ON THE IMMUNOPATHOGENESIS OF HIV INFECTION

Jakob Nilsson

Stockholm 2006
The cover image shows a photomicrograph of the accumulation of FOXP3+ regulatory T cells within the parafollicular area of a tonsil from an HIV infected individual with a progressive infection. FOXP3 protein is stained in brown, cell nuclei are counterstained with HTX in blue. The micron bar in the top left corner indicates 20 microns.

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For Anna
Abstract
CD8+ T cell dependent clearance of microbial infections by perforin mediated killing of infected cells is a central component in the combat against viral infections. In HIV-1 infected individuals the incidence of virus infected cells in lymphoid tissue, which is the major site for virus production, does not drop, but instead slowly increases as the disease progresses. This suggests that the function of HIV-specific CD8+ T cells in the lymphoid tissue of HIV infected individuals is compromised. The work presented in this thesis has focused on the study of the immune response in lymphoid tissue of HIV infected individuals. The relation between granzyme A and perforin expression in lymphoid CD8+ T cells was assessed in HIV infected individuals, at the single cell level. While the expression of granzyme A in lymphoid tissue was extensively increased as compared to uninfected controls, no concomitant increase in the expression of perforin was noted. This was in contrast to expression in lymphoid tissue of individuals with acute EBV infection, where levels of granzyme A and perforin were concomitantly up regulated. The absence of perforin expression in lymphoid tissue of HIV infected individuals was not due to lack of infected cells since we showed that both intracellular HIV DNA and RNA levels were typically 10-100 times higher in lymphoid tissue as compared to peripheral blood. The low expression of perforin may depend on skewed production of cytokines required for the activation and maturation of efficient cytotoxic CD8+ T cells. In order to address this we analyzed the expression of several chemokines and cytokines in the lymphoid tissue of individuals that were undergoing acute symptomatic HIV infection. A profound and early immune activation was evident during acute HIV infection, with increased expression of β-chemokines and also elevated levels of both Th1 and Th2 type cytokines. This immune activation was associated with the accumulation of CD8+ T cells that expressed granzyme A but not perforin. The presence of a selective impairment in the expression of perforin, in spite of increased levels of several immune activating cytokines, lead us to investigate potential factors that could interfere with efficient cell mediated immunity. One such factor is regulatory T cells (T_{reg}), characterized by the constitutive expression of the transcription factor, FOXP3. Indeed, active HIV-replication was associated with a significant accumulation of T_{reg} within lymphoid tissue and subsequently increased expression of mediators associated with T_{reg} suppressive function, such as CTLA-4, TGF-β and the tryptophan catabolising enzyme, IDO. Furthermore, the accumulation of T_{reg} within lymphoid tissue was correlated with plasma viral load in HIV infected individuals, suggesting a link between T_{reg}, viral load and disease progression. We also showed that the accumulation of T_{reg} and mediators associated with T_{reg} function in lymphoid tissue was only evident in individuals with a progressive HIV infection and thus absent in HIV infected subjects with a non progressing type of infection, who are naturally able to control virus replication. Similar results were also obtained in a cohort of SIV infected rhesus macaques. It is likely that the accumulation of T_{reg} can have negative influence on antiviral cell mediated immunity since we found that a high perforin/FOXP3 ratio in lymphoid tissue was associated with a non progressing type of infection in both SIV infected rhesus macaques and HIV infected individuals.

Novel therapeutic approaches aimed at enhancing immune function in HIV-infected patients should likely be targeted to the manipulation of T_{reg} numbers and/or function in order to improve immunity.

Keywords: Human Immunodeficiency Virus-1, Lymphoid tissue, cell mediated immunity, CD8+ T cells, perforin, cytotoxicity, regulatory T cells, FOXP3.

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List of publications

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

Low levels of perforin expression in CD8+ T lymphocyte granules in lymphoid tissue during acute human immunodeficiency virus type 1 infection.
*Journal of Infectious Diseases* 2002 May 1;185(9):1355-8.

Early immune activation in gut associated and peripheral lymphoid tissue during acute HIV infection.
Submitted *AIDS* 2006

The prevalence of regulatory T cells in lymphoid tissue is correlated with viral load in HIV-infected patients.

HIV-1 driven regulatory T cell accumulation in lymphoid tissues is associated with disease progression in HIV/AIDS.
*Blood* 2006 prepublished online August 10, 2006; DOI 10.1182/blood-2006-05-021576
Jakob Nilsson

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<th>Description</th>
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<tbody>
<tr>
<td>AIDS</td>
<td>Acquired Immune Deficiency Syndrome</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
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<tr>
<td>ART</td>
<td>Anti Retroviral Therapy</td>
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<tr>
<td>AZT</td>
<td>Azidothymidine</td>
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<tr>
<td>CCR</td>
<td>CC chemokine receptor</td>
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<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
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<tr>
<td>CTL</td>
<td>Cytotoxic T Lymphocyte</td>
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<tr>
<td>CTLA-4</td>
<td>Cytotoxic T cell Antigen 4</td>
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<td>CXCR</td>
<td>CXC chemokine receptor</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic Cell</td>
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<tr>
<td>DC-SIGN</td>
<td>DC-specific ICAM-3 grabbing non-integrin</td>
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<tr>
<td>EBV</td>
<td>Epstein Barr Virus</td>
</tr>
<tr>
<td>FOXP</td>
<td>Forkhead-winged-helix transcription factor</td>
</tr>
<tr>
<td>GALT</td>
<td>Gut Associated Lymphoid Tissue</td>
</tr>
<tr>
<td>GITR</td>
<td>Glucocorticoid-Induced TNFR family-related Receptor</td>
</tr>
<tr>
<td>HAART</td>
<td>Highly Active Anti-Retroviral Therapy</td>
</tr>
<tr>
<td>HLA</td>
<td>Human Leukocyte Antigen</td>
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<tr>
<td>HIV-1</td>
<td>Human Immunodeficiency Virus 1</td>
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<tr>
<td>HIV-2</td>
<td>Human Immunodeficiency Virus 2</td>
</tr>
<tr>
<td>IDO</td>
<td>Indoleamine 2,3 –dioxygenase</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>LN</td>
<td>Lymph Node</td>
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<tr>
<td>LTNP</td>
<td>Long Term Non Progressor</td>
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<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
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<tr>
<td>MIP</td>
<td>Macrophage Inflammatory Protein</td>
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<tr>
<td>NK</td>
<td>Natural Killer Cell</td>
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<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cells</td>
</tr>
<tr>
<td>pDC</td>
<td>Plasmacytoid Dendritic Cell</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated on T cell Activation, Normal T cell Expressed and Secreted</td>
</tr>
<tr>
<td>RM</td>
<td>Rhesus Macaque</td>
</tr>
<tr>
<td>SIV</td>
<td>Simian Immunodeficiency Virus</td>
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<tr>
<td>SM</td>
<td>Sooty Mangabey</td>
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<tr>
<td>TCR</td>
<td>T Cell Receptor</td>
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<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
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<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
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<tr>
<td>T_{reg}</td>
<td>Regulatory T cell</td>
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<td>UNAIDS</td>
<td>Joint United Nations Program on HIV/AIDS</td>
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<td>WHO</td>
<td>World Health Organization</td>
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Introduction

HIV

The epidemic
The global human immunodeficiency virus (HIV) epidemic has despite intensified prevention and treatment strategies continued to expand and at the end of 2005 it was estimated that 40.3 million people were infected with HIV worldwide. The number of people newly infected during 2005 was believed to be 4.9 million and approximately 3.1 million people died in 2005 due to HIV infection (1). The driving force for the epidemic differs among countries and while heterosexual spread is the main route of transmission in sub-Saharan Africa, intravenous drug use is a major contributor for the epidemics in Asia and Eastern Europe. There is however a great risk for the development of an extensive sexually spreading epidemic in Eastern Europe, since intravenous drug use is often financed by commercial sex work in this region (1). Even though the HIV prevalence in Sweden remains low (2) a call for caution might be warranted, with the rapid increase in Estonian HIV-1 epidemic as a potential risk factor. One should also be aware that the reported condom use in Sweden is low (3) which can also be seen in the massive increase of Chlamydia infection (~90% increase in incidence since 1999) that has taken place during the last years (2).

HIV-1 and HIV-2
There are two separate HIV viruses, HIV-1 and HIV-2 that can spread between humans and cause acquired immunodeficiency syndrome (AIDS). While HIV-1 is the virus responsible for the global HIV epidemic and the vast majority of HIV infected individuals are infected with HIV-1 (1), HIV-2 is most prevalent in western Africa. There are however also significant numbers of HIV-2 infected people in other regions, especially in regions with past socio economical connections with Portugal, like south west India, Angola, Mozambique and Brazil. While HIV-1 has a pandemic profile with rising prevalence in the affected developing countries, HIV-2 is mostly endemic with stable prevalence (4). Apart from having altered epidemiological features the two HIV types also show differences in disease progression and are believed to have a separate origin (4). HIV belongs to the Retroviridae family in the Lentivirus genus. This genus also includes Simian Immunodeficiency Virus (SIV), which has been shown to infect several African monkeys and apes (5). The modern history of SIV actually started some 12 years prior to the recognition of AIDS in humans when there was an outbreak of lymphomas in captive rhesus macaques (RM) that were kept at the California National Primate Research Centre (6). This
outbreak was at that time not thought to be of infectious origin even though immune suppression and opportunistic infections were found. The sick RM were kept together with apparently healthy sooty mangabeys (SM) but it was not until much later that the SM were recognized as the carriers of the SIV that cased the AIDS-like disease in RM (6). The infection of RM with SIVsm from SM is now considered to be the best available model of HIV infection in humans and is widely used for HIV research.

**AIDS as a zoonosis**

Compelling phylogenetic evidence show that the viruses evolving into HIV-1 and HIV-2 were transferred to the human population from SIV infected African monkeys. HIV-1 stems from chimpanzees which harbour the strongly related SIVcpz and HIV-2 originated from sooty mangabeys harbouring SIVsm (7-10). Both of these monkeys are residents of east and central Africa where they are in close contacts with humans, both as pets and as a source of meat (11). These relations enables routes of cross-species transmission, but despite this large exposure of humans to SIV infected monkeys only 10 cross-species transmissions are believed to have occurred during the last century (5). This indicates that there are species restriction factors in place and suggests that cross-species transmitted viruses are not sufficiently adapted to the new host to be able to cause an epidemic. This requirement for virus adaptation is not unique to HIV and a similar situation is present in the avian flu spread into the human population. In a strict sense this also disqualifies the view of HIV as a zoonosis.

**Why did the HIV epidemic start in the 1980s?**

It is believed that the virus responsible for the global HIV (onward HIV-1 is referred to as HIV) epidemic entered the human population somewhere between 1915 and 1940 in central Africa (12). Retrospective analysis of old blood samples have also been able to show presence of HIV in a plasma sample, obtained 1959, in Leopoldville, Belgian Congo, now Kinshasa, Democratic Republic of Congo (13). HIV was also retrospectively found in a Norwegian family consisting of a couple and their daughter. The father of the family was a sailor and visited several east African ports during the early 1960s and was probably infected around this time. He then subsequently infected his wife and during a later pregnancy the infection was vertically transmitted to their daughter, who was born in 1967. All of the family members died from AIDS in 1976 (14). So why did the epidemic not kick off in Norway during the 1970s or even during the 1940s since HIV infected humans appear to have been around for quite a long time? Several explanations for this has been put forward, most of them suggesting deforestation, urbanization, increased travel, prostitution, population growth and changes in social behaviour as major contributors for the emergence of the HIV epidemic during the end of the 20th century (15, 16). An important factor may also be the increased use of injections, with unsterile
needles and syringes, which might promote viral adaptation through serial passages or facilitate other forms of viral adaptation such as recombination (17-19).

