# Interleukin-4-Mediated Regulation of CD25 Gene Expression in Human B Lymphocytes

by

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# Synopsis

This thesis describes the regulation of CD25 gene expression by interleukin-4-activated signal transduction pathways in quiescent human B lymphocytes. In particular, the importance of a cAMP-dependent pathway, which down-regulates binding of a putative transcriptionally repressive protein factor from a negative regulatory element in the promoter region of the CD25 gene, is highlighted. CD25 is the alpha chain of the multi-component interleukin-2 receptor complex and is a requirement for formation of high affinity interleukin-2 receptors. In addition, CD25 is the only unique component of the interleukin-2 receptor and as such it confers rigourous specificity for binding of interleukin-2. To date, interleukin-4 is the only cytokine recognized to be capable of up-regulating CD25 in human B lymphocytes.

The initial aim of this work was to identify signalling pathways which were responsible for up-regulation of CD25 expression, by artificially activating specific second messenger systems. These early studies were based on the limited knowledge of interleukin-4-induced signal transduction pathways available at the time; treatment of resting human B cells with phorbol ester, calcium ionophore and forskolin results in modest increases in cell surface levels of CD25. Additionally, these stimuli and those required for elevation of surface CD23 levels are similar. It seemed possible therefore, that the ability of IL-4 to promote increases in intracellular calcium, protein kinase C activation and cAMP generation might have a role in IL-4-induced CD25 expression. Additionally, chelation of intracellular calcium and down-regulation of PKC, by chronic phorbol ester treatment, attenuates the ability of IL-4 to up-regulate CD25. Similarly, this latter treatment abrogates any increase in CD25 levels observed in response to anti-Immunoglobulin and anti-CD40 antibodies, the only other stimuli capable of inducing CD25 expression in primary human B cells.

Upon binding to its receptor, IL-4 induces a biphasic elevation of intracellular cAMP; a

transient increase which peaks at approximately 2 minutes and is followed by a more sustained increase after 10 minutes. This sustained cAMP generation is maintained until approximately 30 minutes after stimulation, before dropping back to basal levels. Additionally, we have demonstrated that IL-4-induced cAMP production is dose dependent and leads to the activation of the cAMP-dependent protein kinase, PKA, in primary human B cells.

The ability of forskolin to elevate cell surface levels of CD25 suggested that cAMP generation might regulate expression of the CD25 gene. The work presented here demonstrates that IL-4 promotes a decrease in transcription factor binding to a negative regulatory element in the promoter region of the CD25 gene and that this attenuation of DNA binding activity can be mimicked by stimulation of human B cells with agents which increase levels of intracellular cAMP. Additionally, we have demonstrated that inhibition of PKA and protein tyrosine kinase activity, but not PKC, results in the abrogation of the ability of IL-4 to attenuate binding activity at the negative regulatory element. Thus, we have determined that IL-4 promotes the removal of a transcriptional repressor protein from a negative regulatory element in the CD25 promoter, via a cAMP/PKA -dependent pathway, and that this event is at least partially responsible for the ability of IL-4 to up-regulate CD25 expresion.

The mature, EBV transformed EDR B cell line is deficient in response to IL-4 in a variety of ways; i.e., IL-4 does not induce CD25 expression, their ability to promote cAMP generation in response to IL-4 is severely diminished in comparison to primary B cells and IL-4 is unable to promote attenuation of protein binding activity for the negative regulatory element. Investigations into transcriptional activation of the CD25 gene have demonstrated that forskolin, but not IL-4, is able to increase the activity of a chloramphenicol transferase reporter gene under the control of a CD25 promoter fragment in the EDR B cell line and, in addition, forskolin promotes a decrease in DNA binding activity to the negative regulatory element. Co-stimulation of transfected EDR

cells with IL-4 and forskolin results in enhancement of both reporter gene activity and cell surface expression of CD25, compared with forskolin alone. Therefore, by artificially generating cAMP in these cells it possible to overcome a block in an cAMP regulated-IL-4-induced signal transduction pathway and promote transcripitional activation of the CD25 gene. The ability of forskolin and IL-4 to synergise with respect to CD25 expression, suggests that removal of transcription factor binding to the negative regulatory element is a necessary but insufficient signal for a maximal response. Thus, the work presented in this thesis has also tried to identify a candidate for a second IL-4-induced signal transduction pathway, which may be responsible for CD25 up-regulation. In particular, IL-4-mediated regulation of transcription factor binding to a putative IL-4 response element has been examined.

The work presented here has defined a partial mechanism for IL-4-mediated regulation of CD25 up-regulation in mature human B lymphocytes. Thus, an IL-4-induced cAMP generating/PKA activating pathway is responsible for the down-regulation of transcription factor(s) which bind to a negative regulatory element in the promoter of the CD25 gene and it appears that this pathway is necessary for activation of expression of the gene, but is insufficient for induction of a maximal response. Additionally, IL-4 elevates the binding of an as yet unidentified transcriptional complex to a putative IL-4 response element in the CD25 promoter, an event which may act in conjunction with the cAMP-dependent pathway as a co-activational stimulus for the CD25 gene.

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### Declaration

The work described in this thesis was performed personally unless otherwise stated

## Abbreviations

| | |
|---|---|
| APC | Antigen Presenting Cell |
| APRE | Acute Phase Response Element |
| ATL | Adult T Cell Leukaemia |
| BCL | B Cell Chronic Leukaemia |
| cAMP | Cyclic Adenosine 3',5' Monophosphate |
| CRE | cAMP Response Element |
| CREB | cAMP Response Element Binding Protein |
| CTLL | Cytotoxic T Cell Lymphocytic Leukaemia |
| EGF | Epidermal Growth Factor |
| EMSA | Electrophoretic Mobility Shift Assay |
| FACS | Fluorescence Activated Cell Sorting |
| GAS | Interferonγ Activated Sequence |
| $^{125}$I | $^{125}$Iodine |
| hIL-4 | Human Interleukin-4 |
| HIV | Human Immunodeficiency Virus |
| HIV-LTR | Human Immunodeficiency Virus-Long Terminal Repeat |
| HTLV-1 | Human T Cell Lymphotropic Virus-1 |
| IDR | Imperfect Direct Repeat |
| IFN | Interferon |
| IL-2R | Interleukin-2 Receptor |
| IL-4R | Interleukin-4 Receptor |
| IL-4RE | IL-4 Response Element |
| IL-4RS | IL-4 Response Sequence |
| IGF-1 | Insulin Like Growth Factor-1 |
| IRS-1/2 | Insulin Receptor Substrate-1/2 |
| LPS | Lipopolysaccharide |
| MHC | Major Histocompatability Complex |

| | |
|---|---|
| NF-AT | Nuclear Factor-AT |
| NF-IL-4 | Nuclear Factor-IL-4 |
| NF-κB | Nuclear Factor-κB |
| NRE | Negative Regulatory Element |
| $PBu_2$(PDBu) | Phorbol Dibutyrate |
| PDGF | Platelet Derived Growth Factor |
| PDE | phosphodiesterase |
| PI3K | Phosphatidyl Inositol-3 Kinase |
| PKA | Protein Kinase A (cAMP-Dependent Protein Kinase) |
| PKC | Protein Kinase C |
| PMA | Phorbol 12-, 13- Myristate Acetate |
| PRM-1/2 | Proline Rich Motif-1/2 |
| RAG-1/2 | Recombinase Activating Gene-1/2 |
| RARE | Retinoic Acid Response Element |
| SAC | *Staphylococcus aureus* Cowan I |
| SH2/3 | *src* Homology Domain 2/3 |
| SP-50 | Silencer Protein-50 |
| SRE | Serum Response Element |
| SRF | Serum Response Factor |
| STAT | Signal Transducer and Activator of Transcription |
| X-SCID | X-Linked Severe Combined Immunodeficiency Disorder |

# Chapter 1

# Introduction

## 1.1 B Cell Development

The humoral arm of the immune response in mammalian systems i.e., the production of antibody in response to invasion by foreign antigens and pathogens, is mediated by B lymphocytes. The process of development of B cells is both complex and lengthy and can be largely divided into two distinct phases, an antigen independent phase which occurs in the bone marrow and an antigen-dependent phase which occurs largely in the periphery, i.e., the tonsils, lymph nodes, blood etc (Figure 1.1). B cells, like all haematopoietic cells, develop from pluripotent haematopoietic stem cells, a single precursor cell type which arises in the bone marrow [Kee and Paige, 1995]. In addition to being a precursor for B cells, the pluripotent haematopoietic stem cell also develops into T lymphocytes and myeloid cells, including erythrocytes, neutrophils, granulocytes and macrophages. Commitment to the B cell lineage is defined early in lymphoid development and can be identified by the expression of CD19, a pan B cell surface marker which is expressed firstly in early pro-B cells and then throughout B cell development. The development process itself involves maturation from stem cells through a series of intermediate B cell precursor stages to finally become either mature antibody secreting plasma cells, or mature memory B cells. This process of differentiation is marked by the presence of a variety of cell surface proteins, the expression of which are often both transient and dependent upon the specific stage of development of the B cell, and perhaps more importantly by the expression of membrane immunoglobulin (mIg), which forms the B cell antigen receptor (BCR) in association with accessory molecules such as Igα, Igβ and CD19 [Jongstra and Misener, 1993; Pleiman *et al.*, 1994].

