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REGULATION OF ANTIBODY PRODUCTION IN IMMUNOCOMPROMISED PATIENTS

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"*...but the stars are burnin' bright like some mystery uncovered*
I'll keep movin' through the dark with you in my heart..."
Bruce Springsteen, Blood brothers

To Olivia, Hugo and Emil

ABSTRACT

The aim of this thesis was to study B-cell function and the regulation of specific antibody (Abs) production in immunocompromised individuals. As models, we have studied children with acute lymphoblastic leukaemia (ALL) and patients with HIV-1 infection. In the first group, leukaemia by itself causes immunosuppression due to impaired haematopoiesis. In addition, the subsequent treatment with chemotherapy affects the immune system. In the latter group, the viral infection causes immunosuppression and deterioration of immune functions.

Hypergammaglobulinemia is a common finding in autoimmune disease, chronic infections and in lymfoproliferative disorders. The regulation of specific Ab production in HIV and childhood ALL was studied in relation to hypergammaglobulinemia. When analysing anti-measles Abs in these two patient groups an important difference was noted. Specific Ab levels were decreased in HIV-1 infected subjects even in presence of high total immunoglobulinG (IgG) levels whereas the high serum IgG levels in the children with ALL reflected elevated levels of specific Abs. This indicates that the memory B cell compartment in children with ALL is conserved at diagnosis and activated by the disease, while the memory B cell compartment is compromised during HIV-1 infection.

HIV-infected subjects with a reduced memory B-cell pool showed reduced titers of specific Abs compared to HIV-infected subjects with a normal memory B-cell pool and healthy controls. This indicates that long-term immunity is maintained by memory-B-cells. However, hypergammaglobulinemia is present in both groups of HIV subjects irrespectively of the number of memory B-cells. The high total IgG contains high levels of polyspecific Abs in the HIV-infected subjects compared to healthy individuals. The source of the polyspecific Abs in HIV is naïve CD70+ B-cells containing intracellular IgG and secreting IgG *in vitro*.

During chemotherapy plasma cells as well as memory B cells are reduced and in approximately 40% of the ALL children the levels of vaccination induced specific Abs have declined. Memory B-cells and plasma cells in the bone marrow (BM) decreases during therapy but are rapidly restored after treatment. Although the Ab producing cells are regenerated, specific Ab titres remain low at follow up in the ALL children.

Memory B-cells are defined in peripheral blood as being CD27+ B-cells. The soluble form of CD27, sCD27, has been described as an immune activation marker in many different diseases such as HIV and rheumatoid arthritis. The expression of the membrane bound CD27 and its natural ligand CD70 on leukaemic cells was significantly increased as compared to healthy controls and correlated to the levels of sCD27 in serum.. In ALL children with the translocation, t(12;21), in the leukaemic pre B-cell clone, sCD27 was significantly higher compared to other leukaemic subtypes. Blocking CD27-CD70 interaction on the leukaemic cells reduces proliferation, which indicates that expression of CD27 and CD70 on these cells is beneficial for the leukaemia.

The survival of memory B-cells and plasma-cells is mediated by different growth factors, cytokines and cell-to-cell contact through different receptors. Many of these factors are produced from stromal cells and other immune cells in the microenvironment. Nerve growth factor (NGF) regulates B cell activation and differentiation and is an autocrine survival factor for memory B-cells. Since memory B-cells are prone to apoptosis during HIV-1 infection, the plasma NGF level was studied and found to be lower in HIV-1 subjects compared to controls. The addition of recombinant NGF to cultures of purified B cells reduced cell death of memory B cells from HIV-1-infected subjects. Stromal cell-derived factor-1 (SDF-1/CXCL12), a chemokine produced by stromal cells, plays an important role in normal B-cell lymphopoesis, migration and homing of pre-B cells and plasma cells to the BM. At diagnosis of pre-B ALL, serum level of SDF-1 is elevated in children with ALL compared to healthy children. SDF-1mRNA is present in leukaemic cells derived from children with ALL and might be the source of the elevated SDF-1. In addition, recombinant SDF-1 enhances pre-B leukaemic cell proliferation *in vitro*. Cell-to-cell contact between the stromal cells and leukaemic cells regulates the secretion of SDF-1 in our *in vitro* model indicating the importance of the microenvironment for the pathogenesis of childhood leukaemia.

Key words: hypergammaglobulinemia, specific antibodies, memory B- cells, microenvironment

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LIST OF PUBLICATIONS

I. **Nilsson A**, De Milito A, Engström P, Nordin M, Narita M, Grillner L, Chiodi F, Björk O. Current chemotherapy protocols for childhood acute lymphoblastic leukemia induce loss of humoral immunity to viral vaccination antigens. Pediatrics 2002; 109(6): e91-97

II. De Milito A, **Nilsson A**, Titanji K, Thorstensson R, Reizenstein E, Narita M, Grutzmeier S, Sönnerborg A, Chiodi F. Mechanisms of hypergamma-globulinemia and impaired antigen-specific humoral immunity in HIV-1 infection. Blood 2004; 103: 2180-2186

III. **Nilsson A,** De Milito A, Tran Thi Thanh Ha, Winberg G, Mowafi F, Björk O, Chiodi F. Expression of CD27-CD70 in childhood ALL is associated with the t(12;21): implication for tumor growth. *Submitted*

IV. Titanji K, **Nilsson A**, Mörch C, Samuelsson A, Sönnerborg A, Grutzmeier S, Zazzi M, De Milito A. Low frequency of plasma nerve-growth factor detection is associated with death of memory B-lymphocytes in HIV-1 infection. Clin Exp Immunol 2003; 132: 297-303

V. **Nilsson A,** Mowafi F, De Milito A, Scarlatti G, Björk O, Chiodi F. High serum levels of SDF-1 reflect a proliferative CXCR4-SDF-1 loop for malignant pre-B cells during childhood acute lymphoblastic leukaemia. *Manuscript*

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LIST OF ABBREVIATIONS

Ab/s	Antibody/ ies
ADCC	Antibody-dependent cellular cytotoxicity
Ag	Antigen
AIDS	Aquired immunodeficiency syndrome
ALL	Acute lymphoblast leukaemia
BCA-1	B-cell attracting chemokine-1
BCR	B-cell receptor
BM	Bone marrow
CTL	Cytotoxic T-lymphocytes
CXCR4	C-X-C chemokine receptor 4
GC	Germinal centre
IL	Interleukin
HAART	Highly active anti-retroviral therapy
HIV-1	Human immunodeficiency virus-1
MFI	Mean fluorescence intensity
MIP-3α	Macrophage inflammatory protein 3-α
NGF	Nerve growth factor
PBMC	Peripheral blood mononuclear cells
PSA	Polyspecific self-reactive antibodies
sCD27	Soluble CD27
SDF-1	Stromal cell-derived factor-1
TdT	Terminal deoxynucleotidyl transferase
TNF-α	Tumour necrosis factor-α
TLR	Toll-like receptor
WBC	White blood cell count

1 INTRODUCTION

In 1796, Dr Edward Jenner succeeded in preventing smallpox in a small child, and probably without understanding it he had performed the first active immunisation against a life-threatening disease. Two hundred years later, childhood immunisations are part of standard health care. The focus of this thesis was to study the regulation of specific antibody (Ab) production in immunocompromised individuals and to study maintenance of serological memory in these patients. Life-long protection against many pathogens is dependent on serological memory comprising high affinity antibodies that are induced by disease or immunisations.

As models of immunoincompetent individuals, we have studied children with acute lymphoblast leukaemia (ALL) and patients with HIV-1 infection. In the first group, leukaemia by itself causes immune suppression due to impaired haematopoiesis. In addition, the subsequent treatment with chemotherapy affects the immune system both during treatment and after completed chemotherapy. In the latter group, the viral infection causes immunosuppression and deterioration of immune functions. Our studies have focused on the B-cell compartment and antibody production.

2 BACKGROUND

B-CELL DEVELOPMENT

Antibody production and establishment of humoral immunity during B-cell ontogeny

The main function of B cells is to produce Abs against proteins and polysaccharide antigens (Ags). Five major immunoglobulin (Ig) isotypes (IgA, D, M, G and E) are ultimately expressed on the cell surface of B cells or secreted by plasma cells. From infancy and during childhood there appears to be an orderly progression in the development of immunoglobulins. IgM is the first one to be produced as early as *in uteri* by infected foetuses and adult levels of IgM are reached by 1 year of age, while IgG levels are not reached until the age of 5 years (1). Serum IgA develops late and adult levels are reached during puberty.

Memory B cells are absent in cord blood and there is an expansion of the memory B cell pool in blood until adult levels are reached (>12 years of age)(2). In infants and children below 2 years of age, memory B cell are not present in the marginal zone of secondary lymphoid organs. Lack of memory B cells in the spleen may explain why the immune system in small children does not iniate an Abs response after a secondary encounter with an antigen (3). In infant mice, the same pattern is observed with the additional finding that plasma cells fail to migrate to the bone marrow (BM) due to a limited homing capacity of the BM (4).

Antibody effector functions

The Ab comprises of the Ag-binding site and the constant region (Fc). Abs can mediate protection against infection by several mechanisms. Firstly, binding of the Fc region to receptors on macrophages or NK-cells mediates Ab-dependent cellular cytotoxicity (ADCC). Secondly, Ag-Ab complexes induce opsonization and thereby facilitate

phagocytosis of the Ag by neutrophils and macrophages. Thirdly, in one of the simplest effector systems, Abs combat pathogens just by binding to them, and thereby is binding and infection of the host cells prevented.

Early stages of B-cell development occurs in the BM

B-cell development in humans is represented by several checkpoints centred around commitment to B-lineage and the production of a functional Ig gene rearrangement leading to the expression of the B-cell receptor (BCR) on the cell surface (5). The BCR is a complex consisting of an antigen-recognition structure and a membrane-bound Ig. During foetal life human B-lineage cells develop in the liver, but by gestation week 30 the B-lineage development is transferred to the BM (6).

The first checkpoint for B cell development is the commitment of the haematopoietic stem cell to the B-lineage, which occurs in the BM. To date, three transcription factors have been identified as essential for this commitment. Two of them, E2A and EBF, are required for initiation of B-lymphopoesis (5, 7) although they do not definitely determine B cell commitment. The third factor, Pax5, appears to be essential for B-cell commitment, which is illustrated by the fact that Pax5 $^{-/-}$ deficient mice have an absolute block in B cell development at an early stage (8).

Pro-B cells represent the first B-cell precursor and can be identified by the surface expression of CD19 (5). During this stage, rearrangement of the Ig heavy chain locus is initiated by the transcription factors E2A and EBF. D_H-J_H rearrangements occur early in this stage and the subsequent V-DJ rearrangement starts in the late pro-B stage resulting in the surface expression of pre-BCR. The differentiation process of B cells can be followed by the expression of different phenotypic cell surface markers during the different stages (9).

The second checkpoint is when signalling via the pre-BCR induces cell proliferation and differentiation into the pre-B stage. Appropriate signalling via the pre-BCR results in allelic exclusion at the heavy-chain locus and allows the cell to begin the process of light chain rearrangement with the kappa locus and finally the lambda locus (10). However, the mechanism by which pre-BCR signalling mediates B-cell development is unclear. One hypothesis is that aggregation of pre-BCR occurs in lipid rafts at the cell surface and thereby maturation is promoted (11). If the process of V-DJ rearrangement or signalling via the pre-BCR does not occur, the pre-B cell will be deleted.

When heavy and light chain rearrangement is completed, the heavy and light chains are co-expressed on the cell surface to form an antigen-specific surface receptor. The immature B-cells then undergo receptor-mediated negative selection whereby auto-reactive B-cell receptors will undergo apoptosis. In mice, B-cells are produced at a rate of $2x10^7$ cells/ day, but only $2x10^6$ cells are selected for further maturation (7) and these immature B-cells then leave the BM and emigrate primarily to the spleen.

The role of stromal cells in B-cell development

The BM microenvironment consists of stromal cells, haematopoietic cells, vascular-endothelial cells and extra-cellular matrix and it is the site of production of a wide range of cytokines and growth factors. Early B-cell development is not only intrinsic, but is also dependent on the cell-bound and soluble factors, such as interleukin (IL) -7 and stromal cell-derived factor –1(SDF-1), provided by the BM microenvironment. Stromal cells also express many surface antigens important for cell contact and cell-to-cell regulation such as adhesion molecules and MHC class I molecules (12). Fas has also been reported to be expressed on murine BM stromal cells and it is possible that

Fas^+ stromal cells may be involved in selection and regulatory functions in the BM (13).

IL-7 was initially isolated as a growth factor for B-cell precursors, but has also been shown to induce viability and expansion of T-cells (14). Pro-B cells are capable of proliferation in response to IL-7 alone and the IL-7 receptor (IL-7R) is present on the cell surface up to the pre-B stage (15). *In vitro* studies have shown that cells expressing the pre-BCR have an advantage in proliferation at low concentrations of IL-7 and that these cells will survive and outgrow cells without a functional pre-BCR (16) thus being a mechanism for positive selection of B cell progenitors with a productive rearranged BCR. IL-7 is produced from stromal cells, but the mechanisms regulating IL-7 production has not been clearly defined. The release of IL-7 by stromal cells requires cell-to-cell contact with the B precursor cells (17).

Another interesting molecule is SDF-1, a chemokine produced from stromal cells, which has been shown to be crucial for B-lymphopoesis. SDF-1 was first identified as a factor with predominantly chemotactic activity inducing migration. SDF-1 differs from other chemokines as it is highly conserved between species; human and murine SDF-1 differs only by one amino acid (18). Also in contrast to other chemokines, SDF-1 interaction with its receptor, CXCR4 (19), appears to be specific without any cross-reactivity with other chemokines or chemokine receptors. CXCR4 is highly expressed on the cell surface of B-cell precursors and also on mature B-cells, however the chemotactic activity is shown to be higher in early stages of B-cells (20, 21). CXCR4 has attracted a lot of interest, as it is one of the co-receptors for HIV-1 (22) in addition to the major receptor, the CD4 molecule. Gene knockout experiments with mice deficient in either SDF-1 or CXCR4 result in a similar phenotype characterised by

deficient haematopoiesis, abnormal neuronal development in cerebellum, abnormal cardiac development and defective vascularisation in the mesenteric vessels (23 , 24). Both SDF-1 and CXCR4 deficient mice have impaired B-lymphopoesis with a high proportion of immature B cells in the peripheral blood (20, 25), indicating a crucial role for SDF-1 in B-lineage development and homing to the BM. SDF-1 has also been implicated in cell trafficking and tissue micro environmental localization of various lymphocyte classes and subsets such as plasma cells (26).