**Pharmacological treatment of HIV infection**
The introduction of effective anti retroviral therapy (ART) during 1995 and 1996 has dramatically changed the situation for HIV infected people in the industrialized world (20). The first antiretroviral that was developed azidothymidine (AZT, a nucleoside analogue) was approved for use in 1987. Mono therapy with AZT was however often not effective since viral resistance frequently occurred. The addition of two new classes of anti retrovirals (non-nucleoside analogues and protease inhibitors) in 1995 and 1996 made combination therapy possible. This proved to be very effective in prolonging the life of HIV infected persons and preventing opportunistic infections by limiting viral replication (20). While the combination therapy is effective at suppressing virus replication it is not without side effects, such as lipodystrophy and increased risk for myocardial infarction. The current strategy of treatment is to wait as long as possible before introducing ART (21). Therapy is often initiated below a certain CD4 count in peripheral blood, such as 200 CD4+ cells per microliter of blood, and while therapy initiated earlier might “save” more CD4+ cells, especially in mucosal tissues, this has not been associated with a better functioning anti HIV response or a better ability to control viral load at a later treatment interruption (22, 23). Recently, HIV fusion inhibitors have been approved for clinical use and even though this represents an interesting new approach with low toxicity they have to be used in combination with existing drugs to limit the risk for resistance mutations (24). New pharmacological therapies are being developed and the use of small molecule inhibitors to interfere with HIV entry and replication represents an interesting new strategy for the treatment of HIV infection.

**HIV lifecycle**
The HIV virus is a retrovirus and as such the infective HIV virions contain a double stranded RNA which is 9.2kb long. The HIV genome contains 9 genes (encoding 15 proteins), consisting of two regulatory (rev, tat) and four accessory (vif, vpu, nef and vpr) in addition to env, gag and pol which encode the major structural proteins. To enter its host cell HIV utilizes the CD4 receptor, which binds to the viral gp120 (25). This induces a conformational change, which enables binding to either β-chemokine receptor CCR5 (R5 strains) or CXCR4 (X4 strains) as a co-receptor (26).
Figure 1. Schematic illustration of the HIV lifecycle within an infected cell. RT reverse transcriptase, LTR long terminal repeat. Adapted from (27).

Since CCR5 and CXCR4 are often present within lipid rafts of similar composition as the viral lipid bi-layer, the recruitment of these co-receptors aids the viral fusion (28). After fusion is accomplished the virus is unpackaged and the nucleocapsid is dismantled. The reverse transcriptase (RT) then reverse transcribes the viral RNA into DNA, which subsequently enters the cell nucleus and is incorporated into the host cell DNA by the viral integrase. The RT encoded by pol does not have any proof reading capacity and is extremely error prone, generating around one error per replication round (29). This is the basis for the extremely rapid evolutionary speed of HIV that allows it to evade neutralizing antibodies and also quickly develop resistance to pharmacological therapies. The most variable part of the HIV genome is the env that encodes the surface proteins gp120 and gp41. It is a generally accepted fact that if you sequence virus from a patient’s peripheral blood you will never sequence two viruses that have the exact same env sequence. When the virus is actively replicating the structural proteins and the double stranded RNA are assembled into viral particles at the cell membrane, where the new virions finally bud from cholesterol-rich rafts in the plasma membrane (30).
Natural course of HIV infection
Infection with HIV is in most cases (>75%) associated with clinical symptoms of variable severity and duration (31). These symptoms usually occur 12-16 days after infection and most commonly consist of fever, fatigue, sore throat, headache and lymph gland enlargement. HIV viral load can be detected around a week after infection. The viral load then rapidly increases to peak about a week after the onset of symptoms (19-23 days after infection). After this peak there is a decline in viremia during the following two weeks, this is associated with lymph gland enlargement, CD8+ lymphocytosis and the appearance of IgG-antibodies. The viral load then continues to slowly decline and usually reaches a set point 3-4 months after infection (31). A correlation between the peak viral load and the viral set point has been shown, this implies that early events during HIV infection may to a large extent determine disease prognosis (32, 33). The acute phase of infection is then followed by a stage of clinical latency when a steady state of viral production is present (34-36). The disease progression can be visualized by a steady and ongoing drop in peripheral CD4+ T cell counts and if treatment is not initiated progression to AIDS occurs with the emergence of opportunistic infections and subsequent death of the infected individual (37). The time from infection to fatality is variable but take around ten years in most infected individuals (38).

Figure 2. Natural course of HIV infection. The diagram illustrates the relationship between peripheral blood CD4+ T cell count and plasma viral load. R5 and X4 indicates CCR5 and CXCR4 utilizing viruses. Adapted from (39).
Innate immune responses in HIV infection

Innate immunity represents the first line of defence against microbial infections. The innate immune system is activated by conserved structures on invading pathogens and greatly assists in regulating subsequent adaptive immune responses. Several robust effectors of innate immunity could potentially assist in limiting HIV infection, but like many successful pathogens HIV has been shown to be able to disrupt several pathways of the innate immune system. A role for innate immunity in resistance to infection and in limiting viral replication has however been suggested. One of the hottest topics in innate immunity towards HIV is host resistance factors that are able to interfere with the early steps of retroviral infection. Two of the effectors that have attracted a lot of attention are TRIM5-alpha and APOBEC3G (40-42). While TRIM5-alpha is believed to mediate species specific restriction of retroviruses, APOBEC3G can be packaged into virions and cause mutations which are incompatible with further virus replication. A more detailed description of ABOBEC3G function and how it is inhibited by HIV will be presented in a later section of this thesis dealing with the effect of free HIV viral proteins. Other factors that are thought to be able to limit HIV infection are the CCR5 binding β-chemokines, including RANTES (encoded by CCL5). Elevated expression levels of β-chemokines due to genetic polymorphism has been associated with resistance to infection (43, 44). IFN-α is another innate effector with potent antiviral effects that has been shown to limit HIV production in vitro (45). Our own recent data also show that RANTES and IFN-α expressed locally in the cervical mucosa is associated with persistent HIV seronegativity in female sex workers with a high risk behaviour for contracting HIV (46). The beneficial effects of both RANTES and IFN-α in the setting of a disseminated HIV infection can however be questioned. We have in paper II shown a massive β-chemokine expression that is present already prior to peak viremia in individuals undergoing acute HIV infection. Recent studies also implicate IFN-α in the continued loss of CD4+ T cells that is a hallmark of progressive HIV infection (47). Several other innate effectors such as Secretory Leukocyte Protease Inhibitor (SLPI) and human β-defensins 2 and 3 (hBD-2 and hBD-3) have been shown to limit HIV replication in vitro, but their contribution to anti HIV innate immunity in vivo remains to be proven (48, 49). In summary, although many innate effectors have been suggested to possess anti HIV activity the relative effect they have on HIV transmission and disease progression remains to be elucidated. There are probably also several undiscovered innate effectors that participate in limiting HIV replication in vivo, including the elusive Cellular Antiviral Factor (CAF) (50). Furthermore, if these innate effectors are expressed prior to infection they may mediate a more efficient protection against HIV infection as compared to their effect in an already established systemic HIV infection.
**Effects of HIV free viral proteins**

HIV virus replication is an error prone process and it is estimated that only a very small fraction of produced viral products are incorporated into infectious virions. As a consequence of this many HIV proteins are produced in great excess. Apart from the prototypical retroviral Gag, Pol and Env proteins, HIV-1 produces six additional proteins i.e. Tat, Rev, Nef, Vif, Vpr and Vpu. While Tat and Rev are needed for virus production Nef, Vif, Vpr and Vpu are often dispensable for virus growth in many *in vitro* systems and are as such known as auxiliary proteins. They have however important functions for viral replication and pathogenesis *in vivo*. Apart from regulating and facilitating virus production within an infected cell, many of these proteins have also gained immunomodulatory properties. Several HIV viral proteins have been implicated in interfering with innate antiviral responses mounted by infected cells and with adaptive immunity (51). I will discuss a few of these findings in this section.

The HIV accessory protein Viral Infectivity Factor (Vif) has long remained one of the most poorly understood HIV proteins. It has been known for some time that Vif is only needed for viral replication in some cell types such as primary CD4+ T cells and macrophages, while HIV replication in common cell lines such as HeLa and 293T is Vif independent (52). It was not until the identification of the cell factor mediating Vif dependency in non permissive cells that understanding of Vif function was improved (53). The host restriction factor, a cytidine deaminase, is the previously mentioned APOBEC3G (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G) (54). It appears that the function of APOBEC3G is to remove the amino group from cytosine bases and thus create uracil bases (55). APOBEC3G is thought to be incorporated into produced HIV virions in infected cells (54). Incorporation of APOBEC3G into HIV virions restricts HIV infection, probably by inhibiting integration of reverse transcribed HIV cDNA (56). APOBEC3G has been shown to have a broad specificity and is able to block other viral infections (42). APOBEC3G also appears to be conserved in several species, suggesting a central role for APOBEC3G in innate antiviral defense (56). In order to circumvent this antiviral defense mechanism HIV Vif appears to bind directly to APOBEC3G and target it for ubiquitination and subsequent degradation by cellular proteasomes. As an effect of this APOBEC3G is not incorporated into produced virions and infection of new target cells remains uncompromised (57). A further effect of APOBEC3G in mediating the resistance to HIV infection in primary PBMC has also been recently suggested. It has been shown that cells residing in lymphoid tissues or PBMC activated by cytokines such as IL-2 and IL-15 are naturally permissive to HIV infection due to the presence of APOBEC3G in a high molecular mass complex (58). Primary PBMC however have been shown not to have APOBEC3G in a high molecular mass complex.
Even though these data are exciting they remain preliminary findings and further research into this interesting area is needed.

HIV Viral Protein U (Vpu) is specific for HIV-1 and not present in HIV-2, suggesting that it may be involved in the increased virulence of HIV-1. Research on Vpu has gained insight into two major functions for this viral protein. Vpu appears to greatly enhance viral particle release from the cell membrane. Exactly how this is mediated remains to be elucidated but it is believed that Vpu can form an ion channel at the plasma membrane by oligomerisation. It has been suggested that these ion channels might increase particle release by changing the membrane potential (59). The fact that virus particles from engineered viruses lacking Vpu can efficiently release from cells of Simian origin suggests that Vpu somehow interferes with a human factor that compromises the release of viral particles, this human factor has however not been identified (60). The second major action of Vpu that has been described is the ability of Vpu to bind and target intracellular CD4 molecules for degradation. This is believed to enhance viral replication by inhibiting intracellular CD4-HIV envelope binding, which causes non-infectious HIV lacking envelope to form (61). Inhibiting CD4 production might also have several immunological consequences.

