From the Department of Microbiology, Tumor and Cell biology
Karolinska Institutet, Stockholm, Sweden

HIV-1 Infection and Loss of Serological Memory: the Role of Altered Expression of B-Cell Chemokine Receptors, Timing of HAART and Impaired Antibody Affinity Maturation

Alberto Cagigi

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Dedico questa tesi a tutte le persone che mi vogliono bene e che in vari modi mi hanno supportato durante questi anni, prima di tutti, i miei genitori.
Brindo all'amicizia, quella vera, quella al di sopra del tornaconto personale, dell'invidia, dell'ipocrisia e della falsità.

I dedicate this thesis to all those people who love me and who supported me in several ways during the past years, first of all, my parents.
I toast to friendship, the true one, the one above self-interest, envy, hypocrisy and falseness.

ABSTRACT

Currently, big efforts in HIV-1 research are devoted to the design of a HIV-1 vaccine. However, no successful vaccine is yet available, also due to the lack of basic knowledge in HIV-1 pathogenesis which is still profound. HIV-1 infected patients suffer of several abnormalities related to B-lymphocytes, which function is to produce antibodies (immunoglobulins) against common pathogenic viruses and bacteria, and experience loss of serological memory since early stages of infection. In the absence of a HIV-1 vaccine, ensuring a better protection against common diseases in infected adults and children is fundamental to prevent lethal infections of these patients. The research presented in this thesis aimed at improving the general knowledge on HIV-1 pathogenesis and at better understanding the mechanisms behind the B-cell impairments observed during HIV-1 infection. We found that (paper I), the loss of memory B-cells is a progressive event starting early during primary infection, when a partial but irreversible depletion of specific memory B-cells occurs. A possible explanation for this phenomenon is memory B-cell exhaustion and cell death as a consequence of the general (polyclonal) B-cell activation due to the reactivation of many pathogens typical of HIV-1 infection. In this scenario, the death of specific memory B-cells could be compensated through the activation of naïve B-cells by unspecific stimuli which may lead to the production of low-quality (affinity) antibodies. Through the work presented in paper II, we found that naïve B-cells in fact undergo processes of immunoglobulin affinity maturation in the immunoglobulin genes. Another important aspect which might be impaired during HIV-1 infection is lymphocyte trafficking between the periphery and (within) secondary lymphoid organs, for example lymph-nodes and spleen, where immunoglobulin affinity maturation takes place. In paper III, we observed that altered expression of key molecules for B-cell migration, such as the chemokine receptor CXCR5 and the chemokine CXCL13, occurs in B-cells and alters B-cell migration. This may result in defective reactions in the secondary lymphoid organs. In this respect, the application of the currently available antiretroviral therapy (HAART) early during primary infection might help minimizing these detrimental effects. In paper IV, we found that in the milieu of a developing immune system, such as the one of children infected with HIV-1 *in utero*, at birth or through breast feeding (vertically infected), an application of therapy within the first year of life in fact preserves both the development and the function of B-cells. This results in effective and long-lasting immunization upon common childhood vaccinations with measles, tetanus and pneumococcus. Summarizing, the work presented in this thesis enlightens the role of altered expression of B-cell chemokine receptors and impaired immunoglobulin affinity maturation for the loss of serological memory observed during HIV-1 infection and suggests the early initiation of HAART in vertically infected children. The understanding of these phenomena, in the absence of a HIV-1 vaccine, might be the key event for ensuring a better life of HIV-1 infected patients and might help solving the problems encountered so far in the development of a HIV-1 vaccine.

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SOMMARIO

Sebbene la maggior parte della ricerca sull'HIV-1 sia stata fino ad ora dedicata alla scoperta di un vaccino, la mancanza di conoscenze di base sul virus e sulla sua patogenesi, non ha permesso di raggiungere risultati apprezzabili. I pazienti infetti da HIV-1 presentano diverse anomalie dei linfociti B, la cui funzione è quella di produrre anticorpi (immunoglobuline) contro i piu' comuni virus e batteri patogenici, e una perdita di memoria sierologica sin dalle fasi iniziali dell'infezione. In assenza di un vaccino per l'HIV-1, assicurare ad adulti e bambini infetti una protezione migliore contro frequenti malattie è fondamentale allo scopo di prevenire infezioni letali. La ricerca presentata in questa tesi ha avuto lo scopo di migliorare la conoscenza generale sulla patogenesi dell'HIV-1 e di capire meglio i meccanismi alla base dei danni subiti dalle cellule B. Abbiamo scoperto (articolo I) che la perdita di cellule B della memoria è un evento progressivo che avviene già dalle fasi iniziali dell' infezione in cui si può osservare una parziale ma irreversibile deplezione di cellule B della memoria specifica. Una possibile spiegazione per questo fenomeno è l'esaurimento e la morte di queste cellule come conseguenza dell'attivazione generale (policlonale) a seguito della riattivazione di diversi patogeni durante l'infezione da HIV-1. In questo scenario la morte delle cellule B della memoria specifica potrebbe essere compensata dall'attivazione di cellule B prive di precedente esperienza (naïve) da parte di stimoli non specifici che potrebbe risultare in una produzione di anticorpi di bassa qualità (affinità). Con il lavoro presentato nell'articolo II abbiamo infatti scoperto che queste cellule svolgono processi di maturazione dell'affinità nei geni delle immunoglobuline. Un altro aspetto importante che potrebbe essere compromesso durante l'infezione da HIV-1 è la circolazione di linfociti tra la periferia e (all'interno dei) tessuti linfoidi secondari, come i linfonodi e la milza, dove avvengono i processi di maturazione dell'affinità. Nell'articolo III abbiamo osservato che nelle cellule B l'espressione alterata di alcune molecole chiave per la loro migrazione, come il recettore delle chemochine CXCR5 e la chemochina CXCL13, potrebbe appunto comprometterne la migrazione e risultare in reazioni difettose nei tessuti linfoidi secondari. A questo riguardo la somministrazione della terapia antiretrovirale attualmente disponibile (HAART) nelle fasi precoci dell'infezione primaria potrebbe aiutare a minimizzare questi effetti dannosi. Nell'articolo IV abbiamo scoperto che, nel contesto di un sistema immunitario in via di sviluppo come quello dei bambini che vengono infettati *in utero*, alla nascita o con l'allattamento (infettati verticalmente), una somministrazione della terapia entro il primo anno di vita infatti preserva sia lo sviluppo che la funzione delle cellule B risultando in una risposta efficiente e duratura alle comuni vaccinazioni somministrate durante l'infanzia come morbillo, tetano e pneumococco. Riassumendo, il lavoro presentato in questa tesi descrive, nel contesto della perdita di memoria sierologica osservata durante l'infezione da HIV-1, il ruolo dell'alterazione dei recettori delle chemochine nelle cellule B, la compromissione della maturazione dell'affinità delle immunoglobuline e suggerisce l'inizio precoce dell'HAART nei bambini. Capire questi fenomeni, in mancanza di un vaccino per l'HIV-1, potrebbe essere l'evento chiave per garantire una vita migliore ai pazienti e per aiutare a risolvere i problemi finora riscontrati nella progettazione del vaccino stesso.

LIST OF PUBLICATIONS AND MANUSCRIPTS INCLUDED IN THIS THESIS

I. Titanji K, De Milito A, **Cagigi A**, Thorstensson R, Grutzmeier S, Atlas A, Hejdeman B, Kroon FP, Lopalco L, Nilsson A, Chiodi F. Loss of memory B-cells impairs maintenance of long-term serologic memory during HIV-1 infection. Blood. 2006 Sep 1;108(5):1580-7. Epub 2006 Apr 27.

II. **Cagigi A**, Du L, Dang LVP, 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. Manuscript.

III. **Cagigi A**, Mowafi F, Dang LVP, 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.

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OTHER RELATED PUBLICATIONS

Cagigi A, Nilsson A, De Milito A, Chiodi F. B cell immunopathology during HIV-1 infection: lessons to learn for HIV-1 vaccine design. Vaccine. 2008 Jun 6;26(24):3016-25. Epub 2007 Dec 17. Review.

Mowafi F, **Cagigi A**, Matskova L, Björk O, Chiodi F, Nilsson A. The chemokine CXCL12 enhances proliferation in pre B-ALL via STAT5 activation. Pediatr Blood Cancer. 2008 Apr;50(4):812-7.

Ehlin-Henriksson B, Wu L, **Cagigi A**, Mowafi F, Klein G, Nilsson A. Changes in chemokines and chemokine receptor expression on tonsillar B cells upon Epstein-Barr Virus infection. Immunology. Accepted for publication.

LIST OF THE MOST COMMON ABBREVIATIONS

Ab	Antibody
Ag	Antigen
AID	Activation-induced cytidine deaminase
AIDS	Acquired human immunodeficiency syndrome
A. k. a.	Also known as
APC	Antigen presenting cell
ASC	Antibody secreting cell
BCR	B-cell receptor
BM	Bone marrow
CCL	C-C ligand
CCR	C-C receptor
CD	Cluster of differentiation
CHI	Chronic HIV-1 infection
CMV	Cytomegalo virus
CpG	Cytidine phosphate guanosine
CSR	Class switch recombination
CXCL	C-X-C ligand
CXCR	C-X-C receptor
DC	Dendritic cell
ELISpot	Enzyme-linked immunosorbent spot
FDC	Follicular dendritic cell
GC	Germinal center
HAART	Highly-active antiretroviral therapy
HIV-1	Human immunodeficiency virus-1
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LN	Lymph-node
LPS	Lipopolysaccharide
LTNP	Long-term non progressor
MHC	Major histocompatibility complex
MZ	Marginal zone
NOG	NOD/Shi-scid/IL-2Rγ^{null}
PBMC	Peripheral blood mononuclear cell
PC	Plasma cell
PHI	Primary HIV-1 infection
SCID	Severe combined immunodeficiency
SHM	Somatic hyper-mutation
SIV	Simian immunodeficiency virus
TLR	Toll-like receptor
TNF	Tumor necrosis factor
V, D, J and L, H	Variable, diversity, joining and light, heavy
WHO	World Health Organization

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1 INTRODUCTION

1.1 Development of the B-cell compartment, B-cell phenotypes and molecular mechanisms of B-cell differentiation

B-cells arise in the bone marrow (BM) from hematopoietic stem cells. In order to complete their differentiation, B-cells must undergo a complex series of phenotypic, molecular and functional changes (Table 1). Pro- and pre- B-cell precursors differentiate, and acquire surface expression of cluster of differentiation (CD) 10 and interleukin (IL) -7 receptor α. In the presence of IL-7, B-cells rearrange the variable (V), diversity (D) and joining (J) regions of the immunoglobulin (Ig) heavy (H) (VDJ), and light (L) (VJ) chain genes respectively, through a process involving recombination activating gene (RAG) -1 and RAG-2 gene products (Sagaert and De Wolf-Peeters 2003). After Ig gene rearrangement is complete, RAG-1 and 2 gene expression is down-regulated and immature B-cells lose the expression of CD10 and IL-7 receptor α and begin to express IgM as part of a not yet functional B-cell receptor (BCR) (Fry and Mackall 2002; Sagaert and De Wolf-Peeters 2003). At this stage B-cells become activated, up-regulate the surface expression of CD24 and CD38 and are exported to the periphery as immature/transitional B-cells. Here they complete their maturation, co-express IgD and IgM and finally home mainly to the spleen, lymph-nodes (LNs) and mucosa-associated lymphoid tissue (MALT) (Carsetti, Rosado et al. 2004). Transitional B-cells in the spleen marginal zone (MZ) provide a first line defense against quickly replicating pathogens, such as encapsulated bacteria. Evidences for this derive from the fact that stimulation of transitional B-cells through Toll-like receptor (TLR) -9 with bacterial cytidine phosphate guanosine (CpG) DNA motifes *in vitro* causes terminal differentiation of transitional B-cells followed by production of natural antibodies (Abs) with innate anti-pneumococcal specificity (Capolunghi, Cascioli et al. 2008). Human transitional B-cells combine the phenotype and tissue distribution of mouse T1 and T2 B-cells (Carsetti, Rosado et al. 2004). Mature naïve B-cells brightly express surface IgD but poorly IgM and are able to recognize antigens (Ags).

