Regulation of Expression of the Interleukin-2 Receptor Alpha Chain (CD25) in Human Tonsillar B Lymphocytes

by

Heather Margaret Pringle

Division of Biochemistry and Molecular Biology,

IBLS,

University of Glasgow,

G12 8QQ.

A thesis submitted in partial fulfillment of requirements for the degree of Doctor of Philosophy, August 1997.

ABSTRACT

The Interleukin-2 Receptor α chain (IL-2Rα or CD25) plays an important role in B cell activation since it allows formation of the high affinity IL-2 Receptor and thus permits the B cell to respond to physiological concentrations of IL-2. IL-4 appears to be the sole cytokine which can induce IL-2Rα in human tonsillar B cells. In addition, polyvalent anti-Immunoglobulin antibodies and anti-CD40 antibodies can cause up-regulation of IL-2Rα (Burlinson *et al* 1995). However, although anti-Ig, anti-CD40 and IL-4 can increase IL-2Rα expression, this elevation in IL-2Rα levels does not necessarily facilitate subsequent B cell proliferation in the presence of IL-2. Of the three, only anti-Ig antibodies, in combination with IL-2, could cause resting B cells to proliferate to a significant level.

Stimulation of human tonsillar B cells with polyvalent anti-Ig antibodies resulted in more than 80% of them acquiring an IL-2Rα-positive phenotype. IL-2Rα expression on different subsets of B cells present within the tonsillar population was studied by stimulating the cells with isotype-specific antibodies; anti-μ, anti-δ or anti-γ antibodies. Low concentrations of anti-μ antibodies up-regulated expression of IL-2Rα, as did all concentrations of anti-γ antibodies. However, high doses of anti-μ antibodies and any dose of anti-δ antibodies failed to up-regulate IL-2Rα. It is possible that the failure of B cells to respond to high doses of anti-μ antibodies or to anti-δ antibodies by increasing

IL-2Rα expression may cause these cells to become tolerised and thus prevent them from causing an auto-immune response.

Expression of IL-2Rα on the cell surface can be related to events taking place at the promoter region of the IL-2Rα gene. There are at least three positive regulatory regions (PRR_I-PRR_{III}) and two negative regulatory regions (NRE I and NRE II) within the promoter region and we chose to study PRR_I and NREI because these regions are believed to be involved in the induction of IL-2Rα. It was found that an, as yet, unidentified protein, NRE-BP, which binds to NRE I plays a major role in controlling IL-2Rα transcription when cells are stimulated via their sIgM receptors. NRE-BP appears to be involved in the silencing of the IL-2Rα gene and remains bound to the promoter when the B cells are stimulated with high concentrations of anti-μ antibodies. At low concentration of anti-μ antibodies, binding of NRE-BP to NRE I is attenuated. Although anti-γ antibodies up-regulate expression of IL-2Rα on the cell surface, anti-γ antibodies have no effect on the binding of NRE-BP to the promoter. Previous studies have discovered differences in signalling through the sIgM and sIgG complexes (Roifman *et al* 1987; Law *et al* 1992; Shu *et al* 1994). Now we have determined that sIgM and sIgG appear to act differently in the regulation of IL-2Rα and that the binding of NRE-BP to NRE I is important in this variation.

ACKNOWLEDGEMENTS

I am grateful to Professor J.G. Lindsay for allowing me to make use of the facilities at the Biochemistry Department, IBLS, University of Glasgow, for this research. In addition, I would like to thank the nurses at the Royal Hospital for Sick Children, Yorkhill for putting up with my constant demands for tonsils and to all the sickly children in Glasgow who donated tissue to this project.

Most of all, I would like to thank my supervisor, Dr Bill Cushley for his enthusiasm, insight and patience. Over the past three years he has been constantly supportive and encouraging and a tremendous amount of help to me. I would also like to thank Dr Ellen Hewitt for all her help and advice. Both Ellen and Bill were always willing to lend me a hand and uncomplainingly attempted to instill some of their encyclopedic knowledge of all things immunological to me. Thanks also go the other members of the lab: Jon, Sandra, Maureen, Stephen and Catriona.

Finally, I would like to thank those who have had to put up with the indirect effects of my erratic research activities. Thanks to my mother and father who supported me both emotionally and financially throughout my time at Glasgow. Thanks to Etuka, Gordon, Steve and Rachel for all the basketball games. In particular, thanks go to Marianne and Reese who supplied me with continuous support, patiently listened to my complaints and, when it all got a wee bit too much, took me to the pub!

CONTENTS IN BRIEF

CONTENTS

CHAPTER 2: MATERIALS AND METHODS

CHAPTER 3: THE INDUCTION OF IL-2Rα EXPRESSION IN RESTING HUMAN B LYMPHOCYTES BY STIMULATION WITH ANTI-IMMUNOGLOBULIN, ANTI-CD40 AND IL-4

CHAPTER 4: DIFFERENT ISOTYPES OF ANTI-IMMUNOGLOBULIN HAVE DIFFERENT EFFECTS ON THE INDUCTION OF IL-2Rα IN RESTING TONSILLAR B CELLS

LIST OF FIGURES

ABBREVIATIONS

Ab	Antibody
Ag	Antigen
AET	2-aminoethylisothiouronium bromide
AgR	Antigen Receptor
anti-Ig	Anti-Immunoglobulin antibody
AP-1	Activator protein 1
APC	Antigen Presenting Cell
ARAM	Antigen Recognition Activation Motif
ARH-1	Antigen Receptor Homology Motif 1
ATP	Adenosine Triphosphate
ATL	Adult T Cell Leukaemia
Bcl-2	B Cell Lymphoma/Leukaemia-2
BCR	B Cell Antigen Receptor Complex
bp	Base Pair
BSA	Bovine Serum Albumin
Ca^{2+}	Calcium
CaM Kinase II	Calmodulin Kinase II
cAMP	Cyclic Adenosine Monophosphate
CAT	Chloramphenicol Acetyltransferase
CD	Cluster of Differentiation

CD23	Low Affinity Receptor for IgE
CD25	IL-2 Receptor α Chain
cDNA	complementary DNA
cpm	counts per minute
CR2	Complement Receptor 2
CREB	cAMP Response Element Binding Protein
DAG	Diacylglycerol
DE	Downstream Element
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EBS	Ets Binding Site
EBV	Epstein-Barr Virus
EDTA	Ethylenediaminetetra-acetic acid
EGTA	[Ethylene-bis(oxyethylenenitrilo)]tetra-acetic acid
EPOR	Erythropoietin Receptor
ER	Endoplasmic Reticulum
FACS	Fluorescence Activated Cell Sorter
FcR	Fc Receptor
FCS	Foetal Calf Serum
FDC	Follicular Dendritic Cells
FITC	Fluorescein Isothiocyanate
g	Force of Gravity

GAP	GTPase Activating Protein
GAS	γ-activated Sequences
γ_c	Common γ Chain
G-CSFR	Granulocyte Colony Stimulating Factor Receptor
GDP	Guanine Diphosphate
GM-CSFR	Granulocyte-macrophage Colony Stimulating Factor Receptor
GNEF	Guanine Nucleotide Exchange Factor
GPI	Glycosyl-phosphatidyl Inositol
H Chain	Heavy Chain
HCl	Hydrochloric Acid
HIV	Human Immunodeficiency Virus
HMG	High Mobility Group Protein
HTLV-I	Type I Human T Cell Leukaemic Virus
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL-4	Interleukin-4
IL-2R	Interleukin-2 Receptor
IL-4RE	Interleukin-4 Response Element
IP_3	Inositol-1,4,5-trisphosphate
ISGF	Interferon Stimulated Gene Factor

ISRE	Interferon Stimulated Response Element
ITAM	Tyrosine-based Activation Motif
J	Joining (e.g. J Chain, J Exon)
JAK	Just Another / Janus Kinase
kDa	Kilodaltons
L	Light Chain
LTR	Long Terminal Repeat
mAb	Monoclonal Antibody
MAPK	Mitogen Activiated Protein Kinase
MHC	Major Histocompatibility Complex
mIg	Membrane Bound Immunoglobulin
mRNA	Messenger Ribonucleic Acid
NF-κB	Nuclear Factor-κB
NP-40	Nonidet-P-40
NRE	Negative Regulatory Region
NRE-BP	Negative Regulatory Region Binding Protein
PAGE	Polyacrylamide Gel Electrophoresis
pBCR	Pre-B Cell Receptor
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PI	Propidium Iodide
PI-3-Kinase	Phosphoinositide-3-Kinase

PIP_2	Phosphatidylinositol-4,5-bisphosphate
PKA	Protein Kinase A
PKC	Protein Kinase C
PLC	Phospholipase C
PMA	Phorbol Myristate Acetate
PMSF	Phenyl Methyl Sulphonyl Fluoride
pp	Polypeptide
PRR	Positive Regulatory Region
PTK	Protein Tyrosine Kinase
r	Recombinant
R	Receptor
RAG	Recombination Activating Genes
RARE	Retinoic Acid Response Element
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SH_2	*src*-Homology 2
sIg	Surface Immunoglobulin
SOS	Son of Sevenless
SP	Silencer Protein
SRBC	Sheep Red Blood Cells
SRE	Serum Response Element
SRF	Serum Response Factor
STAT	Signal Transducer and Activator of Transcription

TCR	T Cell Receptor
TEMED	N, N, N', N'-Tetramethylethylenediamine
TK-CAT	Thymidine Kinase Promoter-CAT Construct
TLC	Thin Layer Chromatography
TNF	Tumour Necrosis Factor
Tris	Tris(hydroxymethyl)methylamine
UE	Upstream Element
V Region	Variable Region
XSCID	X-linked Severe Combined Immunodeficiency

Chapter One

Introduction

1.1 B Cell Development

1.1.1 The Differentiation Pathway of B Lymphocytes.

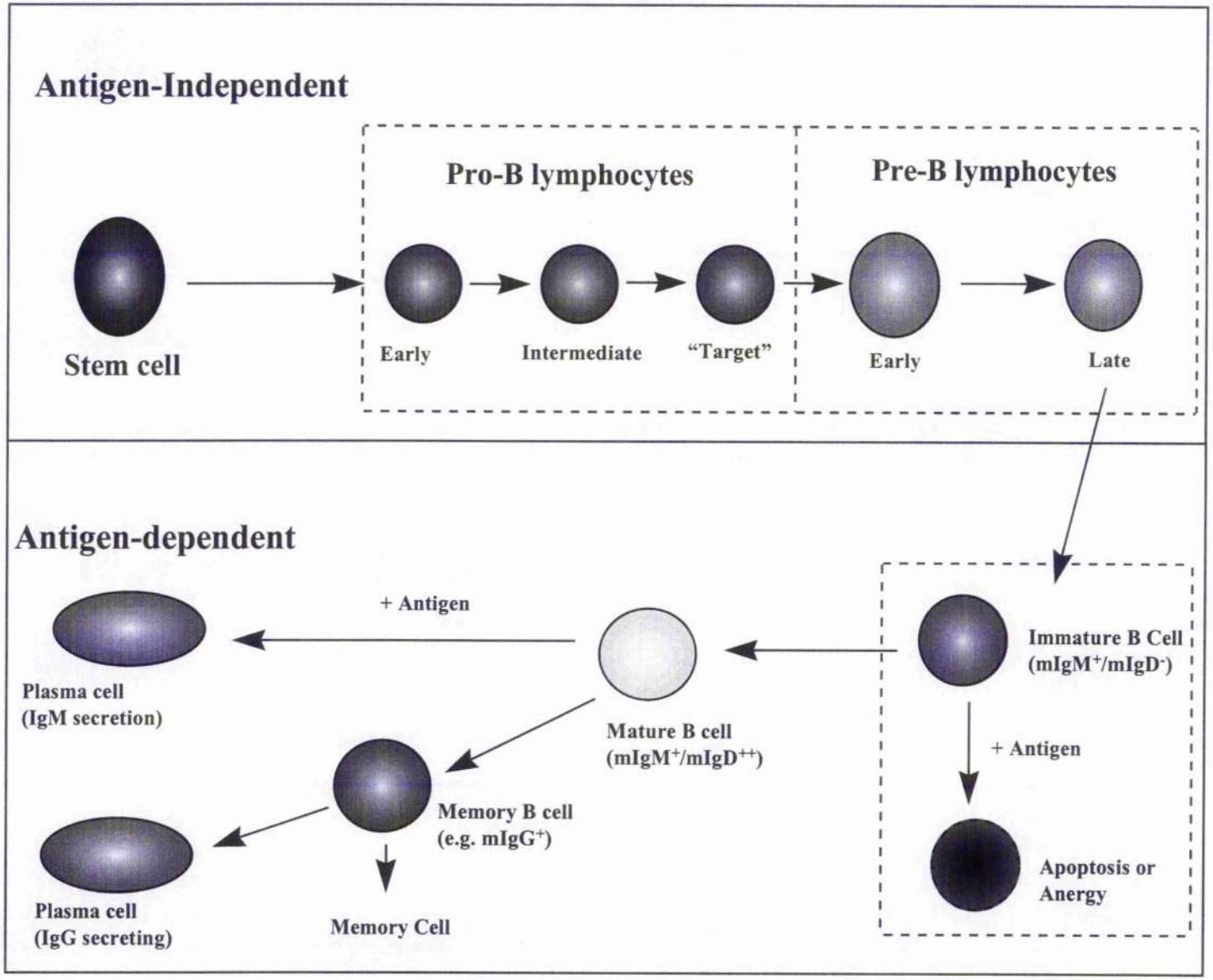
B lymphocytes are derived from the pluripotent stem cells that give rise to the erythroid, myeloid and lymphoid lineages of blood cells (Smith *et al* 1991). During embryogenesis, B cell development occurs in waves, firstly in the placenta and embryonic blood, later in the foetal liver and finally in the spleen and bone marrow (Melchers 1979). Millions of newly formed lymphocytes leave the bone marrow daily (Osmond 1993). Each of these cells either multiplies and differentiates or dies depending on the nature, timing and location of interactions with other cells of the immune system (Liu *et al* 1992; Parker 1993).

B lymphocyte development has traditionally been divided into two phases (Figure 1.1). The first phase is thought to be independent of foreign antigen and takes place in the bone marrow. The first descendent of the pluripotent stem cell that is committed to the B lineage is termed a pro-B cell. These do not express B lineage specific markers and have not yet begun to rearrange the Immunoglobulin (Ig) genes (Davidson *et al* 1984, 1988; Muller-Sieberg *et al* 1986). The next stage is the development of the pre-B cell. These express the pre-B cell Receptor (pBCR) (Reth 1992; Melchers *et al* 1995) which contains the Immunoglobulin (Ig) heavy chains like a mature B cell Receptor but with the light chains replaced by "surrogate" chains, λ_5 and V pre-B (Sakaguchi and Melchers 1986; Kudo and Melchers 1987; Schiff *et al* 1992). The Antigen -Independent phase terminates when Ig is expressed from rearranged Heavy

(H) and Light (L) chain genes on the surface of immature B cells and occurs in the periphery. At this point B cells with Ig specific for self-antigens are either deleted (Nemazee and Burki 1989) or anergised (Goodnow *et al* 1988).

Figure 1.1 The B Cell Development Pathway.

Reproduced from Cushley and Harnett (1993).


Antigen-Independent
Pro-B lymphocytes
Pre-B lymphocytes
Stem cell
Early
Intermediate
"Target"
Early
Late
Antigen-dependent
+ Antigen
Plasma cell
(IgM secretion)
Mature B cell
(mIgM+/mIgD++)
Immature B Cell
(mIgM+/mIgD-)
+ Antigen
Memory B cell
(e.g. mIgG+)
Plasma cell
(IgG secreting)
Memory Cell
Apoptosis or
Anergy

In the second, Antigen-Dependent phase, the remaining surface Ig$^+$ cells can be positively selected in the peripheral circulation and colonise the secondary organs. Depending upon the nature of the antigen encountered, these cells can be stimulated either independently of T cells or with the co-operation of helper T cells (reviewed by Melchers 1989).

The first B cell which has the opportunity to encounter antigen is the mIgM$^+$ "Immature" B cell. This has stable expression of IgM at the B cell surface. Stimulation of the antigen receptor (AgR) on immature B cells results either in anergy or deletion of the clone (Scott *et al* 1987; Goodnow *et al* 1988; Nemazee and Burki 1989; Alez-Martinez *et al* 1991). Thus B cells which produce AgRs which bind to self tissue components are removed, avoiding the secretion of self-reactive antibodies which could lead to auto-immune disease.

The next stage in development is that the immature B cell expresses mIgD. This mIgM$^+$ /mIgD$^+$ B cell is regarded as "mature" and can react positively to activation via the AgR. In the case of thymus-dependent antigen, the response of the B cell is determined not only by antigen but also by T cell-derived cytokines. There are a number of possibilities. Firstly the activated B cell may become a plasma cell; these express no mIg and are solely devoted to the production of the secretory form of IgM. Alternatively, the B cell may become a memory B cell. These cells form a minor subset of B cells and can be distinguished by their somatically mutated antibodies, lack of surface IgD and characteristic surface markers. They are mostly resting, long-lived and recirculating and many have switched isotype from IgM to IgG, IgA or IgE.

Recent evidence suggests that these cells may home preferentially to mucosal tissues (Liu *et al* 1995) where they can rapidly respond to incoming pathogens.

1.1.2 Clonal Selection

The Clonal Selection Theory (Burnet 1959) postulates that B cells express their antibody as a surface receptor and can thus be selected by antigen. Each B lymphocyte expresses a receptor of a single antigen specificity; i.e. antigen receptors are clonally distributed. In mice and humans, different B cells express different receptors at the beginning of their development. This is unlike the situation which occurs in chicken B cell development. Here, all cells initially express the same or similar receptors and diversity is generated through subsequent rounds of gene conversion during proliferation (Reynaud *et al* 1994).

In all vertebrates the genes encoding antibody variable (V) regions are assembled during B cell development from gene segments termed V, D and J (for the *Igh locus*) or κ or λ (for the *Igk* and *Igl loci*) through a process of site-specific recombination. (Tonegawa 1983; Alt *et al* 1987). This involves the introduction of double-strand breaks at specific recognition signal sequences adjacent to the V, D and J elements by the recombination-activating genes RAG-1 and RAG-2 (Oettinger *et al* 1990; McBlane *et al* 1995), followed by double strand repair (Roth *et al* 1995). Numerous V, D and J gene segments are present in both the *Igh* and the two *Igk* and *Igl loci*. Each cell joins a different set of segments so that different cells express different receptors from the beginning. To increase diversity further, there is variation in the

recombination breakpoints and nucleotide deletions and insertions at those points (Alt and Baltimore 1982).

1.1.3 The Antibody Response

Immature B cells expressing conventional surface IgM receptors exit the bone marrow and migrate to the periphery. Here they can become mature (i.e. are positively selected) or undergo apoptosis or anergy. Three-quarters of the peripheral B cell pool are IgM^{+} / IgD^{++} mature B cells, the main cells involved in primary immune responses. The remaining cells are memory cells and B-1 cells (Hayakawa *et al* 1983). B-1 cells possess characteristic cell surface markers such as CD5 and Mac-1 (Hardy *et al* 1994). Early in ontogeny, they dominate the B cell population but later are confined to participation in local gut and lung-associated immune responses. The origin of the cells is controversial; there is evidence that they represent an ancient separate cell lineage (Herzenberg *et al* 1986) and are involved in "natural defence" against common pathogens in the environment (Herzenberg *et al* 1986; Kocks and Rajewsky 1989).

Induction of antibody responses in B-1 and conventional B cells (B-2 cells) requires presentation of antigen in immunogenic form. Multimeric antigens can cause B cells to proliferate and differentiate into antibody secreting plasma cells by efficient cross linking of the antigen receptor (Mond *et al* 1995). Both B-1 and conventional B cells participate in these T-cell-independent responses (Forster and Rajewsky 1987), producing mainly IgM and IgG3 antibodies.

T-cell-dependent responses (Clark and Ledbetter 1994) are elicited by protein antigens which cannot themselves trigger an antibody response. The antigen is first complexed by natural antibodies and components of the complement system and presented to B cells on antigen presenting cells (APC). It is taken up by the B cell through its B cell Receptor Complex (BCR) and processed; i.e. fragmented into peptides inside the cells. These are then presented to T helper cells by class II molecules of the Major Histocompatibility Complex (MHC), triggering the T cell response. T-cell-dependent responses generally involve conventional B cells and it is in these responses that such B cells generate immunological memory and a new antibody repertoire by somatic hypermutation of their antibody genes.

1.1.4 The Germinal Centre Reaction

Affinity maturation and memory generation take place in a specialised microenvironment known as germinal centres (MacLennan 1994). These arise inside follicles composed of naive B cells, on immunisation with T-cell-dependent antigens. There is rapid expansion of an oligoclonal population of antigen-activated B cells in a highly organised environment whose main other components are antigen-specific helper T cells (Fuller *et al* 1993; Zheng *et al* 1994) and Follicular Dendritic Cells (FDC). FDCs are long lived cells of indeterminate origin (Humphrey and Sundaram 1985). They carry antigen complexed to antibodies and components of the complement system on their surface. Antigen can be retained in its native form on FDC's for at least one year. This form of antigen presentation, with signals delivered by the T helper cells is thought to be critical for the selection and maturation of high

affinity memory cells. Interaction of B cells and FDCs leads to the formation of a finely structured germinal centre. Initially, rapid B cell proliferation gives rise to a "dark zone" which is relatively devoid of T cells; these proliferating B cells are known as centroblasts. The maturing B cells then migrate to the "light zone" which is abundant in FDCs with some T cells and become known as centrocytes. Somatic hypermutation is set in motion within the dark zone and high affinity "mutants" are selected within the light zone (Moller 1992). This process occurs only during germinal centre development and at this point there is evidence of massive cell death by apoptosis. B cells with high affinity receptors for antigen are subsequently "selected" and switch from producing IgM to another Ig class.

Unlike naive, resting and memory B cells (Pascual *et al* 1994), proliferating germinal centre cells express low levels of Bcl-2 and are programmed to die unless rescued by signals involving antigen and antigen-specific T cells (MacLennan 1994). Thus cells expressing high affinity antibodies can be positively selected in the microenvironment of the germinal centre. Germinal centre B cells can also be negatively selected by ligands binding to their BCR. They undergo rapid apoptosis on encountering antigen in the absence of T cell help (Linton *et al* 1991; Rathmell *et al* 1995; Pulendran *et al* 1995; Han *et al* 1995). This mechanism may further minimise the risk of autoreactive mutants. Following this selection process, further centrocyte maturation occurs and the cells will either become plasma-blast cell type cells secreting antibody or become long-lived memory cells, ready to perform the functions for which they have been selected.

1.2 B Lymphocyte Antigen Receptor Complex

The B cell antigen Receptor Complex (BCR) consists of a membrane-bound immunoglobulin molecule non-covalently associated with 2 accessory molecules, Ig-α and Ig-β, which occur as disulphide linked heterodimers. The BCR belongs to a class of receptors which includes the T-cell Receptor (TCR) and receptors for the Fc regions of IgE (FcεRI) and IgG (FcRγIIIa) (reviewed by Cambier and Jensen 1993). These receptors possess a complex hetero-oligomeric structure where ligand binding and signal transduction are compartmentalised into distinct receptor subunits. In the BCR, antigen is bound via membrane bound Immunoglobulin (mIg) molecules and the mIg-associated "transducer" structure is comprised of Ig-α and Ig-β (recently designated CD79a and CD79b, respectively).

The mIg molecules are expressed on normal peripheral B cells at 10^4-10^5 copies per cell. They differ from secreted antibody molecules in their heavy (H) chain carboxy terminus which contains spacer, transmembrane and cytoplasmic sequences not found in the secreted molecules (Rogers *et al* 1980; Alt *et al* 1980). All immunoglobulin isotypes can occur as receptors but on the vast majority of peripheral B cells (~90%) only mIgM and mIgD are found (Abney *et al* 1978). In resting high density B cells mIgD molecules are expressed at a 3-10 fold higher level than mIgM molecules (Havran *et al* 1984).

Much effort has been made to define the structure of mIg but the size of the cytoplasmic domains remains unclear. The membrane form of the μ heavy chain (μ_m) was predicted to have 25-26 hydrophobic amino acids, which traverse the bilayer in a helical fashion, and a charged cytoplasmic tail of only 3 residues (Lys-Val-Lys) (Kyte and Doolittle 1982). This small cytoplasmic tail was thought to be present for stable membrane insertion, being too small to express any intrinsic catalytic activity. In contrast, the Klein, Kanehisa and Delisi algorithm (Klein *et al* 1985) predicts that the cytoplasmic tail may consist of 11 amino acids. If correct, these mIg heavy chains have potential sites for interaction with cytoplasmic molecules; however there has been little evidence of this to date and it is generally accepted that the cytoplasmic tail of μm is only three residues long.

1.2.1 The Detection of New Components of the BCR

Since mIg molecules have such limited cytoplasmic structures it was proposed that the BCR must transduce signals via associated molecules. The first indication that the B cell antigen receptor needed accessory molecules came from transfection experiments using myeloma cell lines (Hombach *et al* 1988a, b, 1990). These cell lines do not express mRNA encoding the membrane-bound form of immunoglobulin. A myeloma H chain loss variant cell line, J558L, was transfected with vectors containing the secreted (μs) and membrane (μm) forms of the μ heavy chain and the resultant transfectants analysed. J558Lμs cells produced and secreted large amounts of IgM antibodies whereas the J558Lμm transfectants produced mIgM in the

cytoplasm but not on the cell surface, thus indicating that the cells lack a factor for the surface expression of mIgM (Sitia *et al* 1987; Hombach *et al* 1988a, b). Further studies isolated a spontaneous surface mIgM$^+$ variant (J558Lμm3) of the J558Lμm transfectant. Analysis of these two lines revealed that surface mIgM in J558Lμm3 cells, but not in J558Lμm cells, was associated with a disulphide linked heterodimer. This heterodimer consisted of two glycoproteins with molecular masses of approximately 34kDa and 39kDa and were called Ig-α and Ig-β, respectively.

A different approach to the subject was taken by Campbell and Cambier (1990). They observed that ligation of a small proportion of mIgM or mIgD leads to the inactivation of the signalling ability of the remaining receptors. In other systems such desensitisation is mediated by receptor phosphorylation. However, the cytoplasmic tails of mIgM and mIgD do not contain such potential phosphorylation sites. Thus, an hypothesis was formed that mIg desensitisation is mediated by phosphorylation of its transducer complex. This was investigated by stimulating [^{32}P]-ATP-loaded B cells with anti-IgM, anti-IgD, PMA and Aluminium Fluoride. The cells were lysed and mIg and its associated molecules immunoprecipitated. A complex of three inducibly tyrosine-phosphorylated glycoproteins was co-purified with mIgM. This complex consisted predominantly of a disulphide-linked heterodimer of 32 and 37kDa proteins and, at lower proportion (~10%), a disulphide-linked dimer of 34 and 37kDa proteins. The 32kDa protein was found to be identical to Ig-α and the 37kDa protein was found to be identical to Ig-β.

Subsequently, experiments were performed to identify which genes encoded Ig-α and Ig-β. Amino-terminal sequences were obtained from the Ig-α/Ig-β hetero-dimer expressed in mIgM^{-} J558Lμm3 cells (Hombach *et al* 1990) as well as from pp32, pp34 and pp37 co-purified with mIgM isolated from normal spleen cells (Campbell *et al* 1991). pp32 and Ig-α were found to have an identical amino-terminus to the deduced protein sequence of the *mb-1* gene. pp34, pp37 and Ig-β share an identical amino-terminus with the deduced protein sequence of the B29 gene. Further evidence was obtained from Western blots; antibodies produced against 3 peptides determined from the sequence of *mb-1* reacted specifically with pp32. Similar experiments with B29, pp34 and pp37 provided strong evidence that pp34 and pp37 are products of the B29 gene. Thus, the data indicate that pp32 and Ig-α are identical and are products of the *mb-1* gene. Also, pp37 and Ig-β are identical and are products of the B29 gene. pp34 also has extensive homology to the B29 gene product and has been designated Ig-γ. The difference in molecular weight between pp34 and Ig-β is not a function of phosphorylation or glycosylation (Reth *et al* 1991). Ig-γ is a truncated form of Ig-β lacking the C-terminus. It is expressed on low density splenic B cells and bone marrow cells.

While all classes of sIg associate with apparently identical heterodimers, there are some differences in the glycosylation status of the Ig-α chain. The stoichiometry of the complex formed between sIg and the Ig-α/Ig-β heterodimer is unknown. However, the requirement of Ig-α and Ig-β expression for sIg transport indicates the ratio is at least 1:1 and the bilateral symmetry of sIg would suggest a ratio of 2:1.

Recent studies have investigated the role of mIg in receptor assembly, signal transduction and internalisation. mIg associates with Ig-α and Ig-β via the μm chain CH3 and/or CH4 domains as well as the transmembrane spanning region (Williams *et al* 1990; Hombach *et al* 1990). There is a polar patch (residues TTAST) in the transmembrane region of μm which signals retention of mIgM within the endoplasmic reticulum (ER) unless Ig-α and Ig-β are also expressed (Williams *et al* 1990). This indicates that Ig-α and Ig-β may engage this site, facilitating transport of the receptor through the ER. The C terminus of the μm chain is not necessary for the assembly and transport of the receptor (Parekh *et al* 1992). However, it has been implicated in receptor-mediated signal transduction. Point mutations in the C-terminus of μm do not affect association with Ig-α and Ig-β but do ablate antigen-induced signalling (Pleiman *et al* 1994b). The core of this region (residues YSTTVT within the transmembrane region) was defined as being of particular importance. This sequence is predicted by the Klein, Kanehsisa and Delisi algorithm as being cytoplasmic and therefore is capable of interaction with a cytoplasmic effector. This suggests that the function of mIg extends beyond antigen binding and that the C-terminus of the μm chain could play a critical role in signal transduction.

The two most likely functions for Ig-α and Ig-β are assembly and transport of the B cell antigen receptor complex and signal transduction. As stated previously, transfection studies with the J558L cell line showed that Ig-α was mandatory for surface expression of all Ig isotypes (Sitia *et al* 1987; Hombach *et al* 1988a, b). This is true for mIgM but subsequent experiments have shown that IgD and some subclasses of IgG can be expressed at the cell surface without Ig-α (Venkitaraman *et*

al 1991). There has been much interest in the structure and function of the mIg associated proteins. Both have extracellular domains and are structurally very similar to CD3γ, δ and ε. Each contains the single extracellular Ig-like domain, a single transmembrane region and cytoplasmic tails of 48 (β) and 61 (α) amino acids and are members of the Ig superfamily (Hermanson *et al* 1988; Sakaguchi *et al* 1988). The site of interaction between the Ig-α/Ig-β hetero-dimer and μm is unclear. The transmembrane domains of Ig-α and Ig-β contain a glutamic acid residue and a glutamine residue, respectively. These residues may interact either with each other or with the polar patch motif (TTAST) which is present in the μm chain transmembrane region. As stated earlier, expression of this TTAST motif appears to signal intracellular retention of mIg unless associated with the Ig-α/Ig-β hetero-dimer (Williams *et al* 1990).

Studies have shown that the cytoplasmic tails of Ig-α and Ig-β possess independent signalling functions. They differ quantitatively in their ability to activate specific signalling events; thus signalling events are compartmentalised into different chains. It was recognised initially by Reth (1989) that Ig-α and Ig-β each contain a sequence motif of about 26 amino acids which is common to the cytoplasmic tails of other transducer chains including TCRζ, TCRη, CD3ε, CD3γ, CD3δ, FcεR1β, FcεR1γ, human FcγRIIa and potentially CD22 (LePrince *et al* 1993). This motif has variously been called the Antigen Receptor Homology Motif 1 (ARH-1) (Clark 1993), the Antigen Recognition Activation Motif (ARAM) (Weiss 1993), and the Tyrosine-based Activation Motif (ITAM) (Klausner and Samelson 1991). The motif is characterised by the presence of 6 conserved amino acids in the sequence (where X is an unconserved amino acid):

$$D/E\text{---}X_7\text{ -- }D/E\text{---}X_2\text{ -- }Y\text{---}X_2\text{ -- }L/I\text{---}X_7\text{ -- }Y\text{---}X_2\text{ --}L/I.$$

This motif can activate signal transduction events such as protein tyrosine kinase activation, Ca^{2+} mobilisation and/or IL-2 production (reviewed in Weiss and Littman 1994). Also, the motif exhibits binding activity for enzymes including members of the *src* family of tyrosine kinases (Clark *et al* 1992).

1.2.2 Are There Other Receptor Components?

Ig-α and Ig-β are the most easily recognised proteins associated with mIg. However other receptor components may exist. As noted previously, there is a second gene product, Ig-γ which is similar to Ig-β but with a truncated cytoplasmic tail (Friedrich *et al* 1993). Most of the ARH-1 motif is missing from this molecule, a fact which may have a significant effect on receptor function. This molecule replaces Ig-β on developing B cell such as bone marrow cells.

CD22, a 135kDa B cell restricted glycoprotein, is a possible component of the Antigen Receptor Complex (Clark 1993). Among normal B cells, only those that are CD22 positive can mobilise Ca^{2+} in response to Ig cross linking and mIg-mediated signalling can be enhanced by ligation of CD22 (Pezutto *et al* 1988, 1987). Following cross-linking of mIg CD22 becomes rapidly phosphorylated. CD22 can also be coimmunoprecipitated with mIg under certain circumstances (Schulte *et al* 1992; LePrince *et al* 1993). Finally there are a number of YXXL sequences that are

reminiscent of the ARH-1 motif (Stamenkovic and Seed 1990; Torres *et al* 1992). However, although CD22 obviously plays a role in BCR function, evidence of its association with the complex remains circumstantial.

It has been suggested that CD45, the major lymphocyte membrane protein tyrosine phosphate phosphatase, is a component of the B cell antigen receptor complex. Expression of this surface protein is essential for mIg-mediated signalling leading to calcium mobilisation, $p21^{ras}$ activation and proliferation (Justement *et al* 1991; Kishihara *et al* 1993). CD45 knockout mice do not respond to ligation of the BCR (Kishihara *et al* 1993). *In vitro* CD45 dephosphorylates Ig-α and Ig-β and *in vivo* cross-linking of mIg and CD45 enhances dephosphorylation of Ig-α and Ig-β (Justement *et al* 1991). Finally, extensive cross-linking of CD45 alone causes enhanced phosphorylation of Ig-α and Ig-β (Lin *et al* 1992). However it seems most likely that CD45 is not a part of the BCR complex but is present at a high concentration in the plasma membrane to maintain equilibrium between phosphorylated and non-phosphorylated substrates.

There has been a report by Yellen-Shaw and colleagues of a 56kDa protein associated with mIgM in mature, but not immature B cells (Yellen-Shaw and Monroe 1992). It occurs as a disulphide-linked homodimer, p56-p56, associating with mIgM in a non-covalent manner. It does not appear to associate with mIgD. Other ~50-60kDa species have been recorded, although all seem to vary in some way from p56 described by Yellen-Shaw (Gold *et al* 1991; Petrini *et al* 1983; Koch and Haustein 1983). The function of these proteins is unknown.

1.2.3 Co-Receptors of the BCR.

It is clear that, as with T cells, the response of B cells to antigen will be modified by co-receptor molecules. Three of these molecules are important: the human FcγRIIb, the CD19/CD21 (CR2) complex and CD22. The involvement of these molecules can be negative in some cases and positive in others. As discussed earlier, CD22 may be a component of the BCR complex. However, since CD22 also exhibits ligand binding specificity, its function could also be described as that of a co-receptor. The human FcγRIIb is preferentially expressed on B cells and is a 40kDa member of the Ig superfamily. It has two extracellular Ig-like domains, a single transmembrane spanning region and a 44-76 amino acid cytoplasmic tail (reviewed in Ravetch and Kinet 1991). Experiments have determined that when FcγRIIb is co-ligated in antigen receptor signalling there is premature termination of extracellular calcium influx. This is as a consequence of a termination of IP_3 generation (Bijsterbosch and Klaus 1984) due to the ending of sustained Phospholipase C (PLC) activation (Wilson *et al* 1987). Since PLC is regulated by tyrosine phosphorylation it is likely that the action of FcγRIIb is mediated by an alteration either in protein tyrosine kinase or protein tyrosine phosphatase activity.

Ligation of the CD19/CD21 complex has the opposite effect to that of FcγRIIb ligation. CD21, also known as Complement Receptor 2 (CR2), is a 140kDa protein. The extracellular domain of this protein is made up of 15 short consensus repeats common in complement receptor families (Matsumoto *et al* 1991; Tanner *et al* 1987;

Moore *et al* 1987). There is a single transmembrane region and a 34 amino acid cytoplasmic tail. Among the natural ligands for CD21 are iC3b, C3dg, CD23, IFN-α and the Epstein-Barr virus (EBV) (Bohnsack and Cooper 1988; Dosch *et al* 1990; Aubry *et al* 1992; Delcayre *et al* 1991).CD21 exists as part of an oligomeric complex made up of CD19 which is a 95kDa glycoprotein member of the Ig superfamily, Leu-13, which is a 16kDa cell surface protein and TAPA-1, a 20kDa serpentine molecule containing 4 transmembrane regions (Bohnsack and Cooper 1988; Andria *et al* 1991; Bradbury *et al* 1992; Zhou *et al* 1991). Both Leu-13 and TAPA-1 are expressed ubiquitously whereas CD19 and CD21 are found on B cells only (Moore *et al* 1987; Tedder *et al* 1983; Oren *et al* 1990; Jaffe *et al* 1989; Chen *et al* 1984; Evans *et al* 1990). Involvement of this co-receptor complex in BCR-mediated signalling causes enhancement in B cell activation (Carter *et al* 1991; Carter and Fearon 1992) and the mechanism by which the co-receptor signals is well understood. TAPA-1 and CD19 have extended cytoplasmic structures and are proposed to be the signal transducing subunits (Matsumoto *et al* 1991). CD19, which binds to the tyrosine kinase *lyn*, is strongly phosphorylated on BCR cross linking (van Noesel *et al* 1993; Tuveson *et al* 1993). There are six tyrosine residues flanked by acidic amino acids in the cytoplasmic tail of CD19; it is likely that these are the tyrosine kinase substrate sites. These sequences, if phosphorylated, are consensus binding sites for SH2 domain containing cytoplasmic effectors. Antigen receptor ligation phosphorylates these sites leading to binding of Phosphoinositide-3-Kinase (PI-3-Kinase) (Songyang *et al* 1993). Previous studies suggest that binding of phosphorylated CD19 by PI-3-Kinase may lead to enzyme activation (Shoelson *et al* 1993; Myers *et al* 1992). CD19's cytoplasmic tail is necessary for CD19 enhancement of antigen receptor-mediated

calcium mobilisation. However, some CD19 signalling is independent of its cytoplasmic tail. For example, homotypic adhesion and growth inhibition are dependent on the transmembrane domain which determines the interaction with TAPA-1(Bradbury *et al* 1993). As stated previously, CD19 is of particular importance in antigen receptor-mediated signalling because coligation of CD19 and BCR reduces by 2 orders of magnitude the numbers of antigen receptors that must be ligated to induce B cell proliferation (Carter and Fearon 1992). Thus, antigen in the form of complexes containing complement components may hyperstimulate B cells by virtue of mIg-CR2 cross-linking.

1.2.4 BCR Mediated Signal Transduction

The earliest detectable event following cross-linking of the BCR is an increase in protein tyrosine phosphorylation. This is due to the activation of multiple protein tyrosine kinases. These include members of the *src* family including blk, fyn, lyn and lck (Burkhardt *et al* 1991; Yamanishi *et al* 1992) and the more distantly related syk (previously denoted Ptk72) (Hutcheroft *et al* 1991, 1992; Taniguchi *et al* 1991; Yamada *et al* 1993). The *src*-family kinases interact with the resting BCR primarily via the Ig-α chain (Clark *et al* 1992) which associates with the first 10 N-terminal residues of the kinase (Pleiman *et al* 1994a). The difference in the abilities of Ig-α and Ig-β to bind the *src*-family kinases is due to a sequence of 4 amino acids, DCSM, within the ARH-1 motif of Ig-α (the equivalent sequence in Ig-β is QTAT) (Clark *et al* 1994). Exchanging these sequences can switch the binding ability of Ig-α and Ig-β.

Therefore DCSM seems to determine the ARH-1-mediated binding of *src*-family kinases to the resting receptor.

Immediately following BCR ligation the Ig-α and Ig-β ARH-1 motifs are phosphorylated on tyrosine residues in the amino acid sequences YEGLN and YEDI. On phosphorylation of these residues, there is an approximately 20 fold increase in binding of *src*-family kinases which triggers kinase activation (Clark *et al* 1994). The enhanced association is due to the binding of *src*-family kinase SH2 domain regions to the phosphorylated tyrosine residues in the ARH-1 motif and the concomitant loss of binding to the 10 N-terminal residues (Pleiman *et al* 1994a). Thus, there is apparently a spatial reorientation of the receptor-associated *src*-family kinases so that binding of the kinases changes from N-terminal mediated to SH2 domain mediated.

The protein tyrosine kinase syk is also associated with the resting receptor complex and is immediately phosphorylated following receptor ligation. Originally it was thought that syk associated with sIgM (Hutchcroft *et al* 1992). However, syk binds strongly to phosphorylated Ig-α and Ig-β ARH-1 motif peptides. Recent findings showed that a phosphorylated Y-X-X-L/I sequence is the predicted binding site for the syk SH2 domains (Songyang *et al* 1994) and that the related T cell kinase ZAP-70 binds to the corresponding tyrosine-phosphorylated ARH-1 motifs in the cytoplasmic tails of the TCR complex (Wange *et al* 1993). Therefore syk may be involved in BCR signal transduction at 2 levels; firstly, it binds to the resting receptor via sIg transmembrane/cytoplasmic regions and, secondly, it binds via its SH2 domains to phosphorylated Ig-α and Ig-β and becomes activated.

Studies on the phosphorylation of cellular substrates (Yamanishi *et al* 1992; Hutchcroft *et al* 1991,1992; Brunswick *et al* 1991; Campbell and Sefton 1990,1992, Gold *et al* 1990) and *in vitro* assays on the activity of kinases (Burkhardt *et al* 1991; Yamanishi *et al* 1992; Yamada *et al* 1993) have shown that maximal kinase activity is achieved 15-60 seconds after BCR stimulation. Experiments using kinase inhibitors indicate that all downstream signalling events and the subsequent biological responses are dependent on tyrosine kinase activity (Cambier *et al* 1991; Padeh *et al* 1991; Pure and Tardelli 1992). Although the mechanism by which the BCR signals is finally emerging, it is unclear whether the multiple activated tyrosine kinases act in parallel or as a cascade. It remains uncertain as to how the initial ARH-1 motif phosphorylation is triggered. Initially Hutchcroft *et al* (1991) implicated syk as the first kinase to be activated following ligation of the BCR. Several other lines of evidence have supported this hypothesis. Firstly, in T cells, on cross linking chimeric receptors containing syk cytoplasmic domains but not lck or ZAP-70, there is inducible tyrosine phosphorylation and calcium mobilisation (Kolanus *et al* 1993). In addition, syk associates with sIg in the absence of Ig-α and Ig-β (Hutchcroft *et al* 1992) and a B cell lymphoma with a knockout syk gene results in a virtual cessation of BCR signalling (Takata *et al* 1994). *src*-family kinases have been found to bind phosphorylated syk through their SH-2 domains. This is an event that requires syk to be activated (Cambier *et al* 1994). Finally, syk is activated by TCR ligation of the lck deficient J.CaM1.6 cell line. This occurs in the absence of *src*-family or ZAP-70 kinase activation (Couture *et al* 1994).

However, there is also evidence that *src*-family kinases are phosphorylated prior to syk (Kurosaki *et al* 1994). This would be consistent with studies on the TCR (Chan *et al* 1991, 1992a,b) which showed that the syk-related protein, ZAP-70, bound to TCRζ only after phosphorylation by lck. Thus the identity of the kinases initiating BCR signalling and the mechanisms by which they are activated are unclear.

The most interesting feature of BCR signalling is the number of tyrosine kinases utilised. Recent evidence suggests that different kinases can bind to distinct downstream effectors (Pleiman *et al* 1993) and have different substrate peptide preferences (Goldman *et al* 1994). This raises the possibility that these kinases activate different downstream pathways. After the initial activation of protein tyrosine kinases the signalling pathway diverges into at least three biochemical cascades. The best known of these is the activation of the phosphatidylinositol-4,5,-bisphosphate (PIP_2) specific phospholipase C (PLC) pathway by tyrosine phosphorylation (Coggeshall and Cambier 1984; Coggeshall *et al* 1992; Carter *et al* 1991; Bijsterbosch and Klaus 1985). Although PLCγ1 is present, PLCγ2 is the predominant isoform in B cells (Coggeshall *et al* 1992). PLCγ2 activation leads to the hydrolysis of phospholipid producing Inositol-1,4.5-trisphosphate (IP_3) and Diacylglycerol (DAG) (reviewed in Nishizuki 1992). These mediate the activation of various isoforms of protein kinase C (PKC); the PKC isoforms expressed in B cells are α, β, δ, ζ and η (Mischak *et al* 1991) and of these α, β and δ are DAG regulated. IP_3 mediates calcium influx and the release of calcium from stores in the Endoplasmic Reticulum is caused by IP_3 binding to receptors on the plasma membrane and on the ER membrane (Khan *et al* 1992). The mobilisation of calcium also activates Calcium Calmodulin kinase II.

The biological effects of this are unknown. However nuclear CaM kinase II has been shown to phosphorylate the ets-1 DNA binding protein (Valentine *et al* 1995; Fisher *et al* 1991), perhaps altering its transcription regulating activity. PKC modifies the transcriptional activity of the AP-1 complex, possibly by activating an intermediary phosphatase (Boyle *et al* 1991).

