CHRONIC IMMUNE ACTIVATION
AND LYMPHOCYTE APOPTOSIS
DURING HIV-1 INFECTION

Nicolas Ruffin

Stockholm 2012
“Live as if you were to die tomorrow. Learn as if you were to live forever”

Mahatma Gandhi
ABSTRACT

HIV-1 infected individuals are subject to a chronic immune activation resulting from HIV-1 replication, microbial translocation, and lymphopenia. Despite the great advance of antiretroviral treatment (ART), the immune activation remains associated with poor immune reconstitution during HIV-1 infection. The overall aim of this PhD thesis is to contribute to a better understanding of the causes and consequences of immune activation, possibly leading to the design of improved therapy for HIV-1 infected individuals.

Premature senescence of T cells, as a consequence of immune activation, is thought to be associated with the increased levels of CD28- T cells during HIV-1 infection. In Paper I, the phenotype and functional properties of CD28- T cells from HIV-1 individuals naive to treatment, under ART and uninfected controls were assessed. Despite displaying similar markers of senescence, and late differentiation, we found that whereas CD28- T cells from untreated patients are highly susceptible to both spontaneous and activation-induced apoptosis, the same T cell population from ART-treated patients showed an enhanced capacity to proliferate upon weak TCR stimulation. Importantly, apoptosis of CD28- T cells from untreated patients was correlated with HIV-1 viral load, and their decreased ability to proliferate was associated with a reduced IL-2 production. High levels of CD28- T cells during HIV-1 infection might result from the chronic immune activation, whereas their sustained levels despite ART, is likely to arise from their capacity to proliferate under weak TCR signaling. Furthermore, with a capacity to produce IFN-γ, TNF and perforin, CD28- T cells from HIV-1 infected individuals might also contribute to the immune activation.

The mechanisms underlying the loss of memory B cells and the decline of serological memory during HIV-1 infection remain elusive. As microbial translocation and the associated immune activation have been shown to correlate with T cell depletion, we evaluated, in Paper II, the association between the serum levels of soluble CD14, a marker of microbial translocation, with the loss of resting memory B cells in HIV-1 infected individuals. Soluble CD14 levels were found to correlate with both the decline of resting memory B cells, and their increased expression of IL-21R. IL-21R expression on memory B cells was increased during HIV-1 infection, and also negatively correlated with the levels of circulating memory B cells. Notably, IL-21R positive memory B cells were more prone to apoptosis, measured by higher Annexin V staining and lower Bcl-2 expression, as compared to B cells lacking the receptor. Furthermore, TLR triggering by microbial products resulted in IL-21R expression on memory B cells in vitro. Our results identify a novel role for microbial translocation and the associated immune activation, contributing to the loss of memory B cells during HIV-1 infection.

Lymphopenic conditions are associated with increased IL-7. This cytokine involved in T cell homeostasis, is also found to be elevated in HIV-1 infected individuals concomitantly with low CD4+ T cell counts; although the regulation of IL-7 production is not fully understood in the context of HIV-1 infection. Using human intestinal epithelial (DLD-1) and bone marrow stromal (HS-27) cell lines, we investigated in Paper III, the consequence of pro-inflammatory cytokines on IL-7 production, measured at the mRNA and the protein levels. Whereas IFN-γ induced high IL-7 production in both cell lines, IL-1β treatment led to the opposite effect. We also analyzed the gene expression profiles of HS-27 cells treated with IL-1β and/or IFN-γ using the whole-genome microarray Human Gene 1.0 ST. Both cytokines resulted in enhanced expression of genes implicated in T cell immunity, particularly important during HIV-1 pathogenesis. Our results show that the immune activation can lead to profound change in stromal and epithelial cells, which in turn might shape immune responses.

While IL-7 is known to participate to T cell homeostasis, it has recently been shown that this cytokine possibly contribute to B cell defects, leading through IFN-γ release by T cells, to Fas up-regulation and sensitivity to Fas-mediated apoptosis. We further evaluated IL-7 regulation of T cell survival in Paper IV, and observed that B cells, co-cultured with IL-7 treated T cells, proliferated, displayed a phenotype of differentiated cells and secreted high levels of immunoglobulins (Igs). The Ig secretion was demonstrated to be a consequence of CD70 up-regulation on T cell upon IL-7 treatment. IL-7 led also to BAFF production by T cells, which enhanced B cell survival. In the context of HIV-1 infection, such mechanisms might be implicated in the B cell activation and hypergammaglobulinemia observed in patients.
LIST OF PUBLICATIONS


Other related publications not included in the thesis


CONTENTS

1 30 years of HIV ............................................................................................................. 1
   1.1 A Brief History............................................................................................................ 1
   1.2 HIV characteristics........................................................................................................ 2
   1.3 Anti-retroviral treatment ............................................................................................... 4
2 Pathogenesis of HIV-1 infection ...................................................................................... 5
   2.1 Chronic immune activation ............................................................................................ 6
   2.1.1 Markers of immune activation during HIV-1 infection ........................................... 6
   2.1.2 Lessons from HIV-2 and non-pathogenic SIV-infections ....................................... 8
   2.2 T cell depletion in the gut and microbial translocation ............................................... 12
   2.2.1 Mucosal damages in the early phase of HIV-1 infection ........................................ 12
   2.2.2 Imbalance of T helper cells during HIV-1 infection ............................................... 13
   2.3 T cell exhaustion during HIV-1 infection .................................................................... 15
   2.3.1 Ageing of the immune system and CD28 expression ............................................. 16
   2.3.2 Markers of T cell exhaustion .................................................................................. 17
   2.4 Causes and consequences of lymphopenia ............................................................... 19
   2.4.1 Mechanisms of T cell depletion ............................................................................. 20
   2.4.2 Consequences of Lymphopenia, the role of IL-7 .................................................. 24
   2.5 Chronic activation of B cells and memory B cell loss ............................................... 28
   2.5.1 Humoral response against HIV-1 .......................................................................... 28
   2.5.2 B cell hyperactivation and exhaustion ................................................................... 28
   2.5.3 Loss of memory B cells and serological memory ................................................... 30
3 Aims of the Thesis ............................................................................................................ 32
4 Results and Discussion .................................................................................................... 33
   4.1 Impact of HIV-1 replication ......................................................................................... 33
   4.1.1 T cell senescence and apoptosis – Paper I – ......................................................... 33
   4.1.2 B cell Activation – Paper II – .............................................................................. 38
   4.2 Role of Microbial Translocation ................................................................................ 42
   4.2.1 Loss of memory B cells – Paper II – ................................................................. 42
   4.2.2 IL-7 Regulation – Paper III – ........................................................................... 45
   4.2.3 Immune regulation by stromal cells – Paper III – ............................................... 47
   4.2.4 Lymphopenia and B cell Activation – Paper IV – ............................................... 50
5 Conclusions and Perspectives ........................................................................................ 54
6 Acknowledgements ......................................................................................................... 57
7 References ...................................................................................................................... 60
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>16 S rDNA</td>
<td>Bacterial 16S ribosome DNA</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>AGM</td>
<td>African green monkey</td>
</tr>
<tr>
<td>AICD</td>
<td>Activation-induced cell death</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen-presenting cell</td>
</tr>
<tr>
<td>ART</td>
<td>Antiretroviral therapy</td>
</tr>
<tr>
<td>Bcl</td>
<td>B cell lymphoma</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>CCL</td>
<td>C-C chemokine ligand</td>
</tr>
<tr>
<td>CCR</td>
<td>C-C chemokine receptor</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein succinimidyl ester</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic lymphocyte</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T-lymphocyte antigen 4</td>
</tr>
<tr>
<td>CCL</td>
<td>C-X-C chemokine ligand</td>
</tr>
<tr>
<td>CXCR</td>
<td>C-X-C chemokine receptor</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DR</td>
<td>Death receptor</td>
</tr>
<tr>
<td>EndoCAb</td>
<td>Endotoxin-core antibodies</td>
</tr>
<tr>
<td>FasL</td>
<td>Fas ligand</td>
</tr>
<tr>
<td>FRC</td>
<td>Fibroblastic reticular cell</td>
</tr>
<tr>
<td>GALT</td>
<td>Gut-associated lymphoid tissue</td>
</tr>
<tr>
<td>gp</td>
<td>Glycoprotein</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LTNP</td>
<td>Long-term non-progressor</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MSM</td>
<td>Men who had sex with men</td>
</tr>
<tr>
<td>NAb</td>
<td>Neutralizing antibody</td>
</tr>
<tr>
<td>PC</td>
<td>Plasma cell</td>
</tr>
<tr>
<td>PD-1</td>
<td>Programmed death-1</td>
</tr>
<tr>
<td>RM</td>
<td>Rhesus macaque</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>SIV</td>
<td>Simian immunodeficiency virus</td>
</tr>
<tr>
<td>SM</td>
<td>Sooty mangabey</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Tfh</td>
<td>Follicular T helper cell</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TLM</td>
<td>Tissue-like memory</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TNFR</td>
<td>TNF receptor</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis inducing ligand</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T helper cell</td>
</tr>
</tbody>
</table>
1 30 YEARS OF HIV

The substantial movement of science, political support and community responses has made possible the access of people infected with human immunodeficiency virus (HIV) to therapy and to decrease the number of new infections. Yet, further efforts are needed to better understand the immunological dysfunctions occurring in HIV-infected patients in order to improve therapeutic interventions and the development of a safe and effective HIV vaccine. My PhD studies have been focusing on the mechanisms affecting the immune cells, namely B and T lymphocytes, in relation to the chronic immune activation occurring during HIV-1 infection. The pathogenesis of HIV-1 is driven by T cell depletion, immune activation fuelled by viral replication, microbial translocation due to mucosal damage, and lymphopenia caused by CD4+ T cell depletion. I investigated the role of viral replication on T cell activation and survival; the connection between microbial translocation and loss of memory B cells; and the potential impact of lymphopenia on B cell activation.

1.1 A BRIEF HISTORY

In June 1981, the Centers of Disease Control and Prevention (CDC) reported cases of men who had sex with men (MSM) with pneumonia, documenting for the first time what became known as acquired immunodeficiency syndrome (AIDS) [1]. These patients, previously healthy, were suffering from opportunistic infections and rare cancers due to a damaged immune system. The causative agent responsible for AIDS was isolated in 1983 [2, 3], and later named HIV.

It appeared rapidly that HIV was spread worldwide and the different routes of HIV transmission were soon identified. HIV is transmitted through sexual contact, blood transfusion, the share of infected needles and from infected mothers to their newborns. Thirsty years on, the HIV epidemic has affected more than 60 million individuals and caused an estimated 30 million deaths. At the end of 2010, an estimated total of 34 million people were living with HIV, of which 68% were located in sub-Saharan Africa [4].

The first drug against HIV, AZT was authorized in 1987, and as of today more than 25 anti-retroviral drugs are available. The introduction in 1996 of treatments based on the combination of typically 3 or 4 of these drugs, namely anti-retroviral therapy (ART) led to a substantial improvement for the life of HIV-infected
Indeed, while the median life survival was only 10 months after a diagnosis of AIDS in 1985, it is thought that now, a 28-year-old HIV-infected patient under ART can live up to 80 years of age after diagnosis. However, treated patients remain at risk for other pathologies non-associated with AIDS, e.g. cardiovascular, renal and hepatic disease and malignancies. Also, the access to ART has been limited in low- and middle-income countries; but with great improvement in recent years, 47% of HIV-infected individuals in need of treatment were receiving ART in 2010. Yet, 1.9 million people died of AIDS in 2010 and the HIV epidemic remains a global health challenge.

In 2010, 2.7 million individuals were newly infected worldwide, which is 21% lower than the new infections that occurred in 1997 at the peak of the epidemic. 70% of those newly infected individuals resided in sub-Saharan Africa. These important numbers, together with other challenges, such as the rise of multi-drug resistant virus, the management of individuals co-infected with HIV and other pathogens (e.g. Tuberculosis, malaria...) and the cost of the treatment make vital the quest for an HIV vaccine. After the failure of 2 major phase III clinical trials for a prophylactic HIV vaccine (the rgp120 HIV Vaccine Study and the STEP Study), the RIV144 AIDS vaccine trial brought new optimism for the conception of an effective HIV vaccine that would either prevent infection and/or lead to slowing or preventing disease progression.

1.2 HIV CHARACTERISTICS

HIV is a lentivirus, member of the retrovirus family, for which two types exist, HIV-1 and HIV-2, having 40% differences in their genetic sequences. These viruses most probably originated from cross-species transmissions of simian immunodeficiency virus (SIV). HIV-1 is distributed into groups: M (main), O (outlier), N (new or non-M/O) and P with genetic variations of around 30% between groups. The main group (M) is further divided into subtypes (or clades) A-D, F-H, J and K differing by 15-20% in their genetic sequences. HIV-1 is widely distributed globally while endemic areas for HIV-2 are predominantly situated in West Africa. HIV-2 having lower transmission rates and less pathogenicity, resembles the SIV infection in natural hosts, which I will further discuss below (Section 2.1.2).

The HIV-1 is composed of double-stranded RNA of 10 kilobases encapsulated in a capsid and matrix made of structural viral proteins. The virus also contains viral proteins important for the first steps of virus replication and is surrounded by a
plasma membrane derived from the infected host in which the envelop (env) proteins are anchored. HIV-1 genome comprises 3 major structural genes: group-specific antigen (gag) codes for proteins involved in the assembly and release of the virus and its encapsulation; polymerase (pol) codes for the protease catalyzing the cleavage of protein precursors, the reverse transcriptase (RT) and the integrase, important molecule driving the integration of the viral sequence into the host genome; and envelope (env) codes for gp120 and gp41, indispensable for the binding and entry of the virus into the target cells. Additionally, regulatory and accessory genes are present. Transactivator of viral transcription (tat) and regulator of RNA transport (rev), are regulatory genes, whereas viral infect factor (vif), viral protein R (vir), negative factor (nef), and the viral protein U or X (vpu or vpx) for HIV-1 and HIV-2 respectively are accessory genes. The HIV sequence is flanked by non-coding long terminal repeats (LTRs) important for the HIV integration into the host genome. HIV-1 proteins are crucial for the replication of the virus, but also have important effects on the host immune system as later described in this thesis.

As any virus, HIV needs to infect a cell in order to replicate. HIV-1 cycle begins by the binding of the envelope glycoprotein of the virus, gp120, on the CD4 molecule situated primarily on T cells, but also on macrophages and dendritic cells (DC). CD4 expression is necessary but not sufficient for HIV-1 infection of the host cells; the presence of a co-receptor is required. The conformational changes occurring upon CD4 binding enable gp120 to bind either the chemokine receptor CCR5 or CXCR4. These chemokine receptors are the main co-receptors for HIV-1, but it has been shown that other molecules could act as co-receptors [15]. The usage of either of the co-receptors has an impact on HIV-1 tropism and disease progression. R5 viruses, using CCR5 as co-receptor, are the ones transmitted and predominate during the early and chronic stages of HIV-1 infection. In many HIV-1 infected individuals, progression to late stage of the infection is associated with a switch of HIV-1 in co-receptor specificity, with the appearance of viral variants able to use CXCR4 (X4 viruses) or both CCR5 and CXCR4 (R5X4 viruses) [16]. In addition to gp120, HIV-1 envelope complex comprises gp41, responsible for the fusion of the virus with the cell host membrane after gp120 has bound both CD4 and the co-receptor CCR5 or CXCR4. At this point, viral core containing the genetic material of HIV-1 is transferred to the cytoplasm of the host cell together with some accessory proteins that will enable the initiation of virus replication [17]. The retrotranscription of HIV-1 single stranded RNA into DNA by RT is followed by the transfer of the viral DNA to the cell nucleus for its integration into the host chromosomes. The lack of fidelity and proofreading of the RT during the
retrotranscription lead to a high rate of mutations in HIV-1 sequence that are responsible for virus escape from the immune responses and for the development of drug-resistant viruses.

Once integrated, the virus sequence will be transcribed and translated into proteins necessary for HIV-1 replication and dissemination from the infected cells to new target cells. By not replicating after its integration, HIV-1 can also establish a latent form of infection, thus creating viral reservoirs which make envisioning a cure for HIV-1 infected patients difficult.