### 1.1.1 Immunoglobulin Gene Re-arrangement

The accepted dogma of immunoglobulin expression is that during the antigen-independent phase of B cell development there is no expression of intact Ig on the plasma

membrane of the B cell, but early in this phase somatic rearrangement of immunoglobulin genes occurs (i.e., VDJ rearrangement of heavy chain genes and VJ rearrangement of Kappa or Lambda, light chain genes) [Reviewed by Alt *et al.*, 1992]. This process begins in early pro-B cells with heavy chain gene rearrangement and the expression of a cytoplasmic μ chain (IgM heavy chain) marks progression to the large pre-B cell stage of development. Expression of fully fuctional plasma membrane IgM (mIgM), capable of complex signal transduction [Cushley and Harnett, 1993], does not occur until much later in development and until one of the light chain genes (usually the kappa gene) has undergone successful rearrangement, i.e., until the complete Ig structure is available. However, the discovery that proteins encoded by the lambda-5 and V(pre-B) genes (termed the surrogate light chain) formed a membrane complex with the Ig heavy chain μ protein early in B cell development [Karasuyama *et al.*, 1990], suggested that the expression of membrane Ig molecules occurred earlier than originally thought [Melchers *et al.*, 1993]. Disruption of the lambda-5 gene in murine embryonic stem cells, results in the blockade of B cell development at the pre-B cell stage [Kitamura *et al.*, 1992]. Expression of the μ-λ5−VpreB complex at the surface of pre-B cells acts as an indicator of successful heavy chain gene re-arrangement and a signal for progression to re-arrangement of the kappa light chain gene [Ehlich *et al.*, 1993; Tsubata *et al.*, 1992]. The appearance of mIgM on the B cell surface occurs late in the bone marrow phase of B cell development and defines the immature B cell. This is followed closely by transition of immature B cells from the bone marrow to the periphery, whereupon development becomes antigen-dependent. The initiation of Ig gene rearrangement is a function of commitment to the B cell lineage and a process which can be compared with rearrangement of the genes which encode the T cell receptor (TCR) in T lymphocytes [Borst *et. al.*, 1993]. The signals which are responsible for inducing commitment of stem cells to a particular cell lineage are ill understood; however, recent discoveries have highlighted the importance of DNA-binding proteins encoded by *Ikaros*, E2A and pax-5 genes, with respect to the development of lymphoid cells. Disruption of the *Ikaros* gene results in failure of murine T and B cell development [Clevers *et al.*, 1993]. Similarly,

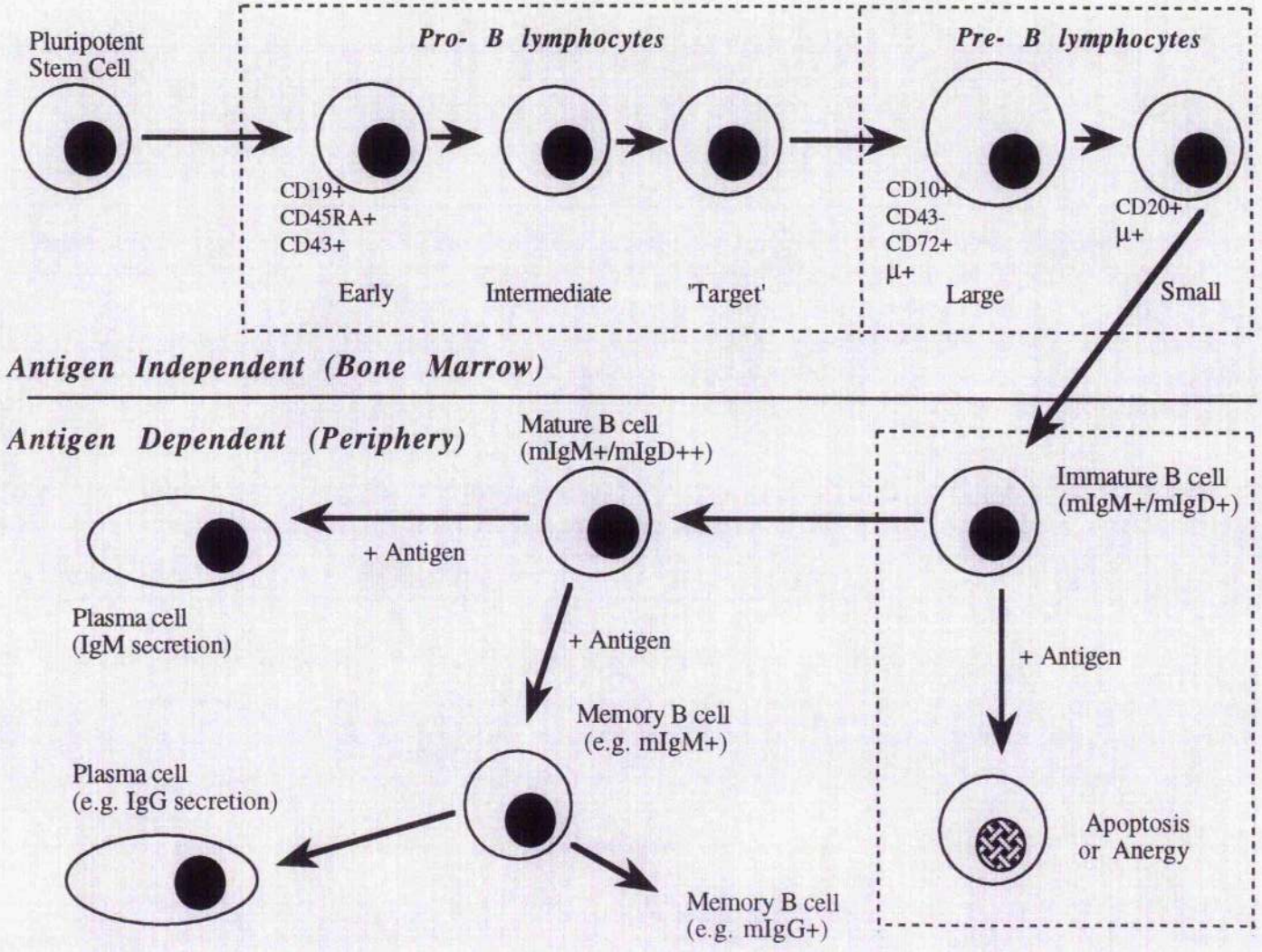



Figure 1.1: B Lymphocyte Development

inactivation of E2A and pax-5 genes results in the arrest of B cell development at the $CD43^+$ pro-B cell phase and total abrogation of Ig gene rearrangement [Urbanek *et al.*, 1994; Zhuang *et al.*, 1994]. The implications of this are, that E2A and pax-5 gene products are able to facilitate recombination of Ig genes. However, the pax-5 gene product, B cell-specific activation protein (BSAP), also regulates expression of CD19 and CD19 transcripts are absent in pax-5 gene knockout mice [Urbanek *et al.*, 1994]. Thus, there is a possibility that CD19 expression is also a requirement for commitment to the B cell lineage and for maturation of B cells. This latter suggestion is supported by the finding that CD19 deficient mice, while having normal levels of bone marrow B cell phases, had reduced levels of peripheral B cells, which did not proliferate in response to mitogenic stimuli, and reduced serum immunoglobulin [Engel *et al.*, 1995]. In recent years the importance of CD19, particularly in the presence of low concentrations of anti-IgM, as a co-activator of B cells has been highlighted and the synergy between membrane IgM and CD19 signal transduction demonstrates that CD19 has a role to play in B cell activation and development [Reviewed by Fearon and Carter, 1995]. All phases of development of B lymphocytes in the bone marrow are regulated by cytokines and growth factors produced by stromal cells, e.g. IL-7 and IL-11, and to a lesser extent by some T cell derived cytokines [Burrows and Cooper, 1993]. It is probable that the effects of such growth factors act in combination with Ig gene rearrangement and expression to promote B cell maturation.

The B cell compartment of the immune system can be divided into B cell subsets, depending upon anatomical location, surface marker expression and biological function. These B cell subsets are characterized by the expression of certain cell surface markers; thus, conventional B-2 cells are $IgM^{lo}$, $IgD^{hi}$, $CD19^{hi}$, $Mac1^-$, $IL\text{-}5R^-$ and $Fc\varepsilon RII^+$ [Waldschmidt *et al.*, 1991], whereas B-1a are $CD5^+$, $IgM^{hi}$, $IgD^{lo}$, $CD19^{lo}$ $Mac1^+$, $IL\text{-}5R^+$ [Kantor, 1991] and $Fc\varepsilon RII^-$ and B-1b cells display a cell surface marker pattern similar to B-1a, but are $CD5^-$. B-1 cells constitute between 5 and 30% of the total B cell

population and are found predominantly in spleen of newborns and within the peritoneal cavity, while being rare in lymph nodes and peripheral blood. Whereas conventional B-2 cells are continuously generated in adult bone marrow, B-1 cells appear to be produced early in ontogeny and constitute a separate B cell lineage; as evidenced by the inability of adult bone marrow to replenish B-1a stocks in irradiated mice [Kantor *et al.*, 1992]. CD5 can be induced in B-2 cells by treatment with anti-IgM and IL-6 and it has been hypothesised that Ig cross-linking in the absence of T cell help triggers pre-B cells to become $CD5^+$ B-1a cells [Haughton *et al.*, 1993]. This correlates with the appearance of B-1a cells early in development and also with the diversity in Ig gene re-arrangement observed between B-1a, B-1b and B-2 cells. B-1a cells have a restricted V gene repetoire and often express self-reactive antibodies, a phenomenon which would favour clonal expansion early in ontogeny, where exposure to self protein is more likely [Pennel *et al.*, 1989; Tornberg and Holmberg, 1995]. As development progresses the B cell repertoire is expanded substantially, due to greater diversity in light and heavy chain re-arrangements and the B-2 subset begins to predominate.

