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ROLE OF IL-7 IN IMMUNE ACTIVATION DURING HIV-1 INFECTION

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ABSTRACT

Viral replication, lymphopenia and microbial translocation at the mucosal surfaces lead to a chronic state of immune activation during HIV-1 infection. Chronic immune activation is believed to impact on the functionality of cell types that are not the main target for virus replication, including CD8+T cells, B cells and NK cells.

In this thesis two main aspects of the pathogenesis of HIV-1 infection were studied: 1) how viral replication and disease progression affect the homeostasis of peripheral NK cell subsets and functionality of CD28- T cells and 2) the impact of the lymphopenia-associated cytokine IL-7 in promoting immune activation via its priming role for T cells proliferation and its indirect effects on B cells and NK cells. Specifically, in **paper I** we show that an altered expression of CD70 and CD27 molecules on NK cells, as well as an altered distribution of NK cell subsets, occurs during HIV-1 infection in parallel with disease progression. Our data suggest that during the chronic phase of HIV-1 infection there is a general increased expression of CD70 on NK cells and an expansion of the immunomodulatory NK cell subset ($CD56^{high}$). Since $CD56^{high}$ NK cells can secrete large amount of proinflammatory cytokines, our data imply that NK cells can potentially contribute to immune activation, via proinflammatory cytokines and bystandard activation of CD27 expressing cells. In **paper II** we demonstrate that IL-7 promotes Fas induced proliferative signals on suboptimally activated T cells. Our group has previously shown that during HIV-1 infection IL-7 induces Fas expression and increases Fas mediated apoptosis of T cells. Since IL-7 is a major inducer of lymphopenia induced homeostatic T cell expansion, and is often found at high level during lymphopenia, we propose that Fas-induced proliferative signals of weakly activated T cells can contribute to T cell activation in HIV-1 infected patients. In **paper III** we investigated the phenotypic, survival and proliferative characteristics of CD28- T cells and analyzed the impact of viral replication on their functionality. Our data suggest that viremia induces accumulation of apoptosis-prone, senescent CD28- T cells. Thus we propose that control of HIV-1 replication with an early initiation of ART, might be beneficial for survival and functionality of this effector/memory subset, often specific for pathogens that establish chronic infections. In **papers IV** and **paper V** we studied the indirect effects of IL-7 on B cell homeostasis, a mechanism which may be potentially important in the settings of HIV-1 infection or in other conditions characterized by increased levels of IL-7. In **paper IV**, we report that IL-7 induces Fas expression on resting B cells and increases their sensitivity to apoptosis via the induction of IFN-γ production by T cells. In **Paper V** we show that IL-7 is able to upregulate CD70 expression on T cells, which can ultimately lead to IgG production by triggering of the CD27 molecules on B cells. Lymphopenia, through the increased IL-7 concentration, may thus confer non-antigen activated T cells with general effector function, as demonstrated by the release of IFN-γ and induction of CD70. Such mechanisms could contribute to improve immunological responses, in a situation when the immune system is weakened by lymphopenia, at the price of less regulated T cell responses contributing to bystander damage of the B cell pool. Overall **paper IV** and **paper V** illustrate novel mechanisms by which IL-7, a T cell trophic cytokine, can contribute to impaired B cell homeostasis; these findings should possibly be considered when using IL-7 therapy aiming at restoring T cells numbers in lymphopenic patients.

LIST OF PUBLICATIONS

I. Titanji K, **Sammicheli S**, De Milito A, Mantegani P, Fortis C, Berg L, Kärre K, Travi G, Tassandin C, Lopalco L, Rethi B, Tambussi G, Chiodi F. *Altered distribution of natural killer cell subsets identified by CD56, CD27 and CD70 in primary and chronic human immunodeficiency virus-1 infection.* Immunology. 2008 Feb;123(2):164-70. Epub 2007 Jul 11.

II. Rethi B, Vivar N, **Sammicheli S**, Fluur C, Ruffin N, Atlas A, Rajnavolgyi E, Chiodi F. *Priming of T cells to Fas-mediated proliferative signals by interleukin-7.* Blood. 2008 Aug 15;112(4):1195-204. Epub 2008 Apr 25.

III. Vivar N, Ruffin N, **Sammicheli S**, Hejdeman B, Rethi B, Chiodi F. *Survival and proliferation of CD28- T cells during HIV-1 infection relate to the amplitude of viral replication.* J Infect Dis. 2011 Jun 1;203(11):1658-67.

IV. **Sammicheli S**, Pham TH, Dang LVP, Pensieroso S, Vivar N, Ruffin N, Hejdeman B, Chiodi F , Rethi B.*IL-7 promotes CD95-induced apoptosis in B cells via the IFN-γ/STAT1 pathway* (Submitted)

V. **Sammicheli S**, Ruffin N, Vivar N, Chiodi F, Rethi B.*IL-7 modulates IgG production inducing CD70 expression on T cells* (Manuscript)

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LIST OF ABBREVIATIONS

ADCC	Antibody dependent cell mediated cytotoxicity
AGM	African Green Monkey
AICD	Activation induced cell death
AIDS	Acquired immunodeficiency syndrome
APC	Antigen presenting cell
ASC	Antibody secreting cell
ART	Anti retroviral treatment
BAFF	B cell activating factor
Bcl	B cell lymphoma
CHI	Chronic HIV infection
CMV	Cytomegalovirus
CTL	Cytotoxic T cells
CTLA	Cytotoxic T lymphocyte antigen
DC	Dendritic cell
DN	Double negative
DP	Double positive
FCRL	Fc receptor-like
FDC	Follicular dendritic cells
FRC	Fibroblast reticular cells
GALT	Gut associated lymphoid tissue
GM-CSF	Granulocyte/macrophage colony-stimulating factor
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HLA	Human leukocyte antigen
HPE	Homeostatic peripheral T cells expansion
IFN	Interferon
IRIS	Immune reconstitution inflammatory syndrome
KIR	Killer cell immunoglobulin-like receptor
KREC	κ-deletion recombination excision circle
LCMV	Lymphocytic choriomeningitis virus
LTNP	Long-term non progressor
MHC	Major histocompatibily complex
MIP	Macrophage inflammatory protein
NCR	Natural cytotoxic receptor
PD	Programmed death
PHI	Primary HIV infection
PRR	Pattern recognition receptor
RM	Rhesus macaque
RT	Reverse transcriptase
RTE	Recent thymic emigrant
SCID	Severe combined immunodeficiency
SIV	Simian immunodeficiency virus
SM	Sooty mangabeys
SP	Single positive
STAT	Signal transducer and activator of transcription
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TSLP	Thymic stromal lymphopoietin

1 INTRODUCTION

1.1 Immunodeficiency virus (HIV)

In 1981, a novel disease characterized by an unusual clustering of rare pathologies, including Kaposi's sarcoma, *pneumocystis carinii* pneumonia and candidiasis, was reported in 4 previously healthy homosexual men. The common anamnesis characterizing the 4 patients was a marked reduction of CD4+ helper T cell number (1). Two years later, the causative agent of this new immunodeficiency syndrome, acquired immune deficiency syndrome (AIDS), was isolated from biological specimens from patients and later on, the virus named human immunodeficiency virus (HIV) (2-4). 30 years after its discovery, HIV infection remains an important problem worldwide, with over 30 million people deceased in AIDS, 33 million adults and children living with HIV/AIDS, 2.6 million of new cases and 1.8 million deaths per year, according to the report of the United Nations Programme on HIV/AIDS (UNAIDS) published in 2010 (www.unaids.org/globalreport).

1.1.1 The virus

HIV is a lentivirus belonging to the family of Retroviridae. Is transmitted as a single-stranded envelope RNA virus and is retrotranscribed to a double stranded DNA by the viral reverse transcriptase (RT) upon entry into the target cells. The viral DNA is subsequently transported to the nucleus and integrated as a provirus into the host cellular DNA by a virally encoded integrase. The HIV-1 infection cycle begins when the envelope (Env) glycoprotein gp120 binds to the CD4 molecule on the surface of target cells. CD4 is expressed primarily by helper T cells, but can be expressed at variable levels also by macrophages and dendritic cells (DCs). Env is a complex composed by the transmembrane gp41 subunit, which is associated with the gp120, the protein anchoring to the CD4 molecule. Binding of gp120 to CD4 initiates the adsorption process; conformational changes of gp120 enable the binding of the gp120 to the coreceptor CCR5 or CXCR4, which ultimately leads to the fusion of the gp41 protein with the membrane of the target cells. Once the virus establishes a productive infection, a large number of viral particles bud from infected cells and can disseminate the infection to new cells.

HIV-1 can remain silent as an integrated provirus for a variable period of time establishing a latent infection of CD4+ T cells. Latent reservoirs represent a serious obstacle for HIV-1 eradication, owing that they can persist for long period of time, with the provirus being formally invisible for the immune system and not targeted by anti retroviral treatment (ART), which only acts on replicating virus (5, 6).

Based on genetic variation, two types of HIV viruses exist, type 1 and type 2. HIV-1 is more virulent, is broadly distributed worldwide and accounts for the majority of HIV infections. Both HIV-1 and HIV-2 represent cross species transmissions of simian immunodeficiency virus (SIV) between primates and humans, with HIV-1 having its origin most probably in the SIV chimpanzee (SIVcpz) and HIV-2 having its root in the SIV of sooty mangabeys species (SIVsm) (7).

1.1.2 Immune response to HIV-1

HIV-1 has evolved several mechanisms to evade immune recognition and, as I will discuss in this thesis, HIV-1 can, directly and indirectly, induce several defects in both the innate and adaptive arms of the immune system. This translates into an inefficient immune response to the virus and favours its evolutionary persistence in the host.

At the interface of mucosal tissues, HIV-1 gets access to DCs. DCs secrete pro-inflammatory cytokines, which initiate an innate immune response, then migrate to the draining lymph nodes where they can activate and condition virus specific T cells responses. DCs can bind HIV-1 via pattern recognition receptor (PRRs), such as Toll-like receptors (TLRs) and C-type lectins such as DC-specific ICAM3-grabbing non-integrin (DC-SIGN). Two types of DCs can bindHIV-1 in peripheral tissues sites: conventional DCs and plasmacytoid DCs (pDCs). Upon endocytosis of HIV-1, pDCs are activated via the binding of viral RNA to TLR-7, and can induce large amount of type I Interferons (IFNs), promoting anti-viral responses. As part of the innate immune response, and being specialized in killing of viral infected cells, Natural Killer (NK) cells are activated early after HIV-1 infection. Nevertheless, as discussed later in this thesis, HIV-1 infection induces defects on NK cells and their responses appear to be “anergic” and unable to protect from viral dissemination (8).

1.1.2.1 Adaptive immune response

The high degree of HIV-1 diversity, and the rapid mutation rate of HIV-1 structural genes, makes it difficult for the adaptive immune system to defeat the virus, and eventually, both the cellular and humoral responses lag behind the HIV-1 rapidly diversifying nature. However, cytotoxic T cell (CTL) responses appear quickly after HIV-1 infection and play a major role in controlling the spreading of the virus during the acute phase of infection (9, 10). A strong, cross-reactive CTL response has been demonstrated to occur in the SIV infection model and was also associated with the capacity to remain uninfected in subjects highly exposed to HIV-1 infection, as well as in long-term non-progressors (LTNP) (11-13). CD4+T helper responses arise few weeks after the establishment of HIV-1 infection, and are associated with control of viremia and with maintenance of antiviral CTLs responses (14, 15). The hallmark of HIV-1 pathogenesis is a gradual loss of CD4+T cells leading to a profound immune deficiency and CD4+T cells specific response slowly decline as a result of the viral infection (15).

The initial antibody response to HIV-1 arises within 4-8 weeks following primary infection and is directed against non-neutralizing epitopes of the envelope protein as well as the core/matrix protein (16). Broadly neutralizing antibodies (bnAb) appear several months after the transmission, in a minority of patients, and generally do not associate with control of viremia (17, 18). The levels of IgA antibodies present at the mucosal sites, where HIV-1 infection is disseminated, are also low when compared to other subclasses of immunoglobulins (19). Overall, antibody responses to HIV-1 infection are clearly ineffective to further control virus dissemination.

The proposed mechanisms by which HIV-1 promote the functionality loss of T cells and B cells will be discussed extensively in the following chapters, but potentially these mechanisms arise as a result of direct effect of the virus and as a result of the virus-induced immune activation.

