

**To my family**



## ABSTRACT

During the course of HIV-1 infection, several B cell dysfunctions occur as result of virus replication and indirect mechanisms of immunopathology. The B cells abnormalities include hypergammaglobulinemia, a decreased number of memory B cells, increased levels of activation markers on B cell surface and plasmacytosis. The molecular bases for these impairments are not fully characterized but may have relevance for designing functional HIV vaccines and improved treatment.

**In paper I** the expression of chemokine receptors/chemokines important for B cell function was determined on cells from HIV-1 infected patients and controls. We studied the CXC chemokine receptor 4 (CXCR4), CXCR5, and CC chemokine receptor 7 (CCR7) and their respective ligands. We found a decreased expression of CXCR5 to be present on blood B cells from patients ( $P < 0.05$ ), in association with low CD4<sup>+</sup> T-cell counts. Interestingly, B cells in blood and lymph nodes from HIV-1-infected patients also displayed an increased expression of the CXC chemokine ligand 13 (CXCL13), the ligand for CXCR5. Upon B-cell activation in vitro, CXCL13 was secreted in culture. The findings suggest that altered CXCR5/CXCL13 expression may participate in B-cell dysfunctions during HIV-1 infection. Loss of memory B cells is a regular finding in the blood of HIV-1 infected patients and the possibility exists that increased apoptosis via the Fas death receptor pathway may participate in this pathological mechanism. Interleukin-7 (IL-7), present to high levels in blood of HIV-1 infected patients, was previously reported to lead to increased Fas expression and Fas mediated apoptosis on T cells. **In paper II**, a novel mechanism responsible for increased B cell apoptosis in presence of the high IL-7 concentration was described. T cells cultured with IL-7 induced high Fas expression on resting B cells together with an increased sensitivity to Fas mediated apoptosis. As the mediator responsible for B cell priming to Fas mediated apoptosis we identified the cytokine IFN- $\gamma$  that T cells secrete in response to IL-7. These results indicate a potential link between IL-7 and the increased B cell apoptosis in HIV-1 infected individuals. During HIV-1 infection, loss of memory B-cells, together with an altered differentiation of naïve B-cells, result in production of low quality antibodies, which may be due to impaired immunoglobulin affinity maturation. **In paper III**, we evaluated the effect of HIV-1 infection on class switch recombination and somatic hypermutation, crucial processes for the generation of functional antibodies, by studying the expression of activation-induced cytidine deaminase (AID) in peripheral B-cells from HIV-1 infected patients and healthy controls. We also studied the phenotype of B cells and their ability to produce immunoglobulins in vitro. Cells from HIV-1 infected patients showed higher baseline levels of AID expression and increased IgA production measured ex-vivo and upon CD40 and TLR9 stimulation in vitro. Moreover, the percentage of CD27(-)IgA<sup>+</sup> and CD27(-)IgG<sup>+</sup> B-cells in blood was significantly increased in HIV-1 infected patients. Interestingly, our results also showed a significantly increased number of somatic hypermutations in the VH genes in CD27(-) cells from patients. Taken together, the results show that during HIV-1 infection, CD27(-) B-cells can produce class switched and somatically hypermutated antibodies. High levels of soluble CD27 (sCD27), a marker of immune activation, are found during HIV-1 infection; whether sCD27 has a biological role on B cells was previously not known. The aim of **paper IV** was to investigate whether sCD27, by binding to CD70, can induce IgG production from B cells. B cells from healthy and HIV-1-infected individuals were cultured with recombinant human sCD27 (rhsCD27) and IgG production was measured in culture. We demonstrated that rhsCD27 induced IgG production from antigen-primed (CD27<sup>+</sup>) B cells. This effect was mediated by rhsCD27 binding to CD70 on B cells leading to activation of Blimp-1 and XBP-1, transcription factors associated with plasma cell differentiation. We found a significant correlation between the levels of serum sCD27 and IgG in HIV-1-infected individuals and healthy controls. The sCD27 may act to enhance immunoglobulin production and differentiation of activated memory B cells, thus providing an activation signal to antigen-experienced B cells. This mechanism may operate during HIV-1 infection when continuous immune activation may lead to up-regulation of CD70 expression and increased sCD27 cleavage and account for increased levels of circulating IgG. In conclusion, in this PhD thesis different mechanisms leading to impairments of B cell function observed during HIV-1 infection, in parallel to abnormal events of immune activation, are characterized.

## LIST OF PUBLICATIONS

- I. Cagigi A, Mowafi F, **Phuong Dang LV**, Tenner-Racz K, Atlas A, Grutzmeier S, Racz P, Chiodi F, Nilsson A. *Altered expression of the receptor-ligand pair CXCR5/CXCL13 in B cells during chronic HIV-1 infection*. Blood. 2008 Dec 1;112(12):4401-10. Epub 2008 Sep 9.
- II. Sammiceli S, **Dang VPL**, Hong TP, Vivar N, Ruffin N, Chiodi F and Rethi B. *IL-7 promotes CD95-induced apoptosis in B cells via the IFN- $\gamma$ /STAT1 pathway* (Submitted)
- III. Cagigi A, Du L, **Dang LV**, Grutzmeier S, Atlas A, Chiodi F, Pan-Hammarström Q, Nilsson A. *CD27(-) B-cells produce class switched and somatically hyper-mutated antibodies during chronic HIV-1 infection*. PLoS One. 2009;4(5):e5427. Epub 2009 May 1.
- IV. **Dang LV**, Nilsson A, Ingelman-Sundberg H, Cagigi A, Gelinck LB, Titanji K, De Milito A, Grutzmeier S, Hedlund J, Kroon FP, Chiodi F. *Soluble CD27 induces IgG production through activation of antigen-primed B cells*. J Intern Med. 2011 Sep 14. doi: 10.1111/j.1365-2796.2011.02444.x.

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## LIST OF ABBREVIATIONS

AFC	Antigen forming cell
AID	Activation-induced cytidine deaminase
AIDS	Acquired immunodeficiency syndrome
ASC	Antigen secreting cell
BAFF	B cell activating factor
Bcl	B cell lymphoma
BCR	B cell receptor
Blimp-1	B lymphocyte induced maturation protein 1
BM	Bone marrow
CLP	Common lymphoid progenitor
CSR	Class switch recombination
DC	Dendritic cell
FDC	Follicular Dendritic cell
GC	Germinal centre
HAART	Highly Active Antiretroviral Therapy
HCDR	Heavy chain complementary determining region
HIV	Human immunodeficiency virus
HSC	Hematopoietic stem cell
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LPS	Lipopolysaccharide
LTNP	Long term non-progressor
MHC	Major histocompatibility complex
Nab	Neutralizing antibody
NHL	Non-Hodgkin lymphoma
Pax 5	Paired box protein 5
PC	Plasma cell
SHM	Somatic hypermutation
SIV	Simian immunodeficiency virus
TD	T-cell dependent
TI	T-cell independent
T(FH)	Follicular helper T
TCR	T cell receptor
TGF	Tumor growth factor
TLR	Toll like receptor
TNF	Tumor necrosis factor
XBP-1	X-box binding protein 1

# 1 INTRODUCTION

The focus of the present PhD thesis has been to investigate mechanisms of B cell immunology and B cell dysfunction occurring during infection with human immunodeficiency type-1 (HIV-1). The data presented in the thesis have been generated analyzing *ex-vivo* biological samples obtained from HIV-1 infected patients but experiments have also been performed *in vitro* to pin-point different mechanisms.

## 1.1 B cell development and differentiation

B cell development, activation and differentiation are tightly regulated processes in order to maintain B cell homeostasis and to generate effective humoral immune responses.

### 1.1.1 B cell development and activation

Human B lymphocytes are generated from hematopoietic stem cells (HSC) through sequential steps of development transforming stem cells to common lymphoid progenitors (CLP), progenitor B cells (pro-B), pre-B and immature B cells. The process of genetic and phenotypic transformation occurs in close contact with stromal cells in the bone marrow (BM) [1]. Immature B cells differentiate to naive B cells through series of transitional stages either in BM or spleen [2], where they become long-lived and are able to generate survival signals through the B cell receptor (BCR) [1].

B cell differentiation in the periphery is typically initiated in response to exogenous stimuli (antigens) and these responses are divided in T-cell dependent (TD) or T-cell-independent responses (TI). In secondary lymphoid tissue, upon encountering an antigen and help from cognate CD4<sup>+</sup> T cells through interactions between the Major

Histocompatibility Complex (MHC)- T cell receptor (TCR), CD40-CD154, CD80/CD86-CD28 and cytokines, B cells are clonally expanded and differentiate [1]. Ruprecht & Lanzavecchia suggested that besides BCR signaling and T cell help, a third signal mediated through Toll like receptors (TLRs) is also essential for differentiation of naive B cells [3]. Memory B cells, in order to differentiate to plasma cells (PC), require a lower number and lower magnitude of triggering signals than naïve B cells [4].

A proportion of fully activated B cells differentiate to extrafollicular short-lived PCs in lymphoid tissue with the capacity to secrete low affinity Immunoglobulin M (IgM) [5, 6]. Alternatively, B-cells migrate back to the B-follicle forming the germinal centre (GC) for further differentiation to memory B cells and long-lived PCs [5]. The molecular events leading to the decision between commitment to extrafollicular PC or GC B cell are not fully characterized. Studies conducted in mouse models showed that B cells with high signaling intensity through the BCR or/and CD40 preferentially differentiate to extra-follicular PCs. Another striking finding suggested that up-regulation of B lymphocyte induced maturation protein 1 (Blimp-1) is also implicated in the formation of short-lived PCs [5, 7].

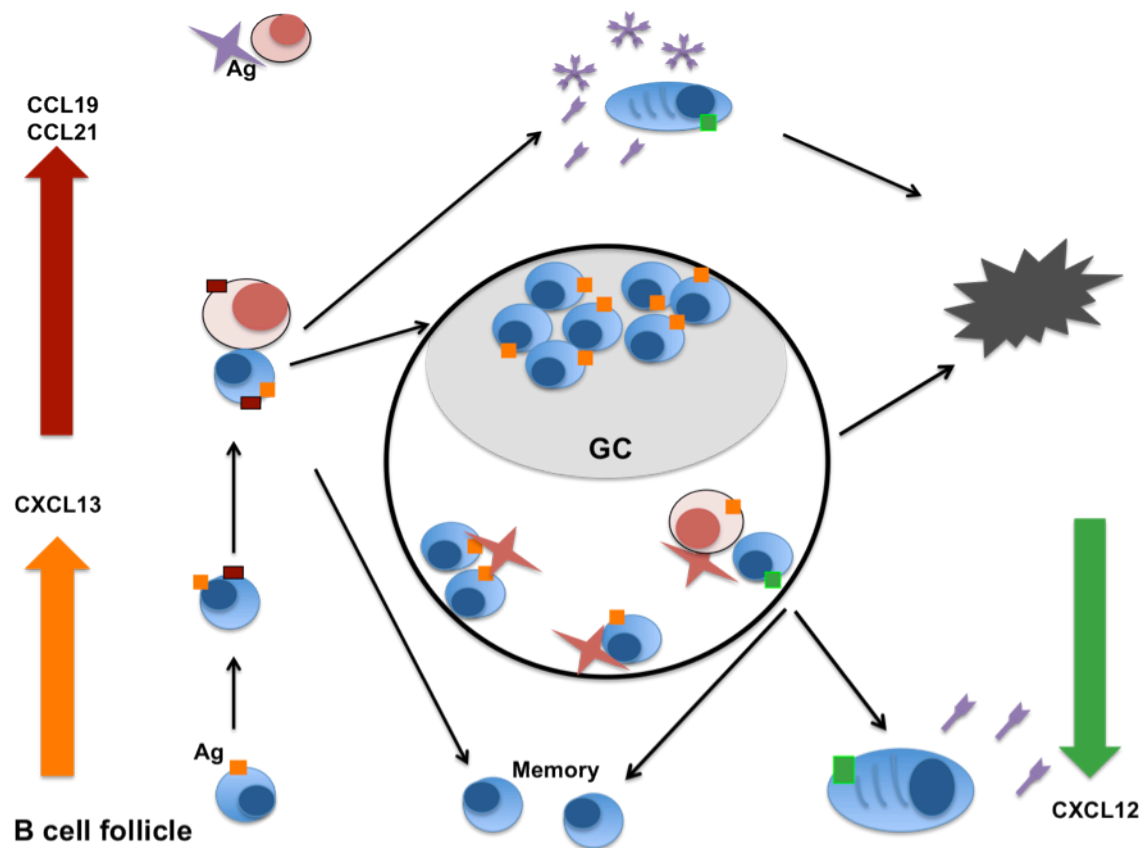
The GC is a specialized microenvironment composed of a dark zone (containing packed proliferating centroblasts) and a light zone (consisting of non-dividing centrocytes, Follicular helper T cells (T(FH)) and a network of Follicular Dendritic cells (FDCs)) [8] (Figure 1). In the dark zone, centroblasts undergo somatic hypermutation (SHM), an Activation-induced cytidine deaminase (AID)-dependent process, and thereby diversify their antigen receptor. By a yet undefined mechanism, B cells stop proliferating and mature into centrocytes. Some of the centrocytes undergo a



