Signalling mechanisms in B cell differentiation

Studies on specific human immune responses *in vitro*

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Contents

Abbreviations

NK cells	Natural Killer cells
APC	Antigen presenting cell
GC	Germinal centre
sIg	surface immunoglobulin
BCR	B cell receptor
TCR	T cell receptor
Th1	T helper cell (type 1)
IL-	Interleukin
TNF	Tumour Necrosis Factor
IFN	Interferon
CD	Cluster of differentiation
DC	Dendritic cell
FDC	Follicular dendritic cell
IDC	Interdigitating dendritic cell
T-zone	T cell zone
ICAM	Intracellular adhesion molecule
VCAM	Vascular cell adhesion molecule
GCDC	Germinal centre dendritic cell
LFA-1	Lymphocyte function associated antigen
TNFR	Tumour Necrosis Factor Receptor
CD40L	CD40 ligand
IL-2R	Interleukin-2 receptor
MALT	Mucosal associated lymphoid tissue
PALS	Periarteriolar lymphoid sheath
HEV	High endothelial venules
TBM	Tingible body macrophages
NFkB	Nuclear factor kappa B
MAP-kinase	Mitogen activated phosphate-kinase
LPS	Lipopolysaccharide
TRADD	TNFR1-associated death domain protein
TRAF	TNFR-associated factor
IRAK	IL-1 receptor kinase
NIK	NFkB inducing kinase
IKK	IkB kinase
mAb	monoclonal antibody
HAMA	Human anti mouse antibody
PBL	Peripheral blood lymphocytes

CDR	Complementary determining region
V genes	variable genes

Original papers

The present thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I Ingvarsson, S., Dahlenborg, K., Carlsson, R. and Borrebaeck, C.A.K. Coligation of CD44 on naive human tonsillar B cells induces a germinal center phenotype. (1998) *Submitted.*

II Ingvarsson, S., Simonsson Lagerkvist, A.C., Carlsson, R. and Borrebaeck, C.A.K. Stimulation of human peripheral lymphocytes via CD3 and soluble antigen abrogates specific antibody production by reducing memory B cell numbers. (1994) *Scand. J. Immunol.* 42, 331-336.

III Ingvarsson, S., Simonsson Lagerkvist, A. C., Mårtensson, C., Granberg, U., Ifversen, P., Borrebaeck, C.A.K. and Carlsson, R. Antigen specific activation of B cells *in vitro* after acquisition of T cell help with superantigen (1995) *Immunotechnology,* 1, 29-39.

IV Franzén, A., Ingvarsson, S., Brady, K., Moynagh, P. and Borrebaeck, C.A.K. (1998). In vitro secretion of specific IgE antibodies is associated with NFkB activation induced by T helper 2 cells. *Manuscript.*

1 Introduction

Our environment contains a large variety of infectious agents such as viruses, bacteria and parasites. These agents can cause great damage and even kill us. The human body has an answer to this problem and it is called the ”Immune system”.

The immune system can be divided into two parts based on their defence mechanisms, namely natural (innate) immunity and acquired (adaptive) immunity. The natural immunity is based on different mechanisms. These are physical barriers like the skin and the mucosal membranes, phagocytes such as macrophages and natural killer cells, the complement system and soluble mediators in the periphery e.g. interferons and tumour necrosis factors. The innate immunity is ”the first line of defence against infectious agents” and it protects us from foreign macromolecules and infectious microbes, without discriminating between foreign substances.

The adaptive immunity is, on the other hand, based on specific stimulation from the foreign substance and is specifically amplified by molecules called antigens. This specific immunity can be divided into cell mediated (cellular immunity) and humoral immunity. Cellular immunity is mediated by T lymphocytes, whereas humoral immunity is based on proteins called antibodies (immunoglobulins), raised against the antigens. B lymphocytes are the antibody factory of the body and the production of antibodies is often dependent on T cell help.

The antibody molecule has often been described to have the shape of the letter Y. It consist of four polypeptide chains, two smaller (light chains) and two larger (heavy chains) held together by covalent and non-covalent forces (see figure 1). There are two forms of light chains called kappa (κ) or lambda (λ) and five different forms of heavy chains termed alpha (α), delta (δ), epsilon (ε), gamma (γ) and my (μ). Both κ and λ light chains can be combined with any of the heavy chain forms where the antibody isotype is named based on the heavy chain it consists of: α-chains; IgA, δ-chains; IgD, ε-chains; IgE, γ-chains; IgG and μ-chains; IgM. Furthermore, the antibody molecule consists of two parts, the Fc part

and the Fab part (figure 1). The Fc part mediates the biological activity of the antibody allowing it to bind to Fc receptors on lymphocytes and by doing so mediating cellular effector functions. The Fab part, however, is responsible for binding the antigen and thereby neutralising it.

The purpose of my work was to study the cellular signalling mechanisms behind the induction of specific antibodies and to design *in vitro* systems allowing generation of antigen specific antibodies of human origin.

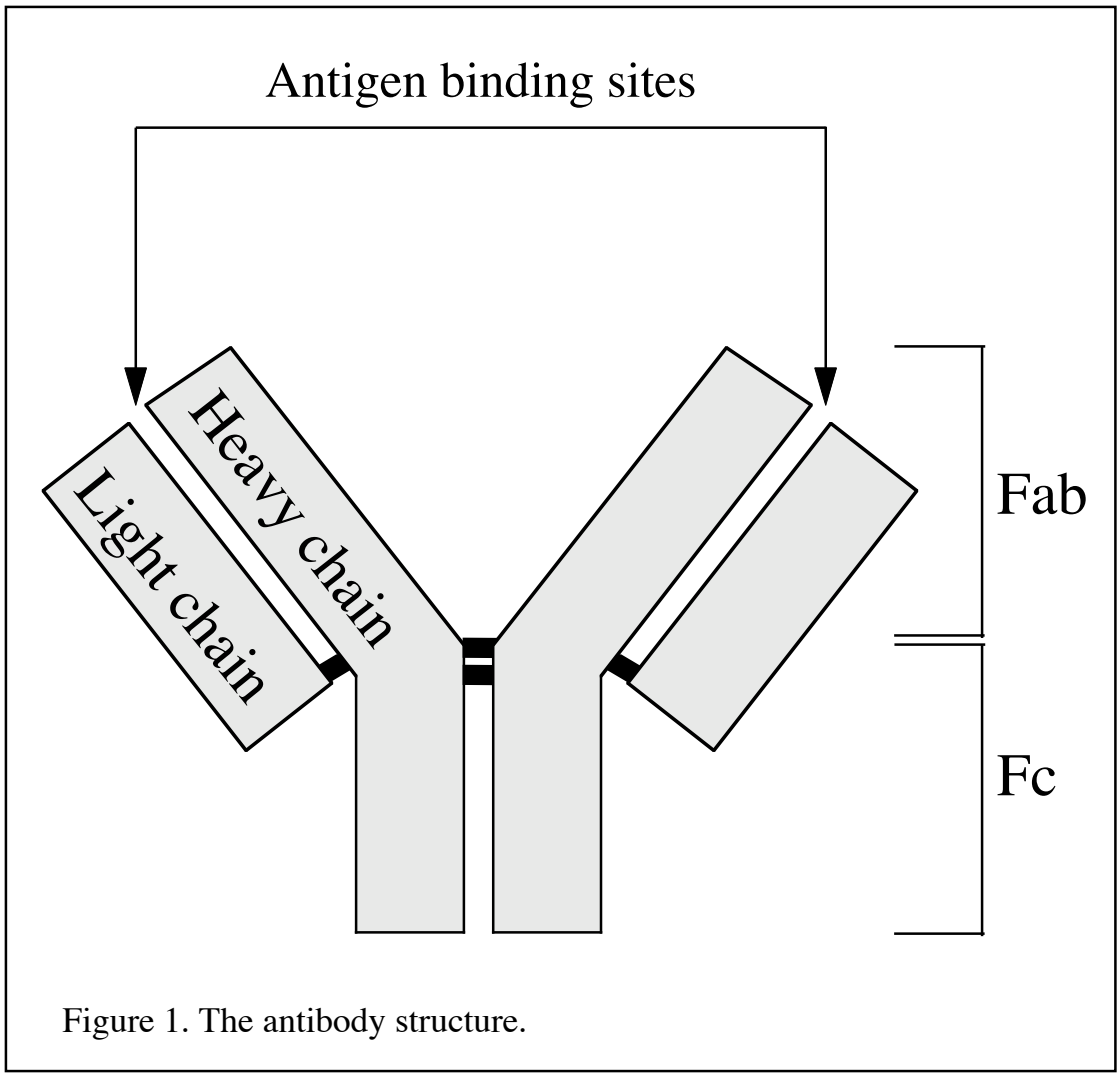

Figure 1. The antibody structure.

2 Cells of the immune system

All cells involved in the immune response arise from pluripotent haemapoietic stem cells. The cells of the immune system, the leukocytes (white blood cells), have been divided into three main categories, namely: 1) lymphocytes, 2) monocytes and 3) polymorphonuclear cells.

1. Lymphocytes are a heterogeneous group of cells, considering their morphology. The two major types of lymphocytes are *B cells* and *T cells,* but other cells like *natural killer cells (NK cells)* also belong to the lymphocyte lineage. The origin of *dendritic cells* is not clear and dendritic cells have been generated in vitro from both myeloid and lymphoid cells. I have chosen to discuss dendritic cells under the chapter on lymphocytes.
2. Monocytes belong to the *mononuclear phagocyte system*. Monocytes are found in blood, but when they migrate into tissue the differentiate into macrophages. Macrophages are responsible for phagocytosing foreign particles, producing cytokines for recruiting inflammatory cells and they can also function as antigen presenting cells (APCs).
3. The granulocytes can be divided into *Neutrophils, Eosinophils, Basophils* and even though they are not specific for any antigen they are effector cells that play an important role in acute inflammation and immediate hypersensitivity reactions.

My thesis deals with the first kind of leukocytes, the *lymphocytes*. B lymphocytes, T lymphocytes and dendritic cells will be described in detail below, while NK cells are not a subject of this thesis.

All cells have a large variety of surface molecules and each cell type and cell subset has a combination of such molecules, that can be used to characterise them. A surface marker, that is known to be lineage specific or identifies a differentiation stage, is called a cluster of differentiation (CD) marker. Identification of CD markers has often been carried out by monoclonal antibodies. Later on, different cell subsets will also be discussed according to their surface marker expression.

