EXPLORING THE COMPLEXITY OF FUNCTIONAL HIV-1 SPECIFIC T CELL RESPONSES AND GLOBAL VIRUS-Host GENETIC VARIABILITY

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To all HIV infected individuals and their families
ABSTRACT

HIV has become one of the biggest health problems up to date. After 30 years of tremendous effort, the development of an HIV vaccine is still far from achieved. One of the main issues is the high mutation rate of the virus which results in the selection of mutated variants. This gives the virus the ability to escape from selective pressures, such as the immune system. Also the correlates for an effective immune response against HIV remain uncertain.

T cells have been proven to play a key role in controlling the virus in HIV infected individuals. T cells recognize small fragments of HIV as antigenic peptides bound and presented on HLA molecules. The HLA molecules are the most polymorphic proteins in the entire human genome. As the HLA-peptide interaction is highly specific, HIV infected individuals will present different peptides from the HIV proteins dependent on their HLA repertoire. In this thesis we explore the complexity of functional HIV-specific T cell responses and global virus-host genetic variability in different study cohorts.

Individuals with frequent exposure to HIV without establishment of infection have been well studied in the hope of finding the secret of their reduced susceptibility. However, no one has studied if exposure to HIV through oral sex can induce T cell responses. Through access to samples from healthy HIV negative individuals living in a relationship with an HIV infected partner we show that oral exposure is enough to mount a systemic HIV specific CD4+ and CD8+ T cell response.

To cope with the enormous variability of HIV sequences and HLA alleles we combined the use of bioinformatic tools with molecular biology, and immunological assays. We successfully identified several highly immunogenic peptides that were recognized in a diverse study population infected with several HIV subtypes. Responses against these peptides were further investigated to address the effects of HIV point mutations on T cell recognition. We show that recognition of the HLA-peptide complex by the T cell receptor is highly sensitive and that one single point mutation will reduce the chance of inducing a response by 40%.

Despite the high mutation rate of HIV, there are some regions that are more conserved within the HIV genome. Mutations in these regions have the potential of reducing viral fitness, why viral variant carrying such mutations may be less pathogenic. We hypothesized that the character of the HIV peptide (i.e. variable or conserved) targeted by CD8+ T cells would influence the quality and quantity of T cell responses, and affect disease progression. We show that patients targeting a conserved peptide in early HIV infection maintain their responses for up to four years, while patients targeting a variable peptide lose their responses over time. Importantly, patients targeting a conserved peptide had a lower viral load and a slower CD4+ T cell decline. The identification of virological and immunological characteristics that influences disease outcome is highly relevant for the development of a therapeutic vaccine.

These studies are based on the access to exclusive patient material through excellent collaborations, and combination of bioinformatics and immunological assays. This thesis has brought new knowledge to the field, but also addressed the complexity of HIV specific T cell responses.
LIST OF ABBREVIATIONS

AIDS    acquired immune deficiency syndrome
APC     antigen presenting cell
CRF     circulating recombinant form
DC      dendritic cell
DNA     deoxyribonucleic acid
ELISPOT enzyme-linked immunospot
ER      endoplasmic reticulum
EUI     exposed uninfected individual
GALT    gut-associated lymphoid tissue
HIV     human immunodeficiency virus
HLA     human leukocyte antigen
IFN     interferon
IL      interleukin
LTNP    Long-term nonprogressors
MHC     major histocompatibility complex
NK      natural killer
NKT     natural killer T
PBMC    peripheral blood mononuclear cells
RNA     ribonucleic acid
RT      reverse transcriptase
SIV     simian immunodeficiency virus
STI     sexually transmitted disease
TAP     transporter associated with antigen processing
TCR     T cell receptor
Th      T helper
TNF     tumor necrosis factor


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CONTENTS

1. Aims

2. Human Immunodeficiency Virus – HIV .............................. 1
   2.1 An Introduction to HIV ........................................ 1
   2.2 The History of HIV .............................................. 1
   2.3 The Origin of HIV ................................................ 1
   2.4 HIV-1 Structure and Protein Functions ........................ 2
   2.5 Genetic Diversity .................................................. 5

3. Immunology ............................................................... 5
   3.1 The Innate Immune System .................................... 6
   3.2 The Adaptive Immune System .................................. 7
      3.2.1 The Cellular Immune Response ......................... 7
         3.2.1.1 Activation of Cellular Immune Responses .... 7
         3.2.1.2 Antigen Binding and Presentation .......... 8
         3.2.1.3 CD4+ T cells ........................................ 9
         3.2.1.4 CD8+ T cells ......................................... 10
      3.2.2 The Humoral Immune Response ......................... 11

4. HIV Infection ............................................................ 12
   4.1 HIV Transmission ................................................ 12
   4.2 Disease Progression in HIV Infection ....................... 12

5. Potentials for a Prophylactic or Protective HIV vaccine .... 14
   5.1 Exposed Uninfected Individuals .............................. 14
   5.2 Factors Associated with Disease Progression ............ 15
   5.3 The History and Current Goals for HIV vaccines ........ 17

6. Results and Discussion ................................................. 19
   6.1 Introduction ..................................................... 19
   6.2 Material and Methods .......................................... 19
      6.2.1 Study Cohorts ............................................ 19
      6.2.2 ELISPOT versus Flow Cytometry .................... 20
6.3 Paper I ................................................................. 21
6.4 Paper II ................................................................. 23
6.5 Paper III ................................................................. 26
6.6 Paper IV ................................................................. 28
6.7 Summary of Important Findings ......................................... 30

7. Concluding Remarks .......................................................... 31
8. Acknowledgements ................................................................. 33
9. References ................................................................. 36
10. Related publication - Populärvetenskaplig artikel ................. 45
    Att identifiera komponenter till ett optimerat hivvaccin
1. AIMS

The overall aims of this thesis were to gain a deeper insight into functional T cell responses during early and chronic HIV infection and the complexity of the virus-host interactions.

Paper I: To investigate if oral exposure is enough to induce a systemic HIV specific T cell response in HIV exposed uninfected individuals living with an HIV infected partner.

Paper II: To identify broadly immunogenic epitopes in an ethnically diverse HIV infected population infected with multiple HIV subtypes.

Paper III: To explore the determinants for recognition of the virus and virus variants by epitope-specific CD8+ T cell responses based on the patients HLA genotype and autologous virus sequence quantitatively.

Paper IV: To investigate whether the character of the immunodominant epitope (variable or conserved) targeted early in HIV infection influences the efficacy of the T cell responses over time and thereby the disease outcome.
2. Human Immunodeficiency Virus - HIV

2.1 An Introduction to HIV

Infection with the human immunodeficiency virus (HIV) has become one of the biggest health problems up to date. Around 35 million people are living with HIV today and it has been estimated that more than 25 million individuals have died from AIDS (UNAIDS, AIDS epidemic update 2010, www.unaids.org). The number of people suffering from and affected by the disease are even higher. This tragedy has demolished entire societies and severely affected family members emotionally as well as financially. Even though HIV is a global problem with a worldwide spread, the distribution is highly unfair. Countries and regions with extreme poverty have in general been the most affected, with the highest prevalence found in sub-Saharan Africa.

2.2 The History of HIV

It all started about 30 years ago when clinicians in New York and California reported an increased prevalence of different opportunistic diseases and some rare forms of cancer, such as Kaposi’s sarcoma, which typically is not seen in healthy individuals [1,2,3]. The observations were first made in young homosexual men, but soon such symptoms were also noted among intravenous drug users (IDU) and hemophiliacs. The disease was given the name acquired immunodeficiency syndrome – AIDS [4]. In 1983, Dr’s Francoise Barre-Sinoussi and Luc Montagnier at the Pasteur Institute in Paris managed to isolate the virus causing AIDS [5]. Shortly thereafter Dr Robert Gallo confirmed these findings [6], and the virus was given the name human immunodeficiency virus (HIV). In 1986, a closely related virus was isolated in West Africa, and was named HIV-2 [7,8]. The pathogenesis of HIV-2 is not as aggressive as that of HIV-1 and the spread has not been as successful. The virus is mainly found in West Africa with the highest prevalence in Guinea-Bissau [9].

2.3 The Origin of HIV

Phylogenetic studies show that HIV has been introduced into the human population several times, and the spread of the pandemic we are suffering from today probably started about 100 years ago [10,11,12]. HIV originates from the simian immunodeficiency virus (SIV) that is found in non-human primates [13]. HIV-1 most likely originates from SIV in chimpanzees (Pan troglodytes troglodytes) [14] while HIV-2 is more closely related to SIV in sooty mangabey monkeys (Cercocebus atys) [15]. HIV-1 is divided into groups M (major group), O (outlier) and N (non-M/non-O) [16]. Recently a new group, P, was identified [17]
that is more closely related to SIV found in wild-living gorillas (*Gorilla gorilla*) [17,18:Van Heuverswyn, 2006 #19]. The M group is further divided into subtypes A, B, C, D, F, G, H, J, K as well as several circulating recombinant forms (CRFs) between different subtypes [19]. Up to date more than 40 CRFs have been identified (www.hiv.lanl.gov).

### 2.4 HIV-1 Structure and Protein Functions

HIV is a lentivirus belonging to the retrovirus family (*Retroviridae*). Retroviruses are enveloped with an RNA genome. The most distinguishing features for retroviruses are their replication through a DNA intermediate, which is transcribed from RNA with the help of the viral enzyme reverse transcriptase, and the integration of the viral genome into the host cell DNA. This integration leads to the establishment of a lifelong chronic infection. Newly produced viral RNA copies and proteins move to the surface of the cells to form new HIV particles. The immature viral particles bud from the cellular membrane and then mature by cleavage of the viral polypeptides by protease.