The ability of B-cells to produce highly specific Abs, upon encountering an Ag, will then be dependent on Ig affinity maturation (Carsetti, Rosado et al. 2004). This process consists in the introduction of somatic hyper-mutations (SHMs) in the Ig V region genes and class switch recombination (CSR) (Revy, Buck et al. 2005; Pan-Hammarstrom, Zhao et al. 2007; Stavnezer, Guikema et al. 2008). CSR and SHM are

both dependent on the up-regulation of the enzyme activation-induced cytidine deaminase (AID) in B-cells (Muramatsu, Kinoshita et al. 2000; Pan-Hammarstrom, Zhao et al. 2007). CSR is a highly regulated process, controlled by T-cell membrane interaction via the CD40 ligand with the CD40 molecule on the surface of activated B-cells and by soluble T-cell cytokines. Ligation of these cytokines to cognate receptors on B-cells causes direct downstream activation of signal transducers and activator of transcription (STAT) -6 and nuclear factor kappa B (NFkB) transcription factors and AID transcription (Stavnezer 1996; Pan-Hammarstrom, Zhao et al. 2007). Switching from IgM to IgG production and secretion is normally a phenomenon restricted to Ag triggered B-cells. Interestingly it has been shown that bacterial CpG and viral double stranded RNA can directly activate B-cells by TLR9 and TLR3 ligation respectively, inducing IgG and IgA CSR (He, Qiao et al. 2004; Xu, Santini et al. 2008). Most TLRs are localized in intracellular compartments and ligate viral nucleic acid in endosomes; this localization ensures that TLRs will not bind to the host nucleic acid which is not normally accessible in these compartments and it does not trigger TLRs (Iwasaki and Medzhitov 2004). Human B-cells express TLR1, TLR3 and TLR6 through TLR10 and their levels increase upon B-cell activation, particularly those of TLR9 and 10 (Bourke, Bosisio et al. 2003; Xu, Santini et al. 2008).

Table 1. Phenotypic, molecular and functional changes during B-cell development.

B-cell stage	Mouse analogue	A. k. a.	Location	Immuno-phenotype	Ig expression	Description
Pro/Pre B-cells		B-cell precursors	BM	CD10+ IL7Rα+	None	Immunoglobulin gene rearrangements ongoing
Immature B-cell			BM	CD5+ CD10+ IL7Rα-	IgM+	VDJ rearranged/ Surrogate BCR
Immature/ Transitional B-cells	T1 and T2 B-cells		BM/periphery/spleen MZ	CD5- CD10+ $CD24^{high}$ $CD38^{high}$	IgD^{low} IgM^{high}	Co-express IgD and IgM
Mature naïve B-cells		Follicle centre B-cells: centrocytes-centroblasts	Secondary follicles: light-dark zone of GC, also MZ/periphery	CD10- $CD21^{high}$ $CD24^{low}$ $CD38^{low}$ CD40+ $CXCR4^{low}$ $CXCR5^{high}$ $CCR7^{high}$	IgD^{high} IgM^{low}	Able to recognize Ags
IgM memory B-cells	B1a and B1b B-cells	Lymphocytic corona and MZ B-cells	Secondary follicles: lymphocytic corona, MZ	CD27+	IgD^{low} IgM^{high}	Secrete natural antibodies/Respond to T-independent Ags
Memory B-cells		Follicle centre B-cells: centrocytes-centroblasts	Secondary follicles: light-dark zone of GC, also MZ/periphery	$CD21^{high}$ CD27+ CD40+ CD80+ CD86+	IgD- IgM- IgA+ or IgE+ or IgG+	CSR and SHM
Short lived plasma cells		MZ derived plasma cells	Secondary follicles: MZ (mainly spleen) /periphery	CD38+ $CXCR4^{high}$ $CXCR5^{low}$ $CCR7^{low}$	sIgM+	Secrete low-affinity antibodies/No CSR but low SHM
Long lived plasma cells		GC derived plasma cells	Periphery/BM	CD38+ CD138+ $CXCR4^{high}$ $CXCR5^{low}$ $CCR7^{low}$	sIgA+ or sIgE+ or sIgG+	Secrete high-affinity antibodies/CSR and SHM

1.1.1 B-cells as antigen presenting cells

Ag-primed B-cells up-regulate CD40 and other co-stimulatory molecules such as CD80 and CD86. This allows B-cells to function also as antigen presenting cells (APCs) through Ag- major histocompatibility complex (MHC) class II complexes since B-cells can internalize Ags through endocytosis. Ag presentation mediated by B-cells is of moderate activity and mainly directed towards T-cells able to recognize the same Ag in the T cell-zone of secondary lymphoid organs (Sagaert, Sprangers et al. 2007).

1.2 Role of chemokine and chemokine receptors during immune responses

Lymphocyte migration between the periphery and secondary lymphoid organs is the key for effective immune responses. Migration is highly regulated by chemokine receptor expression on lymphocytes together with the expression of their respective ligands (chemokines) in different tissue compartments (Rojo, Suetomi et al. 1999; Okada and Cyster 2006). The chemokine receptor C-X-C receptor (CXCR) 4 is largely expressed in BM B-cells, as well as in the periphery, and plays an important role for early B-cell development (Nagasawa, S. et al. 1996; Honczarenko, Lee et al. 2005) and plasma cell (PC) homing to the BM (Tew, DiLosa et al. 1992; Hauser, Debes et al. 2002). Conversely, the chemokine receptor CXCR5 is generally expressed by mature B-cells and plays an important role for the recruitment of naïve B-cells into secondary lymphoid organs (Muller, Hopken et al. 2003) where germinal center (GC) formation takes place and CSR and SHM occur. B-cells also express moderate amount of the chemokine receptor C-C receptor (CCR) 7, which also contributes to the migration within the LNs (Okada, Ngo et al. 2002).

1.2.1 Changes in the organization of secondary lymphoid organs during immune responses

The organisation of GCs into light and dark zones has been attributed to changes in the B-cell chemokine receptor expression of CXCR4, CXCR5 and CCR7 (Okada, Ngo et al. 2002; Allen, Ansel et al. 2004; Allen, Okada et al. 2007). Ag-primed B-cells (also called centroblasts) proliferate and rapidly up-regulate CXCR5 and CCR7. Proliferating B-cells increase their responsiveness to the chemokines C-X-C ligand (CXCL) 13 and C-C ligand (CCL) 19/21 (cognate ligands for CXCR5 and CCR7 respectively), which are secreted by follicular dendritic cells (FDCs), follicular stromal cells (Gunn, Ngo et al. 1998; Ansel, Harris et al. 2002; Carlsen, Baekkevold et al. 2004) and rarely by T-cells (Kim, Lim et al. 2004). Centroblasts migrate to one extremity of the GC forming the dark zone and the resting B-cells (also called centrocytes) including memory B-cells, accumulate at the opposite extremity of the GC forming the light zone (Sagaert, Sprangers et al. 2007). Non-Ag primed naïve B-cells are pushed aside from the follicle and form the mantle zone. The inner part of the mantle zone is called lymphocytic corona which includes a T-cell-zone, while the outer part is called marginal zone, which is mainly visible in the spleen (Sagaert, Sprangers et al. 2007). Upon encounter with Ag specific T-cells in the T-cell zone, activated B-cells

undergo Ig affinity maturation processes, down-regulate the expression of CXCR5 and CCR7 and by up-regulating CXCR4, become susceptible to CXCL12-driven chemotaxis (Okada, Ngo et al. 2002; Allen, Ansel et al. 2004; Allen, Okada et al. 2007). Leaving the GC, activated B-cells finally become long-lived PCs producing high affinity soluble switched Abs or alternatively, become memory B-cells and up-regulate the CD27 expression (Tarlinton 2006; Sagaert, Sprangers et al. 2007). Conversely, MZ B-cells proliferate *in loco* and differentiate into short lived IgM producing PCs and act as a first line defense against pathogens (Sagaert and De Wolf-Peeters 2003). Lymphocytic corona and MZ B-cells, during the early phases of infection and before specific Abs are produced, secrete natural Abs, mostly of IgM type, which bind to Ags with low affinity and then become IgM memory B-cells (Carsetti, Rosado et al. 2004; Carsetti, Rosado et al. 2005). Response against T-independent Ags also takes place in the MZ (Sagaert, Sprangers et al. 2007). In the mouse, natural Abs are produced by B1a B-cells while B1b B-cells are also responsible for the secretion of Abs against T-independent Ags (Alugupalli, Leong et al. 2004; Carsetti, Rosado et al. 2004). A schematic representation of the changes in the organization of secondary lymphoid organs occurring during immune responses is shown in figure 1.

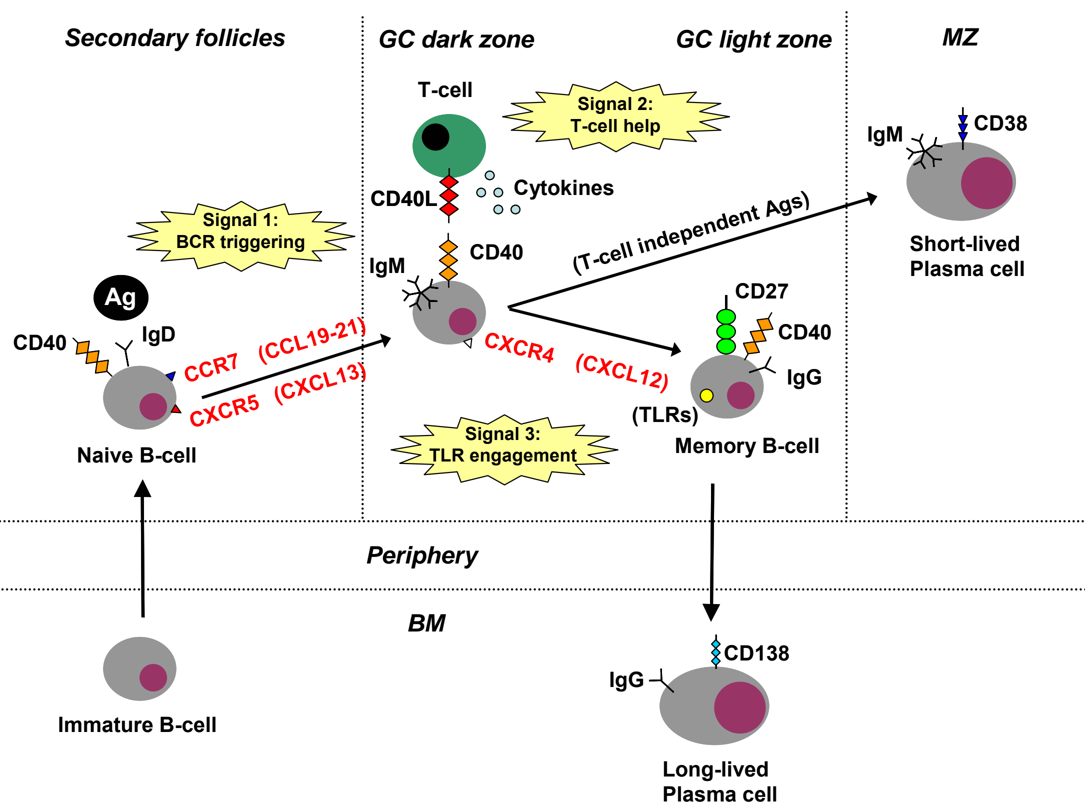

Figure 1. Schematic representation of the changes in the organization of secondary lymphoid organs occurring during immune responses.