second type of recombination called class-switch recombination (CSR), a process mediated by AID, by which B cells replace the Ig heavy chain segments  $C\mu/C\delta$  (IgM/IgD antibody) with the downstream segment  $C\alpha$  or  $C\gamma$  or  $C\epsilon$  (IgA, IgG or IgE antibody) (reviewed in [9]). The T cell help and cytokines determine the exact isotype to which B cells switch. The CSR can also occur in TI responses inducing switch to IgA [10].

Centrocytes will then be selected according to their ability to bind antigen-complexes captured by FDCs and to elicit help from T(FH) cells. The process of selection of centrocytes is not fully elucidated but recent data suggest that the T(FH) cell is one of the main forces to drive selection of high affinity B cells for survival and differentiation [11]. Thereafter, the centrocytes have three developmental options: go back to the dark zone as centroblasts and re-start another round of SHM; leave the GC as memory cells/PCs or undergo apoptosis [9]. Antibody-forming cells can repress IL-21 and B-cell lymphoma 6 (Bcl-6) expression in naive T (FH) cells, thus providing a negative feed-back loop for the GC reaction and thereby controlling the magnitude of the ongoing immune response [12].

The molecular interactions leading to either PC or memory B cell formation are poorly understood and several hypotheses have been proposed in this context. Several authors have suggested the involvement of different members of the tumor necrosis factor (TNF) family where persistent CD40 ligation or CD27 interaction lead to the preferential differentiation into memory B cells or PCs respectively (reviewed in [13]). Tarlinton DM et al. proposed that PC differentiation was triggered in part by high affinity interactions between the BCR and the antigen [14].



**Figure 1. B cell differentiation in secondary lymphoid tissues**

*Naïve B cells migrate to the B cell zone in secondary lymphoid tissue in a CXCR5/CXCL13 dependent manner. After antigen-activation, B cells up-regulate CCR7 and migrate toward the T-B cell border to receive T cell help. Following the extra-follicular B cell response, short-lived PCs develop and secrete low affinity antibodies. B cells migrate back to the B cell follicle and form the GC. In the dark zone of GC, centrocytes proliferate and undergo SHM. Moving to the light zone, the centroblasts undergo CSR and selection through close interaction with FDCs and T(FH) cells. The centroblast leaves the GC to become either a memory B cell or home to the BM as a long-lived PC in a CXCR4/CXCL12 dependent way. Non-selected centroblasts may go back to the dark zone for another SHM round or undergo Fas-induced apoptosis. The small rectangles on B cells indicate chemokine receptors.*

### **1.1.2 Pathways involved in B cell proliferation and differentiation**

#### *1.1.2.1 Receptor-ligand pairs*

Multiple pathways are involved in tuning of B cell responses and different molecules are functionally implicated at distinct stages of B cell development and differentiation. CD40 is ubiquitously expressed on B cells whereas its ligand CD154 is transiently expressed on activated T cells. CD40 signaling promotes GC formation, affinity maturation and the differentiation toward memory and PCs [15]. The importance of CD40 signaling pathway is underlined by studies of CD40-knockout mice and patients with deregulation of CD40 signaling, where high affinity and switched memory B cells are missing [15, 16]. CD27 is shown to act at the sequential step after B cell activation by CD40 ligation and these molecules inhibit the function of one another in a mutually exclusive manner. While CD40 ligation is involved in enhancing B cell proliferation, CD27 signaling induces PC differentiation and Ig production [17-20]. In addition, CD70 can also operate as a receptor, since cross-linking of CD70 leads to proliferation of human malignant B cells *in vitro* and differentiation of B cells *in vivo* in a CD70-transgenic mouse model [21, 22].

#### *1.1.2.2 Transcription factors*

Commitment to PC differentiation requires a number of transcription factors, of which the most important are Bcl-6, Paired box protein 5 (Pax 5), X-box binding protein (XBP-1) and Blimp-1. Bcl-6 is a critical regulator of T(FH) cell formation and GC reaction [23, 24]. Pax 5, on the other hand, is essential for both early development of B cells and during the GC reaction. Pax 5 activates cluster of genes and transcription factors participating in BCR signaling such as CD19 and CD79A, and represses those

genes involved in Ig secretion including IgH, XBP-1 and Blimp-1 (reviewed in [13]). Blimp-1 is detected in PCs in both TI and TD responses, including memory-derived PCs and also in a small subset of GC B cells [25]. Blimp-1 has the dual-function to ensure irreversible differentiation of PCs: i) removal of inhibitors including Pax 5 and Bcl-6 and ii) induction of genes required for proliferation and Ig secretion [13].

#### *1.1.2.3 Cytokines*

The development, differentiation and survival of B cells are governed, among other factors, by cytokines that function in tissues or systemically (reviewed in [26]).

T helper cell-derived cytokines, which are considered to be crucial regulators for B cell differentiation and antibody maturation, consist of Interleukin-2 (IL-2), IL-4, Interferon- $\gamma$  (IFN- $\gamma$ ), IL-21, IL-10 and Tumour Growth Factor- $\beta$  (TGF- $\beta$ ) [26]. While IL-4 is a switch factor for IgG and IgE, TGF- $\beta$ , which is found in mucosal tissues, induces IgA switching. IL-10 has been implicated in the induction of IgG antibodies and IFN- $\gamma$  drives IgG2 class switching [26, 27]. On the other hand, IL-10 also exerts regulatory functions by inhibiting the production of IL-2 and IFN- $\gamma$  from macrophages and activated T cells [28] and IFN- $\gamma$  inhibits the production of IL-4 secreted from T helper type 2 (Th2) cells [26, 29].

IL-7 is produced by stromal cells in lymphoid tissues and plays a non-redundant role in the development and maintenance of T cells [30]. The IL-7 receptor is a heterodimer composed of the IL-7R $\alpha$  chain and the common cytokine-receptor- $\gamma$ -chain [31]. IL-7 is also required for B cell lymphopoiesis in mice; specifically, IL-7 has been shown to participate in the differentiation of CLP to pro-B cells, to promote survival of pro and pre-B cells and to stimulate VDJ rearrangement at distant V<sub>H</sub> gene segments during

early development [30]. B cells in adult mice lacking either chains of the IL-7 receptor cease to develop at the stage of pro-B cells [29]. Interestingly, a few patients suffering from severe combined immunodeficiency (SCID) with identified point mutations in the IL7R genes, leading to undetectable IL7R mRNA level, showed normal or increased B cell numbers which suggests that IL-7 play a different role in B cell lymphopoiesis in humans [30, 32].

B cell activating factor (BAFF), belonging to TNF family, is a crucial factor for the survival of immature and PCs [33, 34]. In mature B cells, BAFF might play a different role since BAFF is shown to induce CD40-independent class switch to IgG1, IgE and IgA antibodies [34].

### **1.1.3 B cell homing**

Chemokine receptors, belonging to the family of G-protein coupled receptors, are the key regulators of leukocyte migration between blood and tissues, and thereby play important roles in dendritic cell (DC), B and T cell maturation as well as in the development of the GC [35]. While some chemokines are produced constitutively in tissue, others are only secreted in response to activation stimuli to recruit functional lymphocytes to the proper site for further development.

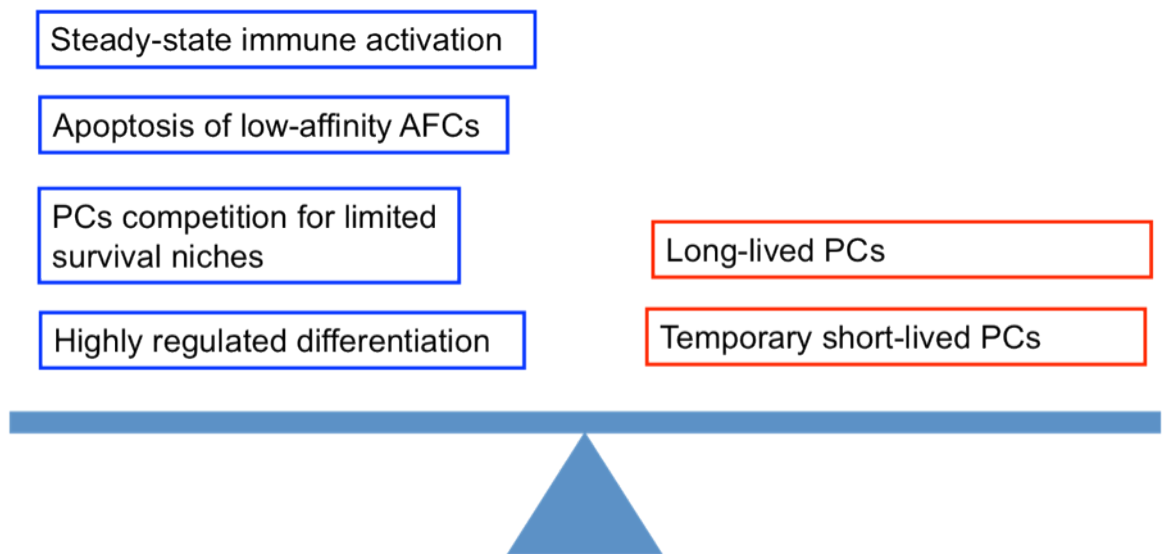
Mature naïve B cells migrate from blood to lymph nodes in a CXCR5-dependent manner toward a higher gradient of the ligand CXCL13 produced by stromal cells in the B cell follicle and from FDCs and T (FH) cells [26]. Following antigen activation, B cells up-regulate CCR7 and increase the responsiveness toward the corresponding ligands CCL19 and CCL21, which facilitates migration toward the border of the B and T cell areas where high levels of CCL19 and CCL21 can be found [36, 37]. After

further differentiation, long-lived PCs or memory B cells leave the lymphoid tissue. CXCR5- and CCR7 expression decrease while CXCR4 expression increases, which induces homing to the red pulp of the spleen or to the medullary cords of lymph nodes where CXCL12 is present (Figure 1). Down-regulation of CXCR5 and CCR7 is enough to drive the PCs out of the white pulp or lymph node cortex but the real driving force for PC homing to the BM is CXCR4 [36, 38]. IgA-secreting cells, however, home to epithelial tissue, a process that involves the chemokine receptors CCR9 and CCR10 [39].

GC maintenance also depends to a certain extent on CXCR4 and CXCR5 expression [40]; mice lacking CXCR5 tend to have small and mislocated GCs [41, 42]. However, the migration of cells may be regulated by responsiveness to the chemokine more than the exact level of receptor surface expression, but how B and T cells interpret and orchestrate two chemokine signals simultaneously is unknown.