HIV is a single stranded positive RNA virus with a spherical envelope, a matrix and a cone shaped capsid proteins, containing the two RNA strains. The HIV genome is about 10 000 nucleotides long and encode nine genes: *gag, pol, env, rev, nef, tat, vif, vpr and vpu*. By alternative splicing and protein degradation, HIV
transcribes fifteen proteins in total; Gag-p6, Gag-p7, Gag-p17, Gag-p24, Polreverse transcriptase (RT), Pol-integrase, Pol-protease, Env-gp120,Env- gp41, Rev, Nef, Tat, Vif, Vpr, and Vpu.

The HIV envelope consists of a lipid bilayer acquired from the cell membrane during budding and the Env proteins gp120 and gp 41, forming the spike and knob structure respectively. The Env protein gp 41 is a transmembrane glycoprotein, while the gp120 protein forms an extracellular trimer, noncovalently bound to the extracellular part of gp41. Binding of the Env proteins to receptors on the cell surface of the host cell leads to docking of the viral particle and fusion with the cell membrane. Initially, the gp120 binds to the CD4 receptor on immune cells. This interaction leads to conformational changes of gp41 and exposes the binding site for the co-receptors, most commonly CCR5 and CXCR4.

The gag gene encodes the precursor protein Gag p55. This protein is further cleaved into four structural proteins; the capsid protein Gag p24, the matrix protein Gag p17, the nucleocapsid Gag p7, and the spacer peptide 2 Gag p6. The p7 and p6 proteins are located inside the capsid to protect the viral RNA.

The pol gene encodes for three enzymes that are crucial for an efficient reproduction of the virus: a protease, a reverse transcriptase (RT), and an integrase. The RT transcribes the viral RNA to a DNA intermediate, the integrase enable integration of the viral DNA into the host genome, and the protease cleaves Gag-Pol precursor proteins into functional proteins enabling the maturation of the viral particle. Once the viral genome has been integrated it is referred to as provirus. Inside infected host cell in a resting state the integrated DNA can be silent and latently embedded for years before the cell is activated and the viral proteins are translated by the host cell machinery.

HIV also encodes the regulatory proteins; Tat, Rev and Nef. Tat is a trans-activator and facilitates initiation and elongation during HIV transcription. It binds to Tat responsive regions (TAR) in the long terminal repeats (LTRs) of the pro-viral DNA and allows for the transport of the unspliced messenger RNA (mRNA) from the nucleus to the cytoplasm. Rev is a protein that stabilizes and facilitates this transportation. Nef is one of the proteins that are expressed earliest during primary HIV infection. This protein has several important functions such as down regulation of CD80/86 and MHC class I and II molecules. This down-regulation inhibits recognition of HIV infected cells by other immune cells. Nef also down-regulates the CD4 molecule, which facilitates the release of progeny virus. The HIV genome also encodes three small accessory proteins, Vpu, Vpr and Vif. Vpu degrades CD4 and enhances the release of new virus particles. Vpr is a trans-activator for host cellular genes and promotes cellular differentiation. Vif prevents the action of the cellular antiviral defense protein.
APOBEC-3G, which promotes the infectivity of the virus [20]. Detailed information of the HIV proteins and their functions can be found at: www.hiv.lanl.gov/content/sequence/HIV/MAP/landmark.html

Figure 2
The life cycle of HIV:
1. Viral attachment through binding of the viral spike protein to the host cell receptors. The attachment to the CD4 receptor induces conformational changes in the viral envelope proteins allowing binding to the co-receptor
2. Fusion with host cell membrane and viral entry
3. Uncoating
4. Reverse transcription of the viral RNA to DNA by the viral enzyme RT
5. Nuclear import of the viral DNA
6. Integration of the viral DNA into the host genome, facilitated by the viral enzyme integrase
7. Transcription of viral mRNA from the integrated DNA
8. Translation of the viral proteins
9. Virion assembly at the cell surface
10. Budding and release of newly formed virion. Maturation through cleavage of the Gag-Pol precursor proteins
2.5 Genetic Diversity

One of the characteristics of HIV is the large population size and the high production of progeny virus. There are about $10^8$ productively infected cells within an HIV infected individual, and about $10^{10}$ new virions are produced every day [21,22]. This fast reproduction together with an enormously high mutation rate makes HIV exceptionally diverse. This high genetic diversity is seen not only at a population level but also within the patients. Estimation shows that the average mutation rate for HIV is 0.1-0.3 mutations per genome and replication cycle [23,24]. In comparison, despite that the influenza virus has a high mutation rate, the viral population found within one chronically infected HIV individual at one time point is as diverse as the influenza variability seen worldwide during a flu season [25]. The successful diversity is mainly due to the RT enzyme, which is error-prone and lacks proof reading ability, thus causing a high proportion of point mutations during the transcription of RNA to DNA. These mutations may be substitutions, insertions and/or deletions of nucleotides. Secondly, RT jumps between the two HIV RNA strands during the transcription and gives rise to a DNA template that is a recombination of two parental RNA strains [26]. Thirdly, the cellular enzyme polymerase II used during translation of the integrated viral DNA also lacks a proof reading function. Fourthly, there are other factors, such as the cellular antiviral proteins APOBEC. The APOBEC proteins are cellular enzymes that induces nucleotide hypermutations during reverse transcription, which can destroy the replicative capacity of the virus and contributes to the variability of HIV [27].

The high diversity of HIV is a major challenge for the immune system as well as for the development of HIV vaccines, as the virus tends to mutate and escape under selective pressure. Selective pressure from the immune system is a strong driving force in HIV evolution.

3. Immunology

The immune system is highly sophisticated and advanced with several alternative ways to combat invading pathogens such as viruses, bacteria and fungi. There are physiological barriers, such as the skin, which is made up by epithelial cells, and mucosal membranes, which cover the respiratory, gastrointestinal, and genital tracts. There are also chemical barriers, e.g. low pH, that act to create an unpleasant milieu for invading pathogens. Also cells of the immune system and soluble factors produced by cells play a key role in our defense against pathogens. The immune system can be divided into two different systems acting in different manners: the innate immune system and the adaptive immune system. Although they differ significantly from each other, there are a lot of interactions between them, and they are to some extent dependent on each other.
3.1 The Innate Immune System

The innate immune system is the first defense against pathogens, and acts within a couple of hours after an infection. It is referred to as the unspecific immune response, and recognizes conserved patterns on pathogens and damaged cells. Activation of the innate immune system by recognition leads to a cascade of events. Among the most important players are the dendritic cells (DC). DCs circulate in the peripheral blood and secondary lymphoid tissue, as well as in mucosa and other organs. DCs are professional antigen presenting cell (APC) that engulf and digest pathogens and cellular debris of infected cells or cancer cells through phagocytosis. When a DC captures an antigen, it becomes activated and homes to a lymph node. The digested antigens are presented as short peptides on major histocompatibility complex (MHC) molecules for recognition and activation of other immune cells, including naïve T cells. Other professional APCs are B cells and macrophages. Neutrophils, basophils, and eosinophils are granulocytes with the ability to phagocytose. However, they cannot present antigens as the APCs, but they are important players in the innate immune system since they engulf pathogens and can release soluble factors, such as histamine, which is produced by basophils and triggers the inflammatory response. Natural killer (NK) cells recognize and kill infected cells and tumor cells through lysis. Recognition can be either direct, via recognition of activation/inhibition receptor expression, or due to antibody-coating of the damaged cell or invading pathogens. NKT cells express features of both T cells and NK cells, and they can recognize glycolipids presented on the CD1d molecules that are expressed on APCs.

Other important factors for the lytic clearance of damaged cells are the complement system, and secreted soluble factors like the anti-microbial peptides (AMP). The α- and β-defensins are important AMPs that are positively charged and can bind to the negatively charged membrane of microbes and thereby cause lysis [28,29]. The innate immune system recognizes damage-associated molecular patterns (DAMP) in cancer cells, as well as pathogen-associated molecular patterns (PAMP), e.g. proteins, DNA, RNA, and sugar motifs. The most important pathogen recognition receptors (PRRs) are the toll-like receptors (TLRs), which are expressed both intracellular and on the cell surface. Upon recognition, activation occurs and sets the immune system into a viral state by production of interferons (IFN), which can be secreted by DCs among other cell types. The most important role of IFNs is acting as communicators between different cells. They induce up regulation of MHC molecule expression, which is important for the adaptive immune system. IFNs also increase protein kinase expression, such as APOBEC, which is an important protease used in the defense against viral infection. Infection also creates an inflammatory state that will lead to the release
of chemokines such as MIP1α, MIP1β and RANTES. Chemokines recruit other immune cells to the site of injury or infection.

3.2 The Adaptive Immune System

The adaptive immune system is highly specific and takes a longer time to react and mature than the innate immune system. This maturation process can take days or even weeks. There are two arms of the adaptive immune system; the cellular and the humoral responses. The cellular immune system is made up by T cells while the humoral immune system is built up by B cells. T and B cells both originate from the same haematopoietic stem cells in the bone marrow but mature at different sites. The T cells mature in the thymus while the B cells have their development in the bone marrow. T and B cells express antigen-specific receptors called T cell receptors (TCR) and B cell receptors (BCR). Upon encounter with the proper antigen, both B and T cells start to proliferate rapidly. This clonal expansion of antigen-specific cells is essential to enable control and depletion of the incoming pathogen. T cells are further divided into CD4+ T cells, CD8+ T cells, and NKT cells.