### 1.1.2 Activation and Differentiation of B Lymphocytes

Following mIgM expression and entry of B cells into the periphery, regulation of development becomes a function of exposure to antigen. In addition, B cell development in the antigen-dependent phase is also regulated by T cell intervention and can be split into T cell-independent and T cell-dependent processes. T cell-dependent regulation is controlled both by soluble protein growth factors known as cytokines and cell-cell contact through cell surface adhesion molecules expressed on T cells and B cells [Jelinek and Lipsky, 1988; Lederman *et al.*, 1993; Patel *et al.*, 1992; Tohma and Lipsky, 1991]. Thus, T cell dependent antigen responses were defined as those processes which required the action of T cell-manufactured cytokines and adhesion molecules to promote B cell development. It is now evident that many of what were originally thought of as T cell-independent developmental processes are regulated in some part by T cell factors, emphasizing that this reliance upon T cell help is even more integral to B cell

development than was first thought [reviewed by Banchereau *et al.*, 1994; Jelinek and Lipsky, 1987].

Exposure to antigen induces immature B cells to either undergo apoptosis, i.e., programmed cell death, or cellular anergy [Ales-Martinez *et al.*, 1991]. This clonal deletion of immature B cells during early exposure to antigen, permits the removal of potentially self-reactive B cells; i.e., B cells which, if allowed to mature, would mount an immune response to self proteins, resulting in the development of autoimmune disorders. Maturation of mIgM positive immature B cells, to mIgM/mIgD double positive mature B cells, results in a positive response when the latter come into contact with antigen; i.e., mature resting B cells are activated in response to antigen. Activation of mature resting B cells, via mIgM or mIgD, results in proliferation (i.e, the expansion of specifically activated B cell clones) and secretion of antibody from the cell [Brink *et al.*, 1992]. Cell division has been deemed a necessary step in the differentiation of B cells into mature antibody secreting cells; treatment of human peripheral blood B cells with inhibitors of DNA synthesis, mitomycin C and hydroxyurea, prevents the generation of Ig secreting cells in response to pokeweed mitogen [Jelinek and Lipsky, 1983]. This requirement is not universal however, as some studies have shown the development of small resting B cells into IgM secreting cells in the absence of DNA synthesis [Andersson *et. al*, 1980]. Thus, it appears that in some cases at least, cell division has a role in B cell activation which goes beyond the expansion of Ig secreting cells. In addition, it has been suggested that on-going Ig synthesis and secretion requires continual proliferation of differentiated B cells [Jelinek *et al*, 1987]. The implications of this are that factors such as cytokines like IL-2, which are essential to promote proliferation of activated B cells following their initial differentiation [Lipsky *et al.*, 1988], are integral in both the extent and maintenance of a specific immune response. In addition to this primary immune response, B cells undergo further processes of differentiation; this includes isotype switching, with resultant IgA, IgE and IgG secretion [Lutzker *et al.*, 1988; Rothman *et al.*, 1988], and somatic mutation of the hypervariable region of the antigen binding sites.

an event which leads to production of higher affinity antibodies [Both *et al.*, 1990; Tonegawa, 1983]. The final stages of the B cell developmental process are terminally differentiated-antibody secreting plasma cells and memory B cells. The latter express very high affinity antigen receptors and are responsible for the initiation of rapid secondary immune responses upon re-infection, whereby re-challenge with the same antigen results in production of high affinity antibodies often of the IgG and IgE classes. It is thought that many rounds of B cell proliferation enhance the rate of Ig secretion by terminally differentiated plasma cells and may also be important in both promoting heavy chain isotype switching and providing opportunities for somatic mutation, which leads to the production of high affinity antibodies. The initial concept of B cell activation was that mature resting B cells were activated in response to antigen stimulation and that following this, T cell factors helped promote proliferation and differentiation. However, the discovery that IL-4 (as a co-stimulant along with anti-Ig antibodies) acts on resting B cells to activate them and promote DNA synthesis, cell division and Ig secretion [Kotowicz and Callard, 1993], suggests that IL-4 'primes' small resting B cells to respond to antigen and demonstrates that T cell intervention is not only a regulator of already proliferating cells, but is integral in the initial processes of B cell activation.

## 1.2 The Importance of IL-2 in B Cell Development

### 1.2.1 The Role of IL-2 in B Cell Activation, Proliferation and Differentiation

B lymphocyte development, as well as being regulated by exposure of the cell population to antigen and activation through the B antigen cell receptor complex, is heavily regulated by cell-cell surface contact by means of adhesion molecules and by the action of soluble protein factors known as cytokines. A subfamily of cytokines, known as lymphokines, are produced by and are particularly responsible for the regulation of development of Haematopoietic cells [Callard, 1990; Paul and Seder, 1994]. All cytokines mediate their

effects by binding to specific receptors expressed on the surface of target cells, and subsequently generating a cascade of intracelluar signalling pathways which ultimately result in the activation or suppression of a specific set of genes [Foxwell *et al.*, 1992; Ihle *et al.*, 1995; Taga and Kishimoto, 1993]. The classification of cytokine receptors allows them to be grouped into a number of superfamilies based on their structural homology to one another. One such superfamily is the haematopoietin receptor superfamily, which is characterized by the presence of four conserved cysteine residues and a WSXWS motif in the extracellular, amino terminal domain of the receptor [Cosman *et al.*, 1990; Foxwell *et al.*, 1992]. Other superfamilies to which cytokine receptors belong include: the Immunoglobulin superfamily, e.g. IL-1 and PDGF receptors ; a tumour necrosis/nerve growth factor receptor superfamily with cysteine rich repeats, eg. TNF α and β receptors; a type II TGFβ receptor superfamily with a serine/threonine kinase domain; an IL-8 receptor superfamily belonging to the G-protein coupled, seven transmembrane domain, receptor supergene family, e.g. IL-8 and platelet factor PF-4 receptors [Taga and Kishimoto, 1993].

One cytokine which plays a key role in lymphoid cell development is interleukin 2 (IL-2), a 15.4 kDa protein, produced and secreted by activated T lymphocytes and originally identified as T cell-growth factor. IL-2 is produced as a result of either mitogenic (by agents such as phytohaemagglutinin), or antigenic (via the T cell receptor complex) stimulation of T cells. Secreted IL-2 acts in both autocrine and paracrine growth stimulating modes upon the surrounding T cell population and is important in controlling their proliferation and differentiation. Interestingly, the stimulation of IL-2 production occurs in conjunction with up-regulation of a high affinity form of the IL-2 receptor complex, thereby allowing T cells to make a maximal response to the cytokine. The discovery that B cells could be induced to express receptors for IL-2, led to the idea that IL-2 might act as more than just a T cell growth factor. Thus, in addition to activation of T cells, IL-2 is also found to induce activation of natural killer cells, proliferation of oligodendroglial cells and growth and differentiation of B lymphocytes. As is the case

with T lymphocytes, the high affinity form of the IL-2 receptor is only expressed on activated B cells of the mature circulating population. A quiescent G0 B cell population is driven into G1 by a variety of stimuli and an integral part of this activation process is the expression of high affinity IL-2 receptors, an event which primes B lymphocytes for further activation; i.e., proliferation and expansion of B cell clones and differentiation into mature memory B cells.

Antigenic stimulation of T and B cells is profoundly different: the T cell antigen receptor binds either a class I or II MHC antigen/peptide complex, which are expressed on the surface of antigen presenting cells (APC); the B cell antigen receptor binds freely occurring antigenic molecules which circulate in the blood and require no prior processing. Similarly, there is a high degree of variability in other stimuli which activate T and B cells; including, the accessory molecule complexes which associate with the T and B cell antigen receptors and are thought to be responsible for mediating signal transduction in reponse to antigen, and reponses to adhesion molecule interactions and to various cytokines. The induction of expression of high affinity IL-2 receptors on these different cell types is also subject to entirely different regulatory mechanisms and will be discussed in this chapter.

