ALTERED T CELL HOMEOSTASIS DURING HIV-1 INFECTION: CONSEQUENCES OF LYMPHOPENIA AND CHRONIC T CELL ACTIVATION

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To my beloved parents
ABSTRACT

Despite all the intense efforts and important progress achieved worldwide by the scientific community and public-health organizations, the HIV pandemic remains without effective solutions. The pathogenic mechanisms underlying the immunodeficiency that follows HIV-1 infection are still poorly understood. This lack of understanding is likely the main reason why at present there is neither a cure nor a vaccine for HIV-1 infection.

In this thesis I address two main aspects of the pathogenesis of HIV-1 infection 1) the enhancement of T cell sensitivity to apoptotic and proliferative signals during lymphopenic conditions and 2) the role of chronic T cell activation in the generation of terminally differentiated T cells that might further contribute to the exacerbation of immune activation observed during HIV-1 infection.

In paper I we showed that IL-7, a cytokine that was primarily associated with anti-apoptotic and proliferative effects of T lymphocytes, can potently induce Fas expression on T cells. This implied also an increased sensitivity to Fas mediated apoptosis. The correlation found between serum levels of IL-7, Fas expression and sensitivity to Fas mediated apoptosis exhibited by T cells from HIV-1 infected individuals strongly indicated a role for IL-7 in the enhanced sensitivity of T cells to Fas-mediated apoptosis observed during HIV-1 infection. In paper II, we demonstrate that Fas, previously associated to cell death, can act as a potent co-stimulatory molecule during HIV-1 infection. Of relevance is that the rates of proliferation greatly exceeded the levels of apoptosis upon Fas signals. Moreover, we demonstrate that IL-7 primes T cells to Fas co-stimulatory signals. Hence, the high levels of serum IL-7 associated with HIV-1 infection may enhance the sensitivity of non-activated T cells to Fas mediated apoptosis, while in the case of T cells able to recognize low affinity antigens it might enhance Fas-mediated proliferation.

In Paper III, we studied the phenotypic and functional characteristics of CD28- T lymphocytes from both healthy and HIV-1 infected individuals treated with HAART or naïve to treatment. We show that these cells exhibit certain characteristics of senescence and an apoptosis prone phenotype, independently if they originated from healthy or HIV-1 infected individuals. Interestingly, only CD28- T cells from untreated patients showed high levels of apoptosis upon TCR triggering whereas the CD28- T cells from patients undergoing HAART exhibited a strong proliferative response. Our findings suggest viral replication as an important factor regulating the homeostasis of CD28- T cells. In Paper IV we show that naturally occurring CD28- cells, either from healthy or HIV-1 infected individuals, contributed to enhance DC activation. This paper provides evidence for a role of CD28- T cell population in the accelerated inflammatory reactions and immune activation through promoting the production of inflammatory cytokines by DCs.

In summary, the work presented in this thesis, possibly provides further insights into the pathogenesis of HIV-1 infection, characterized by a vicious circle formed between lymphopenia-induced rescue mechanisms and chronic immune activation, main inducers of immunodeficiency through the alteration of T cell homeostasis.
LIST OF PUBLICATIONS AND MANUSCRIPT


III. Vivar N, Ruffin N, Sammicheli S, Hejdeman B, Rethi B, Chiodi F. Survival and proliferation of CD28- T cells in HIV-1 infection is determined by the levels of HIV-1 replication. (Manuscript).

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<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen</td>
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<tr>
<td>Ab</td>
<td>Antibody</td>
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<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
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<tr>
<td>BM</td>
<td>Bone marrow</td>
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<tr>
<td>CFSE</td>
<td>Carboxyfluorescein succinimidyl ester</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>EBV</td>
<td>Epstein Barr virus</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>FasL</td>
<td>Fas ligand</td>
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<tr>
<td>HAART</td>
<td>Highly active antiretroviral therapy</td>
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<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
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<tr>
<td>HPE</td>
<td>Homeostatic peripheral expansion</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>LTNP</td>
<td>Long term non-progressor</td>
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<tr>
<td>MCH</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
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<tr>
<td>SIV</td>
<td>Simian immunodeficiency virus</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<tr>
<td>TREC</td>
<td>TCR excision circles</td>
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<td>TLR</td>
<td>Toll like receptor</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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<td>TRAIL</td>
<td>TNF-related apoptosis ligand</td>
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1 INTRODUCTION
1.1 HIV
   1.1.1 Epidemiology

More than 25 years after its discovery, the human immunodeficiency virus (HIV) has become a global health problem of unprecedented dimensions. HIV has already caused an estimated of 25 million deaths worldwide, originating profound demographic changes in the most heavily affected countries. Despite all the intense efforts and important progress achieved worldwide by the scientific community and public-health organizations, the HIV pandemic remains, without effective solutions at hand to confine it.

According to reports from WHO and UNAIDS on the global status of the HIV/AIDS epidemic at the end of 2007, approximately 33.2 million people were living with HIV. 31.2 million corresponded to adults; 2 million were children under 15 years of age. That same year, some 2.5 million people became newly infected, and 2.1 million died of AIDS, including 330 000 children.

The highest prevalence of HIV infection remains in sub-Saharan Africa, accounting for approximately 67% of the total HIV infections in the world. The second highest prevalence is in South and South East Asia with 15% of the total. South Africa (with approximately 5.7 million infections) is the country with the largest number of HIV patients in the world followed by Nigeria (with approximately 2.6 million infections). India has an estimated 2.5 million infections (0.23% of population), making India the country with the third largest population of HIV patients.

Combined antiretroviral therapy (ART) is the only known medical treatment that can improve the prognosis of HIV infected patients. Unfortunately, due to the high costs of such drugs, the access to HIV ART therapy in developing countries is limited. Currently, less than one third of the people in need for therapy are receiving it. The lack of availability of health care infrastructures to distribute the antiretroviral drugs and to follow treated patients also contributes to slow down the goal of global treatment for HIV infection.
1.1.2 The virus

HIV is a lentivirus, member of the family of Retroviridae. It is transmitted as single-stranded enveloped RNA virus. Upon entry into the target cell, the viral RNA genome is converted to double stranded DNA by a virally encoded reverse transcriptase (RT) that is present in the viral particle. The viral DNA is then integrated into the cellular DNA by a virally encoded integrase along with host-cellular co-factors. The genome can be silent for a variable period of time until it is transcribed and viral replication can be initiated.

The infection of host cells by HIV-1 begins with the binding of the viral envelope glycoprotein (gp120) to specific receptors present at the plasma cell membrane. The main receptor for HIV-1 is the CD4 molecule which is expressed by T-helper lymphocytes, macrophages and dendritic cells (DCs). Binding of gp120 to CD4 molecule, initiates the process of HIV-1 adsorption to the target cell membrane followed by conformational changes in gp120 that enable it to bind to a co-receptor, the chemokine receptors CCR5 and CXCR4. The gp120 binding to the co-receptor molecule triggers further conformational changes and consequently the exposure of the hydrophobic region of the viral envelope transmembrane glycoprotein gp41, named fusion peptide, that ultimately leads to viral envelope fusion with target cell membrane.

After the virus has infected the cell, two pathways are possible: either the virus becomes latent and the infected cell continues its physiological functions, or the virus becomes active and replicates, and a large number of virus particles are released through budding from infected cells that can then infect other cells.

There are two types of HIV known to exist: HIV-1 and HIV-2. Both viruses represent cross species transmissions of simian immunodeficiency virus (SIV) from primates to humans, HIV-1 most probably has its origin in the SIVcpz from the common chimpanzee while HIV-2 has its origin in SIVsm from sooty mangabey¹.
HIV-1 is distributed worldwide and is the cause of the majority of HIV infections. On the contrary, HIV-2 is confined to West Africa and Southern and Western India. Sporadic outbreaks of HIV-2 infection have been reported from European countries, from North and South America as well as Korea. Most cases of HIV-2 infection reported in Europe and North America have been among African immigrants. Among European countries Portugal has a high number of HIV-2 cases in the native population and this country appears to be an important dissemination point for the virus within Europe.

HIV-1 is more virulent while HIV-2 is less pathogenic. Also, transmission of HIV-2 has been shown to be much less efficient than HIV-1. It has been suggested that the lower virulence of HIV-2 could be attributed to a reduced functionality of its nef regulatory gene and to a lower viral burden exhibited by HIV-2 infected individuals.

1.2 TRANSMISSION AND NATURAL COURSE OF HIV-1 INFECTION

HIV can be transmitted mainly by three routes: unprotected sexual intercourse, blood transfusion and from mother to child during pregnancy, at delivery or through breast-feeding. In the absence of treatment, HIV-1 infection leads to a progressive loss of circulating CD4+ T lymphocytes which ultimately results in the immune dysfunction clinically defined as AIDS.

During the phase of primary HIV-1 infection, the virus infects a large number of CD4+ T cells. At this stage efficient immune responses against HIV-1 are still not mounted and thus, the viral replication and spreading is uncontrolled and a marked decrease in the numbers of circulating CD4+ T cells occurs. During this period the patient can experience influenza-like symptoms including fever, lymphadenopathy, myalgia and rash; however, in most of the cases this acute event can be confused with other common viral diseases due to the non-specific nature of such symptoms.
Eventually, the appearance of an adaptive immune response consisting of HIV-1 specific CD8+ T cells may control viral replication\textsuperscript{12}. This may allow the regain of CD4+ T cell numbers, ending the acute phase to enter a period of latency in which the patient remains asymptomatic during a variable period of time (from weeks to years). Although a good CD8+ T cell response has been linked to slower disease progression and a better prognosis, CD8+ T cells are not able to eliminate the virus\textsuperscript{12}. During this latent phase of the infection, the virus may not be detectable in the blood stream; however HIV-1 is active within lymphoid organs, where a large amounts of virus becomes trapped in the follicular dendritic cells (FDC) network\textsuperscript{13}.

The capacity of HIV-1 to establish latent infection of CD4+ T cells allows viral persistence despite specific immune responses and successful antiretroviral therapy\textsuperscript{14,15}.