1.3 The maintenance of serological memory

The ability to develop and maintain an intact immunological memory is essential for the development of effective immune responses upon re-infections with previously encountered Ags (Zinkernagel and Hengartner 2001). Maintenance of serological memory is carried out by long-lived PCs and memory B-cells which play an essential role in the production of highly specific Abs by rapidly generating secondary immune responses (Slifka and Ahmed 1998; Zinkernagel and Hengartner 2001; Bernasconi, Traggiai et al. 2002; Tangye, Avery et al. 2003). However, whether maintenance of serological memory throughout life is dependent on Ag persistence is still debated and several theories have been formulated. The lifespan of PCs (Slifka, Antia et al. 1998), the persistence of Ag (Oehen, Waldner et al. 1992) and the possibility of activating memory B-cells upon polyclonal signals (Bernasconi, Traggiai et al. 2002) have been proposed as the possible mechanisms responsible for maintenance of serological memory.

The lifespan of PCs in the bloodstream, in absence of Ag, has been estimated to be around 3 months. However, PC survival throughout life has been suggested to be dependent on PC homing to BM survival niches (Slifka and Ahmed 1998; Hauser, Debes et al. 2002). On the other hand, it has also been suggested that maintenance of protective long-lived IgG levels is related to Ag re-exposure through re-infections, persistent infections and through the presence of immunocomplexes on FDCs (Oehen, Waldner et al. 1992; Zinkernagel and Hengartner 2001). Maintenance of serological memory has also been attributed to polyclonal activation of memory B-cells which then continuously differentiate into PCs. Polyclonal activation occurs in response to bystander T-cell help and microbial products triggering TLRs directly on B-cells in the absence of specific Ag (Bernasconi, Traggiai et al. 2002). TLR stimulation has also been suggested as a third signal required for activation of human naive B-cells in a model where BCR stimulation would be the signal one, T-cell help the signal two and TLR activation would be the third signal (Ruprecht and Lanzavecchia 2006) (Figure 1). In addition, a more recent report suggested that the PC pool is independent of memory B-cells (Ahuja, Anderson et al. 2008). In this murine model, the levels of Ag specific PCs generated during an immune response could be maintained over a period of 16 weeks in the absence of B-cells, previously depleted (Ahuja, Anderson et al. 2008).

1.4 The human immunodeficiency virus-1 (HIV-1)

HIV-1 transmission occurs mainly by fluid exchange during sexual intercourse and through blood contact. Vertical transmission occurs from mother to child *in utero* during pregnancy, at delivery and through breast feeding with a rate of 25% in the absence of treatment (Coovadia 2004). The risk of vertical transmission can however be reduced to under 1% by antiretroviral treatment of the mother during pregnancy, cesarian section at delivery and by avoiding breast feeding after childbirth (Coovadia 2004). HIV-1 infection in humans is pandemic. At 2007, the Joint United Nations Programme on HIV/AIDS (UNAIDS) and the World Health Organization (WHO) estimate that over 33 million people are infected with HIV-1 and that the acquired human immunodeficiency syndrome (AIDS) has killed more than 25 million since its description in 1981.

HIV-1 belongs to the family of retroviruses called lentiviruses. The virus is composed of two copies of positive single-stranded RNA containing 9 genes encoding 19 proteins (gag, pol, and env, tat, rev, nef, vif, vpr and vpu) flanked by 2 repetitive sequences of bases called long terminal repeats (LTR) (Figure 2). *Gag* encodes for the viral core structural proteins, *pol* encodes for the viral enzymes, and *env* encodes for the viral envelope structural proteins. In particular, *env* encodes for the protein gp160 which is subsequently cleaved into gp120 and gp41. Both these surface proteins, especially gp120, have been considered as targets for vaccines against HIV-1. Gp120 is the main molecule interacting with the CD4 receptor on T-cells for entry of the virus into the host cells. The six remaining genes, *tat*, *rev*, *nef*, *vif*, *vpr*, and *vpu*, are regulatory genes for proteins that control the ability of HIV-1 to infect cells, replicate, or cause disease. In particular, the nef protein downregulates CD4, as well as the MHC class I and class II molecules (Garcia and Miller 1991; Schwartz, Marechal et al. 1996; Stumptner-Cuvelette, Morchoisne et al. 2001). Due to the lack of proof-reading by the viral enzyme reverse transcriptase, a high number of mutations is introduced in the viral genome during replication into the host cell. This results in the high HIV-1 genetic variability (Robertson, Hahn et al. 1995). Thus, many new HIV-1 variants are generated in a single individual per day. Genetic recombination may occur when a single cell is simultaneously infected by two or more different strains of HIV-1 although this is a rare event (Thomson, Perez-Alvarez et al. 2002). Three main groups of HIV-1, defined as the M, N, and O clades, have been identified on the basis of

differences in env (Thomson, Perez-Alvarez et al. 2002). Moreover, according to the usage of CXCR4 (X4) or CCR5 (R5) chemokine receptor as co-receptors for entry into the host cells, X4 and R5 tropic HIV-1 can also be identified (Coakley, Petropoulos et al. 2005).

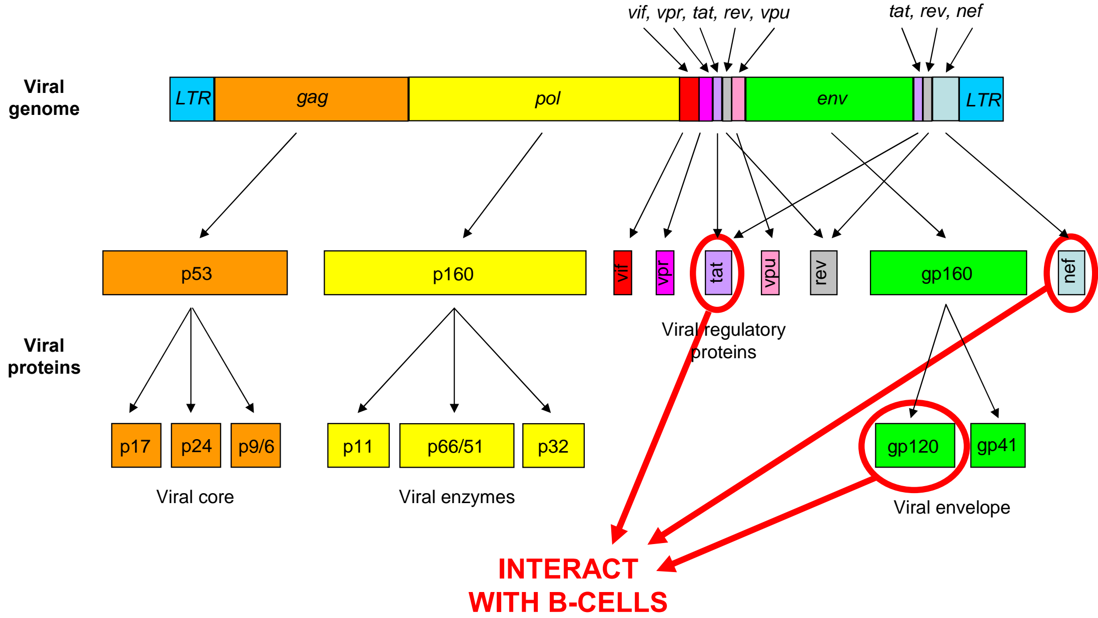

Figure 2. HIV-1 genome and HIV-1 viral proteins interacting with B-cells.

1.4.1 B-cells in HIV-1 infection

HIV-1 infection is associated with several B-cell abnormalities such as phenotypic alterations, polyclonal B-cell activation, increased frequencies of B-cell malignancies, hypergammaglobulinemia and poor Ag-specific primary and secondary immune responses (Opravil, Fierz et al. 1991; Ng, Hurt et al. 1994; Moir, Malaspina et al. 2001; De Milito, Nilsson et al. 2004; Wood and Harrington Jr 2005). Moreover, HIV-1 infection induces follicular hyperplasia in secondary lymphoid tissue with alterations in the GC and splenic MZ architecture (Koopman, Taher et al. 1998; Wilkins, Davis et al. 2003). Defects in the B-cell compartment arise early during primary HIV-1 infection as a decline of B-cell number and increased expression of activation and apoptosis markers (Titanji, Chiodi et al. 2005). A summary of the B-cell defects occurring during HIV-1 infection is represented in figure 3.

HIV-1 is unable to infect B-cells because of the lack of CD4 expression on the B-cell surface. However, most B-cells constitutively express the HIV-1 co-receptor CXCR4 and several viral particles have been shown to be up-taken by B-cells (Moir and Fauci 2008). The mechanisms by which HIV-1 impairs humoral immunity may either be the result of indirect virus-induced B-cell defects, or B-cell polyclonal activation with or without concomitant lack of functional dialogue between B- and T-cells in secondary lymphoid organs. Several viral factors that might have a role in the B-cell impairments have already been described. For instance, interaction between the viral envelope gp120 and the immunoglobulin VH family member 3 (VH3) has previously been related to the depletion of VH3-expressing B-cells in HIV-1 infected individuals (Berberian, Goodglick et al. 1993; Juompan, Lambin et al. 1998). Moreover, also the tat protein, released from infected T-cells *in vivo*, may act on bystander B-cells by up-regulating Fas expression on the B-cell surface (Huang, Li et al. 1997) thus contributing to the increased susceptibility of B-cell to apoptosis during HIV-1 infection (De Milito 2004). Interestingly, tat has also been shown to inhibit proliferation of BCR triggered naïve and memory B-cells (Lefevre, Krzysiek et al. 1999). In contrast, polyclonally activated B-cells from GC origin respond positively to tat-induced proliferation (Lefevre, Krzysiek et al. 1999). More recently, also purified proteins from the HIV-1 *env* and *nef* genes have been shown to interact with B-cells affecting CSR in opposite directions (He, Qiao et al. 2006; Qiao, He et al. 2006). In this respect, it is of particular interest that HIV-1 structural proteins accumulate and persist in patient LNs despite highly-active antiretroviral therapy (HAART) treatment (Popovic, Tenner-Racz et al. 2005).

