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CHEMOKINES AND CHEMOKINE RECEPTORS DURING VIRAL INFECTIONS IN MAN

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Stockholm 2007

"The manual arts have always taken precedence over the fine arts. Remember somebody had to build a ceiling before Michelangelo could go to work"

John Ratzenberger, Actor

ABSTRACT

Chemokines and chemokine receptors are critical mediators of cell migration during immune surveillance, inflammation and development. The generation of antibody responses depends on B and T cell collaboration within germinal centers in lymphoid tissue. Activation of B lymphocytes is accompanied by alternations in chemokine responsiveness that brings together the antigen specific B cells with its cognate CD4 T cell. Several human viral infections have been shown to interfere with chemokine receptor expression and signaling, I have studied two human viruses. Human immunodeficiency virus-1 (HIV-1) infects mainly CD4$^+$ T cells but infection is associated with impaired B cell function with loss of B cell responses to specific antigens and a loss of B cells with memory phenotype. Epstein-Barr virus (EBV) in contrary infects mainly B cells and is associated with a variety of human malignancies.

The aim of this thesis was to i) characterize chemokine receptor expression on different subpopulations of B cells during chronic HIV-1 infection and primary EBV infection; ii) study the effects of altered receptor expression on B cell migration during HIV-1 and EBV infection and iii) clarify the role of CXCL12 for proliferation and signaling in childhood pre-B ALL.

Our main finding in *paper I* was a decreased expression of CXCR5 both at the mRNA and protein level on B cells from HIV-1 infected individuals compared to controls. We could also detect an increase in CXCL13 expression in B cells from HIV-1 infected subjects. In *paper II and III*, tonsillar B cells were infected with EBV and the expression of CXCR4, CXCR5 and CCR7 was followed over time. Already two days after infection, a decrease of surface CXCR4, CXCR5 and CCR7 was detected and after 14 day of infection both CXCR4, CXCR5 and CCR7 was totally absent from the cell surface. EBV infection also caused a decrease in migration towards the respective ligands compared to uninfected B cells. In order to further investigate the CXCR4/CXCL12 pathway in pre B leukemic cells, we found that CXCL12 enhances proliferation of ALL cells and signal transducer and activators 5 (STAT5) was activated upon ligation with CXCL12 (*paper IV*). CXCL12 has together with interleukin-7 (IL-7) been shown to enhance proliferation of leukemic cells and we could detect a higher IL-7 level in a few children with pre-B ALL compared to controls. Interestingly, in *paper V*, we showed that IL-7Rα is down-regulated on T cells in HIV-1 infected individuals and this correlated with depletion of CD4$^+$ T cells in HIV-1 infected subjects.

In summary, little is known about the impact of chemokines and chemokine receptors during viral infection and how it modulates the immune response. In these studies, I have shown that both HIV-1 and EBV influences B cell chemokine receptor expression and migration during infection in humans. The natural evolution of our work would be to study how chemokine and chemokine receptors affect the natural course of infection and evolution of immunological responses in experimental models of the viruses used in this thesis, e.g. HIV-1 and EBV.

Keywords: B cells, Chemokine receptors, Chemokines, HIV-1, EBV

LIST OF PUBLICATIONS

I. **Mowafi F,** Cagigi A, Vu Phuong Dang L, Atlas A, Chiodi F, Nilsson A. Altered expression of the receptor-ligand pair CXCR5/CXCL13 in B-cells during chronic HIV-1 infection. *Submitted*

II. Ehlin-Henriksson B, **Mowafi F**, Klein G, Nilsson A. Epstein-Barr virus infection negatively impacts the CXCR4-dependent migration of tonsillar B cells. Immunology. 2006 Mar; 117(3):379-85.

III. **Mowafi F**, Cagigi A, Klein G, Nilsson A, Ehlin-Henriksson B. Changes in chemokines and chemokine receptor expression on tonsillar B cells upon Epstein-Barr Virus infection *Submitted*

IV. **Mowafi F**, Cagigi A, Matskova L, Björk O , Chiodi F , Nilsson A. The chemokine CXCL12 enhances proliferation in pre B-ALL via STAT5. Pediatric Blood & Cancer 2007 Oct 3; [Epub ahead of print]

V. Rethi B, Fluur C, Atlas A, Krzyzowska M, **Mowafi F**, Grutzmeier S, De Milito A, Bellocco R, Falk KI, Rajnavolgyi E, Chiodi F. Loss of IL-7Ralpha is associated with CD4 T-cell depletion, high interleukin-7 levels and CD28 down-regulation in HIV infected patients. AIDS. 2005 Dec 2; 19(18):2077-86.

LIST OF ABBREVIATIONS

Ag	Antigen
AIDS	Acquired immunodeficiency syndrome
ALL	Acute lymphoblastic leukemia
APC	Antigen presenting cell
ASC	Antibody secreting cell
BCR	B cell receptor
BL	Burkitt lymphoma
BM	Bone marrow
CR2	Complement receptor type 2
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
EBV	Epstein-Barr Virus
ELISA	Enzyme-linked immunosorbent assay
FDC	Follicular dendritic cell
GALT	Gut-associated lymphoid tissue
GC	Germinal center
HAART	Highly active antiretroviral therapy
HEV	High endothelial venules
HIV	Human immunodeficiency virus
HTLV	Human T cell lymphotropic virus
ICAM	Intercellular adhesion molecules
Ig	Immunoglobulin
IL-7	Interleukin-7
IL-7R	Interleukin-7 receptor
LCL	Lymphoblastoid cell line
LFA	Lymphocyte function-associated antigen
LN	Lymph node
LP	Lamina propria
LPS	Lipopolysaccharide
LTNP	Long time non-progressor
mAb	Monoclonal antibody
MadCAM	Mucosal addressin cellular adhesion molecule
MHC	Major histocompatible complex
MLN	Mesenteric lymph node
PBMC	Peripheral blood mononuclear cell
PP	Peyers patches
RGS	Regulators of G-protein signaling
SHM	Somatic hypermutation
SIV	Simian immunodeficiency virus
STATs	Signal transducer and activators
VLA	Very late antigen

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GENERAL B CELL BIOLOGY

B cell development

During embryogenesis, the human B cell lineage develops in the fetal liver but during the second trimester the B lineage development is transferred to bone marrow (BM) [1] and there after continuous B cell production occurs in the BM through out life. B cell development in the BM is represented by several checkpoints centered around the commitment to B lineage and the formation of a functional Ig gene rearrangement leading to the expression of the B cell receptor (BCR) on the cell surface [2]. Further differentiation requires antigen (Ag) stimulation, and occurs in secondary lymphoid organs after B cell emigration from the BM [3]. In the following section, the events leading to antibody production from B cells will be described in more detail.

B cell maturation

The BM microenvironment consists of stromal cells, vascular endothelial cells as well as hematopoetic cells and it is a site for production of chemokines, cytokines and growth factors [4]. Early lymphoid development is not only intrinsic but also dependent on signals derived from the BM microenvironment. The pro-B cells represent the earliest B lineage cells and at this stage, B cell development is defined by rearrangement and expression of the heavy and light chain immunoglobulin genes. The Ig gene rearrangement is dependent on the expression of several genes, among them RAG-1 and RAG-2. The B cells undergo D-J joining on the H chain to become early pro-B cells and at this stage, pro-B cells express CD19, CD45 and Class II MHC [5, 6]. Joining of a V segment to the D-J_H completes the late pro-B cell stage and productive V_H-DJ_H recombination results in cell surface expression of the Ig μ proteins as part of the pre-B cell receptor (pre-BCR). Signaling from the pre-BCR promotes allelic exclusion at the IgH locus, stimulates proliferative cell expansion, and induces

differentiation to small pre-B cells, which start to recombine immunoglobulin light (L)-chain genes [7]. If the process of V-DJ rearrangement or signaling via the pre-BCR does not occur, the pre-B cell will be deleted [8]. Following proliferation, non-dividing pre-B cells undergo V-J joining on the L chain. Once the L chain has been successfully synthesized, it is expressed with the µ chain on the cell membrane and the B cell has developed into an immature B cell. The immature B cells will undergo receptor-mediated negative selection whereby binding to self-Ag in the BM will lead to apoptosis. Thereafter, B cells will express the δ chain and membrane IgD and IgM, and emigrate from the BM as mature naive (resting) B cells [7, 9].

The germinal center reaction

The germinal centers (GCs) are located in the peripheral lymphoid tissue such as lymph nodes, tonsils and spleen. In B cell differentiation, the GC is known to be the major site for somatic hypermutaion (SHM) of the immunoglobulin genes and affinity maturation and GC B cells are progenitors of long-lived antibody producing plasma cells and memory B cells [10]. GCs form as early as 5 days after exposure to a T cell dependent antigen, when antigen-specific B cells and T follicular helper cells (T_{FH}) migrate into peripheral lymphoid tissues [11]. In histological sections, a primary follicle is composed of re-circulating B cells and follicular dendritic cells (FDC) and outside the primary follicle, a T cell zone with re-circulating T cells and MHC class II positive dendritic cells (DCs) is located. The follicle is surrounded by the mantel zone with non-reactive B cells. Upon an immune response, a secondary follicle is formed consisting of the GC with its dark and light zones. In the dark zone, the Ag-specific B cell blast (centroblast) undergoes massive proliferation and SHM before migrating into the light zone where the Ag-specific B cell (centrocyte) undergoes affinity maturation in close contact with a network of FDCs and T cells [11, 12]. FDCs bind Ag-antibody

complexes and together with T_{FH} initiate selection and differentiation of memory-B cells or plasma cells [13].

VIRAL INFECTIONS AND HUMORAL IMMUNITY

Specific humoral immunity is mediated by antibodies produced by plasma cells. These antibodies recognize particular Ags such as viruses [14-16]. The cellular immunity is mediated by T cells which express the T cell receptor (TCR) specific for peptides presented by major histocompatible complex (MHC) molecules [17, 18]. Peptides (derived intracellularly) in a virus infected cell are presented by MHC class I molecules, the Ag-presenting cells (APCs) is then recognized by a CD8^{+} T cell, which induces killing of the infected cell by cytotoxity or/and cytokines. Peptides derived from phagocytized proteins are presented by MHC class II molecules and are then recognized by CD4^{+} T cells which release cytokines [19]. The main function of B cells is to produce viral Ag-specific antibodies, they also function as antigen presenting cells (APCs) through their MHC class II proteins, for CD4^{+} T cells. This process is critical for the cellular interactions underlying T cell help for immunoglobulin production [20]. The complement system, phagocytes, antibody-cellular cytotoxity and neutralizing antibodies (antibodies able to inhibit infection) are all important tools used by the immune system to clear virus particles and infected cells [21-23].

Human immunodeficiency virus-1 (HIV-1) and -2 (HIV-2)

In 1981 the Communicable Disease Center, Atlanta, USA noted an increase in cases of *Pneumocystis carinii* (now classified as *P.jiroveci*) combined with severe depletion of CD4^{+} T cells. This immunodeficiency syndrome appearing without a known cause of disease was referred to as "acquired immune deficiency syndrome" AIDS. In 1983 the causative agent of AIDS was isolated and named human T cell lymphotropic virus

(HTLV-III); later on the virus was renamed to HIV-1. According to the UNAIDS/WHO 2006 AIDS Epidemic Update, an estimated 39.5 million people are living with HIV-1 and 3 million die of AIDS every year throughout the world.