### 1.2.2 The Structure of the IL-2 Receptor

The high affinity form of the receptor for IL-2 (IL-2R) consists of three distinct subunits, a 55kDa α chain (also referred to as the Tac antigen or as CD25), a 75kDa β subunit [Waldmann *et al.*, 1984] and a 64kDa γ subunit, and has a Kd of $10^{-11}$M [Robb *et al.*, 1984]. Two of the subunits found in the IL-2 receptor complex, the IL-2Rβ and γ components, are members of the haematopoietin receptor superfamily, whereas the third component, IL-2Rα, appears to be unique in that it showed no identifiable sequence homology to any other known cytokine receptor component, until the recent discovery of the IL-5 receptor α chain which has a degree of homology to IL-2Rα. In addition to the

high affinity form, IL-2 also binds two receptors of low and intermediate affinity; these receptors are comprised of a IL-2Rα chain (Kd= $10^{-8}$M) and a complex of IL-2Rβ and γ chains (Kd= $10^{-9}$M), respectively [Robb *et al.*, 1984]. One of the key advantages of this multi-component structure with respect to maximising the cellular effects of IL-2, appears to be the combination of certain kinetic features of the individual components. Thus, the high affinity form of the IL-2R demonstrates a very fast association with its ligand, a feature of the IL-2Rα subunit, combined with a slow dissociation rate, a property of the intermediate form of the receptor which was first associated with the IL-2Rβ chain. This fast 'on'/slow 'off' rate of ligand association facilitates a rapid response to IL-2 which can also be sustained to allow a maximal response to the cytokine.

It has become apparent that the extent of constitutive and inducible expression of the α, β and γ components of the IL-2 receptor complex is variable depending upon cell type. Thus, in resting human B cells both the β and γ subunits are constitutively expressed while levels of α (CD25) are both very low and inducible [Waldmann *et al.*, 1984]. In contrast, resting T cells express low levels of γ but not α or β on the cell surface [Nakarai *et al.*, 1994]. The α and β chains are expressed in response to mitogenic stimulation of resting T cells; however, in later stages of development, the levels of β chain are reduced and it is this component which limits the availability of high affinity IL-2 receptors [Nakarai *et al.*, 1994]. The situation is entirely different in natural killer cells, where it is predominantly the γ component of the receptor which is inducible and is, therefore, the limiting factor with respect to expression of high affinity receptors [Nakarai *et al.*, 1994]. IL-2 receptor expression is also tightly regulated in non-lymphoid cells. Thus, in human monocytes IL-2Rβ is constitutively expressed, but IL-2 stimulation does lead to post-transcriptional stabilization of IL-2Rβ mRNA. These cells also constitutively express IL-2Rγ mRNA, which is up-regulated by IFNγ and IL-2, again via a mechanism of post-transcriptional stabilization [Bosco *et al.*, 1994]. IL-2Rα in contrast, is not constitutively expressed on resting monocytes, but is induced by stimulation with IFNγ [Bosco *et al.*, 1994]. Thus it seems that the expression of high affinity IL-2 receptors and, therefore,

responsiveness to IL-2 is dependent upon the level of inducibility of different components of the receptor complex, an event which appears to be differentially regulated depending upon cell lineage and also upon the developmental phase of the particular cell type.

### 1.2.3 Signalling Components of the IL-2 Receptor

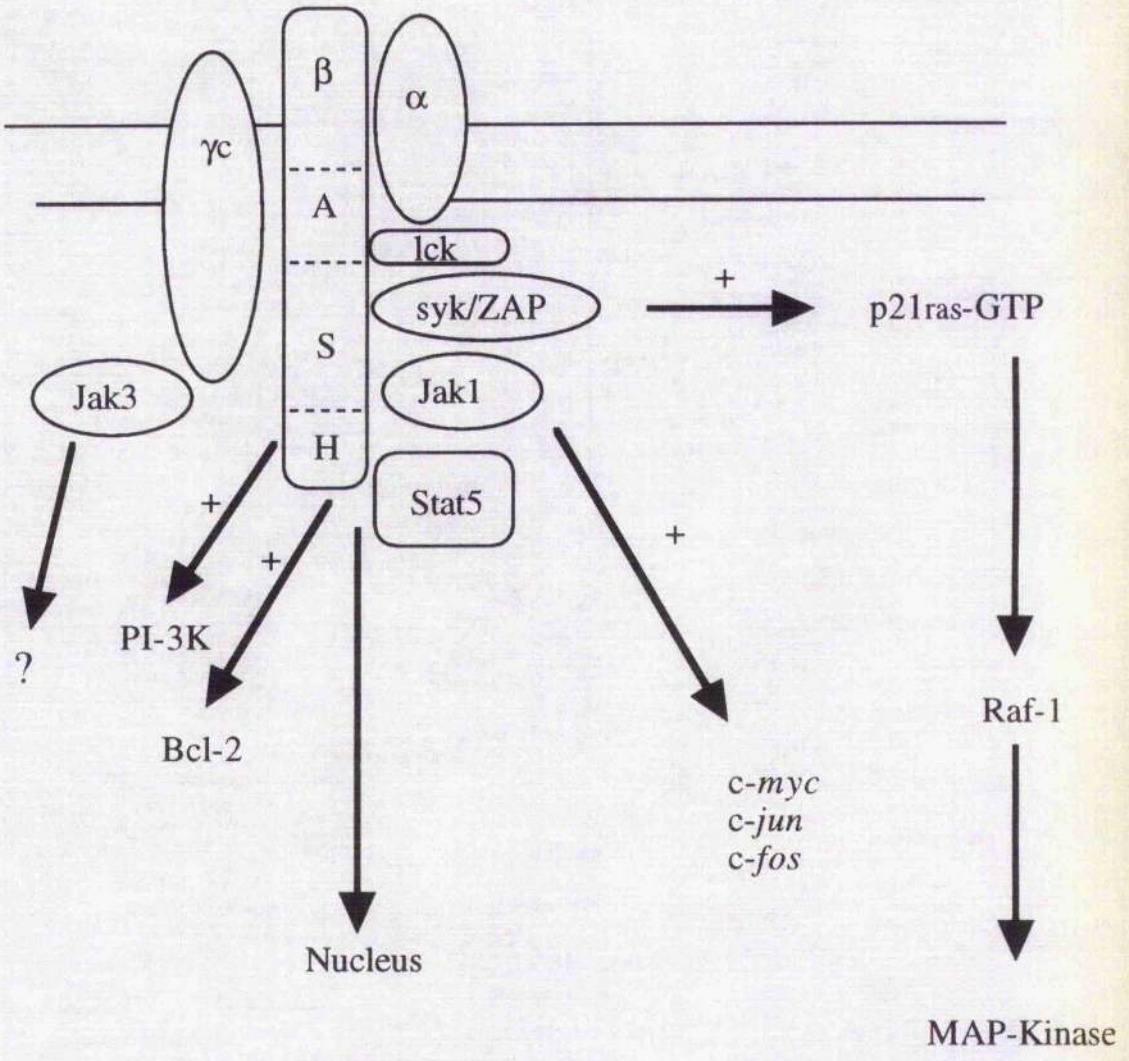
Early evidence suggested that IL-2 signalling pathways are predominantly mediated by the β subunit, however, important intracellular signals activated by the γ component have also been identified. In contrast, it was suggested that the α chain had no signal transduction capability and was purely responsible for regulation of the affinity of IL-2 receptor binding. An initial feature of IL-2 induced signal transduction, like a variety of other cytokines, is tyrosine phosphorylation of IL-2Rβ and direct association of tyrosine kinase activity with the receptor complex. Like other cytokine receptors however, none of the individual components of the IL-2 receptor have protein tyrosine kinase domains within their structure [Reviewed by Taga and Kishimoto, 1993]. Therefore, the premise was that the IL-2 receptor was closely associated with independent cytosolic tyrosine kinases and recent studies have revealed that IL-2 activates a complex array of intracellular signalling molecules. Transfection studies using wild type and mutant forms of IL-2Rβ demonstrated that the transmembrane domain and a central region in the cytosolic domain are dispensable, with respect to signal transduction. However, both functionally unique and redundant elements exist in the proximal and distal regions of the cytoplasmic domain [Goldsmith *et al.*, 1994]. The basis for these studies hinged on the ability of IL-2 to induce a proliferative response in the BA/F3 B cell line [Goldsmith *et al.*, 1994] and it was, therefore, a possibility that regions responsible for induction of signalling pathways unrelated to the proliferative response were overlooked. The cytoplasmic domain of IL-2Rβ contains three regions, designated S, A and H, which appear to have distinct signalling capabilities. IL-2Rβ interacts both physically and functionally with members of the *src* proto-oncogene family; thus, $p56^{lck}$ is activated in peripheral blood lymphocytes in response to IL-2 [Hatakeyama *et al.*, 1991] and in an

analogous manner, $p59^{fyn}$ and $p53/56^{lyn}$ are activated in BA/F3 derived cells [Torigoe *et al.*, 1992]. IL-2Rβ-$p56^{lck}$ association appears to require the A region of the receptor subunit [Hatakeyama *et al.*, 1991]. The S region of IL-2Rβ [Hatakayama *et al.*, 1989] is responsible for activation of Jak1 [Witthuhn *et al.*, 1994], Syk/ZAP-70 [Miyazaki *et al.*, 1994], $p21^{ras}$-GTP binding protein [Satoh *et al.*, 1992], Raf-1 and also the induction of c-*myc* early gene expression [Shibuya *et al.*, 1992]. IL-2 induction of other early response genes, c-*fos* and c-*jun*, appears to correlate with activation of $p56^{lck}$ [Minami *et al.*, 1993]. In addition and more recently, it has been shown that IL-2 induces expression of members of the bcl-2 gene family, including bcl-2, bcl-$x_L$ and bax, and that this induction also requires the S region of IL-2Rβ [Miyazaki *et al.*, 1995]. The H region of IL-2Rβ is an absolute requirement for activation of STAT5, although it is a possibility that this is only in the capacity of physical association, as Jak1 appears necessary to functionally activate STAT5 [Gaffen *et al.*, 1995]. IL-2Rβ also associates with the 85kDa regulatory subunit of phosphatidyl inositol-3-kinase (PI-3 kinase or p85) [Truitt *et al.*, 1994]. In IL-2-stimulated T cell lysates a fusion protein, containing the N- and C-terminal SH2 domains of p85, co-precipitates with an 80kDa tyrosine phosphorylated protein shown to be the IL-2Rβ chain [Truitt *et al.*, 1994]. Therefore, PI-3 kinase physically associates with IL-2Rβ through SH2-phosphotyrosine interactions.