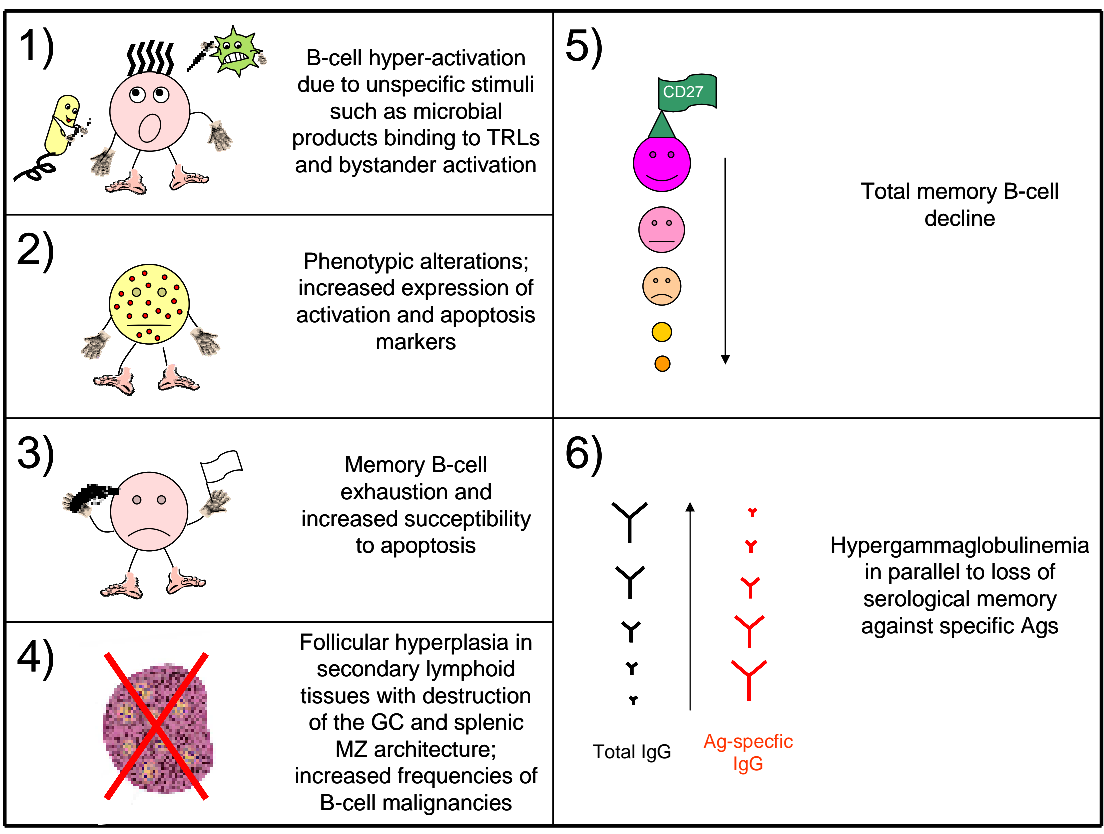

Figure 3. B-cell defects occurring during HIV-1 infection.

1.4.2 B-cells in other immunodeficiencies

Abnormalities during B-cell development may directly cause immunological diseases (LeBien and Tedder 2008). In the X-linked agammaglobulinemia (XLA) which is due to a mutation in the Bruton tyrosine kinase (BTK), there is a block in the transition from pro-B to large pre-B-cells in the BM resulting in a substantial reduction in the percentage of peripheral B-cells (Tsukada, Saffran et al. 1993; Vetrie, Vorechovsky et al. 1993). Common variable immunodeficiency (CVID) is caused by mutations in several genes including the inducible T-cell costimulator (ICOS), the pan B-cell marker CD19, and the tumor necrosis factor (TNF) receptor superfamily member TACI during later stages of B-cell development. Affected individuals have low serum Ig levels and increased susceptibility to infection, memory B-cell reduction, impaired CSR and B-cell activation (Bacchelli, Buckridge et al. 2007). A similar clinical picture is caused by the hyper-IgM syndrome, characterized by elevated serum IgM and a general failure of B-cells to undergo CSR and SHM (Durandy, Taubenheim et al. 2007). The most common mutation causing the hyper-IgM syndrome has been identified in the gene encoding for the CD40 ligand on activated T-cells. However, rare mutations in CD40,

IKK-gamma/NEMO, and uracil-DNA glycosylase have also been reported in B-cells. Of particular interest is that all mutations causing the hyper-IgM syndrome interfere with the function of AID, thus impairing the formation of functional class switched and somatically mutated Abs (Revy, Muto et al. 2000).

1.4.3 Vaccination in HIV-1 infected individuals

Development and maintenance of serological memory upon vaccination is severely impaired in immunocompromised individuals who will remain susceptible to infection with several viral and bacterial pathogens such as measles, tetanus and pneumococcus. As a consequence of T-cell depletion, HIV-1 infected individuals respond poorly to immunization and both adults and children experience rapid waning of immunity (Janoff, Douglas et al. 1988; Janoff, Hardy et al. 1991; Kroon, van Dissel et al. 1994; Arpadi, Markowitz et al. 1996).

Most viruses and live attenuated virus vaccines elicit a T-dependent immune response. Consequently, CD4+ T-cells have a crucial role for the efficacy of immunizations against T-dependent Ags. A widely used vaccine eliciting a T-dependent immune response is represented by the MMR vaccination which is a mixture of three live attenuated viruses against measles, mumps and rubella. MMR is generally administered to children between 9 and 16 months of age. However, a second dose is required since a small number of individuals (2-5%) fail to develop immunity to measles after the first dose (Moreton 2002).

Ab formation against several bacterial Ags, mainly represented by capsular polysaccharides, is a T-independent process and vaccines based on these Ags are poorly immunogenic and frequently elicit poor memory responses (Lesinski and Westerink 2001). Thus, in order to provide increased T-cell help and improved immune responses some bacterial vaccines have been conjugated to toxins for example in the childhood pneumococcal vaccination. In adults, a 23-valent pneumococcus non-conjugated polysaccharide vaccine (PPV) is usually administered once, while children under the age of two years often fail to mount an adequate response to the 23-valent adult vaccine. Instead a 7-valent pneumococcal conjugated vaccine (PCV) is used which requires several booster doses. Immune responses against PPV have been extensively studied in HIV-1 patients by several investigations showing that HIV-1 infected individuals are less likely to respond to PPV than healthy controls (Janoff,

Douglas et al. 1988; Rodriguez-Barradas, Musher et al. 1992; Rodriguez-Barradas, Groover et al. 1996; Kroon, van Dissel et al. 1999). These studies suggest that since pneumococcus Ags are T-independent, the mechanisms behind the lower Ab response to PPV in HIV-1 patients might be associated not only with the clinical status and CD4+ T-cell count, but also with B-cell defects (Subramaniam, Segal et al. 2003).

Nowadays, a large number of researchers are investigating the possibility to optimize DNA vaccination techniques. These techniques rely on the delivery of genetically engineered plasmids encoding for specific vaccination Ags directly into the body of patients. Newly encoded Ags will then be processed by the host cells and displayed on their surface in a MHC class I or class II restricted fashion according to the delivery method and plasmid design (Feltquate, Heaney et al. 1997) and recognized as foreign by the immune system (Alarcon, Waine et al. 1999; Robinson and Pertmer 2000). The most commonly used methods of DNA delivery into the host are the saline injection of DNA into skin or muscle and the gene gun immunization which have been shown to be effective in mice (Robinson and Pertmer 2000). The immunogenicity of these DNA delivery methods in primates can be enhanced by *in-vivo* electroporation (Widera, Austin et al. 2000). However, the application of DNA vaccines is still experimental in the context of a number of viral (including HIV-1), bacterial and parasitic models of disease, as well as in tumor vaccination models (Cui and Huang 2005). Interestingly, plasmid DNA itself has an adjuvant effect on the immune system (Alarcon, Waine et al. 1999; Robinson and Pertmer 2000) due to recognition of CpG motifes by the host TLR9 (Klinman, Yamshchikov et al. 1997).

1.5 Animal models for the study of HIV-1 infection

The simian immunodeficiency virus (SIV), which naturally infects African green monkeys and sooty mangabeys, is genetically related to HIV-1. However, although present at high levels in the blood, SIV triggers only a mild immune response (Holzammer, Holznagel et al. 2001) which does not cause the development of simian AIDS in their natural hosts (Baier, Dittmar et al. 1991). Moreover, SIV does not undergo the extensive mutation and recombination typical of HIV-1 (Baier, Dittmar et al. 1991). Conversely, infection with SIV of heterologous hosts such as rhesus or cynomologus macaques, results in the generation of genetic diversity and simian AIDS (Daniel, King et al. 1984). Thus, SIV infection of rhesus or cynomologus macaques is a suitable animal model for the study of T- and B-cell pathogenesis during SIV/HIV-1

infection by allowing parallel observations in several tissue compartments. However, SIV infection does not fully mimic HIV-1 infection or vaccination against HIV-1 and elevated laboratory costs and ethical issues render research based on these animal models rather complex.

Many researchers study HIV-1 pathogenesis and test new vaccine candidates directed against HIV-1 in mouse models; these models pose different kind of limitations. Nude mice are genetic mutants that lack or have a dysfunctional thymus which results in a greatly reduced number of T-cells (Wortis 1974). Other models are based on mice with severe combined immunodeficiency (SCID) which lack B- and T- cells (McCune 1991) and non-obese diabetic (NOD)/Shi-scid/IL-2Rγ^{null} (NOG) mice (Goldman, Blundell et al. 1998; Hiramatsu, Nishikomori et al. 2003; Traggiai, Chicha et al. 2004), which lack activity of T- B- and natural killer (NK) -cells and have reduced complementary activities. However, mice are generally not permissive for HIV-1 infection, thus modifications of the infecting virus and/or alternative approaches such as DNA vaccination must be performed (Boberg, Brave et al. 2008).

A new frontier in this field has recently been the "humanization" of mice obtained by transferring hematopoietic stem cells into mice which are lethally irradiated, leading to repopulation of the murine depleted tissue compartments by human lymphocytes (Marcus, David et al. 1995). SCID and NOG mice can also accept heterologous cells compared to other non-humanized models such as nude mice but less efficiently than irradiated mice (Boberg, Brave et al. 2008). Once mice have been humanized, they can be infected with HIV-1 as recently demonstrated in a humanized NOG murine model where long-lasting viremia after infection with both R5 and X4 HIV-1 strains could be observed together with anti-HIV-1 gp120- and p24-specific Ab formation (Watanabe, Terashima et al. 2007). Intrarectal transmission of HIV-1 followed by systemic infection and CD4+ T-cell depletion has also been successfully reproduced in humanized NOG/SCID mice (Sun, Denton et al. 2007).

2 AIM OF THE THESIS

Nowadays, many efforts within HIV-1 research are devoted to the design of a HIV-1 vaccine. However, no successful vaccine has yet been designed, probably because lack of knowledge in the field of HIV-1 pathogenesis is still profound. HIV-1 does not directly infect B-cells but yet patients suffer of several B-cell abnormalities including loss of serological memory which might occur early during primary HIV-1 infection (PHI). In the absence of a HIV-1 vaccine, ensuring a better protection against infections in infected adults and children is fundamental.