1.3 ANTI-RETROVIRAL TREATMENT

Anti-retroviral drugs target many steps of HIV-1 replication, from the entry phase to the maturation of the virus. Entry and fusion inhibitors interfere with binding, fusion and entry of HIV-1 to the cell. Non-Nucleoside and nucleotide reverse transcriptase inhibitors (NNRTI) inhibit the RT, as do Nucleoside reverse transcriptase inhibitors (NRTI). The HIV-1 protease and integrase are blocked by protease inhibitors (PIs) and Integrase strand transfer inhibitors (INSTIs) respectively. Maturation inhibitors are also developed, targeting Gag processing. In addition to compounds acting directly on virus protein, CCR5 receptor agonists are also used to prevent HIV-1 binding to target cells. To avoid the emergence of drug-resistant HIV-1 virus, the regimen of infected patients is usually a combination of 3-4 drugs to be taken daily. The treatment in most cases, results in the decrease of viral load to under detectable levels in the blood (<50 copies/ml) leading to an increase in life expectancy of HIV-1 infected individuals, as previously discussed. Another consequence of low viral load in HIV-1 infected individuals under ART is the decreased transmission of the virus [18]. However, despite therapy, some alterations of the immune system are not completely normalized; this thesis will describe some mechanisms underlying persistent immune dysfunctions in ART-treated individuals.
2 PATHOGENESIS OF HIV-1 INFECTION

Sexual mucosal transmission is the major route for HIV-1 acquisition. HIV-1 infection is established by one single founder in 80% of heterosexual transmissions, but certainly more variants in the case of transmissions among MSM or intravenous drug users [19]. The early events leading to HIV-1 infection are not totally elucidated and remain a domain of active research essential for the design of effective preventive intervention. *In vitro* models using human tissues and *in vivo* SIV models gave some evidence on the mechanisms that the virus uses to establish the infection [20]. Although the virus possibly crosses the mucosal epithelium by transcytosis or using dendritic cells, the first cells to be productively infected by HIV-1 are the CD4+ T cells. The integrity and state of inflammation of the mucosa play an essential role in the sexual transmission of HIV. Hence, individuals co-infected with *Neisseria gonorrhoeae*, *Herpes simplex virus* type 2 (HSV2) and human cytomegalovirus (CMV) are more susceptible to HIV-1 infection [21].

Once the infection is established, the virus replicates rapidly until viremia – the amount of circulating virus – reaches a peak. This phase of acute infection lasts for a few weeks after transmission of HIV-1, and can be accompanied by flu-like symptoms for the infected individual. At this stage, immune responses against HIV-1 are mounted and, concomitantly, viremia stabilizes to its set point. This is ensued by a chronic asymptomatic phase that can last for a decade or more. Unless treatment is initiated, the CD4+ T cell numbers will gradually decrease (Figure 1). As of a level below 350 CD4+ T cells/µl of blood, HIV-1 infected patient can experience opportunistic infections and the stage of AIDS is reached when the CD4+ T cell count is below 200 cells/µl of blood [22].

This thesis will focus on the alterations of the immune system during chronic HIV-1 infection, especially on the relationships between immune activation and the homeostasis of B and T cells.
2.1 CHRONIC IMMUNE ACTIVATION

HIV-1 infection is characterized by the progressive destruction of the immune system eventually leading to AIDS. CD4+ T cells are the cells primarily affected, but humoral immunity is also altered during the infection [24]. Yet, HIV-1 replication alone is insufficient to explain all the immune dysfunctions occurring in infected patients. Disease progression and mortality are strongly associated with chronic systemic immune activation [25-28].

The immune activation observed during HIV-1 infection is reflected by B cell hyperactivation and high levels of circulating immunoglobulins (Ig)-G [29]; the expression of activation markers at the surface of both CD4+ and CD8+ T cells [30]; high turn-over of lymphocytes [31]; and increased levels of inflammatory cytokines in the plasma from infected individuals [32].

2.1.1 Markers of immune activation during HIV-1 infection

Several markers are used to measure the activation of immune cells. The high expression of CD38, HLA-DR, CD25, CD69 and Fas (CD95), among others markers, define T cell activation. Expression of CD38 or HLA-DR alone or co-expression of CD38 and HLA-DR on CD4+ and CD8+ T cells from HIV-1 infected patients has been repeatedly associated with CD4+ T cell decline [25, 33] and disease
progression [26, 28], better than the levels of HIV-1 replication, i.e. viral load [25, 26, 33, 34]. Even in HIV-1 infected elite controllers, who display a non-measurable level of viremia (<50 copies/ml of blood), T cell activation correlates with CD4+ T cell counts [27]. After ART initiation the co-expression of CD38 and HLA-DR decreases in parallel with T cell apoptosis, i.e. cell death [35, 36], but is still elevated as compared to uninfected individuals and remains associated with CD4+ T cell levels [27, 37], further confirming a link between T cell activation and their depletion. The loss of IL-7 receptor α (IL-7Rα/CD127) has also been shown to be associated with immune activation and T cell depletion [38, 39]. The activation of B cells is manifested by an increased level of circulating IgG, named hypergammaglobulinemia [29]. HIV-1 infected patients also exhibit markers of activation on their B cells. Both naïve (CD27-) and memory (CD27+) B cells express higher CD38 and Fas levels in patients as compared to controls and these high levels of expression of activation markers persists, although at decreased levels, in ART-treated patients [40, 41]. Another population of B cells, defined by their low expression of CD21, impaired proliferative capacity and high IgG production, has been found in HIV-1 infected individuals [42, 43]. Levels of CD21low B cells correlate with viremia; they also display higher expression of Fas and are more susceptible to apoptosis than CD21+ B cells [42]. Importantly, memory B cells are also depleted during HIV-1 infection [24].

Beside the activated phenotype of leucocytes from HIV-1 infected patients, the immune activation is also assessed through the measurement in the plasma of soluble molecules (β2-microglobulin, sCD27) and markers of inflammation (TNF, IFN-γ, Interleukin (IL)-1β, IL-6, C-reactive protein (hsCRP) and D-dimer). β2-microglobulin is a molecule part of the human leukocyte antigen (HLA) complex that is released by activated T cells; its levels are elevated in HIV-1 infected patients and correlate with CD4+ T cell counts [44, 45]. β2-microglobulin measurement has also been used as a surrogate marker for HIV-1 infection [46]. Soluble CD27 was also proposed as a marker for immune activation [47, 48]. Tumor necrosis factor (TNF) and interferon (IFN)-γ, secreted upon T cell activation, have been shown to be increased in the primary HIV-1 infection [49]. TNF is a potent pro-apoptotic molecule but its role is debated in the context of HIV-1 pathogenesis (see below) [50]. Levels of IL-1β, a pro-inflammatory cytokine, were reported to be increased during the acute phase of HIV-1 infection, both in the gut-associated lymphoid tissues (GALT) and in the serum [51, 52]; however the levels are normalized in the chronic phase of the infection [52, 53]. IL-6 is also an inflammatory cytokine and D-dimer, a pro-inflammatory marker;
both are elevated during HIV-1 infection and related to mortality rate of infected individuals [54].

Immune activation appears early during HIV-1 infection and correlates to CD4+ T cell decline, better than the magnitude of HIV-1 replication [25, 33, 34]. Upon initiation of ART, the levels of many of the activation markers, both soluble and expressed on the T cell surface, are decreased but not normalized [27]. Additionally, the association between T cell activation and CD4+ T cell depletion persist in ART-treated HIV-1 individuals.

The chronic immune activation in HIV-1 infected individuals relies on (1) HIV-1 replication, (2) microbial translocation, and (3) lymphopenia. The presence of ongoing viral replication in infected patients is accompanied by HIV-1 specific immune responses [55, 56]. As the immune system is unable to resolve HIV-1 infection, there is a continuous activation of the immune cells accompanied with a state of inflammation. In addition to the direct effects of the virus on the immune cells, it has been shown that the integrity of the gut mucosa of HIV-1 infected patients is altered [57, 58]. Bacterial products, such as lipopolysaccharide (LPS), cross from the lumen to the circulation. This process, called microbial translocation, has the potential to activate immune cells through toll-like receptors (TLRs) and has been shown to play a role in HIV-1 pathogenesis [58]. Indeed, the depletion of CD4+ T cells in the GALT has been linked to impaired structural integrity of the mucosal epithelium as further discussed in this thesis. A third mechanism can also participate to the chronic immune activation occurring in HIV-1 infection. The depletion of CD4+ T cells induces compensatory mechanisms involving IL-7 in order to keep the homeostasis of the T cell compartment [59]. IL-7 is a key cytokine for T cell-number maintenance, providing survival and proliferative signals. Promoting T cell activation, IL-7 may participate in the increased immune activation observed in HIV-1 infected patients, as this thesis will describe.

2.1.2 Lessons from HIV-2 and non-pathogenic SIV-infections

HIV-2 infection is less pathogenic, and the progression to AIDS is slower, than with HIV-1. On the other hand, SIV infection in their natural host, does not generally lead to AIDS despite a high level of viral replication. The understanding of the mechanisms underlying this lower pathogenicity of HIV-2 and SIV in their natural hosts could uncover new strategies for a functional cure or a vaccine against HIV.
2.1.2.1 HIV-2

Reports from longitudinal studies showed that HIV-2 transmission rates through sexual encounter and from mother to child is lower than for HIV-1 [60]. The low viral load found in HIV-2 infected individuals can explain their lower infectivity, longer asymptomatic phase and slower progression to AIDS as compared to HIV-1 infected patients. Most HIV-2 infected patients display high CD4+ T cell counts, reflecting either a low intrinsic replication capacity of the virus, an effective immune response against the virus, or both [61].

Although lower than in HIV-1 infection [62], the level of immune activation during the course of HIV-2 is also a prognostic marker for death [63]. Importantly, immune activation levels are comparable for similar CD4+ T cell counts in HIV-1 and HIV-2 infected patients, suggesting that immune activation drives CD4+ T cell depletion and pathogenesis in both infections [34].

Strong HIV-2 cellular immune responses are thought to be responsible for the lower viral load in HIV-2 infected patients. Whereas HIV-1 specific CD4+ T cell responses are absent or severely impaired in infected individuals, asymptomatic and non-progressive HIV-2 infection is characterized by the maintenance of functional HIV-2 specific CD4+ T cells [64]. Also, the HIV-2 specific CD8+ T cell population contains cells with a phenotype of early differentiation, expressing both CD27 and CD28 molecules [65]. The percentage of these cytotoxic T lymphocyte (CTL) responses have been shown to correlate positively with CD4+ T cell counts and negatively with immune activation, measured by HLA-DR expression on CD4+ T cells. Additionally, the quality of antibodies produced by HIV-2 infected patients is better than those found in HIV-1 patients. These antibodies are broadly neutralizing (NAbs) and thus participate to the control of HIV-2 replication (Table 1).

2.1.2.2 SIV infection in natural and non-natural host

SIV infection is natural and non-pathogenic in some African non-human primates, e.g. African green monkeys (AGMs), sooty mangabeys (SMs), and chimpanzees. Recent findings showed that chimpanzees in the wild also acquire AIDS [66]. Despite high levels of viremia, AGMs and SMs usually preserve high levels of circulating CD4+ T cells and rarely develop AIDS following SIV-infection [67]. In contrast, non-natural hosts such as rhesus macaques (RMs), when infected by SIV experimentally, rapidly display a decline of their circulating CD4+ T cells eventually leading to AIDS, similar to HIV-1 infection in human. The cytopathic effect of SIV is comparable to HIV-1. Indeed, both natural and non-natural hosts
experience CD4+ T cell depletion in the gut mucosa during acute infection; although CD4+ T cells from natural hosts express remarkably low levels of CCR5, the HIV/SIV co-receptor. In discrepancy with the non-natural hosts, however, AGMs and SMs experience either stabilization or progressive recovery of the gut CD4+ T cells together with absence of microbial translocation. Importantly, the SIV-specific immune responses of infected natural hosts are similar to non-natural hosts and are lower than those found in HIV-1 infected individuals [68, 69]. Cellular responses against SIV in SMs do not participate in the resistance of these animals to the progression to AIDS [69].

Innate responses have been shown to be lower in non-pathogenic SIV-infection both in the acute and chronic phase as compared to pathogenic infection [70]. The mechanisms involve a lower capacity of dendritic cells (DC) from SMs to secrete type I IFN upon SIV exposure ex vivo [70]. These results were corroborated by genetic analyses of the transcriptional profile induced by SIV-infection [71]. Animals undergoing pathogenic SIV-infection displayed a shift toward cellular stress pathways and T helper (Th)-1 responses with strong type I and II IFN responses. In contrast, a strong type I IFN response was induced during acute SIV-infection of AGMs and was rapidly resolved after the peak of viremia [71]. Slow progression of SIV-infected RMs was also associated with a mucosal Th17 response and the lack of Th1 response, confirming a crucial role for the kind of immune response induced by SIV in the outcome of the disease [72]. Non-pathogenic SIV-infection of SMs does not affect the levels of Th17 cells, whereas this CD4+ T cell population is depleted from the gut during HIV-1 infection [73].

The major difference found between non-pathogenic and pathogenic SIV-infection is the association of systemic immune activation with disease progression (Table 1) [58, 74, 75]. Furthermore, the induction of immune activation by injection of LPS to SIV-infected AGMs led to an increase of both viral load and CD4+ T cell depletion [76].
<table>
<thead>
<tr>
<th></th>
<th>HIV-1</th>
<th>HIV-2</th>
<th>SIV pathogenic</th>
<th>SIV non-pathogenic</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Viral load</strong></td>
<td>High</td>
<td>Low in LTNPs</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td><strong>CD4+ T cell depletion in the gut</strong></td>
<td>+++</td>
<td>n.d.</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td><strong>Th17 cells</strong></td>
<td>Affected</td>
<td>n.d.</td>
<td>Affected</td>
<td>Intact</td>
</tr>
<tr>
<td><strong>Recovery of CD4+ T cells in the gut</strong></td>
<td>-</td>
<td>n.d.</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><strong>Viral load</strong></td>
<td>High</td>
<td>Low in LTNPs</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td><strong>Recovery of CD4+ T cells in the gut</strong></td>
<td>-</td>
<td>n.d.</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><strong>Viral load</strong></td>
<td>High</td>
<td>Low in LTNPs</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td><strong>Microbial translocation</strong></td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td><strong>Systemic immune activation</strong></td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td><strong>CD4 decline</strong></td>
<td>Fast</td>
<td>Slow</td>
<td>Fast</td>
<td>-</td>
</tr>
<tr>
<td><strong>T cell apoptosis</strong></td>
<td>+++</td>
<td>Low</td>
<td>+++</td>
<td>Low</td>
</tr>
<tr>
<td><strong>T cell exhaustion</strong></td>
<td>+++</td>
<td>-</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td><strong>Resting memory B cell depletion</strong></td>
<td>++</td>
<td>n.d.</td>
<td>++</td>
<td>n.d.</td>
</tr>
<tr>
<td><strong>Neutralizing Abs</strong></td>
<td>+/-</td>
<td>++</td>
<td>n.d.</td>
<td>-</td>
</tr>
<tr>
<td><strong>T helper cell function</strong></td>
<td>Declined</td>
<td>Maintained</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td><strong>Cytotoxic T Lymphocyte function</strong></td>
<td>+</td>
<td>+</td>
<td>Low</td>
<td>Low</td>
</tr>
</tbody>
</table>

*Table 1. Comparison of HIV-1 infection with HIV-2 and pathogenic and non-pathogenic SIV-infection.* [61, 62, 67, 77]. LTNP: Long term non progressors. n.d, not determined. IFN, Interferon.+++elevated; ++/+intermediate; -low.
2.2 T CELL DEPLETION IN THE GUT AND MICROBIAL TRANSLOCATION

As AIDS patients suffer from enteric infections and frequent diarrhea, early studies showed immune alterations in the gastrointestinal tract with loss of Th cells and bacterial colonization [78, 79]. The loss of CD4+ T cells was observed not only in patients with AIDS but also during early HIV-1 infection [80]. Further studies of acute HIV-1/SIV infections confirmed that the major pathogenic event occurring in early HIV-1 infection is the rapid and profound depletion of CD4+ T cells from the gut [81-83]. The GALT is the largest lymphoid tissue of the body and comprises a majority of memory CD4+ T cells expressing CCR5, the HIV-1 co-receptor. The massive CD4+ T cell depletion in the GALT during acute HIV-1 infection may also partly explain the reduction of the viral load to its set-point by lowering the availability of target cells for HIV-1 replication [67].