#### **1.1.4 Maintenance of serological memory**

The serum Ig levels are maintained at a steady-state level by several mechanisms: i) a tightly regulated process of activation/differentiation and selection of B cell clones for PC development; ii) elimination of antibody secreting cells (ASCs) once the antigen is cleared and iii) the competition of newly produced PCs with the previous PCs residents in BM for cytokines and survival niches [43] (more in figure 2).



**Figure 2. Maintenance of immunoglobulin levels during physiological condition.** *Several mechanisms regulate the production of Ig during physiological conditions, leading to constant level of Igs in serum. AFC: Antibody forming cell.*

Long-term serological memory is maintained by long-lived PCs and memory B cells. Memory B cells are phenotypically diverse, reflecting different developmental pathways (TI and TD responses) [44, 45]. Although, CD27 was once considered to be “the” memory B cell marker many authors have shown that not all human memory B cells express CD27 [46-48]. Recently a CD27 negative memory B cell population, with low number of mutation and tonsil-like phenotype, was found in peripheral blood of humans; their role remains however unclear [47, 49]. In addition a new subset of B220- memory B cells was found in the BM and spleen of mice. These cells showed a greater capacity to secrete antibodies as compared to the conventional B220+ memory cells and displayed a self-replenishing capacity [50].

### **1.1.5 Mature B cell survival and apoptosis**

During the GC reaction, B cells pass a checkpoint in which B cells with low or undesired specificity undergo apoptosis. B cell is rescued from apoptosis only if the BCR efficiently binds to and takes up the antigen, through BCR, and CD40 interactions. The CD40 signal also increases the expression of anti-apoptotic molecules like the Bcl-xl family, which is further augmented by BCR engagement [51]. The fact that the loss of low affinity clones is mediated by apoptosis is indicated by their persistence in Bcl-xl transgenic mice [52]. The role of Fas (also called CD95) is not completely elucidated but down-stream signaling of Fas is induced in GC B cells undergoing apoptosis. However, it is unknown whether B cells need Fas engagement to undergo apoptosis or if they are prone to apoptosis and only escape the elimination when proper surviving signals are provided [53]. In Fas deficient *lpr* mice, the selection of high affinity B cells within the GC is impaired, suggesting that Fas is required for clonal selection [54].

In post GC cells, maintenance of memory B cells and long-lived PCs is not completely understood. Survival of these cells is regulated by a complex interplay between pro-apoptotic and anti-apoptotic molecules of the Bcl-2 family. Thus, Bcl-2 transgenic mice have an expanded memory B-cell compartment due to enhanced size and half-life of memory B cells [55]. However, the importance of other factors such as BAFF, IL-6 and TNF- $\alpha$  has also been shown for memory B cell survival [56]. Whether memory B cells are dependent on BCR triggering by a persistent antigen for long-term survival has also been debated. However, elegant studies in the mouse indicated that the BCR of a memory B cells could be altered to another specificity never seen by the animal with persistence of memory B cells (44). In immunization experiments with titration of the



antigen dose to very low levels, there was no significant difference in formation and persistence of memory B cells [57].

Long-term PCs survive in close contact with stromal cells in BM niches. Several studies have indicated a crucial role for CXCL12, IL-6 and BAFF for PC survival [56]. Recent data from Tarlinton et al. have shown that PC survival is dependent on Mcl-1 (personal communication), an additional anti-apoptotic protein of the Bcl-2 family previously demonstrated to play an essential role for GC formation and B cell memory [58]. This is supported by older findings of Mcl-1 positive PCs in human spleen [59].

## **1.2 HIV-1 infection**

### **1.2.1 Immune activation during HIV-1 infection**

Abnormal levels of immune activation, defined as hyperactivation, occur during HIV-1 infection. The relevance of persistent immune activation for HIV-1 induced pathogenesis has been confirmed in many studies comparing HIV-2 and HIV-1, as well as non-pathogenic and pathogenic simian immunodeficiency viruses (SIV). The hyperactivation is defined by increased lymphocyte activation, increased production of pro-inflammatory cytokines and loss of immune functions, features all associated with increased microbial translocation, destruction of lymphoid architecture and lymphopenia [48].

Although CD4<sup>+</sup> T cells are the main targets for HIV-1 infection, a number of alterations related to direct and indirect effect of HIV-1 have been observed in many lymphocyte populations including B cells. B cell dysfunctions were already reported in 1980s but the pathogenic mechanisms underlying B cell impairments remain unclear. B

cell maturation and differentiation are tightly regulated; therefore, aberration at any stage of development could result in dysfunction of the B cell response as well as perturbation of the B cell compartment.

### **1.2.2 Aberrant activation of B cells in HIV-1 infection**

HIV-1 induced B cell hyperactivation is one of the major pathogenic hallmarks of HIV-1 infection and it is thought to play a critical role in B cell dysfunctions. The aberrant activation of B cells is characterized by many features including up-regulation of activation, proliferation and terminal differentiation markers on circulating B cells, increased polyclonal B-cell activation, impaired response to antigens, elevated serum levels of immunoglobulins and increased differentiation of B cells to plasmablasts/PCs. The evidence that B cell hyperactivity may be, to some extent, directly induced by viral replication is illustrated by many studies, which demonstrate that the vast majority of dysfunctions associated with immune activation are decreased with HAART treatment [60-63]. In addition, the persistence of several B cell dysfunctions during HAART, together with incomplete normalization of factors associated with B-cell hyperactivity might imply that at least part of B cell immunopathology may be due to factors other than viral replication, such as lymphopenia [64].

The peripheral blood B cell compartment is comprised to a large extent of naive B cells, followed in number by memory cells and other subpopulations, including immature transitional B cells, activated B cells, tissue-like memory B cells and plasmablasts. The proportion of different B cell populations might vary among different individuals, but is kept at a relatively constant level in a defined age period in an individual. During HIV-1 infection, however, the relative proportions of the different populations within the B cell compartment are distorted (Table 1).

An increased expression of activation markers including CD38, Fas and CD70 is reported to occur in naive-like CD27<sup>-</sup> B cells. These B cells also display a low level of somatic mutations in the Ig gene and the ability to secrete Igs *in vitro* [60, 65-67]. In accordance with the elevated proportion of CD27-IgG<sup>+</sup> B cells in peripheral blood, Moir et al also reported the expansion of tissue-like memory B cells, cells characterized by the expression of IgG<sup>+</sup>FcRH4, CD27<sup>-</sup> and CD21<sup>low</sup>. Even though these tissue-like memory B cells carry a BCR with high affinity for HIV-1 proteins, these cells are prematurely exhausted, as manifested by the high expression levels of inhibitory receptors including PD1, CTLA4, FcRL4, limited number of mutations in the Ig genes and replication history [68]. The term exhaustion refers to virus-induced functional loss of immune cells [48]. The premature exhaustion of this HIV-1 specific memory B cell population might contribute to the deficient immune response against HIV-1. Conventional memory CD27<sup>+</sup> B cells also show an activated phenotype, including elevated expression of activation markers CD70, Fas and low CD21 expression [65].

Activated/terminally differentiated B cells are also expanded during HIV-1 infection, especially in viremic patients. These cells are characterized by low levels of CD20, CD21 and CD19 expression; increased expression of the activation markers CD86/CD80, CD38 and CD27; enlarged B cell size with plasmacytoid features and up-regulated expression of genes involved in differentiation and proliferation [69-72]. In addition to the expansion of activated/differentiated B cells, PCs characterized by CD20<sup>-</sup>CD21<sup>low</sup>CD27<sup>++</sup>CD38<sup>++</sup> are also over-represented in the peripheral blood of HIV-1 viremic individuals [70], demonstrating that HIV-1 preferentially induces the expansion of B cells associated with antibody secretion.

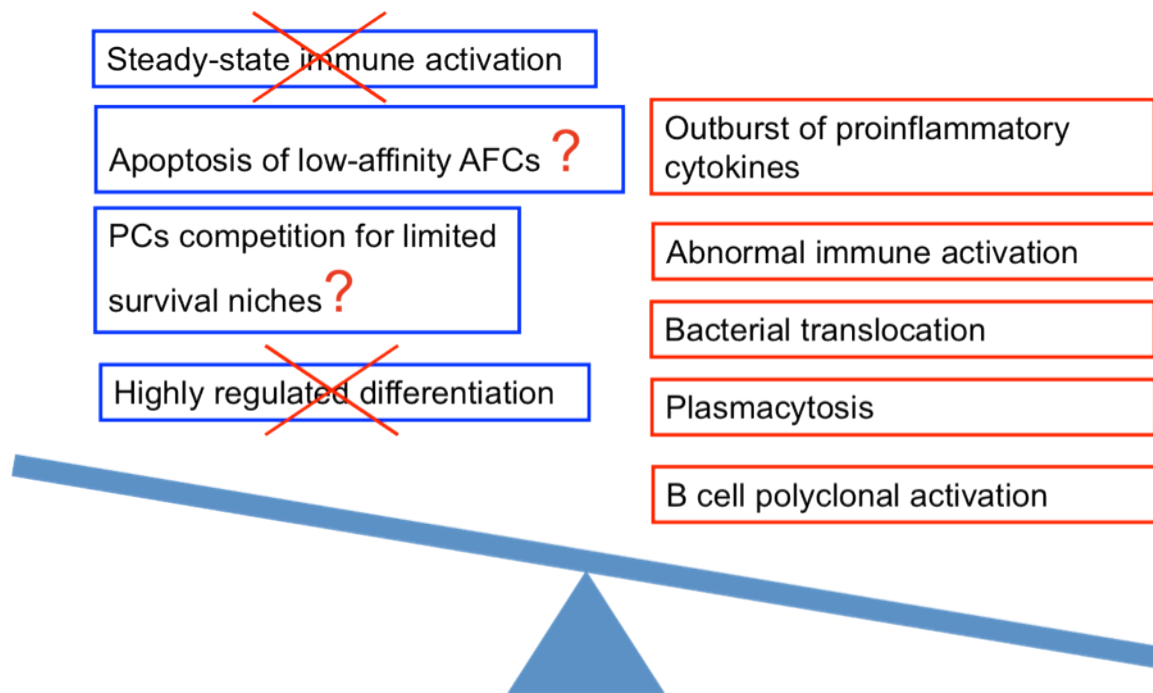
<b>B cell subpopulations</b>	<b>Abnormalities</b>	<b>References</b>
<b>Immature B cells</b>	-Expanded in peripheral blood -Altered expression of CXCR4 chemokine -Increased susceptibility to intrinsic apoptosis	[1-4]
<b>Naive B cells</b>	- Increased number and percentage in peripheral blood -Increased expression of activation markers -High rate of spontaneous apoptosis	[5-10]
<b>Memory B cells</b>	-Decreased number of classical memory B cells -Expanded number of tissue-like memory B cells -High rate of spontaneous apoptosis	[5, 7, 11-22]
<b>Differentiated B cells and Plasma cells</b>	-Increased numbers of circulating CD21 low with the feature of plasmacytoid cells -Plasmacytosis in bone marrow	[12, 23, 24]

**Table 1. Dysfunctions of B cell subpopulations occurring during HIV-1 infection**

Hypergammaglobulinemia is also considered as one of the consequences of cell clonal expansion of both HIV-1 specific and HIV-1 non-specific B cells in secondary lymphoid tissues including GALT, as demonstrated in many studies conducted during HIV-1 and SIV infections [62, 73-75]. One of the mechanisms proposed for B cell polyclonal activation is through the CD27/CD70 pathway, in which the increased expression of the ligand CD70 on CD4+T cells might be responsible for inducing Ig

secretion from CD27<sup>+</sup> B cells [76]. In addition, the increased B cell differentiation to plasmablasts is also facilitated by direct contact with Natural killer cells [77], which are also shown to up-regulate the expression of CD70 during HIV-1 infection [78] (Figure 3).

