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Studies on

Medical and Immunological Interventions in HIV - 1 Infection

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Abstract

The very first HIV-1 infected patients who received antiretroviral combination therapy (HAART) were severely ill and had very low CD4+ T cell counts. We describe a group of severely ill HIV-1 infected patients monitored for the first two years of their HAART. The patients were subdivided retrospectively into viral responders and viral low responders. Memory and naive T cells increased in both groups and membrane bound activation markers decreased. There were no clinical differences in number of deaths or HIV related events during two years follow up in the two groups. However after seven years there were clinical differences. Virological clearance was achieved in half of the patients in the original viral low responder group. Differences in adherence to HAART may explain the diverse outcome in the two groups. **This study argues for continued treatment with HAART in spite of viral failure** and subsequent development of primary and secondary resistance mutations. If treatment with HAART is interrupted the CD4+ T cell count again decreases and viral load increases to the same level as before treatment. A group of HIV-1 infected patients with a history of long time HAART, well suppressed plasma viral loads and recovered CD4+ T cell counts, were followed during long-term treatment interruption (LTI). We found that CD4+ T cell decrease during treatment interruption was an inverse reflection of CD4+ T cell increase during treatment. A close correlation between pre-HAART nadir CD4+ T cell counts (lowest ever), levels of CD4+ memory cells at the start of LTI and the duration of LTI, indicates that **CD4+ T cell memory levels may not be fully recovered even after a long period of effective HAART**, irrespective of absolute CD4+ T cells count reached during treatment.

HAART does not improve the specific immune response against HIV. Therapeutic vaccination would be a valuable addition to antiviral chemotherapy as immune stimulation potentially helps to reduce the need for antiretroviral drugs by strengthen or inducing new immunological responses. We monitored the long-term immune responses of HIV-infected patients immunized with HIV envelope rgp160 before HAART was introduced. **HIV specific T-helper cell responses induced by immunization were maintained at high levels up to 7 years after the last injection.** The addition of HAART in these patients did not alter this HIV-specific response but gave a profound reduction in viral load and increased total CD4+ T cell counts. Immunization with rgp160 was combined with HAART in another group of patients. As controls, patients treated with HAART only, were followed in addition to two groups receiving tetanus as a non HIV specific vaccine (one group with and one without HIV infection). In keeping with previous rgp160 immunization studies, we were able to demonstrate a positive effect of rgp160 on CD4+ T cell count, measurable six to twelve months after the last immunization. The HIV-specific T cell response was maintained at very high levels up to two years in HAART treated patients, but not to the same extent in non-HAART treated patients, despite comparable or even higher CD4+ T cell levels during follow up. CD4 specific responses to recall antigens (tetanus toxoid and tuberculin) were boosted by the rgp 160 immunization. **HIV specific immunization during HAART might thus induce responses potentially beneficial during a future planned treatment interruption.**

In order to control HIV, effective CD4 and CTL responses are needed in addition to sufficient levels of neutralizing antibodies. Plasmid DNA vaccines can stimulate CTL by intracellular protein production, presented via the HLA class I pathway. They may also stimulate B cells to generate antibodies. We describe a study where DNA constructs encoding the ***rev***, ***tat*** and ***nef*** regulatory HIV-1 genes were given to asymptomatic HIV-1 infected patients on stable HAART and with undetectable viral load. The results were compared with a prior non-randomized study where the same genes were given separately in a ten fold lower total dose to patients with similar CD4+ T cell levels but not on HAART. New HIV-specific proliferative responses were found in all immunized patients who lacked this response before immunization. The specific cytolytic capacity decreased in the placebo group but not in the immunized groups. We did not find that HAART per se was important for the immediate response to the chosen DNA plasmids, in patients with comparable CD4+ and CD8+ T cell levels, even though the total DNA dose was ten folds higher. Since both nef and tat have immune suppressive activities, these properties may have been more prominent in a combination of the genes in a higher dose.

We have clinical evidence that long-term antiviral treatment causes viral suppression and clinical benefits in both viral responders and low-responders. An important variable for prediction of successful interruption of treatment appeared to be retained CD4+ memory cells, directly correlated with nadir CD4+ T cell count. HIV immunization together with antiviral treatment enhanced the magnitude and duration of new HIV-specific immune responses. Immunization with HIV antigens alone has improved short-term survival and almost always induces new HIV-specific T cell responses. This shows that new memory cells can be induce by vaccination in the chronic phase of infection, which should permit extended treatment interruption.

List of publications

I. Hejdeman Bo, Lenkei R, Leandersson A-C, Hultström AL, Wahren B, Sandström E, Bratt G. Clinical and immunological benefits from highly active antiretroviral therapy in spite of limited viral load reduction in HIV type 1 infection.
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II. Hejdeman Bo, Koppel K, Boström A-C, Vivar N, Lenkei R, Sandström E, Wahren B and Bratt G
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Manuscript

III. Boström Ann-Charlotte, Hejdeman B, Matsuda R, Fredriksson M, Fredriksson E-L, Bratt G, Sandström E and Wahren B
Long-term persistence of vaccination and HAART to human immunodeficiency virus (HIV).
Vaccine. Volume 22(13-14), 2004, pp.683-1691

IV. Hejdeman Bo, Leandersson A-C, Fredriksson E-L, Sandström E, Wahren B and Bratt G.
Better preserved immune responses after immunization with rgp160 in HIV-1 infected patients treated with Highly Active Antiretroviral Therapy than in untreated patients with similar CD4 levels during a 2 years follow up.
HIV Medicine, Volume 4(2), 2003, pp.101-110

V. Hejdeman Bo, Boström A-C, Matsuda R, Calarota S, Lenkei R, Fredriksson E-L, Sandström E, Bratt G and Wahren B.
DNA immunization with HIV early genes in HIV-1 infected patients on Highly Active Antiretroviral Therapy.
AIDS Research and Human Retroviruses, Volume 20(8), 2004. In print.

List of abbreviations

ABC	abacavir
ADCC	antibody dependent cellular cytotoxicity
AIDS	acquired immunodeficiency syndrome
APC	antigen presenting cell
APV	amprenavir
ART	antiviral therapy
ATA	atazanavir
AZT	zidovudine, azidothymidine, ZDV
CAF	CD8+ cell antiviral factor
CCR-2,-3-5	cystein-cystein linked chemokine receptor 2, 3 or 5
CD	cluster of differentiation
CMV	Cytomegalovirus
CNAR	CD8+ T lymphocyte noncytotoxic antiviral response
CNS	central nervous system
CpG	Cytosin-Phosphate-Guanosine
crm1	a nucleocytoplasmic transport protein
CRFs	circulating recombinant forms
CSF	cerebrospinal fluid
CTL	cytotoxic T lymphocyte
CXCR4	cystein-x-cystein linked chemokine receptor 4
ddC	zalcitabine
ddI	didanosine
d4T	stavudine
DNA	deoxyribonucleic acid
DT	diphtheria toxin and tetanus
DTH	delayed type hypersensitivity
EBV	Epstein-Barr virus
EFV	efavirenz
ELISA	enzyme-linked immunoabsorbent assay
FI	fusion inhibitor
env **/** env	HIV-1 envelope ***gene*** / envelope proteins (gp120 and gp41)
gag / gag	HIV-1 group specific antigen ***gene*** / proteins (proteins 6, 7, 17 and 24,)
GM-CSF	granulocyte macrophage-colony simulating factor
gp	glycoprotein
HAART	highly active antiretroviral treatment / therapy
Hib	Haemophilus influenzae type b
HIV-1	Human immunodeficiency virus type 1
HIV-2	Human immunodeficiency virus type 2
HLA	human leukocyte antigen
HLA DR	MHC class II antigen
IDV	indinavir
IFN	interferon
Ig	immunoglobulin
IL	interleukin
i.m.	intramuscular
IPC	interferon producing cells (=PDC)
LPS	bacterial lipopolysaccharids

LPV	lopinavir + ritonavir
LTNP	long term non progressors
LTR	long terminal repeat
LTI	long-term supervised treatment interruption
MBL	mannose-binding lectin
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
MVA	Modified Vaccinia Ankara virus
nef **/** nef	HIV-1 negative regulatory factor ***gene*** / protein
NK cell	natural killer cell
NNRTI	non nucleoside reverse transcriptase inhibitors
NRTI	nucleoside/tide reverse transcriptase inhibitor
NSI	non syncytium inducing
ODN	oligodeoxyribonucleotides
p	protein
PBMC	peripheral blood mononuclear cell
PMN	polymorphonuclear neutrophilic leukocyte
PCR	polymerase chain reaction
PDC	plasmacytoid dendritic cells (=IPC)
PHA	phytohemagglutinin
PI	protease inhibitor
pol / pol	HIV-1 polymerase ***gene*** / polymerase proteins (proteins 10, 32, and 51/66)
PPD	purified protein derivate of mycobacterium tuberculosis
PWM	pokeweed mitogen
RANTES	regulated upon activation of normal T-cell (expressed and secreted)
rev / rev	HIV-1 regulatory of virion ***gene*** / protein
rgp	recombinant glycoprotein
RNA	ribonucleic acid
RRE	rev response element
RT	reverse transcriptase
RTV	ritonavir
SI	syncytium inducing, stimulation index
SIV	simian immunodeficiency virus
SFV	Semliki Forest Virus
SHIV	Simian-Human Immunodeficiency Virus
SQV	saquinavir
STI	structured treatment interruption; usually and in the text “on and off cycles” of HAART
TAR	transcriptional activation region
tat / tat	HIV-1 transactivator gene / protein
3TC	lamuvidin
TLR	Toll-like receptor
TFV	tenofovir
TNF	tumor necrosis factor
V1 - 5	variable regions 1 to 5 of HIV-1 gp120
vif / vif	HIV-1 viral infectivity factor ***gene*** / protein
vpr / vpr	HIV-1 viral R ***gene*** / protein
vpu / vpu	HIV-1 viral U ***gene*** / protein
WB	Western Blot
ZDV	zidovudine (=AZT)

Contents

Aims of the thesis

General aim:

To study immune reconstitution induced by medical and immunological interventions in patients chronically infected by HIV-1.

Specific aims:

- To study the capacity of long-term Highly Active Antiretroviral Therapy (HAART) to reconstitute the immune system in severely ill patients.
- To analyze the influence of baseline immunological and virological data on the outcome of treatment interruption.
- To study the impact of effective HAART on the preservation of immune responses induced by vaccination of HIV-1 infected patients.

The Pandemic

At the end of 2003, estimates by the World Health Organization indicate that approximately 40 million people were living with HIV type 1 (HIV-1) and more than 20 million had died. Each day, HIV-1 infects another 16,000 persons worldwide. The latest ***AIDS epidemic update*** from UNAIDS in July 2004 reported steady increases in people living with HIV/AIDS as well as in the number of AIDS deaths. The virus has a global spread, with the vast majority (70%) of infected persons in Sub-Saharan Africa. An explosive increase is now occurring in India, China and other countries in Asia. Most people with HIV are infected via sexual contacts but mother-to-child transmission is also contributing to the rapid spread (about 5 % of new HIV-1 infections). In Eastern Europe an extensive use of intravenous drugs contributes to a rapid increase in newly infected individuals. Also in North America and Western Europe infections are on the rise.

The virus

The origin of HIV-1

HIV belongs to the Lentivirus genus of the retroviridae family. Lentivirus is named from the Latin *lentus*, meaning slow, because the resultant disease develops slowly. HIV-1 was first isolated by French researchers in 1983 from a patient with signs and symptoms that often precede AIDS [15] and shortly afterwards by an American group from another patient with fully developed AIDS [80]. A second closely related, but less prevalent and less virulent virus, HIV-2, was discovered in 1986 [47].

HIV-1 evolved with the chimpanzee subspecies *Pan troglodytes troglodytes* but did not cause any disease [81]. The main group of HIV-1 (group M; for definition see section "*Subtypes*") began to spread in human population approximately 70 years ago [224]but may have begun even earlier [124]. It is even less clear when groups O and N of HIV-1 variants were transmitted. HIV-2 is genetically similar to the simian immunodeficiency virus (SIV) that is endemic among the *sooty mangaby*, another African primate [103].

Viral structure and replication

HIV-1 consists of an outer envelope and an inner nucleocapsid protein that encapsulates two plus-stranded copies of RNA together with the enzyme reverse transcriptase (RT) and other HIV proteins. (Figure 1).

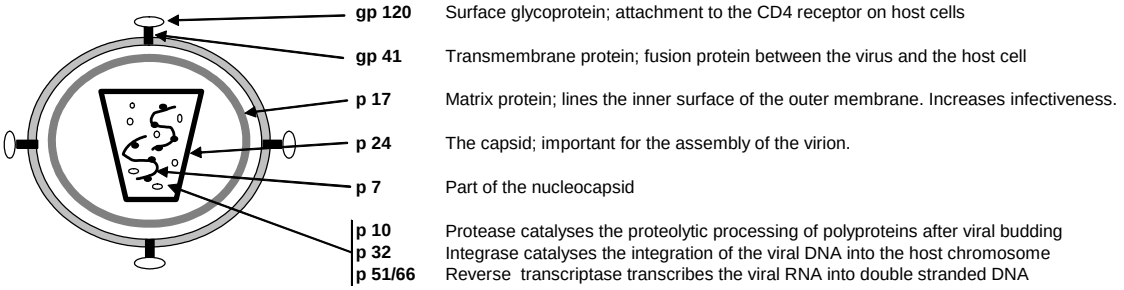

Figure 1 The viral structure with structural proteins and catalyzing enzymes.

The viral RNA genome contains three major genes for structural proteins (***gag, pol*** and ***env***) as well as genes that code for regulatory and accessory proteins (***tat, rev, nef, vif*** and ***vpu***) (figure 2).