2.2.1 Mucosal damages in the early phase of HIV-1 infection

Changed in intestinal bacteria and increased of inflammation are thought to arise early during HIV-1 infection [84]. A recent study showed that in the acute stage of HIV-1 infection, before the viral set-point, an infiltration of both CD4+ and CD8+ T cells occurs in the duodenum [85]. Consistent with the activated phenotype of the CD4+ T cells rendering them prone to activation-induced apoptosis, their frequency and density are subsequently decreased during chronic HIV-1 infection. Indeed, activated memory CD4+ T cells are the direct targets for HIV-1, and the T cell depletion observed in the GALT has been attributed mainly to HIV-1 and to virus-induced Fas-mediated apoptosis [86-88] (see below section 2.4.1). A large proportion of the CD8+ T cells present early during acute infection is expressing perforin, an important molecule for their cytotoxic activity [85]. A correlation was found between the number of perforin-positive CTLs and the frequency of apoptotic epithelial cells, linking the CD8+ T cell response during acute HIV-1 infection to the observed mucosal damage [85]. Pro-inflammatory cytokines, such as TNF, IL-1β and IL-12 were also found at higher levels in GALT from HIV-1 infected individuals during acute infection as compared to uninfected individuals, with an increase of CD8+ T cells expressing granzyme, but not perforin [51]. Importantly, the intestinal functions measured during acute and chronic HIV-1 infection are similar but altered as compared to uninfected individuals: lower resistance, symptomatic of a lower thickness, and increased permeability possibly leading to the passage of microbial products into the circulation [85]. These results are in line with the previously observed decreased expression of genes involved in the epithelial barrier maintenance in primary HIV-1/SIV infection [89,
The importance of mucosal barrier alterations for HIV-1 pathogenesis was established by the correlation of microbial translocation with the immune activation occurring in HIV-1 infected patients [58]. Higher levels of LPS, a component of bacterial cell walls with a strong immuno-stimulatory effect, are found in chronically HIV-1 infected individuals as compared to controls. Notably, LPS levels inversely correlate with CD4+ T cell counts [91] and are also associated with CD8+ T cell activation (CD38+HLA-DR+ phenotype) [58]. The mechanisms of LPS immuno-modulation act on monocytes and macrophages and induce their release of soluble CD14 (sCD14). Accordingly, levels of sCD14 are increased during HIV-1 chronic infection [92] and correlate with LPS levels [58].

Adaptive immune responses are mounted to neutralize LPS activity and endotoxin-core antibodies (EndoCAb) are present in patients suffering sepsis, a condition in which acute microbial translocation occurs [93]. Plasma EndoCAb levels during HIV-1 infection are surprisingly lower as compared to uninfected individuals, and inversely correlate with LPS levels in non-treated patients [58, 91], possibly as a result of B cell dysfunctions (see below). Bacterial 16S ribosome DNA (16S rDNA) is also a marker for microbial translocation, which is found to be elevated in the plasma from HIV-1 infected as compared to uninfected individuals. Bacterial 16S rDNA levels are associated with higher CD8+ T cell activation and lower CD4+ T cell recovery after ART during HIV-1 infection [94, 95].

Importantly, SIV pathogenic infection of RMs induces mucosal damages and immune activation that are not observed in SIV-infected SMs [58, 96]. The disease progression of HIV-1 infected individuals and their mortality has also been associated with the levels of circulating LPS and/or sCD14, further confirming the importance of microbial translocation in HIV-1 pathogenesis [54, 97-100]. Moreover, the microbial translocation measured by LPS, sCD14, EndoCAb or 16S rDNA, is not totally normalized after ART initiation and is associated with lower CD4+ T cell recovery [58, 95, 101, 102].

2.2.2 Imbalance of T helper cells during HIV-1 infection

If the infiltration of perforin-expressing CD8+ T cells can explain the mucosal damages occurring in early HIV-1/SIV infection, the persistence of the epithelial barrier alterations cannot be similarly attributed to CTLs as these cells have been shown to lose their perforin expression during acute and chronic infection [85, 103, 104]. There is, however, an association with CD4+ T cell depletion and epithelial functions in the gut [89]. Additionally, studies on mucosal tissue from SIV-infected SM suggest that Th17 helper T cells could play a role in HIV-1
CD4+ T helper cells are categorized by their cytokine profile, responsible for different regulation of the immune responses against pathogens (Table 2). Th1 and Th2 helper T cells regulate immune responses against intracellular infections and parasites respectively [105]. Th1 cells, by producing IFN-γ, promote cellular responses that enhance CD8+ T effector cells activation. The cytokines produced by Th2 cells favor, on the other hand, humoral immunity by activating B cell responses. Th17 cells, producing IL-17, IL-21 and IL-23, are important for mucosal immunity against extracellular pathogens [106].

<table>
<thead>
<tr>
<th>Cytokine profile</th>
<th>Function</th>
<th>Localization</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Th1</strong></td>
<td>IFN-γ</td>
<td>Intracellular infection</td>
</tr>
<tr>
<td><strong>Th2</strong></td>
<td>IL-4, IL-5, IL-13</td>
<td>Humoral immunity against parasites</td>
</tr>
<tr>
<td><strong>Th17</strong></td>
<td>IL-17, IL-21, IL-22, IL-26</td>
<td>Mucosal immunity against extracellular pathogens</td>
</tr>
<tr>
<td><strong>Tfh</strong></td>
<td>IL-21</td>
<td>Humoral immunity</td>
</tr>
<tr>
<td><strong>Treg</strong></td>
<td>TGF-β, IL-10</td>
<td>Suppression of immune responses</td>
</tr>
</tbody>
</table>

*Follicular T helper cells; **Regulatory T helper cells; IL, interleukin; TGF, transforming growth factor.*

Th17 cells are selectively depleted from the GALT and peripheral blood of HIV-1 infected individuals [73, 107, 108]. Additionally, Th17 cells are also affected in pathogenic SIV-infection, whereas their levels remain similar in non-pathogenic SIV-infection as compared to uninfected animals [72, 73]. Of note, long-term non progressors (LTNPs), rare HIV-1 infected individuals who control HIV-1 replication without ART, have higher levels of circulating Th17 cells than normal progressors [109] and display similar mucosal Th17 numbers as compared to uninfected individuals [110]. These high levels of Th17 in LTNPs and non-pathogenic SIV-infection are associated with an intact mucosa and low microbial translocation. The mechanisms may possibly involve the secretion of IL-17 and IL-22 by Th17 cells, which have been shown to modulate the proliferation of epithelial cells and their up-regulation of antimicrobial protein production [106]. Also, the CD4+ T cell reconstitution in the GALT in HIV-1 infected patients under ART was associated with the presence of Th17 cells [111].
Another Th cell population thought to play a role in HIV-1 pathogenesis is the regulatory T helper cells (Treg). Treg are characterized by the expression of the forkhead family transcription factor Foxp3, but to study these cells is a challenge since a specific lineage marker has yet not been identified on their surface. Ongoing work is being carried out to better identify this discrete population of helper T cells and their possible functions during HIV-1 infection [112]. In vitro depletion of Treg cells from PBMCs suggests a suppressive role of these cells on HIV-specific T cell responses. However, Treg cells have been shown to be progressively depleted during the course of HIV-1 infection, and their levels to correlate with disease progression [113]. A more recent study found opposite results, demonstrating increased levels of Treg cells among CD4+ T cells to correlate with disease severity [114]. Although their role remains elusive during HIV-1 pathogenesis, the balance between Treg and Th17 cells has been shown to be altered in HIV-1 infected patients as compared to uninfected and HIV-1 infected elite controller individuals who exhibited low levels of viremia (<50 copies/ml of blood) despite no treatment [115, 116]. Further studies characterizing both Th17 and Treg cells are needed to clarify their importance in the pathogenesis of HIV-1 infection [117-119].

2.3 T CELL EXHAUSTION DURING HIV-1 INFECTION

The CD4+ T cell depletion is associated with the level of immune activation, probably induced, at least partially, by the microbial translocation from the gut. In parallel, recent studies have pointed out another consequence of T cell activation during HIV-1 infection, namely T cell exhaustion [120-122]. Exhausted T cells are characterized by modifications in the co-stimulatory receptor expression, lower cytokine release and lack of proliferative capacity, i.e. replicative senescence [123].

Although, functional correlates of CTL-mediated protection remain to be further defined, there are evidences suggesting that HIV-1 specific T cell responses are important in the course of the disease. The peak of HIV-1 specific CD8+ T cells is concomitant with the decrease of viral load to its set-point. Also, the association of better viral control in individuals with particular HLAs or strong Gag-specific CTL responses shows the importance of cellular responses during HIV-1 infection [55, 56]. A better understanding of the mechanisms leading to T cell exhaustion may lead to innovative treatment promoting a stronger HIV-1 specific T cell response.
2.3.1 Ageing of the immune system and CD28 expression

Lack of replicative capacity following sustained *in vitro* stimulation of cells is an old observation. The history of a cell replication has been shown to be reflected by the length of the telomeres, which are repetitive DNA sequences found at the ends of a chromosome and aimed at protecting it from degradation or fusion with other chromosomes. Decreased telomere length, as well as replicative senescence, arises also *in vivo*, in animal models of chronic infection [124]. The relevance of this phenomenon has been demonstrated by the study of cells isolated from centenarians, whose T cells displayed an inability to proliferate, a decrease length of their telomeres and phenotypic alterations when compared to cells from young individuals [125]. Importantly, the loss of CD28 expression on T cells upon ageing is associated with a higher susceptibility to infection and lower responses of elderly to vaccination [126, 127].

CD28, part of the Ig superfamily, is a crucial co-stimulatory molecule, expressed on the surface of the majority of resting naïve CD4+ and CD8+ T cells in human peripheral blood. T cell activation usually requires CD28 ligation by CD80/CD86 on antigen-presenting cells (APC), although high doses of antigens can also lead to sufficient TCR stimulation [128, 129]. The CD28 triggering following TCR activation induces the expression of anti-apoptotic proteins and enhances cytokine production such as IL-2 that, in turn, promotes T cell proliferation [130]. On the other hand, sustained T cell activation leads to the down-regulation of CD28 expression [123, 131]. CD28- T cells have been shown to have poor proliferation capacity, in line with their increased expression of CD57, a marker of senescence[132]. In addition to their lower proliferation, *in vitro* generated CD28-T cells have a lower sensitivity to activation-induced cell death (AICD) [133-135]. The resistance of CD28- T cells to undergo apoptosis was proposed as a mechanism leading to their accumulation with age or under chronic inflammation conditions. It has also been shown that a subset of CD8+CD28- T cells have suppressive functions [136]. This distinct Treg population, named T suppressors, inhibits Th proliferation by acting on APCs.

Similarly to elderly, HIV-1 infected individuals display lower CD28 expression on T cells [137-139]. A negative association between markers of disease progression, such as high β2-microglobulin levels, and numbers of circulating CD8+CD28+ T cells in HIV-1 infected patients also suggests the involvement of the increased proportion of CD8+CD28- T cells in HIV-1 pathogenesis[138]. Those CD28- T cells have shorter telomeres and poor proliferative capacity [137]. Recently, CD57 expression was suggested as a better marker for impaired proliferation of HIV-1
specific CD8+ T cells [140]. However, these CD8+CD57+ T cells were also more prone to apoptosis, contrasting with previous data suggesting a resistance to AICD of senescent T cells. The loss of CD28 on T cells from HIV-1 infected patients was also associated with a decreased expression of IL-7 receptor α (IL-7Rα) [141], suggesting an increased susceptibility to apoptosis (see below). Additionally, CD8+CD28- T cells from HIV-1 infected individuals, rather than being suppressors T cells, induce DC activation and are therefore likely to be participating in the induction of chronic immune activation [142]. More studies are needed to elucidate the precise role of CD28- T cells in HIV-1 infection and understand the mechanisms underlying their accumulation.

2.3.2 Markers of T cell exhaustion

Negative immune regulation occurs through the appearance of Tregs, soluble factors and the expression of inhibitory receptors on T cells. T cell exhaustion is a consequence of chronic inflammation, and results from the accumulation of inhibitory receptors associated with impaired effector functions [123]. Those negative immune regulators are also found on T cells during HIV-1 infection [122], and thus may also contribute to the immune deficiency observed in patients, measured by the increased susceptibility to pathogens and decreased efficacy of vaccines [143].

Following T cell activation, CD28 expression is down-regulated while the expression of programmed death (PD)-1 and cytotoxic T-lymphocyte antigen 4 (CTLA-4) increases. These proteins are inhibitory molecules and have been shown to play an important role in HIV-1 infection [144]. Despite being up-regulated on HIV-1 specific CD4+ T cells and associated with markers of disease progression, the blockade of CTLA-4 in SIV-infection did not show any beneficial effects [144]. PD-1 expression is increased on both CD4+ and CD8+ HIV-1 specific T cells in association with T cell exhaustion and disease progression [145-147]. Also, the blockade of PD-1 leads to the restoration of HIV-1 specific T cell responses in vitro. PD-1 expression on CD8+ T cells from HIV-1 infected individuals was associated with spontaneous and Fas-mediated apoptosis [148]. Animal models of SIV-infection also confirmed the role of PD-1 in T cell exhaustion. The inhibition of PD-1 signaling leads to a lower viremia and an increased survival of the infected animals, associated with improved SIV-specific immune responses [77]. On the other hand, a study on non-pathogenic SIV-infection revealed that PD-1 is expressed on T cells early on and may thus participate in the resolution of the immune activation occurring during acute infection [74].
Recent publications have identified other inhibitory molecules that also contribute to T cell exhaustion. T cell immunoglobulin and mucin domain-containing molecule-3 (Tim-3) expression on CD4+ and CD8+ T cells correlates with viral load, levels of CD38+ cells and lower CD4+ T cell counts [149]. Tim-3 positive cells also display impaired effector functions as measured by cytokine production and proliferation. Tim-3 blockade also ameliorates proliferation of both CD4+ and CD8+ HIV-1 specific T cells [149].

The co-expression of several inhibitory receptors leads to a greater degree of T cell exhaustion as shown in chronic infection in mice and humans [123]. These results are also confirmed during HIV-1 infection. Indeed, the expression pattern of PD-1 with CD160 and 2B4, other inhibitory receptors found at increased levels during chronic infection, is associated with T cell exhaustion in HIV-1 infected individuals [150]. CD8+ T cells expressing only 2B4 display higher effector functions than cells expressing 2 or 3 of those inhibitory receptors (Table 3). Similarly, signaling inhibition by several inhibitory receptors induces a greater proliferation and cytokine production [150].

<table>
<thead>
<tr>
<th>IFN-γ</th>
<th>+++</th>
<th>+++</th>
<th>++</th>
<th>+/-</th>
<th>+/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>CTL</td>
<td>+++</td>
<td>++/-</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>IL-2</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Proliferation</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+/-</td>
<td>++</td>
</tr>
</tbody>
</table>

*Table 3. T cell exhaustion during HIV-1 infection.*

Notably, the interaction between the pathogen and the host is important for CD8+ T cell differentiation and exhaustion. Although similar during the acute phase of the infection, the phenotype of memory CD8+ T cells differs according to the infecting viruses in the chronic infection [151]. Based on CD28 and CD27 expression, 3 stages of memory T cell differentiation are defined: CD28+CD27+ for early, CD28-CD27+ for intermediate and CD28-CD27- for late differentiation.
While most Epstein-Barr virus (EBV)- and hepatitis C virus (HCV)-specific CD8+ memory T cells display a phenotype of early differentiation with great ability to proliferate, CMV-specific cells have a greater cytotoxic potential with a phenotype of late differentiation in patients during the chronic phase of infection. HIV-1 specific CD8+ T cells displayed an intermediate phenotype, expressing CD27 but lacking CD28 molecule [151]. The expression of inhibitory receptors also varies depending on the differentiation stage of T cells [150]. When comparing the expression of PD-1, 2B4, and CD160 on T cells from the same patient, HIV-1 specific CD8+ T cells exhibit higher levels of those 3 inhibitory receptors than CMV-specific CD8+ T cells. The initiation of ART induces a decreased expression of PD-1, confirming previous data [145, 147, 150]. CD160 expression, but not 2B4, was also lower on HIV-1 specific CD8+ T cells after ART [150]. Consistent with an improved immunological system of HIV-1 infected individuals under ART; CMV-specific CD8+ T cells also displayed lower inhibitory receptor expression. HIV-1 infected individuals are often affected by other pathogens, such as HCV, mycobacterium tuberculosis, or parasites [152-154]. Further studies dissecting the importance of memory T cell responses and the influence of co-infections are required for the elucidation of the mechanisms underlying the detrimental interactions between HIV-1 and other pathogens.

2.4 CAUSES AND CONSEQUENCES OF LYMPHOPENIA

As previously discussed, CD4+ T cells are the main target of HIV-1, and their depletion is a correlate of disease progression. CD4+ T cells from the GALT are mostly depleted through direct infection by HIV-1 during the acute phase of the infection. HIV-1 infects preferentially activated HIV-1 specific CD4+ T cells [155], and functional HIV-1 specific CD4+ T cells, with IL-2 secretion and proliferative capacity, are lost early in the infection [156, 157]. Yet, non-HIV-1 specific T cells are also depleted and exhibit higher susceptibility to apoptosis than cells from uninfected individuals, despite the low frequency of HIV-1 infected cells [158, 159]. CTL responses against HIV-1 are thought to be important as they are associated with decreased viremia after primary infection [56]. However, as these cells are unable to eradicate the virus, the continuous viral replication, together with the microbial translocation induces a systemic immune activation ultimately causing the progressive T cell exhaustion and depletion [160]. The exceptional regenerative capacity of the immune system is also progressively impaired during HIV-1 infection [161], and, together with the persistent cell death, eventually leads to AIDS.
2.4.1 Mechanisms of T cell depletion

Early studies showed that T cells from HIV-1 infected patients are more prone to cell death as compared to T cells from uninfected individuals [162-165]. Also, levels of CD4+ T cell apoptosis are correlated with and CD4+ T cell counts and viral load [166] and are linked with HIV-1 disease progression [167]. The mechanisms leading to CD4+ T cell depletion are various and involve direct effect of HIV-1 replication, killing by CTL T cells, effects of HIV-1 viral proteins and bystander apoptosis. The signaling pathways taking place during apoptosis are summarized in Figure 2.