The HIV accessory protein that has attracted the most research attention during the last years is probably the Negative Factor (Nef). Nef is the first HIV protein expressed in infected cells where it is subsequently targeted to the cell membrane. Nef was first thought to have a negative effect on viral replication in vitro (as the name implies), but has later evolved as one of the most important viral factors in enhancing viral replication and spread (62). This is exemplified in vivo where infections with HIV that has deletions within the Nef gene show a slower disease progression (63, 64). One of the primary functions for Nef is in the interference with endosome trafficking. This functionally alters the expression of several plasma membrane proteins that use these endocytic pathways. Among these are many molecules with important immunological functions such as; CD4, MHC-I, MHC-II, TNF and CD28 (65). The ability of Nef to down regulate CD4 expression is believed to be important for HIV infection and has recently been linked with HIV disease progression (66). The down modulation of MHC is thought to interfere with cell mediated adaptive immunity, while still sparing infected cells from NK-cell mediated killing. This is suggested to be based on a selective down regulation of HLA-A and HLA-B, while HLA-C and HLA-E remain expressed and as such interacts with inhibitory receptors on NK-cells (67). Apart from its role in interfering with endosome trafficking Nef has major effects on the cell signaling cascades. This is mainly based on the interactions between several key signaling molecules and the Src homology-3 binding domain on Nef. Given that many signal transduction proteins carry a Src homology-3 domain the effects of Nef are
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multiple and often seem to be counteractive (68). It is clear however that Nef has several important effects on cell activation, apoptosis and migration. Nef can also be released into the extra cellular space and enter uninfected CD4+ T cells and macrophages. Internalized Nef is then able to activate Nuclear Factor–kappaB (Nf-kappaB) which subsequently renders the cell more susceptible to HIV infection and optimizes the target cell for production of HIV virions (69). It should be mentioned however that data on the effect of exogenous and endogenous Nef are often contradictory and some studies have indeed found a negative effect on Nf-kappaB expression mediated by Nef (70). Another prominent action of Nef is its ability to enhance viral infectivity. This mechanism remains, despite intensive research, elusive. A recent publication has suggested the modification of the cortical actin skeleton as the principal action of Nef that increases viral infectivity (71). This is an interesting suggestion that might be able to reconcile several previous findings, but further research to clarify this issue is needed. Taken together the effect of Nef has become one of the trickiest issues in HIV research and though much remains to be clarified it is clear that Nef is crucial for HIV pathogenesis.

HIV Rev and Tat do not belong to the classical HIV auxiliary proteins since they need to be present in a virus infected cell for efficient production of virus to occur in vitro. While Rev has important functions in exporting transcripts from the cell nuclei, Tat functions as a highly effective transcription factor (72, 73). Apart from these functions Tat has also been implicated in interfering with anti HIV immunity. Extra cellular Tat has been shown to induce production of several cytokines including IL-2 and TGF-β (74, 75). Tat has also been suggested to be involved in HIV induced neurotoxicity and induction of apoptosis in cultured PBMC (76-78).

In summary HIV viral proteins have in addition to their functions in assisting with virus production also evolved several properties that impede with effective anti HIV immunity. These properties include the ability to counteract robust innate factors that interfere with virus infection and production as well as effects that limit the ability of the adaptive immune system to identify and kill virus infected cells. Aside from these “immunosuppressive” features many of the HIV proteins have evolved additional immune activating features. Since both virus production and infectivity seems to be heavily dependent on the activation status of the susceptible cell this appears to be crucial for efficient virus production and dissemination within the human host.

Humoral immune responses in HIV infection

An efficient mechanism to clear viral pathogens and prevent subsequent infections with the same pathogen is the production of neutralizing antibodies by the human immune system (79). However in trying to elicit neutralizing
antibodies towards HIV the immune system faces an extremely difficult task (80, 81). This is mainly due to two separate reasons; First, the HIV envelope (the natural target for neutralizing antibodies) is heavily glycosylated which makes it poorly immunogenic (25). Secondly, HIV is readily able to mutate around produced neutralizing antibodies by way of its extremely high mutation rate. This is aided by the fact that the envelope tolerates a high portion of diversity without compromising on its function (80). This result in a situation where the humoral immune system is always playing “catch up” with the virus, which has often evolved into a different looking virus by the time that the immune system has managed to produce a sufficient amount of a neutralizing antibody towards a specific viral quasi species. These mechanisms likely explain why the presence of neutralizing antibodies is probably not directly responsible for the early down regulation of viremia and does not generally correlate with protection against disease progression (82, 83). It should however be mentioned that there are a few broadly neutralizing antibodies described in the literature and efforts to synthesize immunogens that will elicit these types of antibodies are underway (84-86). This broadly neutralizing capacity has however only been shown for lab adapted strains and the efficiency of these antibodies in vivo remains an open issue.

**Cell mediated immunity in HIV infection**

The clearance of virus infected cells is dependent on cell mediated immunity. In HIV infection the importance of cell mediated immunity has been demonstrated by several experimental approaches. I will only briefly describe two of these approaches in this section, since a more detailed discussion on the importance of cell mediated immunity will follow in later sections of this thesis (87). Firstly, the depletion of CD8 positive T cells from the immune system during SIV infection of rhesus macaques leads to a massive increase in virus production (88). Secondly, better functioning cell mediated immunity towards HIV has frequently been linked with the ability to control virus production in patients who show slow or halted disease progression (89-95). The specific function of cell mediated immunity that mediates control of HIV viremia has still not been completely elucidated. There are reports on the importance of soluble factors that repress virus replication (96, 97), but evidence for the importance of fully mature poly-functional CD8+ effector T cells in the control of HIV viremia has been mounting (94, 95). Fully mature CD8+ T cells are able to eliminate virus infected cells by the use of cytotoxic granules containing the pore forming molecule perforin and apoptosis inducing serine-proteases termed granzymes (98). This pathway has been shown to be crucial for the ability to eliminate viruses in experimental models (99, 100). Increasing evidence also suggests that perforin expressing CD8+ T cells are important in the control of HIV infection (94). Finding correlates of protection from disease progression in HIV infection is crucial, since the development of an effective therapeutic vaccine will depend
on the vaccines ability to induce protective immunity. In order to generate such a vaccine we will need to assess the relevance of vaccine induced responses in human subjects by means other than a live virus challenge (for obvious reasons). By identifying the correlates of protection in individuals with a slow or halted disease progression we can establish a pattern of immune responses that potential vaccine candidates can be benchmarked against.

Regulatory T cells

History of Regulatory T cells
The idea of a suppressor T cell was first put forward in the 1970s by RK Gershon when he suggested that T cells could act as regulatory cells by suppressing immune responses. During the 1970s and the early 1980s it was mainly believed that suppression was mediated by soluble factors that were encoded by an I-J gene within the major histocompatibility complex (MHC) and immunoglobulin (Ig) genes. When molecular studies during the later part of the 1980s showed that MHC and Ig were encoded by separate genes, and failed to find evidence of I-J determinants, suppressor T cells lost their appeal. The word suppressor T cells became stigmatized in mainstream immunology and only a few immunologists continued to work on T cell mediated suppression (101). It was not until 1995 that suppressor T cells re-entered the stage, when Shimon Sakaguchi described that the depletion of a population of T cells expressing the high affinity interleukin 2 (IL-2) receptor α-chain (CD25) in mice caused several autoimmune disorders and that these could be reversed by the adoptive transfer of CD25 positive cells (102). These cells were termed regulatory T cells (T\text{reg}) by Sakaguchi, probably to avoid the connection with the troublesome history of suppressor T cells (103). Sakaguchi and co-workers were also able to show that T\text{reg} are produced in the Thymus, thus providing an elegant link between central and peripheral tolerance(104). With the identification of mutations within the forkhead-winged-helix transcription factor FOXP3 as the cause for the human X-linked recessive disease IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) and scurvy in mice FOXP3 was discovered as a selective marker for T\text{reg}, that was also able to drive naïve T cells into a regulatory phenotype (105-108). Recent data indicates that FOXP3 mediates its function by binding to the inducible transcription factor NFAT (109). NFAT can also bind to AP-1 and cause immune activation, so it appears that NFAT directs opposing programs of T cell activation and T cell tolerance by recruiting alternate transcriptional partners (AP-1 versus FOXP3). The efforts of these pioneering immunologists have lead an explosive increase in the research on T\text{reg}, which is exemplified by the >2000 publications with relations to regulatory T cells that have been published during the last five years (110).
Central and peripheral T cell tolerance

In order to prevent the “horror autotoxicus” as described by the pioneering immunologist Paul Ehrlich (i.e. the immune system attacking its host) a number of mechanisms are in place that collectively contribute to tolerance to self (111). These have been divided into central and peripheral tolerance, where central tolerance occurs in the thymus and peripheral tolerance, as the name suggests, is maintained in the periphery. Since the random recombinatory mechanisms that result in the development of an almost unimaginable receptor diversity of T cell receptors (TCR) also gives rise to large portion of cells (20-40%) that bind self antigen with a potentially dangerous affinity, these auto reactive cells have to be deleted or kept in check (112). In the thymus this is accomplished by a process termed negative selection in which auto reactive T cells that bind self peptide MHC-complexes with high avidity are deleted. This process acts together with the positive selection of T cells that are able to bind self peptide MHC-complexes with at low or intermediate affinity in order to generate a peripheral T cell compartment that is able to recognize numerous foreign antigens without the risk of autoimmunity. Evidence shows that this process is not perfect however, since auto reactive T cells can be detected in the periphery of healthy individuals (113). These auto reactive cells are in the healthy individuals kept in check by additional mechanisms collectively referred to as peripheral tolerance. T_{reg} can, as previously stated, be developed in the thymus and assist in up holding peripheral tolerance. The mechanisms by which T_{reg} suppress auto reactive cells will be discussed in a later section of this thesis. While several aspects of the other mechanisms that assist in upholding the peripheral tolerance remain unknown some pathways have been elucidated. In order to become functionally active and proliferate T cells need additional signals apart from antigenic stimuli. These additional signals consist of both co-stimulatory molecules on antigen presenting cells and the presence of pro-inflammatory cytokines. The lack of additional signals when a self reactive T cell encounters its cognate antigen will prevent activation and proliferation of that cell, thus assisting in upholding the peripheral tolerance (111). The importance of these functions can be visualized by the fact that manipulation of these systems can lead to the breaking of tolerance. The addition of an agonistic antibody stimulating the important co-stimulatory receptor CD28 for example lead to the onset of acute autoimmunity in a number of unfortunate individuals who were taking part in a novel drug trial (114). The addition of recombinant pro-inflammatory cytokines like IFN-α is also associated with increased risk of developing autoimmune diseases such as autoimmune hyperthyroidism (115).

Natural or induced regulatory T cells

In line with the Linnean obsession of dividing everything into separate entities (despite the knowledge that immune cells seem to be highly dynamic when it comes to the expression of receptors and cytokines), regulatory T cells have also
been subjected to efforts of division into subclasses. T_{reg} expressing FOXP3, CD4 and high levels of CD25 are termed natural regulatory T cells and are believed to originate in the thymus (104). Recent data however indicates that these cells might be generated in the periphery from non-regulatory cells under specific circumstances, such as the presentation of antigen under sub-optimal conditions (116). Regulatory cells expressing little or no FOXP3 that produce preferentially large amounts of interleukin 10 (IL-10) are known as T regulatory 1 cells (Tr1) (117). The intraepithelial T cells of the gut associated lymphoid tissue (GALT), which produce a lot of transforming growth factor β (TGF-β) are known as T helper type 3 cells (Th3) (118). On top of this there are also FOXP3, CD4 positive cells that express low levels of CD25 and whether these represent a special entity of induced T_{reg} or if they are simply natural regulatory T cells that have lost some of their CD25 expression remains to be elucidated but some recent data seem to favour the later explanation (119). Additional markers that have been associated with the T_{reg} phenotype are cytotoxic T cell antigen 4 (CTLA-4) and glucocorticoid-induced TNFR family-related receptor (GITR) (120). A lot of the research on T_{reg} is naturally performed in mice and while the differences between mouse and human T_{reg} have so far been few such differences probably exist and should be kept in mind (116). In summary there is currently a bit of confusion as to the definition of the T_{reg} entity and future research will hopefully generate a more complete picture.