Two different types of HIV have been isolated, HIV-1 which is spread world-wide and HIV-2 mostly localized to West-Africa. HIV-1 is an enveloped retrovirus belonging to the lentivirus family. Retroviruses carry their genetic information in the form of RNA. HIV-1 mainly infects CD4 receptor-bearing cells and the virus enters the host cell by binding the viral envelope protein glycoprotein 120 (gp120) to the CD4 receptor and a chemokine co-receptor on the host cell surface. There are two types of HIV-1 strains, M-tropic viruses and T-tropic viruses. The M-tropic viruses utilize the β-chemokine receptor CCR5 and sometimes the CCR3 co-receptor. T-tropic virus strains utilize the α-chemokine receptor, CXCR4, as their co-receptor. The CCR5-using HIV-1 variants are associated with the process of early virus transmission while disease progression has been associated with HIV-1 variants using the CXCR4 receptor [24].

Each virus particle or virion contains two copies of RNA genome, which are transcribed into viral DNA by the viral enzyme reverse transcriptase in the infected cell and integrated into the host cell chromosome. The integrated DNA is known as the provirus. The HIV-1 genome consists of nine genes of which three is common to all retroviruses. The *gag* gene encodes the structural proteins of the viral core, *pol* encodes the enzymes involved in viral replication and integration and *env* encodes the viral envelope glycoproteins. In CD4$^+$ T cells activated in response to Ag presentation, virus replication is initiated by transcription of the provirus. Activation of CD4$^+$ T cells induces the transcription factor NFκB that binds to cellular DNA and to the viral provirus, initiating transcription of viral RNA by the cellular RNA polymerase. This

transcript is spliced into different mRNAs encoding for the different viral proteins [25, 26].

HIV-1 also establishes a latent infection where the provirus remains quiescent in memory CD4$^+$ T cells and in dormant macrophages and these cells are thought to be a reservoir for the infection. Viral load is measured by detecting the RNA or viral DNA present in mononuclear cells. FDCs in lymphoid tissue have been shown to retain HIV-1 within immune complexes at the surface of these cells even though they are themselves not infected. [25, 26]

A second HIV serotype, HIV-2, was isolated from West African patients suffering from AIDS in 1986 [27]. The two viruses bear considerable homology in structure and sequence, both infect CD4$^+$ T cells but HIV-2 isolates have been shown to use several alternative chemokine receptors in addition to the major co-receptors CCR5 and CXCR4 [28]. Several publications have indicated that HIV-2 infected individuals may progress to AIDS more slowly than HIV-1 infected individuals; intense research efforts are conducted to understand the basis for this slow progression. Attention has also been given to the small proportion of people infected with HIV-1 who maintain normal CD4$^+$ T cell numbers and do not develop symptoms for several years following primary infection. These indivuals are defined as long time non-progressors (LTNPs), although many of these patients will eventually progress to disease. When comparing virological and immunological features of HIV-1 and HIV-2 infections, the plasma viral load is much lower during HIV-2 infection [29], and most HIV-2-infected individuals can be classified as LTNPs. An explanation for the low plasma viral load seen during HIV-2 can be the control of infection by specific host immune responses such as strong HIV-2-specific cytotoxic T lymphocyte response [30].

Humoral immunity during HIV-1 infection

During acute infection HIV-1 spreads into lymphoid organs and causes a strong immune response. This early response involves mostly cytotoxic $CD8^+$ T cells (CTL), which keeps down the viral replication to a steady state whereas neutralizing antibodies have been shown to develop several months after the first encounter with the virus. The viral replication continues even though the patients normally do not present with any clinical direct symptoms. Despite the high rate of replication, the viral load is kept under control by the immune system throughout the chronic phase of infection. The virus eventually breaks through the immune defense of the host resulting in increased viral load and decreased $CD4^+$ T cell numbers. In opposite to plasma levels, examination of lymph nodes have shown high levels of infected cells even in the early stage of infection [31]. Defects in the B cell compartment can be detected already during primary HIV-1 infection [32] and humoral immunity is further impaired during chronic infection [33]. In the simian immunodeficiency virus (SIV) macaque model, a recent study by Zhang *et al.* showed that after SIV inoculation, no obvious depletion of $CD4^+$ T cells was found, either in peripheral blood or lymph node compartments, within 5 months after primary infection with SIV. On the other hand, progressive depletion of proliferating B cells and disruption of the FDC network in GC was evident in the samples collected at as early as 20 days after viral inoculation [34].

Depletion of mucosal $CD4^+CCR5^+$ T cells has been documented during acute HIV-1 infection in humans and in the SIV macaque model of HIV-1 infection [35-39]. This depletion of $CD4^+$ T cells occurs primarily at the mucosal effector sites, particularly in the gut-associated lymphoid tissue (GALT) where a substantial portion of the $CD4^+$T cells in the body are located. A majority of the $CD4^+$ T cells in the intestinal lamina

propria (LP) express CCR5 [40]. Recent studies suggests that massive depletion of CD4$^+$CCR5$^+$ T cells at the mucosal effector sites is mediated by direct viral infection and cell destruction via either viral cytopathic effects or cytotoxic T lymphocyte mediated cytolysis [36, 41].

The antibody response to HIV-1 is generally directed to the structural proteins of the virus. Antibodies against the envelope proteins gp120, gp41 the core protein p24 and the matrix protein p17 become detectable within a few weeks of infection [42-44]. Even though neutralizing antibodies are often considered as the most effective defense against virus infection, during HIV-1 only a fraction of antibodies has been shown to have neutralizing activity [45, 46]. The few neutralizing antibodies to HIV-1 known today have shown to interfere with virus attachment to CD4 or to the co-receptors by binding to domains involved in the fusion of the virus [47, 48].

Epstein-Barr virus (EBV)

The British physician Denis Burkitt identified in the 1950s a previously unknown lymphoma which affected young African children, today known as Burkitt´s lymphoma. In 1964, Michael Anthony Epstein and Yvonne Barr when examining biopsies from Burkitt's lymphoma under an electron microscope detected virus particles morphologically similar to a herpes virus. Today this virus is known as Epstein-Barr Virus, and it was the first human virus to be associated with cancer.

EBV is a double stranded DNA virus of the γ-herpes virus family. In the virus particles the genome exists in a linear form, but after infection the virus DNA forms a circle and persists as an episome (extra-chromosomal viral DNA) in the nuclei of the infected cell [49]. EBV infects mainly B cells but occasionally also other cell types such as epithelial

cells may become infected [50]. EBV is transmitted orally and the virus can be detected in the saliva from immunosuppressed patients, infectious mononucleosis (IM) patients and at lower levels, from healthy EBV-seropositive individuals [51]. EBV infects B cells after interaction of the viral glycoprotein gp350/220 with the complement receptor C3d also called Complement receptor type 2 (CR2/CD21). A complex between the viral proteins gp25 (gL), gp42/38 and gp85 (gH) mediates the interaction between EBV and the major histocompatibility complex II (MHC II), which serves as a co-receptor [52]. The B cells are activated by cross-linking of CD21 and the major surface glycoprotein, gp350/220, which induce Lck activation and Ca^{2+} mobilization following mRNA synthesis, cell adhesion, CD23 expression and interleukin-6 production. When the viral genome is uncoated and transferred to the nucleus, transcription from the Wp promoter leads to expression of the latent EBV genes. The EBV nuclear antigen (EBNA), leader protein [EBNA-5 (LP)] and EBNA2 are responsible for the entry of the infected cell into the G1 phase of the cell cycle. The EBV^{+} cells begin to proliferate and secrete cytokines important for B cell growth. The virus eventually establish latent infection in memory B cells; during this latent phase only a limited number of virus proteins are expressed [53].

EBV latent proteins

If T cells are removed or inhibited, EBV^{+} B cells from peripheral blood of virus carriers can be cultured *in vitro* and give rise to EBV-transformed, immortalized cell lines known as lymphoblastoid cell lines (LCL) [54]. The LCL cells produce several latent proteins including six nuclear antigens (EBNAs 1, 2, 3 , 4 and 5 and EBNA-6) and three latent membrane proteins (LMPs 1, 2A and 2B) [55]. LCLs also express the non-coding small non-polyadenylated RNAs EBERs 1 and 2. The functions of the EBERs are to date not clear. The function of the EBV encoded proteins has been studied in

detail in LCLs. Different types of EBV latency are known to occur in EBV^{+} immortalized B cells and tumors. The different EBV gene expressing patterns are divided into three latency patterns; in the latency III program, the full set of EBV encoded proteins are expressed while in the latency I, the only EBNA gene expressed is EBNA1. In latency II, EBNA1 and the LMP genes are expressed [67].

Viral protein	Expression	Biological function	Others	Reference
EBNA-1	Latency I, II, III	Replication, maintenance of viral episome.	May have a role in oncogenesis of B cell lymphoma.	[56]
EBNA-2	Latency III	Transcriptional activator of cellular and viral proteins. Associated with transformation of the EBV infected B cell.	Up-regulates CD21 and CD23 on B cells as well as LMP1 and LMP2.	[55, 57]
EBNA-3 (3A) EBNA-4 (3B)	Latency III	Important for proliferation and B cell transformation *in vitro*.		[58]
EBNA-6 (3C)	Latency III	Induce up-regulation of CD21 and LMP1.		[59]
LMP1	Latency II, III	Important for EBV-mediated proliferation. May mimic B cell activation by CD40-CD40L signals, retaining the T cell derived activation signal. Induces activated B cell phenotype. Inhibits apoptosis by up-regulation of anti-apoptotic molecules.	Regarded as an oncogene as LMP1 transgenic mice develop B cell lymphomas. Associated with different signaling pathways such as NF-κB, p38/MAPK, JNK/AP-1 and JAK/STAT.	[55] [60] [61] [62], [63, 64]
LMP2A	Latency II, III	LMP2A has ITAMs present like the BCR. LMP2A has been reported to both mimic BCR by activating ITAMs and provide the B cell with a surviving signal as well as prevent activation.		[65] [66]

Table1. The expression of different viral proteins, related to the latency stage and function in EBV infection.

Humoral immunity during EBV infection

There are two different hypothesis concerning the route of EBV infection in B cells. In primary EBV infection the virus enters via the mouth through the epithelium of the tonsil; infects the naïve B cells in the lymphoid tissue underneath the epithelium [68]; the B cells become activated, migrate into the follicle and form a GC [69].

One model suggests that EBV first enters the naïve B cell pool and starts to express its latency proteins corresponding to the latency III in vitro which drives the proliferation. In the GC the EBNA1, LMP1 and LMP2A proteins are expressed (latency II) [70] which substitute CD40 and BCR stimulation [71]. The EBV-infected B cells in the GC undergo clonal expansion and differentiation into memory B cells. The EBV[+] memory B cell pool acts as the long-term reservoir of EBV and these cells do not express any viral proteins (latency 0) and the virus can not be detected by EBV-specific T cells. Many new virus particles can be produced if the memory B cells are activated and differentiate into plasma cells and switch to the lytic life cycle [72, 73].