As described previously, the GALT is a major site for HIV-1 replication during acute infection with an important infiltration of CD8+ T cells [85]. Similarly, the presence of high levels of CD8+ T cells was observed in lymph nodes of HIV-1 infected individuals [168]. The peak of HIV-1 specific CD8+ T cell response coincide with the decrease of the viral load to its set point, suggesting a role for CD8+ T cells in the initial suppression of viral replication [55]. A recent study also showed an association of HIV-1 specific CD8+ T cells and delayed disease progression [169], confirming a beneficial role of cellular response against HIV-1 [55]. This data, together with the low frequency of HIV-1 infected CD4+ T cells, suggest that HIV-1 specific CTL responses are unlikely to participate in the generalized CD4+ T cell depletion symptomatic of HIV-1 infection.

During HIV-1 pathogenesis, the molecules involved in apoptosis are profoundly dysregulated. Indeed, the death receptor Fas has been shown to be up-regulated on both CD4+ and CD8+ T cells from infected patients, and linked to their depletion [170-173]. Other death receptors, the tumor necrosis factor receptors (TNFR) are also implicated in T cell apoptosis and the levels of pro-apoptotic molecule Bcl-2 are altered during HIV-1 infection [171, 174]. During HIV-1 infection, the apoptotic signaling pathways are altered by HIV-1 replication and inflammatory cytokines. The bystander apoptosis of T cells has also been shown to play an important role as I will describe in further detail in the following sections.

2.4.1.1 Direct and indirect effects of HIV-1

The direct infection of CD4+ T cells by HIV-1 can lead to their cell death by the disruption of the cell membrane caused by the virus budding, or by the cellular toxicity induced by the accumulation of RNA/DNA and proteins from the virus [175]. Also the expression of Env proteins on the surface of infected cells allows the binding to another cell expressing the CD4 molecule through a virological synapse, leading to cell-to-cell fusion and the formation of giant multinucleated
Apoptosis or programmed cell death is an active and highly regulated process which occurs at several stages in multicellular organisms: embryo development, homeostasis, immune cell regulation, cell damage (e.g. stress, DNA damage) or infection. Cells undergoing apoptosis stop their protein synthesis, expose phosphatidylserine on their membrane; their mitochondria become dysfunctional and chromatin condensation and DNA fragmentation is initiated. The apoptotic cell shrinks and blebs appear at the surface before the cell breaks up into smaller apoptotic bodies. The membrane protein expression of apoptotic cells is modified to facilitate phagocytosis for degradation without eliciting inflammation (Review [176]).

Apoptosis occurs either in a receptor-dependent (the extrinsic pathway), or in a non-receptor-dependent way (the intrinsic pathway). Besides apoptosis, other types of cell death have been defined by morphological criteria, such as necrosis or autophagy.

To date there are eight identified death receptors (DR); DRs belong to the tumor necrosis factor (TNF) receptor superfamily and are characterized by a common extracellular cysteine rich domain and an intracellular globular protein interaction domain called death domain (DD). All DR ligands function in an endocrine or paracrine manner, and upon binding cause a trimerization of their respective receptors, which is required for apoptosis. The triggering of DRs results in the formation of death-inducing signaling complex (DISC) within seconds of receptor engagement. The DISC contains caspases, which are cysteine proteases that specifically cleave their target sites after aspartic acid residues. Autoproteolytic processing of caspases-8 and -10 (initiator caspases) leads to their activation and release from the DISC. Thus, a cascade of caspase activation occurs, resulting in the proteolytic processing of caspase-3 which is the main apoptotic effector.

The death receptor pathway is connected to the intrinsic pathway via the cleavage of the protein Bid by caspase-8. Truncated Bid (tBid) then translocates to the mitochondria, promoting apoptosis. The mitochondrial or intrinsic pathway also leads to caspase-3 activation. Three classes of proteins act upstream the mitochondria. The anti-apoptotic proteins, so-called Bcl-2 like (e.g. Bcl-2, BclXL, Bfl-1), interact with the pro-apoptotic proteins of the Bax-like family (Bak, Bad, Bid, BclXS) in order to inhibit their function. BH3-only proteins (e.g. Bim, Bmf) form the third class. After a stress, pro-apoptotic proteins are released from the anti-apoptotic proteins and cluster on the mitochondrial membrane to form pores. Followed by its release from the mitochondria, Cytochrome C (CytC) acts on APAF-1, leading to caspase-9 recruitment and formation of the apoptosome to finally activate caspase-3.

\[ \text{Figure 2. Mechanisms of apoptosis} \]
cells called syncytia. Syncytia are not a stable form of living cell and rapidly undergo apoptosis through the mitochondrial pathway and are therefore hardly detectable in HIV-1 infected patients [175, 177]. HIV-1 proteins have been shown to differently impact on the fate of infected cells by regulating molecules involved in the apoptotic signaling pathway. The Env protein can induce cell death via different mechanisms activating the mitochondrial pathway [178]. Infected cells also up-regulate Fas and Fas ligand (FasL) expression through the effect of Nef. Nef protein, as well as Vpr and the HIV-1 protease, induce the activation of caspase-3, an effector molecule of apoptosis. Bcl-2, an anti-apoptotic molecule, is a substrate of HIV-1 protease, and its expression is modulated by Tat [175]. While HIV-1 proteins impact on the survival of infected cells, in vivo data suggest that HIV-1 replication has little cytopathic effect [179]. Indeed the low levels of infected cells observed in the patients cannot explain the high susceptibility of their leukocytes to undergo apoptosis [158]. Additionally, CD8+ T cells that are not infected by HIV-1 are also prone to apoptosis.

A role for non-infectious virions in T cell apoptosis has been suggested for the understanding of the discrepancy between the low rate of infected cells in vivo, on one hand, and the high levels of apoptosis associated with HIV-1 viral load on the other [180, 181]. Importantly, it has been shown that abortive infection also leads to T cell apoptosis [182]. Additionally, with high levels of viral replication, high quantities of circulating HIV-1 proteins are present in the host tissues either directly released from infected cells or on the surface of HIV-1 virions, and could contribute to the apoptosis of all T cell subsets. Indeed, HIV-1 proteins have been shown to directly affect immune cells, participating in their activation and/or depletion [175, 177, 183]. Soluble and membrane-bound gp120 has been shown to induce pro-apoptotic pathway by the up-regulation of Fas/FasL, tumor necrosis factor (TNF) and TNR-related apoptosis-inducing ligand (TRAIL) on non-infected cells. Similarly, other HIV-1 proteins, such as Tat, also induce Fas/FasL expression and interact with Bim, a pro-apoptotic molecule that binds Bcl-2 [184], thus promoting cell apoptosis.

2.4.1.2 Cytokine dysregulation during HIV-1 infection

Cytokines are important molecules for T cell functions but also for regulating apoptosis. The balance of Th1/Th2 cytokines is important in vitro but conflicting data are available on their role in HIV-1 pathogenesis [173, 185, 186]. While IFN-γ, a Th1 cytokine, and IL-12, which favors Th1 responses, rescue T cells from HIV-1
infected patients, Th2 cytokines (IL-4 and IL-10) increase their susceptibility to undergo apoptosis [173]. TNF, a pro-apoptotic cytokine, is also thought to play a role in HIV-1 pathogenesis, as previously mentioned; however, the limited results of anti-TNF therapy question its relevance during the infection [187]. IL-10, which showed a potential in suppressing inflammatory cytokines, such as IFN-γ, TNF or IL-6, was tested for the treatment of HIV-1 infected individuals and also failed to impact beneficially on the CD4+ T cell counts or the viral load [188]. Other cytokines influencing the survival of T cell and studied in the context of HIV-1 infection are IL2, IL-7, IL-15 and IL-21, part of the γ-chain cytokine family [189, 190]. IL-2 is a central cytokine engaged in T cell differentiation, proliferation and survival. IL-2, directly secreted by activated T cells, induces the up-regulation of Bcl-2, but displays both pro- and anti-apoptotic effects depending on the context. In clinical trials, IL-2 treatment of HIV-1 infected patients induced an increase of CD4+ T cell counts. Nevertheless, data failed to show a decrease in mortality and opportunistic infection in the cohort of IL-2 treated patients [191]. The absence of CD4+ T cell recovery in the gut of IL-2 treated HIV-1 infected patients may explain the absence of long term beneficial effects of IL-2 therapy [192]. IL-15 shares many biological effects with IL-2; and, although non consistently, IL-15 serum levels and production by stimulated PBMCs were reported to be dysregulated during HIV-1 infection [190]. Treatment of SIV-infected RM with a combination of ART and IL-15 showed an increase of SIV-specific and total CD8+ T cell responses. CD4+ T cells at the mucosa remained unaffected by IL-15 treatment and a detrimental effect of IL-15 was observed, delaying viral suppression induced by ART [193]. IL-7 is an important cytokine for T cell homeostasis. Through the regulation of the protein members of the Bcl-2 family, IL-7 influences T cell survival [194]. Levels of IL-7 are elevated during HIV-1 infection, in association with low CD4+ T cell counts, i.e. lymphopenia [38, 141, 195, 196]. HIV-1 patients treated with IL-7 also showed an improvement of their CD4+ T cell counts [197]. A detailed description of IL-7 regulation and the involvement of this cytokine in the pathogenesis of HIV-1 infection will be presented below. Of note, recent data on IL-21 treatment of chronically SIV-infected RM [198]. IL-21 is rather a co-stimulating cytokine than a survival factor. IL-21 plasma levels are low in HIV-1 infected individuals as compared to uninfected individuals and ART leads to increase IL-21 levels [199-201]. The ability to produce IL-21 by T cells has been associated with better CTL responses in HIV-1 infected patients and relative control of viral load [202-204]. Furthermore, IL-21 treatment of SIV-infected animals enhances the functions of both CD4+ and CD8+ T cells, although their numbers were unchanged [198].
2.4.1.3 Bystander apoptosis

The importance of HIV-1 proteins in the observed T cell apoptosis is also confirmed by the findings that ART induces a decreased level of cell death in HIV-1 infected patients [35]. However, spontaneous CD3- and Fas-mediated apoptosis of both CD4+ and CD8+ T cells from ART-treated patients with undetectable viremia, remain at higher rates as compared to T cells isolated from uninfected donors [205]. Additionally, despite high levels of viremia, apoptosis of CD4+ T cells is not observed in non-pathogenic SIV-infection [206-208]. These studies point out that the state of lymphocyte activation is crucial in the propensity of T cells to undergo apoptosis, further confirming a role for the chronic immune activation, rather than the direct effect of HIV-1 replication, in T cell depletion [159]. Therefore, T cell apoptosis during HIV-1 infection may be likely to occur through AICD. This mechanism of apoptosis occurs few days after a T cell has been activated through its T cell receptor (TCR) and is essential for the homeostasis of the immune system after the clearance of an infection. AICD occurs through Fas as this molecule is up-regulated upon T cell activation. The described activated phenotype of T cells found in HIV-1 infected patients and the association of these markers of activation with cell death favor this hypothesis [209].

2.4.2 Consequences of Lymphopenia, the role of IL-7

As T cells are depleted by direct and mostly indirect effects of HIV-1 replication and the consequent immune activation, homeostatic mechanisms are taking place to replace those immune cells. The thymus is the organ where T cell progenitors mature before being exported in the periphery as recent thymic emigrants (RTEs). Additionally, peripheral homeostatic signals induce the expansion of the T cells and thus the maintenance of T cell numbers and a broad T cell repertoire [210].

During the thymic development of T cells, TCR rearrangement occurs at the chromosome level and, as a by-product, TCR excision circles (TRECs) are produced. TRECs are small episomal DNA not replicated during cell division. Therefore, the measure of TRECs reflects the replicative history of T cells and is used also to quantify the thymic output, i.e. the production of new naïve T cells [211]. Study of T cells from HIV-1 infected patients showed that TREC levels are decreased, suggesting that the rapid T cell turn-over observed is not fully compensated by RTEs [212, 213]. The low RTE levels have been shown to be the consequence of impaired thymic proliferation, which begins early during HIV-1 infection [212]. With the initiation of ART, the thymic functions are recovered and participate to the CD4+ T cell repletion [161, 212, 214, 215].
The selection of T cells in the thymus is governed by the recognition at low affinity of the major histocompatibility complex (MHC) associated with a self-peptide by the TCR. In addition to this MHC-peptide, the T cells receive signals from cytokines, especially IL-7 [216, 217]. These signals are also vital for T cell survival in the periphery but do not induce cell division in healthy condition. The access to secondary lymphoid tissues has been shown to be important for naïve CD4+ T cells to get survival signal, probably due to the presence of fibroblastic reticular cells (FRCs) producing IL-7 [216].

2.4.2.1 Regulation of IL-7 levels during HIV-1 infection

Besides FRCs, other cell types have been shown to produce IL-7, such as stromal cells, intestinal epithelial cells, keratinocytes, follicular DCs, smooth muscle and endothelial cells [194]. During lymphopenia, such as experienced by HIV-1 infected individuals, the levels of IL-7 are increased [195, 196, 218, 219] and correlate with the CD4+ T cell counts. The high IL-7 levels observed in lymphopenic conditions are thought to arise from the accumulation of the cytokine due to a lack of consumption [220]. However, IL-7 production by keratinocytes and human intestinal cells has been shown to be increased by IFN-γ [221, 222]. Additionally, IL-7 production in lymphoid tissue has also been shown to be increased during HIV-1 infection, as a result of T cell depletion, suggesting a potential feedback mechanism [38]. In contrast, a recent study demonstrated the destruction of FRC network in lymphoid tissues during lymphopenic conditions, resulting in decreased IL-7 production [223]. As IL-7 production arises from multiple sources, further studies are needed to elucidate how IL-7 levels are regulated during HIV-1 infection.

2.4.2.2 IL-7Rα expression during HIV-1 infection

IL-7 is needed at different stages of T cell differentiation: during thymopoiesis and T cell maturation, survival of naïve T cells, T cell activation, and memory T cell generation [220]. IL-7 signaling induces T cell survival by the up-regulation of Bcl-2 and BclXL, important anti-apoptotic molecules, and proliferation [224]. The receptor for IL-7 is a heterodimer composed of IL-7Rα and the γ-chain, common to all γ-chain cytokine family members. Naïve T cells express IL-7Rα constitutively whereas TCR signaling induces its down-regulation [224]. Memory T cells are heterogeneous in terms of IL-7Rα expression with the highest levels found on central memory T cell subset (T_CMO) as summarized in Table 4 [225].
### Table 4. Phenotype of T cells during their differentiation.

<table>
<thead>
<tr>
<th></th>
<th>Naive</th>
<th>Effector (T&lt;sub&gt;E&lt;/sub&gt;)</th>
<th>Effector Memory (T&lt;sub&gt;EM&lt;/sub&gt;)</th>
<th>Central Memory (T&lt;sub&gt;CM&lt;/sub&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD62L</td>
<td>High</td>
<td>Low</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>CCR7</td>
<td>High</td>
<td>Low</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>CD27</td>
<td>High</td>
<td>Low/Intermediate</td>
<td>Low/Intermediate</td>
<td>High</td>
</tr>
<tr>
<td>CD127</td>
<td>High</td>
<td>Low</td>
<td>Low/Intermediate</td>
<td>High</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>Intermediate</td>
<td>Low</td>
<td>Intermediate</td>
<td>High</td>
</tr>
</tbody>
</table>

Levels of IL-7Rα on T cells are also modulated by the presence of the cytokine as demonstrated by the down-regulation of IL-7Rα expression on T cells cultured in the presence of IL-7 [226]. This mechanism may occur during HIV-1 infection as T cells from HIV-1 infected patients exhibit lower IL-7Rα levels as compared to uninfected individuals [141]. Other studies, however, failed to establish a link between IL-7 levels and IL-7Rα expression during HIV-1 infection [227-229]. Additionally, while IL-7 treatment leads to a transient IL-7Rα lower expression in vitro, T cells from HIV-1 infected individuals do not recover IL-7Rα expression upon culture in the absence of the cytokine, pointing out further regulatory mechanisms of IL-7Rα expression prevailing during HIV-1 infection [141]. Among those, the HIV-1 protein Tat has also been proposed to induce IL-7Rα down-regulation [230]. Although the mechanism of IL-7Rα down-regulation remains to be clarified, the T cells of HIV-1 infected individuals with low IL-7Rα expression also exhibited lower levels of Bcl-2, associated with increased susceptibility to apoptosis [227], and inability to respond to IL-7 treatment in vitro [231]. Lower IL-7Rα expressing T cells, particularly in the CD8+ T cell population may arise from the expansion of effector T cells [228]; in line with the observed association of immune activation with the low IL-7Rα expression on T cells [39]. Furthermore, T cells from HIV-1 infected individuals under ART, recover IL-7Rα expression and respond similarly as controls to IL-7 treatment in vitro, measured by proliferation and Bcl-2 up-regulation [232]. Importantly, CD4+ T<sub>CM</sub> expressing IL-7Rα, associated with robust proliferation and IL-2 production, are thought to contribute to controlling HIV-1 infection, and their loss correlates with immune activation and T cell depletion [39]. The importance of IL-7Rα was recently confirmed when an association between its expression and the recovery of CD4+ T cells upon ART was showed [233].
2.4.2.3 Effects of IL-7

As previously described, IL-7 stimulates T cell proliferation and survival. Consequently, IL-7 treatment of HIV-1 infected individuals has been shown to ameliorate the CD4+ T cell recovery in combination with ART [197]. Additionally, IL-7 levels were associated with increased levels of immature transitional B cells (CD10+) during HIV-1 infection [234]. The impact of IL-7 on B cell development was further confirmed by similar associations found in patients suffering idiopathic CD4+ lymphocytopenia [235]. Furthermore, IL-7 treatment in humans enhanced the numbers of transitional B cells [236].