The factors contributing to HIV-1 induced B cell hyperactivity are mostly unidentified. Several soluble cytokines and growth factors including IL-6, IL-10 and BAFF have been implicated in this process [48, 79, 80]. Products of microbial translocation such as lipopolysaccharide (LPS), enter the circulation as the result of damage to the intestine epithelial barrier during HIV-1 infection, activate macrophages and indirectly act as a B cell activating factor [48, 81]. In addition, HIV-1 virus and its associated proteins might also induce B cell hyperactivation through both direct and indirect mechanisms. The complex formed between the HIV-1 virion and the host CD40L, formed upon viral budding from infected PBMCs, is able to induce activation of B cells by up-regulation of the CD80 activation marker and AID expression [82, 83]. In addition, both gp120 and Nef can promote Ig secretion by inducing AID expression and indirectly through Ferritin-secreting macrophages, respectively [79, 84-86]. Nef, on the other hand, was also shown to suppress class switching by interfering with CD40 signaling [87], thus indicating that the role of this protein in Ig production remains to be further investigated.



**Figure 3. Impaired maintenance of immunoglobulin productions during HIV-1 infection.** *The steady-state production of Ig is impaired during HIV-1 infection, due to several immunopathological features.*

### **1.2.3 Functional abnormalities of B cells during HIV-1 infection**

The function of B cells in controlling HIV-1 infection might be more important than previously thought. Huang et al showed that HIV-1 infected patients treated with Rituximab showed a declined production of autologous neutralizing antibodies (Nab), which was associated with elevated plasma viremia, whereas an increase in Nab was accompanied by the decline of plasma viral load [88]. However, during the course of HIV-1 infection, B cells might also act to facilitate infection of T cells: this may occur through direct binding of the virus to CD21, expressed on the majority of resting mature B cells, followed by passage of the virus to T cells [89]. Activated B cells also can induce activation of other immune cells through co-stimulatory molecules such as

CD80/CD86 and CD40 [66, 71, 90-92] and secretion of pro-inflammatory cytokines like IL-6 [48].

Early findings on the function of B cells isolated from HIV-1 infected patients showed that despite being activated, B cells respond poorly to different stimuli in vitro [93]. The poor responsiveness of B cells in vitro might be due to an enlarged proportion of immature/transitional, terminally differentiated and exhausted tissue-like memory B cells among B cells in circulation [64, 68, 91, 94].

The impaired function of B cells occurring during HIV-1 infection might also reflect a defective communication between T and B cells, which subsequently affects the affinity maturation process [64]. A study conducted in rhesus monkeys, however, showed that antibodies against SIV have undergone extensive somatic hypermutation [95]; furthermore, Scamurra et al also confirmed that the density and molecular VH repertoire of mucosal B cells in HIV-1 infection are comparable to that of healthy donors [96]. These data suggest that the SHM process might occur normally in secondary lymphoid tissues and that impaired B cell response during HIV-1 infection is likely due to the loss of memory B cells, improper communication between T and B cells and probably other factors such as impaired homing.

#### ***1.2.4 Defects in B cell homing during HIV-1 infection***

The migration of B cells strictly depends on the responsiveness toward chemokines and to a lesser extent, on the exact expression of chemokine receptors at the cell surface. During HIV-1 infection, CXCR5 is down-regulated on naive B cells [97], while the corresponding ligand CXCL13 is increased and its levels are positively correlated to the levels of plasma viremia [98]. These alterations in both the expression of receptor and

ligand are partially normalized after HAART treatment and thus, might have effect on the expression of the chemokines and the migrating ability of B cells [98, 99]. CXCR4, the co-receptor for the R4 strain of the HIV-1 virus, is down-regulated after infection of T cells [100], but whether a similar mechanism takes place on B cells has not been studied. The CXCR4 corresponding ligand, CXCL12, has been shown not only to act as a ligand involved in B cell homing, but also to increase Ig production in B cells stimulated with gp120 [101]. Gp120-treated B cells showed a decreased migration toward CXCL12 and CCL21 by 40-50%. The expression of CXCR4 is diminished while the expression of CCR7 is preserved during gp120-activation of B cells [102]. Moreover, B cell migration is also affected by alteration in the secondary lymphoid compartment. Specifically, follicular lysis, GC loss in Peyer's patches [73], as well as disruption of FDCs network in GCs, are all observed in acute SIV infection [103].

#### ***1.2.5 B cell apoptosis during HIV-1 infection***

Virus-induced apoptosis is another pathogenic feature of HIV-1 infection on B cells and, on the long-term, increased B cell apoptosis might lead to B cell lymphopenia [104, 105]. Loss of naive and memory B cells both from the circulation and secondary lymphoid tissues has been reported to occur early in HIV-1 and experimental SIV infections [106-108].

The loss of memory B cell populations includes both IgM and switched memory B cells, and was associated with declined plasma levels of pneumococcal, measles and tetanus antibodies [109, 110]. The decreased levels of circulating memory B cells could be the consequence of increased B cell apoptosis, depletion of VH3-expressing memory B cells by HIV-1 gp-120 and activation-induced PC differentiation [65, 66, 111]. Interestingly, Ho A et al reported that immature/transitional B cells are



susceptible to intrinsic apoptosis while mature/activated B cells are prone to undergo extrinsic (Fas-driven) apoptosis [72]. Recently, Titanji et al demonstrated that the depletion of a specific B cell subset, called activated memory B cells (CD27+CD21-), is associated with disease progression in SIV infected macaques and occurs through a PD-1 associated pathway, thus impairing SIV-specific and responses with other specificity [112].

### **1.2.6 Effect of HAART treatment on B cell abnormalities**

HAART can effectively reduce HIV-1 viremia; therefore, the treatment can ameliorate the virus-induced immunological impairments. HAART has been shown to reduce, but not normalize, the levels of soluble markers associated with immune activation including sCD27, IgG, IgA and CXCL13 [99].

The loss of memory B cells is irreversible according to several studies [108, 113, 114], although it was also reported that HAART partially restores the levels of some of the specific memory B cells, including influenza and tetanus, but not HIV-1 specific memory cells [105, 113, 115, 116]. This lack of normalization of the pool of memory B cells is also described in pediatric HIV-1 infection [117, 118], with the occurrence of poor immune responses to both TD and TI antigens during HAART treatment [119, 120]. However, Pensiero et al suggested that early treatment not only preserves memory B cells response to vaccine antigens [121], but also restores functional memory B cells [105].

The percentages of activated/terminally differentiated B cells (CD21 low, CD38+) and other activated B cell populations (expressing CD70, CD80/CD86) [70, 71, 116], as well as immature/transitional B cells [116], are also normalized during HAART

treatment, leading to decreased hypergammaglobulinemia [61, 63, 122, 123]. HAART also leads to a reduced B cell turnover (both cell proliferation and cell death) in HIV-1 and SIV infections [124, 125]. In parallel with decreased hypergamma-globulinemia, HAART also reduces the level of HIV-1 specific antibodies and HIV-1 non specific antibodies [63, 77, 126].

### ***1.2.7 HIV-1 specific B cells and neutralizing antibodies***

HIV-1 specific B cells are present at extremely low frequency in peripheral blood of HIV-1 infected patients, as compared to other virus infections [127]. Nabs are principal components of the humoral immune response but their role in HIV-1 protection deserve further clarification [128]. A pivotal study conducted by Baba TW et al [129], however, clearly demonstrated that treatment of pregnant macaques with the combination of the three anti-HIV-1 monoclonal antibodies (F105, 2G12 and 2F5) with neutralizing capacity protected the animals from intravenous SHIV-vpu+ challenge administered after delivery. In addition, treatment after birth with the monoclonal antibodies indicated above also protected the newborn monkeys from oral SHIV-vpu+ s challenge.

During acute HIV-1 infection, outburst of pro-inflammatory cytokines followed by immunosuppression leads to delayed generation of productive antibody responses [128, 130]. Ironically, the HIV-1 infected subjects with the most pronounced anti-Env responses also have the higher level of GC damage. Furthermore, the decline of anti-Env IgA and IgG in serum upon HAART treatment suggests that the HIV-1 antigens are a driving force for the production of Nabs [73, 128, 131]. Among broadly Nabs against HIV-1, the five most potent shares some similarities including high SHM level, long HCDR3 (heavy chain complementary determining region) and/or poly-reactivity [128, 132]. These properties may reflect defects in SHM regulation and tolerance

mechanisms since the presence of a long HCDR3 has been associated with tolerance [128, 132, 133]. The polyreactive antibodies are able to bind with high avidity since they can bind to both Env and non-virion component.

## 2 AIMS OF THE THESIS

B cell dysfunction is one of the severe consequences of virus-associated immunological perturbations occurring during the course of HIV-1 infection. The persistence of viral replication leads to increased immune activation and elevated levels of soluble factors produced from immune cells. The consequences of this phenomenon are bystander activation of B cells, hypergammaglobulinemia, decreased numbers of memory B cells, increased levels of activation markers on B cells and plasmacytosis. Understanding the molecular basis of these impairments will help in designing functional HIV-1 vaccines and improved treatment.

The aims of this thesis were:

- To investigate the expression of chemokine receptor/ligand pairs on B cells during HIV-1 infection and to study whether changes in the expression of these molecules may affect migration of B cells
- To study whether defects in the process of antibody affinity maturation, consisting of CSR and SHM, occurs during HIV-1 infection
- To clarify some aspects of the mechanisms involved in deregulation of B cell apoptosis during HIV-1 infection
- To study the role of soluble CD27 (sCD27), a molecule released from activated immune cells, in inducing differentiation of B cells toward plasmablasts.

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

### **3 RESULTS AND DISCUSSIONS**

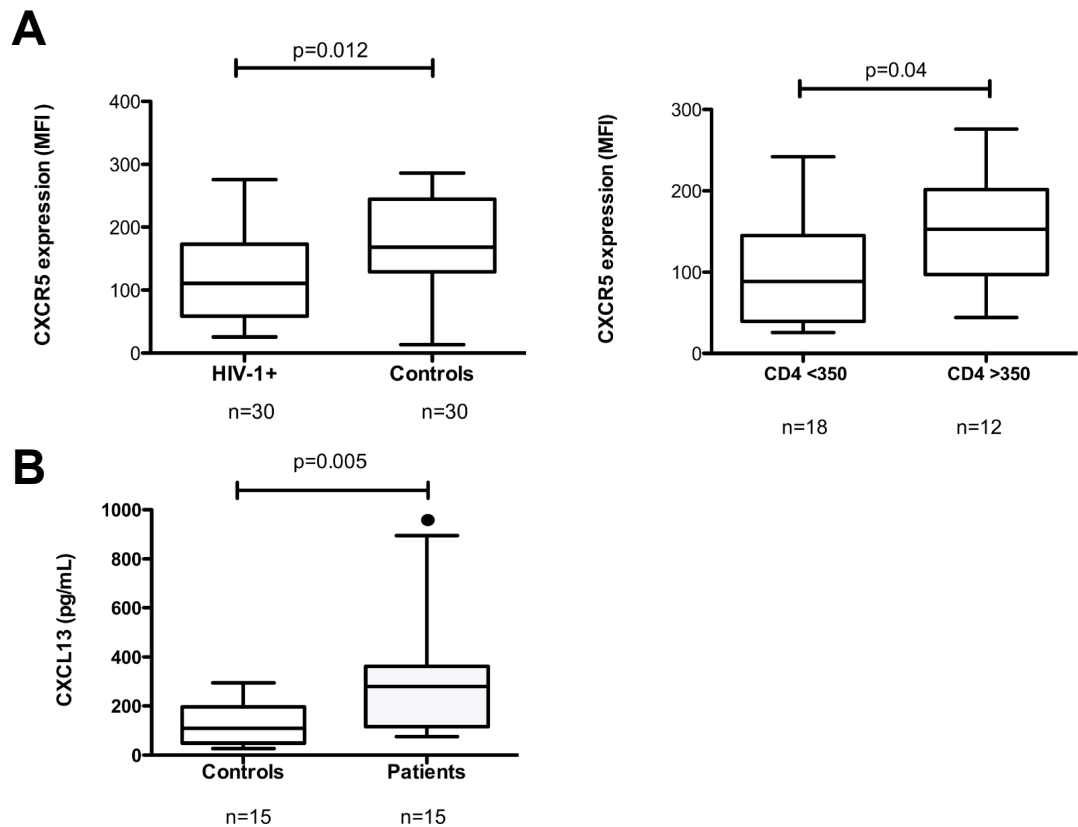
Not only cell-mediated immunity, but also humoral immune responses may play a critical role in reducing HIV-1 spread, as well as controlling other opportunistic and secondary infections. During HIV-1 infection, the immune system is dysfunctional due to both the direct impact of the virus and virus-induced chronic immune activation. Many of the studies on B cells during HIV-1 infection have been conducted on peripheral blood and therefore it has been questioned whether the results would be representative of what happens in the lymphoid tissues; it was recently discussed, however, that the similarity between cells resident in blood and in tissues may be higher than expected [134]. Accordingly, studies conducted on peripheral blood and lymphoid/mucosal tissues of HIV-1 infected individuals, together with data obtained from the SIV model, gradually build up a comprehensive picture of B cell lymphocyte dysfunctions during HIV-1 infection. In the following sections the main results of this thesis will be presented.