The second tyrosine kinase regulated signalling pathway is activated by many receptors, including the BCR, and involves the $p21^{ras}$ oncoprotein (ras) (Harwood and Cambier 1993; Lazarus *et al* 1993; Downward 1992). Ras is a key mediator of tyrosine kinase action and a potent regulator of cell growth and differentiation. Many of the effects of ras appear to be mediated by a protein kinase cascade controlled by ras. Activated ras associates with the Raf-1 serine/threonine kinase (Koide *et al* 1993) and is required for the activation of Raf-1 *in vivo* (Cook and McCormick 1993). In turn Raf-1 phosphorylates and activates MEK (Kyriakis *et al* 1993) which phosphorylates and activates the mitogen activated protein (MAP) kinases (Davis 1993). These may regulate gene expression since they can phosphorylate a number of transcription factors *in vitro* including c-*jun*, c-*fos* and c-*myc*. Ras is a membrane-associated guanine nucleotide-binding protein that exists in an active GTP-bound state and in an inactive GDP-bound state. In B cells ras is regulated by GTPase-activating protein (GAP) which stimulates the GTPase activity of ras favouring the inactive GDP-bound form of ras (Gold *et al* 1992). In contrast, guanine nucleotide exchange factors (GNEFs) stimulate the release of GDP from ras allowing GTP to bind. In B cells ras appears to be regulated primarily by the GNEF, vav (Bustelo *et al* 1992; Gulbins *et al* 1993). Vav is tyrosine phosphorylated and presumably activated

following BCR cross-linking (Bustelo and Barbacid 1992). Several GNEFs have been described in mammalian systems. These include homologues of the *Drosophila* "son of sevenless" protein (mSOS1 and mSOS2 in mouse) (Bowtell *et al* 1992). Cellular ras activity reflects a balance between GNEF activity and GAP activity. mSOS-1 translocates from the cytosol to the membrane where ras is located (Buday and Downward 1993). This is mediated by the adaptor protein Grb-2 which binds mSOS-1 by means of its *src*-homology 3 (SH3) domains (Rozakis-Adcock *et al* 1993). In addition, products of the *shc* gene, $p46^{shc}$ and $p52^{shc}$, have been implicated as upstream regulators of ras. The Grb-2/mSOS-1 complex binds to tyrosine phosphorylated shc via the SH2 domain of Grb-2 (Rozakis-Adcock *et al* 1992). Recent studies have shown that ligation of the BCR stimulates phosphorylation of shc and mSOS-1 (Saxton *et al* 1994). This correlated with the formation of complexes containing shc, mSOS-1, Grb-2 and an unidentified 145kDa tyrosine phosphorylated protein.

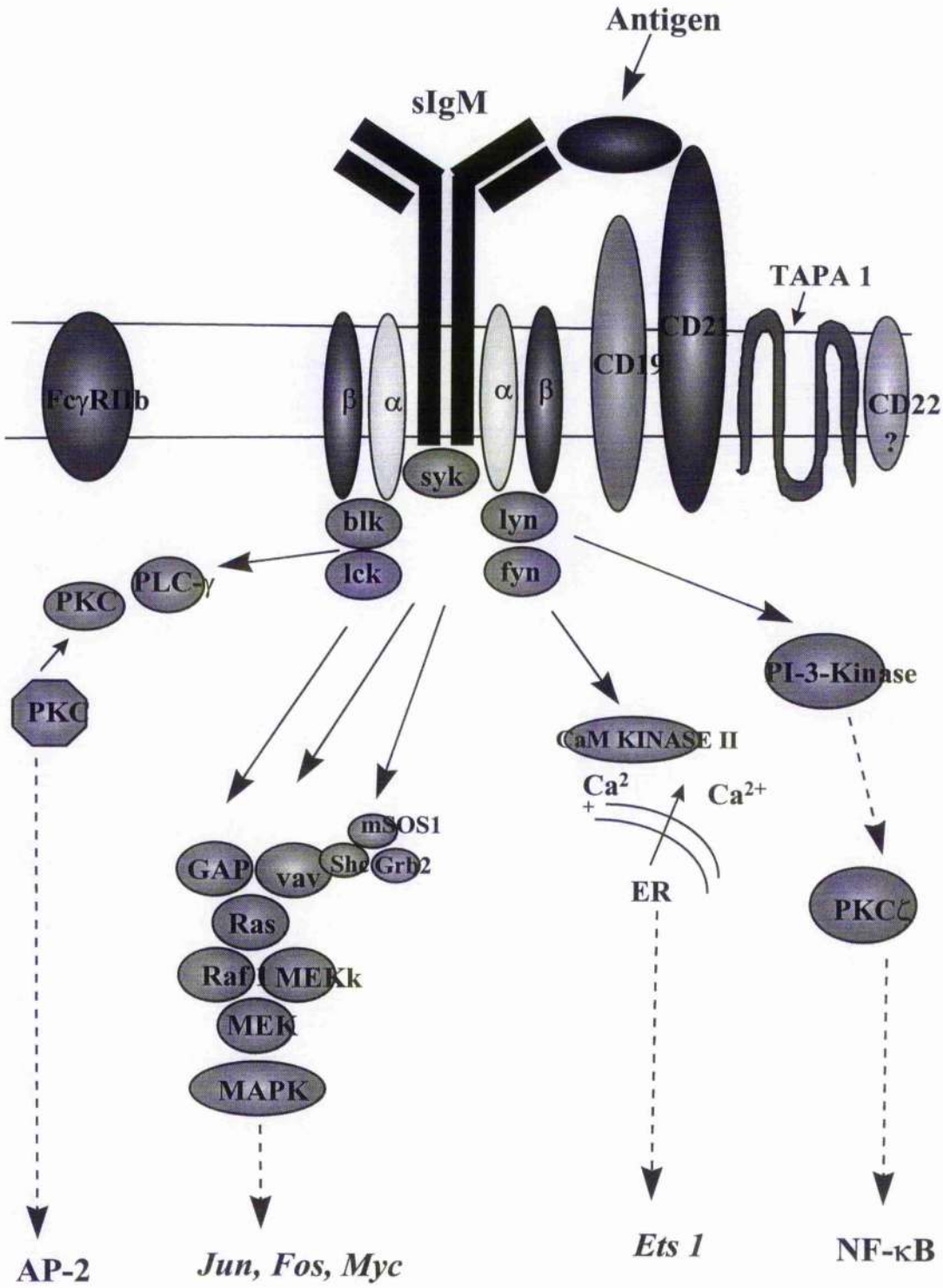
The third cascade activated following BCR cross-linking involves the activation of PI-3-kinase (Burkhardt *et al* 1991; Gold *et al* 1992; reviewed in Cantley *et al* 1991). This produces inositol lipids which activate a unique PKC isoform PKCζ (Nakanishi *et al* 1993). The role of *src*-family kinases in the regulation of downstream signalling is indicated by the discovery that BCR activation leads to kinase association with effectors including PLC-γ-2, GAP, MAPK and PI-3-kinase (Burkhardt *et al* 1991; Pleiman *et al* 1993). PLC-γ-2, GAP and MAPK associate with the N-terminal 10 residues of the *src*-family kinases. However PI-3-kinase binding occurs through an interaction with the SH3 domain of the kinases lyn and fyn but not blk (Pleiman *et al* 1993). The region within PI-3-kinase responsible for this binding is a proline rich area

found in the regulatory p85 subunit (Pleiman *et al* 1994c). Since BCR mediated tyrosine phosphorylation has not been shown, it may be that the binding of the *src*-family kinase SH3 domain regulates PI-3-kinase directly.

In summary, a model of the immediate events in BCR mediated signal transduction was proposed (Cambier *et al* 1994) and **Figure 1.2** indicates the early and late signalling events occurring when the BCR is stimulated with antigen. Stimulation of the BCR leads to the activation of a tyrosine kinase; candidates for this may be found in the resting receptor complex. The first substrates to be phosphorylated are the ARH-1 motif tyrosines of Ig-α and Ig-β. It was hypothesised that phosphorylation of these motifs leads to the reorientation of associated *src*-family kinases so that SH2 domains may now bind. Binding of these SH2 domains to the tyrosine phosphorylated ARH-1 motifs activates the *src*-family kinases. The reorientation of the kinases may allow focusing of effectors such as GAP, PLC-γ-2 or MAPK via binding to the kinase's newly available unique region. The activity of these effectors can be mediated by subsequent phosphorylation. Reorientation of the Ig-α/*src* family kinase complex leads to SH3 domain binding to PI-3-kinase and thus its activation. In addition, phosphorylation of Ig-α and Ig-β may also lead to syk binding to the ARH-1 motifs and to syk activation. Precisely how coreceptors like CD22, FcγRII and the CD21/CD19/TAPA-1 complex may modify BCR signalling remains unclear.

Figure 1.2 Model of Signal Transduction through the B Cell Receptor Complex (BCR)

The B Cell Antigen Receptor is a complex of antigen-binding sIg with the membrane-spanning signal transducing heterodimers Ig-α/Ig-β. Signal transduction by the BCR is triggered by receptor cross-linking and depends upon an ARH-1 in Ig-α and Ig-β which transmits signals from the BCR to the illustrated signalling components. These act in concert with the cytoplasmic and membrane molecules shown.


Antigen
sIgM
TAPA 1
FcγRIIb
β
α
α
β
CD19
CD21
CD22
?
syk
blk
lck
lyn
fyn
PLC-γ
PKC
PKC
PI-3-Kinase
CaM KINASE II
Ca2+
Ca2+
ER
mSOS1
GAP
vav
Shc
Grb2
Ras
Raf 1
MEKk
MEK
MAPK
PKCζ
AP-2
Jun, Fos, Myc
Ets 1
NF-κB

1.3 The Interleukin-2 Receptor

B lymphocyte growth and activation are regulated by signals transmitted after the binding of antigen to sIg and of cytokines to their specific receptors. Cytokines are a set of small proteins whose expression, secretion, or both, is induced as a result of antigen-stimulated cellular activation. These proteins act by binding to high affinity receptors expressed on target cells and by inducing biochemical signals within those cells that profoundly affect their behaviour. The binding of antigen to the BCR is the initial event in B lymphocyte activation. The second signal that has been most thoroughly investigated is that provided by contact with helper T cells and the cytokines secreted by these cells.

One such cytokine, Interleukin-2 (IL-2), has pleiotropic effects on lymphocytes, including T, B and Natural Killer cells (Smith 1988a;b; Taniguchi *et al* 1986; Robertson and Ritz 1992) as well as other hematopoietic cells (Benveniste and Merrill 1984; Malkovsky *et al* 1987; Espinoza-Delgado *et al* 1990, Djeu *et al* 1993). IL-2 was found to be a potent proliferation and differentiation-inducing agent of B lymphocytes after cross-linking of their sIgs (Nakagawa *et al* 1985; Defrance *et al* 1988). The effects of IL-2 on these various cells are mediated through cell surface receptors. Over the past few years, our understanding of the IL-2 Receptor complex (IL-2R) has increased substantially. It is now known that the IL-2R comprises at least three subunits, the α, β and γ chains, encoded by distinct genes. These subunits can be expressed individually or in various combinations resulting in receptors that bind IL-2 with very different affinities. The IL-2R comes in three different forms; the high

affinity IL-2R (Kd = 10^{-11}M) contains the three distinct subunits, the intermediate affinity receptor (Kd = 10^{-9}M) is made up of the β and γ chains and the low affinity receptor (Kd = 10^{-8}M) consists of the α chain alone (Smith 1989).

1.3.1 The IL-2 Receptor α chain (IL-2Rα)

The first IL-2R component to be identified was the α chain, (variously known as CD25, Tac antigen or IL-2Rα). It is a 55kDa membrane glycoprotein (p55) capable of binding IL-2 (Leonard *et al* 1983; Robb and Greene 1983). The deduced amino acid sequence of the human IL-2Rα predicts a mature protein of 251 amino acids with a signal peptide of 21 amino acids in length (Leonard *et al* 1984; Nikaido *et al* 1984; Cosman *et al* 1984). The receptor has a short (13 amino acids) cytoplasmic domain that is positively charged and presumably serves an anchoring function. The protein contains a hydrophobic 19 residue membrane spanning region and the majority of the protein (219 amino acids) is extracytoplasmic. There are two N-linked carbohydrate addition sites and multiple O-linked carbohydrate addition sites.

The α chain is a non-signalling subunit and was thought to be the only cytokine member of a large family of binding proteins whose members include the complement receptor proteins (Davie *et al* 1986; Perkins *et al* 1988). However, Giri *et al* (1995) recently identified and cloned a novel IL-15 binding protein that is structurally related to the α chain of the IL-2R. Together, the α chains of the IL-2 and IL-15Rs define a new cytokine receptor family. Both proteins contain a short consensus repeat called a "sushi domain". Proteins containing this motif, such as Cls, Clr C4BP, Factor XIII,

Factor B and Factor H, are proteins that bind to other proteins. In these proteins, the sushi domains are often found repeated multiple times; complement factor H, for example, consists entirely of 20 sushi domains (Perkins *et al* 1988). The IL-2Rα contains two sushi domains; several residues in the first sushi domain have been identified as being directly involved in IL-2 binding (Moreau *et al* 1987; Robb *et al* 1988). Therefore, the α chains of the IL-2R and IL-15R define a new family of binding proteins for helical cytokines. It remains to be discovered if this family of molecules plays a role in other cytokine receptors.

IL-2Rα has been found to be expressed on $CD4^-$ $CD8^-$(double negative) thymocytes and activated T and B cells (Uchiyama *et al* 1981; Ceredig *et al* 1985; Lowenthal *et al* 1986; Meuer *et al* 1983; Waldmann *et al* 1984) and has been implicated in thymocyte differentiation (Nishi *et al* 1988; Tentori *et al* 1988, Zuniga-Pflucker and Kruisbeek 1990; Carding *et al* 1991) and lymphocyte proliferation (Waldman 1989; Minami *et al* 1993; Taniguchi and Minami 1993). IL-2Rα is also expressed on pre-B and immature B cells in the bone marrow (Chen *et al* 1994) suggesting a potential role for the IL-2R in early B cell differentiation. IL-2Rα is not constitutively expressed in resting T and B lymphocytes and is upregulated by distinct groups of cytokines in both T and B lymphocytes (Greene *et al* 1989, Butcher *et al* 1990; Butcher and Cushley 1991; Tomizawa *et al* 1991; Zola *et al* 1991; Clipstone and Crabtree 1994). As IL-2Rα has no overt signal transducing capacity its main role appears to be in the generation of a specific high affinity receptor for IL-2.

1.3.2 The IL-2 Receptor β chain (IL-2Rβ)

A second component of the IL-2R was subsequently identified (Sharon *et al* 1986; Tsudo *et al* 1986; Teshigawara *et al* 1987; Dukovich *et al* 1987) and was denoted the β chain. IL-2Rβ is a 75kDa protein that is constitutively expressed on lymphocytes. IL-2Rβ cDNA encodes a protein consisting of 551 amino acids (Hatakeyama *et al* 1989). The NH_2 terminal 265 amino acids apparently comprise the signal sequence, leaving 525 amino acids to make up the mature form of IL-2Rβ. Within this, regions of 214, 25 and 286 amino acids in length comprise the extracellular, membrane-spanning and cytoplasmic regions, respectively. Several unique amino acid motifs are found in the extracellular region of IL-2Rβ; their resemblance to sequences found in other cytokine receptors is discussed later. The cytoplasmic region of IL-2Rβ is larger than that of IL-2Rα but does not contain any apparent catalytic motifs such as kinase consensus sequences (Hanks *et al* 1988). It can be divided into 3 sub-regions based upon their amino acid compositions. These have been designated as the "serine rich" region, the "acidic" region and the " proline rich" region.

1.3.3 The IL-2 Receptor γ chain (IL-2Rγ)

The third and most recently identified IL-2R component, IL-2Rγ, is a 64kDa protein that also has structural homology to these cytokine receptors (Takeshita *et al* 1992; Kamio *et al* 1992). IL-2Rγ has a very low affinity for IL-2 by itself (Voss *et al* 1993). However, when expressed with IL-2Rβ, these two chains form an intermediate

affinity receptor for IL-2. In addition, the β and γ chains of the IL-2R are utilised by the IL-15R (Giri *et al* 1994). Moreover, the γ chain has been shown recently to be shared by receptors for several other cytokines (IL-4, IL-7, IL-9) (Kondo *et al* 1993,1994; Noguchi *et al* 1993a, Russell *et al* 1993, 1994) and is thus designated the common γ chain, γc.

A central role for the γ chain is supported by the fact that X-linked severe combined immunodeficiency (XSCID) occurs as a direct result of γ chain mutations (Noguchi *et al* 1993b; Voss *et al* 1994). Patients with XSCID were shown to have mutations in their IL-2Rγ gene resulting in premature termination of the protein. XSCID is a rare congenital disease in man and is characterised by a marked decrease in circulating T cells while B cells are present but poorly functional (Conley 1992). Other haematopoietic cell lineages appear unaffected. Therefore the IL-2Rγ chain plays a critical role in immune system functioning.

The protein structure of IL-2Rγ has been deduced; the NH_2 terminal 22 amino acids comprise the signal sequence with the remaining 347 amino acids making up the mature form of the protein (Takeshita *et al* 1992). Within this chain, regions of 232, 29 and 86 amino acids constitute the extracellular, membrane-spanning and cytoplasmic regions, respectively. The cytoplasmic region of IL-2Rγ is considerably shorter than that of IL-2Rβ; it contains a sequence that appears homologous to an SH2 domain but with no apparent catalytic motifs. Both IL-2Rβ and γc chains appear to participate in signal transduction (Arima *et al* 1992; Kishimoto *et al* 1994).

1.3.4 The Cytokine Receptor Superfamily

Both IL-2Rβ and γc are members of the cytokine receptor superfamily (Bazan 1990a). This is a large family of receptors for cytokines which regulate the immune and haematopoietic system. It is characterised by an extracellular 200 amino acid region of structural homology. This haematopoietic cytokine receptor family includes such receptors as IL-3R, IL-4R, IL-5R, IL-6R, IL-7R, IL-9R, IL-15R, the erythropoietin receptor (EPOR), G-CSFR, GM-CSFR and LIFR, in addition to the IL-2R (β and γ chains) (Bazan 1990a; Miyajima *et al* 1992; Taga and Kishimoto 1992). The family also includes receptor proteins for factors which normally function outside the immune system , for example, growth hormone and prolactin (Bazan 1990a, Davis *et al* 1991). The homologous region common to all members of this family is characterised by four conserved cysteine residues in the NH_2 terminal half of this region and a Trp-Ser-X-Trp-Ser motif (where X is a non-conserved amino acid) at its carboxy-terminal end. The region is composed of 2 fibronectin type III molecules (Patthy 1990). Each consists of about 100 amino acids and contains 7 β strands folded to form a barrel-like structure located within the hinge region connecting the two type III modules and this is predicted to function as the ligand interaction site (Bazan 1990a). Concurrent with finding structural similarities within this receptor superfamily, the respective ligands appear to possess a common architecture consisting of four α helices interconnected by peptide loops (Bazan 1990b).

1.3.5 IL-2 Receptor-Mediated Signal Transduction

IL-2Rβ plays a critical role in transducing mitotic signal(s) in haematopoietic cell lines. From studies with lymphoid lineage BAF-B03 cells expressing mutant IL-2Rβ proteins with deletions in the intracytoplasmic domain, it was shown that the cytoplasmic domain of IL-2Rβ harbours two functional domains. One is the membrane-proximal serine-rich region which is important in the transduction of the IL-2 induced proliferative signals and the other is a more membrane-distal acidic region with which tyrosine kinases are physically associated (Shibuya *et al* 1992). It should be noted that the serine -rich region contains a conserved stretch of hydrophobic amino acids. Replacement of leucine in this stretch with a proline may induce a conformational change in IL-2Rβ because the resultant receptor is no longer capable of transmitting the IL-2-induced mitotic signal (Mori *et al* 1991).

1.3.6. Signalling by the Cytokine Receptor Superfamily: JAKs

Tyrosine phosphorylation of cellular substrates is believed to play a crucial role in the intracellular signal transduction pathways that regulate cellular activation and differentiation (Hunter and Cooper 1985, 1986). Recently, a number of studies showed that cytokine receptors associate with and activate members of the JAK family of protein kinases. JAK was originally an acronym for "just another kinase"; it has also been proposed as an acronym for Janus kinase, Janus being the Roman god of gates and doorways who is depicted with two faces looking in opposite directions.

This refers to the structure of members of the JAK family whose most striking feature is the presence of two kinase domains. The domain proximal to the carboxy-terminal contains all the consensus sequences associated with tyrosine kinases (Hanks *et al* 1988). Immediately amino-terminal to this is a second kinase-like domain. This lacks several residues essential for kinase activity in other kinases and the function of this domain is, as yet , undetermined. Interestingly, there is a lack of readily detectable SH2 or SH3 domains in the sequences amino terminal to the kinase domains. There are at least four JAK family members with identifiable blocks of homology between them (Pawson and Gish 1992). These homologous regions may define the domains responsible for the association of JAKs with members of the cytokine receptor superfamily.

Various studies defined the importance of the JAK family in signalling through cytokine receptors (Reviewed in Ihle *et al* 1993). These studies linked JAKs with specific cytokine receptors. In the case of the IL-2R, IL-2 induces the tyrosine phosphorylation of JAK1 and JAK3. (Kawamura *et al* 1994; Witthuhn *et al* 1994; Johnston *et al* 1994). In fact, JAK1 associates with IL-2Rβ and JAK3 associates with IL-2Rγ (Russell *et al* 1994). This differential association of JAK1 and JAK3 with IL-2Rβ and γc is required to transduce an IL-2 signal. Russell *et al* (1994) also predicted that defective γc-JAK3 association would be found in many XSCID patients. In addition, the activation of JAK3 by all γ_c cytokine receptors is logical since γc physically associates with JAK3. However, the activation of JAK1 by IL-2, IL-4, IL-7 and IL-9 suggests that IL-4R, IL-7R and IL-9R, like IL-2R, all associate with JAK-1. Because the IL-15R also contains β and γc (Giri *et al* 1994), it seems reasonable to

assume that IL-15 will also activate JAK1 and JAK3. Further study determined that JAK1 and JAK3 were selectively associated with the serine-rich region of IL-2Rβ and the carboxy-terminal of γc, respectively. Both regions were essential for IL-2 signalling. However, it is clear that the different signals induced by different cytokines whose receptors contain γc cannot solely be explained by the involvement of JAK1 and JAK3.

These findings show that IL-2 signalling is of a complex nature. In addition to JAK1, IL-2Rβ can recruit multiple protein tyrosine kinases (PTKs), such as *src* family kinases (Hatakeyama *et al* 1991; Kobayashi *et al* 1993; Minami *et al* 1993) and syk PTK (Minami and Taniguchi 1995). There is direct evidence for a physical interaction between IL-2Rβ and lck (Hatakeyama *et al* 1991). The cytoplasmic region of IL-2Rβ containing the acidic region is primarily responsible for the molecular interaction with lck. This can be separated from the additional region responsible for mitotic signalling, the serine-rich region. lck tyrosine kinase is divided into three domains: the N-terminal CD4 or CD8 binding domain, the modulatory domain containing the SH2 and SH3 domains and the kinase domain. The N-terminal half of the kinase domain is responsible for its association with IL-2Rβ. It seems there may be redundant and hierarchical associations with, or activation of, the *src* family PTKs in a cell lineage specific manner;thus, lck, lyn and fyn each seem to participate in IL-2 signalling in different cells (Hatakeyama *et al* 1992; Torigoe *et al* 1992; Kobayashi *et al* 1993).

1.3.7 Other Biochemical Events in IL-2 Signalling.

Following IL-2 stimulation, there is a rapid increase in intracellular pH as a result of the activation of an Na^+/H^+ antiport (Mills and May 1987). It is unlikely that there is involvement of other well-characterised biochemical pathways, such as activation of PKA or PKC or calcium mobilisation (Mills *et al* 1986, 1988; LeGrue 1988; Valge *et al* 1988). Various substrates, including IL-2Rβ itself are phosphorylated on their serine and threonine residues as a result of stimulation with IL-2 (Ishii *et al* 1988). The serine/threonine kinase involved is the ubiquitous cytoplasmic kinase p70-75 Raf-1. This is activated possibly as a result of phosphorylation on the serine, threonine or tyrosine residues following engagement of the IL-2R (Turner *et al* 1991; Carroll *et al* 1990; Zmuidzinas *et al* 1991). Raf-1 is the earliest substrate so far identified that is phosphorylated by tyrosine kinases upon IL-2 stimulation. Since Raf-1 rapidly undergoes tyrosine phosphorylation and lck rapid activation upon engagement of the IL-2R, the activity of these two kinases may be mutually regulated.

In addition, stimulation of T cells by IL-2 activates PI-3-kinase (Augustine *et al* 1991; Remillard *et al* 1991; Merida *et al* 1991). Another possible signalling mechanism involves the hydrolysis of glycosyl-phosphatidyl inositol (GPI). Following IL-2 stimulation, GPI is rapidly hydrolysed generating two potential signal mediators, a glycosylated diacylglycerol and an inositol phosphate glycan (Eardley and Koshland 1991; Merida *et al* 1990). It has also been reported that IL-2R activation may lead to signal transduction pathways involving cyclic AMP (Wickremasinghe *et al* 1987) and

experiments performed within this laboratory have indicated that inducing IL-2Rα causes cAMP levels to increase in B cells (McKay and Cushley 1997)

1.3.8 Multiple Signalling Pathways elicited by a Single Receptor.

Cytokines induce the expression of several genes including nuclear proto-oncogenes in T lymphocytes (Cleveland *et al* 1987; Reed *et al* 1985a, b) and B lymphocytes (Kelly *et al* 1983). On IL-2 stimulation, there is a rapid and transient increase in the number of *c-fos* transcripts, as well as the more stable accumulation of *c-myc* and *c-myb* transcripts (Stern and Smith 1986; Pauza 1987; Trouche *et al* 1991; Hatakeyama *et al* 1992). IL-2Rβ is involved in at least two distinct intracellular signalling pathways mediating nuclear proto-oncogene induction (Shibuya *et al* 1992). One pathway involves tyrosine phosphorylation events mediated by the *src*-family PTKs and leads to induction of the *c-fos*, *c-jun* and other genes of this family (Minami *et al* 1993). There is also a kinase independent pathway which appears linked to *c-myc* induction. From studies (previously mentioned in Section 1.3.5) with lymphoid lineage BAF-B03 cells expressing mutant IL-2Rβ proteins with deletions in the intracytoplasmic domain, it appears that an IL-2R mutant lacking the acidic region is incapable of mediating induction of *c-fos*. It was shown, nevertheless, to be capable of delivering proliferative signals accompanied by induction of *c-myc*. In contrast, mutation of the serine-rich region of IL-2Rβ could neither induce cell proliferation nor *c-myc* induction (Shibuya *et al* 1992). Hence the serine-rich region may elicit an as yet unidentified signal that leads to *c-myc* induction. Studies with fibroblastoid

cells containing reconstituted IL-2R complexes have also suggested the existence of two distinct pathways for *c-myc* and *c-fos* induction generated from the IL-2Rγc chain (Asao *et al* 1993).

1.3.9 The STAT Family of Transcription Factors

One of the important consequences of cytokine stimulation is the induction of gene expression. A novel group of transcription factors known as STAT proteins (signal transducers and activators of transcription) has been shown to be of importance in signalling through receptors of the cytokine receptor superfamily. The STATs were originally identified as components of the interferon (IFN) signalling system (Darnell *et al* 1994). IFN-α/β induces the formation of a transcription complex termed ISGF-3 which binds to the interferon-stimulated response element (ISRE) and activates transcription. The ISGF-3 complex consists of a 48kDa DNA binding component, an 84-91 kDa protein and a 113kDa protein, termed p48, p84, p91 and p113 , respectively: p91 and p84 are alternatively spliced products of the same gene. For formation of the ISGF-3 complex and its migration to the nucleus p91/p84 and p113 must be tyrosine phosphorylated; it is hypothesised that this tyrosine phosphorylation is mediated by a JAK kinase.

Alternatively, stimulation of cells with IFN-γ causes p91 only to become tyrosine phosphorylated. Following phosphorylation, p91 migrates to the nucleus and participates in DNA binding complexes that recognise gamma-activated sequences

(GAS) in genes that are transcriptionally activated by IFN-γ. These complexes do not contain p48 but may contain related proteins (Pearse *et al* 1993).

The p91/p84 and p113 components of the ISGF-3 complex are members of the STAT family. Thus, STAT-1α is p91, STAT-1β is p84 and STAT-2 is p113. The STAT family is characterised by the presence of a carboxy-terminal SH3 domain followed by an SH2 domain. There are additional blocks of homology found in the amino-terminal region of these proteins. A conserved tyrosine is found near the carboxy-terminus that is phosphorylated and essential for function (Shuai *et al* 1993). STATs bind to specific sequences related to the GAS found in the promoter regions of several cytokine sensitive genes. There is wide variation in the sequences bound by specific STATs. However, the elements are palindromic and have the general sequence T-T-$(N)_{3-6}$-A-A (where N is a non-conserved base) (Seidel *et al* 1995).

It was demonstrated through the use of mutants for IFN signalling that functional JAKs must be present to obtain tyrosine phosphorylation of STAT proteins, thus suggesting that STAT proteins are direct substrates of JAKs. JAK1 and JAK2 are associated with the IFN-γ receptor and JAK1 and TYK2 are associated with the IFN-α receptor. Since JAK1 is activated by both IFN-α/β and IFN-γ and STAT-1 is phosphorylated after treatment with each ligand, then it can be hypothesised that JAK1 is the kinase directly phosphorylating STAT-1. Conversely, since STAT-2 is only seen in response to IFN-α/β, STAT-2 could be a specific substrate for TYK2. In fact, STAT-1 has much broader functions than simply the IFN response.

Recent evidence demonstrates that ligand-receptor interactions other than those involving IFNs can activate JAK-STAT proteins and other STAT proteins have been discovered. Thus, it seems likely that JAK-STAT pathways may be of some importance in polypeptide-induced transcription (Sadowski *et al* 1993; Silvennoinen *et al* 1993; Ruff-Jamison *et al* 1993; Bonni *et al* 1993). A STAT-1-related protein has been identified which associates with gp130 and JAK1 and is rapidly tyrosine phosphorylated during the IL-6 response (Lutticken *et al* 1994). It has been designated STAT-3 (Zhong *et al* 1994b; Kishimoto *et al* 1994)

STAT-4 was cloned (Yamamoto *et al* 1994) and found to be functionally like other members of the family in that it can be tyrosine phosphorylated by JAK1 or JAK2 and acquires γ-activated sequences (GAS) binding activity. It is primarily expressed in haematopoietic cells and in developing spermatogonia (Zhong *et al* 1994a). It was recently discovered that IL-12 can induce tyrosine phosphorylation of STAT-4 in human T lymphocytes (Jacobson *et al* 1995; Quelle *et al* 1995).

STAT-5 is expressed in the spleen, bone marrow and thymus. There are two STAT-5 proteins, STAT-5A and STAT-5B which are the products of distinct genes (Azam *et al* 1995; Mui *et al* 1995). Cytokine receptors which contain a common β chain such as IL-3, IL-5 and GM-CSF appear to be involved in signalling through the tyrosine phosphorylation of STAT-5. In the murine system, IL-3, IL-5 and GM-CSF all induce tyrosine phosphorylation of STAT-5 whereas in humans IL-2 activates STAT-5. In humans, STAT-5 is phosphorylated by JAK3, a result entirely consistent with the

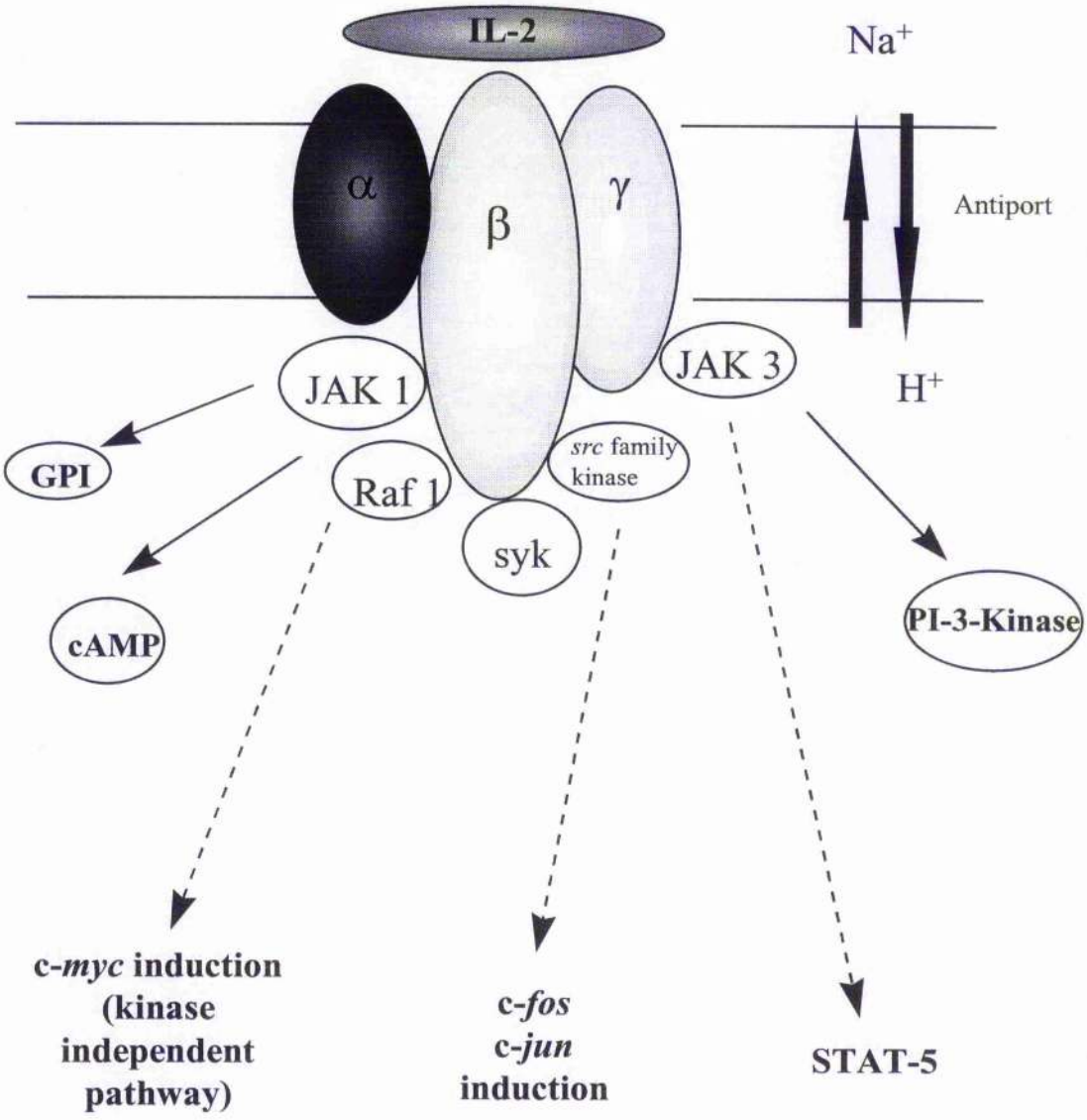
finding that the C-terminal region of the IL-2Rβ chain is required for STAT-5 activation (Fujii *et al* 1995).

Finally, STAT-6 was discovered (Hou *et al* 1994). This is an IL-4 regulated STAT protein; it appears that the α chain of the IL-4 receptor confers specificity for STAT-6 activation. In the case of IL-2 mediated signal transduction, IL-2 does not activate STAT-1, 2 or 3 in T cells (Beadling *et al* 1994). As previously stated, STAT-5 is activated by IL-2 (Fujii *et al* 1995) and STAT-6 also appears to be a substrate for IL-4 -driven tyrosine phosphorylation in T cells (Quelle *et al* 1995). Therefore, since both IL-2 and IL-4 receptors have a common γc subunit that selectively associates with JAK3 (Miyazaki *et al* 1994), it is possible that STAT-5 and STAT-6 activation in T cells is mediated by JAK3.

In conclusion, IL-2 signalling is a complex process involving a large cast of molecules and a schematic diagram outlining the pathways involved is illustrated in **Figure 1.3**. Much remains to be elucidated before we will be able to fully understand the signal transduction pathways that activate target genes in the nucleus in response to stimulation of the IL-2R on the cell surface.

Figure 1.3 Summary of the Signal Transduction Pathways Through the High Affinity Interleukin-2 Receptor.

IL-2 binds to the high affinity heterotrimeric IL-2R, causing Jak-1 and Jak-3 to associate with specific residues within the cytoplasmic tails of the receptor together with Raf-1, syk and *src*-family kinases. These cause signalling via certain second messenger pathways and induction of various genes through the binding of transcription factors.


IL-2
Na+
α
β
γ
Antiport
JAK 1
JAK 3
H+
GPI
Raf 1
src family kinase
syk
cAMP
PI-3-Kinase
c-myc induction (kinase independent pathway)
c-fos c-jun induction
STAT-5

1.4 The IL-2Rα Promoter Region

One approach used to elucidate cytokine signal transduction is the identification of *cis* response elements within genes and their corresponding transcription factors which respond to cytokine signals. The IL-2Rα gene has been well-characterised in T lymphocytes; a number of *cis* regulatory elements within the promoter region have been mapped and their corresponding *trans*-activating factors identified. Therefore, this section will deal largely with what is known in T lymphocytes and then relate this to what is currently being explored in B lymphocytes.

One reason why the IL-2Rα gene has been so extensively studied in T lymphocytes is because infection of $CD4^+$ T lymphocytes with Type 1 Human T Cell Leukaemic Virus (HTLV-1) leads to constitutive IL-2Rα gene expression and cell immortalisation (Gootenberg *et al* 1981; Depper *et al* 1984; Waldmann *et al* 1984; Uchiyama *et al* 1985). This pathogenic retrovirus has been implicated as the cause of aggressive and often fatal adult T cell Leukaemia (ATL) (Reviewed by Wong-Staal and Gallo 1985). Patients with this leukaemia may suffer from epidermal or dermal leukaemic T cell infiltrates, hypercalcemia, osteolytic bone lesions and infection with opportunistic organisms (Bunn *et al* 1983). HTLV-1 encodes a 40kDa trans-acting regulatory protein termed Tax (or alternatively Tat-1, pX, xlor, $p40^x$, TA-1) (Yoshida and Seiki 1987) which activates HTLV-1 long terminal region (LTR) and can induce transcription of various cellular genes including IL-2Rα and IL-2 (Inoue *et al* 1986; Cross *et al* 1987, Muniyama *et al* 1987; Siekivitz *et al* 1987; Ballard *et al* 1988 Bohnlein *et al* 1988, Lowenthal *et al* 1988;).

Early studies of IL-2Rα gene expression indicated a 1352 base pair (bp) fragment 5' to the IL-2Rα gene which was shown to possess promoter activity when fused to a CAT reporter gene (Leonard *et al* 1985). In addition, 5' elements required for IL-2Rα activity in HTLV-1 transformed T cell lines and in Jurkat cells were mapped (Cross *et al* 1987; Greene *et al* 1989). Since data obtained from tumour T cell models and cell lines are not necessarily representative of resting T lymphocytes present in peripheral blood, similar studies on primary T cells were later done (Lowenthal *et al* 1989a; Algarte *et al* 1995). These studies showed that a proximal IL-2Rα promoter/enhancer region (positioned -276 to -274 relative to the transcriptional initiation site) contained 4 putative binding sites for nuclear factors that apparently positively regulate its transcription (NFIL-2RA, NF-κB, SRF and Sp-1). Latterly, this has been designated "Positive Regulatory Region I" (PRR_I) since, in all, there are now at least three positive regulatory regions (PRR_{I-III}) within the human IL-2Rα promoter region. PRR_{II}, like PRR_I, lies close to the transcription initiation site between positions -137 and -64. This region contains binding sites for at least two DNA binding proteins, an ets family protein, Elf-1, (Thompson *et al* 1992) and the non-histone chromatin-associated protein HMG-I(Y) (Elton and Reeves 1986; Johnson *et al* 1989; Lund *et al* 1986). PRR_{III} lies some distance upstream of the 5' cap site, between nucleotides -3780 and -3703. It contains a potential binding site for STAT-5 as well as consensus sequences for Elf-1, HMG-I(Y) and a member of the GATA family. In addition to these positive regulatory regions, there are two negative regulatory regions, NREI and NREII, located between nucleotides -400 and -368 (Smith and Greene 1989) and between nucleotides -317 and -342 respectively (Lowenthal *et al* 1989b). Thus,

multiple regulatory elements are involved in IL-2Rα gene activation and a schematic diagram illustrating the IL-2Rα promoter region is illustrated in **Figure 1.4** (page 60).

1.5 The Positive Regulatory Region I (PRR_I) in the IL-2Rα Promoter.

1.5.1 Upstream Element 1

Upstream element-1 (UE-1, [-289 to -269]) within PRR_I is the binding site for the poorly defined 56kDa nuclear binding factor, NFIL-2RA, which is apparently involved in basal transcription activity (Ballard *et al* 1989; Lowenthal *et al* 1989; Toledano *et al* 1990). Deletion of this upstream element from a fully functional IL-2Rα promotor construct produced a loss of both PMA and TNF-α mediated induction of the IL-2Rα promotor in T cells (Ballard *et al* 1988; Lowenthal *et al* 1989a, b).

1.5.2 The κB Element

Gel retardation studies coupled with chemical footprinting and methylation assays (Ballard *et al* 1988) served to map the binding site for one or more inducible proteins to a region located between bases -267 and -256 (Bohnlein *et al* 1988; Ballard *et al* 1988). Comparison of this 12bp promoter segment with sequences of other known protein recognition sites revealed a striking similarity to the binding site for the transcription factor NF-κB (Bohnlein *et al* 1988, Leung and Nabel 1988; Ruben *et al* 1988, Lowenthal *et al* 1989b). NF-κB is a ubiquitous transcription factor whose

properties seem to be most extensively exploited in cells of the immune system. κB-like regulatory motifs have been detected in a variety of cellular genes involved in the immune response, including those encoding κ light chain Ig (Sen and Baltimore 1986), MHC Class I antigens (Baldwin and Sharp 1988) and IL-2 (Hoyos *et al* 1989).

Five independent genes have been identified which encode transcription activator proteins which recognise the κB motif; all are classified as members of the *rel* family of transcription factors. The Rel protein family has been divided into two groups based on differences in structure, function and mode of synthesis (Baeuerle and Henkel 1994). The first group consists of p50 (NF-κB1) and p52 (NF-κB2) which are synthesised as precursor proteins of 105kDa and 100kDa,respectively. The mature proteins possess a "Rel homology" domain of 300 amino acids which includes DNA binding and dimerisation domains and a nuclear localisation signal. They form functional dimers with other members of the family while dimers containing unprocessed proteins remain in the cytoplasm. The second group of Rel proteins includes p65 (Rel A), Rel (c-Rel), Rel B and the *drosophila* Rel proteins, dorsal and Dif. They are not synthesised as precursors and possess one or more transcriptional activation domains, in addition to the Rel homology domain. The subunit composition of the different complexes defines the fine specificty of binding to the target sequences and their transactivating activity (Perkins *et al* 1992; Lernbecher *et al* 1993).

In the case of IL-2Rα gene expression, Costello *et al* (1993b) reported that in resting T cells the κB region was bound by NF-κB p50 homodimers that were constitutively

present in the nucleus of resting cells. On activation, there was binding by NF-κB p50-p65 heterodimers and probably c-Rel homodimers. Further support for this theory was produced by separate experiments which again suggested that PKC expression, which triggers expression of IL-2Rα in T cells, induced NF-κB activation through the induction of p65 and c-Rel in the nucleus where they bind the IL-2Rα promoter as heterodimers with p50 (Pimental-Muinos *et al* 1994a). Several lines of evidence indicate that this κB site in the IL-2Rα promoter is functionally important. Deletion of this site from a fully active IL-2Rα promoter construct or point mutations within the element results in complete abrogation of PMA and TNF–α inducibility of IL-2Rα (Ballard *et al* 1988, Lowenthal *et al* 1989a, b). Secondly , a fragment containing the κB element conferred mitogen and cytokine inducibility to a thymidine kinase promoter-CAT construct (TKCAT) (Ballard *et al* 1988; Bohnlein *et al* 1988, Lowenthal *et al* 1988, 1989a, b). In fact, the element displayed enhancer-like properties since it functioned independently of orientation and reiteration of the binding site led to an amplification of the induced response. Thus, it is clear that this κB element plays a crucial role in the induction of the IL-2Rα gene in human T cells.

1.5.3 The Serum Response Element

Directly downstream from the κB element is a flanking Serum Response Element (SRE) / CArG box [-253 to -243]. SRE is a major *cis* element responsible for the induction of *c-fos* transcription in response to a variety of mitogens (Treisman 1992) and binds a transcription factor known as the Serum Response Factor (SRF). The proximity of κB and the SRE /CArG box raises the possibility that both elements act

in cooperation, as suggested by previous regulatory element mutagenesis and gel retardation studies (Kuang *et al* 1993). It has been proposed that c-Rel/p50 heterodimers synergise with SRF to give a high level of IL-2Rα promoter activity. In addition, both c-Rel and p65 can cooperate with SRF to activate gene transcription (Pierce *et al* 1995). Thus, more than one member of the Rel family can cooperate with SRF in transcriptional activation. These studies also indicated that p50 homodimers bound strongly to the κB site but did not activate transcription. Thus, it may be that the binding of SRF, readily present in the nuclei of resting T cells, to the SRE / CArG box of IL-2Rα is impaired by stably bound p50 homodimers to the flanking κB element.

Another possibility is that the SRE / CArG box could be occupied by another CArG box-binding protein, YY-1. This recognises the downstream part of the CArG box (Natesan and Gilman 1993). YY-1 is a ubiquitously expressed zinc finger protein with degenerate DNA binding specificity (Hahn 1992) which affects promoter activity positively or negatively by affecting promoter structure rather than directly contacting the transcriptional machinery (Natesan and Gilman 1993). However, the majority of studies favour a role for SRF in human IL-2Rα gene regulation (Lin *et al* 1990; Roman *et al* 1990; Toledano *et al* 1990; Kuang *et al* 1993; Pimental-Muinos *et al* 1994a, b).