During HIV-1 infection, high levels of IL-7 are preferentially found in patients with low CD4+ T cell counts [237]. The mechanisms for such discrepancy are thought to arise from the inability of T cells to consume IL-7 as a consequence of low receptor expression or to an increased production of IL-7. Nevertheless, it has also been shown that high IL-7 may have detrimental effects on T cells. Indeed, Fas expression on T cells was found to be induced by IL-7 treatment in vitro and in vivo [238]. IL-7 levels in HIV-1 infected individuals also correlated with the increased Fas expression and the associated susceptibility to Fas-mediated apoptosis of T cells. On the other hand, the increase of Fas expression has been shown to act as a co-stimulatory signal for T cell proliferation under suboptimal TCR activation [239]. The T cell proliferation induced by Fas co-stimulation was at higher levels than the measured apoptosis. These results suggest that IL-7 may act on T cell peripheral homeostasis, lowering the threshold of T cell proliferation, and thus might participate in the T cell depletion through AICD observed during HIV-1 infection. Furthermore, a new role was proposed for IL-7 regulation of B cell apoptosis [240]. As mature B cells lack the expression of IL-7Rα, IL-7 acts on B cells through the release of IFN-γ from T cells. IFN-γ induces the up-regulation of Fas expression on B cells, rendering them more susceptible to Fas-mediated apoptosis [240]. This mechanism may enlighten some of the B cell dysregulations observed during HIV-1 infection as I will now discuss.
2.5 CHRONIC ACTIVATION OF B CELLS AND MEMORY B CELL LOSS

Similarly to T cells, B cells from HIV-1 infected individuals display impaired functions, a high degree of activation, susceptibility to apoptosis, which eventually lead to the loss of memory B cells and serological memory [241]. Still, B cells are not susceptible to HIV-1 infection, and the exact mechanisms underlying their altered functions remain to be elucidated.

2.5.1 Humoral response against HIV-1

In the weeks following HIV-1 infection, the immune response induces high levels of HIV-1 specific antibodies (Abs) used for diagnostic testing in clinics. The antiviral activity of anti-HIV-1 Abs is mediated either by their capacity to neutralize the virus (NAbs), and thus preventing HIV-1 entry into target cells, or by the induction of effector functions (complement, Ab-dependent cytotoxicity, phagocytosis). The passive administration of anti-HIV-1 NAbs conferred protection from infection in non-human primates, supporting the necessity of NAbs elicitation by an efficient vaccine [11]. Potent NAbs against HIV-1 have been isolated from infected individuals, demonstrating the ability of the immune response to generate such Abs during the natural course of infection. However, these NAbs are not present in the acute phase of the infection when they are most needed. Early Ab responses are against non-neutralizing epitopes of the HIV-1 envelope and when NAbs are formed, the virus escapes rapidly as a consequence of the prominent amount of mutations introduced by RT. Therefore, the B cell responses against HIV-1 are considered ineffective [241, 242]. Nevertheless, the study of memory B cells from HIV-1 infected individuals with high NAbs titers and low-to-intermediate viral load, revealed a broad HIV-1 specific memory B cells with mutated immunoglobulins [243]. A better understanding of B cell biology, in particular at the level of the mucosa, which is the chosen site for HIV-1 transmission, would reveal new strategies for the development of an efficient vaccine against HIV-1.

2.5.2 B cell hyperactivation and exhaustion

The same year of the discovery that HIV-1 was the causative agent of AIDS, report showed an abnormal activation of B cells isolated from infected patients [29]. HIV-1 infected individuals exhibit high concentrations of circulating IgG, a phenomenon known as hypergammaglobulinemia. Further studies have shown that activated T cells may contribute to the secretion of high levels of IgG by B cells.
During HIV-1 infection, T cells up-regulate CD70, which activates B cells through CD27 and leads to a higher IgG production. CD70/CD27 signaling is important for B cell differentiation into plasma cells (PCs), leading to the production of large amount of Abs [245]. B cells lacking CD27 expression also present an increased intracellular IgG content [246]. When looking at CD21 expression, a complement receptor that is down-regulated during B cell activation, it has been shown that B cells from viremic HIV-1 infected individuals are enriched with CD21<sub>low</sub> B cells [42]. Despite displaying low proliferation capacity, these CD21<sub>low</sub> B cells were found to secrete a high amount of IgG and have plasmablast features as observed by electron microscopy [42]. With the development of multicolor flow cytometry, allowing further dissection of B cells into different subsets, the CD21<sub>low</sub> B cell population displaying plasmablast characteristic may likely represent activated memory B cells (CD27+CD21<sub>low</sub>), a B cell subset increased during HIV-1 infection. An accumulation of PCs, based on their morphology, has also been reported in the lymphoid compartment of HIV-1 infected patients, possibly accounting for the hypergammaglobulinemia measured in serum [247, 248].

As described for T cells, the exhaustion of B cells is not surprising in the context of HIV-1 infection. In spite of high levels of circulating Igs in the serum of HIV-1 infected individuals, the capacity of B cells to secrete Ig <em>ex vivo</em> upon stimulation is decreased as compared to uninfected individuals [244]. The proliferative responses of B cells from HIV-1 infected individuals are also decreased [42]. The further study of the CD21<sub>low</sub> B cell population showed that CD27-CD21<sub>low</sub> B cells express the inhibitory receptor Fc-receptor-like-4 (FCRL4), similarly to human tonsilar B cells, and were therefore termed tissue-like memory (TLM) B cells [249]. These TLM B cells display lower capacity to proliferate as compared to naïve and classical memory B cells, lower epitope diversity and a lower number of divisions, suggesting an exhausted state. CD21<sub>low</sub> B cells are also more susceptible to Fas-mediated apoptosis as compared to CD21<sub>+</sub> B cells [250]. A recent publication confirmed the role of inhibitory receptor expression on B cell exhaustion during HIV-1 infection [251]. Following the silencing of inhibitory receptors, such as FCRL4 and sialic acid-binding Ig-like lectin 6 (Siglec-6), B cells displayed higher proliferation and cytokine release. Importantly, while classical memory B cells were seen to comprise influenza-specific memory B cells, this TLM B cell population contained most of the HIV-1 specific memory B cells [249]. The mechanism behind the skewed HIV-1 B cell responses is yet to be elucidated.

HIV-1 individuals undergoing ART exhibit decreasing levels of both HIV-1 specific and non-specific Abs, with normalization of the hypergammaglobulinemia [252,
253]. Consistent with a possible role of HIV-1 in promoting IgG secretion, HIV-1 protein gp120 has been shown to bind to tonsilar and splenic B cells and to lead to their polyclonal activation [254]. HIV-1 gp120 acts directly on B cells through mannose C-type lectin receptors (MCLRs), promoting the activation of class-switch recombination; gp120 also induces B cell-activating factor (BAFF) secretion by monocytes, which support the MCLR up-regulation on B cells. The HIV-1 protein, Nef, appears to mediate multiple and opposite functions. Nef protein expressed on infected macrophages induces the production of ferritin, a protein shown to induce B cell activation [255]. Furthermore, IgG levels in HIV-1 infected individuals correlates with both the levels of ferritin and the viral loads. However, it has also been shown that Nef accumulates on B cells and suppresses immunoglobulin class-switch DNA recombination through the blocking of the CD40 ligand (CD40L) signaling pathway [256].

2.5.3 Loss of memory B cells and serological memory

In addition to an activated phenotype, peripheral blood B cells from HIV-1 infected individuals are also susceptible to apoptosis [159, 257]. Fas expression on memory B cells is enhanced during HIV-1 infection and associated with Fas-mediated apoptosis [258]. Despite high levels of circulating Ig, HIV-1 infected patients have a reduced number of classical memory B cells in blood [24, 258]. Importantly, the levels of specific Abs and memory B cells against measles and tetanus have also been shown to be decreased in HIV-1 infected individuals during acute or chronic infection [24]. These results suggest the rapid loss of long-term serological memory, defined as circulating antibodies to previously encountered pathogens and vaccine antigens, during acute HIV-1 infection [24]. After ART initiation, it has been shown that normal levels of memory B cells are restored in HIV-1 individuals [259]. These results are contrasted by other publications observing lower memory B cells in ART-treated patients, as compared to uninfected individuals [41, 258, 260]. However, early initiation of ART in adult and children is associated with better memory B cell functions [261, 262].

It has been also questioned whether the differentiation of B cells into long-lived PCs could be affected in HIV-1 infected patients [263, 264]. Indeed, results from vaccination studies show that HIV-1 infected individuals are capable of generating an early antibody response similar to those of uninfected individuals [265]. Nevertheless, the levels of specific Abs are decreased several months after immunization in HIV-1 infected as compared to uninfected individuals [266]. These results suggest either a defective generation, or an impaired survival of
memory B cells and PCs [267]. Supporting this hypothesis, a recent case study demonstrated that the levels of total and HIV-1 neutralizing Abs are affected by Rituximab treatment, an anti-CD20 monoclonal antibody, which depletes memory B cells but not long lived PCs [268]. As a consequence of Rituximab treatment of this one HIV-1 infected individual, hypergammaglobulinemia disappeared transiently, as well as HIV-1 NAbs. These data confirm that the high levels of circulating Igs found during HIV-1 infection are most likely to arise from activated B cells as previously discussed, rather than a consequence of increased long-lived PCs cells.

The mechanisms underlying the loss of memory B cells during HIV-1 infection remain to be fully clarified. IL-2 signaling has been shown to be important for memory B cell survival in ART-treated HIV-1 infected individuals [269]. In the absence of IL-2, the transcriptional factor Foxo3a induces the expression of TRAIL on memory B cells. As a consequence of TRAIL expression, memory B cells from those HIV-1 infected individuals were more susceptible to apoptosis. The addition of IL-2, or the silencing of Foxo3a led to increased memory B cell survival [269].

While the phenotype and functions of B cells during HIV-1 infection have been extensively studied, little knowledge is available on the factors triggering their activation, exhaustion and higher apoptosis susceptibility. Since microbial translocation has been associated with T cells activation and depletion, further studies need to establish if similar mechanisms could prompt the B cell dysfunctions observed in the course of HIV-1 infection.
3 AIMS OF THE THESIS

The overall aim of this thesis was to investigate the mechanisms of the immune activation that occur during HIV-1 infection, and contribute to the exhaustion and depletion of lymphocytes. As the state of immune activation, in HIV-1 infected individuals relies on (1) the rate of HIV-1 replication, (2) the microbial translocation, and (3) the lymphopenia, the specific aims of this thesis were:

- To study the impact of HIV-1 replication on:
  - T cell senescence, assessing the functions and phenotype of CD28- T cells – Paper I –
  - on B cell activation – Paper II –

- To evaluate the possible influence of microbial translocation and the associated immune activation on:
  - memory B cell activation and depletion – Paper II –
  - IL-7 production by epithelial and stromal cell lines – Paper III –
  - the expression of genes from stromal cells, implicated in mucosal immunity – Paper III –

- To further investigate the potential role of IL-7, induced by lymphopenia, on B cell activation – Paper IV –

The methods used to verify the aims are described in details in the enclosed articles.
4 RESULTS AND DISCUSSION

4.1 IMPACT OF HIV-1 REPLICATION

HIV-1 replication, measured by the amount of virus in the circulation, i.e. viral load, is directly or indirectly correlated to various defects of the immune system in infected individuals. The hallmark of HIV-1 infection is the progressive loss of CD4+ T cells. For decades, the mechanisms causing CD4+ T cell depletion has been a debate among the scientific community. It is now accepted, as described in the introduction, that the loss of CD4+ T cells results from multiple factors and that immune activation is a better predictor of HIV-1 disease progression than the viral load [160]. However, the efficient suppression of HIV-1 viremia in ART-treated patients, by restoring the CD4+ T cell levels and improving immunity, demonstrates, if needed, that the virus itself is the causative agent of many alterations occurring in the course of HIV-1 infection. To better understand the factors contributing to altered lymphocyte functions during HIV-1 pathogenesis, we studied the association of HIV-1 replication on the activation and differentiation of T and B cells during HIV-1 infection.

4.1.1 T cell senescence and apoptosis – Paper I –

T cells from HIV-1 infected patients, display a premature senescence, defined by poor T cell proliferation associated with a phenotype of late differentiation [120, 209, 270]. Importantly, these cells include memory and effector T cells specific for pathogens, e.g. HIV-1, CMV [151, 209, 271, 272]. The expression of specific markers, such as high levels of CD57 and low levels of CD28 on T cells is associated with immune senescence; and such phenotype is also found in HIV-1 infected individuals [137-140, 148]. As the increased proportion of CD28- T cells in elderly individuals is associated with impaired immune responses [273], it is likely that similar mechanisms are taking place during HIV-1 infection. However, the resistance of CD28- T cells generated in vitro, or isolated from centenarians to undergo apoptosis [274], is in opposition to the high levels of cell death observed during HIV-1 infection. Additionally, while in vitro generated CD8+CD28- T cells were suggested to be suppressor cells [136], the same cells, when isolated from HIV-1 infected and non-infected individuals can induce DC activation [142]. These results underlined the need to further assess the functions of CD28- T cells during
HIV-1 infection in order to better understand the mechanisms leading to their increased levels.

Using multicolor flow cytometry, we thoroughly assessed the phenotype of CD28- T cells from viremic and non-viremic (under ART) HIV-1 infected patients. Consistent with previous reports, CD28- T cells were found in increased numbers in HIV-1 infected as compared to uninfected (n=20) individuals (p<0.05). Notably, ART did not induce a normalization of the CD28- T cell levels. While the vast majority of CD4+ T cells expressed CD28, approximately 60% of CD8+ T cells lacked CD28 expression during HIV-1 infection, as compared to 40% in uninfected individuals (p<0.05). In line with a more differentiated phenotype typical of effector cells, CD28- T cells displayed low levels of CD27 expression, with no difference between the groups of HIV-1 infected and uninfected individuals. The telomere lengths were also shorter in CD28- T cells as compared to CD28+ T cells. CD28- T cells from the 3 groups exhibited similar, high levels of CD57 and Fas expression whereas IL-7Rα expression was low, as compared to CD28+ T cells (p<0.001). There was no statistical difference in Annexin V staining, characteristic of apoptotic cells, whereas lower Bcl-2 expression was found in CD28- T cells, as compared to CD28+ T cells (p<0.001). CD28- T cells from viremic HIV-1 infected individuals displayed the lowest levels of Bcl-2, with more than 50% of cells showing low Bcl-2 expression (p<0.001 as compared to ART-treated HIV-1 infected and uninfected individuals). PD-1 expression was higher on CD28- T cells (p<0.001 as compared to CD28+ T cells), with the highest levels found on CD28- T cells from untreated and viremic HIV-1 infected individuals as compared to ART-treated HIV-1 infected patients (p<0.05). These results showed that CD28- T cells exhibit markers of senescence (e.g. CD57, PD-1) and susceptibility to apoptosis (e.g. Fas, low Bcl-2).