#### **3.1 Altered chemokine/chemokine receptors on B cells of HIV-1 infected patients (paper I)**

Chemokines and their corresponding receptors play an important role in the regulation of lymphocytes migration between peripheral and lymphoid tissues, a process pivotal for efficient immune responses. In order to investigate whether the perturbation of B cell populations observed in peripheral blood during HIV-1 infection could be due to redistribution and altered migration of B cells, we analyzed the surface expression of chemokine receptors that are important for B cell homing to lymphoid tissues, including CXCR5, CXCR4 and CCR7.

We observed a decreased expression of CXCR5 on total B cells from HIV-1 infected patients, as well as on B cell subpopulations including naïve and memory cells and plasmablasts, especially in patients with low CD4<sup>+</sup> T cell counts (Figure 4A). No significant changes in CXCR4 and CCR7 expression were detected on B cells during HIV-1 infection.

To analyze whether down-regulation of these chemokine receptors affected the migration ability of B cells in vitro, we used a migration assay for B cells utilizing the corresponding chemokine ligands including CXCL13, CXCL12 and CCL19/CCL21. Unexpectedly, we did not observe any decrease in the migration of B cells from HIV-1 infected patients as compared to healthy donors. On the contrary, increased responsiveness of B cells to CXCL13, CXCL12 and CCL21 was observed in patients with low CD4<sup>+</sup>T cell counts. IFN- $\alpha$  was previously shown to increase B cell responsiveness toward CXCL12 and since the level of IFN- $\alpha$  is increased during HIV-1 infection [135], the responsiveness of B cells toward CXCL12 is likely to increase accordingly [136]. The increased chemotactic response toward CXCL12 and CCR7 might due to B cell hyperactivation. Badr et al, however, also showed that gp120 binds to CXCR4 and inhibits the chemotactic response of B cells toward CXCL12 [102]. Taken together all these findings raise the possibility that migration of B cells may be affected by the presence of a complex network of soluble molecules which concentration is increased during HIV-1 infection.



**Figure 4. Detection of chemokine and chemokine receptors on peripheral blood B cells in healthy individuals and HIV-1 infected patients.** (A) The expression of chemokine receptor CXCR5 on CD19+ B cells in HIV-1 infected patients and healthy controls is detected by flow cytometry. The expression of CXCR5 receptor in patients is shown in relation to CD4+T cell counts. (B). The chemokine ligand CXCL13 is detected in vitro in cultures of activated B cells isolated from healthy controls and HIV-1 infected patients.

It was previously shown that secretion of CXCL13 from FDCs, T(FH) cells and stromal cells initiates the recruitment of B cells into the B cell follicles of secondary lymphoid tissues [36]. The gradient created by the different concentrations of CXCL13 in the specific areas of the lymphoid tissues is essential for normal trafficking of naive B cells and T(FH) in these tissues, as well as for lymph node formation and organization [137]. We showed that CXCL13 was produced from B cells activated

through CD40 ligation but not through BCR and we also showed that CXCL13 levels are higher in cultures of B cells isolated from HIV-1 infected patients (Figure 4B). These data suggest that during HIV-1 infection, bystander (non BCR-mediated) B cell activation might be responsible for an increased production of CXCL13. The increased peripheral levels of CXCL13 might contribute to down-regulation of its receptor CXCR5 on B cells, as the result of receptor internalization, as also proposed by Widney et al [98]. High levels of CXCL13 are also found in patients suffer from Systemic *lupus* erythematosus (SLE), Nephritis and Non-Hodgkin lymphoma (NHL) in patients with acquired immunodeficiency syndrome (AIDS); cell lines established from patients with AIDS-NHL also secreted low levels of CXCL13 [138, 139]. These results also suggest that the increased CXCL13 secretion observed from B cells during HIV-1 infection might be associated with the development of B cell lymphoma.

The increased levels of CXCL13 in serum might reflect the increased production of CXCL13 in lymphoid tissues, possibly leading to increased chemo-attraction of other lymphocytes to lymphoid tissues. Abnormal migration of cells to the lymph nodes may be one of the mechanisms responsible for enlarged lymph nodes during HIV-1 infection [98]. Alternatively, the increased levels of CXCL13 in serum may be due to the secretion of this chemokine from circulating, activated B cells. In this latter case, the physiological CXCL13 gradient existing between the periphery and the lymphoid tissue could be decreased leading to two possible consequences: i) naive B cells fail to home to the B cell follicle and ii) partially activated B cells are released to the periphery.

Altered homing of B cells was previously reported to occur during HIV-1 infection. These abnormalities include an increased proportion of CD38 expressing cells in



peripheral blood as the result of a reverse trafficking of GC B cells; accumulation of B cells in the spleen of SIV infected macaques; expansion of naive B cells in peripheral blood and altered chemokine expression of tissue-like memory cells [60, 68, 107, 140].

### **3.2 IL-7 leads to Fas induced apoptosis on B cells (paper II)**

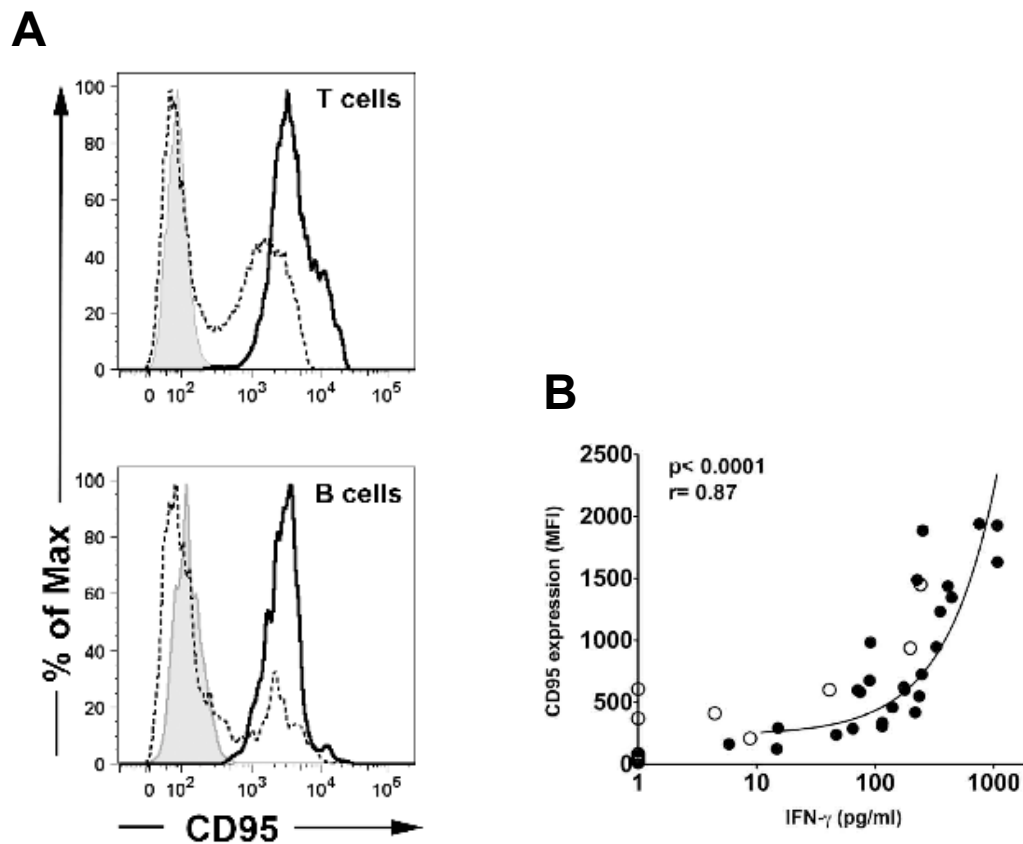
The cytokine IL-7 appears not to have an effect on B cell survival at the pro- and pre-stages of B cell development in human, but it can induce expansion of the pro-B cell compartment [141, 142]. Increased IL-7 levels are positively associated with an increase percentage of immature/transitional B cells in blood during HIV-1 infection, in idiopathic CD4<sup>+</sup> T cell lymphopenia and during IL-7 therapy [94, 143, 144]. The exact mechanism through which IL-7 therapy leads to an increased number of immature B cells remains unclear. It is possible that IL-7 may directly induce expansion of pro- and pre- B cells, or it may function through indirect mechanisms to affect B cell homeostasis.

Accelerated B cell apoptosis, associated with viral replication has been reported to occur both in HIV-1 and SIV infections [72, 73, 107]. These apoptosis-prone B cells express high levels of Fas and low levels of Bcl-2 [65, 110, 114]. CD4<sup>+</sup>T cells may play a role in B cell apoptosis as suggested by the increased B cell turnover associated with diseases characterized by declined CD4<sup>+</sup>T cell counts, including HIV-1 infection [145]. HAART does not normalize the levels of Fas expression on B cells and B cell apoptosis; in addition long term non-progressors (LTNPs), who do not receive HAART and have detectable levels of viremia, have levels of B cell apoptosis comparable to what found in the blood of healthy donors [65, 146]. These findings suggest that viral-independent mechanisms may be involved in B cell depletion during HIV-1 infection.

Fluur et al previously showed that IL-7 up-regulates Fas expression on T cells, a phenomenon also associated with Fas-induced apoptosis [147]. During HIV-1 infection, increased immune activation leads to increased FasL availability, which together with IL-7 induced Fas upregulation on T cells is responsible for increased T cell apoptosis [148]. In our study, we identified a novel mechanism through which IL-7 primes B cell for apoptosis. IL-7 induces IFN- $\gamma$  secretion from resting T cells; IFN- $\gamma$ , in turn, mediates STAT1 phosphorylation and up-regulates Fas expression on most of the B cell populations (Figure 5). This effect seems to be solely due to IFN- $\gamma$  since blocking of IFN- $\gamma$  signaling leads to total inhibition of STAT1 phosphorylation, as well as up-regulation of Fas expression on B cells. These results indicate that IL-7-induced B cell apoptosis might be pronounced during HIV-1 infection due to the excessive availability of IL-7. This mechanism may partly explain increased B cell depletion during HIV-1 infection. In line with this hypothesis, an association between elevated IL-7 levels and reduced number of peripheral mature B cell is found in patients suffering from end-stage renal diseases and during IL-7 therapy in patients with refractory malignancy [144, 149].

