The Germinal Centre Reaction

Genetic and proteomic analysis of factors important for survival and growth of B lymphocytes

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Printed by Intellecta DocuSys AB
Göteborg, Sweden 2008

ISBN 978-91-628-7401-8

Cover illustration:
Hematoxylin and anti-Collagen I stained mouse fibroblasts used in paper I

Abstract

During an immune response the B lymphocytes main function is to produce antibodies in the specific defence against the pathogen. When naïve B lymphocytes become activated by binding of an antigen, the cells differentiate to become antibody producing plasma cells. During this process, some cells form germinal centres after interaction with T lymphocytes, where the immunoglobulin (Ig) genes are differentiated, to evolve high affinity plasma cells or memory B lymphocytes.

We have used the Burkitt lymphoma cell line Ramos to study the germinal centre reaction. Ramos cells are normally cultured in medium containing serum, but we have adapted Ramos cells to long-term survival in a serum-free medium. The serum-free cells are more sensitive to dilution, which indicates a production of autocrine growth factors. We have studied the gene expression changes that occur during adaption to serum-free media by global gene expression analysis, and we found several deregulated genes involved in cell cycle regulation and apoptosis. We also identified a Ramos cell line deficient in MHC class II expression, resembling the situation during Bare lymphocyte syndrome. The cause of this deficiency is studied by examining the function of transcription factors regulating MHC class II expression.

Germinal centre B lymphocytes are highly susceptible to apoptosis unless rescued by survival signals from T lymphocytes and follicular dendritic cells. In an effort to study these interactions we have isolated and identified secreted extracellular proteins produced by serum-free Ramos cells. The expressions of these proteins were also examined in tonsil germinal centre B lymphocytes and the levels were compared with cells in the pre- and post-germinal centre stage of the tonsils.

During the germinal centre reaction the antibody gene of B lymphocytes are differentiated through somatic hypermutation and class switch recombination. These events are dependent on the AID mediated cytidine deamination and involve different DNA repair systems, many of which involve error-prone polymerases. We have studied the function of one of these, Polymerase ζ, by establishing mouse fibroblast cell lines deficient of the Rev3 subunit of Pol ζ. Rev 3 deficient cells are more sensitive to cell cycle arrest caused by UV-radiation or cisplatin treatment than cells with a functional Pol ζ, confirming a function of Pol ζ in the translesion synthesis over DNA nicks and crosslinking lesions.

Original papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals I-IV:

I. Zander L, Bemark M, Immortalized mouse cell lines that lack a functional *Rev3* gene are hypersensitive to UV irradiation and cisplatin treatment.
DNA Repair (Amst). 2004 Jul 2; 3(7):743-52

II. Zander L, Bemark M, Identification of genes deregulated during serum-free medium adaptation of a Burkitt's lymphoma cell line.
Cell Proliferation. 2008; 41:136-55

III. Zander L, Friskopp L, Bäckström M, Bemark M, Proteomic analysis of proteins secreted by germinal centre B lymphocytes.
Manuscript

IV. Zander L, Bemark M, Spontaneous loss of MHC class II expression in a transformed B cell line – a potentially new mutation leading to Bare lymphocyte syndrome.
Manuscript

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Abbreviations

AID	Activation-Induced cytidine Deaminase
APC	Antigen Presenting Cell
BCR	B Cells Receptor
BER	Base Excision Repair
CSR	Class Switch Recombination
CTL	Cytolytic T Lymphocyte
DC	Dendritic Cell
DNA	Deoxyribonucleic acid
DSB	Double Stranded Break
FDC	Follicular Dendritic Cells
HR	Homologous Recombination
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LPS	Lipopolysaccaride
LT	Lymphotoxin
MHC	Major Histocompatibility Complex
MMR	Mismatch repair
MZ	Marginal Zone
nanoLC-FT-ICR MS	nano -liquid chromatography Fourier transform-ion cyclotron resonance mass spectrometry
NER	Nucleotide Excision Repair
NHEJ	Nonhomologous end-joining
NK cell	Natural Killer cell
PCR	Polymerase Chain Reaction
Pol	Polymerase
RAG1/2	Recombination Activation Gene 1/2
RNA	Ribonucleic acid
SHM	Somatic Hyper Mutation
SSA	Single Stranded Annealing
TCR	T Cell Receptor
TdT	Terminal Deoxynucleotidyltransferase
TGF	Transforming Growth Factor
T_H1	T helper cell type 1
T_H2	T helper cell type 2
TNF	Tumour Necrosis Factor
UDG	Uracil DNA glycosylase

Introduction

The Immune Response

An immune response is the body's defence against foreign substances such as bacteria, virus and helminths. The immune system can be divided into an unspecific innate immunity and a targeted specific adaptive immunity. The innate immunity is always active to retain the body from harmful microbes. Cells of the innate immunity are situated in all exposed tissues and are often the first to recognize the pathogen, leading to the killing of microbes or infected cells. Activated innate immune cells also produce signals for initiating inflammation with the recruitment of new leukocytes, which will lead to an extensive cell expansion and differentiation, to evolve microbe specific effector cells of the adaptive immune defence. The microbes are eliminated directly by the innate immunity that will also differentiate the adaptive immunity to produce cytokines and immunoglobulin (Ig) molecules that will mark the pathogen, resulting in a more powerful response from the innate immunity. When an infection is cleared, many cells are no longer needed and must die through apoptosis. Only a few microbe specific cells survive as a long lasting memory of the infection, leading to the ability to more efficiently clear the infection during a secondary exposure.

For the immune defence to function properly, it has to be tightly controlled. A misdirected immune response may lead to hypersensitivity, and, if the immune defence react to the body's own cells, autoimmune diseases may develop. On the other hand, a defective immune system will lead to susceptibility to pathogens, and in some cases the inability to clear infections, which could be lethal.

Innate immunity

The innate immunity is a first rapid defence that responds within hours against an infection. The body is constantly exposed to pathogens, but effective epidermal and mucosal barriers prevent the microbes to enter the body at the exposed sites, such as the skin and the respiratory and gastric tracts. If the

microbes manage to cross the barriers and enter the body, circulating phagocytic neutrophils are the first to be activated[1]. Cells belonging to the innate immune defence recognize molecular patterns that are characteristic for microbial pathogens, but are not expressed by mammalian cells, such as unmethylated DNA motifs (CpG), dsRNA, LPS or microbial glycoproteins and glycolipids (Fig. 1). Neutrophils are, together with macrophages, phagocytic cells that recognize these structures through recognition by receptors such as Toll like receptors (binds CpG, dsRNA and LPS) or mannose receptors (glycoproteins and glycolipids)[2,3]. The microbe is phagocytosed into the cell and is killed by microbicidal products such as free radicals. Infections of intracellular microbes are recognized by natural killer cells (NK cells) by the binding of NKG2D ligands, which are expressed on the cell surface of stressed infected cells[4]. Besides the NKG2D receptors the NK cells also express the inhibitory receptors, killer cell Ig-receptors (KIR) and CD95 lectin-like receptors, that recognize normal cells expressing major histocompatibility complex (MHC) class I and thereby inhibit the cytotoxicity toward these cells. Infected cells are killed using granules filled with perforin that creates pores in the infected cell and thereby target the microbe. NK cells are also one of the main producers of IFNγ, a cytokine that activates macrophages. Macrophages are key regulators of the immune response where it not only work as phagocytic cells in the defence of both intracellular and extracellular microbes, but also activates the adaptive immunity. There is a tight connection between the two immune systems where e.g. macrophage production of IL-12 activates T cells, that in turn activates the innate immunity, by the production of IFNγ[5, 6].

Adaptive immunity

The effector cells in the adaptive immune response are T lymphocytes and B lymphocytes. Unlike the cells of the innate immunity, T and B cells can produce specific, highly diverse receptors for recognition of microbial antigens (T cell receptors and Ig molecules respectively). The T lymphocytes cannot recognize an antigen alone, but has to have a peptide fragment of the antigen presented to it by a major histocompatibility complex (MHC) molecule[7]. There are two classes of MHC that present peptides to T cells[8]. MHC class I (HLA-A, B and C in humans) is expressed on virtually all nucleated cells, and is mainly presenting intracellular peptides to $CD8^+$ cytotoxic T cells (CTLs) in cell-mediated immunity (Fig. 1). This acts as an indicator of infection of the cell by intracellular microbes, mainly viruses. Peptides from the microbe are presented by MHC class I molecules to be recognized by polymorphic T cell receptors (TCRs) on CTLs[9]. The infected

cell is then killed by the CTL through secretion of perforin and proteases that induce apoptosis[10]. This reaction is enhanced by activated $CD4^+$ type I T helper cells[11]. MHC class II (HLA-DO, DQ and DR, in humans) has more restricted expression, and is found on professional antigen presenting cells (APCs), such as dendritic cells, macrophages or B lymphocytes, but also by a few other cell types after induction.

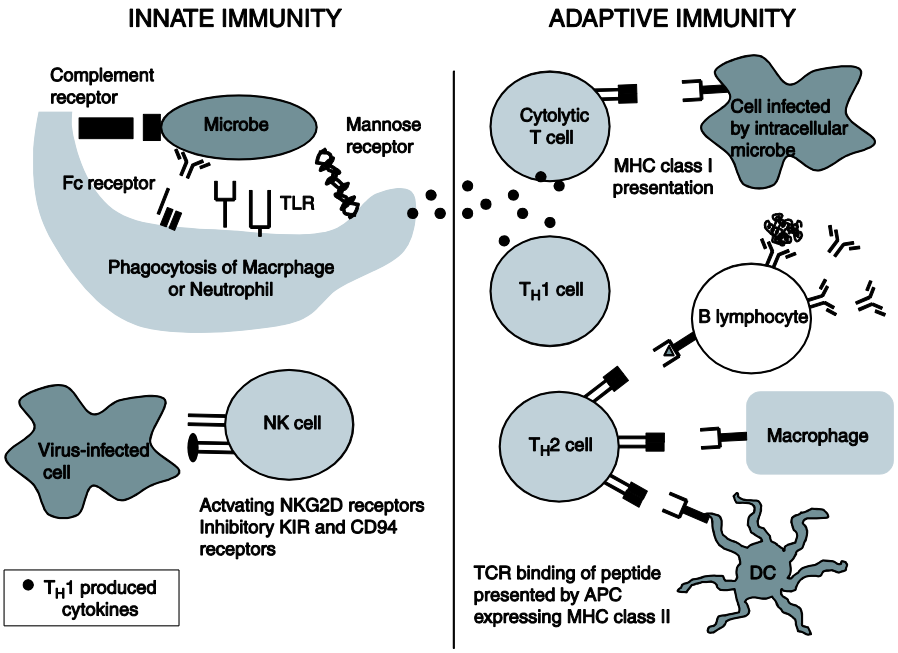

Figure 1. ***Recognition of microbes and activation of the immune defence.*** The innate immune defence contain neutrophils and macrophages that recognize different structures on a microbe by mannose and Toll like receptors. The microbe can also be identified by antibodies or complement molecules, which induce phagocytosis by the neutrophils or macrophages after binding to Fc or complement receptors respectively. NK cells recognize cells that are stressed by virus-infection through the expression of NKG2D ligands. The killing of NK cells are tightly controlled by the inhibitory receptors KIR and CD94 that inhibit the response through binding of MHC molecules.
During an adaptive immune response T cells are activated by the presentation of antigen peptides by MHC molecules. In a cell-mediated response cytolytic T cells kill cells infected by intracellular microbes through the interaction of TCR with MHC class I. T helper 1 cells are specialized cytokine producing cells that contributes to a stronger cell-mediated response as well as innate immune response. T helper 2 cells recognize peptides presented by MHC class II on antigen presenting cells (i.e. B cells, macrophages and dendritic cells). Through this interaction, the T cells become activated and can thereby induce a stronger response by i.e. activate a humoral response with B cell production of soluble antibodies.
TLR, Toll like receptor; NK cell, Natural killer cell; T_H1, T helper cell type 1; T_H2, T helper cell type 2; MHC, Major histocompatibility complex; TCR, T cell receptor; APC, Antigen presenting cell; DC, Dendritic cell

Most naïve (i.e. cells that have not seen antigen) T helper cells are located in lymphoid organs, and are activated when antigen are presented by specialized APCs. Peripheral unmature dendritic cells mature upon binding to an antigen. During this process, they enter the circulation and reach lymphoid organs where they activate T cells and thereby function as antigen presenting cells[12]. The type 1 T helper cells main function is to help CTL by inducing proliferation and to activate macrophages of the innate immunity through the secretion of IFNγ[13]. T helper 2 cells produce cytokines (e.g. IL-4) and other stimuli for the activation of B lymphocytes of the humoral immunity. B lymphocytes are antigen presenting cells that produce antibodies that recognize antigens such as external structures on the microbes or toxins. The antigens can, depending on its origin and characteristics, activate the B lymphocyte in a T cell dependent or T cell independent manner. In both cases, the B lymphocyte differentiate to an antibody secreting plasma cell[14]. The antibodies neutralize antigens by binding and, together with the complement system, activate different cell types of the innate immunity such as macrophages, neutrophils, NK cells and eosinophils. In a T independent response, low affinity IgM antibodies are mainly produced, but in T dependent response, the antibody response can develop into high affinity antibodies of different subclasses. B lymphocytes, as other APCs, express MHC class II on their cell surface, which present peptides from extracellular microbes to T helper cells[9]. Both T lymphocytes and B lymphocytes can differentiate into long lived memory cells following activation that sustain recognition upon secondary infection by the same microbe.