1.5.4 The Downstream Element

Again directly downstream of the κB element is a GC box [-245 to -240] which binds the transcription factor, Sp-1. One of the most striking features of the IL-2Rα promotor is that CArG and GC boxes overlap by three nucleotides. Site directed mutagenesis and transient transfection assays in Jurkat T cells suggest that Sp-1 acts as a repressor of IL-2Rα expression, perhaps by competing for the binding of SRF-like nuclear factors to the overlapping SRE/ CArG box (Roman *et al* 1990). However, this theory remains controversial; other data suggests there is not a repressor role for Sp-1 by steric hindrance (Pimental-Muinos *et al* 1994a, b; Algarte *et al* 1995).

1.6 The Positive Regulatory Region II (PRR_{II}) in the IL-2Rα Promoter.

PRR_I is a potent inducer which at least partially regulates inducible IL-2Rα expression. However, the sequence of this region is not well conserved in mice (Suzuki *et al* 1987). Recently, a second regulatory region was characterised within the human IL-2Rα promoter and designated PRR_{II} (John *et al* 1995). This region is located between nucleotides -137 and -64 relative to the transcription initiation site and contains binding sites for at least two DNA-binding proteins. Deletion of these sites greatly reduced IL-2Rα gene transcription even when the potent PRR_I region was intact. Unlike PRR_I, PRR_{II} is strictly conserved between mice and humans.

Within PRR_{II} is a GGAA purine rich motif (-91 to -94) which is a potential Ets binding site. In fact, the Ets binding protein, Elf-1 was found to bind PRRII *in vitro*. Elf-1 is a protein specific to lymphoid tissues which is closely related to the Drosophila E74 protein (Thompson *et al* 1992; Wang *et al* 1992). In addition, there is a 19 base pair AT rich sequence (-116 to -98) which is a major HMG-I(Y) binding site. HMG-I(Y) proteins are low molecular weight (10-11kDa) high mobility group proteins that bind in the minor groove of AT rich sequences and induce strong bending in the DNA helix (van de Wetering and Clevers 1992; Giese *et al* 1992). There are two further potential HMG-I(Y) binding sites betwen nucleotides -128 and -123 and between nucleotides -90 and -84.

Both Elf-1 and HMG-I(Y) can interact with each other in the absence of DNA and it has been proposed that interaction with HMG-I(Y) may allow Elf-1 to maintain a transcriptionally active confirmation (John *et al* 1995). Elf-1 can also interact with NF-κB p50 and c-Rel, both of which have been implicated in the inducible expression of the IL-2Rα gene (Tan *et al* 1992). Moreover, this interaction is enhanced by the presence of HMG-I(Y). Thus, HMG-I(Y) may act as a "molecular glue" between Elf-1 and NF-κB and its ability to bend DNA (Lehn *et al* 1988) could also facilitate protein-protein interactions promoting the formation of an active transcription complex.

1.7 The Positive Regulatory Region III (PRR_{III}) in the IL-2Rα Promoter.

In mice, the IL-2Rα gene has an IL-2 Response Element located about 1.3 kilobases upstream of the 5' cap site (Sperisen *et al* 1995). A sequence similar to this has recently been identified within the human IL-2Rα promoter (John *et al* 1996; Lecine *et al* 1996) and designated PRR_{III}. It is located between -3780 and -3703 and contains two potential binding sites for STAT family proteins (GAS motifs), a GATA site and two Ets binding site (EBS) motifs. The two GAS sites, GASd [$TTC(N)_3GAA$] and GASp [$TT(N)_5AA$] are located between nucleotides -3181 and -3173 and between nucleotides -3161 and -3153, respectively. GASd overlaps with an EBS motif while GASp overlaps with a GATA site (TGATAA). There is another EBS consensus site located between nucleotides -3138 and -3133, designated EBSp indicating it is the proximal EBS element. Both consititutive and inducible factors bind to these PRR_{III} sites *in vitro*. In T cells, IL-2 is required for the maximal and prolonged expression of IL-2Rα (Sperisen *et al* 1995) and the PRR_{III} has been shown to possess IL-2-dependent enhancer activity (John *et al* 1996; Lecine *et al* 1996). It has been hypothesised that STAT-5 plays a major role in controlling PRR_{III} activity since GASd binds STAT-5a and STAT-5b proteins *in vitro* which are activated by IL-2. The GASd site overlaps with an EBS motif which binds a constitutive complex; this contains Elf-1 as well as Ets-1 and/or Ets-2. On induction of the gene with IL-2, this is believed to be disrupted by binding of the STAT-5 proteins to GASd.

There is also a putative GATA binding motif which overlaps GASp. GATA family proteins contain a highly conserved amino acid domain which binds the GATA

sequence motif (A/TGATAG/A) through a unique C4 zinc finger structure (Orkin 1992). Surprisingly, experiments have suggested that GATA-1 (or possibly a related protein), previously thought only to be found in cells of the erythroid lineage (reviewed in Weiss and Orkin 1995), bound PRR_{III} as part of a complex that also contains STAT-5 and Elf-1 proteins.

It has also been proposed that HMG-I(Y) proteins may play a role in the regulation of PRR_{III} activity, similar to that played in the regulation of PRR_{II}. There are two potential HMG-I(Y) binding sites, one juxtaposed to the EBSp site, the other overlapping the GATA motif. Thus, HMG-I(Y) may also function to modulate the architecture of PRR_{III}, facilitating interactions between the key regulatory proteins like Elf-1, STAT-5 and GATA-1.

1.8 The Negative Regulatory Regions

There are two negative regulatory elements, NRE I and NRE II, within the IL-2Rα promotor located between nucleotides -400 and -368 (Smith and Greene 1989) and between nucleotides -317 and -342 (Lowenthal *et al* 1989b) respectively. NRE I is a 31bp region which contains an 11bp core element (TTCATCCCAGG). This core element is similar to a binding site present in the LTR of the Human Type 1 Immunodeficiency Virus (HIV-1) (nine from eleven bases) that binds *in vitro* to a 50kDa protein (SP50) (Smith and Greene 1989). This HIV-1 LTR has been implicated in the negative regulation of HIV-1 gene expression (Rosen *et al* 1985). Introduction of a point mutation within this 11bp core element enhanced basal and PMA inducible

activity of the IL-2Rα promoter in human T cell lines (Smith and Greene 1989). In addition, the mutation disrupts binding of SP50. Therefore, NRE I apparently acts as a transcriptional silencer. This mechanism could be comparable with that seen in the transcriptional induction of the β-IFN gene which appears to involve the displacement of a silencer protein from the promoter, thus permitting the binding of other transcription factors to adjacent or overlapping positive regulatory domains (Goodburn and Maniatis 1988). Similarly, the IL-2 gene is negatively regulated by a specific protein binding to an NRE. Transcriptional activation depends upon the inactivation or displacement of this protein (Nabel *et al* 1988).

There is a second element within NRE I which is homologous to a retinoic acid response element (RARE). This is located immediately 3' to the 11bp core NRE element (Bhatti and Sidell 1994). The identity of the protein binding to this RARE motif remains to be defined. The RARE site does contain a consensus motif for AP-2 binding.

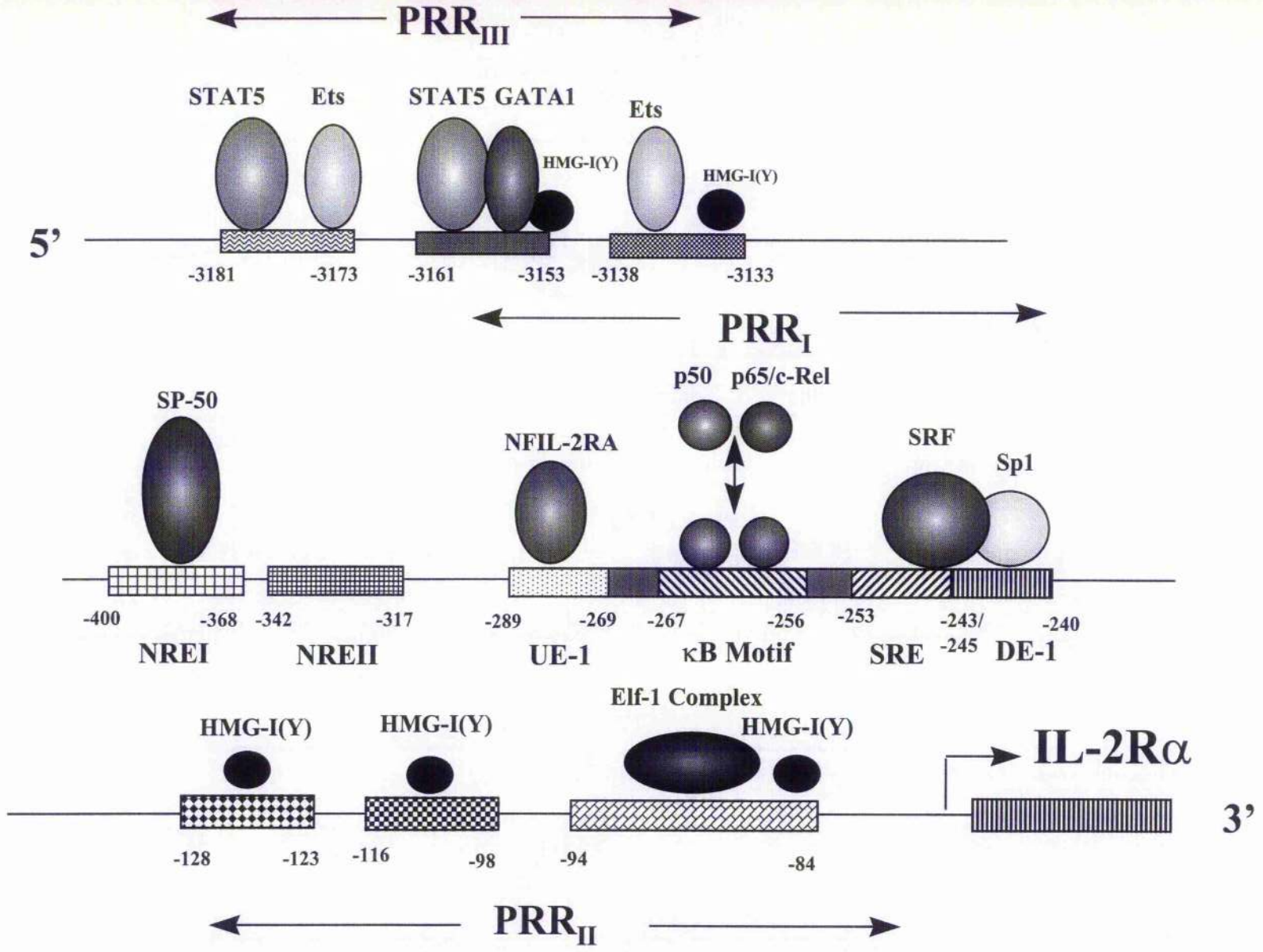
In fact there are several new putative binding sites in the vicinities of both NREs. These contain the consensus binding site [C(A/T)(T/G)ANN(C/T)] of a poorly characterised regulatory element originally described as a γ-IFN response element γ-IRE (Yang *et al* 1990).

In summary, there are numerous regulatory elements involved in IL-2Rα gene activation and these are illustrated in **Figure 1.4**. There is apparently a major role for the NF-κB transcription factors. However, this ubiquitous family of factors lacks the

specificity characteristics required to explain the strict IL-2Rα gene transcription regulation. Therefore it is likely that NF-κB cooperates with SRF to induce IL-2Rα promotor activity. There are two further *trans*-acting factors which may be involved. NFIL-2RA and Sp-1; their involvment in the induction remains uncertain. Together these four DNA-binding sites constitute PRR_I. There are two further positive regulatory regions, PRR_{II} and PRR_{III}. It seems likely that PRR_{II} is involved more in the control of basal promoter activity with PRR_I and PRR_{III} contributing to the inducible regulation of the gene. In addition, there are two NRE regions upstream from the κB motif. Proteins binding to these regions may lead to the functional silencing of the gene.

Figure 1.4 A Schematic Diagram Showing the IL-2Rα Promoter in T Cells.

The main elements of the promoter region of the IL-2Rα gene are illustrated as boxes with distinct styles and the location of the elements relative to the transcription initiation site are shown as numbers along the bottom of the boxes. The complexes of transcriptional regulatory factors which interact with each motif are shown above the boxes.


PRR$_{III}$
STAT5
Ets
STAT5 GATA1
Ets
HMG-I(Y)
HMG-I(Y)
5'
-3181
-3173
-3161
-3153
-3138
-3133
PRR$_{I}$
p50
p65/c-Rel
SP-50
NFIL-2RA
SRF
Sp1
-400
-368
-342
-317
-289
-269
-267
-256
-253
-243/
-245
-240
NREI
NREII
UE-1
κB Motif
SRE
DE-1
Elf-1 Complex
HMG-I(Y)
HMG-I(Y)
HMG-I(Y)
IL-2Rα
3'
-128
-123
-116
-98
-94
-84
PRR$_{II}$

1.9 The IL-2Rα Promoter in B Cells

In comparison to T lymphocytes, there has been relatively little work done on the transcriptional events which facilitate IL-2Rα gene induction in B lymphocytes. Experiments have been performed by this laboratory to determine whether B cells contain DNA-binding proteins which specifically bind to any of these regulatory elements and which receptor-ligand interactions on the B cell surface can lead to induction of the IL-2Rα gene. These studies are discussed in Chapter Five.

An IL-4 Responsive Element (TTCTAAGAA) was identified in the promoter regions of the CD23b and Iε genes (IL-4RE) which is specifically bound by the novel IL-4 induced transcription factor, NFIL-4 (Kohler and Rieber 1993). A sequence homologous to IL-4RE is found in the IL-2Rα promoter between nucleotides -306 and -298. The protein NFIL-4 which binds to this site appears to correspond to the recently identified transcription factor known as STAT-6 (alternatively known as IL-4 STAT) (Hou *et al* 1994). This is a 100kDa protein which, when discovered, was found to have substantial similarity to the STAT family of transcription factors. The most significant segments of sequence similarity corresponded to three regions, one consisting of approximately 50 amino acids located to the NH_2-termini of all STAT proteins and two more centrally located regions which were predicted to specify SH2 and SH3 domains.

1.10 Aims of the Project

The aims of my project were to investigate the factors which could induce expression of IL-2Rα in human tonsillar B lymphocytes. In particular, it was determined that I would focus on how signals through the antigen receptor would affect IL-2Rα expression and thus cause the B cell to proliferate and differentiate. Finally, the role of transcription factors which bound the IL-2Rα promoter region were to be studied in order to determine which were important in causing IL-2Rα to be up-regulated and which, if any, were under the direct control of signals transduced by the antigen receptor.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Chemicals

All routine chemicals were of the highest grade available and were supplied by the Sigma Chemical Company of Poole, Dorset, U.K., unless otherwise stated below.

2.1.2 Human Tissue

Tissue was obtained from juvenile male and female tonsillectomy patients at the Royal Hospital for Sick Children, Yorkhill, Glasgow (courtesy of Mr Carachi). From these we obtained resting B cells. The reason for the removal of the tonsils was not known. However, the children were generally young and it is unlikely that they had been exposed to any chronic infections. In addition, any tissue which appeared infected was immediately discarded.

2.1.3 Cell Culture Materials

The following tissue culture supplies were obtained from Gibco-BRL Life Sciences, Paisley, Scotland, U.K.:-

RPMI-1640 medium

Glutamine

Penicillin / Streptomycin

Foetal Calf Serum

Trypan Blue was obtained from the Sigma Chemical Company, Dorset, U.K..

2.1.4 B Cell Separation Materials

Percoll	Sigma.
Ficoll-Hypaque (1.077g/ml)	Pharmacia, Uppsala, Sweden.
Sheep Red Blood Cells in Alsever's Solution	SAPU, Carluke, Lanarkshire, Scotland, U.K.
2-Aminoethylisothiouronium bromide (AET)	Sigma.

2.1.5 Radiochemicals

The following radiochemicals were obtained from Amersham International plc, Buckinghamshire, U.K.:-

(Methyl-[^{3}H]) Thymidine	44Ci/mmol
γ-[^{32}P]-Adenosine Triphosphate	5000Ci/mmol

[^{14}C]-chloroamphenicol	50-62mCi/mmol

2.1.6 Cytokines

IL-1α, IL-1β, IL-3, IL-5, IL-6 and IL-8	Geneva Biomedical Research Institute. Geneva, Switzerland.
TNF-α	British Biotechnology, Abingdon, U.K.
IL-11	Genetics Institute, Cambridge, MA, USA.
IL-2	Genzyme, Kent, U.K.
IL-4	Immunex, Seattle, WA, USA.
IL-7 and IL-13	R&D Systems, Abingdon, U.K.

2.1.7 Gel Electrophoresis Materials

40% Acrylamide / Bisacrylamide solution (29:1) from Biorad Laboratories, CA, USA.

Ammonium Persulphate and TEMED from Sigma.

2.1.8 Antibodies

Polyclonal Goat anti-human Immunoglobulin	Sigma

Goat anti-human IgM (μ heavy chain specific),	Southern Biotechnology Associates Inc., Birmingham, AL, USA.
Goat anti-human IgD (δ heavy chain specific), $F(ab')_2$ fragment anti-human IgM (μ heavy chain specific), $F(ab')_2$ fragment anti-human IgD (δ heavy chain specific).	
Affinipure Goat anti-human IgG (H&L), Affinipure $F(ab')_2$ fragment Goat anti-human IgG (H&L), Affinipure Fab fragment Goat anti-human IgG (H&L).	Jackson Immunoresearch Laboratories Inc., PA, USA.
Monoclonal Mouse anti-human CD40 antibody	Serotech, Oxford, U.K.
FITC-conjugated monoclonal mouse anti-human IL-2 Receptor, RPE-conjugated monoclonal mouse anti-human CD19.	DAKO, Denmark.
Biotinylated mouse anti-human CD23	The Binding Site, Birmingham, U.K.
Streptavidin-Quantum Red conjugate	Sigma

2.1.9 Oligonucleotides

The following oligonucletides were obtained from Promega, WI, USA:

CREB Consensus Oligonucletide	5' AGA GAT TGC CTG ACG TCA GAG AGC TAG 3'
AP-1 Consensus Oligonucleotide	5' CGC TTG ATG AGT CAG CCG GAA 3'
NF-κB Consensus Oligonucleotide	5' AGT TGA GGG GAC TTT CCC AGG C 3'

The following oligonucletide was obtained from CRUACHEM, Beatson Institute, Scotland, U.K.:

IL-4RE Consensus Oligonucleotide	5' CAG TGC CAA GAA GTG CTT GCT 3'

The following oligonucleotide was obtained courtesy of J. Winnie, Beatson Institute, Scotland, U.K.:

NRE Consensus Oligonucleotide	5' TTC ATC CCA GG 3'

2.1.10 Plasmids

CD25 Reporter Gene Plasmids were constructed by Dr E. Hewitt, Division of Biochemistry and Molecular Biology, University of Glasgow, U.K. (Hewitt *et al* 1997).

2.1.11 Enzymes

T4 Polynucleotide kinase was obtained from Promega, MA, USA.

T4 Polynucleotide kinase 10 x Buffer Stock:-

700mM Tris-HCl, pH7.6

100mM $MgCl_2$

50mM DTT

2.2 BUFFERS

Phosphate Buffered Saline (PBS) pH7.2

170mM NaCl

3.4mM KCl

10mM Na_2HPO_4

1.8mM KH_2PO_4

TBE Buffer

89mM Tris-HCl, pH8.0

89mM Boric Acid

20mM EDTA

Tris-EDTA (TE) Buffer

10mM Tris-HCl, pH8.0

1mM EDTA

2.3 METHODS

2.3.1 Preparation of B Lymphocytes from Human Tonsils

2.3.1(a) AET Treatment of Sheep Red Blood Cells

5ml of sheep erythrocytes were centrifuged and washed twice in incomplete medium (RPMI-1640 medium containing 100 I.U./ml Penicillin / Streptomycin). At the same time, 102mg of 2-aminoethylisothiouronium bromide (AET) was dissolved in 10ml distilled water, adjusted to pH9.0 with 5M NaOH and filter sterilised. 4ml of the AET solution was added to the washed sheep erythrocytes and the suspension incubated at 37°C. After 20 minutes, the cells were washed five times in incomplete medium and then resuspended in 9mls of incomplete medium. The AET-treated Sheep Red Blood Cells (AET-SRBC) were stored at 4°C for up to one week.

2.3.1(b) Isolation of B Cells

Cells were obtained by teasing apart excised tonsils followed by passage through a fine mesh to obtain a single cell suspension. Mononuclear cells were obtained by centrifugation over Ficoll (1 part Ficoll: 1 part cell suspension) at 1400rpm for 15 minutes. The mononuclear cells were removed from the interface and then washed twice in incomplete medium. The cells were then resuspended in incomplete medium (see Section 2.3.1a) and 10% (*v/v*) AET-SRBC was added. After incubating for 15 minutes at 37°C, the suspension was centrifuged at 800g for 5

minutes and 10% (*v/v*) FCS (heat inactivated) was layered on top of the resulting pellet. This was left on ice for 30 minutes. After the incubation, the pellet was gently resuspended by rocking, the sample layered onto Ficoll and centrifuged at 1400g for 20 minutes. B lymphocytes were removed from the interface and washed twice in incomplete medium. The cells were then resuspended in 1.5ml of incomplete medium.

2.3.1(c) Separation of the B Cell Populations by Percoll Gradient Centrifugation

Percoll gradients were prepared as described by Ratcliffe and Julius (1982). Percoll densities of 1.09, 1.08, 1.075, 1.065 and 1.055 g/ml were prepared from an iso-osmotic stock (9 parts Percoll: 1 part 10xPBS) using the formula:

$$V_y = \frac{V_i(\rho_i - \rho)}{(\rho - \rho_y)}$$

where ρ_i = the density of the stock iso-osmotic percoll

ρ = the density of the solution produced

V_y = the volume of diluting medium

V_i = the volume of stock iso-osmotic percoll.

1ml of each density of Percoll solution was layered into a 15ml centrifuge tube starting with 1.09g/ml and decreasing in density to 1.055g/ml. The B cell suspension was layered onto this and a covering layer of 1ml incomplete medium was pipetted on top. The gradients were

centrifuged at 2200rpm for 30 minutes. Resting B cells are small, high density cells and these were harvested from the 1.09/1.08 g/ml interface. After removing the B cells from the interface, the cells were washed three times in incomplete medium and then used immediately. An aliquot of cells was removed at this stage and analysed by FACS analysis using antibodies conjugated to anti-CD3 and anti-CD19 to determine how pure the B cell population was. This method routinely produced a population of cells that was greater than 95% B cells (data not shown).

2.3.2 Cell Culture of Human Tonsillar B Lymphocytes

Cells were maintained in complete medium (RPMI-1640 supplemented with 10% (*v/v*) foetal calf serum, heat inactivated at 56°C for 30 minutes, 2mM L-Glutamine and 100 I.U./ml Penicillin/Streptomycin). Cells were cultured for the times indicated in the text and figure legends and with the appropriate concentrations of cytokines and antibodies. Cells were cultured in $25cm^2$ flasks, 24 well or 96 well plates at 37°C in a 5% CO^2 atmosphere. Preparation and maintenance of the cells was carried out in a Laminar Flow Cabinet.

2.3.3 Determination of Cell Viability.

Cell viability was determined by mixing 1 volume of cell suspension with 1 volume of 0.4% (*w/v*) Trypan Blue. Viability was determined on the basis that dead cells take up Trypan Blue. The numbers of live and dead cells were counted using a Neubauer haemocytometer. The

average number of cells per 16 square grid was multiplied by 10^4 and the dilution factor taken into account to estimate the number of cells per ml of culture.

$$\% \text{ viability} = \frac{\text{number of live cells}}{\text{Total Cell Number}} \times 100$$

2.3.4 Stimulation of Resting B Tonsillar Cells with Anti-Ig, Anti-CD40 and IL-4

Cells were cultured in 24 well plates in complete medium at a concentration of 10^6 cells per well in a final volume of 1ml. Varying concentrations of anti-Ig, anti-CD40 antibodies or IL-4 diluted in complete medium were added to wells either separately or in combination. The cells were then incubated for 24 hours at 37°C at 5% CO_2 atmosphere and harvested for use in FACS analysis.

2.3.4 Flow Cytometric Analysis

2.3.4(a) Two Colour Flow Cytometry

Aliquots of approximately 1 x 10^6 cells were removed from cultures, pelleted, washed twice in ice-cold PBS and resuspended in PBS to give 1 x 10^6 cells/50µl. Two different antibodies which were directly conjugated to fluorescent markers (FITC and PE) were then added to the cells as specified in the text and figure legends. The samples were incubated on ice for 30

minutes. After incubation, the cells were washed twice in ice-cold PBS and finally resuspended in 0.5ml of PBS for analysis by flow cytometry. In each experiment a control was included where no antibody was added and this was used to correct for the autofluorescence of the cells. In addition, prior to the experiment, negative isotype control experiments were done (data not shown).

Immediately prior to data collection, propidium iodide (PI) was added to each sample at a final concentration of 5μg/ml. Antibody staining was analysed using a FACScan flow cytometer (Becton-Dickinson, Mountain View, California, USA) using the FACSCAN programme. Cells were gated on forward and side light scatter, as well as for the PI-emitted fluorescence to facilitate exclusion of non-viable cells from the analysis. Analysis was performed using the LYSYS programme.

2.3.4(b) Three Colour Flow Cytometry

Preparation of the cells was the same as in Section 2.3.4(a) but three antibodies, which were directly conjugated to fluorescent markers (FITC and PE), or to biotin, were added simultaneously to the cells as specified in the text and figure legends. The samples were incubated on ice for 30 minutes, washed twice in ice-cold PBS and resuspended in 50μl PBS. 10μl of Streptavidin-Quantum Red Conjugate was added to the samples and they were again incubated on ice for 30 minutes. The cells were then washed twice in ice-cold PBS and resuspended in 0.5ml PBS. They were analysed using a FACScan flow cytometer as described

before but were gated on forward and side light scatter only to facilitate analysis of live cells. A control sample of cells containing no antibody was also included, as in Section 2.3.4(a) to correct for the autofluorescence of the cells.

2.3.5 Cell Proliferation Studies

Cells were cultured in 96 well round bottomed tissue culture plates at a concentration of 10^5 cells per well. The cells were cultured for three days in complete medium at 37°C at 5% CO_2 over range of concentration of antibodies and cytokines as specified in the text and figure legends; all cultures were established in triplicate. Following this, the cells were pulsed with 0.5μCi of methyl-[^{3}H]-thymidine in a volume of 10μl of complete medium. The tissue culture plate was then incubated for 8 hours at 37°C at 5% CO_2 and then the contents of each well were harvested on to filter mats using a semi-automatic cell harvester (Skatron Instruments Ltd, Suffolk, U.K.). The filter papers were dried, immersed in scintillation fluid and counted by a liquid scintillation counter (Wallac 1209 RACKBETA counter). [^{3}H]-Thymidine incorporation was expressed as mean counts per minute (cpm) + Standard Deviation (SD).

2.3.6 Gel Shift Assays

2.3.6(a) Preparation of Nuclear and Cytoplasmic Cellular Extracts

Buffers:-

Buffer A	**Buffer B**
10mM HEPES, pH7.5	25mM HEPES, pH 7.5
2mM $MgCl_2$	400mM NaCl
15mM KCl	1mM EDTA
0.1mM EDTA	20% Glycerol
0.1mM EGTA	1mM DTT
1mM DTT	0.1% NP40
0.5mM PMSF	0.5mM PMSF
2μg/ml Leupeptin	2μg/ml Leupeptin
2μg/ml Aprotinin	2μg/ml Aprotonin

Primary tonsillar B cells were cultured overnight in fresh medium prior to stimulation for four hours with the appropriate concentrations of cytokines and antibodies as described in the text and figure legends. 5 x 10^6 cells were removed at time zero, and at hourly intervals thereafter, pelleted and washed once in PBS. The samples were then resuspended in Buffer A and left on ice for 30 minutes. NP-40 was added to a final concentration of 0.5% (*v/v*) and the nuclei

immediately separated by centrifuging at 10,000g. The supernatant (the cytosolic extract) was removed and stabilised by adding 60µl of 50% (*v/v*) glycerol while the nuclear pellet was resuspended in 100µl of Buffer B and gently rocked for 30 minutes at 4°C. The suspension was centrifuged at 10,000g and the supernatant removed. This was the nuclear extract. Both nuclear and cytoplasmic extracts were stored at -70°C.

2.3.6(b) Non-Denaturing Gels for Gel Shift Assay

The cellular extracts were resolved by electrophoresis on a 7% (*w/v*) non-denaturing acrylamide gel prepared as follows:-

17.5% acrylamide (40% Acrylamide /Bisacrylamide solution, 29:1)

0.25 x TBE

0.1% Ammonium Persulphate

0.05% TEMED

The gels were pre-run for two hours at 150V using 0.5 x TBE as the reservoir buffer.

Prior to loading, the samples were assayed for protein using the Lowry protein assay method. Extracts containing 5µg of protein were combined with binding buffer in the presence or absence of cold competitor oligonucleotide as required and incubated at room temperature for 10 minutes. 1µl of γ-[^{32}P]-labelled oligonucleotide (0.035-0.35pmol) was added to the mixture and incubated at room temperature for a further 20 minutes.

5 x Binding Buffer Stock:-

20% (*v/v*) Glycerol

5mM $MgCl_2$

2.5mM DTT

250mM NaCl

50mM Tris-HCl, pH 7.5

0.25mg/ml Poly(dI-dC).poly(dI-dC)

The samples were loaded onto the gel and electrophoresed at 150V for 140 minutes. A small amount of dye was run parallel to the sample to allow progress of electrophoresis to be monitored. Free and protein bound oligonucleotide was visualised by autoradiography of the dried gels.

Dye Solution:-

250mM Tris-HCl, pH7.5

0.2% (*w/v*) bromophenol blue

0.2% (*w/v*) xylene cyanol

40% (*v/v*) Glycerol

2.3.6(c) Radio-Labelling of Oligonucletides for Gel Shift Assay

The sense and antisense oligonucleotides were synthesised and annealed by mixing equimolar amounts of the two oligonucleotides, heating to 80°C for 5 minutes in a water bath and then allowing them to cool slowly to room temperature. γ-[^{32}P]-dATP labelled fragments were produced by labelling the 5' terminus of the DNA using T4 polynucleotide kinase. For each probe, 17.5pmol of the appropriate DNA was mixed with 1μl of T4 polynucletide kinase 10 x Buffer, 5-10 units of T4 polynucleotide kinase, 1μl of γ-[^{32}P]-dATP and the volume made up to 10μl with distilled water. The reaction was incubated for 10 minutes at 37°C and then stopped by the addition of 1μl 0.5M EDTA. 89μl of TE Buffer was added and the radio-labelled probe stored at -70°C until needed.

2.3.7 Transfection of Tonsillar B Cells by Electroporation

Prior to electroporation, resting tonsillar B cells were cultured for a minimum of 1 hour after isolation. The cells were then washed and suspended in incomplete medium at a concentration of 6.25 x 10^6 cells/ml. 0.8ml aliquots of this cell suspension were electroporated at an optimum voltage of 0.3-0.35kV, capacitance 960μFD in a 0.4cm Gene Pulser cuvette (Biorad Laboratories, CA, USA) with 40μg of the appropriate plasmid in 40μl of TE buffer (Anderson *et al* 1991). The electroporated cells which had been transfected with the same plasmid were pooled and incubated for a further hour in complete medium. Aliquots were then removed and

stimulated for 48 hours with the appropriate antibodies as indicated in the text and the figure legends.

2.3.8 Reporter Gene Assay

The transiently transfected cells were harvested and washed three times in sterile PBS. Cell extracts were prepared by resuspending the cells in 100µl of Tris-HCl, pH7.8 and then lysing them by immersing them for 5 minutes in a dry ice-ethanol bath followed by immersion for 5 minutes in a 37°C water bath. This freeze-thaw lysis was done three times. The samples were centrifuged at 250g for 10 minutes to pellet the cellular debris and the supernatant removed and heat-inactivated by incubating at 65°C for 10 minutes, to inactivate the cellular acetylases. At this point, the protein content of each cellular extract was determined by the Lowry protein assay. The reporter gene activity was measured by incubating the cell extracts with 5mM Acetyl Coenzyme A and 1µl of [^{14}C]-chloroamphenicol (0.025µCi) at 37°C for 16-20 hours.

The assay was stopped by adding 600µl of ethyl acetate, mixing and centrifuging at 250g for 5 minutes. The upper phase of the ethyl acetate was removed and then lyophilised until no ethyl acetate remained. The lyophilised sample was resuspended in 20µl ethyl acetate and the acetylated products resolved by thin layer chromatography (TLC) on 20 x 20cm silica plates in a 95% chloroform:5% methanol solvent system. Acetylated chloroamphenicol was visualised by autoradiography and quantitated by phosphorimage analysis. Data from the phosphorimage

analysis was expressed as phosphorescence units minus background phosphorescence per mm^2 ($P\text{-}B/mm^2$) and was normalised for cellular protein content.

2.3.9 Lowry Protein Assay

Solution A	**Solution B**
2%Na_2CO_3	16.6% Folins Ciocalteu Phenol Reagent
1% $CuSO_4.5H_2O$	
2% NaK tartrate.$4H_2O$	
2.5% SDS	
0.1N NaOH	

BSA standard protein samples were prepared at concentrations of 5-100 mg in 1ml. An equal volume of Solution A was added to the standard samples and to the samples which contained an unknown amount of protein. These were then mixed and incubated at room temperature for 10 minutes. Following this, 0.5ml of Solution B was added to the samples, they were mixed immediately and incubated at room temperature for a further 30 minutes. The absorbance of the standard samples was read at 750nm with a spectrophotometer (tungsten light setting) and from the values obtained a standard curve was plotted. This was used to determine the amount of protein present in the cellular extracts.

Chapter 3

The Induction of CD25 Expression in Resting Human B Lymphocytes by Stimulation with Anti-Immunoglobulin, Anti-CD40 and IL-4.

3.1 Introduction

Interleukin-2 (IL-2) plays a crucial role in both the differentiation and growth of human B lymphocytes (Callard 1990; Cushley and Harnett 1993) and the effects of the cytokine are mediated through the interaction of IL-2 with the IL-2 Receptor complex (IL-2R) (Smith 1989; Waldmann 1989). To date, three polypeptide chains have been identified as components of the IL-2R, namely the α, β and γ chains. The human IL-2Rα chain was originally defined by the Tac antibody and is now defined by the CD25 antibody cluster. It was identified as a 55kDa membrane glycoprotein (p55) capable of binding IL-2 (Leonard *et al* 1983; Robb and Greene 1983). It was thought to be the only cytokine receptor of a large family of binding proteins whose members include complement receptor proteins (Davie *et al* 1986; Perkins *et al* 1988). However, a novel IL-15 binding protein was recently identified and cloned that is structurally related to the α-chain of the IL-2R (Giri *et al* 1995). The IL-2Rα chain binds IL-2 with low affinity (Kd = 10nM) and is non-functional with respect to IL-2 internalisation and IL-2 signalling (Hatakeyama *et al* 1985; Greene *et al* 1985).

The β chain of the IL-2R is a 75 kDa glycoprotein which, through its cytoplasmic domain, appears to play a critical role in receptor-mediated signalling. The third component of the IL-2R complex is a 64kDa transmembrane subunit, the γ_c chain. The β and γ_c chains are required for ligand internalisation and signal transduction (Arima *et al* 1992; Kishimoto *et al* 1994) and both belong to the haematopoietin receptor superfamily. Mutations in the IL-2Rγ chain appear to be responsible for the X chromosome-linked immunodeficiency SCIDX1 in humans (Leonard *et al* 1994). SCIDX1 patients suffer from severe recurrent infections, fail to thrive and unless treated with curative bone marrow transplantation will die in the first years of life.

The disease is characterised by a marked decrease in circulating T cells and the presence of poorly functioning B cells (Conley 1992). The IL-2Rγ chain also participates in the formation of functional receptors for IL-4, IL-7, IL-9 and IL-15 (Leonard *et al* 1994; Giri *et al* 1994; Kimura *et al* 1995).

Different combinations of these three receptor subunits on human B cells give rise to three affinity classes of receptor. A high affinity IL-2R is formed through the combination of the α, β and γ components in a heterotrimeric complex (Kd ~10^{-11}M) with a receptor of intermediate affinity formed by a combination of β and γ subunits (10^{-9}M); IL-2Rα alone binds IL-2 with relatively low affinity (Kd ~ 10^{-8} M) (Smith 1989; Waldmann 1989; Kishimoto *et al* 1994). The α subunit of the IL-2R is not constitutively expressed and is up-regulated by distinct groups of cytokines in both T and B lymphocytes (Greene *et al* 1989; Clipstone and Crabtree 1994; Butcher *et al* 1990; Butcher and Cushley 1991; Zola *et al* 1991; Tomizawa *et al* 1991). For example, both IL-1 and Tumour Necrosis Factor-α (TNF-α) can promote expression of the α chain in T lymphocytes (Lowenthal *et al* 1986; Lee *et al* 1987). IL-4 and IL-5 control expression of the IL-2R in murine B cells through independent induction of its two chains. It was shown that IL-5 induces these cells to express CD25 while IL-4 induces the β chain (although not always) (Loughnan and Nossal 1989; Nakanishi *et al* 1993). This was the first example of a situation where two chains of a receptor are separately regulated by two different cytokines to permit a coordinated appearance of functional high affinity receptors. By contrast, in human tonsillar B cells, IL-4 has been shown to up-regulate expression of CD25 (Butcher *et al* 1990; Butcher and Cushley 1991; Zola *et al* 1991; Tomizawa *et al* 1991). This effect is apparently

specific to IL-4 and can be inhibited by IFN-γ. As the α chain has no overt signal transduction capacity, its main role would appear to be in the generation of the specific high affinity receptor for IL-2 (Roessler *et al* 1994) which is crucial for the growth and differentiation of B cells.

Initially we sought to identify, in addition to IL-4, any other receptor-ligand interactions on human B cells which would facilitate up-regulation of CD25. The growth and activation of B lymphocytes are regulated by signals transmitted after the binding of antigen to surface immunoglobulin (sIg) and of cytokines to their specific receptors. A number of other B cell-associated surface molecules are also involved. The binding of antigen to sIg is the primary event in B lymphocyte activation. Thus we studied the effect on CD25 induction of stimulating the B cell via its antigen receptor. Anti-Ig antibodies were used as analogs of antigen, with the assumption that anti-Ig will bind to constant regions of membrane Ig molecules on all B cells and have the same biological effects as antigen that binds to the hypervariable regions of membrane Ig molecules only on antigen specific B cells.

We also looked at the effect on CD25 levels on resting human B cells in response to CD40 ligation, as mediated by anti-CD40 antibody. The CD40 antigen is a 45-50kDa glycoprotein which is expressed on B cells, T cells and dendritic cells. It is a member of the TNF receptor superfamily (Banchereau *et al* 1994). The CD40 ligand, a member of the TNF family, is a 39kDa glycoprotein found on T cells, mast cells and basophils. The levels of expression on T cells increase as they mature (Gauchat *et al*

1993; Nonoyama *et al* 1995). CD40 activation is critical for B cell proliferation (Banchereau *et al* 1991), Ig class-switching and the rescue of germinal centre B cells from apoptosis following somatic mutation (Liu *et al* 1989; Zhang *et al* 1991). Therefore we sought to examine the effect of CD40 ligation on CD25 expression. Stimuli were also used in combination to discover if any synergistic effects could be noted.

As stated, B cells can be induced to express IL-2Rα but there are conflicting reports as to whether such B cells can respond to IL-2 and proliferate. In the murine system, unstimulated B cells can respond to high concentrations of IL-2, and IL-5-treated B cells have been shown to have significantly enhanced responses to IL-2 (Nakanishi *et al* 1992). In contrast, Karasuyama *et al* found no synergy between IL-5 and IL-2 in either B cell maturation or growth induction (Karasuyama *et al* 1988). Therefore we sought to discover whether an increase in CD25 expression could induce IL-2 responsiveness in resting human tonsillar B cells.

3.2 The Induction of CD25 Expression in Resting Tonsillar B Lymphocytes.

The B lymphocytes used in this study were prepared from a single cell suspension of freshly spilled tonsillar cells with the removal of T lymphocytes by rosetting with AET-SRBC. The non-rosetting cells were then centrifuged over a discontinuous Percoll gradient and B cells collected from the 1.08/1.09 g/ml interface. This method routinely produced a population of cells that was greater than 95% B cells. B cells purified from the 1.08/1.09 g/ml interface are small and putatively resting in nature.

Following purification and prior to culture only a small proportion (typically 0.5-3%) of the B cell population expresses IL-2Rα (24, 25 , data not shown). Previous studies (Butcher *et al* 1990; Butcher and Cushley 1991; Zola *et al* 1991; Tomizawa *et al* 1991) have demonstrated that IL-4 can induce expression of IL-2Rα on human B cells. We sought to extend these studies by analysing the effect of ligation of surface Ig, CD40 and various cytokines upon IL-2Rα expression in the high density resting population of human tonsillar B cells.

In figure 3.1 resting tonsillar B lymphocytes were cultured with 100u/ml IL-4 and then analysed after 24 hours by two-colour flow cytometry. The dot-plot shows that while only 17.8% of the control population (unstimulated B Cells) express IL-2Rα on their surface, 34% of the IL-4 stimulated B cells express cell surface IL-2Rα. These data are consistent with previous observations that IL-4 induces IL-2Rα expression on resting tonsillar B cells. The ability of other cytokines to promote this effect was then studied.

Firstly the effects of IL-10 and IL-13, two cytokines with potent stimulatory effects towards B cells, were examined. IL-10 is a major immunoregulatory cytokine produced chiefly by T cells and monocytes. It has been shown to enhance expression of IL-2Rα on B cells which have been pre-stimulated with anti-CD40 antibody (Fluckiger *et al* 1993) and has also been shown to up-regulate IL-2Rα expression on cloned T cells of various phenotypes (Cohen *et al* 1994). IL-13 is a T cell derived cytokine which regulates human monocyte and B cell function. IL-13 and IL-4 share

certain functional characteristics including the induction of the macrophage-like dendritic cell morphology (te Velde *et al* 1988) with associated CD23 expression on monocytes (Vercelli *et al* 1988) and stimulation of Ig synthesis by B cells (Van Vlasselaar *et al* 1992). However **Figure 3.2** shows that neither IL-10 nor IL-13 were capable of inducing IL-2Rα expression at the concentrations shown. Dose response titrations were also done using a range of concentrations of these cytokines (data not illustrated) but neither showed an increase in IL-2Rα expression. The concentration ranges used were over at least 5 points and were of a range that had shown appreciable effects in other types of cells. A range of other cytokines was then studied to see if any of these were capable of inducing IL-2Rα expression (**Figure 3.3**). Of particular interest was IL-5 which, as stated previously, has been shown to increase IL-2Rα expression on resting murine B cells. However no other cytokine examined could induce IL-2Rα expression in human tonsillar B cells either at the concentration range shown in the experiment or indeed across a wider concentration range as before (data not illustrated).

Finally, levels of IL-2Rα expression on resting B cells cultured either with polyvalent anti-Ig or anti-CD40 antibodies were examined (**Figure 3.4**). Anti-CD40 antibody (Figure 3.4,panel D) induced a response of similar profile to that of IL-4; i.e. 28.2% of the B cell population were induced to express IL-2Rα on their cell surface. However culture of the cells with anti-Ig antibodies drove a response of considerably greater magnitude. As seen in Figure 3.4, panel C, 68.4% of the B cell population were induced to express IL-2Rα. Although the absolute level of IL-2Rα expression induced varied between individual tonsillar preparations, IL-4, anti-Ig and anti-CD40

consistently increased IL-2Rα expression on high density B cells and anti-Ig routinely induced the greatest number of B cells to become IL-2Rα positive.

3.3 The Effectiveness of IL-4, Anti-Ig and Anti-CD40 Induction of IL-2Rα Expression.

The concentrations of IL-4, anti-Ig and anti-CD40 required to drive half-maximal expression of IL-2Rα were evaluated by testing the response of B cells across a wide concentration range of each stimulus; the concentration of each stimulus which elicited half-maximal elevation of IL-2Rα expression (i.e. the EC_{50} dose) was also determined (Figure 3.5). IL-4, anti-Ig and anti-CD40 all increase IL-2Rα expression in a dose dependent manner; thus the number of B cells becoming positive for IL-2Rα increased in proportion to the concentration of IL-4, anti-Ig or anti-CD40. For IL-4, the EC_{50} was of the order of 40-50 units/ml IL-4 (Figure 3.5, panel A) which is in good agreement with previous data from this and other laboratories. Dose-response titrations of the effects of anti-Ig (Figure 3.5, panel B) and anti-CD40 (Figure3.5, panel C) revealed that the EC_{50} values for these stimuli were approximately 10 μg/ml and 0.2μg/ml, respectively.

These dose-response curves were converted to molar concentrations (Figure 3.6, panel A) to compare the potency of stimuli. This revealed that IL-4 is by far the most potent inducer of IL-2Rα expression in resting human B cells. The amounts of anti-Ig or anti-CD40 stimuli required to effect half-maximal stimulation were 2-3 orders of magnitude greater than that observed with the cytokine. Finally, Figure 3.6, panel B

notes the maximal IL-2Rα response which could be induced by each stimulus. Anti-Ig consistently drives the greatest proportion of B lymphocytes to express IL-2Rα (60-80%). IL-4 and anti-CD40 induced broadly similar responses; they both induced 20-25% of B cells to become IL-2Rα positive.