It is noteworthy that IFN- $\gamma$  induces IL-7 production by epithelial and BM stromal cells [150], suggesting that during HIV-1 infection, a positive regulatory loop of IL-7 and IFN- $\gamma$  productions could be exponentially amplified and lead to further B cell depletion as the result of Fas-induced apoptosis. We also showed that IL-7 can exert its apoptotic effect when presented in a concentrated form on the surface of stromal cells. This mechanism might accelerate the B cell apoptotic level in lymph nodes where a significant production of IFN- $\gamma$  may occur. Consistent with this hypothesis, Peruchon

et al reported an increased apoptosis of B cells in lymph nodes during SIV infection [107], possibly mediated by IL-7 concentrated at high level on stromal cells.



**Figure 5. IL-7 treatment of T cells leads to increased Fas expression on B cells through IFN- $\gamma$  (A).** Representative histogram of Fas (CD95) expression on T and B cells from PBMCs treated with (solid line) and without (dashed line) IL-7 for 5 days. (B) The correlation of IFN- $\gamma$  produced in vitro from T cells isolated from healthy donors and levels of Fas expression on B cells.

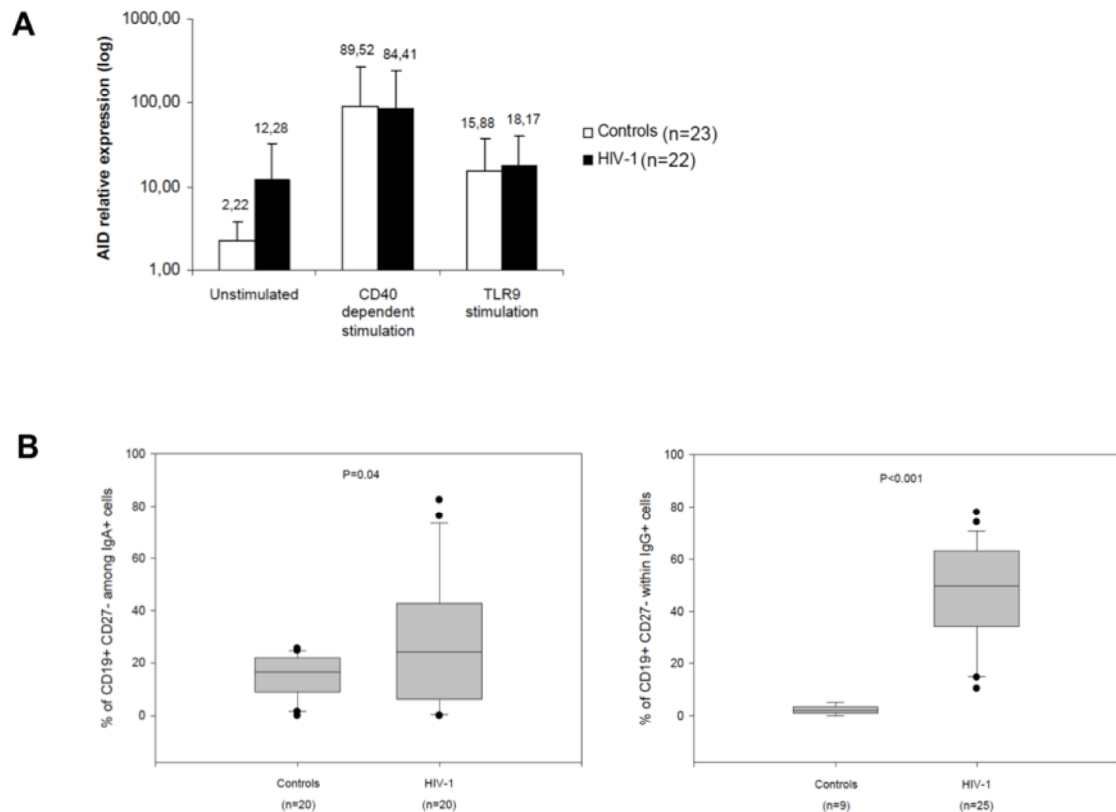
### 3.3 Impaired affinity maturation during HIV-1 infection (Paper III)

The efficiency of T-dependent B cell responses is greatly dependent on the process of antibody affinity maturation including CSR and SHM, which are catalysed by the AID enzyme. During HIV-1 infection, B cells produce excessive levels of Ig although they

are unable to mount proper B cells response to HIV-1 and other antigens, suggesting that the process of affinity maturation may be affected.

We found that peripheral B cells from HIV-1 infected patients are abnormally activated, as characterized by elevated baseline levels of AID. Despite the higher baseline level of AID, the levels of AID after stimulation with CD40L and CpG are similar in both HIV-1 infected patients and controls (Figure 6A). High AID expression has been implicated in the pathogenesis of different types of diffuse large B cell lymphomas [151] and is found in PBMCs from HIV-1 infected patients presenting with NHL [152]. B cells from HIV-1 infected patients have a lower fold-increase of AID expression after activation with CpG and CD40L as compared to cells from healthy donors and, accordingly, we speculate that these cells might be exhausted with a reduced ability to proliferate and differentiate. A decreased induction of AID expression has been previously observed in aged mice and humans [68, 153].

We also detected elevated levels of IgA both at baseline and after stimulation with anti-CD40 and CpG in cultured B cells from HIV-1 infected patients. On the other hand, IgG levels were comparable in non-stimulated B cells from HIV-1 infected individuals and controls and post-CpG stimulation, whereas CD40 ligation induced a significantly higher IgG secretion from purified B cells of HIV-1 infected patients. This increased Ig production might have a link with, or be the consequence of, the increased AID expression in B cells.



**Figure 6. Impaired CSR occurs during HIV-1 infection.** (A). The AID level in B cells is analyzed ex-vivo and after stimulation with anti-CD40 antibody and CpG. (B) The proportions of CD27-IgG<sup>+</sup> and CD27-IgA<sup>+</sup> B cells are analysed in PBMCs from HIV-1 infected patients and healthy donors.

Intriguingly, we detected a moderate correlation between the expression of CD27<sup>+</sup> on B cells and AID level in healthy donors but not in HIV-1 infected individual. Therefore, we postulated that CD27<sup>+</sup> B cells might be responsible for the increased AID expression found in patients. In line with the speculation, an increase of CD27-B cells among IgG<sup>+</sup> cells was found in HIV-1 infected patients as compared to that of healthy controls. It is a possibility that these cells may tissue-like memory B cells with a low level of mutation [47, 49] or/and activated naive-like B cells [60, 66]. In parallel with the elevated levels of CD27-IgG<sup>+</sup> B cells, we also pin-pointed the presence of an increased level of CD27-IgA<sup>+</sup> B cells in HIV-1 infected patients, which is associated

with high level of IgA secretion in vitro (Figure 6B). The IgA<sup>+</sup> B cells are essential for protection and for limiting the spread of virus at the mucosal sites. It has previously been speculated that B cell polyclonal activation might be a positive aspect of the immune response against viruses, possibly inducing activation and antibody production from B cells with a broad range of specificity. It is interesting that HIV-1 antibodies with broad neutralizing capacity show a certain level of polyspecificity [128, 132]. Therefore, an elevated switch to IgA might be an effective tactic of the immune system to control viral replication. During HIV-1 infection, this elevated IgA levels could also be due to increased levels of circulating microbial products like LPS and bacterial 16S ribosome DNA entering the circulation from the damaged intestinal epithelial barrier [81, 154]. Since massive depletion of CD4<sup>+</sup>T cells occurs in mucosal tissue during HIV-1 infection, the elevated IgA level is likely due to TI responses and is associated with low affinity antibodies.

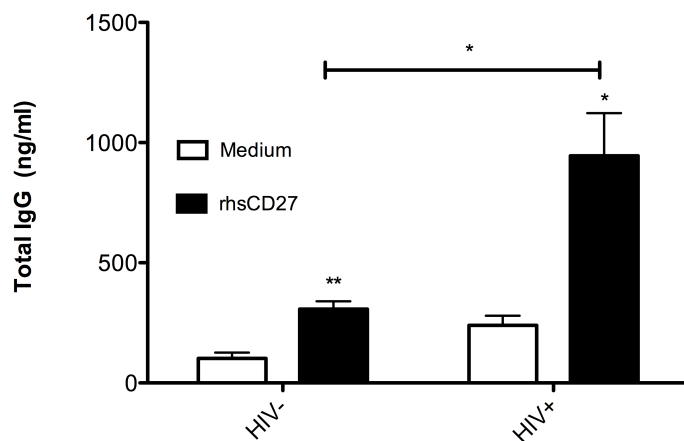
Regarding SHM, we observed an increased degree of mutation in CD27<sup>-</sup> B cells from HIV-1 infected patients, which could be the consequence of enlarged CD27-IgG<sup>+</sup> and CD27-IgA<sup>+</sup> populations. On the contrary, CD27<sup>+</sup> B cells during HIV-1 infection contained a lower level of mutations compared to those of healthy donors, implying a profound impairment of SHM in conventional memory B cells. This could be the consequence of improper T-B cell communication, intrinsic B cell deficiencies (hyperactivated and exhausted), elevated polyclonal B cell activation and depletion of memory B cells taking place during the course of HIV-1 infection.

### **3.4 sCD27 induces differentiation of memory B cells to plasmablasts (paper IV)**

The sCD27 is released from activated T or B cells through a proteolytic process. Elevated levels of sCD27 have been reported in many diseases including HIV-1 infection [155-158]. In paper IV, we detected a significant elevation of sCD27 serum level in HIV-1 infected patients as compared to healthy donors, consistent with higher level of immune activation [159]. In parallel with elevated levels of sCD27, higher levels of IgG were also found in the serum of patients, and these parameters were both negatively correlated to CD4<sup>+</sup>T cell counts. In addition, a strong correlation between sCD27 and IgG serum level was detected in both healthy donors and HIV-1 infected patients; these results suggest a possible role of sCD27 for IgG production under physiological conditions and for hypergammaglobulinemia when the levels of sCD27 are pathologically increased.

We showed that sCD27 binds to CD70 expressed on CD27<sup>+</sup> B cells, followed by phosphorylation of Erk and upregulation of transcription of factors involved in PC differentiation, including Blimp-1 and XBP-1, ultimately, inducing IgG secretion (Figure 7). This effect of sCD27 is not observed in naive B cells but only in memory B cells due to the increased capacity of memory B cells to differentiate to PCs [160]. However, during HIV-1 infection, as mentioned earlier and as reported by several authors [65, 98, 99, 161, 162], sCD27 levels are elevated and CD70 expression is up-regulated on CD27<sup>+</sup> memory B cells, thus enhancing the differentiation of memory B cells toward PCs. This process may ultimately lead to both a decreased percentage of memory B cells and to hypergammaglobulinemia. These observations are further

supported by a study conducted in patients with advanced myeloma showing that a dramatic decline of the CD27<sup>+</sup> memory compartment is linked to plasmacytosis [163].



**Figure 7. Recombinant sCD27 induces IgG secretion from B cells in vitro.** IgG was measured in cultures of B cells isolated from HIV-1 infected patients ( $n=6$ ) and healthy donors ( $n=6$ ) with and without 1000ng/ml sCD27 for 5 days. Higher IgG levels were detected in rhsCD27 treated B cell cultures from both healthy (HIV<sup>-</sup>) ( $p=0.002$ ) and HIV<sup>+</sup> donors ( $p=0.02$ ), as compared to control cultures. IgG production in B cells from HIV-1 infected individuals was significantly higher than in B cells from healthy controls ( $p=0.026$ ).