1.2 Immune activation during HIV-1 infection

In HIV-1 infected individuals it is estimated that a relatively small proportion of circulating CD4+ T lymphocytes carries the virus, ranging between 0.1 and 1%; yet, on a long run, HIV-1 infection leads to the death of a majority of CD4+ T cells, the destruction of the immune system and death of the host by opportunistic pathogens and tumours. Such an overwhelming impact of the virus has been attributed to the chronic and systemic immune activation initiated by the virus, more than solely to the direct cytopathic effect of HIV-1 for T cells (20, 21). Indeed, during HIV-1 infection, several different cell types, including CD8+ T cells, B cells and NK cells suffer from the viral insult despite not being the main targets for virus replication (22).

The important role of immune activation in HIV-1 pathogenesis has been supported by studies on non-human primates infected by SIV, as well as by studies comparing HIV-2 with the more virulent HIV-1 infection (23, 24). On the other hand, within the first weeks of infection, at the peak of viral replication, a massive CD4+ memory T cells depletion occurs in the intestinal mucosa as well as in lymph nodes and blood (25, 26) and this initial CD4+ T cell depletion was associated with a high infection frequency, ranging between 30 and 60% (27, 28). These findings indicate a direct, virus induced immunopathology during early disease stages.

Chronic immune activation is manifested by increased lymphocytes activation and turnover, increased pro-inflammatory cytokine levels, the disruption of intestinal epithelium followed by microbial translocation from the gut to the circulation, the destruction of lymphoid architecture, as well as the loss of thymic functions. There are several mechanisms implicated in the maintenance of immune activation, including lymphopenia itself, the microbial translocation and the ongoing HIV-1 replication, as it will be discussed later (Fig. 1). However, causes and consequences of the generalized immune activation in a disease that paradoxically is characterized by immune deficiency are still subjects of an active debate.

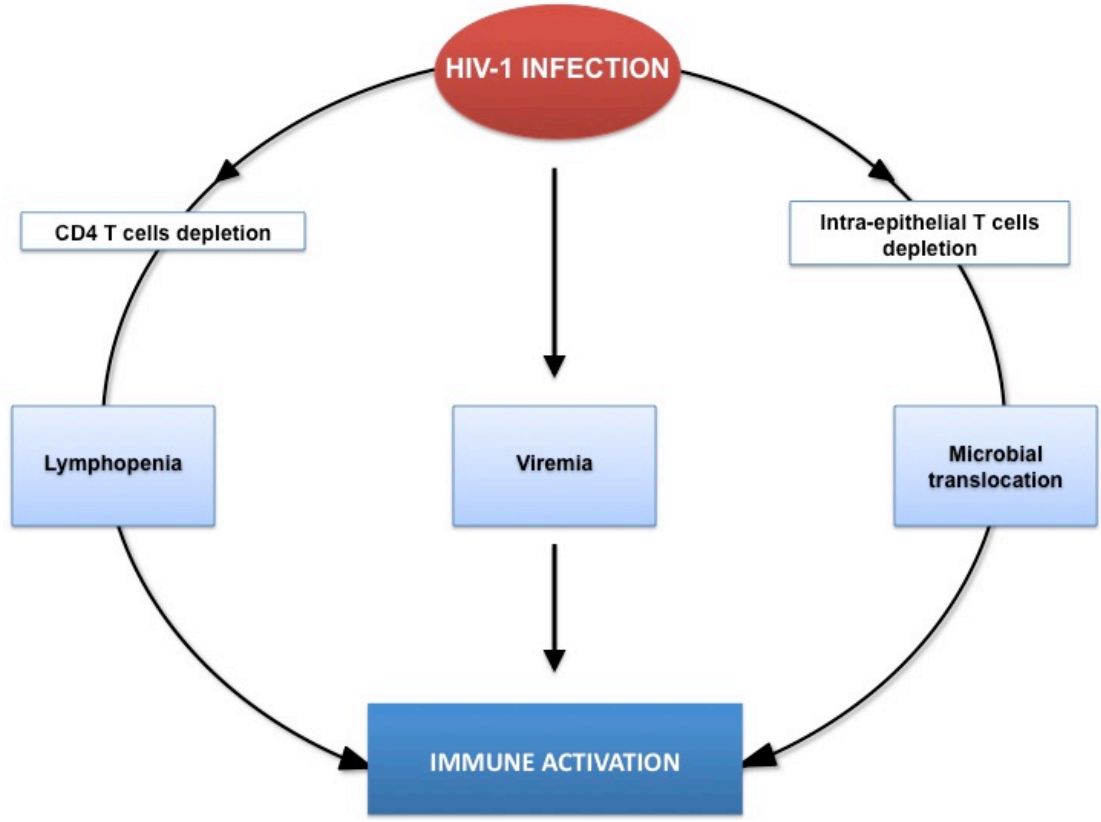

Figure 1. Direct and indirect consequences of HIV-1-induced immune activation. *HIV-1 infection induces chronic immune activation through direct viral replication (viremia), or through at least 2 indirect effects: microbial translocation and lymphopenia. Microbial translocation results from structural disruption of the epithelial barrier mostly in the gut, as a potential consequence to intra-epithelial T cells depletion. Lymphopenia results from total CD4+T cells depletion.*

1.2.1 Disease progression in natural and non-natural hosts of SIV

Important indications for the role of immune activation in HIV-1 pathogenesis have been provided by comparing some natural and non-natural hosts of SIV: sooty mangabeys (SM) or african green monkeys (AGM) and Rhesus macaques (RM) respectively. SMs display high levels of viremia, but a preserved CD4+ T cell homeostasis during the chronic phase of SIV infection, with CD4+T cell numbers that normalize after the acute infection to a level that is comparable with uninfected animals. SMs can be naturally infected by SIV, and live an apparently normal life as the result of co-adaptation between the virus and the host (23, 24). On the other hand, RMs experience an immunopathology similar to that induced by HIV-1 infection in

humans, with high viral load, high levels of immune activation, massive CD4+ T cell depletion and progression to AIDS (23, 29).

The different outcomes of SIV infection in the various hosts has been attributed to several factors, including the severity of intraepithelial CD4+ T cell depletion in early disease stages. In RMs, as well as in humans, memory and activated CD4+ T cells express the CCR5 chemokine receptor at the mucosal sites and, as a result, these cells are targets of SIV and HIV-1 infection and early depletion. In contrast, only a minority of mucosal T cells express CCR5 in SMs, mostly the short lived activated T cells and not the memory cells (23, 24).

1.2.2 Role of mucosal T cell damage in immune activation

The mucosa of the intestinal tract constitutes a unique anatomical and immunological barrier that discriminates commensal colonizing microorganisms from external pathogens. The gut associated lymphoid tissue (GALT) represents the largest of the secondary lymphoid tissues and comprise the majority of the lymphocytes in the body (30-32). Intraepithelial T cell depletion occurring during HIV-1 infection has been associated with damaged intestinal epithelium (25, 28) leading to enteropathy, increased intestinal permeability, malabsorption and to microbial translocation from the intestinal lumen to the tissues and circulation (33-35). In HIV-1 infected patients, as well as in SIV infected RMs, high levels of plasma LPS and sCD14, biomarkers for microbial translocation, can be found. Microbial products are potent immune-modulatory molecules that might contribute to a generalized state of immune activation through their action on TLRs. On the contrary, SMs manifest only a transient appearance of microbial translocation during the acute phase of SIV infection, suggesting an important role of the epithelial damage and the flow of microbial products into the circulation in HIV-1 disease progression (23, 24).

The effects of HIV-1 infection on the gut epithelium have been attributed at least in part to Th17 T cell depletion. Th17 cells can regulate epithelial cell homeostasis, tissue repair and wound healing via IL-22 (36, 37) and are potentially required for the integrity of the epithelium.

Microbial translocation might play an important role in inducing a persistent and widespread stimulation for the innate immune system. Chronic immune activation in non-natural hosts and HIV-1 patients is often associated with the presence of inflammatory cytokines, including IFN type I and II and Tumor Necrosis Factor alpha (TNF-α) (38, 39). Higher expression of IFN-induced genes was observed in the gut mucosa of SIV infected RMs when disease progressors were compared to non-progressors (40). Notably, pDCs of SIV infected SMs have substantially reduced levels of IFN- α induction and a less marked TLR-7 and 9 signalling upon ex vivo activation compared with RMs (41). Recently, pDCs were shown to augment β7 integrins expression during SIV infection possibly increasing their recruitment to intestinal tissues and therefore contributing to the local immune activation (42). The dysregulated production of type I IFNs might lead to increased B cell activation and interfere with B cell tolerance, similarly to mechanisms suggested to take place in systemic lupus erythematosus (43).

1.2.3 Lymphopenia and immune activation

Humans experience lymphopenia as the result of congenital or acquired immunodeficiency syndromes or as the result of cytoreductive therapies (44). T cell regeneration can occur via thymopoiesis or homeostatic peripheral T cells expansion (HPE). Due to thymic involution occurring early in life, the significance of peripheral T cell expansions increases with age (45-47). HPE is initiated as T cells decrease their activation threshold under lymphopenic conditions and weak TCR signals, via recognizing low affinity, often self antigens, can trigger proliferation and acquisition of effector/memory phenotype (48, 49). Prolonged lymphopenia can therefore be considered a trigger of T cell activation and indeed, there are several indications for increased T cell responses in lymphopenic conditions (50). These include the NOD mice where disease progression correlated with levels of lymphopenia or the HIV-1 associated immune reconstitution inflammatory syndrome (IRIS), a pathologic inflammatory response to a previously acquired infections that can accompany the initiation of ART(51, 52). There are several similarities in T cell phenotype between chemotherapy treated and HIV-1 infected individuals, all reflecting increased and generalized immune activation, including the increased ratio of activated or memory cells, the increase of CD8:CD4 ratio, the upregulation of expression of the Fas (CD95)

death receptor and the down-regulation of expression of the Bcl-2 anti-apoptotic molecule (53-55).

The cytokine IL-7 is often implicated in the lymphopenia induced T cell stimulation (56). IL-7 levels are elevated in lymphopenic conditions, including HIV-1 infection, idiopathic CD4+ T cell lymphopenia or therapeutic T cell depletions (57, 58) and the concentration of IL-7 correlates with the severity of CD4+ T cell depletion. Due to the important roles for IL-7 in increasing survival and stimulating T cell responses upon antigen recognition, the high levels of IL-7 might contribute to a generalized T cell activation and HPE in lymphopenic patients (53, 57, 59).

1.2.4 Interleukin-7

IL-7 is a 25 KDa glycoprotein encoded by a six exons-gene segment located in the chromosome 8q12-13. IL-7 is a non-redundant cytokine for T cells development and survival. IL-7 signals through a heterodimeric receptor composed by IL-7Rα chain, that is part of the thymic stromal lymphopoietin (TSLP) receptor as well, and the common γ-chain, shared with other cytokines namely IL-2, IL-4, IL-9, IL-15 and IL-21 (60). The non-redundant role of IL-7 in T cell development and survival is manifested by the absence of T cells in humans with severe combined immunodeficiency (SCID), a genetic inherited syndrome resulting from loss of function mutations in the IL-7 or γc receptors (61, 62).

In mice, where IL-7 was first discovered (63), deficiency in the IL-7 signalling leads to the absence of both T cells and of B cells, suggesting a differential regulation of IL-7 mediated B cells homeostasis between mice and humans. Moreover, in SCID patients B cell development is preserved indicating that IL-7 might not be required for B cells ontogeny in humans (64). On the other hand, IL-7Rα is expressed by the common lymphoid progenitors as well as by the B cell progenitors until the pro-B cell stage in humans. IL-7 mediated signalling modulates the expression of transcription factors involved in B cell lineage commitment and represses Igκ transcription during immunoglobulin gene rearrangements (65-67). Furthermore, in vitro studies have shown that IL-7 can induce the proliferation and survival of immature B cells and

elevated levels of IL-7 in vivo, as a result of lymphopenia or IL-7 therapy, have been associated with the expansion of immature transitional B cells (68, 69). All together these findings suggest that, despite the apparently normal level of B cells in the SCID patients, IL-7 might exert important functions for B cell development in humans.