B-CELL MATURATION WITHIN SECONDARY LYMPHOID TISSUE

The germinal centre reaction

When the immature B-cell leaves the BM, the antigen-specific BCR is assembled and expressed as a low affinity IgM and IgD receptor on the cell surface. These naïve B-cells can differentiate into low affinity Abs-secreting plasma cells following a primary Ag encounter (27). However, in order to produce specific high-affinity Abs, the naïve B-cell need to undergo a series of developmental genetic events leading to the emergence of memory B-cells. These genetic events take place in the secondary lymphoid organs and include the formation of germinal centres (GC) within the follicles of the lymphoid organs. In the antigen-primed B-cell, expression of genes regulating cell cycle control will be altered leading to a clonal expansion of the antigen-specific B-cell (28). During this expansion, somatic hypermutation occurs in the Ig variable gene region creating a high-affinity receptor for the Ag (29, 30). There is also an isotype switch in the constant region of the heavy chain genes with subsequent production of IgG instead of IgM (27). Within the GC, changes in the expression of survival/death genes lead to a negative selection of the somatically mutated cells unless they are selected by their respective Ag (31, 32).

Differentiation towards memory B-cells and plasma cells

The final step of GC B-cell development is the differentiation of high affinity B cells towards memory B-cells or plasma cells (33). In this process, molecules of the TNF receptor family (TNFR) and their respective ligands play a crucial role for the regulation of humoral immune responses (34). Figure 1 summarizes the events leading to memory B-cell or plasma cell formation.

Expression of the cell surface molecules CD40 and CD27 are important in regulating the cognate-dependent contacts with T helper cells during the GC reaction (35-38). After antigen-presentation, CD40L-expressing T-cells initiate the GC-reaction by interaction with CD40 on the naïve B-cell (39) and after somatic hyper mutation and class switch the B cell differentiate into a memory B-cell. Memory B cells are

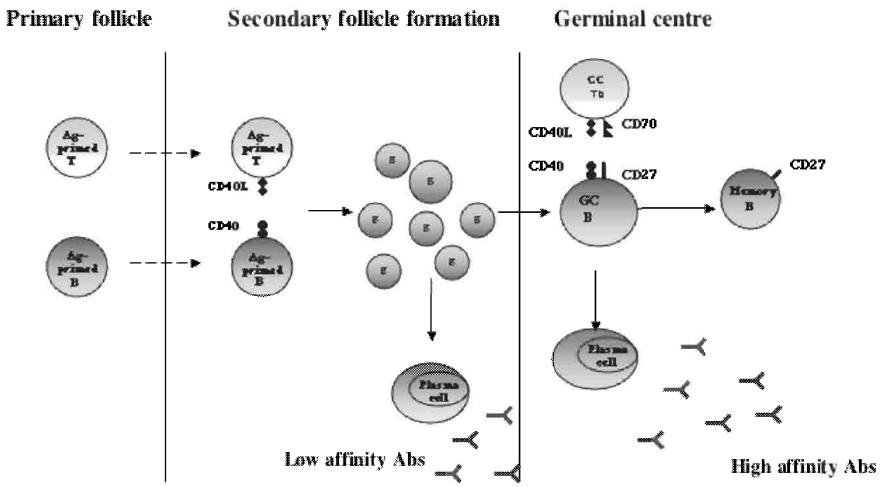

Fig1. Schematic figure of helper T-cell regulated B-cell differentiation to memory B cells and plasma cells. Dendritic cells present the antigen to naïve cells in the T- or B-cell zone and following clonal expansion of Ag-specific T-helper and B-cells, they migrate to the T-B borders to initiate cognate contact with each other. During the formation of the secondary follicle, B-cell differentiation is driven either towards short-lived plasma cells or towards the germinal centre reaction (GC). The GC reaction results in the formation of memory cells that can either differentiate to plasma cells, which produce a high amount of high affinity antibodies, or remain as non-secreting memory cells

characterised by the expression of CD27 (40, 41), which is a molecule commonly expressed by naïve T-cells (42) and NK-cells (43). The ligand for CD27 is the CD70 molecule, which is expressed on activated T helper cells (44), as well as on activated B-cells (45). Interactions between CD27 and CD70 together with withdrawal of CD40L (33, 46)have been shown to promote Ig production and plasma cell differentiation (2, 47-49). The newly generated plasma cells leave the lymphoid organ and migrate to the BM where they complete their differentiation and produce large quantities of specific Abs. Memory B cells are resting cells capable of recirculation between the periphery and the lymphoid tissue and upon re-infection they promptly respond with the production of specific high-affinity Abs.

The microenvironment of secondary lymphoid organ

Formation of memory B-cells and plasma cell differentiation do not only depend on receptor interactions, but also on growth factors and cytokines supplied from immune cells and stromal cells in the microenvironment. The cytokines interleukin –10 (IL-10) and interleukin-4 (IL-4), produced from activated T helper cells, are important cytokines linked to the differentiation of plasma cells from B-cells (50, 51) in secondary lymphoid organs. These cytokines also affect immature cells in the BM. IL-10, together with IL-7, has been shown to induce proliferation of pre-B cells (52) and interestingly, IL-4, together with activated T helper cells has been shown to promote the differentiation of pre-B cells to Ig-secreting plasma cells *in vitro* (53).

Stromal and epithelial cells are also major components of secondary lymphoid tissue in the same manner as in the BM compartment. SDF-1 is present in lymphoid tissue (54) together with several important chemokines such as MIP-3α and BCA-1. These

chemokines together with their respective ligands (CCR7 and CXCR5, respectively) recruit cells of the immune system to the lymphoid tissue and retain them within the appropriate supportive microenvironment. Activation of B-lymphocytes with CD40 ligation and IL-4 results in enhancement of migration in response to chemokines expressed within lymphoid tissues, among them SDF-1 (55). During formation of the plasma blast there is an increase in the chemotactic response towards SDF-1, which facilitates homing of the plasma blast to the BM and the red pulp of the spleen (56). It has also been shown that after a secondary immunisation and in the course of a memory immune response, plasma blasts in the spleen change their responsiveness and increase migration in response to SDF-1 (57). In addition to having a role in chemoattraction, SDF-1 acts as a co-stimulator for activated T helper cells and thereby increases the production of IL-4 and IL-10 (58). Altogether this indicates a role for SDF-1 in promoting plasma cell differentiation, migration and Ig synthesis.

MAINTENANCE OF HUMORAL IMMUNITY AND SEROLOGICAL MEMORY

Memory is a hallmark of immunity and production of Abs can last for a lifetime. Memory represented by Abs is responsible for protection against re-infection for many lethal infectious agents (59) and it is also the basis for the majority of successful vaccines. The mechanisms leading to long-time serological memory are poorly understood and three major hypothesises have been presented over the past decade.

Dr Zinkernagel and colleagues suggested that long-term Abs memory is driven by a constant differentiation of memory B-cells to plasma cells in the presence of antigens (60). Antigen-driven proliferation and differentiation induces high levels of protective Abs; however, this hypothesis would require persistence of infectious antigens within

the human body. Dr Ahmed and colleagues have on the other hand shown that mouse plasma cells can be long-lived and are able to produce Abs for several months in the absence of memory B-cells or antigens (61). It is, however, less likely that a human long-lived plasma cell would sustain Ab production over a human lifespan since that would require plasma cell survival for many years (>70 years).

Dr Lanzavecchia presented an alternative mechanism for maintenance of serological memory in 2002, when his group showed that human memory B-cells proliferate and differentiate into plasma cells in response to polyclonal, non-cognate stimuli (62, 63). The polyclonal stimuli that can activate memory B-cells are of two origins; those which activate B-cells via TLR-4 or TLR-9 (64) such as microbial products (LPS) (65) and single-stranded DNA motifs (CPGs) or secondly, activated T-cells stimulating the B-cells via CD40L and cytokines without the presence of antigen-activation. The in vivo data provided indicate a correlation between specific Abs level and the frequency of antigen-specific memory B-cells under steady-state conditions. Figure 2 summarises the hypothesis by Dr Lanzavecchia.

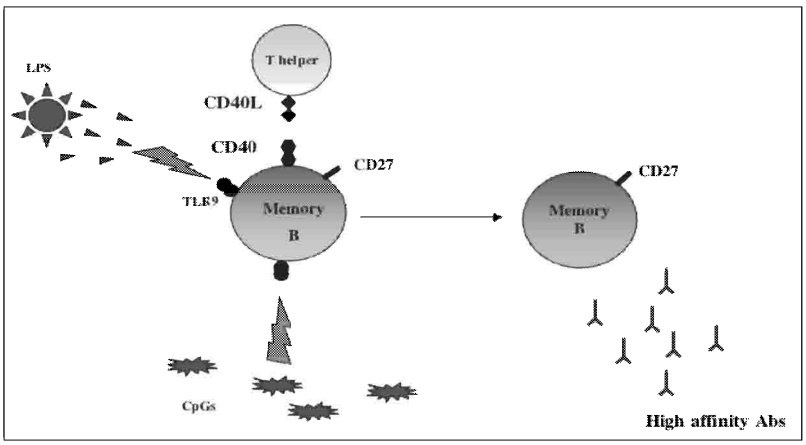

Fig 2. Maintenance of serological memory adapted from Bernasconi et al (62). Memory B cells are activated in a non-cognate fashion by T-helper cells through the CD40-CD40L or through activation by Toll-like receptor 9 by LPS or CpGs. Activation of the memory B-cell leads to the production of high affinity antibodies.

CHILDHOOD ALL

Leukaemia is the most common form of childhood cancer in Sweden and in the western world. Each year, approximately 70 children in Sweden are diagnosed with ALL. The survival of childhood ALL has dramatically improved over the past 30 years and the current chemotherapy protocols results in a high cure rate. The overall survival in childhood ALL is today approximately 80% (66).

Clinical characteristics for stratification of patients into risk-groups

The treatment protocols used today are based on a patient classification into different risk-groups. The improvement of survival has been achieved by tailoring the intensity of treatment to the risk of relapse in each patient. So a child with many poor prognostic factors will have a more intense treatment. In the current chemotherapy protocol four major risk-groups are identified; standard intensity (SI), intermediate intensity (II), high intensity (HI) and very high intensity (VHI) treatment groups.

Age is an important factor for classification into the different risk-groups. The incidence of ALL has an age-peak in early childhood between 2-3 years of age (67). Children between 1 year and 10 years have a favourable prognosis and infants < 1 year have the worst outcome. Girls tend to have a slightly better outcome than boys (68). However, gender is not part of the classification.

Another important clinical parameter used for classification is white blood cell count (WBC) where a high WBC at diagnosis, place the child in a higher intensity group (69). Other markers of high tumour burden such as enlarged lymph nodes, enlarged liver and spleen also correlate inversely to prognosis (70). Infiltration of leukaemic cells in the

CNS is present in approximately 5 % of ALL children at diagnosis and is a poor prognostic factor (71).

Phenotype and genetic alterations in the leukaemic clone

At the diagnosis of ALL, a diagnostic BM aspiration is performed with phenotypic and genetic analysis of the leukaemic clone. In childhood ALL, pre-B phenotype account for 80% of the patients while 20% have a T-cell phenotype. Children with T-cell leukaemia receive a more intense treatment due to the association with adverse prognostic factors like high WBC (72). The intensified treatment protocol for T-ALL has improved the outcome for T-ALL and outcome is now similar to pre-B ALL. Childhood pre-B ALL is characterised by a high expression of CD10 and terminal deoxynucleotidyl transferase (TdT) in the leukaemic cells. In infant ALL (<1year), CD10 is absent from the cell surface of the leukaemic blasts.

Genetic alterations have been shown to be associated with biologically distinct subtypes of ALL (73). The main genetic alterations occurring in childhood ALL are hyperdiploidy in 35% of the cases and the t (12; 21) translocation occurring in 25% of the children. Hyperdiploidy is associated with favourable risk features such as age between 1 and 10 years, a low white blood cell count (WBC) and a pre-B phenotype. The chromosomal gains in hyperdiploidy are restricted to certain chromosomes and the good prognosis has been linked to chromosomes 4, 10 and 17 (74).

The t (12; 21) translocation has also been associated with a good outcome and is mainly found in children in the 3-6 year age group, also with a pre-B phenotype and with the expression of myeloid antigens. The t (12; 21) translocation results in the formation of a hybrid gene involving the Tel gene on chromosome 12p and the AML

gene at 21q22 (73 , 74). The resulting protein acts as a putative transcriptional repressor. An important secondary event in t (12; 21) translocation is the deletion of the normal Tel allele from the chromosome 12 not involved in the translocation. This loss of function may also contribute to leukaemic transformation (74).

There are several other genetic alterations in pre-B ALL with known poor prognosis such as the 11q23 translocation with MLL-rearrangement in infant ALL, the Philadelphia chromosome t (9; 22) translocation in approximately 5% of patients and t(1; 19) translocation also affecting 5% of the ALL population (74). Recent data has also shown that deletion of the INK-4 locus on chromosome 9 is associated with increased risk of relapse (75).

Treatment of ALL

The majority of children with ALL in Sweden are treated with the Nordic Society for Paediatric Haematology and Oncology´s (NOPHO) chemotherapy protocol (76). Only children with known poor prognostic factors (t 9; 22) at diagnosis or children who respond poorly to the initial chemotherapy are scheduled for BM transplantation in first remission. Children with early relapses during or soon after the end of treatment are also transplanted.

The NOPHO protocol consists of three major parts (76); an induction phase over 6 weeks followed by a consolidation period of 2 months and subsequently maintenance treatment until 2-2.5 years after diagnosis. During the induction phase patients in all risk-groups are treated with doxorubicin and vincristine intravenous and high doses of corticosteroids orally. Consolidation therapy with methotrexate is given with 5 g/m^2 for SI and II leukemia and 8 g/m^2 in HI patients. II and HI patients are treated with a re-

induction periods where additional doxorubicin, vincristine and steroids is administered. In the protocol in use today, HI patients are also treated with 2 courses of high-dose cytarabinoside (ara-C).

Immunodeficiency during and after ALL

The immunosuppressive effect of chemotherapy is well known. In childhood ALL, chemotherapy induces severe neutropenia, B- and T-cell depletion which results in clinical complications related to immune incompetence. Infections are the major cause of morbidity during treatment, although the mortality from infections has decreased dramatically the last decade due to improved supportive care with prophylactic antibiotics, anti-fungal and anti-viral drugs (77). Neutropenia ($<1.0 \times 10^9$ cells/mL) is often present at diagnosis as a consequence of the leukaemia infiltration and disruption of haematopoiesis in the BM. Prolonged periods of neutropenia (> 7 days) increase the risk for invasive bacterial infections (78, 79). T-cell depletion in children with ALL increases the risk of opportunistic infections and in particular Pneumocystis carinii (80), however reactivation of viral infections (e.g. herpes zoster virus) also occurs (81). The role of B-cell depletion with low levels of specific Abs and the risk for secondary infections in this patient group is not well defined. There are reports on deficient neutralisation of bacterial toxins and low levels of protective Abs towards specific bacteria (e.g. enterobacteriae) (82, 83). When chemotherapy ceases, the WBC normalises within months and the total B- and T-cell counts resolve quantitatively 6 months to 1 year after cessation of therapy (84-87). Severe infectious complications in ALL children after cessation of therapy have not been reported.

HIV-1 INFECTION

The infection with HIV causes a severe acquired immunodeficiency syndrome (AIDS) affecting approximately 44 million people worldwide and among them an increasing

proportion of children. The introduction of highly active anti-retroviral treatment (HAART) has dramatically improved clinical management of patients, with improved survival and life expectancies as a result. Unfortunately, this is true only for HIV infected subjects in developed countries and not for the majority of infected patients living in Africa and Asia.