2.1 B lymphocytes

B lymphocytes (B cells) are called so because they were first shown to mature in the Bursa of Fabricius in birds. In man, B cells mature in bone marrow where they undergo V-D-J rearrangement of the variable region of their immunoglobulin genes and start to express surface immunoglobulin (sIg). The first B cell subset is called Pro-B cell and at that stage the V-D-J rearrangement takes place. The Pro-B cell then becomes a pre-pre B cell and expresses the μ heavy chain on its surface. By now, the B cell starts to express genes that code for surrogate light chains (λ_5 and V_{preB}) and at that stage they are called pre-B cells. At last the B cells express a functional Ig molecule on its surface with a μ heavy chain and κ or λ light chain and are then referred to as an immature B cells. Thereafter, they enter the periphery and are called mature B cells. Mature B cells can circulate in the periphery for a few days or weeks, where they also die if they do not encounter antigen. These naive (mature) B cells co-express IgD and IgM, but if the B cell binds an antigen it down regulates IgD. After antigen encounter, the B cell enters the lymphoid organs and is activated in the outer T cell zone. Following this activation the B cell can either become a plasma cell, secreting immunoglobulins, or enter primary follicles where it participates in giving rise to germinal centres (GC). The GC is a egg shaped structure containing mostly B cell and it consists of two major areas, the dark zone and the light zone. B cell differentiation and selection takes place in the GC, where the B cell differentiation signals towards plasma cell and memory B cell are provided.

B cells express surface immunoglobulin (sIg) as a complex together with two other molecules, Igα and Igβ, and the function of these molecules is to mediate signals into the B cells, when sIg is ligated. This complex is called the B cell receptor (BCR). sIg is the molecule, that recognises the antigen and each B cell expresses sIg with one certain specificity. Positive as well as negative selection of B cells is based on the binding properties of the BCR, causing elimination of self reactive B cells in the bone marrow and further differentiation of cells with high affinity for the foreign antigen in lymphoid organs. Mature B cells have been divided into five subsets based on phenotype and function (Liu and Banchereau 1996a). These five subsets are called Bm1-5, where Bm stands for mature B cell

subset). Bm1 cells ($IgD^+/CD38^-$) are naive B cells and Bm2 ($IgD^+/CD38^+$) represent germinal centre founder cells. Then there are the germinal centre B cell subsets Bm3 ($IgD^-/CD38^+/CD77^+$), which are the centroblasts and Bm4 ($IgD^-/CD38^+/CD77^-$), represent the centrocytes. Finally there are the Bm5 cells ($IgD^-/CD38^+$), which are the memory B cells. Plasma cells are not included in this classification of mature B cells, since their phenotype is not so well defined. Plasma cells have been described as extra follicular $IgD^-/CD38^+$ high expressing cells being much larger than other B cells. At the GC stage, B cells undergo class switching and terminal differentiation into Ig secreting plasma cells or memory B cells. Memory B cells can survive for months without any antigenic stimulation. They circulate in the periphery and enter the lymphoid organs upon antigenic stimulation and if they are provided with T cell help, they participate in the secondary antibody response.

There is another B cell subset, also found in secondary lymphoid organs. These cells are $IgM^-/IgD^+/CD38^+$ and they can contain up to 50 mutations in their VH genes. How these cells have developed is not known, but they are either activated naive B cells that have been trapped in the dark zone and undergone many rounds of somatic mutations or sIgD positive memory cells having passed many times through germinal centres (Liu *et al.* 1996b).

2.2 T lymphocytes

T lymphocyte (T cell) precursors arise in the bone marrow and migrate into the thymus to give rise to T lymphocytes (thymus derived cells). The thymus consists of lobes, that are divided into lobules and each lobule is made up of a cortex and a medulla. A developing thymocyte migrates from the cortex to the medulla as it goes through maturation in three developmental stages. These are: 1) $CD4^-/CD8^-$ double negative cells, 2) $CD4^+/CD8^+$ double positive cells and then 3) single positive $CD4^+/CD8^-$ or $CD4^-/CD8^+$ cells. Single positive cells then enter the periphery and become either $CD4^+$ T helper cells or $CD8^+$ cytotoxic T cells.

Like B cells, T cells have a receptor specific for antigens (or peptides) called the T cell receptor (TCR). The TCR is a part of a complex called TCR/CD3 complex, that mediates signals into the T cells, during T cell - antigen presenting

cell (APC) interaction. Signalling is initiated by interaction of the TCR with MHC class I or II molecules, presenting antigenic peptides. The TCR consists of two polypeptide chains, α and β chain or γ and δ chain. Most of the T cells that develop in the thymus end up being αβ T cells, but γδ T cells are found in the body especially at specific sites like e.g. skin and gut. CD4 and CD8 are co-receptors of the TCR and in the process of antigen recognition, $CD4^+$ T cells recognise MHC class II molecules and $CD8^+$ T cells recognise MHC class I. The interaction of the co-receptors will be discussed later in this thesis.

T cells are divided into two major populations, based on their function, namely helper T cells ($CD3^+$/ $CD4^+$/$CD8^-$) and cytotoxic T cells ($CD3^+$/$CD4^-$/$CD8^+$). T helper cells are then further divided into T helper 1 (TH1) cells and T helper 2 (TH2) cells after they have differentiated from a naive T cell progenitor called T helper 0 cells (TH0). The T helper cells have been categorised on the basis of their cytokine secretion profile. TH1 cells produce interleukin (IL)-2, IL-3, interferon (IFN)-γ, Tumour Necrosis Factor (TNF)-α and TNF-β, whereas TH2 cells produce IL-4, IL-5 and IL-10. The most discriminating feature of TH1 cells is that they do not secrete IL-4. TH1 cells are important in the activation of cytotoxic cells like macrophages resulting in phagocyte-mediated host defence reactions, whereas TH2 cells activate eosinophils and stimulate IgE production via their IL-4 secretion. Both TH1 and TH2 cells are important in B cell activation resulting in proliferation and Ig secretion.

The CD3 surface molecule of the TCR/CD3 complex is expressed on all T cells and ligation of this molecule/complex has shown to result in a very potent polyclonal activation of T cells (see paper 2). Another surface molecule, expressed on most T cells is the CD2 molecule, which has also shown to efficiently transduce activation signals for T cells *in vitro* (Conrad *et al.* 1992). Another surface molecule expressed on all T cells is the CD45 molecule. This molecule exists in different splice forms called CD45RA and RO. Mature T cells express CD45 on their surface, whereas naive T cells they express the CD45RA isoform, but during activation and differentiation the T cells switch over to CD45RO expression (Kristensson *et al.* 1990).

2.3 Dendritic cells

Dendritic cells (DCs) are thought to be a progeny of bone marrow derived cells, related to mononuclear phagocytes. DCs are morphologically very different from other lymphocytes and they have long membranous dendrites pointing out from the centre of the cell. Functionally they have been described as very potent APCs. Four major subsets of dendritic cells have been identified in humans; Langerhans cells, blood dendritc cells, interdigitating dendritic cells (IDCs) and follicular dendritic cells (FDCs). FDCs are not thought to be of the same origin as the other three subsets, and will be discussed later in the chapter about germinal centre reactions. Langerhans cells are found in skin and they are very potent in taking up antigen. IDC's are found in the T cell-zones (T-zones) of secondary lymphoid tissues and play a major role in priming of T cells. It has been shown, that Langerhans cells take up antigen and transport it via the afferent lymph to the T-zone. Thus it seems that dendritic cells differentiate from being an Langerhans cell in skin or epidermis, becoming blood dendritic cells with a final differentiation of IDC in the T-zone or perhaps migrating into primary follicles to become a germinal centre dendritic cell GCDC (Macatonia *et al.* 1987; Cumberbatch *et al.* 1990; Grouard *et al.* 1996).

3 B - T cell signalling in cognate interaction

Communication between the cells of the immune system occurs via soluble mediators or through interaction between molecules expressed on the surface of these cells. When two cells interact with each other via surface molecules it is called cognate interaction. The following chapter discusses the major group of soluble and surface bound molecules responsible for the dialogue between B and T cells.

3.1 Adhesion molecules

Adhesion and homing molecules are a group of surface markers that are involved in: recognition of endothelial cells, lymphocyte homing and cell - cell adhesion. These molecules are selectins, intergrins, proteoglycans, mucosal addressins and members of the Ig superfamily. During lymphocyte homing it has been demonstrated by video technology that the cells home to their sites using a multistep process (Lawrence *et al.* 1991; von Andrian *et al.* 1991). The first step is rolling of the lymphocyte as it interacts with endothelial adhesion molecules. The second step is triggering of the cell by chemokines and intergrins, while the third step involves a strong adhesion via adhesion molecules, like intracellular adhesion molecule-1 and 2 (ICAM-1 and 2) and vascular cell adhesion molecules, VCAMs. The fourth and last step in this process is migration into tissue and chemotaxis (Mackay *et al.* 1993).

Another function of adhesion molecules is to establish cell-cell contact in lymphocyte activation and deliver early signals in these events. The lymphocyte function-associated antigen-1 (LFA-1) and its naturals ICAM-1 and have been studied extensively in terms of T cell - APC interaction. LFA-1 belongs to the intergrin family and is expressed on T and B cells as well as on some other leukocytes. ICAM-1 and 2 belong to the Ig superfamily and they are expressed on most leukocytes. It has been demonstrated that LFA-1 on T cells facilitates functional triggering of TCR, by binding ICAM-1 on APC's and mediate adhesion (Bachmann *et al.* 1997).

3.1.1 CD44

Another well studied cell surface molecule is CD44. The gene that codes for CD44 has 19 exons and 12 of those 19 exons can undergo alternative splicing. Of at least 18 different CD44 transcripts, the two most common ones are CD44H (hematopoietic) and CD44E (epithelial) (Lesley *et al*. 1993; Lazar *et al*. 1995). Data show that CD44 is involved at different stages in the lifespan of the lymphocyte such, as lymphocyte homing, leukocyte activation as well as tumour metastasis and development (Miyake *et al.* 1990, Jalkanen *et al.* 1986; Shimizu *et al.* 1989; Gunthert *et al.* 1991; Wheatley *et al.* 1993). The major ligand for CD44 is hyaluronate (HA), but other molecules such as collagen and fibronectin have also been shown to bind CD44 (Aruffo *et al.* 1990; Carter 1982; Carter *et al.* 1988). CD44 is expressed on various cell types like B and T cells, monocytes and epithelial cells and seems to work as an organ specific homing receptor for lymphocytes (Lesley *et al*. 1993). One of the phenotypical changes of B cells during differentiation is down regulation of CD44 as they become germinal centre B cells. CD44 is strongly expressed on resting B cells as well as on memory and plasma cells (Kremmidiotis *et al.* 1995).

