Cellular and Molecular Aspects of the Germinal Centre Reaction

Katarina Dahlenborg

Lund 1998

Katarina Dahlenborg
Dept. of Immunotechnology
Lund University
PO Box 7031
S-220 07 Lund, Sweden

ISBN 91-628-3080-5
Printed by Bloms i Lund Tryckeri AB, Lund, Sweden

”At ville forstå naturens finmekanik ved hjælp af vores praktiske tankevaner er som at ta boxehandsker på for at løse en knude på en spindelvævstråd”

Piet Hein, Prosa-Gruk

Contents

Abbreviations

aa	amino acids
ab	antiboy
ADCC	antibody-dependent cell-mediated cytotoxicity
ag	antigen
APC	antigen presenting cell
BAP	B cell receptor associated proteins
BCR	B cell receptor
CB	centroblast
CC	centrocyte
CD	cluster of differentiation
CDR	complementary determining region
cl.	class
CTL	cytotoxic T lymphocyte
D	diversity region
DC	dendritic cell
DZ	dark zone
FDC	follicular dendritic cell
FMZ	follicular mantle zone
FR	frame work region
GC	germinal centre
GC-B	germinal centre B cell
GC-T	germinal centre T cell
GM-CSF	granulocyte macrophage–colony stimulating factor
H	heavy chain
ICAM	intracellular adhesion molecule
IDC	interdigitating cell
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ITAM	immunoglobulin tyrosine-based activation motif
J	joining region
κ	kappa chain
λ	lambda chain
L	light chain
LFA	leukocyte function associated antigen
LPS	lipopolysaccharide
LZ	light zone
MHC	major histocompatibility complex
MZ	marginal zone
NK	natural killer cell
OVA	ovalbumin
PALS	periarteolar lymphoid sheaths
PNA	peanut agglutinin
PTK	protein tyrosine kinase
RAG	recombinase activation genes
sIg	surface/membrane bound immunoglobulin
TDA	T cell-dependent antigen
TCR	T cell receptor

TH	T helper cell
TNF	tumour necrosis factor
V	variable region

Original papers

This thesis is based on the following studies, which will be referred to in the text by their Roman numerals.

I Dahlenborg, K., Pound, J., Gordon, J., Borrebaeck, C. A. K., and Carlsson, R. (1997) Terminal differentiation of human germinal centre B cells *in vitro*. *Cell. Immunol.* 175, 141-149

II Dahlenborg, K., Borrebaeck, C.A.K., and Carlsson, R. (1998) Single cell culture systems for assessment of *in vitro* mutational activity in human germinal centre B cells. *Submitted*

III Dahlenborg, K., Pound, J.D., Gordon, J., Borrebaeck, C.A.K., and Carlsson, R. (1998) *In vitro* accumulation of somatic mutations in human germinal centre B cells. *Submitted*

IV Andersson, E., Dahlenborg, K., Ohlin, M., Borrebaeck. C.A.K., and Carlsson, R. (1996) Immunoglobulin production induced by CD57+ GC-derived helper T cells *in vitro* requires addition of exogenous IL-2. *Cell. Immunol.* 169, 166-173

V Ingvarsson, S., Dahlenborg, K., Carlsson, R., and Borrebaeck, C.A.K., (1998) Coligation of CD44 on naïve human tonsillar B cells induces progression towards a germinal centre phenotype. *Submitted*

1 Introduction

The playground for the biology that this thesis deals with is a complex milieu known as the *immune system*. It is a remarkable organisation of physical, biochemical, cellular and molecular origin with one ultimate goal: to protect its host from harmful infections. With an environment full of bacteria, viruses, fungi and parasites it is not an easy task to maintain host integrity since an infection growing uncontrolled will, eventually, be lethal.

The immune system can be divided into at least two different compartments–the *innate* and the *adaptive*. The innate includes the physical barrier of skin, mucosal surfaces, stomach acid, some cellular components (phagocytes, NK cells) and proteins (lysozyme, complement), whereas the adaptive part consists of cells (T and B) and protein produced by B cells, *i.e. antibodies*. The adaptive part is characterised by a unique feature: the capacity to improve and memorise earlier events. The innate part has a more robust way of clearing foreign *non-self* structures by merely *engulfing* them. This thesis deals with a part of the adaptive immune-defence: how the cells collaborate to produce antibodies and how they improve and create memory for a rapid response upon re-encounter with a non-self structure, known as the *antigen*.

The T and B cells are involved in the process and circulate throughout the body via the blood stream and the lymphatics. When the cells pass through the body and especially the secondary lymphoid organs, such as spleen, tonsils and lymph nodes, they may encounter antigen. B cells have the capacity to take up antigen and to show or *present* it in the form of small pieces or *peptides* to the T cell. This involves an interaction or *recognition* of the antigen via a receptor on the T cell. Such T-B cell interactions will lead to a reciprocal communication and activation of the antigen-specific cells. If such communication proceeds in a correct manner, the B cell will get signals to produce antibodies which act as a powerful ”homing missile” for destruction or clearance of the invading non-self antigen. Ultimately, the B cell will migrate into a highly specialised microenvironment within the lymphoid organ, the *B cell follicle* (Fig. 1). When there, they will grow or *proliferate* clonally, change their appearance or *phenotype*, *i.e. differentiate*, and improve their antibodies by introducing small changes, *somatic mutations*, into their antibody (*immunoglobulin*)

genes. When the B cell follicle is activated it forms a *germinal centre* (GC). Events and cellular collaboration within the GC is the focus of this thesis. The outcome of the GC reaction will be the generation of B cells producing improved antibodies with sometimes up to a thousand times better binding capacity (*affinity*) to the antigen. Cells secreting antibodies are called *plasma cells* or *plasma blasts*. Cells that have seen the antigen, but do not commit to antibody production, reside in the lymphoid organ where they were activated, or in the circulating B cell pool. They are called *memory* cells and are easily activated when they meet the antigen again (*recall antigen response*). Memory T and B cells have at least two features in common; i) they have seen the antigen before and ii) they respond more rapidly and vigorously than their naïve counterpart.

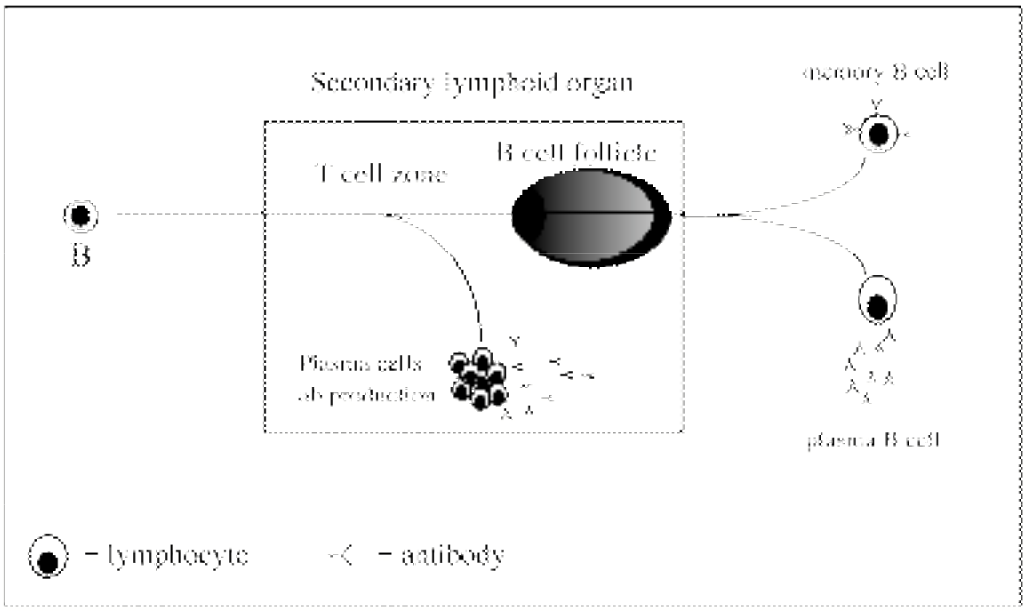


Fig. 1 Schematic overview of the migratory pathway of B cells within a secondary lymphoid organ.

Why do we then study such events? Except for the pure curiosity of a former biology student, a curiosity shared with immunologists all over the world, such knowledge will give us further understanding of how an ordinary vaccination works. Also, the knowledge will help us to reproduce such events in a test-tube (*in vitro*). Why then make it so complicated when we can use animals like rabbits and mice to produce antibodies for us? Well, the animals do the work just fine, but the outcome will be antibodies suited for that particular species, and if we want to use them for diagnosis they will fulfil their task. But, if we want to create antibodies useful for

therapy, such as treating cancer in humans, they have to be ”man-made” to work most efficiently and not to be recognised as foreign by the human immune defence.

2 The Humoral Immune Response

The result of an efficient humoral immune response is the generation of a high affinity (*i.e.* somatically mutated and selected) pool of antibodies to efficiently eliminate the invading non-self antigen. Also, it will produce a pool of memory cells with a capacity to be reactivated rapidly by an antigen seen before (*recall antigen*). To be able to produce high affinity ab to a particular ag, the B cell needs T cell help. Antigens that evoke high affinity antibodies are named T cell-dependent antigens and comprise proteins. B cells recognise ag via their membrane bound ab-molecule (sIg) exposed on the cell surface. The protein is then processed and presented as small peptides on MHC cl. II molecules (Roit *et al.*, 1989), to be recognised by T cell receptors. This recognition will recruit further help from ag-specific T cells. The response to a T cell-dependent antigen (TDA) then continues via a specific interaction of the B cell with antigen specific T cells in a cognate (MHC-ag-TCR) interaction. T cells play a crucial role guiding the immune response and the B cell activation via cell-cell interactions and soluble factors (e.g. interleukins). This communication is in close association with different accessory cells, such as members of the APC population, contributing with both cell bound and soluble co-stimulatory molecules.

2.1 The immunoglobulin molecule

The ability to recognise unprocessed antigen is a unique capacity of B cells equipped with the membrane bound form of the antibody molecule. In its soluble form, the ab plays an important role in clearing the periphery of non-self antigens. The antibody molecule is a heterodimer, consisting of two heavy chains and two light chains covalently joined together with disulphide bridges and can be divided into a constant part and a variable part (Fig. 2). The constant part, the Fc region, assigns the molecule its Ig-class. Thus, antibodies are divided into different Ig-classes (IgM, IgA, IgE, IgG and IgD) due to differences in their constant parts. The different classes have certain biological activities (effector functions) such as complement activation and mediation of cell cytotoxicity (ADCC). On the other hand, the variable (V) regions designate the antigen specificity to the molecule. The variable region can be

divided into framework regions (FR) and hyper-variable regions (complementary determining region, CDR) (Fig. 2). During affinity maturation, mutations are introduced into the variable region of the immunoglobulin gene to generate antibodies with better/higher affinities than the original. Random diversification like this is not a trivial mechanism as it might introduce changes in the specificity of the antibody. In solving this, a sophisticated process of re-confirming specificity and selecting for higher affinity variants expressed on the B cell is undertaken. This process will be discussed more thoroughly in context of the germinal centre reaction.

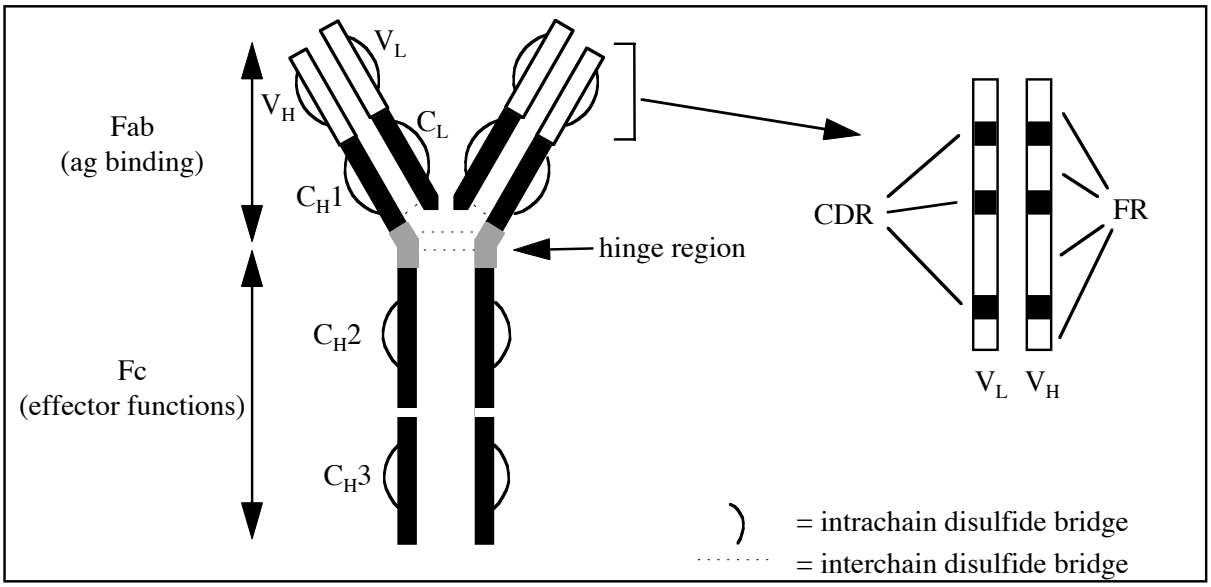


Fig. 2 Schematic picture of the Ig molecule here represented by an IgG molecule. Both the V_L and V_H contain CDR (hypervariable regions) and FR (variable regions).

2.2 B cells - the antibody producers

B cell ontogeny starts in spleen and liver in mammals and proceeds in bone marrow in adult individuals (Roitt *et al.*, 1969). The ”B” giving its name to this lymphocyte population has its origin in the bursa of Fabricius in birds, where proliferating foci of newly generated B cells from primitive progenitor cells were first described (Glick *et al.*, 1956). The hallmark of the B cells is their ability to produce antibodies. To be able to do so, the immature B cells are selected on the basis of fully rearranged immunoglobulin genes as well as on not recognising self antigens. The latter would and result in an autoimmune condition and be fatal for the host. Outlined in figure 3 is the rearrangement of the immunoglobulin genes during B cell lymphoipoesis. A fully rearranged Ig gene, results in the generation of a mature B cell expressing cell

surface bound forms of IgM and IgD molecules (Melchers *et al*., 1994). These newly produced mature B cells are then submitted to the peripheral B cell pool. Passing through peripheral lymphoid tissue, the B cells migrate via T cell rich areas where they might become activated antigen specifically and subsequently expand clonally in the nearby B cell follicles. This gives rise to Ig producing plasma cells and a pool of memory B cells. The process taking place within the secondary B cell follicle is known as the germinal centre reaction (Thorbecke *et al*., 1994). It will be further dissected in detail in the following chapters.

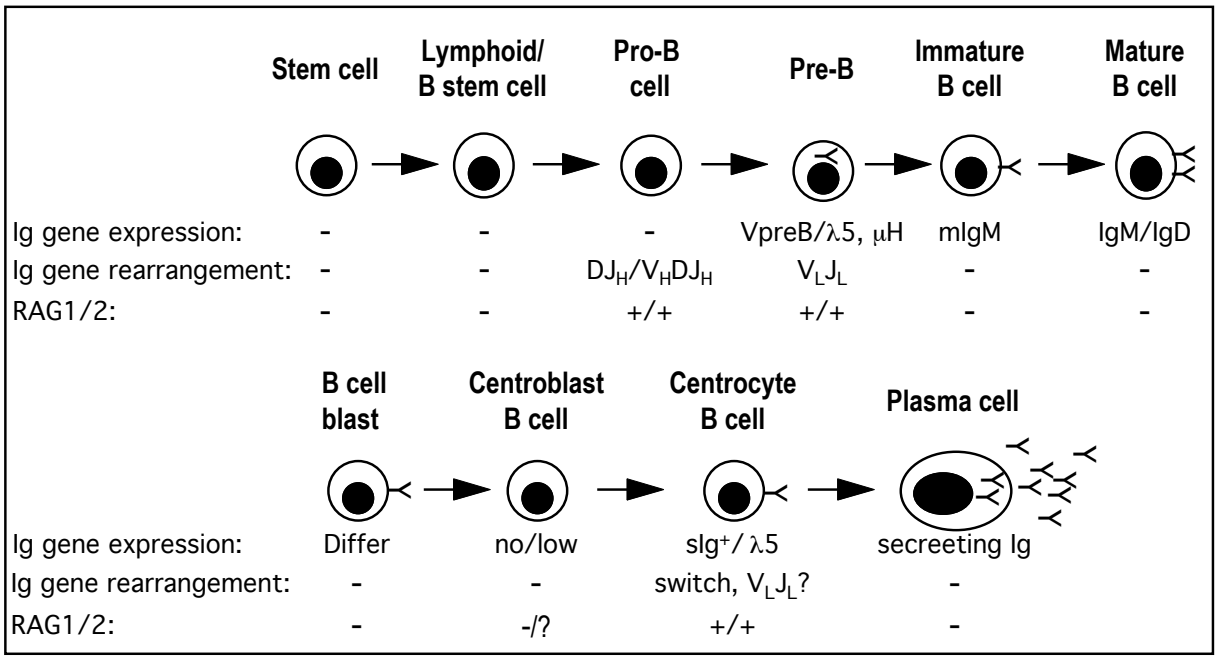


Fig. 3 Ig rearrangement during B cell development. Germinal centre B cells re-express pre-B cell characteristics like RAG1/2 and λ5.

2.2.1 Definition and subsets

B cells are defined as the lymphocytes with the unique capacity of producing antibodies. The mature, naïve B cell expresses properly rearranged IgM and IgD molecules on its surface. Giving the cells the designation ”naïve” refers to their virgin status, still not being antigen specifically activated and expressing non-mutated immunoglobulin germline genes. These B cells recirculate and constitute about 85-95% of the B cells in blood.

The classical way of subdividing B cells include the observation of an heterogeneous expression of the CD5-molecule on extra-follicular B cells. In mice, these two B cell populations have been postulated to belong to different lineages

(Kantor *et al.,* 1991). The conventional B cells (B2 in mice, $CD5^-$ in humans) constitute the recirculating B cell population which in its naïve form express high levels of IgD on their surfaces. Contrasting, the B1 cells (former Ly-1 in mice, $CD5^+$ in humans) constitute a small population, about 1% in mice, with distinct ontogeny and anatomical localisation (Herzenberg *et al.,* 1986). The B1 cells have been correlated with autoimmunity and a predisposition to produce autoantibodies (Casali *et al.*, 1987; Hardy *et al.*, 1987; Jasin 1991; Nakamura *et al.*, 1988). Recent reports have questioned this "different lineage" theory since $CD5^-$ B cells can be induced to express CD5 with IL6 and anti-μ (Yingzi *et al.*, 1990) or PMA (Zupo *et al.*, 1994a).

2.2.2 Activation

The B cell is equipped with a powerful "homing missile" on its surface, the membrane bound form of the immunoglobulin molecule. This tool of recognition, estimated as about 10^5 molecules per resting B cell, makes it possible for the B cell to recognise a foreign antigen, internalise it, process it in lysosomes and endosomes and present peptides on its MHC class II molecules. This antigen-antibody interaction decides the first level of specificity for the B cell. The second level occurs in the context of T cells, and involves TCR-MHC interactions that, again, will confirm antigen specificity of the cells activated. The sIg by itself has rather poor signalling capacity. In solving this problem, the sIgM and sIgD molecules are found in a complex with other B cell specific transmembrane molecules with signalling capacity which, together with sIg, constitute the B cell-receptor complex (BCR; Fig. 4).