1.2.5 IL-7 sources and regulation

IL-7 is produced by non-hematopoietic cells, primarily stromal cells, but several other cell types have also been identified as possible source of IL-7, including fibroblast reticular cells (FRC), intestinal epithelial cells, keratinocytes, peripheral blood and follicular DCs, smooth muscle and endothelial cells (60, 70). The mechanisms that regulate IL-7 production are yet to be fully characterized. The prevailing theory suggests that IL-7 is produced at a fixed and constitutive rate by stromal cells, and the amount of IL-7 is just sufficient to maintain a finite number of T cells. According to this theory, T cells continuously compete for IL-7 and IL-7 levels are regulated by its consumption more than by its production (57, 60, 71, 72). Several lines of data support this hypothesis. *Il-7* mRNA transcripts have been shown to be produced constitutively by stromal cells (72), in lymphopenic conditions the number of T cells inversely correlates with the levels of IL-7 (71, 73) and increased IL-7 concentrations in vivo, due to IL-7 therapy in humans or monkeys or transgenic IL-7 expression in mice, resulted in increased peripheral T cell numbers (71). IL-7 induces the down-regulation of its own receptor and transgenic IL-7Rα expression leads to a decreased number of T cells in the thymus as well as in the periphery, suggesting that IL-7Rα down-regulation might occur as an altruistic mechanism to maximize the availability of IL-7 (74, 75).

On the other hand, other studies proposed an inducible IL-7 production. IFN-γ has been shown to induce IL-7 gene expression and protein release by keratinocytes, human intestinal cells, epithelial and stromal cells lines (76-78). During HIV-1 infection, some studies suggested an increased number of IL-7 producing cells in the lymphoid tissues, at least in certain disease stages (56, 79). Furthermore, it has been shown in mice that hepatocytes can produce IL-7 in an inducible manner (80). TLR ligands upregulated IL-7 production in the liver via a type I IFN dependent manner and the IL-7 released from hepatocytes was able to modulate T cell responses. These results indicated an alternative, inflammatory role of IL-7 in the regulation of the immune responses.

1.2.6 The roles of IL-7 and IL-7Rα in T cell development and homeostasis

Maturation of αβ T cells takes place in the thymus, and involves a well-defined series of stages, including rearrangement of TCR genes, selection of the appropriate TCR repertoire and acquisition of CD4 and CD8 coreceptor expression. The stages through which a T lymphocyte progenitor give rise to a naïve mature T cells progress as follows: double negative (DN) ➔ double positive (DP) ➔ single positive (SP). IL-7Rα is expressed at the DN stage, lost during the DP stage and then re-expressed at the SP stage (81, 82). IL-7Rα expression is considered to be essential for survival and proliferation of developing T cells (72). Indeed, the absence of IL-7Rα expression in the DP thymocytes was initially suggested to be the result of their selection process: DP thymocytes are negatively selected or die by neglect if their TCRs have too high or too low affinity for self peptides, respectively. Therefore DP thymocytes rely strongly on survival signals induced by their TCRs and the expression of IL-7Rα might select inappropriate T cells clones. However, transgenic expression of IL-7Rα on DP cells did not protect them from cell death and could not perturb their selection process (72, 83). Nevertheless, IL-7Ra down-regulation, both in the thymus and in the periphery, occurs when T cells receive stimulatory signals through the TCR molecules.

Mature naïve T cells freshly migrating from the thymus are called recent thymic emigrants (RTEs). IL-7 has a potent survival effect for RTEs and it can induce proliferation in the absence of TCR signalling (84, 85). Indeed cancer patients treated with exogenous IL-7 show a marked increase in the RTEs number (86). Peripheral naïve and memory T cells are maintained by homeostatic cytokines signalling and by the recognition of low affinity peptides presented on MHC molecules. IL-7 is a crucial factor for the maintenance of peripheral T cells. Generally, different subsets of peripheral T cells express different level of IL-7Rα, and as a consequence, are differentially regulated by IL-7 availability (71, 72). Naïve T cells express high levels of IL-7Rα, and their survival is strongly dependent on IL-7 signalling (70). During antigen-specific T cell activation, IL-7Rα is down-regulated and only a small proportion of effector cells will maintain the expression of the receptor. It is suggested that this minority of effector T cells will give rise to the central memory T cells (87, 88). IL-7Rα is expressed at similar level within CD4 and CD8+ T cells (72); however,

during lymphopenia, IL-7 promotes the regeneration of CD8+ T cells more efficiently, as compared to CD4+ T cells . A possible explanation for this differential effect of IL-7 on CD4+ and CD8+ T cells might be the IL-7 induced MHC II down-regulation on IL-7Rα positive DCs that could decrease the homeostatic CD4+ T cell expansion (89).

During HIV-1 infection, as well as in chronic hepatitis C virus (HCV) infection or in aged individuals, T cells show a marked down-regulation of the IL-7Rα (58, 90, 91). The mechanism leading to the IL-7Rα down-regulation during persistent infections is currently unknown. For HIV-1 infection it was proposed that IL-7Rα down-regulation could be a consequence of IL-7 binding or alternatively induced by Tat HIV-1 protein (92, 93). However these mechanisms induced only a transient IL-7Rα down-regulation on T cells, whereas the low level of IL-7Rα is maintained when T cells from HIV-1 patients are kept in culture in absence of IL-7. Moreover IL-7Rα down-regulation is sustained in vivo in T cells from HIV-1 infected patients and is only partially restored by ART treatment (94). IL-7Rα low T cells accumulating during chronic viral infections or in elderly individuals are previously activated, antigen specific T cell clones in late stages of differentiation. Interestingly, acute viral infections, like vaccinia or influenza virus, do not induce down-regulation of IL-7Rα but rather T cells express high level of IL-7Rα indicating an important role for chronic T cell activation in IL-7Ra down-regulation (95, 96). Such hypothesis is supported by the mouse LCMV model where acute infection induced memory T cells maintained by IL-7 and IL-15 whereas chronic infection led to memory T cells maintained by constant antigen-dependent stimuli but not by cytokines (97, 98).

1.2.7 Lymphopenia and impaired lymphoid tissue architecture

Lymph nodes are secondary lymphoid organs anatomically suited to support the contact between T lymphocytes and antigen presenting cells (APCs). Their architecture is formed by a matrix of FRCs and follicular dendritic cells (FDCs). FRCs provide a network of conduits for the lymphocytes to migrate and in addition, these cells stimulate T cell survival via the production of chemokines and IL-7.

HIV-1 infection induces collagen deposition in the lymph nodes, eventually leading to tissue fibrosis that might represent an irreversible obstacle for T cell regeneration. The

level of collagen deposition showed a strong inverse correlation with the number of naïve T cells and early initiation of ART, in conjunction with anti-fibrotic drugs, was suggested as treatment to prevent and improve the loss of naïve T cell niches (79, 99).

Lymphopenia has been directly associated with the collapse of T cell maintenance by the FRC network in a model presented recently (79). Collagen deposition might be initiated by an increased transforming growth factor (TGF)-β production of regulatory T cells. Lymphoid tissue fibrosis inhibits the access of migrating T lymphocytes to the FRC derived survival factors, including IL-7, which leads to decreased T cell survival. Apoptosis of T cells, in turn, decreases lymphotoxin-β production in the lymphoid tissues that acts as a survival factor for FRCs. Based on this scenario, the inhibition of FRC - T cell interactions by tissue fibrosis has a detrimental effect on the maintenance of both cell types. Forming a vicious circle, T cell depletion leads to decreased FRC survival that, in turn, will lead to further T cell depletion (79). Considering the important role of the secondary lymphoid tissues in the maintenance and functionality of T and B lymphocytes, it is conceivable that HIV-1 infection can lead to an overall impairment of the immune system, affecting several cell types that are not directly targeted by the virus.

1.2.8 Altered homeostasis of T cells during HIV-1 infection

The hallmark of HIV-1 pathogenesis is a gradual loss of CD4+ T cells leading to a profound immune deficiency and occurrence of opportunistic tumours and infections (100). HIV-1 infection targets specifically T cells, via the interaction of its envelope protein gp120 with the CD4 and co-receptors. CD4+T cells can be directly modulated by viral factors, like the envelope protein gp120 or nef, the latter inducing intracellular signalling in T cells (101-103). On the other hand, the direct apoptosis induced by viral infection accounts only for part of the T cell dysfunctions occurring during HIV-1 infection (22), as it has been discussed earlier. Paradoxically, the attempt of the immune system to control HIV-1 infection increases immune activation, generates new targets for HIV-1 infection (104), increases T cell turnover (105) and exhaustion (106). HIV-1 is known to replicate more efficiently in activated T cells and HIV-1 specific T cells are eliminated rapidly due to viral infection (107, 108). Immune activation leads to increased proliferation and differentiation of naïve and memory CD4+T cells into

effectors, increased expression of CCR5, which renders CD4+T cells more susceptible to HIV-1 infection (104, 109).

1.2.8.1 Increased T cells activation, senescence and exhaustion

Immune activation, originating from the presence of microbial TLR ligands, viral antigens and lymphopenia induced stimulatory factors, like IL-7, leads to a decreased threshold for activation, increased proliferation, memory/effector differentiation and finally, functional exhaustion and increased apoptosis of T cells.

Increased levels of CD38 and HLA-DR, markers for T cell activation, have been detected on CD8+ T cells in correlation with disease progression (38, 110). T cells with effector or effector memory phenotype accumulate in the circulation of HIV-1 infected individuals on the expenses of the naïve and central memory T cell pool (111, 112). This, on a long run, potentially reduces the capacity of the immune system to generate an efficient immune response to novel antigens.

Using bromodeoxyuridine (BrdU) and deuterated glucose to label the DNA of proliferating T cells, as well as with Ki67 staining, increased T cell proliferation has been detected in HIV-1 infected patients (113-115). Replicative senescence of long term activated T cell clones has been suggested and associated with the down-regulation of the CD28 molecules, with shortening of the telomeres and the upregulation of the CD57 molecules (116, 117). Parallels have been drawn between the ageing of the immune system and the HIV-1 induced T cell pathology (118). Interestingly, expanded clones of cytomegalovirus specific T cells have been detected in both HIV-1 infected and aged individuals (119-122)

A large body of evidence indicates that sustained immune activation induces the expression of inhibitory receptors on T cells during HIV-1 infection, the best characterized of which are PD-1 and CTLA-4 (123, 124). PD-1 expressing T cells have impaired proliferative responses to cognate antigen and have higher susceptibility to spontaneous and Fas-mediated apoptosis. PD-1 and CTLA-4 are typically expressed on HIV-1 specific CD8+ and CD4+T cells respectively, therefore suggesting a strong role of these inhibitory pathways in the impaired functionality of T cells responses against

HIV-1 (123-125). Blockade of PD-1 ligand (PDL-1) binding greatly enhanced the survival of SIV infected RMs and increased SIV specific T and B cell responses. Blocking CTLA-4, on the other hand, promoted SIV replication, most probably because such treatment inhibited Treg functionality that induced a generalized T cell activation and thus, increased the cellular targets for SIV infection and replication (126, 127).

Over the past years, a number of studies have evidenced that the quality, more than the magnitude, of both CD4+ and CD8+ T cells responses are crucial for the control of HIV-1 infection. Comparing HIV-1 progressors or non-progressors patients, it was shown that frequencies of "multifunctional" CD4+ T cells, able to produce IFN-γ, IL-2 and TNF represented over 50% of the total T cells in LTNPs when compared with progressors HIV-1 patients, whose T cells produced IFN-γ only (128, 129). Similarly, CD8+T cells of LTNPs had increased proliferative capacity and perforin-mediated cytolytic activity after in vitro culture as compared with CD8+T cells of HIV-1 progressors (130).

1.2.8.2 T cell apoptosis

Apoptosis is considered as a major contributor for CD4+ and CD8+ T cells depletion caused by HIV-1 infection (105). Mechanisms accounting for increased apoptosis of T lymphocytes are induced directly by the virus or indirectly as a result of the immune activation (105). Direct mechanisms of apoptosis include the cytopathic effect of HIV-1 or interference of viral proteins with apoptotic pathways. Although described only in *in vitro* systems, it has been shown that gp120 down-regulates Bcl-2 on primary CD4+T cells, Nef induces depolarization of the mitochondrial membrane and activation of caspase-3 and Tat mediates the activation of caspase- 8 (131-133).

The levels of Fas expression and soluble form of TNF, FasL and (TNF)-related apoptosis-inducing ligand (TRAIL) are all increased in HIV-1 infected patients and are suggested to participate, as part of the indirect mechanism of damage, in the overall increased sensitivity of T cells for apoptosis (105). Specifically, immune activation correlates in humans with the sensitivity to apoptosis of effector/memory T cells and, in HIV-1 infected chimpanzee, with the sensitivity of T cells to Fas-mediated cell death

(134, 135). Fas is upregulated on CD4+ and CD8+ T cells during HIV-1 infection and is responsible for their enhanced susceptibility to apoptosis (136, 137). HIV-1 infected patients have increased plasma level of TNF and increased sensitivity to TNFR-induced apoptosis, as well as increased level of TRAIL, as result of HIV-1 activation of APCs (138, 139).