It has been questioned if naïve B cells are the primary target for EBV and a second model suggested that the processes of immortalization and latency are independent from the expression of the latency program III [74] and that EBV can enter any B cell and initiate a variety of expression programs . Experimental data has shown that during *in vitro* infection of tonsillar B cells, EBV can infect both memory and naïve B cells [75].

Most of the neutralizing activity in EBV-positive serum is directed against the transmembrane glycoprotein gp350/220 [76, 77]. EBV is also associated with different human malignancies such as Burkitt lymphomas (BL), nasopharyngeal carcinoma and opportunistic B cell lymphomas (NHL) in immunosuppressed and AIDS patient [78].

HUMAN CHEMOKINE RECEPTORS AND CHEMOKINES

The chemokine receptors are integral seven transmembrane glycoproteins, belonging to the group of G-protein coupled receptors. Receptor-ligand interaction leads to signal transduction involving G-proteins which promotes the release of intracellular second messengers such as calcium, cAMP and phophoinositides (reviewed by Sanchez-Madrid and del Pozo, 1999) [79]. Chemokines (chemotactic cytokines) are small heparin-binding proteins that are known to play a crucial role in directing the movement of cells throughout the body. Chemokines and chemokine receptors are also important in DC maturation [80], B and T cell development [81, 82]. Chemokines and their receptors were previously divided into four families (CXC, CC, C and CX3C) based on their different cystein residues in the ligands, but in year 2000 a new system of nomenclature was developed where each ligand and receptor is identified by its subfamily and given a special identifying number [83]. To date, approximately 20 chemokine receptors and 50 chemokines have been discovered [13].

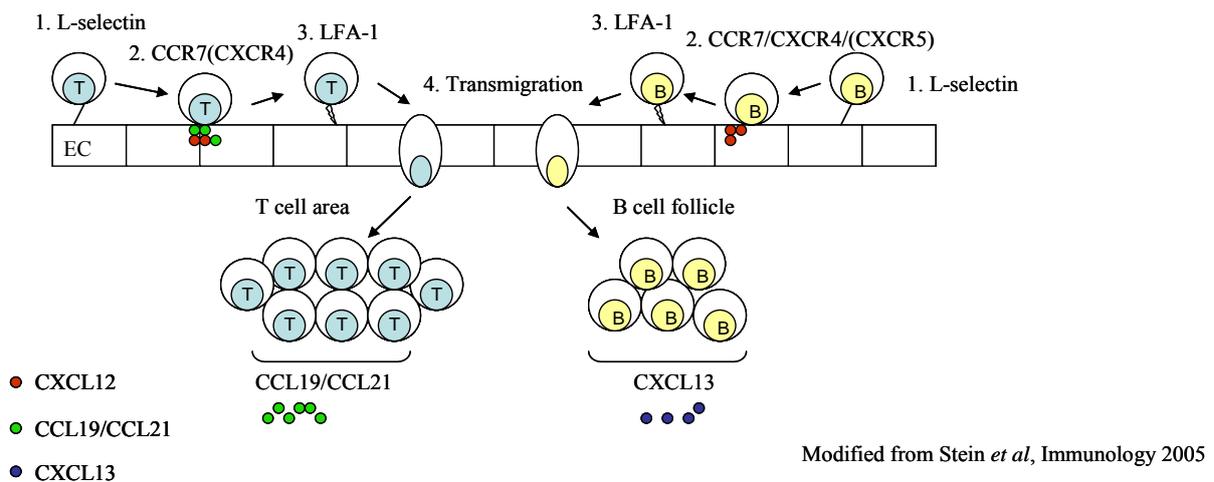**Lymphocyte homing to secondary lymphoid organs**

Fig 1. Chemokines and chemokine receptors during T and B cell homing to secondary lymphoid organs. After L-selectin mediated tethering and rolling of Tand B cells from blood, endothelial cells (EC) expressing CCL21 and CCL19 bind to CCR7 on the rolling T cell and trigger signals to intergrins such as LFA-1. B cells additionally rely on CXCR4- and potentially CXCR5-dependent signals. Chemokine receptor mediated signals induce conformational changes of integrins resulting in firm adhesion. After transmigation B cells are accumulated in CXCL13-expressing B cell follicles and T cells migrates to the T cell area expressing CCL19 and CCL21.

B cell migration

After B cell development in the BM, mature naïve B cells re-circulate the secondary lymphoid organs in search of their specific Ags. Lymphoid migration or "homing" to lymph nodes and Peyers patches (PPs) has been studied by homing experiments and microscopy. In addition to chemokine receptors, lymphocytes express different adhesion molecules such as L-selectin (CD62L) and the lymphocyte function-associated antigen-1 (LFA-1) and these molecules are important for the recruitment of lymphocytes to lymphoid tissue (Fig 1) . Integrin αLβ2 mediates lymphocyte rolling and firm adhesion to the vascular endothelium in post-capillary high endothelial venules (HEV) [84]. In the GALT, which includes PPs and mesenteric LNs (MLNs), the integrin α4β7 and its endothelial ligand mucosal addressin cellular adhesion molecule-1 (MAdCAM-1) play an important role for chemotaxis of lymphocytes and inflammatory cells. In order for the lymphocyte to mediate a firm adhesion to the

vascular endothelium, the adhesion molecules are required to reach a high affinity state by chemokine receptor signaling. This cascade of activation and adhesive events has been described in details for leukocyte trafficking to inflamed tissue as well as for hematopoetic stem cell homing from blood to BM [85-87]. Kunkel and colleagues described the "multi-step process" of leukocyte recruitment and speculated that this paradigm is also true for trafficking of antibody-secreting cells from lymphoid tissue to target effector tissue [86]. In the lymphoid organs the B and T lymphocytes are separated into different zones, the T cell zone and B cell zone respectively. For the lymphocytes to distinguish where to localize when entering the lymph node, the chemokines and the expression of chemokine receptors play an important role (Fig 2). In order to generate an immune response, chemokines are needed to orchestrate the migration of DCs, T cells and B cells.

The CXCR4 receptor and its ligand CXCL12

CXCR4 plays a central role during B cell development and mice lacking the receptor or its ligand, CXCL12, show severe impairment in B cell development [88]. CXCL12 is required for the earliest B cell precursors in fetal liver and BM [89]. CXCR4-mediated signaling prevents premature release of B cell precursors from the BM. Despite similar CXCR4 expression, early B cell precursors and immature B cells show different response to CXCR4 mediated migration and CXCL12 triggers a sustained migration response in pro and pre-B cells. This sustained migration diminishes during B cell maturation in the BM and is absent in circulating mature B cells, which exhibit only transient CXCL12-induced migration [90]. Studies on IgG positive antibody-secreting cells (ASC) show an up-regulation of cell surface CXCR4 and increased response towards CXCL12, but decreased ability to respond to CXCL13 (B zone chemokine), CCL19 and CCL21 (T zone chemokines) [91, 92]. Similar data were shown on mRNA

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levels of ASC in lymph nodes [93]. This change of B cell sensitivity to chemokine induced migration as they differentiate into IgM or IgG ASC, indicates a role for CXCL12 in guiding ASC to return to the BM for antibody production [86, 91]. The signal transduction pathway following CXCR4 activation is not fully understood and a recent publication by Palmesino *et al..* suggested that the different response towards CXCL12 in pre-B cells as compared to mature B cells is depending on different ERK1/2 activation [94]. This result indicates that the magnitude of the CXCR4 response is dependent on downstream accessory proteins.
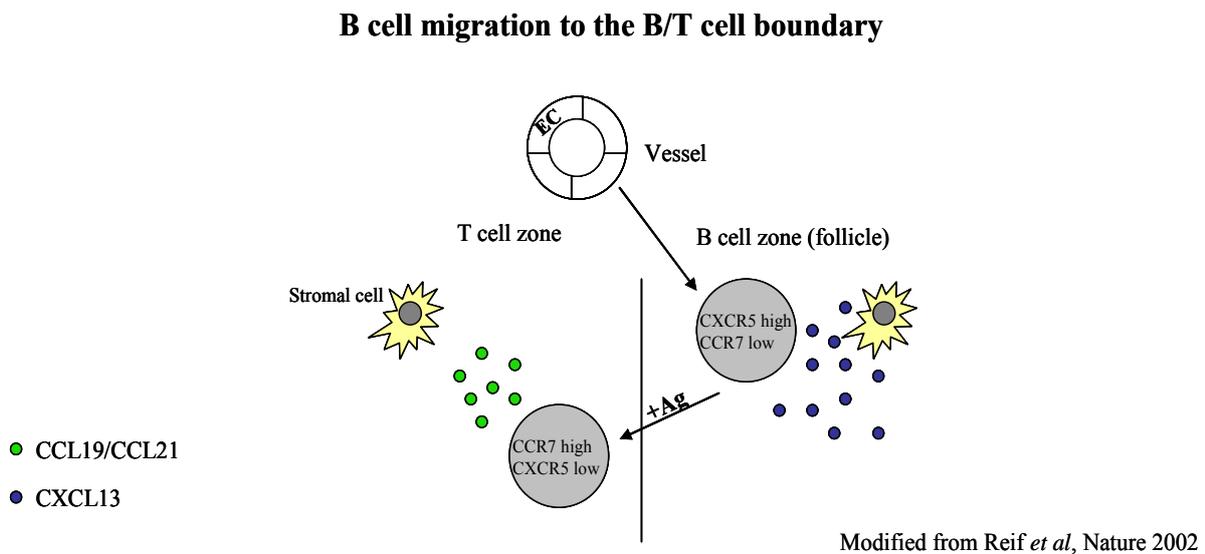
B cell migration to the B/T cell boundary

Modified from Reif *et al*, Nature 2002

Fig 2. B cell migration to the B/T cell boundary in spleen and lymph nodes after BCR engagement. B cells enters spleen and LN through blood vessles. Naive B cells express high levels of CXCR5 and low levels of CCR7 and the responsivness to CXCL13 mediates migration into the follicle. After antigen binding, BCR signaling leads to increased surface CCR7 and the B cell migrates towards the CCR7 ligands CCL19 and CCL21 in the T cell zone.

CXCR5 and its ligand CXCL13

CXCR5 is expressed on mature re-circulating B cells, small subsets of CD4$^+$ and CD8$^+$ T cells, and skin-derived migratory DCs [81, 95]. CXCR5 is responsible for guiding B cells into the B cell zones of secondary lymphoid organs. CXCL13 is expressed by FDCs and also on stromal cells located in the B cell areas of secondary lymphoid organs [96, 97]. Mice deficient in CXCR5 or CXCL13 lack several types of peripheral lymph nodes and the majority of Peyer's patches [97, 98] which indicates the important role of CXCR5 and CXCL13 in the development of secondary lymphoid organs and lymphoid tissue.

CCR7 and its ligands CCL19 and CCL21

Early studies identified the CCR7 receptor as an important homing molecule controlling lymph node entry of naive T cells through HEV and entry of activated mature DCs through afferent lymphatics. LNs of CCR7$^{-/-}$ mice display a disturbed architecture, lacking the characteristic structural segregation into B cell follicles and T cell areas [99]. This phenotype illustrates two important functions of CCR7 and its ligands; the regulation of cell homing [99, 100] and the functional organization of lymphoid organs. In transgenic mice that lack the CCR7 receptor, or lack lymphoid CCL19 and CCL21, antigen engagement of B-cells fails to cause movement to the T zone[101].