Mechanism of suppression by regulatory T cells

In addition to the factors described above, peripheral tolerance can also be upheld by the active suppression of auto reactive cells by T_{reg}. Recent data have postulated several mechanisms that are employed by the T_{reg} to achieve this suppression and while research within this field has been intense during the last years, much remains to be elucidated (121, 122). One of the basic questions that have interested several researchers is the antigen specificity of T_{reg}. It has been argued that once activated they seem to be able to suppress “everything” and thus do not seem to act in an antigen specific way. On the other hand it has been advocated that they do express a TCR so they must be antigen specific. Recent data however seems to be able to somewhat reconcile these two views. It appears that the activation of T_{reg} is indeed antigen specific and they are able to proliferate in response to antigenic stimuli (123, 124). Once activated they appear however, to be able to suppress not only cells specific for the same antigen, but also in a more “bystander” like fashion (121, 125). Studies on the effect of antigen specific T_{reg} indicate that in vivo, unlike in vitro, the T_{reg} specificity seems crucially important, since it enables the homing of T_{reg} to the relevant lymphoid site, such as a lymph node (126, 127). Once in the selected compartment T_{reg} are however also able to suppress bystander cells that are being activated in the same compartment (128).
On the immunopathogenesis of HIV infection

Figure 3. Schematic illustration of possible mechanisms of suppression mediated by regulatory T cells. (a) T_reg may induce the expression of IDO through CTLA-4/CD80 or CD86 interactions with DC. (b) T_reg has also been suggested to express or induce expression of immunosuppressive cytokines like TGF-β or IL-10. (c) T_reg may induce expression of the inhibitory B7-H4 molecule on DC. Adapted from (122).

Another topic that has been heavily debated is whether the suppression mediated by T_reg is contact dependent or conveyed by soluble factors. In a stringent mouse allograft model, tolerance was found to be solely dependent on CTLA-4 interactions, mediated by T_reg, which is contact dependent (129). While the T_reg mediated inhibition of the killing of tumour antigen expressing B-cells, in a mouse lymph node by antigen specific cytotoxic T cells, was found to be dependent on TFG-β (130). Further strengthening this is data showing the need for an intact TGF-β II receptor on cytotoxic CD8+ cells in order for them to be susceptible to T_reg mediated inhibition of target lysis (but not IFN-γ production) (131). Furthermore T_reg have been shown to induce production of indoleamine 2,3 –dioxygenase (IDO) by antigen presenting cells (APC). IDO is an enzyme that degrades the essential amino acid tryptophan and thus suppresses the activation of nearby cells (132, 133). It has been suggested that IDO suppression is somewhat antigen specific, since only cells specific for, and activated by, the antigen presented by the IDO producing APC will be suppressed (134). A “bystander” suppression of cells specific for antigen presented by neighbouring APCs, not expressing IDO, has however also been noted (135). Whether IL-10 functions as an immunosuppressive factor released by T_reg is also not clear, but it should be mentioned that this cytokine has mainly been implicated in suppression mediated by induced regulatory cells such as Tr1 cells (121). In addition to these mechanisms T_reg have also been suggested to kill autoreactive cells via the perforin pathway (136) and recent evidence also suggests that T_reg
might induce the expression of B7-H4 (a newly discovered member of the B7 family of co-stimulatory molecules) on APC which in turn can inhibit antigen specific immune responses (122). In summary T\textsubscript{reg} seem to be able to suppress immune activation through a number of mechanisms that might be very dependent on the experimental setup (especially on the type of compartment studied) and I think that it is safe to predict that several pathways remains to be elucidated.

Regulatory T cells and infections
Even though the most important function conveyed by T\textsubscript{reg} is believed to be the protection from autoimmune reactions, T\textsubscript{reg} have been implicated in modulating the response towards several infectious pathogens (137). In some experimental systems T\textsubscript{reg} have been shown to suppress acute immune responses in such a way that it protects from exaggerated immune activation, that if unchecked induce vast collateral tissue damage (123). The absence of T\textsubscript{reg} in this model has been shown to cause mortality (123). The most investigated role for T\textsubscript{reg} in infectious diseases has nonetheless been the ability of T\textsubscript{reg} to assist in the development of chronic infections (123, 138-140). It has been suggested that T\textsubscript{reg}, expanding early after infection, interfere with the generation of fully mature CD8+ effector T cells and thus limit the ability of the immune system to clear the body of infected cells (139, 140). The enhanced immune responses and increased ability to clear infection that has been observed in several model systems were T\textsubscript{reg} have been depleted suggests that therapy focused on manipulating T\textsubscript{reg} prevalence and function may be important in the treatment of chronic infections (123, 139, 141). Indirect evidence indicates that T\textsubscript{reg} specific for microbial antigens exist and that these cells are able to expand and diminish in an antigen dependent manner (123). Why T\textsubscript{reg} specific for foreign antigens exist is at this time a matter for speculation, but as recent evidence in other fields of immunology has shown, the interplay and co-evolution between the human immune system and our pathogens have likely played an important role (142). The balance between immune activation and suppression appears to be crucial for the outcome of infections. Too much immune activation may cause unwanted collateral damage to the host and influx of T\textsubscript{reg} into the relevant tissue might be a way to prevent this. If the balance is shifted so that the T\textsubscript{reg} accumulate in too high numbers or too early after the onset of infection this might however efficiently hamper immune mediated pathogen clearance. The induction of tolerance towards itself by an invading pathogen can also be viewed as an advanced mechanism of pathogen escape from the immune system. It is therefore highly likely that several microbes have exploited these pathways in order to better survive within the human host.
**Regulatory T cells in transplantation, autoimmunity and cancer**

Even though research on T<sub>reg</sub> in infectious diseases has lately gained pace, the majority of T<sub>reg</sub> related research is performed within other fields of immunology. T<sub>reg</sub> research is especially active in transplantation immunology and autoimmune diseases where much of the research is focused on mechanisms of immunological tolerance. Transplant immunologists are exploring the possibility of inducing T<sub>reg</sub> mediated tolerance towards transplant grafts. One strategy is the design of various protocols for the delivery of antigens under the protection of antibodies that compromise T cell activation and maturation. Blockage of CD4, CD3 and CD28 by monoclonal antibodies have all been shown to enhance tolerance and reduce rejection of grafts in transplant models (143-145). The ultimate goal of this research is naturally the ability to transplant cells and solid organs across MHC barriers without using immunosuppressant’s that compromise host immunity towards microbial infection. In autoimmunity research, studies focusing on why tolerance is not functioning properly are dominating. A deficiency in the function of T<sub>reg</sub> from individuals with an autoimmune disorder has in line with this been linked with the occurrence of several autoimmune disorders (146-148). The prospect of restoring tolerance in patients with autoimmunity or preventing the onset of disease is the ultimate goal of this research. Several new protocols aiming for this are being developed in different models of autoimmune diseases. The *in vivo* conversion of auto-antigen specific T cells into T<sub>reg</sub> by employing antigen presentation under sub optimal conditions represents a promising therapeutic approach (128). In cancer immunology T<sub>reg</sub> have been found to obstruct efficient clearance of tumour cells by the immune system. Studies on patients with ovarian carcinoma has reviled that T<sub>reg</sub>, accumulated within solid tumours, suppress tumour specific immunity and are associated with decreased survival time (149). In line with this a high intra tumour CD8/ T<sub>reg</sub> ratio has been associated with favourable prognosis in patients with ovarian cancer (150). Therapies aimed at depleting the T<sub>reg</sub> population are currently included in adoptive cell transfer protocols aimed at generating more efficient anti-tumour cell mediated immunity against malignancies such as colon cancer and melanoma (151). Even though subsequent autoimmunity is a reality with these kinds of non-selective therapies, the induction of controllable and transient autoimmunity might be tolerable under the circumstance of being able to alter a life threatening disease. In summary research on T<sub>reg</sub> and tolerance immunity is currently very intensive and there is a lot of hope that the manipulation of T<sub>reg</sub> will generate ways to cure a wide array of diseases and revolutionize the field of transplantation.
Studies on HIV infection in lymphoid tissue

Background
Although HIV research is a broad and active research field with over 10,000 new HIV related publications during the last year, the vast majority of the immunological and virological studies performed are based on the analysis of peripheral blood mononuclear cells (PBMC), either obtained from HIV infected individuals or used in *in vitro* systems (110). This is probably a result of several factors, but I will only discuss a few of these in this thesis. Firstly there is no solid inexpensive (small rodent) animal model of HIV infection that allows for the sampling of lymphoid tissues. The best available model is as previously mentioned SIV infection of rhesus macaques (RM) and although this appears to be a relevant model it is expensive and requires specialized facilities, which limits the use of the RM model. With the limitation of animal models much of the HIV research is performed using human specimens. While this in many aspects may be more relevant, it has some obvious restrictions. The sampling of lymphoid tissue (tonsillar tissue, lymph nodes, and gut associated lymphoid tissue) from humans requires a somewhat invasive procedure, since this will always be obtained in the form of a biopsy (disregarding the use of lavages). This may be associated with discomfort and also requires a procedure with trained personnel (e.g. surgeon for the sampling of inguinal lymph-nodes or a gastro-enterologist when obtaining ileal biopsies by coloscopy). The material obtained may also be limited in volume (i.e. the number of lymphoid cells) which makes the task of extracting solid data difficult. All in all the above mentioned contributes to the use of peripheral blood in the study of HIV infection and while this might be easy to obtain it might not always produce relevant answers.

Why is the lymphoid compartment important in HIV infection?
HIV is a lymphotropic virus which preferentially infects cells expressing high amounts of the receptor CD4 together with co-receptors CCR5 or CXCR4. This means that the majority of virus production and infection will take place where the majority of the CD4 positive cells expressing the relevant chemokine receptors reside. With only 1-2% of the body’s total lymphocytes present in peripheral blood, the majority of virus infected cells reside in the lymphoid tissues (152) Furthermore cells isolated from peripheral blood are not easily infected by HIV *in vitro* without prior activation. This is in contrast to cells isolated from lymphoid tissue, which are readily infected with HIV *in vitro* without the addition of activating agents (153). In line with these *in vitro* findings, the number of HIV infected cells in peripheral blood is quite low while infection rates in lymphoid tissue can be very high, especially during acute infection (154). Disease progression during the course of HIV infection is
monitored by assessing the CD4+ T cell frequencies in peripheral blood and while this has proved to be a reliable marker it greatly underestimates the loss of CD4+ T cells. Studies on lymphoid tissue have shown a severe depletion of memory CD4+ T cells that occur extremely early, especially in gut associated lymphoid tissue (GALT) from both SIV infected rhesus macaques and HIV infected individuals (155-160). These studies have collectively provided some important clues to the pathogenesis of HIV infection which studies of peripheral blood would not have been able to elucidate. Studies on the immune responses elicited by HIV infection, which focus on identifying components conferring protection from disease progression, will also benefit from the study of lymphoid tissue. Primarily, since the “targets” for the immune response (i.e. HIV infected cells) reside in lymphoid tissue. Finding the correlates of protection from disease progression in peripheral blood is also an important task, since this will be crucial in the design and evaluation of potential vaccines (161). One should remember however that these factors will be correlates and finding the mechanisms responsible will most certainly require the study of lymphoid tissue.