All together, the immune system is composed by an intricate collaboration of different effector systems that all have the purpose of protecting the body from harmful pathogens. In this thesis, I have studied the function of B lymphocytes and the development of high affinity antigen specific Ig genes.

B lymphocytes

B lymphocytes can be divided into three mature naïve subsets depending on B cell receptor (BCR) specificity and cell location. B1 cells are a unique B cell subset with self-renewal capacity, that is stationated in the peritoneal and pleural cavities[15]. B1 cells mainly produce low affinity IgM antibodies that are specific for polysaccharides and lipids such as LPS. These antibodies, called natural antibodies, are produced even without any signs of infection and is thought to mediate rapid humoral response[16, 17]. B1 plasma cells also seem to participate in specific mucosal immunity producing T cell-independent IgA antibodies, in response to commensal bacteria in the gut[18]. Marginal zone B lymphocytes are found in close contact with blood sinusoids of the spleen and constitute the main effector population against blood born antigens[19, 20]. They, like B1 cells, differentiate into low affinity IgM antibody producing plasma cells in the early response toward thymus-independent antigens[21]. The third B cell subset is the B2 follicular B lymphocytes. Naïve follicular B lymphocytes circulate through secondary lymphoid organs until activated by a protein antigen[19]. They usually respond in a thymus-dependent manner and can differentiate to either long-lived, low affinity producing plasma cells or into a high affinity antibody producing plasma or memory cells.

The B lymphocyte receptor

The B lymphocyte receptor (BCR) is the membrane bound form of antibody expressed by B cells. As a result of antigen binding to the BCR, the naïve B lymphocytes become activated[22]. The BCR is composed of two antigen binding Ig molecules, the Ig heavy and the Ig light chain, and two signal transducing molecules Igα and Igβ[23] (Fig. 2). B lymphocytes bind to antigen through three hypervariable regions, called complementarily-determining regions (CDRs), in the variable part of the Ig gene[24, 25]. When an antigen is bound to the BCR the Ig molecules on the surface interact in a cross-linking resulting in a signalling through Igαβ for activation and proliferation[26]. Soluble antibodies lack the transmembrane tail due to differential splicing and are accessible for Fc receptors that bind to the constant part of the Ig molecule.

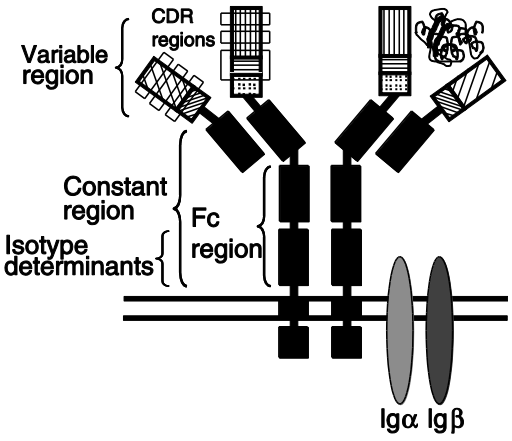

Figure 2. ***The B cell receptor***. The B cell receptor is composed of an Ig heavy and an Ig light chain together with the signalling molecules Igα and Igβ. The heavy chain consists of several constant parts, namely the isotype determinant, which determines the Ig molecules subclass, the Fc receptor-binding site and a transmembrane and cytoplasmic part that anchor the molecule to the cell surface. The variable part of the Ig gene is diversified through V(D)J recombination and is then able to bind an antigen in the CDR regions, which are also the main sites for SHM during affinity maturation.

Development of B lymphocytes

B lymphocytes develop from haematopoietic stem cells in the bone marrow throughout life to maintain a diversified pool of B lymphocytes[27] (Fig. 3). In tight connection with stromal cells, the haematopoietic cells proliferate and differentiate into B lymphocytes in response to IL-7 and by the interaction between the adhesion molecules VLA-4 and VCAM-1[28, 29]. The most critical step during the B lymphocyte development is for the cell to express functional antibodies.

B lymphocytes develop antibodies with capacity to bind different antigen after a random gene expression through V(D)J recombination[30]. In the Ig loci there are hundreds of equivalent gene segments, but only one of each V, D and J are expressed on a single cell[31]. V(D)J recombination is restricted to immature B and T lymphocytes (T cells recombine their T cell receptor for peptide recognition), and is initiated through specific enzymes. Deficiency of these recombination-activation gene 1 and 2 proteins (RAG1 and 2) in mice leads to a complete block in B and T cell development[32, 33]. Initially, RAG1 or RAG2 binds the recombination recognition sequences adjacent to one of the D and J segments in the Ig heavy gene[34] (Fig. 4). The function of RAG1 and 2 is to cut the DNA sites into blunt or hairpin ends and to display these ends to other proteins[35]. Hairpin ends are then opened by a newly identified member of the nonhomologous end joining (NHEJ) repair system called Artemis[36]. Artemis cleaves the hairpin ends and these are then processed from long overhangs to blunt ends. This result in the addition of one or two nucleotides in the coding joints[37]. The double stranded breaks are reconnected by other members of the NHEJ repair system, leaving a switching circle with the additional D and J

segments behind[38]. Artemis and several other members in the NHEJ system have been shown essential for V(D)J recombination[39-42]. The newly formed DJ segment is then connected to one of the V regions to complete the variable part of the heavy chain[34]. To further increase the diversity of Ig genes, the 3' ends of the cleaved gene segments can be elongated by an enzyme called terminal deoxynucleotidyltransferase (TdT)[43, 44]. The light chain of the Ig gene is recombined in a similar way, but do not achieve the same degree of diversity. Light chain genes only consists of V and J elements and TdT is not expressed during light chain recombination[30, 45]. After the V(D)J recombination the Ig gene is transcribed for expression on the cell surface. Due to different splicing forms the Ig molecule can be expressed as either IgM or IgD[46].

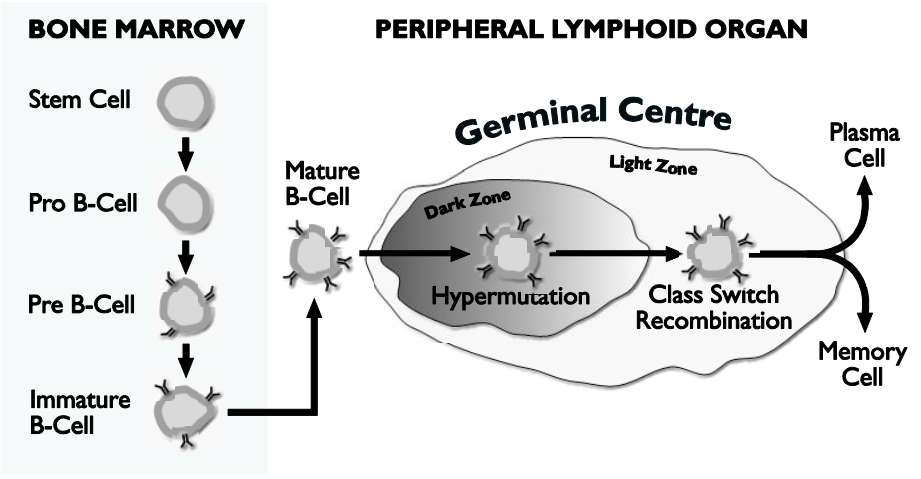

Figure 3. ***B cell development and differentiation.*** B lymphocytes develop from haematopoietic stem cells in the bone marrow. To become a mature B lymphocyte the cells differentiate and start to express Ig molecules. During the pro-B cell stage the V(D)J recombination occurs of the heavy chain, which is expressed and tested during the pre-B cell stage together with the surrogate light chain. A rearranged light chain replaces the surrogate light chain and the antibody is tested for function and reactivity against self-antigens at the immature B cell stage, before the cell enter the circulation as a mature B lymphocyte.
A B lymphocyte that becomes activated by binding of an antigen can, through T cell interaction in a peripheral lymphoid organ, start to proliferate to form a germinal centre. In the germinal centre, the B cell diversifies its Ig genes through somatic hypermutation and class switch recombination, to eventually re-enter the circulation as high affinity antibody producing plasma cells or memory B cells.

At the pro-B cell stage the B lymphocyte starts with the V(D)J recombination of the heavy gene. The B cell first recombinates one of the two heavy chain alleles[28, 45], and if the translocated gene can be transcribed, the other allele is

silenced (allelic exclusion)[47]. If the transcription is out of frame, e.g. because of random incorporated nucleotides by TdT, the other allele is used for V(D)J recombination and transcribed[48]. This mechanism ensures that the cells only express antibodies that bind one type of antigen. If a functional recombination is performed the cell will survive and start to express the heavy chain on the cell surface together with a surrogate light chain indicating that the cell is in a pre-B cell stage[49, 50] (Fig. 3). At this stage the cells also express the Igαβ to test the function of the recombined Ig heavy molecule. Only cells with functional pre-B cell receptor will survive and become a mature B cells[51]. During the immature B cell stage VJ recombination of one of the alleles of the light chains κ and λ occur. A recombined κ and λ light chain replaces the expression of surrogate light chain, and the complete Ig molecule is expressed on the cell surface and tested[52]. Only B lymphocytes with a functional BCR will survive. The immature cells are also tested for their reactivity toward self-antigens through a negative selection[22, 53]. Upon binding of the BCR during this stage, the cells either are going through apoptosis or will have their Ig molecules renewed through receptor editing[54]. When the variable part of the Ig gene is complete it will be expressed as IgM and IgD before the cell leaves the bone marrow and enter the circulation as a mature B lymphocyte.

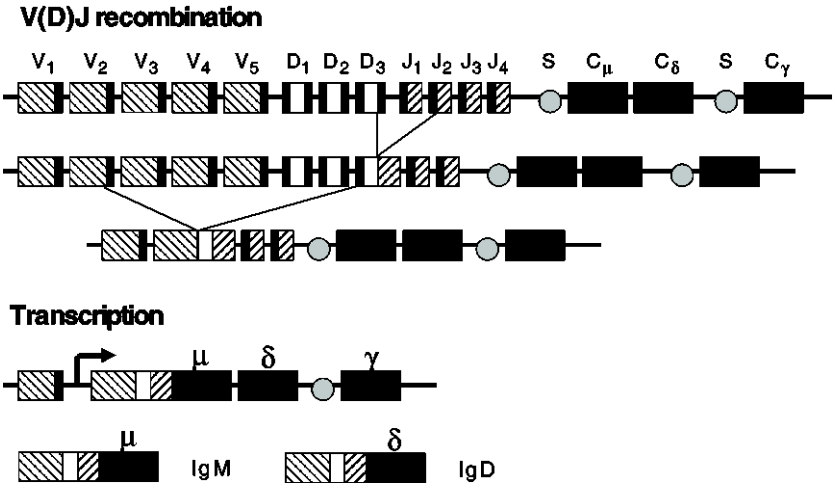

Figure 4. ***V(D)J recombination.*** During V(D)J recombination one of each variable parts (V, D and J) are combined through recombination of gene segments. First one of the D segments is joined with one of the J segments, and then the DJ part is recombined to one of the V segments. V(D)J recombination occurs during early B cell development and antibodies are expressed by naïve mature B lymphocytes as IgM or IgD, which is regulated at a scriptional level through differential splicing.
V, variable; D, Diversity; J, Joining; C, Constant genes; S, Switch elements; E, Enhancer element; Arrow, Primer

Functions of the B lymphocyte in an immune response

The main function of B lymphocytes is to produce antibodies, also called immunoglobulin (Ig) molecules, which has a key function in the humoral immune response. The antibodies, both membrane bound and soluble, are highly polymorphic and can bind a variety of different antigens. Ig genes in B lymphocytes go through three types of genetic alterations, i.e. V(D)J recombination, somatic hypermutation (SHM) and class switch recombination (CSR), to achieve highly diverse antibodies[55]. V(D)J recombination occurs in all B cells during early development while SHM and CSR only takes place in activated cells to achieve high affinity antibodies with specialized functions[56, 57].