CHEMOKINE RECEPTORS IN VIRAL INFECTIONS

Many virus infections have been associated with chemokines and their receptors. For example mice lacking the CCR6 receptor have shown an impaired humoral immune response to rotavirus (RV), a virus which causes severe diarrhea in children [102]. These mice showed a reduced amount of Ag-specific ASCs compared to wild-type mice [103]. In addition, CCR9 and its ligand CCL25 play an important role in the small intestine immune response [86] where CCR9 controls B cell homing to the gut. CCL25 is expressed in the small intestine and it has been shown that RV-specific IgA ASC migrate to CCL25 [104].

During chronic infection with hepatitis C virus (HCV), which causes liver inflammation, elevated expression of CXCR3 and CCR5 on T cells isolated from HCV infected liver has been shown [105]. The ligands for CXCR3 (CXCL9, CXCL10 and CXCL11) are all interferon inducible proteins, and these chemokines are increased in the liver in HCV, as well as the CCR5 ligands CCL3, CCL4 and CCL5 [106]. A study by Humrich *et al.* shows that mature DCs infected with vaccinia virus (VV) are not able to migrate in response to the chemokines CCL19 or CXCL12, chemokines which would normally promote chemotaxis of DCs from tissues to secondary lymphoid organs [107].

Chemokine receptors during HIV-1

The chemokine field has gained a lot of interest since chemokine receptors were found to be co-receptors for HIV-1 entry into target cells [108]. In 1995, Robert Gallo published that chemokines, a class of naturally occurring compounds, can block HIV-1 entry into cells and halt the progression of AIDS [109]. The role that chemokines play in controlling the progression of HIV-1 infection has influenced thinking on how HIV-1 works against the human immune system. Chemokines and their receptors are regarded as having a potential role in possible HIV-1 vaccine development.

The chemokine receptors CXCR4 and CCR5 function as co-receptors for HIV-1 entry into CD4$^+$ cells. During the early stages of HIV-1 infection, viral isolates tend to use CCR5 for viral entry, while isolates from the time when overt immunodeficiency is established tend to use CXCR4. The importance of chemokine receptors for HIV-1 entry and AIDS pathogenesis has been illustrated by numerous publications over the years. Before its identity as a chemokine receptor, CXCR4 was shown to mediate entry of T cell line-tropic (T-tropic) HIV-1 strains [108], which was shown to be inhibited by the CXCR4 ligand CXCL12 [110].

The CC-chemokine receptors CCR5 was shown to mediate entry of macrophage-tropic (M-tropic) HIV-1 strains, a function which is also mediated by CCR3 and CCR2b, although to a lesser extent [111-113]. The importance of CCR5 in HIV-1 pathogenesis is underscored by the observation that individuals deficient in CCR5 are resistant to infection by HIV-1 [114, 115].

HIV-1 is a virus infecting CD4$^+$ cells, resulting in increased viral load and decreased CD4$^+$ T cell numbers, which finally destroys the immune system of the host. Eventhough, HIV-1 is infecting mainly CD4$^+$ T cells, B cell in HIV-1 infected are depleted [116]. The Nef protein of HIV-1, HIV-2 and SIV has been shown to be important in virus replication and contributes to pathogenesis in infection with these lentiviruses [117]. Nef has also been shown to modulate surface levels of several immune receptors such as CD4, CD8, MHC-I, invariant chain of MHC-II (CD74) and DC-SIGN. Recent studies have shown that Nef down-regulates CCR5 [118] and CXCR4 on the surface of primary CD4 T cells [119]. Venzke *et al.* suggested that the down-regulation of CD4 together with CCR5 is an efficient way for the host to protect cells from super-infection with a CCR5-using HIV-1 variant at the level of fusion of the viral envelope with the plasma membrane. In macaques infected with SIV, viral Nef has been shown to both down-regulate CXCR4 and strongly inhibit migration towards CXCL12, and it has been speculated that this is a mechanism for the virus to impair the anti- viral immune response [120] . The CXCR5 receptor has also been suggested to act as a co-receptor for HIV-2 and anti-CXCR5 monoclonal antibodies (mABs) are able to block HIV-2 infection of CXCR5 transduced cells [121].

Tonsillar B cells treated with the HIV-1 viral protein gp120 have shown to decrease their migratory response towards the chemokines CXCL12, CCL20 and CCL21 without affecting the expression or internalization of CCR6 and CCR7. CXCR4 in the same study showed to be down-regulated upon gp120 stimulation [122]. Several studies clearly showed that the expression and the function of chemokine receptors, both on T cells and B cells, are influenced by HIV-1.

Chemokine receptors during EBV

Nakayama and colleagues [123] have monitored chemokine receptor expression in EBV-positive BL lines and receptor expression and migration in EBV-immortalized human B cell lines. Their major finding was down-regulation of CXCR4, CXCR5 and up-regulation of CCR6 and CCR10 in EBV-immortalized B cell lines and they suggested that this impaired chemokine receptor expression could be dependent on the expression of the different EBV latent genes. After transfection of an EBV negative B cell lymphoma line (BJAB) with a vector containing EBNA2 and LMP1, they showed down-regulation of CXCR4, but not CXCR5 on mRNA level. The surface expression of CXCR4 was slightly down-regulated in EBV infected cells, moderately down-regulated in EBNA2 transfectants and clearly down-regulated in LMP1 transfectants. Surface expression of CXCR5 was not altered in the transfectants compared to the parental cell line [123]. On the other hand, Chen and colleagues when constructing genetically manipulated lymphoblastoid cell lines, which were EBNA3B$^-$/3C^{low}, found an up-regulation of CXCR4 at the RNA level and in some cases at the surface level. Migration assay performed on the EBNA3B$^-$/3C^{low} LCLs showed enhanced chemotaxis towards CXCL12 as compared to wildtype LCLs [124].

The Interleukin-7 receptor (IL-7R) and interleukin-7 (IL-7) in HIV-1

Interleukin 7 (IL-7) is a cytokine produced by stromal cells in lymphoid tissue which is crucial in both B and T cell development. IL-7 was first described as a B cell precursor growth factor but has also been shown to have a profound effect on T cells where IL-7 is able to stimulate fetal and adult thymocyte growth in mice [125, 126]. IL-7R signaling occurs when IL-7 cross-links the IL-7Rα and γ_c which causes phophorylation of JAK proteins, creating docking site for different signaling molecules such as STAT5 (signal transducer and activator of transcription 5). Phosphorylation of STAT5 induces dimerization and translocation of these molecules to the nucleus followed by gene transcription. Src family kinases are also shown to be activated by IL-7 [127] as well as PI3 kinase pathway [128].

A recent study by Alves *et al.*. identified a subset of circulating naive CD8$^+$ T cells, which are IL-7Rα low and this T cell pool was increased in HIV-1 infected individuals. It has been suggested that IL-7Rα^{low} naive T cells may constitute a pool of circulating naive cells that recently exited from cytokine-rich niches such as secondary lymphoid organs [129]. In healthy individuals a positive correlation between IL-7Rα expression and the IL-7-induced responses has been shown. This relationship is lost in HIV-1 infected patients, suggesting that a receptor signaling transduction defect in IL-7R function occurs in these patients [130].

Chemokine receptors and childhood ALL

Acute lymphoblastic leukemia (ALL) is a malignant disease of the BM in which early lymphoid precursors proliferate and replace the normal hematopoietic cells. ALL is the most common malignancy in children and pre-B cell ALL constitutes approximately 80% of childhood ALL. The malignant cells of pre-B ALL are lymphoid precursor cells that are arrested in an early stage of development [131] .

Chemotactic responsiveness to CXCL12 is down-regulated during B cell maturation with the earliest B cell precursors being most responsive [132-134]. Several studies on B lineage ALL have shown that the leukemic cells express CXCR4 [135-140] and that CXCR4 and CXCL12 are required for migration of pre-B ALL cells [140] thus directing leukemic cell migration within the BM microenvironment. Mechanisms that facilitate normal and pre-B acute ALL cell migration through stromal layers also include the β1 integrins VLA-4 and VLA-5 which are important molecules in CXCR4 signaling [141, 142].

A recent study by Juarez *et al.* shows that CXCL12 and IL-7 in synergy increase proliferation of B ALL cells and enhances phosphorylation of mitogen-activated protein kinases, ERK-1/2, p38 and AKT *in vitro* [143]. These results suggest a crosstalk between IL-7 and CXCL12 in B ALL cells. By culturing B ALL cells in presence of CXCL12, a significant correlation between IL-7R expression and the proliferative response to IL-7 were found which suggest that CXCL12 is required for optimal response to IL-7 by the IL-7R [143].

In this context, it is interesting that mice lacking the IL-7R or IL-7 gene, show reduced numbers of mature B cell and late pre-B cells [144, 145]. Pro-pre-B cells express the IL-7R and a down-regulation of IL-7R is correlated with rearrangement of κ-light chain [146] and expression of IgM by immature B cells [147]. Mature B cells have been shown not to express the IL-7R. The B cell development in mice and man differs even though the expression of IL-7R during B cell development is similar [148].

AIM OF THE THESIS

The basis of this thesis is that impaired humoral immunity may be a result of defect or lack of interaction between B and T cells in secondary lymphoid organs. Therefore we investigated the expression of chemokines and chemokine receptors important for homing responses in B cells during viral infection. We choose to focus our studies on CXCR4, CXCR5 and CCR7 as they play a critical role in directing B cell migration in lymphoid organs.

The specific aims of this thesis are:

- To characterize chemokine receptor expression on different subpopulations of B cells during chronic HIV-1 infection

- To study the expression of CXCR4 on EBV infected tonsillar B cells

- To study the effects of altered receptor expression on B cell migration during HIV-1 and EBV infection

- To clarify the role of CXCL12 for proliferation and signaling in pre-B ALL

- To assess the IL-7R expression on T cells during HIV-1

MATERIAL AND METHODS

HIV-1 infected patients

In *paper I,* blood specimens from 47 HIV-1 infected individuals and 46 non-infected controls were collected. In the HIV-1 cohort the median CD4$^+$ T cell count was 375 cells/µl (range: 50–1230 cells/µl) and viral load ranged from <20 to 250.000 copies/ml in blood. Thirty-nine patients were undergoing highly active antiretroviral therapy (HAART) and 8 patients were untreated.

In *paper V,* blood samples were obtained from 38 HIV-1 infected patients (29 men, 9 women) and 17 non-infected subjects. The median CD4$^+$ T cell count was 312 cells/µl (range: 20–1000 cells/µl). Of the patients, 11 were treated with reverse transcriptase inhibitors, and 15 were undergoing HAART. Plasma viral load was determined using the NASBA system (*paper I and V*).

Pre-B ALL patients

In *paper IV*, the study population consisted of 37 children diagnosed with pre-B ALL. Sera from 21 healthy children were included as controls. The median age of the ALL children was 4.5 years (0.5 -15) and 9.5 years (4-16) in the controls. The controls were children with cancer undergoing BM aspiration as part of the diagnostic investigation, but with no evidence of malignant cell infiltration. For the proliferation study, 5 children with pre-B ALL, 2 children with T ALL and controls (n=4) were included. Informed consent was obtained from all subjects before enrolment and the ethical committees of the Karolinska University Hospital and Karolinska Institutet approved the studies. For the children, the parents gave informed consent to participate in the study.