Following the identification of γc as the third component of the IL-2 receptor complex [Takeshita *et al.*, 1992a; Takeshita *et al.*, 1992b], it was discovered that the gene for IL-2Rγ (localized to chromosome Xq13) and the locus for X-linked Severe Combined Immunodeficiency (X-SCID) appeared at the same chromosomal position [Noguchi *et al.*, 1993]. A number of unrelated X-SCID patients have mutations in their IL-2Rγ gene, resulting in different premature stop codons which lead to C-terminal truncation mutants [Noguchi *et al.*, 1993]. A variety of internal deletion, truncation and point mutations in the IL-2Rγ of a number of patients were shown to result in an inability to form high affinity IL-2 receptors; thus, a range of different mutant IL-2Rγ chains from many X-

SCID patients all manifested the same symptom, i.e., an inability to associate with IL-2Rβ [DiSanto *et al.*, 1994]. X-SCID is characterized by an absent or drastically reduced T cell population, normal numbers of functionally deficient B cells and severely diminished cell-mediated and humoral immunity. The severity of the disease is in contrast with autosomal SCID, characterized by greatly reduced IL-2 production, where patients develop almost normal numbers of peripheral T cells and the condition can be corrected by administration of exogenous IL-2. The extreme severity of X-SCID, compared with other forms of SCID, and the apparent relationship of the disease with mutations in IL-2Rγ, led to the notion that IL-2Rγ was involved in the receptor mediated signalling of other cytokines, as an inability to produce IL-2 does not in itself result in total abrogation of T cell development. IL-7 is integral in the regulation of early T cell and thymic development and IL-4 is recognized as one of the major B cell growth factors. The discovery that IL-2Rγ is required for expression of high affinity IL-4 and IL-7 receptors [Kawahara *et al.*, 1994; Kondo *et al.*, 1994; Russell *et al.*, 1993], goes some way to explaining the extreme nature of X-SCID; i.e., a number of cytokines which regulate development rely on IL-2Rγ [γ-common or γc] for efficient signal transduction. Indeed, monoclonal antibodies to IL-2Rγ block both T and B lymphocyte development during, and at stages prior to, seeding of the thymus by precursor cells and the appearance of pro-B cells, respectively [He *et al.*, 1995a]. Signalling pathways activated by IL-2Rγ are far less complex than for IL-2Rβ but are nonetheless important; activation of Jak3 is solely attributed to γc and it is tyrosine phosphorylated in response to IL-2, IL-4 and IL-7 [Kondo *et al.*, 1994; Russell *et al.*, 1993]. Co-expression of IL-2Rα, β and γ subunits in fibroblasts and stimulation with IL-2 results in induction of the nuclear proto-oncogenes c-*fos*, c-*jun* and c-*myc*. Regions of the IL-2Rβ chain which are responsible for activation of these genes have been well characterized. However, co-transfection of a mutant IL-2Rγ gene, lacking all but seven of the cytoplasmic domain amino acid residues, and a fos-CAT construct into F7 cells (a BA/F3 derivative, stably transfected with IL-2Rβ) results in a drastic decrease below basal of CAT reporter activity in response to IL-2; compared with cells transfected with the full length wild type IL-2Rγ

Figure 1.2: IL-2 Induced Signal Transduction



receptor, where an increase in reporter gene activity was observed [Kawahara *et al.*, 1994]. This dominant-negative effect also occurs in IL-2-induced c-*myc* expression and cellular proliferation. Therefore, IL-2Rγ is necessary for the efficient transduction of certain signals that have previously been associated with the IL-2Rβ component. In addition, proliferation in response to IL-4 and IL-7 in BA/F3 cells transiently transfected with the IL-4Rα and IL-7Rα chains, was augmented by co-transfection with wild type IL-2Rγ, but inhibited by the IL-2Rγ truncation mutant [Kawahara *et al.*, 1994]. Transfection of CTLL-2 T cells with chimeric IL-2 receptor molecules, i.e., the extracellular domain of c-kit (a receptor which homodimerizes in response to stem cell factor - SCF) and the intracellular domains of IL-2Rβ and IL-2Rγ (named kit/2β and kit/2γ, respectively) demonstrated that a proliferative response to SCF only occurred when both kit/2β and kit/2γ were co-expressed [Nelson *et al.*, 1994]. In addition, co-expression of chimeric IL-2Rβ and IL-2Rγ receptor molecules with different extracellular receptor domains (G-CSFRα and c-kit) resulted in a loss of proliferative responses to either of these ligands [Nelson *et al.*, 1994]. Thus, it appears that the cytoplasmic domains of both IL-2Rβ and IL-2Rγ are necessary for IL-2 to promote an efficient proliferative signal. Furthermore, association of the extracellular haematopoietic domains of IL-2Rβ and γ is required to induce heterodimerization of the cytoplasmic domains and subsequent signal transduction.

### 1.2.4 The Importance of CD25 Expression

CD25 is differentially expressed throughout the various stages of lymphocyte development. Thus, in later stages of differentiation which occur in the periphery, expression of CD25 is observed only on activated, mature T and B cells [Waldmann *et al.*, 1984]. However, CD25 is also found on the cell surface of both T and B cells in the early stages of development, in the thymus and bone marrow respectively. CD25 is expressed on $CD4^-/CD8^-$ thymocytes [Uchiyama *et al*, 1981] and more recently its expression has been demonstrated in the pre-B cell and immature stages of murine B cell differentiation [Chen *et al.*, 1994]. Chen *et. al.* (1994) demonstrated that the onset of

CD25 expression occurs during progression from the pro- to pre-B cell stage and is associated with μ heavy chain expression. CD25 is also expressed in immature murine B cells but is lost during the process of maturation to $IgM^+/IgD^+$ B cells [Chen *et al.*, 1994]. Other studies have demonstrated that expression of CD25 occurs in a transition between pre-B cell subsets and is expressed upon maturation from pre-B I to pre-B II cells in the murine system, an event which is also marked by down-regulation of TdT and c-kit [Rolink *et al.*, 1994]. In addition, in mature resting murine and human B cells levels of CD25 expression are either very low or non-existent, but can be induced to express this IL-2 receptor component in response to various stimuli. In human B cells, CD25 is the only inducible component of the IL-2 receptor, whereas both the β and γ subunits are constitutively expressed [Takeshita *et al.*, 1992a; Waldmann *et al.*, 1984]. Therefore, regulation of cell surface levels of CD25 in B lymphocytes is essential in order for them to express the high affinity form of the IL-2 receptor, a necessity for maximal activation by IL-2. In addition, CD25 is the only unique component of the IL-2 receptor complex; the γ subunit designated γ–common (γc) is also a component of the IL-4, IL-7, IL-9 and IL-15 receptors [Grabstein *et al.*, 1994; Kondo *et al.*, 1994; Russell *et al.*, 1993] and a receptor consisting of the β and γc subunits i.e., the intermediate form of the IL-2 receptor, also binds IL-15 [Giri *et al.*, 1994; Grabstein *et al.*, 1994]. IL-15, despite sharing certain functional cellular effects with IL-2, is structurally distinct and the differential response of some cells to IL-2 and IL-15 is likely regulated by the unique α chain of the IL-15 receptor [Giri *et al.*, 1995]. The α subunit of the IL-2 receptor is not part of the IL-15 receptor complex, therefore, the implications are that it is the IL-2Rα chain (CD25) which confers specificity for IL-2 binding. It is likely, therefore, that CD25 expression is a requirement for the mediation of cellular events uniquely associated with stimulation by IL-2. Both the IL-2R β and γc subunits appear to have important and distinct signal transduction capabilities; in contrast, CD25 has no intrinsic signalling motifs in its structure and does not appear to associate with any intracellular signalling molecules. It was considered therefore, that IL-2 activated intracellular signalling pathways were mediated via the β and γc components of its receptor and that CD25, as an

absolute requirement for formation of high affinity receptor complexes, was limited to modulating IL-2 binding affinity of the IL-2 receptor [Robb *et al.*, 1984; Waldmann *et al.*, 1984]. However, recent studies have suggested that CD25 may be responsible for generating a negative regulatory signal in murine peripheral blood monocytic cells [Kasinrerk *et al.*, 1994; Kumar *et al.*, 1987]. The evidence for this is based on the ability of an anti-IL-2Rα antibody to enhance the proliferative response of lymphocytes to antigen. It must be noted, however, that this antibody-induced enhancement is only observed at sub-optimal concentrations and that at high concentrations the anti-IL-2Rα antibody inhibits a proliferative response. These observations outline the possibility of a role for CD25 which goes beyond simply regulating affinity and specificity of ligand binding, i.e., that it plays an integral part in IL-2-induced signalling pathways.