1.3 Altered homeostasis of B cells during HIV-1 infection

The contribution of HIV-1-induced immune activation in deteriorating the functionality of the immune system is very well manifested by the defects of B cell responses occurring during HIV-1 infection (140). B cells hyperactivation and low level of antibody responses were reported very early after the discovery of HIV-1 as the aetiological agent of AIDS (141-143). B cells are not the main targets of HV-1 infection and the factors contributing to HIV-1-induced B cells dysfunctions remain largely unknown. However, since many of the B cells abnormalities associated with HIV-1 infection can be reverted by ART, it has become clear that viremia plays a major role in B cells pathogenesis of HIV-1 patients (140). B cell defects induced by HIV-1 infection are either direct or indirect as a result of the systemic immune activation (144). Direct effects include activation of B cells via immune-complex of HIV-1 virions binding to the complement receptor CD21 (145), or viral binding on C-type lectins receptor and DC-SIGN (146, 147). Although these mechanisms have been described to occur in vitro, the frequency of virions associated with B cells in vivo is too low to be responsible for the magnitude of B cell pathogenesis occurring in HIV-1 infection. Several cytokines and growth factors, which potentially can activate B cells, including IFN-α, TNF, IL-6, IL-10, CD40L and BAFF, are found increased in the serum of HIV-1 infected patients (41, 146, 148-150).

Interestingly, most of the immune dysfunctions induced on B cells by HIV-1 infection appear to mirror the defects occurring on the T lymphocytes; these include B cells hyperactivation, increased B cells turnover and increased B cells exhaustion. Notably, HIV-1 induces a distinctive pathological signature on B cells, which has a long-term effect and is not reverted by ART, that is the loss of memory B cells and loss of serological memory (140, 151-153).

1.3.1 HIV-1 induced B cells hyperactivation

HIV-1 induces increased level of immunoglobulin in serum (hyper-gammaglobulinemia) and polyclonal B-cell activation as a result of increased B cell activation. Several mechanisms have been proposed to be responsible for hypergammaglobulinemia, including the direct effect of gp-120 binding to C-type lectins receptor (146), or the production of the acute phase protein ferritin. Specifically, HIV-1 infected macrophages were shown to secrete ferritin through a Nef dependent mechanism, and the level of ferritin in the plasma of HIV-1 patients correlated with the extent of hypergammaglobulinemia (154).

Accumulation of activated B cells has been described in highly viremic HIV-1 patients (155). Activated B cells are defined by the low expression of CD21 and can secrete large amount of immunoglobulins and therefore are potentially responsible for the observed hypergammaglobulinemia (155). Hypergammaglobulinemia has been also proposed to result from bystandard CD27 signalling. During HIV-1 infection CD70 has been found upregulated in T cells together with an increased level of bone marrow plasmacytosis. Since CD70 can induce CD27-dependent IgG production, bystandard activation of CD70-CD27 axis could result in hypergammaglobulinemia (156). Interestingly, hypergammaglobulinemia and the accumulation of CD21 low activated B cells are both normalized by ART (144).

Chronic T cells activation by HIV-1 results in their exhaustion. The term exhaustion refers to virus-specific immune cells that have lost the capability to respond to an antigen, due to expression of inhibitory receptors, such as, in the case of T cells, PD-1 and CTLA-4 (123, 124, 157). It is now clear that a similar exhaustion phenomenon can occur in B cells during HIV-1 infection. In HIV-1 viremic patients, there is an accumulation of a B cells subset expressing inhibitory receptors, such as Fc receptor-like-4 (FCRL4), normally present on memory B cells found in tonsils. This population, named tissue like memory B cells, is defined as $CD20^{high}CD10\text{-}CD27\text{-}CD21^{low}$ (158). In addition to the inhibitory receptors, tissue like memory B cells express a profile of trafficking receptors that favour their migration to inflamed tissues, and preclude homing or trafficking in lymph nodes (158).

Tissue like memory B cells were shown to have a shortened replication history, as measured by κ-deletion recombination excision circles (KRECs), and to be enriched in virus specific responses, as measured by the frequency of antibody secreting cells (ASC) (158). The potential role of increased expression of inhibitory receptors for the exhausted functionality of tissue like memory B cells during HIV-1 infection has recently been reinforced. In fact, specific siRNA silencing FCRL4 and sialic acid-binding Ig-like lectin 6 (singles-6) in tissue like memory B cells from HIV-1 infected patients restored tissue like memory B cells functionality and proliferation (159).

There are evidences that PD-1 is involved in exhaustion of B cells, similarly as for T cells. This has been reported by two studies conducted in the SIV infected RMs, where memory B cells were shown to express high level of PD-1 after SIV infection. Interestingly, it was shown that rapid disease progression of SIV infected RMs strongly associated with loss of activated ($CD21^{low}$) B cells expressing high level of PD-1. Nevertheless, blockade of PD-1 in vivo resulted in increased plasma level of SIV-specific antibody, as well as improved B cells survival and proliferation (127, 160).

1.3.2 HIV-1 induced B cells apoptosis

B cells hyperactivation results in increased B cell turnover, as shown by the ratio of Ki67 expressing B cells during HIV-1 infection as well as in SIV infected RMs (112, 161). Most of the Ki67 positive cells are activated $CD21^{low}$ B cells with phenotypical signatures of plasmablasts (158). Increased cell proliferation is physiologically regulated by increased cell death. Several studies have shown that B cells from viremic HIV-1 infected patients are decreased in number and have an apoptotic-prone phenotype (162, 163). Decreased B cell survival in HIV-1 viremic patients has been primarily attributed to an increased susceptibility of B cells for Fas-mediated apoptosis, or to their decreased Bcl-2 expression (152, 164). Specifically, the overall increased in apoptosis susceptibility resulted from the accumulation of two B cells subsets, normally present at very low frequency in healthy individuals, namely activated memory B cells (CD19+CD27+CD21low) and immature transitional B cells (CD19+CD10+CD27-). Activated memory B cells express high level of Fas, low level of BAFF receptor, and are susceptible to Fas-mediated apoptosis. Immature transitional B cells express low level of Bcl-2 and are sensitive to spontaneous apoptosis (158, 161, 162, 164, 165).

Interestingly, occurrence of immature transitional B cells in peripheral blood has been associated with increased level of IL-7, both as a result of HIV-1 infection, and in non-HIV-related idiopathic CD4+T cell lymphocytopenia.

ART induces an increase in B cell number, as a result of reduced frequency of both activated memory and immature transitional B cells. On the other hand, the increased expression of Fas occurring on resting memory B cells, does not normalize after initiation of ART (152). This suggests that other viremia-independent mechanisms might regulate the fate of B cell subsets during HIV-1 infection. Indeed, the mechanisms leading to Fas upregulation on B cells during HIV-1 infection are still elusive.

Memory B cells are regarded as long lived, resting, antigen experienced cells that are rapidly engaged after exposure to cognate antigen (166). Mechanisms by which HIV-1 infection induces loss of memory B cells are yet unsolved. Although ART has been shown to decrease the number of activated $CD21^{low}$ memory B cells, the increase in the number of resting CD27+ B cells is only partial (162, 167). Loss of memory B cells functions was proposed to occur early after HIV-1 infection. Indeed recent findings have shed light for the timing of ART as a crucial parameter in preventing the integrity and longevity of B cells humoral responses during HIV-1 infection. Specifically, in HIV-1 vertically infected children it was shown that initiation of ART within the first year of age translated in the normal development of HIV-1 specific memory B cell responses to HIV-1 gp160, as well as to common vaccination antigens measles and tetanus toxoid (168). These findings on paediatric HIV-1 infection were thereafter confirmed in adults HIV-1 patients, thus providing a strong suggestion that early initiation of ART may prevent memory B cells damage (169).

1.4 Altered homeostasis of NK cells during HIV-1 infection

NK cells constitute 15% of peripheral-blood lymphocytes and function as important mediators of innate immune responses against viruses and tumour cells (170). NK cells express a wealth of surface receptors that can either inhibit or activate their cytotoxic activity. Inhibitory receptors include killer cell immunoglobulin-like receptors (KIRs), which are highly polymorphic and specific for MHC class I molecules, and CD94, non-

polymorphic and recognizing non-classical MHC molecule HLA-E. NK cells activating receptors include NKG2D, the natural cytotoxicity receptors (NCRs) and the FCγ receptor CD16. The complex integration of signalling events from inhibitory or activatory receptors, expressed at the single cell level, determines the quiescence or the activation of a NK cell in response to a target cell (171).

Two distinct subsets of peripheral NK cells can be defined according to the cell surface expression of CD56. $CD56^{low}$ NK cells are the largest population, they have high number of cytolytic granules, abundant levels of KIRs and NCRs and can readily lyse target cells in the absence of prior sensitization. The remaining 10% of peripheral NK cells are $CD56^{high}$; they are poorly cytotoxic but can secrete large amounts of pro-inflammatory cytokines, including macrophage inflammatory protein (MIP) -1α and β, CCL-5, IFN-γ, TNF and granulocyte/macrophage colony-stimulating factor (GM-CSF) (170).

Due to their innate immune capabilities, NK cells have probably a considerable role for the prevention and control of HIV-1 infection. NK cell responses to HIV-1 include direct lysis of infected cells and antibody dependent cell mediated cytotoxicity (ADCC); in addition they can facilitate adaptive immune responses via the induction of pro-inflammatory cytokine and recruitment of lymphocytes to inflamed tissues. Furthermore, NK cells can secrete CCL3, CCL4 and CCL5, the ligands for the chemokine receptor CCR5, therefore inhibiting the entry of HIV-1 via receptor competition (172).

Despite these potential opportunities, NK cells responses are functionally impaired during HIV-1 infection. Theoretically, HIV-1 infected cells are excellent target for NK cell killing, based on the ability of HIV-1 to down-regulate MHC-I molecules (173). Nevertheless, Nef is known to selectively down-regulate HLA aplotypes that are largely targeted only by CTLs, while maintaining the expression of HLA-C and HLA-E, which inhibit NK cell activation (174, 175).

NK cells from HIV-1 infected patients have decreased intracellular stores of perforin and granzyme A and show a phenotype of incomplete activation (176, 177). These features, together with an impaired ADCC activity (178, 179), result in a generalized

decreased cytotoxic capacity of NK cells during HIV-1 infection. Indeed, NK cell mediated suppression of HIV-1 replication inversely correlates with the level of HIV-1 viremia (180).

NK cells from HIV-1 infected patients show low *in vitro* responsiveness to cytokines, and impaired NK cell cytotoxicity after IL-2 activation is associated with higher probability to progress to AIDS (181). Furthermore, during HIV-1 infection there is an accumulation of $CD16^{high}CD56$- NK cells, at the expense of the $CD16^{high}CD56^{low}$ cytotoxic NK population. CD56- NK cells have low cytotoxic activity and fail to condition adaptive immune responses due to their low production of IFN-γ and TNF upon activation (182, 183). Although it was shown that having higher NK cells functionality, in term of both cytotoxicity and immunomodulatory abilities, could predispose to protection from HIV-1 transmission in a cohort of intravenous drug users who remained seronegative despite several years of high-risk exposure (184), the exact contribution of NK cells for HIV-1 protection remains unclear.