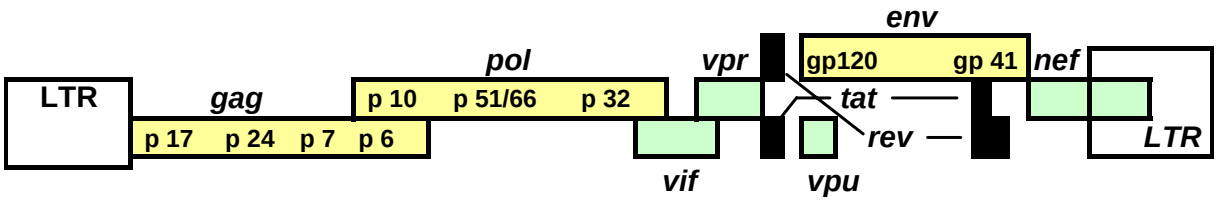

Figure 2 The viral genome with structural, regulatory and accessory genes (modified from [75])
Gene products
Gag – capsid proteins; the matrix protein (p17), the capsid proteins (p24, p7 and p6).
Pol – proteins, the viral enzymes protease (p10), reverse transcriptase (p51/66) and integrase (p32).
***Env – envelope proteins*;** the viral surface glycoprotein (gp120) and transmembrane protein (gp41) which is cleaved from a precursor protein (gp160).
Tat - Transactivator protein speeds up the transcription of viral RNA performed by RNA polymerase. Secreted tat protein upregulates the chemokine receptors of uninfected cells and makes them more susceptible to viral infection.
Rev - Regulator of virion expression protein mediates the transport of unspliced and partly spliced mRNA though nuclear pores into the cytoplasm and mediates binding of ribosome to the viral transcript.
Nef - Negative regulatory factor protein makes infected cells less vulnerable to cytotoxicity by down-regulating the CD4 and MHC class I molecules from the cell surface.
Vif - Virion infective factor protein; important for proviral DNA synthesis, core packing and cell to cell transmission of virus.
Vpr - Viral protein R; induces cell differentiation.
Vpu - Viral protein U; transports the envelope protein to the cell surface.

The envelope, partially formed at the host cell membrane, consists of a lipid bilayer with viral glycoproteins (gp) protruding from its surface. Gp120 binds to the CD4 (**C**luster of **D**ifferentiation) receptor present on T lymphocytes, macrophages and dendritic cells as well as on microglia cells in the nervous system. A conformational change takes place which allows binding of the gp120-CD4 complex to a coreceptor (CCR5 or CXCR4; see below section "*Coreceptor usage*") [46]. Further conformational changes in the transmembrane protein gp41 expose a fusion peptide which is inserted into the cell membrane and triggers the fusion of the viral envelope to the cell membrane. The viral genome is uncoated and enters the cell. The reverse transcriptase (RT) transcribes the viral RNA genome into double-stranded DNA. Viral DNA is integrated into host DNA by the viral integrase forming a provirus [75]. The integrated viral DNA, with functions like cellular DNA transcribed by the cellular machinery, can remain in a latent stage for long periods and later become activated.
The production of infectious virus particles from an integrated HIV provirus is stimulated by a cellular transcription factor, NFκB [199], which binds to promoters in the cellular DNA and the viral LTR (Long Terminal Repeat) [116]. The transcription of viral RNA by a cellular RNA polymerase is then initiated.
Early gene products such as tat and rev proteins, enhance and speed up viral production:

- **tat** by binding to the transcriptional activation region (TAR) in the LTR of the virus, which causes removal of factors that block cellular RNA polymerase II and increases polymerase activity a hundredfold [91].
- **rev** by binding to a specific viral RNA sequence (RRE, rev response element) and to a host nucleocytoplasmic transport protein (Crm1). This engages a host pathway for exporting mRNA through nuclear pores into the cytoplasm [199].
- The reverse transcriptase, or **pol** activity, occurs as an intermediate event.

- Env precursor proteins are glycosylated and cleaved in the Golgi apparatus before they are inserted in the cellular membrane. Both gag and env proteins contribute to the structure and are assembled together with two full RNA genomes at the cell membrane where new viral particle bud out from the membrane.

Genetic variability

White a high rate of viral production (10^{10} particles/day), a generation time of 1 – 2 days [196] and a mutation rate of 3.4 x 10^{-5} per base pairs per cycle during reverse transcription [155], genetic variability is extremely high. The overall rate of nucleotide substitution is in the region of one million times that of the somatic genes [142]. The viral population is relatively homogenous during the initial period of an HIV-1 infection but it diversifies over the course of the infection until genomic sequences differ as much as 15% in a specific area of gp120 (the V3 loop) [171], while the overall intersubtype diversity (see below) is as much as 35% [84]. Very small changes in the viral proteins appear to mediate escape and promote viral replication which results in a high capacity to adapt to the host immune response, develop drug resistance and escape candidate vaccines. The genetic diversity diminishes again in late stage AIDS, probably as a result of reduced pressure from a failing immune system. However, the characteristics of the selective pressure in a person on antiviral treatment may differ from those in drug-naive patients. The presence of drug-resistant mutations in untreated individuals indicates transmission of resistant virus. In the US, over 40% of infected newborns may have HIV strains with mutations associated with reduced drug susceptibility [247]. Most drug resistant mutants have reduced capability to replicate and tend to revert to “wild-type” forms if not exposed to drugs [52]. However, compartmentalization within an HIV-1 infected individual may result in different viral populations in different parts of the body [217].

Coreceptor usage

The HIV-1 co-receptors belong to the chemokine receptor family. These receptors normally react with a group of chemotactic cytokines that direct leukocytes to migrate to sites of inflammation [104] or are important for fetal cell development [223, 250]. Several pathogens use these receptors for cell entry. The fifteen known receptor subtypes are named on the basis of the position of two linked cysteins (C) in the chemokine they specify. The chemokine receptor subtypes mainly used by HIV-1 are:

- **CCR5** (CC receptor 5) predominantly expressed on dendritic cells, macrophages and memory CD4+ T lymphocytes (CD45+RO+CD62L-CD26+). The majority of CD4+ cells in the peripheral blood, spleen and lymph nodes express very little CCR5, whereas high levels are expressed by these cells in the intestine, vagina and rectum. HIV strains mostly associated with virus transmission uses CCR5 and require only a low level of CD4 on the infected cells. The β-chemokines RANTES (regulation-upon-activation), MIP-1α (macrophage inflammatory protein) and MIP-1β are natural suppressors of HIV-1 infection through blocking of CCR5 [5]. The CCR5 receptor level is moderately increased on CD4+ T lymphocytes from HIV-1 infected asymptomatic patients and significantly increased in individuals with advanced HIV infection in parallel with CXCR4 receptors [169].
- **CXCR4** (CXC receptor 4) has an intervening amino acid between the first two cysteins [227]. Variants of HIV-1 that infect CD4+ cells that express CXCR4 require high levels of CD4 on the cells they infect (mainly naive T lymphocytes CD45RA+ CD62L+).

With disease progression the virus expands its coreceptor type usage [49] from initially CCR5 to CXCR4 and further also to CCR3, CCR2β, CCR8, CX3CR1 and others but the significance of these coreceptors is not fully known.

Several classification systems have been used and are often referred to in the literature:
1) Classification based on viral cell growth in peripheral blood mononuclear cells [69]:
- ***Slow/low*** *(SL*)
- ***Rapid/high*** *(RH)*

2) Classification based on induction of syncytia formation in MT-2 cells [97], expressing CXCR4:
- ***Non-syncytium-inducing*** *(NSI)*
- ***Syncytium inducing*** *(SI)*

The MT-2 (Mature T-cell number 2) cell line was established from cord blood lymphocytes that had been co-cultured with leukemia cells from a patient with adult T-cell leukemia. Patients who harbor viruses with the SI phenotype progress more quickly to AIDS than those with NSI viruses [49, 118].
A related classification system is based on the type of cells infected:
- ***Macrophage-tropic*** (M tropic) viruses infect macrophages but not T-cell lines
- ***T-cell line tropic*** (T tropic) viruses infect CD4 T cells with CXCR4 as coreceptors.

The latter definition is however not rigorous since some macrophages can be infected by T tropic viruses and activated T-cells by M tropic viruses.
3) Classification based on coreceptor usage [17]:
- ***R5*** virus uses CCR5
- ***X4*** virus uses CXCR4
- ***R5X4*** virus uses both coreceptors.

Viruses classified as NSI, M-tropic or R5 are virtually synonymous unlike those classified as SI, T-tropic or X4.

Subtypes

On the basis of nucleotide sequences derived from the ***env*** and ***gag*** genes, HIV-1 has been subdivided in three major groups:

1. ***M*** (Main), subdivided into nine sub-subtypes (A–K excluding I and E)
2. ***O*** (outlier)
3. ***N*** (non-M-non-O)

In the ***M*** group, subtype B is the most studied and predominates in Europe, North America and Australia while subtype C is the most spread globally and predominates in South Africa. All ***M*** virus subtypes are found in Central Africa but A and C predominate there; viruses belonging to ***O*** and ***N*** are also found in this region. Recombinant variants of group ***M*** (CRF = *circulating recombinant forms*) are spread epidemically in Central and West Africa [231]. In Uganda and Tanzania more than 30–50% of circulating HIV strains may be recombinant [238]. Also in Eastern Europe recombinant strains have been reported to be responsible for local outbreak among injecting drug users [22]. Recombinant virus consists of sequences from more than one epidemic subtype and mosaic viruses have regions that resemble four or more subtypes. Recombinants occur when the reverse transcriptase jumps back and forth between RNA templates from at least two different subtypes of HIV-1 during the transcription and require the simultaneous infection of a cell with two viruses of different subtypes. A new subtype is defined if **a**) three representative strains are identified in at least three individuals **b**) three representative full-length genomes are sequenced **c**) the new subtype is approximately equidistant from all previously characterized subtypes in all genomic regions [195]. Sub-subtype label are used to describe distinctive lineages that are not sufficiently distant genetically to qualify as a new subtype.
Biological subtype differences may influence properties as virulence, tropism and transmission. This is best demonstrated in the choice of coreceptor [21, 230], but so far there is no convincing evidence related to the rate of disease progression [2, 3, 105] or response to

drug therapy [185]. The effect of vaccines [149, 240] and diagnostic tests may however be dependent on the subtype.

The immune system

Innate immunity

Most microorganisms are detected and destroyed within minutes or hours by defense mechanisms that do not require a prolonged period of induction before they can identify and defeat an infection. Barriers such as skin, mucosa, temperature and pH, as well as a large number of substances that are directly induced by the microorganism, are all a part of this first-line defense called natural or innate immunity. This response does not adapt after repeated exposure to an organism but plays an important part in shaping the subsequent adaptive immune response. Tissue macrophages and polymorphonuclear neutrophilic leukocytes (PMN) play a key role in the innate immunity. Natural killer cells (NK cells) are large granular lymphocytes, able to mediate direct lysis or antibody-dependent cellular cytotoxicity (ADCC) by recognizing virus specific antibodies. They recognize and kill cells with reduced expression of MHC class I molecules (for definition see section *"Adaptive immunity"*) on the surface of the infected or transformed cell. Stimulated by activated monocytes (via IL-15, IL-12), NK cells release several cytokines and chemokines (e.g. macrophage inflammatory protein (MIP)-1α/β) and RANTES) that block the entry of HIV-1 that uses CCR5 coreceptor [125]. Bacterial DNA contains immunostimulatory motifs, consisting of unmethylated CpG dinucleotides, that trigger NK cells as well as B cells and macrophages to proliferate, mature and secrete cytokines [122] (se also section "*Active immunotherapy* "). A key cell type in innate immunity is the one that produces type 1 interferon (IPCs), also called plasmacytoid dendritic cells (PDC). Type interferons α and β (IFN α /β) both have strong adjuvant effects on a variety of immune cell types as monocytes, NK cells and T cells, as well as direct inhibitory properties against HIV [221]. Virus, gram-positive bacteria and mycobacteria all induce cells to produce IFN.

The innate system uses a diversity of receptors, such as *the mannose receptors* and *toll-like receptor*, to recognize and respond to pathogens like HIV [100]. The innate immune system also includes several soluble factors, such as lysozyme, complements and acute phase proteins. Defensins, antimicrobial peptides present in granules of phagocytic cells and in epithelial cells, can block the infectious agent by cell lysis, stimulate chemotaxis and signal to the adaptive immune system [136].

Adaptive immunity

If an infectious organism succeeds in breaking through early lines of defense, clearance of pathogens is undertaken by the adaptive immune responses, mediated by lymphocytes and antibodies. The adaptive immune system is able to recognize both intracellular and extracellular pathogens and create a long-lasting memory. The response is induced by professional antigen presenting cells (APC). The response of T and B lymphocytes can distinguish between different antigens as well as between self and non-self.

B lymphocytes receptors with membrane bound receptors, recognized by different pathogens and their protein products, differentiate into *plasma B lymphocytes* with the capacity to secrete antibodies that are able to bind to the specific pathogen and engage different effector mechanisms in the immune system.

T lymphocytes, which are divided into two major groups, distinguished by the expression of the cell-surface proteins CD4 and CD8, recognize foreign antigens that are displayed on the surface of the body's own cells. Infected cells or cells of the immune system that have taken up foreign antigen, display on their surface peptide fragments derived from pathogen protein delivered by a specialized host-cell glycoprotein, the major histocompatibility complex (MHC).

There are two major classes of MHC molecules – MHC class I and MHC class II – which differ in their structure and expression pattern on tissues of the body:

- *MHC class I* molecules are expressed on all nucleated cells, especially on hematopoietic cells. They present peptides from pathogens synthesized in the cytosol, commonly viruses, to CD8+ cytotoxic T lymphocytes specialized to kill cells that present the same antigen.
- *MHC class II* molecules are expressed particularly by dendritic cells, macrophages and B lymphocytes. The bind peptides and present them to CD4+ T lymphocytes.

CD4+ T lymphocytes play a central role in immune homeostasis. Activated CD4+ T lymphocytes are critical in promoting the survival of B-lymphocyte and antibody production. They also provide helper function to CD8+ T lymphocytes and secrete various kinds of cytokines that have profound immuno-regulatory effects in many disease states.

CD4+ T lymphocytes differentiate into two types of effector T lymphocytes:

- T_H1 differentiation tends to be stimulated by pathogens that accumulate in large numbers inside macrophage and dendritic cells. T_H1 cells produce cytokines such as IL-2 associated with inflammatory signals resulting in T-cell growth, activation of macrophages and NK cells.
- T_H2 production is mainly stimulated by extracellular antigens. T_H2 cells produce IL-4, IL-5 and IL-10, which help B lymphocytes to proliferate and differentiate.

Table 1
Definition of sub-cellular markers on CD4+ and CD8+ T lymphocytes

CD45RO+ CD45RA+/CD62L+ CD45RA+/CD62L-	Naive T cells express high levels of a high molecular weight isoform of **CD45** (called RA), whereas memory T cells express high levels of a low molecular weight isoform (RO) [1, 214]. T cells recycle between blood and lymphoid tissues and then back to blood in a process thought to be dependent on L-selectin (**CD62L**) expression [29, 184].
CD25+	Represents the IL-2 receptor. [89].
CD26+	Involved in T cell activation and function [108].
CD28+	A co-activation molecule which must be present on T cells to elicit their proliferation [89] and which downmodulates during the course of the disease and thereby contributes to cell anergy and sensitivity to apoptosis.
CD38+/HLA DR	Expressed on T cells upon activation [112] often in conjunction with HLA DR (MHC class II antigen).
CD95+	(Fas) capable of inducing cell death by binding to different death-signaling pathways (i.e. monoclonal antibodies or CD95L; belonging to the tumor necrosis factor (TNF) cytokine family) [23].