3.2.1 The Cellular Immune Response

3.2.1.1 Activation of cellular immune responses

The T receptor on the T cells recognizes antigenic peptides, so called epitopes (short fragments of a partly degraded antigen) presented on MHC molecules on APCs. This recognition is highly specific and is dependent on binding to both the MHC molecule and the epitope. The T cells undergo clonal selection including a positive and a negative selection during their maturation in the thymus to ensure that the cells can distinguish self from non-self antigens and not to react against self molecules as they proceed into the circulation. During the positive selection, T cells will have to interact with several cell surface molecules such as MHC molecules, to ensure reactivity and specificity. Cells that are incapable of binding will proceed into programmed cell death, i.e. apoptosis, while cells that express a TCR with a binding capacity will move on to the negative selection. In the negative selection process, the cells will be eliminated if they bind to strongly to the self antigen-MHC complex. There is a delicate threshold between a too week and a too strong binder. Errors in this selection can cause autoimmune responses due to recognition of self-antigens in the tissues.
3.2.1.2 Antigen binding and presentation

Antigens are presented to the TCR on MHC class I and MHC class II molecules. MHC class I molecules are expressed on all nucleated cells, and present intracellular antigens such as viruses. These antigens have been degraded in the cytoplasm by the proteasome into optimally 8-11 amino acid long epitopes. They are transported from the cytoplasm into the endoplasmic reticulum (ER) via the transporter associated with antigen processing (TAP) proteins, where the epitopes are loaded on to a MHC class I molecule. The MHC epitope complex is transported to the cell surface via the Golgi complex.

![Antigenic processing and presentation by MHC class I (A-E) and class II (1-7) molecules.](image)

**Figure 3**

Antigenic processing and presentation by MHC class I (A-E) and class II (1-7) molecules.

A. Degradation of endogenous antigens into peptides by the proteasome
B. Transportation of peptides into ER via TAP proteins
C. Loading of antigen on to the MHC class I molecule in the ER
D. Transportation of MHC class I-peptide complex to cell surface via the Golgi complex
E. Presentation of endogenous antigen to CD8+ T cells

1. Exogenous antigen is taken up by phagocytosis
2. Exogenous antigen is degraded within lysosome vesicle
3. MHC class II molecule associated with an invariant chain is transported from ER to the Golgi complex
4. Invariant chain is degraded within the endosome
5. Fusion of the late endosome and lysosome. Loading of the exogenous antigen on to the MHC class II molecule
6. Transportation of MHC class II-peptide complex to the cell surface
7. Presentation of exogenous antigen to CD4+ T cells
MHC class II molecules are expressed on professional APCs such as DCs, macrophages and B cells. They present exogenously derived antigens that have been phagocytosed by the APCs. The phagosomes fuse with lysosomes where the phagocytosed antigen is degraded into short peptides (usually 11-20 amino acids). The lysosomes fuse with endosomes containing MHC class II molecules, and the MHC epitope complex is transported and presented on the cell surface. In humans the MHC is encoded by genes called human leukocyte antigen (HLA). The HLA class I and II alleles are the most variable genes in the human genome. The HLA class I antigens are further divided into A, B, and C alleles. There are more than 480 HLA-A, 800 HLA-B and 260 HLA-C alleles described in the HLA-database (http://www.ebi.ac.uk/imgt/hla). Each individual has the ability to present three to six different HLA class I alleles. The number of different alleles in each person depends on whether the individual is homozygote or heterozygote at each locus. Different HLA alleles have different binding specificities, i.e. depending on what alleles are represented, individuals will respond against different epitopes from the same pathogen. A heterozygote will have an advantage in most infections, since they are able to present a broader variety of antigens compared to individuals with a homozygote HLA allele composition. However, even if the same epitope is presented on several different HLA alleles [30], the impact on the pathogen might differ in the different individuals, depending on which allele that presents the antigen [31].

3.2.1.3 CD4+ T cells

The CD4+ T cells are called T helper (Th) cells. They secrete cytokines and express co-stimulatory molecules that promote differentiation and maturation of other immune cells after recognition of exogenously processed antigens presented on MHC class II molecules. There are several different types of T helper cells, based on different cytokine secretion profiles upon activation and their expression of intra- and extra-cellular molecules. The identification of CD4+ T cell subsets is a complicated topic and new subsets are emerging as the identification of cytokine production and cellular markers progresses. The Th1 subset produces IFNγ and TNF and plays the bigger role in the control of intracellular pathogens. The Th1 subset is potent producers of IL2, which is a cytokine that facilitates the proliferation of T cells, and promotes the activation and differentiation of CD8+ T cells. The Th2 subset produces IL4, IL5, IL6 and IL13, which are cytokines that facilitate the priming of the humoral response, and are more involved in the clearance of extracellular pathogens. Th17 cells are a subset of IL17 producing cells. Similarly to the Th2 cells, the Th17 cells also act on certain extracellular pathogens, but it has been shown that Th17 cells are also involved in autoimmune diseases and tissue inflammation [32]. Another subset of CD4+ T cells is the regulatory T cells (Treg). These cells are highly important for the maintenance and control of the adaptive immune system. Tregs are also
involved in tolerance, prevention of autoimmune diseases and limit chronic inflammatory diseases (reviewed in [33]). There are some additional subsets of CD4+ T cells, such as Th22 and Th9, which are reviewed in [34].

Figure 4
Overlapping HLA class I epitopes within a short fragment of the HV Gag p17 protein, illustrated by the amino acid sequence of the HXB2 strain. The epitopes are restricted to different HLA class I alleles (given within parenthesis). This shows how one sequence can contain several different HLA restricted epitopes, hence how the same protein can be presented differently dependent on the HLA allele.

3.2.1.4 CD8+ T cells

The CD8+ T cells recognize intracellular antigens presented on MHC class I molecules. Studies have shown that the CD8+ T cells are highly important in the control of the virus in HIV-infection in both humans [35, Borrow, 1994 #40] and in non-human primates [36,37,38]. Activated CD8+ T cells that can kill infected cells are called cytotoxic T cells (CTL) and they produce antiviral cytokines.

To become activated by professional APCs, the CD8+ T cells need three signals. First its TCR will recognize and bind to the proper MHC epitope complex. The second signal is obtained through co-stimulatory molecules, such as CD28 on T cells, interacting with CD80/86 on the DCs. Thirdly; cytokines are needed to facilitate differentiation and proliferation of the antigen-specific CD8+ T cells. Once the T cell is activated it circulates to peripheral tissues, where it can recognize and kill cells containing the same antigen that they were introduced to by the DCs. Upon recognition and activation, there will be a polarization of lytic granules in the CTLs to the contact site with the damaged cell, resulting in the formation of the immunological synapse. The CTL will release the contents of the granules, including perforin and granzymes which will act on the targeted cell and induce apoptosis [39,40]. Perforin is harmless within the granules, but undergoes a conformational change once it is released. It creates circular pores in the cell membrane of the targeted cell or mediate endocytosis enabling granzyme B to enter the target cell [41]. Granzyme A and B are proteases that
cleave several proteins in the cytoplasm. The released the granular contents will act directly on the damaged cell and induce apoptosis, but the CTL also acts indirectly by cytokine signaling. The anti-viral cytokine IFNγ has several important features. It affects the virus and blocks viral replication upon secretion. IFNγ also acts on other immune cells and facilitates activation of macrophages and NK cells, and promotes isotype switching in B cells. Moreover, IFNγ up-regulates the MHC molecules on infected cells, which leads to a more efficient antigen presentation. Apoptosis may also be induced in damaged cells via CD95 (Fas), which is expressed on a variety of cells upon injury. Both CTLs and NK cells express the Fas ligand and bind to Fas on damaged cells, thereby inducing apoptosis.

As mentioned, CD8+ T cells recognize intracellular antigens presented on MHC class I molecules. The DCs have the ability to present exogenous antigens on the same molecules, a phenomenon called cross-presentation, which leads to cross-priming and allows CD8+ T cells to also recognize exogenous antigens [42].

It is known that the recognition of antigens by the TCR is highly specific, but some cross-recognition does occur. Thus, antigens that are very similar but not identical can in some cases be recognized by the same TCR. This can be of advantage for the immune system when it comes to pathogens with high mutation rates, such as HIV, that adopt under selective pressure (notably by mutations within the amino acid sequence) in order to escape the immune system. However, the promiscuity of the TCR recognition can lead to autoimmune disease when a self-antigen is similar to that of a pathogen, and the immune response starts attacking healthy cells expressing those self-antigens [43].

3.2.2 The Humoral Immune Response

The humoral immune response is mediated by B cells. The B cells express an antigen binding receptor, the B cell receptor. This recognition of the antigen by the B cell receptor is highly specific. It recognizes both whole proteins, which are found directly on the pathogen surface or as free soluble antigens in the circulation. Secretion of cytokines from the T helper cells, as well as binding of the B cell receptor to the antigen, activates the B cell and induces differentiation into plasma cells. The plasma cells produce soluble antibodies, which are free forms of B cell receptors. There are several ways for B cells to use the antibodies for elimination of a pathogen. Coating the pathogen with antibodies allows for other immune cells to bind to the Fc part of the antibody and phagocytose, or lyse the pathogen. The antibodies can also neutralize the pathogen by binding to a site used by the pathogen to dock with a target cell, thereby inhibiting infection by the pathogen.
4. HIV Infection

4.1 HIV Transmission

The most common route for HIV transmission is via heterosexual intercourse. The virus can enter the body through the mucosa in the genital, rectal and oral tracts. HIV is also transmitted via blood through blood transfusion, organ transplantation, needle exchange among intravenous drug users, and needle accidents in health care and laboratory settings. HIV can also be transmitted horizontally from mother-to-child during delivery and breast-feeding. The risk of transmission differs depending on the transmission route, the presence of other infections, and the level of viral load exposure. Genital exposure constitutes a considerably lower transmission risk compared to rectal exposure. However, in the presence of other genital infections, HIV may enter more easily through lesions in the mucosa, which increases the risk for infection. Also the level of viral load during exposure makes a difference in the transmission rate as a higher viral load equals a higher risk for infection [44].