**Is cell mediated immunity important in controlling HIV infection?**

**Background**
The work presented in this thesis focuses mainly on the study of cell mediated immune responses in lymphoid tissue of HIV infected individuals. The choice of this subject is derived from the idea that cell mediated immunity is important in the control of HIV infection and though this has been briefly discussed previously in the introduction it deserves a closer look. Several approaches have been used to test the importance of cell mediated immunity and a few of these are discussed below. The ordering of these approaches in this section should not be interpreted as a sign of their individual strength; instead as is quite common in research they all have their pros and cons.

**CD8 depletion in the rhesus macaque model**
Depleting CD8+ T cells from rhesus macaques by use of a monoclonal anti-CD8 antibody has been a frequently used system for assessing the importance of CD8+ T cell mediated immunity in SIV infection (88, 162, 163). These studies have investigated the effect of depleting the CD8+ T cell pool, prior to, or at the time of SIV infection, as well as the effect of CD8-depletion during chronic SIV infection. Collectively they have found an increase in SIV viral load when the CD8+ T cells are reduced, both in the acute and the chronic setting. There is also a temporal correlation in that the viral load often decreases at later time-points
when the CD8+ T cells start to reappear. Although this is a straightforward
approach that strongly suggests a crucial role for CD8+ T cells in controlling
SIV infection it has also received some critique. Depletion of CD8+ T cells has
been suggested to cause immune activation and proliferation of CD4+ T cells
which might facilitate the increased viral load that is observed in the setting of
CD8+ T cell depletion during acute SIV infection (164). This does however not
explain the increase in viral load that has been demonstrated in the setting of a
chronic SIV infection where CD4+ T cells are believed to already be
proliferating in order to compensate HIV induced CD4+ T cell depletion.

Studies of HIV long term non-progressors
Another approach taken to understand the mechanisms that protects against
disease progression has been the study of HIV infected individuals that are
naturally able to suppress viral replication and sustain peripheral CD4+ T cell
counts. These individuals are usually termed HIV long term non-progressors
(LTNP) and though this term has been frequently used, the definition of the
LTNP has been variable (165). It should also be mentioned that the phenotype of
HIV infected individuals is quite variable in terms of disease progression with
patients experiencing fast, normal, slow and non-progressing disease patterns
and every variant in between. Despite the problems in classifying these
individuals several studies have indeed found a correlation between better
functioning cell mediated immunity and LTNP status (166). It has been
previously published that CD8+ T cells from HIV LTNP are able to proliferate
and express perforin in response to HIV antigens in vitro (94). In line with this it
has also recently been shown that HIV non-progressors maintain poly functional
CD8+ T cells that are able to produce a more extensive repertoire of cytokines
when they are re-stimulated in vitro (95). Collectively these findings suggest
that it is the quality and not the quantity of the cell mediated response that seems
to be important in the protection from disease progression. It should be
mentioned however that some recent data indicates that the poly functional
CD8+ T cells are also able to produce a larger quantity of the investigated
cytokines as compared to the more mono functional CD8+ T cells which are
more prevalent in HIV progressors, thus elegantly linking quality and quantity
with better functional capacity (167). Though these studies are exciting it should
be underlined that these findings are only correlates and a major question that
remains to be answers is whether this is responsible for the observed low viral
load and stable CD4 count or merely due to these parameters.

The effect of HLA polymorphism on HIV disease progression
Another aspect in the study of individuals with a slower disease progression has
been the effort to link this with a certain genotype. The focus on the HLA alleles
started with the observation that people that were homozygous for a certain
allele seemed to have a faster progressing disease (168). This subsequently lead
to the finding that some HLA types like HLA-B57, HLA-B27 and HLA-B51 are frequently associated with better prognosis and lower plasma viral loads (169, 170). In line with a HLA mediated impact on disease progression, individuals carrying HLA-B35 often show an accelerated disease progression (168). The fact that different HLA alleles seem to influence disease progression has been viewed as strong evidence for the importance of cell mediated immunity in determining HIV prognosis. It should be remembered however that HLA alleles are closely linked with a large number of genes within the extended HLA complex and it has been very difficult to pinpoint the effect to a single gene.

**Studies of highly exposed seronegatives**

The observation that some individuals who have been exposed to HIV several times (usually commercial sex workers in high prevalence areas) but remain uninfected carry measurable amounts of virus specific CD8+ T cells has contributed to the idea that virus specific CD8+ T cells in the right setting can protect from disease (171). Further publications have also claimed that the HIV specific CD8+ T cells in a group of highly exposed persistent seronegatives (HEPS) differ in their repertoire from HIV infected individuals (172). The same group has however also published the observation that a prolonged period of not being exposed to HIV infection seems to increase the risk for infection at a later exposure (173). This has been interpreted as a need for continuous boosting of CD8+ T cell responses in order for these cells to be protective. On the other hand this observation could also suggest a role for innate immunity in protection from HIV infection, since active sex work could promote innate immune responses that are generally believed to be short lived and lack memory. In line with finding other correlates of protection than cell mediated immunity, protection in HEPS individuals has also been associated with the presence of neutralizing IgA in cervico-vaginal lavages (174). It is also difficult to envisage that the low numbers of HIV specific CD8+ T cells that have been detected in the peripheral blood of these individuals could potentially mediate protective immunity at the mucosal surface. The finding of HIV specific CD8+ T cells in the genital mucosa of these individuals however strengthens the data on a protective role for these cells in HEPS individuals since they seem to be present in the relevant anatomical location (175). Our own recent findings of an increased expression of both RANTES and IFN-α in the cervical mucosa of HIV seronegative commercial sex workers with a high risk for HIV exposure might also suggests that the HIV specific CD8+ T cells could be attracted to and retained within the cervical mucosa for a prolonged period of time (46). This suggestion is based on previous publications demonstrating a central role for both RANTES and IFN-α in attracting and keeping activated lymphocytes from leaving a specific lymphoid compartment (176, 177). One could also speculate that the discontinuation of commercial sex work could be associated with the disappearance of these local factors, owing to decreased cervical mucosal
challenge with HIV or other pathogens. This would then cause the activated HIV specific CD8+ T cells to egress from the cervical mucosa and subsequently render the host less protected from a vaginal challenge with HIV.

Results and discussion

Immunopathogenesis

Background
The central question that I have tried to address in the work presented in this thesis is; why HIV infected cells in lymphoid tissues are not eliminated by cell mediated immunity? This is a question that has been addressed by many scientists across the globe and a lot of contributing factors have up to this time been suggested. I have in the following sections addressed a few of the factors that are believed to be contributing to the hosts’ inability of eliminating virus infected cells. I do not, in this thesis aim to cover this entire (quite extensive) field and it should be kept in mind that this issue is far from resolved and much remains to be elucidated.

Viral escape from cytotoxic T-lymphocyte (CTL) responses by introduction of mutations
Predictions of virus production, the mutation rate of the virus and the size of the viral genome suggests that during active virus replication, each possible mutation could occur every day in an infected individual (178, 179). One might then be lead to believe that the selection pressure imposed by a certain CTL clone would instantly lead to virus escape. This reasoning however does not take the selection pressure that favours conservation of amino-acid sequence into consideration. For any virus, the majority of possible mutations will for the most part be deleterious. In addition to this a large number of mutations will come with a great cost to viral fitness and only a minority of mutations will leave the virus unaffected. Despite these facts CTL escape is a well known strategy employed by HIV to avoid recognition (180). The escape mutations which evolve have been described to disrupt CTL recognition by three different mechanisms. Mutations affecting the HLA binding residues that anchor the peptide in the MCH peptide binding groove may generate a new mutated peptide that will no longer bind (181). Alternatively, mutations affecting the TCR binding residues might result in a reduced TCR affinity for the MHC bound peptide (182). More recent data also suggests that by mutating residues flanking the presented peptide HIV might be able to interfere with processing of the targeted peptides and thereby prevent them from being presented (183, 184). Many of these mutations have a fitness cost for the virus and a pre-existing
compensatory mutation is often needed for the escape mutation to occur (180). Based on this finding, it has been suggested that escape mutations that require compensatory mutations will only occur late into HIV infection simply because the chance of generating these two mutations in the same virus are small (180). One might also speculate that if the viral load is efficiently suppressed the likelihood for these mutations to occur will greatly diminished. It should be mentioned however that most of the data on virus escape from targeted CTL epitopes comes from the study of individuals with a certain known disease altering HLA phenotype, such as HLA-B27 (181, 185). This might cause over interpretation of the importance of this phenomenon, since even in individuals with a progressive HIV infection broadly directed and high frequency CD8+ T cell responses can be measured (186). Several of these responses are also directed against conserved epitopes that are not mutated in autologous sequenced strains (186, 187). This suggests that in a large part of HIV infected individuals, mechanisms other than viral escape through epitope mutation might be causing the inability of the immune system to eliminate virus infected cells. Taken together CTL escape by sequence variation is an indication that cell mediated immunity can apply a strong selection pressure on the virus in at least some individuals. It will be of great importance for any potential HIV vaccine candidate aimed at inducing cell mediated immunity to try and target epitopes which are not easily mutated by the virus.

**Lack of CD4+ T cell help**

Perhaps the most prominent effect of HIV infection the loss of CD4+ T cells does not only cause the immunodeficiency syndrome, but also compromises the host’s ability to mount efficient anti-HIV cell mediated immunity. The CD4+ T cell is a central player in any immune response by supplying stimulatory cytokines and co-stimulation to a wide range of immune cells including DC and CD8+ T cells. CD4+ T cells are vital for the induction of efficient CD8+ T cell responses and for the establishment of CD8+ T cell memory (188). By depleting large numbers of CD4+ T cells HIV efficiently cripples the immune system and limits its ability to respond. In line with this studies focusing on individuals that are able to control virus replication have shown that they are able to maintain robust poly functional (IL-2 and IFN-γ) CD4+ T cell responses (189, 190). Maintenance of HIV specific CD4+ T cell responses have also been described in individuals infected with HIV-2, which, in the majority of cases (>80%), does not result in progression to AIDS (191). HIV has also been suggested to selectively infect and deplete HIV-specific CD4+ T cells which further compromises the hosts HIV specific immune responses (192). It should be mentioned however that HIV specific CD4+ T cells can be detected in individuals with a progressive infection (193, 194). In addition, although typically absent in untreated patients, there are several reports showing that significant proliferative responses to HIV antigens is present in patients
receiving effective antiretroviral therapy, confirming that these cells are not depleted (195, 196). In summary the early and continuing loss of CD4+ T cells during HIV infection greatly impairs the ability of the host to mount efficient cell mediated responses. However the presence of HIV specific CD4+ T cells in individuals with a progressive disease and the increased proliferative ability induced by ART suggests alternative mechanisms that reduce the function of virus specific CD4+ T cells during active viral replication.