Immune responses and factors of importance for viral control

Strong immune responses, including neutralizing antibodies, antibody dependent cellular cytotoxicity and T cell dependent responses develop soon after HIV seroconversion. However, these HIV specific responses are lost early of the infection [241]. Only a minority of HIV

infected individuals have preserved functional immune responses that allow them to remain free of symptoms for decades with high CD4 T-cell counts and low plasma viral loads without antiretroviral treatment. These long-term non-progressors (LTNP) comprise < 1% of HIV-1 infected individuals [70, 135] thou certain criteria used have reported figures as high as 5 – 10 % [34]. The ability of these patients to harbour the virus successfully is believed to be due to favourable combinations of innate and adaptive immune system factors [51, 110].

a) Humoral responses

Strong antibody responses are raised against ***env*** (the V3 loop in gp120 and the immunodominant loop in gp41), ***gag*** (p24 and p17) and ***pol*** gene products. The beneficial role of neutralizing antibodies in the natural course of HIV-1 infection has been questioned. The ability of the virus to evade the antibody response is thought to be the result of its high mutation rate, overlapping hypervariable loops in the V1 and V2 region (antibodies reactive with critical parts of gp120) and also the glycosylation of the envelope which may mask important epitopes. HIV-1 coated with antibodies can still infect new CD4+ T lymphocytes [99].

A relation between levels of neutralizing antibodies and stage of disease has been reported by some authors [38, 94, 168, 236] but the ability of these antibodies to neutralize primary or autologous viral isolates varies [28, 68]. Anti-***tat*** antibodies correlate inversely with disease progression [30, 192] and high prevalence of antibodies to certain epitopes of RT is also associated with asymptomatic infection [130].

A paradoxical hyperactivation of B lymphocytes, resulting in poly- or oligoclonal hypergammaglobulinemia, is accompanied by decreased B lymphocyte proliferative responses to T cell independent B cell mitogens [129]. Virus-specific and polyclonal antibodies are possibly regulated independently, since the HIV-specific response declines as disease progresses, whereas the polyclonal response increases [216].

Autoantibodies as well as autoimmune diseases are increased in HIV infection (reviewed in [248]) but serum levels of immunoglobulin do not correlate with the occurrence of autoantibodies. Abnormal anti-myosion concentrations were found in 19% of HIV positive patiens without heart disease (compared to 3% of HIV negative controls) and 43% of HIV patients with heart disease. Titers of autoantibodies to HLA and other surface markers of CD4+ T lymphocytes appear to increase with progression of disease and might contribute to the pathogenesis of the immunodeficiency that characterizes HIV-1 infection [248]. Structural homologies of HIV-1 env-products and the functional molecules involved in the control of self-tolerance may be the genesis of autoreacitivity.

Defective humoral immunity may account for the late increase in the susceptibility of HIV-infected subjects to bacterial infections (such as pneumococcal polysaccharides). The magnitude of the antibody production in response to vaccination correlates with disease stage [114]. Treatment with highly active antiretroviral drugs (HAART) significantly restores the ability to respond to different vaccines [18, 126]. In a study with both polysaccharide (pneumococcal and Haemophilus influenzae type b, Hib) and protein (diphtheria toxin and tetanus, DT) antigens in HIV-1 sero-converters, with a median CD4+ T cell count of >500 x 10^6/l, a significant increase in vaccine antigen-specific IgG was generated comparable to the level found in HIV-negative controls [113]. During a 12-week follow-up, however, the levels of Hib antibodies decreased more markedly in the HIV-1 infected group. No direct correlation between viral load, CD4+ T cell count and level of antibody responses was observed in this group of newly HIV-1 infected patients. In studies with influenza and tetanus vaccines in HIV-1 infected persons a direct correlation was noted between CD4+ T cell count and antibody responses [127]. In contrast to vaccines against influenza and tetanus, pneumococcal

vaccines are T-lymphocyte independent. However, even responses to pneumococcal vaccines are affected in patients with very low CD4+ T cell counts [174].

b) Cellular responses

CD4+ T lymphocytes are the primary target cells for HIV-1 [53]. The infection is characterized by a progressive qualitative as well as quantitative destruction of these cells, and a subsequently increased risk of developing life-threatening complications [158]. Opportunistic infections and other symptoms become more frequent as the CD4 T lymphocyte count falls, starting at around 350 x 10^6/l in blood and accelerating if the count falls below 200 x 10^6/l.

The qualitative aspects of CD4+ T cell destruction are evident from a number of variables such as:
- skin testing for delayed-type hypersensitivity
- lymphocyte proliferation
- cytokine production
- responses to immunization.

CD4+ T lymphocytes are the major producers of virus in untreated patients. The central role of these lymphocytes means that this affects several aspects of the immune system:
- helper cell (CD4+) reactivity
- priming, maintenance of memory and maturation of CD8+ T lymphocyte function,
- differentiation of NK cells,
- maturation of B lymphocytes,
- migration of lymphocytes to the site of infection.

The direct effects of the virus on T lymphocytes cannot explain the decline of CD4+ lymphocytes, as a very small proportion of these cells are infected [7]. Three dominant mechanisms for the loss of CD4+ T lymphocytes during HIV infection have been suggested;
- a direct HIV-1 toxicity through viral replication, syncytia formation and cell death;
- CD8+ specific cytotoxic lysis of both infected and uninfected CD4+ T lymphocytes;
- an increased susceptibility to the induction of apoptosis – regulated cell death.

In addition several other mechanisms for the decline of CD4+ T cells have been suggested as;
- cell death due to disruption of the cell membrane of infected cells,
- antibody-dependent cellular cytotoxicity,
- autoimmune mechanisms,
- direct effects on the regeneration of mature T lymphocytes from the precursor cell pool in the bone marrow [66, 138].

The resting memory CD4+ T lymphocytes with long half-life, established early during infection [212], form a stable persisting cellular reservoir as they carry DNA with integrated provirus [72, 73]. Other potential reservoirs are persistently infected macrophages with integrated virus and extracellular virus particles trapped on specialized cells in the germinal centers of the peripheral lymphoid tissue [43].

A weak HIV-specific CD4+ T lymphocyte response is generally induced a few months after infection. Strong HIV-specific CD4+ T lymphocyte responses to HIV-1 gag [200, 201] have been associated with control of viral replication. A shift in CD4+ T lymphocyte response from T_H1 to T_H2 (i.e. from a cellular to a humoral response) has been proposed to influence

disease progression in that patients with progressive disease produce low levels of type 1 cytokines (IL-2, IL-12, IL-15) and high levels of type 2 cytokines (IL-4, IL-5, IL-6, IL-13).

CD8+ T lymphocyte count increases in the early stage of HIV-1 infection. This response primarily consists of memory and activated CD8+ T lymphocytes while naive CD8+ T lymphocytes, as well as naive CD4+ T lymphocytes, decline.
As in most acute viral infections, specific CD8+ cytotoxic activity (CTL) rises within 3–4 days after infection and peaks after 7–10 days. In most infections it then declines, while in HIV infection the activation persists. Strong CTL responses are usually detected early in HIV infected patients and are maintained for many years [242] but in the late stage of disease these CTLs can no longer control the infection and subsequently the viral load increases and disease progresses [11]. The lack and perhaps also the dysfunction of CD4+ T cells [98] may account for the loss of HIV specific CTL. In the late stage of infection both memory CD4+ and CD8+ T lymphocytes decline at similar rates [197].

High levels of broadly reactive CD8+ CTLs have been reported to correlate with maintenance of a low viral load and a stable clinical status [172, 180], though this has been questioned [67, 161]. CTL responses that are reported to be low during disease progression to AIDS are:
- anti ***nef*** [203].
- anti ***tat*** [235]. ***Tat***-specific CTL have also been shown to control infection in primate models [6, 61].
- anti ***rev*** [235].

CTL that are reported to control viral load are:
- anti ***env*** (especially in early HIV-1 infection) [170].
- anti ***gag*** (especially in chronic HIV-1 infection) [172].

This means, that most CTL responses contribute to a favorable course of infection.

A defect in the expression of perforin, especially in lymphoid tissue, is established very early during primary infection (an important component of the death machinery of cytotoxic T cells). This is believed to inhibit an effective HIV-specific CTL response [8]. The failure of CD8+ T lymphocytes to express perforin is due to actions mediated by ***nef*** [16]. A connection has been found between a high proliferative capacity of HIV-specific CD8+ T cells, expression of perforin and immunological control of HIV-1 infection without medication [162].

Thus, a highly active CTL response to all proteins in terms of both effector function and number of epitopes recognized may be important for control of virus replication both in humans and in primates. This may lead to equilibrium between CTL responses and HIV viral load, and maintenance of a stable CD4+ T lymphocyte count over many years. A shift from the non-progressor to the progressor state may however occur [67], perhaps as an indication that HIV cannot be fully cleared even in LTNP and that the balance might be vulnerable.

CD8+ T cells can also suppress HIV replication in infected CD4+ T cell without killing the cells [13, 139]. CD8+ cells from patients with no or very slow disease progression are able to suppress replication of all HIV-1 and -2 strains tested in target CD4+ lymphocytes and macrophages through a noncytotoxic mechanism. This CD8+ noncytotoxic antiviral response (CNAR) can completely suppress HIV production, most strongly in healthy individuals while CNAR is lost with disease progression. CNAR appears to be mediated by the secretion of a soluble CD8+ cell antiviral factor (CAF) that blocks HIV transcription, but the nature of CAF

has not been fully identified. Other soluble factors defined to inhibit HIV are defensins and chemokines.

Monocytes/macrophages are the second major targets of HIV-1. These cells are not killed by HIV-1 and may continue to release virus for a long period and also carry HIV-1 into different tissues, including across the blood-brain barrier.

Dendritic cells especially in the mucosa, express high levels of the adhesion molecule DC-SIGN (C-type lectin) that binds to gp120. The dendritic cells transmit infectious virus to activated T lymphocytes in the regional lymph nodes and thereby contribute to the transfer of the virus [86].

c) Immune activation

The persistence of HIV infection leads to a chronic immune activation evident from increased expression of CD38, HLA-DR and Fas (CD95) [85, 88] on both CD4+ and CD8+ T lymphocytes. The chronic and dysregulated immune activation is believed to induce massive cell death and impairment of many immune functions during HIV-1 infection. Fas is a receptor molecule on T lymphocytes belonging to the tumor necrosis factor (TNF) family. Stimulation of Fas by the Fas ligand initiates apoptosis. Upregulated expression of Fas on CD4+ and CD8+ T- and also B lymphocytes might be an indication that these cells are susceptible to apoptosis through the Fas-FasL pathway. A positive correlation was found between markers of immune activation (both cellular and soluble) and the CD4+ T lymphocyte decline during HIV infection [137]. Examples of soluble activation markers are CD27 (from CD4+ & CD8+ T and B lymphocytes), TNF-α (from CD4 & CD8 T lymphocytes and monocytes / macrophages), neopterin (from monocytes / macrophages) and β_2-microglobulin (from MHC class I expressing cells).

d) Other factors of importance for viral control

1) HLA constitution and antigen presentation

The presentation of virus proteins by antigen-presenting cells is important for the early response to HIV. This is related to the polymorphism of the host's HLA genes. Individuals heterozygous at HLA loci are able to present a greater variety of antigenic peptides than are homozygotes, resulting in a more effective immune response to a large array of pathogens [42]. In a cohort of Nairobi prostitutes, who appeared resistant to HIV infection, uncommon variants of HLA genotypes were found [152]. A highly significant association between HLA class I homozygosity and rapid progression to AIDS has been observed in both Caucasians and African Americans. Several HLA alleles, such as HLA-B27 and B-57, have been associated with slow progression (although this was not significant after correction for multiple tests) [40]. In contrast HLA-B35 is associated with accelerated progression to AIDS [41].

2) Co-receptor differences

A genetic mutation in co-receptors on T cells that seems to affect disease progression is the CCR2-V64I mutation [220] and a 32 base-pair deletion that prevents the expression of the CCR5 receptor [56]. The latter mutation is largely confined to Caucasians and is extremely rare in Africans [249]. Individuals heterozygous for CCR5Δ32 show delayed disease progression [27] and homozygous individuals are highly resistant demonstrated by the very low frequency of this genotype in infected populations [107].

The HIV-1 coreceptor usage strongly parallels malaria pathogenesis, where Duffy (a multi specific, non-signaling chemokine-binding protein on erythrocytes) is used as a receptor for cell entry by Plasmodium vivax. As in HIV, absence of or defects in entry receptors correlate with protection against vivax malaria [229]

3) Soluble factors

Increased levels of interferon (INF) and β-chemokines, including RANTES from CD4+ T lymphocytes and the macrophage inflammatory proteins MIP-1α, MIP-1β, have been associated with low viral load and retarded disease progression [234] but these findings have been questioned by others [128].

4) Natural Type 1 Interferon

The numbers and functions of interferon producing cells (IPC) are increased in LTNPs. The number of circulating IPCs is negatively correlated with HIV viral load [221]. Studies have indicated that HIV-infected healthy subjects with low CD4+ T lymphocyte counts ($<100 \times 10^6$/l) but conserved IPCs ($>2 \times 10^6$/l) do not develop opportunistic infections or cancer [222]. The causal relationship between loss of IPCs and occurrence of opportunistic infection remains to be clarified.

5) Early HIV RNA peak levels

A clinical observation is that individuals with a moderate to severe symptomatic primary infection may experience more progressive HIV-1 disease [213]. This may indicate that a high dose of virus infection spread rapidly in to many organs. It also indicates that the degree of inflammatory responses during the very first weeks of infection is a reflection of early HIV RNA peak levels. The level of HIV RNA early in infection has been shown to have a predictive value for the rate of CD4 decline and subsequently also for the clinical outcome of the patient [119].

6) Viral factors

The most important viral factor that helps the virus to avoid the human immune defense is the massive attack on CD4+ T cells including down-regulation of MHC and CD4 molecules on host cells [4, 167]. But a large genetic heterogeneity together with viral variations, make it possible for the virus to avoid and escape from both humoral and cellular responses [76, 93, 117, 143, 198].