It was initially suggested that the latent HIV-1 reservoir in the resting CD4+ T cell compartment is virologically quiescent in the absence of activating stimuli; however, it was shown later that low levels of ongoing viral replication persisted in patients receiving ART despite they were consistently aviremic\textsuperscript{16}. Furthermore, substantially higher levels of HIV-1 pro-viral DNA were found in activated CD4+ T cells rather than in resting CD4+ T cells, probably due to ongoing reactivation of latently infected, resting CD4+ T cells, favouring the virus spread by activated CD4+ T cells in these patients. Latent HIV-1 infection was also detected in mature CD4+ and CD8+ thymocytes, indicating that the virus must initially infect immature double positive (CD4+CD8+) thymocytes which then differentiate into either mature CD4+ or CD8+ (single positive) T cells\textsuperscript{17}.

Importantly, viral reactivation can occur in the absence of cellular DNA synthesis since it appears that activation but not necessarily proliferation is required to promote HIV-1 transcription. These events, by retuning the half-life of the latently infected CD4+ T cells, may allow intermittent refilling of the viral reservoirs despite prolonged periods of aviremia. Although present at low frequency, latent reservoirs persist for a long period of time\textsuperscript{18} and therefore they represent a serious obstacle to virus eradication.
1.3 PATHOGENESIS OF HIV-1 INFECTION

The main features defining HIV-1 pathogenesis are the gradual loss of peripheral CD4+ T cells and progressive immune deficiency that leads to opportunistic infections, malignancies and eventually death. However, the mechanisms underlying the severe lymphopenia induced by the infection are yet poorly understood.

From a general perspective, chronic infections in mouse and man cause persistent immune activation and consequent enhanced apoptosis that eventually leads to lymphopenia. HIV-1 infection provides a chronic immunologic stimulus that can enhance lymphocyte apoptosis; and in addition it has the ability of inducing lymphocyte apoptosis through direct or indirect mechanisms which are distinct from immune activation alone.

Several mechanisms have been proposed to explain CD4+ T cell depletion during HIV-1 infection; these include both direct and indirect pathogenic effects of the virus on mature CD4+ T cells as well as on their progenitors, a decreased production of new T cells, alteration of T cell homeostasis due to the lymphopenic environment and finally the paradoxical effect of chronic hyper-activation of the immune system.

1.3.1 T cell apoptosis during HIV-1 infection

There is strong evidence that T cell depletion during HIV-1 infection has its roots in increased apoptosis of both CD4+ and CD8+ T cells. As only a minor fraction of apoptotic lymphocytes are directly infected by HIV-1, the enhanced apoptosis observed during the course of infection cannot be explained solely by the direct effect of the HIV-1 infection. Indeed, HIV-1 encoded proteins have been shown to induce apoptosis of both infected and uninfected cells through various mechanisms.

On the other hand, unlike the gradual decline of CD4+ T cell numbers observed in the peripheral blood of HIV-1 infected humans\textsuperscript{19-21} and SIV-infected macaques\textsuperscript{22,23}, in the gut and other mucosal sites a massive and accelerated CD4+ T cell depletion occurs within the first weeks of infection\textsuperscript{19,21}. It was shown that such massive depletion of
CD4+ T cells from the gut during the acute phase of SIV infection in rhesus monkeys was associated to high infection frequency\textsuperscript{22,23}. The extensive CD4+ T cell apoptosis found in those reports was attributed to the direct viral infection, with cell destruction via either viral or cytotoxic T lymphocyte (CTL) mediated cytolysis or Fas-mediated apoptosis\textsuperscript{24,25}. Furthermore, it was shown that such massive depletion of the cellular targets for HIV-1 replication correlated with the drop in plasma viral load, which was observed after the initial viral replication. This observation suggests that improvements of CD4+ counts following the primary infection may be due to a temporary decline of the viral load due to depletion of the targets of viral replication and that HIV/SIV specific CD8+ T cells may not be the only determinants for control of viral replication\textsuperscript{22,26}.

Apoptosis is a highly regulated and coordinated cellular death process that is crucial for cellular homeostasis. Changes in the expression and function of the factors regulating apoptosis may account for the development of immune dysfunction observed during HIV-1 infection. Some of those regulators of apoptosis and their alterations are briefly described below.

\textit{1.3.1.1 Bcl-2}

Bcl-2 molecules play a key role in the regulation of lymphocyte death. Regulation of Bcl-2 expression might be crucial for the development and persistence of a memory T cell response following immune activation\textsuperscript{27}. Decreased levels of Bcl-2 expression were detected in CD8+ T cells from HIV-1 patients as compared to healthy controls. This low expression of Bcl-2 was associated with enhanced sensitivity to spontaneous and Fas-mediated apoptosis\textsuperscript{28}. Bcl-2 expression can be modulated by various factors including Interleukin-2 (IL-2) which can up-regulate Bcl-2 expression\textsuperscript{29}. During HIV-1 infection a defective production of IL-2 has been documented, and this was associated to the progressive depletion of the main source of IL-2, the CD4+ T cells\textsuperscript{30}. In addition, it was shown that HIV-1 replication in susceptible CD4+ T or monocytic cell lines induced the decrease of Bcl-2 expression, allowing an initial boost of replication\textsuperscript{31}. 
1.3.1.2 Fas / FasL

Fas molecule (CD95) belongs to the tumor necrosis factor receptor (TNFR) superfamily; it is characterized by three extra-cellular cystein-rich domains and an intracellular death domain shown to transduce signals for apoptosis\textsuperscript{32}. Direct \textit{in vitro} infection with HIV-1 or stimulation with viral proteins such as gp120, Tat and Nef induced up-regulation of FasL (Fas ligand, CD95L) on CD4\(^+\) T cells and on antigen presenting cells (APCs)\textsuperscript{33,34}. With progression of HIV-1 infection, an increasing level of Fas expression is detected in both CD4\(^+\) and CD8\(^+\) T cells in association with increased susceptibility to Fas-mediated apoptosis\textsuperscript{35,36}.

Increased expression of FasL by blood mononuclear cells and high plasma levels of soluble FasL are found in HIV-1 infected patients in correlation with viral load\textsuperscript{35,37}. FasL is also up-regulated on both CD4\(^+\) and CD8\(^+\) T cells from patients, thus converting those cells into possible effectors of apoptosis. Indeed activated CD4\(^+\)T cells expressing FasL can kill Fas-expressing CD8\(^+\) T lymphocytes\textsuperscript{38,39}. In addition, the lack of CD4\(^+\) T cell depletion observed in HIV-1 infected chimpanzees is associated with the lack of susceptibility of their T lymphocytes to Fas-mediated apoptosis, arguing for a role of the Fas pathway in CD4\(^+\) T cell depletion.

1.3.1.3 TNF

The regulation of both TNF and TNF receptors is also altered in HIV-1 infected patients\textsuperscript{40}. Elevated serum TNF levels are seen in symptomatic HIV-1 infected patients but not in asymptomatic patients\textsuperscript{41,42}. Both cognate receptors for TNF (p75 and p55) are expressed in a variety of cell types\textsuperscript{43}. \textit{In vitro} experiments have shown that HIV-1 infection of lymphocytes or monocytes induces TNF production\textsuperscript{44}. It is known that TNF activates HIV-1 transcription through activation of the transcription factor NF\(\kappa\)B, originating an autocrine loop that results in high levels of TNF production and increased levels of HIV-1 transcription\textsuperscript{45,46}.

Serum levels of soluble TNFR (p75) are predictive of HIV-1 disease progression, independently of other immunologic or virologic prognostic markers\textsuperscript{47}. Evidence for a role of TNF as a mediator of HIV-1 disease was also provided by the partial reduction of cell mediated killing of uninfected CD4\(^+\) T cells induced by the administration of
soluble TNFR decoys. A role for TNF/TNFR pathways was confirmed by the enhanced TNFR-mediated cell death found in T cells from HIV-1 infected individuals. Such enhanced cell death was associated to alteration of Bcl-2 expression and activation of caspases.

1.3.1.4 TRAIL

Another member of the TNF superfamily, the TNF-related apoptosis-inducing ligand (TRAIL) has been shown to be involved in CD4+ T cell death. TRAIL has two death receptors (DR) that induce apoptosis, DR4 (TRAIL-R1) and DR5 (TRAIL-R2); the other three TRAIL receptors TRAIL-R3, TRAIL-4 and osteoprotegerin lack the death domain and therefore are not able to initiate apoptosis.

It has been shown that HIV-1 induces apoptosis of uninfected CD4+ T cells by a TRAIL/DR5. This mechanism is triggered by type I IFN produced by HIV-1 stimulated plasmacytoid dendritic cells (pDC). TRAIL protein is expressed on the cell membrane (mTRAIL) or is secreted (sTRAIL) and both forms induce apoptosis of cells expressing functional DRs. TRAIL may contribute to HIV-1 immunopathogenesis because CD4+ and CD8+ T cells from HIV-1 infected patients are more susceptible to TRAIL-induced apoptosis in vitro than T cells from healthy donors. Also, HIV-1 Tat protein can indirectly induce apoptosis of CD4+ T cells through the stimulation of TRAIL production by monocytes.

All these findings indicate that TRAIL may contribute to the enhanced levels of T cell apoptosis observed during the course of HIV-1 infection. However, a recent study showed that recombinant TRAIL can actually help to reduce the HIV viral burden, probably by inducing apoptosis of cells that harbour latent HIV reservoirs.

1.3.2 Impaired regenerative capacity of the immune system during HIV-1 infection

Even though the immune system possesses an exceptional regenerative capacity, this property may not be boundless. Indeed, the life span of T cells in vivo is limited and after a certain number of divisions they reach a state of growth arrest suggestive of replicative senescence. The replicative history of a cell is commonly assessed by the length of their telomeres which are repetitive DNA sequences (TTAGGG in
vertebrates) located at the end of eukaryotic chromosomes and are critical for genomic stability. Telomeric DNA cannot be fully replicated and thus it is shortened during each round of cell division. Significant shortening of telomere length in both CD4+ and CD8+ T cells have been detected in HIV-1 infected patients.

Studies of the immunological changes occurring in aged individuals have shown phenotypical and functional alterations in T cells that are thought to be the cause of the generalized decline in immune responses and increased susceptibility to infections seen in the elderly population. Such a status termed immunosenescence is thought to occur as a consequence of persistent T cell activation and proliferation driven by repeated antigenic exposure experienced throughout life. Therefore, it has been proposed that during HIV-1 infection, the continuous viral replication which unceasingly stimulates the immune system may lead to an accelerated aging of T cells.

Despite intense T cell expansion upon antigenic stimulation the telomere length can be maintained by up-regulating the activity of telomerase, which is an enzyme that adds specific DNA sequence repeats to the 3' end of the telomeres. Telomerase activity, however also decreases after repeated antigenic stimulation. Continuous T cell stimulation induced by chronic HIV-1 infection may lead in this way to an eventual decreased replicative capacity of T cells.