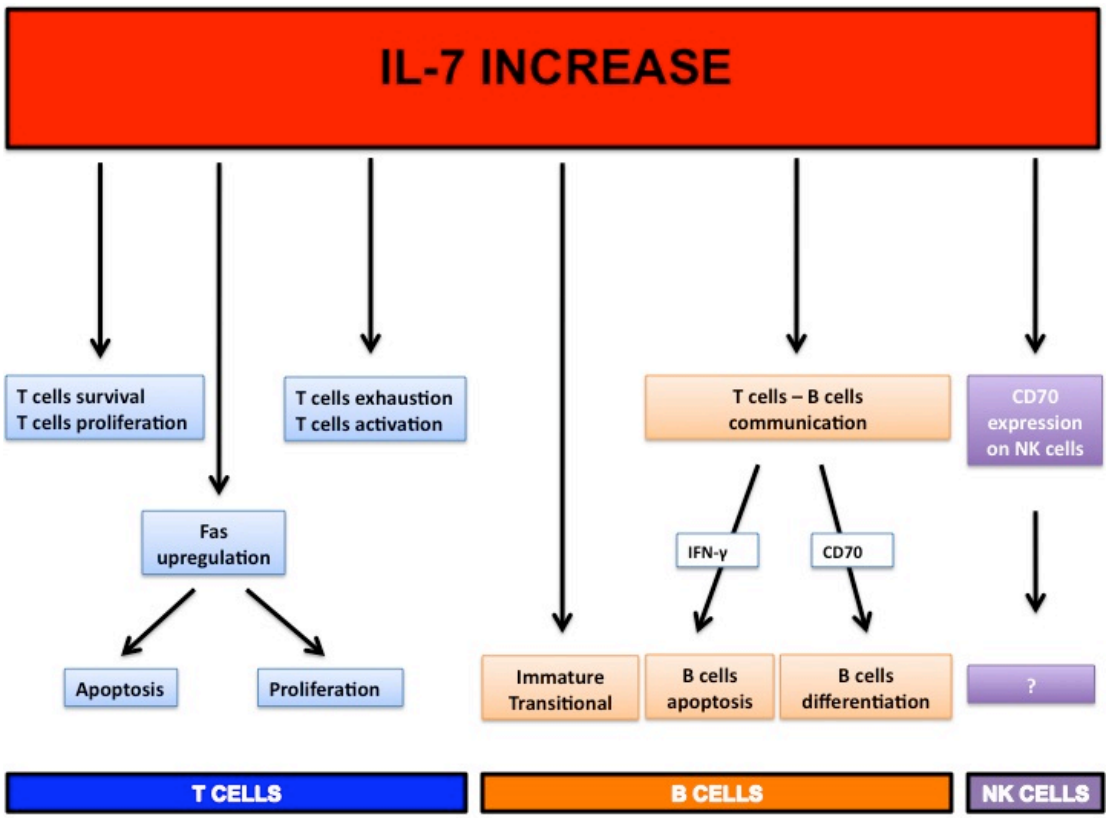

Figure 2. Potential consequences of increased IL-7 levels.
T cells*: IL-7 induces T cells survival by increasing Bcl-2 expression and proliferation through upregulation of CD25 and induction of IL-2. IL-7 can also induce Fas expression on T cells, and prime T cells to context dependent Fas signalling, inducing proliferation of suboptimal activated T cells, or apoptosis in non- activated T cells. IL-7 can also induce expression of activation markers on T cells (CD45RO, CD40L and CD70) and markers associated with T cell exhaustion (PD-1).*
B cells*: High IL-7 levels have been associated with accumulation of Immature Transitional B cells and, as discussed in this thesis, IL-7 impacts on survival and differentiation of B cells, respectively, via secretion of IFN-γ and upregulation of CD70 by T cells.*
NK cells*: IL-7 induces CD70 expression on NK cells, most probably through an indirect effect mediated from IL-7 using cells; upregulation of CD70 expression may possibly induce bystandard activation of CD27-expressing cells.*

2 AIM OF THE THESIS

The present thesis focuses on unravelling how IL-7 can impact on the level of immune activation during HIV-1 infection and how IL-7 and immune activation relate with the homeostasis and dysfunctions of the immune system.

The specific aims of this thesis are:

- Study the impact of disease progression and IL-7 in subset distribution of NK cells during HIV-1 infection

- To evaluate the priming role of IL-7 for Fas proliferative signals of suboptimally activated T cells

- To study the phenotype and functional characteristics of CD28- T cells in relation to disease progression and viral replication

- To dissect the mechanism by which IL-7 can induce Fas upregulation on B cells and enhance their susceptibility to Fas mediated apoptosis

- To evaluate the mechanism by which IL-7 induces IgG production by B cells

All methods used to verify the aims are described in details in the enclosed articles.

3 RESULTS AND DISCUSSION

3.1 Distribution of NK cell subsets identified by CD56, CD27 and CD70 in primary and chronic HIV-1 infection (PAPER I)

NK cells are cytotoxic lymphocytes constituting a major component of the innate arm of the immune system. NK cells play a prominent role in the rejection of tumours as well as in killing of viral infected cells. Due to their functional abilities, NK cells should have a considerable role in the prevention of HIV-1 infection; nevertheless NK cells present with phenotypical and functional impairments during HIV-1 infection (185, 186). Peripheral NK cells are defined within two major groups, based on the expression of CD56: the majority being $CD56^{low}$ and accounting for the NK cells characterised by cytotoxic activity, and around 10% of peripheral NK cells being $CD56^{high}$ with the primary features of cytokine producing cells.

HIV-1 infection has been associated with an altered ratio of $CD56^{low}$ and $CD56^{high}$ NK cell subsets possibly indicating the presence of NK cell dysfunction during HIV-1 infection (182, 183). Therefore we sought to analyze NK cell subsets in a cross sectional study in HIV-1 infected patients, during primary and chronic phases of HIV-1 infection (PHI and CHI, respectively) in order to follow the distribution of NK cells subsets during disease progression.

The ratio of NK cells in PBMCs was not affected by HIV-1 infection or disease progression. The $CD56^{high}$ population contracted during PHI, whereas the same subset became enlarged in patients with CHI as compared with PHI or healthy controls. In addition, the dysregulation of $CD56^{high}$ and $CD56^{low}$ subsets was not corrected by ART during CHI. In parallel with the expression of CD56, we studied the expression of CD27 and CD70 during disease progression, a receptor-ligand pairs belonging to the TNF family (187, 188). It has been demonstrated that NK cells could be divided into $CD27^{high}$ cells, that possess strong effector functions, and $CD27^{low}$ cells, characterised by a higher threshold of activation through their NK inhibitory receptors (189). We found increased CD27 and CD70 expression in both the $CD56^{high}$ and $CD56^{low}$ NK cell

subsets during CHI, as compared to non-infected individuals. We studied the effects of the lymphopenia induced cytokine IL-7 on NK phenotype and we showed that IL-7 can upregulate CD70 expression of NK cells in PBMCs of both healthy and HIV-1 infected individuals.

Based on our results, NK cell functionality might change during disease progression. In chronic HIV-1 patients $CD56^{high}$ NK cells are expanded at the expense of the $CD56^{low}$ subset. Since $CD56^{high}$ NK cells are mainly immunomodulatory and express lower level of inhibitory receptors, this could result in a less regulated NK cells activation with low cytotoxic function but potentially able to increase the level of immune activation via cytokine release. In addition, CD70 upregulation might contribute to immune activation by increasing the activation of CD27 expressing T, B and NK cells. IL-7 may participate in CD70 upregulation on NK cells during HIV-1 infection; however, since the NK cells do not express the IL-7Rα, the effect of IL-7 is most probably indirect, mediated by another, IL-7 sensitive cell type.

3.2 IL-7 priming to T cells proliferation during HIV-1 infection (PAPER II)

Naïve and memory T cells in lymphopenic hosts proliferate in response to low affinity antigens presented on MHC molecules by professional APCs (190, 191). IL-7 is a major regulator of this mechanism, known as HPE, since it was shown that grafting depleted host with IL-$7R^{-/-}$ T cells abolished the proliferative response of T cells to low affinity antigens (59, 192). On the other hand, this ability of IL-7 to enhance T cell proliferation to low affinity peptides during lymphopenia might promote the system to less regulated tolerance; indeed IL-7 mediated T cell activation has been implicated in several autoimmune diseases (50, 193). Due to its harmful potentials in predisposing the immune system to autoimmunity, HPE is controlled by several negative regulators, including activation induced cell death (AICD) and competition for homeostatic cytokines (54, 72).

Fas is involved in the maintenance of homeostasis and tolerance in the immune system, by transmitting apoptotic signals to repeatedly activated T cells as well as to auto-reactive B cells (194, 195). Our group has shown previously that IL-7 can induce Fas expression and Fas mediated apoptosis of resting T cells, and that the plasma levels of

IL-7 correlated with Fas expression and Fas-mediated apoptosis of T cells in HIV-1 infected patients (196). On the other hand, Fas has also been shown to act as a costimulatory molecule for T cell activation, inducing IL-2 production and proliferation upon suboptimal TCR triggering (197, 198). Fas has been extensively studied in T cell apoptosis during HIV-1 infection, however, little is known on Fas involvement in T cells proliferation under lymphopenic conditions.

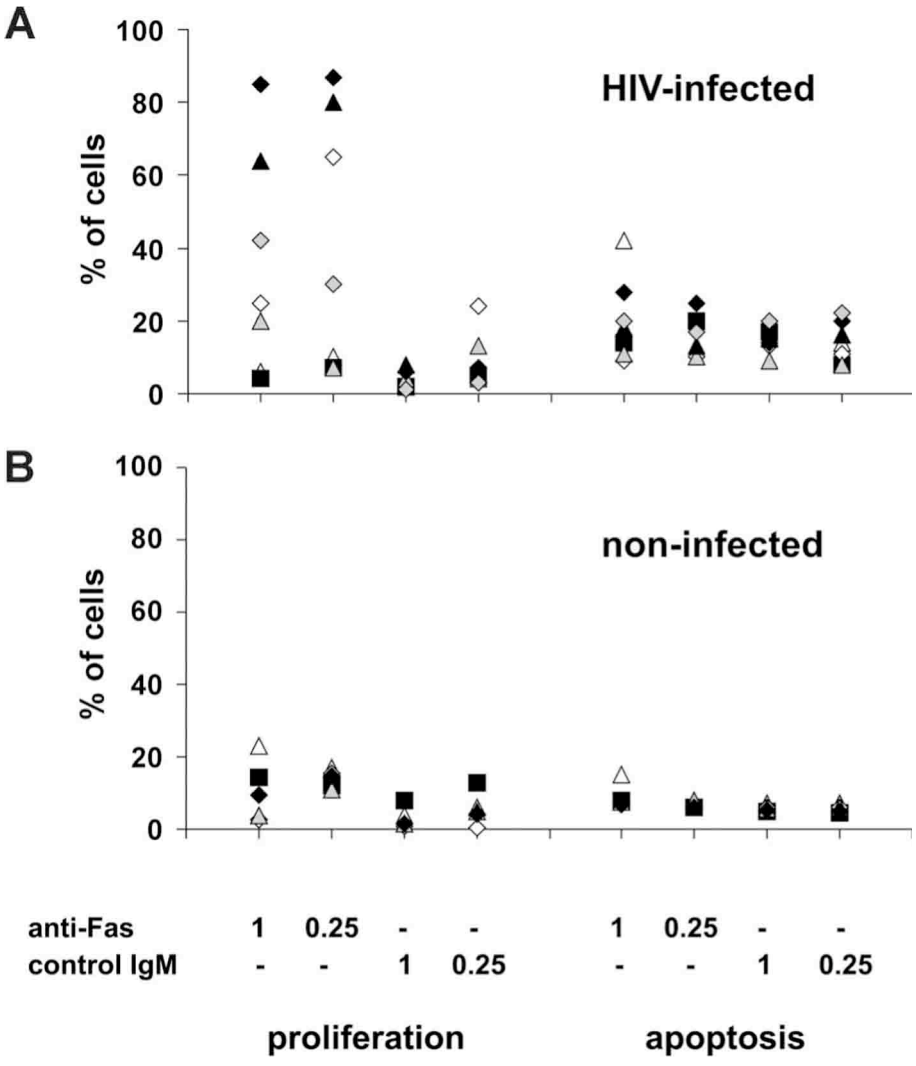

Figure 3. Fas-induced proliferation and apoptosis of purified T cells from HIV-1 infected individuals.
Proliferation and apoptosis of suboptimally anti-CD3 activated T cells from (A) HIV-1 infected patients (n=7) and (B) healthy controls (n=5) combined with an anti-Fas antibody or isotype matched antibody control, at the indicated concentrations.

In this project we studied whether Fas could act as a costimulatory receptor on T cells of HIV-1 infected individuals and whether the lymphopenia induced cytokine IL-7

could be a priming signal sensitizing T cells to stimulatory Fas signals. We found that T cells isolated from HIV-1 infected patients, showed an enhanced proliferation in response to suboptimal TCR triggering in the presence of Fas cross-linking as compared to healthy individuals. Proliferation greatly overcame the Fas induced apoptosis in the same conditions, indicating that Fas contributes primarily to proliferation of weakly activated T cells in HIV-1 infected patients (Fig. 3).

As IL-7 has been shown to increase T cells proliferation and we have previously demonstrated its modulatory effect on Fas expression of T cells (57, 196), we tested whether pre-treatment of T cells from healthy donors with IL-7 for 5 days could prime their Fas-proliferative ability upon suboptimal dose of anti-CD3. Indeed, IL-7 greatly enhanced Fas induced proliferation of suboptimally activated T cells (Fig. 4).