The most important viral factor, apart from viral load, that correlates to disease progression is the viral phenotype. HIV-1 variants that induce syncytium formation are closely associated with a rapid progression compared to non-syncytium inducing variants [49, 118].

Some studies suggest that non-progression in a subgroup of patients may be the result of infection with attenuated viruses [55]:

- Single amino acid changes in the ***nef*** gene have been shown to be unique for LTNP and individuals infected with deleted ***nef*** are described as LTNP [55, 156].
- Mutations in a highly conserved part of the ***rev*** gene are associated with asymptomatic infection [106, 111].
- Deletion of ***vpx*** and ***vpr*** genes produces a similar picture in rhesus macaques, infected with SIV, as in some HIV-1 infected LTNP humans [87].
- Defects in accessory genes as ***vif*** and ***vpu*** have been shown to be partially accountable for non-progression of disease in some HIV-infected individuals [160].

Specific background to paper I - Antiretroviral therapy

The introduction of antiretroviral combination therapy (HAART) in the mid of 1990, led to a dramatic change in the morbidity and mortality of treated HIV-1 infected individuals [179], Figure 3. Today, HIV-infected individuals, starting HAART with a CD4+ T cell count above 200 x 10^6/l, have been reported to have mortality rates comparable to other chronic diseases [115].

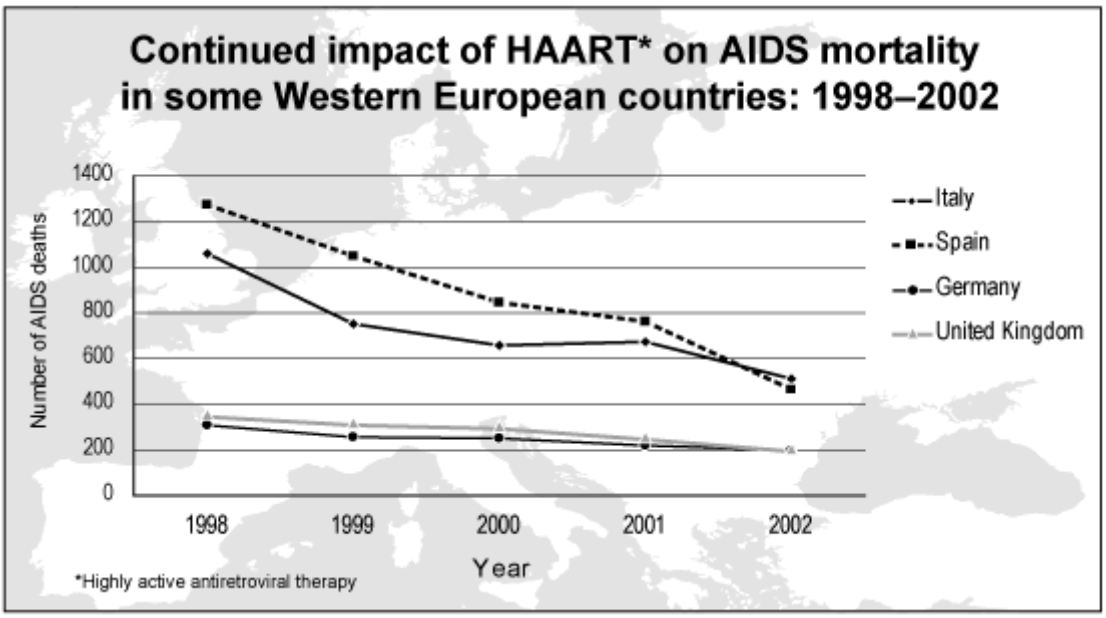

Figure 3 Source: HIV/AIDS surveillance In Europe (2002), WHO. End-of-year report. Data compiled by the European Centre for the Epidemiological Monitoring of AIDS.

> ***A cohort of 202 advanced HIV-1 infected patients started HAART in 1996 at Venhälsan (I)*** (previously unpublished data). At the start of HAART the median duration of their HIV infection was 82 months [range $_{5-95\ perc.}$ 8 - 141] and 72% were previously treated with nucleoside analogs. One third of the patients had had an earlier AIDS-defining event and 44% were MT-2 positive. CD4 T cell count was 190 [range $_{5-95\ perc.}$ 6 - 479] and log HIV RNA 4.83 [range $_{5-95\ perc.}$ 2.70 – 5.95] at the start of HAART.
> Mortality during the first year of treatment was 0% and during the following years 5%, 2%, 1.5% and 0.5% respectively. Half of the patients were estimated to have an AIDS-related event as the cause of death. Patients who died because of AIDS started HAART at a lower CD4 T cell count than those with other causes of death (p=0.008).

The European Union has a centralized process of drug registration through the European Agency for the Evaluation of Medicinal Products. The aim is to harmonize access to new drugs throughout Europe. Currently approved antiretroviral agents and their interaction in the viral replication cycle are presented in Table 2. Chemokine analogs which interfere with attachment and integrase inhibitors, which interfere with the integration of the viral genome, are also being developed.

Table 2
Antiretroviral agents (approved in EU)

Class	Generic name	Abbreviation	Trade name	Company
NRTI	abacavir	ABC	Ziagen	GlaxoSmithKline
	didanosine	ddI	Videx	Bristol-Myers Squibb
	emtricitabin	FTC	Emtriva	Swedish Orphan
	lamivudine	3TC	Epivir	GlaxoSmithKline
	stavudine	d4T	Zerit	Bristol-Myers Squibb
	tenofovir	TDF	Viread	Swedish Orphan
	zalcitabine	ddC	Hivid	Roche
	zidovudine	AZT, ZDV	Retrovir	GlaxoSmithKline
NNRTI	efavirenz	EFV	Stocrin	Merck Sharp & Dohme
	nevirapine	NVP	Viramune	Boehringer Ingelheim
PI	amprenavir	APV	Agenerase	GlaxoSmithKline
	atazanavir	ATA	Reyataz	Bristol-Myers Squibb
	fosamprenavir	fAPV	Telzir	GlaxoSmithKline
	indinavir	IDV	Crixivan	Merck Sharp & Dohme
	lopinavir + ritonavir	LPV	Kaletra	Abbott
	nelfinavir	NFV	Viracept	Roche
	ritonavir	RTV	Norvir	Abbott
	saquinavir	SQV	Fortovase, Invirase	Roche
FI	enfuvirtide	T-20	Fuzeon	Roche

NRTI (nucleoside/tide reverse transcriptase inhibitors) inhibit the reverse transcriptase (RT) by competing with building elements of the growing DNA chain for binding to the RT and causing chain termination.
NNRTI (non- nucleoside reverse transcriptase inhibitors) bind to the RT and make it less flexible and thereby blocking the building of the DNA chain.
PI (protease inhibitors) bind to the protease enzyme and block the cleavage of viral protein precursors (gag and gag-pol polyproteins).
FI (fusion inhibitor) interfere with the fusion or binding of HIV particles to host cells.

The currently recommended combination of therapies consist primarily of a backbone of two NRTI, complemented by either one NNRTI or one PI (boosted PI, i.e. use of low-dose ritonavir to enhance blood levels of other PI) but drugs from all three groups can be used simultaneously if these combinations do not have the intended effect. However, the potency of only triple NRTI as a first-line regime in patients with high viral load has been questioned and this combination is presently recommended only as a treatment simplification in patients with fully suppressed viral replication and a low risk of resistance to thymidine analogue RT inhibitors, for example patients without previous mono or dual therapy with NRTI [60]. Keeping further therapeutic options open is of vital importance when choosing first-line therapy. A history of exposure to resistant virus or the result of resistance testing showing key mutations should be taken into account.

Cohort of 202 advanced HIV-1 infected patients started HAART in 1996 at Venhälsan (II)
In the cohort 55% had a switch or the addition of new NRTI at the start of HAART. 70% started with AZT+3TC+IDV as first-line HAART and another 17% started with RTV or IDV as protease inhibitors in combination with AZT and/or 3TC and/or DDI. Stavudin (d4T) was used as first line NRTI in 5% of the patients. At follow-up visit after 60 months (n=175) 13% of the patients were on treatment interruption. Of patients still on HAART at follow up, 39 % had a combination that did not include protease inhibitors. The most frequently used PI was NFV (27 %), followed by RTV boosted IDV (11 %) or LPV (9 %). 52% had more than one switch in the PI and/or NNRTI added to the NRTI backbone (a median 2 switches per patient during 60 months of follow up [range $_{5\&\ 95}$ 0 – 5]).

In chronic HIV-1 infection there is a general consensus that treatment should be initiated if HIV-related clinical signs and symptoms have occured [60, 208]. In the absence of HIV-related conditions the main criterion for commencing therapy is the CD4+ T cell level. Treatment should be considered if CD4+ T cell counts are between 200 and 350 x 10^6/l The decision to start therapy in this CD4+ T cell range should be influence by factors like the rate of the CD4+ T cell decline and the plasma HIV RNA load along with the patient's willingness to begin treatment. If CD4+ T cell count is <200 x 10^6/l treatment should always be offered. A patient is considered to be in the chronic phase when infection is diagnosed > 6 months after risk exposure (or undefined duration). Treatment should can also be considered for patients with an acute primary HIV infection as early treatment theoretically may delay disease progression and preserve the cellular immune effector T cells as well as anti-HIV humoral immune responses against HIV [157, 200]; so far only one clinical study has suggested that short-term HAART during primary infection can alter future disease progression (discussed in a recently published review by Smith et al [219]). The Swedish consensus group [208]only advocate treatment of acute infection within clinical studies or close consultation with specialist.

Today many doctors wait longer before introducing antiretroviral medication. This has to do with the possibility that adverse events related to medication (see below) are only partly reversible [59]. However, a low nadir CD4+ T lymphocyte count, besides being associated with a greater risk of opportunistic complications [165], carries a greater risk of virologic failure [57] and, as discussed below (paper II), might cause irreversible damage to the immune system.

The effect of medication is usually monitored by measuring HIV-1 RNA levels (= the number of virions HIV-1 in plasma) and CD4+ T lymphocyte counts in blood. The main goal is to depress viral load to very low levels (< 50 copies/ml blood). It is, however, not unusual for patients who are doing well on antiretroviral therapy to experience occasional "blips" of transient viremia with subsequent resuppression [164].

The initiation of HAART is normally followed by a decreased viral load. A three-phase decrease in HIV-1 RNA levels has been described [183];

- ***a first phase*** with a 99% decrease in viremia (reflecting the rapid inhibition of active viral replication in CD4+ T lymphocytes and death of virus producing cells);
- ***a second phase*** with a slower decrease (reflecting viral inhibition and elimination of infected cells with longer life i.e. HIV infected macrophages);
- ***a third phase*** with a very slow decay rate (reflecting eradication of long-lived memory CD4+ T lymphocytes that harbor latent HIV provirus) [181]. In this phase, the half-life of infected cells is estimated to be as long as 44 months or longer.

Eradication of HIV is not yet feasible, as best demonstrated by the rapid viral rebound that occurs if treatment is interrupted. As mentioned previously, reservoirs of latently infected cells are established early after infection and integrated HIV-1 DNA is not accessible to the currently available antiviral drugs. There is also the phenomenon of compartmentalization: not all drugs have access to every part of the body, e.g. the central nervous system. Even if medication does reach all compartments, the concentration of certain drugs may be suboptimal so that, in a cellular perspective, mono-therapy might be common. The NRTIs zidovudine, stavudin and abacavir penetrate fairly well into the CSF as well as the NNRTI nevirapine [90]. CSF concentrations above IC50 levels are reached by the PI indinavir (IDV), but not by ritonavir, saquinavir, or nelfinavir. However, measurements of CSF drug

concentrations should be interpreted with caution as CSF levels of antiretroviral drugs do not usually reflect drug levels within the brain parenchyma accurately.

Cohort of 202 advanced HIV-1 infected patients started HAART in 1996 at Venhälsan (III)
In the cohort, with log HIV RNA 4.83 [range $_{5-95\ perc.}$ 2.70 – 5.95] at the start of HAART, **74 %*** of patients still on HAART reached undetectable HIV RNA levels during the first year and thereafter **78 %***, **85%*** (73%**), **85%** (77%**), and **88%*** (75**) during the following years.
(* < 500 copies/ml, ** < 50 copies/ml)

The proposed mechanisms that allow CD4+ T cell recovery during HAART are redistribution of memory CD4+ T cells, regeneration of memory CD4+ T cells from the thymus and reduction of immune activation (reviewed [36]). However the quantitative and qualitative effects and the timing of the recovery of immune cells differ, depending on the state of the HIV-1 infection. Within weeks after the initiation of HAART, significant increases occur in the numbers of B cells, CD4+ and CD8+ T lymphocytes in blood. These initial increases are due to lymphocyte redistribution, not proliferation [178], as demonstrated by a decrease in proliferation markers during early infection [206]. A large number of B and T lymphocytes may be trapped in peripheral sites during active viral replication and the degree of trapping tends to increase as disease progresses [197]. This could explain why the initial response to HAART seems to be proportionally greater in individuals with lower CD4+ T lymphocyte counts. However, the number of naive T lymphocytes increases slowly after initiation of HAART in chronically infected persons [10, 178], as demonstrated in paper I., but earlier if treatment is introduced at primary infection [35]. As also described in paper I, individuals treated with HAART do not achieve the same degree of immune restoration. Factors that predict the magnitude of CD4+ T lymphocyte increase include levels of HIV replication and the rate of CD4+ T lymphocyte decrease before the initiation of HAART. Individuals with higher HIV RNA levels and more acute pre-therapy CD4+ T lymphocyte decline have been reported to have better CD4+ T lymphocyte increases than individuals with lower pretreatment HIV RNA levels and more subtle CD4+ T lymphocyte declines [50, 193]. The pre-existence of multi-resistant viruses is also of importance for the effect of HAART as all drugs in the combination may not have the intended antiviral effect.

Cohort of 202 advanced HIV-1 infected patients started HAART in 1996 at Venhälsan (IV)
CD4 T cell increases during the five-years follow up of HAART treated patients are visualized in figure 4. Approximately 50% of the total CD4 T cell gain was registered after 12 months. We did not find any correlation between CD4 T cell slope before and after the initiation of HAART but high HIV RNA levels correlated with a step CD4 T cell increase during HAART ($p<0.005$). However, these figures might have been influenced by the fact that 72% of the patients were treated with one or two nucleoside analogues when HAART was initiated and this may have tended to modify pre-HAART CD4 T cell levels but obviously to a lesser degree HIV RNA levels.