Upon exposure, HIV infects target cells via lesions in the mucosa, via transcytosis in the epithelial cells, or directly at the infection site. The virus can also dock on DCs without infecting the cells by binding to DC-SIGN, and then be transported to lymph nodes where it encounters and infects other target cells. The virus uses the CD4 receptor as the main receptor and the chemokine receptors CCR5 or CXCR4 as co-receptors for docking and fusing with the host cell. There are other cells, like DCs, macrophages and microglia in the brain that also express these receptors and may become infected. HIV preferentially targets activated CD4+ T cells [45] and the loss of these cells leads to immunodeficiency and the emergence of opportunistic diseases. These features constitute the hallmark of disease progression and the onset of AIDS.

4.2 Disease progression in HIV infection

During the acute stage of infection the viral load reaches a peak level and there is a massive destruction of CD4+ T cells [46,47]. The most severe loss of T cells is in the gut associated lymphoid tissue (GALT), where around 80% of the CD4+ T cells are depleted [48,49]. Within a couple of weeks, the adaptive immune response increases, the peak viremia drops to a steady state level [50], and the CD4+ T cell count recovers to some extent. The HIV-specific CTL responses play an important role in viral control [35, Borrow, 1994 #40,36,37,38]. The level of viral load at the peak and during steady state is a good predictor of disease progression, i.e. that a low viral load predicts a better disease outcome with a slower progression [44]. Many individuals experience flu-like symptoms at this first acute phase [51].
After the acute infection and a stabilization of the viral load, HIV infected individuals enter a chronic phase that can last for years. During this time, the immune system may partially control the virus but it is not able to clear the infection, due to several factors such as the integration of the viral genome into the host cell DNA, and the high reproduction rate of the virus. During the chronic infection there is a constant depletion of CD4+ T cells throughout the course. This imbalance in the immune cell distribution dampens several other immune cells and leads to an impairment of the immune response. Also, during the chronic phase the immune system is constantly activated due to the ongoing viral replication, which eventually will lead to exhaustion and a defect responsiveness of the immune cells [52].

If left untreated, the chronic phase most commonly last for seven to ten years. Once the CD4+ T cell count is too low, and the patient starts to suffer from several opportunistic diseases, such as rare cancer forms like Kaposi’s sarcoma and different herpes virus infections. These are diseases that a healthy immune system normally combats, but at this severe stage of the HIV infection the immune system is too hampered and inefficient. Ultimately, the CD4+ T cell count drops below 200 cells per μl, and the patients develop opportunistic diseases and AIDS. Without treatment the patient will eventually die from these AIDS related infections.

Figure 5
The clinical course of HIV infection.
5. Potentials for a Prophylactic or Protective HIV vaccine

5.1 Exposed uninfected individuals

Some individuals are less susceptible to HIV infection, and are referred to as exposed uninfected individual (EUI). The first report describing this phenomenon was published in 1989 [53]. Since then there have been numerous observations of individuals that are exposed to HIV without getting infected. These individuals have been found among homo- and heterosexual discordant couples [54,55,56], commercial sex workers [57], and children born to HIV positive mothers [58]. EUIs have been intensely studied in the hope of identifying factors associated with protection. Such knowledge might be applicable in clinical settings and contribute to the discovery of a protective immune response needed for development of an effective vaccine.

Despite the large number of studies on EUIs, there is no single factor that applies to all. There have been reports showing elevated levels of soluble factors such as RANTES [59,60] and alpha defensins [61,62] in EUIs. Also genetical [61,63] and immunological factors related to the cellular [64,65,66,67], and humoral immune responses [68,69] have been identified (reviewed in [70]). Several studies have shown neutralization of HIV with antibodies of unknown specificity [56,68,69,71]. HIV specific T cell responses have been found in EUIs although they remain seronegative. Also, some HLA alleles are seen at a higher frequency in EUIs compared to HIV infected individuals [72]. Interestingly, Kaul et al show that the targeted T cell epitopes differed in the EUI group compared to the HIV infected individuals, while they expressed identical HLA alleles [73]. Adaptive immune responses towards the HLA molecules of the partner, targeting allo-antigens have also been detected since these molecules are expressed on the surface of the virus [54,55]. We showed in paper I in this thesis that HIV exposure via oral sex is enough to mount a systemic HIV specific T cell response in exposed uninfected partners of discordant couples [74]. The development of HIV specific immune responses in these EUIs indicates that their immune system has recognized viral particles but that the virus has not been able to establish an infection.

Among these different observations in EUI studies, there is only one factor that is clear and solid when it comes to reduced susceptibility, and that is the delta 32 mutation in the CCR5 gene [75]. Individuals with two copies of the CCR5 delta 32 mutation are almost completely protected against sexual transmission of HIV [76], and even those with one copy of the mutation (i.e. heterozygous) do have some partial protection against infection [77]. This mutation is more common in the western parts of the world with the highest prevalence in the Nordic countries, where the mutation (homo- and heterozygote) is found in up to 14% of the population, and homozygotes in 1-2.5% [78].
Another factor that has been proven to strongly decrease the risk for HIV transmission is male circumcision. Studies have shown that circumcised male has a reduced susceptibility to HIV transmission with more than 50% compared to uncircumcised men [79,80].

5.2 Factors associated with disease progression

Although clearance of the virus in an HIV infected individual is not achievable, there are differences as to the level of viral replication and speed of the disease progression (reviewed in [81]). Certain individuals, i.e. elite controllers and long-term nonprogressors (LTNP), are able to control the infection and have a slow disease progression. These controllers manage to keep the viral load below detectable levels (<50 copies/ml) and can be infected for decades with sustained CD4+ T cell counts [81,82,83]. Identifying viral characteristics and immunological and/or genetic factors that are responsible for the enhanced control in these populations have been intensely studied. Several immunological factors have been associated with disease progression. Some of the most important findings are highlighted and discussed in this section.

As described in the previous section, CCR5 is the co-receptor that HIV uses for viral binding and fusion to the host cell. The CCR5 delta 32 mutation results in a truncated form of CCR5, which prevents virus attachment. Homozygotes for the CCR5 delta 32 mutation (inherited from both parents) are protected against sexual transmission of HIV whereas heterozygote’s only have a partial protection. However, during an HIV infection heterozygous for the mutations have a lower viral load and a slower disease progression due to the truncated co-receptor expression which inhibits the viral binding and fusion with new cells [77].

It is well known that the CD8+ T cells play a key role in the control of viral replication in acute [35,84], and chronic infection [85,86]. It has also been shown that certain HLA class I molecules presenting the antigens for CD8+ T cells are important for disease progression [87]. Being homozygous for the HLA-A, -B, and/or C alleles is a disadvantage, while heterozygous individuals have an advantage in terms of disease progression [88,89]. A diversity of HLA alleles gives more options for antigen presentation and is better for targeting the pathogen from several angles. Some alleles, such as HLA-B57/58, and HLA-B27, are more frequently found in slow progressors [90,91,92], while HLA-B35 has been found at a higher frequency in individuals with a more rapid disease progression [88,93,94]. The mechanism behind these associations seems to be highly delicate, and even minor amino acid differences between HLA alleles might break this association. Despite that only three amino acids differs in the genomic composition, HLA-B5801 is found at a higher frequency in subjects with slow disease progression, while HLA-B5802 does not have any association with
disease outcome [95]. Similarly, HLA-B3502 and B3503 are associated with faster disease progression while HLA-B3501 is not, even though there is only three and one amino acid difference between the two alleles, respectively [96,97].

Kosmrlj et al. found that individuals carrying protective HLA alleles had a larger fraction of naïve T cell clones recognizing viral epitopes and epitope-variants presented by these HLA alleles [98]. Several studies show that alleles, which are associated with better disease progression, are more prone to bind to epitopes located in the more conserved regions of the HIV proteome, such as the capsid protein Gag-p24 [99]. Regions that are more conserved among HIV subtypes and strains are crucial for the viral fitness, why escape mutations under immunological pressure are more difficult for the virus to cope with and are thus less likely to persist. Another observation is that epitopes targeted by alleles associated with better disease progression are similar to epitopes targeted by chimpanzees during SIV infection [100,101]. This is interesting since despite susceptibility to SIV infection and establishment of chronic infection, the chimpanzees do not develop AIDS.

The character of the immunologically targeted epitopes is one of the most important factors in viral control. HIV infected individuals with a broad response targeting epitopes in the Gag region, which is more conserved, have a lower viral load compared to those who only target one or no Gag epitopes in chronic infection. The opposite phenomenon is seen for in the targeting of Env epitopes, which is a highly variable protein. HIV positive subjects with broad Env responses have a significantly higher viral load and lower CD4+ T cell count than subjects with few or no Env responses [102].

In addition to the importance of epitope character, there have also been qualitative differences associated with progression. Patients with slow disease progression such as LTNP and individuals infected with HIV-2 have a higher fraction of polyfunctional T cells that are capable of producing multiple cytokines, such as MIP-1β, TNF, IL2 and IFNy, at the same time upon stimulation [103,104,105]. This is in comparison to progressors who have more of a monofunctional T cell profile. Controllers also have a higher proliferative capacity [106]. The level of IL2 has been associated with the proliferative capacity of both CD4+ and CD8+ T cells [107]. There is also a higher frequency of cells producing perforin and granzyme B in controllers, which gives them a greater cytolytic ability [108,109]. Activation markers, such as CD38 and HLA-DR are elevated in HIV infection and are found to correlate with disease progression [110,111]. Slow progressors have also been identified with lower levels of immune exhaustion compared to progressors [106,108,109,112,113]. However, it is not clear whether these qualitative differences are the cause or the effect of a better disease outcome and a more intact immune system. Antigen sensitivity seems to be involved in the protective role of polyfunctional T cell
responses [114,115]. A low viral load also means less exhaustion and damage of cells, while an impairment of the CD4+ T cell repertoire gives a less effective immune response. It is also known that when progressors are put on anti-retroviral treatment (ART) they increase the CD4+ and CD8+ T cell functions, but this does not mean that they are able to control the viral replication once the treatment is withdrawn [116,117]. The increased number of functions is rather a result of lowering the antigen exposure [117,118].