The impaired production of new T cells during HIV-1 infection may be due to direct or indirect alterations induced in early progenitors. Studies performed on bone marrow progenitor cells showed a decreased number of lineage-restricted colony forming units and in some cases infection and/or apoptotic death of CD34+ progenitors. Although the mechanisms behind those findings remain obscure, the alterations induced in the progenitors by the virus could be reversed upon ART.

In addition, HIV-1 infection of the thymus has been detected in both children and adults, possibly contributing to the suppressed thymic function observed in HIV-1 infected patients. Accordingly, a diminished restoration of naïve T cell numbers, as indicated by the decreased frequency of T cells bearing a naïve phenotype (CD45RA+CD62L+) and T cells bearing TCR excision circles (TREC; markers of
recent intrathymic TCR rearrangement) was found in association to disease progression\textsuperscript{70,71}. Thymocyte depletion and cortical and medullar architectural changes have been observed in association with viral replication within thymocytes\textsuperscript{72}. Further evidence for the HIV-1 induced thymic dysfunction is provided by several studies showing a regained thymopoyesis after treatment of some individuals upon effective ART\textsuperscript{73-75}.

The peripheral lymphoid organs also suffer alterations upon HIV-1 infection. Asides depleting both CD4+ and CD8+ T cell populations, HIV-1 infection leads to an altered structure of T cell niches in lymphoid tissues, disrupted by fibrosis related to chronic immune activation and inflammation\textsuperscript{76,77}. This process may interfere with T cell trafficking within the reticular cell network that produces survival factors such as Interleukin-7 (IL-7) and consequently limiting the access to important survival signals.

In summary, the regenerative capacity of mature T cells is progressively lost during HIV-1 infection, particularly at the later stages, either due to insufficient regeneration of central memory T cells or as a result of excessive differentiation, cell death, or progressive destruction of lymph node architecture associated with chronic inflammation.

### 1.4 T CELL HOMEOSTASIS DURING LYMPHOPENIA

There are two primary pathways for T cell regeneration, one is the thymic-dependent differentiation of bone marrow (BM) derived progenitors and the second is the thymic independent antigen-driven peripheral expansion of mature T cells.

#### 1.4.1 The thymic-dependent pathway

Initial evidences for thymic-dependent T cell regeneration in humans came from studies performed in children with severe combined immunodeficiency who were treated with BM transplantation (BMT). In those cases, the T cell regeneration was often complete, with the restoration of numbers and functions of the peripheral blood T
cells to normal. Further evidence of the relevance of the thymic pathway in T cell regeneration was provided by studies on patients receiving intensive chemotherapy, which induces a profound CD4+ T-cell depletion with a complete loss of the naïve subsets. In those studies children, but not young adults, showed a recovery of CD4+ T cell numbers within 6 months from cessation of chemotherapy. In addition, the CD4+ T cell recovery was accompanied by the reappearance of naïve (CD45RA+CD45RO-) CD4+ T cells, which is a useful clinical marker for thymically-derived CD4+ T cell populations.

Interestingly, the children showed a significant enlargement of the thymus, even above baseline values, which has been termed thymic rebound. Since similarly treated young adults showed a persistent CD4+ T cell depletion for over one year or more from the treatment, age appeared to be the main factor determining CD4+ T-cell regenerative capacity. Similar results were observed in children and adults after allogeneic BMT, where the total CD4+ T-cell numbers in the peripheral blood and the ability to generate naïve-type CD4+ T cells is inversely related to age. Thymic-dependent pathway of T-cell regeneration displayed by children is characterized by the relatively rapid rise of peripheral blood naïve CD4+ T cells and the normalization of peripheral blood total T-cell numbers over the course of several months. Moreover, these paediatric patients regain an immunocompetent status, as demonstrated by the normalization of functional T-cell responses and the ability to respond to neo-antigen via vaccination. Interestingly, the loss of CD8+ naïve T cells (which parallels the decline in total CD4+ T-cell numbers) has been observed in children with progressive HIV infection. This suggests that HIV-1 infection, by targeting thymic tissue, may impair thymic-dependent T-cell regenerative pathways even in young children.

1.4.2 Thymic-independent pathways

Because thymic involution occurs early in life, when T cell depletion occurs in adulthood the thymic-dependent T cell regeneration is limited. Even when thymic recovery can occur, it is delayed for at least one year following lymphopenia. Therefore, T cell regeneration following the onset of lymphopenia relies mainly upon thymic-independent mechanism known as homeostatic peripheral expansion (HPE).
During lymphopenic conditions, the degree of expansion exhibited by T cells upon encountering of cognate antigen (Ag) exceeds the expansion found in replete hosts in response to the same Ag\textsuperscript{83}. Also, the degree of expansion in response to cognate Ag exceeds that occurring in response to Ag with lower affinities for the TCR\textsuperscript{84}. Hence, upon HPE, there is a tendency of T cell repertoires to be oligoclonal and skewed toward dominant Ags\textsuperscript{83}. Besides the exaggerated response to cognate Ag, it was demonstrated that HPE involves the proliferation of T cells toward low affinity Ags, which were represented by both self-Ags and low affinity cross-reactive environmental Ags\textsuperscript{84,85}. Additionally, it was shown that IL-7 was required for the proliferation of naïve cells in response to low affinity Ags during HPE\textsuperscript{86,87}, whereas IL-15, IL-4 and other cytokines tested were not required. Importantly, the role for IL-7 in the induction of HPE appears to be consistent regardless of the method by which lymphopenia is induced\textsuperscript{86,88}.

Several studies in mice show that naïve T cells acquire phenotypical and functional features of memory T cells during HPE\textsuperscript{89,90}. In those studies, the proliferation of naïve T cells in lymphopenic hosts resulted in increased expression of memory markers, although up-regulation of the activation markers CD69 and CD25 did not occur\textsuperscript{90-92}. Also, the memory phenotype of those cells was shown to be stable and they became more responsive to specific Ag. In addition, T cells generated upon HPE exhibited an increased sensitivity to apoptosis. In the case of the CD4+ T cells, their increased sensitivity to apoptosis was due to decreased IL-7R and Bcl-2 expression, while the apoptosis of CD8+ T cells was death receptor mediated (Fas/FasL pathway)\textsuperscript{93}. Furthermore, a role for cytokine production was proposed for the increased sensitivity to apoptosis, as IFN\textgreek{g} was shown to up-regulate Fas and FasL on CD8+ T cells leading to receptor mediated apoptosis. IFN\textgreek{g} also induced down-regulation of IL-7R and Bcl-2 expression on the CD4+ T cells. IL-2 may also play a role in the increased sensitivity to apoptosis through the down-regulation of IL-7R and Bcl-2 in CD4+ T cells\textsuperscript{94}.

Remarkably, the CD4+ and CD8+ T cell subsets show different patterns of expansion during HPE. CD8+ T cells regenerate at a more rapid rate than CD4+ T cells after chemotherapy induced depletion\textsuperscript{95,96}. CD8+ T cell subsets also present a heterogeneous pattern of recovery. It was shown that CD8+ CD28+ and CD8+CD45RA+ subsets exhibited a moderate and slow pattern of recovery, respectively. On the contrary, CD8+CD28- and CD8+CD57+ subsets displayed a very high rate of recovery, reaching
values that were even higher than the levels shown prior to chemotherapy\textsuperscript{95}. CD4+ T cells, on the other hand, have a propensity for prolonged deficiencies after depletion in vivo, either due to their requirement of Thymic-dependent pathways for their rapid recovery or due to short lived apoptosis prone CD4+ T cells generated upon HPE\textsuperscript{96}.

T cell populations following lymphocyte depletion induced either by chemotherapy regimes or by HIV-1 infection share many common features. In both cases all T cell populations present with an increased frequency of memory phenotype (CD45RO\textsuperscript{+}) in parallel with an enhanced expression of activation markers (HLA-DR and CD38)\textsuperscript{25,81}. The CD4+:CD8\textsuperscript{+} T cell ratios may decrease due to both an increased CD4\textsuperscript{+} T loss or to the more effective recovery exhibited by CD8\textsuperscript{+} T cells, resulting in the prolonged T cell subset imbalance observed in lymphopenia induced by chemotherapy treatment or HIV-1 infection. Similarly to HIV-1 infection, the TCR repertoire upon BMT shows also evidence of oligoclonal expansions or loss of repertoire diversity\textsuperscript{97,98}. Furthermore, alike T cells from HIV-1 infected individuals, T cells from chemotherapy treated patients are also susceptible to apoptosis upon activation. Such increased susceptibility to apoptosis may be responsible for the decline in CD4\textsuperscript{+} T cell numbers observed in those patients.

### 1.4.3 Interleukin-7

Human interleukin-7 (IL-7) is a 25KDa protein encoded by a six exons-gene located on the chromosome 8q12-13. IL-7 signals through a heterodimer composed by the common cytokine signalling gamma chain (\(\gamma_c\)) and the IL-7R\(\alpha\) (CD127). Due to the ubiquitous expression of the \(\gamma_c\) by lymphocytes, the responsiveness to IL-7 is controlled by the expression of IL-7R\(\alpha\).

Different cell types have shown the ability of producing IL-7 such as bone marrow and thymic stromal cells, thymic epithelial cells, dendritic cells, keratinocytes and the intestinal epithelium\textsuperscript{99}. Recently, a specialized type of stromal cells – the T zone fibroblastic reticular cells (FRCs) - was identified as the main source of IL-7 in lymph nodes\textsuperscript{100}. Whether these are relevant sources of IL-7 production \textit{in vivo} and whether
such production occurs at a constitutive rate or whether it is susceptible of regulation is not well defined.

IL-7 is a crucial factor for the survival, proliferation and function of T cells at different stages of differentiation. The mechanisms through which IL-7 exerts its functions are not fully understood and it is believed that IL-7 acts through the maintenance of basic cellular homeostasis (i.e. transport mechanisms, metabolic activity) and through the regulation of anti-apoptotic and pro-apoptotic Bcl-2 family member proteins. In addition, IL-7 can act as a costimulatory molecule for T cell activation induced by cognate antigens. Furthermore, it has been shown that IL-7 induces homeostatic peripheral expansion of T cells in response to low affinity antigens in lymphopenic hosts and promotes memory formation.