The membrane bound Ig molecules are connected to other molecules in a B cell activation complex called the B lymphocyte receptor, while secreted antibodies are diffusing freely throughout the body. The antibodies can be divided into five subclasses depending on the constant part of the Ig gene (IgM, IgD, IgA, IgG and IgE) that has different functions[58]. Complement receptors and Fc receptors expressed by cells in the innate immune system such as neutrophils, macrophages and NK cells recognize the soluble antibodies bound to microbes, and these antibodies are thereby inducing killing of the pathogen[59-61]. IgM is the first isotype synthesized by a B lymphocyte. IgM (and to some degree also IgG and IgA) activates the complement system leading to phagocytosis of the microbe by neutrophils and macrophages[62]. IgG is the most abundant isotype in serum and binds to Fc receptors on both phagocytotic cells and NK cells. IgG is also, together with IgA (that is mainly found in the gut and in secretions), specialized on neutralization of toxins. IgE can, together with eosinophils, provide a protection against infection of helminths[63].

Secondary lymphoid organs

The secondary lymphoid organs are the spleen, lymph nodes and lymph node-like structures as the tonsils and the Peyer's patches in the gut. They are composed of T cell rich areas and B cell follicles, and circulating B lymphocytes first enter the T cell zone and then migrate to the B cell follicle. Naïve follicular B lymphocytes are located in the follicles of secondary lymphoid organs, where they stay about a day before they re-enter the blood circulation again either directly or via the efferent lymph[64]. Apart from the T and B cell areas, there is a marginal zone in the spleen surrounding the

lymphoid structure. This is comprised by a mixture of marginal zone B lymphocyte and memory B cells, as well as several macrophage subtypes[65, 66]. The marginal zone B lymphocytes respond rapidly to blood borne thymus independent antigens with high IgM production[67]. The memory B lymphocytes, on the other hand, react as circulating memory cells do, to antigens in a thymus dependent response that differentiate the cells to proliferation and Ig secretion[68]. Which memory B cells that sustain the memory B cell phenotype and which differentiate into plasma cells seems to be dependent Ig isotype, as differentiated plasma cells are mostly producing IgG and the memory B cells produce IgM as well as switched isotypes[69]. In a thymus dependent response, the activation of B cells is dependent on T cells that differentiate them into either short lived extrafollicular plasma cells or into proliferating germinal centre B lymphocytes that re-enter the follicle. The cells then eventually return to the circulation as memory B cells or long lived plasma cells[70, 71].

The formation and compartmentalization of secondary lymphoid organs depend on proteins from the tumour necrosis factor (TNF) family and interaction with APCs such as dendritic cells and macrophages that produce signals and transport antigens for activation of B cells inside the lymphoid organ[72, 73]. Different TNF family interactions, together with BCR specificity, also determine B cell fate of immature B cells and the differentiation to either MZ cells or follicular B lymphocytes. BCR engagement also seems to determine whether the naïve cells will become short lived plasma cells in an extrafollicular response or develop into GC B lymphocytes[74, 75]. The TNF member lymphotoxin is necessary for establishment of T and B cell zones in lymphoid tissues[76], while BAFF and APRIL have roles in the development of mature follicular B cells, GC B cells and Ig production[77-79]. BAFF is expressed by several different cell populations such as T cells, dendritic cells, macrophages and FDC, and cab bind to three different receptors BAFF-R, BCMA and TACI, all expressed by B cells [80-82]. Deficiency in the main BAFF receptor, BAFF-R, leads to a severe reduction in mature B lymphocytes[81], and the three receptors for BAFF have different functions during a germinal centre reaction. Thus, BAFF seems to be involved in both B lymphocyte maturation and germinal centre differentiation. BAFF-R is expressed throughout the B cell maturation, but is downregulated when the B cells differentiate to become GC B cells[80]. At the same time the B cells start to express BCMA, the main receptor for Ig secreting cells, and traces of TACI appears to be an inhibitory BAFF receptor that primarily functions in B1 cells and marginal zone B cells [83, 84].

The B cell interaction with T cells

T lymphocytes function as direct effectors in the defence against intracellular microbes, as well as activators of B lymphocytes and macrophages. B lymphocytes in the lymphoid follicle present antigens that can be recognized by the T cell receptor, which binds to the peptide to specifically activate the T cell. B lymphocytes that encounter an extracellular antigen through interactions with its BCR are activated and start to process this antigen for presentation to T cells. The antigen is cleaved into small peptide fragments that can be displayed on the cell surface by MHC class II molecules, and upregulation of co-stimulatory molecules such as B7 molecules takes place[85, 86]. When a $CD4^+$ T cell recognize a peptide, the MHC class II molecule also interact with CD4, and with the help of signalling through the co-stimulatory molecules, the T cell is activated and start to proliferate. Activated T cells are in turn necessary for B cell differentiation to Ig secreting plasma cells, both for an extrafollicular response and germinal centre formation, through CD40-CD40L interaction and the production of cytokines[87, 88]. Thus, a normal MHC class II function is essential for a normal adaptive immune response and MHC class II deficiency, seen in Bare lymphocyte syndrome, leads to the failure to mount immune responses and the development of functional B lymphocytes[87].

The Germinal Centre Reaction

Mature follicular B lymphocytes circulate through peripheral lymphoid organs until they recognize an antigen. Upon antigen binding the cells become activated and migrate towards T cell areas in the lymphoid tissue. The antigen is processed inside the B lymphocyte, to be presented to T lymphocytes on MHC class II molecules[89]. Signals provided by activated T cell promote the B lymphocytes to proliferate, and many cells differentiate into early IgM-producing plasma cells.

Specific interaction with the T cell will also direct some cells to enter B cell follicles, where they start to proliferate rapidly to form germinal centres. The major part of the antibody diversity is created in the bone marrow through V(D)J recombination, which creates a pool of cells able to bind to different antigens. The antibody-antigen interaction is, however, fine-tuned during an immune response to yield higher binding affinities, a phenomenon known as affinity maturation. This process takes place in the germinal centres. The activated B lymphocytes proliferate vigorously and diversify their antibody genes through antibody gene-specific random mutagenesis (somatic hypermutation)[90]. Cells with increased affinities to an antigen are then selected, and high affinity cells leave to form memory B lymphocytes and antibody secreting plasma cells.

The formation of a germinal centre

For a germinal centre to form, the B lymphocyte first has to recognize an antigen. The B cell then need to interact with T cells which gives the CD40-CD40L interaction that is necessary for both germinal centre formation and memory B cell development[91, 92]. Circulating B cells express several proteins from the TNF family, i.e. $LT\alpha_3$, $LT\alpha_1\beta_2$ and $TNF\alpha$, which are essential for compartmentalization of lymphocytes within secondary lymphoid organs[76]. $LT\alpha_1\beta_2$ expressed by B lymphocytes is important for development and differentiation of FDCs in the secondary follicle that upregulates the expression of CXCL13 to attract CXCR5 positive B lymphocytes that migrates into the follicle[76, 93]. Thus, follicles form and is maintained through a positive loop of signals between B cells and FDCs. The FDC also express the adhesion molecule VCAM-1, also seen on bone marrow stromal cells, that together with ICAM-1 bind LFA-1 on B cells, to attach the B cell to the FDC and keep the B cell in the follicle[94]. It is also thought that the FDC provide

activated B lymphocytes with signals that makes them differentiate to centroblasts and start to proliferate to form a dark zone of the germinal centre[95]. In the dark zone, the B cells hypermutate their Ig gene before they migrate to the light zone when they differentiate into non-proliferating centrocytes. In the light zone, B cells carrying high affinity antibody molecules are selected for further survival and the cells also go through class switch recombination that changes the constant part of the Ig gene from IgM to IgA, IgE or IgG. Both of these events are dependent on the expression of activation-induced cytidine deaminase (AID)[96, 97]. AID deaminates cytosine in the Ig gene, which is necessary for both mutations during somatic hypermutation (SHM) and breaks during class switch recombination (CSR). Eventually, the B lymphocytes will differentiate into long-lived plasma cells or memory B lymphocytes[98]. This differentiation is mediated by signals from T cells, such as IL-4 and IL-10. IL-4 drives differentiation of B cells to memory B lymphocytes and IL-10 toward plasma cells. CD40L and the two cytokines together with FDC have shown to be enough to differentiate GC B lymphocytes to memory B cells and plasma cells *in vitro*[99].

The role of follicular dendritic cells

FDC and T cells are necessary for survival and differentiation of B lymphocytes at different stages of the germinal centre reaction. The FDC seems to provide the B lymphocytes with signals for rapid proliferation as well as survival signals throughout the germinal centre reaction. The newly formed proliferating centroblasts are highly susceptible to apoptosis, due to high expression of pro-apoptotic factors such Fas and low level expression of anti-apoptotic proteins such as Bcl-2[100]. Rescue signals from FDC are thus essential for B cells to avoid apoptosis and to sustain proliferation[99, 101]. The cellular selection in the germinal centres is thought to operate through survival signals given to high-affinity cells, with low-affinity cells following a default apoptotic pathway[102]. The B lymphocytes that evolve high affinity antibodies are rescued from apoptosis through an intricate process involving competition for limiting amounts of antigen. FDC can bind immunocomplexes (soluble antigen-antibody complexes) by their complement (CD21 and CD35)[103] or Fc receptors (CD23 and CD32)[104, 105] for several months for presentation to B cells. B cells with high affinity antibodies will compete successfully for FDC presented antigens, and will survive, probably due to signals provided by the FDC as well as interaction with T cells. The immunocomplexes that are captured and retained by the receptors are delivered to the neighbouring B cells in the form of iccosomes[106]. Iccosomes are immunocomplex-coated bodies, delivered to germinal centre B lymphocytes for subsequent presentation to T cells.

Somatic hypermutation

Somatic recombination of the V(D)J genes results in high diversity among antigens and the possibility to bind a variety of antigens. The primary antibodies however, usually bind antigens with a modest affinity and specificity, which can be improved by somatic hypermutation[90]. Random mutations are introduced in the variable part of the Ig gene where the antibody binds to the Ig gene[107] (Fig. 5). In the complementarily-determining regions of the variable part of the Ig gene there are palindromic hotspot sequences composed of RGYW/ WRCY motifs (where R is A or G, Y is C or T and W is A or T), in which the somatic mutations are concentrated[108]. The modification of the genome by SHM results in point mutations, insertion and deletion of nucleotides[90, 109].

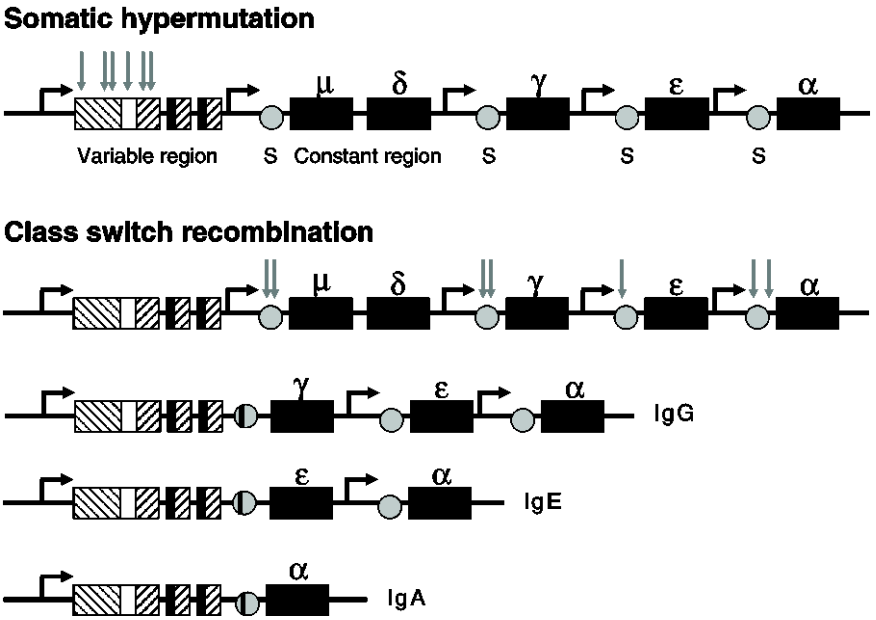

Figure 5. ***Somatic hypermutation and class switch recombination.*** During somatic hypermutation, mutations are introduced in the variable part of the Ig gene to achieve variability in the antigen binding site of the antibody. Mutations are also introduced in the switch regions (S) between the constant regions of the Ig gene, but also contributing in isotype switch from IgM to IgG, IgE or IgA.