HIV establishes a chronic infection in the host

The primary infection with HIV can either be asymptomatic or cause mild mononucleosis-like disease with generalised lymphadenopathy. After the primary infection a period of clinical latency (<10years) will follow where there will be an ongoing virus replication in the lymphoid organs (88). HIV-I infects primarily CD4+ T helper cells and cells of the macrophage/monocytic lineage including macrophages, monocytes and dendritic cells. A decrease in peripheral CD4+ T helper cells occurs during the acute infection. T helper cells have a central role in the immune system through mediating differentiation of B- and T- cells and thereby controlling bacterial and viral infections. The immunodeficiency associated with HIV-1 results from loss of T helper cells and an increasing susceptibility to opportunistic and secondary infections (88).

HIV-1 and B cell dysfunction

HIV-1 induces perturbations in B-cell function early in the time course of infection with hypergammaglobulinemia in patients and high spontaneous secretion of Ig *in vitro* (89) as hallmarks of B cell dysfunction. There is also an increased incidence of B cell malignancies in HIV-1 infection compared to uninfected controls (88, 90). At the same time, hyperactivation is accompanied by refractory B cell response to stimulation *in*

vitro by T cell dependent (e.g. pokeweed mitogen, PWM) and T cell independent mitogens (e.g. Stafylococcus aureus-Cowan strain, SAC) (91).

Several mechanisms, both direct and indirect, may account for B cell dysfunction during HIV-1 infection. There have been reports of HIV-1 infecting primary B cells after CD40-ligation and the resulting up-regulation of the HIV-1 receptors CD4 and CXCR4 on B cells (92). The virus itself and several viral proteins (Nef, Tat and gp41) induce proliferation and differentiation of B cells *in vitro* (93-95). The viral protein gp120 has on the other hand an inhibitory effect on normal B cell function *in vitro* (96). Viral gp120 is also associated with the selective deletion of memory B cells expressing the V_H3-gene (97). Since the V_H3 family encodes for 50% of all Abs, this may represent one mechanism for loss of antigen-specific Abs (98) during HIV-1 infection.

T-cell activation may induce B-cell dysfunction

B cell dysfunction is an early event in the infection and is detected before any significant loss of T helper cells can be detected (91). This suggests that B cell dysfunction is intrinsic, however, several investigators have reported that T helper cells have a role in driving B cell activation. CD4+ T helper cells have been shown to stimulate IgG secretion through a non-cognate, contact-depend mechanism (99). There have also been reports on a higher percentage of CD40L+ and CD70+ T cells in HIV-1 infected subjects that may contribute to hypergammaglobulinemia (100, 101).

Impaired humoral immunity in HIV-1

HIV-1 infection can induce both virus-specific and poly-specific B cell responses with the production of Abs not only against the virus but also high amounts of polyspecific auto Abs. Among these are IgG known as polyspecific self-reactive Abs (PSA) directed against self-antigens and lymphocyte antigens (102). The production of specific Abs is impaired which is demonstrated by a poor response to vaccination with new and/or recall antigens. Low levels of specific Abs against pneumococcal polysaccharides, pneumocystis carinii, measles and tetanus toxoid have been found (103, 104) and these low levels persist after attempts to re-immunise (105-107). Impaired humoral immunity with loss of protective and specific Abs may lead to secondary infections that are normally controlled by the host.

3 AIM OF THE THESIS

The primary aim of this thesis was to investigate B-cell function in children with ALL and to study whether these children need re-immunisations after chemotherapy in order to maintain immunity to the childhood immunisations currently used in Sweden. Secondly, we wanted to study the mechanisms involved in the regulation of long-term serological memory. The effects of leukaemia and chemotherapy, as well as the effect of HIV-1, on B-cell function and antibody production was analysed in relation to:

- The relationship between total IgG and specific antibodies
- The maintenance of specific antibodies and memory B-cells
- Survival factors for memory B-cells and plasma cells
- Production of growth and survival factors from stromal cells in the bone marrow
- Ab response after re-immunisation with measles, mumps and rubella (MMR) vaccine in children treated for ALL

4 PATIENTS

Children with ALL

All children (n=105) included in these studies were admitted to the Paediatric Cancer Unit at Astrid Lindgren Children Hospital at Karolinska University Hospital, for diagnosis and treatment of ALL. In *paper 1* children (n=43) treated between 1986-1996 were included for retrospective analysis of antibodies against viral vaccination antigens. The cohort in this study consisted of 5 children with T-ALL and the remaining 38 children all had pre-B ALL. In the following studies (*paper 3 and 5*), children with pre-B ALL, diagnosed between 1996 and 2003, were included. Patient characteristics are summarized in table 1. Eighty healthy age-matched children undergoing minor surgery at Astrid Lindgrens Children Hospital were included in the studies as healthy controls (Table 1).

Table 1. ALL-patients characteristics in the thesis.

	Risk-groups	***Gender (m/f)***	***Age (years)***	***WBC (10^9/l)***
Thesis	SR=47	20/27	4 (2-8.5)	4.8 (0.8-14)
N=105	IR=42	25/17	4.5 (1-17.5)	13.7 (2.5-44.5)
	HR=21	10/11	7 (0.5-16)	32 (2.2-255)
Paper 1				
N=43	SR=27	6/21	3.5 (2-9)	3.9 (1.2-13.2)
	IR=8	4/4	4 (1.5-15)	12.6 (2.9-39.4)
	HR=8	2/6	8 (4-15)	64.5 (6.5-255)
Paper 3				
N=70	SR=27	10/17	5 (2-8.5)	3.2 (0.7-12.5)
	IR=31	17/4	7 (1.5-17)	15 (2.5-44.5)
	HR=12	6 /6	6 (0.5-16)	11 (2.2-123)
Paper 5				
N=37	SR=14	9/5	3.5(2.5-8.5)	8 (1.9-12.5)
	IR=23	15/8	4.5 (1.5-15)	17.5 (3.7-44.5)
	HR=0			
Controls				
N=80		34/48	5(1.5-13)	n.d

HIV-infected patients

In *paper 2,* 72 HIV-1-infected subjects and 23 blood donors of similar age were included. The median CD4+ T cell count in the HIV-1 subjects was 400 cells/μL (range 40-1130). In the patients' cohort, 13 subjects were treatment-naïve, 5 were treated with reverse transcriptase inhibitors and 54 were undergoing highly active antiretroviral therapy (HAART), a combination therapy containing inhibitors of the viral protease and reverse transcriptase.

In *paper 4,* a total of 131 HIV-1-infected and 108 uninfected age-matched blood donors were included. The median CD4+ T cell count among the patient population was 320 cells/μl (range 16-869). One hundred subjects were undergoing antiretroviral treatment while 31 patients were drug- naïve.

Ethics

The ethics committees at the Karolinska Institutet North or the Karolinska Institutet South approved the studies. All samples were collected after informed consent from the patients and controls. For children, informed consent was obtained from the parents.

5 METHODS

SEROLOGY

Determination of specific antibodies and total IgG

The amount of plasma IgG in patients and controls was measured by nephelometry and the amount of IgG secretion in vitro was measured by sandwich IgG ELISA (108). Measles Ab concentrations were analyzed using a standard in-house indirect quantitative enzyme immunoassay as previously described (109). For quantitative measurement the World Health Organization (WHO) 2nd International Standard for Anti-Measles Serum was included. The cut-off for protective levels was set to > 200 mIU/mL (109). Avidity for measles Abs was tested using the Enzygnost Measles virus IgG detection ELISA kit (Dade-Behring, Behringwerke, Germany) (110) and avidity (%) was calculated as the ratio of the absorbance values with and without urea treatment.

For detection of rubella Abs, an automated micro particle enzyme immunoassay (Rubella IgG 2.0 IMx, Abbott, Abbott Park, IL) was carried out in an IMx Analyzer according to the manufacturers' instructions. The cut-off for protective levels was set to >10 IU/mL (111). Antibody levels to tetanus toxoid were evaluated by an accredited modified Delfia test previously described (112).

Determination of anti-HIV-1 Ab titres was performed by using the Enzygnost ® HIV 1/2 ELISA (Dade-Behering, Behringwerke, Germany) following the manufacturers instructions.

ELISA

Quantification of plasma nerve growth factor(NGF)

The amount of plasma NGF was measured by sandwich ELISA following the manufacturer's recommendations (Boehringer Mannheim, Germany). Samples were tested in triplicate and reconstitution experiments were performed on 10 control samples by adding known amount of recombinant NGF to plasma samples in order to test for the sensitivity and reproducibility of the assay. The detection limit of the ELISA was 10 pg/ml and samples with NGF below the threshold were considered negative (arbitrary value of 5 pg/ml).

Soluble CD27 ELISA

Quantification of sCD27 was performed with a commercially available sandwich-ELISA kit (CLB, Amsterdam, Netherlands) as previously described (113). All patient samples were tested in duplicates and the sCD27 concentration in sera was calculated by using the mean value of the optic density (OD value) plotted against the standard curve.

SDF-1 measurements in serum and culture supernatants

The SDF-1 serum concentration in children with ALL was quantified with a commercially available sandwich ELISA (R&D, Minneapolis, MN, USA) as previously described (114). Culture supernatants were analyzed using the human SDF-1α Quantikine ® kit (R&D Systems, Abingdon, UK) according to the manufacturers instructions.

FLOW CYTOMETRY

Phenotyping of lymphocyte populations

Using two- or three-color flow cytometry, phenotyping of peripheral blood and BM cells was performed with a FACScan Instrument (Becton-Dickinson, Mountain View, CA) using the CellQuest software (Becton-Dickinson). At diagnosis of childhood ALL, the expression of cell surface molecules such as CD27, CD70 and CXCR4 on leukaemic cells was analysed on $CD19^+$ cells without using any additional pre-B leukaemic marker as there was a high leukaemic cell infiltration of the BM, with an average infiltration of > 93% (70-100%) blasts.

Cell death

Apoptosis was measured by flow cytometry (115) using an Annexin V staining kit (Pharmingen, San Diego, Ca) according to the manufacturers recommendations. Lymphocytes were detected by forward and side-scatter and parameters were collected for 20,000 live gated cells/sample.

Determination of poly-specific self-reactive antibodies (PSA)

Measurement of PSA was performed as previously described (116). Binding of PSA to target lymphocytes obtained from buffy coats was analysed by flow cytometry and the reactivity was expressed as mean fluorescence intensity (MFI).

SDF-1 REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR)

Cellular RNA was extracted using the Rneasy Mini kit (Qiagen Sciences, Maryland, USA) according to the manufacturers instructions. The RT-reaction was performed using Ready to go You-Prime First-Strand-Beads (Amersham Biosciencies, New

Jersey, USA), containing the necessary reagents. The PCR reaction was performed with primers for SDF-1 and actin published previously (117, 118). Detection of the amplified PCR-product was performed by electrophoresis on a 2% agarose gel (Gibco) followed by ethidium bromide staining.

BIOLOGICAL ASSAYS

Vaccination

All children without protective immunity after chemotherapy were offered revaccination (1 booster). Fourteen children received one booster dose of vaccine against MMR and 3 children refused vaccination. The children were immunized with MMR II (Pasteur Merieux MSD, Copen-hagen, Denmark), a live attenuated vaccine containing the Edmonton measles strain (≥1000 CCID 50), the Jeryl Lynn mumps strain (≥2000 CCID50) and the RA 27/3 rubella strain (≥1000 CCID 50). Sera were collected prior and 3 months after vaccination for analysis of specific Ab titres.

Proliferation assay

Proliferation of cells, measured as thymidine incorporation, was evaluated by adding 1μCi/well ^{3}H-labeled thymidine (Nordic Biosite, Sweden) into the cultures for the last 18 hrs. All cultures were performed in triplicate wells and a mean value for proliferation was calculated.

Transwell culture system

To evaluate whether polyclonal B-cell activation is dependent on cell-to-cell contact the following model system was studied. Un-fractionated PBMC were cultured at a cell concentration of 0.5 x 10^{6} cells/mL. Purified B cells were cultured in Transwell plates (Corning Incorporated, NY) with polycarbonate membrane with pore size 5.0

μm as follows. B cell-depleted PBMC were layered in the bottom chamber and in the upper chamber purified B cells were added at the same proportion as in the un-fractionated PBMC. In parallel, a similar number of purified B cells as in the total PBMC were cultured alone. Supernatants for measurement of IgG secretion were collected after 7 days in culture.

STATISTICS

Statistical analysis was performed with the software GraphPad Prism® (GraphPad Software Inc, San Diego, CA). Normal distribution of the data was tested by the Kolmogorov-Smirnov test. The differences within patients at different time-points were analyzed using Wilcoxon signed rank test for matched pairs. Differences between the patients and controls were analyzed using Mann-Whitney test and data in the text are shown as median (range), if not otherwise stated. Differences between more than three groups were analyzed by one-way ANOVA followed by Dunn's or Bonferronis post-hoc test.

6 RESULTS

Hypergammaglobulinemia and specific antibodies

One of the aims of this thesis were to analyse how maintenance of specific humoral immunity in immunosuppressed patients, i.e. children with pre-B ALL and HIV-1 infected subjects, is regulated. In children with ALL, samples for analysis were taken at diagnosis as part of a longitudinal study and before any treatment with blood products or chemotherapy was given. In some patients, samples were also taken at follow-up (> 6 months) after completed chemotherapy. In the studies on the chronically HIV-1 infected patients, samples were collected at different time-points during disease progression as a cross-sectional study.

We started by analysing the relationship between the total amount of IgG and anti-measles IgG in these two patients groups. In the ALL children, total IgG was elevated at diagnosis compared to the normal controls [8.7 g/L (3.40-20.3) vs. 5.9 g/L (2.0-13.1), p<0.0001]. The HIV-1 infected subjects also had higher levels of total IgG than the controls [13.8 g/L (8-25.6) vs. 9.25 g/L (7.1-13.7), p<0.0001], which are in accordance with previously published studies (119) (120) (Fig 3).

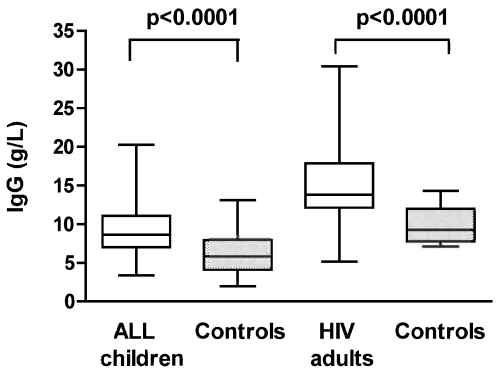

Fig 3. Total IgG in children with ALL (n= 61) was significantly higher than in healthy age-matched controls (n=80). Hypergammaglobulinemia was also present in HIV-1 subjects (n= 72) compared to the adult controls (n= 23).