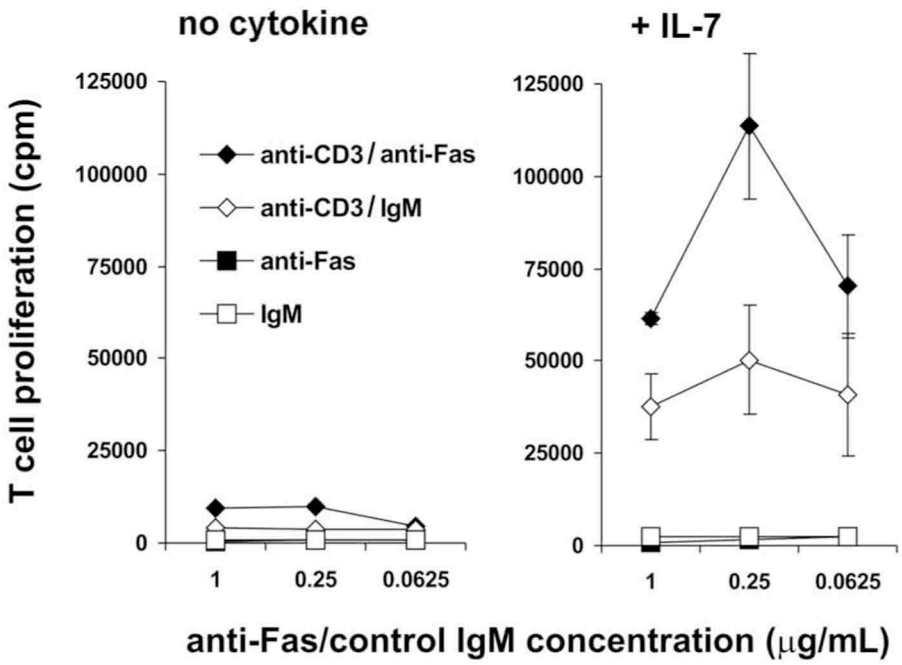

Figure 4. IL-7 priming of suboptimally activated T cells to Fas-mediated proliferative signals
A representative experiment of purified T cells from healthy individuals (total n=3) pre-treated with IL-7 for 5 days (right panel) or left untreated (left panel) and thereafter activated with a suboptimal concentration of anti-CD3 combined with anti-Fas antibody or isotype matched control. Thymidine incorporation measured at day 3 of culture.

The costimulatory action on IL-7 treated T cells by Fas was reflected by the ability of Fas to increase IL-2 production as well as by stimulating CD25 expression on T cells. We studied the apoptotic and costimulatory roles of Fas molecules on different T cell

subsets following IL-7 treatment and we showed that memory T cells were more sensitive to Fas triggering than naïve T cells and that among CD8+ T cells, the Fas-induced T cell proliferation exceeded greatly the level of apoptosis.

As homeostatic proliferation occurs in lymphopenic hosts after recognition of low affinity peptide/MHC complexes presented to T cells by APCs (190, 191), we set up a model to address the contribution of Fas molecules in T cells activation driven by self-antigens. IL-7 pre-treated, or freshly isolated T cells, were cultured together with autologous DCs for 4 days, in the presence or absence of recombinant FasL. Interestingly, Fas mediated signals increased the proliferation of IL-7 treated CD4+ T cells in the presence of autologous DCs further demonstrating the costimulatory role of Fas molecules on IL-7 treated T cells.

Overall, in this project we demonstrated a context dependent regulation of T cell apoptosis and proliferation by Fas molecules. IL-7 increases the sensitivity of resting T cells to Fas signals and the presence of Fas ligand molecules can lead to increased apoptosis of non-activated T cells. On the other hand, increased IL-7 levels promote Fas induced proliferation of T cells that receive weak TCR stimuli, possibly contributing to homeostatic proliferation in T cell depleted hosts.

3.3 Loss of CD28 expression on T cells as a marker of immune activation during HIV-1 infection (PAPER III)

Excessive antigen-driven activation has been proposed as a mechanism inducing progressive loss of T cell effector functions when the immune system reacts with pathogens that are able to establish chronic long-term infections, including HIV-1, HCV, HBV and CMV (199, 200). Persistent antigen activation causes a sustained induction of T cell proliferation, which results in an increased ratio of T cells with a senescent, exhausted memory/effector phenotype (117, 118, 201). CD28- T cells have been reported as antigen experienced T cells with a limited TCR variability, arising from activation of their CD28+ T cells precursors (202, 203). CD28- T cells display shorten telomeres length and show an impaired proliferative ability in response to antigens (117, 204). CD28- T cells have been reported to be relatively resistant to apoptosis, and, as a result, they are found accumulated in several chronic infections

(205), in aged individuals (206) or in patients with diverse autoimmune diseases (207, 208).

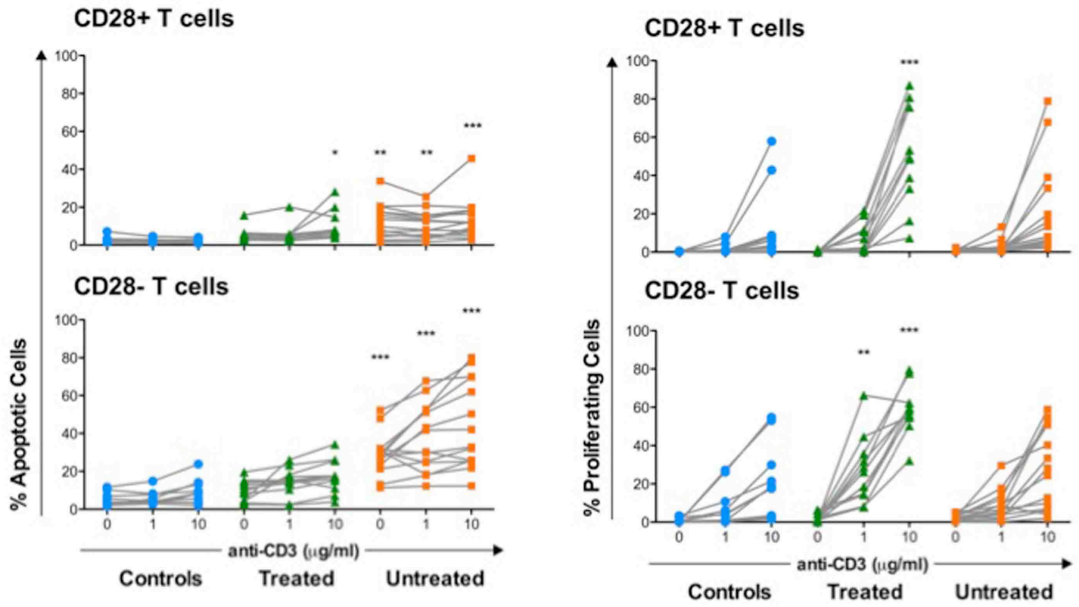

Figure 5. Apoptosis and proliferation property of CD28+ and CD28- T cells
Purified T cells from healthy individuals (n=10, blue) and treated (n=11, green) or untreated (n=14, orange) HIV-1 infected patients. Percentage of apoptotic (left panels) or proliferating (right panels) T cells among CD28+ (upper panels) and CD28- (lower panels) T cells cutured with anti-CD3 at the indicated concentration.

CD28- T cells comprise a large number of T cells in HIV-1 infected patients, and therefore they potentially account for T cell disorders observed during HIV-1 infection. In addition, the majority of CD8+ T cells specific for HIV or CMV derived antigens are CD28-, suggesting that the CD28- subset might represent a memory pool against pathogens that can establish persistent infections. Therefore, functionality of the CD28- T cells can strongly determine immunity against chronic infections. We decided to investigate the phenotypic, survival and proliferative characteristics of CD28- T cells in HIV-1 infected patients either under ART, or naïve to treatment, in order to evaluate the impact of viral replication for CD28- T cells functionality.

According to published reports, CD28- T cells isolated *ex vivo*, or generated *in vitro* through the induction of several rounds of proliferation, were resistant to apoptosis and exhibited impaired proliferative ability (203, 209). CD28- T cells of our studied cohorts, displayed a senescent and apoptotic prone phenotype, with shorter telomere

length, high expression of CD57, Fas and PD-1 and low level of IL-7Rα and Bcl-2 expression.

Functionally, CD28- T cells of patients naïve to treatment showed a low threshold for both spontaneous and activation-induced apoptosis, while in patients under ART the values were comparable to those of healthy individuals. On the other hand, CD28- T cells of patients under ART showed the highest induction of T cell proliferation, using low or high levels of anti-CD3 stimulation, strongly arguing against the association of the CD28- phenotype with replicative senescence (Fig. 5).

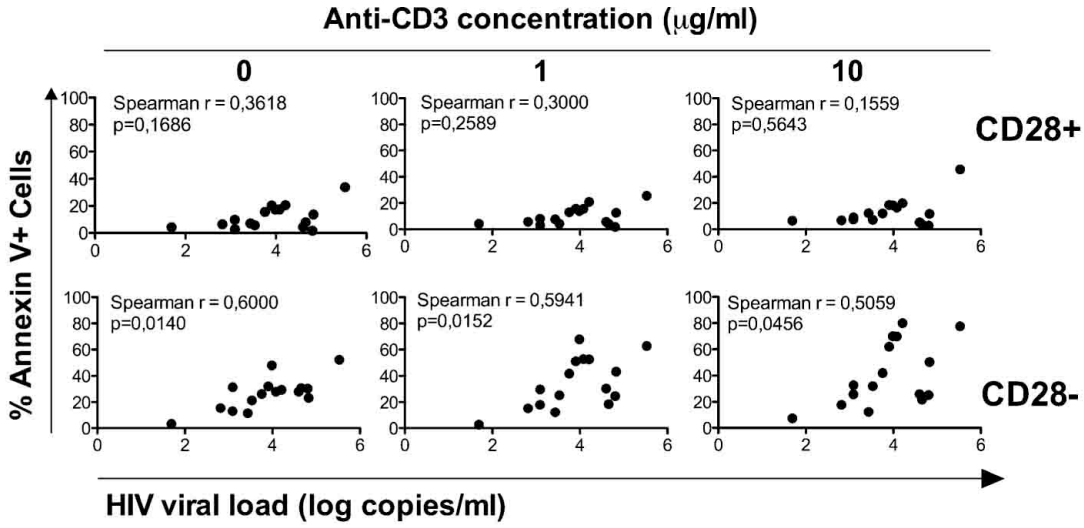

Figure 6. Correlation between viremia and apoptosis of CD28+ and CD28- T cells from HIV-1 infected patients.
Correlation of HIV-1 viral load with spontaneous and activation-induced T cell apoptosis measured on CD28+ (upper panels) and CD28- (lower panels) T cells from HIV-1 viremic patients (2 under treatment and 14 naïve to treatment) activated at the indicated anti-CD3 concentrations. Calculated Spearman r and P values are indicated for each anti-CD3 treatment inside the panels.

Interestingly, the level of viral replication correlated with both spontaneous and activation induced apoptosis of CD28- T cells (Fig. 6). Thus our data suggest that the control of HIV-1 replication with an early initiation of ART might be beneficial for HIV-1 infected patients by preserving highly functional, effector and memory T cells.

3.4 IL-7 promotes Fas-induced apoptosis in B cells via the IFN-γ/STAT1 pathway (PAPER IV)

B cells are not the main targets of HIV-1 infection; nevertheless they display a complex array of dysfunctions during HIV-1 infection (144). Disturbances in B cells responses are manifested by their increased turnover, activation, accumulation of immature, exhausted and apoptosis prone B cell subsets in the circulation as well as hypergammaglobulinemia, loss of memory B cells and reduced level of pathogen-specific antibodies (144, 151, 158, 161, 210). Viral replication and viremia-induced immune activation are considered to be the primary causes of the B cell dysfunctions occurring in HIV-1 infection (144, 161). On the other hand, B cell disturbances are not fully restored by ART indicating the role of viremia independent mechanisms in B cell dysfunctions during HIV-1.

Fas upregulation and sensitivity to Fas mediated apoptosis have been often implicated in the accelerated B cell and T cell depletion during HIV-1 infection (105, 144, 161). Our group has previously shown that IL-7 upregulates the expression of Fas on T cells and render them more sensitive to Fas mediated apoptosis. In HIV-1 infected patients, IL-7 levels correlated with Fas expression on T cells, and with their sensitivity to Fas mediated apoptosis, suggesting that IL-7 might have a role in the accelerated T cells apoptosis in HIV-1 infected patients (196). We have also noticed that IL-7 stimulation of PBMCs induced Fas upregulation on B cells, similarly to T cells (Fig. 7). IL-7 increased Fas expression on all subpopulations of B cells defined by the cell surface markers CD19, CD27, CD10 and CD21, namely naïve, resting memory, activated memory, tissue like memory, germinal center founder and immature transitional B cells. We showed that in addition to increasing Fas expression, IL-7 primed B cells to Fas mediated apoptosis (Fig. 8). Peripheral B cells do not express IL-7Rα and, accordingly, Fas upregulation was not induced by recombinant IL-7 protein if the cytokine was added to purified B cells, but only if T cells were present in culture.