The aims of this thesis were

- To investigate the dynamics and the mechanisms behind loss of serological memory by:
  - Evaluating the loss of specific Abs during HIV-1 infection in relation to specific memory B-cells (Paper I and IV)
  - Studying the molecular mechanisms of Ig affinity maturation in HIV-1 infected individuals (Paper II)
  - Measuring chemokine and chemokine receptor expression on B-cells and patterns of B-cell migration (Paper III)
- To investigate whether timing of HAART in pediatric HIV-1 infection could be helpful to preserve the memory B-cell compartment (Paper IV)
- To define new markers of disease progression and new methods to evaluate the B-cell compartment during HIV-1 infection (Paper I and IV)

3 RESULTS AND DISCUSSION

3.1 Loss of serological memory during HIV-1 infection (Paper I)

HIV-1 infection results in impaired ability of patients to mount both primary and secondary immune responses depending on the immune system status and disease progression (Ballet, Sulcebe et al. 1987; Janoff, Hardy et al. 1991; Kroon, van Dissel et al. 1994; Kroon, van Dissel et al. 1997; Kroon, Rimmelzwaan et al. 1998; Kroon, van Dissel et al. 1999; Kroon, van Dissel et al. 1999; Kroon, van Dissel et al. 2000; Malaspina, Moir et al. 2005). As a result, long-term serological memory is severely impaired as suggested by vaccination studies (Nielsen, Kvinesdal et al. 1998; Malaspina, Moir et al. 2005) and by studies of the memory B-cell compartment during HIV-1 infection (De Milito, Morch et al. 2001; Chong, Ikematsu et al. 2004; De Milito, Nilsson et al. 2004). The maintenance of a functional memory B-cell compartment is crucial not only for secondary immune responses but also for protection against opportunistic infections in HIV-1 infected patients. This has been demonstrated by *in vivo* studies with SIV infection models showing that cytomegalovirus (CMV) reactivation is associated with loss of CMV-specific CD4+ T-cells and the subsequent decline of CMV-specific Ab production by B-cells (Kaur, Kassis et al. 2003). Loss of Ag-specific Abs in humans is also associated with reduced levels of CD4+ T-cells and total memory B-cells during HIV-1 infection (De Milito, Morch et al. 2001; De Milito 2004).

In order to characterize the dynamics of loss of serological memory during HIV-1 infection, we measured serum Ab titers to measles and pneumococcus, a T-dependent and a T-independent Ag, in HIV-1 infected patients at different stages of infection including PHI, chronic HIV-1 infection (CHI) and long-term non progressors (LTNPs). We found that loss of specific memory to these Ags is a progressive event occurring in the course of HIV-1 infection since it was not detectable at the early stages of PHI but clearly detected in patients with CHI. Interestingly, LTNPs had specific Ab levels to measles and pneumococcus similar to those of healthy controls. In parallel, we also evaluated the percentage of total memory B-cells and found a strong correlation to the CD4+ T-cell count in patients with CHI (paper I). Based on these data we suggest the percentage of total circulating memory B-cells as an additional marker of HIV-1 immune dysfunction. On the other hand, the percentage of circulating memory B-cells was still preserved in both PHI and LTNPs (paper I). Normal levels of memory B-cells

in these patients are probably accompanied by a declining, but still relatively high CD4+ T-cell count. Thus, a partially preserved helper T-cell function could still induce a good Ab response to vaccination in early stages of PHI and in LTNPs (Weiss, Wallace et al. 1995; Kroon, van Dissel et al. 1999; Kroon, van Dissel et al. 2000; Malaspina, Moir et al. 2005). However, the dramatic reduction in Abs to pneumococcus, a T-independent Ag, suggests that an intrinsic defect takes place in the B-cell compartment early during HIV-1 infection. Reduction of specific Abs to pneumococcus is already detectable during PHI and this is in line with a previous observation that peripheral memory B-cells are phenotypically altered but normal in frequency during PHI (Titanji, Chiodi et al. 2005). A possible contribution to a direct B-cell damage during HIV-1 infection is HIV-1 gp120, which is able to crosslink membrane IgM on the surface of B-cells, acting as a superantigen and inducing their activation, thus contributing to the depletion of VH3+ B-cells (Karray and Zouali 1997). The VH3 gene family encodes for approximately 50% of all Abs, including those against pneumococcal Ags (Kruetzmann, Rosado et al. 2003; Vinuesa, Sze et al. 2003). Thus, the depletion of VH3+ memory B-cells might contribute to the decrease of anti-pneumococcal Abs that we observed (paper I).

The response against pneumococcal Ags is well characterized and known to trigger short-lived PC and IgM+ memory B-cell formation in a T-independent fashion in the spleen MZ (Kruetzmann, Rosado et al. 2003; Vinuesa, Sze et al. 2003). Also IgM+ memory B-cells are reduced in PHI (Titanji, Chiodi et al. 2005). Conversely, a response against T-dependent Ags such as measles should generate memory B-cells and long-lived PCs residing in the BM (Hauser, Debes et al. 2002). Given that the total decline of memory B-cells goes in parallel with the loss of serological memory during HIV-1 infection, the question that comes along is: what is the impact of Ag-specific memory B-cells in the maintenance of Ag-specific Ab titers over-time? We addressed this question by using a measles-specific B-cell enzyme-linked immunosorbent spot (ELISpot), modified from a previously described method (Crotty, Aubert et al. 2004), to detect and enumerate measles-specific memory B-cells in patients upon polyclonal stimulation of peripheral blood mononuclear cells (PBMCs) *in vitro*. Interestingly, we found that in parallel to the reduction of anti-measles Ab levels, the number of measles-specific antibody secreting cells (ASCs) was also significantly reduced in CHI. This strongly correlated to both the total memory B-cell percentages and the plasma anti-measles Ab titers (paper I). Taken together, our data suggest that the reduced numbers

of circulating specific memory B-cells during CHI reflects an actual functional defect in specific serological memory. In support of our hypothesis is the fact that measles Ab titers correlate to the memory B-cell percentages also in healthy individuals (Amanna, Carlson et al. 2007). Although the percentage of measles-specific memory B-cells was significantly reduced in PHI (paper I), the total number of circulating memory B-cells in these patients, was comparable to healthy controls (Titanji, Chiodi et al. 2005). This might be due to the fact that the few memory B-cells that are lost during PHI are likely to be non-HIV-1 specific such as measles. Altered expression of TNF receptors on memory B-cells (Moir, Malaspina et al. 2004) and HIV-1-associated B-cell exhaustion leading to a dysfunctional memory B-cell compartment occur in HIV-1 infected viremic individuals (Moir, Ho et al. 2008) when HIV-1-specific memory B-cells are probably still in the process of being formed. HIV-1-associated B-cell exhaustion has been indicated for a population of tissue-like memory B-cells in peripheral blood, lacking the expression of CD27 and presenting low levels of surface CD21 (Moir, Ho et al. 2008). This B-cell population showed patterns of chemokine and inhibitory receptors similar to those described for antigen-specific T-cell exhaustion and had low Ig diversity compared with memory B-cells, indicating that these B-cells may be part of a dysfunctional memory B-cell compartment (Moir, Ho et al. 2008).

The strong association between loss of specific Abs against measles and loss of measles-specific memory B-cells finally raises the question on whether such B-cell deficiency may be corrected by HAART. For this reason, we investigated the presence of specific Ab titers to measles and pneumocuccus in patients with PHI prior to and upon 6 months of HAART. Interestingly, we found that therapy does not improve serological memory neither if initiated during PHI or after established infection (paper I). This strongly suggests that PHI might be the crucial event for the elimination of Ag-specific pools of memory B-cells and that Ag re-encounter might be needed for the restoration of Ag-specific memory B-cells at this stage (Figure 4). Interestingly, treated patients also experience a reduction in HIV-1-specific Abs (paper I). Memory B-cell response to HIV-1 may thus require the presence of circulating Ag as demonstrated by the disappearance of HIV-1-specific Abs in patients on HAART (Morris, Binley et al. 1998; Notermans, de Jong et al. 2001; Fondere, Huguet et al. 2003).

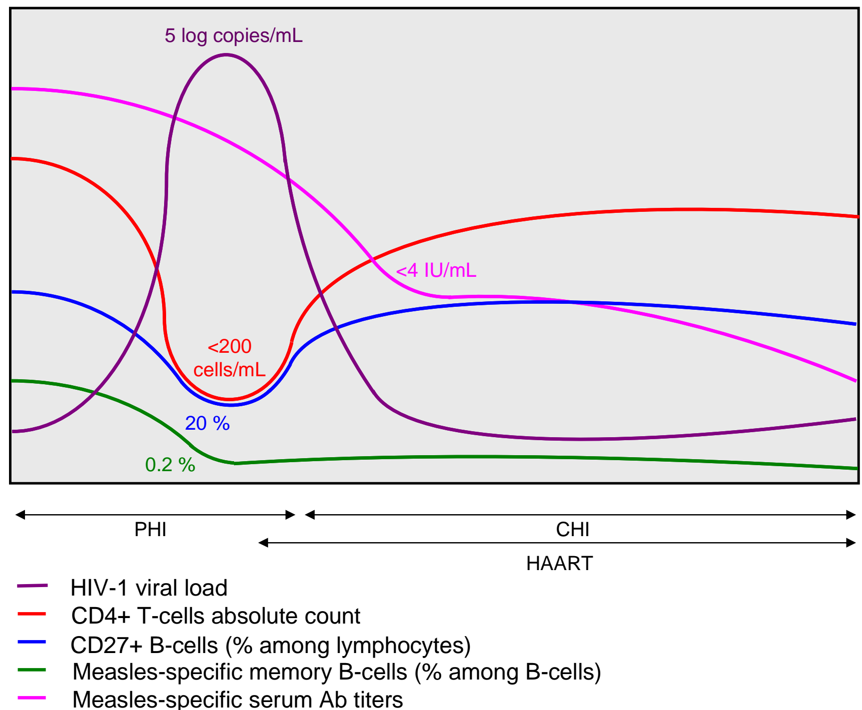

Figure 4. Dynamics of memory B-cell death and loss of serological memory. HIV-1 infection leads to total and measles-specific memory B-cell death early during PHI causing loss of serological memory. While total memory B-cell percentages are recovered upon HAART initiation, the measles specific memory B-cells remain low suggesting that Ag re-encounter might be needed for their formation *de novo*. The values shown in this figure are based on the median values observed in our study groups while scales are arbitrary.

3.2 Impaired antibody affinity maturation contributes to the reduction of specific antibodies observed during HIV-1 infection (Paper II)

Despite that successful HAART is able to normalize CD4+ T-cell counts and B-cell subpopulations percentages in blood (Moir, Malaspina et al. 2008), many opportunistic micro-organisms still remain more active within the mucosa (Nannini and Okhuysen 2002; Laurence 2005). This may result in the increased circulation of microbial products within body compartments during both PHI and CHI (Brenchley, Price et al. 2006). In this scenario, B-cells may be more susceptible to activation and differentiation into ASC by polyclonal stimuli (Bernasconi, Traggiai et al. 2002) thus contributing to hypergammaglobulinemia (Fondere, Huguet et al. 2004). Bacterial CpG DNA motifes activate B-cells by TLR9 ligation inducing IgG CSR (He, Qiao et al. 2004). Interestingly, also viral double stranded RNA has been shown to induce production of class switched Abs through the engagement of TLR3 in the upper respiratory tract (Xu, Santini et al. 2008) but whether viral RNA from HIV-1 impacts on the production of class switched Abs is not known. However, purified proteins from the HIV-1 *nef* and *env* can interact with B-cells *in vitro* and affect the key enzyme for inducing Ig affinity maturation AID (He, Qiao et al. 2006; Qiao, He et al. 2006). The interaction of B-cells with nef inhibits AID expression (Qiao, He et al. 2006) whereas the interaction with env has been shown to increase AID expression in a CD40 independent manner and only in a subset of B-cells expressing mannose C-type lectin receptors (He, Qiao et al. 2006). Whether the cumulative effect of HIV-1 infection *in vivo* is to cause long-term defects on AID in B-cells is not known.