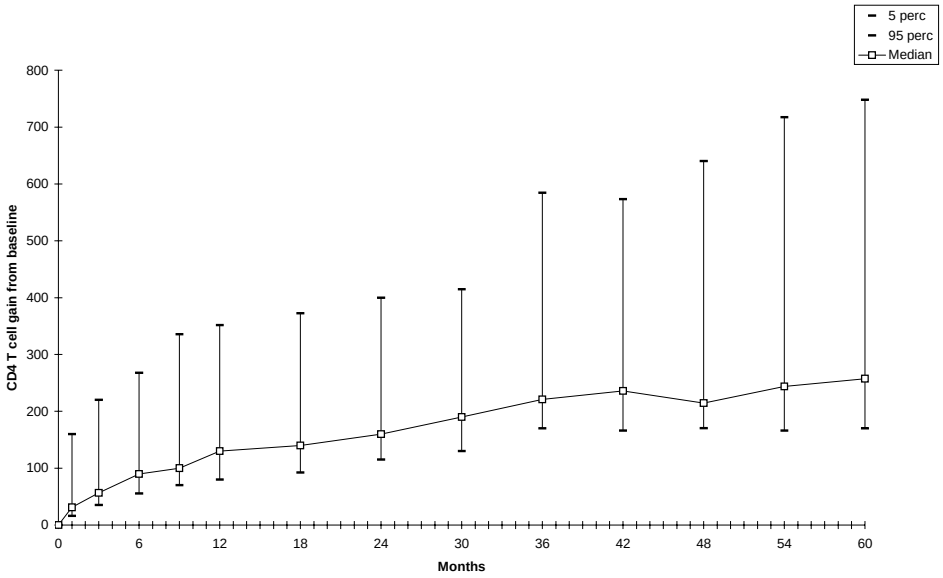

Figure 4. CD4+ T cell gain from the start of HAART in 202 patients followed during 5 years. Only data from patients still in the study are visualized at each time point.

A long-lasting recovery of CD4+ T lymphocyte function during HAART has been shown, even in advanced HIV-1 disease, to antigens to which the host is highly exposed (i.e. Cytomegalovirus and M. tuberculosis) [141] but previously not to antigens with less frequent exposure (i.e. tetanus). This is further discussed in paper III where significant increases in recall responses to tetanus were seen in non immunized HAART treated patients and in paper IV where 3 of 10 non immunized patients had a significant increase during HAART. HIV-specific CD4+ T lymphocytes lost early during infection are not restored in chronically infected patients on successful HAART [202]. Treatment with HAART during or shortly after acquisition of HIV infection permits a greater restoration of immune function, including a preservation of HIV-specific CD4+ T lymphocyte responses [133, 154, 200]. Even though some viral replication persists during HAART, compartmentalization of immune cells in lymphoid tissue, limits appropriate stimulation of T cells [43].

A tendency towards normalization of virus induced elevated activated CD4+ and CD8+ T lymphocytes (HLA DR+) following HAART has been observed in both early [20] and late [10] initiation of treatment. Long-term HAART may be required to fully normalize immune activation, since some activation markers, e.g. soluble Fas (sFAS), show just a minimal decrease after one year of treatment [54]. Persistent T cell activation during HAART is associated with decreased CD4 T cell gains [109].

The need for life-long treatment has to be weighed against the long-term risks of therapy. Replacement of one drug by another is recommended if an adverse event can reasonably be attributed to the former. Nucleoside analogues are associated with hyperlactatemia due to mitochondrial dysfunction [245]. The etiology of metabolic disturbances, like peripheral fat wasting, central fat accumulation, hyperlipidemia, decreased insulin sensitivity [39, 163] and osteoporosis [33], is probably more complex. The roles of different classes of drugs are not well established but probably NRTI, NNRTI and PI are involved. There is also an individual predisposition. The increased risk of coronary heart disease [65, 77, 123] underscores the importance of reducing other cardiovascular risk factors as overweight, smoking, high blood pressure etc. as continued treatment of HIV is often more urgent.

Cohort of 202 advanced HIV-1 infected patients started HAART in 1996 at Venhälsan (V)
Of the patients with a follow up visit month 60 (n=175) 90% had a history of changes in PI and/or NNRTI added to the NRTI backbone during the observed period. Approximately half of the patients had to alter their medication because of side-effects and the remaining half because of viral failure. In total 28 % of all patients interrupted treatment during a five year follow-up mostly due to side effects; 80% of these had only one period of treatment interruption. Treatment interruption was less frequent during the first year (5%) but was distributed uniformly thereafter, with 10-12% of the patients yearly.

These side-effects have drastically altered the clinical perspective, from an initially cheerful optimism related to the dramatic HAART-related change in morbidity and mortality, to a more balanced view where effective HAART is not the only important goal when dealing with HIV-1 infected patients.

Paper I - Immune reconstitution during HAART

The aim was to study the development of the absolute numbers and relative proportions of CD4+ and CD8+ T lymphocyte subsets, reflecting maturation and activation, in relation to the viral response to protease inhibitor based HAART.
The studied individuals (n=42) belonged to a group of patients with advanced HIV infection and a very low CD4+ T lymphocyte count at the start of HAART. A majority had experienced an earlier AIDS-related event, high viral baseline levels and CD4 T lymphocyte counts below 100 x 10^6/l. All but one were nucleoside analogue experienced.
After two years with HAART the viral load had decreased by less than one log (median value) from baseline in half of the studied patients (defined as viral low responders, vLR) and to below the detection limit in the remaining half (viral responders, vR).
No differences in HAART efficacy in relation to baseline viral load, CD4+ T cell count and the number of new nucleoside analogues at the start of HAART were observed in our study, possibly because all these patients were in an advanced stage of the disease.
After seven years (unpublished data) we saw clinical differences between vR and vLR. Twelve of 19 vLR patients are still alive (six have died and one is lost to follow up) whereas 22 of the 23 vR patients are still alive (one is lost to follow up), p=0.015. All but one patient are still on HAART. For the patients who are still followed at the clinic, the CD4 T lymphocyte count has reached 518 x 10^6/l (18%) in the original vLR group and 443 x 10^6/l (22%) in the vR group, p=ns. The viral load is below 50 copies / ml in 10 of the 12 original vLR patients and in 18 of 22 vR patients.
Whether immunologic improvement in the vLR group is related to the generation of multi-resistant, less fit viral particles, switch of phenotype or other less-well studied direct effects of HAART (e.g. decreased activation and/or apoptosis) was not studied in our work. Other authors have described a reduced replicative capacity in virus with primary genotypic mutations within the protease gene (particularly V82A, I84V, L90M and D30N) and the reverse transcriptase mutation M184I/V [12, 58].
We conclude that despite the presence of reduced drug susceptibility, antiretroviral drug therapy can provide immunologic and virologic benefits in patients in whom drug therapy fails to completely suppress HIV. These benefits from HAART reflect continued antiviral-drug activity, probably as a result of reduced replicative capacity of mutated virus as pointed out by Lisziewich [145] and later by Hicks [101]. This may contribute to prolonged control of the HIV-infection, potentially adding more years of survival. Benefits of suboptimal antiretroviral regimen would probably not be sustained indefinitely. There is a risk that the

effect of the new drugs will be negated by the accumulation of resistant mutations. Continued treatment pending a novel, more potent, drug combination, however, resulted in ultimate virological success in 10 of the original 19 vLR patients.

We found that the different outcome in viral low responders compared with viral responders might be explained by differences in adherence to HAART. While adherence is one of the main issues behind virological failure [74, 78, 186], the occurrence of resistance, poor drug absorption, altered distribution, excretion and metabolism and the development of cellular factors might impair the efficacy of the antiviral drugs at the site of action [233]; factors that can accumulate sequentially. Mutations are rarely seen when a drug concentration is constantly high since the selective process is then retarded. Mutated virus may exist before the initiation of HAART; previous exposures to only one or two nucleoside analogues or transmission of drug-resistant virus [148] may lead to suboptimal virologic response to HAART. Suboptimal drug concentration, high viral production combined with a high error rate of HIV-1 RT are all factors that result in selective pressure, with an increased probability of the virus accumulating resistant mutations. Resistance can be determined either genotypically or phenotypically and such analysis is recommended at the start of HAART [225] and in all patients failing therapy [60]. If resistance mutations are found, three options are available; 1) to change only the drug(s) for which resistance is documented 2) to change the whole regimen (if minor resistance, not detected, is suspected) 3) stop all therapy (see section Treatment interruption) or 4) continue failing therapy (if no safe options left).

In a period when growing problems with drug resistance and drug toxicity, as well as rising costs, are liable to render an optimal drug regime unfeasible, the knowledge that continued medical intervention is often meaningful in spite of viral failure might help our patients to survive at least until new drugs are available. Many doctors who treated HIV patients before the era of HAART have observed that several patients survived in good health for many years with very low CD4+ T lymphocyte counts. Moreover, the positive effects that HAART induces on health are often seen before the increase in CD4+ T lymphocyte count becomes measurable. These observations indicate that we probably have mechanisms that, at least in part, compensate for missing functions of CD4+ T lymphocytes. Perhaps innate immunity, a genetically very much older system, contributes to this compensation. Certainly the immune system has an enormous “overcapacity” whereby an extensive functional loss can occur before negative effects on health are apparent.

Specific background to paper II - Treatment interruption

Interruption of medication has been studied in relation to three clinical scenarios: acute and chronic drug-suppressed infection and virologic drug failure [83, 146, 175, 189, 226]). Treatment interruption can be performed in an intermittent way (on-and-off cycles of HAART, so called *Structured treatment interruptions* (STI)), for specified periods or as long as possible *Long term supervised treatment interruption* (LTI).

Treatment interruption in the form of STI has been seen as a way of boosting immunity to HIV. This notion originated in an anecdotal report of the “Berlin patient” who was able to control HIV viral replication for more than four years after two cycles of on-and-off therapy [147]. Analyses of his blood revealed strong HIV-specific CD4+ T lymphocyte responses as well as CTLs but no HIV-neutralizing antibodies. This observation was followed by a study where increases in the quantity and quality of CD4+ T lymphocyte responses were observed in eight patients where treatment during acute infection was followed by repeated STIs [200]. In the acute infection it thus appears successful to do repeated STIs. The connection between

enhanced CTL induced by STI and increased control of viral rebound was confirmed in a primate model using simian immunodeficiency virus (SIV) [150].
Among patients who start HAART during the chronic phase of HIV-1 infection, the HIV-specific T cell responses mobilized during treatment interruption are only transient [37]. The CD4+ T lymphocyte count decreases and the viral load returns to the set-point that existed before the initiation of therapy. In the Swiss-Spanish Study [64], the largest ongoing prospective clinical trial of STI in chronically infected patients, no improvement was observed in viral load rebound during repeated therapy interruptions (2 weeks off and 8 weeks on HAART). Other investigators have found that some chronically infected patients may be able to reconstitute the immune system during STI and even improve viral control [182, 204, 205] but the majority does not.
The development of drug resistance, repopulation of cellular virus reservoirs and possible acute retroviral syndrome may be considered as draw-backs during short-term STI but none of the referred studies reported any of these obstacles.

The rationale of LTI is to limit and even reverse adverse effects (i.e. serum lipids, body fat changes and other metabolic disturbances such as insulin resistance and factors associated with an increased risk of cardiac complications) and thereby improve quality of life and improve long-term compliance. Pill fatigue has also become a major argument for initiating a drug holiday. In the majority of patients there is a rapid return of virus replication combined with a continuous fall in CD4+ T cells [166]. An increased incidence of HIV-related events and only a limited effect on drug-induced body fat changes have raised doubts about the safety and rationale of LTI.

Parameters that have been reported to predict the response to treatment interruption are nadir CD4+ T cell count, pre-HAART CD4+ T cell loss, baseline levels of memory CD4+ T cells and pre-HAART viral load [62, 82, 153, 189]. Differences in baseline characteristics and end-point values make it difficult to compare the conclusions made by different authors.

Paper II - Immune deterioration during interruption of HAART

The aim was to identify indicators that may predict the outcome of treatment interruption as a guideline for clinicians and as a preparation for further studies with immune-based therapeutic vaccines preceding LTI.
The studied individuals were 27 HIV-1 infected patients with a history of long term HAART, well suppressed plasma viral loads and restored CD4+ T cell counts. The patients were subdivided retrospectively into two groups based on the duration of the treatment interruption. Ten patients had still not restarted HAART when the study data base was frozen. HAART restart was initially planned when CD4+ T cell counts had decreased to less than 50 % of baseline levels in two consecutive samples. However, as several patients wished to continue their LTI for as long as possible, this endpoint criterion was not always followed.
A steep downward CD4 T cell slope during LTI correlated with 1) steep CD4+ T cell increase during HAART; illustrated in Figure 5, 2) relatively high CD4+ and CD8+ T cell counts at baseline, 3) relatively high absolute levels and/or percentages and/or proportions of CD4+ and CD8+ naive T cells at baseline, 4) relatively low proportions of CD4+ memory T cells and low percentages and/or low proportions of CD8+ memory T cells at baseline, and 5) high viral load during LTI.

High viral replication during LTI correlated with 1) low nadir CD4+ T cell counts and percentages, 2) relatively high proportions of CD4+ naive T cells at baseline, and 3) relatively low percentages of CD4+ memory T cells at baseline.

A short total period of treatment interruption was associated with 1) previous AIDS events, 2) long period with HAART, 3) low nadir CD4+ T cell counts and percentages, 4) relatively low absolute levels and/or percentages and/or proportions of CD4+ memory T cells at baseline, 5) relatively high percentages of CD8+ naive T cells at baseline, and 6) high viral load during LTI.

We conclude that before considering LTI in a patient performing well on HAART, the physician should study the CD4+ T cells levels before HAART and CD4+ T cell increase during HAART, rather than the current CD4+ T cells value. If previous CD4+ T cell counts are not available, analysis of CD4+ and CD8+ naive and memory T cell levels, may serve as a guide to the outcome of LTI. These parameters should also be considered before evaluating LTI after immunological interventions such as IL-2 treatment or therapeutic vaccines, as they may influence the outcome of such studies and thus conceal the potential benefits of immunotherapy. Our data also underline the importance of starting (and restarting) HAART before the CD4+ T cell count declines below recommended levels.