![Figure 1. Levels of serum IL-7 in relation to CD4+ and CD8+ T cell counts.](image)

The modulation of IL-7 that accompanies the alteration of CD4+ and CD8+ T cell counts occurs during both the primary and chronic phases of HIV-1 infection. Whether T cells can benefit from the increased availability of IL-7 is yet to be clarified.

During HIV-1 infection, as well as in other lymphopenic conditions (idiopathic CD4+ T lymphocytopenia or cyto-reductive therapies for cancer, autoimmune diseases or bone marrow transplantation) a negative correlation has been found between
plasma levels of IL-7 and CD4+ T cell counts. Such increase of circulating IL-7 has been documented over all stages of the disease\textsuperscript{109} including in patients with primary HIV-1 infection\textsuperscript{110,111}, probably as a result of early T cell depletion (Figure 1). More recently, it was shown that long-term non-progressors (LTNP) exhibited significantly lower serum levels of IL-7 compared to progressors and the LTNP who lost their non-progressor status\textsuperscript{112}.

Two models have been proposed to explain the high levels of IL-7 found in association to T cell depletion. The first one, a homeostatic model, suggests that the IL-7 producer cells sense the lymphopenia and respond with an increased production of IL-7, originating an increased availability of the cytokine which will in turn enhance survival and antigen-driven expansion of the residual T cells, aiming at restoring T cell homeostasis\textsuperscript{102,106}. Evidence of a role for IL-7 in the immune reconstitution of T cells during HIV-1 infection was provided by studies showing that in untreated patients at advanced stages of HIV-1 infection the levels of IL-7 in serum were elevated and inversely correlated with CD4+ and CD8+ T cell counts. In the case of treated patients the levels of IL-7 were undetectable, only when the patients responded to HAART. On the contrary, in the cases of HAART failure the levels of IL-7 were comparable to those found in the group of untreated patients IL-7 and HAART.\textsuperscript{113}

The second model suggests that serum IL-7 levels increase passively as the T cells consuming IL-7 are depleted. According to this model IL-7 is produced at a fixed constitutive rate which limits the size of the T cell pool and the levels of IL-7 are regulated through consumption rather than production\textsuperscript{101,114}. This may be the case during chronic HIV-1 infection, in which IL-7 accumulation occurs concomitantly with severe T cell depletion and with a decreased efficiency of IL-7 in inducing T cell reconstitution. Such scenario might be worsened by the IL-7Rα down regulation observed on the T cells in parallel with increased serum IL-7 levels\textsuperscript{115}.

Although the mechanisms underlying the elevated IL-7 concentration found in the serum of HIV-1 infected individuals are not completely understood. The increase of IL-7 has been interpreted as a mechanism that might stimulate the regeneration of the T cell pool by promoting maintenance and proliferation at various stages of T cell differentiation. However, in chronic HIV-1 infection, the highest IL-7 levels in plasma are observed predominantly when CD4+ T cell number falls below 200/µl, a late stage
of HIV-1 infection when T cells seem to be incapable of spontaneous regeneration and high IL-7 levels may not be sufficient to counteract T cell depletion\textsuperscript{106,109,117}. Therefore, the regenerative effects of lymphopenia-induced IL-7 in HIV-1 infected individuals remain mostly speculative, based on animal models and in vitro data. So far, a positive role for IL-7 on T cell restoration has been suggested only during the early phase of HIV-1 infection\textsuperscript{111} and in sporadic cases during chronic infection\textsuperscript{112}. The mechanisms that hold back T cell recovery despite the high levels of IL-7 during HIV infection are yet to be defined.

### 1.5 T CELL HYPER ACTIVATION DURING HIV-1 INFECTION

HIV-1 infection is characterized by a generalized state of immune activation that persists throughout the entire course of the infection. Importantly, the levels of immune activation correlate to disease progression\textsuperscript{118-120}. Thus it is currently believed that such chronic over activation of the immune system plays a major role in HIV-1-induced pathogenesis and the ensuing development of AIDS\textsuperscript{121}.

![Figure 2. High levels of immunoactivation are directly correlated to activation induced apoptosis during HIV-1 infection.](image)

Several parameters have been used to quantify the levels of T cell activation in vivo, including cell surface expression of CD38, HLA-DR, CD25, CD69, neopterin, TNFR type II and $\beta_2$-microglobulin\textsuperscript{118,122-124}. Among these, CD38 expression has shown to
have a high prognostic significance\textsuperscript{125}. High levels of T cell activation, determined by the expression of HLA-DR and CD38 in CD4+ and CD8+ T cells correlate better to disease progression than viral load\textsuperscript{119,120,125,126}.

### 1.5.1 Role of immunoactivation in HIV-1 pathogenesis

Indirect evidences of the fundamental role of immunoactivation in the pathogenesis of HIV-1 infection have been provided by studies of SIV-infection in primates, by studies of HIV-2 infection and finally by the impact of HAART on immunoactivation.

#### 1.5.1.1 Primate models

Important indications of the role of immune activation on HIV-1 pathogenesis have been provided by comparative studies of two simian models of SIV infection: the non-pathogenic SIV infection of sooty mangabeys (SMs) occurring under natural conditions, and the pathogenic SIV-infection of rhesus macaques (RM) under experimental conditions, which leads to the development of a disease similar to AIDS in humans. The SIV-infected SMs present with a preserved CD4+ T cell homeostasis, do not develop immunodeficiency and exhibit low levels of T cell activation, despite manifested viral replication. In contrast, the RM, like HIV-1 infected humans, experience gradual CD4+ T cell loss and progression to AIDS and in parallel present with high levels of T cell activation\textsuperscript{127,128}.

The finding that both SMs and RM suffer similarly of massive deletion of CD4+ T cells from gut mucosa early upon SIV-infection\textsuperscript{129} and that the level of mucosal CD4+ T cells remains stable in SMs while it gradually declines in RM, suggests that the differences in mucosal immune function observed between SMs and RM develop later during the chronic phase of infection.

Another difference between SIV-infected SMs and RM is in the expression of CCR5 by CD4+ T cells. In RM (and in humans) approximately 10-20% of blood and >50% of mucosal tissues CD4+ T cells express CCR5, whereas the fraction of CD4+CCR5+ T cells in SMs is much lower, 1-5% in both blood and mucosal associated lymphoid tissue (MALT)\textsuperscript{130}. Although the low expression of CCR5 on CD4+ T cells does not protect SMs from infection, the apparent restricted expression of CCR5 to effector/activated CD4+T cells will limit SIV-infection to short lived, dispensable CD4+ T cells, preserving in this way the central memory CD4+ T cells from
infection. Low expression of CCR5 may also prevent homing of CD4+CCR5+ T cells to inflamed tissues in which they would otherwise became a target for SIV-infection.

1.5.1.2 HIV-2

Another source of evidence for a role of immune activation in the development of HIV-1 pathogenesis is provided by studies on HIV-2 infection. Most HIV-2 infected individuals exhibit a slow disease progression; they usually display lower levels of immune activation as compared to HIV-1 infected patients. It was shown that in both HIV-1 and HIV-2 infected patients, CD4+ T cell depletion was similarly correlated to the levels of immune activation, despite a large difference in viremia levels. In addition CD8+ T cells expressing the early activation marker CD69 were more frequent in HIV-1 infected patients than in HIV-2 infected patients.

1.5.1.3 The impact of HAART and adjunct therapy on immunoactivation

Further evidence for a role of immunoactivation in the progression of the HIV-1 infection is that upon highly active antiretroviral therapy (HAART), the increase in CD4+ T cell counts appears to be better correlated with the reduction of immune activation and apoptosis rather than with suppression of HIV-1 replication. In this context, the use of cytostatics as adjuncts to antiretroviral therapies have been shown to have beneficial effects by limiting the levels of immunoactivation and reducing the number of activated HIV-1 target cells through their immunomodulatory effects.

1.5.2 Possible causes of immunoactivation during HIV-1 infection

Despite the substantial evidence showing that immunoactivation plays an important role in HIV-1 pathogenesis, the mechanisms responsible for this phenomenon remain obscure. Nevertheless, multiple factors have been found that could possibly contribute to the generalized immunoactivation observed during the course of HIV-1 infection.

1.5.2.1 Chronic Activation by HIV-1

The establishment of immune activation and inflammation during HIV-1 infection involves mechanisms that are directly or indirectly related to viral replication. Even though the eventual appearance of specific immune response is able to control viral replication, HIV-1 is never eliminated completely. Therefore, it has been suggested
that as with lymphocyte expansion and contraction in response to conventional antigen challenge, chronic activation by HIV-1 triggers bursts of lymphocyte proliferation, differentiation and death and that this superposition of bursts leads to the relatively constant overall T cell turnover observed during the course of the infection\textsuperscript{138,139}.

In addition, several studies suggest that HIV-1 antigens, in the absence of direct infection, can induce activation of T cells and APCs resulting in the production of pro-inflammatory cytokines and chemokines\textsuperscript{140-142}. The envelope protein gp120 binds to CD4 and/or CCR5, resulting in intracellular signalling and activation of immune cells in the absence of direct infection\textsuperscript{52,143}. A similar effect was induced in lymphocytes by the protein Nef\textsuperscript{144,145}.

1.5.2.2 T cell activation by other pathogens

The sustained antigen-induced immunoactivation occurring during HIV-1 infection may also be due to other viruses such as CMV and EBV. In healthy individuals, intermittent CMV reactivation appears to occur, as evidenced by the increased numbers of CD69\textsuperscript{+} CMV-specific cells indicative of recent in vivo activation\textsuperscript{146}. In HIV-1 infected individuals, the observed lymphopenia may result in the deficient immune control of these persistent viruses and thus allow their replication and reactivation. Furthermore, the inflammatory conditions present during HIV-1 infection may also participate in the reactivation of latent forms of CMV and EBV. This is supported by recent studies showing a significant activation of EBV and CMV-specific CD8\textsuperscript{+} T cells during HIV-1 acute infection\textsuperscript{147,148}.

In addition, other pathogens including those causing opportunistic infections during the later stages of the disease, might also play roles in the HIV-1 associated immunoactivation\textsuperscript{149,150}. Infections by helminthes may also result in a more rapid progression to AIDS, possibly by augmenting the level of activation of the immune system\textsuperscript{151}.