3.1.2 MHC - TCR

A mature αβ T cell co-expresses either the CD4 or the CD8 molecule together with the TCR. The TCR binds MHC displaying antigenic peptide on APCs. CD4 expressing T cells recognise antigen displayed on MHC class II, whereas CD8 expressing T cells bind antigen on MHC class I (Janeway *et al*. 1988). (The interaction between TCR/CD8 and MHC I will not be discussed in this thesis). When antigen is taken up by an APC, it is processed and presented as a short peptide (12-25 amino acids) on the MHC class II molecule. The peptide is placed in a groove on the part of the MHC molecule, that interacts with the TCR (figure 2). The co-receptors (CD4 or CD8) play an important role in the signalling via TCR as they facilitate 100 times increase in T cell activation, when they are ligated to MHC II and MHC I respectively. This means that T cell activation can be induced with limited amounts of antigen (Springer 1990). This is similar to how CD19 and CD40 ligation can lower the threshold for sIg signalling in B cell

activation (Carter *et al.* 1992; Wheeler *et al.* 1993) (see chapter on signalling in B-T cell interaction). To be fully activated T cells need two signals according to Bretcher and Cohn's "Two signal theory" (Bretscher and Cohn 1970), a signal via TCR and a co-stimulatory signal (Bretscher 1992; Bachman *et al*. 1997) (also discussed in chapter on CD28).

3.1.3 CD40 - CD40L

The CD40 molecule was discovered by monoclonal antibodies raised against B cells (Paulie *et al.* 1985; Clark *et al.* 1986). It belongs to the tumour necrosis factor receptor (TNFR) family and is expressed on all B cells from the pre-B cell stage to mature B cell stage. A significant discovery was that B cells could be cultured for a longer period of time *in vitro*, by crosslinking the CD40 molecule with antibodies against CD40. In these studies the anti-CD40 antibodies were bound to CD32 transfected fibroblasts which allowed crosslinking of CD40 (Bancherau *et al.* 1991). This system, usually referred to as the CD40 system, enabled immunologists for the first time to study B cell development and differentiation *in vitro*.

The CD40 ligand (CD40L) was first cloned from the EL-4 murine thymoma cell line (Armitage *et al.* 1992). It belongs to the tumour necrosis factor (TNF) family and is expressed on activated T cells. CD40L expression can be induced in five minutes and a transient expression can be maintained upon cognate interaction (Casamayor-Palleja *et al.* 1995). The CD40L is necessary for B cell activation and differentiation and seems to play a crucial role in immunoglobulin class switching. This was discovered when it was shown that a defect in the CD40L gene was responsible for X-linked hyper IgM syndrome, a disorder described with elevated levels of IgM and dramatically decreased concentration of IgG, IgE and IgA in serum (Allen *et al.* 1993; Notarangelo *et al.* 1996).

3.1.4 CD80/CD86 - CD28 /CTLA-4

CD28 is the best characterised co-stimulatory molecule expressed on resting T cells and it belongs the Ig superfamily. CTLA-4 is another costimulatory molecule expressed on T cells which also belongs to the Ig superfamily (Lenschow *et al.* 1996). Both CD28 and CTLA-4 share a conserved amino acid sequence in their

variable domain (MYPPPY), which is necessary to bind the B7-1 molecule (Peach *et al.* 1994). While CD28 is constitutively expressed on resting T cells and comparatively distributed over the cell surface, CTLA-4 is expressed at almost undetectable levels, but its expression is rapidly increased following TCR signalling (Chambers *et al.* 1997). CTLA-4 expression can also be increased by CD28 signalling and IL-2 stimulation (Linsley *et al.* 1992; Perkins *et al.* 1996). As mentioned before, T cells need a co-stimulatory signal for successful T cell activation and CD28 has been suggested as the most prominent costimulatory molecule in this case.

CD80 (B7-1) and CD86 (B7-2) both belong to the immunoglobulin superfamily and these molecules are the natural ligands for CD28 as well as CTLA-4, but their binding properties are very different. CD80 was discovered in 1981 and was identified as the ligand for CD28 in 1990 (Yokochi *et al.* 1982; Linsley *et al.* 1990). CD86 was however not discovered until 1993 (Azuma *et al.* 1993; Freeman *et al.* 1993) and its similarities to CD80 indicated that it might also bind to CD28. CD80 as well as CD86 have been found expressed on activated B cells, dendritic cells, Langerhans cells, activated monocytes and on activated T cells.

3.2 Cytokines

Cytokines have an important role in lymphocyte activation. The list of cytokines is long and their roles in lymphocyte activation are many. Here only a few of them, relevant to B cell activation will be discussed. Early in the activation process of T cells, IL-2 is produced. IL-2 binds to the IL-2R on that very same T cell (an autocrine effect) or to other by-stander lymphocytes. IL-2 induces proliferation of T cells resulting in clonal expansion (Smith 1986). IL-4, IL-5 and IL-13 together with TGF-β induce immunoglobulin class switching upon CD40 ligation (Paul *et al.* 1987, Takatsu *et al.* 1988; Coffman *et al.* 1989). IL-3, IL-6 and IL-10 are involved in differentiation of B cells towards plasma cells (Liu 1997a). IL-13 is very important in NK cell activation as well as priming of T helper cells for a type 1 profile, by inducing IL-10 and IFN-γ production in these cells (Trinchieri *et al.* 1996).

3.3 Signalling events in B-T cell cognate interaction

After the antigen binds sIg on B cells, or is taken up by other APC, it is degraded and processed into small peptides to be presented to the TCR by MHC II molecules. Interaction between cell adhesion molecules belong to the first events in B-T cognate interactions. When LFA-1 on T cells binds its ligand, ICAM-1, on APCs, it facilitates T cell activation by lowering the amount of antigen required for T cell activation (Bachman *et al.* 1997). CD86 is expressed on B cells after only 6 hrs of stimulation and it has been shown that its ligation of CD28 can upregulate CD40L expression on T cells as well as induce IL-2 production. TCR triggering is however sufficient for upregulation of CD40L (de Boer *et al.* 1993). When a B cell receives a signal via CD40, it rapidly upregulates both CD80 and CD86 (Ranheim *et al.* 1993). Thus, there is a reciprocal dialogue between those two receptor ligand pairs, but other signals are also important in the regulation of the signalling pathways of these molecules (Roy *et al.* 1995). A group of surface molecules have been reported to be expressed as a complex in close vicinity of the BCR. These molecules are CD19, CD21 and CD81. Together with CD22 they are involved in modulating the response delivered through the BCR (Fearon *et al.* 1995; O'Keefe *et al.* 1996). It has e.g. been demonstrated that crosslinking of CD19 as well as CD40 lowers the amount of antigen needed for sIg stimulation, whereas CD22 ligation raises this threshold (Carter *et al.* 1992; Wheeler *et al.* 1993). As mentioned earlier, CD28 and CTLA-4 have different binding properties and it has been demonstrated that CTLA-4 has 10-fold higher affinity for the B7 molecules than CD28. Blocking CTLA-4 binding to CD80/CD86 resulted in increased T cell proliferation, indicating an inhibitory role for CTLA-4 by inhibiting IL-2 production, indirectly causing apoptosis (Krummel *et al.* 1996; Walunas *et al.* 1996; Chambers *et al.* 1997). CD44H seems to play a role in lymphocyte activation as it has been shown that CD40 ligation of B cells rapidly upregulates CD44 (Guo *et al.* 1996) and ligation of CD44 seems to have strong synergy with CD2 and CD3 signalling in T cell activation (Denning *et al.* 1990; Conrad *et al.* 1992). Figure 2 shows a possible order of the signalling events in B-T cell cognate interaction.

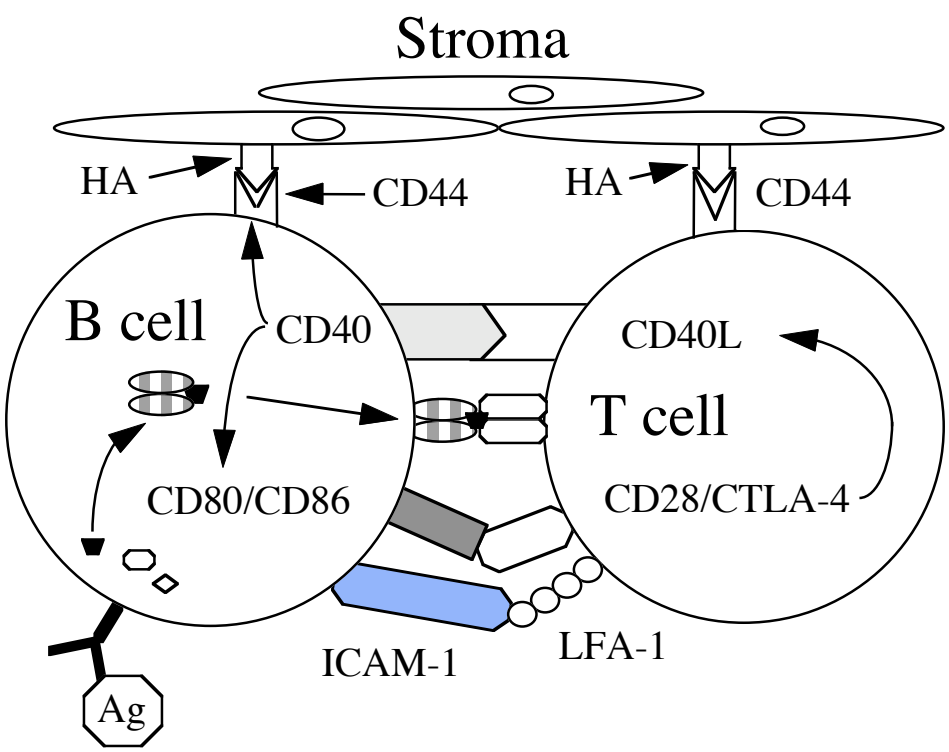

Figure 2. Signalling in B-T cell interaction.

Other receptor ligand pairs are also involved in B-T cell interactions such as OX40-OX40L and CD27-CD70, and both these pairs belong to the TNFR and TNF superfamilies.

4 T cell dependent antibody response

Secondary lymphoid tissues i.e. lymph nodes, spleen, and mucosa-associated lymphoid tissue (MALT) are the sites for initiation of the immune response. Two of the major functions of the secondary lymphoid tissues are antigen collection and lymphocyte recruitment into specialised microenvironments. Figure 3 outlines some the different areas of a spleen.