5.3 The history and current goals for HIV vaccines

Although 30 years has passed since the discovery of HIV, we still do not have a vaccine against the virus. Several vaccines against other pathogens are delivered as attenuated pathogens. However, vaccination with attenuated HIV is not possible due to the risk of reversion to an infectious virus particle that regains its pathological features. Therefore, most HIV vaccine candidates have been delivered either as recombinant soluble proteins in order to induce neutralizing antibodies (NAbs), or as HIV genes via viral DNA vectors to stimulate a T cell response. There have been several changes of focus in the HIV vaccine field during the years. Initially, the field was focusing on induction of NAbs to block viral entry into the host cell. Even though some success was seen in *in vitro* cultures against lab adapted HIV strains, the induced NAbs had no effect on primary HIV isolates [119]. Then, the importance of T cells for viral control in early infection was revealed [35, Borrow, 1994 #40,84] and combined with repeated failures in NAb protection, the field shifted towards T cell based vaccine candidates [120]. Today the field has switched again, and the current general belief is that an efficient HIV vaccine needs to be able to induce both humoral and cellular immune responses [121].

The first approved HIV vaccine trial was executed in October in 1987 [122]. At this time, there was not enough insight about the complexity of this virus, and the field was rather optimistic to succeed. Since then the disappointments have been many, with more than 170 phase I or II clinical trials all over the world that all have failed to induce any protection against infection, nor lowered the viral load, or slow down the CD4 decline in HIV infected vaccinees [120,123,124,125,126]. In 2009, the world’s first positive results were presented after a phase IIb HIV vaccine trial performed in Thailand [127]. This was a randomized, multicenter, double-blinded, placebo-controlled efficacy trial, using a heterologous prime-boost approach. The subjects were primed with a recombinant canarypox vector vaccine (ALVAC-HIV [vCP1521]), and boosted twice with a recombinant glycoprotein 120 subunit vaccine (AIDSVAX B/E) administered through injections. This study showed a 31.2% reduction in the infection rate in vaccinated volunteers compared to the placebo group. The efficacy was higher during the first year post vaccination, but had a decreased efficacy over time. Also, due to numerous caveats and interpretation of the
results, it has been debated whether the reduction really is significant, and what the factors responsible for this potential protection could be. The biggest problem with the development of an HIV vaccine is the high mutation rate of the virus together with the genetic diversity of the immune system in humans [128,129]. How well the vaccine from the Thai trial would cope with the global diversity of HIV strains and the human HLA-variation is still not known.

One solution to the problem of virus diversity might be the use of a mosaic vaccine. The mosaic construct was presented in 2007 by Fisher et al, and is an \textit{in silico} optimization method for maximization of the coverage of potential T cell epitopes [130]. Since then, two vaccine trials have been published [131,132]. Both of them were performed in rhesus monkeys, with two different delivery strategies. Both studies showed promising results and induced responses of high broadness (i.e. number of targeted epitopes), and depth (i.e. subtype variants of the same epitope) without compromising the magnitude of the response. This approach has similarities to our study that was published in 2008, where we used an \textit{in silico} method to identify broadly immunogenic peptides that would be recognized in individuals of different ethnical backgrounds infected with different HIV subtypes (paper II) [133]. We found that the vast majority of our identified elite epitopes were represented in the mosaic construct. The differences are that the mosaic construct is a whole protein approach that does not consider antigen presentation, while our method is focusing on identifying optimal epitopes to target, and address antigen presentation capacity by taking HLA-binding, proteosome cleaving, and TAP-transportation into account. Human trials of the mosaic vaccine are ongoing and it will be very interesting to see whether this novel strategy is superior for protection against HIV infection or not.
6. Results and discussion

6.1 Introduction

The main task of this thesis was to identify factors that influence the efficacy of an HIV-specific immune response directed by CD8 T+ cells. A better understanding of the complexity of the interaction between the immune response and the virus is important for the development of HIV vaccines. The main goal would be a prophylactic vaccine that could prevent new infections. There are some rare individuals who are naturally less susceptible and remain uninfected despite being repeatedly exposed to the virus. These EUI have been heavily studied in the hope of finding the key to their natural defense. One potential factor is through immune responses induced by repeated exposure to the virus. In paper I we studied the prevalence of anti-HIV cellular immune responses in a cohort of EUI who had been exposed through oral sex by their HIV infected partner.

The main problem when it comes to the development of a vaccine or identifications of an effective immune response is the high mutation rate of HIV. The emergence of viral variants is a great challenge for the immune response. Also the highly variable HLA molecules, presenting antigen to antigen-specific T cells within the human population makes it even more difficult. In papers II and III we combined bioinformatic tools with immunological assays to cope with this high variability of the virus and the host and studied the HLA-peptide-TCR recognition in more detail.

If the goal of a protective vaccine is not reachable, another achievement would be to find a vaccine that would work therapeutically and lower the viral load in HIV positive individuals. A lower viral load would slow down disease progression and also limit the spread by reducing the risk for transmission. There are some HIV positive individuals who are infected but seem to be coping with the virus. They are called long-term nonprogressors or elite controllers. Even within progressors there are huge differences in disease progression in terms of viral load and CD4+ T cell decline. In paper IV we are identifying some immunological and virological factors that seem to affect disease outcome.

Although each study included in this thesis has contributed with important information to the field, each answer has raised several new questions. Even though this is where this thesis ends, it is only the beginning of finding all the answers to really understand the complexity of functional HIV specific T cell responses and global virus-host genetic variation.

6.2 Materials and Methods

6.2.1 Study Cohorts

Paper I
We were fortunate enough to get access to PBMC samples from 25 discordant couples attending the Gay Men’s Health Clinic in Stockholm, Sweden, in order to study the immune responses in EUIs. We received PBMC’s from the HIV negative partner, i.e. the EUI, and clinical data of the HIV positive partner. The EUIs had answered a questionnaire regarding their sexual behavior, and been examined by clinicians every sixth months for detection of HIV and other STI’s. Cells had been collected at each examination time point during 2.5 years. None of the 25 volunteers were detected with HIV or other STI during the study period. Ethical approval for this study was given by the ethical committee at Karolinska Institutet, and all participants gave their informed consent.

Papers II and III

For paper II and III we wanted to study immune responses toward a predicted set of HLA class I restricted HIV epitope variants targeted by individuals of various ethnical backgrounds infected by diverse HIV subtypes. We selected 31 HIV-1 infected patients attending the HIV clinic at the Karolinska University Hospital. The only inclusion criterion was a detectable viral load (>50 copies/ml) to ensure detectable T cell responses. This study cohort was idealistic for our study purpose. They differed in route and state of infection, age, gender, treatment history and ethnicity as they originated from 13 countries and were infected with different HIV subtypes. This study was approved by the Regional Ethical Council in Stockholm, Sweden, and all volunteers provided written informed consent.

Paper IV

Through our collaboration with Prof Hecht we got the privilege to work with exclusive patient material from the OPTIONS cohort at the University of California, San Francisco (UCSF) in USA. We were able to examine early T cell responses and monitor them over time. Longitudinally drawn PBMC and plasma samples were obtained from 13 HIV-1 subtype B infected men followed for at least three years without any treatment. We had access to all clinical data (CD4 and viral load) during the study period as well as information regarding previously identified early T cell responses against the HIV-Gag region. The study was approved by an ethical committee and all patients provided written informed consent.

6.2.2 ELISPOT versus Flow Cytometry

Two different immunological assays were used for the studies in this thesis to identify HIV specific T cell responses – the ELISPOT IFNγ assay and the ICS flow cytometry assay. The ELISPOT IFNγ assay is a fairly easy, cheap and straightforward method that detects IFNγ producing cells upon stimulation with an antigen (e.g. peptide). The ICS flow cytometry assay is more complicated and demands more exclusive and expensive equipment. However, it also provides highly sophisticated data and gives the opportunity to distinguish between
different cells at a single cell level and to simultaneously identify production of several cytokines and chemokines. One drawback with the flow based assay is the number of cells needed as the ICS flow cytometry assays require at least 500,000 cells per test, compared to ELISPOT that only require 150,000-200,000 cells per test. Also, the ELISPOT assay is more sensitive and is able to pick up weaker responses than the flow cytometry assay. Thus, when working with very limited numbers of cells, weak responses, or screening of responses, the ELISPOT assay is preferred. Whereas the ICS flow cytometry assay has the great advantage to simultaneously identify several phenotypic and functional markers needed to understand the characteristics of an immune response and to compare these to other responses in intra- and inter-patients studies. In this thesis, assay selection was dependent on the aim of each study. The benefits and drawback of these assays are further discussed in [118,134]. Additional details can be found in the papers I-IV included in this thesis.