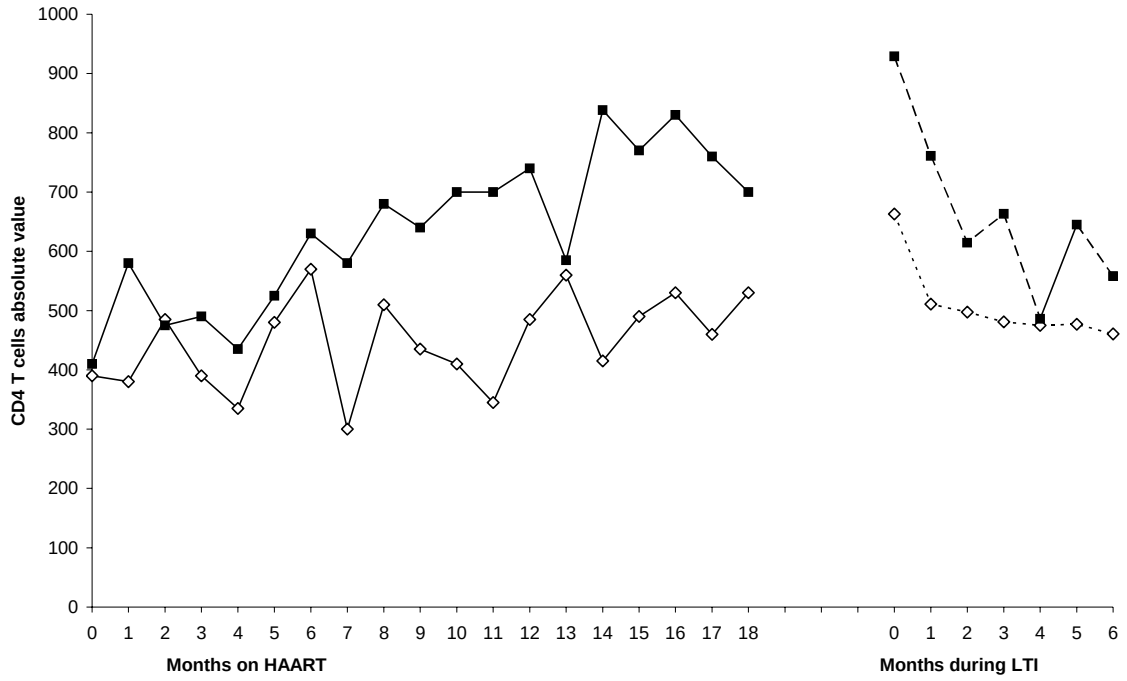

Figure 5. CD4+ T cell increase during the 15 first months on HAART (full line) and CD4+ T cell decrease during the first 6 months of LTI (dotted line) in patients with CD4 T cell slopes during HAART over median value (-■-) and equal or below median value (-□-) during HAART.

All patients in this study have CD4 T cell counts within what are considered to be "normal values" at baseline (= the start of LTI). Median values for CD4+ memory cells are above the "normal range" in patients with long LTI, a moderate CD4+ T cell decrease during LTI and a viral load in the lower region after treatment interruption. Concerning CD4+ naive cells, most patients have values within the "normal range" but for those with a moderate CD4+ T cell decrease the values are at the lower limit of this range. This raises questions about what should be considered as "normal values" or perhaps "favorable values" during treatment and recovery of a chronic infection like HIV. All but one patient with treatment interruption

longer than 24 months had a CD4+ memory count > 330 x 10^6/l at baseline and no patient in this group had < 60% in the proportional level of these cells.
The study also raises questions about the quality and function of those CD4 T cells we interpret as positive effects of HAART. In an extended analysis of the same patients, we found a positive correlation between a steep CD4+ T cell increase during HAART and relatively high CD4+ naive T cell count at the start of LTI but no similar correlation with CD4+ memory cells. As the level of memory cells at baseline correlates with nadir CD4+ T cell count, it might be asked whether immunological losses incurred before HAART might ever be restored, even after several years of "successful" HAART. However, we end up with the clinical observation that once the CD4+ T cell count has risen above 200 x 10^6/l, the risk of HIV-related complications decreases and we can often withdraw prophylactic treatment for different types of opportunistic infections. This ability to withstand secondary infections might be a result of an enormous overcapacity in the immune system, with several compensatory mechanisms involved.

Specific background to paper III, IV and V - Active immunotherapy

a) Rgp160 vaccines

The development of therapeutic vaccines against HIV-1 is important, not only with a view to enhancing the immunity of already infected persons, but also as a step in the evolution of prophylactic vaccines. Observations obtained during prophylactic vaccine development might also be valuable for future treatment of already infected individuals.

The first large-scale HIV vaccine with recombinant envelope protein for therapeutic use was based on recombinant gp120 [63]. This phase II study was discontinued prematurely when no beneficial effects were noted on several clinical parameters. Gp120 has five variable regions (V1 – V5). The V3 loop is the principal domain responsible for most immunological responses in vivo, involved in both CD4 ligand interaction and in coreceptor binding [45, 48]. Concerns that the gp120 might have immunosuppressive effects by binding to the CD4 receptor and thereby suppressing antigen-specific activation [44, 177], prompted an examination of the immunologic properties of gp160. Three international phase II – II/III studies performed with recombinant gp160 before the era of HAART demonstrated that the immunogen is persistently immunogenic, significantly increasing the ability of HIV-1 infected persons to mount new proliferative HIV-specific T lymphocyte responses [19, 188, 191, 232]. These comparatively small studies did not show clinical benefits. Inspired by the positive effects on T cell proliferation and the results of another rpg160 study, (Vac 02, performed at Venhälsan, Stockholm Söder Hospital), where the induction of rgp160-specific responses correlated with good prognosis and an additional capability to respond to other antigens [131], a randomized multi-center double blind placebo-controlled trial (Vac04) with 835 HIV-1 infected individuals was performed with the intention to further establish possible clinical benefit of rgp160 [209]. The outcome of this study was that significantly fewer vaccine-group patients than placebo-group patients reached the primary immunological endpoint of a decrease of more than 30% from baseline CD4+ T lymphocyte count. A larger proportion of the vaccine patients had CD4+ T lymphocyte counts above baseline at six months. HIV-1-specific T cell immune reactivity was induced in all vaccine recipients studied. Significantly fewer deaths were observed among the vaccine recipients compared with the placebo patients at two years, but not at the end of the study at three years. At this time, HAART was not available, and the increased two-year survival could be associated to the repeated vaccination. One explanation for the additive immunogenicity of rgp160 is that

rgp160 of HIV-1_{LAI} differs from the prevailing native HIV-1 envelops in that it lacks CD4 binding [237].
A possible limitation of envelope subunit vaccines is that they fail to induce cross-neutralizing antibodies against primary HIV-isolates. However, it has been demonstrated that strong T-helper cell responses in asymptomatic HIV-1 carriers infected with different subtypes (B – G) can be induced by immunization with a rgp160 immunogen derived from a virus of genetic subtype B [132]. This might indicate that a particular immunogen can be produced with the potential to be effective against many different HIV strains. How effective these immune responses are remains do be investigated.

Results from a phase II trial of another therapeutic vaccine candidate, REMUNE, were published recently [194]. The vaccine, a gp120 depleted, whole inactivated virus preparation, was administered to HIV-1 infected patients on ART (antiretroviral therapy) with CD4+ T cell counts > 250 x 10^6/l and HIV RNA < 500 copies/ml. The HIV-1 specific immune responses that were augmented were confined to those contained in the immunogen, with no induction or responses to envelope protein (removed during the preparation of the antigen). A transient reduction in viral load during treatment interruption has been demonstrated in a small group of patients immunized with REMUNE [71].

Table 3
Studies with gp160 in HIV-1 infected persons performed in collaboration between Venhälsan, Stockholm Söder Hospital & Swedish Institute for Infectious Disease Control (SMI).

Year	Study	Substances	Administration	No of patients
1990	**Vac 01**	gp 160 + intermittent AZT/placebo	i m inj	40
1992 - 1994	**Vac 02**	gp 160 + best possible antiviral therapy	20 pat. i m inj every 2 months 20 pat. i m inj every 6 months	40 (from Vac 01)
1994 - 1999	**Vac 02 (continued)**	gp 160 + best possible antiviral therapy	i m inj every 2 months	40
1992 - 1999	**Vac 03**	gp 160 + best possible antiviral therapy	i m inj every 3 months in a compassionate protocol	13
1993 - 1996	**Vac 04 Nordic Study**	gp 160 / placebo + best possible antiviral therapy	i m inj / double blind	835
1994 - 1996	**Vac 04 endpoint**	gp 160 / placebo + best possible antiviral therapy	i m inj double blind	
1994 - 1996	**Immuno**	gp 160 + best possible antiviral therapy	i m inj	12
1998 - 2000	**Vac 05**	gp 160 + HAART tetanus + HAART only HAART tetanus (healthy controls)	i m inj i m inj i m inj	10 10 10 10

b) HIV-1 DNA vaccines

It is likely that for intracellular organisms such as malaria, tuberculosis and HIV, both cellular and humoral immune responses are required for control of an established disease as well as for protection against infection. Only vaccines derived from replicating organisms induce cellular immunity efficiently. From a safety standpoint, live or attenuated vaccines raise several issues, especially for a virus like HIV with an enormous potential to mutate back to the wild type. DNA vaccines, containing genes for the antigens of interest, are under intense investigation because of their ability to mimic the effects of live attenuated vaccines and induce both humoral and cellular immune responses. DNA vaccines can be relatively easy redesigned to address the numerous HIV subtypes, recombinants, immune escape variants and drug-resistant virus populations, if necessary. The vaccine can also be manufactured in a relatively cost-effective manner and stored with relative ease, eliminating the need for a cold chain.

DNA vaccines consist of the foreign gene of interest cloned into a bacterial plasmid. The plasmid includes an origin of replication (allowing for growth in bacteria), a strong promoter for optimal expression in mammalian cells (e.g. CMV), sequences for stabilization of mRNA transcripts and often a bacterial antibiotic resistance gene (for plasmid selection during bacterial culture),. The DNA is taken up by host cells and travels to the nucleus, where it is expressed using the host machinery. The induced response has been reported to last up to one year after immunization in mice [228].

At least three mechanisms by which DNA plasmids are processed and presented to elicit an immune response have been described [95]:

- direct transfection of professional antigen-presenting cells,
- antigen secretion by somatic cells (myocytes, keratinocytes or others),
- transfer of protein produced by transfected somatic cells and taken up by professional antigen presenting cells leading to T lymphocyte activation (cross-priming).

The expressed protein will have the same naive conformation and other characteristics as during natural infection of the host cell and the antigen produced is presented by both MHC class I and II molecules. The DNA might also activate innate immunity by CpG motifs (see also section *Augmentation of vaccine-induced immune responses)*. Thereby all types of immune responses are seen, both innate and adaptive. The exact nature of the induced immune response depends on several factors:

- the design of the DNA construct resulting in an enhanced MHC class I presentation or a MHC II processing,
- the use of naked DNA or DNA cloned into a live vector (se below),
- the route of administration,
- the timing of the immunization,
- the use of adjuvants or other immunomodulatory substances (e.g. cytokines or chemokines)

A variety of routes have been proposed for DNA injection; those that have been studied most are intramuscular, subcutaneous and intradermal. From 10 to 100 μg of plasmid DNA is required to elicit responses in mice but higher doses may be necessary in humans depending on the antigen given. In a study with DNA vaccine encoding a malaria antigen, doses of plasmid DNA in the 500 to 2500 mg range were given [243]. By contrast, DNA immunization by gene-gun often requires only 0.1 – 1 μg of plasmid DNA to induce antibody or CTL responses. Gene-gun technology uses a gas-driven bombardment device that propels gold particles coated with plasmid DNA directly into the skin or muscle [246] and leads to direct

transfection of antigen presenting cells. Transfection of Langerhans cells in the skin may result in enough antigens to trigger an antibody response while processing in muscle cells preferentially results in a cellular response. Alternatively, studies in mice have demonstrated that intranasal administration of HIV-1 gag DNA adsorbed onto cationic polylactide coglycolide micro particles (PGL-DNA) can induce prolonged expression of gag protein in local and systemic lymphoid tissues [218]. Also intra-gastric vaccination with a Salmonella env DNA vaccine vector has the capacity to induce env-specific CD8+ T lymphocytes, in both mucosal and systemic lymphoid tissue [215]. In humans mucosal administration of DNA can induce local cell-mediated responses [151].

A problem in the development of therapeutic HIV vaccines is that different CTL epitopes appear to be recognized during different phases of infection. Acutely infected patients recognize diverse epitopes, while chronically infected persons mainly recognize CTL epitopes of gag, but with a strong negative association between the magnitude of the response and viral load in progressive infection [92]. The ease with which virus escapes CTL response is illustrated by vaccine failure if the vaccine sequence varies from the virus strain by as little as a few amino acids [14]. One strategy to avoid CTL escape is to provide many conserved epitopes from several subtypes or recombinant strains in the same vaccine.

Several genes in the HIV-1 genome are of interest for the development of DNA plasmid vaccines, for both prophylactic and therapeutic purposes:

Env

Partial protection from challenge has been demonstrated in primates after vaccination with env DNA constructs [190]. Partial protection is defined here as low virus titers in infected animals or a prolonged period to development of AIDS after challenge of immunized animals. One problem with the ***env*** gene is the large variability, especially in the five hypervariable regions of the gp120 subunit, an obstacle which may be overcome by plasmids containing env DNA from several different subtypes. Immunizations with DNA with env and rev genes have resulted in a lowered viral load in chimpanzees [26].

Gag

As mentioned earlier, gag-specific CTL responses have been inversely associated with viral load in chronic HIV-1 infection [172]. Gag products, such as p24 and p17, are probably the best CTL-inducing peptides [102].

Pol

Approximately 80% of HIV-1 infected patients have CTL recognition of HIV-1 pol products (RT and/or integrase and/or protease) [96]. Pol products like RT and protease are of specific interest because of the development of drug escape mutations in these genes. CTL targeting domains of RT containing drug-induced mutations could be used to put additional pressure on the virus, acting in synergy with the drug [239]. RT is also likely to stimulate cross-clade immune responses together with p24 [159].

Regulatory genes

CTL against tat and rev are associated with less rapid disease progression to AIDS [235]. In a small pilot study using recombinant SFV (Semliki Forest Virus) as one vector and MVA as another vector, both expressing the same SIVmac 32H ***rev*** and ***tat*** genes, partial protection was elicited against homologous intravenous SIV challenge [176]. Since the tat protein in itself has immunosuppressive effects vaccines with detoxified HIV-1 tat protein (tat toxoid) have been produced in order to avoid the potentially negative effects of a pure tat vaccine or genes expressing these products [79]. It was shown that the tat protein alone partially protect

against SHIV challenge [30]. A high level of nef-specific CTL has been found in a group of Gambian women who remained seronegative for HIV-1 infection despite repeated exposure [203]. Also small accessory proteins like Vpu, Vif and Vpr are essential for viral replication and of interest as targets in vaccine development.