B cells up-regulate CD70 expression upon *in vitro* stimulation through BCR and CD40, but not CpG, and this effect is further augmented in combination with certain proinflammatory cytokines including IL-10 and IL-12. These data indicate that the subpopulations targeted by sCD27 are B cells activated through BCR and CD40 ligation and that TD B cell responses might be mainly affected by sCD27 activation. This possibility is also supported by the striking finding that both measles specific ASCs and measles antibodies levels are profoundly reduced in HIV-1 infected patients [110]. The effect of sCD27 on IgG production might be limited under physiological condition due to the low available level of sCD27. During an immune response, a large



amount of sCD27 is produced in tissue where a massive level of T and B cells undergo activation; consequently, sCD27 could serve as a co-stimulatory factor to produce a fast and high level of Igs to amplify primary, as well as secondary responses in lymphoid tissue.

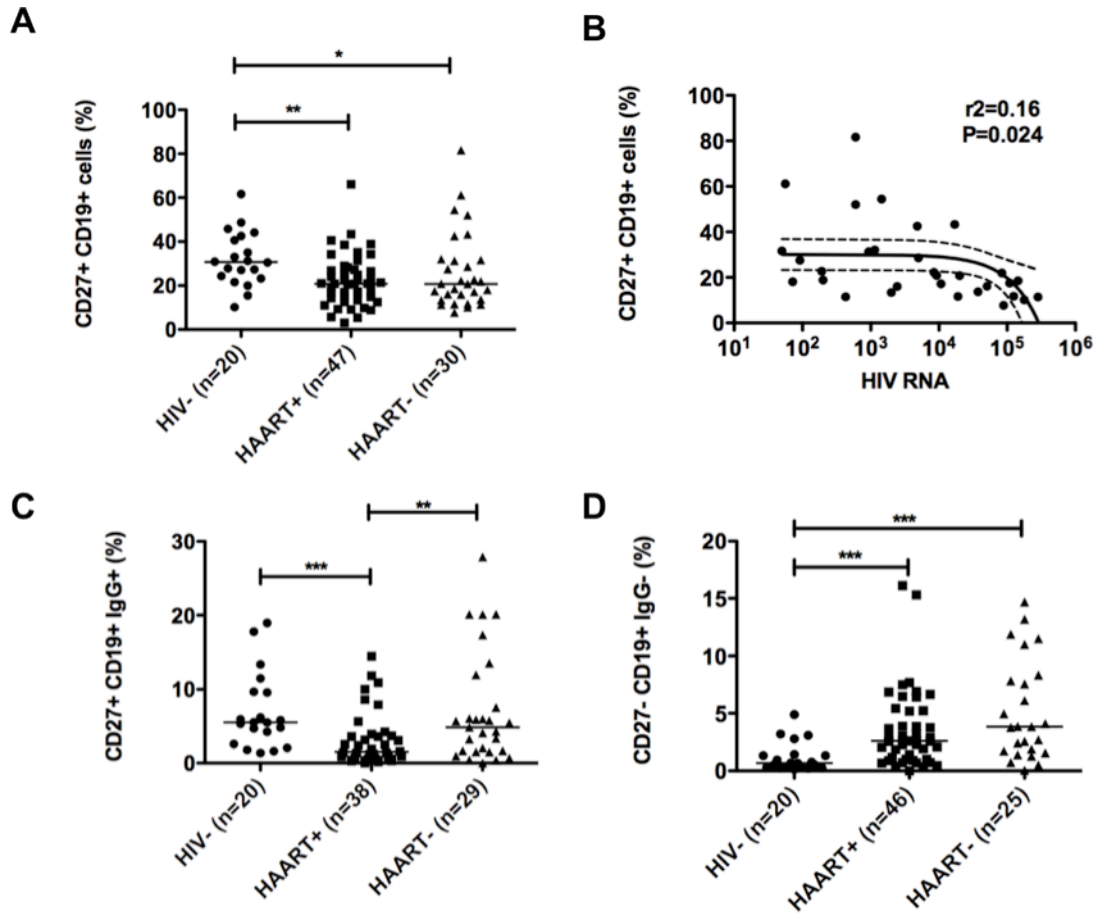
The effect of sCD27 is more pronounced during HIV-1 infection due to the presence of high level of immune activation and to an outburst of production of inflammatory cytokines. Interestingly, a positive correlation between the levels of expression of CD70 and CD27 on B cells is observed only during HIV-1 infection, although the exact mechanism underlying this correlation is unclear. This data suggests that CD70 is preferentially up-regulated on CD27<sup>+</sup> B cells, which are in turn the subject of activation-induced B cell differentiation mediated by sCD27 triggering. Consistent with this hypothesis, during HIV-1 and SIV infection, several authors detected increased B cell differentiation followed by an increase of ASC numbers and plasmacytosis [62], polyclonal expansion of B cells producing env-specific antibodies [74] and polyclonal activation and terminal differentiation of B cells in peripheral blood and GALT [73].

Since it has been suggested that immune activation declines once HAART is started, we monitored the level of sCD27 in untreated and HAART treated HIV-1 infected patients and found that the sCD27 serum levels are significantly higher in HAART naive patients; interestingly, the serum levels of IgG are also significantly elevated in untreated patients. We propose that sCD27 exerts a direct impact on production of Ig *in vivo* and thus partially explains the phenomenon of hypergammaglobulinemia associated with viral replication. However, the serum levels of IgG and sCD27 are not fully normalized after HAART, suggesting the involvement of other viral-independent pathways in inducing sCD27 and IgG production.

In conclusion, sCD27 might have a positive effect during normal physiological conditions for induction of antibody production from less-differentiated B cells during the course of immune activation, when a fast and efficient antibody production needs to take place. However, during HIV-1 infection, abnormal levels of immune activation and its associated factors including pro-inflammatory cytokines induce elevated levels of sCD27 and further contribute to hypergammaglobulinemia, a phenomenon associated with HIV-1 pathogenesis.

### **3.5 Deregulation of B cell subsets in peripheral blood during HIV-1 infection (unpublished results)**

In line with previous findings [76, 108, 114], we also found a significant reduction of conventional CD27<sup>+</sup> memory B cells in the peripheral blood of HIV-1 infected individuals, regardless of treatment (Fig 8a). This data is supported by studies showing the delayed reconstitution of the number of memory B cells after starting HAART [106, 113]. In addition, we detected a moderate negative correlation between the levels of CD27<sup>+</sup> memory B cells and HIV-1 viral load in our untreated cohort ( $r^2=0.16$ ,  $P=0.024$ ) (Fig 8b), suggesting a negative impact of persistent viral replication on the level of peripheral memory B cells. A number of HIV-1 viral proteins (Nef and Env) has been shown to directly affect B lymphocytes, but their impact on the survival of CD27<sup>+</sup> B cell levels remains unclear [87, 102, 164]. Acute HIV-1 infection seems to be a crucial period in which memory B cells are massively depleted [65]; starting HAART treatment at this early period might compensate for virus-dependent B cell depletion, but may not rescue from virus-independent alterations.

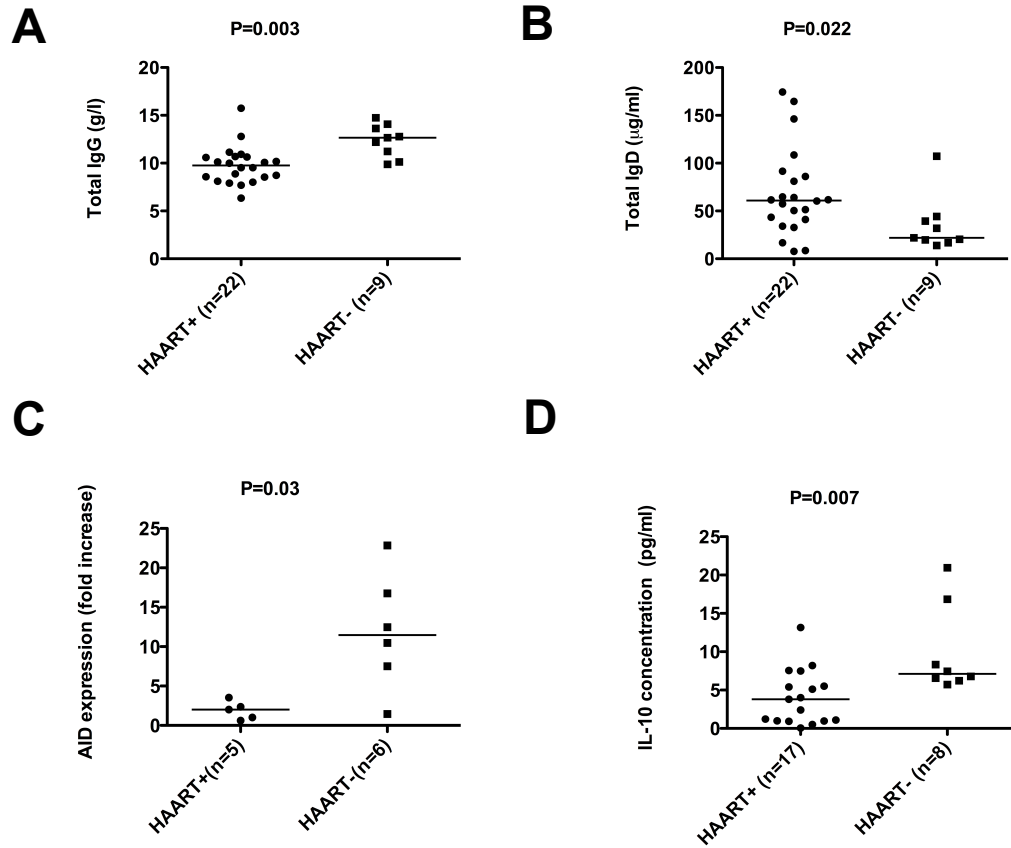


**Figure 8. The C27+ and CD27- B cells in controls and in naive and HAART treated HIV-1 infected patients.** (A) Elevated levels of CD27+CD19+ B cells are detected in healthy controls as compared to HIV-infected HAART-treated ( $P=0.014$ ) and HAART-naive patients ( $P=0.044$ ). (B) A negative correlation is found between levels of CD27+CD19+ B cells and plasma viremia. (C) The level of CD27+IgG+CD19+B cells is significantly higher in healthy controls compared to HAART-treated patients ( $P=0.0003$ ), while the level of CD27+IgG+ CD19+B cells is lower in HAART-treated compared to HAART-naive patients ( $P=0.0068$ ) (D) The proportion of CD27-IgG+ B cells is significantly increased in HAART-treated and HAART-naive patients as compared to healthy controls ( $P=0.0003$  and  $P=0.0001$ , respectively). \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\* $P < 0.001$ .

In our cohort, we found that the level of CD27+IgG+ B cells in HAART-naïve patients is significantly higher ( $p=0.0068$ ), as compared to HAART treated patients and is comparable to the level of these cells found in healthy controls (Fig 8C). We also detected higher CD70 expression in this population of CD27+ CD19+IgG+ B cells in untreated patients, compared to the level in treated patients and healthy controls. The CD27 marker may not be enough to distinguish between resting memory or activated B cells; therefore the proportion of activated B cells may be higher in HAART naïve patients than in healthy controls. On the contrary, CD27+ B cells may represent resting memory B cells in the healthy controls. HAART is reported to reduce the level of hypergammaglobulinemia and the percentage of B cells producing Ig [63, 65, 165]. In this regard, HAART might decrease CD27+CD19+ activated B cells, while retaining the number of resting memory B cells.

CD27- B cells are also able to secrete Igs [47, 49]; therefore we studied the frequencies of CD27-IgG+ in patients in relation to HAART treatment. HAART-naïve patients display elevated frequencies of CD27-IgG+ in comparison to HAART-treated, although this difference is not statistically significant (Fig 8D). In addition, the frequency of CD27-IgG+ B cells is significantly higher in patients compared to healthy donors, suggesting that HAART does not revert the expansion of this population and/or the increased size of this population might be due to viral independent mechanisms. In addition, an increased number of CD27-IgG+ B cells was also observed in the peripheral blood of elderly people [166, 167]. These findings, taken together, might lead to the speculation that B cells during HIV-1 infection suffer from functional exhaustion. In accordance with high levels of IgG+ expressing cells, we also detected higher IgG secretion (Figure 9A) in naïve patients, suggesting that both CD27-IgG+

and CD27+IgG+ B cell sub-populations might be responsible for elevated IgG production measured in serum.