6.3 Paper I

**Induction of systemic HIV-1-specific cellular immune responses by oral exposure in the uninfected partner of discordant couples**

HIV is a chronic infection and so far we do not seem to be able to clear the infection. However, there are some individuals that are less susceptible to HIV infection. This fascinating group of people has been exposed to HIV through different routes i.e. intravenously, sexually, and horizontally. They have been given many names throughout the years, such as highly exposed persistently seronegative (HEPS), exposed seronegative (ESN), and exposed uninfected (EU) individuals. In this thesis they are referred to as exposed uninfected individuals (EUI). The phenomenon of frequent exposure without establishment of infection has been well studied in the hope of finding the secret of their reduced susceptibility. However, no study of T cell responses on EUIs with exposure only via oral sex had been previously performed. Through our collaboration with Professor Broliden and Dr Hasselrot, we got the opportunity to study T cell responses in a unique cohort consisting of 25 discordant couples who in detailed interviews with their physician reported that they practiced protected anal intercourse but had unprotected oral sex with their HIV infected spouse.

We received PBMC’s collected longitudinally during two and a half years time from the 25 HIV-uninfected partners of the discordant couples. We also had access to all clinical data (CD4 count and viral load) from the HIV infected partner. The IFNγ ELISPOT assay was used to identify HIV specific T cell responses using stored PBMC samples. We detected T cell responses against HIV in three of the 25 tested EUI’s. The frequency of individuals with detected HIV-specific T cell responses in other EUI cohorts have differed depending on the study population, rate and route of potential exposure as well as assay use. The number of identified responders in
our study is comparable to the study by Suy et al., where two out of the 20 tested EUIs in heterosexual discordant couples had an HIV specific T cell response [67].

One of the responders in our study had a strong HIV-Gag response. This individual was highly interesting since his partner had a treatment interruption due to side effects during the study period. During this time his viral load went from an undetectable level to 286,000 copies/ml. The treatment scheme was changed and his viral loads decreased back to undetectable levels. We had the unique opportunity to monitor the HIV-specific T cell responses in the HIV negative partner before, during, and after the treatment interruption and to study how the detected responses correlated to the changing antigen exposure (figure 6a). Intracellular cytokine staining and flow cytometry analysis were used to distinguish between CD4+ and CD8+ T cells and to identify multiple cytokine, i.e.

**Figure 6**

Viral exposure and multiple cytokine production by HIV-Gag specific CD4+ and CD8+ T cells. PBMCs collected at two different time points (marked with black arrows). Viral load and treatment history in the partner reveal HIV-exposure (a). PBMCs were stimulated with the HIV-Gag p55 peptide pool and activation was measured using the ICS-assay. HIV-specific T-cell responses were detected in CD4+ T cells (b) and CD8+ T cells (c) at the first time point (T0). One year later (T12) the responses were still detectable in the CD4+ T cells (d), but not in the CD8+ T cell population (e). ART, antiretroviral therapy. (Figure modified from [74])
IFNγ, TNFα, MIP-1β and IL2, production. Interestingly, this individual had strong CD4+ and CD8+ HIV-specific T cell responses, and produced several cytokines upon HIV Gag antigen stimulation (figure 6b-e). The detection of HIV-specific CD4+ T cells was not too surprising, as the cells generally recognize antigens that have been phagocytosed and presented by HLA class II molecules on professional APC. Thus, it is likely that the CD4+ T cell responses were induced by incoming cell debris or free viral particles from the exposure that did not establish an infection. However, the strong CD8+ T cell response is not as easily explained as it recognizes endogenous antigens presented by HLA class I molecules. These antigens are generally intracellular antigens, such as viruses or self-antigens in cancer cells. Hence, a CD8+ T cell response normally indicates replication of the pathogen and would be expected after an HIV infection. However, an alternative explanation is that the CD8+ T cells were activated through cross-presentation by the HLA class II molecules on professional APCs, i.e. the antigen is presented via the extracellular route to the CD8+ T cell on a MHC class II molecule instead through the classical MHC class I pathway. We also show that the HIV-specific CD4+ T cell response was sustained for at least one year. Even though transmission through breast feeding occurs via the oral route, oral sex is not considered to be a high risk factor since the task of the normal oral flora is to neutralize and block invading pathogens [135]. In this study we show that local HIV exposure via oral sex is enough to mount a systemic CD4+ and CD8+ T cell response. To answer the question of whether these responses have any protective features or if they are merely a trace left by antigen exposure, this topic needs to be further investigated.

6.4 Paper II

Broadly immunogenic HLA class I subtype-restricted elite CTL epitopes recognized in a diverse population infected with different HIV-1 subtypes

The diversity within HIV as well as the genetic variations of the human immune system (i.e. HLA and TCR diversity) make it difficult to develop an HIV vaccine as well as to study the immune responses against HIV, but needs to be considered and evaluated in detail. Validation of vaccine candidates and studies on antigen specific T cells might be hampered by the choice of antigens used in the immunological assays. Peptide pools corresponding to consensus sequences are commonly used, but they might underestimate or even fail to evoke a specific response. In this study we aimed at identifying broadly immunogenic epitopes that are recognized in an ethnically variable population infected with different HIV subtypes. To select epitopes we collaborated with Prof Ole Lund and his group at the Technical University of Denmark. They are developing and working
with advanced bioinformatic tools including a prediction tool for HLA-class I restricted CD8+ T cell epitopes, called NetMHC[136]. Using the bioinformatic tools we could work with large datasets and handle the immense genetic diversity of the virus and the host. For us, the main benefit was to select epitopes likely to be recognized by a large numbers of subjects independent of which HIV type they were infected with, and to work with a limited number of optimal 9 amino acid long HLA class I restricted epitopes. During the time for the set-up of this study, the concept of HLA supertypes was commonly used to simplify the enormous amount of HLA alleles present within the human population. A large number of HLA alleles were divided into twelve supertypes based on the similarity of the peptide they are prone to bind [137,138].

The prediction method used in this study, NetMHC 1.0, predicts all possible optimal peptides from a given sequence. The prediction is mainly based on the likelihood of binding to a certain HLA-supertype, but also takes into account the TAP-transport efficiency and the proteasomal cleavage. In this study we used a set of 322 different full-length sequences obtained from the HIV Molecular Immunology database (www.hiv.lanl.gov), including the main subtypes and circulating recombinant forms. From the sequences 5652 unique peptides were predicted to bind to the HLA supertypes. To limit the number of peptides another novel algorithm called EpiSelect was used to select peptides, that would give the highest coverage possible and an equal distribution of epitopes within the different HIV subtypes, as well as to be able to bind the major HLA supertypes [137,138]. We ended up with a total of 184 predicted peptides covering the main proteins encoded by the HIV-genome.
In collaboration with Karolinska University Hospital, we received PBMC from HIV positive patients who volunteered for this study. Patients enrolled in this cohort were infected with different HIV subtypes and originated from Europe, Africa, Asia, North America and South America. This gave us access to the desired genetically variable study population, which was particularly suitable for the aim of this study. The immunogenicity of the 184 predicted peptides were tested by an IFNγ ELISPOT assay using PBMC samples obtained from the 31 study subjects. Impressively, as the majority of the predicted peptides were immunogenic. We found that 114 out of the 184 tested peptides were recognized in by least one of the study subjects, and 45 of these were novel epitopes that had not been described previously. A median of six epitopes were recognized by each patient (figure 7a). The data show that the HIV-Nef epitopes were the most immunogenic; 96% of tested epitopes were recognized by at least one patient (figure 7b). Also, more than 50% of the tested peptides in the HIV-Gag, -Pol and -Env regions were recognized, which also indicates a very high immunogenicity. In addition, we found 21 epitopes that were highly immunogenic and recognized by four or more patients infected with different HIV subtypes. Thus, by using a highly limited number of epitopes it is possible to study immunological factors of importance for clinical outcome with a global perspective. Ultimately such knowledge will help to design future vaccine antigens.

This study is a good example of the benefits of translation research to gain a deeper knowledge in the field. However, bioinformatic tools will only be useful if they are accurate and give true information. In this study we got the opportunity to use bioinformatic tools to limit the set of desired epitopes for experimental work, but also to validate the novel algorithm by testing the immunogenicity of the predicted and selected peptides. We found that 66% of the immunologically identified responses could be explained by the patients HLA allele belonging to the predicted HLA supertype. By extending our analysis and instead use the HLA allele prediction tool there was a huge improvement and we could explain 85% of the detected responses. Thus, we found that the use of HLA supertypes may not be beneficial as it tend to oversimplify the specificity of the HLA-peptide binding. Since this study was conducted the HLA class I and II prediction tools have been immensely improved, and it is now even possible to predict binding to unknown alleles. Further information on this topic can be found in the review written by Lundegaard C et all [139].

At the time of publication of this paper, Fisher et al used another approach to combat the high variability within HIV, by inventing the so called mosaic proteins for use as a polyvalen vaccine [130]. This was also conducted by in silico methods to get the best coverage in broadness of HIV strains, but also depth, meaning inclusion of several epitope variants. Since then, these mosaic proteins have been used in vaccine trials on rhesus macaques and showed to induce broad and deep HIV-specific T cell responses [131,132]. Interestingly, although we were using another model and only predicted epitopes giving an optimized viral and human
coverage, the vast majority of our 21 elite epitopes were found in the mosaic protein. The mosaic vaccine is currently tested on humans and it will be very interesting to see whether this increase in response depth and broadness will have any effect on HIV-specific responses and disease outcome.