1.5.2.3 Role of mucosal antigens in chronic immune activation

As mentioned before, unlike the gradual decline of CD4\textsuperscript{+} T cell numbers observed in the peripheral blood of HIV-1 infected humans\textsuperscript{19-21} and SIV-infected macaques\textsuperscript{22,23}, in the gut and other mucosal sites a massive and accelerated CD4\textsuperscript{+} T cell depletion occurs within the first weeks of infection. The disruption of the mucosal barrier originated in that way may result in a systemic microbial translocation from the intestinal lumen to
the systemic circulation, where they can activate the immune system\textsuperscript{152,153}. Translocation of bacterial products is very likely to result in activation of the innate immune response through interaction with APCs by binding of toll like receptors (TLRs) which in turn will induce the production of pro-inflammatory cytokines such as TNF, IL-6 and IL-1\(\beta\), eventually leading to systemic activation and differentiation of lymphocytes. This model is supported by increased levels of plasma LPS found during HIV-1 infection, moreover a positive correlation was found between plasma LPS levels and levels of immune activation\textsuperscript{152}. However, these findings are still controversial and need further confirmation with studies involving large clinical cohorts\textsuperscript{154}.

1.5.2.4 Bystander activation

Another potential factor contributing to immunoactivation during HIV-1 infection is the non-antigen specific bystander activation of T and B lymphocytes. This phenomenon is caused by increased production of pro-inflammatory cytokines (TNF and IL-1\(\beta\)) and lymphopenia induced regulators (like IL-7). Although the mechanisms of this ‘bystander’ activation are still not clear, it is possible that they also involve the up-regulation of apoptosis related molecules (CD95, TRAIL, DR4/5) on the surface of T cells, rendering them prone to activation-induced cell death\textsuperscript{34,52,155,156}.

1.5.2.5 Depletion/dysfunction of regulatory T cells

A special T cell subset, the CD4+CD25+ regulatory T cells (\(T_R\)) can suppress immunoactivation via direct cell-cell contact, production of cytokines, and inhibition of DC activity. The depletion or dysfunction of these cells is another potential factor accounting for the enhanced immunoactivation during HIV-1 infection\textsuperscript{157}. However, several studies regarding the role of \(T_R\) in HIV-1 and SIV infection suggest that \(T_R\) may play a dual role. \(T_R\) may be protective if suppressing the chronic immune activation but detrimental if inhibiting effective T-cell responses\textsuperscript{158-161}.

1.5.3 Consequences of immune activation during HIV-1 infection

Again, although a growing body of evidence underscores chronic immune activation as a key determinant of immunodeficiency in HIV-infected individuals, the exact mechanisms by which this phenomenon contributes to CD4+ T-cell depletion and
disease progression remain unknown. Nevertheless, some hypothetical mechanisms have been proposed:

1.5.3.1 **Generation of available targets for HIV-1 replication and elimination of HIV-1 specific T cells**

The activation, proliferation and differentiation of naïve and memory CD4+ T-cell leads to increased CCR5 expression that renders these cells more susceptible to infection\(^{162}\). Also, HIV-1 is known to replicate more efficiently in activated CD4+ T lymphocytes, therefore the preferential activation, infection and killing of HIV-specific CD4+ T cells\(^{163}\) results in the loss of CD4+ T-cell help. This potentially contributes to the impairment of CTL responses to the virus.

1.5.3.2 **Alteration of the homeostasis of the T-cell pool leads to gradual depletion of T cells**

The chronic T cell activation induced during the course of HIV-1 infection implies an increased and accelerated differentiation of naïve T cells to T cells with effector/memory phenotypes. With disease progression there is a decrease in the proportion of naïve (CD45RA+CD62L+) T cells and an increase of activated memory/effector (CD45R0+CD62L-) T cells. This expanded activated memory/effector populations are short-lived and occur among both CD4+ and CD8+ lineages\(^{164,165}\). The expansion of such phenotype of CD4+ cells may cost the reduction of the naïve and memory T-cell pools, resulting in a reduced capacity of the immune system to generate effective responses to new or previously encountered antigens. Chronic immunoactivation may also lead towards the proliferative senescence of the T cell pool, giving place to an interesting view of AIDS as a disease characterized by the premature aging of the immune system\(^{62}\). HIV-1 infection, in the same way as other chronic inflammatory conditions, is characterized by the increased numbers of a population of T cells lacking CD28 expression. CD28- T cells are regarded as antigen-experienced cells that have reached terminal stages of differentiation; they have been reported to display features of senescence, including shortened telomeres and expression of CD57, which is expressed on the majority of CD28- T cells, particularly for HIV-1 specific CD8+ T cells\(^{166,167}\). Other studies analysing antigen-specific T cell responses indicated that HIV-1 specific CD8+ T cell clones, characterised by PD-1 expression and impaired proliferation upon encountering of specific antigens, down-regulated CD28 expression\(^{168}\).
In addition the expansion of activated memory/effector cells might be accompanied by the production of pro-inflammatory and pro-apoptotic cytokines that complete the vicious cycle sustaining the generalized immune activation associated with pathogenic HIV-infection.

1.5.3.3 Disruption of lymphoid T cell niches
As mentioned before, during HIV-1 infection the structure of T cell niches in lymphoid tissues may be disrupted by fibrosis related to chronic immune activation and inflammation\textsuperscript{76,77}. Such disruption of the T cell niches may restrain not only the antigen-dependent T cells expansion but the access to important survival signals.

1.5.3.4 Impairment of the regenerative capacity of the immune system
The regenerative capacity of mature T cells is progressively lost during HIV-1 infection, particularly at the later stages, due to either insufficient regeneration of central memory T cells, excessive differentiation, cell death or progressive destruction of lymph node architecture associated with chronic inflammation. In addition, immunoactivation may also impair the regenerative capacity of the immune system at the levels of bone marrow, thymus, and lymph nodes \textsuperscript{68,69,76}.

1.5.4 CD28- T lymphocytes

1.5.4.1 Characteristics and origin
CD28 is a member of the immunoglobulin super family, which is normally expressed on the majority of CD4+ T cells and CD8+ T cells in human peripheral blood\textsuperscript{169}. CD28 is a key co-stimulatory molecule, which is down-regulated upon T cell activation. Although there is a clear association between persistent inflammatory conditions and enlargement of the CD28- T cell population, the mechanisms through which these cells are originated remain unclear. Increased numbers of T cells lacking CD28 molecule are found in HIV-1 infected patients\textsuperscript{60} (Figure 3), as well as in patients suffering of other chronic inflammatory conditions such as rheumatoid arthritis\textsuperscript{170}, Wegener's granulomatosis\textsuperscript{171} and multiple sclerosis\textsuperscript{172}, and in the elderly populations \textsuperscript{60,173}. Thus, the enlarged size of CD28- T cell population observed during HIV-1 infection, which can represent more than 50\% of the peripheral T cell pool\textsuperscript{60,62,173,174}, is probably the result of several factors including persistent antigen-specific activation and chronic
bystander activation prevailing during the course of the disease. There is strong evidence indicating that CD28- T cells originate from CD28+ precursors that have undergone repeated antigenic stimulation\textsuperscript{175,176,177}. In addition CD28- T cells have been shown to exhibit a restricted TCR diversity \textsuperscript{170,178,179} and the shortening of telomeres\textsuperscript{180,181}, further suggesting their origin upon antigenic stimulation \textit{in vivo}. Interestingly, it has been shown that the expression of CD28 molecules can also be modulated by other factors such as HIV-derived proteins\textsuperscript{182} and by cytokines like TNF\textsuperscript{175} and IL-12\textsuperscript{183}.

![Graph showing CD28 expression in HIV- and HIV+ conditions](image)

**Figure 3. The number of CD28- T cells is increased during HIV-1 infection**

\textbf{1.5.4.2 Functionality}

CD28- T cells are regarded as antigen-experienced cells that have reached terminal stages of differentiation. CD28- T cells have been shown to exhibit shortened telomeres and expression of CD57 that have been proposed as markers of senescence\textsuperscript{166,167}. Furthermore, the loss of CD28 expression has been associated to the impaired capacity of T cells to undergo cell division\textsuperscript{174,184-186}. Because the \textit{in vitro}- induced loss of CD28 expression also coincides with their resistance to activation-induced apoptosis\textsuperscript{187,188}, it has been proposed that the apparent expansion and persistence of the CD28- T cell population \textit{in vivo} upon aging or under chronic inflammatory conditions, occurs as a result of accumulation.

In addition, a distinct subset of human T regulatory cells (T\textsubscript{R}) characterized by their CD8+CD28- phenotype and termed T suppressors (T\textsubscript{S}) has been reported to act as negative regulators of the immune response\textsuperscript{189}. \textit{In vitro} generated CD28- T cells appear to inhibit the antigen-presenting function of DCs by inducing inhibitory receptors that will render DCs tolerogenic.
Studies analysing antigen-specific T cell responses indicated that HIV-1 specific CD8+ T cell clones, characterised by PD-1 expression and impaired proliferation upon encountering of specific antigens, down-regulated CD28 expression\textsuperscript{168}. In addition, in aged individuals, antigen experienced CD8+ T cell clones with limited TCR diversity, low IL-7R expression, increased sensitivity to \textit{ex vivo} apoptosis and impaired proliferative abilities have been shown to accumulate and these cells were also characterized by CD28 down-regulation\textsuperscript{190}. Another study indicated that the lack of CD28 expression may not necessarily correlate with the low proliferative ability of T cells in HIV-1 infected patients\textsuperscript{166}.

The divergent observations regarding the association between CD28 loss and impaired survival and/or proliferation could be explained by the different experimental settings in which the functionality of those cells were tested and the way in which those cells were obtained (clonal expansion in long term cultures or \textit{ex vivo} isolated). This indicates a context dependent regulation of the CD28- T cell population that may be influenced by antigen-specificity, level of immune activation or disease stages.
2 AIMS OF THE THESIS

The present thesis is focused on two main aspects of the pathogenesis of HIV-1 infection 1) the lymphopenia-induced enhancement of T cell sensitivity to apoptotic and proliferative signals and 2) the role of immune activation in the induction of a terminally differentiated phenotype of T cells that might further contribute to the exacerbation of inflammatory reactions and chronic T cell activation observed during HIV-1 infection.