**Interference with dendritic cell function**

Dendritic cells (DC) are important presenters of antigens to T cells. They acquire antigens, which are subsequently presented as peptides loaded on MHC II for the interaction with CD4+ T cells and on MHC-I for interactions with CD8+ T cells. The presented peptides on the relevant MHC molecule are recognized by the TCR on a given T cell. In addition to the MHC-TCR interaction, DC activate the T cells via co-stimulatory molecules expressed on DC that find their corresponding ligands on T cells (197). Cytokines such as IL-12p70, IL-10, IFN-α produced by the DC may also regulate the actions of interacting T cells. The expression of CD80 and CD86 on DC and production of IL-12 is thought to be crucial for the induction of antigen specific CTL (198). The full maturation of DC and its ability to efficiently prime CTL responses is thought to be dependent on CD40-CD40L interactions (199). Both CD80 and CD86 can bind to CD28 and CTLA-4, but CD86 is thought to mediate the stimulatory signal to T cells by binding to CD28 and CD80 has been implicated as the functional ligand for CTLA-4 and as such induce a suppressive signal (200). As there is a deficiency in the cell mediated immunity in individuals with progressive HIV infection, the DC population has also been shown to have a compromised function (201). DC with significantly lower levels of CD80 and CD86 have been shown to accumulate in lymph nodes of individuals undergoing acute HIV infection as compared to DC that accumulate during acute EBV infection (201-203) We have also in paper III shown an increased expression of CD80 but not CD86 mRNA in lymphoid tissue of individuals with a progressive HIV infection as compared to HAART treated individuals. The expression of the immunosuppressive enzyme IDO was also shown to be increased in individuals with a progressive HIV infection, both as compared to individuals on HAART and individuals with a non-progressive type of infection (paper IV). Our preliminary data implies that the observed expression of IDO was associated with DC within lymphoid tissue. This could suggest a role for T_{reg} in mediating the observed DC dysfunction, perhaps through interactions with B7 family receptors (CD80, CD86 and others). *In vitro* studies have shown a selective defect in IL-12 production by DC infected with HIV but other studies have shown a more generalized lack of responsiveness in DC from HIV infected individuals, especially in their ability to respond to CD40L stimuli (201, 203)The possibility of inducing effective cell mediated immunity by the addition
of in vitro primed, antigen loaded DC has also been shown in a pioneering study where a reduction of viral load was observed (204). The longevity for this effect on the viral load and subsequent disease progression however remains to be elucidated. Since DC express both CD4 and CCR5 they can be infected by HIV, but whether the observed abnormalities in DC function is due to direct infection of DC remains an open issue. Several studies have also found no obvious differences between DC from HIV infected and uninfected individuals and the successful effect of adding autologous antigen loaded DC in the previously mentioned study also argues for the presence of functionally intact DC during HIV infection (204).

The DC have also been viewed as something of a Trojan horse during HIV infection (152). This is due to the ability of DC at mucosal surfaces to be infected by HIV or capture infectious virus through lectin receptors such as DC-SIGN. DC may subsequently leave the mucosal site and migrate in to a draining lymph node where they interact with T cells (152). Through antigen presentation DC may deliver HIV preferentially to the CD4+ T cells which are specific for the antigen presented by the DC (205). The DC-T cells contacts can facilitate the infection of the participating CD4+ T cell, which in turn greatly amplifies the infection and leads to subsequent loss of antigen specific CD4+ T cells (206). In summary our findings together with previously published work suggest that DC in the context of progressive HIV infection might not be functioning optimally. Our finding of an enhanced production of IDO in individuals with a progressive HIV infection also suggests that DC in this context may assist in suppressing immune responses in lymphoid tissue during HIV infection.

HIV specific CD8+ T cells are dysfunctional in progressive HIV infection
The fact that HIV infected cells are not eliminated from lymphoid tissues during progressive infection, but instead seem to increase as the disease progresses has questioned the functional ability of HIV specific CD8+ T cells (207, 208). Numerous studies have been carried out in order to characterize the phenotype and function of HIV specific CD8+ T cells in HIV infected individuals and with the introduction of the tetramer technology this area of research has been further advanced. Despite the previously discussed data that highlight the importance of anti-HIV cell mediated immunity, the frequency or breadth of virus specific CD8+ T cells has not been found to be inversely correlated with viral load and disease prognosis (209, 210). This is something that one might assume to be the case if cell mediated immunity carried out by CD8+ T cells is important in limiting active replicating HIV infection. Another interpretation of this data is that it is not the quantity, but the quality of CD8+ effector T cells that is important and several studies have indeed found a correlation between the ability to mount poly functional CD8+ T cell responses and a on non-progressing type of disease, which is in support of this interpretation (94, 95).
If the HIV specific CD8+ T cells have a compromised functional ability in individuals with progressive disease, what crucial function/functions do they lack?

We have studied the expression of granule mediated cytolytic proteins in CD8+ T cells from the lymphoid tissue of both acutely and chronically HIV infected untreated individuals within a wide range of viral loads and disease status. From these studies we have previously and in both paper I and II shown that acute and chronic HIV infection is characterized by the accumulation of CD8+ T cells that express granzyme A but not perforin in lymphoid tissue (211). Perforin release is clearly important in the elimination of virus infected cells, which has been verified in studies on perforin knockout mice (212). Furthermore children with familial, hemophagocytic lymphohistiocytosis, linked to a defective perforin gene, often die of overwhelming viral infections (213). Our finding of a selective impairment in the expression of perforin in lymphoid tissue during acute as well as chronic HIV infection is also in contrast with what we observed in lymphoid tissue during acute EBV infection where the vast majority of virus infected cells were also efficiently eliminated. Furthermore, individuals with a defective perforin gene have also been shown to be unable to clear EBV infected cells (214). We found that during acute EBV infection there was a massive infiltration of granzyme A and perforin expressing CD8+ T cells. These cells expressed granzyme A in granules, which was visualized with immunohistochemistry. During perforin dependent cytotoxicity, granzymes are trans-located via endosomes to the cell nuclei of the target cell, where they induce apoptosis (215). This could also be visualized by us with immunohistochemistry and during acute EBV infection, typically around 50% of granzyme A expressing cells in lymphoid tissue showed a nuclear pattern of expression, indicating that they were virus infected cells succumbing to successful perforin mediated cytotoxicity. In contrast, during acute and chronic HIV infection only a very small fraction of granzyme A expressing cells had a nuclear expression (Figure 4). Thus, these data further strengthens the importance of perforin for effective in vivo lysis of virus infected cells.
Fig 4. Expression of granzyme A and perforin in lymphoid tissue (LT) from individuals undergoing acute HIV (aHI) (a,c) or acute EBV (aEBV) infection (b,d). Increased expression of granzyme A in LT during both aHI (a) and aEBV (b) infection. Perforin is however only concomitantly increased during aEBV (d) infection. Note the nuclear translocation of LT granzyme A positivity in aEBV infection (b) which is found in up to 50% of granzyme A expressing cells. This is however only found in less than 10% of LT granzyme A expressing cells from individuals undergoing aHI (a), further suggesting a lack of functional CD8+ T cells that are able to kill virus infected cells.

The lack of perforin expression was not visualized in peripheral blood, where typically 19-24% of all mononuclear cells concomitantly expressed perforin and granzyme A. This is higher than expression in uninfected controls (14-17%), but significantly lower compared to perforin and granzyme A expressing cells in patients during acute EBV infection (28-32%). The numbers of perforin and granzyme A expressing cells in peripheral blood should be interpreted with the knowledge that natural killer cells (NK-cells) typically made up 14-17% of total PBMC in our studied patients, while the number of NK-cells in lymphoid tissue in our experience was generally below <1%. The lack of perforin expression in lymphoid tissue of HIV infected individuals was not due to lack of relevant target cells, since the frequency of virus infected cells was typically one to two logs higher in lymphoid tissue as compared to peripheral blood (paper I). If perforin expressing cells are not induced, even during acute HIV infection this
seems to be contradictory to previous suggestions of immune exhaustion as the causative mechanism for defective CD8+ T cell function.

Instead one might question the ability of HIV in generating an immune response that would lead to the development of virus specific, fully mature CD8+ T cells. This question was addressed by us in paper II, where we investigated the expression of cytokines and chemokines in surgically obtained biopsies from both peripheral lymphoid tissue and GALT in individuals with acute HIV infection (day -3 to 48 from the onset of acute symptoms). In this study we were able to show that there was indeed an early and broad immune activation in both lymphoid compartments during acute HIV infection. The immune activation in GALT seemed to precede activation in peripheral lymphoid tissue, which is consistent with earlier published work on acute SIV infection (155). We also found significantly increased expression of IL-2, IFN-γ, TNF-α, Mip1-α/β and IL-12 all of which are thought to be central for the induction of CD8+ T cells. In addition to this we also observed increased expression of IL-4 and IL-10, which are believed to primarily drive Th2 type responses. The concomitant expression of both Th1 and Th2 type cytokines in peripheral lymphoid tissue and GALT during acute HIV infection gives the impression of a somewhat un-coordinated response that might be less efficient in promoting effective CD8+ T cell responses. The expression of the investigated chemokines and cytokines was associated with the accumulation of CD8+ T cells within both of the studied compartments. The accumulated CD8+ T cells were able to express granzyme A but not perforin. This suggests that even at the initiation of the anti HIV immune response fully mature virus specific CTL are not allowed to be developed.

Accumulation of regulatory T cells in HIV infection
The first studies that investigated the presence of $T_{reg}$ within HIV infected individuals collectively found that HIV infected individuals seemed to have fewer circulating $T_{reg}$ as compared to uninfected controls (216, 217). This was suggested to be dependent on selective depletion of $T_{reg}$ by HIV, since these cells typically express CD4 together with CCR5 and are easily infected by HIV in vitro (216). The relative lack of $T_{reg}$ in HIV infected individuals was then also suggested to contribute to the immune activation associated with active replicating HIV infection (216-218). In line with the above findings, a study that tried to correlate $T_{reg}$ functional capacity and HIV replication in treated and untreated HIV infected individuals found that individuals with more measurable $T_{reg}$ responses towards HIV seemed to have lower plasma viral loads (219). Collectively these studies advocated for a beneficial role of $T_{reg}$ during HIV infection and the data seemed to contribute to the idea that suppression of exaggerated immune responses is beneficial for HIV infected individuals. Two other studies have however demonstrated that depletion of $T_{reg}$ from HIV infected individuals PBMC increased in vitro HIV specific T cell responses
(141, 219). These data seemed contradictory; since better functioning cell mediated immunity has been frequently linked with improved disease prognosis.