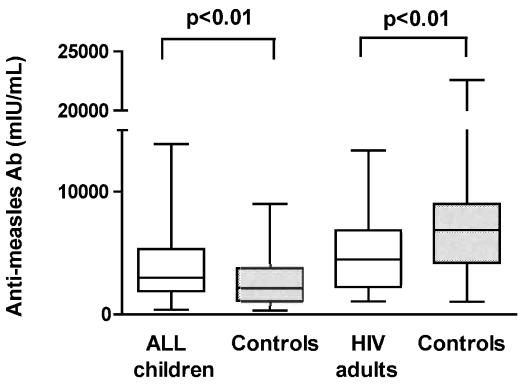

Fig 4. Specific anti-measles antibodies were also higher in the ALL children (n=37) compared to the controls (n=80) but in the HIV-1 patients (n=37) the anti-measles antibody titers were lower than in the controls (n=20).

The levels of specific measles Abs were also elevated in the ALL children compared to the age-matched controls [2 985 mIU/mL (390-13 900) vs. 2 150 mIU/mL (320-9 000), p<0.02]. There was a significant correlation between the level of total IgG and the titre of specific measles Abs (r=0.43, p<0.05) in the ALL patients, but not in the age-matched controls. In contrast, the HIV-infected patients had lower measles Ab levels [4 480 mIU/mL (1 100-10 990) vs. 6 875 mIU/mL (1 465-20 090), p< 0.02] compared to the age-matched controls (Fig 4).

Specific Ab production is maintained from memory B-cells

In *paper 1*, the effect of chemotherapy on specific Abs levels was studied. During treatment there is a significant decrease in Ab levels against several vaccination antigens. Specific measles Ab titres before and 2 years after treatment were compared in 16 patients and the titres of anti-measles Abs declined in all patients [7 580 mIU/mL (600-20 000) vs. 440m IU/mL (0-8 600); p< 0.001]. All children who had retained protective levels of antibodies showed high avidity (>30%)Abs against measles. There was also a decline in specific anti-rubella Ab titres in all patients after chemotherapy.

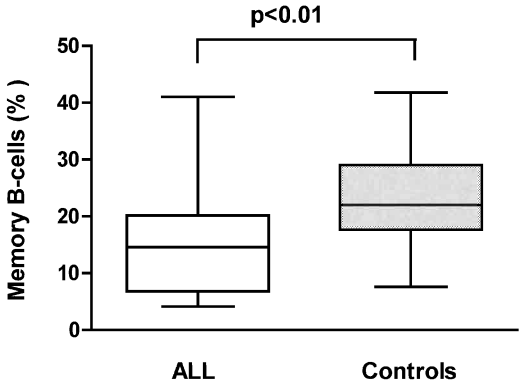

Fig 5. Six months after completed chemotherapy the memory B-cell population, characterised as $CD27^+$ $CD19^+$ cells, is still significantly decreased in ALL patients (n= 15) than in controls (n=43).

Serological memory is dependent on Abs generation and production from memory B-cells and terminally differentiated plasma cells. In *paper 1*, we demonstrated that plasma cells in the BM are deleted during chemotherapy; however, the plasma cell pool is rapidly regenerated within 6 months after treatment (0.3% vs. 1.3 %, p<0.01) and at this time-point the levels of plasma cells are comparable to the controls. Memory B-cells are also depleted during chemotherapy and at follow-up 6 months after completed treatment patients still have a significantly lower proportion of memory B-cells in peripheral blood compared to controls [14.6%(4.1-41) vs. 22% (7.6-41.8), p<0.01](Fig 5). Although the Ab producing cells are regenerated specific Ab titres remain low at follow up in the ALL children.

Memory B-cells in HIV-infected patients were analyzed in relation to specific anti-measles Ab titres. In *paper 2*, we demonstrated that memory B lymphocytes are primed for apoptosis and that the percentage (and number) of memory B-cells are reduced during HIV-1 infection, compared to uninfected controls [20% (6.8-43) *vs.* 34.3% (21.1-60.7), p< 0.001]. The HIV-1-infected subjects were grouped with regards to the median percentage of memory B cells into subjects carrying normal (NM, >20%) or low (LM, <20%) memory B cells. Among the infected individuals, anti-measles Ab

titres in HIV-1 LM subjects [3 300 IU/mL (1120- 6 680)] were significantly lower compared to the HIV-1 NM subjects [5 610 IU/mL (1 060-13 360), $p< 0.05$] and healthy controls ($p < 0.01$). This was also shown for the anti-tetanus toxoid Abs. In addition, the anti-measles Ab titres were positively correlated to the percentage of memory B-cells in the HIV-1 subjects. The BM plasma cell population was not quantified in these patients, but previous studies have shown that BM plasmacytosis occurs in HIV-1 infection (121, 122).

Naïve CD70$^+$ B-cells as a source of IgG

Although hypergammaglobulinemia are present in HIV-1 patients, they have low specific Ab titres as compared to the ALL children. Thus, we attempted to better characterize the nature of the IgG in HIV-1 and analysed the amount of poly-specific self-reactive Abs (PSA) in HIV-1 subjects and controls.

Human PSA from HIV-1-infected subjects had a higher reactivity (defined as mean fluorescence intensity (MFI)) compared to healthy subjects [11.8 (4.6-53.9) *vs.* 7.8 (4.1-12.6), $p< 0.01$]. In the HIV-1 population, the MFI of PSA correlated with the plasma IgG ($r = 0.547$, $p<0.001$). However, the PSA reactivity was similar between HIV-1 NM subjects and HIV-1 LM subjects [15.9 (10.3-23.8) *vs.* 7.5 (7-21.6), $p= 0.226$]. Interestingly, the PSA reactivity was positively correlated to the percentage of CD70+ naïve B- lymphocytes ($r = 0.36$, $p< 0.05$).

To determine whether naïve B-cells are activated *in vivo* and thus are a source of IgG production, B-cells were purified from 3 untreated patients and 3 donors. The intracellular content of IgG in naïve B-cells in relation to the expression of CD70 (an activation marker) and LAIR1 (a differentiation marker) was analyzed. Naïve B cells

from HIV-1-infected patients more often expressed intracellular IgG and in the population of $CD70^+$ naïve B-cells the fraction of IgG^+ cells was higher in HIV-1-infected compared to uninfected subjects (47.6±2.4% *vs.* 13.1±1.9%). HIV-1-infected persons had a similar proportion of IgG^+ cells in the $LAIR1^+$ naïve B-cells compared to healthy donors (7.8±3.5% *vs.* 9.2±0.9%). These observations indicate that activated naïve B-cells from HIV-1-infected patients might have undergone an IgM-IgG class-switch process.

CD27 and CD70; two important molecules for antibody production

Interactions between CD27 on B-cells and CD70 on T-cells are important for memory cell differentiation after antigen-stimulation of the B-cell. Therefore, the expression of CD27 and CD70 on the cell surface of B- and T-cells in children at diagnosis of pre-B ALL was studied similar to the HIV-1 patients. In *paper 3*, we showed that there is an aberrant expression of CD27 and CD70 in ALL (Fig 6). The percentage of B cells in peripheral blood expressing CD27 and CD70 [43.5% (1-94) and 6.7% (0.1-83)] was significantly higher in these patients compared to controls [22% (7.6-42) and 5.5% (1-14.3), p<0.01).

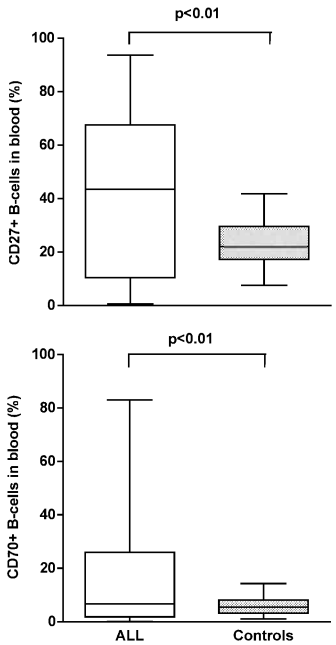

Fig 6. The proportion of $CD27^+$ and $CD70^+$ B-cells is higher in the ALL children (n=21) at diagnosis than in the controls (n=43).

The expression of CD27 and CD70 molecules on leukaemic cells in the BM correlated to leukaemic cells in blood ($r=0.78$, $p<0.001$ and $r=0.90$, $p<0.001$) and to the levels of soluble CD27 in serum at diagnosis ($r=0.78$, $p<0.001$ and $r=0.61$, $p<0.05$).

The expression and release of CD27 was different within the different genetic subtypes of childhood ALL (Table 2). Children with the translocation (t 12; 21) involving chromosome 12p, presented with significantly higher levels of sCD27 compared to those without the translocation [1275 U/mL (199-2177) vs. 424 U/mL (131-2005) respectively, $p<0.01$]. The breakpoint of the translocation is located on the same segment as the CD27gene and the up-regulation of CD27 occurs most likely as an epigenetic phenomenon. We found that CD4, also located on chromosome 12p, is present on leukaemic cells carrying the t (12; 21) translocation.

The biological importance of the CD27-CD70 interactions on leukaemic cells was studied *in vitro* through blocking of the receptor-ligand pair. When blocking the CD27-CD70 interaction with a monoclonal anti-CD70 Ab, the leukaemic cell proliferation was reduced, which suggests that the up-regulation of CD27 and CD70 is beneficial for these cells.

Table 2. Soluble CD27 in the different genetic sub-types of childhood ALL.

Genetic subtype	*Number of patients*	*SolubleCD27 (U/mL)*
Hyperdiploidy	20	352 (131-1814)
Translocation 12; 21	16	1275 (199-2177)
Normal karyotype	14	620 (185-1649)
Other genetic alterations	16	584 (144-2005)

CD27 and CD70 are also expressed on T-cells. Upon T-cell activation, CD70 is expressed on the cell surface (123) and $CD70^+$ T-cells have the ability to stimulate memory B cells into IgG secretion and plasma cell formation (124, 125). Our group has previously shown that HIV-1 patients have a high expression of CD70 on T-cells (101) as a result of chronic activation. In children at diagnosis of ALL, there was an increase in the percentage of $CD70^+$ T-cells compared to healthy age-matched controls [5%(0.3-58.7) vs. 1.2%(0.4-11.1), p<0.02] (Fig 7). However, there was not a significant correlation between the CD70+T-cells and IgG in the limited number of patients investigated (n=20) (p=0.21).

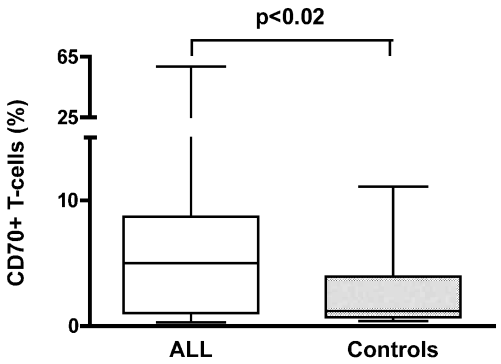

Fig 7. At diagnosis of ALL, patients (n=21) have a higher proportion of activated T-cells in peripheral blood than controls (n=43).

Soluble factors mediating survival of Ab producing cells

The survival of memory B-cells and plasmacells is mediated by different growth factors, cytokines and cell-to-cell contact through different receptors. In ALL children, chemotherapy significantly reduces the memory B-cell compartment. In HIV-1 infection the memory B-cells pool is decreased compared to normal controls as demonstrated in *paper 2* and *paper 4*. Several mechanisms have been suggested for the depletion of memory B-cells during HIV-1 infection. B-cell activation leads to a higher susceptibility to apoptotic stimuli in B-cells (126) with a concomitant up-regulation of

Fas and Fas L on memory B-cells (101). B-cell activation may also lead to an increase in differentiation into plasma cells (124).

In *paper 4*, the effect of NGF on the survival of memory B-cells in HIV-1 patients and controls were studied. NGF is an autocrine survival factor for memory B-cells and NGF also inhibits apoptosis in memory B-cells (127, 128). We found a lower median plasma NGF in HIV-1-infected subjects [5 pg/ml (5-112)] compared to healthy controls [14 pg/ml (5-175)] ($p=0.003$). The frequency of NGF detection in plasma was also decreased in the HIV-1 infected subjects compared to healthy controls (33.6% vs. 63.6%, $p<0.001$). In addition, when recombinant NGF was added to purified B-cells from HIV-1 subjects cultured over-night, a low but statistically significant reduction of apoptosis of memory B cells was induced. The highest concentration of NGF (1000 ng/ml) induced a 20% reduction of memory B cell death compared to cells cultured in the absence of NGF [(mean ± SEM) 24.0±3.0 vs. 17.4±1.3 %, $p=0.001$]. In contrast, the addition of NGF did not have any significant effect on cell death of naïve cells.

SDF-1 was first identified as a pre-B cell growth factor (19, 24) with a predominantly chemotactic activity; however, SDF-1 has recently also been shown to mediate plasma cell survival (129) and an increase in IgG production from activated B-cells *in vitro* (130). In *paper 5*, we analysed the SDF-1 levels in children at diagnosis of pre-B ALL and the ALL children have elevated levels of SDF-1 in serum compared to controls [4.8 (0-32) ng/mL vs. 0 (0-3.2) ng/mL, $p< 0.0001$]. In twentysix (70.2%) children with ALL the SDF-1 serum concentrations were above 3.5 ng/mL (mean± 2SD of SDF-1 levels in the control group), whereas 11 (29.8%) had values below 3.5 ng/mL. In the controls, there were no samples with an SDF-1 concentration above 3.5 ng/mL (Fig 8).

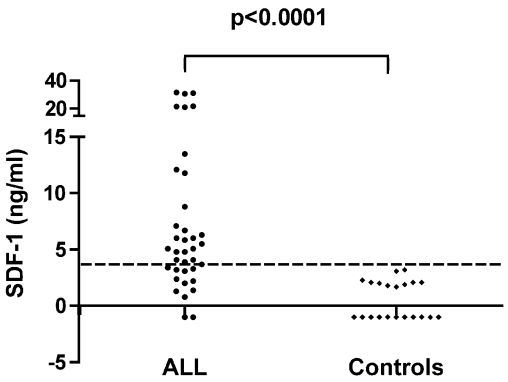

Fig 8. SDF-1 levels in children with pre-B ALL (n=37) is higher than in the controls (n=21). The dotted line represent a cut-off based on the mean SDF-1 ± 2SD in the controls.

SDF-1 induced proliferation of leukaemic pre-B cells, but not in normal pre-B cells, when added to the leukaemic BM cultures *in vitro*. This suggests that SDF-1 contributes to maintenance and possibly expansion of the leukaemic clone and not only to migration of leukaemic cells.

Stromal cells BM may function as important regulators of B-cell fate

In the BM, stromal cells produce growth factors, cytokines and chemokines important for haematopoiesis and maturation of early progenitors. The regulation of cytokine production from stromal cells has not been extensively studied but *in vitro* studies indicates that cytokine production is induced from stromal cells (131, 132) by different stimuli such as cell-to-cell contact and inflammatory cytokines. Human BM stromal cells have been found to produce NGF (133) as well.