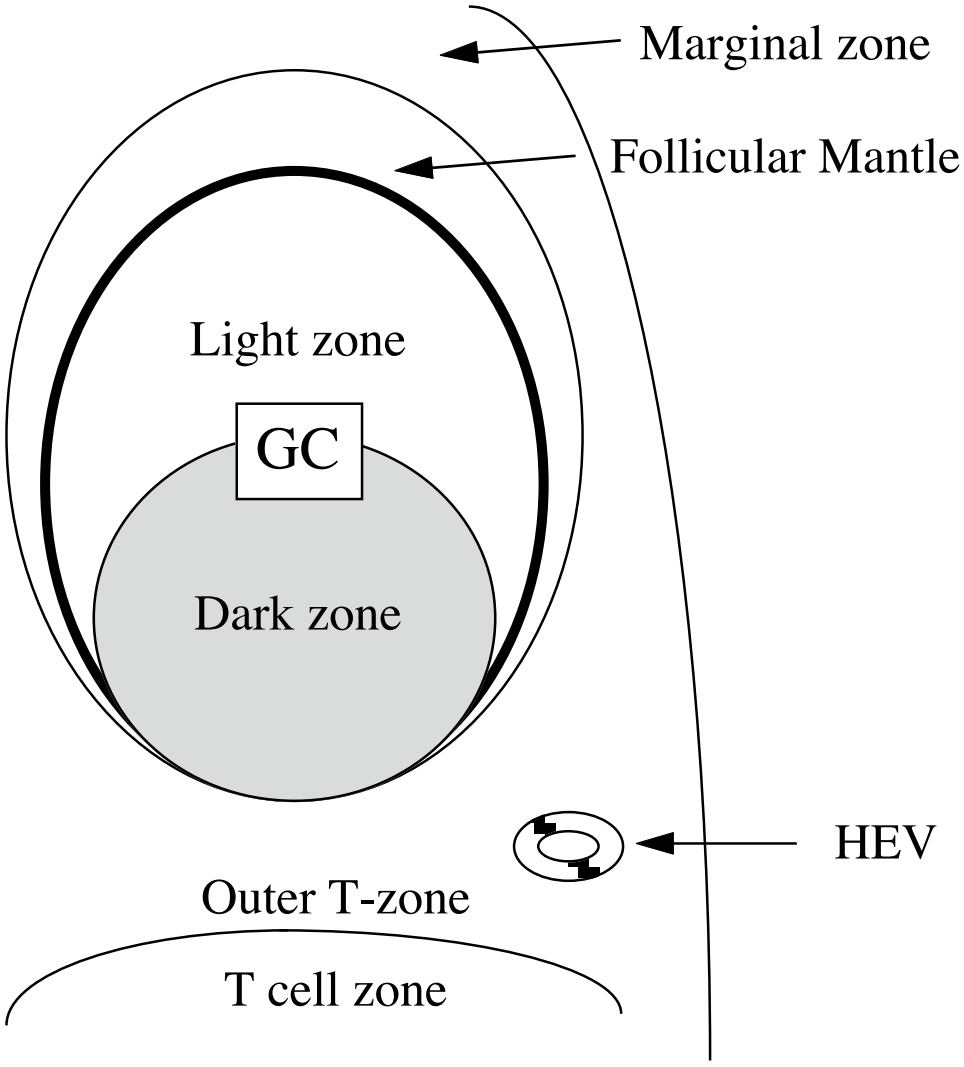

Figure 3. The different compartments of secondary lymphoid tissue (spleen).

Dendritic cells (DCs), like e.g. the Langerhans cells of the skin, pick up antigens and differentiate during their migration to the lymphoid organs (Larsen *et al*. 1990; Steinman *et al.* 1997). The DCs enter the T cell rich zone of the lymphoid organs via the afferent lymphatics (Lukas *et al*. 1996). These T-zones have different names depending on the lymphoid organs; in spleen they are referred to as periarteriolar lymphoid sheaths (PALS), in lymph nodes they are called deep cortex and paracortex and in the Peyer's patches of MALT they are named the inter follicular zones. The DCs in the T cell areas are usually referred to as interdigitating cells (IDCs) (Veldman 1970).

4.1 Primary immune response

Priming of T cells takes place in the T-zone of the lymphoid organs by cognate interaction with IDC's (Larsen *et al.* 1990). Lymphocytes enter the lymphoid organs via high endothelial venules (HEV) in the T cell zone. After priming, T cells can either leave the lymphoid organs to become effector cells or memory T cells (Powrie *et al.* 1989) or they can migrate to the outer T-zone and provide help together with memory T cells in the secondary immune response (Powrie *et al*. 1989; Akbar *et al.* 1988.; Beverly 1990). The marginal zone, which surrounds the follicular mantle, is populated with virgin and memory B cells (MacLennan *et al*. 1997). These marginal zone B cells can be found 3 days after immunisation and are present even a year later (Liu 1996a). In spleen, the marginal zones are rich in blood sinusoids (Herman 1980), which makes it easy for the marginal zone B cells to pick up antigen from the blood. When marginal zone B cells get a signal via their sIg, they migrate to the T-zone (Liu *et al.* 1988; Liu *et al.* 1991; Toellner *et al.* 1996), where they establish cognate interactions with primed T cells (figure 4).

The number of newly formed virgin B cells specific for a single antigen is very low and since they enter the T-zones during their normal migration it is difficult to analyse if their migration to the T-zones is antigen driven or not (Howard *et al*. 1972; Lortan *et al*. 1987). The virgin B cell probably comes in contact with the antigen in the blood as it migrates into the secondary lymphoid

organs, although there is no evidence for this. During cognate interaction of virgin B cell, or antigen activated marginal zone B cell and a primed T cell, the B cell receives signals via MHC, by interacting with the TCR complex. There is not much knowledge about which other signals take place during this cognate interaction in the outer T-zone, but it has been shown that signalling through CD40 (Foy *et al.* 1994) and CD80/CD86 (Ronchese *et al.* 1994) is essential for the formation of germinal centres.

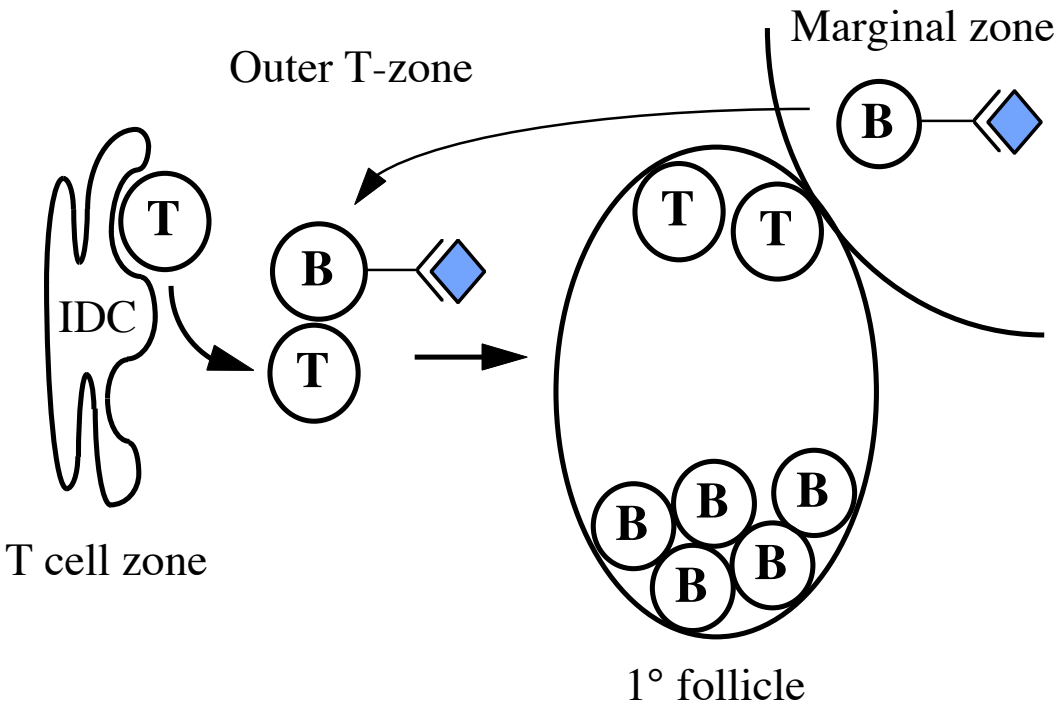

Figure 4. Primary immune response.

A B cell, that has been stimulated by a primed T cell, can either enter extrafollicular foci to become a short lived plasma cells (Ho *et al.* 1986; Smith *et al.* 1996) or enter primary follicles and form germinal centres. It is still not known what induces the B cells to enter a primary follicle (figure 4), but the same cell might be the progenitor of both early plasma cells and GC founder B cells, as it

has been shown that cloned B cells of both follicular and extra follicular origin share junctional diversity of their Ig variable region (Jacob *et al.* 1992). It has been shown that OX40 is expressed on activated T cells and OX40L has strong expression on activated extrafollicular B cells. *In vitro* studies on murine B cells show that signalling via OX40L induces proliferation and differentiation into plasma cells indicating the importance of the OX40-OX40L receptor-ligand pair in early plasma cell differentiation (Stüber *et al.* 1995; Stüber *et al.* 1996). Histochemical stainings for OX40 show that the expression is mainly extra follicular, indicating a role for OX40 in plasma cell differentiation during the primary response (Stüber *et al.* 1996). Cytokines, such as IL-3, IL-6 and IL-10 are also likely to be involved in the direction of the primed B cells towards plasma cells (Liu *et al.* 1997a). It has also been shown that *in vitro* cultured naive B cells together with IDCs give rise to IgM secreting plasma cells (Björck *et al.* 1997).

4.2 Germinal centre formation and somatic mutations

After cognate interaction in the outer T-zone, some B cells migrate to the primary follicles, as mentioned earlier. The T cells also migrate to the follicles, but whether they migrate separately or as a B-T cell complexes is not known (MacLennan *et al.* 1997). Inside the follicles the primed B cells proliferate at an exponential rate and the follicle is filled from the T-zone end towards the follicular mantle (FM) and a germinal centre (GC) is formed (see figure 4). GCs are formed quickly after an immune challenge. Studies in rats show that the first proliferating B blasts (specific for the antigen) can be detected after 24 hours (Liu *et al.* 1991). For the next 3 days, rapid proliferation can be detected with a peak around 36 hrs and by day four mature GCs have developed.