SHM is initiated by the transcription of the variable part of the Ig gene and by the binding of AID[110] (Fig. 6). AID is similar to the mammalian RNA editing deaminase APOBEC-1, the catalytic component of a complex that changes ribocytidine to uridine in apolipoprotein B mRNA in gastrointestinal tissue[111]. Unlike APOBEC-1, AID introduces mutations in germinal centre B

lymphocytes, where it binds to the nontemplate single stranded DNA during transcription[107, 112, 113]. The proteins do, however, share the deamination capacity of cytidine and the incorporation of uracil[114]. The incorporated uracil base can be replicated over, causing the reversion to C/G, or uracil can be transcribed as a thymidine causing T/A mutations. Most SHM mutations are acquired in this pathway, which is evident by the number transitions on C/G bases[115]. Uracil residues can also be recognized and removed by the base excision repair enzyme uracil DNA glycosylase (UDG) resulting in an abasic site[116, 117]. The abasic site can then be replicated over resulting in any of the four nucleotides, resulting in transversions at C/G sites. The mutations at A/T sites, together with the insertions and deletions found in connection to SHM[118], seems not to be targets by AID itself but rather an effect of the mismatch repair system (MMR). MMR is a repair system that recognizes lesions in newly synthesised DNA, and while repairing this defect introducing new mutations in SHM by the usage of error prone polymerases (described in the Gene mutations and repair section). There are also evidence for blunt end double stranded breaks during SHM[119, 120]. The direct function of these is not clear but it is suggested that they are repaired by homologous repair (HR) with the involvement of error-prone polymerases causing mutations around the lesion.

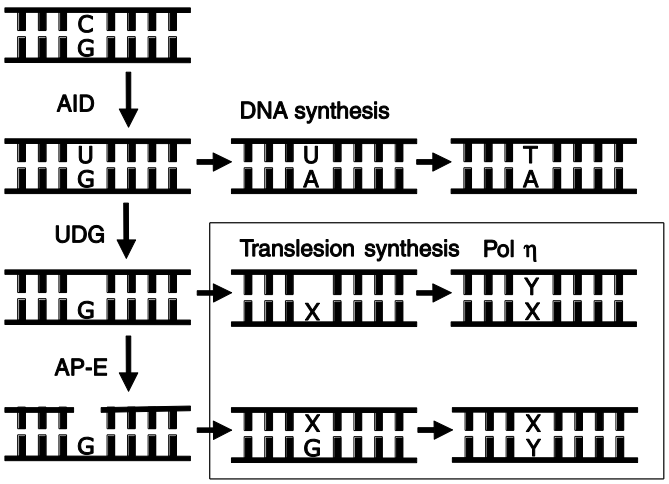

Figure 6. ***Ig gene diversification model.*** The mutations in SHM are initiated by the deamination of cytosine to uracil by AID. The uracil can then be replicated over, forming a T/A mutation, or, if the other strand is replicated, the lesion will be repaired. By the use of base excision repair proteins, an abasic site can be formed by UDG and the sugar-phosphate chain can be cleaved by AP-endonuclease (AP-E). These lesions are thought to be replicated over by Polymerase η as a part of the translesion synthesis system causing a mutation to any of the four base pairs T/A, A/T, C/G or G/C.

Class switch recombination

Isotype switch recombination is a process where the variable chain segment (VDJ) is associated with a different constant region, from Cμ to Cα, Cε or Cγ (Fig. 5). In humans there are four genes of the IgG subtype ($C\gamma_{1\text{-}4}$) and two different IgA genes ($C\alpha_{1,\ 2}$). The recombination occurs when B lymphocytes differentiate to become centrocytes in the light zone of the germinal centre[121]. Which type of antibody that are produced depends on the cytokine milieu, and e.g. IL-4 induce the B cells to IgG and TGF-β to IgE[122, 123]. The cytokines direct the class switching by inducing sterile transcription of one of the subclass switch regions and thereby permitting the binding of AID[124, 125]. The transcription will, like SHM, initiate the introduction of AID induced mutation in the transcribed switch region and the formation of abasic sites by UDG[97, 116]. The mutations found in switch regions are, like the mutations in the variable Ig regions, concentrated to C/G nucleotides in RGYW hotspots and mainly result in transitions[126]. Some of the abasic sites are, however, presumed to be targets of AP endonuclease that cleaves the sugar-phosphate chain developing a single stranded break in the DNA[127]. Two nearby nicks will lead to a double stranded break (DBS) in the transcribed switch regions resulting in a DNA loop with the intervening constant regions deleted[128]. The cleaved switch regions are then repaired by the nonhomologous end joining (NHEJ) system, a system not required in SHM[129, 130].

Gene Mutations and Repair

The genomic stability is constantly challenged by accidental attacks of intermediates produced during normal metabolism as well as by the exposure of environmental DNA damaging agents. The main external mutagenic affects on the body are UV-radiation and the inhalation or ingestion of chemic carcinogens. Specialized DNA editing systems persistently repair these mutations, or if these systems fail, the replication is stopped and the cell dies[131]. Occasionally a mutated strand is used as a DNA template during replication and the mutation is thereby preserved in that cell linage. This requires the use of error-prone polymerases that are involved in a translesion synthesis system that can tolerate DNA damage during replication in a final rescue for the survival of a mutated cell.

DNA repair systems

Cellular DNA is susceptible to accidental damage from normal metabolites such as hydroxyl or oxygen radicals[132, 133]. Simple lesions i.e. the addition of a miscoding alkylation (e.g. O^6-metylguanine), are repaired by a plain damage removal by specialized enzymes. Most DNA lesions though, have to be repaired by the removal of the damaged base or a short DNA segment. Base excision repair (BER) recognizes a variety of damaged bases such as some of those produced by oxygen radicals (Fig. 7). DNA glycosylases are a group of enzymes operating in BER that each recognise a specific lesion in the DNA strand[134]. UDG, also functional in SHM, is one of these DNA glycosylases that recognizes uracil in the DNA strand[133]. The DNA glycosylase removes the damaged base and attracts AP endonuclease to the site that then cleaves the sugar-phosphate chain at the abasic site[135]. The gap is filled by the incorporation of an undamaged nucleotide by polymerase β and the single stranded break is repaired by the XRCC1- Ligase III complex[136-138], or, if more than one base have been replaced, by Pol δ or Pol ε together with Ligase I[139].

The most abundant types of UV-induced damage are nucleotide dimers, thymidine-thymidine cyclobutane pyrimidine dimers and thymidine-cytosine photoproducts[140]. These are all causing adducts in the DNA chain that seem to be recognized by the XPC-hHR23B protein of the nucleotide excision repair system (NER)[141-143]. NER is a repair system that is specialized on recognizing

a variety of helix-disorting DNA lesions, and are initiated by forming a TFIIH complex that will separate the two strands around the lesion[144, 145]. A fragment of 24-32 bp of the damaged strand is then cut out by two nucleases, XPG and ERCC1-XPF creating a single stranded gap[146, 147]. Finally the gap is refilled by polymerase δ or ε and sealed by Ligase I[148].

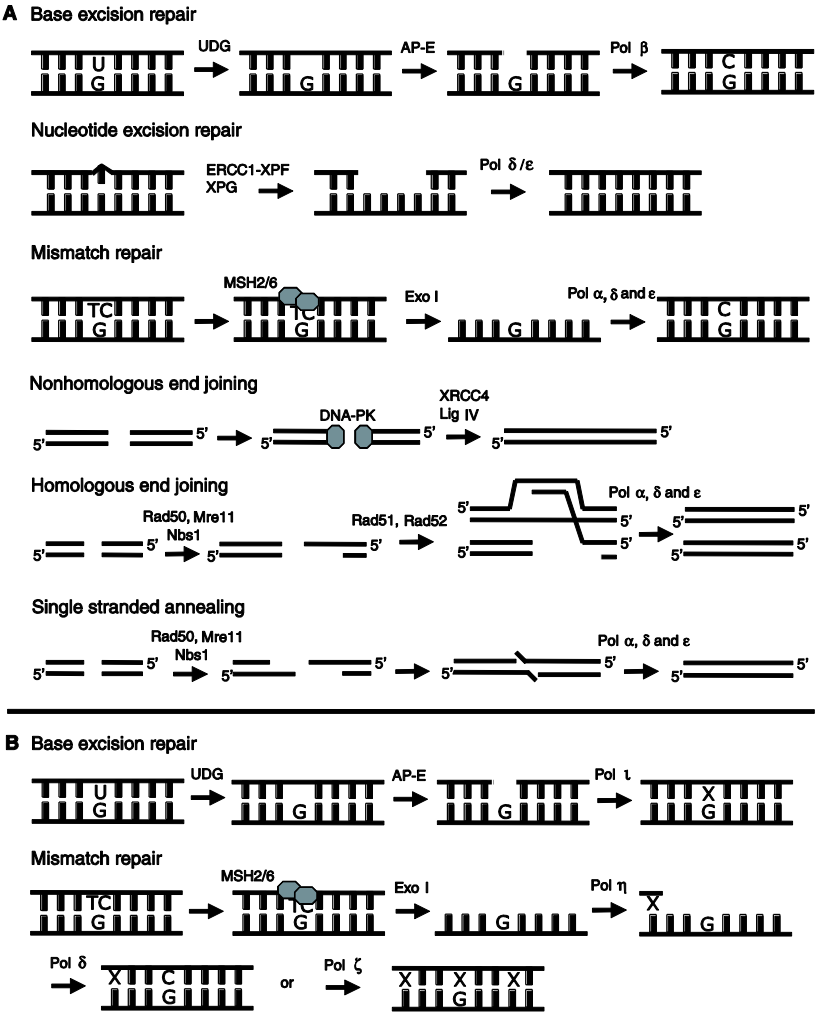

Figure 7. ***Schematic description of the DNA repair systems and their use in SHM.*** (A) An overview of the DNA repair systems (for details, see text). (B) The function of BER and MMS in somatic hypermutation. In SHM the repair systems are altered to, instead of repairing the lesion, introduce mutations using error-prone polymerases. In BER Pol β is thought to be replaced by Pol ι or Pol η and in MMR mutations can be introduced around the lesion by Pol η. The replication can then be extended by the conventional Pol δ or by the error-prone Pol ζ causing additional mutations.

During replication, high fidelity polymerases (i.e. Pol α, δ and ε) that seldom introduces mutations are used. Nevertheless, incorrect nucleotides can be inserted in the newly synthesised strand. These are recognized and repaired by the mismatch repair system (MMR). MMR can also recognize incorporated lesions such as UV induced nucleotide dimmers that are incorrectly replicated over[149]. The newly synthesised strand is distinguished from the template strand by an unknown mechanism, but recognition of nicks in the misincorporated strand has been suggested[150]. The DNA lesion is then recognized by one of the MSH heterodimers together with the efficiency enhancers MLH1 together with MLH2/ 3or PMS1/ 2[151-153]. The MSH heterodimer MSH 2/ MSH6 mainly recognize substitutions and one base insertions or deletions, while the MSH2/ MSH3 binds all kinds of insertions and deletions[154, 155]. The bound MSH complex then seems to recruit exonuclease I that forms a 100-200 bp long single stranded gap around the DNA lesion, which can then be repaired by the normal replication machinery[156, 157].

Double stranded breaks (DSB) are probably the most dangerous lesions to the cell, as they provide opportunity for inappropriate translocations. They are caused during ionizing radiation, by exposure of certain chemicals and indirectly as a product of blocked replication. The major repair system of DSB is the NHEJ pathway, but the DSB can also be repaired during replication by homologous recombination (HR) or by single stranded annealing (SSA). During nonhomologous end-joining the DNA ends are recognized and bound by the Ku70 and Ku80 proteins that attract activated DNA-PK to the ends[158, 159]. Single stranded overhangs can often form hairpin and other damaged ends that are processed by the protein Artemis to facilitate the repair[36]. The DNA-PK complex makes the rejoining by the DNA Ligase IV and Xrcc4 heterodimer easier and the break is repaired in a mechanism that enables excision of intermediate DNA fragment, seen in both V(D)J recombination and CSR[41, 42, 160]. In homologous recombination the DSB is repaired without any loss of information, but this requires an additional gene copy of the DNA, only accessible during replication. The DSB is bound by the Rad50, Mre11 and Nbs1 complex that reduce one strand of the DSB ends forming 3' single stranded overhang. The 3' single stranded ends can then be bound by Rad52 that attract further proteins including the strand exchange protein Rad51[161]. Rad52 act as a facilitator for strand invasion and annealing of the single stranded overhang to the undamaged chromosome[162]. The replication is then continued by the ordinary polymerases and the two fragments can be reconnected. In single stranded annealing, the 3' overhangs are produced in the same way as in HR but are directly annealed by short homologous regions in the overhang. The gaps are filled by polymerase α, δ

and ε[163], and the excessive overhangs are trimmed by the MMR system before all single stranded gaps are repaired by a ligase.