Our findings of elevated levels of SDF-1 in serum at diagnosis of ALL prompted us to study the interaction between leukemic cells and stromal cells *in vitro*. In *paper 5*, we present a model for cell-to-cell interactions in the BM that results in increased SDF-1 production from the human BM stromal cell line HS-27. When cultured together with

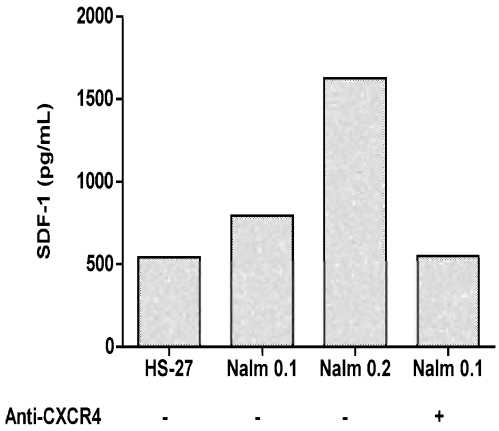

Fig 9. SDF-1 levels in culture supernatants from the cell line HS-27 (column 1) increases when HS-27 is cultured in combination with 0.1x 10^6 (column 2) and 0.2x10^6 Nalm-6 cells (column 3). The induction of SDF-1 is blocked when an anti-CXCR4 Ab is added to the culture (column 4).

increasing amounts of the human pre-B leukemic cell line Nalm-6, SDF-1 in the culture supernatants is increased. The increase in SDF-1 is not mediated by soluble factors but is dependent on cell-to-cell contact. To test whether the up-regulation of SDF-1 production is mediated via the receptor, CXCR4, a monoclonal anti-CXCR4 Abs was added to the culture system. When the Nalm-6 cells were cultured together with an anti-CXCR4 Abs there was no up-regulation of SDF-1 production (Fig 9).

The cellular source of the elevated SDF-1 remains to be identified as SDF-1 mRNA can be detected in stromal cells and in primary leukaemic cells, derived from children with ALL, and from the leukaemic cell line Nalm-6 . However, there is no release of SDF-1 when Nalm-6 is cultured alone.

Re-immunisation of children treated for ALL

One of the aims for this thesis was to investigate whether children after treatment against ALL needed to be re-immunized against measles, mumps and rubella. We have shown that Ab production and maintenance of humoral immunity is impaired in these children.

In *paper 1*, 14 children without immunity against measles were revaccinated and serum Ab levels were analyzed after 3 months. The median Ab level was somewhat increased compared to before the booster, however not statistically significant. Eight children responded with increased Ab titres after one booster of vaccine however 6 of the 14 without immunity failed to achieve protective levels of specific Abs.

To further study the type of vaccination response, an additional ELISA with concomitant measles IgG avidity tests was performed on sequential samples after treatment and after revaccination. In responders, avidity and Ab titres indicated a primary immune response in 4 children and in 2 children a secondary immune response. In the non-responders, avidity and Ab titres were unchanged in spite of the booster.

Eleven children were also non-immune against rubella and they were given a booster vaccination. Compared to before the booster the Ab level was significantly elevated [47.8 IU/mL (3.2-150) vs. 12.4 IU/mL (1.2-150); $p<0.01$]. However, despite he revaccination 3 children failed to achieve protective levels of Abs against rubella.

7 DISCUSSION

The primary aim of this thesis was to study B-cell function and long-term immunity in children who had undergone chemotherapy for treatment of ALL and in HIV-1 infected subjects. By studying specific Abs at diagnosis and after treatment, it became evident that the regulation of Ab production is altered already at diagnosis of these diseases.

Hypergammaglobulinemia is a common finding in autoimmune disease (134, 135), chronic infections (136, 137) and in lymfoproliferative disorders (138). In this thesis, we have studied the regulation of specific Ab production in two different disorders (HIV and childhood ALL) in relation to hypergammaglobulinemia. When analysing anti-measles Abs in these two patient groups an important difference was noted. Specific Ab levels were decreased in HIV-1 infected subjects even in presence of high total IgG levels whereas the high serum IgG levels in the children with ALL reflected elevated levels of specific Abs. This indicates that the memory B cell compartment in children with ALL is conserved at diagnosis and activated by the disease, while the memory B cell compartment is compromised during HIV-1 infection.

Maintenance of serological memory has been under debate for many years (60, 61, 139) and one of the current hypothesis is that non-cognate activation by T-cells via CD40-CD40L interactions with memory B cells sustains specific Ab titres in the host (62). In peripheral blood at diagnosis of childhood ALL, there is an increase in the proportion of CD70+ activated T-cells that may be responsible for triggering specific Ab production from memory B cells and thus contributing to hypergammaglobulinemia (45).

HIV-1 establishes a chronic infection (88) in the host characterised by immune activation (140). Recently, it was proposed that hypergammaglobulinemia in murine viral infections is induced by activated T helper cells stimulating naïve B cells, independently of the BCR, to switch and produce polyspecific IgG antibodies. In this murine model, activation of naïve B cells requires CD40-CD40L interaction (via B-T helper cells) and presentation of viral peptides in conjunction with the MHC class II on B cells (141). In *paper 2* we present evidence of a similar polyspecific IgG production from naïve B cells in HIV-1 infection. The phenotype of these naïve B cells indicate recent activation with subsequent up-regulation of CD70 (45) and a decrease in LAIR-1 expression (142). The correlation of IgG levels to the proportion of CD70+ B cells in the HIV-1 infected subjects further strengthens our observation of IgG secretion from naïve B cells during chronic HIV-1 infection.

The cellular mechanisms leading to isotype switch of naïve B cells have only partly been characterised (143). Interactions between CD40-CD40L are crucial for isotype switch and CD40L- deficiency leads to the hyper-IgM syndrome without the production of IgG, IgA or IgE (144-146). Whether the activation and switch to IgG production from HIV-1 naïve B cells is followed by differentiation to plasma cells has not been extensively studied. However, there are several reports on plasmacytosis in the BM of HIV-1 infected subjects (121, 122, 124) but it is not characterised whether the plasma cells produce high affinity versus low affinity Abs. If HIV-1 naïve cells differentiate to plasma cells it would be of interest to characterise the chemokine receptor profile of these plasma cells. This could implement our knowledge on whether these cells may home to the BM compartment. In fact, homing to the BM (57, 147) is dependent on SDF-1 induced migration via a functional and responsive CXCR4.

The hypergammaglobulinemia and loss of specific Abs during HIV-1 is accompanied by a decrease in the proportion and numbers of memory B cells. Memory B cells are more prone to apoptosis in HIV-1 (*paper 2*) and there is also a lack of growth factors during HIV-1 infection (*paper 4*) resulting in a reduction of the memory B cell compartment. The concomitant decrease of memory B cells and antigen-specific Abs in HIV-1 indicates a crucial role for memory B cells in maintaining a specific serological memory. In Fig 10, our current understanding of the mechanisms of hypergammaglobulinemia in childhood ALL and HIV-1 infection are summarised.

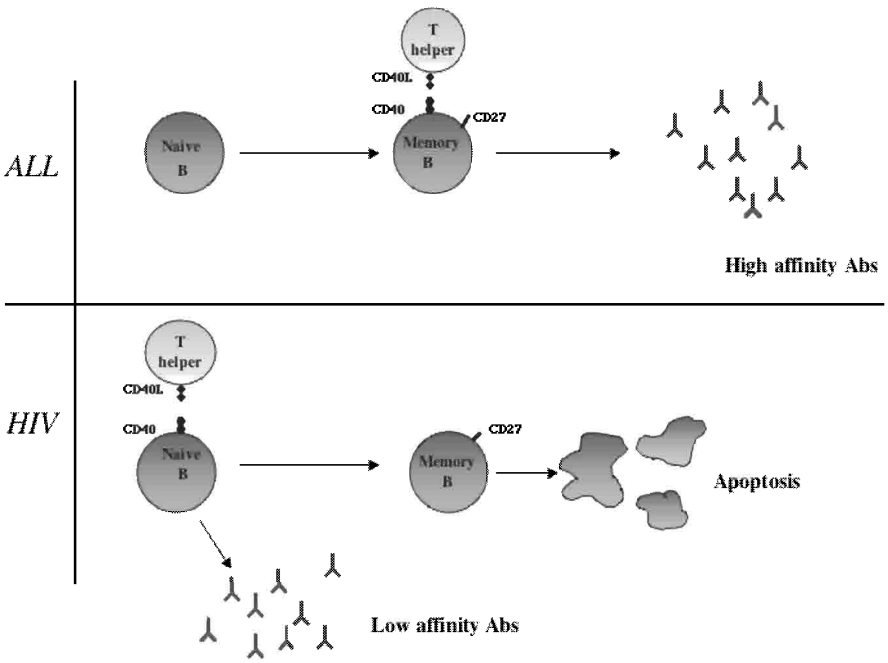

Fig 10. A proposed model for the relation between specific, high affinity antibodies, hypergammaglobulinemia and the memory B-cell compartment. In children with ALL, memory B-cells are activated by the disease to produce high amounts of specific antibodies that accounts for the high total IgG present in sera at diagnosis. In HIV-1 infection, memory B-cells are prone to apoptosis and the immune activation leads to production of poly-specific, low affinity antibodies from naïve B-cells.

During chemotherapy plasma cells as well as memory B cells are reduced and in approximately 40% of the ALL children the levels of vaccination induced specific Abs have declined (*paper1*). In our cohort, young age (< 7 years) at diagnosis of ALL was associated with a reduction of specific Ab titres below levels considered being protective. The humoral arm of the immune system is still evolving during infancy and early childhood (1, 3, 4) and it is likely that the memory B cell compartment in the young child is less developed and therefore more vulnerable during chemotherapy.

Our study and a recent study by Ek and co-workers (148) indicate that children after completed chemotherapy have lost protective levels of vaccination induced specific Abs. When re-immunising the ALL children, the specific Ab response measured as Ab titres and avidity remains poor in approximately 50% of the children. Further studies are needed where specific Ab titres are measured one week and one month after re-immunisation including quantification of specific Abs-secreting cells in order to better characterise the specific Ab response in these children. Several mechanisms such as sub-optimal Ag-presentation or deficient T cell help with perturbations in B-T cell interactions may explain the poor Ab response after immunisation in the former ALL patients. If our current data are reproduced, new strategies for re-immunisations in children treated with chemotherapy need to be set in place.

Immune responses after immunisations in the HIV-1 infected population are also impaired with rapid loss of Ag-specific Abs following in vivo immunisations (103, 105) and this phenomena is not corrected for with HAART (149). Considering that the majority of HIV-1 infected individuals live in areas with a high incidence of infections *per se* successful immunisations of children and adults would decrease overall morbidity in infections (150, 151).

In normal B lymphoid development, the expression of CD27 and CD70 is tightly regulated (40, 45) and represents memory B cells (CD27) (41, 152) and recently activated B cells (CD70) (45). In contrast to this restricted and tightly regulated pattern of expression, we found a high proportion of $CD27^+$ and $CD70^+$ B-cells in the blood and BM of children with pre-B ALL (*paper 3*). The soluble CD27, sCD27, mirrored the cell bound expression of CD27 and CD70 on the leukaemic cells in the BM and we found a significantly higher level of sCD27 in children with the t (12; 21) translocation. When CD27-CD70 interaction in cells from ALL is blocked, the proliferation of the leukaemic cells *in vitro* was reduced indicating a possible role for CD27-CD70 interactions in the pathogenesis of ALL.

It is not clear whether the expression of CD27 and CD70 in different malignancies supports the expansion of the tumour or whether it represents a mechanism for immune surveillance or immune escape. The expression of CD70 (ligand) on brain gliomas induces apoptosis of cytotoxic T-cells through the CD27 receptor thus preventing an effective immune response against the tumour (153). In CD70+ nasopharyngeal carcinomas, CD27+ T-cells are present in the infiltrating stroma of the tumours indicating that tumour and T-cell interactions via CD27-CD70 occur and may be of importance in tumour development (154). Several studies have shown that pre-B leukaemic cells express CD40 and also CD40L indicating that these cells can interact with other immune cells (155, 156) Other members of the TNFR-family have also been associated with tumour immune escape such as Fas and TNFR (157).

On the other hand, CD27-CD70 interactions have emerged as an important mechanism for NK-cell mediated tumour rejection and induction of tumour specific T cell memory (158). NK cells express CD27 constitutively and when activated through this receptor,

IFN-γ production increases and perforin-mediated cytotoxicity is enhanced (159, 160). It would be interesting to investigate whether the increased expression of CD27 and CD70 in the t (12; 21) ALL elicit a stronger innate immune response or an increased cytotoxic T-lymphocyte (CTL) response (161) via CD27-CD70 interactions.

NK-cells function as a link between innate and adaptive immunity in participating in the regulation of Ab responses (162). It has also been suggested that NK-cells can program B cells for switching to IgG2a production (163) independently of CD40-CD40 ligand interactions. In HIV-1, NK-cells promote immunoglobulin production and plasma cell differentiation (164) involving CD27-CD70 interactions.

B-cell differentiation, from the early stages in the BM to the terminally differentiated plasma cells, require receptor-signalling, adhesion molecules, soluble growth factors and chemokines for the localisation to the appropriate environment for further maturation. Many of the growth factors, cytokines and chemokines important for B lymphoid development such as IL-7, SDF-1, NGF (165) are produced by stromal cells (17, 166). In this context, it would be interesting to study the establishment of long-lived serological memory in relation to structural changes in secondary lymphoid organs occurring during ontogeny.

In HIV-1 infection a gradual destruction of the secondary lymphoid organs by the virus has been described (167) and the architecture of lymph nodes is only partly restored by HAART (168). Tonsils from HIV-1 infected subjects are characterised by a general cellular depletion, accumulation of plasma cells and absence of germinal centres. A dysfunction in the interplay between immune cells and stromal cells in secondary lymphoid organs could be one of the explanation behind the low levels of NGF

associated with HIV-1 (*paper 4)*. In normal lymphopoesis, NGF induces immunoglobulin production (169) and promotes differentiation of B cells into plasma cells (170). NGF has also been associated with malignant lymphomas of B cell type (171) in adults. In this context, it is interesting that NGF regulates the production of neuropeptide Y (NPY), a peptide shown to be elevated in plasma of children with ALL (172).

It is not completely understood how the production and secretion of cytokines and chemokines are regulated from stromal cells. Apparently, cell-to-cell contact is necessary for the induction of SDF-1 production (*paper 5*) and this holds true also for other soluble factors (131, 132, 173) . The production of the chemokine, SDF-1, from stromal cells affects migration within the microenvironment and may facilitate organ infiltration in ALL as well leukaemic cell proliferation. It is also conceivable that the elevated SDF-1 might influence immunoglobulin production and homing of plasma cells to the BM and thereby contribute to the hypergammaglobulinemia seen at diagnosis of ALL. The interaction between stromal cells and immune cells is also a possible target for immunotherapy in childhood ALL. Inhibitors of CXCR4 reduce leukaemic cell proliferation *in vitro* (174) and prevent BM metastasis from other solid tumours (175, 176).

8 FUTURE PERSPECTIVES

My PhD project started with a very basic and clinically relevant question whether children with ALL maintained protective levels of vaccination induced Abs or not after chemotherapy. By addressing that question my interest in immunology, on receptors and ligands, growth factors and chemokines has increased. And now, after five years, there are still more questions that need to be answered!