The area of the GC, closest to the T-zone, is called dark zone and it is filled with densely packed cells in cycle (figure 5). The B cells within the dark zone are called centroblasts. The centroblasts are sIg negative and proliferate extensively after the dark zone has been filled giving rise to non-proliferating centrocytes, that migrate into and populate the light zone.

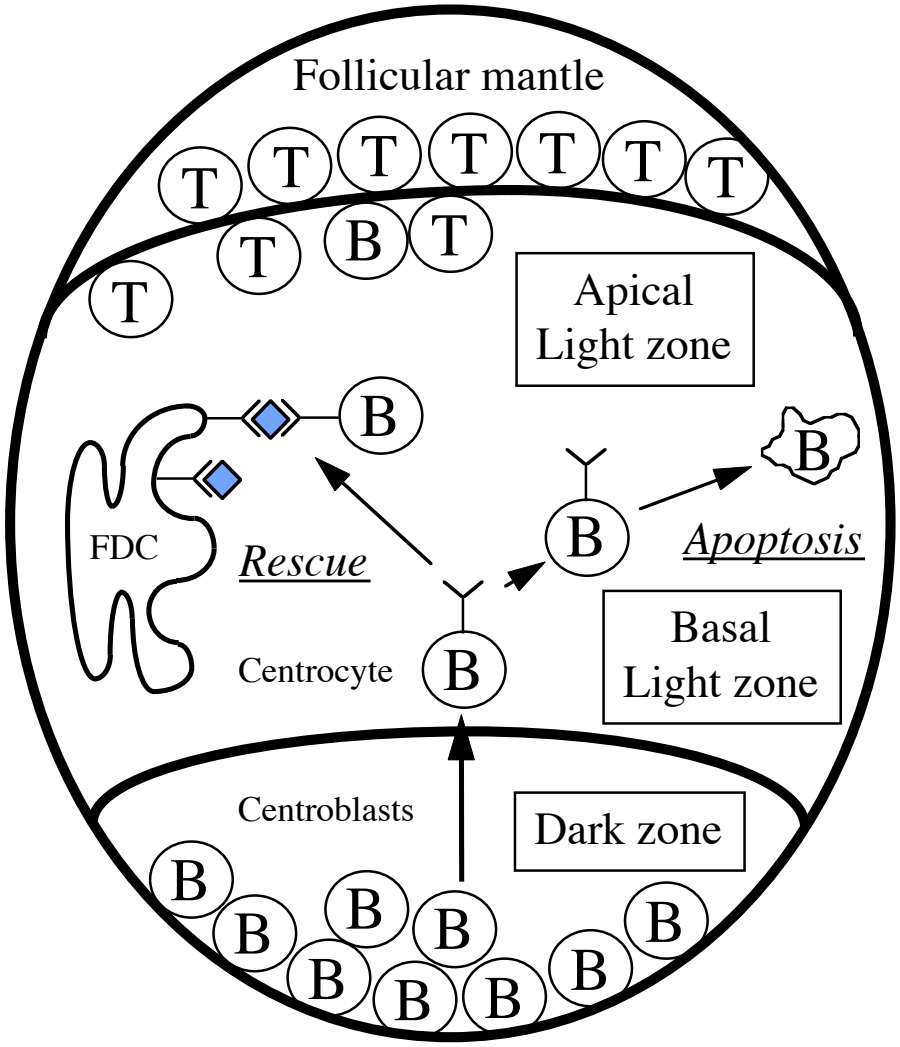

Figure 5. The structure of a germinal centre.

The majority of the cells in GC are B cells, but T cells represent about 10-15% of the cells in the GC (Liu *et al.* 1997b). The T cells within GCs are found as a broad band in the upper part of the light zone (apical light zone) and more dense in the outer zone (Hardie *et al.* 1993; MacLennan *et al.* 1997). The structure of a germinal centre is shown in figure 5. A few percent of GC cells are called tingible body macrophages (TBM). The TBMs are found in the light zone and contain chromatin fragments (tingible bodies) from phagocytosed cells, that have

undergone apoptosis (Chan *et al.* 1993). A unique feature of the GC is the network of cells called follicular dendritic cells (FDCs). This FDC network is fine and widely spread in the dark zone, but it becomes more dense in the light zone. Recent evidence suggest that the FDC's are of bone marrow origin (Szakal *et al.* 1995) and have the unique feature to hold antigen on their surface in the form of immune complexes for as long as 11-12 months (Mandel *et al.* 1980). A distinct morphological phenomenon of the FDC's is that at the end of their dendrites they form bead shaped structures called iccosomes, that have immune complexes on their surface. The GC B cells take up antigen from the FDCs by internalising the iccosomes, when their BCR binds the immune complex with sufficient affinity (Tew *et al.* 1989). As mentioned earlier, an additional type of dendritic cells have been located in GCs. These cells are $CD4^{+}CD3^{-}$ and they have the ability to stimulate GC T cells (Grouard *et al.* 1996).

When the GC is fully developed, somatic point mutations start to occur in the variable region of the immunoglobulin genes (Berek *et al.* 1988) and this process is initiated in the centroblast population (Pascual *et al.* 1994). There is not much known about the signals that initiate the mutation process, but it has been demonstrated that the GC formation and somatic mutation process are separate events and that the degree of mutations is dependent on the amount of T cell help available (Miller *et al.* 1995a). It has also been shown that the mutation rate is lower in aged mice, which seems to partly depend on CD86 expression (Miller *et al.* 1995b). Moreover addition of anti-CD86 antibodies lowers the amount of mutations in normal mice (Han *et al.* 1995a), indicating that signalling via CD86 is directly or indirectly involved in the onset of the mutation process.

4.3 Positive and negative selection in germinal centres

The rate of centrocytes entering apoptosis (programmed cell death) is high in GCs and in the area closest to the dark zone, the death rate is highest. It has been shown, that unless these newly formed centrocytes can bind immune complexes on FDCs via their sIg, they enter apoptosis (Liu *et al.* 1989; Lindhout *et al.* 1993). However, ligation of the sIg is not enough to rescue the centrocyte from apoptosis

and the following chapter will discuss the possible candidates for the second signal.

It has been demonstrated that only few minutes after sIg triggering, adhesion molecules are activated and LFA-1:ICAM-1 and VLA-4:VCAM-1 adhesion takes place (Hedman *et al.* 1992). Attempts have been made to rescue centrocytes from apoptosis by *in vitro* crosslinking molecules such as LFA-1, VLA-4, CD21, CD40 and sIg, but none of these attempts could prevent B cells from undergoing apoptosis (Liu *et al.* 1989; Koopman *et al.* 1991; Bonnefoy *et al.* 1993; Lindhout *et al.* 1995).

One of the major questions about the second signal seems to be whether CD40 has a major role in the immediate rescue after the B cells enter the light zone or not. Evidence supporting CD40 involvement are e.g. 1) Co-cultures of freshly isolated GC B cells and memory T cells resulted in downregulation of CD77 and upregulation of CD44, which is characteristic for B cells after rescue from apoptosis (Casamayor-Palleja *et al.* 1996), 2) CD40 ligation of centrocytes caused a delay of apoptosis by 48 hrs (Holder *et al.* 1993; Casamayor-Palleja *et al.* 1996) and 3) Freshly isolated GC T cells did not express CD40L, but a transient expression of the ligand can be induced in 5 minutes upon cognate interaction (Casamayor-Palleja *et al.* 1995). There are however data indicating that CD40 may not be involved this early in the selection stage such, as 1) As mentioned earlier, stainings for T cells in GCs show that the majority of the T cells are found as a broad band in the upper part of the light zone (apical light zone) and more dense in the outer zone. This indicates that when the centrocytes migrate into the light zone there are no or very few T cells available to provide the CD40 signal, 2) CD40 and sIg ligation only delays apoptosis, but does not prevent it (Liu *et al.* 1989), 3) If the CD40-CD40L interaction is blocked at the selection stage in GC, the death rate in GCs is not increased (Foy *et al.* 1994; Han *et al.* 1995a and b; Gray *et al.* 1996) and 4) CD40 ligation of GC B cells suppresses their differentiation into plasma cells (Arpin *et al.* 1993), indicating that if all centrocytes are rescued from apoptosis by CD40 ligation they all get a differentiation signal towards memory cells.

A CD40 signal is almost certainly vital for centrocytes after rescue from apoptosis, but it is more likely to be important during switch and differentiation rather than positive selection. It has been demonstrated in mice, that the T cells within GCs, are specific for the immunising antigen (Fuller *et al.* 1993). This may indicate that T cells participate in the selection of B cells after immediate rescue by FDCs. There are however other signals that might be important in the immediate rescue of GC B cells from apoptosis. A specific cysteine proteinase inhibitor, Cystatin A, has been shown to be actively transported from FDCs to GC B cells and this inhibitor seems to block the apoptotic cascade (Van Eijk *et al.* 1997). A redistribution of sIg, CD19, CD21, CD22 and CD11c towards the contact area between the B cell and the FDC also shows that these molecules might play a role in the selection process (Lindhout *et al.* 1997).

4.4 Class switching and terminal differentiation of B cells

After being rescued from apoptosis, B cells bearing high affinity BCR need signals for terminal differentiation. They migrate into the apical light zone towards the outer zone, which is loaded with T cells. CD40 is very likely to play a central role at this stage. As mentioned earlier, the mutation machinery and the switching mechanism are separate events and we know that CD40 signalling has a major role in immunoglobulin switching (Allen *et al.* 1993). Isotype switching occurs within germinal centres in the centrocyte population (Bm 4) (Liu *et al.* 1996c). Together with CD40 signalling, cytokines have been strongly suggested to contribute to class switching. Interleukin-4 (IL-4) and IL-13 are e.g. switch factors for IgE (Vercelli 1995), whereas TGF-ß induces IgA switching in human B cells (Islam *et al.* 1991). It should also be mentioned that some cytokines, such as IFN-γ, have inhibiting effects on switching to certain isotypes (Stavnezer 1996). The duration of signals such as CD40 are also likely to be important in B cell differentiation as was demonstrated employing *in vitro* cultured human B cells. Germinal centre B cells were cultured on CD40L expressing L cells together with IL-2 and IL-10 for 3 days. These cultures were then continued for another 4 days with or without CD40 stimulation. Cells with continued CD40 stimulation developed a memory B cell phenotype, whereas removal of CD40 stimuli caused plasma cell

differentiation (Arpin *et al.* 1993). Therefore, it may be the duration of CD40L expression on T cells that controls terminal differentiation of GC B cells.