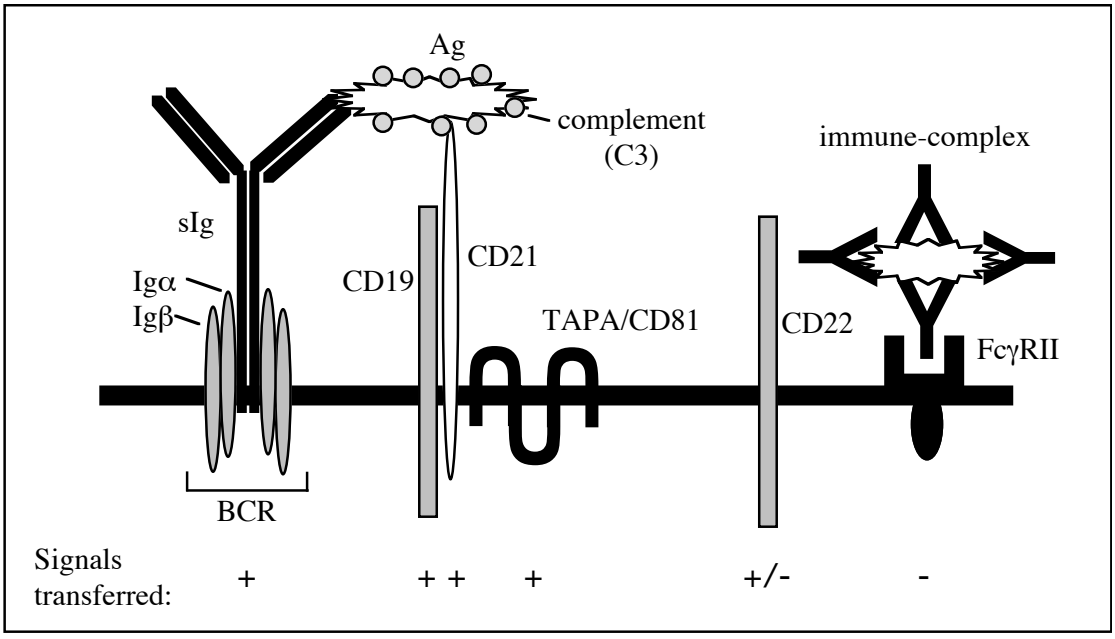


Fig. 4 The BCR (sIg, Igα, Igβ) and associating co-receptors (CD19, CD21, CD22, CD81/TAPA, FcγRII). CD19, CD21 and TAPA hace been suggested as positive regulators of the BCR, compared to FcγRII that mediate negative feedback.

Anti-Ig

Crosslinking of the sIg leads to activation of protein kinases (PK), phosphorylating of cytoplasmatic tyrosine residues (Carter *et al.,* 1991; DeFranco, 1992), and activation of protein kinase C (PKC) in combination with an elevation of intracellular free calcium levels via the phosphoinositide signalling pathway (Gold *et al.*, 1992, Grupp *et al.*, 1987, Norton *et al.*, 1993; Wilson *et al.*, 1987). As a result of these intracellular events, the cells leave G_0 and mostly enter G_1 phase of the cell cycle, increase their size and initiate both DNA and RNA-synthesis (Julius *et al.*, 1984; Monroe *et al.*, 1985; Walker *et al.*, 1986).

This interaction of ag with sIg on the B cell is frequently mimicked *in vitro* by crosslinking of sIg with monoclonal antibodies. Though facilitating *in vitro* studies one must keep in mind that anti-sIg are not proper antigen molecules. The affinity of the antibody used and the degree of crosslinking of sIg is of vital importance (Davis *et al.*, 1986; Mongini *et al.*, 1991 and 1992; Parry *et al.*, 1994). Whereas some anti-Ig monoclonal antibodies stimulate proliferation, others do not without the addition of second signals such as IL4 or ab specific for CD40 (Mongini *et al.*, 1991; Norton *et al.*, 1993). Hyper-crosslinking of sIg may also induce apoptosis in mature B cells (Parry *et al.*, 1994).

The B cell receptor and associated co-stimulatory molecules

The sIg with only a three amino acid (aa) cytoplasmatic domain lack signalling capacity, and therefore needs to rely on associated molecules in this respect (Fig. 4). To solve this problem the sIg is non-covalently associated with disulphide-linked heterodimers of CD79a (Igα, mb-1) and CD79b (Igβ, B29) (Hombach *et al.*, 1990). These heterodimers have extended cytoplasmic signalling tails (61 and 48 aa, respectively) compared to the sIg (Clark *et al.*, 1992; Flaswinkel *et al.*, 1994; Kim *et al.*, 1993; Persin *et al.*, 1994; Reth *et al.*, 1990). The heterodimers also contain a common sequence motif (*I*mmunoglobulin *T*yrosine-based *A*ctivation *M*otif; ITAM) for intracellular protein tyrosine kinases (PTK) (Hombach *et al.*, 1990). By studying the function of these non-antigen binding molecules in mutant and chimeric cell lines the cytoplasmatic domains of the proteins could be functionally linked to the extracellular and transmembrane parts of the sIg (Kim *et al.*, 1993; Lankester *et al.*, 1994; Law *et al.*, 1993). Mice lacking the Igα unit show an impaired early B cell development and a block in peripheral B cell generation. This indicates a checkpoint in B cell maturation for functional BCR expression (Torres *et al.*, 1996). Other *B* cell receptor-*a*ssociated *p*roteins (BAP) have been defined and characterised. Especially, two proteins associating with the IgM (BAP37 and BAP32) and IgD proteins (BAP31 and BAP29) respectively have been described (Kim *et al.*, 1994; Terashima, 1994). They probably contribute to the functional difference in signalling observed between the sIgM and sIgD molecules.

Other co-receptor molecules associated to the BCR (CD19, CD21/C3dR, CD22, CD81/TAPA-1 and FcγRIIb1) give an additional signalling capacity to the BCR complex to promote or reduce cell activation (Frearon *et al.*, 1993). Furthermore, they contribute with avidity effects and further crosslinking of the antigen-BCR interaction e.g. via CD21-complement-ag interactions (Fig. 4). Whereas co-ligation of CD19 has been shown to lower the threshold for anti-Ig stimulation in B cells (Fearon *et al.*, 1992) the FcγRII possesses a suppressive quality when co-ligated with BCR, as might occur when B cells encounter antigen in a complex with IgG (Gergely, 1996; Takai *et al.*, 1996). CD19 deficient mice show a severe impairment in germinal centre formation and poorly affinity matured antibodies, further highlighting the importance of the CD19 molecule in T cell-dependent antibody formation (Rickert *et al.*, 1995; Engel *et al.*, 1995). The ligand for CD19 is not known, although CD77 has been implicated (Maloney *et al.*, 1994). The CD19 molecule associates with CD21 and CD81 thereby recruiting further positive signal transduction pathways, decreasing the threshold for cellular activation. CD21 is the receptor for C3d of the complement cascade and has been shown to play a pivotal role in modulating the signalling via the BCR in T cell-dependent antibody responses (Croix *et al.*, 1996; Luxembourg *et al.*, 1994). CD23 has also been shown to possess the capability of serving as a ligand to CD21 (Aubry *et al.*, 1994).

The expression of the CD22 molecule, as for CD19, is B cell specific and the receptor is similarly phosphorylated upon mIg ligation (Leprince *et al.*, 1993). Its effects in lowering the activation threshold through sIg have been shown with anti-CD22 ab on beads (Doody *et al.*, 1995). Other studies have also revealed that CD22 interacts with negative regulators after BCR cross linking, in contrast to the CD19 molecule (Sato *et al.*, 1997). CD22 has been shown to interact with CD45R0 on T cells and CD75 on T and B cells (Stamenkovic *et al.*, 1991), implicating a role in T cell-dependent B cell activation.

Signalling via the BCR and its associated molecules discussed above induce events that influence other receptors such as the CD80/CD86 and CD40 molecules. These receptors further regulate the cellular responses. Such signalling events and their role in T-B collaboration will be further discussed in following chapters (Fig. 5).

2.3 T cells - the guides

The response to a TDA must, of course, include T cells. The ag is presented to the T cell as a peptide-MHC complex by professional APC, such as dendritic cells (section 2.4). This occurs in the T cell zone of secondary lymphoid organs and results in the recruitment of antigen specific T cells. The role of the T cell is to activate the B cell properly in an antigen-specific manner, guiding the B cell activation. The T cell confirms antigen specificity via its TCR, that recognises peptides in complex with MHC cl. II. This APC-T-B cell interaction may result in the formation of a germinal centre and the subsequent generation of high affinity antibodies specific for the antigen originally recognised by sIg on the B cell.

2.3.1 Definitions and subsets

Mature αβ TCR T cells are divided into $CD8^+$ and $CD4^+$ cells (Swain, 1983). CD4 and CD8 are co-receptors to the TCR, binding to the antigen presenting MHC molecule stabilising and contributing quantitatively to the interaction between the antigen presenting cell and the T cell (Doyle *et al.*, 1987, Garcia *et al.*, 1996, Norment *et al.*, 1988, Viola *et al.*, 1997) (Fig. 5). This molecular configuration will bring tyrosine kinases such as Lck, into close proximity to the TCR (Veillette *et al.*, 1988, Weiss *et al.*, 1994). The CD4 subset contains the helper T cells involved in specific B cell activation by TDA. They recognise exogenous non-self antigen in context with MHC class II. In contrast, the CD8 subset mainly contains T cells recognising endogenous antigen presented on MHC class I. The latter cells also possess a cytotoxic function and have the ability to lyse antigen presenting target cells either by use of perforin alone or in combination with the Fas/FasL interaction (reviewed by Kägi *et al.*, 1996).

In mice, CD4 T helper cell subsets have been defined according to lymphokine secretion profiles (Mosmann *et al.*, 1986). Two major subsets, named TH1 and TH2, have been identified. The TH1 cells produce mainly IL-2, IFNγ and TNF-β whereas the TH2 cells are responsible for IL-4, IL-5 and IL-10 production. In man the identification of such subsets does not seem as clear-cut as in mice (Romagnani *et al.*, 1991). A TH0 phenotype was identified, secreting a mixture of interleukins characteristic of both subsets. Functional studies showed that the majority of TH1 clones but a minority of TH2 clones had the capability of cytotoxic activity, while all TH2 clones studied exhibited capacity to help autologous B cells to secrete Ig (Romagnani *et al.*, 1991 and 1992).

Another way of identifying T cell subsets is via identification of different isoforms of the tyrosine phosphatase CD45. On human cells, the large isoform CD45RA denotes functionally ”naïve” T cells and a shorter splicing form, R0, the

”memory” T cell population. This was postulated since it was shown that RA cells acquire R0 upon activation *in vitro* at the expense of RA expression (Akbar *et al.*, 1988, Kristensson *et al.*, 1992) and the majority of T cells responding to recall antigens was found within the R0 subset (Merkenschlger *et al.*, 1988). We now know that this is a truth with modification, since a conversion from R0 back to RA has been shown to exist in mice (Lightstone *et al.*, 1993), rat (Bell *et al.*, 1990) and man (Michie *et al.*, 1992). Still, this classification is widely used and accepted.

Within the human CD45R0 T helper cell population, the CD30 molecule may be used in further subdividing the cell subset. This molecule belongs to the same nerve-growth factor receptor (NGFR) superfamily, as CD40, OX40, CD27 and TNF receptors, all shown to be involved in regulation of lymphocyte activation. The $CD30^{+}CD45R0$ T cells have been shown to exhibit an enhanced helper activity for B cell Ig production than their $CD30^{-}$ counterpart (Alzona *et al.*, 1994). The CD30 expression on T cells shows no clear cut correlation to the TH2 population described above (Bengtsson *et al.*, 1995), but triggering of the CD30 molecule has been shown to promote differentiation of T cells preferentially secreting TH2 cytokines (Del Prete *et al.*, 1995; Romagnani *et al.*, 1995).

2.3.2 Activation

T cells are activated via their TCR, though signalling via the molecule is usually not enough to drive the cells from the G_0 stage of the cell cycle. To accomplish this, the T cell needs further co-stimulation that may be provided via other cell surface receptor/ligand pairs or via soluble mediators such as interleukins (e.g. IL-2). This has been suggested in a hypothesis for lymphocyte activation known as ”the two signal model” (Cohn *et al.*, 1990). The model suggests, that if the T (or B) cell sees the antigen (signal 1) without further co-stimulation (signal 2), the cell will enter a state of unresponsiveness or anergy. Though, if a proper signal two is provided, the stimulated cell will be turned on. Naïve T cells circulate and do not enter peripheral tissue, whereas memory cells do. In this way, many tissue-specific antigens are not seen by the naïve cells, and are, thus, ignored by the immune system without deletion or anergy. This hypothesis also defines the secondary lymphoid organs as the proper place for activation, where the T cells are given both the cognate antigen signal and proper co-stimulation (e.g. IL-2, CD40, CD80/CD86).

T-B cell collaboration

Without T cells, B cells would not get properly activated merely by binding antigen to sIg, when responding to TDA. The T-B cell collaboration can be divided into an antigen specific and non-specific part. The TCR-MHC class II interaction ensures a proper antigen specific activation of both the T and the B cell. Along with this, several ag non-specific receptor-ligand pairs have been identified contributing to the

T-B cell cross-talk, modulating the interaction positively or negatively (Fig. 5). Of these, the CD40-CD40L (Grewal *et al*., 1995) and CD28-CD80 interactions (Linsley *et al*., 1992; Zhang *et al*., 1997) have received most attention. The CD40 molecule is expressed continuously on B cells (and other APC such as dendritic cells and macrophages), whereas its ligand is upregulated on activated T cells (Armitage *et al*., 1992). Van Essen and co-workers (1995) have also shown that T cells primed in the absence of CD40 will be deficient in their capacity to give B cell help with lack of germinal centre formation as a consequence. It has been shown that CD40L expressing T cells will induce the expression of CD80 on resting B cells (Ranheim *et al*., 1993), as well as the reciprocal scenario (Clark *et al*., 1994). This makes the picture quite complex, referring to the ”hen and the egg” phenomenon in context of cell signalling, since resting cells hardly express the counter-receptor but upregulate it when activated. The scenario needs an initiator. Which cell is activated first; the T or the B cell? Does it matter, as long as one is? What contributes to initiate the cascade in T-B cell collaboration?

Other cell-surface adhesion molecules such as integrins (CD11/CD18) and members of the immunoglobulin superfamily also contribute to the signalling events. Figure 5 shows some interactions between activated T and B cells. In addition to the membrane-bound molecules, there is also an autocrine and paracrine contribution of T and B cell derived interleukins participating in the process.

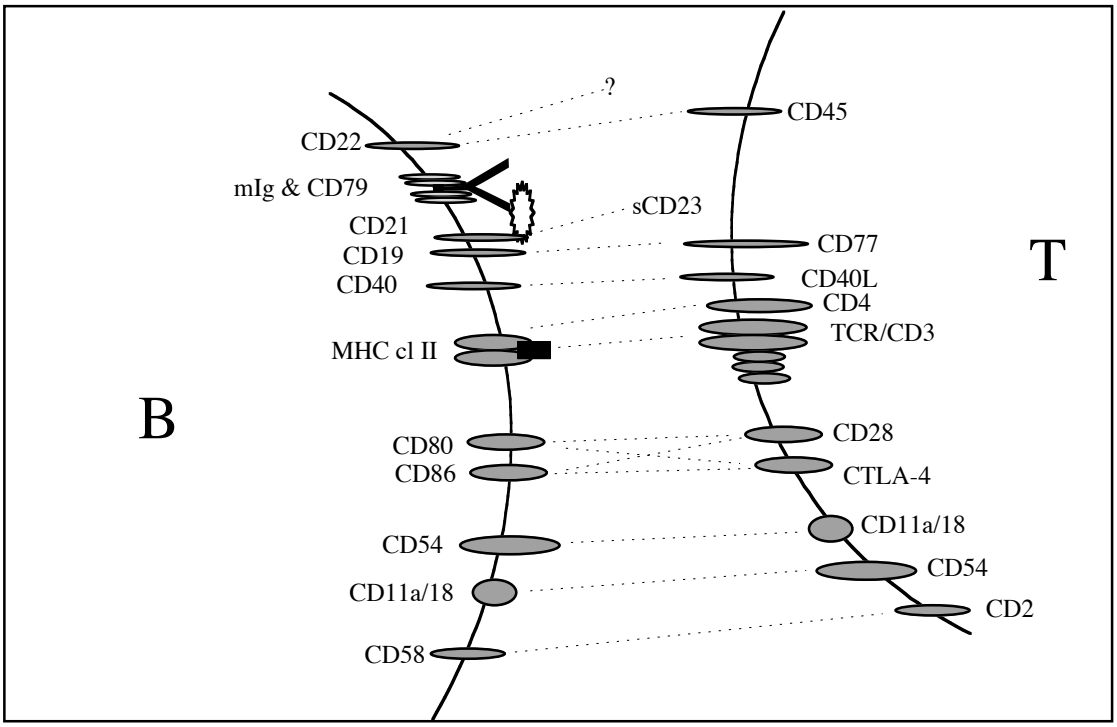


Fig. 5 Receptor-pairs known to participate in T-B cell collaboration. In addition, interleukins contribute in an autocrine and paracrine fashion.

2.4 Dendritic cells - the third party

A candidate initiator of T-B cell collaboration is the dendritic cells (DC) found in the T cell areas of peripheral lymphoid organs (reviewed by Banchereau *et al.*, 1998; Steinman *et al.*, 1997). Naïve resting T cells require a stringent set of co-stimulatory molecules to become fully activated. Dendritic cells, as well as activated B cells and macrophages, have been shown to fulfil these requirement. In mice, the DC have been shown to be the most efficient type of cell in presenting antigen to naïve T cells (Croft *et al.*, 1992).

The origin of DC is diverse. The cells change in phenotype during their life-span and have different tissue distribution. They are found in low cell numbers, hampering the isolation and further studies of these cells. The debate still concerns the origin of the cells. Do they constitute a separate lineage or belong to the monocyte/macrophage lineage (Peters *et al.*, 1996; Steinman *et al.*, 1997)? In tissues DC exists in an immature form, *e.g.* Langerhans cells in skin, that show a high phagocytic capacity to internalise antigen, process it and, later on, expose it on their MHC class II molecules. The immature forms of DC, mainly found in tissue and blood, are the most potent cells in capturing and processing antigens (Romani *et al.*, 1989). The antigen is internalised by phagocytosis, macropinocytosis and adsorptive endocytosis using lectin receptors and Fcγ and Fcε receptors. The immature form of DC is a poor T cell activator, mainly due to low MHC and accessory molecule expression. As in tissue, different DC subsets can also be found in blood, probably reflecting different differentiation stages of the cells (Thomas *et al.*, 1994). In the T cell zone, the DC exist in a mature form called *i*nter *d*igitating *c*ells (IDC). They have lost their capacity to internalise and process antigen but have peptides presented on their MHC class II together with high levels of co-stimulatory molecules, such as CD40, CD54, CD58, CD80 and CD86 making them very potent T cell activators.

In vitro-generated DC have been obtained from interleukin supplemented (GM-CSF, IL-4 and/or TNFα) cultures of bone-marrow derived $CD34^+$ progenitor cells (Szaboles *et al.* 1995) and from human blood monocytes (Romani *et al.* 1994, 1996) facilitating further functional and lineage studies of the cells.