In this study, we measured the AID expression in B-cells from patients with CHI during successful HAART. By comparing the results to what obtained in specimens from healthy controls, we could observe a higher baseline AID expression in HIV-1 infected patients. AID expression did not correlate with the CD27+ B-cell percentage in these patients suggesting that CD27- B-cells might be involved in the processes of CSR (paper II). Moreover, higher baseline levels of AID might reflect an increased responsiveness of B-cells to polyclonal stimulation *in vitro*. Therefore, we studied the AID expression upon CD40-dependent polyclonal stimulation and upon stimulation through TLR9. Interestingly, we found that AID expression on B-cells can be up-regulated to similar levels in both patients and controls (paper II). This may suggest that, in patients with CHI, the general ability of B-cells to induce CSR is not completely impaired.

In parallel, we measured Ig production and found high IgA levels in cultures from unstimulated and stimulated patient specimens (paper II). TLR-driven activation of B-cells might normally be a common phenomenon in the gut where the majority of Abs produced to react with commensal bacteria are of IgA type (Fagarasan, Kinoshita et al. 2001; Cerutti 2008). Thus, the increased ability of B-cells from HIV-1 infected patients to respond to polyclonal stimulation in the absence of T-cell help with the production of IgA, might reflect an increased response to microbial products. Taken together, our results on AID expression and IgA production *in vitro* raise important evidences for the participation of CD27- B-cells in the production of class switched Abs during HIV-1 infection. In order to confirm this, we studied the surface expression of CD27 among all cells expressing intracellular IgA and IgG. Both CD27- IgA+ and CD27- IgG+ B-cells were found to be significantly increased in patients with CHI (paper II). Previously, CD27- B-cells have been found to be IgG+ and able to proliferate in response to CpG in patients with systemic lupus erythematosus (SLE) (Wei, Anolik et al. 2007). In healthy individuals, CD27- B-cells have been defined as a new B-cell memory subtype expressing VH genes with low frequency of SHM (Fecteau, Cote et al. 2006).

Since we detected class switched (IgA+ and IgG+) CD27- B-cells, we investigated the possibility that the VH genes encoding for these Abs might have undergone processes of SHM. We found that the Ig VH region of transcripts from CD27- B-cells sorted from HIV-1 infected patients carry a high number of SHMs. Moreover, from the analysis of the Ig VH region of CD27+ B-cells sorted from patients, we could observe a decrease in the number of SHMs compared to healthy controls (paper II). The low levels of Ag-specific Abs observed in parallel with hypergammaglobulinemia in HIV-1 infected patients may thus be due to both altered CD27- and exhausted CD27+ B-cells which might have impaired mechanisms of CSR because of HIV-associated B-cell exhaustion (Moir, Ho et al. 2008).

These data add relevant information to previous observations (De Milito, Morch et al. 2001; De Milito, Nilsson et al. 2004; Titanji, Chiodi et al. 2005) and suggest a model (Figure 5) where CD27- B-cells are more prone to respond to unspecific signals such as polyclonal bystander activation and TLR triggering during HIV-1 infection. Higher levels of microbial products (Brenchley, Price et al. 2006) due to increased pathogen

reactivation during HIV-1 infection might in fact activate CD27- B-cells in order to overcome the lack of CD27+ B-cells which are exhausted (Moir, Ho et al. 2008) or have died by apoptosis (De Milito, Morch et al. 2001). This would result in the expansion of CD27-IgA+ and IgG+ B-cells (Fecteau, Cote et al. 2006) in blood. The levels of AID and the presence of a high number of SHMs in the Ig VH region of CD27- B-cell transcripts demonstrated in fact that these B-cell subpopulations have undergone Ig affinity maturation (paper II).

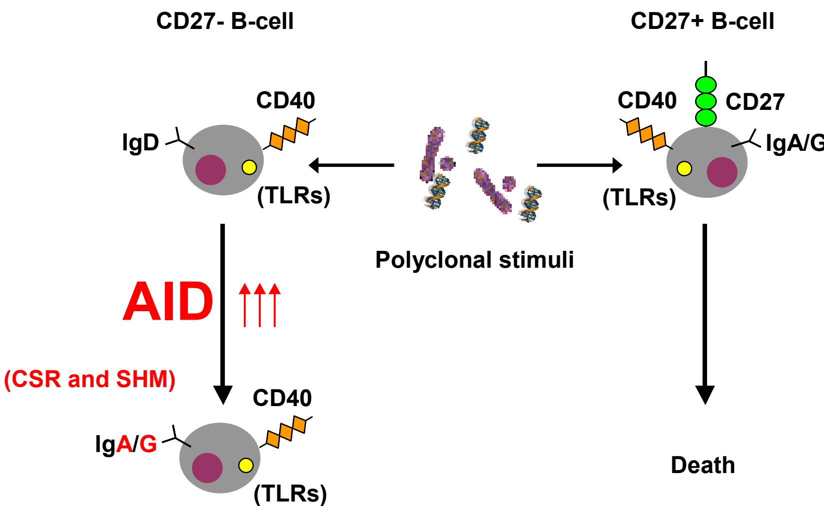

Figure 5. Model for CSR and SHM in CD27- B-cells. CD27- B-cells increase AID expression and might produce class switched and somatically hyper-mutated Abs in response to unspecific stimuli, such as bystander activation or TLR agonists. In contrast CD27+ B-cells might become exhausted and die during chronic HIV-1 infection.

3.3 HIV-1 alters chemokine and chemokine receptors expression on B-cells (Paper III)

The quality of immune responses against T-dependent Ags is regulated by the interaction between T- and B-cells in secondary lymphoid organs. Changes in the B-cell chemokine receptor expression of CXCR4, CXCR5 (Allen, Ansel et al. 2004; Allen, Okada et al. 2007) and CCR7 (Okada, Ngo et al. 2002) during immune responses allow GC formation and B-/T-cell interactions in the secondary lymphoid organs.

In this study we evaluated the expression of CXCR4, CXCR5 and CCR7 chemokine receptors in B-cell subpopulations during chronic HIV-1 infection. We found that naïve, memory B-cells and pre-PCs from HIV-1 infected patients had a decreased expression of the CXCR5 receptor as compared to healthy controls. This phenomenon was more pronounced in patients with a low CD4+ T-cell count indicating that disease progression may lead to dysregulation of B-cell chemokine receptor expression (paper III). Altered CXCR5 expression might have a role in the disruption of GC architecture observed during HIV-1 or SIV infection (Racz, Tenner-Racz et al. 1990; Popovic, Tenner-Racz et al. 2005). CXCR5 mediates migration of B-cells into spleen and lymph nodes (Ansel, Ngo et al. 2000) and together with CXCR4, GC organization (Muller, Hopken et al. 2003; Allen, Ansel et al. 2004). Reduced expression of CXCR5 has been previously described on naïve B-cells during HIV-1 infection (Chong, Nabeshima et al. 2004) in parallel with the appearance of CXCR5 negative B-cells in the blood of HIV-1 positive individuals (Forster, Schweigard et al. 1997). Modulation of the cell surface expression of CXCR4 and CXCR5 occurs upon binding with their respective ligands, leading to rapid internalization and endocytosis of the receptors (Dar, Goichberg et al. 2005). A possible mechanism for the reduced cell surface expression of CXCR5 on B-cells during HIV-1 infection could be the elevated serum levels of CXCL13, the ligand for CXCR5, found in patients (Widney, Breen et al. 2005) which may bind to CXCR5 and lead to its internalization. In order to confirm this hypothesis we measured and compared the cell surface expression and the gene expression of CXCR5 after incubation of cells with CXCL13. With this experiment, we could observe a transient increase in CXCR5 mRNA expression followed by a decrease in the levels of CXCR5 mRNA (paper III). This suggests that CXCR5 expression is in part regulated at the post transcriptional level through internalization.

The main sources of CXCL13 in healthy individuals are FDCs and follicular stromal cells (Gunn, Ngo et al. 1998; Ansel, Harris et al. 2002; Carlsen, Baekkevold et al. 2004). In order to screen B-cells for the expression of chemokines and chemokine receptors, we performed cDNA gene profiles of B-cells from HIV-1 infected individuals and healthy controls using a microarray system detecting 96 chemokine and chemokine receptor genes. Interestingly, we found that high CXCL13 mRNA levels are present in B-cells from HIV-1 infected patients (paper III). To further investigate if this could lead to CXCL13 production and secretion from B-cells, we cultured B-cells sorted from patients and controls *ex-vivo*. Upon polyclonal stimulation, B-cells secreted high levels of CXCL13 (paper III). CXCL13 expression also occurs in GC-derived human CD4+ T-cells upon ligation of the T-cell receptor (TCR) (Kim, Lim et al. 2004). However, BCR ligation did not induce secretion of CXCL13 (paper III). Therefore, it is possible that the high degree of unspecific immunoactivation occurring during HIV-1 infection (De Milito, Aleman et al. 2002), may be a contributing factor leading to up-regulation of mRNA expression and secretion of CXCL13 in B-cells. As a consequence, the cell surface expression of CXCR5 on B-cells may be decreased by autocrine or paracrine secretion of CXCL13 as shown by our *in vitro* data (figure 6). In diseases characterized by a high degree of immunoactivation and inflammation, such as rheumatoid arthritis and neuroborreliosis, high levels of CXCL13 have been reported in association with lymphoid neogenesis and immunoglobulin production (Weyand and Goronzy 2003; Narayan, Dail et al. 2005). Therefore, we also studied the CXCL13 expression in lymph nodes from HIV-1 infected patients and healthy controls. Interestingly, CXCL13+ B-cells could also be detected in lymphoid tissue from HIV-1 patients but not in controls, in addition to CXCL13+ dendritic cells (DCs) and immature DCs expressing CD1a (paper III). In previous studies, *in vitro* derived DCs have been shown to up-regulate the expression of CXCL13 mRNA after stimulation with lipopolysaccharide (LPS) (Perrier, Martinez et al. 2004). Moreover, also infection with Bartonella henselae *in vitro* induced secretion of CXCL13 from DCs (Vermi, Facchetti et al. 2006). Therefore it is possible that also CXCL13 produced from immature DCs participates in the down-regulation of the CXCR5 receptor on B-cells.