A few DNA vaccines have been tested for safety and immunogenicity in humans. Ongoing trials have shown that immune responses are obtained without evident adverse effects. A problem with therapeutic vaccines in HIV infected patients is that the recipients have functional deficits in their immune systems. Treatment with HAART has led to an overall improvement in the immune system but the specific immune responses to HIV appear to remain low [131, 187]. A summary of studies with HIV-1 DNA plasmid performed at Venhälsan, Stockholm Söder Hospital and the Swedish Institute for Infectious Diseases Control is presented in table 4.

Table 4
Studies with HIV-1 DNA plasmids in HIV-1 infected patients performed in collaboration between Venhälsan, Stockholm Söder Hospital & Swedish Institute for Infectious Disease Control (SMI).

Year	Study	Substances	Administration	No of patients
1996	Plasmid (Trial A)	DNA (*rev* or *tat* or *nef*)	i m inj (3 injections) (300 mikrogram)	9 (from Vac 02)
1998	Plasmid	DNA (*rev* & *tat* & *nef*)	i m and booster in oral mucosa (300 mikrogram)	8 (from Plasmid, Trial A)
1999 - 2000	Vac 06 (Trial B)	DNA (*rev* & *tat* & *nef*) placebo	i m inj (100 -> 300 -> 600 mikrogram)	10 5

c) Augmentation of vaccine-induced immune responses

The short-lived memory, even with a continuous use of HAART, might be lengthened by means of a live vector. It is possible to clone the antigen-encoding gene into a live vector such as adenovirus, poxvirus (Canary pox virus, Fowl pox virus, Vaccinia and modified vaccinia ankara, MVA), or alpha virus (Venezuelan equine encephalitis virus, Sindbis virus, and Semliki forest virus). The use of boost regimes has also been based on the use of vector viruses as well as on recombinant proteins and peptides.

Most vaccines require the addition of *adjuvants,* i.e. substances that enhance the immunogenicity of antigens and trick the immune system into responding as though there was an active infection. The most commonly used adjuvant in human vaccines contains aluminum salts that preferentially stimulate antibody responses. Other important adjuvants, used in experimental animals, are sterile constituents of bacterial cell walls, heat-shock proteins and also DNA. Unmethylated cytidine-phosphate-guanosine (*CpG*) dinucleotide is a specific sequence motif in bacterial DNA, which might be present in DNA plasmid. Certain CpG motifs directly activate B lymphocytes to proliferate or secrete antibody, induce professional antigen-presenting cells to secrete cytokines, stimulate T lymphocytes and activate natural killer (NK) cells [122]. The addition of CpG motifs in the plasmid or the separate delivery of these sequences (ODN- phosphotiorate oligodinucleotides) enhances the immune response as measured by IFN-γ and IL-4 secretion, IFN-α, IFN-β, and IFN-γ activation and by NK and CTL activities [210]. The CpG motifs stimulate Toll-like receptor 9 activation and have been more successful together with proteins than together with DNA immunogens.
In addition several investigators have used plasmid DNA encoding various cytokine costimulatory molecules to enchance or bias the immune response by DNA vaccination [134].

IL15 has been found to induce a robust and long-lived CD8+ T lymphocyte mediated memory in vaccine trials in mice and man [24, 173]. The use of *GM-CSF* (granulocyte macrophage – colony stimulating factor) (reviewed in [244]) has been shown to enhance both cellular and humoral immune responses by recruiting antigen-presenting cells to the site of immunization [207] as well as innate immunity by mobilizing IPCs from bone marrow to peripheral blood [9].
Recently, the topically applied imidazoquinoline (*imiquimod)*, registered for treatment of external genital warts, was shown to work as an efficient adjuvant for DNA immunization by inducing the synthesis of IFN-α, IL-12 and IFN-γ [121] besides activating Toll-like receptors 7/8.

Paper III – Long-term persistence of immunization

Our aim was to monitor the immune responses in HIV-infected patients previously immunized with gp160 (Vac 04 in table 3) or DNA vaccines (Vac 06 in table 4) to analyze whether the introduction of HAART would affect the persistence of immunity.
The studied individuals were **1)** patients who had participated in randomized trials of therapeutic vaccination with gp 160 or DNA vaccines, **2)** chronically infected patients with long-term HAART, and **3)** 21 patients who were sampled at 1–5 different time points per patient. 8 samples were pre-HAART, 56 samples were from successful HAART (19–70 months) and 5 samples from 11–15 months of treatment interruption.
Evaluation of immune responses revealed that HIV-specific T-helper cell responses, induced by rgp160 vaccination, are maintained at high levels for several years (up to 7 years) after the last vaccine injection. The addition of HAART in these patients did not alter this HIV-specific response but gave a profound reduction in viral load and increased total CD4+ T cell counts. Not only the response to the HIV gp160 immunogen but also the recall response to HIV p24 was improved and maintained at a high level in immunized patients. Direct positive effects of HAART on recall responses towards CMV, measles and tetanus antigens were also seen. Cells with HIV-specific interferon-γ (IFN- γ) production were retained or increased in long-term HAART treated patients. Immunizations with HIV DNA during HAART treatment permitted persistence or development of innate (NK), CD4+ and/or CD8+ immune responses.
We conclude that it is possible to induce strong and very long-term persistent immune responses in HIV-infected individuals, which gives hope that vaccination prior to therapy interruption might be beneficial in HIV-infected patients.

Paper IV – Immunization with rpg160 during HAART

Our aim was to investigate the impact of HAART before and during immunization with rgp 160 and to compare immunogenicity with a non-HIV immunogen (tetanus) as well as with previous, pre-HAART studies with rpg160.

The studied individuals (Vac 05 in table 3) consisted of 30 HIV-1 infected patients on HAART with undetectable viral load and CD4+ T lymphocytes counts above 200 x 10^6/l for at least 6 months before study start. The patients were randomized into three groups: group A, 10 patients receiving HIV-1 rgp160 vaccine; group B, 10 patients only monitored; and group C, 10 patients immunized with tetanus toxoid. A control group, group D, consisted of 10 HIV negative volunteers immunized with tetanus vaccine. The results were compared with a rgp160 vaccine study performed at the same clinic before the era of HAART in patients with comparable CD4+ T cell levels (Vac 01 in table 3).

The vaccine program consisted of six doses (160 µg each) of rgp160 formulated in aluminium phosphate (Vaxsyn, MicroGeneSys, now ProteinSciences) administered i.m. in the left deltoid muscle during 26 weeks (weeks 0, 1, 4, 8, 17 and 26) in group A. The tetanus vaccine was a commercial standard vaccine (SBL Vaccin, Solna, Sweden) with aluminum phosphate as adjuvant. The subjects in groups C and D were immunized with three 0.5 ml doses i.m. in the left deltoid muscle at weeks 0, 8 and 32. All patients were monitored for two years for T-cell proliferative responses, T cell subset levels, serum IgG and viral load**.**

At follow-up we were able to demonstrate a positive effect on CD4+ T cell count measurable six to twelve months after the last immunization. High levels of HIV-specific T cell responses were maintained up to two years in the HAART treated, but not in the non-HAART treated patients despite comparable or even higher CD4+ T cell levels during follow-up.
In addition, the rgp 160 immunization boosted the CD4 specific responses to certain other antigens (tetanus toxoid and tuberculin). No spontaneous increase in the T cell proliferative responses to rgp160 was observed in the HIV-1 infected HAART treated, but non-rgp160 immunized, control groups. This contrasts the increased T cell proliferative responses to influenza and CMV in those groups. Influenza is an antigen to which the immune system is intermittently exposed and increased antigen-specific T cell responses were observed in all HAART treated groups. A decreased T cell proliferative response to influenza was observed in the non-HAART group, despite active immunization with influenza during early follow up. CMV is a latent virus to which most individuals are exposed continuously. Most HAART patients, but not untreated patients, had strong responses to CMV, with a further increase only in the non-immunized HAART treated group. In the case of CMV, it is thus possible to ascribe the recall of T cell reaction to HAART alone. The patients had a poor Multitest reactivity, which did not improve in spite of HAART.

We conclude that immunization with rgp 160 during HAART leads to positive T helper cell responses. This includes CD4+ T cell levels, induction of new HIV-specific responses and recall responses to other antigens *in vitro.* HAART strengthens the magnitude and persistence of such responses. Viral load might have a negative influence on T cell proliferative ability as well as on the actual absolute numbers of T cells, without any direct correlation between these two malfunctions. However, T cell responses *in vivo* (Multitest reactivities) did not improve, perhaps as a result of long-lasting changes in antigen-presenting cell functions in the skin.
The impact of HIV infection is thus not confined to just the absolute levels of T cells but also their function. Immunization with HIV gp160 before interruption of HAART has a potential to prolong antigen-specific T cell proliferation to both specific (gp160) and recall antigens (tetanus).

We found that immune responses induced before HAART was introduced are preserved for up to seven years irrespective of HAART. However, in paper IV, induced immune responses to rgp160 are described as better preserved during a two-year follow up in a group of rpg160 immunized HAART treated patients than in historical controls consisting of immunized individuals with no HAART. The T cell proliferation index (to the antigen given) in the historical control had a high median value of 70 six months after the last vaccine dose was given and just below 4 one year later (table 5). Patients discussed in paper III, also immunized with rgp160 but with no HAART, had a median SI index around 10 during follow up. This indicates that immune responses measured early after immunization (six months) are high, and measurable responses are found even after several years, probably as long as CD4 T cells levels are kept at ‘normal’ levels. This occurred irrespective of HAART. However, if immunization is performed during HAART when viral load is low, as in one group in paper IV, the magnitude of these responses seems to be even stronger and the persistence seems to

be further enhanced. This was best demonstrated by a median SI index of 140 in the two-year of follow up. Data from a three-month follow-up of patients on treatment interruption (paper IV) indicate that these high responses, induced during HAART, might be retained even without medication. Longer follow-ups are, however, necessary in order to study how long these strong responses may be preserved.

Table 5
T-cell proliferation to gp160 (SI index) during follow up in HIV infected patients immunized with rgp160 only and rpg160 + HAART; Median, 25 & 75-percentils.

	6 monhts	18 - 24 months after immunization	up to 7 years
rgp160 (no HAART)	70 [9 - 120]	4 [7 - 70]	10 [1 - 110]
rgp 160 + HAART	110 [50 - 210]	140 [20 - 540]	

Paper V – DNA immunization during HAART

The aim was to evaluate the immunological responses induced by a combination DNA plasmids containing HIV regulatory genes administered to HIV-1 infected patients on HAART.

The study was double-blind, randomized and placebo-controlled and included fifteen asymptomatic HIV-1 infected patients on stable HAART for at least 6 months and with plasma HIV RNA levels below 50 copies/ml. Ten patients received a combination of ***rev, tat*** and ***nef*** i.m. at weeks 0, 4 and 16 at increasing doses, giving totals of 300 (100 x 3) ug, 900 (300 x 3) ug and 1800 (600 x 3) ug DNA. Five patients received saline in the same amounts i.m.

During the follow-up, new T lymphocyte proliferative responses were induced in the vaccine group. Antigen-specific CTL levels were preserved or increased in the vaccine group. Patients in the placebo group showed a decreased specific lysis to both rev and nef separately and also to pooled samples of all three antigens over the study period. New T lymphocyte proliferative responses were also induced in the vaccine group. No increase in antibody levels was noted. Despite a ten-fold higher vaccine dose, patients on HAART did not respond to vaccination better than the non-HAART patients included in a previous study where the genes were administered separately (Figure 6).

We conclude that HAART per se is not important for the immediate response to the chosen HIV genes in patients with comparable CD4+ and CD8+ T cell levels, even if the total DNA dose is ten-fold higher. Despite a ten-fold higher vaccine dose, patients on HAART did not respond better to vaccination than the non-HAART patients in a previous study where the genes were administered separately. Combining the regulatory genes ***rev***, ***tat*** and ***nef*** in increasing doses may reduce the anticipated augmentation of HIV-specific T cell proliferative and CTL responses. Viral suppression did not seem to further improve the initial vaccine responses of patients with comparable CD4 levels.

Since both ***nef*** and ***tat*** have immune suppressive activities, these properties may have been reciprocally more prominent in a combination of the genes in a higher dose. The ***tat*** gene was shown to have suppressive capacity when combined with ***nef*** [120]. One might speculate whether multi-DNA constructs interact with each other, leading to non-optimal immunological responses for components in the vaccine. Another possible explanation is that

responses in some patients were already maximal and a further improvement could not be expected. Perhaps only patients with an initially low response have the potential to respond [31]. A lower frequency of new antigen-specific CTLs in patients who already demonstrated CTL responses has been described in earlier DNA vaccine trials [25, 32].
Although no comparisons were made with patients immunized with HIV DNA without HAART, we found that immune responses, in the form of p24 specific IFN-γ, induced by HIV DNA plasmids were retained or increased during prolonged HAART for up to 70 months and that p24 specific T-cell proliferation tended to be higher in samples with low plasma viral RNA (paper III).