In an *in situ* study by Ingulli and co-workers (1997), the actual physical contact of antigen (OVA) pulsed DC and naïve antigen-specific T cells was reported showing tangible evidence for what earlier had been suggested for the secondary lymphoid organs, but without any direct antigen-specific demonstration.

The IDC shows a phenotype complementary to the T cell with high levels of MHC class II, CD40, CD80, CD86, CD58/LFA-3, CD54/ICAM-1. Furthermore, IDC have the capacity to upregulate those molecules to higher levels after CD40 ligation *in vitro*, similar as the mature IDC in the T cell area of lymphoid tissue (Caux *et al.*, 1994). This implicates a predominant role of these cells in T cell activation, actually

resembling the B cell. Upon activation, the DC produce an array of interleukins and chemokines (TNF-α, IL-12, IL-8, MIP-1α, MIP-1β) (Caux *et al.*, 1994) with the capacity to affect the neighbouring B and T cells. Also, the different adhesion molecules expressed on the DC can act like physiological "glue" with the capacity to hold the B cell in close contact facilitating the T-B cell interaction. In line with this, a three-cellular complex (T-B-DC) has been suggested as an initiator of the antigen specific interaction in lymph nodes and the initiator of the following follicular reaction, bringing antigen-specific T and B cells, together with antigen, into the primary B cell follicle (Grouard *et al.*, 1996). The effect of DC on B cells has also been studied, showing its role in extrafollicular plasma cell differentiation (Björk *et al.*, 1997). A bit more confusing is the finding that DC produces high levels of bioactive IL-12 upon CD40 triggering favouring a TH1 response, hardly compatible with the present dogma of TH2 cells being most suited for B cell activation (Cella *et al.*, 1996).

2.5 Antibody formation

The production of antigen specific antibodies to a T-dependent antigen reaches two phases during an immune response. Firstly, the primary response gives rise to low affinity antibodies produced by plasmacytoid B cells generated from the initial T-B cell collaboration in the T cell zone. This initial production of antibody secreeting cells is extrafollicular without any affinity-maturation, but might still involve isotype switch in the B cells. Secondly, a few B cells are committed to a follicular expansion. This route generates B cells with a plasmacytoid phenotype. Such cells migrate to bone marrow or mucosal tissue where they reside as terminally differentiated Ig producing plasma cells. The follicular expansion of B cells is a route to produce both memory and plasma cells (Arpin *et al.*, 1995).

Not all antibody production involves T cells. The T cell independent route of B cell activation and plasma cell differentiation will not be further discussed here, since it is beyond the scope of this thesis.

3 The Germinal Centre Reaction

Till now, this thesis has mainly been introducing some of the participants of interest to this study. The following chapter will focus on the generation and selection of B cells with affinity matured antibodies. Identifying the precise collaboration and signalling events between cells, that facilitate a T cell-dependent immune response, has been a challenging task to many immunologists. Today we know that, at least, the secondary lymphoid organs such as spleen and tonsils, provide this highly specialised milieu that enables a few committed B cells to undergo to affinity maturation. This process is initiated in the T cell rich zone and reaches its completion within a nearby B cell follicle during an event known as the germinal centre (GC) reaction (Fig. 6). The result of this is the generation of plasma and/or memory B cells. Memory B cells either recirculate or reside in an area adjacent to the follicle, known as the marginal zone (MZ) in spleen (Liu *et al.*, 1988) and subepithelial area in tonsils (Liu *et al.*, 1995), and respond more vigorously upon a secondary antigenic challenge. B cells reaching the terminal stage of differentiation producing antibodies, *i.e.* plasma cells, migrate differently and have various life spans depending on when they are generated. If generated during a primary immune response, the plasma cells usually reside in the red pulp of the spleen (or medullary cords of lymph nodes) with only a few days of life span (Ho *et al.*, 1986). In contrast the plasma cells obtained during a secondary (or subsequent) response migrate to bone marrow or mucosal lamina propria where they reside and produce antibodies for several weeks (Benner *et al.*, 1981).

3.1 The germinal centre

Over hundred years ago, Flemming (1885) observed cell division in lymph nodes. Though his observation was correct, we now know that the conclusions drawn from this were less true; that the cells originate/germinate within the follicles. But still, he thereby named the proliferating follicles within the lymph nodes as germinal centres, a term still used.

3.1.1 The T cell zone - pre-germinal centre events

The secondary lymphoid organs serve as filters for antigen and the lymphocytes that patrol our bodies in search of non-self structures. Recirculating T and B cells meet and are provoked, if receiving proper antigenic activation, to a primary or recall humoral immune response. Basically, the secondary lymphoid tissue can be divided into an interfollicular T cell zone and several follicular B cell rich zones. Within the spleen, the T cell zone is named the periarteriolar lymphoid sheath, PALS, and can be divided into outer parts of PALS and inner PALS. Techniques, such as immunohistochemistry, allowing identification of hapten-specific B cells have in recent *in situ* studies performed in mice revealed the dynamics and site of responding antigen specific B cells in primary and secondary responses (Han *et al*., 1995a; Jacob *et al*., 1991, 1993; Jacob and Kelsoe, 1992; Liu *et al*., 1991, 1997).

Via its migratory pathway within the spleen the B cell enters a T cell rich area, the T cell zone, where the T cell-dependent B cell response is initiated. In the outer PALS, the B cells have been shown to arrest due to BCR occupancy by using anti-hen egg lysozyme immunoglobulin transgenic B cells (Cook *et al*., 1997). It was also shown that a critical number of BCR molecules must be ligated to achieve B cell arrest. Further, differences in antigenic concentrations determined the fate of the activated B cells. B cells arrested transiently, due to low antigen concentrations, only underwent an abortive follicular proliferation. In contrast B cells activated/arrested in PALS at high antigen concentrations continued with a full follicular response. Other studies have shown the role of outer PALS in eliminating self-reactive B cells (Cyster *et al*., 1994, 1995), a process that seems to be dependent on interaction with Fas/CD95 on $CD4^+$ T cells (Rathmell *et al*., 1995). Also, the outer PALS seem to regulate the pool of newly produced naïve B cells, since the majority of the cells appear to die within here (MacLennan and Gray, 1986; Lortan *et al*., 1987). These studies show the role of BCR signalling in several B cell events; i) T cell-dependent B cell response ii) the fate of such stimulated B cell and iii) in regulating the entire B cell pool.

3.1.2 The B cell follicle

The primary follicle contains a mixture of recirculating naïve and memory B cells. Upon B cell activation in the T cell zone, a few committed antigen specific B cells migrate into the follicle and expand clonally. Why some B cells commit to this route and others to proliferation and differentiation in the T cell zone, is currently not known but it has been suggested that the cells belong to two different B cell lineages (Berek *et al*., 1993; Linton *et al*., 1989, 1992). Jacob *et al*. (1992) on the other hand, showed that proliferating foci in PALS and GC are clonally related arguing against this possibility.

Studies in the rat have revealed that low numbers of ag-specific B cells can be seen within the follicles in the primary response as early as 36h post immunisation (Liu *et al.*, 1991). These cells then proliferate and filled the follicle after 72-84h, giving rise to a secondary follicle. Small, non-cycling follicular B cells are then moved closer together, forming a follicular mantle (FM). Within four days, the secondary follicles polarise and form a germinal centre consisting of a dark zone with rapidly proliferating centroblasts and a light zone with none/low-proliferating centrocytes (Fig. 6).

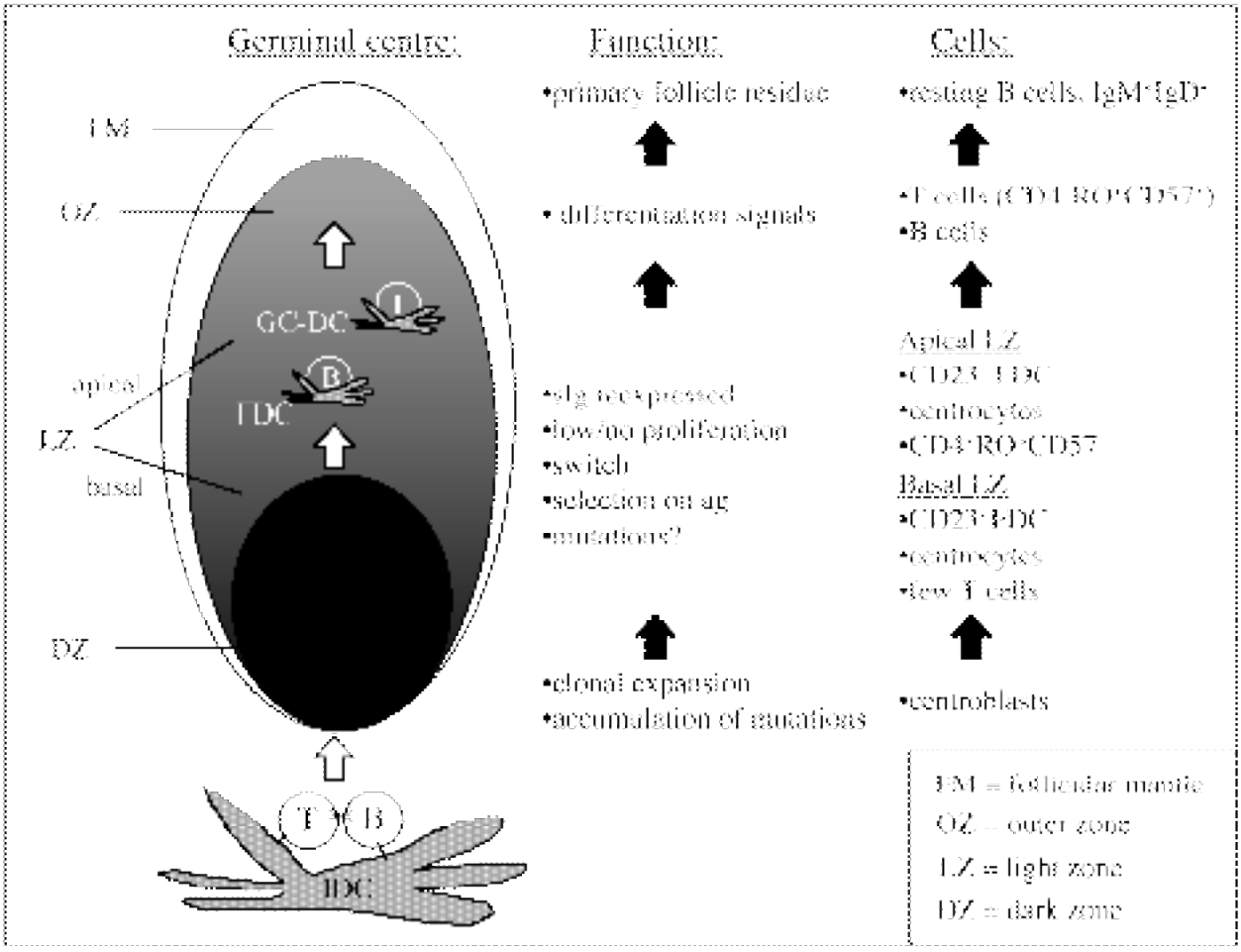


Fig 6 Overview of the germinal centre and its different compartments.

The follicles are formed oligoclonally from a few progenitor cells (Kroese *et al.*, 1987) and might develop oligospecific with respect to antigen. Using immunisation protocol with two haptens, oligoclonallity was shown within one follicle with respect to reactivity to both haptens (Liu *et al.*, 1991). The B cells within the germinal centre are interspersed with immune-complex carrying follicular dendritic cells

(FDC), a few antigen specific T helper cells and tingible body macrophages (TB-MΦ) (Fuller *et al.*, 1993 and Keller *et al.*, 1995). In addition, a newly described dendritic cell population, resembling IDC in the T cell zone can be found (Grouard *et al.*, 1996). TB-MΦ has been shown to contain condensed DNA from cells that have been in cell cycle within the last 12h. Thus, it is believed that these cells acts as a scavenger cells (Liu *et al.*, 1991a). The dark zone centroblast population shows a rapid cell cycling time, calculated to only 6-7h (Liu *et al.*, 1991a and Zhang *et al.*, 1988). Similarly, the non-cycling centrocytes were shown to be derived from cells that had been in cell cycle during the previous 12h (Liu *et al.* 1991a).

The role of T cells in germinal centre formation is definite, since irradiated rats, reconstituted with B cells and antigen, do not form germinal centres (Hunt *et al.*, 1990). This study also revealed that reconstitution with $CD4^+$ T cells in combination with antigen is sufficient for GC formation.

The GC gradually decrease in size, to finally disappear 3-4 weeks post immunisation.

3.1.3 Post-germinal centre

The B cell has at least two possibilities upon exit from the follicle; forming either long-lived memory cells or immunoglobulin secreeting plasma cells. What decides the fate of the B cell has been studied mainly in *in vitro* cultures. The results indicate a role for both soluble factors, such as IL1α, sCD23 and IL2 (Liu *et al.*, 1991b), as well as certain receptor ligand pairs, most importantly that of CD40/CD40L (Arpin *et al.*, 1995; Gray *et al.*, 1994). T cells definitely play a role in determining which pathway of differentiation the B cell chooses. T cells within the germinal centres belong to the $CD4^+CD45R0^+$ (memory) subset and are found predominantly in the apical parts of the light zone (Bowen *et al.*, 1991; Kelly *et al.*, 1995). Another possibility for the centrocytic GC-B cells has also been suggested. This involves the possibility that centrocytes re-adopt the phenotype of centroblasts again for cyclic re-entry of GC-B cells into the dark zone for further affinity maturation (Casamayor-Palleja *et al.*, 1996: Kepler *et al.*, 1993). This re-entry to a centroblastic stage has been suggested to involve T cell, suggesting other roles for the T cell within the GC than plasma cell and memory B cell differentiation.

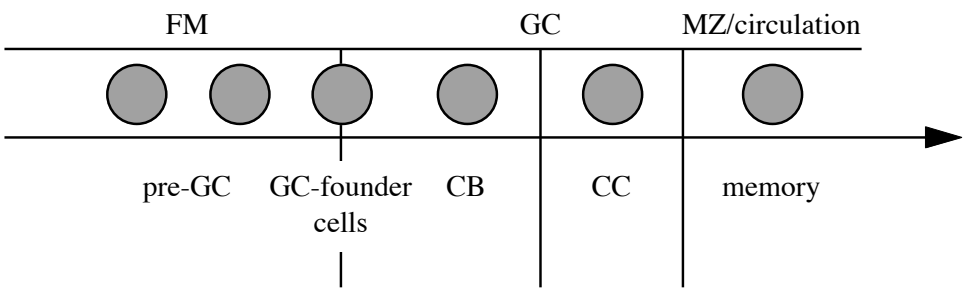
3.2 Cellular collaboration

During the clonal expansion in the GC, the B cells accumulate somatic mutations within their immunoglobulin genes. The cellular and molecular processes regulating such mutational on/of signals is currently unknown and the process has been difficult to reproduce *in vitro*, thus being a challenging task for immunologists. Known to be

particularly important and required for the germinal centre formation is the interaction via the CD40/CD40L (Foy *et al.*, 1994) and the need for CD28/B7 interaction (Fergusson *et al.*, 1996)
In the following chapters, cell-cell interaction and communication relevant for the germinal centre reaction will be discussed from a molecular point of view.

3.2.1 The differentiation and phenotypic changes of an antigen activated B cell

Human tonsillar B cells have been grouped into at least five different populations and subgroups thereof depending on their phenotype, stage of class switching and accumulation of somatic mutations (Feuillard *et al.*, 1995; Hardie *et al.*, 1993; Küppers *et al.*, 1993; Lagresle *et al.*, 1993; Madassery *et al.*, 1991; Pascual *et al.*, 1994). Although not without some controversy a consensus of the different B cell subsets can be reached as outlined in figure 7.



CD38	-	-	+	+	+	-
IgD	+	+	+	-	-	-
CD10	-	-	+	+	+	-
CD23	-	+	-	-	-	-
CD39	+	+	?	-	-	+
CD44	+	+	±	-	±	+
CD71	-	-	+	+	+	-
CD77	-	-	±	+	+/-[b]	-
IgM	+	+	+/-[a]	±	+/-	+/-
sIg	+	+	+	±	+	+
Ki67	-	-	+	+	+	-
Fas	-	-	+	+	+	-
Som. mut.	-	-	+	+	+	+
Switch activ.	-	-	-	-	+	-
bcl-2	+	+	-	-	-	+

[a] The GC-founder population can be further divided into an IgM^+ and an IgM^- population, where the IgM^+ cells show low or no mutations in their Ig genes. The other population show hypermutated Ig genes.
[b] CB and CC can be devided according to CD44 or CD77 expression.

Fig 7. Characteristic markers on different B cell populations pre-, within and post-GC.

The naïve B cell

The naïve B cell subset can be divided into a $CD38^{-}IgD^{+}CD23^{+}$ or $CD23^{-}$ population of small resting cells. They are naïve with respect to somatic mutations which they lack (Klein *et al.*, 1993; Pascual *et al.*, 1994). The cells are found in the follicular mantle surrounding the B cell follicle and have not encountered antigen yet. They do not express any known GC-B restricted markers (CD10, CD77, low/no expression of CD39 and CD44) nor the intracellular proliferation associated antigen Ki67 (Fig. 7). The significance of CD23 is not fully elucidated. CD23 is upregulated rapidly (5-6h) on B cells *in vitro*, via signalling through CD40 (Banchereau *et al.*, 1993) especially with IL4 (Defrance *et al.*, 1987) or after treatment with phorbol esters (Gordon *et al.*, 1986). CD23 positive naïve B cells might therefor have received some degree of activation, compared to their $CD23^{-}$ counterpart.

The GC founder cell

The GC-B founder cell represents an intermediate stage between naïve resting and GC-B cells (Fig. 7). While keeping their IgD expression, they have typically upregulated the surface markers characteristic of GC-B cells such as CD10 and CD77, in combination with less GC specific activation markers such as CD38 and CD71. The cells are in cell cycle, are proliferating ($Ki67^{+}$) and are prone to apoptosis, as they have down-regulated the anti-death protein bcl-2. Such cells are located within a secondary follicle before GC formation or within the dark zone of the GC as shown by immunohistochemistry. A subset of the cells being of large size, co-expressing CD38, sIgM and sIgD has been described to contain a low frequency of mutated transcripts. They thereby represents a fraction of precursor cells in which the mutation machinery has been turned on, compared to the other medium sized cells of same phenotype found to contain non-mutated Ig genes. (Lebecque *et al.*, 1997; Billian *et al.*, 1996). An early form of founder GC-B cells co-expressing both IgD and CD70 have also been described. These cells do not co-express any known GC-marker. The cells are non-cycling and still show naïve B cell expression levels of CD44 and CD39 and is found occasionally within GC and might represent an early GC-founder cell (Lens *et al.* 1996). GC-B founder cells are difficult to define since they represent a population of B cells that migrate from the T cell zone, into the B cell follicle. The cells then proliferate extensively within the secondary follicle before forming a GC structure. All these different stages of B cell populations are included in the GC-B founder cell population.