3.4 The Effects of IL-4, Anti-Ig and Anti-CD40 Combinations on IL-2Rα Expression.

Treatment of resting tonsillar B cells with each of IL-4 , anti-Ig or anti-CD40 increased IL-2Rα expression. In the following studies, the effects of these factors in combination on IL-2Rα levels was studied. Figure 3.7, panel A shows CD25 expression in cells cultured over a range of concentrations of anti-Ig in the presence or absence of IL-4. An IL-4 concentration of 10 units/ml was employed as this induces submaximal IL-2Rα up-regulation. The data show that IL-2Rα expression is increased in a dose dependent manner. IL-4 recruited approximately 10% of B lymphocytes to the IL-2Rα response. Modest doses (1-5μg/ml) of anti-Ig showed a modest additive effect in the presence of IL-4. However, at the maximum concentration of anti-Ig (25μg/ml) IL-2Rα was expressed by 60% of the B cell population and IL-4 did not enhance receptor expression. In conclusion, the combination of submaximal levels of IL-4 and anti-Ig increased IL-2Rα in only an additive manner and no synergistic effects were apparent.

Figure 3.7, panel B shows the combined effects of anti-CD40 and IL-4. Again there was an increase in IL-2Rα levels in response to increasing concentrations of anti-CD40. This was seen whether IL-4 was present or not. The same concentration of IL-4 was used as in figure 3.7A; again 10 units/ml of IL-4 alone elevated IL-2Rα expression by approximately 10%. In combinations of anti-CD40 over the concentration range of 0.1-6.4 μg/ml with IL-4 the levels of IL-2Rα were enhanced by approximately 5% of that expected for a purely additive effect.

A third study was performed to assess the combined effects of anti-Ig and anti-CD40 on IL-2Rα expression by B cells (Figure 3.7, panel C). Anti-Ig was titrated over a concentration range of 0-25 μg/ml where 1-5 μg/ml gave submaximal stimulation of IL-2Rα expression. On culture of B cells with anti-CD40 antibody (0.5 μg/ml) alone IL-2Rα expression was seen to increase by approximately 15%. The combination of anti-Ig and anti-CD40 antibodies gave an additive effect. At lower concentrations of anti-Ig, IL-2Rα expression increased by approximately 15% on addition of anti-CD40. However at 25 μg/ml of anti-Ig, addition of anti-CD40 increased IL-2Rα levels by only 5%. This suggests that IL-2Rα had been induced to the maximum level by anti-Ig stimulation and that no further up-regulation could be achieved.

3.5 The Effects of Anti-Ig antibodies, Anti-CD40 antibodies and IL-4 in combination with IL-2 on B cell proliferation.

Resting, human B lymphocytes can be induced to express IL-2Rα by culturing them with anti-Ig antibodies, anti-CD40 antibodies and IL-4. For B cells to proliferate and differentiate in response to physiological levels of IL-2 they must express the high affinity form of the IL-2R consisting of the constitutively expressed β and γ_c chains, in association with the inducible α chain. IL-2Rα expression is not only essential for this but also confers specificity on the receptor for IL-2 as the β and γ_c are shared with the IL-15R. To determine whether increased IL-2Rα expression results in significant proliferation of resting B cells, we stimulated the cells with a range of concentrations of anti-Ig, anti-CD40 and IL-4 in the presence or absence of IL-2.

Figure 3.8, panel A shows that IL-2 and anti-Ig synergise to induce a strong proliferative response in the resting B cell population. Anti-Ig, in the presence of IL-2 increased proliferation in a dose dependent manner. Cross-linking of surface Ig alone or in the presence of IL-4 showed a weak proliferative response. Figure 3.8, panel B illustrates that anti-CD40 in the presence of IL-2 increases proliferation in a dose dependent fashion at low concentrations. However the strong proliferative response seen on stimulation of cells with anti-Ig antibodies and IL-2 was not observed; indeed high concentrations of anti-CD40 antibodies (3.2-6.4μg/ml) in combination with IL-2 exerted an anti-proliferative effect on the cells. Anti-CD40 antibodies alone or in the presence of IL-4 were unable to induce the cells to proliferate.

Figure 3.8 panel C shows the effects of IL-4 upon B cell proliferation. A range of concentrations of IL-4 had no effect on the proliferative response of the resting B

lymphocytes. In the presence of IL-2, slight proliferation was observed at 1-10 units/ml IL-4 but this was negligible compared to the strong proliferative response seen with anti-Ig and IL-2 and did not even compare to that seen with anti-CD40 antibodies in the presence of IL-2.

3.6 Discussion

The data from this chapter support previous observations (Butcher *et al* 1990; Butcher and Cushley 1991; Zola *et al* 1991; Tomizawa *et al* 1991) that IL-4 is the only cytokine which appears capable of driving IL-2Rα expression in resting human B lymphocytes. However, the data also indicate that cross-linking of the antigen receptor complex or of the CD40 antigen, structures which have a positive influence on B cell activation, also results in enhanced IL-2Rα expression. Stimulation of B lymphocytes with combinations of anti-Ig, anti-CD40 and IL-4 resulted in some additive effects at the level of IL-2Rα expression but no striking synergistic effects were noted. Furthermore, the data demonstrate that anti-Ig induces IL-2 responsiveness. Anti-Ig and IL-2 synergised to produce a strong proliferative response. A lesser response was seen when the resting B cells were treated with anti-CD40 mAb and IL-2 and there was minimal proliferation when a combination of IL-4 and IL-2 were used.

IL-2Rα expression is inducible in both T and B lymphocytes, and available data suggest that non-overlapping groups of cytokines regulate expression of the IL-2Rα gene in the two lymphoid subsets. The data from this chapter confirms this theory.

Thus IL-1α, TNF-α and IL-6 are all known to promote IL-2Rα expression in T lymphocytes (Greene *et al* 1989; Clipstone and Crabtree 1994) but from experiments performed in this laboratory, appear to have no effect on quiescent B cells; similarly IL-4 has no effect upon IL-2Rα levels in quiescent tonsillar T lymphocytes (Butcher *et al* 1990).

Experiments were done to investigate whether IL-10 and IL-13 were capable of promoting IL-2Rα expression in quiescent B lymphocytes. IL-10 is produced chiefly by T cells and monocytes. It was originally designated "cytokine synthesis inhibitory factor" as it inhibits the production of Th1 type cytokines and antigen and mitogen specific T cell activation (Fiorentino *et al* 1991; De-Waal-Maleft *et al* 1991; Taga and Tosatol 1992; Del-Prete *et al* 1993). However, in the human system IL-10 stimulates DNA synthesis of tonsillar B cells which have been activated either via their antigen receptor or via cross-linking of surface CD40 antigen (Rousset *et al* 1992). It has also been reported that IL-2 and IL-10 synergise to induce the proliferation and differentiation of normal and leukaemic B cells activated by anti-CD40 by upregulating the expression of high affinity IL-2 receptors (Fluckiger *et al* 1993).

The spectrum of biological activities of IL-13 largely overlaps those previously ascribed to IL-4; i.e. it enhances expression of CD23 and class II MHC antigens on resting B cells. It also stimulates B cell proliferation in combination with anti-Ig and anti-CD40 antibodies and it induces IgE synthesis (DeFrance *et al* 1994). However, neither IL-10 nor IL-13 could induce IL-2Rα expression in resting B cells.

Not only is the regulation of IL-2Rα lineage specific, it also appears to be species specific. In the murine system, IL-2Rα is up-regulated by IL-5 (Loughnan and Nossal 1989). While IL-5 has clear-cut activities as an eosinophil differentiating factor in mouse and man, there is some controversy concerning the role of IL-5 in B cell differentiation. A number of experimental systems have shown the effects of IL-5 on human B cells (Pene *et al* 1988) whereas others have not (Clutterbuck *et al* 1987). However there is no doubt that IL-5 has no effect on the induction of IL-2Rα in human quiescent B cells.

The data suggest that the 140kDa IL-4 receptor element (Idzerda *et al* 1990) possesses critical structural motifs involved in translation of the IL-4 binding event to activation of IL-2Rα transcription. A role for the γ_c chain in this process would seem less likely as the IL-2 and IL-7 receptor complexes include γ_c and neither of these cytokines promotes IL-2Rα expression in quiescent B cells. It was of interest to discover whether IL-15, a newly discovered cytokine, would have any effect on IL-2Rα induction. Subsequent results in this laboratory indicated that IL-15 has no effect on IL-2Rα expression in human B lymphocytes. IL-15 shares biological activities with IL-2 and can also interact with the β and γ components of the IL-2R complex (Giri *et al* 1994). The discovery of an IL-15 specific binding protein (Giri *et al* 1995) further extended the analogy between IL-2 and IL-15 and their receptor systems. The receptor for IL-15 shares structural similarities with the IL-2Rα subunit. The α chains of the IL-15R and the IL-2R have defined a new family of binding proteins for helical cytokines. It was previously thought that the sole role for CD25 was to generate the

high affinity receptor for IL-2; however, it is also essential in generating the specific receptor for IL-2 since complexes of β and γ_c chains lacking the α chain can also comprise the IL-15 receptor.

CD40 cross-linking induces a similar number of B cells to express IL-2Rα, approximately 25-30%, as observed with IL-4 stimulation. However, only a single anti-CD40 mAb was used in this series of experiments and is clear from a number of studies in murine models the two rat mAbs directed against distinct CD40 epitopes can promote different responses. Thus, one of the two anti-CD40 reagents caused both upregulation of various B cell surface markers and cellular proliferation, while the other failed to do so (although this reagent could synergise with submitogenic amounts of anti-IgM to promote B cell proliferation) (Heath *et al* 1994). Therefore, while the data from this chapter indicate that anti-CD40 mAb can promote IL-2Rα expression in a small subset of quiescent tonsillar B cells, it is clearly possible that mAbs to distinct epitopes may give a different response. Thus, Burlinson *et al* investigated whether the CD40 ligand itself induced expression of IL-2Rα. Their data indicated that both ligation of CD40 with either trimeric soluble CD40 or anti-CD40 mAb induced expression of IL-2Rα to broadly similar levels (Burlinson *et al* 1996).

Stimulation of B lymphocytes with anti-Ig resulted in more than 80% of the quiescent cells acquiring a IL-2Rα positive phenotype. This situation has parallels with anti-CD3 driven IL-2Rα expression in T lymphocytes or in Jurkat cells, where stimulation with anti-CD3ε or anti-TcR reagents is a potent inducer of transcription through the

IL-2Rα locus (reviewed in Greene *et al* 1989; Clipstone and Crabtree 1994). This result is entirely in keeping with the cells responding to a committing stimulation with antigen and expressing receptors for cytokines which can shape their subsequent response to that antigen; in the case of B cells, expression of IL-2 receptors permits the cells to display enhanced growth and/or accelerated immunoglobulin secretion in response to binding IL-2.

Although anti-Ig, anti-CD40 and IL-4 can all up-regulate IL-2Rα expression, this elevation in IL-2Rα levels does not necessarily facilitate subsequent B cell proliferation in the presence of IL-2. This suggests that IL-2Rα induction under these circumstances either fails to form a functional high affinity IL-2R or, more likely, that the ligation of the high affinity heterotrimeric IL-2R by IL-2 does not fully stimulate mitogenic signal transduction pathways within resting B cells. Cross-linking of sIg in combination with IL-2 induces proliferation in resting B cells. As stated cross-linking of the antigen receptor also induces up to 80% of cells to express IL-2Rα. Thus, cross-linking of sIg induces the formation of functional high affinity IL-2Rs and induces the B cells to be responsive to IL-2. A combination of IL-2 and anti-Ig may reflect some physiological significance; extensive cross-linking of sIg on B cells which are in the germinal centre is likely to occur through interaction with follicular dendritic cells (FDC). This response indicates a requirement for highly repetitive or polyvalent ligands, such as antigen in immune complexes on FDC. The data are consistent with a general model of B cell activation whereby there is synergy between cellular interactions and cytokines.

In contrast, anti-CD40 did not synergise with IL-2 to produce a strong proliferative response. However there was an increase in proliferation seen when cells were stimulated with a combination of IL-2 and anti-CD40, suggesting perhaps that a small population of B cells could be induced to express IL-2Rα and respond to IL-2 as a result of stimulation with anti-CD40.

IL-4, while inducing 25-30% of cells to express IL-2Rα, did not synergise with IL-2 to produce proliferation. These data taken together suggests that the IL-4-driven increase in expression of IL-2Rα may be independent of B lymphocyte proliferation.

One of the most striking findings of this chapter is that on stimulating the B cells via their antigen receptor, up to 80% of them were induced to express cell surface IL-2Rα. The following chapter expands upon this finding to determine whether different isotypes of anti-Ig antibodies could have different effects on the up-regulation of IL-2Rα and whether the degree of cross-linking of the antigen receptor could alter IL-2Rα expression. In addition, by using $F(ab')_2$ fragments of these antibodies, it was possible to discover whether Fc receptor-mediated inhibition (which is explained fully in Chapter 4) plays a role in the expression of IL-2Rα in resting human B cells.

Figure 3.1 The Effect of IL-4 on IL-2Rα Expression.

High density resting tonsillar B cells were cultured with 100 Units/ml IL-4 in complete medium for 24 hours. The cells were stained with FITC-anti-CD25 plus PE-anti-CD19 for 30 minutes on ice. After washing, propidium iodide was added to a final concentration of 2μg/ml to facilitate exclusion of dead cells and 5000 live gate events collected. The two colour dot-plots show green fluorescence (FITC) on the x-axis and red fluorescence (PE) on the y-axis. The percentage of B lymphocytes positive for CD25 was calculated as previously described (Butcher *et al* 1990) according to the following equation:

$$\% \text{ CD25}^+ \text{ B Cells} = \frac{\text{CD25}^+ / \text{CD19}^+ \text{ Cells}}{(\text{CD25}^- / \text{CD19}^+ \text{ cells}) + (\text{CD25}^+ / \text{CD19}^+ \text{ cells})} \times 100$$

Panel A: Control (17%)

Panel B: 100 U/ml IL-4 (34%)

The figures shown in brackets are the percentage of B cells positive for CD25 expression.

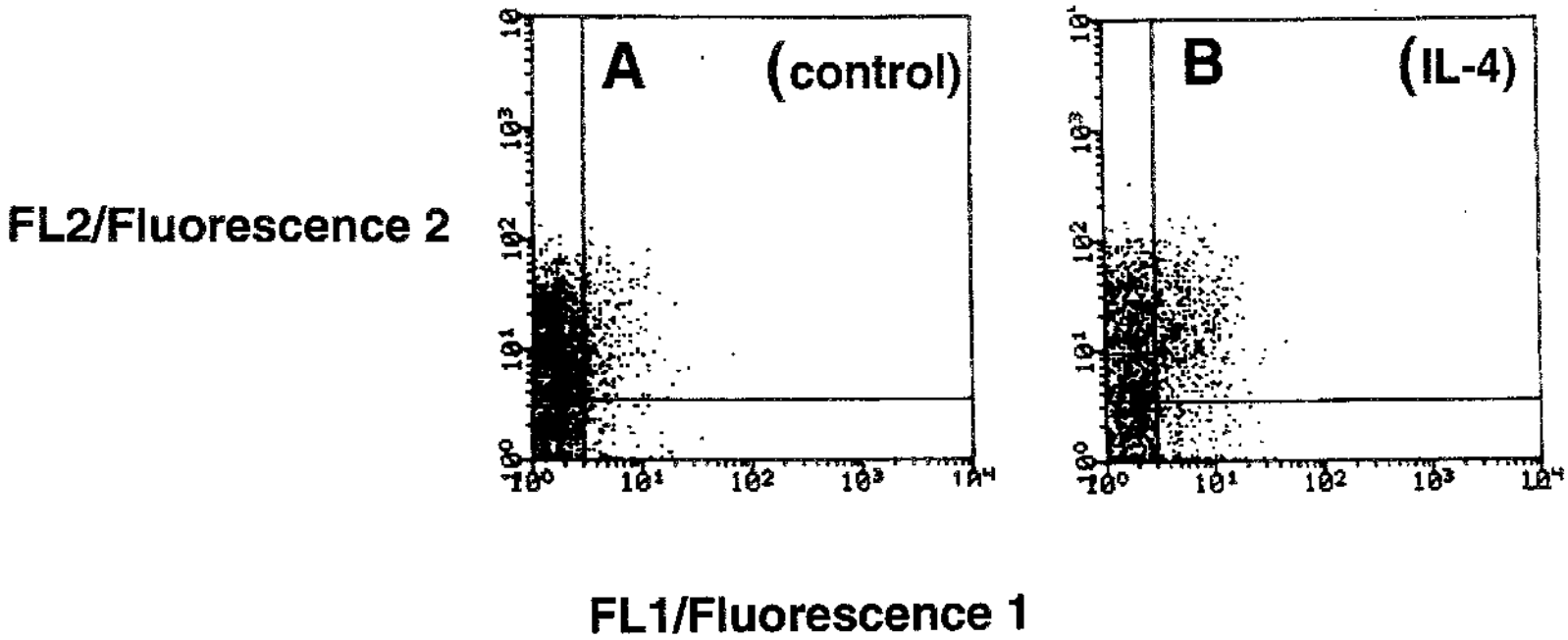

A
(control)
B
(IL-4)
FL2/Fluorescence 2
FL1/Fluorescence 1

Figure 3.2 The Effect of IL-10 and IL-13 upon IL-2Rα Expression.

High density resting tonsillar B cells were cultured with the appropriate cytokines and then stained and the percentage CD25 positive B cells determined as described in the legend to Figure 3.1. The data shown for IL-10 and IL-13 are from independent experiments. Panel A: Control (15.1%); Panel B 100 U/ml IL-10 (18.4%); Panel C: Control (4.5%); Panel D: 100 ng/ml IL-13 (5.4%). Again, the figures shown in brackets are the percentage of B lymphocytes positive for CD25 expression. These experiments were performed 3 times; no statistical analysis was done on the results as it is not possible to compare one tonsillar population with another.

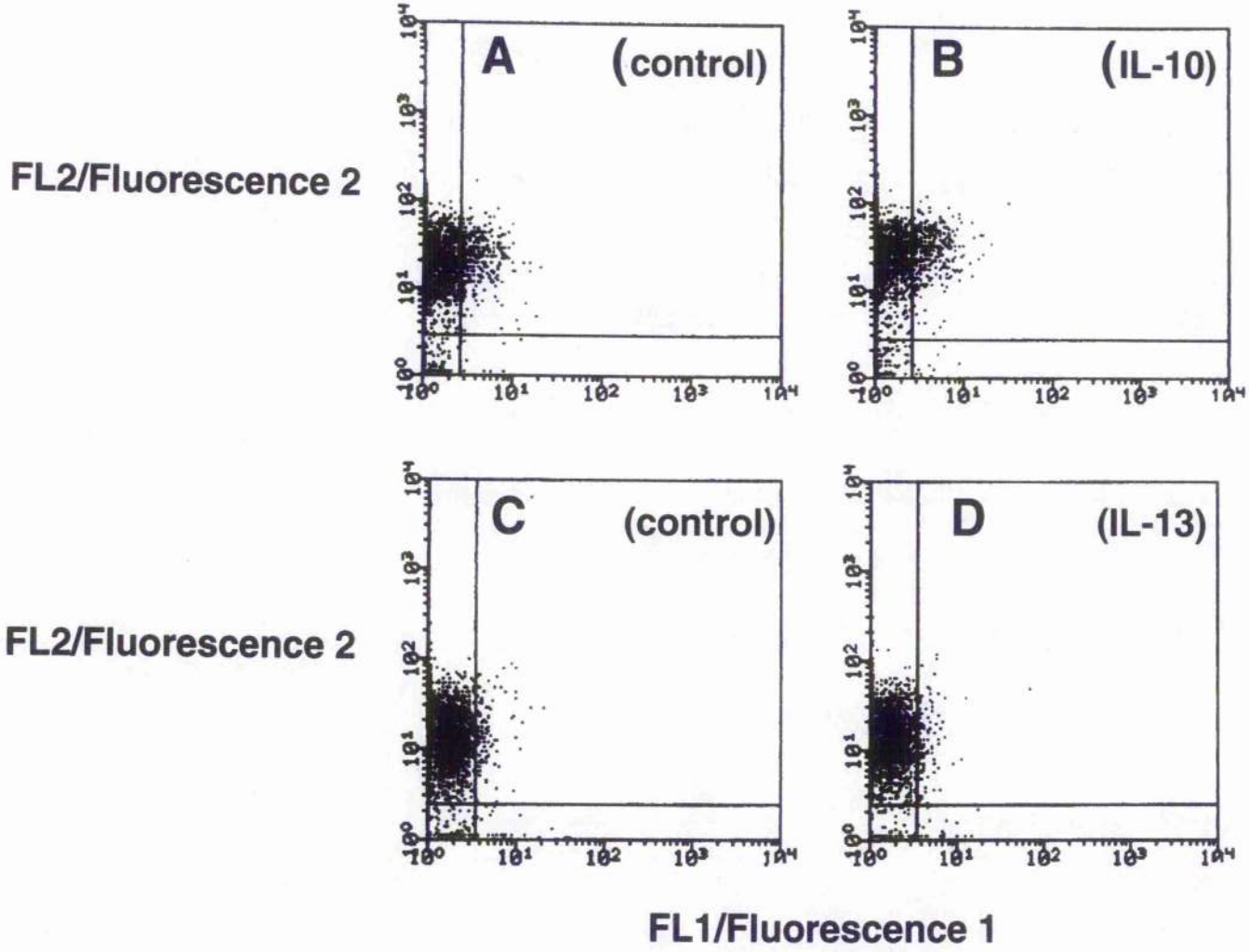

A
(control)
B
(IL-10)
C
(control)
D
(IL-13)
FL2/Fluorescence 2
FL2/Fluorescence 2
FL1/Fluorescence 1

Figure 3.3 The Effect of Various Recombinant Cytokines upon IL-2Rα Expression.

High density resting B cells (not all from the same tonsillar preparation) were cultured with a range of recombinant human cytokines as detailed on the bar chart. The cells were stained and the percentage of CD25 positive B cells was determined as described in the legend to Figure 3.1. 3% of cells cultured in medium alone will express CD25 (data not shown). Each cytokine was tested at least twice on independent tonsillar preparations.

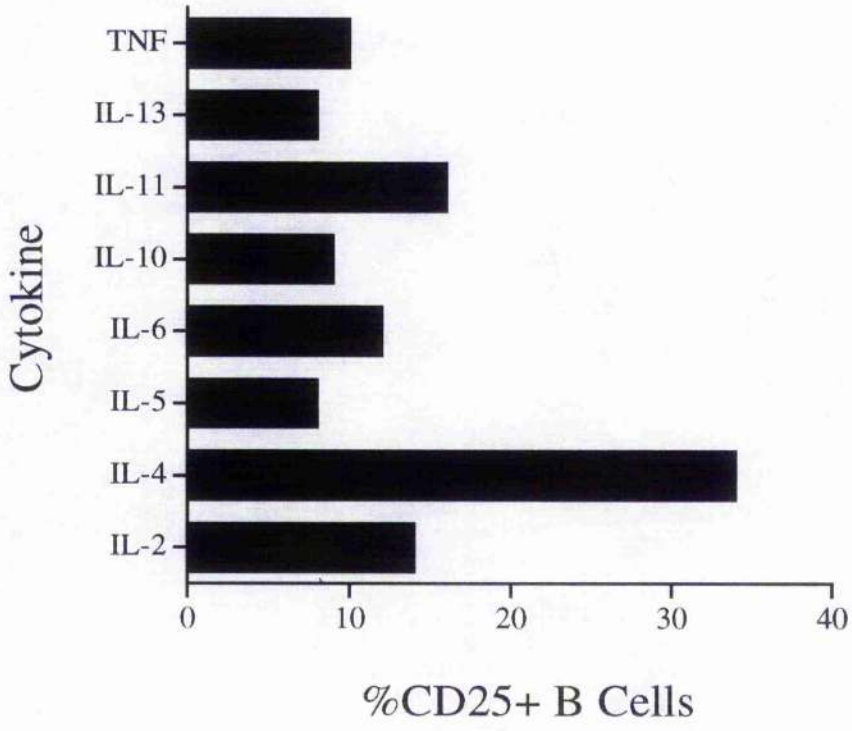

Cytokine
TNF
IL-13
IL-11
IL-10
IL-6
IL-5
IL-4
IL-2
0
10
20
30
40
%CD25+ B Cells

Figure 3.4 The Induction of IL-2Rα by Different Stimuli.

High density resting tonsillar B cells were cultured with the indicated stimuli for 24 hours in complete medium, stained and the percentage of CD25 positive B cells determined as described in the legend to Figure 3.1. Panel A: Control (17.8%); Panel B: 25 μg/ml Polyvalent anti-Ig (68.4%); Panel C: 1 μg/ml anti-CD40 (28.2%). Again, the figures shown in brackets are the percentage of B lymphocytes positive for CD25 expression.

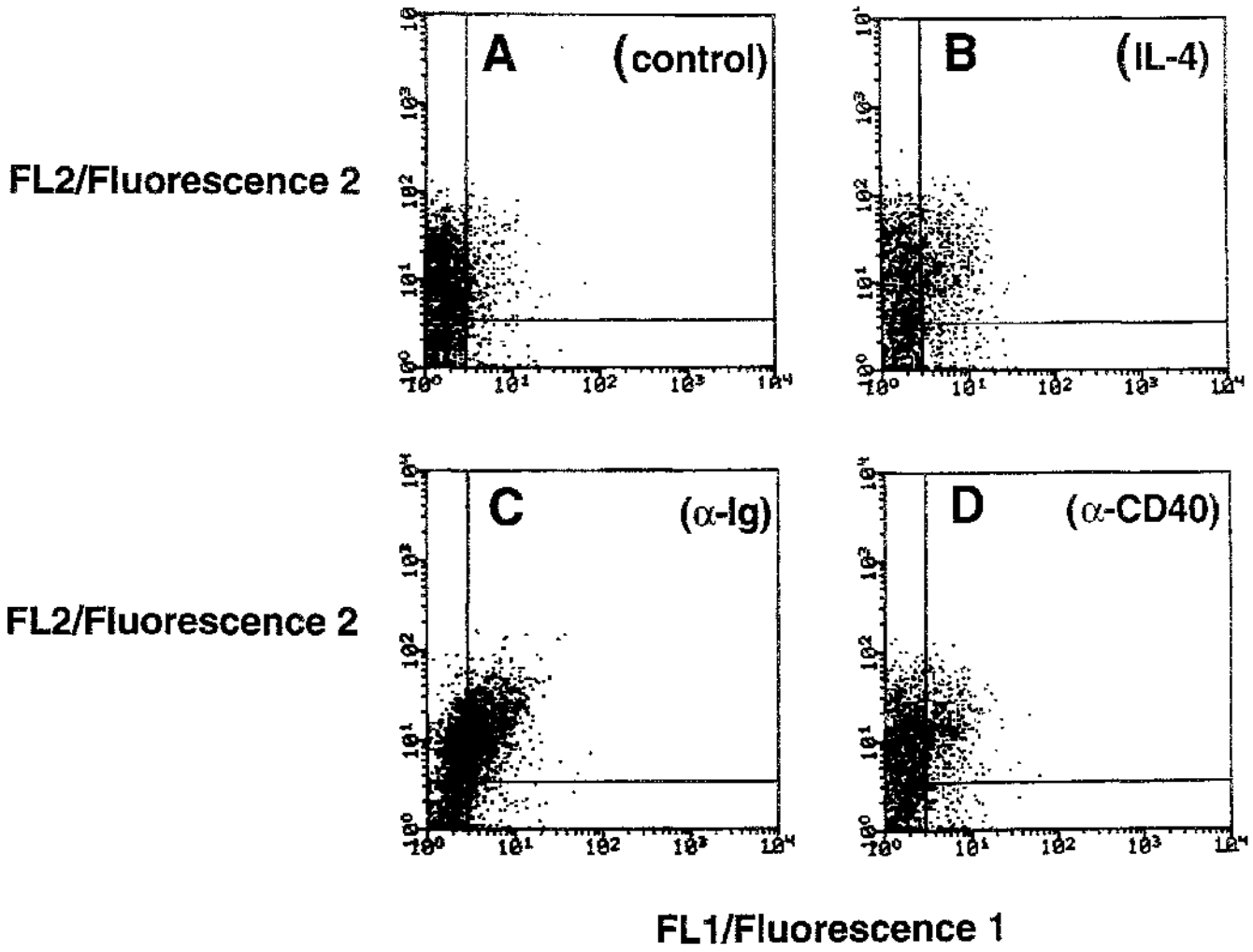

A (control)
B (IL-4)
C (α-Ig)
D (α-CD40)
FL2/Fluorescence 2
FL2/Fluorescence 2
FL1/Fluorescence 1

Figure 3.5 Dose Response Titrations for IL-4, Anti-Ig and Anti-CD40.

High density resting tonsillar B cells were cultured with the indicated concentrations of IL-4 (A), anti-Ig (B) or anti-CD40 (C). CD25 expression was determined by flow cytometry as described in the legend to Figure 3.1. The data shown are representative of 6 independent tonsillar B cell preparations.

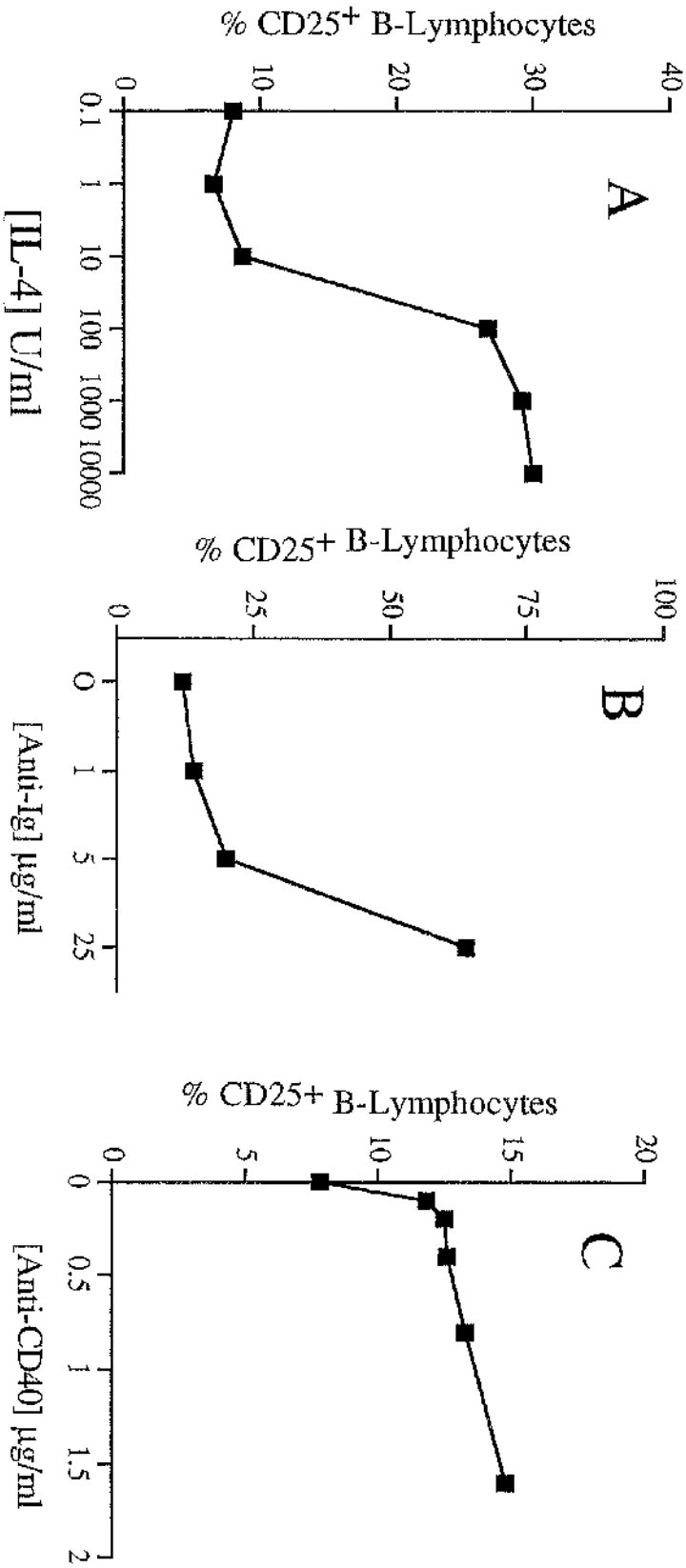
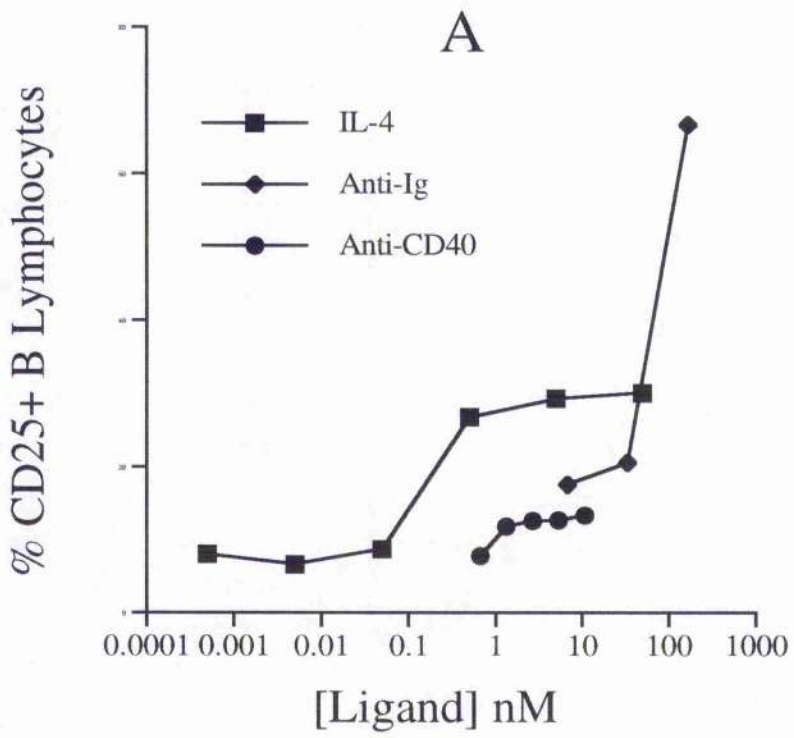

% CD25+ B-Lymphocytes
0
10
20
30
40
A
0.1
1
10
100
1000
10000
[IL-4] U/ml
% CD25+ B-Lymphocytes
0
25
50
75
100
B
0
1
5
25
[Anti-Ig] µg/ml
% CD25+ B-Lymphocytes
0
5
10
15
20
C
0
0.5
1
1.5
2
[Anti-CD40] µg/ml

Figure 3.6 Comparison of Potency of Stimuli.

The percentage of CD25 positive B cells was calculated over a range of IL-4, anti-Ig and anti-CD40 concentrations as described in the legend to Figure 3.1 and the data expressed in terms of the molar concentrations of each stimulus applied (Panel A). Panel B notes the maximal CD25 response induced by each stimulus.


A
% CD25+ B Lymphocytes
IL-4
Anti-Ig
Anti-CD40
0.0001 0.001 0.01 0.1 1 10 100 1000
[Ligand] nM

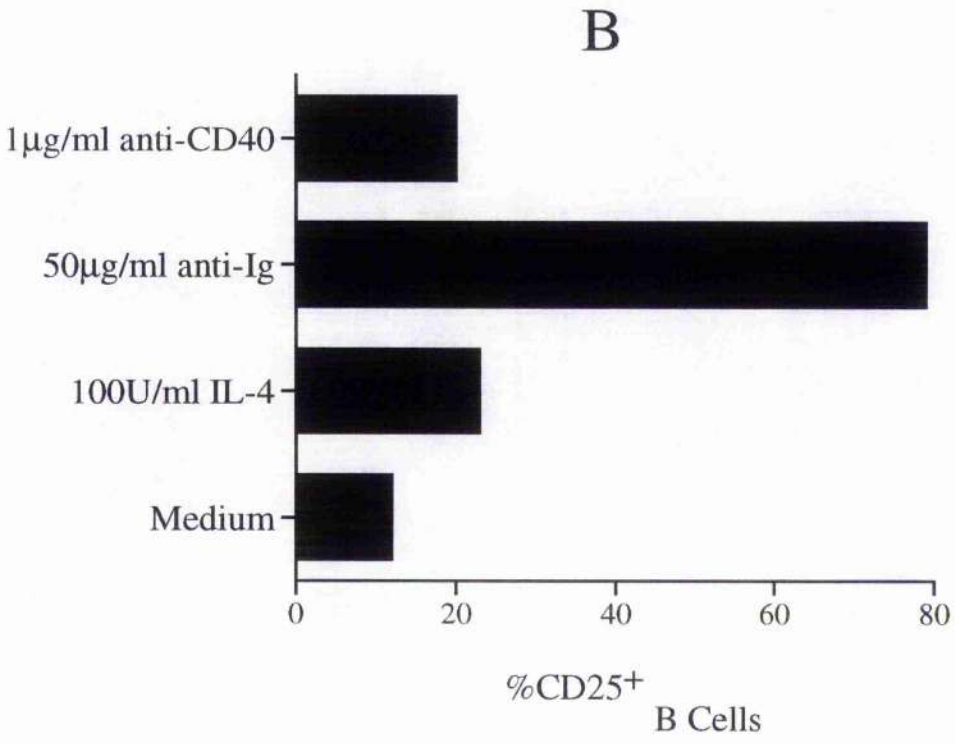

B
1µg/ml anti-CD40
50µg/ml anti-Ig
100U/ml IL-4
Medium
0 20 40 60 80
%CD25+ B Cells

Figure 3.7 The Effect of Combinations of IL-4, Anti-Ig and Anti-CD40 upon IL-2Rα Expression.

High density resting tonsillar B cells were cultured for 24 hours with either anti-Ig (A) or anti-CD40 (B) at the concentrations indicated in the presence and absence of IL-4 (10 U/ml). In addition, anti-Ig was titrated in the presence or absence of anti-CD40 (0.5 μg/ml). The percentage of CD25 positive B cells was assessed as described in the legend to Figure 3.1.

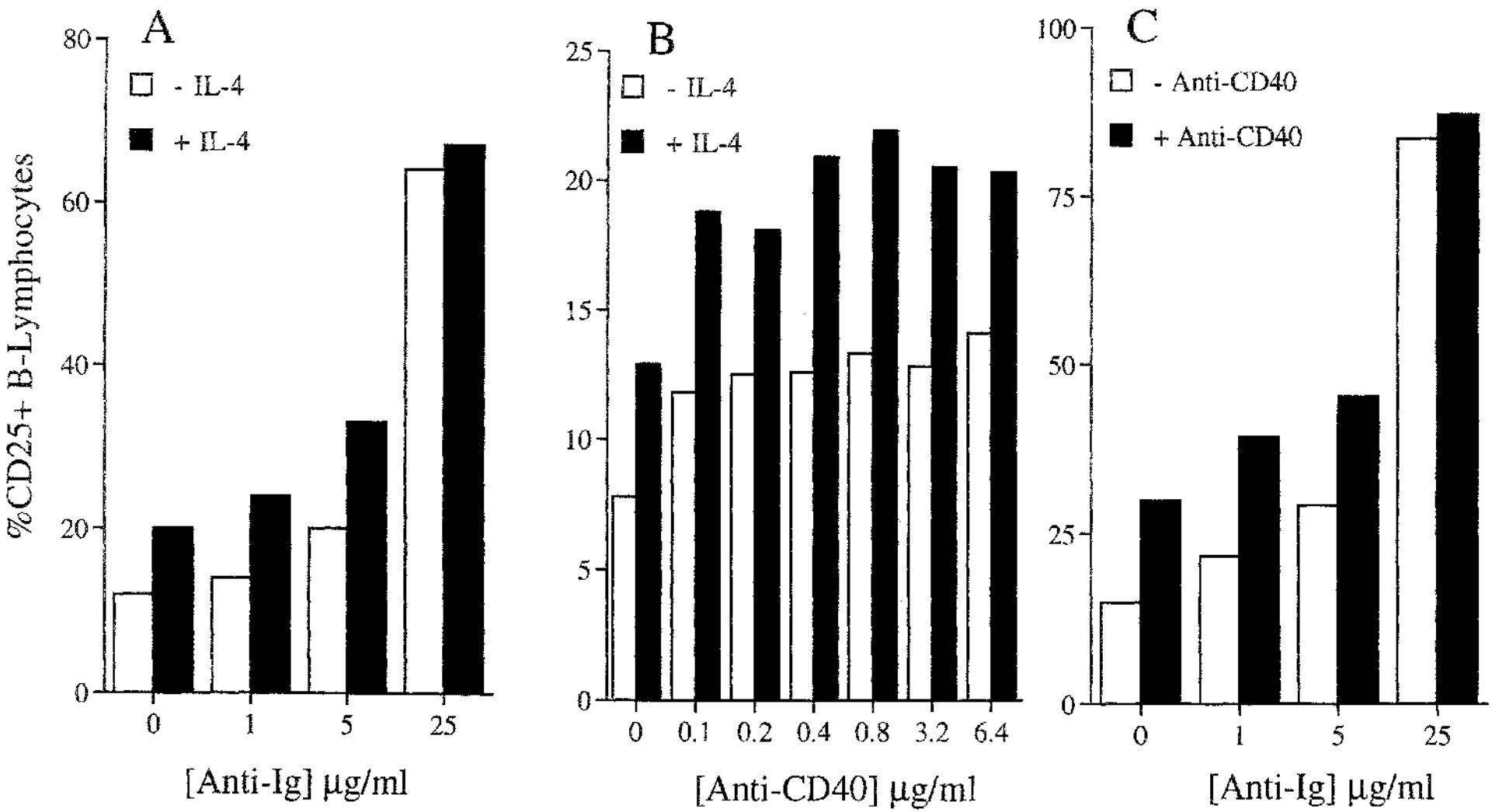

A
- IL-4
+ IL-4
%CD25+ B-Lymphocytes
80
60
40
20
0
0
1
5
25
[Anti-Ig] µg/ml
B
- IL-4
+ IL-4
25
20
15
10
5
0
0
0.1
0.2
0.4
0.8
3.2
6.4
[Anti-CD40] µg/ml
C
- Anti-CD40
+ Anti-CD40
100
75
50
25
0
0
1
5
25
[Anti-Ig] µg/ml

Figure 3.8 The Effect of anti-Ig, anti-CD40 and IL-4 in combination with IL-2 on B Cell Proliferation

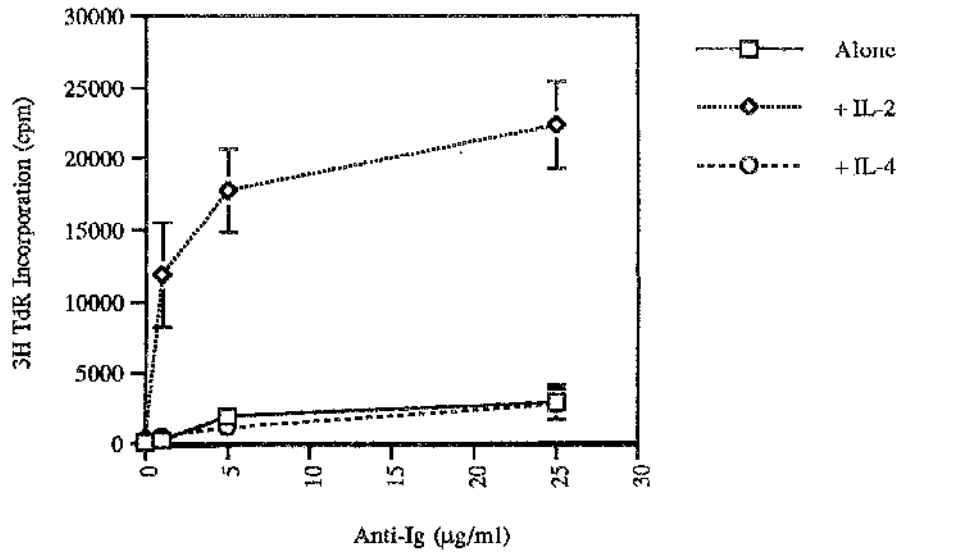
Resting tonsillar human B cells were cultured for 72 hours in complete medium with the following range of stimulants:

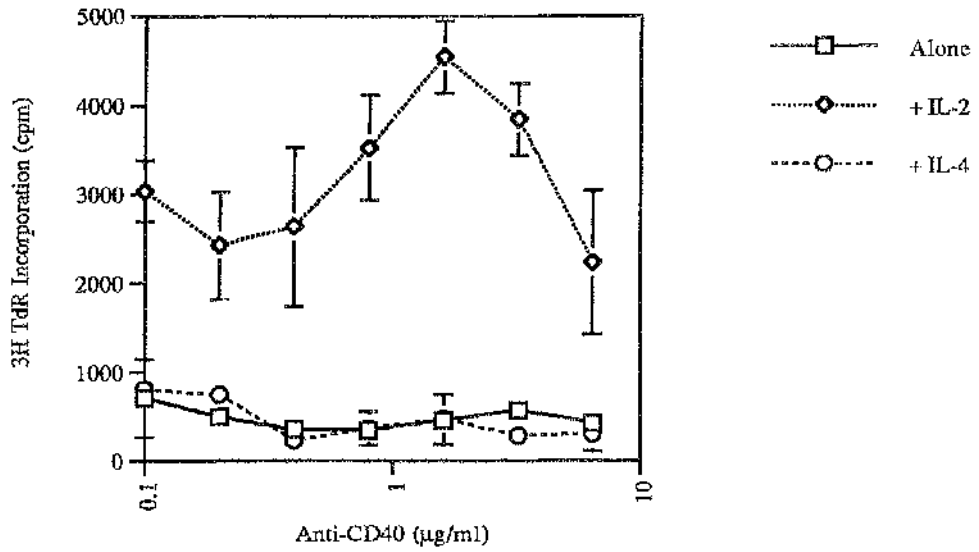
Panel A:0-25μg/ml anti-Ig alone, with 50 units/ml IL-4 or with 50 units/ml IL-2.