To further evaluate the propensity of CD28- T cells to undergo proliferation and apoptosis ex vivo, we stimulated T cells with an anti-CD3 Ab (1 or 10μg/ml) for 24 hours or 4 days (Figure 3). T cells from viremic, untreated patients displayed higher levels of spontaneous apoptosis as compared to both ART-treated patients and uninfected individuals, with the highest rate of apoptosis measured on CD28- T cells (Figure 3a). Upon activation, levels of apoptosis were also greater for CD28- T cells from untreated HIV-1 infected individuals as compared to CD28- T cells from both uninfected or ART-treated HIV-1 infected patients (p<0.001). Examining the proliferation capacity of T cells by means of carboxyfluorescein succinimidyl ester (CFSE) dilution, we observed that CD28- T cells displayed higher levels of proliferation when exposed to both a low and high dose of anti-CD3 Ab as compared to CD28+ T cells. CD28- T cells from ART-treated patients
were especially susceptible to TCR triggering, showing a high rate of proliferation from a low dose of anti-CD3 Ab stimulation (p<0.01 as compared to CD28- T cells from uninfected or untreated HIV-1 infected individuals). The proliferation of CD28- T cells from ART-treated patient was further enhanced at a high dose of anti-CD3 Ab (10µg/ml). For the CD28+ T cell counterpart, we observed that cells from ART-treated patients proliferated at higher rates than CD28+ T cells from viremic patients or uninfected individuals at a high dose of anti-CD3 Ab (10µg/ml). The proliferation of CD28+ T cells was minimal at a low dose of anti-CD3 Ab (1µg/ml), with no difference between the 3 groups (Figure 3b). These results show that CD28- T cells from viremic HIV-1 infected patients are highly susceptible to spontaneous and activation-induced apoptosis. Importantly, the same population of CD28- T cells isolated from ART-treated patients, is not more prone to apoptosis as compared to uninfected individuals, but display greater capacity to proliferate, especially at low dose of activation. Importantly, apoptosis and proliferation of CD4+ and CD8+ T cells were similar, based on their expression of CD28.

**Figure 3.** Increased apoptosis sensitivity of CD28- T cells from untreated HIV-1+ individuals vs. enhanced proliferative responses of CD28- T cells from ART-treated individuals. Purified T cells from control individuals (n=10) and treated (n=11) or untreated (n=14) HIV-1 infected patients were cultured in 96-well plates coated with anti-CD3 Abs at indicated concentrations. Apoptosis was measured by Annexin V binding after 24h and proliferation by CFSE dilution after 4 days of culture. Individual percentages of (a) apoptotic and (b) proliferating cells among CD28+ (upper panels) and CD28- T cells (lower panels) are presented. *p<0.05; **p<0.01; ***p<0.001.
Since the presence of HIV-1 replication was associated with a higher level of CD28- T cell apoptosis, we evaluated the correlation between viral load and apoptosis (Figure 4). We found a significant correlation between both the spontaneous and activation-induced apoptosis of CD28- T cells from untreated HIV-1 infected patients and the viral loads (p<0.05). No correlation was observed between the levels of viremia and the apoptosis rate of CD28+ T cells. Our results suggest a direct or indirect role of HIV-1 replication leading to CD28- T cell apoptosis.

**Figure 4. HIV-1 viremia correlates with spontaneous and activation-induced apoptosis of CD28- T cells.** Correlation of HIV-1 viral load with spontaneous or activation-induced T cell apoptosis measured on CD28+ (upper panels) and CD28- (lower panels) T cells from HIV-1 viremic patients (n=16). Calculated Spearman r and p values are indicated for each anti-CD3 treatment inside the panels.

When measuring IL-2 production from T cells cultured with anti-CD3 Abs using ELISA, we found that T cells from ART-treated patients produced the highest levels of IL-2 (p<0.01 at 1µg/ml anti-CD3 as compared to uninfected individuals; and p<0.001 at 10µg/ml anti-CD3 as compared to both uninfected and untreated HIV-1 infected individuals). Intracellular staining for IL-2 revealed that the CD28+ T cells are responsible for the IL-2 production. CD25 expression, the high affinity subunit of IL-2 receptor, has been shown to be up-regulated on CD28- T cells after TCR triggering with similar kinetics in the ART-treated or untreated patient group, both at a higher rate than in the control group. In contrast, CD25 expression was higher on CD28+ T cells from ART-treated patients as compared to both untreated HIV-infected and uninfected individuals. Therefore, CD28- T cells from ART-
treated HIV-1 infected patients may benefit from a higher availability of IL-2 rather than a differential expression of CD25.

The effectors functions of T cells were also evaluated, measuring the release of TNF, IFN-γ and perforin in cultured of total T cells or purified CD28- and CD28+ T cells stimulated by anti-CD3 Abs. Consistent with their phenotype of effector T cells, CD28- T cells from the 3 groups produced higher amounts of TNF, IFN-γ and perforin as compared to CD28+ T cells. Of note, upon activation, TNF and perforin production by total T cells from untreated HIV-1 infected patients was significantly higher than by T cells from uninfected individuals. The production of TNF and perforin may possibly participate to the observed higher apoptosis of these T cells (Figure 3).

<table>
<thead>
<tr>
<th></th>
<th>Uninfected Individuals</th>
<th>HIV-1 infected Individuals under ART</th>
<th>Untreated HIV-1 infected Individuals</th>
</tr>
</thead>
<tbody>
<tr>
<td>% CD28- T cells*</td>
<td>-</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Phenotype*</td>
<td>High degree of differentiation, short telomeres, Higher expression of PD-1, CD57, Fas, Low expression of CD27, IL-7Rα</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bcl-2*</td>
<td>+</td>
<td>+</td>
<td>Low</td>
</tr>
<tr>
<td>Apoptosis**</td>
<td>-</td>
<td>+/-</td>
<td>+++</td>
</tr>
<tr>
<td>Proliferation**</td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>IL-2 production**</td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>CD25**</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TNF, IFN-γ, Perforin secretion**</td>
<td>Relatively similar upon TCR stimulation</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*measured ex vivo or **after TCR stimulation with anti-CD3 Abs.

Our results, summarized in Table 5, show that CD28- T cells, a cell population found at higher levels in HIV-1 infected individuals, display rather similar features of late differentiation, senescence and predisposition to apoptosis in both infected and uninfected individuals. In contrast to the reported resistance of CD28- T cells to undergo apoptosis [133-135], CD28- T cells from untreated HIV-1 infected individuals, displayed higher susceptibility to undergo spontaneous and activation-induced apoptosis *ex vivo*. The higher levels of apoptosis were in line...
with a lower Bcl-2 expression observed in those cells. Importantly, susceptibility of CD28- T cells to undergo apoptosis was correlated with the levels of HIV-1 viremia. Therefore, HIV-1 replication and the associated immune activation may trigger T cell activation, leading to T cell differentiation toward an effector phenotype with CD28 down-regulation. In the context of an ongoing viral replication, CD28- T cells would thus display higher susceptibility to AICD, consistent with our observations. These results are consistent with the higher susceptibility of T cells to undergo apoptosis during HIV-1 infection and confirm the role of AICD in the T cell depletion occurring in infected individuals [158, 159, 165].

Despite ART and the suppression of HIV-1 replication, the CD28- T cell population is still increased in treated HIV-1 infected individuals. The normalized levels of spontaneous and activation-induced apoptosis of CD28- T cells from ART-treated patients suggest a decreased of immune activation under ART as previously shown [35, 36]. However, CD28- T cells isolated from ART-treated HIV-1 infected individuals were more prone to proliferation as compared to cells from untreated HIV-1 infected and uninfected individuals. This higher proliferative capacity was associated with lower PD-1 expression, a marker for T cell exhaustion. These results show that levels of CD28- T cells may be sustained by their ability to proliferate upon low TCR triggering, and therefore be induced by, and also participate to the continuous immune activation associated with mortality in HIV-1 infected patients, even in the ART era [99, 120, 275].

4.1.2 B cell Activation – Paper II –

B cells are important actors for the immune system, providing Abs to combat infections. In the context of HIV-1 infection, various B cell alteration occur, responsible for the loss of serological memory and the inability of B cells to respond appropriately to pathogens and vaccines [241, 276]. As Th cells are providing signals for B cells activation and differentiation toward memory B cells and PCs, the CD4+ T cell depletion, hallmark of HIV-1 infection, can impact on B cell responses. However, as T cells, the B cell compartment presents activation and exhaustion features. We were thus interested to study the relations between B cell activation and HIV-1 replication and the associated immune activation.

The phenotypical analysis of differentiation markers on B cells confirmed previously published data [249](Figure 5). As compared to uninfected individuals, HIV-1 infected patients naïve to treatment (i.e. untreated) exhibited a slight decrease of total B cell and naïve B cell levels, whereas the transitional B cells
(CD10+) were expanded. Concerning the memory B cells, classical memory (CD27+) B cells were decreased, as were resting memory (RM: CD27+CD21+) B cells (p<0.01). On the contrary, consistent with the immune activation, activated memory (AM: CD27+CD21-) and TLM (CD27-CD21-) B cells were found at higher rate in untreated HIV-1 infected individuals as compared to controls (p<0.001). These alterations are reversed upon ART, as previously observed [259]. In viremic HIV-1 infected individuals, memory B cells display a phenotype of activation and exhaustion. To examine better this state of activation, we measured the expression of CD38 and IL-21 receptor (IL-21R). While constitutively expressed on naïve B cells, IL-21R on memory B cells is mostly expressed upon activation and plays a role in the generation of long-lived Ab responses in human [277, 278].

**Figure 5. B cell phenotype during HIV-1 infection.** The phenotype of B cells was assessed by multicolor flow cytometry on PBMCs from healthy controls (n=20) and HIV-1 infected patients under treatment (n=20) or naïve to treatment (n=20). (a) Representative plots from a control (left panels), an HIV+ patient under treatment (middle panel) and a patient naïve to treatment (right panel) displaying live Vivid-CD19+ B cells (upper panels), memory and naïve B cell subpopulations gated on CD19+CD10- B cells (lower panels). (b) Individual percentages with means and standard deviations of total (CD19+) B cells, naïve (CD19+CD10-CD27-CD21+) B cells, classical memory (CD19+CD10-CD27+) B cells, resting memory (RM:CD19+CD10-CD27+CD21+) B cells, activated memory (AM:CD19+CD10-CD27+CD21-) B cells and tissue-like memory (TLM:CD19+CD10-CD27-CD21-) B cells. Statistical analyses were performed using Kruskal-Wallis tests followed by Dunns comparison test: *p<0.05, **p<0.01, ***p<0.001.
Figure 6. Association of CD38 expression on B cells with CD4+ T cell counts and viral load in HIV-1 infected patients. Percentages of CD38 expression on total, naïve, classical (CD27+) memory, resting memory (RM), activated memory (AM) and tissue-like memory (TLM) B cells were correlated with (a-b) CD4 counts and (c) HIV-1 viremia measured in total HIV-1 infected patients (n=40, a) and patients naïve to treatment (n=20, b-c). Data were analyzed using Spearman correlation test.
Higher levels of CD38 expression were found on B cells from HIV-1 infected patients naïve to treatment as compared to ART-treated and uninfected individuals, especially on classical memory (CD27+) B cells (p<0.05). B cells from treated patients and uninfected individuals expressed low levels of CD38.

In uninfected individuals, classical memory and RM B cells expressed low levels IL-21R (<20%). IL-21R levels were higher on activated memory (30%) and on TLM (60%) B cells, consistent with their activation state. Viremic HIV-1 infected individuals showed higher levels of IL-21R expression on both classical memory and TLM B cells, as compared to control. Higher expression of IL-21R was also observed on memory B cells from HIV-1 infected individuals under ART, but not statistically significant as compared to uninfected controls.

HIV-1 infection is associated with the activation of memory B cells, characterized by enlarged ratio of activated and TLM B cells, which also expressed CD38 and IL-21R at higher rate. To determine the role of viremia and T cell depletion, in B cell activation, we analyzed the correlation of CD38 and IL-21R expression with the viral load and CD4+ T cell counts. No correlation was found between HIV-1 replication or CD4+ T cell counts and IL-21R expression on B cells, suggesting an independent mechanism for IL-21R regulation. Importantly, CD38 expression on all B cell subsets, but activated memory, negatively correlated with CD4+ T cell counts in HIV-1 infected patients, naïve and treated (Figure 6). Similarly, analyzing cells from untreated HIV-1 infected patients separately, CD38 expression negatively correlated with CD4+ T cell counts for all the B cell subsets, and positively correlated with HIV-1 viral load for all subsets but resting memory and TLM B cells.

These results point out a role for HIV-1 replication and the associated immune activation in general B cell activation characterized by CD38 expression. However, this mechanism does not seem to be specific for a particular B cell subset and thus is unlikely to participate in the preferential loss of resting memory B cells observed in HIV-1 infected individuals. This hypothesis is further supported by the lack of association between CD38 expression and the levels of circulating resting memory B cells. As microbial translocation has been shown to be a better predictor for HIV-1 disease progression [279] and since B cells can also be activated by microbial products [280], we investigated the possible link between B cell activation, memory B cell loss and microbial translocation in our cohort of HIV-1 infected individuals.
4.2 ROLE OF MICROBIAL TRANSLOCATION

The chronic immune activation occurring in HIV-1 infection has been associated with elevated levels of microbial products in the circulations, e.g. LPS, bacterial 16S rDNA [58]. The passage of microbial products from the gut lumen to the circulation is attributed to the high HIV-1 replication and the associated immune response, which lead to mucosal damages and CD4+ T cell depletion during the acute phase of the infection [85, 279]. Levels of bacterial products can also be assessed through the measurement of molecules released by the host cells in response to TLR stimulation. For instance, CD14, a molecule expressed on monocytes, is shed as a result of stimulation by LPS. Indeed, sCD14 levels are associated with LPS levels in the circulation of HIV-1 infected individuals and were found to be associated with disease progression and mortality [97, 100]. The relation between microbial translocation and T cell activation has been established but it is unknown how this phenomenon impacts on the B cell compartment. Additionally, other markers of inflammation are found at the mucosal site, in relation to epithelial damages. We investigated the possible role of inflammatory cytokines in modulating important molecules for the immune response and T cell homeostasis, such as IL-7.

4.2.1 Loss of memory B cells – Paper II –

One of the hallmarks of HIV-1 infection is the depletion of memory B cells and the decline of serological memory. As the causes of the loss of memory B cell remain elusive, we investigated a possible role for microbial translocation and the associated immune activation occurring in HIV-1 infected individuals in memory B cell activation and differentiation.

Figure 7. Plasma sCD14 are increased during HIV-1 infection. Levels of sCD14 were assessed by ELISA on plasma samples from healthy controls (n=19) and HIV-1 infected patients under treatment (n=20) or naïve to treatment (n=19). Data represent individual levels in ng/ml of plasma with means and standard deviations of sCD14. *p<0.05, **p<0.01, ***p<0.001, Kruskal-Wallis tests followed by Dunns comparison test.
Soluble CD14 is a good surrogate for microbial translocation, as it has been shown to correlate with LPS levels found in the plasma [58]. Consistent with previous data, our cohort of HIV-1 infected individuals exhibited elevated sCD14 levels in serum as compared to uninfected individuals (Figure 7). Importantly, sCD14 levels were also higher in ART-treated patients (p<0.05 as compared to controls). To evaluate the impact of immune activation, as measured by sCD14, on B cells, we correlated the levels of sCD14 with the different levels of B cell memory subsets and their expression of IL-21R and CD38 (Figure 8). Notably, sCD14 levels correlated with the increased amount of circulating AM B cells (p=0.01) and also with the decreased levels of RM B cells (p=0.02). Furthermore, IL-21R and CD38 expression on classical memory B cells were both correlated with sCD14 levels. CD38 expression on AM B cells, but not RM B cells, was also correlated with sCD14 levels (p=0.03). On the contrary, IL-21R expression on RM B cells, but not AM B cells, correlated with sCD14 levels in our cohort (p=0.01).

![Graphs](image)

**Figure 8. Plasma sCD14 correlates with both activation and loss of memory B cells.**

(a) The percentages of circulating classical memory (CD27+) B cells (left panels), activated memory (AM, middle panels) and resting memory (RM, right panels) B cells and their respective levels of (b) IL-21R and (c) CD38 expression were correlated with the levels of sCD14 found in plasma. Data were analyzed using Spearman correlation test.
These results suggest that the immune activation associated with the microbial translocation might contribute to higher ratio of AM B cells and to the loss of RM B cells. We further explored the association between IL-21R and CD38 expression with the loss of RM B cells during HIV-1 infection. Importantly, we found that IL-21R expression, on classical memory and RM B cells from treated (p<0.05) and untreated (p<0.01) HIV-1 infected individuals negatively correlated with the levels of these cells in the circulation (Figure 9). On the contrary, CD38 expression on memory B cells was not correlated with their decreased levels in any of the cohorts.

To further assess the relation between IL-21R expression and the loss of RM memory B cells, we measured molecules involved in apoptosis on those cells. Annexin V staining showed that all memory B cells subsets carrying IL-21R have higher levels of apoptosis than memory B cells lacking the receptor. Naïve B cells, on the other hand, did not display a differential apoptosis susceptibility in relation to IL-21R expression. Moreover, IL-21R positive memory B cells expressed lower levels of Bcl-2 as compared to IL-21 negative cells. These results suggest that IL-21R positive memory B cells are more susceptible to apoptosis and are in line with the association between IL-21R expression and the decreased levels of memory B cells found in HIV-1 infected individuals.