The specific aims of this thesis are:

- To verify the involvement of IL-7 in the increased Fas expression by T cells and in the increased propensity to Fas-mediated apoptosis during HIV-1 infection.
- To corroborate the existence of a co-stimulatory role for Fas signals during HIV-1 infection, and to investigate whether there is a link between elevated IL-7 and the sensitivity of T cells to Fas induced proliferation.
- To evaluate the influence of disease progression in the regulation of survival and activation of CD28- T lymphocytes during HIV-1 infection.
- To evaluate the role of CD28- T lymphocytes in inflammatory reactions and immune activation through the modulation of DC and T cell responses during HIV-1 infection.
3 RESULTS AND DISCUSSION

3.1 IL-7 AS A DRIVING FORCE OF FAS-MEDIATED SIGNALS DURING HIV-1 INFECTION: A RESCUE MECHANISM OR A PATHWAY TO EXHAUSTION?

High levels of serum IL-7 are detected in parallel with decreasing numbers of CD4+ T cell counts during HIV-1 infection and lymphopenia of other aetiologies\(^{105,106,108,109,113,117}\). Such increase of IL-7 availability is expected to have beneficial effects on T cell restoration; however during HIV-1 infection the benefits of such elevated levels of IL-7 on T cell repopulation are questionable.

IL-7 has also been implicated in the up-regulation of the death receptor Fas, and consequently in the increased sensitivity to Fas mediated signals\(^{191-193}\). It is well known that Fas expression and sensitivity to Fas mediated apoptosis of T cells is enhanced during HIV-1 infection\(^{28,35,194-197}\). On the other hand, Fas appears to have other distinct functions than as mediator of cell death. These additional functions include a role for Fas signals in tissue repair\(^{198-200}\), activation of APCs\(^{201}\) and chemo-attraction of neutrophiles\(^{202}\). Importantly, a co-stimulatory role of Fas was demonstrated upon suboptimal doses of anti-CD3\(^{203-207}\).

The concomitant existence of elevated serum levels of IL-7 and increased expression of Fas molecules observed in HIV-1 infected individuals suggested a role for IL-7 in the modulation of Fas-mediated signals during the course of the infection. In papers I and II we evaluated the effect of high IL-7 levels in the sensitivity of T cells to respond to either apoptotic or co-stimulatory signals induced by Fas, during HIV-1 infection.

3.1.1 The survival factor IL-7: an inducer of Fas-mediated apoptosis?
(Paper I)

We started by evaluating the effect of high doses of IL-7 on Fas expression by T cells isolated from healthy individuals. After 5 days culture in the presence of IL-7, T cells exhibited an increased expression of Fas molecules. This effect of IL-7 was detected in both naïve and memory T cell subsets.
The up-regulation of Fas appeared to occur at the post-transcriptional level as the levels of Fas mRNA did not change in the IL-7 treated cells. In addition, the total amount (at the cell surface and intracellular) of Fas protein did not change upon IL-7 treatment. This latter result suggests that the IL-7 induced Fas up-regulation occurs as a result of redistribution of Fas molecules from the intracellular compartments to the cell membrane, or may be due to stabilization of Fas in the cell membrane rather than increased Fas production.

Fas mediated apoptosis requires the polarization of Fas receptors, an event that occurs through an ezrin-mediated association with the actin cytoskeleton\textsuperscript{208-210}. Upon activation, Fas molecules are recruited to the uropod, a characteristic pole of the cell involved in cell-cell communication. As IL-7 induces a morphological polarization of T cells in culture that results in the development of one or two dominant protrusions similar to the uropod of activated T cells, we investigated whether IL-7 treatment could induce the polarization of Fas receptors. Immunofluorescence staining and microscopic analysis showed that IL-7 induced Fas polarization in both naïve and memory T cells. No polarization was observed on untreated naïve T cells and only a weak polarization occurred on untreated memory T cells. Only T cells which polarized Fas molecules showed a colocalization of ezrin and Fas. As expected, a similar polarization of Fas molecules was observed on T cells activated with anti-CD3 antibody (Ab).

CD43, another molecule known to be recruited to the uropod of activated T cells through association with ezrin\textsuperscript{211}, also colocalized with Fas on IL-7 treated T cells, further indicating a similar molecular organization induced by IL-7 and anti-CD3. The direct interaction between Fas and ezrin induced by IL-7 suggested the predisposition of the IL-7 treated T cells to Fas-mediated apoptosis.

We next tested the potential effects of IL-7 on Fas expression \textit{in vivo}. Cynomologous monkeys were injected daily with different doses of IL-7 during 10 days and Fas expression was measured before and after treatment. A significant Fas up-regulation was induced in both CD4+ and CD8+ T cells by the highest doses of IL-7. The levels of Fas expression normalized 10 days after IL-7 treatment ceased.
The Fas up-regulation induced by IL-7, in vitro and in vivo, supported the hypothesis that elevated serum levels of IL-7 could account for the high levels of Fas expression and increased sensitivity to Fas mediated apoptosis observed in HIV-1 infected patients. To confirm this, we measured serum IL-7 levels in parallel to Fas expression on T cells from a group of HIV-1 infected patients and both parameters were compared to CD4+ T cell counts. In line with previous reports, serum IL-7 concentrations and Fas expression on T cells increased in parallel with CD4+ T cell depletion. Remarkably, IL-7 levels positively correlated with Fas expression on naïve and memory T cell subsets, strongly suggesting IL-7 as an inducer of Fas expression during HIV-1 infection.

Subsequently, we investigated whether the increased Fas expression induced by IL-7 could determine an enhanced sensitivity to Fas mediated apoptosis. T cells from healthy individuals that were cultured during 5 days in the presence of IL-7 and then stimulated with anti-Fas Abs exhibited an enhanced sensitivity to apoptosis (Figure 4). When looking at the different T cell subsets, we observed no differential sensitivity to apoptosis between CD4+ and CD8+ T cells; the memory subset, however, showed to be more sensitive to Fas mediated apoptosis that the naïve subset.

Figure 4. Fas mediated apoptosis in vitro is enhanced by IL-7 treatment. Fas expression was measured by flow cytometry on T cells from healthy individuals at day 0 and at day 5 of culture in the presence or absence of IL-7. The rate of apoptosis is indicated by the percentage of Annexin V positive cells.
Finally, we evaluated the possibility that increased levels of IL-7 in HIV-1 infected individuals could be linked to an increased sensitivity of T cells to Fas-induced apoptosis. The levels of apoptosis upon Fas cross-linking were tested in ex vivo isolated T cells from HIV-1 infected patients and the IL-7 concentration was measured in the serum of the same patients. We found that the ratio of apoptotic cells positively correlated with the serum IL-7 concentration in both naïve and memory T cell subsets. Altogether our findings confirmed our hypothesis of a role for IL-7 as an inducer of Fas expression and Fas-mediated apoptosis during HIV-1 infection.

3.1.2 Fas-costimulation during HIV-1 infection and the implication of increased availability of IL-7 induced by lymphopenia (Paper II)

Our working hypotheses for this study were 1) T cells from HIV-1 infected individuals which express high levels of Fas molecules could benefit of the Fas costimulatory effects upon suboptimal TCR stimulation and 2) high levels of IL-7 could prime T cells to Fas-mediated proliferative signals during HIV-1 infection. In order to test our hypotheses we first sought to determine the optimal experimental condition in which Fas signals could sustain T cell proliferation. We found that the sub-optimal T cell activation induced by a low concentration of anti-CD3 Abs (1 μg/ml) could be modulated by Fas co-stimulatory signals.

T cells isolated from a group of healthy and HIV-1 infected donors were labeled with CFSE and activated with the sub-optimal dose of anti-CD3 (1μg/ml) alone or in combination with two different doses of anti-Fas Abs (1 and 0,25μg/ml). Interestingly, T cells from HIV-1 infected donors exhibited a narrow threshold for proliferation, as both CD4+ and CD8+ T cells showed an increased proliferation in response to low dose of anti-CD3 as compared to T cells from healthy controls. Administration of anti-Fas Abs 1μg/ml in parallel with sub-optimal TCR triggering increased the proliferation of T cells from both groups of donors, although the T cells from HIV-1 infected patients presented with a stronger proliferative response. Importantly, when decreasing the concentration of anti-Fas Abs to 0,25μg/ml the proliferative effect of Fas signals on T cells of the non-infected controls was completely abolished while T cells from HIV-1 patients still exhibited a pronounced proliferation at the same anti-Fas concentration.
Given the possibility that the observed high rates of proliferation may as well be accompanied by massive apoptosis, a phenomenon associated with HIV-1 infection, we analyzed whether Fas mediated apoptosis or Fas-mediated proliferation would predominate under similar experimental conditions (Figure 5). We found that Fas cross-linking on sub-optimally activated T cells from HIV-1 infected individuals induced proliferative rates that greatly exceeded the rates of apoptosis. T cells from healthy individuals showed low levels of both proliferation and apoptosis upon Fas and concomitant sub-optimal TCR triggering. These results confirmed our hypothesis that Fas molecules expressed on T cells of HIV-1 infected patients act as potent costimulatory receptors upon sub-optimal TCR triggering. Moreover, such co-stimulatory effect of Fas predominated over the induction of Fas mediated apoptosis.

![Figure 5. Fas mediated co-stimulation prevails on Fas-mediated apoptosis during HIV-1 infection.](image)

Owing to the elevated levels of IL-7 found in the serum of HIV-1 patients and our findings in paper I showing that IL-7 induced Fas up-regulation, we thought of IL-7 as a potential candidate for priming T cells to Fas-mediated proliferative signals during HIV-1 infection. In order to assess this scenario, we cultured T cells from healthy individuals in the presence of IL-7 during 5 days and measured the costimulatory effect of Fas on those cells, on freshly isolated T cells and on T cells cultured during 5 days in the absence of the cytokine. Interestingly, Fas cross-linking considerably enhanced the
proliferation of IL-7 treated T cells upon sub-optimal activation, whereas only a minor co-stimulatory effect of Fas was observed on untreated T cells. These results further implicate Fas as a potent T cell co-stimulatory molecule under conditions associated with increased IL-7 levels.

Seeking for mechanisms to explain the enhanced Fas mediated proliferation displayed by T cells after IL-7 treatment, we evaluated the levels of IL-2 secretion and CD25 (IL-2Rα chain) expression, as both are prerequisite for activated T cells to undergo high rates of division. We found that Fas signals increased IL-2 production upon sub-optimal activation only in the case of IL-7 treated T cells. Similarly, Fas signals induced an enhanced and sustained expression of CD25 only on IL-7 treated T cells. This indicated that IL-7–induced Fas molecules may confer proliferative ability to sub-optimally activated T cells due to increased IL-2 and IL-2R expression.