We have shown that the concept of hypergammaglobulinemia is different depending on the cellular mechanisms triggering naïve or memory B cells in different diseases. Present studies in the laboratory are focusing on the possible differentiation of plasma cells from these HIV-1 naïve B cells and the chemokine repertoire of these cells. The high production of low affinity Abs in HIV-1 is detrimental to the host since many of these auto Abs are directed against CD4+ T helper cells and other cells of the immune system. To revert this process, would be of benefit to the host and potentially increase the level of specific Abs production as well.

By the initial studies of CD27 and CD70 on B cells we became aware of the deregulated expression of these molecules on leukaemic cells, especially in t (12; 21) ALL. In the future studies, we want to focus on the generation of cytotoxic cells (NK-cells or CD8+ T cells) via CD27-CD70 interactions in ALL. The aberrant expression of CD27 and CD70 is also a possible target for therapy – and in this context it is important to remember that so far the most successful immune therapies are the antibody-mediated therapies against cell surface molecules.

Stratification of patients into the different treatment groups is based on clinical characteristics and biological properties of the leukaemic cells. To increase our knowledge on tumour biology and factors regulating proliferation and survival of leukaemic cells (such as SDF-1 or CD27- CD70) could be of benefit when stratifying children into the treatment groups based on biological features of the leukaemia. This should be done by including more patients and perform prospective studies in relation to these molecules in the future.

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11 REFERENCES

1.**Andersson U**. Development of B lymphocyte function in childhood. Acta Paed Scand 1985; 74:568-573.

2.**Agematsu K**, Nagumo H, Yang FC, Nakazawa T, Fukushima K, Ito S, Sugita K, Mori T, Kobata T, Morimoto C, Komiyama A. B cell subpopulations separated by CD27 and crucial collaboration of CD27+ B cells and T helper cells in immunoglobulin production. Eur J Immunol 1997;27:2073-2079.

3.**Zandvoort A**, Lodewijk ME, de Boer NK, Dammers PM, Kroese FGM, Timens W. CD27 expression in the human splenic marginal zone: the infant marginal zone is populated by naive B cells. Tissue antigens 2001; 58:234-242.

4.**Pihlgren M**, Schallert N, Tougne C, Bozzotti P, Kovarik J, Fulurija A, Kosco-Vilbois M, Lambert P, Siegrist C. Delayed and deficient establishment of the long-term bone marrow plasma cell pool during early life. Eur J Immunol 2001;31:939-946.

5.**Wang LD**, Clark MR. B-cell antigen-receptor signalling in lymphocyte development. Immunology 2003; 110:411-420.

6.**Kamps WA**, Cooper MD. Microenvironmental studies of pre-B and B-cells in human and mouse fetuses. J Immunol 1982; 129:526-531.

7.**Rolink AG**, Schaniel C, Andersson J, Melchers F. Selection events operating at various stages in B cell development. Curr Opinion Immunol 2001; 13:202-207.

8.**Nutt SL**, Heavey B, Rolink AG, Busslinger M. Commitment to the B lymphoid lineage depends on the transcription factor Pax5. Nature 1999;401:556-562.

9.**van Oers MHJ**, Pals ST, Evers LM, van der Schoot CE, Koopman G, Bonfrer JMG, Hintzen RQ, von dem Borne AEG, van Lier RAW. Expression and release of CD27 in human B-cell malignancies. Blood 1993; 82:3430-3436.

10.**Stoddart A**, Fleming HE, Paige CJ. The role of the preBCR, the interleukin-7 receptor and homotypic interactions during B-cell development. Immunol Rev 2000; 175:47-58.

11.**Guo B**, Kato RM, Garcia-Lloret M, Wahl MI, Rawlings DJ. Engagement of the human pre-B cell recepotor generates a lipid raft-dependent calcium signalling complex. Immunity 2000;13:243-253.

12.**Bertrand FE**, Eckfeldt CE, Fink JR, Lysholm AS, Pribyl JAR, Shah N, leBien TW. Microenvironmental influences on human B-cell development. Immunol Rev. 2000;175:175-186.

13.**Lepri E**, Delfino DV, Migliorati G, Moraca R, Ayroldi E, Riccardi C. Functional expression of Fas on mouse bone marrow stromal cells, upregulation by tumour necrosis factor-alpha and interferon-gamma. Exp Hematol 1998;13:1202-1208.

14.**Namen AE**, Lupton S, Hjerrild K, Wignall J, Mochizuk iDY, Schmierer A, Mosley B, March CJ, Urdal D, Gillis S. Stimulation of B-cell progenitors by cloned murine interleukin-7. Nature 1988;333:571-573.

15.**Hayashi S**, Kunisada T, Ogawa M, Sudo T, Kodama H, Suda T, Nishikawa S, Nishikawa S. Stepwise progression of B lineage differentiation supported by interleukin 7 and other stromal cell molecules. J Exp Med 1990;171:1683-1695.

16.**Fleming HE**, Paige CJ. Pre-B cell receptor signaling mediates selective response to IL-7 at the pro-B to pre-B cell transition via an ERK/MAP kinase-dependent pathway. Immunity 2001;15:521-531.

17.**Stephan RP**, Reilly CR, Witte PL. Impaired ability of bone marrow stromal cells to support B-lymphopoesis with age. Blood 1998;91:75-88.

18.**Shirozu M**, Nakano T, Inazawa J, Tashiro K, Tada H, Shinohara T, Honjo T. Structure and chromosomal localization of the human stromal cell-derived factor 1 (SDF1) gene. Genomics 1995;28(3):495-500.

19.**Bleul CC**, Fuhlbrigge RC, Casasnovas JM, Aiuti A, Springer TA. A highly efficious lymphocyte chemoattractant, stromal cell derived factor-1/SDF-1. J Exp Med 1996;184:1101-1109.

20.**D´Apuzzo M**, Rolink A, Loetscher M, Hoxie JA, Clark-Lewis I, Melchers F, Baggiolioni M, Moser B. The chemokine SDF-1, stromal cell-derived factor 1, attracts early stage B cell precursors via the chemokine receptor CXCR4. Eur J Immunol 1997;27:1788-1793.

21.**Honczarenko M**, Douglas RS, Mathias C, Lee B, Ratajczak MZ, Silberstein LE. SDF-1 responsiveness does not correlate with CXCR4 expression levels on developing human bone marrow B cells. Blood 1999;94:2990-2998.

22.**Feng Y**, Broder CC, Kennedy PE, Berger EA. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. Science 1996;272:872-877.

23.**Nagasawa T**, S. H, K. T, Takakura N, Nishikawa S, Kitamura Y, Yoshida NK, H., Kishimoto T. Defects of B-cell lymphopoesis and bone marrow myelopoesis in mice lacking the CXC chemokine PBSF/SDF-1. Nature 1996;382:635-638.

24.**Ma Q**, Jones D, Borghesani PR, Segal RA, Nagasawa T, Kishimoto T, T. BR, Springer TA. Impaired B-cell lymphompoesis, myelopoesis and derailed cerebellar neuron migration in CXCR4- and SDF-1 deficient mice. Proc. Natl. Acad. Sci 1998;95:9448-9453.

25.**Ma Q**, Jones D, Springer TA. The chemokine receptor CXCR4 is required for the retention of B lineage and granulocytic precursors within the bone marrow microenvironment. Immunity 1999;10:463-471.

26.**Yoshie O**, Imai T, Nomiyama H. Chemokines in immunity. Adv. Immunol 2001;78:57-110.

27.**Liu YJ**, Malisan F, de Bouteille rO, Guret C, Lebecque S, Banchereau J, Mills FC, Max EE, Martinez-Valdez H. Within germinal centers, isotype switching of immunoglobulin genes occurs after the onset of somatic mutation. Immunity 1996;4:241-250.

28.**Liu YJ**, Zhang J, Lane PJ, Chan EY, Maclennan ICM. Sites of specific B cell activation in primary and secondary responses to T cell-dependent and T cell-independent antigens. Eur J Immunol 1991;21:2951-2962.

29.**Berek C**, Berger A, Apel M. Maturation of immune responses in germinal centers. Cell 1991;67:1121-1129.

30.**McHeyzer-Williams MG**, McLean MJ, Lalor PA, Nossal GJV. Antigen-driven B-cell differentiation in vivo. J Exp Med 1993;178:295-307.

31.**Martinez-Valdez H**, Guret C, de Bouteiller O, Fugier I, Banchereau J, Liu YJ. Human germinal center B cells express the apoptosis-inducing genes Fas, c-myc, P53, and Bax but not the survival gene bcl-2. J Exp Med 1996;183:971-977.

32.**Klein U**, Tu Y, Stolovitzky GA, Keller JL, Haddad JJ, Miljkovic V, Cattoretti G, Califano A, Dalla-Favera R. Transcriptional analysis of the B cell germinal center reaction. Proc Natl Acad Sci 2003;100:2639-2644.

33.**Arpin C**, Dechanet J, Van Kooten C, Merville P, Grouard G, Briere F, Banchereau J, Liu YJ. Generation of memory B cells and plasma cells in vitro. Science 1995;268:720-722.

34.**Gravestein LA**, Borst J. Tumor necrosis factor receptor family members in the immune system. seminars in Immunol 1998;10:423-434.

35.**Lens SMA**, Tesselaar K, van Oers M, van Lier RAW. Control of lymphocyte function through CD27-CD70 interactions. Seminars in Immunol 1998;10:491-499.

36.**Renshaw BR**, Fanslow WC, Armitage RJ, Campbell KA, Liggitt D, Wright D, Maliszewski CR. Humoral immune responses in CD40 ligand deficient mice. J Exp Med 1994;180:1889-1900.

37.**Laman JD**, Claassen E, Noelle RJ. Functions of CD40 and its ligand, gp39 (CD40L). Crit Rev Immunol 1996;16:59-108.

38.**Defrance T**, Casamayor-Palleja M, Krammer PH. The life and death of a B-cell. Adv Cancer Res 2002;86:195-225.

39.**Galibert L**, Burdin N, de Saint-Vis B, Garrone P, Van Kooten C, Banchereau J, Rousset F. CD40 and B cell antigen receptor dual triggering of resting B lymphocytes turns on a partial germinal center phenotype. J Exp Med 1996;183:77-85.

40.**Maurer D**, W. H, madjic O, F. FG, Knapp W. CD27 expression by a distinct subpopulation of human B lymphocytes. Eur J Immunol 1990;20:2679-2684.

41.**Klein U**, Rajewsky K, Kuppers R. Human immunoglobulin (Ig) M+IgD+ peripheral blood B cells expressing the CD27 cell surface antigen carry somatically mutated variable region genes: CD27 as a general marker for somatically mutated (memory) B cells. J Exp Med 1998;9:1679-1689.

42.**Hintzen RQ**, de Jong R, Lens SMA, van Lier RAW. CD27; a marker and mediator of T-cell activation. Immunol Today 1994;15:307-311.

43.**Yang FC**, Agematsu K, Nakazawa T, Mori T, Ito S, Kobata T, Morimoto C, A. K. CD27/CD70 interaction directly induces natural killer cell killing activity. Immunology 1996;88:289-293.

44.**Hintzen RQ**, Lens SM, Koopman G, Pals ST, Spits H, van Lier RA. CD70 represents the human ligand for CD27. Int Immunol 1994;6:477-480.

45.**Lens SMA**, de Jong R, Hooibrink B, Koopman G, Pals ST, van Oers MHJ, van Lier RAW. Phenotype and function of human B cells expressing CD70. Eur J Immunol 1996;26:2964-2971.

46.**Lane PJ**, Burdet C, McConnell F, Lanzavecchia A, Padovan E. CD40-ligand-independent B cell activation revealed by CD40-ligand-deficient T cell clones: evidence for distinct activation requirements for antibody formation and B cell proliferation. Eur J Immunol 1995;25:1788-1793.

47.**Lens SMA**, de Jong R, Hintzen RQ, Koopman G, van Lier RAW, van Oers MHJ. CD27-CD70; unraveling its implication in normal and neoplastic B cell growth. Leuk Lymphoma 1995;18:51-59.

48.**Nagumo H**, Agematsu K, Shinozaki K, Hokibara S, Ito S, Takamoto M, Nikaido T, Yasui K, Uehara Y, Yachie A, Komiyama A. CD27/CD70 interactions augments IgE secretion by promoting the differentiation of memory B cells into plasma cells. 1998.

49.**Agematsu K**, Nagumo H, Oguchi Y, Nakazawa T, Fukushima K, Yasui K, Ito S, Kobata T, Morimoto C, Komiyama A. Generation of plasma cells from peripheral blood memory B cells. synergistic effect of interleukin 10 and CD27/CD70 interaction. Blood 1998;91:173-180.

50.**Agematsu K**, Hokibara S, Nagumo H, Shinozaki K, Yamada S, Komiyama A. Plasma cell generation from B-lymphocytes via CD27/CD70 interaction. Leuk Lymphoma 1999;35:219-225.

51.**Fecteau JF**, Neron S. CD40 stimulation of human peripheral B lymphocytes: distinct response from naive and memory cells. J Immunol 2003;171:4621-4629.

52.**Saeland S**, Duvert V, Moreau I, Banchereau J. Human B cell precursors proliferate and express CD23 after CD40 ligation. J Exp Med 1993;178:113-120.

53.**Punnonen J**, Aversa G, de Vries JE. Human pre-B cells differentiate into Ig-secreting plasma cells in the prescence of interleukin-4 and activated T cells or their membranes. Blood 1993;82:2781-2789.

54.**Casamayor-Palleja M MP**, Amara A, Bella C, Dieu-Nosjean MC, Caux C, Defrance T. Expression of macrophage inflammatory protein-3alpha, stromal cell-

derived factor-1, and B-cell-attracting chemokine-1 identifies the tonsil crypt as an attractive site for B cells. Blood 2001;97:3992-3994.

55.**Brandes M**, Legler DF, Spoerri B, Schaerli P, Moser B. Activation-dependent modulation of B lymphocyte migrtaion to chemokines. Int Immunol 2000;12:1285-1292.

56.**Hargreaves DC**, Hyman PL, Lu TT, Ngo VN, Bidgol A, Suzuki G, Zou YR, Littman DR, Cyster JG. A coordinated change in chemokine responsiveness guides plasma cell movements. J Exp Med 2001;194:45-56.

57.**Hauser A**, Debes GF, Arce S, cassese G, Hamann A, Radbruch A, Manz RA. Chemotactic responsiveness towards ligands for CXCR3 and CXCR4 is regulated on plasma blasts during the time course of a memory immune response. J Immunol 2002;169:1277-1282.

58.**Nanki T**, Lipsky PE. Cutting edge: Stromal cell-derived factor-1 is a co-stimulator for CD4+ T cell activation. J Immunol 2000;164:5010-5014.

59.**Steinhoff U**, Muller U, Schertle A, Hengartner H, M. A, Zinkernagel RM. Antiviral protection by vesicular stomatitis virus-specific antibodies in alpha/beta interferon receptor-deficient mice. J Virol 1995;69:2153-2158.