Damage tolerance and error-prone DNA polymerases

DNA with damaged nucleotides cannot be transcribed by the normal replication machinery composed of the high fidelity polymerases α, δ and ε[164]. To minimize cell death resulting from replication blockage, a damage tolerance system where error prone polymerases can replicate over a lesion has evolved. The first discovered polymerase involved in damage tolerance was Pol ζ that was first identified in yeast[165]. Since then, up to 15 new polymerases have been discovered in humans with different fidelity degrees and functions. Some of the polymerases discovered have almost as high fidelity as the conventional polymerases but are involved in damage tolerance as they can insert a nucleotide opposite a lesion. Pol η is coded by the XPV gene and has been shown to be involved in the repair of UV lesions. Pol η is not a part of the NER system but can insert a nucleotide opposite a UV initiated thymidine-thymidine dimers, and XPV deficiency has shown to cause replication defects in response to UV radiation[166, 167]. The replication can then be continued by the conventional Pol δ or by the error prone Pol ζ [168]. Pol η is also involved in MMR, where it can incorporate a nucleotide with higher fidelity than Pol δ opposite some lesions, and in the insertion of an A or C opposite an abasic site[169, 170]. Another polymerase that seems to be involved in the insertion of A or C during MMR of an abasic site is Rev1[170]. In both cases, the replication can be continued by the polymerases Pol δ or Pol ζ.

Other polymerases are highly error prone with the capability to insert incorrect nucleotides to undamaged DNA. Pol κ is a highly error-prone polymerase that with a high rate incorporates T to G transversions on undamaged DNA[171]. It has the possibility not only to incorporate one nucleotide but to extend the replication with spontaneous mutations[172]. Pol ι is a polymerase that can both incorporate incorrect nucleotides opposite undamaged DNA and lesions, and extend wrongly synthesized lesions[173]. It preferable incorporates a G opposite a T instead of an A and also adds a G in an abasic site[174]. Pol ζ is a polymerase with low fidelity, that can insert a nucleotide, preferentially an A, opposite an abasic site, but do not incorporate nucleotides opposite other lesions efficiently itself[168]. Pol ζ is rather specialised on extending the replication after an incorrect basepair caused by one of the other error-prone polymerases as Pol η or Pol ι[175].

DNA repair in somatic hypermutation and class switch recombination

In SHM, a high mutations rate is observed in the Ig genes of germinal centre B lymphocytes. These are not corrected by the normal repair system, either due to a specific inactivation of the system or because of the high mutation load. CSR is also dependent on mutation introducing double strand beaks for initiation, but so far the function of different repair system in antibody diversity is not clear.

Previous studies have shown that the ordinary repair systems are functional during SHM and CSR. The base excision repair system has a function in both systems, as it is involved in the formation of abasic sites in response to the deamination of AID (Fig. 6). It seems though that the system is altered, as it does not correct the abasic site efficiently. It has also been shown that germinal centre B lymphocytes have a normal mismatch repair system, and that MMR deficiency, rather than increasing the mutation rate, skews the rate toward G/C mutations [176-178]. The mutations are then also more concentrated to the hotspot fragments in accordance with AID deamination of cytidine. Thus, the MMR system is during SHM and CSR important for a high mutation rate especially at A/T base pairs and the development of new Ig subclasses, instead of repair[179, 180]. Nucleotide excision repair, on the other hand, have not been found to be involved in either somatic hypermutation or class switch recombination[181].

The establishment of mutations is thought to be by the substitution of conventional polymerases by error-prone polymerases. The error-prone polymerases involved in SHM and DSB mutation have been suggested to be the starting polymerase η, possibly in cooperation with Pol ι opposite a lesion, and then the synthesis is continued by the extension polymerase ζ [182-185]. Polymerase μ is a polymerase that is expressed in low levels during replication, but is upregulated in germinal centre B lymphocytes, indicating a possible function in SHM or CSR[186]. Pol μ is so far the only polymerase suggested to be specific for germinal centre reactions, unexpectedly, no major effects in mutations in centroblasts were seen in Pol μ deficient mice indicating that Pol μ is dispensable for both SHM and an efficient immune response [187]. On the other hand has deficiency of Pol κ not shown any changes in mutation distribution and seems not to be involved in SHM[188].

By a diminished expression of Pol β in the BER repair system and instead using the repair of an error-prone polymerase such as Pol ι and Pol η, AID introduced G/C mutations are retained from being corrected[189, 190] (Fig. 7).

However, it does not explain either the A/T mutations or why mutations are not repaired by the mismatch repair system. Also in MMR error-prone polymerases seem be the explanation for the altered function of the repair system. The MSH2/MSH6 complex, that recognize substitutions or one base insertions and deletions, is active together with exonuclease I, that creates a single stranded gap around the AID introduced deamination[191, 192]. In normal MMR, the conventional polymerases are filling the gap, while in SHM and CSR it seems to be repaired by error prone polymerase. These mutations can be both A/T and G/C mutations, and are not only restricted to hot spot sequences, which could then explain the mutation pattern seen in somatic hypermutation. In fact, deletion of Pol η or Pol ζ has shown to decrease at least A/T mutations and may be essential for normal hypermutation. Also, in the repair of DSBs seems error-prone polymerases, foremost Pol ζ, to be important for the initiation of mutations around the cleavage site[193, 194].

Transformation of Cells

Cancer is a family of diseases that involve uncontrolled cell division and tissue invasiveness (metastasis). When a cell is transformed to a tumour cell it becomes immortalized due to deregulation of cell growth and cell cycle regulation. This transformation occurs as a consequence of alterations causing oncogene activation and tumour suppressor gene inactivation. Oncogenes are genes involved in cell cycle progression, such as c-myc and Bcl-2, that when overexpressed promote uncontrolled cell growth[195, 196]. Tumour suppression genes functions as regulators of cell cycle progression or promotes cell death, e.g. p53[197, 198], and have in tumour cells a reduced expression or deficient function. These gene alterations lead to cell growth that is not controlled by the ordinary cell cycle signals, such as growth factors and hormones. The transformed cells also often influence the surrounding cells in the tissue to produce growth factors, or are able to produce these factors themselves, in an autocrine manner to induce proliferation (extensively studied in e.g. myeloma cells[199]). Accordingly, the growth of tumour cells is not as stringently controlled as the growth of primary cells. Still most tumour cell lines are still dependent on serum-containing medium when cultured *in vitro*. The serum provides the cells with growth factors, hormones and other nutrients. The limited growth of tumour cells in serum-free medium likely reflects a need for external growth factors.

Mutations and translocations

The transformation of a cell is the result of several gene alterations that together promote cell cycle deregulation and uncontrolled cell growth[200]. It has been proposed that cancer cells need the ability to self-produce growth signals as well as insensitivity to growth inhibitory signals. Essential for transformation is also to have limitless replicative potential and an ability to avoid apoptosis. These features makes the cells grow in an uncontrolled manner, but for efficient tumour formation the cells also need to be able to sustain angiogenesis and perform tissue invasion and metastasis[201]. These changes can be the consequence of e.g. different genetic alterations generated by environmental factor such as UV-radiation, alterations in chromosome numbers, gene amplifications and/or chromosome translocations. Different retroviruses can also promote malignancy by infecting cells and thereby induce the production of different oncogenes[202]. The major cause for the formation of lymphomas is through translocations. Chromosomal

translocations are initiated by double stranded breaks that are not correctly repaired[203]. Since DSBs are frequently introduced during diversification of B cell and T cell receptors, these genes are often targets of translocations in neoplastic B and T cells[204]. The induction of DSB in B cells has, together with the inactivation of NHEJ repair system and deficiency of the tumour suppressor gene p53, shown to induce oncogenic translocations in B lymphocytes[205].

Burkitt lymphoma

Many B cell lymphomas correspond to the same developmental stage as germinal centre B lymphocytes. Burkitt lymphoma is one of these and is, because it is easy to culture *in vitro*, often used to study germinal centre B cell biology. Burkitt lymphoma has a cell division rate among the highest of any human cancer types. A hallmark is a translocation of the c-myc gene to one of the immunoglobulin genes, which results in deregulated expression of c-myc[206, 207] (Fig. 8). The Myc protein family (to which c-myc belongs) are all nuclear transcription factors that regulate cellular proliferation, differentiation, apoptosis and transformation, that are often involved in oncogenesis[196, 208].

There are two forms of Burkitt lymphomas defined by the origin of the cancer. Endemic Burkitt lymphomas are primarily found in Africa and correlates with EBV infection, while sporadic cases can be found all over the world and are not related to EBV. In both types, the c-myc gene is translocated; in about 80% of the cases into the Ig heavy gene, and in the rest to either of the Ig light chain genes[209]. The c-myc translocations differ between endemic and sporadic cases, but they both appear to be aberrant by-products of the genetic alterations that take place in the Ig gene. In endemic Burkitt lymphoma, the whole c-myc gene is usually translocated to one of the Ig heavy chain joining regions. Several possible explanations for this type of translocation have been proposed. Thus, the translocation may have occurred at a pre-B cell stage when double stranded DNA breaks are introduced by RAG proteins during V(D)J recombination, but may only result in transformation when the cells are activated in germinal centres or there is re-expression of RAG proteins in germinal centre B lymphocytes that rearrange c-myc[210]. Alternatively, the translocation occurred during AID-mediated somatic hypermutation of the immunoglobulin genes in the germinal centres[211, 212]. With regard to the sporadic Burkitt lymphomas, these typically have a translocation between the heavy chain switch region and the first, noncoding intron of the c-myc gene[213]. This type of translocation is assumed

to occur when double stranded breaks introduced are erroneously repaired during AID-mediated class switch recombination[214].

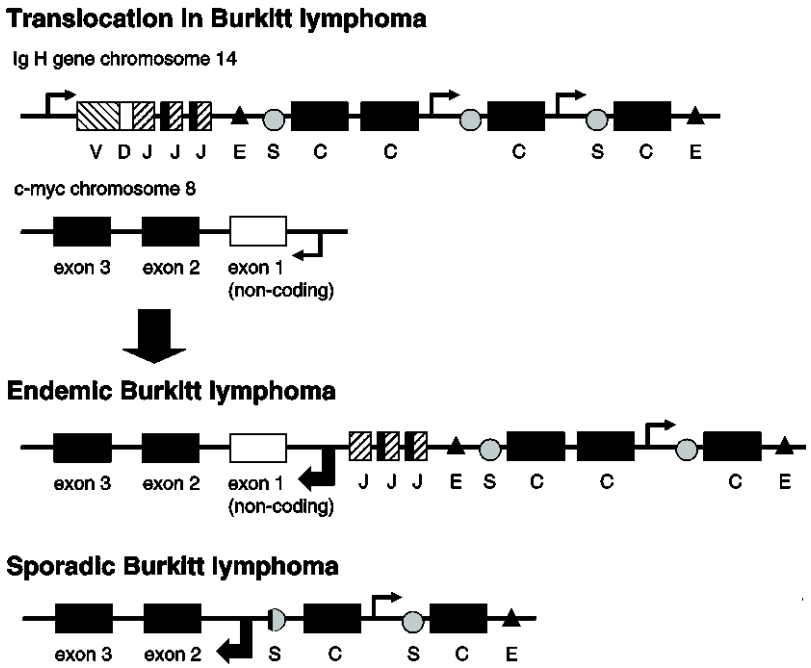

Figure 8. ***Translocation of c-myc in Burkitt lymphoma.*** In Burkitt lymphoma the oncogene c-myc is translocated into one of the Ig genes, causing an overexpression of c-myc by the transcription enhancers in the Ig gene. In endemic Burkitt lymphoma the whole c-myc gene is translocated into the variable region of the Ig gene, while the two coding exons are often replaced into the switching regions in sporadic Burkitt lymphoma. Due to the translocation site into the variable part in endemic Burkitt lymphoma, the rearrangement is thought to occur during V(D)J replication, while the rearrangement into the switch region of sporadic Burkitt lymphoma seems to involve SHM.
V, variable; D, Diversity; J, Joining; E, Enhancer element; S, switch region; C, Constant region

When c-myc is translocated into an immunoglobulin gene, this allele is the only c-myc allele to be transcribed[207, 215]. The normal promoters are located in the first exon of c-myc, but in the sporadic type of Burkitt lymphoma the first exon is not translocated, so an alternative promoter located in the first intron is usually the active site for transcriptional initiation. The immunoglobulin enhancers are thought to be the conclusive factor for c-myc to become an oncogene and the evolvement of Burkitt lymphoma. The immunoglobulin intron enhancer is not translocated in sporadic Burkitt lymphoma because the translocation break is located in the switch region. Enhancer elements in the 3' part of the Ig gene (MHS1-MHS4) therefore appear to be the major cause for the upregulation of the c-myc gene[216, 217].