## 1.3 The Role of IL-4 in B Cell Development

### 1.3.1 Production and Biological Roles of IL-4

Interleukin 4 (IL-4) is a 15-19kDa cytokine principally derived from helper T cells, but also produced by mast cells, certain myeloid cell lines, some B cell lymphomas, B cells from SLE patients and certain bone marrow stromal cell lines [Howard *et al.*, 1982; O'Garra *et al.*, 1990; Okayama *et al.*, 1995; Toellner *et al.*, 1995]. IL-4 has a number of key roles in the development and differentiation of many lymphoid cell lineages, acting as a co-stimulator for activated B cells, T cells and mast cells. In addition, IL-4 also induces growth and differentiation of haematopoietic progenitor cells in the presence of additional cytokines. However, the major role of IL-4 and therefore, the principal interest with respect to the activity of this cytokine, lies in its involvement in B lymphocyte development and differentiation. Thus, IL-4 exerts a number of effects on B cells which include: enlargement of the B cell, a feature which is generally associated with activation; increased expression of surface IgM and IgD, CD23, CD25, CD40, LFA-1, and MHC Class II; isotype switching to produce IgE and certain IgG isotypes;

reduction of cell surface CD5 expression [Reviewed by Callard, 1991]. Such effects result in increased differentiation of B lymphocytes and many lead to the heightened responsiveness of the B cell to subsequent stimuli.

IL-4 enhances the responsiveness of B lymphocytes to anti-Ig reagents by elevating levels of surface IgM and IgD and, in addition, by inducing isotype switching [Kotowicz and Callard, 1993]. At high concentrations of IL-4, LPS stimulated B cells undergo isotype switching and synthesis of both IgE and $IgG_4$ is promoted [Rothamn *et al.*, 1988]. In tonsillar B cells, IL-4 can promote elevation of levels of membrane CD23 in greater than 70% of the B cell population [Defrance *et al.*, 1989] and triggers release of soluble CD23, which is shed from the B cell surface following proteolytic cleavage of membrane CD23 [Bonnefoy *et al.*, 1988]. CD23 is recognised as the low affinity receptor (FcRεII) for IgE and soluble CD23 is known to act as an autocrine growth factor for normal B cell blasts and EBV transformed B lymphoblasts [Swendeman and Thorley-Lawson, 1987]. An additional receptor for CD23 is CD21, the B cell specific receptor for EBV and C3d (Complement Receptor 2), and a novel CD23 receptor expressed on the cell membrane of a pre-B cell line has recently been discovered [White *et al.*, 1996]. Antibodies to CD23 inhibit IgE production, suggesting a link between CD23 and IL-4 regulation of IgE secretion [Bonnefoy *et al.*, 1990]. IL-4 promotes expression of Class II major histocompatablity antigen (MHC) in B cells [Noelle *et al.*, 1984], an event which enhances the capacity of B cells to act as antigen presenting cells, i.e., in their ability to present peptide antigen to $CD4^+$ T cells. CD5 is a pan T cell marker which is expressed on a subset of normal B cells and on most B-CLL cells. IL-4 down-regulates surface expression of CD5 on tonsil B cells and B-CLL cells, but not T cells [Defrance *et al.*, 1989; Freedman *et al.*, 1989]. This down-regulation of CD5 appears to be exclusively regulated by IL-4 and can be blocked by anti-IL-4 neutralizing antiserum.

Resting B cells i.e., those in G0, express only a few hundred high affinity IL-4 receptors. Stimulation with IL-4 results in activation of B cells but is unable to promote

DNA synthesis or cell division. *In vitro* stimulation with high concentrations of anti-Ig antibodies initiates a transition from the G0 to G1 phase of the cell cycle, an event which can be detected by an increase in the size of B cells and elevated levels of cytoplasmic mRNA, and the cells become committed to DNA synthesis. In contrast, low levels of anti-Ig antibodies or stimulation with protein antigen, cannot promote this proliferative response. Co-stimulation of B lymphocytes with anti-Ig antibodies and IL-4 promotes DNA synthesis and cell division [Reviewed by Callard, 1991]. In physiological circumstances levels of circulating protein antigen are likely to be relatively low, and so the responding B cell population requires additional stimuli to promote a proliferative response. Previously, it had been shown that culturing normal human B cells in the presence of irradiated T cells, monocytes and Pokeweed Mitogen (PWM) resulted in elevated levels of CD25 at the cell surface [Waldmann *et al.*, 1984]. Such responses indicated that regulation of CD25 expression on human B lymphocytes was a feature of T cell-dependent B cell activation but gave no indication of any specific stimuli which regulated this response. It has since been demonstrated that IL-4 is the sole cytokine capable of inducing CD25 expression in primary resting human B cells [Burlinson *et al.*, 1995; Butcher and Cushley, 1991; Butcher *et al.*, 1990; Zola *et al.*, 1991], an observation which to some extent explains the ability of IL-4 to act as a co-stimulant for B cell activation. Thus, it may be considered that as well as responding to antigen or anti-Ig, B cells are 'primed' by IL-4 to respond to additional activation stimuli, probably by eliciting some of the cellular responses which have been described: for example, increasing CD23 and release of soluble CD23, thereby promoting its autocrine growth effects on B cells, and elevating cell surface levels of CD25 and promoting formation of high affinity IL-2 receptors, allowing a maximal response to IL-2.

In addition to initiating activation of B cells, IL-4 can, in certain circumstances, promote an anti-proliferative response particularly with respect to IL-2. IL-2-induced stimulation of proliferation, but not differentiation, of B-CLL and follicular lymphoma cells is inhibited by IL-4 [Defrance *et al.*, 1989; Shields *et al.*, 1989]. In addition, the activity of

IL-2 in specific antibody responses to the influenza virus is attenuated by IL-4 [Callard *et al.*, 1991] and immunoglobulin secretion in response to IL-2, SAC and T cell replacing factor is also inhibited [Jelinek and Lipsky, 1988; Splawski *et al.*, 1989]. It has been suggested, that the ability of IL-4 to inhibit IL-2-specific proliferative responses is mediated by inhibiting the expression of high affinity IL-2 receptors. As IL-4 displays both agonistic and antagonistic effects on proliferation of normal B cells it was suggested that IL-4 and IL-2 stimulate distinct B lymphocyte populations and IL-4 blocks proliferation of an IL-2 responsive population. However, such an hypothesis does seem unprecedented if one considers that IL-4 is the only cytokine capable of up-regulating CD25 in human B cells. This latter observation would suggest an increase in potential responsiveness of these B cells to IL-2, as CD25 is an absolute requirement for formation of high affinity IL-2 receptors. IL-4 only promotes CD25 expression of 30-40% of the resting B cell population [Burlinson *et al.*, 1995; McKay and Cushley, 1995], perhaps indicating that only a specific sub-population of B cells are primed to respond to IL-2, whereas it may be that IL-2 responses of the remaining B cell population are actively inhibited by IL-4.

### 1.3.2 IL-4-Induced Signal Transduction

The receptor for IL-4, like many cytokine receptors, is a multi-subunit structure consisting of a 140kDa α chain (IL-4Rα) [Harada *et al.*, 1990; Idzerda *et al.*, 1990; Mosley *et al.*, 1989] and the common γc chain [Russell *et al.*, 1993]. The IL-4Rα chain has been identified as a member of the Haematopoietin Receptor Superfamily and contains a number of signalling motifs within its cytoplasmic domain. The γc chain is a component of a number of other cytokine receptors, as mentioned previously, and as such, intracellular signals which are a feature of this subunit appear to be common to the signal transduction pathways of many of these cytokines. A comprehensive understanding of IL-4 signal transduction in human B cells remains to be elucidated and present knowledge shows it to be a complex process involving several second messenger systems.