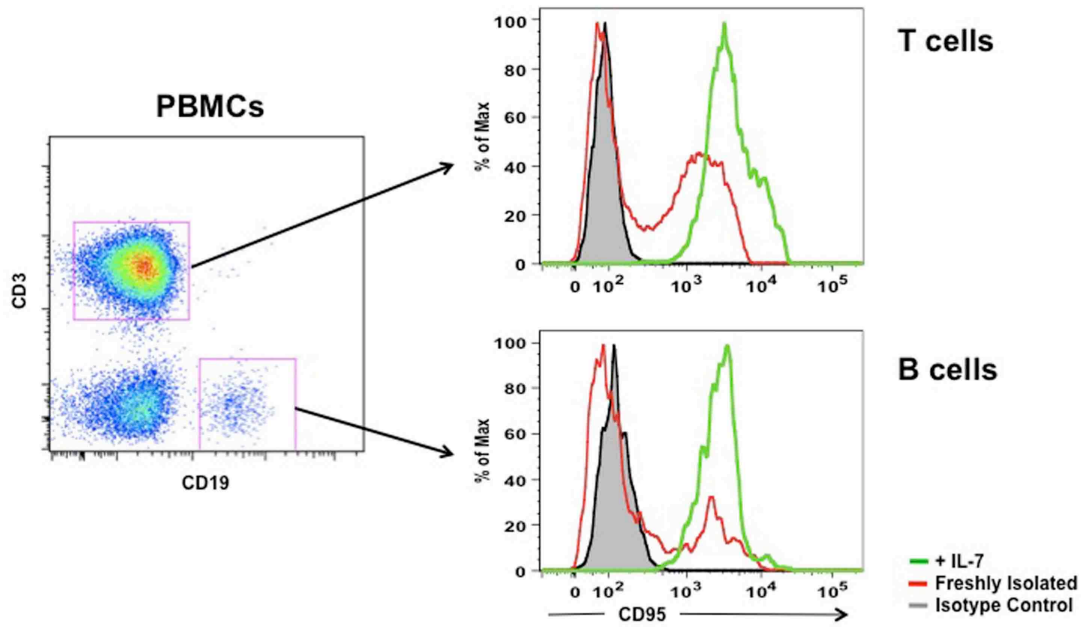

Figure 7. Fas expression on T cells and B cells of IL-7 treated PBMCs
Representative histograms of Fas expression on T cells (CD3+ CD19- PBMCs) and B cells (CD3-CD19+ PBMCs). Fas expression on freshly isolated (red) or after 5 days of IL-7 treatment of PBMCs (green) on T cells (upper histogram) or B cells (lower histogram). Isotype control staining is depicted in gray.

Using IL-7 treated T cell supernatants and transwell experiments we concluded that IL-7 induced Fas upregulation on B cells via a soluble factor released by T cells. To identify the IL-7 induced mediator molecule, we studied a broad range of intracellular phosphorylation events in B cells receiving supernatants from IL-7 treated or untreated T cell. The IL-7 treated T cell supernatants induced STAT1 phosphorylation and, indeed, the upregulation of Fas could be blocked using a STAT1 inhibitor. STAT1 is a canonical signalling component in the IFN-γ pathway. We demonstrated that IL-7 induces IFN-γ secretion from resting T cells and IFN-γ acts as the mediator molecule that upregulates Fas expression on B cells in response to IL-7. IL-7 shared the ability to induce Fas expression on B cells via induction of IFN-γ production with the other γ-chain using cytokines IL-2 and IL-15, but not with IL-4 and IL-21. When T cells were cultured with stromal cells, the efficiency of IL-7 to induce IFN-γ production by T cells was readily increased.

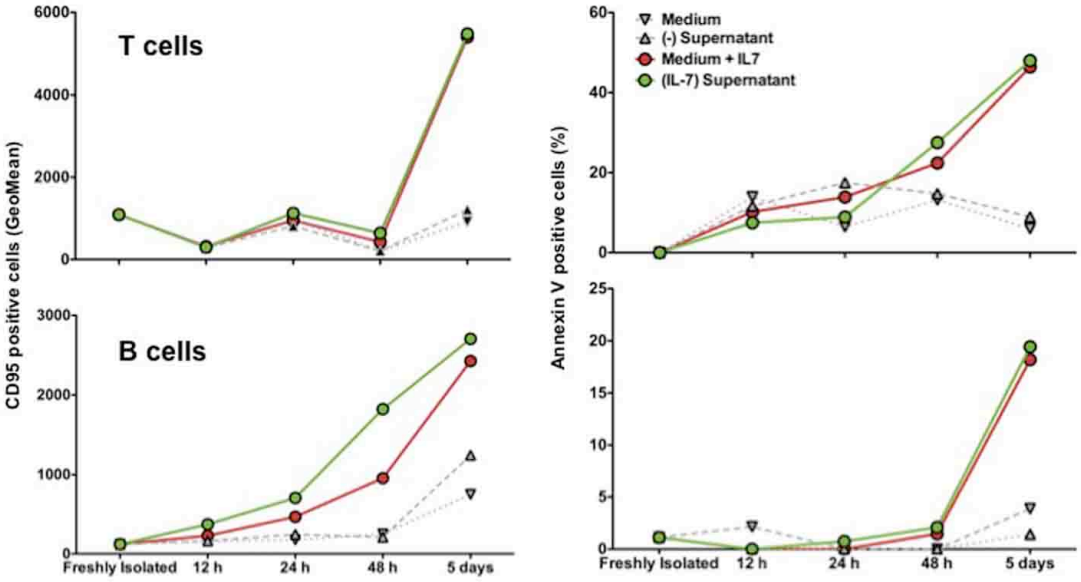

Figure 8. Enhanced Fas mediated apoptosis of T cells and B cells upon IL-7 treatment.
Kinetics of Fas expression (left panels) and Fas-mediated apoptosis (right panels) on CD3+ T cells (upper panels) and CD19+ B cells (lower panels), measured in PBMC cultures. Apoptosis was induced using recombinant FasL for 24 hours in cultures. Data are representative of 3 independent experiments.

Finally we studied the potential role of IL-7 in the regulation of B cell survival during HIV-1 infection, and we correlated plasma levels of IL-7, IL-2 and IFN-γ with Fas expression B cells. We found a strong positive correlation of IL-7 and IL-2 with IFN-γ concentrations and, in the case of ART patients, the levels of all the three cytokines correlated with Fas expression on B cells. As previously reported, highly viremic patients showed increased level of Fas expression and there was no apparent role of IL-2, IL-7 or IFN-γ to induce further Fas expression. Overall, our results suggest a potential role of IL-7 in increasing viremia-independent susceptibility of B cells to Fas mediated apoptosis.

3.5 IL-7 modulates IgG production (PAPER V)

Increased level of IL-7 are often associated with conditions of lymphopenia, including HIV-1 infection or in patients under cytoreductive drugs (58). Owing to its potent ability in increasing T cell survival and proliferation, IL-7 is considered an excellent candidate for the treatment of T cell depleted individuals, with the aim of improving T cells regeneration (69, 211, 212). It is not known how IL-7 levels modulate B cells

responses. IL-7Rα is not expressed by resting B cells, yet we have demonstrated that IL-7 exerts an indirect effect for B cells survival via the induction of IFN-γ (Paper IV). In addition, high levels of IL-7 associate with an increased ratio of immature transitional B cells in HIV-1 infection, during CD4-T lymphocytopenia as well as during IL-7 therapy (69, 165, 213).

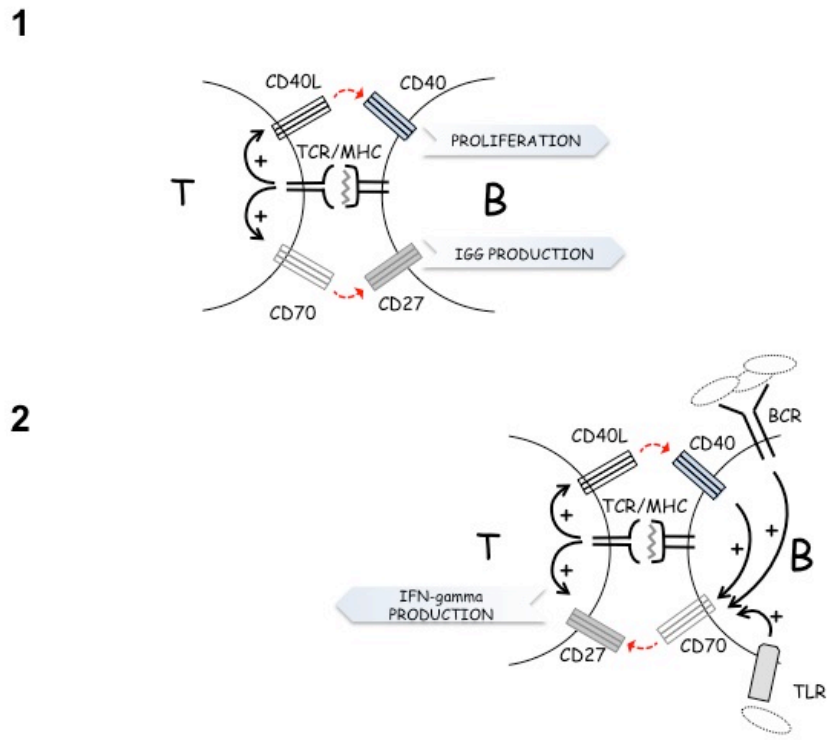

Figure 9. CD70-CD27 costimulatory pathway
CD70-CD27 pathway is involved in T cells - B cells communications. CD70 (ligand) expression is activation dependent, while CD27 (receptor) can be expressed in resting cells. Since CD70 and CD27 can be expressed by T cells and B cells, CD70-CD27 can induce intracellular signalling on both T cells and B cells. (1) CD70-CD27 interaction has been shown to increase IgG production and plasma cells maturation. (2) Activated B cells can efficiently present antigens and costimulate T cells to induce large production of IFN-γ via CD70-CD27 costimulation.

We found that IL-7 treated T cells induced IgG production. This was mediated via an increased survival and activation of B cells, as measured by ratio of $CD20^{-}CD38^{+}$ plasmablasts and proliferating B cells induced by IL-7 treated T cells. Selective blocking IFN-γ, IL-6 and CD40L, all molecules inducible by IL-7 and involved in T cell dependent B cell activation (214, 215), did not lead to the reduction of IgG production mediated by IL-7.

IL-7 treated T cells strongly mediated CD27 down-regulation on B cell cultures. The CD27-CD70 co-stimulatory pathway can enhance the antibody production by purified peripheral B cells and induce plasma cell maturation (216, 217) (Fig. 9). Owing the ability of IL-7 to induce CD70 expression on NK cells, we analyzed the expression of CD70 in IL-7 treated T cells. Indeed, IL-7 induced upregulation of CD70, primarily in memory and activated CD4+T cells. The presence of a CD70 blocking antibody decreased IgG levels, as well as the ratio of plasma blasts and proliferating B cells induced by IL-7 treated T cells. There was no impact on survival of B cells in the presence of the neutralizing CD70 antibody. Altogether our data define a novel mechanism by which, via CD70 upregulation on T cells, IL-7 can modulate antibody production (Fig. 10).