60.**Ochsenbein AF**, Pinschewer DD, Sierro S, Horwath E, Hengartner H, Zinkernagel RM. Protective long-term antibody memory by antigen-driven and T help-dependent differentiation of long-lived memory B-cells to short-lived plasma cells independent of secondary lymphoid organs. Proc Natl Acad Sci 2000;97:13263-13268.

61.**Slifka MK**, Antia R, Whitmire JK, Ahmed R. Humoral immunity due to long-lived plasma cells. Immunity 1998;8:363-372.

62.**Bernasconi NL**, Traggiai E, Lanzavecchia A. Maintenance of serological memory by polyclonal activation of human memory B cells. Science 2002;298:2199-2201.

63.**Traggiai E PR**, Lanzavecchia A. Antigen dependent and independent mechanisms that sustain serum antibody levels. Vaccine 2003;21:35-37.

64.**Bernasconi NL**, Onai N, Lanzavecchia A. A role for Toll-like receptors in acquired immunity: up-regulation of TLR9 by BCR triggering in naive B cells and constitutive expression in memory B cells. Blood 2003;101:4500-4504.

65.**Poltorak A**, He X, Smirnova I, Liu M, Van Huffel C, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B, Beutler B. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science 1998;282:2085-2088.

66.**Gustafsson G**, Schmiegelow K, Forestier E, Clausen N, Glomstein A, Jinmundsson G, Mellander L, Makipernaa A, Nygaard R, Saarinen-Pihkala UM. Improving outcome through two decades in childhood ALL in the Nordic countries. The impact of high-dose methotrexate in reduction of CNS-irradiation. Leukemia 2000;14:2267-2275.

67.**Gustafsson G,** Kreuger A. Incidence of chilhood leukemia in Sweden 1975- 1980. Acta Paed Scand 1982;71:887-892.

68.**Lanning M**, Garwicz S, Hertz H, Jonmundsson G, Kreuger A, O. LS, Moe PJ, Salmi TT, Schroder H, Siimes MA. Superior treatment results in females with high-risk acute lymphoblastic leukemia in childhood. Acta Paediatr 1992;81:66-68.

69.**Rivera GK**, Pinkel DS, Simone JV, Hancock ML, Crist WM. Treatment of acute lymphoblastic leukemia. 30 years' experience at St. Jude Children's Research Hospital. N Engl J Med 1993;329:1289-1295.

70.**Pui CH**, Dodge RK, Dahl GV, Rivera G, Look AT, Kalwinsky D, Bowman WP, Ochs J, Abromowitch M, Mirro J. Serum lactic dehydrogenase level has prognostic value in childhood acute lymphoblastic leukemia. Blood 1985;66:778-782.

71.**Mahmoud HH,** Rivera GK, Hancock ML, Krance RA, Kun LE, Behm FG, Ribeiro RC, Sandlund J, Pui CH. Low leukocyte counts with blast cells in cerebrospinal fluid of

children with newly diagnosed acute lymphoblastic leukemia. N Engl J Med 1993;329:314-319.

72.**Crist W**, Shuster J, Look T, Borowitz M, Behm F, Bowman P, Frankel L, Pullen J, Krance R, Steuber P. Current results of studies of immunophenotype-, age- and leukocyte-based therapy for children with acute lymphoblastic leukemia. The Pediatric Oncology Group. Leukemia 1992;6:162-166.

73.**Greaves MF**, Wiemels J. Origins of chromosome translocations in childhood leukemia. Nature reviews 2003;3:1-11.

74.**Harrison C**. The detection and significance of chromosomal abnormalities in childhood acute lymphoblastic leukemia. Blood Reviews 2001;15:49-59.

75.**Calero Moreno TM**, Gustafsson G, Garwicz S, Grander D, Jonmundsson GK, Frost B, Makipernaa A, Rasool O, Savolainen ER, Schmiegelow K, Soderhall S, Vettenranta K, Wesenberg F, Einhorn S, Heyman M. Deletion of the Ink4-locus (the p16ink4a, p14ARF and p15ink4b genes) predicts relapse in children with ALL treated according to the Nordic protocols NOPHO-86 and NOPHO-92. Leukemia 2002;16:2037-2045.

76.**Gustafsson G**, Kreuger A, Clausen N, Garwicz S, Kristinsson J, Lie SO, Moe PJ, Perkkiö M, Yssing M, Saarinen-Pihkala UM. Intensified treatment of acute childhood lymphoblastic leukemia has improved prognosis, especially in non-high risk patients. the Nordic experience of 2648 patients diagnosed between 1981-1996. Acta Paediatr 1998;87:1151-1161.

77.**Pizzo PA**, Rubin M, Freifeld A, Walsh TJ. The child with cancer and infection. I. Empiric therapy for fever and neutropenia and preventive strategies. J Pediatr 1991;119:679-694.

78.**Santolaya ME**, Alvarez AM, Becker A, Cofre J, Enriquez N, O'Ryan M, Paya E, Pilorget J, Salgado C, Tordecilla J, Varas M, Villarroel M, Viviani T, Zubieta M.

Prospective, multicenter evaluation of risk factors associated with invasive bacterial infection in children with cancer, neutropenia, and fever. J Clin Oncol 2001;19:3415-3421.

79.**Rahiala J**, Perkkio M, Riikonen P. Infections occurring during the courses of anticancer chemotherapy in children with ALL: a retrospective analysis of 59 patients. Pediatr Hematol Oncol 1998;15:165-174.

80.**Linden V**, Karlen J, Olsson M, Palmer K, Ehren H, Hente rJ, Kalin M. Successful extracorporeal membrane oxygenation in four children with malignant disease and severe Pneumocystis carinii pneumonia. Med Pediatr Oncol 1999;32:25-31.

81.**Poulsen A**, Schmiegelow K, Yssing M. Varicella zoster infections in children with acute lymphoblastic leukemia. Pediatr Hematol Oncol 1996;13:231-238.

82.**Nossal GJV**. Current concepts; immunology; the basic components of the immune system. N Engl J Med 1987;316:1320-1325.

83.**Pizzo PA**, Lee JW. Infections in immunocompromised infants and children: Churchill Livingstone Inc.; 1992.

84.**Alanko S**, Pelliniemi T-T, Salmi TT. Recovery of blood B-lymphocytes and serum immunoglobulins after chemotherapy for childhood acute lymphoblastic leukemia. Cancer 1992;69:1481-1486.

85.**De Vaan GAM**, Van Munster PJJ, Backeren JA. Recovery of immune function after cessation of maintenance therapy in acute lymphoblastic leukemia of childhood. Eur J Pediatr 1982;139:113-117.

86.**Mackall CL**, Fleisher TA, Brown MR, Andrich MP, Chen CC, Feuerstein IM, Horowitz ME, Magrath IT, Shad AT, Steinberg SM. Age, thymopoiesis, and CD4+ T-lymphocyte regeneration after intensive chemotherapy. N Engl J Med 1995;332:143-149.

87.**Mackall CL**, Fleisher TA, Brown MR, Andrich MP, Chen CC, Feuerstein IM, Magrath IT, Wexler LH, Dimitrov DS, Gress RE. Distinctions between CD8+ and CD4+ T-cell regenerative pathways result in prolonged T-cell subset imbalance after intensive chemotherapy. Blood 1997;89:3700-3707.

88.**Levy JA**. Pathogenesis of human immunodeficiency virus infection. Microbiol Rev 1993;57:183-289.

89.**Lane HC**, Masur H, Edgar LC, Whalen GR, A. H., Fauci AS. Abnormalities of B-cell activation and immunoregulation in patients with the acquired immunodeficiency syndrome. N Engl J Med 1983;309:453-458.

90.**Bossolasco S**, Nilsson A, de Milito A, Lazzarin A, Linde A, Cinque P, Chiodi F. Soluble CD23 in cerebrospinal fluid: a marker of AIDS-related non-Hodgkin's lymphoma in the brain. AIDS 2001;15:1109-1113.

91.**Terpstra FG**, Al BJ, Roos MT, De Wolf F, Goudsmit J, Schellekens PT, Miedema F. Longitudinal study of leukocyte functions in homosexual men seroconverted for HIV: rapid and persistent loss of B cell function after HIV infection. Eur J Immunol 1989;19:667-673.

92.**Moir S**, Lapointe R, Malaspina A, Ostrowski M, Cole CE, Chun TW, Adelsberger J, Baseler M, Hwu P, Fauci AS. CD40-Mediated induction of CD4 and CXCR4 on B lymphocytes correlates with restricted susceptibility to human immunodeficiency virus type 1 infection: potential role of B lymphocytes as a viral reservoir. J Virol 1999;73:7972-7980.

93.**Schnittman SM**, Lane HC, Higgins SE, Folks T, Fauci AS. Direct polyclonal activation of human B lymphocytes by the acquired immune deficiency syndrome virus. Science 1986;233:1084-1086.

94.**Chirmule N**, Oyaizu N, Saxinger C, Pahwa S. Nef-protein of HIV-1 has B-cell stimulatory activity. AIDS 1994;8:733-734.

95.**Rautonen J**, Rautonen N, Martin NL, Wara DW. HIV type 1 Tat protein induces immunoglobulin and interleukin-6 synthesis by un infected peripheral blood mononuclear cells. AIDS Res Hum Retroviruses 1994;10:781-785.

96.**Karray S**, Zouali M. Identification of the b cell superantigen binding site of HIV-1 gp120. Proc Natl Acad Sci 1997;94:1356-1360.

97.**Scamurra RW**, Miller DJ, Dahl L, Abrahamsen M, Kapur V, Wahl SM, Milner EC, Janoff EN. Impact of HIV-1 infection on VH3 gene repertoire of naive human B cells. J Immunol 2000;164:5482-5491.

98.**Huang C**, Stewart AK, Schwartz RS, Stollar BD. Immunoglobulin heavy chain gene expression in peripheral blood b lymphocytes. J Clin Invest 1992;89:1331-1343.

99.**Macchia D**, Parronchi P, Piccinni MP, Simonelli C, Mazzetti M, Ravina A, Milo D, Maggi E, Romagnani S. In vitro infection with HIV enables human CD4+ T cell clones to induce noncognate contact-dependent polyclonal B cell activation. J Immunol 1991;146:3413-3418.

100.**Wolthers KC**, Otto SA, Lens SM, van Lier RA, Miedema F, Meyaard L. Functional b cell abnormalities in HIV type 1 infection: role of CD40L and CD70. AIDS Res Hum Retroviruses 1997;13:1023-1029.

101.**De Milito A**, Morch C, Sonnerborg A, Chiodi F. Loss of memory (CD27) B lymphocytes in HIV-1 infection. AIDS 2001;15:957-964.

102.**Ditzel HJ**, Barbas SM, Barbas cF, Burton DR. The nature of the auto-immune antibody repertoire in human immunodeficiency virus type 1 infection. Proc Natl Acad Sci 1994;91:3710-3714.

103.**Kroon FP**, van Dissel JT, Ravensbergen E, Nibbering PH, van Furth R. Antibodies against pneumococcal polysaccharides after vaccination in HIV-infected individuals: 5-year follow-up of antibody concentrations. Vaccine 1999;18:524-530.

104.**Laursen AL**, Andersen PL. Low levels of IgG antibodies against pneumocystis carinii among HIV-infected patients. Scand j infect Dis 1998;30:495-490.

105.**Kroon FP**, van Dissel JT, de Jong JC, van Furth R. Antibody response to influenza, tetanus and pneumococcal vaccines in HIV-seropositive individuals in relation to the number of CD4+ lymphocytes. AIDS 1994;8:469-476.

106.**Carson PJ**, Schut RL, Simpson ML, O'Brien J, Janoff EN. Antibody class and subclass responses to pneumococcal polysaccharides following immunization of human immunodeficiency virus-infected patients. J Infect Dis 1995;172:340-345.

107.**Arpadi SM**, Markowitz LE, Baughman AL, Shah K, Adam H, Wiznia A, Lambert G, Dobroszycki J, Heath JL, Bellini WJ. Measles antibody in vaccinated human immunodeficiency virus type 1-infected children. Pediatrics 1996;97:653-657.

108.**Samuelsson A**, Yari F, Hinkula J, Ersoy O, Norrby E, persson MA. Human antibodies from phage libraries; neutralising activity against human immunodeficiency virus type 1 equally improve after expression as Fab and IgG in mammalian cells. Eur J Immunol 1996;26:3029-3034.

109.**Lee M-S**, Cohen B, Hand J, Nokes JD. A simplified and standardized neutralization enzyme immunoassay for quantification of measles neutralizing antibody. J Virol Methods 1999;78:209-217.

110.**Narita M**, Yamada S, matsuzono Y, Itakura O, Togashi T, Kikuta H. Immunoglobulin G avidity testing in serum and cerebrospinal fluid for analysis of measles virus infection. Clin Diagn lab Immunol 1996;3:211-215.

111.**Forghani B**, Schmidt NJ. Antigen requirements, sensitivity and specificity of enzeyme immunoassays for measles and rubella viral antibodies. J Clin Microbiol 1979;9:657-664.

112.**Aggerbeck H**, Norgard-Petersen B, Heron I. Simultaneous quantification of diphteria and tetanus antibodies by double antigen, time-resolved fluorescence immunoassay. J Virol Methods 1996;190:171-183.

113.**Hintzen RQ**, van Lier RAW, Kuijpers KC, Baars PA, Schaasberg W, Lucas CJ, Polman CH. Elevated levels of a soluble form of the T cell activation antigen CD27 in cerebrospinal fluid of multiple sclerosis patients. J Neuroimmunol 1991;35:211-217.

114.**Derdeyn CA**, Costello C, Kilby JM, Sfakianos G, Saag MS, Kaslow R, Bucy RP. Correlation between circulating stromal cell-derived factor-1 levels and CD4+ cell count in human immunodefiency virus type 1-infected individuals. AIDS Res Hum Retroviruses 1999;15:1063-1071.

115.**Mazzini G**, Ferrari C, Erba E. Dual excitation multi-fluorescence flow cytometry for detailed analyses of viability and apoptotic cell transition. Eur J Histochem 2003;47:289-298.

116.**Dorsett BH**, Cronin W, Ioachim HL. Presence and prognostic significance of antilymphocyte antibodies in symptomatic and asymptomatic human immunodeficiency virus infection. Arch Intern med 1990;150:1025-1028.

117.**Winkler C**, Modi W, Smith MW, Nelson GW, Wu X, Carrington M, Dean M, Honjo T, Tashiro K, Yabe D, Buchbinder S, Vittinghoff E, Goedert JJ, O´Brien TR, Jacobson LP, Detels R, Donfield S, Willoughby A, Gomperts E, Vlahov D, Phair J, O´Brien SJ. Genetic restriction of AIDS Pathogenesis by an SDF-1 chemokine gene variant. Science 1998;279:389-393.

118.**Elovaara I**, Sabri F, Gray F, Alafuzoff I, Chiodi F. Upregulated expression of Fas and Fas ligand in brain through the spectrum of HIV-1 infection. Acta neuropathol 1999;98:355-362.