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Figure 5. The figure illustrates the dynamics of FOXP3 expression in peripheral T cells (a) and tonsil tissue (b) of HIV infected and uninfected patients. Active replicating HIV infection leads to the accumulation of FOXP3 expressing cells within lymphoid tissue. A reciprocal reduction of FOXP3 expressing cells can subsequently be seen in peripheral blood. The introduction of HAART which efficiently limits viral replication appears to restore FOXP3 expression to levels close to what is seen in HIV uninfected individuals. FOXP3 mRNA levels are expressed as relative units (RU) and were normalized on expression of GAPDH.
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All of the studies that are discussed above were performed on PBMC from HIV infected individuals and as previous data has repeatedly indicated; studies on peripheral blood might not generate a complete picture. In paper III we were able to put forward an alternative theory by investigating the prevalence of T_{reg} within lymphoid tissue of chronically HIV infected (treated and untreated) individuals. We showed like other studies, that untreated HIV infection was associated with decreased T_{reg} levels in peripheral blood and that this seemed to “normalize” after ART treatment. Contradictory to the previously published suggestions we found however that active replicating HIV infection was associated with an increased prevalence of T_{reg} within the lymphoid tissue and
that this was “normalized” in ART treated individuals (Figure 5). Furthermore
the prevalence of T_{reg} within lymphoid tissue was significantly correlated with
plasma viral load (Figure 6). Together with these data we also found evidence
for increased expression of factors associated with T_{reg} mediated suppression in
the lymphoid tissue of untreated HIV infected individuals. These factors
included increased expression of CTLA-4, IDO and TGF-β, which have all been
implicated as important for T_{reg} suppressive action. Our data collectively
indicated that active T_{reg} mediated suppression was present in the lymphoid
tissue of individuals with untreated HIV infection. Taken together this suggested
an alternative role for T_{reg} in HIV infection as compared to previous published
work. We speculated that active HIV infection retains T_{reg} within lymphoid
tissues, which subsequently leads to decreased T_{reg} levels in peripheral blood.
Long term treatment with (2 years with a plasma viral load of <50 copiels/ml)
ART that reduced viral load and immune activation within lymphoid tissues
resulted in T_{reg} egress from these compartments and increased T_{reg} levels could
as a result of this be detected in peripheral blood. Our observation of a direct
correlation between T_{reg} numbers in lymphoid tissue and plasma viral load
together with the finding that T_{reg} numbers can be altered by treatment with
ART suggested an interesting link between viral load, T_{reg} and disease
progression.

![Figure 6. The amount of both FOXP3 protein and mRNA in lymphoid tissue is correlated with plasma viral load of HIV infected untreated individuals. FOXP3 mRNA is expressed as relative units normalized on expression of GAPDH.](image-url)
The fact that there was an indication of active suppression and not just presence of T$_{\text{reg}}$ in lymphoid tissue of HIV infected untreated individuals also suggested that there might be a role for T$_{\text{reg}}$ in HIV immunopathogenesis. Another research group also recently published results similar to ours, where they showed an accumulation of T$_{\text{reg}}$ in GALT from HIV infected individuals (220). A study on T$_{\text{reg}}$ in SIV infected RM also suggests that the accumulation of T$_{\text{reg}}$ occurs early after infection, with increased levels of T$_{\text{reg}}$ detectable in GALT only 7 days post infection (221). Why are then T$_{\text{reg}}$ not eliminated by direct HIV infection, when they have been described to be easily infected in vitro? A recent study suggests that T$_{\text{reg}}$ are very poor at replicating HIV in vivo, since active expression of FOXP3 was found to repress retroviral transcription (222). This was dependent on FOXP3 mediated reduced activation of both NF-kappaB and CREB (cAMP-responsive element binding protein) and since it is known that HIV replication depends heavily on NF-kappaB activation this would severely compromise HIV replication within T$_{\text{reg}}$. Though not proven in vivo, this finding implies that T$_{\text{reg}}$ might be relatively resistant to HIV induced apoptosis, or at least to death induced by massive HIV replication within a susceptible cell.

In paper IV we followed up on our initial observations in paper III and tried to focus on the effect that accumulation of T$_{\text{reg}}$ within lymphoid tissues could have on disease progression. This was addressed by the analysis of lymphoid tissue biopsies from patients who had a non-progressing type of HIV infection as defined by the ability to suppress virus production and maintain stable CD4 counts for a minimum of 5 years. This was compared to the analysis of lymphoid tissue biopsies obtained from HIV infected untreated individuals with a progressive type of infection as well as biopsies from uninfected controls. Our data from paper IV clearly showed that in our patient group only progressive HIV infection was associated with accumulation of T$_{\text{reg}}$ within lymphoid tissues. This was also true for our previously identified T$_{\text{reg}}$ associated suppressive effectors. To further expand our findings we wanted to investigate whether the same situation was evident in SIV infected RM. Here we also investigated a group of RM that showed a non-progressing type of disease with controlled viremia and stable CD4 counts and compared them to RM with progressive SIV as well as uninfected RM. It should be mentioned however that the non-progressing RM were not able to control their viremia as well as our HIV infected patients, of whom several had maintained undetectable viral loads for up to 15 years. In line with our findings in HIV infected humans, accumulation of T$_{\text{reg}}$ and T$_{\text{reg}}$ associated suppressive factors was only evident in the RM with a progressive SIV infection. These data suggested that accumulation of T$_{\text{reg}}$ within lymphoid tissue is a further factor that is similar between HIV infection and the SIV model. This further strengthens the relevance of SIV research and adds to previously observed immunological species similarities.
Another crucial question that we tried to look closer at in paper IV was why T_{reg} accumulated in lymphoid tissue during active virus replication. Our previous data indicated a direct role on these cells for HIV viremia and we therefore developed an in vitro system, where we used PBMC from HIV uninfected blood donors to test the effect of HIV on T_{reg}. By using AT-2 treated HIV, which retains the conformation of the viral envelope but renders HIV replication deficient we were able to show that together with a limited amount of IL-2, HIV was able to drive the accumulation of T_{reg} in our in vitro system. This was in our system dependent on gp120-CD4 interactions and could to some extent be mimicked by using CD4 binding antibodies. Another question that our in vitro system allowed us to answer was; which mechanism was responsible for the observed accumulation of T_{reg}? Did HIV convert non- T_{reg} into T_{reg}? Was there an HIV dependent expansion of T_{reg} that were already present within the investigated PBMC or did HIV provide selective survival signals for T_{reg}? The results from paper IV suggested that in our in vitro system the selective accumulation of T_{reg} was dependent on a reduced rate of T_{reg} apoptosis in cultured stimulated cells. The accumulated T_{reg} were also found to have an equal ability to suppress T-cell proliferation as compared to HIV unexposed T_{reg}, which indicated that exposure to HIV did not compromise T_{reg} suppressive ability. The results from our in vitro system propose a model where HIV delivered selective survival signals to T_{reg}, which rendered them less prone to apoptosis. Taken together with previous data indicating that HIV gp120-CD4 interactions in conjunction with Type I interferon selectively induces apoptosis in CD4+ T cells our data suggested that the surviving CD4+ T cell population might be heavily skewed towards a suppressive phenotype in lymphoid tissue of HIV infected individuals with a progressive type of disease (47). These data also further emphasized the connection between virus replication, T_{reg} accumulation and disease progression.

We also wanted to investigate whether our observed accumulation of T_{reg} had an effect on cell mediated immune responses within the lymphoid tissue of HIV and SIV infected humans and RM (paper IV). We found that while accumulation of CD8+ T cells, expression of IFN-γ and granzyme A mRNA within lymphoid tissue of HIV and SIV infected humans and RM (CD8+ T cell frequencies were not investigated in our RM although previous data indicate that a similar situation as in the HIV infected humans was present) did not differ between non-progressors and progressors the non-progressing RM expressed significantly more perforin mRNA within lymphoid tissue. The HIV infected non-progressors did however not express significantly more perforin mRNA as compared to the HIV progressors. This could potentially be due to the fact that the majority of our HIV non-progressors were very efficient in suppressing viremia. The only HIV non-progressor with a viremia >1000 copies/ml in line
with this also showed a 10-fold up regulation of perforin mRNA, indicating the presence of fully mature perforin expressing CTL within lymphoid tissue. This increased expression of perforin mRNA was not noted in our HIV progressors or SIV progressors despite the fact that many of them show viral loads in the range of 100 000 – 1 000 000 copies/ml. This finding further strengthens our previous observations of an impaired expression of perforin in lymphoid tissues during progressive HIV infection (paper I and II). It should however be kept in mind that our data are only correlations and does not prove direct functional effects of accumulated T_{reg} within lymphoid tissue. It is our belief however that these data implicate T_{reg} as central players in HIV pathogenesis and merits further investigations into this area of research.

Recent data employing CTLA-4 blocking antibodies in addition to conventional ART treatment during experimental SIV infection of RM also showed a promising effect with further reduction of lymphoid tissue viral load, reduced expression of IDO and TGF-β together with increased cell mediated immune responses (223). Another previously mentioned study on SIV infection that showed an accumulation of T_{reg} already 7 days post infection also found that the accumulated T_{reg} produced large amounts of TGF-β (221). Expression of large quantities of TGF-β within lymphoid tissue might be tightly associated with the deficiency of CD8+ T cells since it has been previously demonstrated that TGF-β is able to inhibit perforin dependent lysis of target cells in a mouse in vivo model (130). The accumulation of T_{reg} was also shown to be negatively correlated with the development of SIV specific CD8+ T cells (221). Taken together with our own data this might suggest a model where T_{reg}, accumulated early during HIV infection continuously interfere with the CTL mediated elimination of virus infected cells. Suppression of cell mediated immunity could be accomplished both through direct inhibition of virus specific CD8+ T cells or by induction of “tolerogenic” DC that express inhibitory instead of activating co-stimulatory molecules and cytokines. Our finding that a high perforin/FOXP3 ratio is correlated to non-progressing infection in both SIV infected RM and HIV infected individuals further suggests that the balance between tolerance and immunity is crucial in determining the outcome of these infections(paper IV). In line with this a high intra-tumour CD8/ T_{reg} ratio was also found to be a strong predictor for favourable prognosis in patients with ovarian cancer (150).
Concluding remarks

Tolerance as a solution to escape pathology
As studies within on SIV infection of sooty mangabeys (SM) has shown, active viral replication with high plasma viral loads and even initial CD4+ T cell depletion does not always result in disease progression, development of AIDS and subsequent death (224). Recent data also advocate that the replication dynamics of SIV does not differ between non-human primate hosts experiencing pathogenic or non-pathogenic infection (225). This suggests that it is the hosts’ immune response towards the virus, instead of the virus replication itself that in the end determines the outcome of infection. Is then immunosuppression of HIV infected individuals an option? While the view that it is the immune system rather than the pathogen that causes a lot of the pathogenesis is appealing, the lack of examples from HIV infected individuals where immunosuppression has proven to be beneficial greatly discourages this idea. The only known factors that prevent from disease progression are better functioning immunity or mutations that compromise virus infectivity. A hallmark of the non pathogenic SIV infection of SM seems to be the lack of immune activation and the selective ignorance of virus, mediated by plasmacytiod dendritic cells (pDC) (226). The pDC from SM unlike pDC from RM do not produce IFN-α upon exposure to SIV (227). This is also in contrast to human pDC who produce a lot of IFN-α in response to HIV exposure. With previous and recent findings implicating IFN-α as a central causative in the continues depletion of CD4+ T cells during HIV infection it is tempting to speculate that this is one of the underlying mechanisms that separates pathogenic from non-pathogenic SIV infection (47, 228). It should also be mentioned that strategies to block IFN-α action by active immunization suggests a beneficial effect on disease progression even though it is associated with several risks (229). In summary, data from non-pathogenic SIV infection of natural hosts suggests that immune activation might be central in causing the pathogenesis of pathogenic lentiviral infections such as HIV-1 infection of humans. A careful characterization of the mechanisms that contribute to the immune activation during pathogenic lentiviral infection might generate novel therapeutic strategies aimed at mimicking the non-pathogenic infection that is observed in several natural hosts’ lentiviral infection.