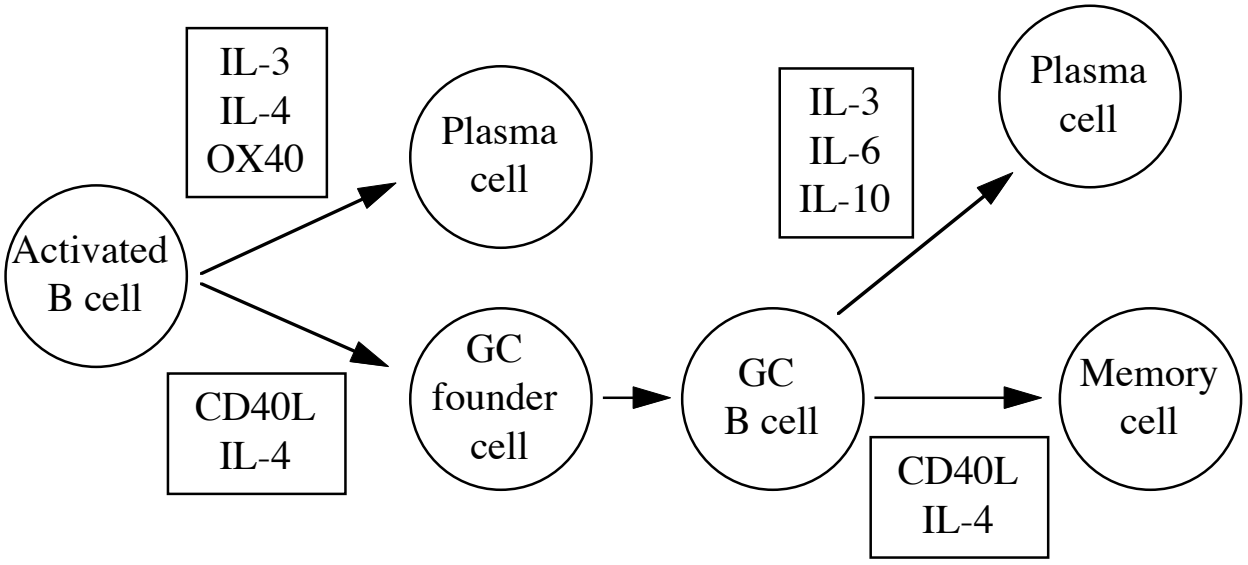

Figure 6. Signals involved in extrafollicular and GC B cell differentiation.

Different signals seem to be required for extra follicular and GC induced plasma cell differentiation. OX40 ligation has been shown to play a major role in promoting B cells to undergo plasma cell differentiation early in the immune response (see page 16). *In vitro* studies of human B cells show that IL-3, IL-6 and IL-10 promote Ig secretion, whereas IL-4 induces B cell proliferation (Arpin *et al.* 1993, Rousset *et al.* 1995). A summary of signals involved in memory and plasma cell differentiation is shown in figure 6. (Adapted from fig. 1 in Liu *et al.* 1997a).

5 Transcription factors in lymphocyte activation

In paper IV of this thesis, I have looked at the use of transcription factor NFκB in B cell activation. The transcriptional activity of the Ig genes is regulated by promoters and enhancers. These are genetic sequences that can bind specific proteins called transcription factors. Other transcription factors, that play important roles in B and T cell biology are e.g. Oct1, Oct2 and AP-1. Oct1 is ubiquitously expressed whereas Oct2 is lymphoid specific and activates Ig gene transcription. The transcription factor AP-1 consists of two subunits fos and jun, which are both proto-oncogenes. AP-1 is necessary for transcription of the IL-2 gene. The activation pathway of NFκB will be discussed below.

5.1 NFκB

The transcription factor NFκB binds to specific DNA sites and was first described to bind to the intron enhancer of the κ light chain gene (Sen *et al.* 1986). NFκB has been suggested to be important in immune and inflammatory responses, cell adhesion growth control and apoptosis (Baldwin *et al.* 1996; Baeuerle *et al.* 1996). The NFκB protein consists of two subunits (p50 and p65) and it belongs to the NFκB/relB transcriptional regulator protein family. Activation of NFκB can be achieved in many ways, e.g. by TNFR- or IL-1R-ligation, LPS stimulation or T and B cell antigen receptor crosslinking (Verma *et al.* 1995; Baeuerle *et al.* 1996). In almost all cells, except for B cells, NFκB is found in the cytoplasm bound to an inhibitory class of proteins known as the IκB family (figure 7) (Verma *et al.* 1995). In the event of appropriate stimuli the IκB is phosphorylated and subsequently degraded leading to NFκB activation (Verma *et al*. 1995; Baldwin 1996). The heterodimeric p50/p65 complex can now be transported into the nucleus. NFκB is constitutively present in the nucleus of mature B cells and was therefore initially suggested to be lymphoid specific (Verma *et al*. 1995). The activation of NFκB has been studied quite extensively and today, several but not all of the links in this intracellular signalling pathway are known. Three of these pathways are shown in figure 7. Signalling via TNFR results in interaction

between the TRADD adaptor protein and TRAF2. TRAF2 is a member of the TRAF signalling adaptor family, which today includes 6 members (Lee *et al.* 1997). Ligation of IL-1R however results in activation of IL-1 Receptor Kinase (IRAK), which leads to interaction with TRAF6. Both TRAF2 and TRAF6 can interact with and activate the NFκB Inducing Kinase (NIK). NIK then binds IKKa, which causes IκB phosphorylation and NFκB activation. The third pathway, shown in figure 7, involves the mitogen activated protein (MAP) kinase cascades. Through cytokine stress signals, mitogen or other unknown signals, a MAP kinase pathway is utilised to activate pp90rsk, which causes IκB phosphorylation (Stancovski *et al.* 1997).

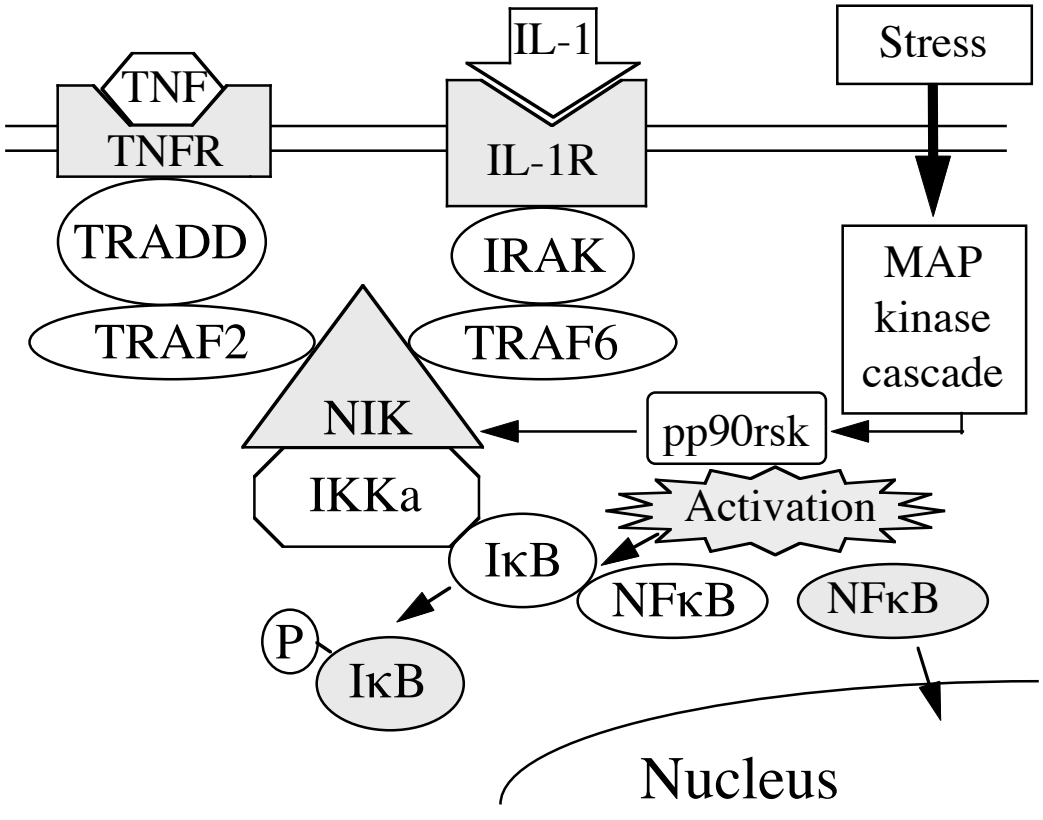

Figure 7. Three pathways leading to NFκB activation.

6 *In vitro* generation of specific antibodies

Antibodies are powerful molecules and their unique property to bind other molecules with certain specificities make them interesting as analytical tools and for therapeutic applications. Each B cell produces only one type of antibodies, i.e. single specificity. B cells were, however, difficult to grow *in vitro* and it was impossible to get them to produce large amounts of antibodies with a single specificities. Köhler and Milstein came up with the solution to this problem and their publication in Nature 1975 called ”Continuous cultures of fused cells secreting antibody of pre-defined specificity” (Köhler *et al.* 1975), revolutionised the field of antibody generation. What they did was to fuse a normal B cell with a myeloma cell line and then clone a cell line producing an antibody with a single specificity, a monoclonal antibody (mAb). In the beginning, mice were immunised with the antigen of interest and B cells from the mouse were used for making the mAbs. This technology, called the hybridoma technology, progressed quickly and resulted in production of murine monoclonal antibodies. These murine antibodies were tested for therapeutic purposes and even if they showed some positive effects in therapy, human antibodies were raised against them causing a so called HAMA (Human-Anti-Mouse Antibody) response. The HAMA response inhibited the effect of the antibodies in therapy and forced scientists to start to design methods to produce human antibodies, but since immunising men and women is not a feasible option, *in vitro* immunisation technology progressed. The first *in vitro* immunisations of human peripheral blood lymphocytes (PBL) resulted in low affinity IgM antibodies (Danielson *et al.* 1987, Borrebaeck *et al.* 1987; Borrebaeck *et al*. 1988), but in 1995 an *in vitro* immunisation procedure was presented showing for the first time isotype switching of antigen specific B cells (Chin *et al.* 1995).