Figure 9. High expression level of IL-21R on B cells is associated with declined percentages of memory B cells during HIV-1 infection. Correlation of IL-21R expression on CD27+ (upper panels) and RM (lower panels) B cells with their percentages in circulation in controls (n=20), HIV-1 infected patients treated (n=20) and naïve from treatment (n=20). Data were analyzed using Spearman correlation test.
The direct impact of microbial product on B cell phenotype was also evaluated by measuring IL-21R and CD38 expression on B cells cultured in the presence of various TLR ligands. We found that TLR2 and TLR9, and to a lesser extent TLR4 triggering induced the up-regulation of IL-21R on both naïve and memory B cell populations, when B cells where cultured alone or in the presence of PBMCs. Importantly, CD38 expression was not increased upon TLR stimulation of purified B cells. However, higher CD38 levels were observed on B cells in the PBMC cultures, in the presence of TLR3, TLR4, TLR7/8 and TLR9 ligands. Our results reveal a direct effect of microbial products in IL-21R up-regulation on B cells, whereas the increased CD38 expression is likely the consequence of monocyte activation.

We demonstrate for the first time, a possible association between microbial translocation in regulating B cell activation and survival, leading to the loss of memory B cells during HIV-1 infection. Our data suggest that microbial translocation occurring in HIV-1 infected patients may lead to IL-21R up-regulation on memory B cells. Furthermore, IL-21 levels have been shown to be decreased, in association with the CD4+ T cell depletion in HIV-1 infected individuals [200]. The low levels of IL-21 could impaired B cell responses, as this γ-chain cytokine provides important signals for B cell activation and differentiation [277, 281]. We hypothesize that during HIV-1 infection, the presence of microbial products induces the activation of memory B cells, which, as a result of low IL-21 levels, might lead to their depletion through similar mechanisms as AICD.

4.2.2 IL-7 Regulation – Paper III –

The possible impact of microbial translocation and the associated immune activation on IL-7 production remains elusive in the context of HIV-1 infection. IL-7, an important cytokine for T cell homeostasis [194], is secreted by multiple cell types, and the regulation of IL-7 production in HIV-1 infected individuals remains speculative. The concomitance of increased IL-7 levels and CD4+ T cell depletion in HIV-1 infected individuals led to the hypothesis that the accumulation of IL-7 may originate from either a lack of consumption, or by the sensing of T cell loss by stromal cells [38]. The accumulation may occur as a result of the depletion of IL-7Rα expressing cells and the down-regulation of this receptor, as observed during HIV-1 infection [39, 141].

Additionally, elevated IL-7 levels are found in HIV-1 infected individuals during the primary infection, when immune activation is the highest [237]. Indeed, the
acute phase of HIV-1 infection is characterized by high levels of pro-inflammatory molecules, both in the GALT and in the peripheral blood of infected individuals [51, 52]. Among those, IFN-γ, TNF and IL-1β were reported to enhance IL-7 production. Human epithelial cells in the gut, as well as bone marrow stromal cells constitutively produce IL-7 [282, 283]. Using human colon carcinoma-derived cell lines, IL-7 production has been shown to be up-regulated by IFN-γ [222].

To better understand the regulation of IL-7 production in inflammatory conditions, as experienced in HIV-1 infected individuals, we cultured intestinal epithelial (DLD-1) and bone marrow stromal (HS-27) cell lines with IL-1β, IFN-γ, TNF and IL-2 (Figure 10).

Consistent with previous report [222], IFN-γ treatment (50ng/ml) resulted in a 3,5- and a 3-fold increase in IL-7 messenger RNA (mRNA) expression in DLD-1 and HS27 cells respectively (p≤0.001) after a 6-hour culture (Figure 10a-b). Contrarily, IL-1β (10ng/ml), consistently reduced IL-7 messenger expression by approximately 2 folds (p≤0.001). This effect was evident from a concentration of 1ng/ml. TNF (20ng/ml) and IL-2 (10ng/ml) did not show any effect on IL-7 mRNA levels, in line with the lack of TNFR and IL-2 receptor measured on DLD-1 and

![Figure 10. Cytokine regulation of IL-7 production by epithelialiaDLD-1 and HS27 cells. (a) and (b) IL-7 mRNA relative expression measured by real-time PCR in DLD-1 and HS27 cells with and without treatment with different cytokines for 6 h. (c) and (d) IL-7 protein levels measured by quantitative ELISA in culture supernatants of DLD-1 and HS-27 cells with different cytokines and in control cultures at 24 h. Data represent mean values and standard deviations of four different experiments.](image)
HS27 cells. Interestingly, IL-1β treatment of the cells abrogated the effect of IFN-γ, partially on DLD-1 (p=0.006), but entirely on HS27 cells (p<0.001). To confirm these observations, IL-7 levels were measured in the culture supernatants after 24 hours (Figure 10c-d). The levels of secreted IL-7 were consistent with the mRNA levels observed. The spontaneous IL-7 production by the DLD-1 and HS27 cells was increased by IFN-γ, whereas IL-1β treated cells displayed lower secretion levels. The combination of IL-1β and IFN-γ, induced an IL-7 production lower than cells cultured with IFN-γ alone. The presence of TNF or IL-2 in the cell cultures had no effect on IL-7 production.

These results demonstrate a role of inflammation in IL-7 production by stromal and epithelial cells. Further studies on human tissues from HIV-1 infected individuals would elucidate the relevance of this mechanism in the mucosal damage. Indeed, IL-7 participates in mucosal immunity as shown by the enhanced IFN-γ production of activated lamina propria T cells upon IL-7 treatment [284]. IL-7 also promotes the secretion of pro-inflammatory cytokines, such as IL-1β, IL-6 and TNF from monocytes [285], suggesting a role for IL-7 in fuelling the immune activation. Additionally, it has been reported that IL-7 could inhibit the anti-CD3-mediated proliferation of intestinal T cells, whereas it enhances the proliferation of peripheral blood T cells [282]. Since IL-7 has also been reported to induce Fas expression and subsequently, Fas-mediated apoptosis on T cells [238], high IL-7 in the GALT during the acute phase of HIV-1 infection might be detrimental and participate to the dramatic T cell depletion occurring in the gut.

### 4.2.3 Immune regulation by stromal cells – Paper III –

To determine if the secretion of other molecules involved in the immune regulation by epithelial and stromal cells are also regulated by IL-1β and IFN-γ, the gene expression profile of the HS-27 cells exposed to these cytokines was measured, using the whole-genome microarray Human Gene 1.0 ST available in the Affimetrix platform. The average gene expression values were derived from 12 samples analyzed, including control cells (n=3), cells cultured with IL-1β (n=3), IFN-γ (n=3) or the combination of the 2 cytokines (n=3).

An important component of immunity is the recruitment of the cells to the tissues where the infection is taking place. This process is under the control of molecules, called chemokines, which are classified according to a particular motif, CC or CXC. Cells expressing the appropriate chemokine receptor will migrate toward an increasing gradient of chemokines. Interestingly, out of 18 gene profiles of
chemokine included in the gene array, 14 were found to be dysregulated in HS27 cells upon treatment with IL-1β, IFN-γ or the combination of the 2 cytokines. The gene expression of the chemokine (C-C motif) ligand (CCL)-8, CCL20, chemokine (C-X-C motif) ligand (CXCL)-9, CXCL10 and CXCL11 were increased more than 500 folds (Figure 11). CCL5 gene expression was also increased by IL-1β and further by the combination of IL-1β and IFN-γ. These results were confirmed by ELISA, measuring CCL5 in the culture supernatants of HS-27 cells. CCL5, also known as RANTES, is an important chemokine involved in T cell recruitment. CCL5 has also been shown to inhibit the entry of HIV-1 into the target cell by binding to the HIV-1 co-receptor CCR5 [286]. High CCL5 has recently been shown to enhance HIV-1 entry at the mucosa by recruiting T cells to this site [287]. Similarly to CCL5, CCL20 gene expression and protein levels in culture supernatants were found to be elevated in the presence of IL-1β, and with the combination of IL-1β and IFN-γ. CCL20-production by epithelial cells upon inflammatory signals attracts T cells and immature DCs [288]. The elevated CCL20 levels observed in HIV-1 infected individuals [289] may be a direct consequence of inflammation in the gut. Moreover, the gene expression and protein secretion of CXCL11, a chemo-attractant for activated T cells, was elevated in the presence of IFN-γ. The combination of IL-1β and IFN-γ promoted further CXCL11 production.

Figure 11. Microarray gene expression profile of chemokines in HS-27 cells. Change in gene expression profiles of chemokines upon 6-hour treatment of HS-27 cells with IL-1β, IFN-γ or the combination of the 2 cytokines, as compared to control cells without stimulation.
The gene expression of some TLRs, such as TLR2, TLR3, TLR4 and TLR6 was also altered in HS27 cells treated with IFN-γ alone, or in combination with IL-1β. Whereas the gene expression of TLR2, TLR3 and TLR4 was increased, the TLR6 gene was down-regulated by similar treatment (p<0,01). The expression of TLRs is important for epithelial functions and the regulation of mucosal immunity [290]. The impact of inflammatory cytokines on TLR expression of epithelial cells and its consequences for HIV-1 pathogenesis remain to be further understood; and can prove to be relevant, especially in the context of microbial translocation observed during HIV-1 infection.

Additionally, the genes of proteins participating to immunity and Th17 cell formation were found to be elevated by the cytokine treatment of HS-27 cells (Table 6). PD-1 ligand (PDL1/CD274) and IL-15 gene expression were found to be increased by IL-1β, and further enhanced by IFN-γ and the combination of the 2 cytokines. IL-6, IL-23 and IL-24 gene expression were enhanced by IL-1β. IL-16 gene expression was also increased by IFN-γ. PDL1 expression is an important factor leading to T cell exhaustion as its blockade was demonstrated to improve T cell responses [145]. Also, recent studies suggest a potential association between PDL1 expression and the balance Treg/Th17 [291], thought to be important in HIV-1 pathogenesis. Furthermore, IL-23, in conjunction with IL-6 and TGF-β1 drive the differentiation of naive CD4+ T cells to Th17 cells [292], which could be important in the control of HIV-1 infection as discussed in the previous sections. There is currently no data on IL-24 in the context of HIV-1 infection, but serum levels of IL-24 have been shown to be decreased in patients infected with TB [293]. In animal models, IL-24 injection has also been shown to promote CD8+ T cell responses in the context of *Mycobacterium tuberculosis* infection [293]. It would be interesting to validate these results by examining the levels of cytokines in tissues from HIV-1 infected individuals and study their impact on the Treg/Th17 cell balance.

<table>
<thead>
<tr>
<th></th>
<th>IL-1β</th>
<th>IFN-γ</th>
<th>IL-1β + IFN-γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD-L1</td>
<td>6,84***</td>
<td>12,56***</td>
<td>38,57***</td>
</tr>
<tr>
<td>IL-6</td>
<td>23,43***</td>
<td>2,98***</td>
<td>26,29***</td>
</tr>
<tr>
<td>IL-15</td>
<td>1,58*</td>
<td>4,13***</td>
<td>3,77***</td>
</tr>
<tr>
<td>IL-23</td>
<td>19,28***</td>
<td>-1,18</td>
<td>5,68***</td>
</tr>
<tr>
<td>IL-24</td>
<td>18,34***</td>
<td>-1,06</td>
<td>9,31***</td>
</tr>
</tbody>
</table>

*Table 6. Change in gene expression of factors relevant for mucosal immunity and Th17 differentiation. HS27 cells were treated with IL-1β and/or IFN-γ for 6 hours. Fold changes in gene expression, as compared to control cells without stimulation. *p<0,05; **p<0,01; ***p<0,001.*
4.2.4 Lymphopenia and B cell Activation – Paper IV –

Lymphopenia is a condition when the lymphocyte count is low. Humans experience lymphopenic conditions either as a result of congenital or acquired immunodeficiency, or following cytoreductive therapies. Levels of IL-7, a key cytokine supporting T cell homeostasis, are increased during lymphopenia. As discussed previously, high IL-7 levels in HIV-1 infected individuals are associated with CD4+ T cell depletion [237]. IL-7 therapy in HIV-1 infected individuals leads to an increased level of circulating CD4+ T cells, demonstrating a beneficial effect of the cytokine [197]. Also, IL-7 has an impact on B cells development, as shown by the association of IL-7 levels with the increased levels of immature transitional B cells (CD10+) during HIV-1 infection [234], other lymphopenic condition [235], as well as in patients undergoing IL-7 therapy [236]. Despite the fact that mature B cells do not express IL-7Rα, an indirect role for IL-7 in affecting B cell biology was established by the finding that Fas expression on B cells is modulated by the release of IFN-γ from IL-7-treated T cells [240]. The increased Fas expression further sensitized B cells to undergo Fas-mediated apoptosis. In Paper IV, we further describe the indirect role of IL-7 in regulating B cell survival and activation.

B cells cultured with IL-7 pre-treated T cells (25ng/ml, 5 days), showed an enhanced production of IgA, IgG and IgM, as measured by ELISA. The effect was detectable after 5 days of T-B co-culture, but was more pronounced after 10 days (p<0,005). IL-7 alone had no effect on immunoglobulin production, but IL-7-pre-treated T cells induced the secretion of Ig to similar levels as by CD40L stimulation (Figure 12a).

Importantly, T cells also enhanced the viability of B cells, measured by flow cytometry (Annexin V-Vivid-). At day 5, B cells cultured alone or in the presence of IL-7 displayed low viability (<20%), while the addition of T cells led to an increased survival of B cells (>35%, p<0,005). IL-7 pre-treatment of T cells further enhanced B cell viability to 60% (p<0,005 as compared to T cells alone). This additional effect of IL-7 was lost at day 10; still, B cells co-cultured in the presence of T cells showed a better viability than B cells alone (Figure 12b).
Figure 12. Immunoglobulins production and survival of peripheral blood B cells in the presence of IL-7 treated T cells. B cells were cultured for 5 or 10 days, alone or with IL-7 (25ng/ml), or co-cultured with non-treated T cells or with IL-7 treated (25ng/ml, 5 days) T cells and IL-7. (a) Immunoglobulin concentrations were measured in culture supernatant by ELISA. Representative results of 9 (IgG) or 6 (IgM and IgA) experiments are shown (b). B cell survival was analyzed using Vivid and Annexin V staining. Data represent mean values and standard deviations.

The better survival of B cells, in co-culture with IL-7 pre-treated T cells, was accompanied by B cell proliferation. Indeed, CFSE-low staining, characteristic of proliferative cells, was found on B cells co-cultured with IL-7 pre-treated T cells. Furthermore, CD38 expression, a marker of activation was induced on B cells. The proliferating B cells expressed higher CD38 expression, and also lower CD20 levels, typical of plasmablasts. These results demonstrate that IL-7 treatment induces changes in T cells, which in turn, enhance B cell activation and differentiation toward a plasmablast phenotype. When B cells were separated according to their CD27 expression, only CD27+ B cells co-cultured with T cells secreted IgG and IgM in the presence of IL-7 pre-treated T cells.

There are different molecules involved in B cell activation during an immune response, among which the members of the TNF and TNFR family have been shown to be crucial. The activation of the CD40/CD40L pathway induces B cell activation and differentiation [294]. CD27, a member of the TNFR family, is a marker for memory B cells and is an important receptor for T-dependent B cell activation [295]. CD27 triggering by its ligand, CD70, participates in B cell differentiation toward PC and Ig secretion. Additionally, the B cell activating factor
(BAFF) promotes B cell survival [296]. Importantly, in our study CD40L expression on T cells remained negative upon IL-7 treatment, and blocking the CD40/CD40L pathway did not abrogate the increased IgG secretion by B cells co-cultured with IL-7 treated T cells. However, CD70 expression, the ligand for CD27, was up-regulated on T cells in the presence of IL-7 (Figure 13a). IL-7 treatment also induced BAFF production by T cells, measured by ELISA (Figure 13b).

The relevance of these pathways on B cell activation was evaluated measuring IgM and IgG secretion in the B-T cell co-cultures in the presence of an anti-CD70 blocking Ab (10µg/ml) and a soluble BAFF receptor (BAFFR; 10µg/ml) (Figure 13c). The blocking of CD70 signaling induced a decrease in Ig secretion by B cells cultured with IL-7 pre-treated T cells. On the other hand, the anti-CD70 blocking Abs did not alter B cell survival, whereas the neutralization of BAFF led to decreased B cell survival. Indeed, IL-7 pre-treated T cells led to a 2.8-fold increased B cell viability in the absence of soluble BAFFR as compared to B cells co-cultured with IL-7 pre-treated T cells in the presence of BAFFR. The presence of soluble BAFFR in the B-T cell co-cultures did not affect Ig production. Also, the effect of anti-CD70 blocking Ab was not enhanced by the addition of soluble BAFFR. Taken together, these results indicate that IL-7 regulates B cell activation and survival through distinct mechanisms, by inducing both CD70 up-regulation and BAFF production in T cells.