Aims

The main objective is to study the differentiation of B lymphocytes into high affinity antibody producing cells in the germinal centre reaction. We have focused on three parts:

1. The neoplastic features, in particular the serum dependence, of the Burkitt lymphoma cell line Ramos.

2. The complex interactions between B lymphocytes and other cells inside the germinal centre through identification of secreted extracellular proteins.

3. The involvement of the error-prone polymerase ζ in repair of DNA lesions that may play roles during somatic hypermutation

Methological Considerations

Cell Material

Ramos

Germinal centre B lymphocytes enter apoptosis rapidly *in vitro* unless they are given proper stimulation. Such stimulations will, however, differentiate them from their GC phenotype[218] To study germinal centre B lymphocytes, we have instead used a germinal centre like Burkitt lymphoma cell line called Ramos. The Ramos cell line is a typical sporadic Burkitt lymphoma with a translocation of the c-myc gene to the switch region of the Ig heavy chain gene[219]. The c-myc gene is, however, intact in the cell line, with all three exons transcribed. This translocation is likely to have occurred in a germinal centre reaction, just before or during class switch recombination, as Ramos expresses IgM from the non-translocated locus. Ramos has retained its germinal centre phenotype and hypermutates its immunoglobulin genes[215].

We have managed to get the Ramos cell line to survive and proliferate in medium that does not contain any serum. Two serum-free cell cultures, SFI and SFII, were used to evaluate the serum dependency and the alterations in gene expression, involved in the adaption to serum-free media (Paper II). Ramos cells cultured in serum-free medium were also used for the isolation of extracellular proteins, to ensure that the protein material was not contaminated by serum proteins (Paper III). One of the serum-free Ramos clones (SFI) was found to express no MHC class II on the cell surface, thus mimicking Bare lymphocyte syndrome. The cause of the deficiency was evaluated in paper IV.

Rev3 deficient cells

To study the role of polymerase ζ, cell lines from REV3 gene targeted mice were generated and studied (the mice have previously been described[220]).

REV3 is one of two subunits of polymerase ζ, and has previously shown to be essential for embryonic development in mice and consequently no homozygously targeted mice are born due to midgestion abortions. Therefore, adherent cells were isolated from $REV3^{N/N}$ $REV3^{+/N}$ and $REV3^{+/+}$ embryos and these were cultured *in vitro* to study the functions of polymerase ζ. The immortalized cell lines lost p53 expression during the transformation (Paper I, Fig 2d). p53 deficiency is a common gene alteration in human cancers and is also a common deficiency in immortalized fibroblastic cell lines[221, 222].

Tonsil B lymphocytes

B lymphocytes were isolated from human tonsils and sorted into a pre-GC ($CD38^{int}$ IgD^{+}), GC ($CD38^{hi}$) and post-GC ($CD38^{int}$ IgD^{-}) subpopulation to evaluate the expression of genes encoding extracellular proteins in normal germinal centre B lymphocytes (Paper III).

Evaluating Cell Characteristics

Immunohistochemistry

The $REV3^{N/N}$ $REV3^{+/N}$ and $REV3^{+/+}$ cells were labelled for expression of Collagen I, an extracellular matrix protein expressed by fibroblasts.

Cell proliferation

The proliferation of Ramos lines were measured by counting plated cells for a total of ten days using a Bürker chamber. To investigate the effect the serum medium have on Ramos cells the three cultures, one serum dependent and two serum-free cultures (SFI and SFII), were grown in both serum medium and serum-free medium (Paper II).

To study if polymerase ζ deficiency has any impact on cell proliferation, plated $REV3^{N/N}$ $REV3^{+/N}$ and $REV3^{+/+}$ cells were counted for 3 or 5 days. Cloning efficiency of the cells was also tested by plating one, three or ten cells per well, and after three weeks scoring wells with cell growth (Paper I).

Cell cycle analysis

We studied cell cycle progression of the different cell cultures to see how serum deprivation (Paper II) or polymerase ζ deficiency (Paper I) effected the cells. To do so we used a BrdU technique where the different stages in the cell cycle (G_1, S, G_2 and apoptotic cells) are separated based on the incorporation of BrdU and leakage into cells of the dye 7-AAD.

Flow cytometric cell analysis

To evaluate the expression of different membrane proteins and cell markers, such as IgM (Paper II), HLA-DR, HLA-DP and MHC class I (Paper IV) and CD markers such as CD20 and CD38 (Paper III), flow cytometric cell analysis of cells labelled with fluorescently labelled antibodies against these marker were used.

Protein Identification

2D-gels

Two dimensional gel electrophoresis was used to separate extracellular proteins produced by Ramos serum dependent cells and the two serum-free cultures SFI and SFII according to isoelectric point and protein size. Protein secreted from the cell cultures were separated on gels and the gels were stained for protein density evaluation (Paper III).

nanoLC-FT-ICR MS

A mixture of extracellular proteins from the two serum-free Ramos cultures was used for protein identification using nanoLC-FT-ICR MS technology. The protein mixture was trypsinated, and the peptide masses were measured for a peptide mass fingerprint that could be compared to the NCBI and Swissprot protein databases for identification (Paper III).

Mutation Analysis

DNA damage

To study the involvement of polymerase ζ in repairing different kind of DNA lesions, the $REV3^{N/N}$, $REV3^{+/N}$ and $REV3^{N/N}$ fibroblasts were subjected to different treatments that either introduce base lesions (hydrogen peroxidase), single stranded nicks (UV-radiation), crosslinking (cisplatin) or double stranded breaks (Zeocin) and compared these with the effect of Blasticidin an antibiotic that inhibits protein synthesis (Paper I).

Plasmid expression experiment

pEF6v5-His vectors expressing the transcription factors CIITA, RFXANK, RFXAP, RFX5 and the CBP inhibitory protein CRI1 were used in an attempt to restore the expression of MHC class II in Ramos SFI cells. The EF-1α promoter seems to be preferable compared to CMV promoter during Ramos transfection (data not shown). The vector inserts were cloned using PCR technique and were fully sequenced before transfection into the cells. To test the plasmids, COS-7 cells were transiently transfected, before the plasmid was electroporated into Ramos cells. Stable transfectants were selected and anti-v5 western blots were used to identify clones expressing the inserts. The expression of MHC class II was tested using flow cytometry of the cells (Paper IV).

DNA sequencing

PCR analysis of DNA from IgM negative cells from the three Ramos cultures, serum dependent cells, SFI and SFII cultures, were preformed to study the mutations causing the loss of IgM. The variable part of the Ig heavy gene was amplified and the DNA sequence was sequenced and compared to a functional IgM sequence (Paper II).
A similar experiment was preformed to establish if there were any mutations in the transcription factors, CIITA, RFXANK, RFXAP and RFX5, and other proteins deregulated in SFI cells causing the MHC class II deficiency. These regulatory gene segments were amplified and sequenced from SFI cells and compared to the sequence of serum dependent cells (Paper IV).

Gene Expression

Micro array analysis

The global gene expression of Ramos cells culture in serum was studied using Affymetix Gene Chip technology and compared with the gene expression of serum-free cells. Genes that were differently expressed in cells adapted to serum-free medium were studied in relation to gene expression of serum-free cells that had been transferred back to serum medium for two days. The proposed functions of the differently expressed genes were studied using the NCBI data base (Paper II).

Northern blot

Northern blot was used to verify the micro array results. Gene expressions were studied in the three different Ramos cultures together with the changes in expression during re-addition of serum medium to SFI and SFII (Paper II).

Real-time PCR

Gene expression of some of the identified extracellular proteins produced by serum-free Ramos cells was studied in different subpopulations of normal tonsil B lymphocytes using real time PCR. The gene expressions were compared between the subpopulations as well as between tonsils and Ramos cells (Paper III).

Real time PCR was also used to examine the changes in gene expression from the micro array results together with northern blot (Paper II)

Results and Discussion

We have used the Burkitt lymphoma cell line Ramos to study germinal centre B lymphocytes. Ramos cells have a germinal centre phenotype and hypermutate their Ig genes constantly[215]. Ramos cells are, like most cell lines, normally cultured in medium containing foetal calf serum to provide the cells with growth factors and nutrients. Serum medium contains a diversity of unknown components, which may vary between different serum batches. Culture in serum containing medium can therefore be disadvantageous in many cases. We have managed to adapt Ramos cells to long-term culture in medium without external growth factors. The serum dependent cells were adapted to serum-free medium through successive dilution, which initially led to cell cycle arrest and extensive cell death. In the majority of cases, no living cells were seen but approximately one in 15 million cells did survive, and started to proliferate (Fig. 9). Two different serum-free cultures (SFI and SFII) were established that had similar growth rate and cell cycle progression as serum dependent Ramos cells. Cells grown under serum-free conditions are, however, more sensitive to dilution. The apoptosis associated with dilution can be avoided if conditioned media is added. Thus, it appears that the cells are producing autocrine growth factors that are needed for survival.

In **paper II** we studied the changes the cells must go through to be able to survive in the absence of external growth factors in serum-free media. The global gene expression of the two serum-free cultures, SFI and SFII, was studied and compared with the expression of serum dependent Ramos cells using micro array technology. To avoid clonal alterations, SFI and SFII were grouped and compared both with serum dependent cells and serum-free cells transferred back to serum containing medium. We found some genes that were deregulated, and about twice as many of these were upregulated as downregulated in the serum-free cells (23 and 12 respectively; p value < 0,005, quotient 2,0). The differently expressed genes were mainly involved in cell growth and survival and the expression changes of most of the genes were not altered when the cells were transferred back to serum containing medium,

this indicates that the alterations were steady changes that were selected during the adaption to serum-free medium.

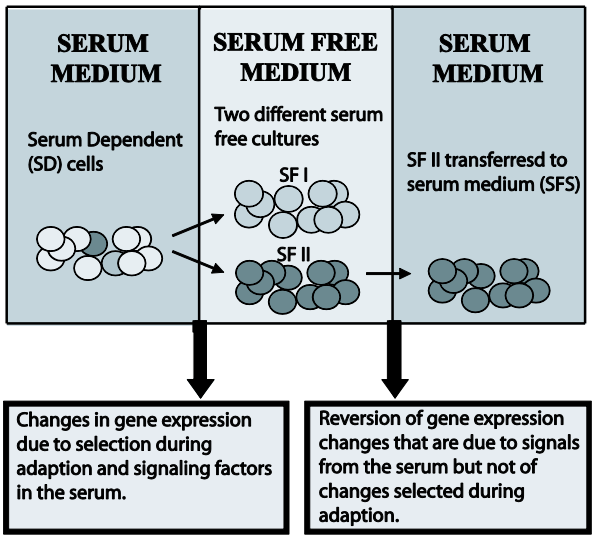

Figure 9. ***Schematic figure demonstrating the experimental setup in paper II.*** Serum-dependent Ramos cells (SD) were adapted to serum-free medium, which resulted in two distinct serum-free cultures (SFI and SFII). SFII cells were then recultured in medium containing serum to evaluate if the genetic changes were restored in the response to serum.

The usage of global expression analysis gives us the opportunity to identify new alterations and whole pathways instead of only evaluating a few already known growth factors. This new information gives us a hint about the capability of transformed cells to survive in a self-stimulating way resembling the tumour growth in the body. By comparing the serum-free cultures with serum-free cells transferred back to serum containing medium we can also establish if the factors are produced in response to the direct response to the lack of serum components or if it is stable adaptions necessary for survival without external growth factors (Fig. 9). Two growth factors have previously been shown to be produced by Ramos cells grown in serum-free medium, growth hormone (GH) and prolactin-like protein[223-225]. A polyclonal anti-GH antibody was shown to inhibit cell growth, but neither human GH alone nor GH in combination with human prolactin-like protein, affected proliferation of serum-free cells. Thus, in these studies, the identified factors do not seem to be responsible for the survival in the absence of external growth factors. This is also indicated from our results as expressions were similar in serum and serum-free cells. No single alternative growth factor was found in the micro array results that could explain the survival of Ramos cells in serum-free medium, or the sensitivity to dilution of the conditioned medium. An

explanation could be that all Ramos cells produce similar amounts of the growth factors as serum cells, but only in cells that are stressed by serum deprivation, the factors are essential for survival. It could also be that the two serum-free cultures SFI and SFII have adapted to serum-free conditions in two different ways resulting in the production of different growth stimulating factors in the two populations.