An alternative way to obtain specific human antibodies is to use the phage display systems and the library technology, and these methods have developed very fast during the past few years. Phage display technology is based on ligating the gene coding for the variable regions of the antibody to the end of the coding

sequence for the phage coat protein pIII. The phage then expresses its protein together with the binding part of the antibody. By coating the antigen of interest on a surface, phages can be selected in terms of their binding ability. The chances of finding a binder depend among other things on the size of the antibody library (Hoogenboom 1997). Different kinds of libraries can be used, such as a naive library obtained from B cells of unimmunised donors, PBL, bone marrow or spleen, or synthetic libraries, which are generated by randomising CDR regions of germ-line segments or rearranged V genes. (Marks *et al.* 1991; Gram *et al.* 1992; Hoogenboom *et al.* 1992: Barbas *et al.* 1992; Söderlind *et al.* 1995; Kobayashi *et al.* 1997).

7 The present investigation

The goals of this study were (i) to investigate the signals required for differentiation of naive B cells towards a germinal centre phenotype and for those cells to acquire the features of germinal centre centroblasts and (ii) to design an *in vitro* immunisation protocol resulting in production of antigen specific antibodies.

i) In order to generate GC B cells from naive B cells, which is a T cell dependent and antigen driven process, we used the so called CD40 system to provide the T cell signal and anti-IgM antibodies to provide signals via the BCR (B cell signal). Anti-CD44 antibodies were also added to generate an extra costimulatory signal. The three antibodies were crosslinked by Fc receptors expressed on transfected fibroblasts. The first paper describes the phenotypical changes that occur on naive B cells when they are stimulated via CD40, sIgM and CD44 and the physiological properties of these cells, i.e. proliferation and apoptosis induction (paper I).

ii) Papers II-IV describe three different *in vitro* immunisation protocols, that were designed or utilised for generation of human antigen specific antibodies. In the first protocol, anti-CD3 stimulated PBL, from newly immunised individuals, were introduced to a recall antigen to study antigen specific antibody production (paper II). In the second protocol, the superantigen staphylococcal enterotoxin A (SEA) was used to provide TCR-MHC class II interaction and the B cells were given the BCR signal by crosslinking their sIg with antigen or pseudo antigen signals (paper III). The third approach was based on using a system that has previously been shown to generate switch from IgM to IgG. This system was used to analyse the effects T cell secreted cytokines, T cell subsets and transcription factors on specific IgE production (paper IV). These data indicate that we have identified the first definite role of CD44 in B cell maturation.

7.1 Paper I.

In order to investigate the signals, responsible for initiation of the somatic mutation process, we studied the requirements for B cells to acquire the phenotype of a centroblast. Our culture system was based on earlier reports (Galibert *et al.* 1995; Wheeler *et al.* 1996) showing that a partial GC phenotype could be obtained by stimulating the B cells via CD40 and surface IgM. The fact, that CD44 down regulation has never been observed *in vitro* and that the involvement of CD44 in homing, adhesion and signalling events of lymphocytes is evident (Lesley *et al.* 1993) lead us to investigate if CD44 signalling was needed for differentiation towards GC B cells. A mAb against CD44 was therefore included in the system (figure 8).

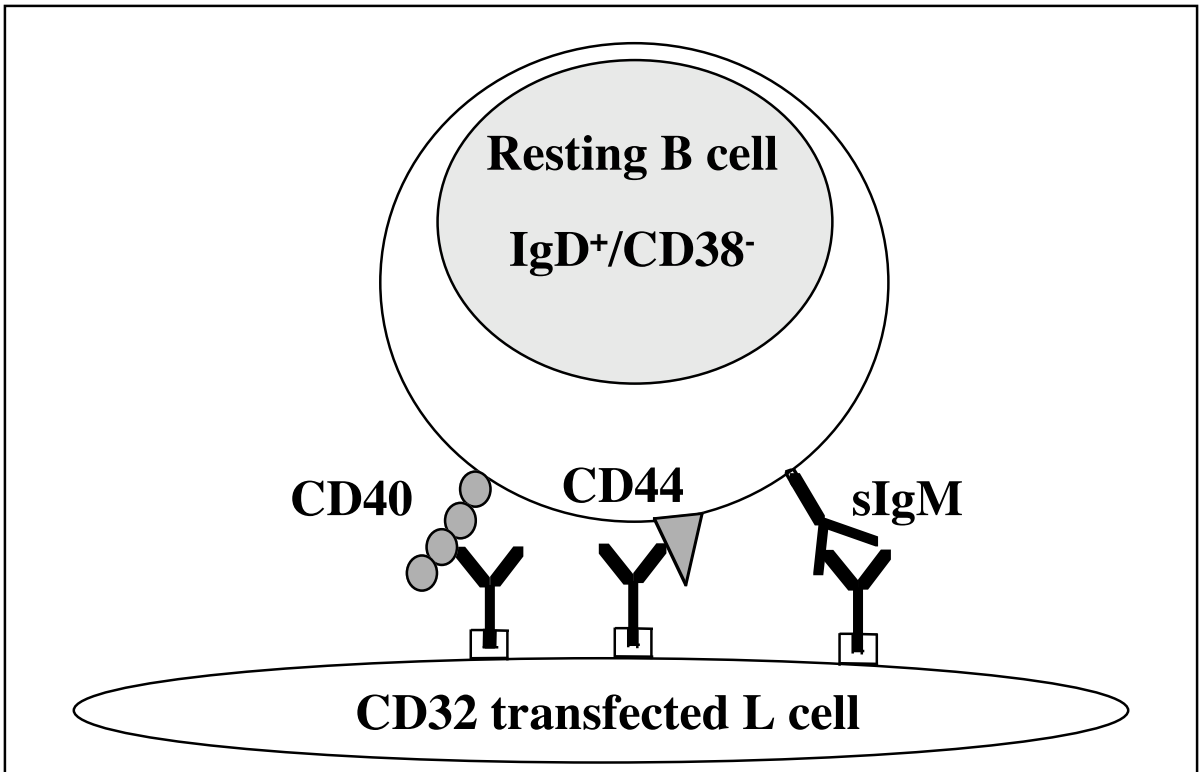

Figure 8. The CD44 culture system.

Our data show that addition of the anti-CD44 mAb induces an upregulation of typical GC markers such as CD10, CD38, CD77 and CD95 whereas CD39 and

CD24 are downregulated, which is also characteristic for GC B cells. CD44 and sIg are downregulated on GC B cells, but we could not analyse for their expression, since mAbs against these surface molecules were used to stimulate the naive B cells. Instead of inducing CD23 downregulation we observed an increase in CD23 expression. This could be explained by *in vitro* studies that have shown that CD40 ligation upregulates CD23 (Mangeney *et al.* 1995).

We analysed the proliferation at different time points after initiation of cultures and observed that the proliferation was at least 5 times lower in cultures without sIg stimulation. That indicates an antigen driven proliferation of the B cells, in congruence with what would have been expected. Since GC B cells are destined for apoptosis we investigated if the *in vitro* generated GC B cells were apoptotic. We could see that the CD10 positive cells from CD44 stimulated cultures were apoptotic (about 50%), whereas less than 10% of all cells in cultures without anti-CD44 were apoptotic.

7.2 Paper II.

This investigation was based on earlier reports stating that B cells could be activated with anti-CD3 stimulated T cells, causing B cell proliferation and differentiation into plasma cells secreting high levels of antibodies (Stohl et.al 1987; Vernino *et al*. 1992). Donors, that had been immunised against the recall antigen tetanus toxoid (TT), were used to obtain higher frequency of antigen specific B cells. The anti-CD3 stimulation increased the levels of total as well as specific antibodies in our cultures, but addition of the antigen totally inhibited the specific response, whereas the total IgG production was not altered. We made several attempts to restore the abrogated response by e.g. adding cytokines and antibodies against costimulatory molecules and removing the antigen without success. We could also see that the frequency of B cells, secreting specific antibodies, was lower in the cultures with soluble antigen. By crosslinking the antigen, bound to the B cells, with murine anti-TT antibodies, the specific B cells could be induced to produce antibodies in the same amount as the cells in cultures without antigen (figure 9).

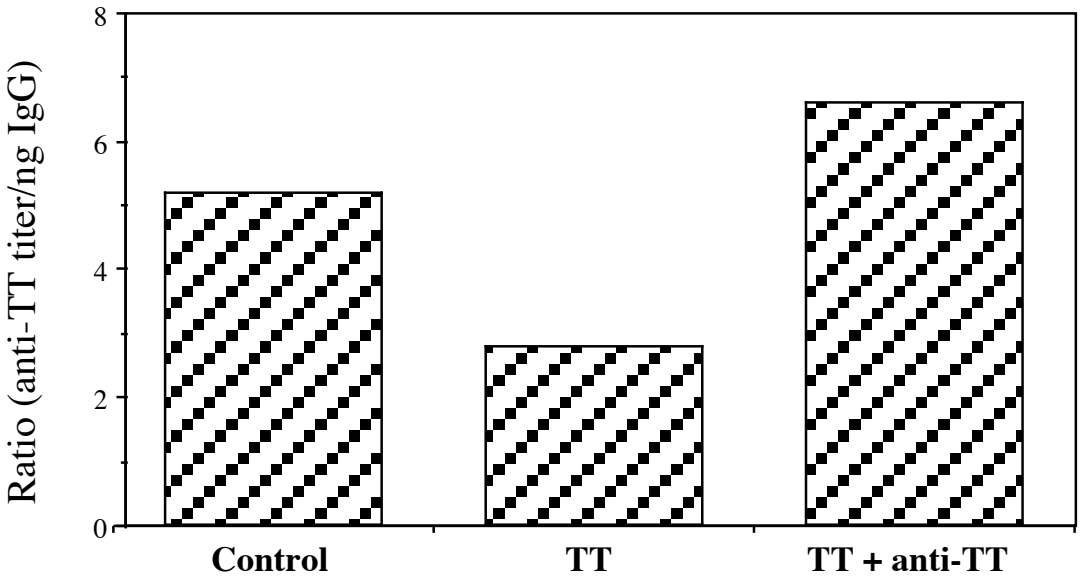

Figure 9. Tetanus toxoid alone reduces the specific antibody production but crosslinking of the antigen, bound to sIg, restores the inhibited response

Soluble antigen has been shown to reduce the number of memory B cells, even after challenge immunisation (Nossal *et al.* 1993), which could explain our results. Another possibility is that when a memory B cells reencounters antigen it migrates into the T cell zone. There it can become a plasma cell, but the plasma cell differentiation signal is absent in our cultures, causing tolerance. One might even speculate, that in the absence of a differentiation signal towards plasma cells, the cell starts to acquire a GC phenotype such as eliminating Ig production and becoming sIg negative, but lacking the additional signals for full differentiation. Our results may therefore indicate that the B cells we stimulate are memory B cells receiving the antigenic signal, but lacking the plasma cell differentiation signal causing either tolerance or apoptosis.