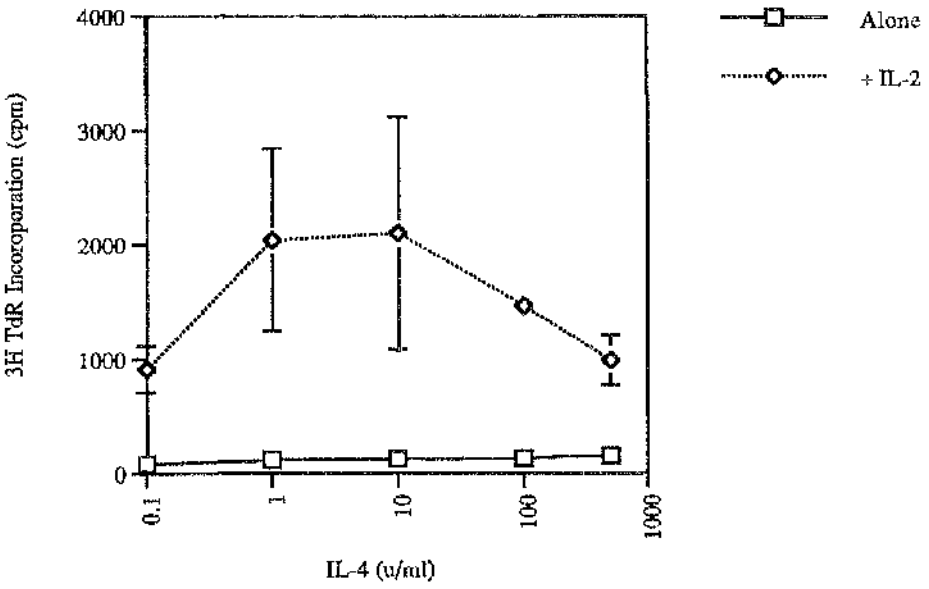
Panel B: 0-6.4μg/ml anti-CD40 alone, with 50 units/ml IL-4 or with 50 units/ml IL-2.

Panel C: 0-1000 units/ml IL-4 either alone or with 50 units/ml IL-2.

Following this, the cells were pulsed with 0.5μCi of methyl-[^{3}H]-thymidine, incubated for 8 hours and then harvested. [^{3}H]-thymidine incorporation is expressed as counts per minute (cpm) and each well was set up in triplicate so that the standard deviation (SD) could be calculated.


30000
25000
20000
15000
10000
5000
0
3H TdR Incorporation (cpm)
0
5
10
15
20
25
30
Anti-Ig (µg/ml)
Alone
+ IL-2
+ IL-4


5000
4000
3000
2000
1000
0
3H TdR Incorporation (cpm)
0.1
1
10
Anti-CD40 (µg/ml)
Alone
+ IL-2
+ IL-4


4000
3000
2000
1000
0
3H TdR Incorporation (cpm)
0.1
1
10
100
1000
IL-4 (u/ml)
Alone
+ IL-2

Chapter 4

Different Isotypes of Anti-Immunoglobulin have Different Effects on the Induction of IL-2Rα in Resting Tonsillar B Cells

4.1 Introduction

A common means of activating B cells so that they are able to respond to cytokines is to stimulate them via their surface immunoglobulin receptors. Experiments in Chapter 3 showed that stimulation of tonsillar B lymphocytes with polyvalent anti-immunoglobulin resulted in more than 80% of the cells acquiring a $CD25^+$ phenotype. This is entirely in keeping with the cells responding to a committing stimulation with antigen and expressing receptors to cytokines which can shape their subsequent response to that antigen. The majority of cells isolated from tonsils are $mIgM^+$ /$mIgD^{++}$ with relatively low numbers of $mIgG^+$ B cells in the tonsillar population. Thus, it was important to determine whether each population of cells could increase expression of IL-2Rα on their cells and the magnitude of this response.

Most mature B lymphocytes co-express two classes of antigen receptor, IgM and IgD. Differences in the signal transduction pathways between them remain a matter of controversy. Both classes of receptor have the same short cytoplasmic sequence (KVK) and are expected to communicate with the same intracellular molecules. Several studies suggest that the engagement of mIgM and mIgD induce similar B cell responses (Steckmann 1980; Mond *et al* 1981; Brink *et al* 1992). This is true for early biochemical responses such as the activation of PTK (Gold *et al* 1990; Burkhardt *et al* 1991) and hydrolysis of inositol phospholipids (Klaus *et al* 1989; Harnett *et al* 1989). Other studies show that mIgM but not mIgD can induce negative responses in B cells such as anergy and apoptosis (Bell and Goodnow 1994; Tisch *et al* 1988; Ales-Martinez *et al* 1992; Kim *et al* 1991, 1992; Carsetti *et al* 1993). Thus it was important

to determine whether stimulation of mIgM or mIgD could induce the B cells to express IL-2Rα, permitting them to respond to IL-2 and causing them to proliferate and differentiate.

Membrane IgG-bearing cells constitute a more mature memory B cell population (reviewed in Vitetta *et al* 1991; Mackay 1993). It has been suggested that memory B cells are derived from a precursor subset that is distinct from precursors of primary or mIgM-bearing cells (Linton *et al* 1989). Many early second messenger pathways are similar in mIgM and mIgG positive cells (Mittelstadt and Defranco 1993; Gold *et al* 1992; Tamaki *et al* 1992; Choquet *et al* 1993) but there are also significant differences. In addition, unlike mIgM and mIgD, mIgG has a cytoplasmic tail of 28 amino acids. Thus, experiments were carried out to determine whether memory B cells were capable of inducing surface IL-2Rα.

Receptors specifically recognising and binding to the Fc part of IgG (FcγR) are members of the immunoglobulin superfamily. Three main types of FcγR have been described; the FcγRI, FcγRII and FcγRIII. Of these, FcγRII (also known as CD32) are expressed on the surface of B cells (Ravetch and Kinet 1991). There are six different transcripts of FcγRII (Van de Winkel and Capel 1993) and FcγRIIb is the major isoform represented on human B cells. It is known to regulate B cell responses. In fact, activated B cells are subject to negative regulation by circulating antibodies which bind to FcγRII (Hunziker *et al* 1990). Binding to the FcRII sends a dominant negative signal to the B lymphocyte that prevents or aborts lymphocyte activation (Klaus *et al* 1984) triggered through the ARH1 motifs of the signal transduction units,

Igα and Igβ (Muta *et al* 1994). Thus, it was necessary to compare the effect of intact anti-Ig antibodies with F(ab'), anti-Ig antibodies on IL-2Rα induction. Intact antibodies co-cross-link the sIg and the Fc receptor whereas $F(ab')_2$ antibodies lack the Fc fragment which contains the sites for binding to the Fc receptor. It was important to determine whether the IL-2 responsiveness induced on the B cell by crossing of surface Ig was subject to negative feedback regulation by Fc receptor binding.

Whether a sIg-mediated signal results in activation of a B cell has been suggested to depend upon several factors; the state of differentiation of the B cell is important (Defranco *et al* 1982; Boyd *et al* 1981; Scott *et al* 1985; Hasbold and Klaus 1990) as is the presence of certain lymphokines (Pecanha *et al* 1991). In addition, the isotype of the sIg delivering the signal may be crucial (Goodnow *et al* 1989; Tisch *et al* 1988; Ales-Martinez *et al* 1988) and finally, the degree of cross-linking achieved by a particular ligand is also a factor (Pecanha *et al* 1991; Russell *et al* 1991). Therefore, in this chapter we sought to address whether the isotype of sIg delivering the signal is important in inducing expression of IL-2Rα and whether there is an Fc-mediated inhibition of this induction on resting B cells.

4.2 Different Isotypes of Anti-Immunoglobulin Antibodies Have Significantly Different Effects on the Induction of IL-2Rα Expression in Resting Tonsillar B Cells.

In Chapter 3 we determined that stimulation of resting B cells with polyvalent anti-Ig caused a massive induction of IL-2Rα expression on the surface of the cells. We sought to extend this study by stimulating populations of resting B cells with the following different isotype-specific anti-Ig antibodies, anti-μ, anti-δ and anti-γ. In **Figure 4.1**, resting tonsillar B cells were cultured with low (0.5 μg/ml) or high (25 μg/ml) doses of anti-μ antibodies and then analysed for IL-2Rα expression after 24 hours by two colour flow cytometry. The dot plots show that, of the control population, only 23% express IL-2Rα on the cell surface. However, when the cells are stimulated with a low dose of anti-μ this percentage increases to approximately 35%. This concentration of antibody appears to produce the maximum level of IL-2Rα expression and no other concentration of anti-μ can induce higher levels of IL-2Rα. Interestingly, high doses of anti-μ do not induce the cells to express IL-2Rα and, in fact, in some cases the percentage of B cells expressing IL-2Rα is slightly lower than that of the control population. The cells, which had been subjected to a high dose of anti-μ, were not dead. Viability checks using Trypan Blue were performed (data not shown) and these confirmed that there was no change in the amount of dead cells present when compared to a control population. These results were noted consistently in independent tonsillar preparations.

The results observed when resting B cells are cultured with anti-μ are in marked contrast to those seen when the cells are cultured with anti-δ. **Figure 4.2A** shows again that approximately 20% of the control population express cell surface IL-2Rα. However, anti-δ was unable to increase IL-2Rα expression above the level of the control either at low doses of the antibody (data not shown) or at high doses (**Panel B**). Again, this was demonstrated in multiple independent tonsil preparations.

Finally, resting B cells were cultured with anti-γ antibodies (**Figure 4.3**) and, again, this showed a different result to that seen with either anti-μ or anti-δ. 20% of the resting B cell population expressed IL-2Rα when unstimulated. However, a high concentration of anti-γ induced 43% of these cells to express IL-2Rα at the cell surface. This was the maximum level of IL-2Rα that could be induced by anti-γ and no further up-regulation could be achieved. The data were representative of several experiments performed on independent tonsil preparations.

To confirm these results, extensive dose response experiments were performed using each antibody over a wide concentration range (**Figure 4.4**). Stimulating the cells with varying concentration of each antibody gave distinct and separate curves. At low concentrations anti-μ induced IL-2Rα expression to a maximum level of approximately 35-40%. In most cases (although not always), the concentration of anti-μ which gave the maximum induction of IL-2Rα expression was 0.5-1 μg/ml; sometimes 5 μg/ml gave the maximum level of IL-2Rα expression. When the cells were cultured with a high dose of anti-μ (25-50 μg/ml) an increase in IL-2Rα expression was never seen. In fact, on some occasions there was inhibition of IL-2Rα

expression with the percentage of cells expressing IL-2Rα falling below that of the control. This distinctive profile was observed in all independent tonsil preparations. Anti-δ, however, was never demonstrated to up-regulate IL-2Rα expression whether at high or low doses of the antibody. The percentage of cells expressing IL-2Rα never increased beyond the level seen in the control population.

When the cells were stimulated with anti-γ antibodies they up-regulated IL-2Rα in a dose dependent manner. This was comparable to the result seen with polyvalent anti-Ig. However, only 50% of the cells at most were driven to express surface IL-2Rα when cultured with 25-50 μg/ml anti-γ, unlike the situation observed with polyvalent anti-Ig when a maximum of 80% of the cells were driven to express IL-2Rα.

4.3 Do Different Isotypes of Anti-Immunoglobulin Antibodies Have a Similar Effect on CD23 Expression?

CD23 is the 45kDa low affinity form of the IgE Receptor, FcRεII, on B cells and is potently induced by IL-4 (DeFrance *et al* 1987). Since IL-4 also induces IL-2Rα on B cells, it was of interest to determine whether other inducers of IL-2Rα would have an effect on CD23 expression. Thus, the same dose response experiments were performed using each isotype antibody and the levels of CD23 expression determined using three colour flow cytometry. Resting B cells already express high levels of CD23 on the cell surface in comparison with IL-2Rα (between 50-70% depending on the tonsillar preparation). When cells were cultured with a wide concentration range of anti-μ, at low concentrations there was an approximate increase of 5% in CD23

expression which was not seen when the cells were cultured with high doses of anti-μ. Thus, this reflects the results seen with IL-2Rα expression in response to anti-μ stimulation, although the increase in CD23 expression is smaller in magnitude than that of IL-2Rα expression. Culturing the cells with anti-γ causes an increase in CD23 expression in a dose-dependent fashion, again reflecting the effect seen when the cells are cultured with anti-γ and stained for IL-2Rα expression.

The only difference seen between the induction of IL-2Rα and CD23 came when the cells were cultured with anti-δ. In this case, there was a slight induction of CD23 expression (of 2-5%) at the higher concentrations of anti-δ antibody. As has been stated previously, no concentration of anti-δ could drive resting B cells to induce IL-2Rα.

4.4 Does Signaling via the Fc Receptor affect IL-2Rα Expression?

The Fc receptor on B cells, FcγRIIb (β1 isoform) helps modulate B cell activation triggered by the surface Ig complex (Ravetch and Kinet 1991; Mellman 1988). Therefore, it was of interest to determine whether Fc receptor-mediated signaling could play a role in the lack of induction of IL-2Rα expression seen when cells are cultured with anti-δ or with high concentrations of anti-μ antibodies. This was done by comparing the effects seen with intact antibodies with those seen when $F(ab')_2$ fragments of antibody were used. Figure 4.6 shows a comparison of intact anti-μ antibodies and $F(ab')_2$ anti-μ antibodies. Both sets of antibodies gave the same result; both could enhance expression of IL-2Rα at low concentrations but high concentrations of the antibodies caused no induction of IL-2Rα.

This was in sharp contrast to the results seen when anti-δ antibodies were used (Figure 4.7). Intact anti-δ antibodies failed to up-regulate IL-2Rα. However $F(ab')_2$ fragments of this antibody reflected the results seen when the cells were stimulated with anti-μ (intact or $F(ab')_2$ fragments; that is, low doses of $F(ab')_2$ anti-δ antibodies up-regulated levels of IL-2Rα to almost 40%. At high concentrations however, no increase in IL-2Rα expression could be detected. This indicates that Fc receptor-mediated inhibition is playing a part in sIgD signaling.

Figure 4.8 shows a comparison of the effects of intact, $F(ab')_2$ fragments and Fab fragments of anti-γ antibodies on IL-2Rα expression. Both intact and $F(ab')_2$ antibodies induced IL-2Rα expression in a dose dependent manner. Fab fragments, which are unable to cross-link receptors did not up-regulate expression of IL-2Rα at all. This is consistent with the theory that cross-linking of any isotype of antigen receptor is necessary for signals to be transduced.

4.4 Discussion

One of the most interesting findings from Chapter 3 was that when B cells are stimulated with polyvalent anti-Ig antibodies almost 80% of them are induced to up-regulate IL-2Rα. Thus, the next step was to determine what happens to IL-2Rα expression when tonsillar B cells are stimulated with different isotypes of anti-Ig antibodies. The cells were cultured with intact anti-μ, anti-δ and anti-γ antibodies; these will stimulate different subsets of cells present within the resting tonsillar

population. Culturing tonsil B cells with anti-μ antibodies will stimulate two different populations of cells. Firstly, if there are any immature IgM^{+}/IgD^{-} cells present, anti-μ will bind to the antigen receptors on these cells. In addition, mature B cells which express both sIgM and sIgD will bind this antibody. Anti-δ antibodies will also bind to receptors on mature B cells while anti-γ antibodies will bind to IgG receptors on memory B cells which are present in small numbers within the resting tonsillar population.

Mature B cells express two classes of membrane-bound immunoglobulin, sIgM and sIgD, while immature B cells express only sIgM (Goding *et al* 1977; Vitetta and Uhr 1977). Whether the two classes of sIg have different functions on B cells is a matter of controversy. A number of studies have suggested that cross-linking sIgM and sIgD induces similar B cell responses (Sieckmann 1980; Mond *et al* 1981; Brink *et al* 1992). In particular, this is true for early biochemical responses such as the activation of PTKs (Gold *et al* 1990; Burkhardt *et al* 1991) and the hydrolysis of phospholipids (Cambier *et al* 1987; Klaus *et al* 1987; Harnett *et al* 1989). However, other studies have revealed functional differences between sIgM and sIgD. sIgM, but not sIgD, induces negative responses in B cells such as anergy and apoptosis (Cambier *et al* 1976; Vitetta *et al* 1976; Ales-Martinez *et al* 1988; Tisch *et al* 1988; Mongini *et al* 1989; Kim *et al* 1991, 1992; Carsetti *et al* 1993). Findings from the experiments performed in this chapter have shown that sIgM and sIgD play different roles in the up-regulation of IL-2Rα. Cross-linking of sIgM with low doses of anti-μ antibodies can induce expression of IL-2Rα while engaging sIgD with anti-δ antibodies does not affect levels of expression of IL-2Rα at all.

Immature B cells are extremely sensitive to tolerance induction whereas mature B cells are more difficult to tolerise (reviewed in Nossal 1983). This has led to the development of the controversial theory that antigen induced signaling through sIgM provides a negative or tolerogenic signal. In fact, ligation of sIgM has also been reported to inhibit the proliferation and differentiation of mature cells (Maruyama *et al* 1985; Kearney *et al* 1978; Anderson *et al* 1974; Ralph 1979; Rothstein and Kolber 1988; Kim *et al* 1992). These experiments would initially appear to oppose this theory since up-regulation of IL-2Rα allows formation of the high affinity IL-2 Receptor and thus leaves the B cell primed to proliferate and differentiate in the presence of other signals. However, only low concentrations of anti-μ antibody cause IL-2Rα to become up-regulated and this effect is not seen with higher doses of the antibody. One explanation for this could be that extensive cross-linking of sIgM could be mimicking the effect seen with auto-antigen within the germinal centre and cause the B cells to become tolerised. Put simply, the signal delivered by foreign antigens trapped on the surface of FDCs might be lower than that delivered by high doses of ubiquitous auto-antigen. Thus, the failure of tonsillar B cells to up-regulate IL-2Rα in response to high doses of anti-μ antibodies may represent a "fail-safe" device for eliminating clones that exhibit auto-reactivity and that will provoke a damaging auto-immune response.

On most mature B cells IgD is the dominant antigen receptor and is expressed 2-10 times more abundantly on the cell surface than IgM (Havran *et al* 1984; Carsetti *et al* 1993). As discussed above, studies have suggested that sIgM, but not sIgD, can induce negative responses in B cells and it has been hypothesised that engagement of

sIgD confers a dominant positive signal for B cell activation. However, knockout mice lacking the IgD antigen receptor are not drastically immunodeficient which suggests that sIgM may functionally replace sIgD in these mice (Nitschke *et al* 1993; Roes and Rajewsky 1993). If this is true then it seems surprising that cross-linking sIgD does not enhance expression of IL-2Rα and thus allow high affinity receptors for IL-2 to form. This suggests that additional signals would be required and that ligating sIgD is not enough to activate the cells.

A comparison of the effects of intact and $F(ab')_2$ fragments of antibodies on IL-2Rα expression indicated that Fc receptor-mediated inhibition played a role in functional sIgD signaling. Intact anti-δ antibodies could not induce IL-2Rα expression whereas low concentrations of $F(ab')_2$ anti-δ antibodies could. In fact, $F(ab')_2$ anti-δ antibodies gave a result identical to that seen when cells were cultured with intact anti-μ antibodies. In the cases of anti-γ and anti-μ antibodies, $F(ab')_2$ fragments give similar responses to those seen with the respective intact antibodies. Work has been done which shows the Fc-dependent suppression of antigen-specific B cell responses can be overcome by T cells or T cell-derived lymphokines (Hoffman and Kappler 1978; Lees and Sinclair 1975; Kolsch *et al* 1980) and IL-4 has been shown to reverse Fc Receptor-mediated inhibition in mice (O' Garra *et al* 1987). It has been hypothesised that FcγRIIb is predominantly responsible for protection of the resting state of B cells preventing inadequate auto-antibody production (Sarmay *et al* 1995). This would prevent B cells from being activated in the absence of suitable T cell help.

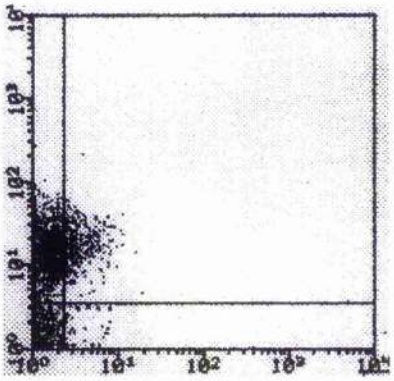
Cross-linking of sIgG on memory B cells causes IL-2Rα to be up-regulated in a dose-dependent fashion. This is a different result to those seen on engaging sIgM or sIgD but one which is of some significance. Memory B cells must be able to proliferate in response to lower amounts of antigen than naive cells (Yefenof *et al* 1986). For them to do this, they would obviously need to be able to form high affinity IL-2Rs, requiring increased IL-2Rα expression. These memory B cells have already been selected against foreign antigen, not self, and therefore there is no need for IL-2Rα levels to be decreased when high doses of antibody are added to prevent an auto-immune response.

The next chapter goes on to discuss events at the promoter region of the IL-2Rα gene. It is important to relate the events that are occurring at the cells surface to those happening at the molecular level. In particular, there is a protein which binds to a negative regulatory region within the IL-2Rα promoter which has the ability to silence expression of this gene and it was of interest to discover whether signals through the antigen receptor could affect the binding of this protein to the promoter region.

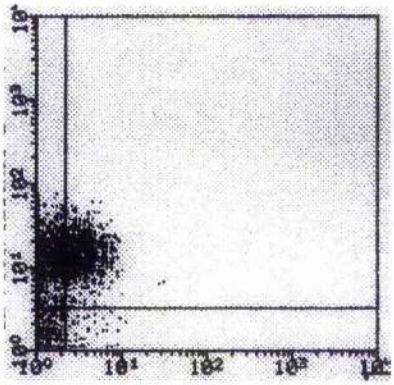
Figure 4.1 The Effect of Anti-μ Antibodies upon IL-2Rα Expression.

High density resting tonsil B cells were cultured in complete medium (Panel A) or with either 0.5 μg/ml anti-μ (Panel B) or with 25 μg/ml anti-μ (Panel C) for 24 hours. The cells were stained with FITC-anti-CD25 and PE-anti-CD19 and analysed by two colour flow cytometry. Propidium Iodide was added to facilitate exclusion of dead cells and 5000 live gated events were collected. The two colour dot plots show green fluorescence (FITC) on the x-axis and red fluorescence (PE) on the y-axis. The percentage of B cells positive for IL-2Rα was calculated as previously described in the legend to Figure 3.1. These experiments were repeated 4 times and a representative experiment shown.

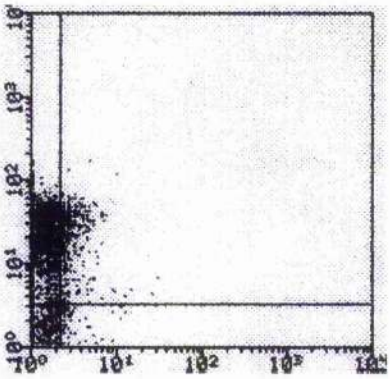
A



B



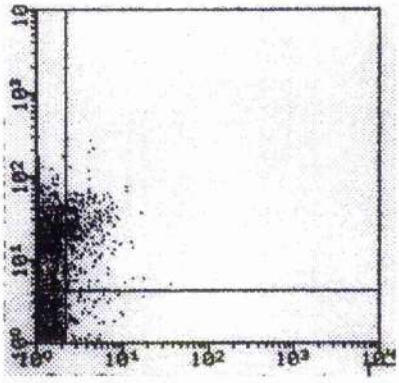
C



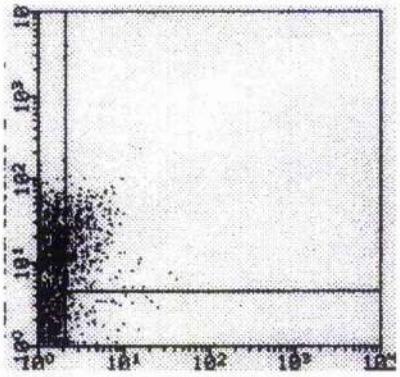
4.2 The Effect of Anti-δ Antibodies on IL-2Rα Expression.

High density resting tonsillar B cells were cultured in either complete medium, Panel A, (19%) or with 25 μg/ml anti-δ, Panel B,(20%) for 24 hours. The cells were stained and the percentage of B cells positive for IL-2Rα was determined as previously described in the legend to Figure 3.1. The figures shown in brackets are the percentage of B cells positive for IL-2Rα expression. Again this was repeated four times and a representative experiment shown.

A



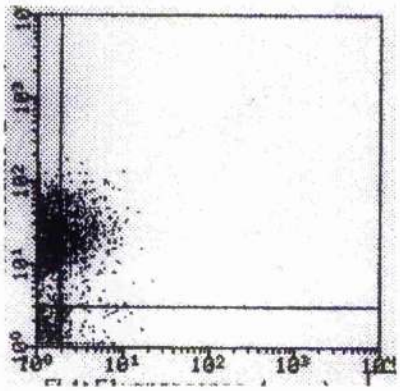
B



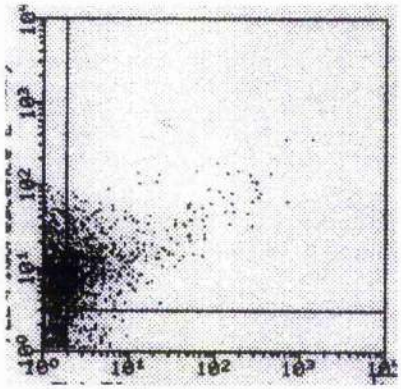
4.3 The Effect of Anti-γ Antibodies on IL-2Rα Expression.

High density resting tonsillar B cells were cultured in either complete medium, Panel A, (21%) or with 25 μg/ml anti-γ, Panel C (51%) for 24 hours. The cells were stained and the percentage of B cells positive for IL-2Rα expression was determined as described in the legend to Figure 3.1. The figures shown in brackets are the percentage of B cells positive for IL-2Rα expression. Again this was repeated four times and a representative experiment shown.

A

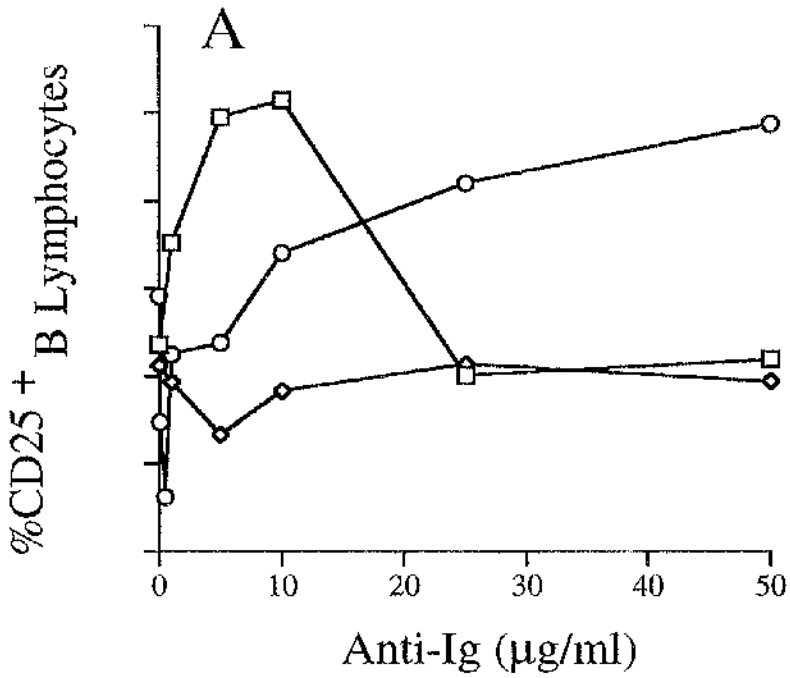


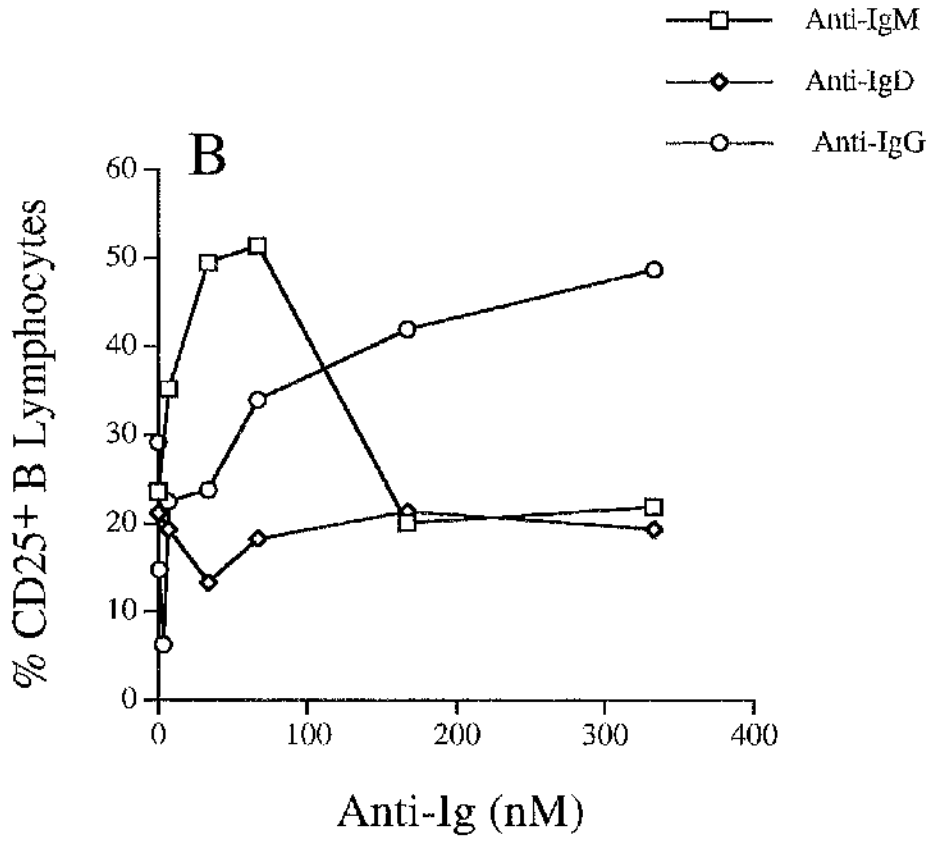
B



4.4 Dose Response Titrations of Anti-μ, Anti-δ and Anti-γ Antibodies.

High density resting tonsillar B cells were cultured with the indicated concentrations of anti-μ, anti-δ and anti-γ antibodies for 24 hours (Panel A). IL-2Rα expression was determined by flow cytometry as described in the legend to Figure 3.1. The data shown are representative of six independent tonsillar B cell preparations. Panel B shows the data expressed in terms of the molar concentrations of each stimulus applied.


A
%CD25 + B Lymphocytes
0
10
20
30
40
50
Anti-Ig (µg/ml)


Anti-IgM
Anti-IgD
Anti-IgG
B
% CD25+ B Lymphocytes
60
50
40
30
20
10
0
0
100
200
300
400
Anti-Ig (nM)

4.5 The Effect of Anti-μ, Anti-δ and Anti-γ Antibodies on CD23 Expression.

High density resting tonsillar B cells were cultured for 24 hours with the indicated concentrations of anti-μ, anti-δ or anti-γ. Cells were then stained with FITC-anti-CD25, PE-anti-CD19 and Biotin-conjugated anti-CD23 followed by Streptavidin Quantum Red to detect the biotinylated antibody. Levels of CD23 expression were determined in the same way as described previously for IL-2Rα expression in the legend for Figure 3.1. Panel A shows the data expressed in terms of the μg/ml of antibody used whereas Panel B shows the data expressed in terms of molar concentrations. This experiment was repeated 3 times and the results show a representative experiment.

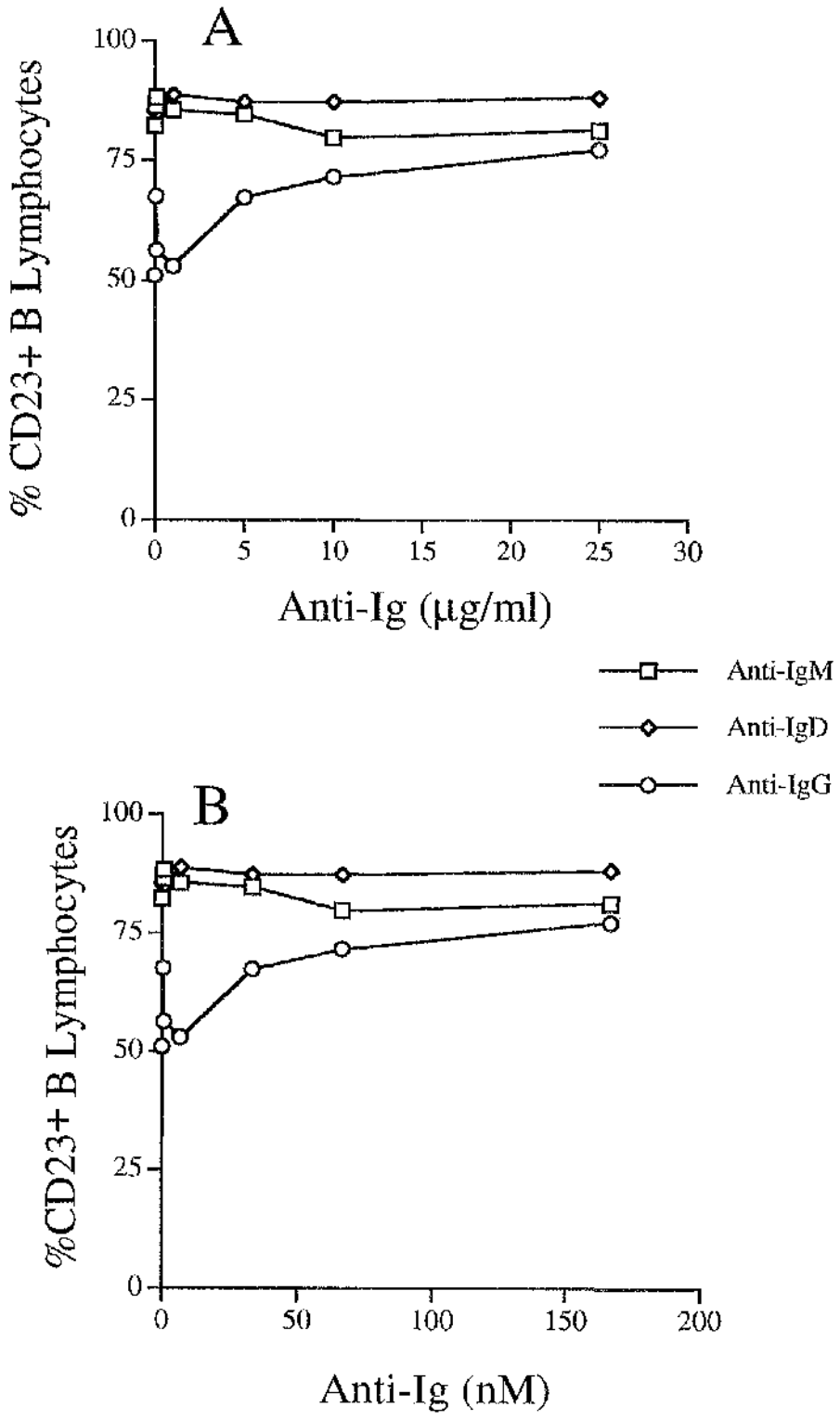

A
% CD23+ B Lymphocytes
100
75
50
25
0
0
5
10
15
20
25
30
Anti-Ig (µg/ml)
Anti-IgM
Anti-IgD
Anti-IgG
B
%CD23+ B Lymphocytes
100
75
50
25
0
0
50
100
150
200
Anti-Ig (nM)

Figure 4.6 A Titration Curve Comparing the Effects of Intact Anti-μ with $F(ab')_2$ Fragments of Anti-μ on IL-2Rα Expression.

High density resting tonsillar B cells were cultured for 24 hours with the indicated concentrations of either intact anti-μ or $F(ab')_2$ fragments of anti-μ. Levels of IL-2Rα expression were determined as described previously in the legend to Figure 3.1.The data are expressed in terms of the molar concentration of each antibody.

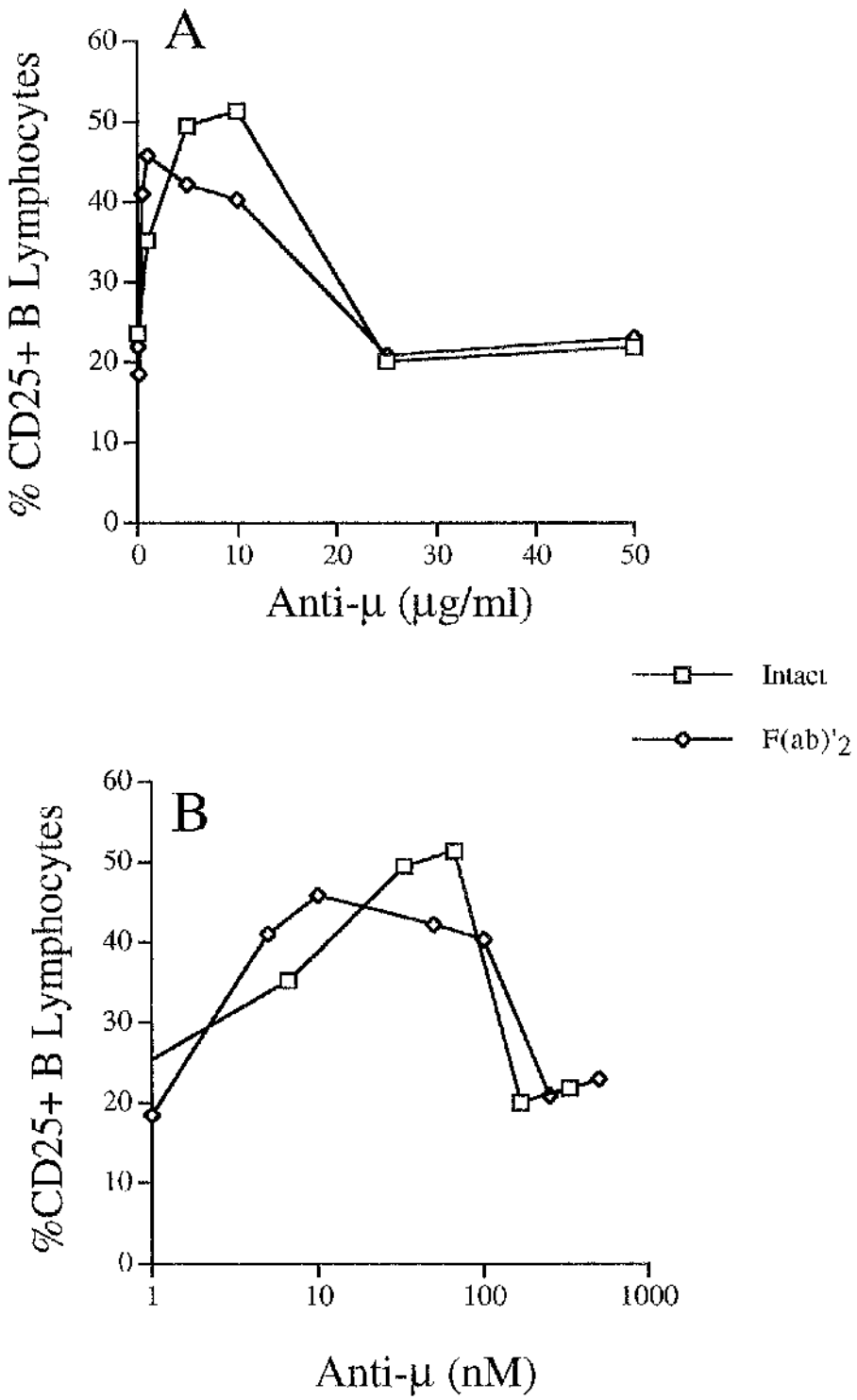

A
% CD25+ B Lymphocytes
60
50
40
30
20
10
0
0
10
20
30
40
50
Anti-μ (μg/ml)
Intact
F(ab)'2
B
%CD25+ B Lymphocytes
60
50
40
30
20
10
0
1
10
100
1000
Anti-μ (nM)

Figure 4.7 A Titration Curve Comparing the Effect of Intact Anti-δ with $F(ab')_2$ Fragments of Anti-δ on IL-2Rα Expression.

High density resting tonsillar B cells were cultured for 24 hours with the indicated concentrations of either intact anti-δ or $F(ab')_2$ fragments of anti-δ. Levels of IL-2Rα expression were determined as shown previously in the legend to figure 3.1. The data are expressed in terms of the molar concentration of each antibody.

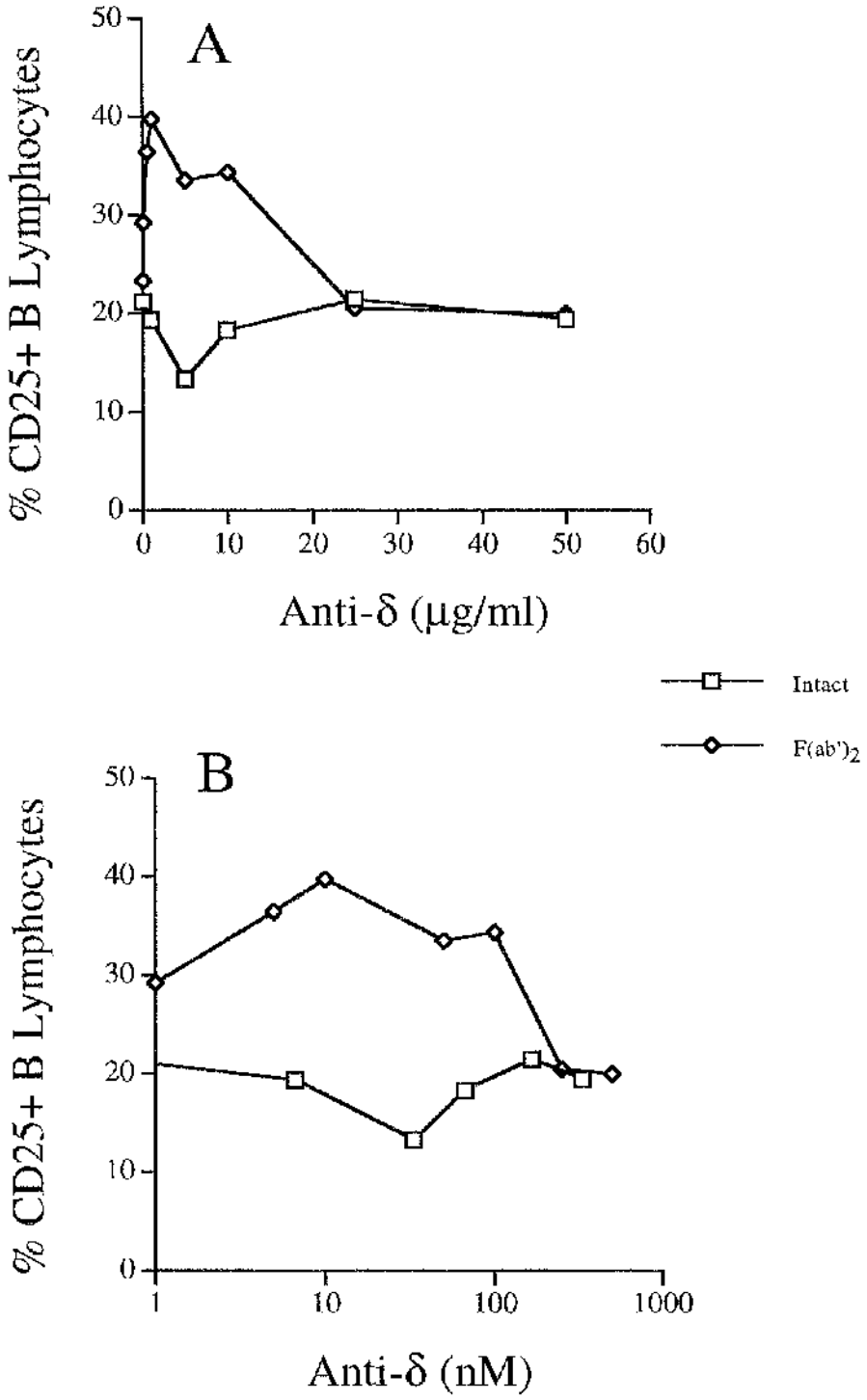
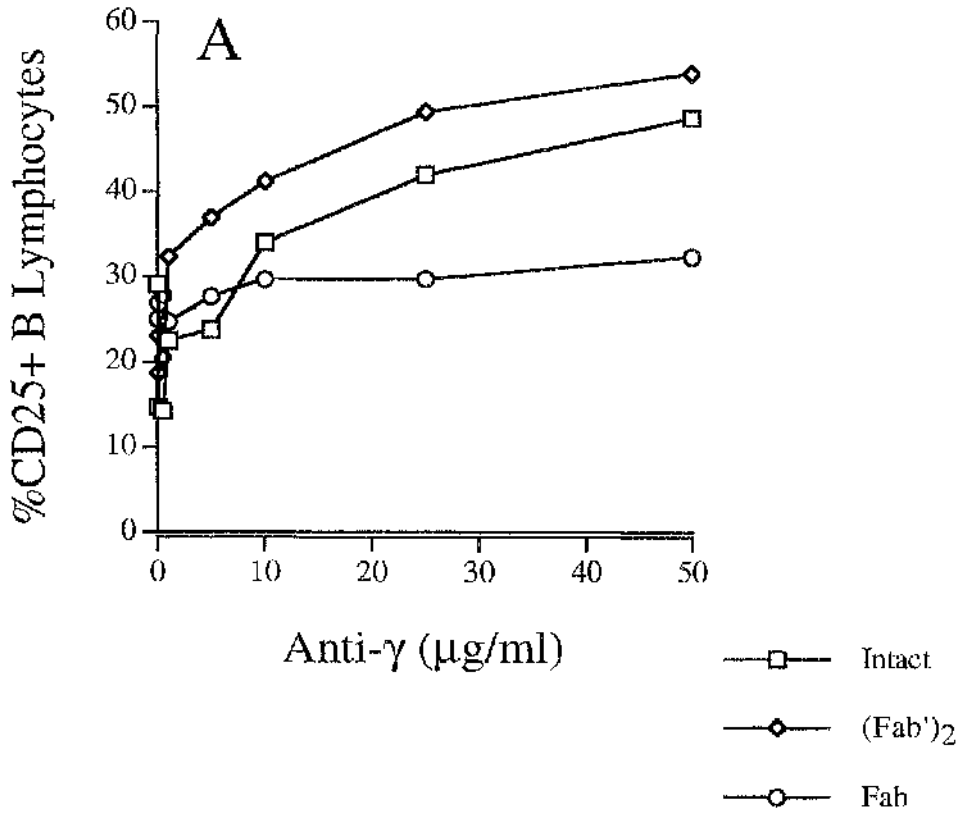
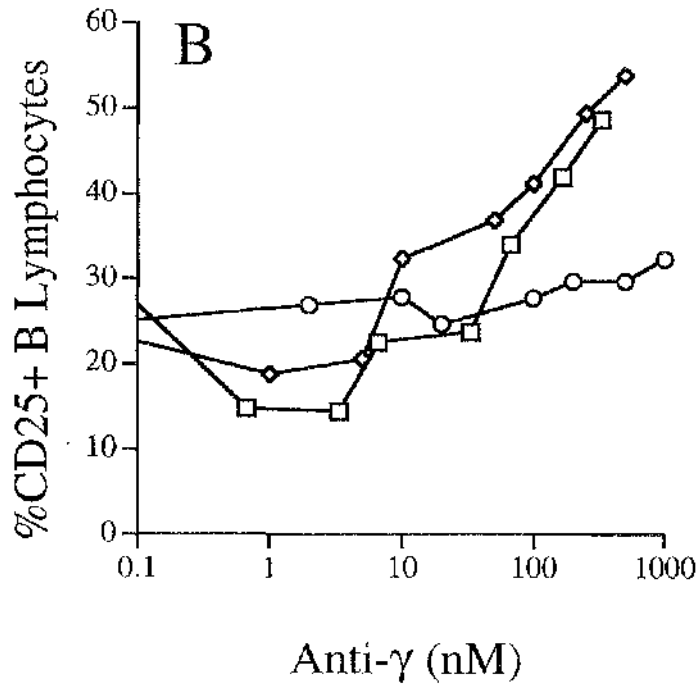

A
% CD25+ B Lymphocytes
50
40
30
20
10
0
0
10
20
30
40
50
60
Anti-δ (μg/ml)
Intact
F(ab')2
B
% CD25+ B Lymphocytes
50
40
30
20
10
0
1
10
100
1000
Anti-δ (nM)

Figure 4.8 A Titration Curve Comparing the Effect of Intact Anti-γ, $F(ab')_2$ Fragments of Anti-γ and Fab Fragments of Anti-γ on IL-2Rα Expression.