The centrocyte and the centroblast

The GC-B cells can be divided into rapidly proliferating dark zone centroblast (CB) cells, and their progeny the non-cycling, light zone centrocyte (CC) (Fig. 6 and 7). GC-B cells are mainly characterised by expression of CD10, CD77, which are GC-specific markers, in combination with a down-regulated or low expression of CD44, no expression of IgD, CD39, CD23, and low levels of sIg. Less GC-specific markers, generally found on activated B (and T) cells such as CD38, CD71, Fas, and Ki67 are also upregulated on GC-B cells (Fig. 7). The high level expression of CD38 on human GC-B cells is in striking difference to murine CD38 expression, which is down-regulated on their GC-B cells. (Oliver *et al.*, 1997). CB cells show mutated Ig V-gene segments indicating that the mutation machinery is targeting these cells (Pascual *et al.*, 1994; Küppers *et al.*, 1993). The GC-B cells show some similar characteristics to pre-B cells. They upregulate CD10 expression and re-express genes involved in V(D)J recombination, RAG1 and RAG2. Some of the cells also begin to transcribe λ5, a component of the pre-B cell complex and double-stranded recombination signal sequence breaks in the κ-locus (Han *et al.* 1996, 1997). Why the GC-B cells undergo receptor-editing is not known. It has been suggested as a mechanism to rescue GC-B cells that normally fail and are eliminated from GC due to gained autoreactivity, low or no ag specificity.

IgH switching is an independent process, and proceeds both within GC and extrafollicular in the T cell zone. It has been suggested to start after the process of somatic mutations. Switch recombination circles, as well as sterile transcripts, have been detected within the centrocyte population (Liu *et al.*, 1996d). A certain controversy whether CD77 or CD44 could be used to divide the human GC-B cell population into centrocytes and centroblasts is found in the literature. In favour of CD44, the lack of CD44 expression correlates better to the expression of the proliferation marker Ki67 than do the expression of CD77. It thereby represents a more suitable marker in relation to the rapidly proliferating centroblast cell population (Feuillard *et al.*, 1995). CD77 (Gb3) has been correlated to B cells undergoing apoptosis within the germinal centre (Mangeney *et al.*, 1991).

Other markers have been described in relation to human GC-B cells, in particular concerning the apoptotic characteristic of the cells. The apoptosis-related gene Fas (CD95) is found both in CC and CB, whereas c-myc is found on proliferating CB and bax as well as p53 in CC (Martinez-Waldez *et al.*, 1996). GC-B cells are prone to apoptosis and described to be low or negative in their expression of the anti-death protein bcl-2 (Liu *et al.*, 1991c). Fas, Bax, c-myc as well as p53 are all proteins linked to apoptosis induction in lymphocytes (Krammer *et al.*, 1994; Oltvai *et al.*, 1993; Shi *et al.*, 1992, Hermeking *et al.*, 1994). In line with this, transfection of c-myc into a lymphoblastoid B cell line induced a centroblastic

phenotype including up-regulation of CD10 and CD38 accompanied by down-regulation of CD39 and CD23 (Cutrona *et al.*, 1995).

Other features of GC-B cells is the expression of the cell surface, zinc dependent, CD10 related protease named carboxypeptidase M (de Saint-Vis *et al.*, 1995), and down-regulation of the negative cell cycle regulator SHP-1 (SH2 domain-containing phosphotyrosine phosphatase) (Delibrias *et al.*, 1997).

3.3 Affinity maturation

One of the hallmarks of the germinal centre reaction is the introduction of somatic mutations into IgV genes followed by an efficient selection procedure that ensures selection of antibodies with higher affinity (Berek *et al.*, 1985; Sharon *et al.*, 1989; Berek *et al.*, 1991). Most vertebrates use a mechanism to further diversify their functionally rearranged immunoglobulin genes, either in their primary rearranged repertoire or in the secondary, affinity-matured antibody repertoire. The two mechanisms known, acting either alone or in combination, is introduction of mainly single base pair mutations (somatic mutations) or gene conversion. In mouse and man, the main mechanism used is introduction of single base pair mutations (Rudikoff *et al.*, 1984; Sablitzky *et al.*, 1985; Wysocki *et al.*, 1986) whereas chicken and rabbit use mainly gene conversion, with some contribution of point mutations (Reynaud *et al.*, 1995; Weinstein *et al.*, 1994). Why the one or other mechanism is chosen is not known, though even among mammals, there is a preferential difference of choice. The scope of action differ between the two mechanisms. When introduction of point mutations depends upon antigenic stimulation and is introduced postnatal, the process of gene conversion seems to act mainly during foetal or early postnatal life and act without the influence of antigen.

The mechanism of introducing mutations to create more variation in Ig genes was suggested over 30 years ago (Lederberg 1959; Brenner and Milstein 1966), but the real proof came from sequencing of λ-light chains of mouse myeloma immunoglobulin genes (Weigert *et al.* 1970). The outcome of somatic mutations and the subsequent selection procedure will be an increased affinity of the serum antibodies produced during the immune response to the antigen. Such improvements can be brought about in different ways. The nature of affinity maturation in Ig V-genes has been studied carefully by immunising mice with haptens and further dissection of B cells with molecular biology techniques. Sequencing of hybridomas revealed at least two types of changes. Firstly, a phase during which incorporation of mutations, with an increasing number over time after immunisation, is seen (Griffiths *et al.* 1984). Secondly, a change in germline usage is seen in the late phase of the response, a phenomenon known as repertoire shift (Bothwell *et al.* 1981; Reth *et al.* 1978; Berek *et al.* 1985; Berek and Milstein 1987,

Jacob *et al.,* 1993). In the study of Jacob *et al*. (1993), four to ten different V(D)J joining sequences were identified in each follicle during early GC formation showing that each GC is populated by several clones of activated GC-founder B cells. At day ten, only one or two of those unique V(D)J joinings could be recovered showing a reduction in genetic variability.

The change in affinity for the antigen of interest is achieved in different ways. The overall affinity is a combined readout of kinetic characteristics as on- and off-rate of the antibody. Following immunisation with 2-phenyl-5-oxazolone (PheOx) in mice, the primary improvement in affinity is due to a decrease in off-rate for the antibodies. Alltough, the overall increase in affinity to PheOx is 100-fold, this includes a late repertoire shift towards antibodies with higher on-rates (Foote and Milstein 1991).

3.3.1 Somatic mutations *in vivo* and *in vitro.*

Most studies of affinity maturation have been performed in mice. Some clonal analysis of the human response has been performed, e.g. in the study by Ikematsu *et al*. (1993) where healthy individuals were immunised with inactivated rabies virus vaccine and the process of somatic mutations formally demonstrated. The nature of the response was studied using molecular biology techniques, sometimes accomplished with hybridoma technology. Studies of somatic mutations in human immunoglobulin genes have been hampered by the fact that immunisation is normally not possible and the lack of proper cell culture systems allowing induction of mutations activity. Also, since germinal centre B cells are programmed for apoptosis, *in vitro* culture of such cells has proven difficult, a problem that we have addressed in paper I and II.

One way of overcoming the problem of immunising humans and still analysing the response in human immunoglobulin genes was to introduce a human immunoglobulin mini-loci into mice. This approach generates antigen specific and somatically mutated affinity-matured human antibodies. Still, the majority of the antibodies produced to a particular antigen were mostly of murine origin, the human ab made were gycosylated in a ”non-human” way and suffered from being derived from a ”mini-loci” which hampered the possible variability of the antibody repertoire created from these mice (Wagner *et al*., 1994, Taylor *et al*., 1994). Another approach has been to study *in vitro* mutating follicular lymphoma cell lines where distribution and nucleotide biases of human Ig-genes may be elucidated (Wu *et al*., 1995; Wu and Kaartinen, 1996). Although this will be a biased study, at a clonal level in the same way as an antigen restriction might bias a study in mice, it will add to knowledge of somatic mutations in human immunoglobulin genes.

On studying mutations in human cells *in vitro*, several attempts have been made to induce naïve, human B cells to mutate in cell cultures. This will evidently

involve at least two different problems; i) to induce a GC-B cell phenotype *in vitro* in naïve human B cells and ii) to induce somatic mutations in such cells. Several attempts have been made to induce mutations in human B cells *in vitro* both from naïve and memory B cells. By using mitogens such as LPS (McHeyser-Williams 1991), LPS and dextran sulphate (Manser, 1987) or MHC cl II cross linking (Wysocki *et al.,* 1992) this question has been addressed. Such activation will mostly lead to proliferation and differentiation, and not to induction of the mutational machinery. One of the questions has been studied in detail in paper V, that is *in vitro* induction of a GC-B cell phenotype from naïve, resting human tonsillar B cells. Other attempts to induce a GC-B cell phenotype *in vitro* have in all cases only resulted in a partial phenotype. Dual triggering of the CD40 molecule (substituting T cells) and sIg (for antigenic signalling), have only resulted in a partial GC-B phenotype (Galibert *et al.,* 1996a; Wheeler *et al.,* 1996, paper V), probably resembling some stage of a GC-founder B cell phenotype. This indicates that signals given *in vitro* were insufficient to mimic antigenic activation of a naïve B cell and adds in a wider aspect of signals delivered from other cells, and, by the micro-milieu that is unique in secondary lymphoid organs. This is further discussed in paper V. Using murine B cells, similar observations have been made *in vitro*. There is an up-regulation of GC-specific PNA-binding capacity as well as an upregulation of non-specific GC B cell molecules B7-2 and MHC cl. II after BCR crosslinking and stimulation via CD40 (Lahvis and Cerny, 1997). The role of soluble factors for differentiation to a GC-B cell phenotype has been investigated by Cerutti *et al.* in a human IgM^+IgD^+ B cell line. This study highlights the importance of IL10, IL4 and IL6, in combination with CD40 signalling, for GC and plasmacytoid B cell differentiation (Cerutti *et al.*, 1998 and Paper III). Similarly, Caligaris-Cappio *et al.* (1989) showed that normal human $CD5^+$ B cells can, by the addition of soluble IL2, IL4, ILl or IL2 in combination with a low molecular weight B-cell growth factor, acquire a partial GC-B/B cell blast phenotype characterised by up-regulation of CD38 and CD10 in combination with sIgD and CD5 down-regulation.

Studies of somatic mutations *in vitro* has so far mainly been performed in human cell lines, whereas only one study has been described using naïve tonsillar B cells. In 1995, Gailbert *et al.* showed that anti-CD40 crosslinking via CD32 transfected murine L cell (Banchereau and Rousset, 1991) in combination with IL-4 only resulted in isotype switched cells without accumulation of somatic mutations. In 1997, Razanajaona *et al.* showed *in vitro* triggering of somatic mutations in human $sIgD^+CD23^+$ tonsillar B cells, though at a low frequency. Whether the usage of CD23 expressing cells in this study might have affected the ability to induce mutations, *i.e.* that the cells were pre-activated to some extent, is not known but might be a possibility. Also in this study, CD40-stimulation was provided with an anti-CD3 activated T cell-clone. Similarly, using a Burkitt's lymphoma B cell line co-

cultured with a cloned TH2 cell line, somatic mutation was shown to occur *in vitro* in a human system after crosslinking of sIg (Denépoux *et al.,* 1997). Murine *in vivo* primed spleenic B cells have also been shown to sustain their mutational activity *in vitro* (Källberg *et al.*, 1996). This study was also performed using sIg crosslinking and a cloned T cell line delivering the CD40/CD40L interaction.

3.3.2 Molecular and cellular requirements for somatic mutations

To study the requirements for mutational activity in Ig V-genes, several studies have been made in mice defect for the molecule of interest, or, via blocking experiments *in vitro* or *in vivo*. Clearly T cells, and particularly the CD40/CD40L interaction, has proven to be required for the formation of GC, (Ferguson *et al.*, 1996, Han *et al.*, 1995b; Foy *et al.*, 1994). Interestingly, studies in mice have shown that $CD4^+$ T cells are required for somatic mutations (Miller *et al.*, 1995a). The CD80/CD86 (B71/B72) and ligands (CD28/CTLA-4) has also gained interest for GC (Ferguson *et al.*, 1996) where anti-B7-2 treatment of mice in established GC reduce Ig hypermutation (Han *et al.*, 1995b). Still, absence/reduction of B7-2 expression can not be the cause of suppression of somatic mutation activity in aged mice, since they still express normal levels of B7-2 on B cells. Interestingly, such aged mice do affinity-mature their antibody response, though being limited to the intrinsic affinities on germline encoded antibodies (Miller and Kelsoe, 1995b and Yang *et al.*, 1996). As such, affinity maturation takes place, although to a limited extent. It thereby uncouples the process of somatic hypermutation and selection. Mice overproducing a soluble form of mouse CTLA-4, have impaired response to T-dependent antigens. They show defective germinal centre formation and reduced amount of somatic mutations (Lane *et al.*, 1994). As suggested, this might be an effect of blocking CD40L up-regulation on T cells. Similarly, mice lacking both B7-1 and B7-2, or B7-2 only, lack germinal centres (Bordello *et al.*, 1997). An extended collaboration by several groups revealed that terminal deoxynucleotidyl transferase, CD23, IL4, IgD and CD30 were not needed for the induction of somatic mutations as shown by studies in mice deficient for these molecules (Texidó *et al.*, 1996). Mice lacking the B cell expressed forms of FcγR by targeted inactivation of the gene, do not alter somatic mutation and affinity-maturation (Vora *et al.*, 1997). In a recent review by Song *et al.* (1998), the need for immune-complexes in regulating the onset of somatic mutations is discussed. They argue that a probable feedback mechanism might exist, since the primary (germline-encoded) switched IgG antibodies are known to augment the germinal centre formation and the onset of somatic mutations.

Different transcription factors have also been implicated as putative regulators of the germinal centre reaction. Such proteins are involved in transcription of

immunoglobulin genes (OBF-1/OCA-B/Bob-1) and other early mediators of immune and inflammatory responses (NF-κB). Disruption of the genes coding for the proteins, normally disrupts the micro-architecture of secondary lymphoid organs and leads to impaired humoral responses with no germinal centres being formed. Still, normal B cell maturation and memory B cell formation seems to occur (Caamaño *et al.*, 1998, Franzoso *et al.*, 1998, Shubart *et al.*, 1996).

A receptor/ligand pair that recently has gained interest in the study of germinal centre formation is the TNF/TNFR. The soluble factors TNFα and LTα (TNFβ) bind to the same receptors TNFRI (CD120a) and TNFRII (CD120b) (Beutler and van Huffel, 1994) whereas a trimeric form of the LTα and LTβ molecules binds to the third receptor TNFRβ (Crowe *et al.*, 1994). Studies showed that TNFα deficient mice failed to form germinal centres (Taniguchi *et al.*, 1997) and LTα deficient mice developed without detectable secondary lymphoid organs. As a consequence, such mice are deficient in GC-formation as well (Togni *et al.* 1994, Matsumoto *et al.*, 1996b). Interestingly, it has been shown that such mice deficient for LTα can produce affinity matured antibodies when immunised with high doses of antigen (in this case NP-OVA) to the same extent as normal mice. This study showed affinity-maturation without the architecture of germinal centres, at least not found in the spleen, lymphnodes and Peyer's Patches (Matsumoto *et al.*, 1996a). Further studies in the LTα knockout mice have revealed that the animals have the capacity to form lymphnode-like structures, including T, B and FDC cells, and the formation of GC cannot be excluded here (Banks *et al.* 1995). The basic question if germinal centres are necessary for somatic mutations, has been further highlighted in studies in amphibians such as Xenopus. They mutate their Ig-genes after immunisation though to a low degree, but they do not generate germinal centre structures (Wilson *et al.*, 1992). The need for the GC structure/milieu for somatic mutation in Ig V-genes will further affect the possibility to reproduce such events *in vitro* (Paper III).

3.3.3 Characteristics of somatic mutations at the DNA-level

It is known that V gene rearrangement is a prerequisite for activation of the mutation machinery (Roes *et al.*, 1989), though the gene does not have to be rearranged into a functional immunoglobulin gene. The process of hyper-mutations has also been correlated to initiation of transcription and has been suggested to act via transcription-coupled repair (Peters and Storb; 1996). They are mainly single base pair substitutions, although insertions and deletions have recently been reported (Wilson *et al.*, 1998; Ohlin and Borrebaeck, 1998). Most Ig genes contain from a few mutations up to around 10 per immunoglobulin gene. Highly mutated $IgD^{+}CD38^{+}IgM^{-}$ B cells with Ig genes harbouring up to 80 mutations have been found (Liu *et al.* 1996b). Since antigen-responses in mice are easily induced and can

be studied by molecular biology techniques, there is now a growing pool of information about the regulatory elements at the DNA level necessary for the mutator machinery to operate. Starting with the boundaries for distribution of mutations, they have been mapped both up- and down-streams of the Ig heavy and light (κ) chain with accumulation of mutations beginning just 5' of the rearranged V(D)J and peaking in the V(D)J coding sequence. The frequency of the mutations is then gradually declining from 5' to 3' in the 3' V(D)J flanking region. The downstream boundary approximately 1.000-1.500 bp 3' of the J-segment, excluding the C region as a target, for both the heavy and light chain (Both *et al.*, 1990; Lebecque *et al.*, 1990; Rada *et al.*, 1994; Rothenfluh *et al.*, 1993). Upstream, the boundary has been located to the leader intron in the Vκ of anti-oxazolone (VκOx1-Jκ gene combination) and also in different B cell lines (Rada *et al.*, 1994; Rogerson *et al.*, 1994). That the upstream boundary seem to be within the leader intron, with no mutations above the transcription initiation (CAP) or promotor, further highlights the suggested correlation between transcription and mutational activity.

As the C-region is excluded as a target for mutations (Gerhart and Bogenhagen, 1983) despite belonging to the same transcriptional unit, it is of interest to know what limits the mutational activity to the 1-2 kb region in which the V(D)J sequences is included. To identify such sequence features involved in the somatic hypermutation process, studies have been made where DNA has been deleted, or changed to non-Ig genes to examine the role of *cis*-acting elements of the Ig loci. Studies where a 2 kb stretch of bacteriophage DNA had been inserted between the Ig promotor and the leader exon, abolished the mutation machinery (Winter *et al.*, 1997). Just deleting VJ in a kappa gene coding sequence, did not abolish mutations (Winter *et al.*, 1997). This emphasises the need for the promotor to be close to the leader exon, whereas the VJ exon is not necessary. The promotor itself in not a target for mutational activity, and can be replaced by another heterologous promotor still preserving the hypermutation function (Betz *et al.*, 1994; Tumas-Brundage and Manser, 1997). The promotor has been implicated in directing the frequency and distribution of mutations (Betz *et al.*, 1994; Peters and Storb, 1996; Tumas-Brundage and Manser, 1997). The importance of both the J-C intron enhancer and 3' enhancer for mutational activity has been shown (Betz *et al.* 1994, Tumas-Brundage *et al.* 1997). Substituting the V(D)J with foreign DNA has shown that the hypermutation machinery targets non-Ig DNA as well if placed downstream of the promotor and leader sequence (Peters and Storb *et al.*, 1996; Tumas-Brundage and Manser, 1997; Yelamos *et al.*, 1995).

There have been several studies devoted to find motif that are preferentially targeted by hypermutations. Despite the fact that mutations primarily accumulate in the CDR as compared to FR, it is difficult to distinguish such tendency from a bias by antigenic selection. Different studies have now shed some light on the issue (Fig.

8). Extended structural analysis have revealed a bias towards accumulation in residues and loops that are involved in contact areas towards the antigen (Cothia *et al*., 1992) and by analysing passenger genes of non-Ig origin hot spots can be revealed that are not biased by subsequent ag-selection (Betz *et al*., 1993, Yelamos *et al*., 1995). Similarly, studies on out-of-frame Ig-genes have revealed knowledge of mutational hot-spots not biased by intrinsic selection by antigen (Dunn-Walters *et al*., 1998). A summary of DNA sequence motifs, targeted by somatic mutations, delineated from different studies is found in Table 1 (Rogozin *et al*., 1992; Smith *et al*., 1996; Wagner *et al*., 1995). To find consensus motifs as targets for mutational activity is difficult. Except from the DNA sequence surrounding the mutated position, and above discussed cis-acting elements, there probably is a certain contribution from secondary structures of the DNA that is difficult to evaluate by sequence analysis.