The major role of chemokines and chemokine receptors is to allow lymphocyte recirculation between the periphery and secondary lymphoid organs during immune responses (Honczarenko, Douglas et al. 1999; Brandes, Legier et al. 2000). Our data on the altered expression of the receptor/ligand pair CXCR5/CXCL13 during chronic

HIV-1 infection raises the question on whether this has an impact on the migration capacity of B-cells from these patients. In this respect, a newly described tissue-like population of memory B-cells with altered chemokine receptor expression in blood has been suggested to have an altered homing capacity (Moir, Ho et al. 2008). Therefore, we set-up chemokine specific migration assays. Surprisingly, we observed that specific migration to CXCL13 but also to CXCL12, ligand for CXCR4, and to CCL19 and 21, ligands for CCR7, was increased in B-cells from patients with low CD4+T-cell count, compared to controls and patients with high CD4+ T-cell count (paper III). This may reflect *in vivo* polyclonal stimulation of B-cells since it has been shown that B-cell activation via CD40-ligation increases CXCL12-mediated migration without changing the chemokine receptor expression (Brandes, Legier et al. 2000; Roy, Kim et al. 2002; Ehlin-Henriksson, Mowafi et al. 2006) and that B-cell activation with LPS also increases chemokine-induced migration (Brandes, Legier et al. 2000). Also type 1 interferons (IFNs) have been shown to increase CXCL12- and CCL21-mediated migration (Badr, Borhis et al. 2005), which may be relevant for HIV-1 pathogenesis as the levels of IFNs are elevated with disease progression (Gringeri, Santagostino et al. 1996).

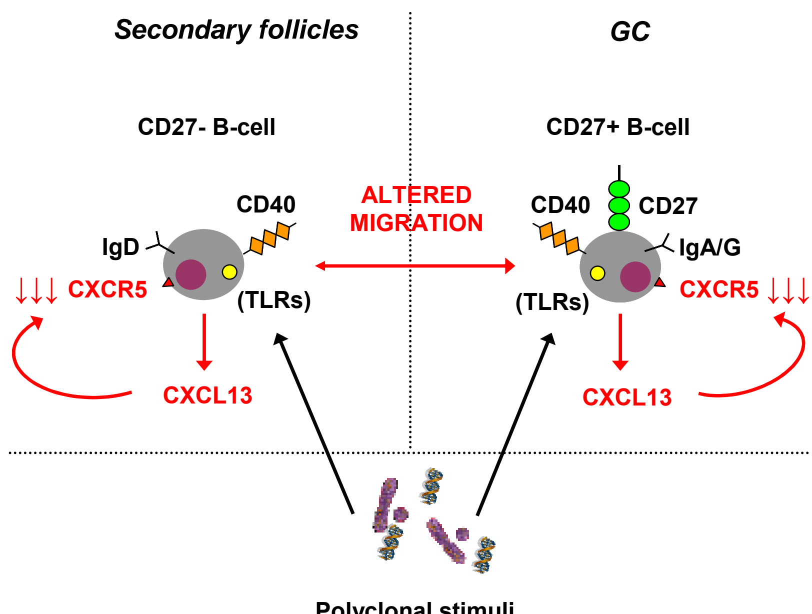

Figure 6. Model for CXCR5 down-regulation and impaired B-cell migration. Polyclonal B-cell activation *in vivo* may lead to autocrine or paracrine CXCL13 secretion by B-cells which might down-regulate CXCR5. In addition, increased B-cell migration due to B-cell hyper-activation might impair GC reactions during HIV-1 infection.

3.4 The timing of HAART impacts on serological memory in vertically HIV-1 infected children (Paper IV)

Perinatally HIV-1 infection is acquired in the milieu of a developing immune system. Neonatal PHI is associated with higher HIV-1 viremia as compared to adults in the absence of antiviral treatment (Sharland, Blanche et al. 2004). The subsequent decline in plasma viral load, typical of late stages of PHI, also requires considerably longer time in infancy resulting in higher systemic viral exposure during the first 2 years of life. The maturation process of the immune system in presence of an active virus replication as HIV-1 remains poorly studied. Previous studies showed that in vertically infected children initiation of antiretroviral treatment within 3 months and less than one year from birth is associated with the normal development of the T-cell repertoire and with a HIV-1 specific long-term cellular response (Romiti, Cancrini et al. 2001; Palma, Romiti et al. 2008; Zanchetta, Anselmi et al.). Thus, early viral suppression through HAART during neonatal PHI might be crucial for preservation of both T- and B-cells, since most beneficial effects on the B-cell compartment are obtained when therapy is applied during PHI in adults (Titanji, Chiodi et al. 2005).

In this study, we performed B-cell phenotyping in a large cohort of children vertically infected with HIV-1 and found that application of HAART within the first year of life was able to preserve normal percentages of total memory B-cells (paper IV). In order to assess the Ag-specific response we performed measles-specific and HIV-1 gp160-specfic B-cell ELISpot upon polyclonal stimulation of PBMCs *in vitro* (Crotty, Aubert et al. 2004) (paper I). Exposure to measles through vaccination, or natural infection generates an Ab response which has been shown to be directly correlated to the total memory B-cell percentage (Amanna, Carlson et al. 2007). On the other hand, in the context of vertical infection HIV-1 particles are present since birth. HIV-1 memory B-cell formation has not been previously evaluated in neonatal infection. Specimens from all the early treated patients were able to form spots against measles. In contrast, B-cells from patients treated later in life as well as patients naïve to treatment formed a significantly reduced number of measles-specific spots (paper IV). Therefore, Ag-specific memory B-cells are preserved through the early application of HAART, fully functional and able to differentiate into ASC upon polyclonal stimulation *in vitro*. Moreover, B-cells from early treated patients showed a high ability to produce spots against HIV-1 despite the low anti-HIV-1 Abs in the plasma of these patients (paper IV).

Low levels of anti-HIV-1 Abs are commonly found in HIV-1 infected infants receiving HAART since early life (Luzuriaga, McManus et al. 2000; Vigano, Trabattoni et al. 2006; Zanchetta, Anselmi et al. 2008). The apparent paradox of HIV-1 infected individuals being HIV-1 seronegative with standard serologic measurements is a peculiarity resulting from the introduction of early HAART protocols (Kassutto, Johnston et al. 2005; Adalid-Peralta, Grangeot-Keros et al. 2006). Therefore, the HIV-1 specific B-cell ELISpot, may represent an additional important method for evaluating the presence and quality of HIV-1 specific memory B-cells even in the absence of detectable anti-HIV-1 Abs. Taken together, our results suggest that the application of HAART within the first year of life is able to preserve the memory B-cell compartment which remains functional for a representative common vaccination Ag, measles, and for an Ag present since birth, HIV-1 gp160 (paper IV).

The normal development and maintenance of memory B-cells due to an early application of HAART in children vertically infected with HIV-1 might be the key for the maintenance of specific Abs to routine childhood immunizations over time. Primary Ab response to vaccination seems to be equivalent in HIV-1 infected children and healthy controls; however, the maintenance of humoral responses is remarkably lower in patients despite successful HAART treatment (Luzuriaga, McManus et al. 2004; Ching 2007). Whether immunization programs for HIV-1 infected children should be revised is still debated since the administration of certain viral or bacterial vaccines may be associated with an increased risk of adverse events in these children (Moss, Clements et al. 2003). On the other hand, since the risk of disease from most vaccine-preventable disease far outweighs the risk of vaccine-related adverse events, the WHO currently recommends routine vaccination of HIV-1 infected children (Moss, Clements et al. 2003). In order to avoid adverse events correlated to vaccines and to obtain the best response upon childhood vaccinations, a preserved cellular response might be essential.

Therefore, we analyzed the anti-measles, tetanus and pneumococcus Ab titers in blood. In agreement with our previous results, we observed an effective response and maintenance of protective Ab titers in vaccinated HIV-1 infected children treated with early HAART. Children treated within 1 year from birth were able to develop and maintain anti-measles and tetanus Abs above protective levels (Chen, Markowitz et al.

1990; Dietz, Galazka et al. 1997) for up to 9 years after vaccination (paper IV). On the contrary, a significant reduction in specific Ab titers and below the cut-off for protective levels, was observed in patients treated later in time. In the late treated groups, protective Ab levels were lost already after 1 year from the last vaccination or boost. However, the difference in anti-pneumococcus Ab titers was less clear between the early and the late treated groups (paper IV). It has been shown that post vaccination pneumococcal IgG levels might be reduced in naïve and HAART treated HIV-1 patients (Rodriguez-Barradas, Musher et al. 1992; Luzuriaga, McManus et al. 2004). Moreover, immunogenicity of pneumococcal vaccination does not necessarily translate into long-term clinical protection. Ab quantification *in vitro* may not correspond to Ab efficacy *in vivo* where protection may be related not only to Ab concentration but also to Ab affinity and avidity (Kamchaisatian, Wanwatsuntikul et al. 2006).

Nevertheless, the normal development and maintenance of both memory B-cells and specific Ab titers against childhood vaccination Ags can be obtained through the application of an early antiretroviral therapy as shown in this study (figure 7). Conversely, a late HAART schedule does not seem to be able to induce recovery of an impaired B-cell compartment (paper IV). As a consequence, long-lasting humoral responses after childhood vaccinations are impaired. Therefore, compressed booster doses should be considered for these patients who lost protection in order to reinforce defense against common pathogens.

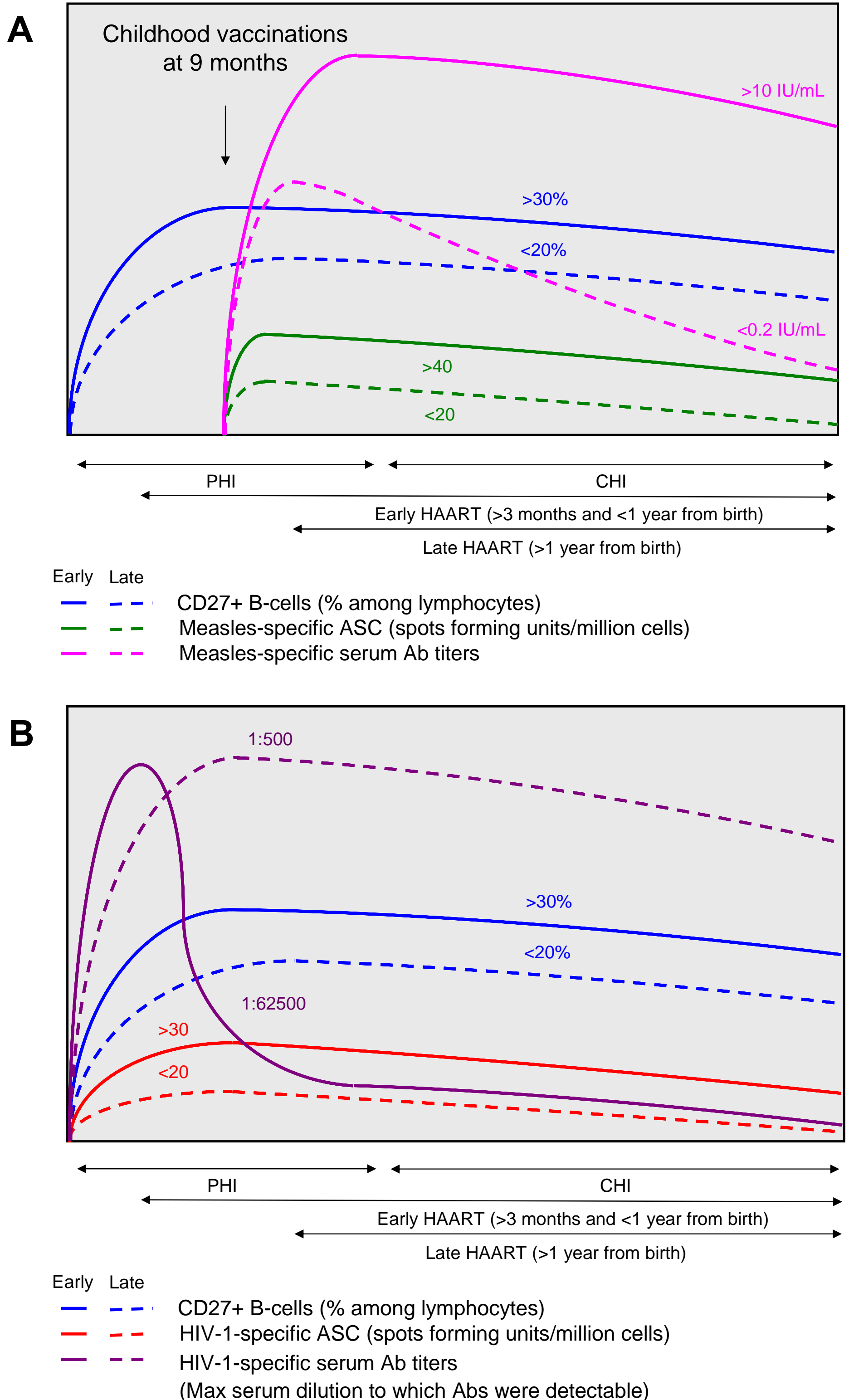

Figure 7. Dynamics of memory B-cell preservation due to an early or late HAART. Early application of HAART during vertical HIV-1 infection preserves the memory B-cell compartment and the formation of Ag-specific memory B-cells and Ab titers as shown for the measles vaccination **(A)**. Formation of specific-HIV-1 memory B-cells as measured by ELISpot is also preserved upon an early application of HAART. However, anti-HIV-1 Abs are not detectable in the serum of these children **(B)**. The values shown in this figure are based on the median values observed in our study groups while scales are arbitrary.