Early studies into signal transduction in resting, human tonsillar B cells revealed that, following IL-4 binding to its receptor, there is a transient increase in $IP_3$ production and consequent calcium mobilization with intracellular levels returning to basal within a minute of ligand binding. This initial phosphoinositide hydrolysis is followed, after a lag period of approximately 10 minutes, by a sustained elevation in intracellular cAMP [Finney *et al.*, 1990]. As well as $IP_3$ production, $PIP_2$ hydrolysis results in the production of diacyl glycerol (DAG), a second messenger which subsequently activates PKC and induces its translocation to the plasma membrane [Reviewed by Cook and Wakelam, 1992]. It has been reported that, in human monocyte cells, IL-4 induces translocation of PKC to both membrane and nuclear fractions of the cell and has a dose-dependent effect on increasing PKC activity [Atruda and Ho, 1992]. In addition, it has been possible, using agents such as phorbol ester, calcium ionophores and the diterpene forskolin, to mimic the ability of IL-4 to increase soluble CD23 production by primary human B cells [Finney *et al.*, 1990]. These data are in direct contrast with IL-4 induced signal transduction in primary, murine B cells. Thus, IL-4 fails to induce $PIP_2$ hydrolysis and resultant $IP_3$ or DAG production in murine B cells, nor does it promote calcium mobilization [Justement *et al.*, 1986; Mizuguchi *et al.*, 1986; O' Garra *et al.*, 1987]. It has also been reported that high concentrations of EGTA in the culture medium were unable to abrogate IL-4 induced expression of MHC Class II in murine B cells, a result which is in direct contrast to the inhibition of anti-IgM induced MHC Class II expression in murine B cells in the presence of relatively low levels of EGTA [Mizuguchi *et al.*, 1986]. Stimulation of both human and murine B cells with anti-Ig antibodies results in $PIP_2$ hydrolysis, $IP_3$ and DAG production and $Ca^{2+}$ mobilization. Co-stimulation of murine and human B cells with anti-Ig and IL-4 results in DNA synthesis and cell division [Howard *et al.*, 1982]. Reports that IL-4 failed to induce $PIP_2$ hydrolysis suggested that, in the murine system at least, this particular signalling pathway was not responsible for this synergistic, proliferative response. However, further studies revealed that co-stimulation of murine B cells with IL-4 and the phorbol

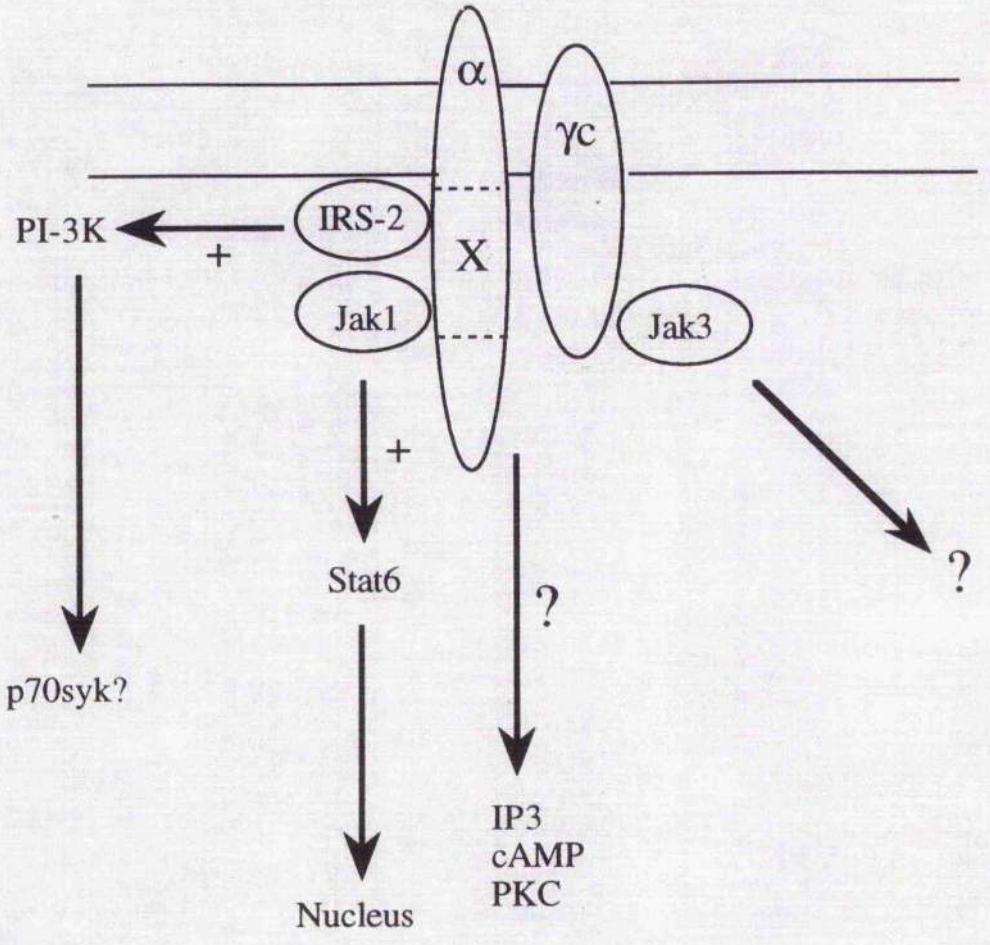
esters PMA and $PBu_2$ induce DNA synthesis, an effect not observed upon co-stimulation with IL-4 and calcium ionophore [Klaus and Harnatt, 1990]. Therefore, it seems that PKC may have a role to play in the cross-talk between the signalling pathways activated during the co-stimulation of murine B cells by anti-Ig and IL-4. Initial studies concerning the ability of IL-4 to activate protein tyrosine kinase activity revealed, that in murine B cells, IL-4 induced tyrosine phosphorylation of a 42-44kDa intracellular protein [Justement *et al.*, 1986; McGarvie and Cushley, 1989a]. Similar experiments in the human Daudi B cell line showed no phosphorylation of a protein of this particular molecular weight [Justement *et al.*, 1986]. IL-4 was also shown to induce tyrosine phosphorylation of a 75-80kDa molecular weight protein murine splenic B cells, an effect which was unique to stimulation with IL-4 in these cells [McGarvie and Cushley, 1989b]. The implication of all these studies is, that there exist distinct, species-specific, signalling mechanisms which are employed by IL-4 in both primary B cells and B cell lines in murine and human systems.

More recently, the nature of IL-4 signal transduction, particularly with respect to protein tyrosine kinase activity, has been partially elucidated. Studies have revealed a number of pivotal IL-4-induced signal transduction pathways, common to both human and murine systems. IL-4 stimulates tyrosine phosphorylation of a 170kDa protein (initially designated 4PS) in two IL-13-dependent myeloid progenitor cell lines, FDC.P2 and 32D, which proliferate in response to IL-4 [Keegan *et al.*, 1994; Wang *et al.*, 1992]. In addition, phosphorylated bands of 130-140kDa, 120kDa and 97kDa were also observed in these cells [Wang *et al.*, 1992]. The 140kDa protein is the IL-4R$\alpha$ chain and it now seems probable that the 97kDa protein is Stat6. Thus, IL-4 induces tyrosine phosphorylation of its receptor, probably via association with intracellular tyrosine kinases. It was at first considered that 4PS (now designated IRS-2), as a target for cytokine-mediated signalling, was unique to IL-4-linked signal transduction, however, it has recently been shown that IL-9 also activates IRS-2 and that it is present in a range of cells lines as well as in normal primary cells. Analysis of the amino acid sequence of

IRS-2 revealed a large degree of sequence homology with IRS-1 (insulin receptor substrate-1), a protein which is phosphorylated in a number of cell types in response to stimulation with either insulin or IGF-1 (insulin-like growth factor-1) [Wang *et al.*, 1993]. Indeed, treatment of certain IL-4 responsive cell lines with insulin and IGF-1 results in phosphorylation of IRS-2. Treatment of the myeloid progenitor cell line FDC.P2 with IL-4 leads to a dramatic association of IRS-2 and the 85kDa subunit of PI-3 Kinase (p85), although this interaction does not involve the phosphorylation of p85 itself [Wang *et al.*, 1992]. Interestingly, the doses of IL-4 required to initiate this interaction and the mitogenic response in these cells, are very similar. In addition, it has been demonstrated that IL-4 can activate IRS-1, which has been over-expressed in 32D cells [Keegan *et al.*, 1994], and that activation of IRS-1 in these cells is necessary for PI-3 Kinase and $p70^{syk}$ activation by insulin, IGF-1 and IL-4 [Myers *et al.*, 1994]. However, it must be noted that activation of $p70^{syk}$ in response to IL-4 is weak when compared with IL-4 induced activation of PI-3 kinase and, therefore, may not be as important in terms of IL-4 signal transduction pathways. Like insulin, IL-4 induces association of GRB2/Sos with activated IRS-1, but unlike insulin, fails to activate Ras and extracellular signal regulated kinases (ERKs), or induce GRB2 association with Shc [Pruett *et al.*, 1995].