	Target sequence:	Ig genes forming basis of the study[d]:
A. Preferred di-nucleotide:	GC, TA	murine, noncoding V-genes
B. Preferred tri-nucleotide:	AGC, TAC, GCT, GTA	" "
C. Consensus motif:	RGYW[a]	selected human V-genes
D. Extended motif analysis:	AANB	human out-of-frame V-genes
(targeted nucleotide	WDCH[b]	" " "
underlined)	DGHD[c]	" " "

[a] R=A/G; W=A/T; Y=C/T; D=A/G/T; H=A/C/T; B=C/G/T
[b] extennded motifs: (A, not G or C)(not C)C(T not G)
[c] " " : (A, not C)G(not G)(T, notC)
[d] refs. A+B. Smith *et al*. (1996) *J. Immunol*. **156**; 2642
C. Rogozin *et al*. (1992) *Biochem. Biophys. Acta* **1171**; 11
D. Dunn-Walters *et al*. (1998) *J. Immunol*. **160**; 2360

Fig. 8 Suggested target-sequences for somatic mutations.

3.4 Selection of better binders - a role for GC-FDC and GC-T cells

Since a single base pair mutation within the Ig-gene may result in at least three different outcomes with respect to affinity, an efficient re-selection of the B cell, to

re-confirm antigen specificity has to take place. A mutation may give rise to a decrease in affinity as well as an increase or no change at all. The selection of a newly mutated B cell is thought to take place among the centrocyte population within the light zone of the germinal centre, since these cells start to re-express sIg. Involved in such procedures are the follicular dendritic cells (FDC), bearing the antigen held as immune complexes.

The FDC are cells located exclusively in B cell follicles, and their origin (hematopoetic or stromal) has for a long time been a question of controversy. They are characterised by their unique capacity to trap antigen in the form of antigen-antibody complexes and by their capacity to retain such complexes for a long period of time by the aid of Fc and complement receptors (Gajl-Peczalaska *et al.*, 1969; Nossal *et al.*, 1968; Tew *et al.*, 1980; Yoshida *et al.*; 1993). Because of this capacity, they are thought to play an important role in maintenance of immunological memory and selection of affinity matured B cells in a T cell dependent antigen-response. Within germinal centres, two types of dendritic cells have been characterised i) cells of a putative stromal origin representing follicular dendritic cells (FDC), mainly affecting B cells and ii) germinal centre dendritic cells (GC-DC) of haematopoetic origin that primarily interact with and activate T cells (Grouard *et al.*, 1996; Liu *et al.*, 1996).

Germinal centre B cells are programmed to apoptosis, and have, as previously discussed, down-regulated the anti-death protein bcl-2. This protein is rapidly upregulated initiating rescue from apoptosis upon crosslinking of several surface molecules on the GC-B cell. Shown to be involved in the rescue is engagement of CD40 (Liu *et al.*, 1989; Holder *et al.*, 1993), sIg (Liu *et al.*, 1989), CD20 (Holder *et al.*, 1995), CD38 (Zupo *et al.*, 1994b). Also, different adhesion molecules provided by FDC cells (Lindhout *et al.*, 1993, 1995) and soluble factors such as the combination of IL1α and CD23 (Liu *et al.*, 1991b). The sequential order of such rescue signals is still under intense investigation but it has showed to involve up-regulation of bcl-2 after 12-24h (Bonnefoy *et al.*, 1993; Zupo *et al.*, 1994b; Liu *et al.*, 1991b; Liu *et al.*, 1991c) and the more rapidly induced (5-6h) rescue protein bcl-X_L (Tuscano *et al.*, 1996). Suggestions based on *in vitro* studies have revealed that B cells are capable of taking up antigen from FDC and to present it to T cells (Tosco *et al.*, 1988). This interaction possibly involves CD40-CD40L signalling since T cells in the outer zone of GC have been shown to contain pre-formed CD40L that is rapidly (within 15-20 min) expressed on the surface upon stimulation (Casamayor-Palleja *et al.* 1995). Further on, the signals delivered by the T cells in the outer zone, both via cell-cell interaction and soluble factors, contribute and guide the B cell to further differentiation to a plasma cell or memory cell (Liu *et al.,* 1991b, Arpin *et al.,* 1995).

During the selection process B cells, re-expressing sIg, compete for antigen held in its native form on FDC. These cells carry immune-complexes where antigen is surrounded with antibodies from earlier, primary or previous recall, responses. B cells that have generated antibodies with higher affinity will compete successfully for the limited amount of antigen present on FDC. The B cells will be selected initially by receiving rescue signals via BCR. GC-B cells and in particular the CC, but not extra-follicular B cells, are sensitive to soluble antigen via receptor cross-linking. This has been suggested as a mechanism to prevent centrocytes to interact with FDC when the antigen concentration is high (Han *et al*., 1995a; Pulendran *et al*., 1995; Shokat and Goodnow, 1995; Galibert *et al*., 1996b; Billian *et al*., 1997). This is most pronounced in the sIg expressing CC population. Thus, when the antigen concentration decreases, high affinity variants are selected on FDC. Suggestions have been put forward for both a positive and negative selection of the B cells with newly generated specificities (for reviews consult Kelsoe *et al*. 1996; Liu *et al*., 1996; 1997; Nossal *et al*., 1994). One of the plausible outcomes of somatic diversification is the generation of auto-reactive B cells. The need, and mechanism, for a positive selection of affinity-matured B cells is easily justified. But how are the autoreactive B cells taken care of? Are they eliminated due to lack of positive signals in sense of subsequent cognate T-B cell interaction? That mutations might give rise to loss or change in antigen specificity or even auto-reactivity has been shown (Casson *et al*., 1995a, 1995b, Chen *et al*., 1992, 1995). The high expression of Fas (CD95) on GC-B cells has suggested AICD (activation-induced cell death) to play an important role in the negative selection mechanism (Lagresle *et al*., 1995; Möller *et al*., 1993, Smith *et al*., 1995, Watanabe *et al*., 1995) Speaking against this is the fact that *lpr* (CD95, Fas)-defective mice seem to have a normal hypermutation and selection mechanism (Jacob *et al*., 1993, Smith *et al*., 1995).

The T cells within germinal centres have been studied extensively as well. The number of T cells found in these structures are very low, and they are mainly located in the apical light zone of the GC. They are specific for the immunising antigen (Fuller *et al*., 1993) and the majority have a phenotype of a memory ($CD4^+CD45RO^+$) T helper cell (Bowen *et al*., 1991; Kelly *et al*., 1995). 70-90% of the T cells in the GC express the co-receptor CTLA-4 (Castan *et al*., 1997; Vyth-Dreese *et al*., 1995), one of the ligands to B7 (CD80/CD86) on B cells, that has been implicated as an important regulator of the immune response (Walunas *et al*., 1994; Waterhouse *et al*., 1995). A sub-fraction of the GC-T cells co-express the CD57 molecule and the function of this population has been examined (paper VI; Bowen *et al*., 1991; d'Angeac *et al*., 1994; Toellner *et al*., 1995). The $CD4^+CD57^+$ subset only produces few cytokines *in vitro* compared to the $CD4^+CD57^-$ subset (Butch *et al*., 1993; Toellner *et al*., 1995, Paper V). Still, they co-express the activation marker CD69 together with a high expression of CD45RO, indicating some kind of

activation of the cells. The CD57-marker is normally found on NK-cells, and a subset of $CD8^+$ T cells (Mollet *et al.*, 1998; Arai 1998) and some B cells (Mast *et al.*, 1998). The $CD57^+$ T cells seem to differ in their capacity to give B cell help from classical T cells. They do not enhance B-cell proliferation and differentiation without the addition of exogenous IL2 (Bouzahzah *et al.* 1995; Paper V). Several groups have suggested that the CD57-marker defines a late activation stage of T-cells, particularly in respect to TH2/IgE immune responses (Brinkmann *et al.*, 1997; d'Angeac *et al.*, 1994, Vollenweider *et al.*, 1995). The $CD4^+CD57^+$ subset has also been found in the periphery, where they constitute only a minor population (0.5-17%) of the total $CD4^+$ T cells (Andersson *et al.*, 1995). They are also reported to play a role in the pathogenesis of rheumatoid arthritis (Imberti *et al.*, 1997) and constitute a major site for HIV infection and replication (Hufert *et al.*, 1997). Still, the precise function of these cells remain unknown.

T cells in GC have also been shown to accumulate somatic mutations in their TCR genes. The T cells that do so are prone to apoptosis, as GC-B cells, suggesting they might be able to affinity-mature here similar to GC-B cells (Zheng *et al.*, 1994). T cells within GC selectively expand for particular TCR α and β CDR3 lengths in the same manner as GC-B cells preferentially expand particular clones in GC (Zheng *et al.*, 1996a and b). This definitely adds an extra dimension to the GC-reaction and focuses attention on the problem of selection, and, especially of autoreactive clones if both the T and the B cell compartment can mutate.

4 Other Contributors to the Germinal Centre Reaction

The lymphoid organs such as spleen, lymph nodes and Peyer's patches constitutes a specialised milieu in which the T cell and B cell meet, get activated and differentiate. Little is known about the interaction between the lymphocytes and the micro-environment within the secondary lymphoid organs. Most studies describe the interaction between lymphocytes and dendritic cells (DC), other antigen presenting cells (APC) as well as follicular dendritic cells (FDC) (Lederman *et al.*, 1992; McHeyzer-Willams, 1996). It is known, that the migration of lymphocytes from the circulation into the different secondary organs via specialised high endothelial post-capillary venuels (HEV) is highly selective. This process involves carbohydrate structures in the stromal cells, where mannose recognition has been described as participating in lymphocyte entry into the spleen (Weston *et al.*, 1991, 1992).

Since this micromilieu contributes with signals that are more or less unknown, the contribution to B and T cell activation in a humoral immune response remains an enigma. Efforts have been made to reconstitute such a milieu *in vitro* by use of cell-lines of stromal/reticular origin which only provide parts of the microenvironment (Castro *et al.*, 1997). The lymphoid organs are divided into different compartments, contributing with signals that make the circulating T and B cells to migrate in a regulated way. Adhesion molecules have been described and quantified in an effort to elucidate their functional involvement in such dynamically cell trafficking process and in the immune response (Leite *et al.,* 1995).

A molecule involved in homing of lymphocytes that has recently gained interest in relation to cell activation and differentiation is the CD44 molecule. It is a widely expressed cell surface glycoprotein (lymphocytes, monocytes, granulocytes, fibroblasts, epithelial cells, erythrocytes) and has been ascribed a multitude of functions. Examples are: differentiation antigen involved in early lymphopoesis, adhesion and homing for lymphocytes in cell-cell (hetero- and homotypic) and cell-matrix interactions, activation/signal transduction of lymphocytes. It has also been described as a potential marker for metastasis. The list can be made long, and the variety of functions explained by the fact that CD44 exists in many different

isoforms due to alternative exon-splicing of at lest 20 different exons. Also, different glycosylation patterns of the molecule contribute to the variety of functions (for recent reviews read Borland *et al*., 1998; Kincade *et al*., 1997; Lesley *et al*., 1993; Stauder and Günthert, 1995). The CD44 molecule has been implicated from several studies to participate in lymphocyte activation, and particularly T and B cell activation (Conrad *et al*., 1992; Guo *et al*., 1996; Haynes *et al*., 1989; Kremmidiotis *et al*., 1995; Lutz *et al*., 1995; Rafi *et al*., 1997). The expression of certain variant isoforms of the molecule can be modulated by mitogenic stimulation or by cytokines such a TNFα and IFNγ (Mackay *et al.,* 1994). Not all ligands to CD44 are known, a point which was recently highlighted by Guo *et al*. (1996). Ligation of CD40 on B cells rapidly (within 3h) induced a costimulatory molecule characterised as CD44H (haematopoetic form of the molecule) which co-stimulated T cell proliferation in a CD80/CD86 and CD28 independent way (Guo *et al*., 1996). Different CD44H expression pattern on B cells within a tonsil has been described thoroughly (Zola *et al*., 1995) Characteristic of CG-B cells is a down-regulation of CD44 within the dark zone centroblasts and a low expression on centrocytes within the light zone. The expression of the molecule and its different isoforms has been suggested to reflect the variable needs for its function (Zola *et al*., 1995; Murakami *et al*., 1991). As such, its involvement in T-B cell collaboration, as a novel costimulatory molecule to facilitate an antigen response, is of major interest (Guo *et al*., 1996; Paper IV).

5 The Present Investigation

To be able to further dissect the events during a human germinal centre reaction, cell culture conditions allowing for growth of GC-B cells *in vitro* have to be established. In a further attempt to reproduce the GC reaction *in vitro*, not only growth of the cells but induction of a complete GC-phenotype from naïve cells, *i.e.* the T cell zone events, has to be mimicked. The aims of this investigation have been to a) design a cell culture system for growth of human germinal centre B cells b) to study such cells *in vitro* under single cell culture conditions c) to evaluate different conditions and analyse if such allows somatic mutations to proceed during the cell culture d) to induce human naïve B cells towards a germinal centre B cell phenotype and e) to elucidate the role of a subset of human germinal centre T cells expressing the CD57 molecule in the GC-reaction.

5.1 Paper I

In this paper the growth of human tonsillar germinal centre B cells together with the murine EL-4 thymoma cells was examined. Such cells have previously been described as providing conditions for efficient growth of both human and murine naïve B cells. The system allows growth of single B cells and for analysis of the cells at a clonal level (Wen *et al.*, 1987; Zubler *et al.*, 1985; Zhang *et al.*, 1990). This prompted us to study the capacity of these cells to support germinal centre B cells for proliferation and further *in vitro* accumulation of somatic mutations. The phenotype of human germinal centre B cells, prepared by negative selection, cultured together with irradiated EL-4 cells and additives (PMA, IL2, T cell replacing factor (PWM-TRF) and 2-mercaptoethanol) revealed that the *in vivo* characteristic of the GC-B cells were kept during the first days upon culture. The cells continued with proliferation with a peak around day 4 and preserved the characteristic heterogeneous GC-B expression of CD77 (Fig. 9). This was followed by a terminal differentiation to Ig secreeting plasma blasts as has been described for resting human and murine B cells cultured in the system. As such, the EL-4 system fulfilled the criteria for growth of human germinal centre B cells at a single cell mode (Paper I). The CD40/CD40L interaction, now well known to be of importance for

germinal centre and memory B cell formation (Foy *et al*., 1994; Arpin *et al*., 1995), is also likely to be important in the EL-4 system. It has been shown that human soluble Ig-CD40 prevents proliferation of human naïve B cells in the system. Similarly, the EL-4 system in our study provides the human GC-B cells with the CD40L, thereby probably contributing to a plasmacytoid differentiation of the GC-B cells as suggested by Arpin *et al*. (1995).

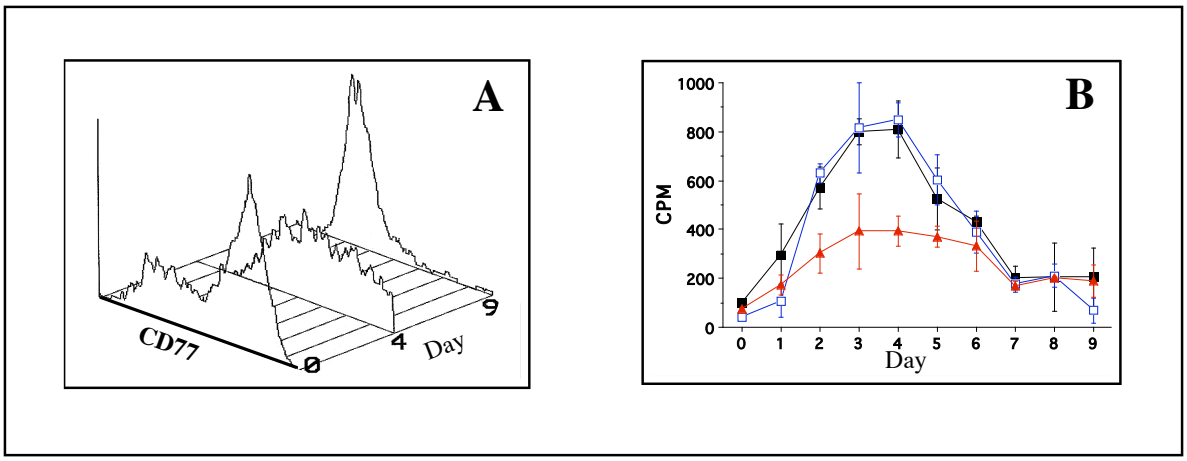


Fig. 9 Preservation of CD77 expression of GC-B cells cultured together with EL-4 thymoma cells (A). Day 0 represents expression on freshly isolated GC-B cells. B shows [^{3}H]Thymidine incorporation by cultured GC-B (■), CB-B (▲) and resting B (□) cells.

5.2 Paper II

Since culture of the human GC-B cells together with the murine EL-4 cells allowed for manipulation of the cells in a single cell fashion and such cultured cells initially kept characteristic features of GC-B cells, we studied the capacity of the GC-B to continue their mutational activity *in vitro*. The single cell concept allowed for assessment of mutational activity at a clonal level, facilitating the mutation analysis. By the use of Single-Strand Conformation Polymorphism (SSCP) analysis (Orita *et al*., 1989) as a rapid tool for screening of *in vitro* mutational activity, single human GC-B cell clones were analysed. The SSCP-assay was set up and characterised using two hybridomas producing mAb (Dahlenborg *et al*., 1997), with known sequence differences. This allowed for detection of 2.5% mutated material with respect to cell input numbers. mRNA extracted from the cultures were analysed with a nested RT-PCR for the presence of VH3 IgH family. The results obtained, showed no evidence of sustained mutational activity *in vitro*. This result was in a way contradictory to the

fact that, at least, the extracellular GC-B cell phenotype initially was preserved to some extent. An extensive analysis of the cultured GC-B cells was then performed to analyse for the intracellular anti-death protein bcl-2, known to be involved in early rescue of the GC-B cells (Liu *et al.* 1989 and 1991c). In this study we show an up-regulation of the anti-death protein bcl-2 after 16-20h of culture, indicating that the cells have been given an early rescue signal. This might prevent further apoptosis, as well as re-route the cells for differentiation to plasmablasts. We conclude from the studies in paper I and II, that the EL-4 cells in combination with SSCP and the single cell concept for growth of human GC-B cells, constitute a useful tool in dissecting *in vitro* mutational activity. But, culture of the GC-B cells together with the EL-4 cells and appropriate additives alone, do not supply with conditions for preservation of mutational activity *in vitro* (Paper II).