4 CONCLUSIONS

In the absence of a HIV-1 vaccine, the loss of serological memory to non-HIV-1 Ags observed during HIV-1 infection might have severe consequences for the health of HIV-1 infected adults and children.

In paper I, we investigated whether loss of serological memory was related to disease progression by evaluating the frequency of total memory B-cells, plasma Abs to measles and pneumococcus and by enumerating measles-specific ASC in patients with PHI, CHI and in LTNPs. We also evaluated the effect of HAART on memory B-cells and Ag-specific Abs in PHI and CHI. The results indicated that the frequency of memory B-cells might represent an additional marker of disease progression since their levels correlated with the CD4+ T-cell counts. Moreover, we found that patients with PHI and CHI had severe defects in serological memory and that despite successful HAART, serological memory could not be restored. The work in paper I enlightens a scenario in which the loss of serological memory might occur early during PHI, due to a partial but irreversible depletion of Ag-specific memory B-cells. This phenomenon might be due to memory B-cell exhaustion and apoptosis as a consequence of polyclonal B-cell activation which is increased during HIV-1 infection (De Milito 2004; Moir and Fauci 2008). In this setting, the death of specific memory B-cells might be compensated by CD27- B-cells triggered by unspecific stimuli to produce class switched and somatically hyper-mutated Abs of low affinity.

In order to test this hypothesis, in paper II we evaluated the effect of HIV-1 infection on CSR and SHM by studying the expression of AID in a cohort of CHI patients as compared to a group of healthy controls. In parallel, we also characterized the phenotype of B-cells and Ig production upon stimulation of PBMCs *in vitro*. Cells from HIV-1 infected patients showed higher baseline levels of AID expression and increased IgA production as measured both *ex-vivo* and upon polyclonal stimulation *in vitro*. Moreover, we found that the percentage of CD27- B-cells expressing intracellular IgA+ and IgG+ was significantly increased in the blood of HIV-1 infected patients as compared to controls. Finally, we also found altered patterns of SHM between patients and controls. Taken together, these results showed that during HIV-1 infection, CD27- B-cells can also produce class switched and somatically hypermutated Abs.

In paper III, we investigated whether expression of chemokine receptors and their ligands may be altered and play a role in the establishment of B-cell dysfunctions during HIV-1 infection. We found that the expression of CXCR5 on different B-cell subpopulations from HIV-1 infected patients was significantly decreased as compared to healthy controls. Moreover from the gene expression analysis of 96 genes belonging to the chemokine and chemokine receptor family, we identified the mRNA for the chemokine CXCL13 as over-expressed in B-cells from HIV-1 infected patients. Interestingly, CXCL13 could also be secreted in culture upon B-cell activation, and CXCL13 positive B-cells could also be found in the lymph nodes of HIV-1 infected patients. Finally we tested B-cell migration towards CXCL13 and also CXCL12 and CCL21 and found that B-cells from HIV-1 infected patients had increased responsiveness to all these chemokines. Taken together, results from paper III indicated that altered expression of CXCR5 and CXCL13 together with B-cell hyper-activation may cause altered B-cell migration resulting in defective B-/T- cell contacts during GC reactions in the secondary lymphoid organs.

In paper IV we investigated whether the application of HAART early during vertical infection might help minimizing the detrimental effects observed in the B-cell compartment during HIV-1 infection. We evaluated B-cell phenotype and enumerated specific ASC and plasma Ab levels for common vaccination Ags such as measles, tetanus and pneumococcus, and HIV-1 antigens in a large cohort of HIV-1 vertically infected children treated with different HAART schedules. Results indicated that initiation of HAART within the first year of life permits the normal development and maintenance of the memory B-cell compartment. On the contrary, both total and Ag-specific memory B-cells from patients treated later in time, were remarkably reduced regardless of viral control. This resulted in the loss of protective Ab titers against vaccination Ags in late treated patients. Thus, early initiation of HAART preserves both the development and the function of B-cells resulting in effective and long-lasting immunization upon childhood vaccinations.

The conclusions reached with the work presented in this thesis are integrated in the model represented in figure 8.

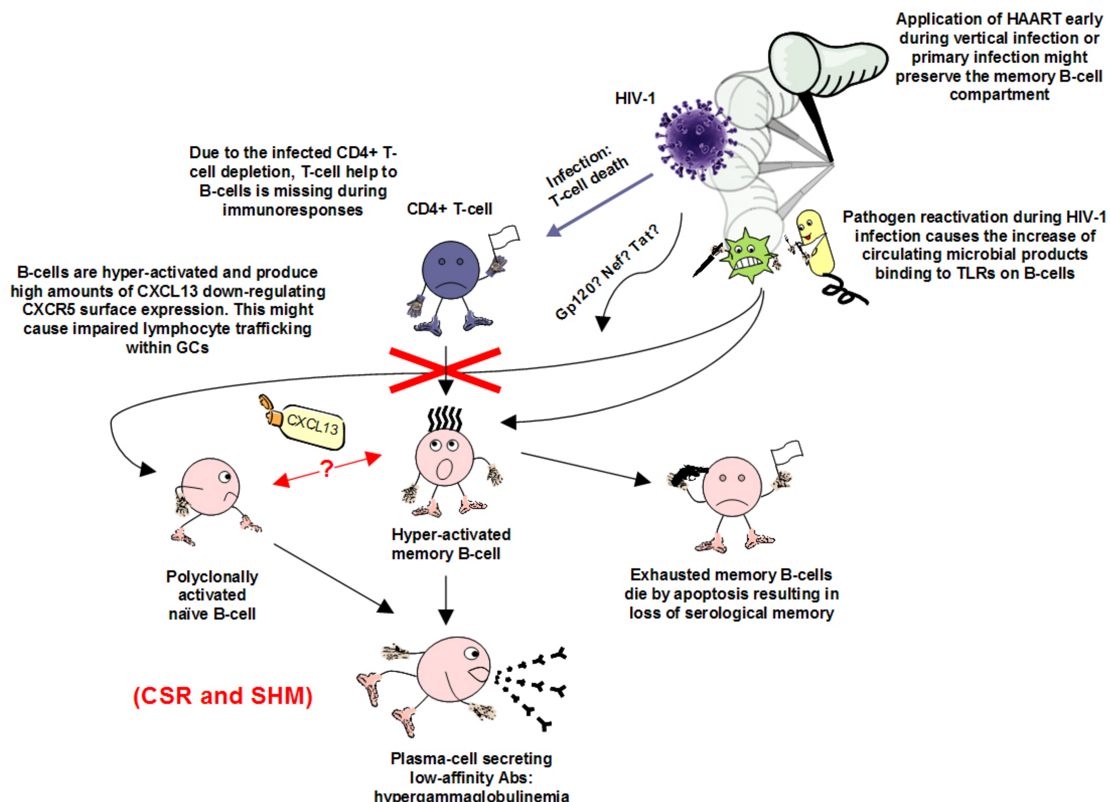

Figure 8. HIV-1 infection and loss of serological memory: the role of altered expression of B-cell chemokine receptors, timing of HAART and impaired Ab affinity maturation.

5 FUTURE PERSPECTIVES

Our data from paper IV hopefully will encourage clinicians to periodically check specific Ab levels in HIV-1 infected children with compromised immune system possibly due a late initiation or failure of HAART. Compressed schedule of booster vaccinations must be taken into account in this population in order to reduce disease morbidity and mortality due to preventable infectious diseases. In this respect, it would be interesting to follow over time the immune response upon re-vaccination of the late HAART treated patients (paper IV) against measles, tetanus and pneumococcus. This could help modifying the standard childhood vaccination protocol for HIV-1 infected children in accordance with their clinical status but also with the status of their memory B-cell compartment which could be evaluated by Ag-specific B-cell ELISpot as shown in our studies (papers I and IV).

The disruption of GC architecture has been observed both during HIV-1 or SIV infection (Racz, Tenner-Racz et al. 1990; Popovic, Tenner-Racz et al. 2005). Thus, the use of animal models, rhesus or cynomologus macaques infected with SIV, might favor further investigations on the role that a possible treatment of HIV-1 infected patients with chemokine analogues or with chemokine receptor neutralizing Abs, might have in correcting impaired humoral immunity. In this respect, it has recently been shown that *in vivo* administration of anti-CXCR3 neutralizing Abs could reduce the infiltration of alloreactive CD8+ T-cells into target organs in a mouse model of graft versus host disease (GVHD) (He, Cao et al. 2008). Therefore, it is possible that the treatment of HIV-1 infected patients with anti-CXCL13 Abs might compensate the detrimental effects of high serum levels of CXCL13 observed in patients or possibly contrast an impaired CXCL13 production by hyper-activated B-cells as shown in paper III. This might help the interactions between B- and T-cells during GC reactions thus preserving GC architecture.

Finally, in the prospect of designing a HIV-1 vaccine based on the use of TLR agonists as adjuvants, it should be considered that B-cell polyclonal activation may possibly be due to impairment in the TLR responsiveness to microbial products, as discussed in paper II, should also be considered. Excess of TLR agonists in HIV-1 infected patients might in fact enhance the incidence of autoimmune disorders already occurring during HIV-1 infection (Onlamoon, Pattanapanyasat et al. 2005). There is increasing evidence

that TLRs, reactive with autologous ligands, may play a major role in these events (Meyer-Bahlburg and Rawlings 2008). It would be interesting to evaluate the consequences of repeated administration of TLR agonists to an immunocompromised host with respect to the appearance of related autoimmune phenomena. Whether the blockade of TLRs might instead help reducing immunoactivation and preserve B-cells from exhaustion and apoptosis observed in HIV-1 infected patients, should also be investigated in relevant models of HIV-1 infection.

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