High density resting tonsillar B cells were cultured with the indicated concentrations of either intact anti-γ, $F(ab')_2$ fragments of anti-γ or Fab fragments of anti-γ. Levels of IL-2Rα expression were determined as shown previously in the legend to Figure 3.1. The data are expressed in terms of the molar concentration of each antibody.


A
%CD25+ B Lymphocytes
60
50
40
30
20
10
0
0
10
20
30
40
50
Anti-γ (μg/ml)
Intact
(Fab')2
Fab


B
%CD25+ B Lymphocytes
60
50
40
30
20
10
0
0.1
1
10
100
1000
Anti-γ (nM)

Chapter 5

Expression of the IL-2Rα Gene in Human Tonsillar B Lymphocytes

5.1 Introduction.

The preceding chapters have demonstrated that IL-2Rα expression can be induced in human tonsillar B lymphocytes by stimulating the cells with either IL-4, anti-Ig or anti-CD40 antibodies. In particular, the isotype of anti-Ig which is used to stimulate the cell is of importance; both anti-μ and anti-γ cause IL-2Rα to be up-regulated on the B cell surface, although this up-regulation is abrogated at high doses of anti-μ. By contrast, induction of expression is not seen when cells are cultured with anti-δ. In order to define the molecular levels of control regulating the IL-2Rα promoter, a series of experiments consisting of gel shift assays and transient transfection studies were performed to determine whether IL-4 or anti-Ig antibodies could induce IL-2Rα promoter activity.

In T lymphocytes the IL-2Rα promoter region has been well characterised and the major *cis* acting sequences identified. A 1352 base pair fragment 5' to the IL-2Rα gene has been shown to have promoter activity when fused to a reporter gene (Leonard *et al* 1985). Several *cis* regulatory elements within this region have been mapped and their corresponding *trans*-activating factors identified. There are believed to be at least 3 positive regulatory elements, PRR_{I-III}, involved in IL-2Rα promoter activity. Within PRR_I, located -276 to -244 relative to the major transcription control site, there are four putative binding sites for nuclear factors that positively regulate transcription (NFIL2RA, NFκB, SRF and Sp-1) (Bohnlein *et al* 1988; Leung and Nabel 1988; Ballard *et al* 1989; Cross *et al* 1989; Lowenthal *et al* 1989; Lin *et al* 1990; Roman *et al* 1990; Toledano *et al*

1990; Algarte *et al* 1995; John *et al* 1995,1996). PRR_{II} (-137 to -64) (John *et al* 1995) contains binding sites for at least two DNA binding proteins, an *Ets* family protein, Elf-1 , (Thompson *et al* 1992) and the non-histone chromatin-associated protein, HMG-I(Y) (Elton and Reeves 1986, Johnson *et al* 1989; Lund *et al* 1986). PRR_{III} is located some distance upstream of the 5' cap site, between nucleotides -3780 and -3703; it has a binding site for STAT-5 as well as consensus sequences for Elf-1, HMG-I(Y) and a member of the GATA family of DNA binding proteins (John *et al* 1996). It seems likely that PRR_{II} contributes towards basal promoter activity and cell-type specificity of expression of the gene whereas PRR_{I} and PRR_{III} contribute more potently to the inducibility of the IL-2Rα promoter.

In addition to the positive regulatory regions of the IL-2Rα promoter, there are two negative regulatory regions which lie within 400 base pairs of the transcription initiation site. NRE I is located between nucleotides -400 and -368 (Smith and Greene 1989) and NRE II is located between nucleotides -317 and -342 (Lowenthal *et al* 1989b). NRE I contains an 11 base pair core element (TTCATCCCAGG) which binds *in vitro* to a 50kDa protein (SP50). This is thought to mediate the negative regulation of the gene; displacement or inactivation of SP50 is associated with transcriptional activation (Smith and Greene 1989).

Thus, the IL-2Rα promoter contains multiple regulatory elements which together orchestrate a coordinated response to mitogenic or growth factor stimulation. Since IL-4

and anti-Ig antibodies can induce surface IL-2Rα expression on human B cells, experiments were performed to determine whether this up-regulation was mediated by interactions at sites within the promoter region. High concentrations of anti-μ can inhibit the induction of IL-2Rα and it was of particular interest to ascertain whether anti-μ - driven inhibition of IL-2Rα in B cells regulates the activity of the protein(s) capable of binding to the NRE I element.

5.2 Analysis of DNA Binding Protein Activity Specific For the NF-κB Region of the IL-2Rα Promoter in Resting B Cells.

NF-κB is a ubiquitous transcription factor whose properties are extensively exploited by cells of the immune system. Several lines of evidence have indicated that the κB site in the IL-2Rα promoter plays a critical role in the induction of the IL-2Rα gene in human T cells (Ballard *et al* 1988; Lowenthal *et al* 1988, 1989a, b; Bohnlein *et al* 1988). Thus it was important to determine whether B cells, like their T cell counterparts, contained DNA-binding protein activities which could bind to the κB motif. Nuclear extracts were prepared from resting tonsil B cells and were found to contain a protein (or proteins) which recognised and retarded labelled NF-κB oligonucleotide (Figure 5.1, lanes 1 and 6) and which were sensitive to competition by a 10- or 100-fold excess of cold NF-κB oligonucleotide (Figure 5.1, lanes 2 and 3 respectively). Binding was insensitive to the

presence of a 10- or 100-fold excess of an non-specific oligonucleotide which corresponded to an AP-2 consensus binding site (Figure 5.1, lanes 4 and 5 respectively).

5.3 The Effects of Anti-μ Antibodies and IL-4 on NF-κB Specific DNA Binding Protein Activity in B Cell Extracts.

A range of concentrations of anti-μ gives a distinctive profile of IL-2Rα induction; that is, at low concentrations of anti-μ IL-2Rα expression is induced in resting tonsil B cells whereas at high concentrations this effect is completely abrogated. Thus, B cell extracts were prepared from tonsillar B cells which were cultured either with a low concentration (0.5μg/ml) or a high concentration (25μg/ml) of anti-μ. These have been shown to induce or inhibit cell surface expression of IL-2Rα, respectively (see Chapter 4). At time 0 there was oligonucleotide binding activity in both sets of extracts (Figures 5.2A and B) and this activity was not significantly altered on addition of either concentration of anti-IgM. NF-κB specific DNA binding protein activity was also studied on extracts which had been cultured with intermediate concentrations of anti-IgM (1 and 10μg/ml) (data not shown). Again, there was no significant alteration in DNA-binding protein activity over a four hour time course. Similar experiments were performed using B cell extracts cultured with varying concentrations of anti-δ and anti-γ antibodies (data not shown), again with an identical result.

Since IL-4 also potently induces IL-2Rα expression in resting B cells, we next determined whether IL-4 exerted any effect on NF-κB protein binding activity in tonsillar B cells (Figure 5.2C). An identical result was obtained to those obtained with anti-μ; that is, there was no alteration in protein binding activity at the κB region when the cells were stimulated with 2nM IL-4 (400U/ml).

5.4 The Effects of Anti-μ Antibodies and IL-4 on NFIL-4 DNA Binding Protein Activity in B Cell Extracts.

We have found IL-4 to be the sole cytokine that will up-regulate IL-2Rα expression in resting tonsillar B lymphocytes. However, the molecular mechanisms of IL-4 action in this response are still obscure. IL-4 also tightly regulates induction of FcεRII (CD23), the low affinity receptor for IgE. An IL-4 responsive element was identified within the CD23 promoter region (DeFrance *et al* 1987; Vercelli *et al* 1988) and an IL-4 dependent nuclear binding factor, NFIL-4, was found to interact specifically with this novel 9 base pair motif (Kohler and Rieber 1993). NFIL-4 also interacts with a homologous sequence within the promoter of the germline Iε transcript. A consensus sequence for the NFIL-4 binding site was proposed to be 5' T(T/C)C(T/C)(A/G)(A/G)GAA 3' and a sequence corresponding to this was found in the IL-2Rα promoter in B cells (-306 to -298). Thus, we next determined whether IL-4 and/or anti-μ exerted any effects on NFIL-4 protein binding activity in tonsillar B cells.

At time 0, NFIL-4 oligonucleotide binding activity was clearly detected in B cell extracts by gel shift assay (Figure 5.3A, B and C). However, both low and high concentrations of anti-μ (Figure 5.3A and B respectively) failed to have any effect on NFIL-4 binding activity to the IL-4RE, as might have been expected. In contrast, NFIL-4 binding activity was increased over a four hour period following addtion of 2nM IL-4. Two mobility shift bands were evident in the analysis and both were shown to be sensitive to competition by excess cold IL-4RE oligonuceotide (data not shown). Thus, binding of NFIL-4 to IL-4RE in the IL-2Rα promoter seems to be under the sole control of IL-4 and, as expected, is not affected by signals delivered via the IgM antigen receptor.

5.5 Does the Negative Regulatory Region Play a Role in Induction of the IL-2Rα Promoter When Cells Are Stimulated With Anti-μ?

Low concentrations of anti-μ will induce expression of IL-2Rα in human tonsillar B cells while high concentrations of the same antibody inhibits this effect completely. This phenomenon is seen solely when cells are stimulated with the anti-μ isotype. A possible explanation for this differential effect could lie in the capacity of anti-μ to influence the activity of the NRE binding protein (NRE-BP) in B cells. To test this notion, it was firstly investigated whether B cells, like their T cell counterparts, contained proteins which could bind to the 11 base pair core region of NRE I.

Nuclear extracts from resting tonsil B cells which had been cultured with either anti-μ (Figure 5.4A) or IL-4 (Figure 5.4B) were found to contain protein(s) which recognised and retarded labelled NRE oligonucleotide (Lanes 1 and 4). This was sensitive to competition by 100-fold excess of cold NRE oligonucleotide (Figure 5.4A and B, lanes 2) but insenstive to the presence of 100-fold excess of an oligonucleotide corresponding to the AP-1 consensus binding site (Figure 5.4A and B, lanes 3). Experiments performed by others in this laboratory confirmed that oligonucleotides corresponding to the consensus binding sites for Oct-1, NF-κB, CREB and TFIID were also unable to compete for binding to the NRE oligonucleotide. Two sequence specific bands were evident; both showed sensitivity to competition with unlabelled NRE oligonucleotide. The gel shift profile is essentially identical to that noted for Jurkat T cell Lines and to that reported previously (Smith and Greene 1989; Hewitt *et al* 1997).

Once it had been established that B cells did indeed possess protein(s) capable of binding to the 11 base pair core motif of NRE I, the next step was to discover whether culturing the B cells with anti-μ had any effect on the binding of these protein(s) to this region. Therefore, extracts were prepared at hourly intervals from resting tonsillar B cells stimulated either with a low concentration of anti-μ (0.5μg/ml) or a high concentration of anti-μ (25μg/ml) and the DNA binding protein activity for the NRE I analysed by gel shift assay (Figure 5.5A and B respectively). It was found that low concentrations of anti-μ significantly decreased NRE oligonucleotide binding activity over a four hour period. However, in sharp contrast, high concentrations of anti-μ did not affect the binding of

proteins to NRE oligonucleotide. This differential effect might partially explain the failure of high concentrations to drive IL-2Rα expression in resting tonsillar B cells. Thus, the distinct induction and then inhibition of IL-2Rα expression seen at the surface of the cell in response to a range of concentrations of anti-μ is reflected by DNA binding events at the NRE I region of the promoter.

Once this effect had been determined, studies were performed to assess the effect that culturing B cells with IL-4 would have on NRE binding protein activity. Extracts were prepared from B cells which had been cultured with 2nM IL-4 for up to four hours. Following addition of IL-4, NRE oligonucleotide binding activity (clearly detectable at time 0) was again reduced over the four period (Figure 5.5C). This was essentially an identical result to that obtained when the cells were cultured with low concentrations of anti-μ and is consistent with the hypothesis that ligands which induce IL-2Rα expression cause loss of NRE-BP activity.

5.6 Do Other Isotypes of Anti-Ig Antibodies Affect NRE Binding Protein Activity?

Anti-γ antibodies can also induce IL-2Rα expression on the B cell surface; unlike anti-μ this induction is not reversed at high concentrations of the antibody. It was therefore of interest to see if varying concentrations of anti-γ would have any effect on the protein(s) binding to NRE I. Extracts were again prepared from resting B cells which in this case

had been cultured with low and high concentrations of anti-IgG and gel shift assays performed (Figure 5.6A and B respectively). However this time there was no alteration in the binding of proteins to the NRE oligonucleotide. At time 0 there was detectable NRE oligonucleotide binding activity and this did not increase or decrease over the four hour time period (or indeed after 24 hours - data not shown).

Culturing resting B cells with anti-IgD antibodies had no effect on the levels of IL-2Rα expression on the cell surface and, in distinct contrast to the results seen with low concentrations of anti-IgM and with IL-4, there was no change in the protein binding activity to NRE I (Data not shown).

5.7 Analysis of the Activity of IL-2Rα Deletion Reporter Constructs.

A reporter plasmid (pCD25-CAT-E) had been constructed previously in this laboratory (Hewitt *et al* 1997). This contained a 592 base pair fragment of the IL-2Rα promoter region of the human IL-2Rα gene located upstream of the chloroamphenicol acetyl transferase gene. The vector also contained an SV40 enhancer sequence which facilitates detection of low levels of reporter activity in human B cells (Hewitt *et al* 1997). The 592 base pair fragment inserted into the plasmid comprised the minimal promoter/enhancer region of the IL-2Rα gene which extends from -472 to +109 and contains the 11 base pair NRE I sequence. In addition, a deletion construct was made which lacked this 11 base

pair region (pΔNRE-CAT-E). Each plasmid was transfected into tonsillar B cells and the reporter gene activity assessed after stimulation with IL-4. Mock-transfected cells were used as a control. Reporter gene activity was assessed by TLC analysis (Figure 5.7A) and quantitated by phosphorimage analysis (Figure 5.7B). Figure 5.7A, lanes 1 and 2 give the results of cells mock-transfected as a control: lane 1 shows unstimulated cells, lane 2 shows cells stimulated with IL-4). B cells transiently transfected with the full length IL-2Rα promoter construct, pCD25-CAT-E, resulted in a low level of reporter gene activity (Figure 5.7, lane 3). Somewhat surprisingly, there was no induction of activity after stimulation of the cells with 2nM IL-4 (Figure 5.7, lane 4). However, on transfecting the cells with the NRE-deletion construct, pΔNRE-CAT-E, the basal level of reporter gene activity was increased approximately 10-fold on stimulating the cells with IL-4 (Lane 6) compared to the activity seen in the unstimulated cells (Lane 5). Figure 5.7B shows a phosphorimage quantitation of the TLC data.

However, the most striking results came when resting B cells were either mock-transfected, transfected with pCD25-CAT-E or transfected with pΔNRE-CAT-E and then cultured for 48 hours with anti-μ antibodies. Figure 5.8, lanes 1,2 and 3, give the results of the mock-transfected cells which were cultured in either complete medium, with 0.5μg/ml anti-μ or with 25μg/ml anti-μ respectively. As expected, these lanes show no acetylated forms of chloroamphenicol. On transfecting the cells with pCD25-CAT-E, there is a 20-25 fold induction of reporter gene activity in cells cultured with 0.5μg/ml anti-μ (Figure 5.8, Lane 5) compared to control cells cultured in complete medium

(Figure 5.8, lane 4). Cells cultured with 25μg/ml anti-μ show no increase in basal gene activity (Lane 6), as might have been predicted from the results of previous FACS and gel shift experiments.

Resting B cells were also transfected with the deletion construct, pΔNRE-CAT-E, and then cultured either in complete medium (Figure 5.8, lane 7), with 0.5μg/ml anti-μ (Figure 5.8. lane 8) or with 25μg/ml anti-μ (Figure 5.8, lane 9). Again at low concentrations of anti-μ there is induction of reporter gene activity. However, most importantly, high dose anti-μ stimulation does not suppress expression of the pΔNRE-CAT-E reporter construct; in fact, there is a significant induction of reporter gene activity. Figure 5.8B shows a phosphorimage quantitation of the TLC data.

These data are consistent with the interpretation that, firstly, protein(s) binding to NRE I inhibits basal activity of the IL-2Rα promoter. Secondly, expression of the IL-2Rα induced by low doses of anti-μ correlates with attenuation of the NRE protein binding activity. Finally, inhibition of IL-2Rα induction by high concentration of anti-μ is caused (at least in part) by the binding of protein(s) to NRE I, presumably resulting in suppression of transcription of the gene.

5.8 Discussion

The IL-2Rα promoter region in B cells possesses multiple regulatory regions. Of these, we chose to study, firstly, the positive regulatory region PRR_1 and, secondly, the negative regulatory region NRE I. The key finding to emerge from this study is that transcription of the IL-2Rα gene in human tonsillar B lymphocytes is subject to negative regulation by an 11 base pair NRE which is a potent suppressor of transcription. In T cells, this NRE I is bound by a 50kDa protein (SP-50) which is, as yet, undefined. It is not known whether the same protein is present in B cells. However, given that the gel shift patterns are similar in B cells, T cells and Jurkat cells, it seems likely that the same protein is involved in each case. Low concentrations of anti-μ which were found to induce expression of IL-2Rα on the cell surface also caused attenuation of the NRE binding protein (NRE-BP) activity. However, at high concentrations of anti-μ there was no attenuation of NRE-BP activity, correlating with the fact that high concentrations of anti-μ do not drive B cells to express surface IL-2Rα.

Regulation of the human IL-2Rα is very complex and the most extensive study of promoter regulation has been in T cells. Recent studies using band shift and DNase I footprinting assays (Algarte *et al* 1995) indicated that there is apparently a major role played by the NF-κB transcription factor in the positive regulation of the IL-2Rα promoter. It was proposed that in resting T cells the κB element is occupied by a constitutive NF-κB homodimer which impairs SRF binding to the flanking SRE/CArG

box. On activation of T cells, the κB element is occupied by a p50-p65 heterodimer which is associated with an SRE/CArG box DNA binding factor. By contrast, resting B cells do not appear to possess such constitutive protein-DNA interactions at the IL-2Rα locus. Again, unlike T cells, activation of B cells either via the antigen receptor or by IL-4 fails to affect the binding activity of proteins to the κB element. In any case the anti-μ data represented here would fail to distinguish between the binding of p50 homodimers and p50-p65 heterodimers to the κB motif; further gel supershift assays would need to be performed. However it is clear that other elements within the promoter must be playing a significant role in IL-2Rα gene regulation in B cells.

One such candidate for this might be the IL-4RE, located -305 to-298 to the transcription initiation site. Sequences in the promoter regions of the CD23 and Iε genes (which are controlled by IL-4) were found to be bound by an IL-4-activated nuclear factor NFIL-4 (recently demonstrated to be STAT-6; Hou *et al* 1994; Kohler and Rieber 1993). A sequence similar to these was found in the IL-2Rα promoter in B cells; the cells also possess a DNA-binding protein which interacts with oligonucleotides containing an IL-4RE sequence. Treatment of the B cells with IL-4 resulted in an increase in activity of the protein binding to the IL-4RE. The identity of this protein in B cells has yet to be defined; early experiments carried out by others in this laboratory indicated that IL-4RE is bound by a phospho-tyrosine containing protein which is sensitive to STAT-6 oligonucleotides. However they were unable to supershift the protein-oligonucleotide complex with anti-STAT-6 antibodies. This suggests that NFIL-4 might be made up of a complex

containing STAT-6 plus another, as yet unidentified protein. The fact that anti-μ antibodies fail to induce binding of NFIL-4 to IL-4RE could mean that another regulatory element within the IL-2Rα promoter region plays a role in the induction of IL-2Rα by anti-μ antibodies.

Thus attention was focused on the role that the negative regulatory elements might play in controlling transcription of this locus. In T cells, the NRE I region of the promoter is active and involved in suppressing transcription of the IL-2Rα gene. The 11 base pair element is bound by SP-50 in T cells, silencing IL-2Rα transcription. B cells also possess a protein capable of binding an oligonucleotide containing this 11 base pair region and, indeed, low concentrations of anti-μ which induce IL-2Rα expression were found to attenuate NRE binding protein activity. High concentrations of this antibody did not appear to alter binding protein activity at this region, a fact which correlates with the observation that high doses of anti-μ will not induce IL-2Rα expression on the cell surface. Furthermore, deletion of the NRE from the promoter sequence increased basal reporter gene activity in B cells treated with both low and high doses of anti-μ. These findings would seem to suggest that NRE I plays a major role in controlling IL-2Rα transcription when cells are stimulated via their IgM antigen receptors. The situation where cells are stimulated via the IL-4 receptor seems to be more complex with a variety of positive and negative regulatory factors all playing a part.

It should be noted that negative regulation of IL-2Rα transcription at NRE I actually encompasses 31 base pairs; the 11 base pair core of NRE I already mentioned and a Retinoic Acid Response Element (RARE). Treatment of B cells with IL-4 results in loss of DNA binding protein activity for RARE specific oligonucleotides (Hewitt *et al* 1997). However, this element does not appear to be under the control of signals transduced via the IgM antigen receptor since treating the cells with a variety of concentrations of anti-μ failed to alter the binding activity of proteins to the RARE oligonucleotide (data not shown).

Therefore it simply remains to speculate on the nature of the protein(s) that might bind to the NRE I 11 base pair core element. In T cells, as has been stated, SP-50 remains undefined. There are no specific homologies to consensus binding sequences for a range of transcription factors. A possible candidate was thought to be YY1, a ubiquitously expressed zinc finger protein which requires only a core CAT motif for DNA binding (Hyde-DeRuyscher *et al* 1995) and it has been known to act both as an activator and as a suppressor of transcription. However, recent studies in this laboratory have appeared to rule out YY1 as a protein which will bind the NRE I element. Other transcription factors which were thought to be possible candidates included CREB, an *ets* family member or HMG-I(Y). All have been discounted, due to competition assays (data not shown), supershift assays and molecular mass, respectively. Thus, although it is known that the proteins which bind NRE I silence transcription and play a dominant role in induction of IL-2Rα by the antigen receptor, it remains to be determined how signals from the antigen

receptor are linked to a protein acting at NRE I and whether this protein belongs to an already defined family of transcription factors or in fact is a novel factor which has not previously been characterised.

Figure 5.1 Identification of NF-κB-specific DNA Binding Activity in Tonsil Resting B Cells.

Nuclear and cytoplasmic cell extracts were prepared from the resting tonsillar B cells (5 x 10^6) which had been cultured overnight in complete medium. The binding activity of 5μg aliquots of protein to γ-[^{32}P]-labelled NF-κB oligonucleotide was assessed by gel shift assay in the absence of cold competitor oligonucleotide (lane 1) or in the presence of 10-fold or 100-fold excess of cold NF-κB oligonucleotide (lanes 2 and 3 respectively) or in the presence of 10-fold or 100-fold excess of a non-specific oligonucleotide, AP-2 (lanes 4 and 5 respectively). Lane 6 shows a repeat of the binding activity of γ-[^{32}P]-labelled NF-κB oligonucleotide in the absence of cold competitor oligonucleotide. Specific binding activities are indicated with horizontal arrows.

Figure 5.1

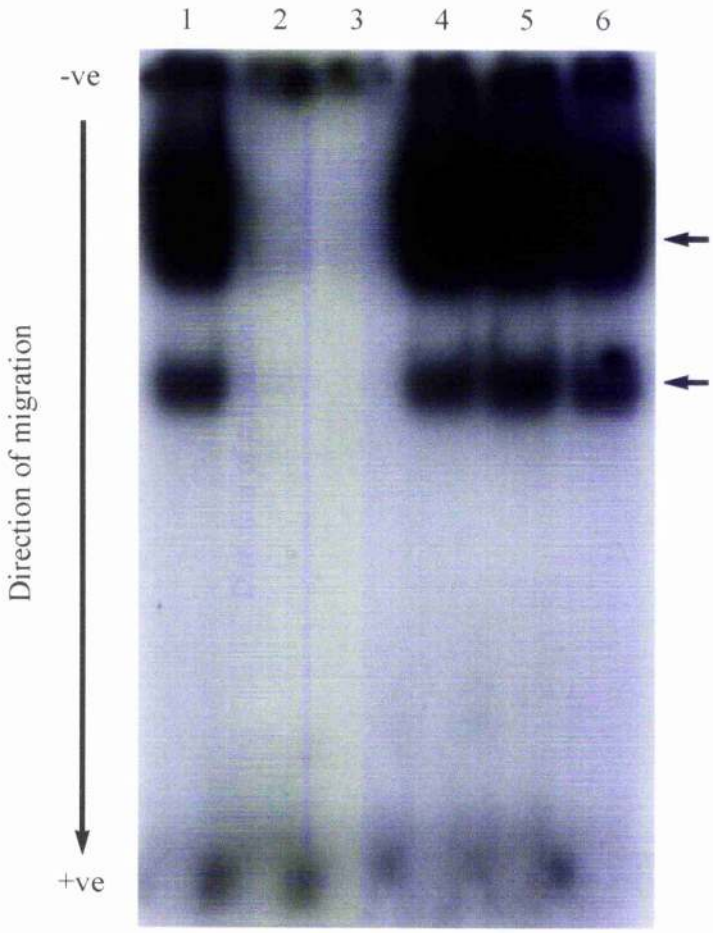


Figure 5.2 Effects of Anti-μ and IL-4 Stimulation on NF-κB Binding in Tonsil Resting B Cells.

Nuclear and cytoplasmic extracts were prepared from resting tonsillar B cells which had been cultured overnight in complete medium and then stimulated with either 0.5μg/ml anti-μ (A), 25μg/ml anti-μ (B) or 400 U/ml IL-4 (C) for up to four hours. 5μg of protein extract was analysed by gel shift assay for binding to γ-[^{32}P]-labelled NF-κB oligonucleotide. Specific binding activities are illustrated by horizontal arrows.

Figure 5.2

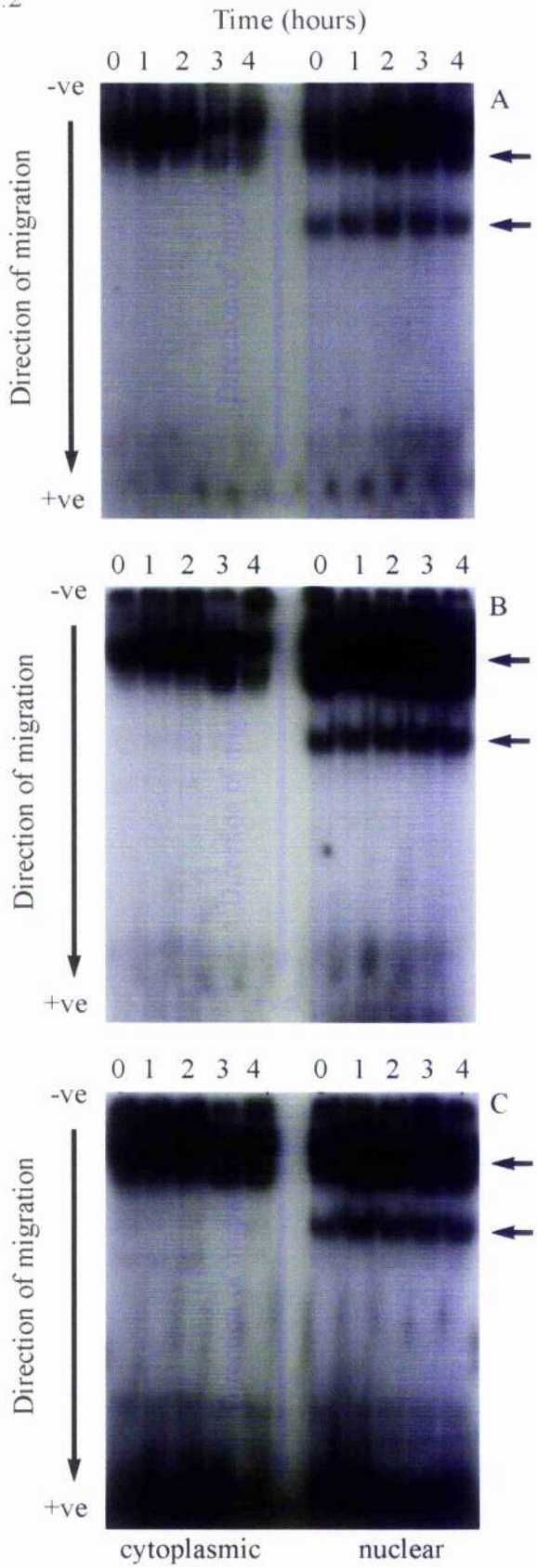

Time (hours)
0 1 2 3 4 0 1 2 3 4
-ve
Direction of migration
+ve
A
B
C
cytoplasmic
nuclear

Figure 5.3 Effects of Anti-μ and IL-4 Stimulation on NFIL-4 Binding in Tonsil Resting B Cells.

Nuclear and cytoplasmic extracts were prepared as described in Figure 5.2, that is cultured overnight in complete medium and then stimulated with either 0.5μg/ml anti-μ (A), 25μg/ml anti-μ or 400 units/ml IL-4 for up to four hours. The binding activity of 5μg of protein to γ-[^{32}P]-labelled NF-IL-4 oligonucleotide was assessed by gel shift assay. Specific binding activities are illustrated with arrows.

Figure 5.3

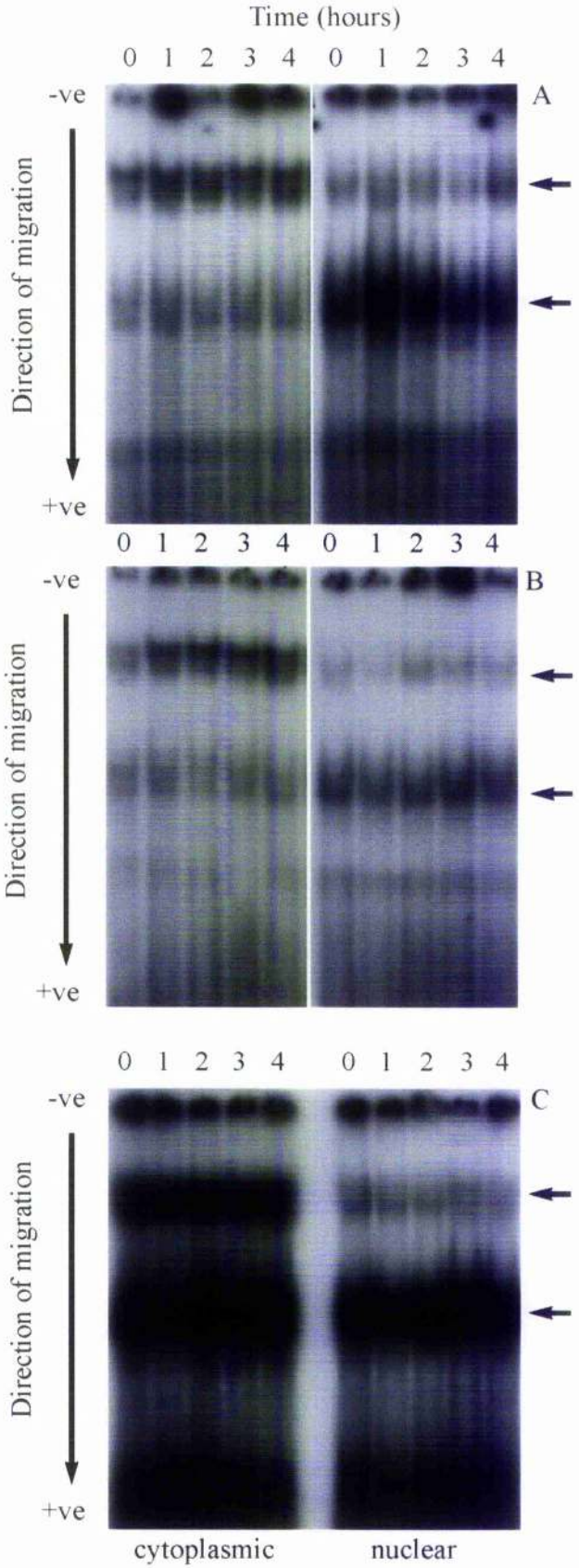

Time (hours)
0 1 2 3 4 0 1 2 3 4
-ve
Direction of migration
+ve
A
0 1 2 3 4 0 1 2 3 4
-ve
Direction of migration
+ve
B
0 1 2 3 4 0 1 2 3 4
-ve
Direction of migration
+ve
C
cytoplasmic
nuclear

Figure 5.4 Identification of NRE specific DNA Binding Activity in Resting Tonsil B Cells.

Nuclear and cytoplasmic extracts were prepared from resting tonsil B cells which had been cultured overnight in complete medium and then stimulated with 25μg/ml anti-μ(A) or 400 units/ml IL-4 (B). The binding activity of 5μg aliquots of protein (nuclear extracts only shown) to γ-[^{32}P]-labelled NRE oligonucletide was assessed by gel shift assay in the absence of cold competitor oligonucleotide (Lane 1), or in the presence of 100-fold excess of cold NRE oligonucleotide (Lane 2) or in the presence of 100-fold cold non-specific oligonucleotide (AP-1) (Lane 3). Lane 4 shows a repeat of the binding activity of γ-[^{32}P]-labelled NRE oligonucleotide in the absence of cold competitor oligonucleotide. Specific binding activities are indicated by horizontal arrows.

Figure 5.4

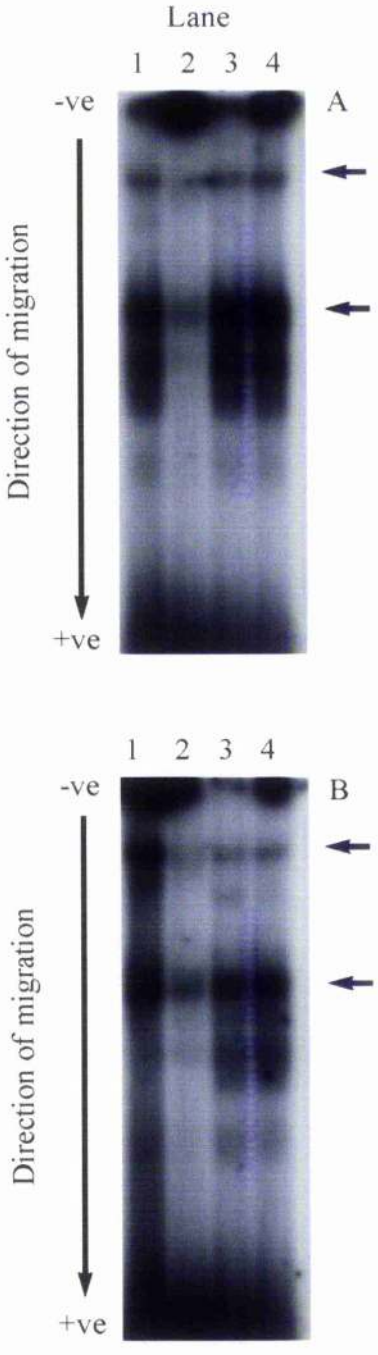

Lane
1 2 3 4
-ve
A
Direction of migration
+ve
1 2 3 4
-ve
B
Direction of migration
+ve

Figure 5.5 Effects of Anti-μ and IL-4 Stimulation on NRE-specific Binding Activity in Resting Tonsil B Cells.

Nuclear and cytoplasmic extracts were prepared as described in Figures 5.2 and 5.3. Cells were cultured overnight in complete medium and then cultured for up to 4 hours with either 0.5 μg/ml anti-μ (A), 25 μg/ml anti-μ (B) or 400 units/ml IL-4(C). The binding activity of 5μg aliquots of protein to γ-[^{32}P]-labelled NRE oligonucleotide was assessed by gel shift assay. Specific binding activities are indicated with horizontal arrows. This experiment is reprasentative of 4 tonsillar preparations.

Figure 5.5

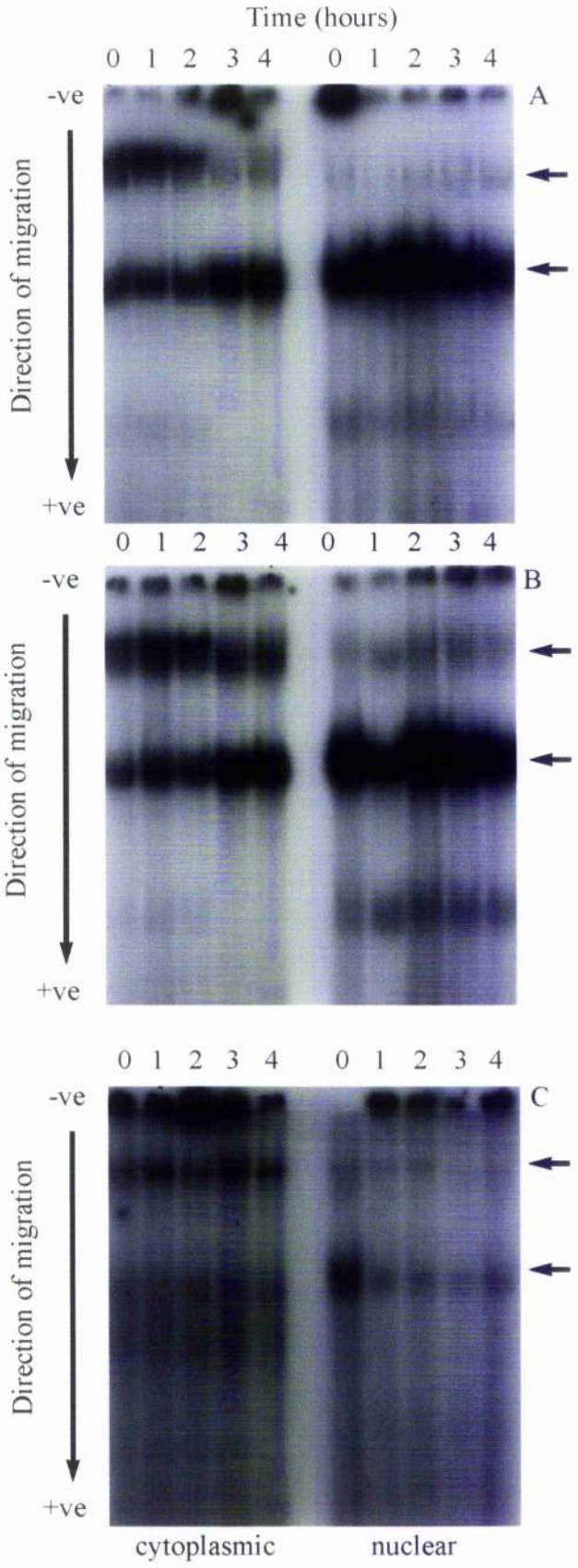

Time (hours)
0 1 2 3 4 0 1 2 3 4
-ve
+ve
Direction of migration
A
B
C
cytoplasmic
nuclear

Figure 5.6 Effects of Anti-γ Stimulation on NRE Binding Activity in Resting Tonsil B Cells.

Nuclear and cytoplasmic extracts were prepared as described in previous figures. Cells were cultured overnight in complete medium and then cultured for up to four hours with 0.5 μg/ml anti-γ (A) or 25 μg/ml anti-γ (B). The binding activity of 5μg aliquots of protein to γ-[^{32}P]-labelled NRE oligonucleotide was assessed by gel shift assay. Specific binding activities are indicated with horizontal arrows.

Figure 5.6

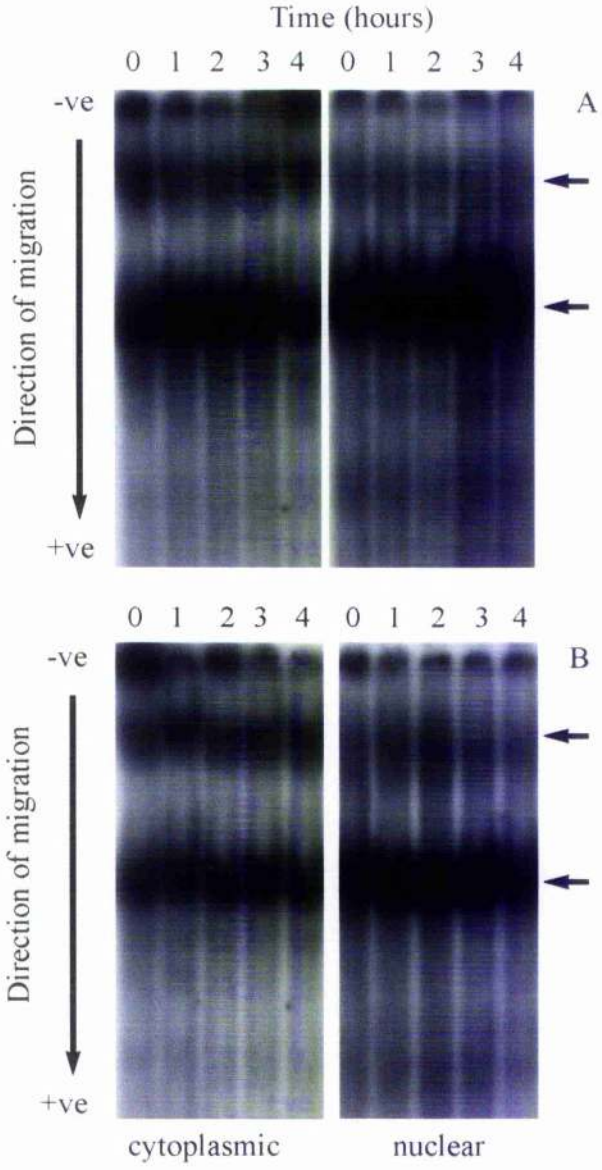

Time (hours)
0 1 2 3 4 0 1 2 3 4
-ve
Direction of migration
+ve
A
0 1 2 3 4 0 1 2 3 4
-ve
Direction of migration
+ve
B
cytoplasmic
nuclear

Figure 5.7 Activity of CAT Reporter Gene Constructs in Tonsil B cells cultured with IL-4

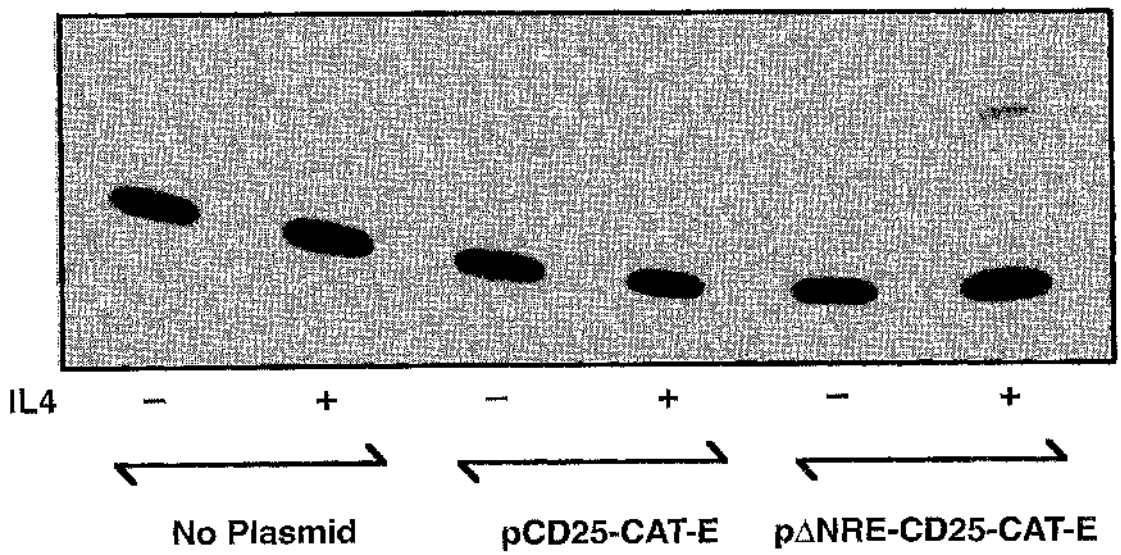
Resting tonsil B cells were transfected with 40 μg of the following plasmid DNAs:

Lanes 1 and 2: No plasmid

Lanes 3 and 4 pCD25-CAT-E plasmid

Lanes 5 and 6 pΔNRE-CAT-E plasmid.