Human tonsils

Tonsil B cells were prepared from human tonsils obtained through routine tonsillectomy. Tonsils were cut into fragments and dispersed into cell suspension. T cells were removed by E rosetting, followed by separation with standard Ficoll-Hypaque gradient centrifugation (*paper II and III*). These cells contained > 90% CD19-positive cells.

Cell preparation and cell cultures

PBMCs from HIV-1 infected patients and pre-B ALL patients were prepared from blood by standard Ficoll-Hypaque gradient centrifugation. B lymphocytes were separated using magnetic cell sorting. The purity of the separated B cells was >95% as verified by anti-CD19 staining. Cells were cultured in serum-free X-Vivo 15 culture medium (*paper I*). T lymphocytes were separated using magnetic cell sorting. Cells were cultured in RPMI 1640 with L-glutamine containing 10% fetal calf serum and antibiotics at a cell concentration of 1×10^6 cells/ml. Cells were cultured in presence of 1, 3 and 10 ng/ml IL-7 or without IL-1 for 7 days (*paper V*).

Flow cytometry studies

Cells were incubated with conjugated mAbs to the relevant surface antigens and data was acquired using FACScan or FACSort. Data was analyzed using the CellQuest Pro software. For the intracellular stainings, cells were first permeabilized and fixed using DAKO Intrastain Kit before incubation with the relevant antibodies.

Transmigration assay

A Transwell culture system with a 5-µm-diameter pore filter was used. 2.5×10^5 cells were re-suspended in medium and loaded into the upper chamber of the Transwell. Thereafter, 600µl of medium containing the respective chemokines were added to the lower well and the plate was incubated at 37°C for 4 h. Migrated B cells were collected and counted by flow cytometry. Migration is presented as an index of migrated cells towards the individual chemokine divided by spontaneous cell migration *(paper I)* or as percentage of migrated cells compared to input population after subtraction of background migration to medium *(paper II and III)*.

cDNA expression array

B cells were collected and RNA was extracted using the RNeasy Mini Kit. Total RNA was evaluated by spectrophotometer. RNA was purified from each sample and cDNA expression microarray analysis was performed using the GEArray Q series Human Chemokines and Receptors Gene Array. The array measures 96 genes encoding for the Small Inducible Cytokine Subfamily A (Cys-Cys) (n=23), Subfamily B (Cys-X-Cys) (n=14), other subfamily members (n=5), chemokine receptor family (n=28), chemokine-like factor superfamily (n=7) and other related genes (n=19).

Total RNA was reverse-transcribed using Ampolabelling (LPR) Kit in the presence of biotin-16-dUTP Amplification of cDNA was performed during 30 cycles. The biotinylated cDNA probes were denatured and added to the hybridization solution. GEArray Q Series membranes were pre-hybridized at 60°C for 2 hours and thereafter hybridized overnight with the cDNA probes. Membranes were then washed, blocked and incubated with alkaline phosphatase-conjugated streptavidin. The labeled biotin on

the membrane was detected by chemoluminescence using GEArray Chemoluminescence Detection Kit.

Analysis of cDNA microarray

cDNA probes were analyzed by GEArray Expression Analysis Suite software (SuperArray, Frederick, MD, USA). *(paper I and III).*

Western Blot

The cells were lysed in lysis buffer and then denatured by boiling. Total cell lysates were loaded in each well and the samples were electrophoresed on SDS-PAGE gel and transferred to PVDF membranes at 80 V for 2 hr. After blocking for 1 hr with 7.5% non-fat dried milk in PBS-Tween 20, the membranes were incubated with the primary antibodies overnight at 4°C. The blots were then incubated with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit Ig antibody or HRP-goat anti-mouse Ig antibody and detected with ECL Plus detection reagent. Primary antibodies were as follows: mouse anti-EBNA2, mouse anti-LMP1 and mouse anti-β actin.

Real-time q-PCR

PBMCs were incubated with CXCL13 for 12 hours. Cell surface expression of CXCR5 was examined by flow cytometry and gene expression levels investigated by q-PCR. The PCR reaction contained 10 μl of master mix, 1 μl RT reaction product including 100 ng of each of the primers containing MGB probe, cDNA and water to final volume of 20 μl. The reaction was performed in ABI Prism 7900 and the reaction conditions were 50 °C for 2 min, 95 °C for 10 min and then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The minor groove binder (MGB) probes were labelled with the fluorescence

labelled dye FAM. The final results were analyzed using ABI Prism 7900 SDS software.

ELISA

Plasma and BM concentrations of CXCL12 in ALL children were measured by a commercially available sandwich ELISA *(Paper V)*. IL-7 concentrations in HIV-1 infected subjects, pre-B ALL children and controls were determined with Quantikine high sensitive immunoassay according to manufacturer's recommendations *(Paper IV)*.

Statistical analysis

Statistical analyses were performed using Sigma Stat for Windows software (SPSS, Chicago, IL, USA). Differences between patients and controls were analysed by parametric (t test*)* or non-parametric (Mann-Whitney U test) tests. Comparison of 3 or more subgroups was performed by one-way ANOVA tests.

RESULTS AND DISCUSSION

Chemokine receptors during HIV-1 infection

Both HIV-1 and EBV infections are associated with B cell abnormalities [149, 150].

Even though B cells are not the main direct target of HIV-1 infection, B cells are

affected during infection and abnormalities are manifested by polyclonal B cell

activation, increased frequency of B cell malignancies, hypergammaglobulinemia and

poor antigen-specific responses [151-154].

In HIV-1 infected individuals, one feature of the pathology is changes in the lymph

nodes (LN) such as persistent lymphadenopathy [155, 156]. LN displays follicular

hyperplasia during primary infection, which gradually progresses to follicular

involution (nearly complete destruction of the FDC network within GCs) during the

chronic stage of infection. These changes in the LN have been shown to result in an

inability to mediate antibody production and antigen-specific T cell responses

ultimately leading to AIDS [155]. The disturbed GC architecture can in part be

corrected by HAART [157]. Since LN architecture is progressively destroyed during

HIV-1 infection, it is possible that normal B cell trafficking maybe impaired as well.

The CXCR5 ligand CXCL13 is a major regulator of B cell trafficking and serum levels

of CXCL13 have been shown to increase during HIV-1 infection [158].

It has also been reported that CXCR4-deficient mice show an altered GC organization

likely due to the observation that CXCR4 is essential for GC dark and light zone

segregation. In contrast CXCR5 and its ligand CXCL13 are required to determine the

correct position of FDCs, B and T cells in the light zone. Analysis of LN in B cell

deficient mice injected with CXCR4 knockout B cells showed that FDC and CXCL13

light zone polarity is lost in the GC [159].

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In *paper I,* we studied the expression of CXCR4, CXCR5 and CCR7 on B cells and we show that CXCR5 is significantly down-regulated on peripheral blood B cells from HIV-1 infected subjects as compared to healthy controls (Fig 3). Although CXCR5 is expressed on all mature circulating B cells in healthy individuals [81], HIV-1 infected individuals have been shown to carry a CXCR5 negative B cell pool in blood [160]. CXCR5 expression is down-regulated in naïve B cells from the peripheral blood from HIV-1 infected patients [161]. Surface expression of chemokine receptors may in part be regulated by chemokines since upon ligation with the respective ligands, CXCR4 and CXCR5 are internalized and endocytosis of the receptors occur [162]. This is an interesting observation as we show that HIV-1 infected patients have elevated levels of CXCL13 in B cells, both at protein and mRNA levels. CXCL13 mRNA expression in B cells has previously not been shown whereas several reports indicate that FDCs and stromal cells are the source of CXCL13 [96, 163, 164]. Monocytes from healthy individuals have shown to up-regulate CXCL13 mRNA in response to lipopolysaccharide (LPS) and accordingly, the up-regulation of CXCL13 seen in patients may be a consequence of the immune activation occurring in HIV-1 infection. In turn, the increased expression of CXCL13 in B cells from HIV-1 infected may be a driving force for the down-regulation of CXCR5 through an autocrine or paracrine loop.
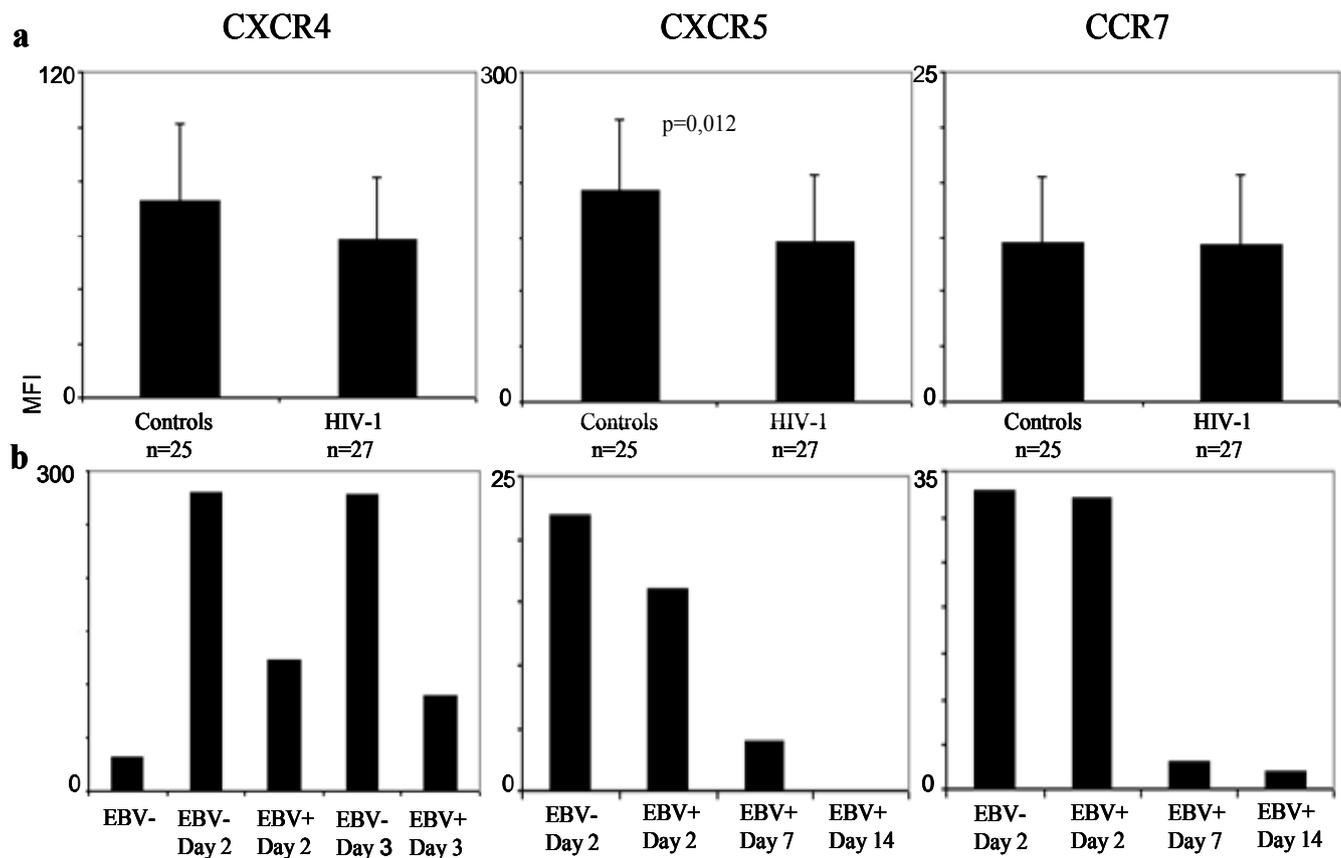
Fig 3. Expression of the chemokine receptors CXCR4, CXCR5 and CCR7 on **a)** CD19+ B cells from blood of HIV-1 infected individuals compared to controls. **b)** Tonsillar B cells infected with EBV; CXCR4 was monitored day 2 and 3, CXCR5 and CCR7 was monitored day 2, 7 and 14 after infection.