Among the changes in gene expression, we found several genes involved in apoptosis that were either upregulated or downregulated. For example we found several antiapoptotic genes with decreased expression in the serum-free cultures, such as Huntingtin interacting protein 2 (HIP2), Defender against cell death 1 (DAD1) and BIRC3. This unexpected result could be due to a stress-induced reaction of the cells when they were transferred into serum-free medium that was later compensated for during the adaption process in other ways. We could not find any obvious gene alteration responsible for the survival in the absence of external growth factors, but some alterations that were all connected to the same signalling pathway and thus could collaborate to allow survival. We did e.g. find a downregulation of the TNF/TNF-R family members LTα, CD27L and Fas together with an upregulation of Herpesvirus entry mediator (HVEM). Fas is a receptor that when bound by FasL activates caspase-induced apoptosis[226]. The same pathway can also be activated by LTα bound to TNFR1, while LTα signalling through HVEM rather inhibits apoptosis by the activation of NF-κB[227, 228]. Together this could lead to a avoidance of apoptosis in the serum-free cells (Fig. 10). The Activating transcription factor 5 (ATF5) is also expressed in a higher level in serum-free cells and this has been shown to directly inhibit apoptosis without interfering with cell cycle regulation[229]. The CD27L and its receptor CD27 are both membrane bound proteins that are expressed by Ramos cells as well as many other malignant B lymphocytes. The function of CD27-CD27L interaction is not clear and has been shown to both activate TRAF mediated NF-κB and JNK activation inducing transcription, and function as a proapoptotic signalling pathway[230-232]. It has also been suggested that the co-expression of CD27 and CD27L on malignant cells can result in a survival inducing signalling through CD27L[233]. It would be interesting to study the function of the deregulation of CD27-CD27L in our serum-free cultures, to evaluate if the reduced expression of CD27L rescues the cells from apoptosis or if the inhibited signalling has the opposite effect, and the cells survive despite this due to other mechanisms.

The Wnt signalling pathway is a transcription activation system deregulated in many forms of cancers by the inactivation of the protein APC[234, 235]. Wnt molecules bind to different FZD receptors in the cell membrane that induce an

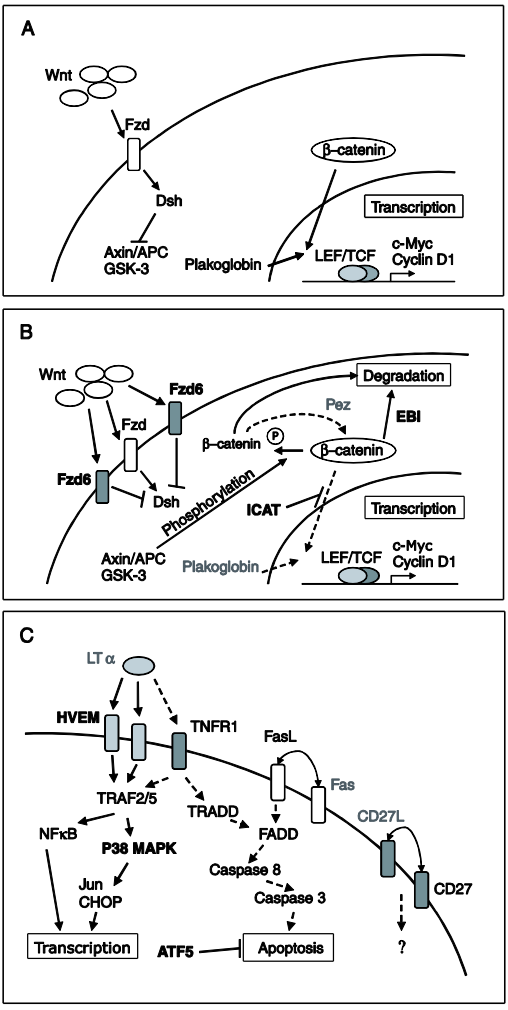

Figure 10. ***Modifications in gene expression during adaption of Ramos cells to serum-free medium.*** (A) The Wnt signalling pathway. (B) Genes deregulated in serum-free Ramos cells, compared to cells cultured in serum containing medium, involved in the Wnt signalling pathway. (C) Gene members of the TNF family deregulated in serum-free medium.
Genes that are upregulated in the serum-free cells are marked in bold and downregulated genes are marked in grey. Arrows indicates a functional pathway and the dotted arrows are pathways downregulated in the cells cultured in a serum-free medium (for details, see text)

signalling cascade through the activation of the Dsh protein that in its turn inhibit the function of the Axin/APC and GSK-3 complex. The GSK-3 protein phosphorylates β-catenin in the absence of Wnt signalling resulting in a degradation of β-catenin. Accordingly, Wnt signalling will inhibit the GSK-3 and thereby keep the b-catenin in an active form that can be transported into the nucleus and there activate TCL/LEF induced transcription. β-Catenin is a main regulator transcription and essential for Wnt signalling. We found an upregulation of the inhibitory Wnt receptor FZD6 in the Ramos cells cultured in the absence of external growth factors compared to serum dependent cells, which will lead to increased phosphorylation of β-catenin[236]. We also found an increased expression of EBI that induce degradation of β-catenin and a downregulation of Pez that have a function in dephosphorylation of β-catenin[237, 238]. This together with an increased expression of ICAT, a protein that prevents β-catenin activation of TCF/LEF in the nucleus[239], indicates a inhibition of Wnt signalling pathway and transcription that has been TCF/LEF induced genes in cells cultured in serum-free medium. Plakoglobin is a protein shown to be able to induce TCF/LEF transcription independently of β-

catenin[240]. Also plakoglobin has a diminished expression in the serum-free Ramos cells. A mutation in one of the proteins in the Wnt signalling pathway as APC or β catenin is often seen in cancer cells leading to the activation of the pathway and enhanced transcription activity by TCF/LEF[235]. An increased transcription of the target genes c-myc and cyclin D1 can be seen that is associated with cell cycle progression and proliferation. Which purpose the reduced TCF/LEF activation has in the serum-free Ramos cells is not known but would be interesting to further evaluate.

The gene expression levels in SFI were also compared with those in SFII. Differences between the two cell populations could be seen, supporting the notion that the serum-free cells are two distinct cultures and probably clonal. We could not see any obvious gene expression differences giving the two cultures two different pathways of survival, but we did find a few genes that were not only downregulated, but even seemed to be deficient in the SFI culture. Among these were all genes coding for MHC class II resulting in a phenotype of SFI resembling the Bare lymphocyte syndrome[241].

In **manuscript IV** we studied the lack of expression of MHC class II in the SFI cells and evaluated the cause of the deficiency. The transcription of MHC class II is regulated by master regulation protein CIITA, which binds to three different transcription factors, the RFX complex, CREB and the NF-Y complex (Fig. 11). The CIITA protein also binds a variety of other proteins including chromatin remodelling proteins and the conventional transcription machinery. The transcription factors previously known to be mutated in Bare lymphocyte syndrome patients, CIITA, RFXANK, RFXAP and RFX5, were investigated together with CRI1 also altered in the SFI culture, which could have a function in MHC class II gene expression regulation.

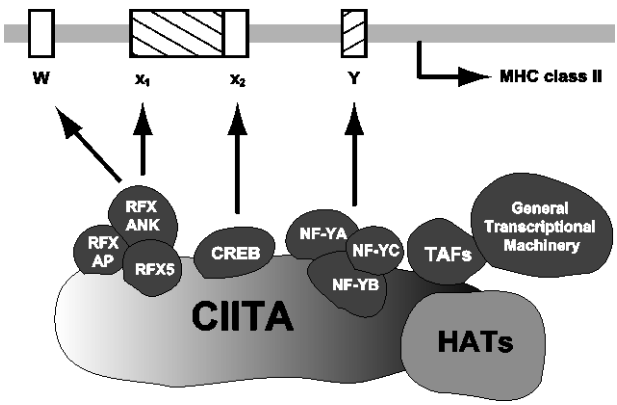

Figure 11. ***The transcription machinery of MHC class II.*** MHC class II expression is regulated by a transcription regulating protein CIITA that binds the transcription factor complexes RFX (RFXANK, RFXAP and RFX5), NF-Y (NF-YA, B and C) and the transcription factor CREB. The transcription factors bind to four different promotor regions in the MHC class II gene initiating transcription. CIITA also binds other proteins necessary for transcription of MHC class II as the chromatin remodelling proteins (HATs) and the conventional transcription machinery.

Despite the numerous gene alterations in the serum-free Ramos cultures, the cells still behaved similarly as serum-dependent Ramos cells both in proliferation and cell cycle progression. They also seemed to have kept their germinal centre phenotype as they, as the serum-dependent cells, showed a small IgM negative subpopulation indicating that the cells still are hypermutating (Fig. 12). The somatic hypermutating machinery was indeed active and resulted in an ongoing mutation that generated stop codons in the Ig heavy gene, resulting in the deficiency of IgM expression. The serum-free cultures had a lower mutations frequency than the serum dependent cells, probably due to a decreased expression of AID (indicated by our micro array data and previously shown as an effect of serum deprivation[242]). Thus, our data shows that somatic hypermutation can occur in Ramos cells in the absence of external serum stimuli.

For a germinal centre to form the activated B lymphocyte has to interact with $CD4^+$ T cell for a normal germinal centre formation and B cell differentiation, and the proliferating germinal centre B lymphocyte is dependent on survival signals from FDC to avoid programmed cell death[92, 99, 101]. However, it has been shown that small germinal centre-like structures can develop that have ongoing hypermutation in the absence of T cell help, but that these quickly disappear due to the lack of signals that rescue the cells from apoptosis[243].

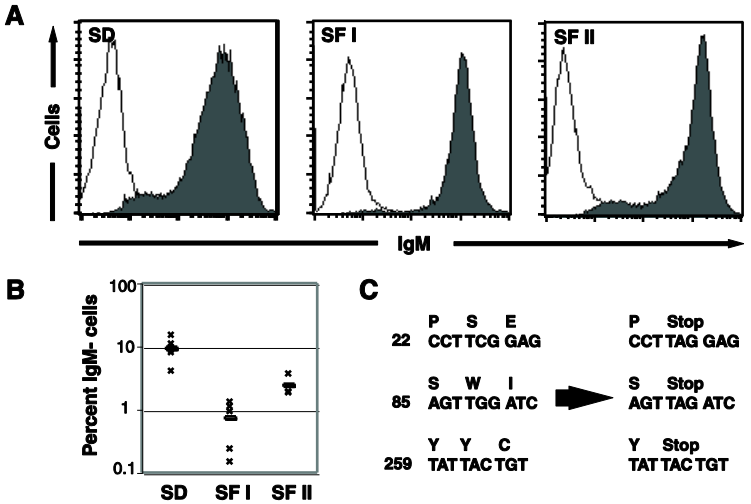

Figure 12. ***Loss of IgM expression in serum-dependent Ramos cells and the serum-free cultures SFI and SFII due to somatic hypermutation.*** (A) IgM surface expression of serum-dependent Ramos cells and the SFI and SFII serum-free cultures. (B) Percentages of cells that do not express IgM seven weeks after subcloning are shown. (C) Stop codons identified in IgM-negative cells generated during culture of subclones. Three examples of stop codon identified are shown.

To more thoroughly examine the germinal centre reaction and the interaction between B cells and FDC, in **manuscript III** we studied the signals produced by germinal centre like B lymphocytes. Extracellular proteins produced by serum-free Ramos cells were isolated and identified using nanoLC-FT-ICR MS technology. The expressions of these extracellular proteins were then examined in normal tonsil B lymphocytes divided into pre-GC, GC and post-GC cell populations. Except from the identification of proteins that can have a function as important signalling molecules in the germinal centre reaction, we also identified a couple of proteins that were not expressed in normal tonsil B lymphocytes, but in the Ramos cells. Both of these proteins have previously shown to have an increased expression in different forms of cancers and one of them have shown to have a function as a growth and survival factor. These extracellular proteins can thus be potential autocrine factors that contribute to the ability of Ramos cells to survive in the absence of external growth factors.