HIV immunopathogenesis: Simultaneous Immune activation and suppression
The accumulation of T_{reg} within lymphoid compartments together with the continued production of IFN-α during HIV infection might be central in HIV immunopathogenesis. While T_{reg} could assist in the impairment of cell mediated immune responses towards HIV, the effects of Type I interferon might be crucial in maintaining a status of continued immune activation which is a
hallmark of HIV infection. This might also be suggested to assist in the development of the clinical symptoms associated with HIV infection. HIV infected individuals typically experience impairment of cell mediated immune responses towards other pathogens even when they have normal CD4 counts and treatment with ART in some cases leads to the development of an immune reconstitution syndrome. This could be dependent on the selective accumulation of $T_{\text{reg}}$ within lymphoid tissue which might globally impair induction of cell mediated immune responses. Treatment with ART has also been shown to be associated with the normalization of $T_{\text{reg}}$ levels within lymphoid compartments. This might lead to induction of immune responses towards many antigens that were previously suppressed. The continued immune activation and inflammation induced by IFN-α would then also mediate the fatigue and weight loss that is often seen during HIV infection. This is something that has been observed during IFN-α treatment of Hepatitis C virus (HCV) infection. An accelerated loss of CD4+ T cells has also been described during IFN-α treatment of HCV/HIV co-infection (230). The effects of the HIV viral proteins in driving the observed immune activation should also be kept in mind. A recent elegant study ascribes the immune activation observed during pathogenic lentiviral infection (HIV-1 and a number of closely related SIVs) to a mutation that abrogates the ability of the viral accessory protein nef in down modulating the TCR-CD3 complex in infected cells (231). This would protect infected cells from activation induced cell death due to TCR activation and subsequently diminish overall immune activation. The fact that SIVsm (sooty mangabey) infection of RM is still pathogenic despite having a retained TCR-CD3 down modulating ability however suggests that the hosts related mechanisms may be more important in determining the outcome of lentiviral infection. The finding that individuals and RM infected with an HIV, or SIV, virus with a deleted nef gene often experience a non-progressing type of disease, with low viral load, also implicates nef as something of a double edge sword in HIV pathogenesis. Where nef seems to mostly accelerates, but may in some circumstance be beneficial for, disease progression (63, 232). It is a well established fact that elderly HIV infected individuals generally have a faster disease progression as compared to young persons. The fact that elderly individuals are believed to have a decreased thymic output has been suggested to be in part responsible for this phenomenon. Recent studies have however shown an increased prevalence of $T_{\text{reg}}$ in the circulation of elderly individuals and it has been speculated that this is the cause of the diminished ability to mount efficient immune responses that generally is a feature of ageing (233). Taken together these data imply that an expanded $T_{\text{reg}}$ population present prior to infection, might shift the balance between immune activation and suppression and thereby accelerate HIV disease progression. Further studies on individuals carrying pathogens, prior to infection with HIV, that are known to induce increased amounts of $T_{\text{reg}}$ such as helminth infections could potentially assist in elucidating these mechanisms (142).
Implications for vaccine design
As better functioning cell mediated immunity, with retained poly-functional antigen-specific CD4+ and CD8+ T cell responses, (with the knowledge of today) appears to be the best way of prolonging the life of HIV infected individuals, finding the correlates of protection from disease progression and constructing vaccines that induce these correlates will be vastly important. As a slower rate of disease progression is also tightly associated with a decreased viral load, vaccines that are able to induce protection from disease progression will also potentially have the ability to change the rate of the HIV epidemic, by altering infectivity. It should be kept in mind however that correlates of protection from progression are correlates and not mechanisms. The induction of the correlates for protection by a given vaccine might merely induce these correlates and not the responsible mechanism. As a consequence, elucidating the mechanisms of protection remains an extremely important task. The inability of the HIV infected host to eliminate virus infected cells from lymphoid tissue is a central deficiency that any successful therapeutic vaccine will have to remedy. As this thesis argument, the inability to eliminate virus infected cells appears to be dependent on an impaired CTL function of virus specific CD8+ T cells, possibly mediated through the early and extensive induction of tolerance by regulatory T cells. Inducing effective CTL responses and avoiding or actively reducing the induction of tolerance is a potentially very important aspect. Observations from patients with autoimmune disorders, with a flare like kinetics, like systemic lupus erythematosus (SLE) also imply that tolerance and immunity are highly active dynamic processes. In line with this an immune, response generated by a highly immunogenic vaccine that manages to override tolerance, might not be sustained in the face of active viral replication and immune activation. In order to improve the success rate, these aspects should be addressed in the construction of vaccine regiments as well as immune reconstitution regiments that depend on cell mediated immunity. The importance of analyzing these parameters in lymphoid tissue, the relevant site for HIV pathogenesis, can not be overestimated.

Final remark
That autoimmunity is the price paid for efficient protection against pathogens is a known statement. However the cost of tolerance in providing advanced pathogens with an elegant route of immune escape might previously have been underestimated.
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On the immunopathogenesis of HIV infection


On the immunopathogenesis of HIV infection


Populärvetenskaplig sammanfattning


Normalt sett klarar kroppens immunförsvar av att eliminera virusinfekterade celler, met hjälp av s.k. CD8+ T-mördar celler som både kan identifiera en infekterad cell och sedan döda den. Vid HIV infektion vet man att antalet infekterade celler i lymfoid vävnad, där det flesta infekterade cellerna finns, inte minskar under infektionens förlopp utan snarare ökar. Det tyder på att de CD8+ T-mördar cellerna inte klarar av att eliminera virusinfekterade celler från lymfoid vävnad. Den här doktorsavhandlingen har försökt besvara varför CD8+ T-mördar cellerna inte klarar av att eliminera celler infekterade med HIV. Vi har bl. a. undersökt biopsier från lymfoid vävnad (framförallt tonsill och tjocktarmsbiopsier) från olika typer av HIV-smittade patienter och jämfört dessa med biopsier från patienter infekterade med EBV (ett virus som orsakar körtelnsfeber) och normala friska icke-infekterade kontroller. Vi har funnit att CD8+ T-mördar celler som finns i lymfoid vävnad från hiv-infekterade patienter inte innehåller proteinent perforin, ett protein som dödar virusinfekterade celler genom att det ger upphov till porer i cellmembranen på målcellerna. Som jämförelse finns det ingen brist på perforin i CD8+ T-mördar celler i lymfoid vävnad från patienter med akut EBV infektion. Under EBV infektion elimineras också virusinfekterade celler på ett effektivt sätt vilket inte sker vid HIV infektion. Att det inte bildas något perforin beror inte på att det saknas HIV infekterade celler i lymfoid vävnad hos HIV infekterade patienter. Vi har visat att både antalet
infekterade celler och den mängd virus som produceras (virus DNA och RNA) är 10-100 gånger högre i lymfoid vävnad jämfört med vad nivåerna är i blod. Trots den stora mängden virus infekterade celler så blir CD8+ T-mördar cellerna inte tillräckligt stimulerade för att uttrycka det viktiga proteinet perforin. Man skulle kunna tänka sig att det beror på att infektion med HIV virus inte genererar rätt typ av stimulerande ämnen i lymfoid vävnad för att leda till perforin uttryck hos de CD8+ T-mördar cellerna. Vi har studerat graden av inflammation i lymfoid vävnad hos individer som nyligen infekterats med HIV för att försöka svara på den frågan. Våra data visar att infektion med HIV leder till en klar ökning av flera inflammatoriska ämnen som visar att kroppens immunsystem aktiverats. Aktiveringen ledde också till att CD8+ T-mördar celler ansamlades och expanderade i den lymfoida vävnaden, något som indikerar att kroppen reagerat som den normalt gör vid en virus infektion. De ansamlade CD8+ T-mördar cellerna blev aktiverade men uttryckte trots det inte det viktiga proteinet perforin och visade alltså på samma brist som vi tidigare observerat.

Eftersom det inte verkade vara brist på stimulerande faktorer försökte vi nu undersöka andra faktorer som kan inverka negativt på CD8+ T-mördar cellers förmåga att döda andra celler. En sådan faktor är en annan typ av immunförsvars cellul som kallas för regulatorisk T cell (tidigare benämnd suppressorcell). Den regulatoriska T cellen har till uppgift att uppehålla den perifera toleransen så att immunsystemet inte angriper normala kroppsegnader. Regulatoriska T celler är något som det forskas mycket på nu, framförallt inom autoimmunitets forskning som syftar till att bota autoimmuna sjukdomar så som reumatism och multipel skleros, där man tror att sjukdomen kan bero på att de regulatoriska T cellerna inte fungerar som de ska. Regulatoriska T celler har nämligen förmågan att dämpa immunförsvar så att det inte skall ge sig på något som är kroppseget eller så att det inte skall överreagera vid en infektion, vilket kan leda till stor skada på omkringliggande vävnader.

Vi fann att regulatoriska T celler ackumulerades i den lymfoida vävnaden hos HIV infekterade patienter och att antalet regulatoriska T celler i lymfoid vävnad var positivt korrelerat till mängden HIV virus i blodet. Mängden virus som en HIV infekterad patient har i sitt blod är avgörande för hur länge som individen kan överleva infektionen. Ju mer virus en HIV infekterad individ har i sitt blod desto större är risken för att tidigt utveckla AIDS. Vi fann att en ansamling av regulatoriska T celler i lymfoid vävnad hos HIV infekterade patienter var dåligt för individens förmåga att försvara sig mot infektionen.

Det finns en liten grupp HIV infekterade individer som klarar av att kontrollera HIV virus produktionen, vilket leder till att de har icke-detekterbara virus nivåer i blod eller bara mycket lite virus (så som 100-1000 virus kopior/ml blod). Dessa individer lever ofta mycket länge med HIV infektion utan att få antiviral
behandling. Vissa har levit över 20 år utan att drabbas av immunbrist och risken
för att dessa individer skall sprida HIV viruset vidare anses också som mycket
liten, då risken för att sprida virus är mycket större om man har höga nivåer av
virus i blodet. Vi undersökte en grupp av sådana individer. De uppvisade inte
någon ökning av regulatoriska T celler i lymfoid vävnad vilket ytterligare
stärker kopplingen mellan en ansamling av regulatoriska T celler och dålig
prognos. En grupp amerikanska forskare använder en modell för HIV infektion
där en viss typ av rhesus apor infekteras med ett närbesläktat virus som heter
SIV. Vi fick från de här experimenten fram samma typ av svar som från våra
tidigare försök vilket ytterligare stärkte våra data. Vi noterade också att de
CD8+ T-mördare cellerna från de apor som inte hade en ackumulerad mängd av
regulatoriska T celler i lymfoid vävnad kunde uppreglera uttrycket av perforin.
Detta fynd visar på en association mellan förmågan att försvara sig effektivt mot
SIV infektion och perforin uppreglering hos CD8+ T-mördare celler. Vi
spekulerar att HIV viruset på något sätt inducerar regulatoriska T celler som
effektivt hindrar de virus infekterade cellerna från att elimineras. I individer och
apor som lyckas att kontrollera HIV och SIV infektion ökar inte antalet
regulatoriska T celler i lymfoid vävnad och de här individerna och aporna verkar
också ha en förmåga att eliminera virusinfekterade celler.

Nya behandlingar som syftar till att manipulera de regulatoriska T cellernas
funktion eller antal kan förhoppningsvis hjälpa till med att öka immunförsvarets
förmåga att eliminera HIV virusinfekterade celler, vilket kan leda till en bättre
prognos för HIV infekterade individer.