7.3 Paper III.

In this investigation we wanted to activate B cells via sIg and by providing cognate interaction with T cells using suboptimal concentrations of SEA as a T cell

mitogen. Since SEA binds TCR after binding to MHC class II, pseudo antigen specific signal is provided to the T cell causing CD40 upregulation. CD40 ligation of B cells has been shown to lower the threshold for sIg activation. Isolated B cells were pre-incubated with pseudo B cell antigen (anti-IgM), primary as well as recall antigens, to preferentially activate B cells specific for the desired antigen. The anti-IgM or the antigen were then crosslinked with antibodies and/or antibody coated beads (figure 10).

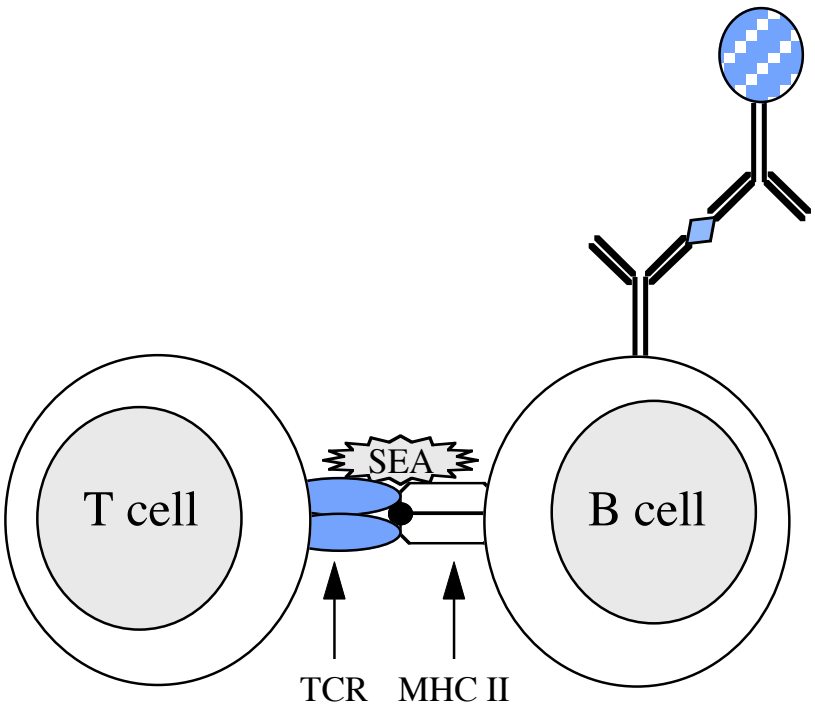

Figure 10. The Crosslinking system.

Our results show, a synergistic effect of sIg stimulation and SEA activation and that the degree of crosslinking is important for specific antibody production. Only specific IgM antibodies were produced in the case of primary antigens, whereas mainly IgG antibodies could be detected against the recall antigen, so there is no evidence for class switching in this system. The antibody production

was dependent on CD28-CD86 and CD40-CD40L interaction and blocking of both signals almost completely abrogated the Ig response.

7.4 Paper IV.

Here we took advantage of a system, capable of generating switch from μ to γ, to generate specific IgE antibodies and to study the required signalling mechanism behind the IgE production. This system is based on using a heterotope peptide with a T cell epitope of a recall antigen and a B cell epitope of a primary antigen. In the initial study (Chin *et al.* 1995), specific IgG antibodies were detected only after secondary immunisation with a continuos CD40 signal. Figure 11 shows the outline of the experimental setup and the results. We obtained both total and specific IgE antibodies after primary as well as secondary immunisation, although the frequency of IgE positive wells was usually higher after the primary immunisation. One donor produced however high amounts of IgE and specific IgE production was completely dependent on addition of IL-4. T cell analysis showed that during primary immunisation, over 50% of the cells were IL-4 positive whereas only about 10% were positive for intracellular IFN-γ. After secondary immunisation, a change of profile seemed to have taken place as the majority of the T cells were now of a TH1 phenotype and exogenous IL-4 could not maintain the TH2 phenotype, that had developed during the primary immunisation.

Analysis of NFκB activation revealed strong activation during primary immunisation in both B and T cells. NFκB was activated in the presence of IL-4 in the secondary immunisation, but cultures lacking IL-4 showed decreaed NFκB activation in B and T cells. These data suggest that NFκB activation in lymphocytes is IL-4 dependent and that the T cells in the primary immunisation provide enough IL-4 for this activation, whereas the dominating TH1 cells in the secondary immunisation do not. Although exogenous IL-4 did not result in sustained TH2 phenotype during secondary immunisation it was sufficient for activating NFκB in both T and B cells and to induce specific IgE secretion.

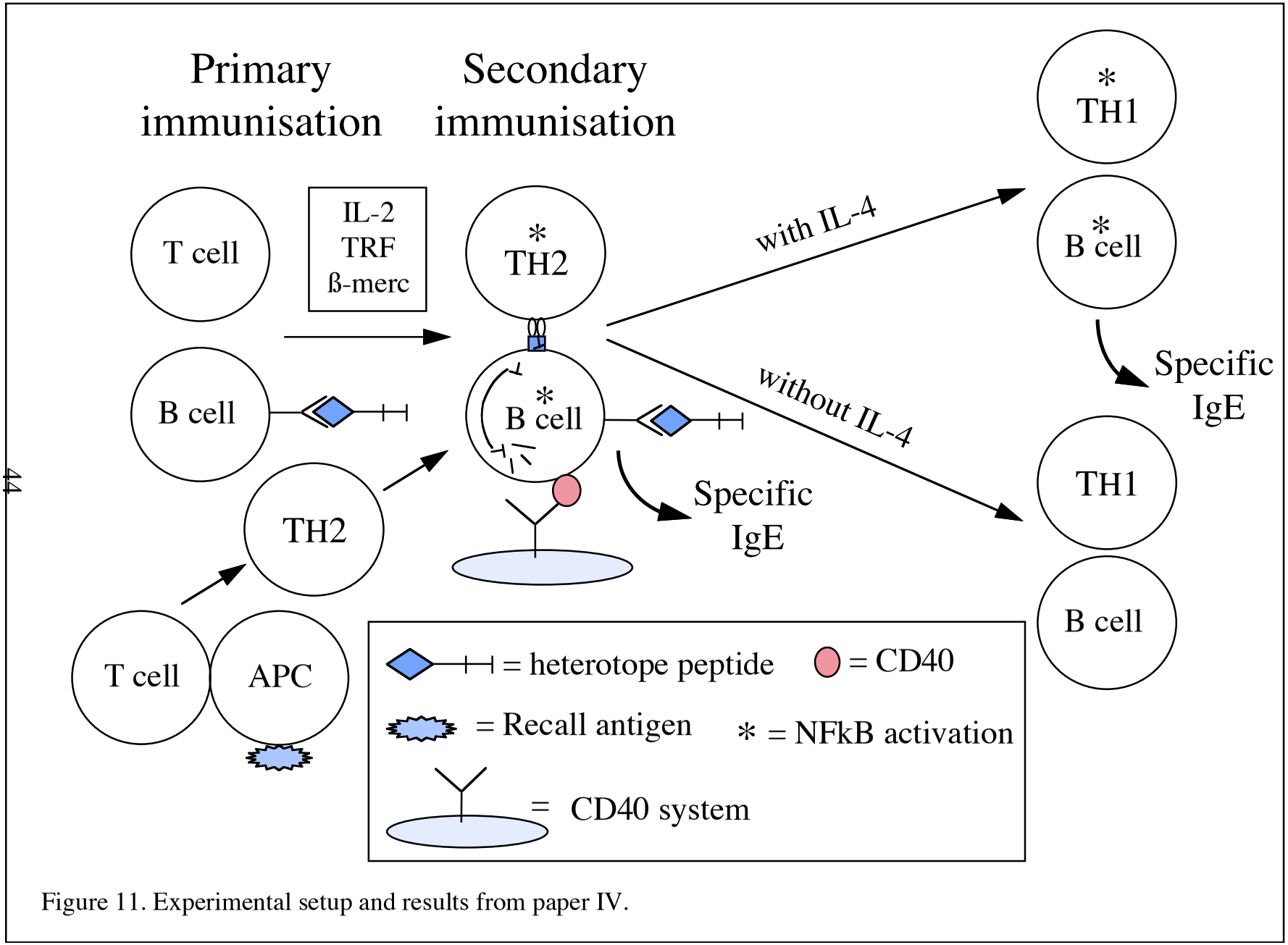

Figure 11. Experimental setup and results from paper IV.

8 Concluding remarks

This thesis involves studies on the signalling mechanism in B cell differentiation, allowing them to produce specific antibodies with high affinity. It describes three different *in vitro* immunisation technologies to obtain antigen specific antibodies of human origin.

The *in vitro* system, using CD3 activation of PBL, demonstrated the importance of what form the antigen is presented to the B cells. The ”crosslinking system” presents an efficient procedure for primary immunisation and shows, that the degree of crosslinking effects the signalling via BCR. Using the *in vitro* immunisation system, that previously had been reported to induce switch from μ to γ, demonstrated that IgE switch is dependent on T cell secreted cytokines for NFκB activation. We demonstrate for the first time the significance of NFκB activation in T-B cell collaboration and its effect on specific IgE switch *in vitro*.

To make the *in vitro* immunisation system more efficient one needs to understand the differentiation stages of peripheral B cells and try to mimic the *in vivo* events as much as possible. Attacking this problem from the very beginning I wanted to differentiate naive B cells allowing them to acquire a germinal centre phenotype. This was achieved by introducing a signal via the CD44 molecule and for the first time showing a role for this molecule in B cell differentiation as well as generating a full germinal centre phenotype. The upregulation of CD77 and the induction of apoptosis, which are related features of germinal centre B cells, strongly suggests that functional GC B cells have developed in our cultures. The next steps will be to study the signals required for maintaining that phenotype and what causes onset of the somatic mutation process. These investigations then need to be followed by trying to understand the rescue and selection signals in positive selection of germinal centre B cells. Finally, These discoveries could pave the way for pivotal studies on mechanisms underlying the somatic hypermutation process

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