To investigate the function of the receptors studied in our cohort of HIV-1 infected patients we performed transmigration assay on B cells (Fig 4). The migration of B cells towards CCL21 showed no difference when comparing specimens from controls and HIV-1 infected subjects. However, a minimal increase in migration of B cells towards CXCL12 and CXCL13 was shown in the HIV-1 infected subjects as compared to controls. This finding is interesting as we show that activation with CD40 and IL-4 increase migration towards CXCL12 in tonsillar B cells (*paper II*). This is in accordance with previous studies showing that LPS stimulated B cells increase their migration towards CXCL12 [165, 166]. Altogether, these findings suggest that the

increased migration towards CXCL12 and CXCL13 may reflect polyclonal stimulation of B cells during HIV-1 infection.

A recent study reported the identification of four additional human CXCL12 isoforms derived from alternative splicing events, CXCL12γ, CXCL12δ, CXCL12ε and CXCL12φ. These CXCL12 splice variants all share the same first three exons, but contain a different fourth exons [167]. Interestingly, Altenburg and colleagues constructed and expressed all of the CXCL12 splice variants in *Escherichia coli*. Recombinant proteins were purified and when their biological properties were analyzed, CXCL12γ induced a weak chemotactic activity of CXCR4 as compared to the other isoforms [168]. Studies have recently shown that also CXCR7 is a receptor for CXCL12 in T cells [169, 170] although contradictory results have been presented in this respect. In fact, Altenburg et al. showed that $CXCR7^+/CXCR4^-$ T cells migrated towards all CXCL12 isoforms [168] whereas other studies showed that CXCL12 does not induce signaling through CXCR7 [170, 171]. In addition, CXCR7 has been shown to form functional heterodimers with CXCR4 and to enhance CXCL12-induced signaling [171].

Chemokine receptors during EBV infection

In *papers II and III,* we wanted to investigate how EBV infection affects the chemokine receptor expression on tonsillar B cells. In *paper II* we showed that the expression of CXCR4 decreased already 2 days after infection and further declined after 3 days as compared to non-infected tonsillar B cells (Fig 3). At these time points the total EBNA expression was 35% and 52%, respectively. We could also detect an increase in CXCR4 expression in the uninfected cells after 2 days compared to cells stained immediately after preparation accordingly to previously reported data from Brandes *et al.* [165].

Upon EBV infection the cell size increases due to activation. By using flow cytometry, resting and activated cells were separated according to cell size. In the large EBV infected cells the expression of CXCR4 was lower than in uninfected control cells. The lower expression of CXCR4 in infected cells might either be due to the fact that the virus infects cells with moderate CXCR4 expression or that EBV might infect cells with variable expression of CXCR4 followed by down-regulation of the receptor in the established lymphoblastoid cell lines. The viral encoded proteins EBNA2 and LMP1 have been shown to down-regulate CXCR4 in the B cell lymphoma line BJAB [123]. The mechanism of down-regulation of CXCR4 by EBNA2 and LMP1 is not known, however infection with herpes virus 6 and 7 in human T cells has also shown to down-regulate CXCR4 [172].

In *paper III* , we investigated further the expression of CXCR5 and CCR7 on tonsillar B cells. Interestingly, we found that both CXCR5 and CCR7 totally disappeared from the cell surface after 14 days following primary EBV infection. CXCR5 has not previously been shown to be down-regulated by EBNA2 or LMP1 in transfected cells, but low expression of CXCR5 is found in EBV-immortalized B cell lines [123]. One striking difference was the difference in CXCR5 expression on B cells isolated from blood (*paper I*) compared to CXCR5 expression on tonsillar B cells (*paper III*) in terms of mean fluorescence intensity (MFI) (Fig 3). Plasmablasts has been shown to express low levels of CXCR5 and it is possible that the reduced CXCR5 expression in EBV infected tonsillar B cells compared to uninfected cells reflects the activated plasmablast-like stage of EBV infected B cells [91, 123].

CCR7 expression both on the cell surface and at mRNA levels was significantly decreased 7 days after EBV infection. This was an unexpected result as CCR7 has been previously shown to be up-regulated by EBV in the ER/EB2-5 model system where EBNA2 is induced via estrogen-mediated activation in different cell lines [173-176]. In order to further investigate this phenomenon we studied the expression of CCR7 in the ER/EB2-5 system and our experiments confirmed the induction of CCR7. This suggests that CCR7 is differently regulated in B cell lines immortalized by EBV than in human tonsillar B cells. This observation may in part be related to the previous findings that upon EBV-infection, different EBV-related genes will be expressed depending on the differentiation stage of the infected B cell population [70, 177]. It is conceivable that different EBV related genes may be expressed in tonsillar B cells versus B cell lines and that this may influence CCR7 expression**.** The impact of differentiation stage and the expression of chemokine receptors have also been demonstrated in studies of regulatory T cell isolated from different tissue compartments [178].

The chemokine receptors CXCR4, CXCR5 and CCR7 all play an important role for B cell trafficking [159, 179, 180]. In order to investigate if EBV could interfere with CXCL12, CXCL13 and CCL21 mediated migration we performed migration studies towards the respective chemokines. In *paper II* , we show that CXCR4 mediated chemotaxis is influenced by EBV infection. Cells cultured with the B95-8 a, sub-strain of EBV, showed decreased migration towards CXCL12 as compared to tonsillar B cells cultured in medium (Fig 4). In order to investigate if the EBNA positive cells in the cultures were responsible for the lower migration, migrated and non-migrated fractions were stained for EBNA expression. The percentage of EBNA positive cells in the migrated fraction was lower than in the total input population indicating that EBV-infected cells have reduced migration capacity towards CXCL12.

In *paper III,* we investigated further the chemotactic reponse towards CXCL13 and CCL21 in EBV infected tonsillar B cells and we demonstrated that EBV interferes with CXCL13 and CCL21 mediated migration already after 2 days from infection. Interestingly, it has been shown that regulators of G-protein signaling (RGS) proteins known to regulate G-coupled chemokine receptors, can down-regulate migration towards CXCL13 and CCL21 [181]. RGS1 is induced by EBNA2 and RGS13 is down-regulated by EBV encoded genes [182] and could therefore interfere with migration. In patients with IM, EBV infected B cells are detected mainly in the interfollicular regions and do not migrate to the GCs [183, 184]. The reduced migration seen in EBV infected tonsillar B cells may exclude infected cells from the B cell follicles, and thus retain cells in the interfollicular areas of the tonsil.
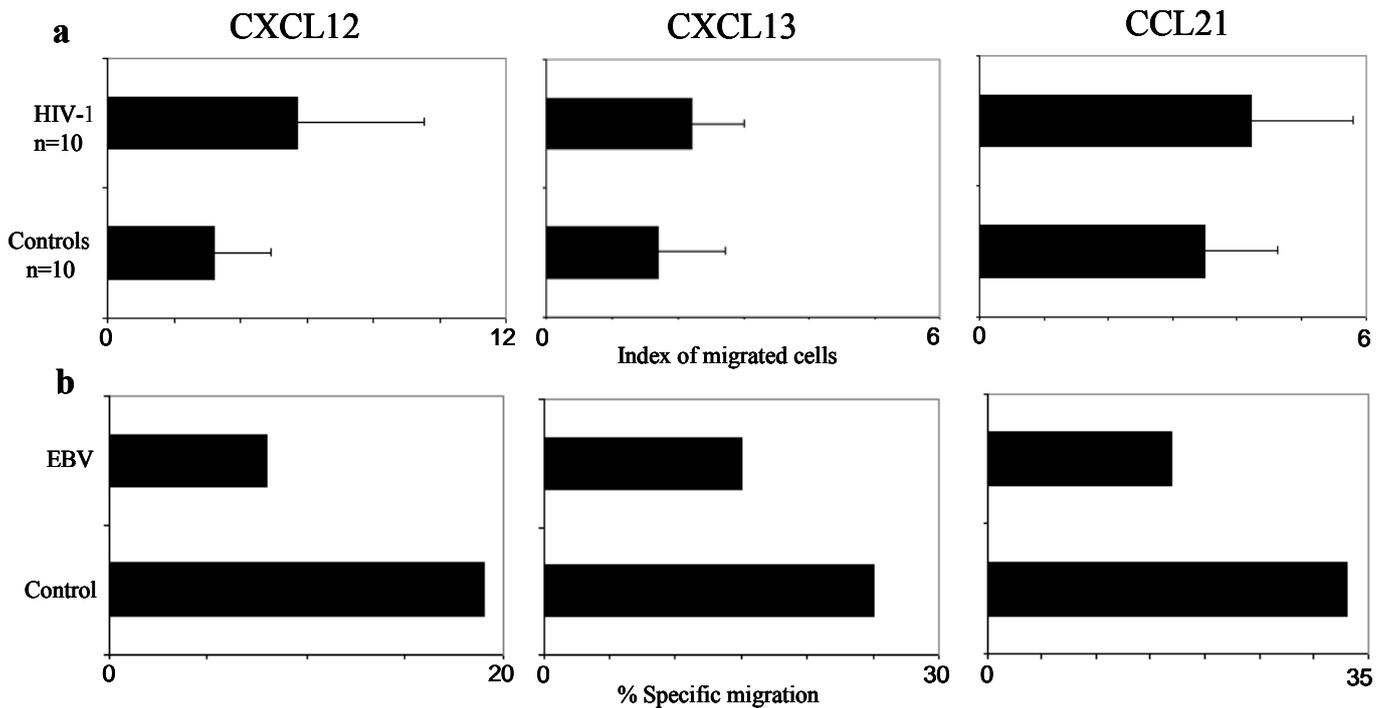
Fig 4. Migration of B cells towards CXCL12, CXCL13 and CCL21 during HIV-1 and EBV infection. **a)** Migration of primary B cells from HIV-1 infected patients and controls. Index of migrated cells is presented as migrated cells towards the specific chemokine divided by spontaneous migration. **b)** Migration of tonsillar B cells infected with EBV and uninfected control cells. Percentage of specific migration is presented as percentage of cells migrated to respective ligand compared to input population after subtraction of background migration.