**Figure 9. Increased IgG production in HAART-naïve HIV-1 infected patients is associated with high levels of IL-10 and AID expression.** (A) Increased levels of IgG and (B) decreased levels of IgD in serum of HAART-naïve patients. (C) AID levels are measured by RT-PCR in PBMCs of HAART naïve and treated patients. (D) Serum IL-10 levels are measured by ELISA.

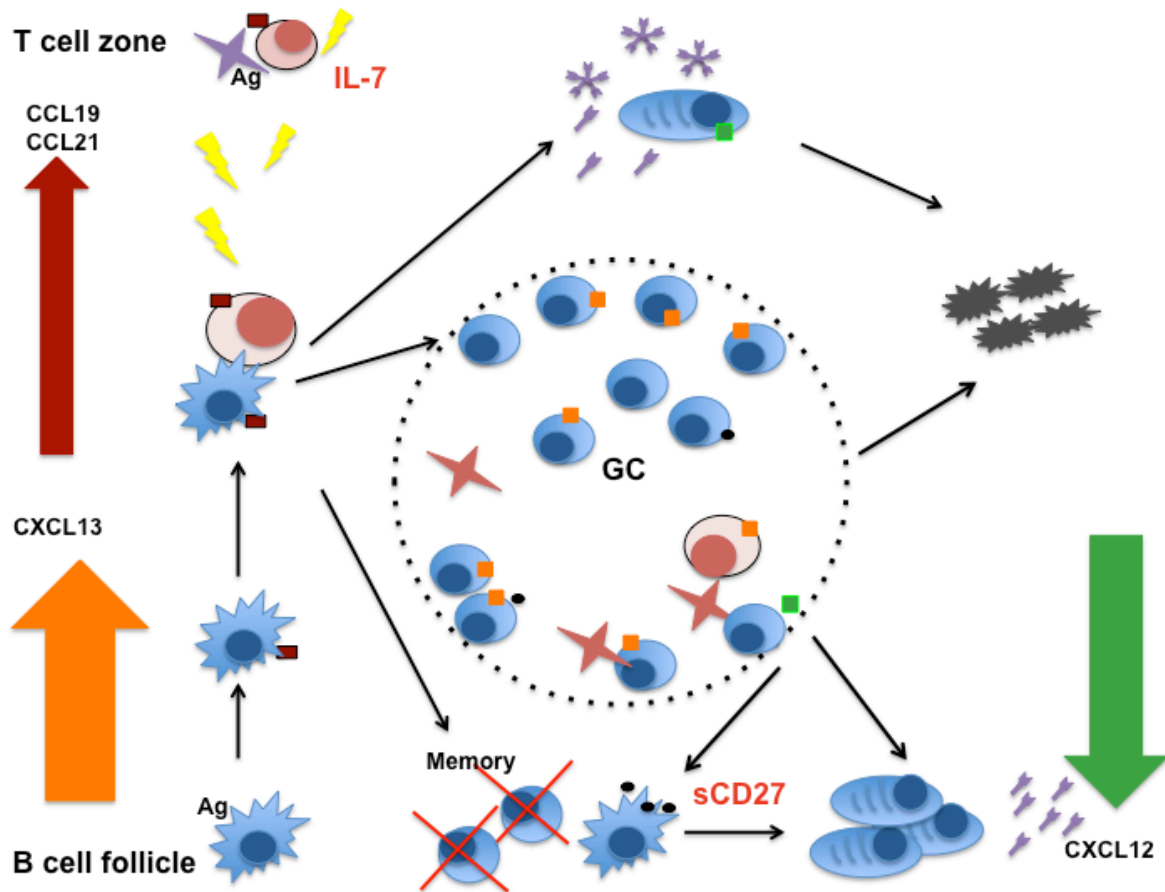
Even though the level of IgD memory B cells is similar in untreated and treated patients, serum IgD level is lower in HAART-naïve as compared to HAART-treated patients (Figure 9B). The function of IgD is still controversial; it was previously shown that IgD can induce basophils to secrete BAFF, and thus IgD may be implicated in B cell survival [168]. In line with this finding, mice deficient in IgD production have a reduced B cell number [169, 170]. Accordingly, HAART induced IgD increase might be associated with increased B cell survival in treated patients.

This expansion of IgG-expressing B cells might be the consequence of increased CSR occurring during HIV-1 infection. We measured AID expression in PBMCs from patients and detected a significant elevation of AID levels in HAART naïve compared to HAART treated patients, suggesting that the CSR might be increased in untreated patients (Figure 9C). Class switching to IgG is facilitated by other factors including direct contact with T cells and cytokines including IL-10 [171]. We also detected higher levels of IL-10 in untreated patients (Figure 9D), and this is further correlated to plasma viremia ( $p=0.006$ ). In addition, other soluble molecules, including sCD27 may facilitate the secretion of IgG from CD27<sup>+</sup> B cells; we found higher levels of sCD27 in the serum of untreated patients and accordingly, this elevated sCD27 may lead to increased IgG production (paper IV). All together these mechanisms might lead to increased levels of IgG<sup>+</sup> B cells, as well as increased IgG secretion in patients without treatment.

## 4 CONCLUSIONS AND FUTURE PERSPECTIVES

In the papers included in this thesis, we reported that a number of B cell perturbations occurs during HIV-1 infection, including altered expression of chemokine receptors and ligands (**paper I**), increased apoptosis of B cells (**paper II**), impaired affinity maturation (**paper III**) and increased B cell commitment to PC differentiation (**paper IV**) (Figure 10). These impairments, taken together, contribute to alter the physiology and immunology of the B cell compartment leading to decreased numbers of memory B cells and hypergammaglobulinemia, which have been considered as important components of HIV-1 associated pathogenesis.

We reported in **paper I** that B cells in HIV-1 infected subjects change their responsiveness toward different chemokine ligands, including CXCL12, CXCL13 and CCL21, a phenomenon associated with low CD4+T cell counts. In addition, activated B cells during HIV-1 infection produce CXCL13 and this might have the pathological effect of mediating disturbances in B cell migration in vivo. Since B cell migration ability is impaired during HIV-1 infection, the question is whether patients could benefit from interventions aimed at correcting, or at least ameliorating, this migration disturbance. For example, chemokines delivered at the mucosal side can increase immune responses locally. Castelletti et al suggested to use CCL28 as a component of a mucosal vaccine since gastrointestinal mucosal IgA-ASCs are significantly elevated in mice immunized with VSV and receiving CCL28 [172]. Although we found CXCL13 expressing B cells in the lymph nodes of HIV-1 infected patients, our initial findings on impaired levels of CXCR5/CXCL13 in B cells during HIV-1 infection need to be confirmed in an in vivo model where dynamics of B cell homing can be followed in presence of the HIV-1 or SIV virus, and correctly interpreted.



**Figure 10. Schematic figure on how the findings in HIV-1 infection presented in this thesis may impact on B cell differentiation and PC formation.** *Homing to secondary lymphoid tissue may be impaired during HIV-1 infection due to altered expression of CXCR5 and its ligand CXCL13. Dysregulation of chemokine receptors and their ligands may also disturb the lymph node architecture. Immune activation associated with HIV-1 leads to accumulation of activated B cells in the periphery, with altered migration as a consequence. The tightly regulated process of SHM and CSR in the GC is impaired, likely affecting selection and PC differentiation with increased production of low-affinity antibodies and hypergammaglobulinemia. During immune activation, increased levels of sCD27 can ultimately lead to increased IgG production through sCD27 mediated differentiation of PCs from CD70+ memory B cells. Finally, increased level of IL-7 also triggers T cells to produce IFN- $\gamma$ , which in turn can lead to increased apoptosis of B cells. The small rectangles on B cells indicate chemokine receptors.*



The decreased number of memory B cells during HIV-1 infection is not only the result of redistribution of B cells to different compartments, including spleen and lymph nodes, but is possibly also due to B cell apoptosis. In **paper II**, we presented a novel mechanism of bystander B cell apoptosis triggered by Fas; the up-regulation of expression of this receptor and increased Fas mediated apoptosis is induced through IFN- $\gamma$  production from IL-7-primed resting T cells. These data not only explain the accelerated B cell apoptosis observed during HIV-1 infection but also shed light on novel roles that IL-7 may play on B cell homeostasis. In spite of the fact that IL-7 is implicated in T cell proliferation and survival upon physiological condition, this cytokine can also induce T and B cell depletion in conditions associated with lymphopenia and characterized by an excessive level of IL-7.

IL-7 therapy has been suggested as a possible treatment during HIV-1 infection since it has a positive role on T cell survival and proliferation [144, 173]; this cytokine can, however, also have a negative impact on the levels of T and B cell populations through the induction of Fas-mediated apoptosis. On the contrary, whether the blocking of the excessive IL-7 levels found in the serum of lymphopenic HIV-1 infected patients could lead to reduced levels of B cell apoptosis needs further investigation. It is likely that using IL-7 as therapy during HIV-1 infection might have a double-sword effect for killing and rescuing T and B cells; therefore, therapy with IL-7 should be carefully evaluated in this respect before being administered to HIV-1 infected patients.

Beside improper homing and increased priming for apoptosis, B cells during HIV-1 infection also suffer from intrinsic molecular defects of mechanisms involved in antibody affinity maturation, including CSR and SHM. In **paper III**, we reported that naive-like CD27<sup>-</sup> B cells carry increased levels of SHM as well as elevated levels of

CSR, a finding supported from the higher levels of CD27-IgG<sup>+</sup> and CD27-IgA<sup>+</sup> B cells observed in the peripheral blood of HIV-1 infected patients. A more detailed characterization of the phenotype of the CD27-IgG<sup>+</sup> B cells identified by us in paper III would be important to pin-point the developmental origin of these cells. B cells during HIV-1 infection are activated as characterized by the high level of baseline AID, as well as by the increased level of expression of the activation marker CD70. Accordingly, understanding the intrinsic B cell defects taking place during HIV-1 infection might help to generate a comprehensive picture of B cell immunopathology, to improve treatment as well as the design of functional HIV-1 vaccines.

It can be speculated that the cumulative result of defects in B cell homing properties and defective affinity maturation may lead to lack of production of fully functional memory B cells in HIV-1 infected patients. The already formed memory B cells, on the other hand, are subjected to activation-induced differentiation. In **paper IV**, we showed that memory B cells up-regulated CD70 expression as the result of activation, which binds to sCD27 to induce PC differentiation and IgG production. This effect is pronounced during HIV-1 infection, where the levels of both receptor and ligand CD70 and sCD27 are elevated and even more significant in HAART-naïve patients where the level of immune activation is higher. The relevance of this finding on the role of sCD27 in inducing IgG production needs to be further explored in diseases characterized by elevated IgG levels in blood. Thus in addition to triggering of TLRs, memory B cells can be easily induced to produce IgG; this finding may represent a tool to induce IgG production from defined population of memory B cells.

Control of viral replication upon HAART leads to normalization of virus-associated immune deficiencies but has a limited impact on the control of immune activation.

Since LTNPs are able to control abnormal immune activation biological specimens from these patients should be further used to analyze the impact of immune activation on B cell dysfunctions. In this thesis I propose different mechanisms leading to impairments of B cell functions observed during HIV-1 infection. The different molecular pathways examined in this thesis are associated, directly or indirectly, with immune activation. Whether interfering with any of these pathways may compensate for the immunological damage occurring in B cells during HIV-1 infections needs to be verified.

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