., Gursel, M., Takeshita, F., and Klinman, D. M. (2001). Human peripheral blood cells differentially recognize and respond to two distinct CPG motifs. *J Immunol* **166**(4), 2372-7.
- Verthelyi, D., Kenney, R. T., Seder, R. A., Gam, A. A., Friedag, B., and Klinman, D. M. (2002). CpG oligodeoxynucleotides as vaccine adjuvants in primates. *J Immunol* **168**(4), 1659-63.
- Vödrös, D., Thorstensson, R., Biberfeld, G., Schols, D., De Clercq, E., and Fenyö, E. M. (2001). Coreceptor usage of sequential isolates from cynomolgus monkeys experimentally infected with simian immunodeficiency virus (SIVsm). *Virology* **291**(1), 12-21.
- Wahren, B., and Brytting, M. (1997). DNA increases the potency of vaccination against infectious diseases. *Curr Opin Chem Biol* **1**(2), 183-9.
- Wahren, B., Morfeldt-Mansson, L., Biberfeld, G., Moberg, L., Ljungman, P., Nordlund, S., Bredberg-Raden, U., Werner, A., Lower, J., and Kurth, R. (1986). Impaired specific cellular response to HTLV-III before other immune defects in patients with HTLV-III infection. *N Engl J Med* **315**(6), 393-4.
- Walker, C. M., Moody, D. J., Stites, D. P., and Levy, J. A. (1986). CD8+ lymphocytes can control HIV infection in vitro by suppressing virus replication. *Science* **234**(4783), 1563-6.
- Wang, R., Doolan, D. L., Le, T. P., Hedstrom, R. C., Coonan, K. M., Charoenvit, Y., Jones, T. R., Hobart, P., Margalith, M., Ng, J., Weiss, W. R., Sedegah, M., de Taisne, C., Norman, J. A., and Hoffman, S. L. (1998). Induction of antigen-specific cytotoxic T lymphocytes in humans by a malaria DNA vaccine. *Science* **282**(5388), 476-80.
- Watanabe, M., Boyson, J. E., Lord, C. I., and Letvin, N. L. (1992). Chimpanzees immunized with recombinant soluble CD4 develop anti-self CD4 antibody responses with anti-human immunodeficiency virus activity. *Proc Natl Acad Sci U S A* **89**(11), 5103-7.
- Watanabe, M., Levine, C. G., Shen, L., Fisher, R. A., and Letvin, N. L. (1991). Immunization of simian immunodeficiency virus-infected rhesus monkeys with soluble human CD4 elicits an antiviral response. *Proc Natl Acad Sci U S A* **88**(11), 4616-20.
- Weber, L. W., Bowne, W. B., Wolchok, J. D., Srinivasan, R., Qin, J., Moroi, Y., Clynes, R., Song, P., Lewis, J. J., and Houghton, A. N. (1998). Tumor immunity and autoimmunity induced by immunization with homologous DNA. *J Clin Invest* **102**(6), 1258-64.

- Widera, G., Austin, M., Rabussay, D., Goldbeck, C., Barnett, S. W., Chen, M., Leung, L., Otten, G. R., Thudium, K., Selby, M. J., and Ulmer, J. B. (2000). Increased DNA vaccine delivery and immunogenicity by electroporation in vivo. *J Immunol* **164**(9), 4635-40.
- Wildbaum, G., Youssef, S., and Karin, N. (2000). A targeted DNA vaccine augments the natural immune response to self TNF- alpha and suppresses ongoing adjuvant arthritis. *J Immunol* **165**(10), 5860-6.
- Wolff, J. A., Malone, R. W., Williams, P., Chong, W., Acsadi, G., Jani, A., and Felgner, P. L. (1990). Direct gene transfer into mouse muscle in vivo. *Science* **247**(4949 Pt 1), 1465-8.
- Wu L, LaRosa G, Kassam N, Gordon Cj, Heath H, Ruffing N, Chen H, Humblis J, Samson M, Parmentier M, Moore Jp, and Mackay Cr. (1997a). Interaction of chemokine receptor CCR5 with its ligands: multiple domains for HIV-1 gp120 binding and a single domain for chemokine binding. - *J Exp Med* - **186**(- 8), - 1373-81.
- Wu L, Paxton Wa, Kassam N, Ruffing N, Rottman Jb, Sullivan N, Choe H, Sodroski J, Newman W, Koup Ra, and Mackay Cr. (1997b). CCR5 levels and expression pattern correlate with infectability by macrophage-tropic HIV-1, in vitro. - *J Exp Med* - **185**(- 9), - 1681-91.
- Wyatt, R., and Sodroski, J. (1998). The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens. *Science* **280**(5371), 1884-8.
- Zuber, B., Mäkitalo, B., Kjerrström Zuber, A., and Wahren, B. (2002). A novel potent strategy for induction of immunity to HIV-1 reverse transcriptase in primates. *AIDS* **16**, 1-10.
- Åsjö, B., Morfeldt-Manson, L., Albert, J., Biberfeld, G., Karlsson, A., Lidman, K., and Fenyö, E. M. (1986). Replicative capacity of human immunodeficiency virus from patients with varying severity of HIV infection. *Lancet* **2**(8508), 660-2.
- Örvell, C., Unge, T., Bhikhabhai, R., Backbro, K., Ruden, U., Strandberg, B., Wahren, B., and Fenyö, E. M. (1991). Immunological characterization of the human immunodeficiency virus type 1 reverse transcriptase protein by the use of monoclonal antibodies. *J Gen Virol* **72**(Pt 8), 1913-8.