119.**Notermans DW**, de Jong JJ, Goudsmit J, Bakker M, Roos MT, Nijholt L, Cremers J, Hellings JA, Danner SA, de Ronde A. Potent antiretriviral therapy initiates

normalization of hypergammaglobulinemia and a decline in HIV type 1-specific antibody responses. AIDS Res Hum Retroviruses 2001;17:1003-1008.

120.**Moir S**, Malaspina A, Ogwaro KM, Donoghue ET, Hallahan CW, Ehler LA, Liu S, Adelsberger J, Lapointe R, Hwu P, Baseler M, Orenstein JM, Chun TW, Mican JA, Fauci AS. HIV-1 induces phenotypic and functional perturbations of B-cells in chronically infected individuals. Proc Natl Acad Sci 2001;98:10362-10367.

121.**Marche C**, Tabbara W, Michon C, Clair B, Bricaire F, Matthiesen L. Bone marrow findings in HIV-1 infection: a pathological study. Prog AIDS Pathol 1990;2:51-60.

122.**Harris CE**, Biggs JC, Concannon A, J,, Dodds AJ. Peripheral blood and bone marrow findings in patients with acquired immune deficiency syndrome. Pathology 1990;22:206-211.

123.**Brugnoni D**, Airo P, Marino R, Notarangelo LD, van Lier RA, Cattaneo R. CD70 expression on T-cell subpopulations, study of normal individuals and patients with chronic immune activation. Immunol Lett 1997;55:99-104.

124.**Nagase H**, Agematsu K, Kitano K, Takamota M, Okubo Y, A. K, Sugane K. Mechanism of hypergammaglobulinemia by HIV infection: circulating memory B-cell reduction with plasmacytosis. Clin Immunol 2001;100:250-259.

125.**Jacquot S**, Kobata T, Iwata S, Morimoto C, Schlossman SF. CD154/CD40 and CD70/CD27 interactions have different and sequential functions in T-cell-dependent B cell responses: enhancement of plasma cell differentiation by CD27 signaling. J Immunol 1997;159:2652-2657.

126.**Samuelsson A**, Broström C, van Dijk N, Sonnerborg A, Chiodi F. Apoptosis of CD4+ and CD19+ cells during human immunodeficience virus type 1 -infection-correlation with clinical progression, viral load and loss of humoral immunity. Virology 1997;238:180-188.

127.**Torcia M**, Bracci-Laudiero L, Lucibello M, Nencioni L, Labardi D, Rubartelli A, Cozzolino F, Aloe L, Garaci E. Nerve growth factor is an autocrine survival factor for memory B lymphocytes. Cell 1996;85:345-356.

128.**Torcia M**, De Chiara G, Nencioni L, Ammendola S, Labardi D, Lucibello M, P. R, Marlier LN, Bonini P, Dello Sbarba P, Palamara AT, Zambrano N, Russo T, Garaci E, Cozzolino F. Nerve growth factor inhibits apoptosis in memory B lymphocytes via inactivation of p38 MAPK, prevention of Bcl-2 phosporylation and cytochrome c release. J Biol Chem 2001;276:39027-39036.

129.**Cassese G**, Arce S, Hauser A, Lehnert K, Moewes B, Mostarac M, Muehlinghaus G, Szyska M, Radbruch A, Manz RA. Plasma cell survival is mediated by synergistic effects of cytokines and adhesion-dependent signals. J of Immunol 2003;171:1684-1690.

130.**Nance CL**, Shearer WT. SDF-1 regulates HIV-1-gp120 induced changes in CD79b surface expression and Ig production in activated human B-cells. Clin Immunol 2002;105:208-214.

131.**Nakao T**, Kim S, Ohta K, Kawano H, Hino M, Miura K, Tatsumi N, Iwao H. Role of mitogen-activated protein kinase family in serum-induced leukemia inhibitory factor and interleukin-6 secretion by bone marrow stromal cells. British Journal of Pharmacology 2002;136:975-984.

132.**Lorgeot V**, Rougier F, Fixe P, Cornu E, Praloran V, Denizot Y. Spontaneous and inducible production of leukemia inhibitory factor by human bone marrow stromal cells. Cytokine 1997;9:754-758.

133.**Chen X**, Katakowski M, Li Y, Lu D, Wang L, Zhang L, Chen J, Xu Y, Gautam S, Mahmood A, Chopp M. Human bone marrow stromal cell cultures conditioned by traumatic brain tissue extract, growth factor production. J Neurosci Res 2002;69:687-691.

134.**Chan OT**, Madaio MP, Schlomchik MJ. The central and multiple roles of B cells in lupus pathogenesis. Immunol Rev 1999;169:107-121.

135.**Wouters CH**, Ceuppens JL, Stevens EA. Different circulating lymphocyte profiles in patients with different subtypes of juvenile idiopathic arthritis. Clin Exp Rheumatol 2002;20:239-248.

136.**Poels LG**, van Niekerk CC. Plasmodium berghei: immunosuppression and hyperimmunoglobulinemia. Exp Parasitol 1977;42:235-247.

137.**Kafetzis DA**. An overview of paediatric leishmaniasis. J Postgrad Med 2003;49:31-38.

138.**Kogawa K**, Hisai H, Morii K, Horimoto M, Kuya T, Sakamaki S, Watanabe N, Niitsu Y. T-cell mediated polyclonal B-cell activation in a case of B-cell lymphoma associated with polyclonal hypergammaglobulinemia. Int J Hematol 1995;62:253-257.

139.**McHeyzer-Williams MG**, Ahmed R. B cell memory and the long-lived plasma cell. Curr Opinion Immunol 1999;11:172-179.

140.**De Milito A**. Immune activation during HIV-1 infection: implication for b cell dysfunctions and therapy monitoring. 2002.

141.**Hunziker L**, Recher M, Macpherson AJ, Ciurea A, Freigang S, Hengartner H, Zinkernagel RM. Hypergammaglobulinemia and autoantibody induction mechanisms in viral infections. Nat Immunol 2003;4:343-348.

142.**van der Vuurst de Vries AR** CH, Logtenberg T, Meyaard L. Leukocyte-associated immunoglobulin-like receptor-1 (LAIR-1) is differentially expressed during human B cell differentiation and inhibits B cell receptor-mediated signaling. Eur J Immunol 1999;29:3160-3167.

143.**Nagumo H**, Agematsu K, Kobayashi K, Shinozaki K, Hokibara S, Nagase H, Takamoto M, Yasui K, Sugane K, Komiyama A. The different process of class

switching and somatic hypermutation; a novel analysis by CD27- naive cells. Blood 2002;99:567-575.

144.**Bhushan A**, Covey LR. CD40:CD40L interactions in X-linked and non-X-linked hyper-IgM syndromes. Immunol Res 2001;24:311-324.

145.**Van Kooten C**, Banchereau J. CD40-CD40L. J Leukoc Biol 2000;67:2-17.

146.**Oka Y**, Rolink AG, Andersson J, Kamanaka M, Uchida J, Yasui T, Kishimoto T, Kikutani H, Melchers F. Profound reduction of mature B cell numbers, reactivities and serum Ig levels in mice which simultaneously carry the XID and CD40 deficiency gene. Int Immunol 1996;11:1675-1685.

147.**Nakayama T**, Hieshima K, Izawa D, Tatsumi Y, Kanamaru A, Yoshie O. Cutting edge: profile of chemokine receptor expression on human plasma cells account for their efficient recruitment to target tissues. J Immunol 2003;170:1136-1140.

148.**Ek T**, Mellander L, Hahn-Zoric M, Abrahamsson J. Intensive treatment for childhood acute lymphoblastic leukemia reduces immune responses to Diphtheria, Tetanus and Haemophilus influenzae type b. Manus 2004.

149.**Pakker NG**, Kroon ED, Roos MT, Otto SA, Hall D, Wit FW, Hamann D, van der Ende ME, Claessen FA, Kauffmann RH, Koopmans PP, Kroon FP, ten Napel CH, Sprenger HG, Weigel HM, Montaner JS, Lange JM, Reiss P, Schellekens PT, Miedema F. Immune restoration does not invariably occur following long-term HIV-1 suppression during antiretroviral therapy. INCAS Study Group. AIDS 1999;13:203-212.

150.**Moss WJ**, Monze M, Ryon JJ, Quinn TC, Griffin D, F. C. Prospective study of measles in hospitalized, human immunodeficiency virus (HIV)-infected and HIV-uninfected children in Zambia. Clin Infect Dis 2002;35:189-196.

151.**Perry RT**, Mmiro F, Ndugwa C, Semba RD. Measles infection in HIV-infected African infants. Ann N Y Acad Sci 2000;918:377-380.

152.**Tangye S**, Liu Y-J, Aversa G, Phillips J, deVries J. Identification of functional human splenic memory B cells by expression of CD148 and CD27. J Exp Med 1998;9:1691-1703.

153.**Wischhusen J**, Jung G, Radovanovic I, Beier C, Steinbach JP, Rimner A, Huang H, Schulz JB, Ohgaki H, Aguzzi A, Rammensee H-G, Weller M. Identification of CD70-mediated apoptosis of immune effector cells as a novel immune escape pathway of human glioblastoma. Cancer Res. 2002;62:2592-2599.

154.**Agathanggelou A**, Niedobitek G, Chen R, Nicholls J, Yin W, Young LS. Expression of immune regulatory molecules in Epstein-Barr virus-associated nasopharyngeal carcinomas with prominent lymphoid stroma. Evidence for a functional interaction between epitelial tumor cells and infiltrating lymphoid cells. Am J Pathol 1995;147:1152-1160.

155.**Renard N**, Lafage-Pochitaloff M, Durand I, Duvert V, Coignet L, Banchereau J. Demonstration of functional CD40 in B-lineage acute lymphoblastic leukemia cells in response to T-cell CD40 ligand. Blood 1996;87:5162-5170.

156.**Kebelmann-Betzing C**, Körner G, Badiali L, Buchwald D, Möricke A, Korte A, Köchling J, Wu S, Kappelmeier D, Oettel K, Henze G, Seeger K. Characterisation of cytokine, growth factor receptor, costimulatory and adhesion molecule expression patterns of bone marrow blasts in relapsed childhood B cell precursor ALL. Cytokine 2001;13:39-50.

157.**Reichmann E**. The biological role of the Fas/FAsL system during tumor formation and progression. Semin Cancer Biol 2002;12:309-315.

158.**Kelly JM**, Darcy PK, Markby JL, Godfrey DI, Takeda K, Yagita H, Smyth MJ. Induction of tumor-specific T cell memory by NK-cell mediated tumor rejection. Nat Immunol 2002;3:83-90.

159.**Yang FC**, Agematsu K, Nakazawa T, Mori T, Ito S, Kobata T, Morimoto C, Komiyama A. CD27/CD70 interaction directly induces natural killer cell killing activity. Immunology 1996;88:289-293.

160.**Takeda K**, Oshima H, Hayakawa Y, Akiba H, Atsuta M, Kobata T, Kobayashi K, Ito M, Yagita H, Okumura K. CD27-mediated activation of murine NK cells. J of Immunol 2000;164:1741-1745.

161.**Yotnda P**, Garcia F, Peuchmar M, Grandschamp B, Duval M, Lemonnier F, Vilmer E, Langlade-Demoyen P. Cytotoxic T cell responses against the chimeric ETV-AML1 protein in childhood acute lymphoblastic leukemia. J Clin Invest 1998;102:455-462.

162.**Blanca IR**, Bere EW, Young HA, Ortaldo JR. Human B cell activation by autologous NK cells is regulated by CD40-CD40 ligand interaction: role of memory b cells and CD5+ B cells. J of Immunol 2001;167:6132-6139.

163.**Gao N**, Dang T, Yuan D. IFN-gamma-dependent and independent iniation of switch recombination by NK cells. J of Immunol 2001;167:2011-2018.

164.**Fournier AM**, Fondere JM, Alix-Panabieres C, Merle C, Baillat V, Huguet MF, Taib J, Ohayon V, Zembala M, Reynes J, Vendrell JP. Spontaneous secretion of immunoglobulins and anti-HIV-1 antibodies by in vivo activated B lymphocytes from HIV-1-infected subjects: monocyte and natural killer cell requirement for in vitro terminal differentiation into plasma cells. Clin Immunol 2002;103:98-109.

165.**Simone MD**, De Santis S, Vigneti E, Papa G, Amadori S, Aloe L. Nerve growth factor; a survey of activity on immune and hematopoetic cells. Hematol Oncol 1999;17:1-10.

166.**Gronthos S**, Simmons PJ. The growth factor requirements of stro-1 positive human bone marrow stromal precursors under serum-deprived conditions. Blood 1995;85:929-940.

167.**Pantaleo G**, Graziosi C, Demarest JF, Cohen OJ, Vaccarezza M, Gantt K, Muro-Cacho C, Fauci AS. Role of lymphoid organs in the pathogenesis of human immunodeficiency virus (HIV) infection. Immunol Rev 1994;140:105-130.

168.**Macias J**, Japon MA, leal M, Saez C, Pineda JA, Segura DI, Ortega J, Lissen E. Structural normalization of the lymphoud tissue in asymptomatic HIV-infected patients after 48 weeks of potent antiretroviral therapy. AIDS 2001;15:2371-2378.

169.**Brodie C**, Gelfand E, W. Nerve growth factor specifically induces human IgG4 production. J Immunol 1992;148:3492-3497.

170.**Otten U**, Ehrardt P, Peck R. Nerve growth factor induces growth and differentiation of human B lymphocytes. Proc Natl Acad Sci 1989;86:10059-10063.

171.**Labouyrie E**, Parrens M, de Mascarel A, Bloch B, Merlio JP. Distribution of NGF receptors in normal and pathologic human lymphoid tissue. J Neuroimmunol 1997;77:161-173.

172.**Kogner P** EA, Barbany G, Persson H, Theodorsson E, Bjork O. NeuropeptideY (NPY) synthesis in lymphoblasts and increased plasma NPY in pediatric B-cell precursor leukemia. Blood 1992;80:1324-1329.

173.**Auffray I**, Chevalier S, Froger J, Izac B, Vainchenker W, Gascan H, Coulombel L. Nerve growth factor is involved in the supportive effect by bone marrow derived stromal cells of the factor dependent human cell line UT-7. Blood 1996;88:1608-1618.

174.**Juarez J**, Bradstock KF, Gottlieb DJ, Bendall LJ. Effects of inhibitors of the chemokine receptor CXCR4 on acute lymphoblastic leukemia cells in vitro. Leukemia 2003;17:1294-1300.

175.**Muller A**, Homey B, Soto H, Ge N, Catron D, Buchanan ME, McClanahan T, Murphy E, Yuan W, Wagner SN, Barrera JL, Mohar A, Verastegui E, Zlotnik A. Involvement of chemokine receptors in breast cancer metastasis. Nature 2001;410:50-56.

176.**Libura J**, Drukala J, Majka M, Tomescu O, Navenot JM, Kucia M, Marquez L, Peiper SC, Barr FG, Janovska-Wieczorek A, Ratajczak MZ. CXCR4-SDF-1 signaling is active in rhabdomyosarcoma cells and regulates locomotion, chemotaxis and adhesion. Blood 2002;100:2597-2606.