6.5 Paper III

Interdisciplinary analysis of HIV-specific CD8+ T cell responses against variant epitopes reveals restricted TCR promiscuity

Recognition of a peptide by the TCR is highly sensitive. A virus changes its appearance through mutations, which lead to changes in the secondary structure, and result in the development of viral variants. The high mutation rate is one of

![Figure 8](image_url)

Figure 8
Similarity between tested and autologous peptides influences recognition (a). Fraction of responders among the predicted binders that show zero, one, or multiple substitutions between tested and autologous peptides (b). Reduction of response magnitude relative to the wild type (i.e. autologous) epitope for variants differing at one or more than one position, respectively (c). (Figure modified from [140])

26
the biggest obstacles in understanding the correlates for an effective immune response. Amino acid substitutions can lead to structural changes in the epitope which in turn can revoke TCR recognition, allowing the virus to escape the immunological pressure. On the other hand, cross-recognition between epitope variants can occur when the TCR recognizing a particular HLA-peptide complex are more flexible, and tolerate some changes within the epitope. However, the extent of this cross-recognition was not well understood at the time for this study, and had not been investigated in a quantitative manner in a genetically diverse population. In this study we once again used bioinformatic tools to understand and explain the patterns of epitope-specific CD8+ T cell responses against variant epitopes. The results of this study are based on the T cell responses described in paper II. We also sequenced the autologous virus (the gag, pol and nef genes) in all study subjects and obtained the high-resolution HLA types for the vast majority of the study subjects. By having the knowledge of the patients HLA class I alleles and the autologous sequences of the virus we used the bioinformatics tool NetMHCpan to investigate how peptides were cross-recognized. We compared tested peptide sequences with the autologous viral sequences to find out how potential differences influenced the detected immune responses as well as the binding capacity to the subjects HLA alleles. As expected, we found that the tested peptide sequences were significantly more similar to the autologous sequences in patients with detectable immune responses than in subjects without a response against the tested peptide (figure 8a). Strikingly, if the peptide differed from the autologous sequence with only one single amino acid the chance of inducing a response was reduced by 40%. One additional substitution further reduced recognition by 50% (figure 8b). This show how fragile the TCR-peptide-HLA recognition is and how one single substitution can revoke the immune response.

We next wanted to see how well tolerated mutations were at different positions of the peptide. Therefore we extended the study by selecting sets of peptide-patient pairs where the autologous and the tested immunogenic peptide sequences were identical. Immune responses were tested against epitope variants including commonly found mutations at different positions. We also included alanine substitutions for all positions influencing recognition by the TCR. We found that substitutions at the different positions were not equally tolerated. Substitutions in the third position (P3) seemed to be most well tolerated. Additionally, we looked at how the mutations affected the strength of the response, i.e. SFU/million cells. We found that the number of mutations will not only affect whether there is a response or not, but also the strength of the response (figure 8c).

Overall this study shows that an interdisciplinary approach is useful to understand complex interactions between a genetically diverse virus and host immune system, and help us to quantitatively investigate how sensitive these interactions are.
6.6 Paper IV

Immunodominant targeting of conserved epitopes in early HIV infection is associated with lower plasma viral load and slower CD4+ T cell depletion

The Swedish cohort at Karolinska University Hospital had the advantage of containing HIV infected patients from all over the world which gave us the opportunity to study a cohort with subjects that represented the global diversity in both ethnicities as well as in virus subtypes and strains. However, although the clinicians are making an excellent job in collecting clinical data on these patients, it is not possible to know how long they have been infected. Neither did we have access to longitudinal samples from the study subjects. Through an established collaboration with Prof Douglas Nixon and Prof Rick Hecht at the University of California in San Francisco (UCSF), we had access to plasma and PBMC samples from HIV infected individuals included in the OPTIONS cohort. This cohort consists of more than 600 patients that have been monitored longitudinally from

Figure 9
The mean percentage of IFN-γ producing CD8+ T cells in subjects targeting a conserved (square) or a variable epitope (triangle) is given at four time points (T1 to T4). The IFN-γ production against conserved epitopes was sustained over time, while it decreased significantly in patients targeting a variable epitope (a). Targeting of a conserved epitope in early infection is associated with lower plasma viral load (b) and slower CD4+ T cell depletion (c) over time. (Figure modified from paper IV)
acute or early infection. This gave us the opportunity to study the interaction between the immune system and the virus and see how they affected each other over time. We also wanted to see how the character (conserved or variable) of the targeted antigen affected clinical outcome and disease progression. We selected study subjects based on previous identified responses against the HIV-Gag region from our study subjects in early infection [141]. We also extended the number of tested epitopes by including highly immunogenic HLA-matched peptides from our previous study (paper I) [133]. All tested peptides were thus located in the Gag region and were HLA matched for each patient. Inclusion criteria for the study subjects (n=13) were that they had been longitudinally monitored for at least three years without any antiretroviral treatment and that we had access to cell and plasma samples from three to four time points, drawn approximately at one year intervals. The ICS flow cytometry assay was used to detect HIV specific CD8+ T cell responses producing IFNγ, TNFα, MIP-1β and IL2.

We showed that patients targeting a conserved peptide did maintain the magnitude of their epitope-specific CD8+ T cell responses throughout the entire study period, while patients targeting a variable peptide had a decrease in the magnitude of the responses over time (figure 9a). Despite the loss of magnitude against variable epitopes we could not detect any differences in the quality of the responses, i.e. the frequency of antigen-specific T cells that produce more than one cytokine. Previous studies has shown a significantly higher frequency of such polyfunctional antigen specific T cells in HIV infected individuals who have a slower disease progression such as LTNP, elite controllers, and HIV-2 infected individuals, compared to patients with a faster disease progression and a higher viral load. In our study, all patients are progressors, which might explain why we did not detect any differences in the frequency of polyfunctional T cells. However, another explanation might be that the loss of polyfunctionality occurs at a later time-point during chronic infection due to exhaustion of the immune system by constant antigen exposure. Most interestingly, the character of the immunodominant peptide targeted in primary infection did influence the level of viral load (figure 9b) and CD4+ T cell depletion (figure 9c). Hence, patients targeting a conserved peptide did have a significantly lower viral load and slower CD4+ T cell depletion over time. Despite that they were all progressors, there were still significant differences due to the targeted peptide character. The existence of a reverse correlation between CD4+ T cell decline and viral load is well known, and is an important predictor for disease outcome [46,47]. This study shows a clear beneficial role for targeting a conserved peptide in early infection. This is likely dependent on the low mutation rate in the epitope region making it more difficult for the virus to evade recognition by the development of escape mutations. Conserved regions are crucial for viral replication, and mutations in these regions have frequently been found to reduce viral fitness.
Sequencing of the autologous virus from plasma samples drawn over time is ongoing, and will reveal important insight in viral escape mutations and T cell cross recognition.

In conclusion, in the current study we show the importance of targeting the appropriate antigen in early infection. Targeting of conserved peptides will not only give the subject a more sustained immune response, but it will also affect the CD4+ T cell loss and the viral load, hence give an improved disease outcome. Thus, it is of importance to consider what antigen to include in future vaccine design. Although pre-exciting HIV specific T cell response toward conserved epitopes might not protect from infection, it could enable a more sustained control of the virus and lead to a slower disease progression in the vaccinees.

6.7 Summary of Important Findings

- We show that exposure of HIV via oral sex is enough to mount systemic HIV specific CD4+ and CD8+ T cell responses in the exposed uninfected partner of discordant couples

- We identified several highly immunogenic HLA class I restricted epitopes that are recognized by CD8+ T cells in a ethnically diverse population infected with different HIV subtypes

- We show that one single mutation in a HLA class I restricted epitope recognized by a CD8+ T cell reduces the chance for recognition with 40%

- We found that the character of the epitope targeted early in HIV infection affects the T cell responses and the disease progression over time. The CD8+ T cell responses against conserved epitope were sustained over time. Most importantly, subjects targeting a conserved epitope had lower viral load and a slower CD4+ T cell decline
7. Concluding remarks

Today we have very effective antiviral drugs against HIV that keep the viral load under detectable levels and stop disease progression. However, these drugs are not a cure. They demand a lifelong treatment involving psychological and physical issues, such as side effects. There is also a financial aspect, with high costs and distribution difficulties of the drugs in developing countries. Therefore, despite the success in the drug development, we still urge for an HIV vaccine. An optimal HIV vaccine would work prophylactically and prevent HIV infection. This seems to be very hard to achieve. The development of a therapeutic vaccine might be a more realistic goal. Such vaccine would control the virus and keep the viral load low and prevent immunological exhaustion. A high viral load equals a higher risk for transmission, why lowering the viremia limits the spread of HIV. For maximum efficacy of a vaccine, it probably should induce both the innate and adaptive immune systems, and stimulate T cell and neutralizing antibody responses [142]. Some cytokines and chemokines produced by cells from both the innate and the adaptive immune system, like MIP1α, MIP1β, and RANTES have a direct effect on the ability of the virus to infect target cells as they bind to the main co-receptor used by HIV and interfere with viral docking and fusion with the host cell [143]. However, the main issue is finding the proper antigen for immunization. As we showed in paper IV, the immunological targeting of a conserved peptide in early infection is beneficial and leads to a lower viral load and a slower CD4+ T cell depletion in HIV infected progressors. The identification of antigens that reduce viral fitness is crucial for a better disease progression, but finding the weakness of the virus is not enough. We also need to consider the ability of presenting such antigen in the infected individual, since the high variability of HLA alleles makes antigen presentation vary in different individuals. In paper II we proposed a way of handling the enormous variability within both the virus as well as the HLA alleles within the human population. The approach that we used to identify broadly immunogenic conserved epitopes could be useful not only for HIV vaccine design, but also for validation of vaccine trials.

Some studies have shown an adaptation of HIV on a population level. In populations with a higher frequency of certain HLA alleles, the circulating virus in that area contains HLA escape mutations corresponding to those alleles i.e. genetic footprint. This indicates that the pressure from CTLs is driving the HIV evolution [95,144,145]. Other studies suggest that the observed epitope clusters seen locally are a result of having a common ancestor during rapid transmission within a population, and not an adaptation of the virus [146,147]. These two theories are not necessarily contradictory. There is no doubt that viral evolution within an individual is driven by the cellular immune response in early and chronic infection.
There are several viral features that make HIV successful and hard to combat. 1) HIV has an extremely high mutation rate, which allows escape mutations upon pressure from the immune responses and antiretroviral drugs; 2) HIV targets and infects immune cells that become hampered, reducing the efficacy of the immune response; 3) the recruitment and accumulation of target cells upon infection and activation of the cell facilitate the spread to other target cells; 4) the viral genome is integrated within the host-cell DNA, establishing latency and enabling the virus to hide in infected cells for years; and 5) there is a long asymptomatic period after HIV infection. A person can therefore live with HIV for years without knowing, and transmit it to several individuals during this time.