Following this, the cells were rested for an hour and then cultured with either complete medium (Lanes 1, 3 and 5) or with 400 Units/ml IL-4 (Lanes 2, 4, and 6) for 48 hours. Then they were harvested and assayed for CAT activity by TLC. Panel A shows an autoradiograph of the TLC plate and Panel B presents a phosphorimage analysis of the TLC data. The units of relative reporter activity are expressed as phosphorescence units minus background phosphorescence per mm^2 . Plasmids were constructed by E. Hewitt (Hewitt *et al* 1997)


IL4
–
+
–
+
–
+
No Plasmid
pCD25-CAT-E
pΔNRE-CD25-CAT-E

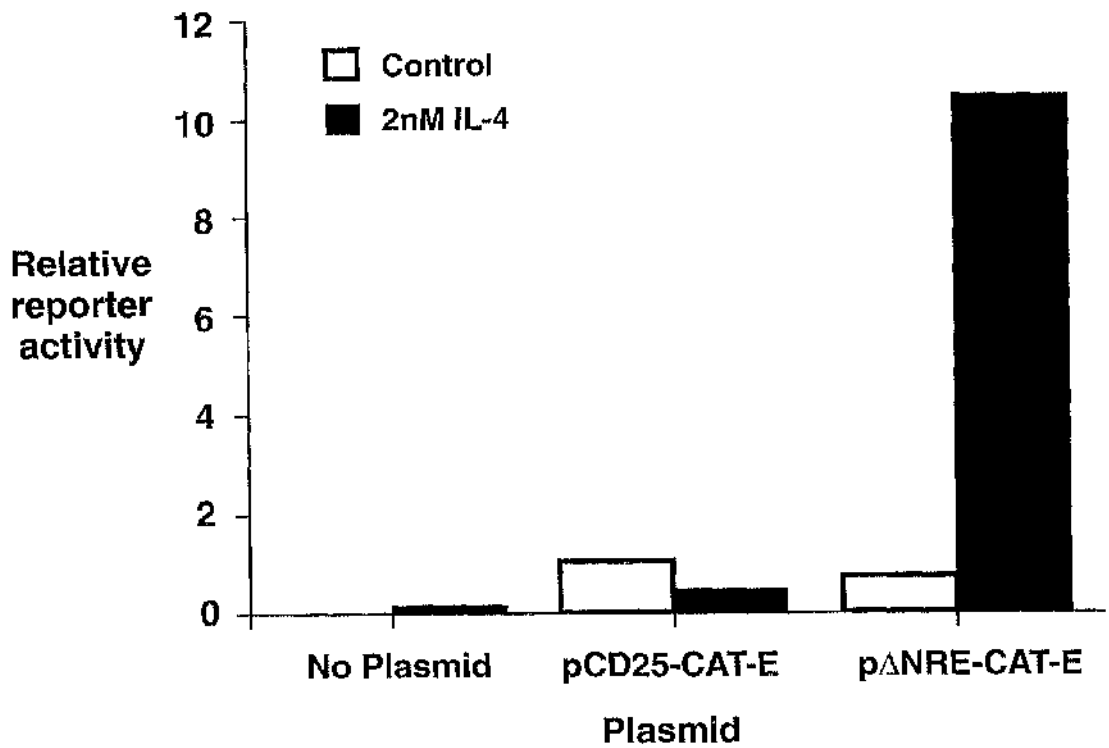

12
10
8
6
4
2
0
Control
2nM IL-4
Relative reporter activity
No Plasmid
pCD25-CAT-E
pΔNRE-CAT-E
Plasmid

Figure 5.8 Activity of CAT Reporter Gene Constructs in Resting Tonsil B Cells Cultured with Anti-μ.

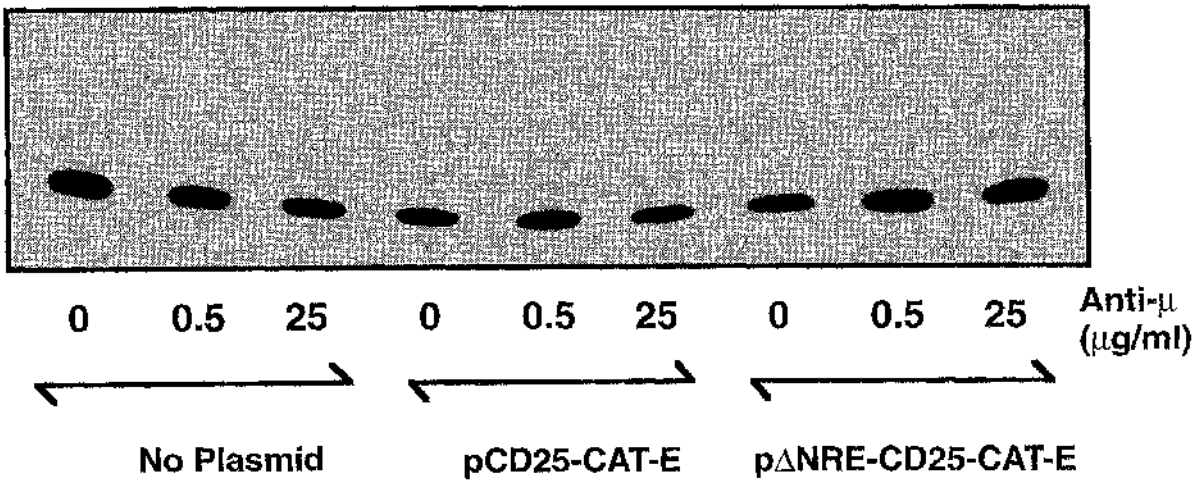
Resting tonsil B cells were electroporated with 40μg of the following plasmid DNAs:

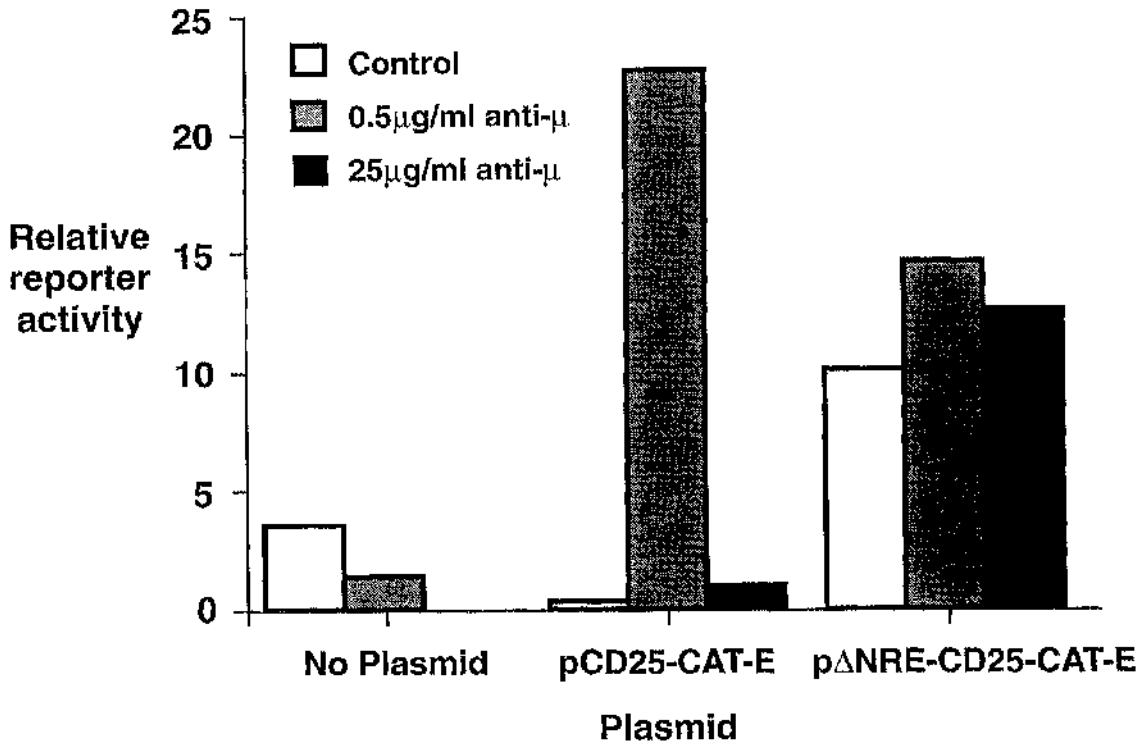
Lanes 1, 2 and 3 No Plasmid

Lanes 4, 5 and 6 pCD25-CAT-E

Lanes 7, 8 and 9 pΔNRE-CAT-E.

They were then rested for an hour before being cultured with either complete medium (Lanes 1, 4 and 7), 0.5 μg/ml anti-μ(Lanes 2, 5 and 8) or 25 μg/ml anti-μ (Lanes 3, 6 and 9). Following this, they were harvested and assayed for CAT activity by TLC. Panel A shows an atuoradiograph of the TLC plate and panel B presents a phosphorimage analysis of the TLC data. Plasmids were prepared by E. Hewitt (Hewitt *et al* 1997)


0 0.5 25 0 0.5 25 0 0.5 25
Anti-μ (μg/ml)
No Plasmid
pCD25-CAT-E
pΔNRE-CD25-CAT-E


25
20
15
10
5
0
Relative reporter activity
Control
0.5μg/ml anti-μ
25μg/ml anti-μ
No Plasmid
pCD25-CAT-E
pΔNRE-CD25-CAT-E
Plasmid

Chapter 6

General Discussion

The experimental data presented in this thesis provide new insights into the regulation of IL-2Rα expression in tonsillar B lymphocytes. In summary, cross-linking of the antigen receptor in B cells can increase IL-2Rα expression, although anti-Ig antibodies are not such potent inducers as IL-4. Engaging the antigen receptor alone cannot cause B cells to proliferate; additional signals are required. Furthermore, ligating sIg on different subsets of B cells causes variations in the expression of IL-2Rα on these cells. Finally, cell surface expression can be related to events at the level of the IL-2Rα promoter.

6.1 The Importance of IL-2Rα in B Cell Activation.

IL-2Rα is not thought to be involved in IL-2 receptor signalling. However, it is an important B cell molecule since its presence allows the high affinity IL-2 receptor to form and therefore permits the B cell to respond to physiological concentrations of IL-2. Thus, factors which can regulate expression of IL-2Rα play a major role in the fate of the B cell. Data from Chapter 3 show that polyvalent antibodies, anti-CD40 antibodies and IL-4 can all induce expression of IL-2Rα in human tonsillar B cells. This induction appears to be both lineage and species specific. It would be of some interest to discover whether any other B cell activators can increase IL-2Rα expression. Ligation of B cell MHC class II antigen causes signal transduction events within the B cell and can cause up-regulation of IL-2Rα in a human B cell line, although this alone cannot cause the cells to proliferate (E.Hewitt, personal communication).

In the experiments on primary B cells presented here, the physiological relevance of IL-2Rα was confirmed. Only B cells which had increased levels of IL-2Rα were able to respond to IL-2 and proliferate significantly. An additional experiment which could be performed to confirm that the presence of IL-2Rα is crucial for B cell growth would be to repeat the proliferation experiments from Chapter 3 but also adding an anti-IL-2Rα antibody. If this inhibited proliferation, it would indicate that IL-2Rα is indeed necessary for B cell proliferation. It is presumably necessary for B cell differentiation also, although no experiments were performed within this laboratory to determine this.

6.2 IL-2Rα Expression in Different B Cell Subsets.

The data presented in Chapter 4 appear to support the theory that sIgD and sIgM have different functions in B cell signalling, since anti-μ antibodies are capable of increasing expression of IL-2Rα while anti-δ antibodies are not. It has been suggested that sIgM plays a negative role in B cell signaling while ligation of sIgD provides a dominant positive response. However, another model suggests that the critical parameter which determines whether a B cell is tolerised or activated via its sIg is not whether sIgM or sIgD is engaged but rather the maturational stage of the B cell upon encounter with the antigen (Norvell and Monroe 1996). Cells isolated from tonsils will mostly be mature, with small numbers of immature or memory B cells (Butcher and Cushley 1991). Adding anti-μ antibodies to tonsillar B cells will stimulate both immature and mature B cells and

it was found that low concentrations of anti-μ antibodies up-regulate IL-2Rα expression while this effect was not seen at high concentrations of the same antibody.

Previous studies in mice have shown that stimulation of germinal centre (GC) B cells with large amounts of soluble antigen can mimic a situation where mature GC cells become auto-reactive and apoptose. B cells outside the follicle do not die but expand and differentiate into antibody secreting cells (Pulendran *et al* 1995 a, b). A similar argument can be used when studying the effects of anti-μ antibodies on IL-2Rα expression in resting tonsillar B cells. The majority of B cells in the tonsil will be GC B cells. Cross-linking their sIgM with large amounts of antibody could provide a signal which mimics that of large amounts of auto-antigen on FDCs. Thus, GC B lymphocytes may be able to discriminate between foreign and self antigen simply by determining the size of the signal received by sIg. This hypothesis is supported by the finding that short term sIg triggering may protect GC cells from apoptosis (Liu *et al* 1989) whereas prolonged stimulation leads to death (Shokat and Goodnow 1995; Pulendran *et al* 1995a; Galibert *et al* 1996). Therefore, it would be interesting to determine whether the cells subjected to high doses of anti-μ antibodies were apoptosing. In addition, further work needs to be done to separate the immature and mature B cells to determine whether differences in the expression of IL-2Rα can be seen.

The reason that high doses of anti-μ antibodies fail to up-regulate IL-2Rα expression has been explained above. However, the failure of B cells to significantly up-regulate IL-2Rα

in response to sIgD is less easy to explain physiologically. In the murine system, anti-IgD antibodies cause a substantial decrease in the number of spleen and lymph node B cells, when T cell help is blocked with anti-CD4 antibodies (Finkelman *et al* 1995). The loss of B cells occurs slowly over a period of 2-7 days. A likely interpretation of this is that sIgD cross-linking in the absence of additional signals inactivates and eventually deletes most auto-reactive B cells regardless of whether the auto-reactivity results from expression of germline or somatically mutated Ig genes. Any slight increase in IL-2Rα expression that could be seen as a result of ligating sIgD can thus be explained by the presence of any T cells which may be contaminating the B cell preparation.

When differentiating into memory B cells, GC B lymphocytes recover the ability to proliferate in response to sIg triggering. Thus, the discovery that anti-γ antibodies can up-regulate expression of IL-2Rα in a dose-dependent manner is unsurprising, since these antibodies would be acting on the sIg of memory B cells and there would be no need for these cells to become tolerised on encountering large amounts of antigen. Memory B cells have been positively selected and are not generally involved in auto-immune reponses. Further investigations into the responses of different subsets of B cells within tonsils would be of much interest. It is possible to separate immature and mature B cells from each other by panning with anti-IgD antibodies. In addition, naive and memory B cells can be purified according to the surface expression of IgD, IgG, IgA, CD38 and CD44 (Liu and Banchereau 1996). These experiments should give pure cultures of each B cell

subset and help provide a clearer picture of how each population of B cells responds when their sIg is ligated.

6.3 $CD5^+$ B Cells.

The 67kDa CD5 molecule is present on B1a lymphocytes. These $CD5^+$ cells produce low avidity auto-reactive antibodies (Hayakawa *et al* 1984; Casali *et al* 1987; MacKenzie *et al* 1991). The function of CD5 on B cells remains unclear but it is thought that $CD5^+$ cells form a separate subset of B cells. $CD5^+$ cells express more sIgM and less sIgD than conventional B cells. If we assume that there are two populations of B cells present within a tonsillar population ($CD5^+$ and $CD5^-$ cells) then it would be interesting to discover whether the cells responding to ligation of sIgM by up-regulating IL-2Rα are $CD5^+$ or not. Studies have shown that $CD5^+$ B cells activated through their antigen receptor fail to proliferate (Zupo *et al* 1991) and require IL-2 to enhance their proliferative response (Zupo *et al* 1991; Defrance *et al* 1992). Experiments in this thesis have shown that anti-μ antibodies fail to up-regulate IL-2Rα at high concentrations; in addition anti-μ antibodies require IL-2 to cause B cells to proliferate. Therefore it would be of interest to determine whether we have been studying the responses of $CD5^+$ cells. This could be done by purifying $CD5^-$ B cells from tonsil B cells by dual colour fluorescence FACS sorting. However, two points suggest that the responses studied have been those of conventional (i.e. $CD5^-$) B cells. Firstly, any changes in IL-2Rα expression seen when the cells were stimulated via sIg were mimicked by alterations in CD23

expression. B cells which express CD23 do not express CD5 (Wortis *et al* 1995) and therefore the fact that CD23 and IL-2Rα are being regulated in the same manner suggests that we have been looking at $CD5^-$ cells. In addition, a recent paper has shown that both $CD5^+$ and $CD5^-$ cells require both anti-μ antibodies and IL-2 to become activated and proliferate. Therefore, it seems likely that the population of B cells responding to anti-μ antibodies by altering their expression of IL-2Rα are $CD5^-$ B cells.

6.4 Events at the IL-2Rα Promoter.

Knowledge of the structure and regulatory properties of the IL-2Rα promoter region has increased greatly over the past few years. There are at least three positive regulatory regions and two negative regulatory regions, all of which are capable of binding large numbers of transcription factors which may (or may not) act cooperatively and which may be influenced by various signals from the cell surface. The experiments within this thesis have focused on NRE I and PRR_I since these, together with PRR_{III}, are believed to be involved in the induction of IL-2Rα expression and therefore of greatest interest. The most clear-cut finding to emerge was that ligating sIgM with low doses of antibody caused NRE-BP to be released from NRE I. NRE-BP is a protein which appears to be involved in the silencing of gene transcription. At high concentrations of anti-μ antibody, NRE-BP remains bound to the promoter. These experiments were confirmed by reporter gene assays which used deletion constructs of the IL-2Rα promoter region. Thus, it is

possible that NRE-BP could play a role in B cell tolerance, when sIgM is stimulated by large amounts of antigen, preventing an auto-reactive response.

As expected, on culturing B cells with anti-δ antibodies, NRE-BP remained bound to the promoter. However, it was surprising that anti-γ antibodies failed to affect NRE-BP activity. These antibodies may be acting potently upon proteins which are bound to another site within the promoter. Previous studies have compared signaling through the sIgM and sIgG complexes and discovered several differences. It was found that the combination of anti-μ antibodies and a phorbol ester was mitogenic in tonsil B cells but that anti-γ antibodies and phorbol ester did not elicit a proliferative response (Roifman *et al* 1987). In addition, protein tyrosine phosphorylation patterns were studied in a variety of B cell lines representing different stages of B cell maturation and there were distinct differences between sIgM and sIgG bearing cells (Law *et al* 1992). Finally, it was discovered that anti-μ and anti-γ antibodies modulate *c-fos* RNA levels differently in human B lymphocytes (Shu *et al* 1994). Now we have determined that sIgM and sIgG are also acting differently in the regulation of IL-2Rα and have pinpointed a transcription factor (NRE-BP) which appears to play a role in this variation. It may be that sIgG acts upon another factor binding to a different site which is able to induce IL-2Rα gene expression even though NRE-BP remains bound to the promoter.

However, there may be another explanation for the differences seen. Both sIgM and sIgG are associated with the transmembrane proteins, Ig-α and Ig-β/γ (Reth 1992). In addition,

it was discovered that sIgG can also be expressed at the cell surface in another form. It is fixed in the membrane by a glycosyl phosphatidylinositol anchor instead of Ig-α and Ig-β/γ (Williams *et al* 1993). Thus, the differences observed may simply be due to the configuration in which sIgG exists in these cells. It would be interesting to discover whether sIgE+ and sIgA+ memory B cells behave in the same way as sIgG+ cells; however such behaviour would be difficult to assess as they are only present in the tonsil in tiny numbers.

6.5 Summary

The experiments within this thesis have shown that, together with IL-4, anti-Ig antibodies and anti-CD40 antibodies are capable of up-regulating cell surface IL-2Rα on human tonsillar B cells. When different subsets of the B cell population are stimulated via their respective sIg receptors there are differences in the expression of IL-2Rα seen. In particular, stimulating immature and mature B cells through sIgM up-regulates expression when low concentrations of antibody are used. This response is abrogated when high concentrations of antibody are used. It is possible that the use of high concentrations of anti-μ antibodies is mimicking the effects seen when large amounts of auto-antigen is present in the germinal centre; the B cells become tolerised and will not provoke a damaging auto-immune response. By studying the binding of NRE-BP to NRE I within the IL-2Rα promoter region, we have discovered that this transcription factor is

under the control of signals transduced via sIgM and therefore may be playing a crucial role in B cell tolerance.

There is much work remaining to be done in the area of IL-2Rα gene regulation. Experiments within this laboratory are currently being undertaken to determine the identity of NRE-BP and the intermediate signalling events which affect the binding of this factor to the promoter. However, control of IL-2Rα expression is undoubtedly extremely complex and multiple elements are acting cooperatively to allow induction of IL-2Rα; it may be several years before a clear picture of the regulation of IL-2Rα expression in B cells emerges.

REFERENCES

Abney, E.R., Cooper, M.D., Kearney, J.F., Lawton, A.R. and Parkhouse, R.M.E. (1978) *J. Immunol.* **120**, 2041-2049.

Ales-Martinez, J., Warner, G.L. and Scott, D.W. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 6919-6923.

Ales-Martinez, J.E., Cuende, E., Martinez, A.C., Parkhouse, R.M.E., Pezzi, L. and Scott, D.W. (1991) *Immunol. Today* **12**, 201-205.

Ales-Martinez, J., Scott, D.W., Phipps, R.P., Casnelli, J.E., Kroemer, G., Martinez, C., and Pezzi, L. (1992) *Eur. J. Immunol.* **22**, 845-850.

Algarte, M., Lecine, P., Costello, R., Plet, A., Olive, D. and Imbert, J. (1995) *EMBO. J.* **14**, 5060-5072.

Alt, F.W. and Baltimore, D. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 4118-4122.

Alt, F.W., Blackwell, T.K. and Yancopoulos, G.D. (1991) *Science* **238**, 1079-1087.

Alt, F.W., Bothwell, A.L.M., Knapp, M., Siden, E., Mather, E., Koshland, M. and Baltimore, D. (1980) *Cell* **20**, 293-301.

Alt, F.W. and Yancopoulos, G.D. (1987) *Nature* **327**, 189-190.

Anderson, J., Bullock, W.W. and Melchers, F. (1974) *Eur. J. Immunol.* **4**, 725-730.

Andria, M.L., Hsieh, C., Oren, R., Francke, U., and Levy, S. (1991) *J. Immunol.* **147**, 1030-1036.

Arima, N., Kamio, M., Imada, K., Hori, T., Hattori, T., Tsudo, M., Okima, M. and Uchiyama, T. (1992) *J. Exp. Med.* **176**, 1265-1272.

Armitage, R.J., Ziegler, S.F., Friend, D.J., Park, L.S. and Fanslow, W.C. (1992) *Blood* **79**, 1738-1745.

Asao, H., Takeshita, T., Ishii, N., Kumaki, S., Nakamura, M. and Sugamura, K. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 4127-4131.

Aubry, J.P., Pochon, S., Graber, P., Jansen, K.U. and Bonnefoy, J.Y. (1992) *Nature* **358**, 505-507.

Augustine, J.A., Sutor, S. and Abraham, R.T. (1991) *Mol. Cell. Biol.* **11**, 4431-4440.

Azam, M., Erdjument-Bromage, H., Kreider, B.L., Xia, M., Quelle, F., Basu, R., Saris, C., Tempst, P., Ihle, J.N. and Schindler, C. (1995) *EMBO.J.* **14**, 1402-1411.

Baldwin, A.S. and Sharp, P.A. (1988) *Proc. Natl. Acad. Sci. U.S.A* **.85**, 723-777.

Baeuerle, P.A. and Henkel, T. (1994) *Ann. Rev. Immunol.* **12**, 141-179.

Ballard, D.W., Bohnlein, E., Hoffman, J.A., Bogerd, H.P., Dixon, E.P., Franza, B.R. and Greene, W.C. (1989) *New. Biol.* **1**, 83-92.

Ballard, D.W., Bohnlein, E., Lowenthal, J.W., Wano, Y., Franza, B.R. and Greene, W.C. (1988) *Science* **241**, 1652-1655.

Banchereau, J., Bazan, F., Blanchard, D., Briere, F., Galizzi, J.P., Vankooten, C., Liu, Y-J., Rousset, F. and Saeland, S. (1994) *Ann. Rev. Immunol.* **12**, 881-922.

Banchereau, J., DePaoli, P., Valle, A., Garcia, E. and Rousset, F. (1991) *Science* **251**, 70-72.

Bazan, J.F. (1990a) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 6934-6938.

Bazan, J.F. (1990b) *Immunol. Today* **11**, 350-354.

Beadling, C., Guschin, D., Witthuhn, B.A. Ziemecki, A., Ihle, J.N., Kerr, I.M. and Cantrell, D.A. (1994) *EMBO. J.* **13**, 5605-5615.

Bell, S.E. and Goodnow, C.C. (1994) *EMBO. J.* **13**, 816-826.

Benveniste, E.N. and Merrill, J.E. (1984) *Nature* **321**, 610-613.

Bhatti, L. and Sidell, N. (1994) *Immunol.* **81**, 273-279.

Bijsterbosch, M.K. and Klaus, G.G.B. (1985) *J. Exp. Med.* **162**, 1825-1836.

Boyd, A.W., Goding, J.W. and Schrader, J.W. (1981) *J. Immunol.* **126**, 2461-2465.

Bohnlein, E., Lowenthal, J.W., Siekevitz, M., Ballard, D.W., Franza, B.R. and Greene, W.C. (1988) *Cell* **53**, 827-836.

Bohnsack, J.F. and Cooper, N.R. (1988) *J. Immunol.* **141**, 2569-2576.

Bonni, A., Frank, D.A., Schindler, C. and Greenberg, M.E. (1993) *Science* **262**, 1575-1579.

Bowtell, D, Fu, P., Simon, M. and Senior, D. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 6511-6515.

Boyle, W.M., Smeal, T., Defize, L.H.K., Angel, P., Woodgett, J.R., Karin, M. and Hunter, T. (1991) *Cell* **64**, 573-584.

Bradbury, L., Goldmacher, V.S. and Tedder, T.F. (1993) *J. Immunol.* **151**, 2915-2927.

Bradbury, L., Kansas, G., Levy, S., Evans, R.L. and Tedder, T.F. (1992) *J. Immunol.* **149**, 2841-2850.

Brink. R., Goodnow, C.C., Crosbie, J., Adams, E., Eris, J., Mason, D.Y., Hartley, S.B. and Basten, A. (1992) *J. Exp. Med.* **176**, 991-1005.

Brunswick, M., Samelson, L.E. and Mond, J.J. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 1311-1314.

Buday, L. and Downward, J. (1993) *Cell*, **73**, 611-620.

Burlinson, E.L., Graber, P., Bonnefoy, J-Y., Ozanne, B.W. and Cushley, W. (1996) *Eur. J. Immunol.* **26**, 1069-1073.

Burlinson, E.L., Pringle, H.M., Ozanne, B.W. and Cushley, W. (1995) *Immunol. Lett.* **45**, 93-97.

Bunn, P.A., Schechter, G.P., Jaffe, E., Blayney, D., Young, R.C., Matthews, M.J., Blattner, W., Broder, S., Robert-Guroff, M. And Gallo, R.C. (1983) *N. Engl. J. Med.* **309**, 257-264.

Burkhardt, A.L., Brunswick, M., Bolen, J.B. and Mond, J.J. (1991) *Proc. Natl. Acad. Sci. U.S.A.* 88, 7410-7414.

Burnet, M.F. (1959) *The Clonal Selection Theory of Acquired Immunity*, Vanderbilt University Press.

Bustelo, X.R. and Barbacid, M. (1992) *Science* **256**, 1196-1199.

Bustelo, X.R., Ledbetter, J.A. and Barbacid, M. (1992) *Nature* **356**, 68-71.

Butcher, R.D.J. and Cushley, W. (1991) *Immunol.* **74**, 511-518.

Butcher, R.D.J., McGarvie, G. and Cushley, W. (1990) *Immunol.* **69**, 57-64.

Callard, R.E. (1990) In "*Cytokines and B Lymphocytes*", Academic Press, London, 121-150.

Campbell, K.S. and Cambier, J.C. (1990) *EMBO. J.* **9**, 441-448.

Campbell, K.S., Hager, E.J., Friedrich, R.J. and Cambier, J.C. (1991) *Proc. Natl. Acad. Sci. U.S.A* .**88**, 3982-3986.

Campbell, M.A. and Sefton, B.M. (1990) *EMBO. J.* **9**, 2125-2131.

Campbell, M.A. and Sefton, B.M. (1992) *Mol. Cell. Biol.* **12**, 2315-2321.

Cambier, J.C. and Jensen, W.A. (1993) *Curr. Opin. Genet. Dev.* **4**, 55-63.

Cambier, J.C., Justement, L.B., Newell, M.K., Chen Z.Z., Harris, L.K., Sandoval, V.M., Klemsz, M.J. and Ransom, J.T. (1987) *Immunol. Rev.* **95**, 37-57.

Cambier, J.C., Keltman, J.R., Vitetta, E.S. and Uhr, J.W. (1976) *J. Exp. Med.* **144**, 293-298.

Cambier, J.C., Morrison, D.C. Chien, M.M. and Lehmann, K.R. (1991) *J. Immunol.* **146**, 2075-2082.

Cambier, J.C, Pleiman, C.M and Clark, M.R. (1994) *Ann. Rev. Immunol.* **12**, 457-486.

Cantley, L.C., Auger, K.R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R. and Soltoff, S (1991) *Cell* **64**, 281-302.

Casali, P., Burastero, S.E., Nakamura, M., Inghirami, G. and Notkins, A.L. (1987) *Science* **236**,77-81.

Carding, S.R., Hayday, A.C. and Bottomley, K. (1991) *Immunol. Today* **12** 239-245.

Carroll, M.P., Clark-Lewis, I., Rapp, U.R. and May, W.S. (1990) *J. Biol. Chem.* **265**, 19812-19817.

Carsetti, R., Kohler, G. And Lamers, M.C. (1993) *Eur. J. Immunol.* **23**, 168-178.

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. U.S.A.* **88**, 2745-2749.

Carter, R.H., Tuveson, D.A., Rhee, S.G. and Fearon, D.T. (1991) *J. Immunol.* **147**, 3663-3671.

Ceredig, R., Lowenthal, J.W., Nabholz, M. and MacDonald, H.R. (1985) *Nature* **314**, 98-101.

Chan, A.C., Irving, B.A., Fraser, F.D. and Weiss, A. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 9166-9170.

Chan, A.C., Irving, B.A. and Weiss, A. (1992a) *Curr. Opin. Immunol.* **4**, 246-251.

Chan A.C., Iwashima, M., Turk, C.W. and Weiss, A. (1992b) *Cell* **71**, 649-662.

Chen, J., Ma, A., Young, F. and Alt, F.W. (1994) *Int. Immunol.* **6**, 1265-1268.

Chen, Y.X., Welte, K., Gebhard, D.H. and Evans, R.L. (1984) *J. Immunol.* **133**, 2496-2501.

Choquet, D., Partiseti, M., Amigorena, S., Bonnerot, C., Fridman, W. and Korn, H. (1993) *J. Cell Biol.* **121**, 355-365.

Clark, E.A. (1993) *J. Immunol.* **150**, 4715-4718.

Clark, E.A. and Ledbetter, J.A. (1994) *Nature* **367**, 425-428.

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, M.R., Johnson, S.A. and Cambier, J.C. (1994) *EMBO. J.* **13**, 1911-1919.

Cleveland, J.L., Rapp, U.R. and Farrer, W.L. (1987) *J. Immunol.* **138**, 3495-3504.

Clipstone, N.A. and Crabtree, G.R. (1994) *Ann. Rev. Biochem.* **63**, 10451083.

Clutterbuck, E., Shields, J.G., Gordon, J., Smith, S.H., Boyd, A., Callard, R.E., Campbell, H.G., Young, I.G. and Sanderson, C.J., (1987) *Eur. J. Immunol.* **17**, 1743-1750.

Coggeshall, K.M. and Cambier, J.C. (1984) *J. Immunol.* **133**, 3382-3386.

Coggeshall, K.M., McHugh, J.C. and Altman, A. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5660-5664.

Cohen, S.B.A., Katsikis, P.D., Feldmann, M. and Londei, M. (1994) *Immunology* **83**, 329-332.

Conley, M.E. (1992) *Ann. Rev. Immunol.* **10**, 215-238.

Cook, S.J. and McCormick, F. (1993) *Science* **262**, 1069-1072.

Cosman, D., Ceretti, D.P., Larsen, A., Park, L., March, C., Dower, S., Gills, S. and Urdall, D. (1984) *Nature* **312**, 768-771.

Costello, R., Cerdan, C., Lipcey, C., Algarte, M., Martin, Y., Baeuerle, P.A., Olive, D. and Imbert, J. (1993a) *Cell Growth Differ.* **4**, 947-954.

Costello, R., Lipcey, C., Algarte, M., Cerdan, C., Baeuerle P.A., Olive, D. and Imbert, J. (1993b) *Cell Growth Differ.* **4**, 329-339.

Couture, C., Baier, G., Altman, A. and Mustelin, T. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 5301-5305.

Cross, S.L., Feinberg, M.B., Wolf, J.B., Holbrook, N.J., Wong-Staal, F. And Leonard, W.J.(1987) *Cell* **49**, 47-56.

Darnell, J.E., Kerr, I.M. and Stark, G.R. (1994) *Science* **264**, 1415-1421.

Davidson, W.F., Fredrickson, T.N., Rudikoff, E.K., Coffman, R.L., Hartley, J.W. and Morse, H.C. (1984) *J. Immunol.* **133**, 744-753.

Davidson, W.F., Pierce, J., Rudikoff, S and Morse, H.C. (1988) *J. Exp. Med.* **168**, 389-407.

Davie, E.W., Ichinose, A. and Leytus, S.P. (1986) *Cold Spring Harbor Symp. Quant. Biol.* **51**, 509-514.

Davis, R.J. (1993) *J. Biol. Chem.* **268**, 14553-14556.

Davis, S, Aldrich, T.H., Valenzuela, D.M, Wong, V., Furth, M.E., Squinto, S.P. and Yancopoulos, G.D. (1991) *Science* **253**, 59-63.

Defrance, T., Aubry, J.P., Rousset, F., Vandervliet, 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.

Defrance, T., Carayon, P., Billian, G., Guillemot, J.C., Minty, A., Caput, D. and Ferrara, P. (1994) *J. Exp. Med.* **179**, 135-143.

Defrance, T., Vandervliet, B., Aubry, J.P. and Banchereau, J. (1988) *J. Exp. Med.* **168**, 1321-1329.

Defrance, T., Vandervliet, B., Durand, I., Briolay, J. and Banchereau, J. (1992) *Eur. J. Immunol.* **22**, 2831-2839.

Defranco, A.M., Davis, M and Paul, W.E. (1982) *In "B and T Cell Tumours"* E. Vitetta and C. Fox, editors Academic Press, New York, 445-471.

Delcayre, A., Salas, F., Mathur, S., Kovats, K., Lotz, M. And Lernhardt, W. (1991) *EMBO. J.* **10**, 919-926.

Del-Prete, G., DeCarli, M., Almerigogna, F., Giudizi, M.G., Biagotti, R. and Romagnani, S. (1993) *J. Immunol.* **150**, 353-360.

Depper, J.M., Leonard, W.J., Kronke, M., Waldmann, T.A. and Greene, W.C.(1984) *J. Immunol.* **133**, 1691-1695.

DeWaal-Malefyt, R.D., Haanen, J., Spits, H., Roncarolo, M.G., Teveldc, A., Figdor, C., Johnson, K., Kastelein, R., Yssel, H. and DeVries, J.E. (1991) *J. Exp. Med.* **174**, 915-924.

Djeu, J.Y., Liu, J.H., Wei, S., Rui, H., Pearson, C.A. Leonard, W.J. and Blanchard, D.K. (1993) *J. Immunol.* **150**, 960-974.

Dosch, H.M., Lam, P., Hui, M.F., Hibi, T. and Cheung, R.K. (1990) *Int. Immunol.* **2**, 833-848.

Downward, J. (1992) *Bioessays* **14**, 777-784.

Dukovich, M., Wano, Y., Thuy, J.B., Katz, P., Cullens, B.R., Kehrl, J.H. and Greene, W.C. (1987) *Nature* **327**, 518-522.

Eardley, D.D. and Koshland, M.E. (1991) *Science* **251**, 78-81.

Elton, T.S. and Reeves, R. (1986) *Anal. Biochem.* **157**, 53-62.

Espinoza-Delgado, I., Ortaldo, J.R., Winckler-Pickett, R., Sugamura, K., Varesio, L. and Longo, D.L. (1990) *J. Exp. Med.* **171**, 1821-1826.

Evans, S.S., Lee, D.B., Han, T., Tomasi, T.B. and Evans, R.L. (1990) *Blood* **76**, 2583-2593.

Fanslow, W.C, Spriggs, M.K., Rauch, C.T., Clifford, K.N., MacDuff, B.M., Ziegler, S.F., Schooley, K.A., Mohler, K.A., March, C.J. and Armitage, R.J. (1993) *Blood* **81**, 2998-3005.

Finkelman, F.D., Holmes, J.M., Dukhanina, O.I. and Morris, S.C. (1995) *J. Exp. Med.* **181**, 515-525.

Fiorentino, D.F., Zlotnik, A., Vieira, P., Mosmann, T.R., Howard, M., Moore, K.W. and O'Garra, A. (1991) *J. Immunol.* **146**, 344-3451.

Fisher, C.L., Ghysdael, J. and Cambier, J.C. (1991) *J. Immunol.* **146**, 1743-1749.

Fluckiger, A.C., Garrone, P., Durand, I., Galizzi, J.P. and Banchereau, J. (1993) *J. Exp. Med.* **178**, 1473-1481.

Forster, I. and Rajewsky, K. (1987) *Eur. J. Immunol.* **17**, 521-528.

Friedrich, J., Campbell, K. and Cambier, J.C. (1993) *J. Immunol.* **150**, 2814-2822.

Fu, X. and Zhang, J. (1993) *Cell* **74**, 1135-1145.

Fujii, H., Nakagawa, Y., Schindler, U., Kawahara, A., Mori, H., Gouilleux, F., Groner, B., Ihle, J.N., Minami, Y., Miyazaki, T. and Taniguchi, T. (1995) *Proc. Natl. Acad. Sci. U.S.A* **92**, 5482-5486.

Fuller, K.A., Kanagawa, O. and Nahm, M.H. (1993) *J. Immunol.* **151**, 4505-4512.

Galibert, L., Burdin, N., Barthelemy, C., Meffre, G., Durand, I., Garcia, E., Garrone, P., Rousset, F., Banchereau, J. and Liu, Y.J. (1996) *J. Exp. Med.* **183**, 2075-2085.

Gauchat, J.P., Henchoz, S., Mazzei, G., Aubry, J.P., Brunner, T., Blasey, H., Life, P., Talabot, D., Floes-Romo, L., Thompson, J., Kishi, K., Butterfield, J., Dahinden, C. and Bonnefoy, J.Y. (1993) *Nature* **365**, 340-343.

Giese, K, Cox, J and Grosschedl, R. (1992) *Cell* **69**, 185-195.

Giri, J.G., Ahdieh, M., Eisenman, J., Shanebeck, K., Grabstein, K., Kumaki, S., Namen, A., Park, L.S., Cosman, D and Anderson, D. (1994) *EMBO. J.* **13**, 2822-2830.

Giri, J.G., Kumaki, S., Ahdieh, M., Friend, D.J., Loomis, A., Shanebeck, K., Dubose, R., Cosman, D., Park, L.S. and Anderson, D. (1995) *EMBO. J.* **14**, 3654-3663.

Goding, J.W., Scott, D.W. and Layton, J.E. (1977) *Immunol. Rev.* **37**, 152-163.

Gold, M.R., Crowley, M.T., Martin, G.A., McCormick, F. And Defranco, A.L. (1992) *J. Immunol.* **150**, 377-386.

Gold, M.R., Law, D.A. and Defranco, A.L. (1990) *Nature* **345**, 810-813.

Gold, M.R., Matsuchi, L., Kelly, R.B. and Defranco, A.L. (1991) *Proc. Natl. Acad. Sci. U.S.A* **88**, 3436-3440.

Goldman, F., Jensen, W. Johnson, G.L., Heasley, L. and Cambier, J.C. (1994) *J. Immunol.* **153**, 2905-2917.

Goodbourn, S and Maniatis, T. (1988) *Proc. Natl. Acad. Sci. U.S.A* **85**, 1447-1451.

Goodnow, C.C., Crosbie, J., Jorgensen, H., Brink, R.A. and Basten, A. (1989) *Nature* **342**, 385-391.

Gootenberg, J.E., Ruscetti, F.W., Mier, J.E., Gazdar, A. and Gallo, R. (1981) *J. Exp. Med.* **154**, 1403-1417.

Greene, W.C., Bohnlein, E and Ballard, D.W. (1989) *Immunol. Today* **10**, 272- 278.

Greene, W.C., Robb, R.J., Svetlik, P.B., Rusk, C.M., Depper, J.M. and Leonard, W.J. (1985) *J. Exp. Med.* **162**, 363-368.

Gulbins, E., Coggeshall, K.M., Baier, G., Katzav, S., Burn, P. and Altman, A. (1993) *Science* **260**, 822-825.

Hahn, S. (1992) *Curr. Biol.* **2**, 152-153.

Han, S.H., Zheng, B., Porto, J.D. and Kelso, G. (1995) *J. Exp. Med.* **182**, 1635-1644.

Hanks, S.K., Quinn, A.M. and Hunter, T. (1988) *Science* **241**, 42-52.

Hardy, R.R., Carmack, C.E., Li, Y.S. and Hayakawa, K. (1994) *Immunol. Rev.* **137**, 91-118.

Harnett, M.M., Holman, M.J. and Klaus, G.G.B. (1989) *Eur. J. Immunol.* **19**, 1933-1939.

Harwood, A.E., and Cambier, J.C. (1993*) J. Immunol.* **151**, 4513-4522.

Hasbold, J. and Klaus, G.G.B. (1990) *Eur. J. Immunol.* **20**, 1685-1691.

Hatakeyama, M., Minamoto, S., Uchiyama, T., Hardy, R.R., Yamada, G. and Taniguchi, T. (1985) *Nature* **318**, 467-470.

Hatakeyama, M., Tsudo, M, Minamoto, S, Kono, T, Doi, T., Miyata, T., Miyasaka, M. and Taniguchi, T. (1989) *Science* **244**, 551-556.

Hatakeyama, M, Kono, T., Kobayashi, N., Kawahara, A., Levin, S.D., Perlmutter, R.M. and Taniguchi, T. (1991) *Science* **252**, 1523-1528.

Hatakeyama, M., Kawahara, A., Mori, H., Shibuya, H. And Taniguchi, T. (1992) *Proc. Natl. Acad. Sci. U.S.A* **89**, 2022-2026.

Havran, W.L., DiGiusto, D.L. and Cambier, J.C. (1984) *J. Immunol.* **132**, 1712-1716.

Hayakawa, K, Hardy, R.R., Honda, M., Herzenberg, L.A., Steinberg, A.D. and Herzenberg, L.A. (1984) *Proc. Natl. Acad. Sci. U.S.A* **81**, 2494-2498.

Hayakawa, K., Hardy, R.R., Parks, D.R., and Herzenberg, L.A. (1983) *J. Exp. Med.* **157**, 202-218.

Heath, A.W., Wu, W.W. and Howard, M.C. (1994) *Eur. J. Immunol.* **24**, 1828-1834.

Hermanson, G.G., Eisenberg, D., Kincade, P.W. and Wall, R. (1988) *Proc. Natl. Acad. Sci. U.S.A* **85**, 6890-6894.

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.

Hewitt, E.L., Ozanne, B.W. and Cushley, W. (1997) *Cytokine* In Press.

Hoffman, M.K. and Kappler, J.W. (1978) *Nature*

Hombach, J., Leclerq, L., Radbruch, A., Rajewsky, K. and Reth, M. (1988a) *EMBO. J.* **7**, 3451-3456.

Hombach, J., Sablithky, F., Rajewsky, K. and Reth, M. (1988b) *J. Exp. Med.* **167**, 652-657.

Hombach, J., Tsubata, T., Leclerq, L., Stappert, H. and Reth, M. (1990) *Nature* **343**, 760-762.

Hou, J., Schindler, U., Henzel, W.J., Ho, T.C., Brasseur, M. and McKnight, S.L. (1994) *Science* **265**, 1701-1705.

Hoyos, B., Ballard, D.W., Bohnlein, E., Sickevitz, M. and Greene, W.C. (1989) *Science* **244**, 457-460.

Humphrey, J.H. and Sundaram, V. (1985) *Adv. Exp. Med. Biol.* **186**, 167-170.

Hunter, T and Cooper, J.A. (1985) *Ann. Rev. Biochem.* **54**, 897-930.

Hunter, T. and Cooper, J.A. (1986) In "*Enzymes 17, part A*" San Diego Academic Press, 192-246.

Hunziker, W., Koch, T., Whitney, S.A. and Mellman, I. (1990) *Nature* **345**, 628-632.

Hutchcroft, J.E., Harrison, M.L. and Geahlen, R.L. (1991) *J. Biol. Chem.* **266**, 14846-14849.

Hutchcroft, J.E., Harrison, M.L., and Geahlen, R.L.(1992) *J. Biol. Chem.* **267**, 8613-8619.

Hyde-DeRuyscher, R.P.E., Jennings, E. and Shenk, T. (1995) *Nucleic Acids. Res.* **23**, 4457-4465.