These results confirm a role for CD70 expressed by T cells in promoting the hypergammaglobulinemia observed in HIV-1 infected individuals, as postulated by previous studies [244, 258]. BAFF levels have also been shown to be elevated during HIV-1 infection, in association with high levels of circulating Abs [297-299]. Therefore, a role for IL-7 elevated levels in the context of HIV-1 infection may be to enhance T cell expression of CD70 and the production of BAFF, contributing to the B cell activation and exhaustion observed in infected individuals. This newly observed role of IL-7 in promoting B cell activation needs further assessment, as it may potentially lead to new strategies for restoring B cell functions in HIV-1 infected patients.
Figure 13. Contribution of CD70 and BAFF from IL-7 treated T cells in B cell activation.

(a) Representative CD70 staining is shown on freshly isolated T cells or on T cells cultured with or without IL-7 for 5 or 8 days. (b) BAFF concentration measured by ELISA in the supernatant of T cells in the presence (white dots) or absence (gray dots) of IL-7. New medium and cytokine was added to the cells at day 2, 5 and 8. Results of five independent experiments are shown; mean and SEM values are indicated. (c) B cells were cultured alone, with IL-7, with untreated T cells or with IL-7 pre-treated T cells and IL-7 for 5 days and the levels of IgM and IgG antibodies were measured in the supernatants. The contribution of CD70 and BAFF in the activation of B cells was studied using CD70 neutralizing antibodies and soluble BAFF-R respectively in the presence of IL-7 treated T cells. Representative results of three independent experiments are shown.
5 CONCLUSIONS AND PERSPECTIVES

The destruction of the immune system is gradual and starts on the first days of HIV-1 infection. Damages at the gut mucosa occurring in acute HIV-1 infection induce profound perturbations of immunological functions of this tissue [51, 84, 85]. The increased permeability of the mucosa allows microbial translocation from the gut to the circulation, which, together with HIV-1 replication, contributes to the activation of the immune system [279]. The role of immune activation in HIV-1 pathogenesis is further confirmed by the study of HIV-2 infected individuals [34] and non-pathogenic animal models [279].

We described in Paper I, that the T cells from HIV-1 infected individuals are enriched with T cells lacking CD28 expression. We demonstrated that CD28- T cells are highly susceptible to spontaneous and activation-induced apoptosis in untreated individuals. Importantly, levels of apoptosis of CD28- T cells, but not CD28+ T cells, positively correlate with the levels of HIV-1 viral load. Upon ART, CD28- T cell levels are not normalized, possibly as a consequence of their increased sensitivity to proliferate and their lower susceptibility to apoptosis. The increased levels of CD28- T cells could arise from HIV-1 replication, the high levels of inflammation and antigens. The propensity of CD28- T cells to undergo AICD may illustrate the activated state of these cells in vivo in relation to HIV-1 replication and the associated immune activation. Since CD28- T cells, despite displaying markers of senescent and late differentiation, are capable of secreting TNF, IFN-γ and perforin, their increased proportion in HIV-1 infection, might also participate to the chronic immune activation.

HIV-1 replication is also associated, as discussed in Paper II, with the increased activation of the B cell compartment. We found that CD38 expression is positively correlated with HIV-1 viral load and negatively correlated with CD4+ T cell counts during HIV-1 infection. However, CD38 is not associated with the observed loss of memory B cells occurring in infected individuals; rather, IL-21R expression on resting memory B cells correlates with their decreased levels in the circulation of both ART-treated and untreated HIV-1 infected individuals. IL-21R positive B cells are more prone to apoptosis and express lower levels of Bcl-2. Importantly, we found that IL-21R expression is induced directly by TLR triggering on B cells; and that levels of sCD14, elevated during HIV-1 infection, correlated with both IL-21R expression on resting memory B cells and the decreased levels of those cells in
HIV-1 infected individuals. We thus define a novel role for microbial translocation and the associated immune activation, possibly contributing to the loss of memory B cells during HIV-1 infection. Pro-inflammatory cytokines, present at elevated levels in viremic HIV-1 infected individuals, are also thought to induce B cell activation [241]. However, experimental data are lacking in the context of HIV-1 infection and further studies are needed to identify the factors implicated in B cell activation.

Epithelial and stromal cells are an important source of IL-7, a key cytokine for T cell activation and homeostasis. In the context of HIV-1 infection, it is believed that IL-7 is increased due to CD4+ T cell depletion, as in other lymphopenic conditions, in order to increase T cell numbers. However, high levels of IL-7 are generally found together with low CD4+ T cell counts [237], suggesting either the inability of T cell to respond to IL-7 or other possible detrimental effect of IL-7. IL-7 levels are also shown to be increased during acute HIV-1 infection, when the inflammation in the gut and other secondary lymphoid tissues is at its highest level [219]. To further assess the regulation of IL-7 by inflammatory molecules, in Paper III we used relevant cell lines stimulated by IL-1β and/or IFN-γ. We found that IFN-γ induces a substantial increase of IL-7 production by epithelial cells (DLD-1) and stromal cells (HS-27). This effect decreases by incubating the cells with the combination of IL-1β and IFN-γ, whereas IL-1β alone inhibits IL-7 production by epithelial and stromal cells. A gene expression profile of HS-27 cells was carried out and revealed profound alterations induced by IL-1β and/or IFN-γ treatment. Indeed, chemokine, cytokine and TLR gene expressions were found dysregulated. In addition, stromal cells are important for survival of PCs in the bone marrow, and inflammatory cytokines also disturb the gene expression of factors implicated in plasma cell survival [267]. These results highlight the important role of epithelial and stromal cells in shaping the capacity of the immune system to respond to pathogens and call for more studies, especially in the context of HIV-1 infection where mucosal immunity is profoundly impaired [57, 119].

The effect of IL-7 has been preferentially examined on T cells; however in Paper IV, we studied the potential impact of elevated levels of IL-7 on B cell activation and survival. Despite the lack of expression of IL-7Rα, IL-7 was proposed to act on B cells through the release of IFN-γ by T cells [240]. IL-7 pre-treated T cells induced Fas expression on B cells, rendering them sensitive to Fas-mediated apoptosis. We also found that IL-7 pre-treated T cells stimulate B cell activation and proliferation. In the presence of IL-7 pre-treated T cells, B cells produce large amount of IgA, IgM and IgG, and display a plasmablast phenotype.
(CD20lowCD38high). This effect was mediated by the up-regulation of CD70 on IL-7 treated T cells, as confirmed by CD70 blocking experiments. These results confirm previous publications linking the hypergammaglobulinemia found during HIV-1 infection to the CD70 expression on T cells [244, 258]. Additionally, IL-7 pre-treated T cells enhance B cell survival through the production of BAFF. Blocking BAFF signaling did not affect Ig secretion by B cells but increased B cell apoptosis. Notably, BAFF levels are also elevated in HIV-1 infected patients [297-299] and may thus participate to the increased survival of activated B cell leading to the high levels of circulating IgG present in patients. Our results revealed a new role for IL-7 in regulating B cell functions, which needs further evaluation in the context of HIV-1 infection.

The pathogenesis of HIV-1 infection is complex and it is often hard to distinguish when the alterations of B and T cell functions are the result or the cause for the observed systemic activation of the immune system. With the introduction of ART, the life of HIV-1 infected individuals has greatly changed, and in most cases associated with the restoration of immune functions. However, immune activation persists in ART-treated individuals and is still associated with increased mortality in treated HIV-1 infected individuals. Therapeutic strategies including immunosuppressive drugs have been proposed in the context of HIV-1 infection [300], but their efficacy remains to be proven. Impaired immune reconstitution of HIV-1 infected individuals has been also suggested to originate from the loss of IL-7Rα expressing CD4+ T cells [39]. Recent data also showed that rather than IL-7 levels, the lack of T cell responsiveness to the cytokine, seems to be responsible for impaired CD4+ T cell reconstitution [301]. Indeed, some ART-treated individuals, despite successful viral suppression, do not recover their CD4+ T cells [302]. The lymphoid tissue fibrosis occurring in the course of HIV-1 infection is associated with poor T cell recovery after ART [303]. These studies suggest the necessity for ART initiation early after the onset of HIV-1 infection, in order to preserve the tissues from destruction. Indeed, it has been shown that HIV-1 infected individuals starting ART early display preserved immune responses and longer survival [261, 262, 304-306]. In addition, the role of microbial translocation and depletion of Th17 cells from the gut during HIV-1 pathogenesis has also led to new therapeutic strategies aiming at restoring normal gastrointestinal flora or decreasing signaling through TLRs [119]. A better understanding of immune alterations occurring at the mucosa during HIV-1 infection may lead to new therapeutic interventions and help uncovering the mechanisms necessary for the design of an effective vaccine against HIV-1.
6 ACKNOWLEDGEMENTS

The work presented in this thesis was performed at the Department of Microbiology, Tumor and Cell Biology (MTC) at Karolinska Institutet. My PhD scholarship was granted by the FP6 EU Europrise Network of Excellence (EC grant LSHP-CT-2006-037611) and additional support was provided by the Swedish Medical Research Council (Vetenskapsrådet), the Swedish International Development Agency (SIDA-SAREC), the Fp6 EU Europrise and the regional agreement on medical training and clinical research (ALF) between Stockholm County Council and Karolinska Institutet.

I would like to acknowledge all the patients and volunteers who have kindly participated in the studies by donating their samples and time; and the staff from Venhålsan for their fantastic work.

I would like to express my gratitude to my main supervisor, Professor Francesca Chiodi; thank you so much for accepting me into your group – “the family”. It has been a great experience to work with such a determined and creative person, supporting me both personally and professionally during these years.

To my co-supervisor, Professor Martin Cranage; thanks for the scientific discussions and the time in your laboratory in London.

To Bence Rethi, my co-supervisor and a scientific role model; thanks for making us look at the Universe (from dendritic cells, to immunology, stocks, European history and Japanese food...and so much more) with such enthusiasm. It is always a real pleasure, and sometimes disconcerting to discuss with you.

To the members of the Europrise Network of Excellence; thank you for giving me the opportunity to participate in this fantastic journey. Special thanks to Natasha Polyanskaya, the project manager for your help and your enthusiasm; Britta Wahren, for your passion and dedication with the PhD School; Robin Shattock, Frances Gotch and Gabriella Scarlatti for the discussions and the inspiration. And to all the Europrise students for the good time!

To Lyda Osorio; you were my first supervisor ever; it is thanks to you that I am here today. Thanks for believing in me!

To Professor Julien Fellah, merci for making me loving immunology.

To the members of the Chiodi group, present and past: you are all exceptional and I feel so lucky to have been able to learn, work, laugh and be, at times, so French; always with you at my side, caring and supportive! Nancy Vivar, my doctor; from those years working together, trying to figure out the fate of CD28-T cells, to the Maltese bus lines or the best sake, I discovered a strong-willed, generous and passionate friend. Stefano Sammicheli, caro! I would say that eventually it has been too short! You are an amazing, kind and devoted person that made our group so special; you also made Tegoia a home for us...grazie e bacio a mamma! Linh Dang, thank you for caring about us, and bringing laughter and happiness! Rebecka Lantto; thanks for your vitality, kindness and honesty...but I am not rude! Thang Pham Hong, you taught me patience and
perseverance, and helped with this thesis right up to its last day. Thank you! Simone Pensieroso; thanks for the good times looking at B cells and discovering New Mexico. Many thanks also to Miriam Kiene, Hanna Ingelman-Sundberg, Anna Nilsson, Carina Bengtsson, and the former members: Malgorzata Kryzowska, Alberto Cagigi, Liv Eidsmo, Carolin Fluur, Frida Mowafi and Simone Becattini.

To my collaborators and co-authors: D Brodin, PD Cam, Bo Hejdeman, NT Hien, Rebecka Lantto, Lucia Lopalco L, Simone Pensieroso, Thang Pham Hong, Stefano Sammicheli and Nancy Vivar.

To all the people at MTC that made my life nicer and happier.

To Adyl, Anna-Maria, Hannes & My fellow at MSA.

I am indebted to Europrise, KI Travel funds and the Travel and Research Grants Sven Gards funds for permitting me to attend conferences, symposiums and courses. With more than 1.5 times around the world travelled, I gained important knowledge and input on immunology, infections and HIV vaccines and microbicides, extremely valuable for the development of the work presented in this thesis.

This work has been a big part of the last years, and would not have been conceivable without the support of my friends and family. I wouldn’t have made Stockholm my home without having crossed the path of wonderful people that I deeply love and who are a part of my life wherever we may be. From Jägargatan:

Susan, my favorite Dutch girl, I could have married you! Alice, I miss your big laughs and the morning espresso! Elena, my opera-mate. Nina, always kind and carrying...so lovely! Ylva, Aude, for shining! Venkatramanan, Mathieu D.

Grazie a la Famiglia D’Amato, for your happiness and le budella de Palermo Wendy, thanks for your happiness and kindness, muchos cariños.

Romain, for everything you do...tu es trop gentil pour être un vrai rebelle! Thanks to my flatemates who have made my mornings so specials: Stefano (same as above) + thank you for your patience (sometimes) and generosity (always); Αγαπητοί, thank you for living in such a beautiful world inside yourself and sharing it with me! Mathieu T: “La meilleure philosophie, relativement au monde, est d’allier, à son égard, le sarcasme de la gaieté avec l’indulgence du mépris.” Chamfort te vas bien...merci pour ce bout de vie partager.

Gracias a mi familia desde América del Sur en Estocolmo, por el apoyo and the fun. Ustedes son grandes! Georges, Andres, Argenis, Carlos F, Euclides.

I have been the happiest during these years sharing all those special moments, thanks guys for being supportive and always there! Carlos, Helder, André, Cage, Johan G, Johan R, José, Markus F, Riccardo...you are all so wonderful!
Viveca, David, Miriam & Astrid, ma famille de Suède...et d’ailleurs. Merci pour être présents, me faire vivre les joies d’un bonheur familial, chantant, sautant, riant autour d’un arbre, ou sur la plage...que du bonheur !

Eugénie, nous n’avons peut-être pas Un Air de Famille, Dans Paris nous aurions pu nous rencontrer pour partager Les Amours Imaginaires ou Les chansons d’Amours, merci pour Le Fabuleux Destin partagé, c’était Une Époque Formidable !... à bientôt en Flandres!!!

Emma, Emma, Emma !!! Ces 6 années, pour nous deux ont été boulversifiantes, émouvantes, frustrantes, étonnantes, entraînantes, voyageantes, et énamourantes...merci ma sœur d’être à mes côtés ! Tu es so wonderful aussi !

Je tiens aussi à remercier ma famille (de France), pour son inconditionnel soutien. Merci d’avoir été là pour moi quand j’en ai eu besoin. Je vous dois beaucoup. Corinne & Benoit, sans vous je ne serais pas parvenu jusqu’ici, merci de votre joie de vivre, de votre générosité ! Virginie, mon autre sœur, merci pour ces moments partagés, accoudés au bar ou devant Mr le Maire, tu es la force et la joie incarnée ! Carole, Michel et les cousins, merci pour venir me rendre visite et rendre ma vie encore plus orange ! Mes frères et leurs familles, Diégo pour sa joie de vivre et ses rires ! Et tous les autres...vous remplissez mon cœur.

Un grand merci à mes parents, Martine & Yves, pour croire en moi et me soutenir. Merci pour avoir éveillé en moi la curiosité de découvrir le monde.

A ti Ronald, gracias por estar aquí, y hacer de mi vida una experiencia maravillosa...hasta la Luna !
7 REFERENCES


eta2 microglobulin ed HIV infection.

62

75.

74.

73.

72.

71.

70.

69.

68.

67.

66.

65.

64.

63.

62.

61.

60.

59.

58.

57.

56.

55.

54.

53.

52.

51.

50.

49.

48.

47.

46.

45.

44.

43.

42.

41.

40.

39.

38.

37.

36.

35.

34.

33.

32.

31.

30.

29.

28.

27.

26.

25.

24.

23.

22.

21.

20.

19.

18.

17.

16.

15.

14.

13.

12.

11.

10.

9.

8.

7.

6.

5.

4.

3.

2.

1.


104. Dion, M.L., R.P. Sekaly, and R. Cheynier, 


301. Bellistri, G.M., et al., Increased bone marrow interleukin-7 (IL-7)/IL-7R levels but reduced IL-7 responsiveness in HIV-positive patients lacking CD4+ gain on antiviral therapy. PLoS One, 2010. 5(12): e15663.