In the context of lymphopenia, recognition of self-peptide/MHC complexes by T cells are thought to contribute to homeostatic T cell expansion under lymphopenic conditions84,91. Also, autologous DCs, a source of self-peptide/MHC, are able to trigger partial T-cell activation and proliferation212. Based on these evidences we explored the possibility of a stimulatory role of Fas in lymphopenia-induced T-cell expansion. With that purpose we set up a model system for self-antigen–driven T-cell activation using autologous monocyte-derived DCs as APCs and a high dose of IL-7. IL-7 pre-treated or freshly isolated T cells were cultured together with autologous DCs for 4 days in the presence of Fas triggering and thereafter T-cell proliferation was analyzed. Triggering through Fas receptors on IL-7 pre-treated T cells resulted in an enhanced CD4+ T-cell proliferation in the presence of autologous DCs, whereas CD8+ T cells did not proliferate under these conditions. Freshly isolated T cells did not proliferate in the presence of autologous DCs. This experiment showed that Fas molecules expressed on IL-7 exposed T cells are able to transduce proliferative signals to T cells stimulated by low-affinity self antigens, indicating a role for Fas signals in T cell expansion induced upon lymphopenic conditions.

We compared the sensitivity of different T-cell subpopulations to Fas-mediated apoptosis or costimulation after IL-7 treatment. We found that IL-7 treated naive CD4+ and CD8+ T cells exhibited minimal level of apoptosis or proliferation to sub-optimal TCR triggering irrespective of the presence or absence of Fas signals. The
CD4+T cell memory pool exhibited comparable rates of apoptosis and proliferation upon Fas signalling. Remarkably, the strongest proliferative effect of Fas cross-linking was exhibited by the sub-optimally activated CD8+ memory T cells and such effect greatly exceeded the levels of apoptosis, possibly reflecting the strongest regenerative ability of this subset observed in chemotherapy-treated patients\textsuperscript{95} or in chronic HIV-1 infection\textsuperscript{213}.

Finally, seeking to determine possible differences in known key regulatory pathways in T cells undergoing apoptosis or proliferation upon concomitant TCR and Fas cross-linking, we evaluated the role of caspase-3 and caspase-8 activity in cultures used for apoptosis and proliferation assays. We found that the activation of both caspases was strictly associated with the induction of apoptosis, indicating a role of both caspases in T-cell apoptosis but not in proliferation. Accordingly, application of caspase-8 and caspase-3 inhibitors to IL-7–treated T cells that were exposed to concomitant Fas signals and sub-optimal TCR triggering resulted in reduced apoptosis but did not affect the proliferative response to Fas-mediated activation.

Altogether our findings indicate an important role for IL-7 and Fas-mediated signals in the regulation of T cell homeostasis. The high levels of IL-7 associated with lymphopenic conditions may simultaneously induce the sensitivity of non-activated T cells to Fas-mediated apoptosis while enhancing the proliferative Fas signals in case of T cells able to recognize low affinity antigens (Figure 6).

![Figure 6](image-url)

**Figure 6.** High IL-7 levels associated with lymphopenic conditions may let non-activated T cells die through Fas-mediated apoptosis while enhancing proliferative Fas signals in case of T cells that are able to recognize low affinity antigens.
3.2 CD28- T LYMPHOCYTES: A TERMINALLY DIFFERENTIATED PHENOTYPE THAT CONTRIBUTES TO IMMUNE ACTIVATION DURING HIV-1 INFECTION?

The persistent antigen-driven activation and the chronic bystander activation that characterizes HIV-1 infection are probable inducers of the increasing numbers of CD28- T cells. In addition, the loss of CD28 expression has been associated to impaired functionality of T lymphocytes. CD28- T cells have been reported to exhibit a limited TCR diversity\textsuperscript{170,179,190}. Some reports showed that these cells display an impaired proliferative ability in response to specific Ags\textsuperscript{168} and resistance to apoptosis\textsuperscript{176,188}, while others indicated a lack correlation between CD28 down-regulation and low proliferative ability of T cells from HIV-1 infected patients\textsuperscript{166} and an increased sensitivity to apoptosis\textsuperscript{190}. In addition a distinct subset with CD8+CD28- phenotype has been reported to possess a potential suppressor activity\textsuperscript{116,214,215}.

Altogether, these findings suggest a possible implication for this growing population in the immune disorders observed during the course of HIV-1 infection. Therefore, we were prompted to investigate the phenotypic and functional characteristics of CD28- T lymphocytes during HIV-1 infection. Moreover, we wanted to evaluate the impact of disease progression in the regulation of survival and proliferation of CD28- T cells during HIV-1 infection. Finally, we studied whether the supposedly suppressive effect of CD28- T cells on DC and T cell activation occurs during HIV-1 infection.

3.2.1 The impact of disease progression in the regulation of survival and Proliferation of CD28- T lymphocytes during HIV-1 infection (Paper III)

Certain CD28- subsets isolated ex-vivo or generated in vitro have shown to became relatively resistant to apoptosis\textsuperscript{176,177,187,188}. On the other hand, it is known that CD4+ and CD8+ T cells from HIV-1 patients are prone to both spontaneous and activation induced apoptosis. When evaluating the propensity of CD28- T cells to apoptosis in comparison to their counterparts CD28+ T cells we found that CD28- T cells HIV-1 infected individuals and from healthy donors displayed a phenotype of senescent cells with a propensity to apoptosis. This was indicated by the higher levels of CD57
expression, the shorter telomere length and the higher percentages of Annexin-V positive cells together with the lower levels of expression of IL-7R and Bcl-2 expressed by CD28-T cells as compared to CD28+ T cells.

Given our finding that CD28- T cells from healthy and HIV-1 infected individuals display a phenotype of senescent cells with a tendency rather than resistance to apoptosis, we decided to assess whether CD28 down-regulation would be also associated to an increased susceptibility to apoptosis upon TCR triggering. For this purpose, T cells isolated from two groups of HIV-1 infected individuals, one under HAART and the other treatment naïve, or from non-infected controls were activated using low (1 μg/ml) or high concentrations (5 and 10 μg/ml) of anti-CD3. The level of apoptosis was evaluated on both CD28+ and CD28- subpopulations by Annexin-V binding assay following one day of activation. The CD28- T cells from all three groups exhibited higher levels of activation induced apoptosis than their counterparts CD28+ T cells; the highest susceptibility to activation-induced apoptosis, however, was mainly found in CD28- T cells from patients naïve to treatment. In the case of HAART-treated patients, CD28- T cells showed low levels of apoptosis although at rates higher than CD28- T cells from non-infected donors (Figure 7A). Importantly, the increased levels of apoptosis observed in CD28- T cells from untreated patients occurred already at the lowest concentration of anti-CD3 (1μg/ml).

As the high levels of apoptosis were found only on the CD28- T cells from patients who were naïve to treatment and who also were the ones presenting with detectable levels of viremia, we evaluated the association of spontaneous and activation-induced apoptosis with HIV-1 viral loads. A significant positive correlation was found between the percentages of Annexin V positive cells and viral loads in the presence of 1 and 10μg/ml of anti-CD3 antibody. Taken together, these results indicate a role for viral replication on the susceptibility of CD28- T lymphocytes to apoptosis upon TCR-triggering.

Given the increased expression of CD57 molecules and relative shortening of telomeres which we found in the CD28- T cell population, we decided to investigate whether such phenotype could be translated into impairment of the proliferative ability of these cells. The proliferative abilities of CD28+ and CD28- T cells isolated from healthy
individuals and HIV-1 patients undergoing HAART or naïve to treatment were evaluated and compared upon TCR triggering.

T cells isolated from the same group of HIV-1 infected individuals and non-infected controls were labeled with CFSE and activated using low (1 µg/ml) or high (5 and 10 µg/ml) concentrations of anti-CD3 antibodies; the percentage of proliferating T cells was calculated following 4 days of activation. Interestingly, only CD28- T cells of untreated patients showed a poor proliferative ability upon TCR triggering, while the same cell population in the case of treated patients exhibited a strong proliferative response (Figure 7b). CD28- and CD28+ T cells from treated patients showed similar levels of proliferation at high concentrations of anti-CD3 (5 and 10 µg/ml); however, at the lowest concentration (1µg/ml), CD28- T cells were readily proliferating, indicating an apparent advantage for CD28- T cell proliferation in response to suboptimal signals.

Figure 7. Upon TRC-triggering, CD28- T cells from HIV-1 infected patients naïve from treatment exhibited higher sensitivity to apoptosis whereas CD28- T cells from treated patients displayed high proliferative rate. Purified T cells from control individuals (n=10) and HIV-1 infected patients undergoing treatment (n=12) or naïve from treatment (n=14) were cultured for 24 or 72 hours on wells coated with anti-CD3 (10µg/ml). A. Percentage of Annexin V positive cells on total T cells, CD28+ and CD28- T cells as measured by Annexin V staining. B. Percentage of proliferative total T cells, CD28+ and CD28- T cells as measured by CFSE dilution. Data represent mean and standard deviation.
As the induction of both IL-2 production and the expression of the high affinity IL-2 receptor (CD25) are required for T cell proliferation upon TCR triggering, we analysed whether the differential ability of CD28- T cells of viremic and aviremic patients to proliferate upon TCR-mediated stimulation is associated with differences in IL-2 production and in the levels of CD25 expression by these cells. Levels of IL-2 were measured by ELISA in the supernatants of the same cultures assessed for apoptosis. Remarkably, only T cells isolated from patients undergoing HAART and characterised by undetectable viral loads produced high levels of IL-2 upon TCR-stimulation. Intracellular staining with anti-IL-2 antibodies in similar experimental settings showed that the main source of IL-2 was the CD28+ population and that IL-2 production was inducible in CD28- T cells through TCR cross-linking although at very low levels. The surface expression of CD25 was measured at 24hrs and 3 days after TCR triggering. Already at 24 hours a marked up-regulation of membrane CD25 on both CD28+ and CD28- T cells was induced in a dose-dependent manner by anti-CD3 antibody, although CD28+T cells showed higher levels of CD25 expression than the CD28- counterpart. Interestingly, the production of high doses of IL-2, as well as increasing CD25 expression, occurred in parallel with the enhanced proliferative ability of T cells isolated from aviremic patients. These findings suggest that immune activation associated with HIV-1 infection may be related to an enhanced ability of T cells to produce IL-2; however such ability of T cells is impaired by T cell exhaustion associated with high levels of viral replication.