Even though the complexity of the virus can seem overwhelming and the development of an effective HIV vaccine still lies in the far future, we must remain hopeful. Who knows what tomorrow brings? The constant development of techniques and assays improves our research and things we are not able to do today might be possible in the near future. Also, there may still be unsolved enigmas concerning factors associated with protections in the population, such as EUI and HIV controllers. New insights might bring knowledge that will help us to win this battle. Until then, the single most effective way to stop the spread via sexual transmission, the main route of infection, is the use of condoms. The increase of HIV and other sexually transmitted infections (STI), especially in the younger population, shows that the consequences of sexual behavior still need to be addressed. Information and easy access to condoms are extremely important and could reduce the spread of HIV. Further effective actions to reduce HIV transmission include needle exchange programs for intra venous drug users as easy access to clean needles reduces transmission in this risk group (http://whqlibdoc.who.int). We also need to change the attitude towards HIV and work to limit the stigmatization caused by the disease, which is a severe problem that millions of people living with HIV have to face every single day.
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My gratitude is beyond any words I know
So just know that I know
and that I love you for everything you are
9. References


10. Related publication - Populärvetenskaplig artikel

I Pérez CL, Karlsson AC. Att identifiera komponenter till ett optimerat hivvacin. Perspektiv på HIV, nr 21/2010
Att identifiera komponenter till ett optimerat hivvaccin

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Hur känner immunförsvaret igen en mikroorganism?

kallade HLA-molekyler på cellytan av antigen-presenterande celler (APC). De hiv-spesifika T-cellerna mognar, cirkulerar i blod och vävnad där de känner igen hiv-infekterade celler som visar samma fragment bundet till HLA-molekylerna som T-cellerna utbildats för att känna igen (Figur 1). Det finns väldigt många olika varianter av HLA-molekyler och vilka fragment av hiv som kan presenteras via de olika varianterna är väldigt specifikt och skiljer sig mycket åt. Det betyder att alla individer som får en infektion inte kommer att presentera och känna igen samma fragment av mikroorganismen eller reagera på samma sätt.

De utbildade specifika T-cellerna går ut i strid (de kallas effektorceller) och kan fanga in och oskadliggöra mikroorganismen direkt eller döda de celler som blivit infekterade, samt tillkalla förstärkning. Vissa av de utbildade immuncellerna blir i stället så kallade minnesceller. Minnesceller kan leva länge i kroppen och har redan all kunskap om mikroorganismens karaktär, vilket gör att nästa gång de kommer i kontakt med denna så kan dessa celler snabbt gå ut i strid direkt med full kraft utan att behöva utbildas innan. Vissa sjukdomar har vi lättare att bilda minnesceller mot dessa och blir vi därför inte sjuka av andra gången vi träffar på dem.

**Hur fungerar ett vaccin?**


**Varför behövs det ett hivvaccin?**

Att identifiera komponenter till ett optimerat hivvaccin

Men ett behandlande (även kallat terapeutiskt) vaccin som hjälpte den som re

dan smittats med att utbilda ett bättre immunsvar så att virusnivåerna hålls nere,

skulle också innebära stora framsteg. Vid låga virusnivåer går utvecklingen mot

aids långsammare. Den smittade skulle då kunna leva längre utan mediciner, vil-

ket skulle minska risken för biverkningar och utveckling av resistens.

Svårt utveckla ett vaccin mot hiv

då viruset ständigt förändras

Det största problemet med hiv är att virus-

set förändras väldigt snabbt och dess ut-

seende ändras så pass mycket att immun-

systemet har svårt att känna igen det. Vi-

ruset är så variabelt att de varianter som

can ses hos en enda infekterad individ är

fler än hela den världsomspännande va-

riationen av influensavirus under en epi-

demi. Det betyder att trots att cellerna gör

sitt jobb och utbildar immunsystemet för

to känna igen viruset, hinner det inte

med att lära om i samma hastighet som

viruset förändras, utan ligger hela tiden

steget efter.

Tanken med ett hivvaccin är att man i

stället ska ligga steget före, men än så

länge har ingen lyckats framställa ett så-

dant vaccin. Mycket forskning har foku-

serat på att utveckla ett hivvaccin och flera

kandidater har sett lovande ut samt gett

upphov till hivspecifika immunceller i de

vaccinerade individerna. Men när den

vaccinerade senare träffat på viruset på

riktigt så har inte dessa immunsvar kun-

nat stoppa infektionen. Efter det hitills

mest lyckade vaccinförsöket, Thai fas III-

förövning (RV144), såg man ett visst läg-

gradigt skydd mot infektion i vaccingrup-

pen, men ingen skillnad i sjukdomsför-

loppet efter att man hade blivit infekte-

rad. Hittills har inga av de andra

vaccinförsöken heller lyckats med att hål-

la nere virusnivåerna i dem som smittats.

Ett av de större vaccinförsöken, STEP, av-

bröts i november 2007 då det inte visade

gig ge något skydd emot hivinfektion

utan snarare tvärtom. På en stor virus-

konferens (CROI) som hölls i San Fran-

cisco i februari tidigare i år presenterades

de senaste resultaten från fortsatta stu-

dier på de som blivit smittade trots att de

vaccinerats. De kunde då se att viruset

Figur I. Hivspecifik T-cell känner igen virusinfekterad cell. Den hivinfekterade cellen visar upp

e ett litet fragment av viruset för T-cellen genom att binda och presentera det på en HLA-molekyl

på cellens yta. Då T-cellen binder och känner igen fragmentet kan den döda den virusinfekte-

rade cellen samt tillsamma hjälpa och oskadliggöra virus.
Att identifiera komponenter till ett optimerat hivvaccin

såg lite annorlunda ut hos de infekterade individer som vaccinerats jämfört med de som inte fått vaccin. Detta kan betyda att de upplärda immuncellerna antingen stoppade infektion av virus som var likt just det de blivit utbildade för, men inte hade något skydd emot alla andra varianter. Ett mer troligt alternativ är att de upplärda immuncellerna gjorde sitt jobb efter att individen blivit infekterad, men att immunförsvaret inte klarade av att känna igen viruset när det förändrade sig. Återigen låg immunsystemet steget efter viruset. I båda fallen visar detta ändå att vaccinet hade haft någon typ av påverkan på hiv, men att viruset inte hade haft några större problem att byta utseende och gömma sig för de utbildade immuncellerna.

**Finns det någon lösning?**


En annan viktig faktor att ta hänsyn till är variationen mellan människors HLA-molekyler, vilket gör att inte alla kan presentera och känna igen samma fragment. Även om man skulle identifiera fragment som är kritiska för viruset, så skulle de kanske inte kunna presenteras för immunsystemet om man inte hade "rätt" HLA-molekyl. Idealt vore att hitta fragment från ursprungliga delar av viruset som kunde kännas igen, samt vara möjliga att presenteras i majoriteten av den mänskliga befolkningens HLA-molekyler.

**Identifiering av optimerade T-cells svar**

I dag vet vi ännu inte vilka kvalitéer ett effektivt immunsvar mot hiv behöver för att kontrollera virusproduktionen. Den enorma variationen som finns mellan olika hivvarianter och olikheten mellan människors HLA-molekyler gör det hela väldigt komplicerat att förstå. Men genom att samarbeta med bioinformatiker har vi i vår grupp kunnat ta hänsyn till dessa faktorer och identifierat hivsvar som är mer stabila och inte lika påverkbara av hivvariation och olikheter mellan HLA-molekyler. I studien som publicerades i tidskriften *Journal of Immunology* (C. L Pérez et al, 2008) lyckades vi med hjälp av bioinformatiska modeller identifiera ett mindre antal hivfragment. Fördelen med dessa fragment är att de: 1) presenteras och känns igen i ett stort antal hivpositiva individer med ursprung från olika delar av världen och som uttryckte många olika HLA-molekyler, 2) känns igen av patienter infekterade med hivvarianter tillhörande många olika subtyper, och 3) är väldigt konserverade (ursprungliga) hos alla olika virusvarianter och subtyper. Det betyder att viruset troligtvis kommer att få svårt att fly från immunsvar som riktas mot dessa fragment och att de kommer att kunna presenteras i många hivinfekterade individer trots att de är smittade med olika varianter av hiv.

Hiv består av mer än 3 000 byggstenar, medan de fragment som presenteras på HLA-molekylen optimalt är nio byggstenar långa. Genom att på ett effektivt sätt skära ner på storleken av viruset och bara fokusera på de bitar som faktiskt kommer att påverka immunsvaret gör vi det
Att identifiera komponenter till ett optimerat hivvaccin


Vad har vi oss att vänta i framtiden?

Trots att man inte nått vårt mål ännu och fortfarande har en lång väg kvar till ett effektivt hivvaccin får vi inte glömma alla de framsteg som gjorts. Den snabba utvecklingen av ny teknik gör det möjligt att söka och erhålla kunskap vi inte hade möjlighet till för bara några år sedan, då vi inte hade rätt verktyg och metoder för den typen av studier. Vår förhoppning är självklart att vi till slut ska kunna hitta ett sätt att översluta hiv och förhindra smittspridningen. Men i dagsläget så är det fortfarande användandet av kondom som är det absolut mest effektiva sättet att undvika infektion vid sexuell smitta.