5.3 Paper III

With the single cell concept developed in paper I and II in combination with SSCP analysis, other cell culture systems known to preserve the GC-B phenotype were assessed for mutational activity. A culture system described by Pound and Gordon (1997) showed that human GC-B cells can be maintained in cultures with human CD32 transfected murine L cells and anti-CD40 monoclonal antibody (the "CD40 system") if supplemented with appropriate interleukin combinations. Human GC-B cells maintained in such cultures, partially preserved a phenotype characteristic of centroblast cells. Particularly, the characteristic low/absence of CD44 expression could be seen in combination with a high CD38 expression in such cultures. We analysed two of the interleukin combinations, i) IL-1β, IL-2 and IL-10 or ii) IL-4, IL-7 and IL-10, both combinations known to sustain GC-B cell proliferation in CD40 cultures (Pound and Gordon, 1997). By combining initially five days of culture in the interleukin supplemented CD40 cultures, with a subsequent clonal expansion and differentiation using the EL-4 cells and additives as in paper I and II, *in vitro* mutational activity was analysed (Fig. 10). The expansion and

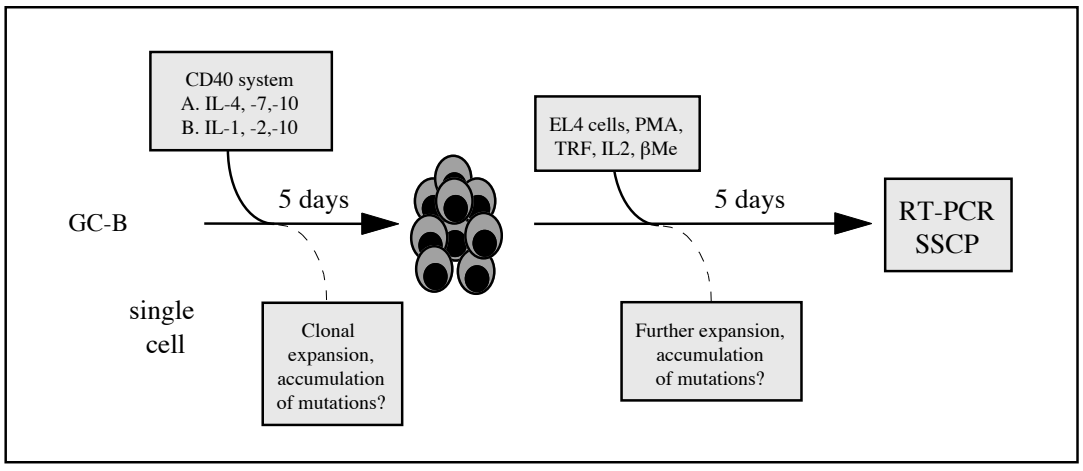


Fig. 10. Combination of the CD40 and EL-4 system used in Paper III.

differentiation using EL4 cells, and additives, facilitated further RNA extraction to perform RT-PCR and subsequent SSCP mutation analysis. We show for the first time that supplementing CD40 cultures with IL-1β+IL-2+IL-10 allowed accumulation of somatic mutations to proceed and detected using SSCP. By sequencing, some mutations were confirmed as outlined in figure 11 (Paper III). Few other studies of somatic hypermutation have been performed on non ag-selected human Ig V genes (Dunn-Walters, 1998). The result in this paper indicate that cytokines might regulate the process of somatic hypermutation. We believe that such cultures will facilitate further studies of human GC-B cells involved in the germinal centre reaction and mimicking somatic hypermutation *in vitro*.

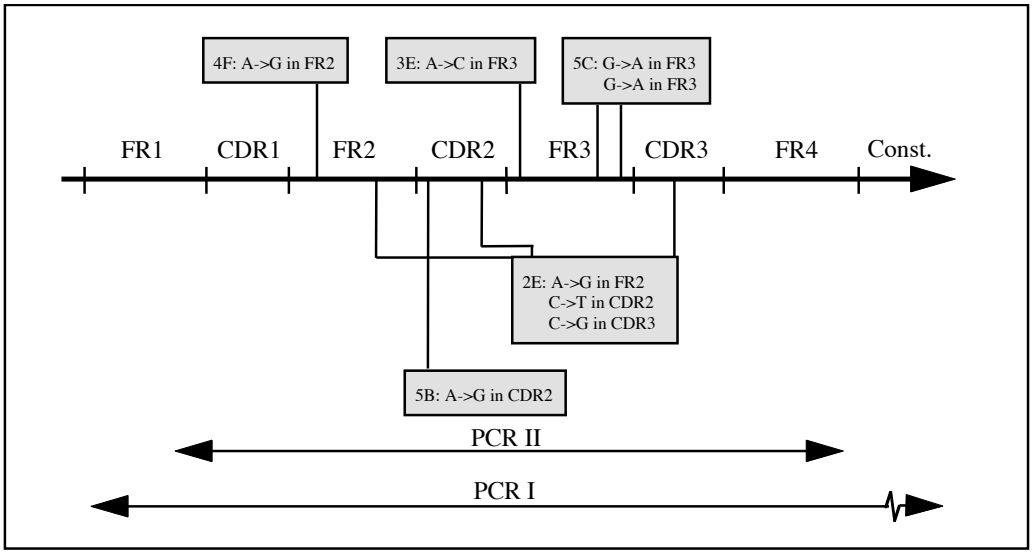


Fig. 11 Distribution of mutations found in clones cultured in the CD40 system supplemented with IL1β, IL2 and IL10.

5.4 Paper IV

In an attempt to understand the role of a sub-population of germinal centre T cells (GC-T) that co-express the CD57 molecule in the GC, we analysed th capacaty of these cells to interact with B cells. The relevance of CD57 expression on these cells is still an enigma. T cells expressing the molecule are mainly found within the light zone of the germinal centre, but a proportion may as well be seen in peripheral blood (Andersson *et al.*, 1995). Based on their co-expression of CD45RO and the early activation marker CD69 (Butch *et al.*, 1993) the cells appear to be activated to some extent. We investigated if such cells were able to derive help to B cells, after activation. In contrast to ordinary $CD4^+$ T cells, such activated $CD4^+CD57^+$ only

induced B cells to Ig secretion in the presence of exogenous IL-2. Unactivated $CD4^+CD57^+$ were not found to contain any mRNA for IL-2, IL4 or IFNγ, nor to be able to produce it upon activation with SEA and APC or a combination of PMA and iononomycin. The cells were also analysed for the contents of CD40L without finding mRNA coding for such. In T cells found in the outer zone, and in isolated $CD4^+CD45RA^-CD57^+$, preformed CD40L has been detected (Casamayor-Palleja *et al*., 1995). IL-2, IL-4 and IL-10 IFNγ and TNFα have in another study by Toellner *et al*. (1995) been reported to be produced by unstimulated GC-T cells at a single cell level. The $CD57^+$ T cells did help GC-B cells to survive in the cultures to the same extent as its $CD57^-$ counterpart. It has been suggested that CD57 is a late activation marker of highly differentiated GC-T cells, and as such, have lost some of their original capacity to help B cells (d'Angeac *et al*., 1994). We make a similar conclusion in our study, and, that some of their B cell helping capacity might be restored with the addition of exogenous IL-2. Another possibility is that they might have a specific function in GC, involving rescue and/or subsequent differentiation of GC-B cells, a fact difficult to explain since the cells do not, in our study, seem to express any of the studied cytokines nor the CD40L. Though, at least cytokines, might be supplemented from other cells such as the $CD57^-$ counterpart and/or FDC.

5.5 Paper V

In an attempt to induce naïve B cells to progress towards a germinal centre B cell phenotype, we used the known fact that ligation of the CD40 molecule in combination with crosslinking of sIg, using anti-IgM antibodies, induces a partial GC-B phenotype (Wheeler *et al*., 1996, Galibert *et al*., 1996a). Dynamical changes of the CD44 expression during B cell activation and differentiation, particularly within tonsils (Kremidiotis *et al*., 1995) lead us to speculate that the molecule may participate in B cell activation. Of particular interest to this study is a paper by Guo *et al*. (1996) which shows that CD44H is rapidly upregulated (within 3h) on CD40 activated B cells (Fig. 12). Such cultured cells will costimulate proliferation of T cells in a CD80/CD86 and CD28 independent manner. The ligand on the B cell is still to be determined. The CD44 molecule fulfils several criteria that makes it suitable for co-stimulatory activities; it exists in several isoforms distributed on a variety of cells, is linked to the cytoskeleton via its cytoplasmatic domain and has the capacity to become phosphorylated when crosslinked (reviewed by Lesley *et al*., 1993). The molecule has been shown to promote human T cell activation and proliferation when co-ligated together with CD2 (Conrad *et al*., 1992) and has also been shown to be involved in murine B cell activation (Rafi *et al*., 1997). The variation in CD44 isoform expression has been suggested to reflect the need for the molecule of particular cell type (Kremidiotis *et al*., 1995).

We used co-ligation of CD44 in combination with anti-IgM and anti-CD40 stimulation on human naïve tonsillar B cell. In such cell cultures we observed a progression towards a GC-B cell phenotype in respect to certain phenotypical changes on the cell surface. The B cells upregulated CD10, CD71, CD77, CD95 and CD38, being characteristic of human GC-B cells, as well as downregulated the expression of CD24 and CD39. Of particular interest is the heterogeneous expression of CD77 as being one of the hallmarks of germinal centre B cells as well as an up-regulation of CD10, only seen on cells co-stimulated with CD44. The *in vitro* activated cells also showed a progression towards apoptosis, as was determined with annexin V stainings. The binding of annexin V to the cells correlated with CD10 upregulation on the same population. This suggests that CD44 is involved in antigen-specific B cell activation and further progression towards the germinal centre B cell phenotype, but whether this signal is provided by stromal cells or lymphocytes, such as the cognate T cells, remains to be resolved (Paper IV; Fig. 12).

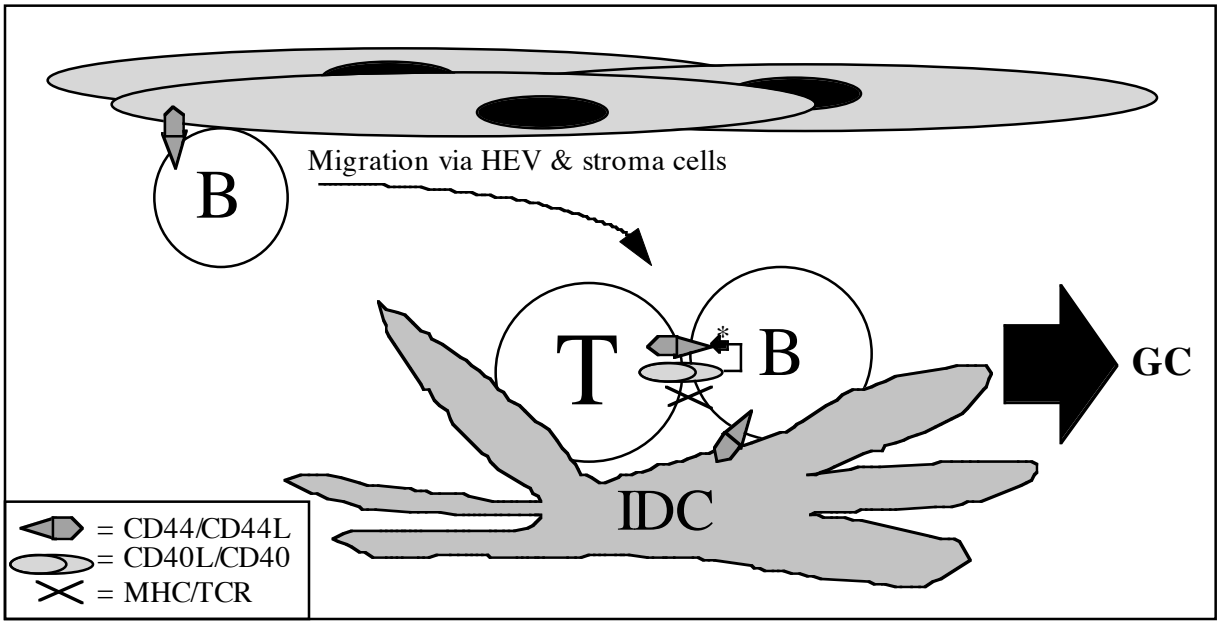


Fig. 12 Possible CD44/CD44L interaction. A B7 independent costimulation has been described (*) for T cells via anti-CD40 stimulated B cells (Guo *et al*., 1996).

6 Concluding remarks

Since the different studies included in this thesis began to take form, and throughout the years in which they were performed, a huge pool of knowledge concerning the B cell response to a T cell dependent antigen along with the formation of the germinal centre reaction and affinity maturation has been collected. Still, the reproduction of such events *in vitro* is not an easily performed task which leave us with the thought that somehow, ”Mother Nature” has not yet given us all the answers. Studies of human B cells, particularly regarding the germinal centre reaction and affinity maturation, have been hampered by the fact that such cells do not seem to thrive *in vitro* and the lack of a proper cell culture system to study such cells in, at a clonal level. This fact probably reflects the complex truth about the cells we are trying to study; that they depend upon the highly specialised micro-milieu, the secondary lymphoid organs with different compartments, in which they are found *in vivo*.

The studies included in this thesis have contributed to this field with different tools to study GC-B cells. This will help in further dissection of cellular and molecular events involved in the germinal centre reaction and affinity maturation. Further studies of the cultures described here, in combination with others, will help us to further understanding of events involved in the germinal centre reaction.

And ultimately to be able to reproduce such events *in vitro*.

7 Acknowledgements

I would like to express my gratitude to all the people that have made the years at BioInvent Int. AB and Dept. of Immunotechnology enjoyable, friendly and creative in making all these days, and nights, possible with this thesis as a result. Since starting, some colleagues have left, new have come with a few being around all the years. All of you have contributed the thesis in different ways: ”fredagsfika”, Tuesday (gourmet!) lunches, ”torsdagsbulle”, pub evenings, dinners, friendship and the daily atmosphere.

I would like to express my gratitude to Roland Carlsson, for excellent supervision with a never ending flow of ideas in designing experiments and for introducing me to the world of T and B cells. I am gratefull for your enthusiasm, great knowledge, critisism, encouragement and support during the years although deserting from the T and B cells to phages...

I am indebted to Carl Borrebaeck for giving me the opportunity to start at the department as a graduate student a few years ago and for the support and guidance the latest few years I spent at Wallenberglab.

I would like to thank Bo Jansson for a never ending flow of enthusiastic discussions about the Thomsen-Friedenreich antigen and other, more or less immunology related, areas. Also, I am thankful to Mats Ohlin for allways being around with a helping hand, source of knowledge and for finding logical critisism to everything.

My warmest gratitude to John Pound and family, for friendship and collaboration, scientific discussions, concerts, encouragement and a never ending support. We have probably contributed to crashes of the mail system and server in Birmingham, but why use telephones...

There are a few people in my near surrounding that I would like to thank warmly. During the last year I have had the benefit of sharing room with the best roommate ever, Bengt, my ”dag-sambo”, contributing with all kind of discussions. I am looking forward to a revised version of Fundamental... Camilla for the never ending flow of mails, smiling as ever. Will there be any possible subscription to continue ”Djurstunden”? Siggi for being a happy, smiling friend and the travel partner No. one during these years, teaching me how to survive without my maps...Frida for being a friend and translating Icelandic with ”skånska” in exchange. She knows that maps do not always help me... I am indebted to her for all the hours she spent on the layout of this thesis.

People oustside the lab have a special role, participating in good and bad experiments as spectators. As being a source of support to keep me aware of life outside the cell-lab. I would especially like to thank Lykke & Ulf and Karin & Claes as former colleagues (Karin and Lykke) and friends (all of you!). For warm friendship, pasta evenings, chat, coffee (!), Pigham and potato support... Furthermore, Susanne & Douglas for trips after work and wildlife distractions...

My beloved family for participation, support and engagements in cells that do not want to grow and for cells that do grow weekends as well. To Johanna and Kristina, for flavouring my life with ”antikloppar”.

And last, but not least, to my Olof. For the continuous love, support and encouragement and for being a part of my life.

8 References

Alzona, M., Jäck, H-M., Fisher, R. I., Ellis, T. M. (1994) J. Immunol. 153; 2861-2867

Akbar, A. N., Terry, L., Timms, A., Beverly, P. C., and Janossy, G. (1988) J. Immunol. 140; 2171-2178

Andersson, E., Ohlin, M., Borrebaeck, C. A. K., and Carlsson, R. (1995) Cell. Immunol. 163; 245-253

Andersson, E., Dahlenborg, K., Ohlin, M., Borrebaeck, C. A. K., and Carlsson, R. (1996) Cell. Immunol. 169; 166-173

Arai, K., Yamamura, S., Seki, S., Hanyu, T., Takahashi, H. E., and Abo, T. (1998) Clin. Exp. Immunol. 111; 345-352

Armitage, R. J., Fanslow, W. S., Strockbine, ., Sato, T. A., Cliffird, K. N., Macduff, B. M., Andersson, D. M., Gimpel, S. D., Davis-Smith, T., Maliszewski, C. R., Clark, E., Smith, C. A., Grabstein, K. H., Cosman, D., Spriggs, M. K. (1992) Nature 357; 80-82

Arpin, C., Déchanet, J., Van Kooten, C., Merville, P., Grouard, G., Brière, F., Banchereau, J., and Liu, Y.-J. (1995) Science 268; 720-722

Aubry, J. P., Pochon, S., Gauchat, J. F., Nueda-Marin, A., Holers, V. M., Graber, P., Siegfried, C., and Bonnefoy, J. F. (1994) J. Immunol. 152; 5806-5813

Banchereau, J., Rousset, F (1991) Nature 17; 678-679

Banchereau, J., and Steinman, R. M. (1998) Nature 392; 245-252

Banks, T. A., Rouse, B. T., Kerley, M. K., Blair, P. J., Godfrey, V. L., Kuklin, N. A., Bouley, D. M., Thomas, J., Kanangat, S., Mucenski, M. L. (1995) J. Immunol. 155; 1685-1693

Bell, E. B., and Sprshott, S. M. (1990) 348, 163-166

Benner, R., Hijmans, W., and Haaijman, J. J. (1981) Clin. Exp. Immunol. 46; 1-8

Bengtsson, Å., Johansson, C., Tengvall-Linder, M., Halldén, G., Van der Ploeg, I., and Scheynius, A. (1995) J. Leuc. Biol. 58; 683-689

Berek, C., Berger, A., and Apel, M. (1991) Cell 67; 1121-1129

Berek, C., Griffiths, G. M., and Milstein, C. (1985) Nature 316; 412-418

Berek, C., and Milstein, C. (1987) Immunol. Rev. 96; 23-41

Berek, C., and Ziegner, M. (1993) Immunol. Today 14; 400-411

Betz, A., Milstein, C., and Gonzalez-Fernandezn ,A., Pannell, R., Larson, T., and Neuberger, M. S. (1994) Cell 77; 239-248

Betz, A. G., Rada, C., Pannell, R., Milstein, C., and Neuberger, M. S. (1993) Proc. Natl. Acad. Sci. U.S.A. 90; 2385-2388

Beutler, B., and van Huffel. C. (1994) Science 264; 667-669

Billian, G., Bella, C., Mondiére, P., and Defrance, T. (1996) Eur. J. Immonol. 26; 1712-1719

Billian, G., Mondiére, P., Berard, M., Bella, C and Defrance, T. (1997) Eur. J. Immunol. 27; 405-424

Björk, P., Florens-Romo, L., and Liu, Y.-J. (1997) Eur. J. Immunol. 27; 1266-1274

Bonnefoy, J.-Y., Henchoz, S., Holder, M. J., Hardie, D., and Gordon, J. (1993) Eur. J. Immunol. 23; 969-972