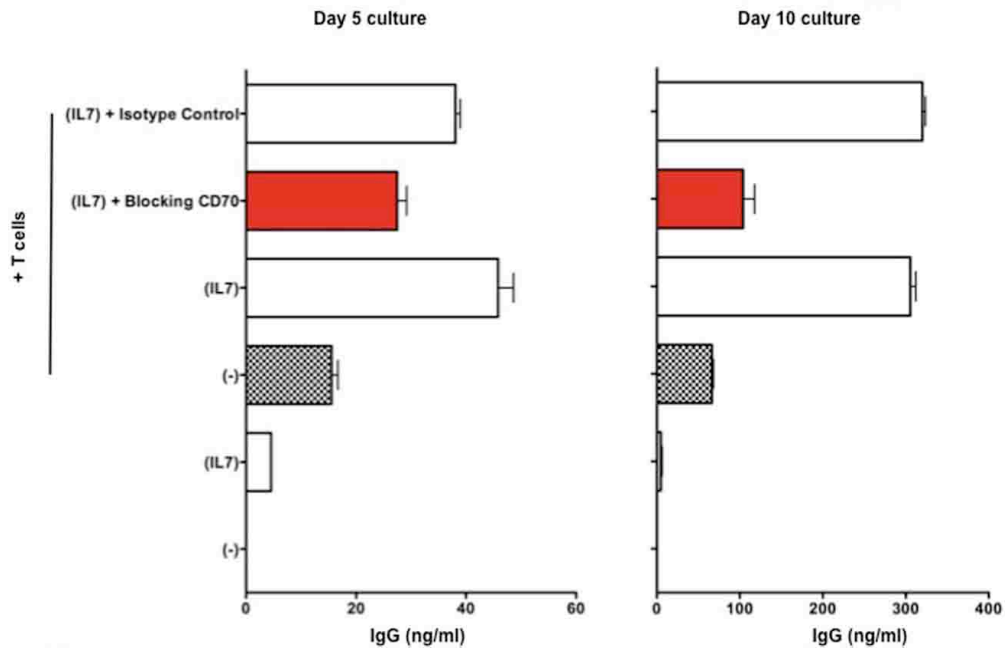

Figure 10. Blockade of CD70-CD27 interaction reduces IL-7 induced IgG production

IgG concentrations measured by ELISA from B cells cultured for 5 or 10 days at different conditions as indicated by the figure.

4 GENERAL CONCLUSIONS AND DISCUSSIONS

In the present thesis I have studied how immune activation influences the homeostasis of NK cells, T cells and B cells during HIV-1 infection, with a special emphasis on the effects of the lymphopenia induced cytokine IL-7.

In **paper I** we studied the role of viral replication and disease progression in regulating the biology of NK cells by analysing the expression of CD56, CD27 and CD70. The message drawn from the project is that in ART treated patients there is an expansion of $CD56^{high}$ NK cells at the expense of the $CD56^{low}$ subpopulation. Since $CD56^{high}$ NK cells have mainly immunomodulatory functions and express lower level of inhibitory receptors (170), their increased ratio might lead to impaired NK cell functionality during chronic HIV-1 infection. NK cells from chronically infected patients expressed high levels of CD70, an activation marker belonging to the TNFR superfamily members. Interestingly, the lymphopenia-induced cytokine IL-7 was able to upregulate CD70 expression on NK cells from PBMC cultures. Since CD27 is expressed on different T, B and NK cell subsets, CD70 upregulation by IL-7 can lead to increased bystandard activation of CD27 expressing cells in conditions characterized by high level of IL-7, including HIV-1 infection.

In **paper II** we demonstrate that IL-7 promotes Fas-induced proliferative signals on suboptimally activated T cells. This study complemented a previous publication of our group, where it was shown that IL-7 can induce Fas expression and sensitize T cells to Fas-mediated apoptosis during HIV-1 infection (196). IL-7 is a major regulator of lymphopenia induced HPE (57). We showed here that T cells from HIV-1 infected patients were sensitive to proliferative Fas signals and that IL-7 primed T cells of healthy individuals to Fas-induced proliferation upon suboptimal activation with anti-CD3 antibodies. These results indicated that Fas-mediated costimulatory signals might contribute to HPE. Altogether it seems that during HIV-1 infection there is a context dependent regulation of T cells by the IL-7 induced Fas molecules. Fas triggering can lead to increased apoptosis of non activated T cells, or can promote Fas induced proliferation of T cells receiving weak TCR stimulation. As the IL-7 therapy clearly showed, high IL-7 levels can lead to increased peripheral T cell numbers in both

chemotherapy treated or HIV-1 infected patients via boosting thymic output, HPE and T cells survival (71). The stimulatory effects of IL-7 on T cells are associated with similar feedback mechanisms that can limit or terminate antigen-specific T cell responses, including the increased sensitivity to Fas mediated apoptosis or the IL-7 induced upregulation of PD-1, a molecule often associated with a functional exhaustion of T cells (196, 218).

In **paper III** we investigated the phenotypic, survival and proliferative characteristics of CD28- T cells and analyzed the impact of viral replication on their functionality. Our data suggest that the proliferative ability and apoptosis sensitivity of CD28- T cells are variables that correlate more with the level of active viral replication of the patients, than with markers of functional exhaustion and replicative senescence. The CD28- subset includes a big part of the CD8+ memory T cells specific for pathogens that can establish persistent infections, like HIV or CMV (205) and therefore, functionality of these cells can strongly determine immunity against chronic infections. Our data suggest that viremia leads to impaired T cell functionality via accumulation of CD28- T cells prone to apoptosis and unable to proliferate upon activation. Thus control of HIV-1 replication with an early initiation of ART, might therefore be beneficial for T cells survival and functionality.

In **paper IV** and **paper V** we studied the indirect effects of IL-7 on B cell homeostasis, potentially important in the settings of HIV-1 infection or in other conditions characterized by increased levels of IL-7. In **paper IV**, we demonstrate that IL-7 stimulates Fas expression on B cells and increase their sensitivity to apoptosis via the induction of IFN-γ production by T cells. In **Paper V** we show that IL-7 is able to upregulate CD70 expression on T cells, which can ultimately lead to IgG production by triggering CD27 molecules on B cells. These results may contribute to a better understanding of the mechanisms leading to impaired B cell functionality in HIV-1 infected individuals.

Fas-induced apoptosis has been reported to be associated with high level of viral replication (161) and we have also detected a strong increase of Fas expression on B cells of viremic patients. On the other hand, when viremia was controlled by ART, plasma levels of IL-7 correlated with IFN-γ concentrations, suggesting that the

production of IFN-γ might be regulated by IL-7. In addition, the concentrations of IL-7 and IFN-γ correlated with Fas expression on B cells in the ART treated patients group, indicating a potential viremia-independent mechanism regulating the susceptibility of B cells to apoptosis during HIV-1 infection. To our knowledge, this is the first work showing that IL-7, a T cell trophic cytokine, can modulate sensitivity to Fas-mediated apoptosis of B cells. It has been shown that increased levels of IL-7 correlate with the occurrence of immature transitional B cells in conditions of lymphopenia, including HIV-1 infection and non-HIV-related idiopathic CD4+T cell lymphocytopenia (165, 213). Since peripheral B cells do not express IL-7Rα, the mechanism of immature transitional B cell accumulation is not known. Our data indicate that IL-7 can indirectly impact on B cell homeostasis via its action of T cells. In line with this hypothesis, IL-7 therapy in humans resulted in a significant decline of peripheral B cell numbers that was reverted 1-2 weeks after cessation of the therapy (69). Although the mechanism for such IL-7 induced B cell decline has yet not been clarified, whether it reflects redistribution or cell death, our results indicate that high IL-7 levels may lead to accelerated B cell apoptosis which in turn could contribute to the decreased number of circulating B lymphocytes.

Lymphopenia, through the increased IL-7 concentration, may thus confer non-antigen activated T cells with general effector function, as demonstrated by the release of IFN-γ. Such a mechanism could contribute to a better immunity, in a situation when the immune system is weakened by lymphopenia, at the price of less regulated and less localized T_H1 type responses that could lead to bystander damage of the B cell pool.

The CD27-CD70 co-stimulatory pathway enhances T and B cells activation, promoting survival and proliferation of T cells or IgG induction from B cells (216, 219). Our data suggest that IL-7 can enhance the B cell stimulatory potential of resting T cells via the upregulation of CD70, possibly contributing to a generalized B cell activation in conditions associated with chronically elevated IL-7 levels. Indeed, enhanced CD70 expression found on T cells from HIV-1 infected patients was suggested as a possible mechanism inducing hypergammaglobulinemia (156). Due to its potent stimulatory effect on T cell proliferation, IL-7 has been considered as an adjuvant for therapeutic vaccines aiming at eradication of tumours (220, 221). These latter works mainly analyzed T cells responses boosted by IL-7. However, our data indicate that IL-7 might

also promote B cell responses. As discussed in **paper I**, IL-7 induces CD70 expression in NK cells, most probably via an indirect mechanism that at the moment remains unknown. Notably, CD70 transgenic mice succumb by opportunistic infections, such as *Pneumocystis carinii* pneumonia, after a fatal T cells immunodeficiency. Excessive CD27 signalling induced effector T cell differentiation at the expense of naïve T cells loss (222). IL-7, by upregulating CD70 expression on T and NK cells could possibly contribute to immune activation, which eventually exacerbates immunodeficiency. Indeed, it has been already reported that homeostatic cytokines can promote effector/memory T cell differentiation from naïve cells in the absence of antigen specific stimulation (214, 223). IL-7 therapy might be beneficial for T cell regeneration, but our data argue against prolonged treatments, in order to avoid the potential effects of IL-7 on abnormal immune activation.

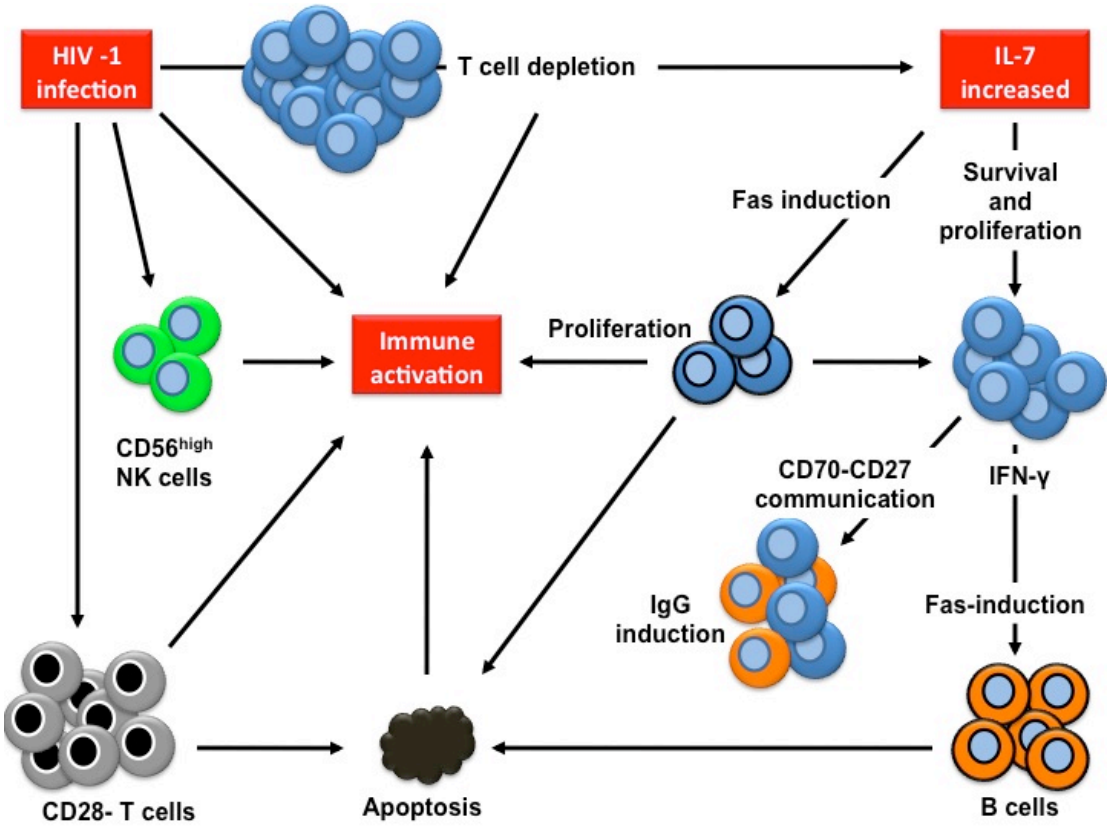

Figure 11. Summary of the thesis findings with focus on potential pathways inducing immune activation during HIV-1 infection.

HIV-1 influences the accumulation of CD28- T cells and CD56^{high} NK cells. The former have proliferative senescence and apoptosis prone phenotypes which impact in immune activation as functionally exhausted T cells. CD56^{high} NK cells are immunomodulatory and therefore can secrete large amount of pro-inflammatory cytokines potentially also fuelling immune activation.

IL-7 is crucial for T cells homeostasis, inducing their survival and proliferation in conditions of lymphopenia. On the other hand, when IL-7 signalling is sustained and dysregulated, it can induce Fas expression on T cells, which can result in either increased Fas-mediated apoptosis of non-activated T cells, or in proliferation of T cell activated with weack TCR signaling. Overall these mechanisms can increase T cells turnover, and therefore, immune activation.

Sustained IL-7 signalling induces IFN-γ production and CD70 expression by T cells. This in turn, can lead to increased suceptibility for apoptosis of B cells and to increased B cell differentiation.

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