Idzerda, R.L., March, C.J., Mosley, B., Lyman, S.D., Vandenbos, T., Gimpel, S.D., Din, W.S., Grabstein, K.H., Widmer, M.B., Park, L.S., Cosman, D. and Beckmann, M.P. (1990) *J. Exp. Med.* **171**, 861-870.

Ihle, J.N., Witthuhn, B.A., Quelle, F.W., Yamamoto, K., Thierfelder, W.E., Kreider, B. and Silvennoinen, O. (1994) *TIBS* **19**, 22-227.

Inoue, J., Seiki, M., Taniguchi, T., Tsuru, S. and Yoshida, M. (1986) *EMBO. J.* **5**, 2883-2888.

Ishii, T., Takeshita, T., Numata, N. and Sugamura, K. (1988) *J. Immunol.* **141**, 174-179.

Jacobson, N.G., Szabo, S., Webernordt, R.M., Zhong, Z., Schreiber, R.D., Darnell, J.E. and Murphy, K.M. (1995) *J. Exp. Med.* **181**, 1755-1762.

Jaffe, E.A., Armellino, D., Lam, G., Cordon-Cardo, C., Murray, H.W. and Evans, R.L. (1989) *J. Immunol.* **143**, 3961-3966.

John, S., Reeves, R.B., Lin, J.X., Child, R., Leiden, J.M., Thompson, C.B. and Leonard, W.J. (1995) *Mol. Cell. Biol.* **15**, 1786-1796.

John, S., Robbins, C.M. and Leonard, W.J. (1996) *EMBO J.* **15**, 5627-5635.

Johnson, K.R., Lehn, D.A. and Reeves, R. (1989) *Mol. Cell. Biol.* **9**, 2114-2123.

Johnston, J.A., Kawamura, M., Kirken, R., Chen, Y.Q., Blake, T.B., Shibuya, K., Ortaldo, J.R., McVicar, D.W. and O'Shea, J.J. (1994) *Nature* **370**, 151-153.

Justement, L.B., Campbell, K.S., Chien, N.C. and Cambier, J.C. (1991) *Science* **252**, 1839-1842.

Kamio, M., Arima, N., Tsudo, M., Imada, K., Ohkuma, M. and Uchiyama, T. (1992) *Biochem. Biophys. Res. Commun.* **184**, 1288-1293.

Karasuyama, H., Rolink, A. and Melchers, F. (1988) *J. Exp. Med.* **167**, 1377-1390.

Kawamura, M., McVicar, D.W., Johnston, J.A., Blake, T.B., Chen, Y.Q., Lal, B.K., Lloyd, A.R., Kelvin, D.J., Staples, J.E., Ortaldo, J.R. and O'Shea, J.J. (1994) *Proc. Natl. Acad. Sci. U.S.A* **91**, 6374-6379.

Kearney, J.F., Klein, J., Bockman, D.E., Cooper, M.D. and Lawton, A.R. (1978) *J. Immunol.* **120**, 158-165.

Kelly, K., Cochran, B.H., Stiles, C.C. and Leder, P. (1983) *Cell* **35**, 603-610.

Khan, A.A., Steiner, J.P., Klein, M.G., Schneider and M.F. Snyder, S. (1992) *Science* **257**, 815-818.

Kikutani, H., Suemura, M., Owaki, H., Nakamura, H., Sato, R., Yamasaki, K., Barsumian, E.L., Hardy, R.R. and Kishimoto, T. (1986) *J. Exp. Med.* **164**, 1455-1469.

Kim, K.M., Ishigami, T., Hata, D., Higaki, Y., Morita, M., Yamaoka, K., Mayumi, M. and Mikawa, H. (1992) *J. Immunol.* **148**, 29-34.

Kim, K.M., Yoshimura, T., Watanabe, H., Ishigami, T., Nambu, M., Hata, D., Higaki, Y., Sasaki, M., Tsutsui, T., Mayumi, M. and Mikawa, H. (1991) *J. Immunol.* **146**, 819-825.

Kimura, Y., Takeshita, T, Kondo, M., Ishii, N., Nakamura, M., Vansnick, J. and Sugamura, K. (1995) *Int. Immunol.* **7**, 115-120.

Kishihara, K., Penninger, J., Wallace, V.A., Kundiz, T.M., Kazuhiro, K., Wakeham, A., Timms, E., Pfeffer, K., Ohashi, P.S., Thomas, M.L., Furlonger, C., Paige, C.J. and Mak, T.W. (1993) *Cell* **74**, 143-156.

Kishimoto, T., Taga, T. and Akira, S. (1994) *Cell* **76**, 253-260.

Klaus, G.G.B., Bijsterbosch, M.K., O'Garra, A., Harnett, M.M. and Ripley, K.P. (1987) *Immunol. Rev.* **99**, 19-37.

Klaus, G.G.B., Harnett, M.M. and Ripley, K.P. (1989) *Adv. Exp. Med. Biol.* **254**, 95-100.

Klaus, G.G.B., Hawrylowicz, C.M., Holman, M.J. and Keeler, M.D. (1984) *Immunology* **53**, 693-698.

Klausner, R.D. and Samelson, L.E. (1991) *Cell* **64**, 875-878.

Klein, P., Kanehisa, M. And Delisi, C. (1985) *Biochem. Biophys. Acta* **815**, 468-476.

Kobayashi, N., Kono, T., Hatakeyama, M., Minami, Y., Miyazaki, T., Perlmutter, R.M. and Taniguchi, T. (1993) *Proc. Natl. Acad. Sci. U.S.A* **90**, 4201-4205.

Koch, N. And Haustein, D. (1983) *Mol. Immunol.* **20**, 33-37.

Kocks, C. and Rajewsky, K. (1989) *Ann. Rev. Immunol.* 7, 537-559.

Kohler, I. And Rieber, G.P. (1993) *Eur. J. Immunol.* **23**, 3066-3071.

Koide, H., Satoh, T., Nakafuku, M. And Kaziro, Y. (1993) *Proc. Natl. Acad. Sci. U.S.A* **90**, 8683-8688.

Kolanus, W., Romeo, C. and Seed, B. (1993) *Cell* **74**, 171-183.

Kolsch, E., Oberbarnscheidt, J., Bruner, K. and Heuer, J. (1980) *Immunol. Rev.* **49**, 61-80.

Kondo, M., Takeshita, T., Ishii, N., Nakamura, M., Watanabe, S., Arai, K-I. and Sugamura, K. (1993) *Science* **262**, 1874-1877.

Kondo, M., Takeshita, T., Higuchi, M., Nakamura, M., Sudo, T., Nishikawa, S-I. and Sugamura, K. (1994) *Science* **263**, 1453-1454.

Kotanides, H. and Reich, N.C. (1993) *Science* **262**, 1265-1268.

Kuang, A.A., Novak, K.D., Kang, S.M., Bruhn, K. and Lenardo, M.J. (1993) *Mol. Cell. Biol.* **13**, 2536-2545.

Kudo, A. and Melchers, F. *EMBO. J.* (1987) **6**, 2267-2272.

Kurosaki, T., Takata, M., Yamanashi, Y., Inazu, T., Taniguchi, T., Yamamoto, T. and Yamamura, H. (1994) *J. Exp. Med.* **179**, 1725-1729.

Kyriakis, J.M., App, H., Zhang, X-F., Banerjee, P., Brautigan, D.L., Rapp, U.R. and Avruch, J. (1993) *Nature* **358**, 417-419.

Kyte, J. and Doolittle, R.F. (1982) *J. Mol. Biol.* **57**, 105-132.

Larner, A.C., David. M., Feldman, G.M., Igarishi, K.I., Hackett, R.H., Webb, D.S.A., Sweitzer, S.M., Petricon, E.F. and Finbloom, D.S. (1993) *Science* **261**, 1730-1733.

Law, D.A., Gold, M.R. and Defranco, A.L. (1992) *Mol. Immunol.* **29**, 917-926.

Lazarus, A.H., Kawauchi, K., Rapoport, M.J. and Delovitch, T.L. (1993) *J. Exp. Med.* **178**, 1765-1772.

Lecine, P., Algarte, M., Rameil, P., Beadling, C., Bucher, P., Nabholz, M. and Imbert, J. (1996) *Mol. Cell. Biol.* **16**, 6829-6840.

Lee, J.C., Trunch, A., Smith, M.F. and Tsang, K.Y. (1987) *J. Immunol.* **139**, 175-179.

Lees, R.K. and Sinclair, N.R. (1975) *Cell. Immunol.* **17**, 525-531.

LeGrue, S.J. (1988) *Lymphokine Res.* **7**, 187-200.

Lehn, D.A., Elton, T.S., Johnson, K.R. and Reeves, R. (1988) *Biochem. Int.* **16**, 963-971.

Leonard, W.J., Depper, J.M., Uchiyama, T., Smith, K.A., Waldmann, T.A. and Greene, W.C. (1982) *Nature* **300**, 267-269.

Leonard, W.J., Depper, J.M., Crabtree, G.R., Rudikoff, S., Pumphrey, J., Robb, R.J., Kronke, M., Svetlik, P.B., Peffer, N.J., Waldmann, T.A. and Greene, W.C. (1984) *Nature* **311**, 626-631.

Leonard, W.J., Depper, J.M., Kanehisa, M., Kronke, M., Peffer, N.J., Svetlik, P.B., Sullivan, M. and Greene, W.C. (1985) *Science* **230**, 633-639.

Leonard, W.J., Depper, J.M., Robb, R.J., Waldmann, T.A. and Greene, W.C. (1993) *Proc. Natl. Acad. Sci. U.S.A* **80**, 6957-6961.

Leonard, W.J., Noguchi, M., Russell, S.M. and McBride, O.W. (1994) *Immunol. Rev.* **138**, 61-86.

LePrince, C., Draves, K.E., Geahlen, R.L., Ledbetter, J.A. and Clark, E.A. (1993) *Proc. Natl. Acad. Sci. U.S.A* **90**, 3236-3240.

Lernbecher, T., Muller, U. and Wirth, T. (1993) *Nature* **365**, 767-770.

Leung, K. and Nabel, G.J. (1988) *Nature* **343**, 776-778.

Lin, B.B., Cross, S.L., Halden, N.F., Roman, D.G., Toledano, M.B. and Leonard, W.J. (1990) *Mol. Cell. Biol.* **10**, 850-853.

Lin, J., Brown, V.K. and Justement, L.B. (1992) *J. Immunol.* **149**, 3182-3190.

Linton, P-J., Decker, D.J. and Klinman, N.R. (1989) *Cell* **59**, 1049-1056.

Linton, P-J., Rudie, A. and Klinman, N.R. (1991) *J. Immunol.* **146**, 4099-4104.

Liu, Y-J., Joshua, D.E., Williams, G.T., Smith, C.A., Gordon, J. and MacLennan, I.C.M. (1989) *Nature* **342**, 929-931.

Liu, Y-J., Johnson, G.D., Gordon, J. and MacLennan, I.C.M. (1992) *Immunol. Today* **13**, 17-21.

Liu, Y-J., Barthelemy, C., DeBouteiller, O., Arpin, C., Durand, I. and Banchereau, J. (1995) *Immunity* **2**, 239-248.

Liu, Y-J. and Banchereau, J. (1996) Human Peripheral B Cell Subsets In "*Handbook of Experimental Immunology*" Editors D. Weir, C. Blackwell and L. Hersenberg, Blackwell Scientific Publications Ltd, Oxford, 93.1-93-9.

Loughnan, M.S. and Nossal, G.J.V. (1989) *Nature* **340**, 76-79.

Lowenthal, J.W., Howe, R.C., Ceredig, R. and MacDonald, H.R. (1986) *J. Immunol.* **137**, 2579-2586.

Lowenthal, J.W., Bohnlein, E., Ballard, D.W. and Greene, W.C. (1988) *Proc. Natl. Acad. Sci. U.S.A* **85**, 4468-4472.

Lowenthal, J.W., Ballard, D.W., Bogerd, H., Bohnlein, E. and Greene, W.C. (1989a) *J. Immunol.* **142**, 3121-3128.

Lowenthal, J.W., Ballard, D.W., Bohnlein, E. and Greene, W.C. (1989b) *Proc. Natl. Acad. Sci. U.S.A* **86**, 2331-2335.

Lund, T., Holtlund, J., Skalhegg, B. and Laland, S.G. (1986) *J. Chromat.* **369**, 341-348.

Lutticken, C., Wegenka, U.M., Yuan, J.P., Buschmann, B., Schindler, C., Ziemecki, A., Harpur, A.G., Wilks, A.F., Yasukawa, K., Taga, T., Kishimoto, T., Barbieri, G., Pellegrini, S., Sendtner, M., Heinrich, P.C. and Horn, F (1994) *Science* **263**, 89-92.

McBlane, F., Vangent, D.C., Ramsden, D.A., Romeo, C., Cuomo, C.A., Gellert, M. and Oettinger, M.A. (1995) *Cell* **83**, 387-395.

McKay, C.E. and Cushley, W. (1996) *Cytokine* **8**, 305-310.

MacLennan, I.C.M. (1994) *Ann. Rev. Immunol.* **12**, 117-139.

Mackay, C.R. (1993) *Adv. Immunol.* **53**, 217-227.

MacKenzie, L.E., Youinou, P., Hicks, R., Yuksel, B., Mageed, R.A. and Lydyard, P.M. (1991) *Scand. J. Immunol.* **33**, 329-335.

Malkovsky, M., Loveland, B., North, M., Asherson, G.L., Gao, L., Ward, P. and Fiers, W. (1987) *Nature* **325**, 262-265.

Maruyama, S., Kubagawa, H. and Cooper, M.D. (1985) *J. Immunol.* **135**, 192-199.

Maruyama, S., Shibuya, H., Harada, H., Hatakeyama, M., Seiki, M., Fujita, T., Inoue, J., Yoshida, M. and Taniguchi, T. (1987) *Cell* **48**, 343-350.

Matsumoto, A.K., Kopicky-Burd, J., Carter, R.H., Tuveson, D.A., Tedder, T.F. and Fearon, D.T. (1991) *J. Exp. Med.* **173**, 55-65.

Melchers, F. (1979) *INSERM Symp.* **10**, 281-289.

Melchers, F. (1989) In "*Immunoglobulin Genes*" Editors T. Honjo, F.W. Alt and T.M. Rabbitts. New York Academic Press, 23-44.

Melchers, F., Rolink, A., Grawunder, U., Winkler, T.H., Karasuyama, H., Ghia, P. and Andersson, J. (1995) *Curr. Opin. Immunol.* **7**, 214-227.

Mellman, I. (1988) *Curr. Opin. Immunol.* **1**, 16-25.

Merida, I., Diez, E. and Gaulton, G.N. (1990) *J. Immunol.* **147**, 2202-2207.

Merida, I., Pratt, J.C. and Gaulton, G.N. (1990) *Proc. Natl. Acad. Sci. U.S.A* **87**, 9421-9425.

Meuer, S.C., Hogdon, J.C., Hussey, R.E., Protentis, J.P., Schlossman, S.F. and Reinherz, E.L. (1983) *J. Exp. Med.* **158**, 988-997.

Mills, G.B., Girard, P., Grinstein, S. and Gelfand, E.W. (1988) *Cell* **55**, 91-100.

Mills, G.B. and May, C. (1987) *J. Immunol.* **139**, 4083-4087.

Mills, G.B., Stewart, D.J., Mellors, A. and Gelfand, E.W. (1986) *J. Immunol.* **136**, 3019-3024.

Minami, Y., Kono, T., Yamada, K., Kobayishi, N., Kawahara, A., Perlmutter, R.M. and Taniguchi, T. (1993) *EMBO. J.* **12**, 759-766.

Minami, Y. Kono, T., Miyazaki, T. and Taniguchi, T. (1993) *Ann. Rev. Immunol.* **11**, 245-268.

Minami, Y., and Taniguchi, T (1995) *Curr. Opin. Biol.* 7, 156-162.

Mischak, H., Kolch, W., Goodnight, J., Davidson, W.F., Rapp, U., Rose-Joh, S. and Mushinski, J.F. (1991) *J. Immunol.* **174**, 3981-3987.

Mittelstadt, P.R. and DeFranco, A.L. (1993) *J.Immunol.* **150**, 4822-4829.

Miyajima, A., Kitamura, T., Harada, N., Yokota, T. and Arai, K-I. (1992) *Ann. Rev. Immunol.* **10**, 295-331.

Miyazaki, T., Kawahara, A., Fujii, H., Nakagawa, Y., Minami, Y., Liu, Z.J., Oishi, I., Silvennoinen, O., Witthuhn, B.A., Ihle, J.N. and Taniguchi, T. (1994) *Science* **266**, 1045-1047.

Moller, G. (1992) *Immunol. Rev.* **126**, 5-178.

Mond, J.J., Lees, A. and Snapper, C.M. (1995) *Ann. Rev. Immunol.* **13**, 655-692.

Mond, J.J., Seghal, E., Kung, J. and Finkelman, F.D. (1981) *J. Immunol.* **127**, 881-888.

Mongini, P.K.A., Blessinger, C., Posnett, D.N. and Rudich, D.N. (1989) *J. Immunol.* **143**, 1565-1574.

Moore, M.D., Cooper, N.R., Tack, B.F. and Nemerow, G.R. (1987) *Proc. Natl. Acad. Sci. U.S.A* **84**, 9194-9198.

Moreau, J-L., Nabholz, M., Diamantstein, T., Malek, T., Shevach, E. and Theze, J. (1987) *Eur. J. Immunol.* **17**, 929-935.

Mori, H., Barsoumiamm E.L., Hatakeyama, M. and Taniguchi, T. (1991) *Int. Immunol.* **3**, 149-156.

Mui, A-F., Nakao, H., O'Farrell, A-M., Harada, N. and Miyajima, A. (1995) *EMBO. J.* **14**, 1166-1175.

Muller-Sieberg, C.E., Whitlock, C.A. and Weissman, I.L. (1986) *Cell* **44**, 653-662.

Muta, T., Kurosaki, T., Misulovin, Z., Sanchez, M., Nussenzweig, M.C and Ravetch, J.V. (1994) *Nature* **368**, 70-73.

Myers, M.G.J., Backer, J.M., Sun, X.J., Shoelson, S., Hu, P., Schlessinger, J., Yoakim, M., Schaffhausen, B. and White, M.F. (1992) *Proc. Natl. Acad. Sci. U.S.A* **89**, 10350-10354.

Nabel, G., Gorka, C. and Baltimore, D. (1988) *Proc. Natl. Acad. Sci. U.S.A* **85**, 2934-2938.

Nakagawa, T., Hirano, T., Nakagawa, N., Yashizaki, K. and Kishimoto, T. (1985) *J. Immunol.* **134**, 959-966.

Nakanishi, H., Brewer, K.A. and Exton, J.H. (1993) *J. Biol. Chem.* **268**, 13-16.

Natesan, S. and Gilman, M.Z. (1993) *Genes Dev.* **7**, 2497-2509.

Nemazee, D.A. and Burki, K. (1989) *Nature* **337**, 562-566.

Nikaido, T., Shimizu, A., Ishida, N., Sabe, H., Teshigawara, K., Maeda, M., Uchiyama, T., Yodoi, J. and Honjo, T. (1984) *Nature* **311**, 631-635.

Nishi, M., Ishida, Y. and Hondo, T. (1988) *Nature* **331**, 267-270.

Nishizuka, Y. (1992) *Science* **258**, 607-614.

Nitsche, L., Kosco, M.H., Kohler, G. and Lamers, M.C. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 1887-1891.

Noguchi, M., Nakamura, Y., Russell, S.M., Ziegler, S.F., Tsang, M., Cao, X. and Leonard, W.J. (1993a) *Science* **262**, 1877-1880.

Noguchi, M., Yi, H., Rosenblatt, H.M., Filipovich, A.H., Adelstein, S., Modi, W.S., McBride, O.N. and Leonard, W.J. (1993b) *Cell* **73**, 147-152.

Nonoyama, S., Penix, L.A., Edwards, C.P., Lewis, D.B., Ito, S., Aruffo, A., Wilson, C.B. and Ochs, H.D. (1995) *J. Clin. Invest.* **95**, 66-75.

Norvell, A. and Monroe, J.G. (1996) *J. Immunol.* **156**, 1328-1332.

Nossal, G.J.V. (1983) *Ann. Rev. Immunol.* **1**, 33-67.

Oettinger, M.A., Schatz, D.G., Gorka, C. and Baltimore, D. (1990) *Science* **248**, 1517-1523.

O'Garra, A., Rigley, K.P., Holman, M., McLaughlin, J.B. and Klaus, G.G.B. (1987) *Proc. Natl. Acad. Sci. U.S.A* **84**, 6524-6528.

Oren, R., Takahashi, S., Doss, C., Levy, R. and Levy, S. (1990) *Mol. Cell. Biol.* **10**, 4007-4015.

Orkin, S.T. (1992) *Blood* **80**, 575-581.

Osmond, D.G. (1993) *Immunol. Today* **14**, 34-37.

Padeh, S., Levitzki, A., Gazit, A., Mills, G.B. and Roifman, C.M. (1991) *J. Clin. Invest.* **87**, 1114-1118.

Parikh, V.S., Bishop, G.A., Liu, K.J., Do, B.T., Ghosh, M.R., Kim, B.S. and Tucker, P.W. (1992) *J. Exp. Med.* **176**, 1025-1031.

Parker, D.C. (1993) *Ann. Rev. Immunol.* **11**, 331-360.

Pascual, V., Liu, Y-J., Magalski, A., DeBouteiller, O., Banchereau, J. and Capra, J.D. (1994) *J. Exp. Med.* **180**, 329-339.

Patthy, L. (1990) *Cell* **61**, 13-14.

Pawson, T. and Gish, G.D. (1992) *Cell* **71**, 359-362.

Pauza, C.D. (1987) *Mol. Cell. Biol.* 7, 342-348.

Pearse, R.N., Feinman, R., Shuai, K., Darnell, J.E. and Ravetch, J.V. (1993) *Proc. Natl. Acad. Sci. U.S.A* **90**, 4314-4318.

Pecanha, L.M.T., Snapper, C.M., Finkelman, F.D. and Mond, J.J. (1991) *J. Immunol.* **146**, 833-839.

Pene, J., Rousset, F., Briere, F., Chretien, I., Wideman, J., Bonnefoy, J.Y. and DeVries, J.E. (1988) *Eur. J. Immunol.* **18**, 929-935.

Perkins, N.D., Schmid, R.M., Duckett. C.S., Leung, K, Rice, N.R., and Nabel, G.J. (1992) *Proc. Natl. Acad. Sci. U.S.A* **89**, 1529-1533.

Perkins, S.J., Haris, P.I., Sim, R.B. and Chapman, D. (1988) *Biochemistry* **27**, 4004-4012.

Petrini, M., Emerson,. D.L. and Galbraith, R.M. (1983) *Nature* **306**, 73-74.

Pezzutto, A., Dorken, B., Rabinovitch, P.S., Ledbetter, J.A., G., Moldenhauer, G. and Clark, E.A. (1987) *J. Immunol.* **138**, 2793-2799.

Pezzutto, A., Rabinovitch, P.S., Dorken, B., Moldenhauer, G. and Clark, E.A. (1988) *J. Immunol.* **140**, 1791-1795.

Pierce, J.W., Jamieson, C.A., Ross, J.L. and Sen, R. (1995) *J. Immunol.* **155**, 1972-1980.

Pimental-Muinos, F.X., Mazana, J. and Fresno, M. (1994a) *J. Biol. Chem.* **269**, 24424-24429.

Pimental-Muinos, F.X. Munoz-Fernandez, M.A, and Fresno, M. (1994b) *J. Immunol.* **152**, 5714-5722.

Pleiman, C.M., Clark, M.R., Gauen, L.K.T., Winitz, S., Coggeshall, K.M., Johnson, G.L., Shaw, A.S. and Cambier, J.C. (1993) *Mol. Cell. Biol.* **13**, 5877-5887.

Pleiman, C.M., Abrams, C., Timson-Gauen, L.K. et al (1994a) *Proc. Natl. Acad. Sci. U.S.A* **91**, 4268-4272.

Pleiman, C.M., Chien, N.C. and Cambier, J.C. (1994b) *J. Immunol.* **152**, 2837-2844.

Pleiman, C.M., Hertz, W.M. and Cambier, J.C. (1994c) *Science* **263**, 1609-1612.

Pulendran, B., Kannourakis, G., Nouri, S., Smith, K.G. and Nossal, G.J. (1995a) *Nature* **375**, 331-334.

Pulendran, B., Smith, K. and Nossal, G.J. (1995b) *J. Immunol.* **155**, 1141-1150.

Pure, E. and Tardelli, L. (1992) *Proc. Natl. Acad. Sci. U.S.A* **89**, 114-117.

Quelle, F.W., Shimoda, K., Thierfelder, W., Fischer, C., Kim, A., Ruben, S.M., Cleveland, J.L., Pierce, J.H., Keegan, A.D., Nelms, K., Paul, W.E. and Ihle, J.N. (1995) *Mol. Cell. Biol.* **15**, 3336-3343.

Ralph, P. (1979) *Immunol. Rev.* **48**, 107-173.

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.

Ravetch, J.V. and Kinet, J.P. (1991) *Ann. Rev. Immunol.* **9**, 457-492.

Reed, J.C., Sabath, D.E., Hoover, R.G. and Prystowsky, M.B. (1985a) *Mol. Cell. Biol.* **5**, 3361-3368.

Reed, J.C., Nowell, P.C. and Hoover, R.G. (1985b) *Proc. Natl. Acad. Sci. U.S.A* **82**, 4221-4224.

Remillard, B., Petrillo, R., Maslinski, W., Tsudo, M., Strom, T.B., Cantley, L. and Varticovski, L. (1991) *J. Biol. Chem.* **266**, 14167-14170.

Reth, M. (1989) *Nature* **338**, 383-384.

Reth, M. (1992) *Ann. Rev. Immunol.* **10**, 97-121.

Reth, M., Hombach, J., Wienands, J., Campbell, K.S., Chien, N., Justement, L.B. and Cambier, J.C. (1991) *Immunol. Today* **12**, 196-201.

Reynaud, C.A., Bertocci, B., Dahan, A. and Weill, J.C. (1994) *Adv. Immunol.* **57**, 353-378.

Robb, R.J. and Greene, W.C. (1983) *J. Exp. Med.* **158**, 1332-1337.

Robb, R.J., Rusk, C.M. and Neeper, M.P. (1988) *Proc. Natl. Acad. Sci. U.S.A* **85**, 5654-5658.

Robertson, M.J. and Ritz, J. (1992) In "*NK Cell Mediated Cytotoxicity: Receptors, Signaling and Mechanisms*" Editors E. Lotzova and R.B. Herberman CRC Press Inc, Boca Raton, 183-206.

Roes, J. and Rajewsky, K. (1993) *J. Exp. Med.* **177**, 45-55.

Roessler, E., Grant, A., Ju, G., Tsudo, M., Sugamura, K. and Waldmann, T.A. (1994) *Proc. Natl. Acad. Sci. U.S.A* **91**, 3344-3349.

Rogers, J., Early, P., Carter, C., Calame, K., Bond, M., Hood, L. and Wall, R.(1980) *Cell* **20**, 303-312.

Roifman, C.M., Benedict, S.H., Cheung, R.K. and Gelfand, E.W. (1987) *Eur. J. Immunol.* **17**, 701-706.

Roman, D.G., Toledano, M.B. and Leonard, W.J. (1990) *New Biol.* **2**, 642-647.

Rosen, C.A., Sodrowski, J.G. and Haseltine, W.A. (1985) *Cell* **41**, 813-823.

Roth, D.B., Lindahl, T. and Gellert, M. (1995) *Curr. Biol.* **5**, 496-499.

Rothstein, T.L. and Kolber, D.L. (1988) *J. Immunol.* **141**, 4089-4096.

Rousset, F., Garcia, E., Defrance, T., Peronne, C., Vezzio, N., Hsu, D.H., Kastelstein, R., Moore, K.W. and Banchereau, J. (1992) *Proc. Natl. Acad. Sci. U.S.A* **89**, 1890-1893.

Rozakis-Adcock, M., Fernley, R., Wade, J., Pawson, T. and Bowtell, D. (1993) *Nature* **368**, 83-85.

Rozakis-Adcock, M., McGlade, J., Mbamalu, G., Pelicci, G., Daly, R., Li, W., Batzer. A., Thomas, S., Brugge, J., Pelicci, P.G., Schlessinger, J. and Pawson, T. (1992) *Nature* **360**, 689-691.

Ruben, S., Poleat, H., Tan, T-H., Kawakami, K., Roeder, R.G., Haseltine, W. and Rosen, C.A. (1988) *Science* **241**, 89-92.

Ruff-Jamieson, S., Chen, K. and Cohen, S. (1993) *Science* **261**, 1733-1736.

Russell, D.M., Dembic, Z., Morahan, G., Miller, J.F., Burk, K. and Nemazee, D. (1991) *Nature* **354**, 308-310.

Russell, S.M., Johnston, J.A., Noguchi, M., Kawamura, M., Bacon, C.M., Friedmann, M., Berg, M., McVicar, D.W., Witthuhn, B.A., Silvennoinen, O., Goldman, A.S., Schmalstieg, F.C., Ihle, J.N., O'Shea, J.J. and Leonard, W.J. (1994) *Science* **266**, 1042-1045.

Russell, S.M., Keegan, A.D. and Harada, N. (1993) *Science* **262**, 1880-1883.

Sadowski, H.B., Shuai, K., Darnell, J.E. and Gilman, M.Z. (1993) *Science* **261**, 1739-1744.

Sakaguchi, N. And Melchers, F. (1986) *Nature* **324**, 579-582.

Sakaguchi, N., Kashiwamura, S., Kimoto, M., Thalman, P. and Melchers, F. (1988) *EMBO. J.* **7**, 3457-3464.

Sarmay, G., Rozsnay, Z., Koncz, G. and Gergely, J. (1995) *Immunol. Letters* **44**, 125-131.

Satoh, T., Fantl, W.J., Escobedo, J.A., Williams, L.T., Minami, Y., Taniguchi, T. and Kaziro, Y. (1993) *Cell. Biochem.* **S17A**. 240-247.

Saxton, T.M., Van Oosteen, I., Bowtell, D., Aebersold, R. and Gold, M.R. (1994) *J. Immunol.* **153**, 623-629.

Schiff, C., Milili, M., Bossy, D. and Fougereau, M. (1992) *Int. Rev. Immunol.* **8**, 135-145.

Schulte, R.J., Campbell, M.A., Fischer, W.H. and Sefton, B.M. (1992) *Science* **258**, 1001-1004.

Scott, D.W., Chace, J.H., Warner, G.L., O'Garra, A., Klaus, G.G.B. and Quill, H. (1987) *Immunol. Rev.* **99**, 153-172.

Scott, D.W., Tuttle, W.J., Livnat, D., Haynes, W., Cogswell, J. and Keng, P. (1985) *Cell. Immunol.* **93**, 124-129.

Seidel, H.M., Milocco, L.H., Lamb, P., Darnell, J.E., Stein, R.B. and Rosen, J. (1995) *Proc. Natl. Acad. Sci. U.S.A* **92**, 3041-3045.

Sen, R. and Baltimore, D. (1986) *Cell* **46**, 705-716.

Sharon, M., Klausner, R.D., Cullen, B.R., Chizzonite, R. and Leonard, W.J. (1986) *Science* **234**, 859-863.

Shibuya, H., Yoneyama, M., Ninomiya-Tsuji, J., Matsumoto, K. and Taniguchi, T. (1992) *Cell* **70**, 57-67.

Shoelson, S.E., Sivaraja, M., Williams, K.P., Hu, P., Schlessinger, J. and Weiss, M.A. (1993) *EMBO J.* **12**, 795-802.

Shokat, K.M. and Goodnow, C.C. (1995) *Nature* **375**, 334-338.

Shu, J., Benedict, S.H. and Chan, M.A. (1994) *Cell. Immunol.* **159**, 170-183.

Shuai, K., Stark, G.R., Kerr, I.M. and Darnell, J.E. (1993) *Science* **261**, 1744-1746.

Siebenlist, U., Franzoso, G. and Brown, K. (1994) *Ann. Rev. Cell. Biol.* **10**, 405-455.

Sieckmann, D.G. (1980) *Immunol. Rev.* **52**, 181-210.

Siekevitz, M., Feinberg, M.B., Holbrook, N., Wong-Staal, F., Gallo, R.C. and Greene, W.C. (1987) *Proc. Natl. Acad. Sci. U.S.A* **84**, 5389-5393.

Silvennoinen, O., Schindler, C., Schlessinger, J. and Levy, D.E. (1993) *Science* **261**, 1736-1739.

Sitia, R., Neuberger, M.S. and Milstein, C. (1987) *EMBO. J.* **6**, 3969-3977.

Smith, K.A. (1988a) *Science* **240**, 1169-1173,

Smith, K.A. (1988b) In "*Interleukin-2*" Academic Press Inc, San Diego, 1-35.

Smith, K.A. (1989) *Ann. Rev. Cell. Biol.* **5**, 397-435.

Smith, L.G., Weissman, I.L. and Heimfeld, S. (1991) *Proc. Natl. Acad. Sci. U.S.A* **88**, 2788-2792.

Smith, M.R. and Greene, W.C. (1989) *Proc. Natl. Acad. Sci. U.S.A* **86**, 8526-8530.

Songyang, Z., Shoelson, S.E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W., King, K., Roberts, T., Ratnofsky, S., Lechneider, R.J., Neel, B.G., Birge, R.B., Fajardo, J.E., Chou, M., Hanafusa, H., Schaffhausen, B. and Cantley, L.C. (1993) *Cell* **72**, 767-776.

Songyang, Z., Shoelson, S.E., McGlade, J., Olivier, P., Pawson, T., Bustelo, X.R., Barbacid, M., Sabe, H., Hanafusa, H., Yi, T., Ren, R., Baltimore, D., Ratnofsky, S., Feldman, R.A. and Cantley, L.C. (1994) *Mol. Cell. Biol.* **14**, 2777-2785.

Sperisen, P., Wang, S.M., Soldaini, E., Pla, M., Rusterholz, C., Bucher, P., Corthesy, P., Reichenbach, P. and Nabholz, M. (1995) *J. Biol. Chem.* **270**, 10743-10753.

Stamenkovic, I. And Seed, B. (1990) *Nature* **345**, 74-77.

Stern, J.B. and Smith, K.A. (1986) *Science* **233**, 203-206.

Suzuki, N., Matsunami, N., Kanamori, H., Ishida, N., Shimizu, A., Yaoita, Y., Nikaido, T. and Honjo, T. (1987) *J. Biol. Chem.* **262**, 5079-5086.

Taga, T. and Kishimoto, T. (1993) *FASEB. J.* **6**, 3387-3396.

Taga, T. and Tosato, G. (1992) *J. Immunol.* **148**, 1143-1148.

Takata, M., Sabe, H., Hata, A., Inazu, T., Homma, Y., Nukada, T., Yamamura, H. and Kurosaki, T. (1994) *EMBO. J.* **13**, 1341-1349.

Takeshita, T., Asao, H., Ohtani, K., Ishii, N., Kumaki, S., Tanaka, N., Munakata, H., Nakamura, M. and Sugamura, K. (1992) *Science* **257**, 379-382.

Tamaki, T., Kanakura, Y., Kuriu, A., Ikeda, H., Mitsui, H., Yagura, H., Matsumara, I., Druker, B., Griffin, J., Kanayama, Y. and Yonezawa, T. (1992) *Cancer Res.* **52**, 566-574.

Tan, T-H., Huang, G.P., Sica, A., Ghosh, P., Young, H.A., Longo, D.L. and Rice, N.R. (1992) *Mol. Cell. Biol.* **12**, 4067-4075.

Taniguchi, T., Kobayishi, T., Kondo, J., Takahashi, K., Nakamura, H., Suzuki, J., Nagai, K., Yamada, T., Nakamur, S.I. and Yamamura, H. (1991) *J. Biol. Chem.* **266**, 15790-17796.

Taniguchi, T., Matsui, H., Fujita, T., Hatakeyama, M., Kashima, N., Fuse, A., Hamuro, J., Nishi-Takaoka, C.and Yamada, G. (1986) *Immunol. Rev.* **92**, 120-169.

Taniguchi, T. and Minami, Y. (1993) *Cell* **73**, 5-9.

Tanner, J., Weiss, J., Fearon, D., Whang, Y. and Kieff, E. (1987) *Cell* **50**, 203-213.

Tedder, T.F., Clement, L.T. and Cooper, M.D. (1983) *J. Immunol.* **133**, 678-683.

Tentori, L., Longo, D.L., Zuniga-Pflucker, J.C., Wing, C. and Kruisbeek, A.M. (1988) *J. Exp. Med.* **168**, 1741-1746.

Teshigawara, K., Wang, H-M., Kato, K. and Smith, K.A. (1987) *J. Exp. Med.* **165**, 223-238.

Tevelde, A.A., Klomp, J.P.G., Yard, B.A., DeVries, J.E. and Figdor, C.G. (1988) *J. Immunol.* **140**, 1548-1554.

Thompson, C.B., Wang, C.Y., Ho, I-C., Bohjanen, P.R., Petryniak, B., June, C.H., Miesfeldt, S., Zhang, L., Nabel, G.J., Karpinski, B. and Leiden, J.M. (1992) *Mol. Cell. Biol.* **12**, 1043-1053.

Tisch, R., Roifman, C.M. and Hozumi, N. (1988) *Proc. Natl. Acad. Sci. U.S.A* **85**, 6914-6918.

Toledano, M.B., Roman, D.G., Halden, N.F., Lin, B.B. and Leonard, W.J. (1990) *Proc. Natl. Acad. Sci. U.S.A* **87**, 1830-1834.

Tomizawa, K., Ishizaka, A., Kojima, K., Nakanishi, M., Sakiyama, Y. and Matsumoto, S. (1991) *Clin. Exp. Immunol.* **83**, 492-499.

Tonegawa, S. (1983) *Nature* **302**, 575-581.

Torigoe, T., Saragovi, H.U. and Reed, J.C. (1992) *Proc. Natl. Acad. Sci. U.S.A* **89**, 2674-2678.

Torres, R.M., Law, D-L., Santos-Argumedo, L., Kirkham, P.A., Grabstein, K., Parkhouse, R.M.E. and Clark, E.A. (1992) *J. Immunol.* **149**, 2641-2649.

Treisman, R. (1992) *TIBS.* **17**, 423-426.

Trouche, D., Robin, P., Robillard, O., Sassone-Corsi, P. and Harel-Bellan, A. (1991) *J. Immunol.* **147**, 2398-2403.

Tsudo, M., Kozak, R.W., Goldman, C.K. and Waldmann, T.A. (1986) *Proc. Natl. Acad. Sci. U.S.A* **83**, 9694-9698.

Turner, B., Rapp, U., App, H., Greene, M., Dobashi, K. and Reed, J. (1991) *Proc. Natl. Acad. Sci. U.S.A* **88**, 1227-1231.

Tuveson, D.A., Carter, R.H., Soltoff, S.P. and Fearon, D.T. (1993) *Science* **260**, 986-989.

Uchiyama, T., Brader, S. and Waldmann, T. (1981) *J. Immunol.* **126**, 1293-1300.

Uchiyama, T., Hori, T., Tsudo, M, Wano, Y., Umadome, H., Yodoi, J., Maeda, M., Sawami, H. and Uchino, H. (1985) *J. Clin. Invest.* **76**, 446-453.

Valentine, M.A., Czernik, A.J., Rachie, N., Hidaka, H., Fisher, C.L., Cambier, J.C. and Bomsztyk, K. (1995) *J. Exp. Med.* **182**, 1943-1949.

Valge, V.E., Wong, J.G.P., Datlof, B.M., Sinskey, A.J. and Rao, A. (1988) *Cell* **55**, 101-112.

Van de Wetering, M. And Clevers, H. (1992) *EMBO. J.* **11**, 3039-3044.

Van de Winkel, J.G.J. and Capel, P.J.A. (1993) *Immunol. Today* **14**, 215-221.

Van Noesel, C.J.M., Lankester, A.C., and Van Lier, R.A.W. (1993) *Immunol. Today* **14**, 8-11.

Van Vlasselaer, P., Gascan, H., Malefyt, R.D. and DeVries, J.E. (1992) *J. Immunol.* **148**, 1674-1648.

Venkitaraman, A.R., Williams, G.T., Dariavach, P. and Neuberger, M.S. (1991) *Nature* **352**, 777-781.

Vercelli, D., Jabara, H.H., Lee, B.W., Woodland, N., Geha, R.S and Leung, D.Y.M. (1988) *J. Exp. Med.* **167**, 1406-1416

Vitetta, E.S., Berton, M.T., Berger, C., Kepron, M., Lee, W.T. and Yin, X.M. (1991) *Ann. Rev. Immunol.* **9**, 193-245.

Vitetta, E.S., Cambier, J.C., Ligler, F., Kettman, J. and Uhr, J.W. (1976) *J. Exp. Med.* **146**, 1804-1805.

Vitetta, E.S. and Uhr, J.W. (1977) *Immunol. Rev.* **37**, 50-56.

Voss, S.D., Hong, R. and Sondel, P.M. (1994) *Blood* **83**, 626-631.

Voss, S.D., Leary, T.P., Sondel, P.M. and Robb, R.J. (1993) *Proc. Natl. Acad. Sci. U.S.A* **90**, 2428-2437.

Waldmann, T.A (1989) *Ann. Rev. Biochem.* **58**, 875-922.

Waldmann, T.A., Goldman, C.K., Robb, R.J., Depper, J.M., Leonard, W.J., Sharrow, S.O., Bongiovanni, K.F., Korsmeyer, S.J. and Greene, W,C. (1984) *J. Exp. Med.* **160**, 1450-1457.

Waldmann, T.A., Greene, W.C., Sarin, P.S., Saxinger, C., Blayney, D.W., Battner, W.A., Goldman, C.K., Bongiovanni, K., Sharrow, S., Depper, M., Leonard, W.J., Uchiyama, T. and Gallo, R.C. (1984) *J. Clin. Invest.* **73**, 1711-1717.

Wang, C-Y., Petryniak, B., Ho, I-C., Thompson, C.B. and Leiden, J.M. (1992) *J. Exp. Med.* **175**, 1391-1399.

Wange, R.L., Malek, S.N., Desiderio, S. and Samelson, L.E. (1993) *J. Biol. Chem.* **268**, 19797-19801.

Weiss, A. (1993) *Cell* **73**, 209-212.

Weiss, A. and Littman, D.R. (1994) *Cell* **76**, 263-274.

Weiss, M.J. and Orkin, S.H. (1995) *Exp. Haematol.* **23**, 99-107.

Wickremasinghe, R.G., Mire-Sluis, A.R. and Hoffbrand, A.V. (1987) *FEBS Lett.* **220**, 52-56.

Williams, G.T., Dariavich, P., Venkitaraman, A.R., Gilmore, D.J. and Neuberger, M.S. (1993) *Mol. Immunol.* **16**, 1427-1437.

Williams, G.T., Venkitaraman, A.R., Gilmore, D.J. and Neuberger, M.S. (1990) *J. Exp. Med.* **171**, 947-952.

Wilson, H.A., Greenblatt, D., Taylor, C.W., Putney, J.W., Tsien, R.Y., Finkelman, F.D. and Chused, T.M. (1987) *J. Immunol.* **138**, 1712-1718.

Witthuhn, B.A., Silvennoinen, O., Miura, O., Siewlai, K., Cwik, C., Liu, E.T., and Ihle, J.N. (1994) *Nature* **370**, 153-157.

Wong-Staal, F. and Gallo, R.C. (1985) *Nature* **317**, 395-403.

Wortis, H.H., Teutsch, M., Higer, M., Zheng, J. and Parker, D.C. (1995) *Proc. Natl. Acad. Sci. U.S.A* **92**, 3348-3352.

Yamada, T., Taniguchi, T. Yang, C., Yasu, S., Sauto, H. and Yamamura, H. (1993) *Eur. J. Biochem.* **213**, 455-459.

Yamanishi, Y., Fukui, Y., Wongsasant, W., Kinoshita, Y., Ichimori, Y., Toyoshima, K. and Yamamoto, T. (1992) *Proc. Natl. Acad. Sci. U.S.A* **89**, 1118-1122.

Yamamoto, K., Quelle, F.W., Thiefelder, W.E., Kreider, B.L., Gilbert, D.J., Jenkins, N.A., Copeland, N.G., Silvennoinen, O. and Ihle, J.N. (1994) *Mol. Cell. Biol.* **14**, 4342-4349.

Yang, Z., Sugawara, M., Ponath, P.D., Wessendorf, L., Banerji, J., Li, Y. and Strominger, J.L. (1990) *Proc. Natl. Acad. Sci. U.S.A* **87**, 9226-9230.

Yefenof, E., Sanders, V.M., Uhr, J.W. and Vitetta, E.S. (1986) *J. Immunol.* **137**, 85-90.

Yellen-Shaw, A.J. and Monroe, J.G. (1992) *J. Exp. Med.* **176**, 129-137.

Yoshida, M. and Seiki, M. (1987) *Ann. Rev. Immunol.* **5**, 541-560.

Zheng, B., Xue, W. and Kelsoe, G. (1994) *Nature* **352**, 556-559.

Zhong, Z, Wen, Z. and Darnell, J.E. (1994a) *Proc. Natl. Acad. Sci. U.S.A* **91**, 4806-4810.

Zhong, Z, Wen, Z. and Darnell, J.E. (1994b) *Science* **264**, 95-98.

Zhou, L-J., Ord, D.C., Hughes, A.L. and Tedder, T.F. (1991) *J. Immunol.* **147**, 1424-1432.

Zmuidzinas, A., Mamon, H.J., Roberts, T.M. and Smith, K.A. (1991) *Mol. Cell. Biol.* **11**, 2794-2803.

Zola, H., Weedon, H., Thompson, G.R., Fung, M.C., Ingley, E. and Hapel, A.J. (1991) *Immunol.* **72**, 167-174.

Zuniga-Pflucker, J.C. and Kruisbeek, A.M. (1990) *J. Immunol.* **144**, 3736-3743.

Zupo, S., Dono, M., Azzoni, L., Chiorazzi, N. and Ferrarini, M. (1991) *Eur. J. Immunol.* **21**, 351-359.