Borland, G., Ross, J. A., Guy, K. (1998) Immunology 93; 139-148

Borriello, F., Sethna, M. P., Boyd, S. D., Schweitzer, A. N., Tivol, E. A., Jacoby, D., Strom, T. B., Simpson, E. M., Freeman, G. J., and Sharp. A. H. (1997) Immunilty 6: 303-313
Both, G. W., Taylor, L., Pollard, J. W., Steele, E. (1990) Mol. Cell. Biol. 10; 5187-5196
Bothwell, A. L. M., Paskind, M., Reth, M., Imanishi-Kari, T., Rajewsky, K., Baltimore, D. (1981) Cell 24; 625-637
Bouzahzah, F., Bosseloir, A., Heinen, E., Simar, L. J. (1995) Dev. Immunol. 4; 189-197
Bowen, M. B., Butch, A. W., Parvin, C. A., Levine, A., and Nahm, M. H. (1991) Hum. Immunol. 31; 67-75
Brenner, S., and Milstein, C. (1966) Nature 211; 242-243
Brinkmann, V., Kristofic, C. (1997) Immunology 91; 541-547
Butch, A. W., Chung, G. H., Hoffman, J. W., and Nahm, M. H. (1993) J. Immunol. 150; 39-47
Caamaño, J., H., Rizzo, C. A., Durham, S. K., Barton, D. S., Raventós-Suárez, C., Snapper, C. M., and Bravo, R. (1998) J. Exp. Med. 187; 185-196
Caligaris-Cappio, F., Riva, M., Tesio, L., Schena, M., Gaidano, G.-L., and Bergui, L. (1989) Blood 73; 1259-1263
Casali, P., Burastero, S. E., Nakamura, M., Inghirami, G., and Notkins, A. L. (1987) Science 236; 77-81
Casamayor-Palleja, M., Feulliard, J., Ball, J., Drew, M., and MacLennan, I. C. M. (1996) Int. Immunol. 8; 737-744
Carter, R. H., and Fearon, D. T. (1992) Science 256; 105-107
Carter, R. H., Park, D. J., Rhee, S. G., and Fearon, D. T. (1991) Proc. Natl. Acad. Sci. USA 88; 2745-2749
Casamayor-Palleja, M., Khan, M., and MacLennan, I, C. M. (1995) J. Exp. Med. 181; 1293-1301
Casson, L.P., and Manser, T. (1995a) J. Immunol. 155; 5647-5654
Casson, L.P., and Manser, T. (1995b) J. Exp. Med. 182; 743-750
Castan, J., Tenner-Racz, K., Racz, P., Fleischer, B., and Broker, B. M. (1997) Immunology 90; 265-271
Csatro, A., Bono, M. R., Simon, V., Vargas, L., and Rosemblatt, M. (1997) Eur. J. Cell Biol. 74; 321-328
Caux, C., Massacrier, C., Vanbervliet, B., Dubois, B., Van Kooten, C., Durand, I., and Banchereau, J. (1994) J. Exp. Med. 180; 1263-1272
Cella, M., Scheidegger, D., Palmer-Lehmann, K., Lane, P., Lanzavecchia, A., and Alber, G. (1996) J. Exp. Med. 184; 747-752
Cerutti, A., Zan, H., Schaffer, A., Bergsagel, L., Harindranath, N., Max, E. E., and Casali, P. (1998) J. Immunol. 160; 2145-2157
Chen, C., Roberts, V. A., and Rittenberg, M. B. (1992) J. Exp. Med. 176; 855-866
Chen, C., Roberts, V. A., Stevens, S., Brown, M., Stenzel-Poore, M. P., and Rittenberg, M. B. (1995) EMBO J. 14; 2784-2794
Clark, M. R., Campbell, K. S., Kazlauskas, A., Johnson, S. A., Hertz, M., Potter, T. A., Pleiman, C., and Cambier, J. C. (1992) Science, 258; 123-126
Clark, E. A., and Ledbetter, J. A. (1994) 367; 425-428
Cohn, M., and Langman, R. E. (1990) Immunol. Rev. 115; 11-147
Cong, YZ, Rabin, E, Wortis, H.H. (1990) Int. Immunol. 3; 467-476
Conrad, P., Rothman, B. I., Kelley, K. A., and Blue, M.-L. (1992) J. Immunol. 149; 1833-1839
Cothia, C., Lesk, A. M., Gherardi, E., Tomlinson, I. M., Walter, G., Marks, J. D., Llewelyn, M. B., and Winter, G. (1992) J. Mol. Biol. 227: 799-817
Croft, M., Duncan, D., and Swain, S. D. (1992) J. Exp. Med. 176; 1431-1437
Croix, D. A., Ahearn, J. M., Roengard, A. M., Han, S., Kelsoe, G. Ma, M., and Carroll, M. C. (1996) J. Exp. Med. 183; 1857-1864
Crowe, P. D., Van Arsdale, T. L., Walter, B. N., Ware, C. F., Hession, C., Ehrenfels, B., Browning, J. L., Din, W. S., Goodwin, R. G., and Smith, C. A. (1994) Science 264; 707-710
Cutrona, G., Ulivi, M., Fais, F., Roncella, S., and Ferrarini, M. (1995) J. Exp. Med. 181; 699-711
Cyster, J. G., and Goodnow, C. C. (1995) Immunity 3; 691-701
Cyster, J. G., Hartley, S. B., and Goodnow, C. C. (1994) Nature 371; 389-395

Dahlenborg, K., Hultman, L., Carlsson, R., and Jansson, B. (1997) Int. J. Cancer. 70; 63-71
Dahlenborg, K., Pound, J. D., Gordon, J., Borrebaeck, C. A. K., and Carlsson, R. (1997) 175; 141-149
d'Angeac, A. D., Monier, S., Pilling, D., Travaglio-Encinoza, A., Reme, T., and Salmon, M. (1994) Eur. J. Immunol. 24; 1503-1511
Davis, L., Vida, R., and Lipsky, P. E. (1986) 137; 3758-3767
Defrance, T., Aubry, J. P., Rousset, F., Vaubervliet, B., Bonnefoy, J. Y., Arai, N., Takebe, Y., Yokota, T., Lee, F., Arai, K., De Vries, J., and Banchereau, J. (1987) J. Exp. Med. 165; 1459-1467
DeFranco, A. L. (1992) Eur. J. Biochem. 210; 381-338
Delibrias, C. C., Floettemann, J. E., Rowe, M., and Fearon, D. T. (1997) J. Exp. Med. 186; 1575-1583
Del Prete, G., De Carli, M., D'Elios, M. M., Daniel, K. C., Almerigogna, F., Alderson, M., Smith, C. A., Thomas, E., and Romagnani, S. (1995) J. Exp. Med. 182; 1655-1661
Denépoux, S., Razananajaona, D., Blanchard, D., Meffre, G., Capra, J. D., Bachereau, J., and Lebecque, S. (1997) Immunity 6; 35-46
Doody, G. D., Justement, L. B., Delibrias, C. C., Matthews, R. J., Lin, J., Thomas, M. L., and Fearon, D. T. (1995) Science 269; 242-244
Doyle, C., and Strominger, J. L. (1987) Nature 330; 256-259
Dunn-Walters, D. K., Dogan, A., Boursier, L., MacDonald, C. M., and Spencer, J. (1998) J. Immunol. 160; 2360-2364
Engel, P., Zhou, L.-J., Ord, D. C., Sato, S., Koller, B., and Tedder, T. F. (1995) Immunity 3; 39-50
Fearon, D. T. (1993) Curr. Opin. Immunol. 5; 341-348
Ferguson, S. E., Han, S., Kelsoe, G., and Thompson, C. B. (1996) J. Immunol. 156; 4576-4581
Feuillard, J., Taylor, D., Casamayor-Palleja, M., Johnson, G. D., and McLennan, I. C. M. (1995) Int. Immunol. 7; 121-130
Flaswinkel, H., and Reth, M. (1994) EMBO J. 13; 83-89
Flemming, W. (1885) Arch. Mikrosk. Anat. 24; 355-
Foy, T. M., Laman, J. D., Ledbetter, J. A., Aruffo, A., Claassen, E., and Noelle, R. (1994) 180; 157-163
Foote, J., and Milstein, C. (1991) Nature 352; 530-532
Franzoso, G., Carlson, L., Poljak, L., Shores, E. W., Epstein, S., Leonardi, A., Grinberg, A., Tran, T., Sharton-Kersten, T., Anver, M., Love, P., Brown, K., and Siebenlist, U. (1998) 187; 147-159
Fuller, A., Kanagawa, O., and Nahm, M. H., (1993) J. Immunol. 151; 4505-4512
Gajl-Peczalaska, K. J. Fish, A. J., Meuwisse, H. J., Frommel, D., and Good, R. A. (1969) J. Exp. Med. 130; 1367-1374
Galibert, L., Burdin, N., de Saint-Vis, B., Garrone, P., Van Kooten, C., Banchereau, J., and Rousset, F. (1996a) J. Exp. Med. 183; 77-85
Galibert, L., Burdin, N., Barthélémy, C., Meffre, G., Drurand, I., Garcia, E., Garrone, P., Rousset, F., Banchereau, J., and Liu, Y.-J. (1996b) J. Exp. Med. 183; 2075-2085
Galibert, L., Van Dooren, J., Durand, I., Rousset, F., Jefferis, R., Banchereau, J., and Lerbecque, S. (1995) Eur. J. Immunol. 25; 733-737
Garcia, K. C., Scott, C. A., Brunnmark, A., Carbone, F. R., Peterson, P. A., Wilson, I. A., and Teyton, L. (1996) Nature 384; 577-581
Gergeley, J., and Sármay, G (1996) Scand. J. Immunol. 44; 1-10
Gearhart, P. J., and Bogenhagen, D. F. (1983) Proc. Natl. Acad. Sci. U.S.A. 80; 3439-3443
Glick, B., Chang, T.S., and Jaap, R. G. (1956) Poult. Sci. 35; 224-226
Gold, M. R., Chan, V. W.-F., Turck, C. W., and DeFranco, A. L. (1992) J. Immunol. 148; 2012-2022
Gordon, J., Webb, A., Walker, L., Guy, G. R., and Rowe, M. (1986) Eur. J. Immunol. 16; 1627-1630
Gray, D., Dullifore, P., and Jainandunsing, S. (1994) J. Exp. Med. 180; 141-155
Grewal, I. S., Xu, J., and Flavell, R. A. (1995) Nature 378; 617-620
Grouard, G., Durand, I., Filgueira, L., Banchereau, J., and Liu, Y.-J. (1996) Nature 384; 364-367
Grupp, S. A., Snow, E. C., and Harmony, J. A. K. (1987) Cell. Immunol. 109; 181-191

Guo, Y., Wu, Y., Shinde, S., Sy, M.-S., Aruffo, A., and Liu, Y. (1996) J. Exp. Med. 184; 955-961
Han, S., Zheng, B., Dal Porto, J., and Kelsoe, G. (1995a) J. Exp. Med. 182; 1635-1644
Han, S., Hathcock, K., Zheng, B., Kepler, T. B., Hodes, R., and Kelsoe, G. (1995b) J. Immunol. 155; 556-567
Han, S., Zheng, B., Schatz, D.G., Spanopoulou, E., and Kelsoe, G. (1996) Science 274; 2094-2097
Han, S., Dillon, S. R., Zheng, B, Shimoda, M., Schlissel, M. S., and Kelsoe, G. (1997) Science 278; 301-305
Hardie, D. L., Johnson, G. D., Khan, M., and MacLennan, I. C. M. (1993) Eur. J. Immunol. 23; 997-1004
Hardy, R. R., Hayakawa, K., Shimuzu, M., Yamasaki, K., and Kishimoto, T. (1987) Science 81-83
Hermeking, H., and Eick, D. (1994) Science 265; 2091-2093
Herzenberg, L. A., Stall, A. M., Lalor, P. A., Sidman, C., Moore, W. A., Parks, D. R., and Herzenberg, L. A. (1986) Immunol. Rev. 93; 81-102
Ho, F., Lortan, J. E., MacLennan, I. C. M., and Khan M. (1986) Eur. J. Immunol. 16; 1279-1301
Holder, M. J., Wang, H., Milner, A. E., Casamayor, M., Armitage, R., Spiggs, M. K., Fanslow, W. C., MacLennan, I. C. M., Gregory, C. D., and Gordon, J. (1993) Eur. J. Immunol. 23; 2368-2371
Holder, M. J., Grafton, G., MacDonald, I., Finney, M., and Gordon, J. (1995) Eur. J. Immunol. 25; 3160-3164
Hombach, J., Tsubata, T., Leclercq, L., Stappert, H., and Reth, M. (1990) Nature 343; 760-762
Hufert, F. T., van Lunzen, J., Janossy, G., Bertram, S., Schmitz, J., Haller, O., Racz, P., and von Laer, D. (1997) AIDS 11; 849-857
Hunt, S. V., and Vonderheide, R. H. (1990) Immunology 69; 487-489
Ikematsu, H., Harindranath, N., Ueki, Y., Notkins, A., and Casali, P. (1993) J. Immunol. 150; 1325-1337
Imberti, L., Sottini, A., Signorini, S., Gorla, R., and Primi, D. (1997) Blood 89; 2822-2832
Ingulli, E., Mondino, A., Khoruts, A., and Jenkins, M. K. (1997) J. Exp. Med. 185; 2133-2141
Ingvarsson, S., Dahlenborg, K., Carlsson, R, and Borrebaeck, C. A. K. (1998) submitted
Jacob, J., Kassir, R., and Kelsoe, G. (1991) J. Exp. Med. 173; 1165-1175
Jacob, J., and Kelsoe, G. (1992) J. Exp. Med. 176; 679-687
Jacob, J., Przylepa, J., Miller, C., and Kelsoe, G. (1993) J. Exp. Med. 178; 1293-1307
Jasin, H. E. (1991) Cell. Immunol. 136; 133-141
Julius, M. H., Heusser, C. H., and Hartmann, K.-U. (1984) Eur. J. Immunol. 14; 753-757
Kantor, A. B. (1991) Immunol. Today 12; 389-391
Keller, K. A., Bucy, R. P., and Nahm, M. H. (1995) Cell. Immunol. 163; 206-214
Kelly, K. A., Bucy, R. P., and Nahm, M. H. (1995) Cell. Immunol. 163; 206-214
Kelso, G. (1996) Immunity 4; 107-111
Kepler, T. B., and Perelson, A. S. (1993) Immunol. Today 14; 412-415
Kim, K.-M., Alber, G., Weiser, P., and Reth, M. (1993) Eur. J. Immunol. 23; 911-916
Kim, K.-M., Adachi, T., Nielsen, P. J., Terashima, M., Lamers, M. C., Köhler, G., and Reth, M. (1994) EMBO J. 13; 3793-3800
Kincade, P. W., Zheng, Z., Katoh, S., and Hanson, L. (1997) Cur. Opin. Cell Biol. 9; 635-642
Klein, U., Küppers, R., and Rajewsky, K. (1993) Eur. J. Immunol. 23; 3272-3277
Kosco, M. H., Szakal, A. K., and Tew, J. G. (1988) J. Immunol 140; 354-360
Krammer, P. H., Dhein, J., Walczak, H., Behrmann, I., Martani, S., Matiba, B., Fath, P. T., Daniel, E., Knipping, E., and Westerndorp, M. O. (1994) Immunol. Rev. 142; 175-191
Kremmidiotis, G., and Zola, H. (1995) Cell. Immunol. 161; 147-157
Kristenson, K., Borrebaeck, C. A. K., and Carlsson, R. (1992) Immunology 76; 103-109
Kroese, F. G. M., Wubbena, A. S., Seijen, H. G., and Nieuwenhuis, P. (1987) Eur. J. Immunol. 17; 1069-1072
Küppers, R., Zhao, M., Hansmann, M.-L., and Rajewsky, K. (1993) EMBO J., 12; 4955-4967
Kägi, D., Lederman, B., Bürki, K., Zinkernagel, R. M., and Hengarten, H. (1996) Annu. Rev. Immunol. 14; 207-232
Källberg, E., Jainandunsing, S., Gray, D., and Leanderson, T. (1996) Science 271; 1285-1289

Lagresle, C., Bella, C., and Defrance, T. (1993) Int. Immunol. 5; 1259-1268
Lagresle, C., Bella, C., Daniel, P. T., Krammer, P. H. and Defrance, T. (1995) J. Immunol. 154; 5746-5756
Lahvis, G. P., and Cerny, J. (1997) J. Immunol. 159; 1783-1793
Lane, P., Burdet, C., Hubele, S., Scheidegger, D., Müller, U., McConnel, F., and Kosco-Vilbois, M. (1994) J. Exp. Med. 179; 819-830
Lankester, A. C., van Schijndel, G. M. W., Frommé, J., Cordell, J. L., van Lier, R. A. W., and van Noesel, C. J. M. (1994) J. Immunol. 152; 2157-2162
Law, D. A., Chan, V. W.-F., Datta, S. K., and DeFranco, A. L. (1993) Curr. Biol. 3; 645-657
Lebecque, S., de Bouteiller, O., Arpin, C., Banchereau, J., and Liu, Y.-J. (1997) J. Exp. Med. 185; 563-571
Lebecque, S., and Gearhart, P. J. (1990) J. Exp. Med. 172; 1717-1727
Lederberg, J. (1959) Science 129; 1649-1653
Lederman, S., Yellin, M. J., Inghirami, G., Lee, J., Knowles, D. M., and Chess, L. (1992) J. Immunol. 149; 3817-3826
Leite, R. P., Carmo-Fonsca, M., Cabeçadas, J., Parreira, A., and Parreira, L. (1995) J. Clin. Pathol.: Mol. Pathol. 48; M93-M100
Leprince, C., Draves, K. E., Geahlen, R. H., Ledbetter, and J. A., and Clark, E. A. (1993) J. Immunol. 90; 3236-3240
Lens, S. M. A., Keehnen, R. M. J., van Oers, M. H. J., van Lier, R. A. W., Pals, S. T., and Koopman, G. (1996) Eur. J. Immunol. 26; 1007-1011
Lesley, J., Hyman, R., and Kincade, P. W. (1993) Adv. Immunol. 54; 271-335
Lightstone, L., and Marvel, J. (1993) Clin. Science 85; 515-519
Linsley, P. S., Wallance, P. M., Johnson, J., Gibson, M. G., Greene, J. L., Ledbetter, J. A., Singh, C., Tepper, M. A. (1992) Science 257; 792-795
Lindhout, E., Mevissen, M. L. C. M., Kwekkeboom, J., Tager, J. M., and de Groot, C. (1993) Clin. Exp. Immunol. 91; 330-336
Lindhout, E., Lakeman, A., and de Groot, C. (1995) J. Exp. Med. 181; 1985-1995
Linton, P.-J., Decker, D. J., and Klinman, N, R. (1989) Cell 59; 1049-1059
Linton, P.-J., Lo, D, Lai, L, Thorbecke, G. J., and Klinman, N. R. (1992) Eur. J. Immunol. 22; 1293-1297
Liu, Y.-J. (1997) J. Exp. Med. 5; 625-629
Liu, Y.-J., and Banchereau, J. (1996a) Immunologist 4; 55-66
Liu, Y.-J., de Bouteiller, O., and Fugier-Vivier, I. (1997) Cur. Opin. Immunol. 9; 256-262
Liu, Y.-J., Barthelemy, C., de Bouteiller, O., Arpin, C., Durad, I., Banchereau, J. (1995) Immunity 2; 239-248
Liu, Y.-J., de Bouteiller, O., Arpin, C., Briére, F., Galibert, L., Ho, S., Martinez-Valde, H., Banchereau, J., and Lebecque, S. (1996b) Immunity 4; 603-613
Liu, Y.-J., Grouard, G., de Bouteiller, O., and Banchereau, J. (1996c) Int. Rev. Cytol. 166; 139-179
Liu, Y.-J., Malisan, F., de Bouteiller, O., Guret, C., Lebecque, S., Banchereau, J., Mills, F. C., Max, E. E., and Martinez-Valdez, H. (1996d) Immunity 4; 241-250
Liu, Y.-J., Mason, D. Y., Johnson, G. D., Abbot, S., Gregory, C. D., Hardie, D. L., Gordon, J., and MacLennan, I. C. M. (1991c) Eur. J. Immunol. 21; 1905-1910
Liu, Y.-J., Oldfield, S., and MacLennan, I. C. M. (1988) Eur. J. Immunol. 18; 355-362
Liu, Y.-J., Zhang, J., Lane, P. J. L., Chan, E. Y.-T., and MacLennan, I. C. M. (1991a) Eur. J. Immunol. 21; 2951-2962
Liu, Y.-J., Cairns, J. A., Holder, M. J., Abbot, S. D., Jansen, K. U., Bonnefoy, J. Y., Gordon, J., and MacLennan, I. C. M. (1991b) 21; 1107-1114
Liu, Y.-J., Joshua, D. E., Williams, G. T., Smith, C. A., Gordon, J., and MacLennan, I. C. M. (1989) Nature 342; 929-931
Lortan, J. E., Roobottom, C. A., Oldfield, S., and MacLennan, I. C. M. (1987) Eur. J. Immunol. 17; 1311-1316
Lutz, M. B., Winzler, C., Aßmann, C., and Ricciardi-Castagnoli, P. (1995) Fund.Clin. Immunol. 2; 413-417