In **paper I** we have studied the function of Polymerase ζ using immortalized fibroblast cell lines that lack functional Rev3 genes. Polymerase ζ is an error prone polymerase thought to be involved in translesion synthesis during replication. It is here shown to be essential for embryonic development although it is apparently not necessary for cell proliferation. The Rev 3 deficient cells did, however, show a slower proliferation rate and lower survival threshold than cells with a functional Rev 3 gene, proposing a function of Pol ζ in tolerance towards endogenous DNA damage. Our results also show that Rev 3 deficient cells are more sensitive to exogenous damage, such as UV-radiation and cisplatin treatment. UV-radiation and cisplatin cause DNA lesions that appear to be recognized by the nucleotide excision repair or the mismatch repair systems respectively[143, 149, 244]. UV-radiation causes induction of apoptosis and cell cycle arrest with increased frequency of cells at the G_2/M phase and a non-synthesising population with a DNA content between $G_1$and G_2. The phenotype was much more severe in the Rev 3 deficient cells as compared to wild type cells (Fig. 13). The effect on cell cycle progression was, however, similar in the Rev 3 deficient population compared to the cells with a functional Rev 3 gene, indicating that both endogenous and exogenous DNA damage are tolerated at the same stage of the cell cycle using Pol ζ. The increased frequency of cells between the $G_1$and G_2 phases can be interpreted as that the replication has begun, but due to the lack of TLS the cell replication stops at the site of the DNA lesion. We also observed that cells with a functional Pol ζ had the ability to repair and overcome the cell cycle arrest caused by UV-radiation over time, while the Rev 3 deficient cells could not. Cisplatin did not induce as many apoptotic cells, but lower concentrations of the cytotoxic agent were needed for cell

cycle arrest at the M/G_2 stage in the Rev 3 deficient cells compared to proficient cells. Thus, Polymerase ζ seems to be important for the repair of lesions caused by cisplatin, enabling cells to divide and thereby increase the survival rate during the induction of these crosslinking lesions.

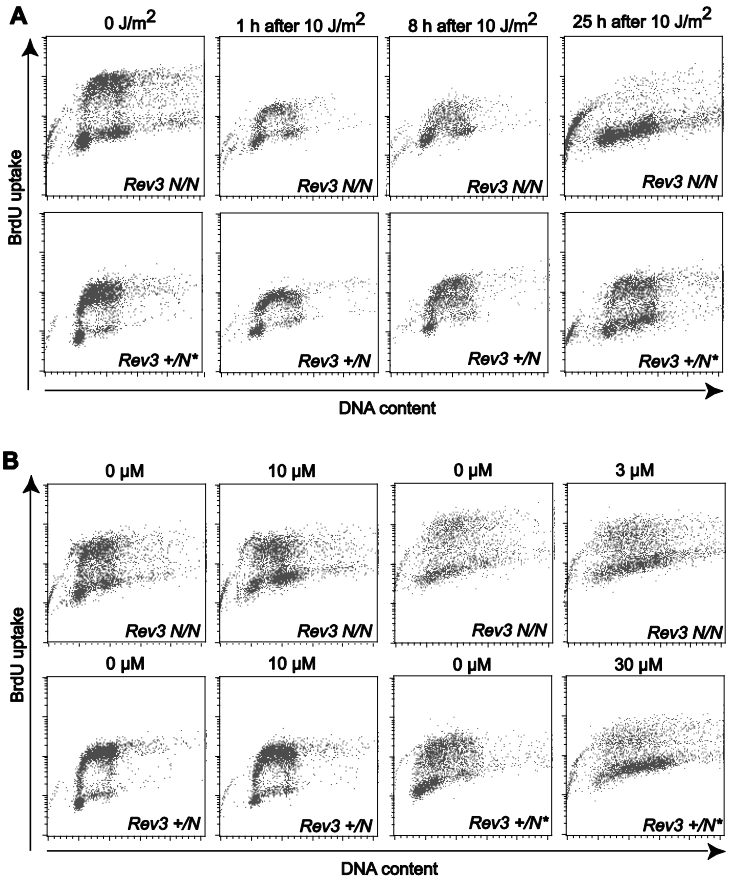

Figure 13. ***Cell cycle analysis of UV-radiation and cisplatin treated Rev3 deficient cells.*** (A) Cell cycle profiles of Rev 3 $^{N/N}$ and Rev3$^{+/N}$ cells after 10 J/m^2 UV-radiation. The cell cycle analysis was performed on untreated cells and 1, 8 and 25 h after UV-radiation. The Rev 3 $^{N/N}$ cells are more sensitive for UV-radiation induced cell cycle arrest and apoptosis than Rev3$^{+/N}$ cells, and the cell cycle progression cannot be restored with time. (B) Cell cycle profiles of Rev 3 $^{N/N}$ and Rev3$^{+/N}$ cells treated with 3, 10 or 30 µM cisplatin for 25h. The results show that of Rev 3 $^{N/N}$ cells are more susceptible to cisplatin treatment than Rev3$^{+/N}$ cells.

Polymerase ζ are here shown to be important for the repair of single stranded alterations, as UV-induced DNA nicks and crosslinking by cisplatin, which are typical lesions repaired by the translesion synthesis system. The

evaluation of the Rev 3 deficient cells also showed that Pol ζ is not essential for the repair of either single base lesions or double stranded breaks. During somatic hypermutation, single base lesions are introduced to achieve variability of the Ig gene. The mutations induced are not repaired by the BER or MMS systems normally active during reparation of these lesions. Polymerase ζ is thought to be active also during hypermutation, but then in the error-prone incorporation of nucleotides during MMR or in the extension of DNA synthesis after double stranded break repair. It is thus possible that Pol ζ is induced in hypermutating cells to be functional in other repair systems than TLS, as a participant of the induction of mutations during somatic hypermutation[245].

Conclusions and Future Perspectives

- **The Burkitt lymphoma B cell line Ramos can survive and proliferate in the absence of external growth factors.**

We have managed to adapt Ramos cells to long term growth in serum-free medium. Cells grown without the addition of external stimuli in the form of unknown serum factors can be useful in a variety experiments were you want to study the cell function and characteristics.

- **Gene expression alterations occur in cells to be able to survive without the stimulation of external growth factors.**

The serum provides the cells with nutrients and growth factors that the cells grown in serum-free medium have to compensate for. Dilution sensitivity of the serum-free cells indicates that the cells likely are producing an autocrine growth factor and have altered their cell capabilities to avoid apoptosis. These gene alterations were evaluated using global gene expression methods and possible alterations in signalling pathways are discussed. The results can be of interest in other studies of serum-free cell cultures and in studies in which the growth abilities of transformed cells are investigated.

- **We have identified a cell culture deficient in MHC class II expression that resemble Bare lymphocyte syndrome. This cell line may carry a novel mutation in MHC class II deficiency.**

Bare lymphocyte syndrome is a disease that is associated with severe combined immune deficiency. The discovery of new regulation factors in MHC class II expression can facilitate the understanding and the approach for a treatment in Bare lymphocyte syndrome patients.

- **Secreted extracellular proteins produced by a germinal centre like B cell line have been identified and the expression of some of these proteins have been evaluated in normal tonsil germinal centre B lymphocytes.**

Germinal centre B lymphocytes are hard to study because they cannot be cultured *in vitro* without differentiation inducing stimulation. To examine which signals germinal centre B lymphocyte produce, the extracellular proteins secreted from serum-free grown Ramos cells were identified. The gene expression levels of some of these proteins were then measured in normal tonsil pre-GC, GC and post-GC B lymphocytes. The proteins identified can be used for further examination of normal germinal centre B lymphocytes. Such studies may include determinants of expression in centroblasts versus centrocytes and for evaluation of protein function in the germinal centre reaction.

- **Polymerase ζ is essential for embryonic development but not for proliferation of immortalized fibroblasts.**

The involvement of Pol ζ in embryonic development and endogenous DNA damage has been established previously but the cell line allow for the function to be further studied. This was the first mammalian Pol ζ deficient cell line immortalized for long-term cell culture *in vitro*.

- **Polymerase ζ is involved in translesion synthesis and repair of UV-radiation and cisplatin induced DNA damage**

Rev 3 deficient cells are more sensitive to UV-radiation and cisplatin than cells with a functional Polymerase ζ, with enhanced frequency of apoptosis and cell cycle arrest. The exact function of Polymerase ζ in repair and TLS would be interesting to study as well as the function of Polymerase ζ in somatic hypermutation. The Rev 3 deficient cell lines are useful for both these types of experiments.

Acknowledgements

Jag skulle vilja tacka alla mina kollegor, vänner och min familj som har hjälpt och stöttat mig under min tid som doktorand. Framför allt vill jag tacka,

Dig **Mats,** för ett gott samarbete under de här åren. Jag har haft turen att ha en handledare som förstår vikten av att fika och ha roligt, inte på raster utan, medan man arbetar. Din stora entusiasm för forskningen och din glädje att dela dina kunskaper har inspirerat mig till att inte bara börja doktorera, utan även att slutföra det. Tack för att du har lärt mig så otroligt mycket!

Linda F, vad hade jag gjort utan dig? Tack för all hjälp med tonsiller och blodprover samt alla många och långa samtal om både experiment, metoder, barn och allt annat.

Nils, Mary-Jo, Paul och Bettan, tack för alla små tips och trevliga vetenskapliga diskussioner.

Tack **Maria O** för att du tog hand om mig när jag började på avdelningen. Tack även för alla roliga samtal om livet, alla promenader, alla pysselkvällar och alla fikor därefter. Jag saknar dig!

Tanya, glada, goa, kaksugna Tanya. Tack för alla öppenhjärtliga samtal och för alla glada skratt. Tack för att du är min vän.

Tack **Anja**, för att du är en omtänksam och stöttande vän i alla väder. Du har ett gott hjärta och jag är glad att ha en vän som slår mig med hästlängder i att prata mycket.

Tack, **Britt-Marie**, för att du alltid är så positiv. **Andrea** för alla trevliga pratstunder på sena kvällar och helger då vi varit de enda tappra, och tack för grapefrukten när jag var gravid. Jag tror aldrig jag ätit något så gott. **Mirjana** tack för alla trevliga samtal om både PCR och annat.

Peter tack för alla intressanta B cells diskussioner och för att du ordnat alla roliga events med tillhörande öldrickning, det har varit mycket uppskattat. **Stina** för alla 10 minuters inkubationer du hjälpt mig genomlida med trevliga samtal. **Kristina** för alla tänkvärda diskussioner om världsliga ting. **Maria S** för att du varit på min sida i striden om mycket och högljutt spelande av P3 på labb. **Anna** för att du ställt upp och lyssnat på mig nu på slutet när jag behövt prata av mig. **Malin S** för all praktisk hjälp med ansökningar osv. **Malin B** för alla tänkvärda ord både vad gäller jobb och annat. **Linda Y** för alla trevliga samtal om barn. **Ellen**, det är alltid roligt att prata med dig, trots engelskan. **Ann-Charlotte, Karin S, Carin B och John** för sällskapet på cell-lab. Så mycket som jag pratar så förstår ni ju hur glad jag varit att inte behöva sitta där själv.

Tack även till alla andra som arbetar och som kommit och gått på avdelningen för klinisk immunologi och avdelningen för mikrobiologi och immunologi. Tack för ni gjort det till en trevlig arbetsplats.

Tack **Pierre och Jessica** för all grafisk hjälp inte minst med bokomslaget, men framför allt för alla trevliga vinkvällar, biljardkvällar och bowlingkvällar under årens lopp. De flesta gångerna har jag faktiskt kunnat koppla bort doktorerandet… och bara haft roligt!

Maria och resten av **Karlstad-gänget**, tack för att jag alltid har vänner att komma hem till. Oavsett hur långt det går mellan gångerna så finns det alltid en tjejsoffa att sitta och prata strunt över ett glas vin i. Tack!

Jenny C, tack för att du är en så bra vän, en riktigt bra, omtänksam och härlig vän. Hoppas vi kan sitta där en dag och säga att vi varit vänner i 80 år…

Sanna och **Ann-Marie** utan er hade jag nog aldrig börjat fundera på att doktorera.

Jag vill även tacka hela min **Svärfamilj** dels för ert intresse av mitt projekt, men framför allt vill jag tacka för er vänskap och för att jag blivit så välkomnad in i er familj.

Farmor och Farfar tack för ni alltid trott på mig och funnits där för mig. **Bittan** för att du hjälpt en Karlstad-tös som jag att känna sig hemma här Göteborg och tack **Lena** för ditt stora intresse och din värme.

Min syster Jenny, tack för att du är du och för att du finns.
Tack för de orden och för att du är en syster man alltid kan prata med, alltid kan fika och shoppa med, men framför allt, alltid kan skratta med. Kram

Mamma och Pappa, tack för att ni alltid finns där för mig. Ni har alltid stöttat mig i allt jag tagit mig för och ert intresse och uppmuntran betyder mycket för mig. Pappa du hade rätt om stjärnorna, tack för att du är så klok. Mamma din omtanke gör mig rörd, med er som föräldrar känner man aldrig riktigt ensam.

Min familj…

Jimmy, tack för att allt ditt stöd, för din uppmuntran och all din hjälp. Utan dig hade många bilder och denna bok sett helt annorlunda ut. Tack även för många, långa och sena nätter du ställt upp för att få ihop vardagen, vilket har gjort det möjligt för mig att slutföra det här.
Tack för att du finns i mitt liv, du är min trygghet och min glädje.

Lova, världens bästa tjej, jag är så glad att du är du och att du är här!

… ni gör mig lycklig.

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