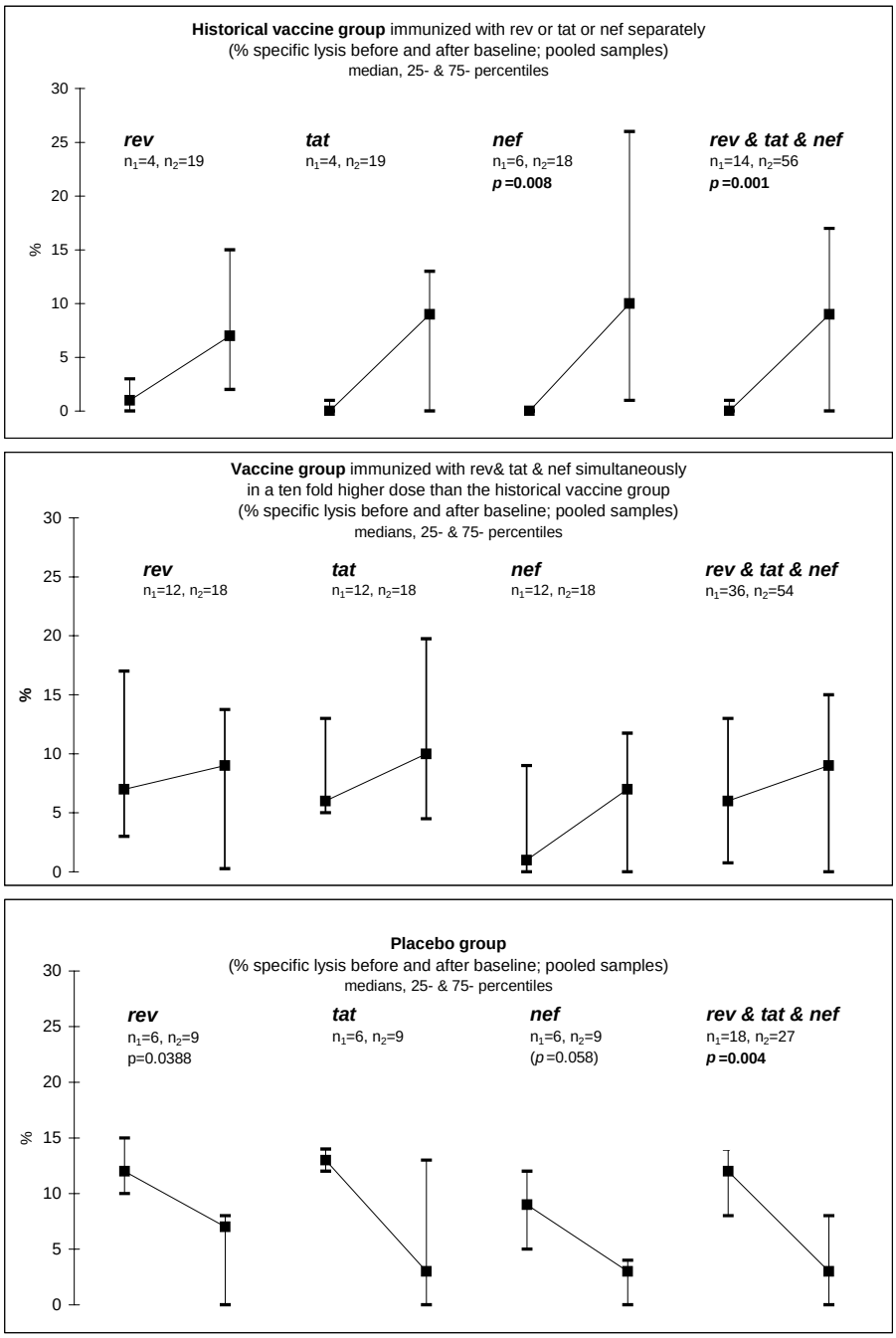

Figure 6. CTL activity expressed as % specific lysis determined by subtracting the percent lysis of control targets from that of antigen-expressing targets. All patients and samples before and after baseline are pooled (n_1= total number of samples before immunization, n_2= total number of samples during follow-up).

Active immunotherapy followed by treatment interruption

In the studies presented in **papers III – V** it was hoped that an immune response would be induced that was sufficiently strong to control HIV-1 infection. In the study presented in **paper II**, we found that patients with previous immunotherapy (rgp160 or DNA) were not more frequent in the group with a longer period of LTI. When HAART was interrupted, the CD4+ T cell decrease and viral load increase were of the same magnitudes in non-immunized, rgp 160 and HIV-1 DNA immunized patients (figure 7). As mentioned earlier, a preserved T cell anti rgp160 proliferative response was documented three months after treatment interruption in patients presented in **paper IV**, in keeping with some persistence even in non-HAART patients.

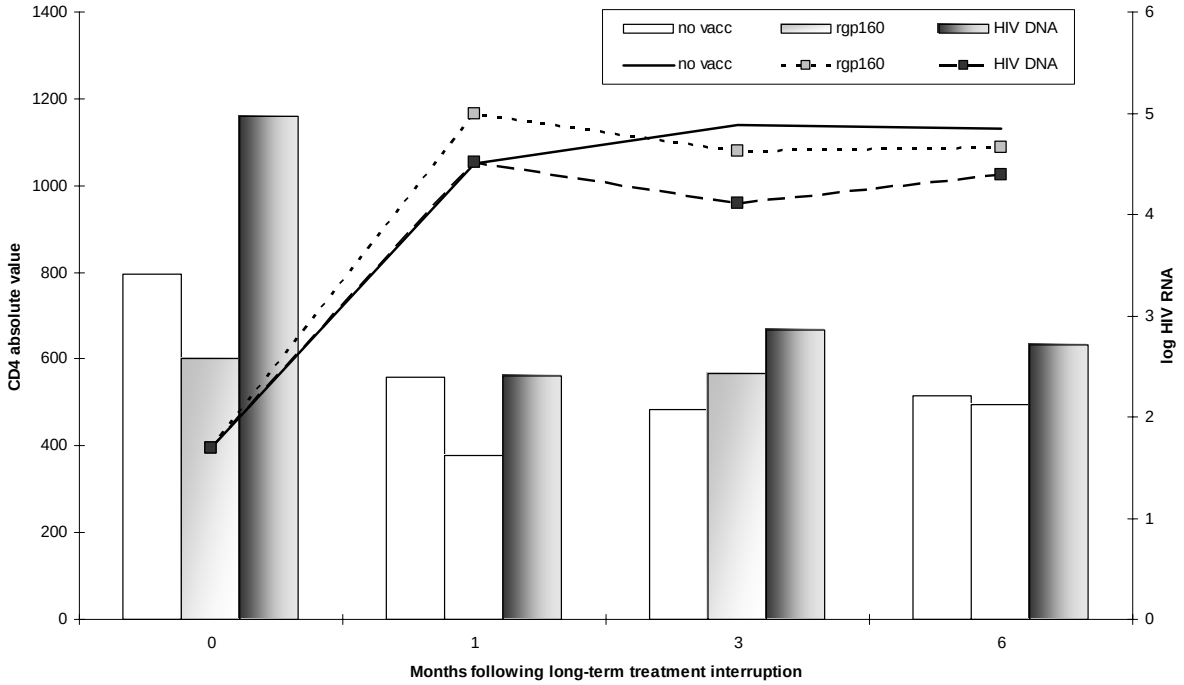

Figure 7 Treatment interruptions in 3 patients with no pre-HAART immunization, 3 patients with pre-HAART rpg 160 immunization and 3 patients with pre-HAART HIV-DNA immunization. Patients are matched for CD4+ T cell increase during HAART.

From these observations we conclude that it is possible to induce long-term persistent immune responses in HIV-infected individuals. The studies cannot predict whether this also translates into a longer symptom-free period during treatment interruption. The development of CD4+ T cell count and viral load in the patients is not promising but it should be born in mind that these studies were not designed to study the effect of treatment interruption after immunization. We did not have standardized time periods between the last immunization and the start of treatment interruption; we did not give any booster dose after HIV-DNA immunization, we did not continue immunization after therapy was interrupted, we did not use earlier knowledge of the impact of repeated exposure to the virus by STI (on-and-of cycles in HAART). However, encouraging results have been reported with therapeutic DNA vaccine together with HAART followed by STI in experiments with 4 macaques chronically infected with SIV (figure 8) [144, 145]. The vaccine, consisting of plasmids containing DNA

expressing all but the integrase protein of the Simian-Human Immunodeficiency Virus (SHIV), was applied to the surface of slightly exfoliated skin. The DNA was considered to be picked up by epidermal Langerhans cells and transported to the lymph nodes. Dendritic cells present DNA-derived antigens to naive T cells and induce HIV-specific CD4+ and CD8+ T lymphocytes that are responsible for the elimination of infected cells. HAART started 14 months post infection and was given in 3 weeks on / 3 weeks off cycles. After 6 cycles of STI-HAART the vaccine was administered during the HAART phase of the next 4 consecutive STI-HAART cycles. The median peak of viral rebound was progressively reduced during consecutive therapy interruptions. Control animals with STI-HAART without vaccine did not reduce viral rebound during corresponding treatment interruption.

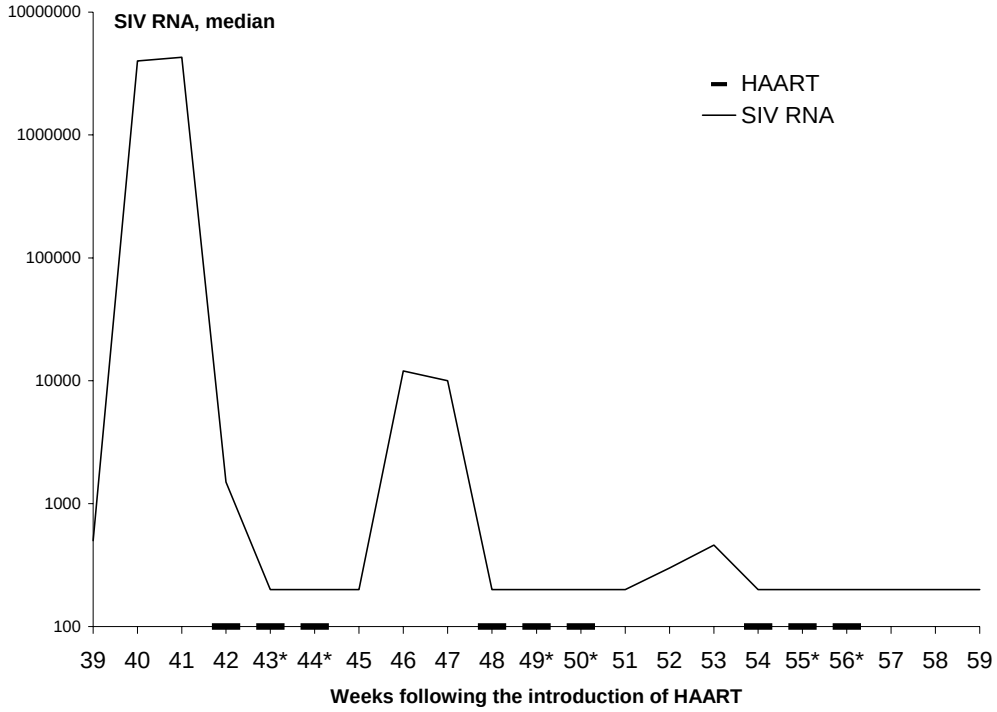

Figure 8. STI-HAART in combination with plasmids containing DNA expressing all but the integrase protein of the Simian-Human Immunodeficiency Virus (SHIV) *) vaccine given (Modified from [145])

At the 10th Conference on Retroviruses and Opportunistic Infections, Levi and colleagues [140] reported the results of a study using a dual-vaccine approach, comprising ALVAC-VIH 1443 and HIV lipopeptides combined with IL 2. The vaccine combination included different parts of HIV, such as ***nef***, ***gag env***, protease and ***pol*** antigens. Seventy HAART-treated volunteers with CD4+ T lymphocytes > 350 x 10^6/l cells and fully suppressed HIV-1 RNA levels were randomized to receive HAART alone or HAART plus the vaccine including IL 2. After immunization both groups stopped HAART after 40 weeks. At week 52, 5% of the controls and 24% of the vaccinated patients had viral loads < 10 000 copies/ml. An association was noted between the positive effects of the vaccine and specific immunologic responses. Data were not provided on any immunological differences between the two groups before study start.

The existence of patients who survive HIV-1 infection for several years without treatment and with spontaneous control of viral replication indicates that the immune system has a potential for "self-defense". The immunological war that is waged in a HIV-1 infected person is a balance between full control and loss of control. The outcome is probably settled very early

on during the infection. However, the intra- and inter-subtype diversity in HIV-1 creates specific difficulties that have to be overcome. A heterogeneous virus population normally poses difficulties for the development of vaccine, as seen with the influenza virus infection, where viral diversity is only 2% compared with HIV's up to 35% intersubtype diversity [84]. The introduction of HAART in the early hours of HIV infection even before antibodies are detectable, results in at least a short-term improvement in virological control and greater immune recovery compared with no therapy. As most patients are identified during symptomatic or late-stage HIV infection [211] and as infection is detected from the existence of antibodies, very early introduction of HAART is only possible in persons with a suspected infection. But the knowledge that immune control is achievable allows us to hope that a combination of HAART and immunization with different HIV-1 specific antigens (perhaps from several different clades) might teach the immune system to recognize vulnerable parts of the virus even in chronically infected persons. Different adjuvants and viral vectors could also contribute, as well as various routes of antigen delivery.

Conclusive remarks and future perspectives

We have clinical evidence that long-term antiviral treatment causes viral suppression and clinical benefits in both viral responders and low-responders. An important variable for prediction of successful interruption of treatment appeared to be retained CD4+ memory cells, directly correlated with nadir CD4+ T cell counts. HIV immunization together with antiviral treatment enhanced the magnitude and duration of new HIV-specific immune responses. Immunization with HIV antigens alone has improved short-term survival and almost always induces new HIV-specific T-cell responses. This shows that new memory cells can be induce by vaccination in the chronic phase of infection, which should permit extended treatment interruption.

Strategies that might be used with the intention of improve immune control in already HIV infected individual are:

1) to start HAART well before T cell count reach below 200x 10^6/l in order to preserve as many HIV specific immune responses as possible;
2) to use HIV specific immunization with as many immunogenic epitopes as possible, preferably from several different clades, together with effective immune-stimulating agents;
3) to use DNA vaccines and live-vector based vaccines in prime-boost regimens in order to assure a broad, long-lasting immune-responses;
4) to use structured treatment interruption (on and off therapy) with continued HIV specific immunization.

However, antiretroviral therapy is today, nearly 20 years after the discovery of the first HIV-specific medication, only available for a minority of infected individuals. Immune based therapy is still a young science and will probably need several years of development. Even if scientific progresses are continuously made and the number of HIV infected individuals who have access to antiretroviral therapy is growing, the spread of the infection goes even faster. The development of a prophylactic vaccine is urgent and might, in the long run, be the only way to slow down the spread. The first prophylactic vaccine candidates will probably not be effective enough to hinder a person to get infected with HIV, but might improve the possibility of self-defense and thereby at least delay time to develop AIDS. Hopefully "pre-vaccinated", HIV infected individuals will have lower viral load which might result in a decrease probability for further transfer of the infection. An obstacle is, however, that human behavior might negate these potential benefits of an early prophylactic vaccine. Vaccinated

individuals may feel “safe” which could result in more risk taking. Only closely monitored clinical trials with prophylactic vaccines will have a possibility to answer what is possible to accomplish. HIVIS is such a trial with the intention to study the optimal delivery modes of plasmid DNA as a prime prior to MVA boosting. The first part of the study is a safety trial of the DNA vaccination, which is to be followed by a safety trial of the MVA immunogen in Sweden. With this safety record it is planned to proceed to a phase 1/2 trial in Tanzania to study the two most promising modes of DNA delivery followed by MVA. This study will be my concern during the next few years in parallel with daily clinical work.

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