Luxembourg, A. T., and Cooper, N. R. (1994) J. Immunol. 153; 4448-4457
McHeyzer-Williams, M. G. (1996) Current Opin. Immunol. 8; 321-326
McHeyzer-Williams, M. G., Nossal, G. J. V., and Lalor, P. A. (1991) Nature 350; 502-505
Mackay, C. R., Terpe, H.-J., Stauder, R., Marston, W. L., Stark, H., and Günthert, U. (1994) J. Cell Biol. 124; 71-82
MacLennan, I. C. M., and Gray, D., (1986) Immunol. Rev. 91; 61-85
Madassery, J. V., Gillard, B., Marcus, D. M., and Nahm, M. H. (1991) J. Immunol. 147; 823-829
Maloney, M. D., and Lingwood, C. A. (1994) J. Exp. Med. 180, 191-201
Mangeney, M., Richard, Y., Coulard, D., Tursz, T., and Wiels, J. (1991) Eur. J. Immunol. 21; 1131-1140
Manser, T. (1987) J. Immunol. 139; 234-238
Martinez-Waldez, H., Guret, C., de Bouteiller, O., Fugier, I., Banchereau, J., and Liu, Y.-J. (1996) J. Exp. Med. 183; 971-977
Mast, J., and Goddeeris, B. M. (1998) Cell. Tissue Res. 291; 107-115
Matsumoto, M., Lo, S. F., Carruthers, C. J. L., Min, J., Mariatasan, S., Huang, G., Plas, D. R., Martin, S. M., Geha, R. S., Nahm, M. H., and Chaplin, D. D. (1996) Nature 382; 462-466
Matsumoto, M., Mariathasan, S., Nahm, M. H., Branyay, F., Peshon, J. J., and Chaplin, D. D. (1996b) Science 271; 1289-1291
Melchers, F., Haasner, D., Rawunder, U., Kalberer, C., Karasuyama, H., Winkler, T., and Rolink, A. G. (1994) Annu. Rev. Immunol. 12; 209-225
Merkenschlager, M., Terry, L., Edwards, R., and Beverley, P. (1988) Eur. J. Immunol. 18; 1653-1661
Michie, C., McLean, A., Alcock, C., and Beverley, P. (1992) Nature 360; 264-265
Miller, C., Stedra, J., Kelsoe, G., and Cerny, J. (1995a) J. Exp. Med. 181; 1319-1331
Miller, C., and Kelsoe, G. (1995b) J. Immunol. 155; 3377-3384
Mollet, L., Sadat-Sowti, B., Duntze, J., Leblond, V., Bergeron, F., Calvez, V., Katlama, C., Debre, P., and Autran, B. (1998) Int. Immunol. 10; 311-323
Mongini, P. K. A., Blessinger, C. A., and Dalton, J. P. (1991) J. Immunol. 146; 1791-1800
Mongini, P. K. A., Blessinger, C. A., Highet, P. F., and Inman, J. K. (1992) J. Immunol. 148; 3892-3901
Monroe, J. G., and Kass, M. J. (1985) J. Immunol. 135; 1674-1682
Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A., and Coffman, R. L. (1986) J. Immunol. 136; 2348-2357
Murakami, S., Miyake, K., Kincade, P. W., and Hodes, R. (1991) Immunol. Res. 10; 15-27
Murphy, J. J., and Norton, J. D. (1993) Eur. J. Immunol. 23; 2876-2881
Nakamura, M., Burastero, S. E., Notkins, A. L., and Casali, P. (1988) J. Immunol. 141; 4165-4172
Norment, A. M., Salter, R. D., Parham, P., Engelhard, V. H., and Littman, D. R. (1988) Nature 336; 79-81
Nossal, G. J. V. (1994) Cell 76; 229-239
Nossal, G. J. V., Abbot, A., Mitchell, L., and Lummus, Z. (1968) J. Exp. Med. 127; 277-
Ohlin, M. and Borrebaeck, C. A. K. (1998) Insertions and deletions in hypervariable loops of antibody heavy chains contribute to molecular diversity. Mol. Immunol. (in press).
Oliver, A. M., Martin, F., and Kearney, J. F. (1997) J. of Immunol. 158; 1108-1115
Oltvai, Z. N., Milliman, C. L., and Korsmayer, S. J. (1993) Cell 74; 609-619
Orita, M., Suzuki, Y., Sekiya, T., and Hayashi, K. (1989) Genomics 5; 874-879
Parry, S. L., Hasbold, J., Holman, M., and Klaus, G. G. B. (1994) J. Immunol. 152; 2821-2829
Pascual, V., Liu, Y.-J., Magalski, A., de Bouteiller, O., Banchereau, J., and Capra, J. D. (1994) J. Exp. Med. 180; 329-339
Persin, C., Korn, M., Vogel., and Haustein, D. (1994) Mol. Imunol. 31; 503-510
Peters, J. H., Gieseler, R., Thiele, B., and Steinbach, F. (1996) Immunol. Today 273-278
Peters, A., and Storb, U. (1996) Immunity 4; 57-65
Pound, J., Gordon, J. (1997) Blood 89; 919-928
Pulendran, B., Kannourakis, G., Nouri, S., Smith, K. G. C., and Nossal, G. J. V. (1995) Nature 375; 331-334

Rada, C., González-Fernandez, A., Jarvis, J. M., and Milstein, C. (1994) Eur. J. Immunol. 24; 1453-1457
Rafi, A., Nagarkatti, M., and Nagarkatti, P. S. (1997) Blood 89; 2901-2908
Ranheim, E. A., and Kipps, T. J. (1993) J. Exp. Med. 177; 925-936
Rathmell, J. C., Cooke, M. P., Ho, W. Y., Grein, J., Townsend, S. E., Davis, M. .M., and Goodnow, C. C. (1995) Nature 376; 181-184
Razanajaona, D., Denepoux, S., Blanchard, D., de Bouteiller, O., Liu, Y.-J., Banchereau, J., and Lebecque, S. (1997) J. Immunol. 159; 3347-3353
Reth, M., Hämmerling, G. L., and Rajewsky, K. (1978) Eur. J. Immunol. 8; 393-400
Reynaud, C.-A., Garcia, C., Hein, W. R., and Weill, J.-C. (1995) Cell 80; 115-125
Rickert, R. C., Rajewsky, K., and Roes, J. (1995) 376; 352-355
Roes, J., Hüppi, K., Rajewsky, K., andd Sablitzky, F. (1989) J. Immunol. 142; 1022-1026
Rogerson, B. J. (1994) Mol. Immunol. 31; 83-98
Rogozin, I. B., and Kolchanov, N. A. (1992) Biochem. Biophys. Acta. 1171; 11-18
Roitt, I., Brostoff, J., and Male, D. (1989) Immunology, second edition
Roitt, I.M., Torrigiani, G., Greaves, M. F., Brostoff, J., and Playfair, J. H. (1969) Lancet 2; 367-371
Romagnani, S. (1991) Immunol. Today 12; 256-257
Romagnani, S. (1992) Immunol. Today 13; 379-381
Romagnani, S., Del Prete, G., Maggi, E., Chilosi, M., Caligaris-Cappio, F., and Pizzolo, G. (1995) J. Leuc. Biol. 57; 726-730
Romani, N., Koide S., Crowley M., Witmer-Pack M., Livingstone A.M., Fathman C.G., Inaba K., and Steinman R.M. (1989) J. Exp. Med. 169; 1169-1178
Romani, N., Gruner S., Brang D., Kampgen E., Lenz A., Trockenbacher B., Konwalinka G., Fritsch P.O., Steinman R.M., and Schuler G. (1994) J. Exp. Med. 180; 83-93
Romani, N., Reider D, Heuer M, Ebner S, Kampgen E, Eibl B, Niederwieser D, and Schuler G (1996) J. Immunol. Meth. 196; 137-151
Rothenfluh, H. S., Taylor, L., Bothwell, A. L. M., Both, G. W., and Steele, E. J. (1993) 23; 2152-2159
Rudikoff, S., Pawlita, M., Pumphery, J., and Heller, M. (1984) Proc. Natl. Acad. Sci. USA (1984) 81; 2162-2166
Saeland, S., Duvert, V., Moreau, I., and Bancherau, J. (1993) J. Exp. Med. 178; 113-120
Sablitzky, F., Wildner, G., and Rajewsky, K. (1985) EMBO J. 4; 345-350
de Saint-Vis, B., Cupillard, L., Pandrau-Garcia, D., Ho, S., Renard, N., Grouard, G., Duvert, V., Thomas, X.,Galizzi, J. P., Bancherau, J., and Saeland, S. (1995) Blood, 86; 1098-1105
Saizawa, K., Rojo, J., and Janeway, C. A., Jr. (1987) Nature 328; 260-262
Sato, S., Jansen, P. J., and Tedder, T. F. (1997) Proc. Natl. Acad. Sci. USA 94; 13158-13162
Sharon, J., Gefter, M. L., Wysocki, L. J., and Margolies, M. N. (1989) J. Immunol. 142; 596-601
Schubart, D. B., Rolink, A., Kosco-Vilbois, M. H., Botteri, F., and Mathias, P. (1996) Nature 383; 538-541
Sharpe, M. J., Milstein, C., and Jarvis (1991) EMBO J 10; 2139-2145
Shi, Y., Glynn, J. M., Guilbert, L. J., Cotter, T. G., Bissonnette, R. P., and Green, D. R. (1992) Science 257; 212-214
Shokat, K. M., and Goodnow, C. C. (1995) Nature 375; 334-338
Smith, D. S., Creadon, G., Jena, P. K., Portanova, J. P., Kotzin, B. L. and Wysocki, L. J. (1996) J. Immunol. 156; 2642-2652
Smith, K. G. C., Nossal, G. J. V., and Tarlinton, D. M. (1995) Proc. Natl. Acad. USA 92; 11628-11632
Song, H., Nie, X., Basu, S., and Cerny, J. (1998) Immunol. Rev. 162; 211-218.
Stamenkovic, I., Sgori, D., Aruffo, A., Sy, M. S., and Andersson, T. (1991) Cell, 66; 1133-1144
Steinman, R. M., Pack, M., and Inaba, K. (1997) Immunol. Rev. 156; 25-37
Staudert, R., and Günthert, U. (1995) Immunologist 3; 78-83
Swain, S. L. (1983) Immunol. Rev. 74; 129-142
Szaboles, P., Moore, M. A. S., and Young, J. W. (1995) J. Immunol. 154; 5851-5861

Taniguchi, T., Takata, M., Akemi, I., Momotani, E., and Sekikawa, K. (1997) Lab. Invest. 77; 647-658
Takai, T., Ono, M., Hikida, M., Ohmori, H., and Ravetch, J. V. (1996) Nature 379; 346-349
Taylor, L. D., Carmarck, C. E., Huszar, D., Higgins, K. M., Mashayekh, R., Sequar, G., Schramm, S. R., Kuo, C.-C., O'Donnell, S. L., Kay, R. M., Woodhouse, C. S., and Longberg, N. (1994) Int. Immunol. 6; 579-591
Terashima, M., Kim, K.-M., Adachi, T., Nielsen, P. J., Reth, M., Köhler, G., and Lamers, M. C. (1994) EMBO J. 13; 3782-3792
Tew, J. G., Phipps, R. P., and Mandel, T. E. (1980) Immunol. Rev. 53; 175-201
Texidó, G., Jacobs, H., Meiering, M., Kühn, R., Roes, J., Müller, W., Gilfillan, S., Fujiwara, H., Kikutani, H., Yoshida, N., Amakawa, R., Benoist, C., Mathis, D., Kishimoto, T., Mak, T. W., and Rajewsky, K. (1996) Eur. J. Immunol. 26; 1966-1969
Thomas, R., and Lipsky, P. E. (1994) J. Immunol. 153; 4016-4028
Thorbecke, G. J., Amin, A. R., and Tsiagbe, V. K. (1994) FASEB J. 8; 832-840
Toellner, K.-M., Scheel-Toellner, D., Sprenger, R., Duchrow, M., Trümper, L. H., Ernst, M., Flad, H.-D., and Gerdes, J. (1995) 7; 344-354
Togni, P. D., Goellner, J., Rudde, N. H., Streeter, P. R., Fick, A., Mariathasan, S., Smith, S. C., Carlson, R., Shornick, L. P., Strauss-Schoenberger, J., Russell, J. H., Karr, R., and Chaplin, D. D. (1994) Science 264; 703-707
Torres, R. M., Flaswinkel, H., Reth, M., and Rajewsky, K. (1996) Science 272; 1804-1808
Tumas-Brundage, K. M., Vora, K. A., Manser, T. (1997) Mol. Immunol. 34; 367-378
Tumas-Brundage, K., and Manser, T. (1997) J. Exp. Med. 185; 239-250
Tuscano, J. M., Druey, K. M., Riva, A., Pena, J., Thompson, C. B., and Kehrl, J. H. (1996) Blood 4; 1359-1364
van Essen, D., Kikutani, H., and Gray, D. (1995) Nature 620-623
Veillette, A., Bookman, M. A., Horak, E. M., and Bolen, J. B. (1988) Cell 55; 301-308
Vora, K. A., Ravetch, J. V., and Manser, T. (1997) J. Immunol. 159; 2116-2124
Vyth-Dreese, F. A., Dellemijn, T. A. M., Majoor, D., and de Jong, D. (1995) Eur. J. Immunol. 25; 3023-3029
Wagner, S. D., Popov, A. V., Davies, S. L., Xian, J., Neuberger, M. S., and Brüggemann, M. (1994) Eur. J. Immunol. 24; 2672-2681
Wagner, S. D., Milstein, C., and Neuberger, M. S. (1995) Nature 376; 732
Walker, L., Guy, G., Brown, M., Rowe, M., Milner, A. M., and Gordon, J. (1986) Immunology 58; 583-589
Walunas, T.L., Lenschow, D.J., Bakker, C.Y., Linsley, P.S., Freeman, G.J., Green, J.M., Thompson, C.B., and Bluestone, J.A. (1994) Immunity, 1; 405-413
Watanbe, D., Suda, T., and Nagata, S. (1995) Int. Immunol. 7; 1949-1956
Waterhouse, P., Penninger, J.M., Timms, E., Wakeham, A., Shahinian, A., Lee, K.P., Thompson, C.B., Griesser, H., Mak, T.W. (1995) Science 270; 985-988
Wegener, A.-M. K., Letourneur, F., Hoeveler, A., Brocker, T., Luton, F., and Malissen, B. (1992) Cell 68; 83-95
Wen, L., Havanich, M., Werner-Favre, C., Browers, N., Perrin, L. H., and Zubler, R. H. (1987) Eur. J. Immunol. 17; 887-892
Weigert, M., Cesari, I. M., Yankovich, S. I., and Cohn, M. (1970) Nature 228; 1045-1047
Weinstein, P. D., Andersson, A. O., and Mage, R. G. (1994) Immunity 1; 647-659
Weiss, A., and Littman, D. R. (1994) Cell 76; 263-274
Weston, S. A., and Parish., C. R. (1991) J. Immunol. 146; 4180-4186
Weston, S. A., and Parish., C. R. (1992) Eur. J. Immunol. 22; 1975-1981
Wheeler, K., and Gordon, J. (1996) Int. Immunol. 8; 815-828
Winter, D. B., Sattar, N., Mai, J.-J., and Gearhart, P. J. (1997) Mol. Immunol. 34; 359-366
Wilson, H. A., Greenblatt, D., Poeine, M., Finkelman, F. D., and Tsien, R. Y. (1987) J. Exp. Med. 166; 601-606

Wilson, P. C., de Bouteiller, O., Liu, Y.-J., Potter, K., Banchereau, J., Capra, J. D., and Pascual, V. (1998) J. Exp. Med. 187; 59-70
Wilson, M., Hsu, E., Marcuz, A., Courtet, M., Du Pasquier, L., and Steinberg, C. (1992) EMBO J. 11; 4337-4347
Vollenweider, I., Lazzrato, M., and Groscurth, P. (1995) Scand. J. Immunol. 42; 381-386
Wu, H., and Kaartinen, M. (1996) Scand. J. Immunol. 43; 193-210
Wu, H., Pelkonen, E., Knuutila, S., and Kaartinen, M. (1995) Eur. J. Immunol. 25; 3263-3269
Wysocki, L. J., Creadon, G., Lehmann, K. R., and Cambier, J. C. (1992) Immunology 75; 116-121
Wysocki, L., Manser, T., and Gefter, M. L. (1986) Proc. Natl. Acad. Sci. USA 83; 1847-1851
Yang, X., Stedra, J., and Cerny, J. (1996) J. Exp. Med. 183; 959-970
Yelamos, J., Klix, N., Goyenechea, B., Lozano, F., Chui, Y. L., Gonzalez-Fernandez, A., Pannell, R., Neuberger, M. S., and Milstein, C. (1995) Nature 376; 225-229
Yoshida, K., Van den Berg, T. K., and Dijkstra, C. D. (1993) Immunology 80; 34-39
Zhang, J., MacLennan, I. C. M., Liu, Y.-J., and Lane, P. J. L. (1988) Immunol. Lett. 18; 297-299
Zhang, Y.-Q., Joost van Neerven, R. J., Van Gool, S. W., Coorevits, L., de Boer, M., Ceuppens, J. L. (1997) Int. Immunol. 9; 1095-1102
Zhang, X., Hauser, C., and Zubler, R. H. (1990) J. Immunol. 144; 2995-2960
Zheng, B., Xue, W., and Kelsoe, G. (1994) Nature 372; 556-559
Zheng, B., Han, S., and Kelsoe, G. (1996a) J. Exp. Med. 184; 1083-1091
Zheng, B., Han, S., Zhu, Q., Goldsby, R., and Kelsoe, G. (1996b) Nature 384; 263-266
Zubler, R. H., Erard, F., Lees, R. K., von Laer, M., Mingari, C., Moretta, L., and MacDonald, H. R. (1985) J. Immunol. 134; 3662-3668
Zupo, S., Dono, M., Massara, R., Taborelli, G., Chiorazzi, N., and Ferrarini, M. (1994a) Eur. J. Immunol. 24: 1426-1433
Zupo, S., Rugari, E., Dono, M., Taborelli, G., Malavasi, F., and Ferrarini, M. (1994b) Eur. J. Immunol. 24; 1218-1222

9 Paper I-VI