We performed mRNA microarray on a large set of chemokines and chemokine receptors in order to investigate whether EBV influences the expression of several genes. EBV infected tonsillar B cells were cultured for 2 and 7 days and compared to uninfected cells cultured for 2 days. CCR9, a chemokine receptor involved in homing to mucosal tissue, was shown to be up-regulated upon EBV infection. It has been previously shown that IgA+ antibody producing cells express high levels of CCR9 in mucosal lymphoid tissue [185, 186] and that the ligand of CCR9, CCL25, which is produced by epithelial cells in tonsils, may induce homing of EBV infected IgA+ B cells during latent EBV infection[187]. An interesting finding was the high expression of C5AR1 (CD88), a gene encoding the receptor for complement C5a. The CD88 has

shown to mediate migration of naïve and memory B cell from purified tonsils but not GC B cells [188]. These data indicate that EBV induces the expression of genes important for homing to mucosal lymphoid tissue. In addition, C5a has been associated to inflammatory disease and IgG immune complex disease [189, 190] although less is known on the direct impact of C5a on immunoglobulin production.

We also found up-regulation of CCL20 upon EBV infection; CCL20 has been shown to be important for migration of regulatory T cells in blood [191]. The mRNA for the inflammatory chemokine CCL11 was found to be up-regulated in our study as well and this is in line with previous findings of CCL11 in EBV positive Hodgkin disease [192] The elevated mRNA levels of the inflammatory chemokines CCL20 and CCL11 suggest that EBV influences the level of expression of chemokines important for T cell dependent immune responses. CXCL2, a chemokine that mediates recruitment of NK cells and cytotoxic T cells, also known to be produced upon infection with influenza virus [193], was shown to be down-regulated in our study upon EBV infection. Those results may indicate that EBV has developed mechanisms for altering chemokine expression in order to evade the host immune response in the tonsil.

CXCL12 in pre-B ALL

In addition to facilitate homing to BM, CXCR4/CXCL12 interaction has also been reported to stimulate proliferation and survival of pre-B ALL cells [194, 195]. We were interested in the signaling pathway downstream from CXCR4 and in addition to BM samples from children with pre-B cell ALL, we also studied the pre-B leukemic cell line Nalm-6 to further investigate the signaling pathway involved in CXCR4/CXCL12 activation.

In *paper IV,* we show that children with pre-B ALL have elevated serum levels of CXCL12 as compared to healthy controls (p<0,001) (Fig 6) and that the high serum levels seen at diagnosis decline to levels comparable to healthy controls upon treatment (p<0,02). In order to investigate further if CXCL12 had a role in proliferation of ALL cells we cultured pre-B leukemic BM together with recombinant CXCL12 and measured the rate of proliferation. In the short time cultures (2 days) CXCL12 increased the proliferation of pre-B leukemic BM as compared to leukemic cells cultured without CXCL12 (p<0,05). In the control BM cultures there were no difference in proliferation between cells cultured with or without CXCL12. To monitor the long-term effect of CXCL12, primary ALL cells were kept additional 7 days in culture and, upon these conditions, an additional increase of proliferation was detected. The CXCL12 induced proliferation was restricted to pre-B ALL cells since we could not detect any increased proliferation in cells from children with T ALL. The CXCR4 expression on the leukemic cells was monitored in presence or absence of CXCL12 in the cultures at day 2, 7 and 10. CXCL12 induced down-regulation of the receptor but interestingly, in the pre-B ALL cells, the decrease of the receptor was more evident than in the controls.

Recent findings have shown that CXCR4/CXCL12 signaling activates the transcription factor STAT5 [196, 197]. Based on our findings we wanted to investigate further downstream signaling in leukemic cells and therefore examined activation of STAT5 in the BM cells of ALL patients and in the pre-B cell leukemic cell line Nalm-6. We performed immunohistochemistry with anti-STAT5 specific antibodies on BM cells from patients and on Nalm-6 cells cultured with CXCL12. Activated STAT5 was observed in the nucleus of 15-20% of the leukemic BM derived cells and similar results were obtained when Nalm-6 cells were cultured with CXCL12 (Fig 5). This data indicates a role for STAT5 in CXCL12 signaling in ALL.
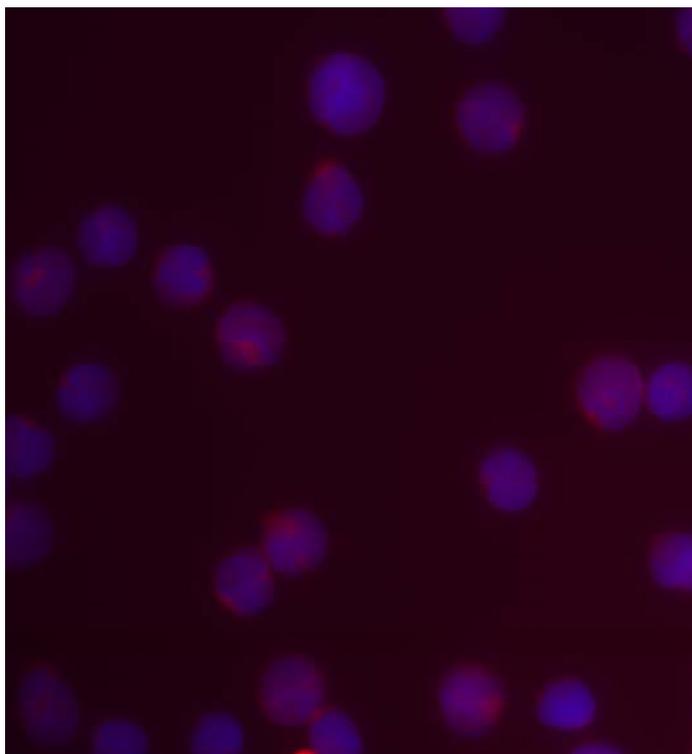
Fig 5. BM aspiration from one ALL patient. Primary leukaemic cells were stained with rabbit antibodies against pTyr694 on STAT5 and anti-rabbit-Alexa 568 as a secondary Ab. Phosphorylated STAT5 is shown in red and nuclei is stained with Hoechst (blue).

A recent study has shown that deficiency of STAT5 impairs early B cell development but does not affect B cell apoptosis, cell cycle entry or Ig production in mice [198]. STAT5 is activated by a broad spectrum of cytokines and growth factors, including IL-7 [199]. Dai *et al.* demonstrated that complete STAT5 deficiency directly blocks early B development at the pro-B cell stage, matching the phenotype of IL-7R$^{-/-}$ mice [200] and it was suggested that STAT5 plays a major role in IL-7 signaling involved in early B cell development. There have been several reports indicating that CXCL12 enhances survival of pre-B cells together with IL-7 [132, 143, 194]. We measured serum levels of IL-7 in 41 children with pre-B ALL and we could not detect any significant changes in IL-7 levels in patients compared to controls (Fig 6). However, the study by Juarez *et al.* showed a relation between IL-7 and CXCL12 in the proliferation of pre-B ALL cells. In this context it would be interesting to expand the number of patients studied.
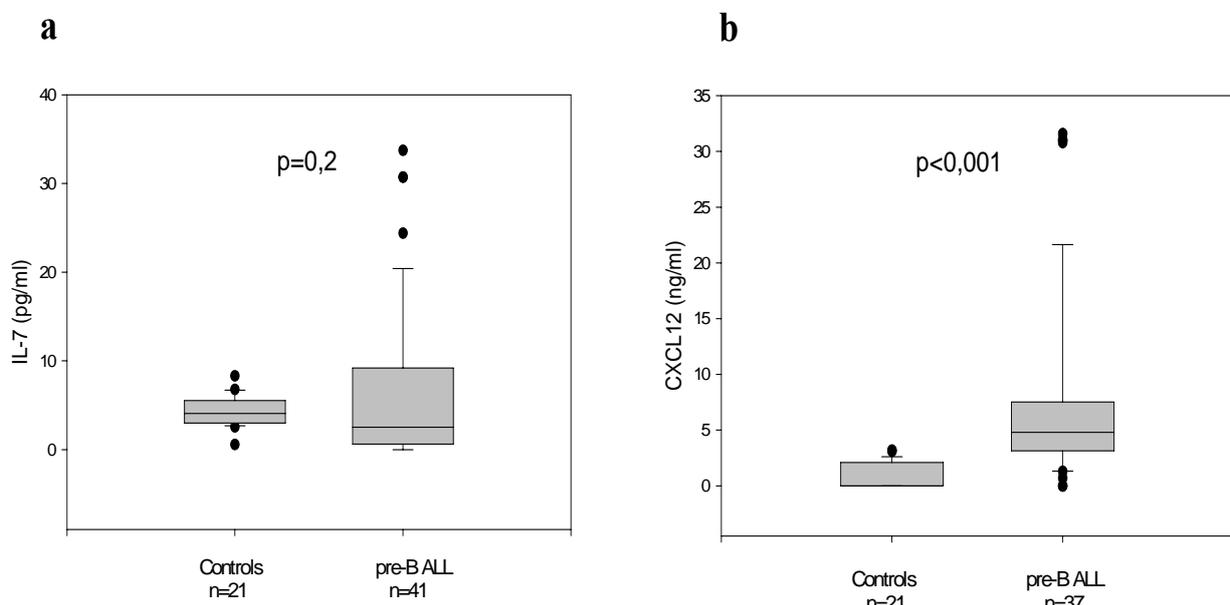
a **b**

Fig 6. Since IL-7 and CXCL12 are growth factors for early B cell progenitors we investigated the serum levels of IL-7 and CXCL12 in children with pre-B ALL by ELISA. **a)** IL-7 in children with pre-B ALL was not significantly elevated compared to controls. **b)** On the other hand CXCL12 in children at diagnosis of pre-B ALL was significantly higher.

IL-7Rα on T cells during HIV-1 infection

A decrease in IL-7R expression on T cells has been previously demonstrated during HIV-1 infection [201-203]. In *paper V,* we analyzed the expression of IL-7Rα on T cells isolated from HIV-1 infected patients (n = 38) at different clinical stages of HIV-1 infection and from non-infected controls (n = 17). A high proportion of T cells from several of the HIV-1 infected patients showed a decreased IL-7Rα expression. In HIV-1 infected patients the ratio of the IL-7Rα low/negative T cells was significantly higher within the CD3 lymphocytes as compared to controls (P < 0.001). When incubating T cells *in vitro* from uninfected controls with IL-7, a decline of the IL-7Rα could be noticed; upon on removal of the cytokine the receptors were again up-regulated. We aimed at verifying whether IL-7 complexed to its receptor in vivo could be responsible for the down-regulation of the IL-7Rα during HIV-1 infection. To verify this scenario we cultured T cells from HIV-1 infected individuals in absence of IL-7; upon these conditions we could not measure a larger proportion of IL-7Rα positive cells, indicating that the presence of high IL-7 levels during HIV-1 infection may not be the only factor contributing to down-regulation of the receptor.

Virus infections and modulation of surface receptors

In order for a CD4^{+} T cell to become optimally activated it requires signals from both the TCR-CD3 complex and the co-stimulatory molecule CD28. CD28 binds to B7 on activated B cells and is required for activation of CD4^{+} T cells [204, 205]. In our study, T cells with memory phenotype from HIV-1 infected subject showed a decreased IL-7Rα expression in association with CD28 down-regulation (*Paper V*).   A study by Secchiero *et al.* shows that stimulation with anti-CD28 mAbs induces a small increase in expression of surface CXCR4 on CD4^{+} T cells. Moreover, stimulation with anti-CD3 showed a progressive down-regulation of surface CXCR4 and stimulation with anti-CD3 and anti-CD28 showed intermediate levels of CXCR4 [206]. Activation of T cells via anti-CD3 stimulation decreases CXCR4 expression but that was in part abrogated by simultaneous anti-CD28 stimulation in vitro. Whether this will translate into different biological properties in vivo is not known to date. The expression of CXCR4 and CXCR5 on B cells after CD28/B7 interaction has not yet been studied.