Finally, due to the apparent increased sensitivity to either proliferative signals (in the case of treated patients) or to apoptotic signals (in the case of untreated patients)) of CD28- T cells upon suboptimal TCR triggering (anti-CD3 at1μg/ml) we decided to evaluate the role of Fas signals in this context. These experiments were also conducted in view of our previous findings on the increased susceptibility of T cells from HIV-1 infected individuals to proliferate upon concomitant Fas and suboptimal TCR triggering (Paper II). As mentioned above, the increased levels of apoptosis observed in CD28- T cells upon suboptimal TCR-triggering occurred especially in the case of patients that were naïve to treatment. The addition of Fas signals in this condition induced only a slight enhancement of apoptotic responses of those cells.

Regarding the proliferative responses to Fas costimulatory signals upon suboptimal TCR triggering, both CD28+ and CD28- T cells from treated patients exhibited a
similar trend of response to Fas-costimulation, although CD28+ T cells showed a
greater rate of proliferation induced by Fas-costimulation than their counterparts CD28-
T cells. In the case of treatment-naïve patients proliferative responses in response to
Fas-costimulatory signals were absent.

Altogether, our findings suggest that increased activation of CD28+ T cells may lead to
the accumulation of CD28- T cells generated upon CD28 down-regulation. In addition,
in the absence of viral replication, CD28- T cells are able to expand in response to
weak or strong antigenic signals. The persistent antigenic stimulation may therefore
represent a critical factor in the regulation of CD28- T cell homeostasis.

3.2.2 CD28- T lymphocytes as potential inducers of
immunoactivation during HIV-1 infection (Paper IV)

Given the various reports of suppressor T cells with a CD8+CD28- phenotype and the increasing numbers of CD28- T cells (which are mostly CD8+) during HIV-1 infection, we hypothesized that this enlarged population could exert a suppressive role thus contributing to the immune deficiency observed during the course of the infection.

CD28- and CD28+ T cells isolated from healthy and HIV-1 infected individuals were
co-cultured with monocyte-derived DCs; 24 hours later DCs were stimulated with LPS
or left without any further treatment another 24 hours. Evaluation of the maturation
markers HLA-DQ, CD86 and CD83 revealed that the solely presence of both CD28+ and CD28- T cells was enough to induce a mature DC phenotype, as indicated by the
up-regulation of the above mentioned molecules (Figure 8a). Interestingly CD28- T
cells induce the same levels of activation as their CD28+ counterparts. In addition,
neither of the T cell populations was able to inhibit the LPS-induced maturation.

We also evaluated the cytokine production by DCs in the same experimental conditions and found that the production of both pro-inflammatory cytokines IL-12 and TNF induced by LPS was not blocked but rather enhanced by the CD28- T cells. The production of the anti-inflammatory cytokine IL-10 was not affected by the presence of either of the T cell populations (Figure 8b)
Figure 8. CD28- T cells induce the expression of maturation markers on DCs and enhance the production of LPS-induced pro-inflammatory cytokines by DCs. Monocyte-derived DCs were cultured alone or in the presence of isolated CD28+ and CD28- T cells during 24 hours. LPS was then added and the cultures were continued for additional 24 hours. Panel a shows the expression of maturation markers CD83 and HLA-DQ by DCs in the absence of LPS. Panel b shows the concentrations of TNF and IL-12 in the supernatants of DC-T cell co-cultures in presence of LPS.

The next step was to evaluate whether CD28- T cells from healthy and HIV-1 infected individuals were able to affect the proliferation of naïve T cells induced by DCs and stimulation with anti-CD3. In order to better define if the effects of CD28- T cells are specific for this subset and since the loss of CD28 expression characterizes T cells at late differentiation stages, CD28+CCR7- effector/memory cells were isolated and compared with CD28- T cells and CD28+ in DC:T cell co-cultures. All three subsets pre-incubated with DCs affected only minimally the proliferation of the third party T cells as compared to the ones that were cultured in the presence of untreated DCs. However, there was a slight reduction in the number of proliferating cells in the presence of both CD28- T cells and CD28+CCR7- memory subset, probably indicating the presence of negative feedback signals delivered by antigen-experienced T lymphocytes that might limit further T cell activation.
In this study we show that naturally occurring CD28- cells do not possess suppressor functions; on the contrary, these cells contribute to increased DC activation. Moreover, the CD28- T cell mediated DC activation and the lack of any CD28- T cell specific suppression was equally observed in specimens from both HIV-1 infected and healthy donors. Taken together, these results indicate that the accumulation of CD28- T cells during HIV-1 infection may not lead to DC or T cell suppression but that this population may rather contribute to accelerated inflammatory reactions and immune activation through promoting the production of inflammatory cytokines by DCs.
4 CONCLUDING REMARKS

The work presented in this thesis, possibly provides further insights into the pathogenesis of HIV-1 infection, characterized by a vicious circle formed between lymphopenia and chronic immune activation main inducers of immunodeficiency through the alteration of T cell homeostasis.

In paper I we demonstrate that IL-7, a cytokine that was primarily associated with anti-apoptotic and proliferative effects on T lymphocytes, can potently induce the expression of Fas molecules on T cells in vitro and in vivo. The increased expression of Fas molecules upon IL-7 treatment might occur as a result of translocation of Fas molecules from intra cellular compartments to the cell surface or due to increased stabilization of Fas in the cell membrane, as indicated by the invariable levels of total Fas protein and mRNA in the IL-7 treated cells in comparison to the untreated cells. In addition, IL-7 treated T cells were characterized by an increased sensitivity to Fas mediated apoptosis in vitro. Further analysis of the effects of IL-7 on the naïve and memory T cell subpopulations showed that the memory subsets presented with the highest sensitivity to Fas-induced cell death.

The enhanced Fas expression observed in macaques upon IL-7 administration together with the correlation found between serum levels of IL-7, Fas expression and ex vivo sensitivity to Fas mediated apoptosis exhibited by T cells from HIV-1 infected individuals, strongly supported the role of IL-7 in the enhanced sensitivity of T cells to Fas-mediated apoptosis observed during HIV-1 infection. Hence, the well known positive effects of IL-7 on survival of proliferation of T cells might be associated somehow to a negative feedback through Fas-mediated signals in HIV-1 infected individuals.

Although Fas is known to act both as a death receptor and as a costimulatory molecule, the increased expression of Fas observed during lymphopenic conditions has been solely interpreted as a mechanism that leads to T cell depletion. However, in paper II we demonstrate the existence of high sensitivity to Fas induced proliferation during HIV-1 infection. Importantly, when comparing the rates of apoptosis and proliferation
by the T cells upon concomitant Fas signals and suboptimal TCR triggering the proliferative rates greatly exceeded the levels of apoptosis in the majority of the cases. Given our findings in paper I, indicating a role for IL-7 in the induction of increased sensitivity to Fas ligation, we evaluated the effect of IL-7 treatment on the sensitivity of T cells to Fas mediated proliferation. IL-7 enhanced the response of T cells to Fas costimulatory signals in the presence of self-antigen bearing DCs or alternatively by suboptimal doses of anti-CD3 Abs. IL-7 primes T cells to respond with high sensitivity to Fas-mediated signals, probably through the enhancement of IL-2 production and CD25 expression. Thus, IL-7 appears to enhance the sensitivity of T cells to Fas signals without playing any instructive role on determining the outcome of either apoptotic or proliferative responses. The high levels of serum IL-7 associated with HIV-1 may simultaneously induce the enhanced sensitivity of non-activated T cells to Fas mediated apoptosis while in the case of T cells able to recognize low affinity antigens it might enhance Fas-mediated proliferation.

In Paper III, we studied the phenotypic and functional characteristics of CD28- T lymphocytes from both healthy and HIV-1 infected individuals. Moreover, we evaluate the impact of disease progression in the regulation of survival and proliferation of CD28- T cells during HIV-1 infection.

The CD28- T cells have been previously described as a senescent population which is characterized by their resistance to apoptosis and impaired proliferative ability. However, in this paper we show that although these cells bear certain characteristics of senescence (high expression of CD57 and shortened telomeres), they exhibit a phenotype of cells that are prone to apoptosis (indicated by high percentages of Annexin V binding cells and low levels of BcL-2 and IL-7R expression). Such phenotype was common to CD28- T cells from both healthy and HIV-1 infected individuals. Interestingly, when evaluating the sensitivity of these cells to activation-induced apoptosis, we found that only CD28- T cells from untreated patients showed high levels of apoptosis.

Since the observed phenotype could also implicate an impaired proliferative ability, we tested the proliferative response of CD28- T cells from the same group of donors upon TCR triggering. Strikingly, we observed a great proliferative response of CD28- T cells in the case of patients that received HAART.
Our findings identify viral replication as an important factor involved in the homeostasis of the CD28- T cell population, thus, supporting the scenario of a context-dependent regulation of the CD28- T cell population that may be influenced by antigen-specificity, level of immune activation or disease stages.

In Paper IV we evaluated the supposedly suppressive effect of CD28- T cells on DC and T cell activation. Interestingly the naturally occurring CD28- cells either in healthy or HIV-1 infected individuals lacked completely any suppressor function. Moreover, these cells contributed to increase DC activation as suggested by the increased expression of maturation markers and enhanced production of pro-inflammatory cytokines exhibited by LPS-stimulate DCs that were previously cultured with CD28- lymphocytes. When evaluating the ability of the DCs pre-cultured in the presence of CD28-T cells, to induce proliferation of naïve T cells, we could not detect any specific suppression either.

Both observations, the CD28- T cell mediated DC activation and the lack of any CD28-T cell specific suppression occurred independently of the health status of the donors. This paper provides evidence for a role of CD28- T cell population in the accelerated inflammatory reactions and immune activation through promoting the production of inflammatory cytokines by DCs.

**Model of AIDS pathogenesis**

HIV → Hyperactivation → Immune deficiency → AIDS

Fas ↑
Fas-L ↑

High levels of IL-7
Fas-mediated costimulation

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**Figure 9. Contribution of IL-7 and Fas signalling to AIDS pathogenesis.** Hyperactivation of the immune system occurring during HIV-1 infection results in increased Fas-mediated apoptosis leading to T cell depletion. High levels of IL-7 associated with lymphopenia become a driving force of Fas mediated signals. This enhancement of Fas mediated signals has a dual role, contributing at the same time to Fas-mediated depletion and to increased Fas-mediated costimulation of T-cells.
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