Park *et al.* showed that HIV-1 infected adults have lower numbers of CD3^{+} lymphocytes expressing L-selectin and VLA-4 and higher numbers of CD3^{+} lymphocytes expressing LFA-1 than healthy controls [207]. Lymphocyte adhesion molecules include the selectin, integrin, and immunoglobulin gene superfamilies [208, 209]. Lymphocyte function-associated antigen 1 (LFA-1) is an integrin expressed on peripheral blood leukocytes which is involved in lymphocyte-endothelial cell adhesion [209]. The ligands for LFA-1 are two members of the immunoglobulin gene superfamily, intercellular adhesion molecules 1 (ICAM-1) and -2 (ICAM-2). Very late antigen 4 (VLA-4) is expressed on T cells and is involved in lymphocyte adhesion to endothelial cells. L-selectin (CD62L) plays a major role in lymphocyte recirculation to peripheral lymph nodes [208, 209]. The same study showed that the frequency of

CD3$^+$ CD28$^+$ lymphocytes and CD3$^+$ L-selectin$^+$ lymphocytes were positively correlated whereas the frequency of CD3$^+$ CD28$^+$ lymphocytes and CD3$^+$ LFA-1$^+$ lymphocytes were negatively correlated [207].The results of this study suggest that HIV-1 infection is associated with altered expression of adhesion molecules. We, together with others, have shown that HIV-1 infection is associated with loss of CD28 surface antigen on T lymphocytes [210]; in this context it is very interesting that adhesion molecules might be affected by the CD28 down-regulation seen during HIV-1 as these adhesion molecules are very important in transmigration of both B cells and T cells and CD28 seems to influence the CXCR4 expression on T cells.

Altered cell surface expression of several molecules has also been reported during EBV infection. EBV encoded viral interleukin-10 (vIL-10) was shown to inhibit IFN-γ-induced HLA-class II, ICAM-1, ICAM-2 and B7.1 (CD80), and B7.2 (CD86) up-regulation on monocytes/macrophages. Complete inhibition of HLA-class I expression has been shown to occur when vIL-10 is present two hours prior to the addition of IFN-γ in vitro, and Salek-Ardakani *et al.*. suggested that vIL-10 affects an early step in the IFN-γ signaling pathway. They propose that vIL-10-mediated impairment of antigen-presenting function could be an escape mechanism for the virus-infected cells to avoid detection by the host T cells [211]. Several herpes viruses encode homologues of chemokines and chemokine- receptor related G-protein-coupled receptors but the role of these proteins for virus replication in the infected host has not yet been defined. However, preliminary data indicate a role for these proteins in immune evasion or in activating cellular elements that facilitates viral replication [212].

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Signaling via JAK/STAT pathway

In my thesis, I have studied the relation between chemokine receptor expression and B cell migration in vitro. However, several studies have indicated the importance of downstream signaling for chemotactic responses. Jak3-deficient mice show a phenotype which is very similar to that of IL-7 and IL-7R and γ_c chain-deficient mice [213]. Jak3 has been shown to be involved in CCR9- and CXCR4-mediated signaling in the thymus and Jak3-deficient BM progenitors and thymocytes show a decreased chemotactic response towards the chemokines CCL25 and CXCL12. This study suggests that the absence of Jak3 affects T cell development, not only through impaired IL-7R-mediated signaling, but also through impaired chemokine-mediated responses, which are crucial for thymocyte migration and differentiation [214].

Altogether these studies show that downstream signaling is important for chemokine-mediated responses and may be studied further in B cells from HIV-1 infected subjects. We detected STAT5 activation in pre-B ALL cells and also in the cell line Nalm-6 after treatment with CXCL12. Activation of the JAK/STAT pathway has been implicated in IL-7R signaling and it was shown that STAT1, STAT5, and STAT3 are activated upon stimulation of precursor B cells with IL-7 in mice [215]. We also show that in the serum of children with pre-B ALL both high levels of IL-7 and CXCL12 could be detected. In addition, EBV viral protein LMP1 has been shown to have consensus sites (proline-rich sequence) characteristic for interaction with members of the Jak family and especially Jak3 [64]. Active STATs has also been found in a variety of EBV-associated malignancies [216-218] and LMP1 expression has been associated with an increase in activated STAT3 and STAT5 in epithelial cells infected with EBV [219].

Future perspectives

In the present thesis the pattern of expressions of chemokines and their receptors was studied upon EBV infection of B cells *in vitro* and *ex vivo* in blood specimens obtained from HIV-1 infected subjects. These results are mostly descriptive in that they measure chemokine and chemokine receptors biology in a relatively simplified system *in vitro* or at one time point in the infected host. The natural evolution of our work would be to study how chemokine and chemokine receptors affect the natural course of infection and evolution of immunological responses in experimental models of the viruses used in this thesis, e.g. HIV-1 and EBV. To study tissue specimens from HIV-1 infected patients obtained through biopsy or post-mortem should be also part of future studies to obtain a comprehensive picture of homing dysregulation during viral infections. In the following paragraphs I briefly summarize some of the questions that could be further developed departing from the results obtained in my thesis.

Mueller *et al.* recently showed that CCL21 and CXCL13 are down-regulated within the lymphoid tissue in mice infected with lymphocytic choriomeningitis virus [220]. Little is known about the impact of chemokines and chemokine receptors during viral infections and how they affect lymphocyte trafficking within lymphoid tissue. It is known that CXCR5 is expressed on peripheral blood cells and is involved in migration of immune cells to lymphoid tissue. We and others have shown that CXCR5 is down-regulated on B cells during HIV-1 [161] and during this infection we could also detect increased levels of CXCL13 in peripheral blood B cells both at mRNA and protein levels. Since the architecture of lymphoid tissue has been reported to be dramatically impaired during HIV-1 [221] it would be of interest to study the expression of chemokines and chemokine receptors in LN and GALT during HIV-1 infection.

It has also been shown *in vitro* that CXCR5 acts as a co-receptor for HIV-2, but not for HIV-1 or SIV. Anti-CXCR5 monoclonal antibody and the ligand for CXCR5, CXCL13 inhibited HIV-2 infection suggesting that CXCR5 is a co-receptor for HIV-2 also *in vivo* [121]. It would be interesting to study the chemokine receptor expression and their function on B cells from HIV-2 infected individuals as well as in LTNPs to investigate if these parameters are associated to the less virulent form of HIV, HIV-2 and to the clinical, immunological and virological control of HIV-1 infection.

The majority of cancers affecting HIV-1 infected subjects are those established as AIDS-defining: Kaposi's sarcoma (KS), non-Hodgkin's lymphoma (NHL), and invasive cervical cancer (ICC). Lymphoma is the most common HIV-1 associated malignancy and it can occur also as a primary CNS lymphoma; EBV has been found in the cerebrospinal fluid of the majority of cases of AIDS associated CNS lymphomas [222]. In industrialized countries people infected with HIV remain at increased risk for malignancies despite the introduction of HAART in the clinic; HAART has not had a significant impact on NHL incidence, particularly systemic NHL. The mechanisms by which depressed immunity could increase the risk for cancer are unclear although there is a strict associated with a low CD4 T cell count. HIV-1 may contribute to the development of malignancies through several mechanisms including impaired immune surveillance and imbalance between cellular proliferation and differentiation. Treating cancer in HIV-infected patients remains a challenge because of drug interactions and the potential effect of chemotherapy on CD4 T cell count and HIV-1 viral load. A better knowledge of viral mechanisms of immune evasion and manipulation will provide the basis for a better management and treatment of the malignancies associated with chronic viral infections. Studies recently conducted in SCID mouse reconstituted with human cells demonstrated that CXCL12 expression may be associated with EBV

infection and suggested that the CXCL12 and its receptor may contribute to development of EBV-associated lymphoma in immunodeficient hosts. Thus studies aimed at understanding if HIV-1 and EBV affect in synergy the expression and function of chemokine receptors ultimately leading to development of lymphomas during HIV-1 are relevant to further pin-point the pathogenesis of these cancer forms associated with AIDS and improve their treatment.

One of the earliest responses to infection is the release of molecules that mediate the immune responses. Cytokines, chemokines and adhesion molecules are all molecules that direct the migration and evoke local infiltration of cells of the immune system. Altered expression of these proteins is likely to prevent or even inhibit immune responses leading to clearance of infection. To interfere with the expression and function of chemokine and chemokine receptors on host immune cells could be considered as a successful strategy for the viruses to avoid an immune response. In general a more detailed knowledge on how modulation of chemokine expression and function serves the purpose of virus induced immune escape may offer novel opportunities for HIV-1 vaccine design.

Kerstin Andreasson and *Lena Norenius* for always being helpful in the P3-lab.
Birgitta Wester for all help and nice conversation in the FACS room. *Wendy Murillo*, for always smiling and letting me in to the PCR facility. *Mia Ehnlund* for always being nice and helping out with stainings. *Gösta Winberg* for teaching me RT-PCR*, Anna Pasetto,* for nice lunches and for taking care of Alberto, *Sándor Feldötö, Alex Candia, Anders Bergqvist, Anna Aleman, Lyda Osorio, Vanoohi Fredriksson, Kenth Andersson, Greger Blomqvist,Torbjörn Laggar, Håkan Wikland, Birgitta Karlsson, Katalin Benedek.*

Marie Arsenian-Henriksson, Roland Möllby, Anneka Erhnst

All my co-authors: *Georg Klein, Barbro Ehlin-Henriksson, Wu Liang, Sven Grutzmeier, Olle Björk, Ludmila Matskova, Alberto Cagigi, Linh Vu Phuong Dang, Ann Atlas, Bence Rethi, Caroline Fluur, Malgorzata Kryzyzowska, Angelo De Milito, Rino Belloco, Kerstin Falk, Eva Rajnavölgyi.*

My family; especially my father *Halim Mowafi* how teached me that everything is possible and the most important thing is to focus on the goal. My mother *Krisitina Mowafi,* for always being happy and your endless energy. My sisters *Laila* and *Mona*, we are all so busy but I look forward to next family reunion in Hurghada. *Sarah and Sofia* the sweetest nieces in the world. *Nicke, Hasse,* you are all important to me. I look forward to our Sunday family dinners. And last *Mormor Ulla* you would have been proud!

My second family, *Åsa, Peter, Marcus* och *Anton*. Åsa for our long telephone calls every evening (and sometimes mornings). For always supporting me, picking up the girls when necessary, preparing food when I was too tired to cook, but mostly for your eternal friendship.

Gunnar, Katarzyna and *Camilla* for our nice friendship.

Kaj, *Berit* and *Annika* for taking me in to your family.

Finally, *Jörgen* my life companion, for your encouragement and understanding and your endless love for your three girls.

Norah and Clara, my precious daughters.

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