The discovery of the Janus family of tyrosine kinases revolutionised understanding of cytokine receptor signal transduction (see section 1.4) and it is now known that IL-4 stimulates activation and tyrosine phosphorylation of two members of this family, Jak1 [Yin *et al.*, 1994] and Jak3 [Witthuhn *et al.*, 1994], in lymphoid cells. Jak1 is phosphorylated in response to IL-4 and associates with both the IL-4Rα chain and IRS-2 [Pernis *et al.*, 1995a], whereas Jak3 associates with the γc subunit of the IL-4 receptor complex. Many cytokine receptors regulate gene expression by activation of a family of proteins known as Stats (Signal Transducers and Activators of Transcription) and these proteins are often tyrosine phosphorylated by members of the Janus family of kinases. IL-4 activation of a Stat transcription factor was first reported in murine T cells, where

Figure 1.3: IL-4 Induced Signal Transduction




X = 41 amino acid critical signal transduction domain (amino acids 433-472; numbering from carboxy terminus)

IL-4-activated DNA -binding activity was observed to have a strong affinity for an IFNγ-activation site (GAS) [Schindler *et al.*, 1994]. This activity was isolated and characterized in the Thp-1 T cell line, where extracts from IL-4 activated cells were found to have DNA-binding activity for an IL-4 response element in the promoter of the human FcγRI gene [Hou *et al.*, 1994]. This activity was subsequently purified and found to be a protein of ~100kDa and described as IL-4Stat protein, now designated Stat6 [Hou *et al.*, 1994]. Stat6 binding sites have since been identified in the promoter regions of a number of IL-4 inducible genes. IL-4 induces tyrosine phosphorylation of Stat6 via Jak1, an event which is followed by formation of Stat6 homodimeric complexes termed STF-IL-4 [Quelle *et al.*, 1995]. STF-IL-4 in turn translocates to the nucleus of the cell and binds to IL-4 responsive elements in the promoters of IL-4 inducible genes. Despite the reported association of Jak1 with IRS-2 upon IL-4 stimulation, the activation of Stat6 does not appear to rely on IRS-2 signalling pathways [Kotiandes *et al.*, 1995]. Thus, IL-4 seems to stimulate two major, independent signal transduction pathways via activation of Jak1 protein tyrosine kinase.

### 1.3.3 Critical Signalling Domains of the IL-4 Receptor

A major endeavour in recent years has been to characterize the IL-4Rα chain and define regions of the cytoplasmic domain which are critical for IL-4 induced signal transduction. One of the initial problems was the relatively low expression of IL-4 receptors on the majority of cell types, between 500-5000 receptors per cell. A subclone of the CTLL-2 T cell line, CTLL-19.4, was isolated by fluorescence activated cell sorting and was found to over-express IL-4 receptors on the cell surface (~$10^6$ receptors/cell) [Mosley *et al.*, 1989]. This discovery facilitated the partial purification and isolation of a full length cDNA clone of the receptor. The full length form of the receptor is a 140kDa protein with extracellular, transmembrane and intracellular domains and the full length receptor cDNA encodes a protein of ~ 810 amino acids [Mosley *et al.*, 1989]. IL-4 responsive

cells, as well as expressing a 140kDa IL-4 receptor protein, were also shown to express a 60-70kDa protein which associated with biotinylated IL-4 [Harada *et al.*, 1990]. More recent studies have identified this second binding protein as the γc subunit of the receptor. The extracellular domain of the 140kDa receptor chain is 220 amino acids in length and features two cysteine residues and a WSXWS motif, both of which are characteristics of the haematopoietin receptor superfamily [Harada *et al.*, 1990]. Initial studies of the cytoplasmic domain of the IL-4 receptor demonstrated no particular homology with other cytokine receptors. However, more recently, short consensus sequences termed PRM1 and 2 (proline rich motif 1 and 2), first identified as the gp130 box 1 in the gp130 component of the IL-6 receptor [Murakami *et al.*, 1991], have been located in the cytoplasmic domains of the majority of haematopoietin cytokine receptors [O'Neal and Yu-Lee, 1993].

A number of studies using transfected forms of the murine pro-B cell line BA/F3 have helped identify critical signalling regions of the human IL-4Rα chain. Transfection of wild type and a variety of deletion and truncation mutants of the human IL-4 receptor into this cell line, revealed that a 41 amino acid sequence located between amino acids 433 and 473 (numbering from the carboxy terminus) was critical for IL-4-induced growth signal transduction [Harada *et al.*, 1992]. This region is highly conserved between human and mouse but is non-homologous with other cytokine receptors. Other studies have revealed the importance of this membrane proximal region and also a membrane distal region, both of which have glutamate and serine rich sequences within them, in IL-4-induced proliferation of BA/F3 cells [Seldin and Leder, 1994]. In addition, it is possible to inhibit IL-4 induced proliferation of this cell line by the introduction into the cell of a synthetic peptide (SP1); a 41 amino acid peptide which corresponds to the 41 amino acid, critical growth signal transduction region of the IL-4 receptor [Izuhara *et al.*, 1995]. In contrast, SP1 was unable to inhibit IL-4-induced proliferation in the HT2 and CTLL-2 T cell lines or the erythroleukaemic cell line TF1, indicating that different regions of the IL-4Rα cytoplasmic domain may be responsible for growth signal

transduction in different lymphoid and myeloid cell types [Izuhara *et al.*, 1995]. The amino acid sequence of the IL-4Rα chain revealed that it has no intrinsic tyrosine kinase activity in its cytoplasmic domain, however, despite this it was shown to have two potential tyrosine phosphorylation sites and one PKC phosphorylation site [Harada *et al.*, 1990]. Scatchard analysis of IL-4 binding to various IL-4Rα chain truncation mutants demonstrated that the cytoplasmic domain is non-essential for forming high affinity binding sites in the extracellular domain. Therefore, association of the IL-4Rα chain and γc is likely to be mediated by the transmembrane and/or extracellular domains of IL-4Rα [Harada *et al.*, 1990]. In addition to growth signal transduction, regions responsible for other IL-4 induced signalling pathways and cellular events have also been identified. The 32D myeloid progenitor cell line expresses few IL-4 receptors and does not mount a mitogenic response to IL-4; however, over expression of IRS-1 cDNA in these cells (32D-IRS-1) results in a mitogenic response to IL-4. Truncation mutants of the human IL-4Rα chain, which lack amino acids 437-557 of their cytoplasmic domain, are unable to induce tyrosine phosphorylation of IRS-1 in these cells [Keegan *et al.*, 1994]. There is, within this region, a particular motif which is also found in the insulin receptor and the receptor for insulin like growth factor 1 (IGF-1). Site directed mutagenesis of a tyrosine at position 497 (Y497) in this motif of the IL-4Rα chain resulted in almost total abrogation of IL-4-induced phosphorylation of IRS-1 in 32D-IRS-1 cells [Keegan *et al.*, 1994]. This 437-557 amino acid region of the IL-4Rα chain is also a requirement for STF-IL-4 activation and, interestingly, Jak3 activation [Pernis *et al.*, 1995a]. A previous report has indicated that neither IL-4-induced IRS-2 nor Stat6 activation involves the association of these two molecules with one another [Kotiandes *et al.*, 1995]. Therefore, a possible interpretation is that either IRS-2 and Stat6 associate the 437-557aa region independently or the commonly associated Jak1 kinase associates with this region and in turn activates both IRS-2 and Stat6. Jak3 is found to be associated with the γc chain of a number of cytokine receptors including the IL-4 receptor complex. Therefore, it is possible that the association of IL-4Rα chain and γc in response to IL-4 requires this 437-557aa region and that deletion mutants are unable to mediate this association, with a

resultant failure to phosphorylate and activate Jak3. Recombinant human IL-4 is able to induce both proliferation and inhibition of apoptotic cell death in human IL-4Rα-transfected BA/F3 cells. Human IL-4Rα constructs with deletions of either the gp130 box1 or the acidic amino acid 333-365 region fail to stimulate IL-4 induced growth or inhibition of apoptosis [Deutsch *et al.*, 1995]. A point mutation in the gp130 box1 (P242S) results in loss of growth stimulation via the IL-4 receptor but does not affect the ability of IL-4 to inhibit apoptosis or to induce expression of c-*myc* RNA [Deutsch *et al.*, 1995]. Other IL-4-induced biological responses, e.g. isotype switching from IgM to IgG1 and IgE, can also be linked to specific regions of the IL-4Rα chain. An Abelson virus-transformed, murine pre-B cell line A20, transfected with the human IL-4Rα, induced production of germline Cε (IgE heavy chain) transcripts. In contrast, complete cytoplasmic domain truncation mutants and mutants lacking the previously described 41 amino acid, critical growth signal transduction region [Izuhara *et al.*, 1995] failed to induce germline Cε transcripts [Schultz *et al.*, 1995]. Therefore, it appears that the critical regions for IL-4 induced growth and differentiation signal transduction are one and the same.

## 1.4 Janus Kinase/Stat Signalling Pathways in Lymphocytes

In recent years the discovery of the *Janus* (or Jak) family of protein tyrosine kinases and their relationship to cytokine signal transduction, has provided great insights into one of the underlying mechanisms of control and regulation of development of lymphoid and myeloid cell lineages. In addition, the relationship between Jak kinases and their ability to activate Stat proteins has uncovered important links in cytokine regulated gene expression. It has been known for some time that many cytokines, upon binding to their receptors, have the capacity to induce tyrosine phosphorylation - both phosphorylation of intracellular proteins and also phosphorylation of the receptor components themselves. However, as none of these receptors have intrinsic tyrosine kinase activity within their cytoplasmic and transmembrane domains, it was assumed that association of

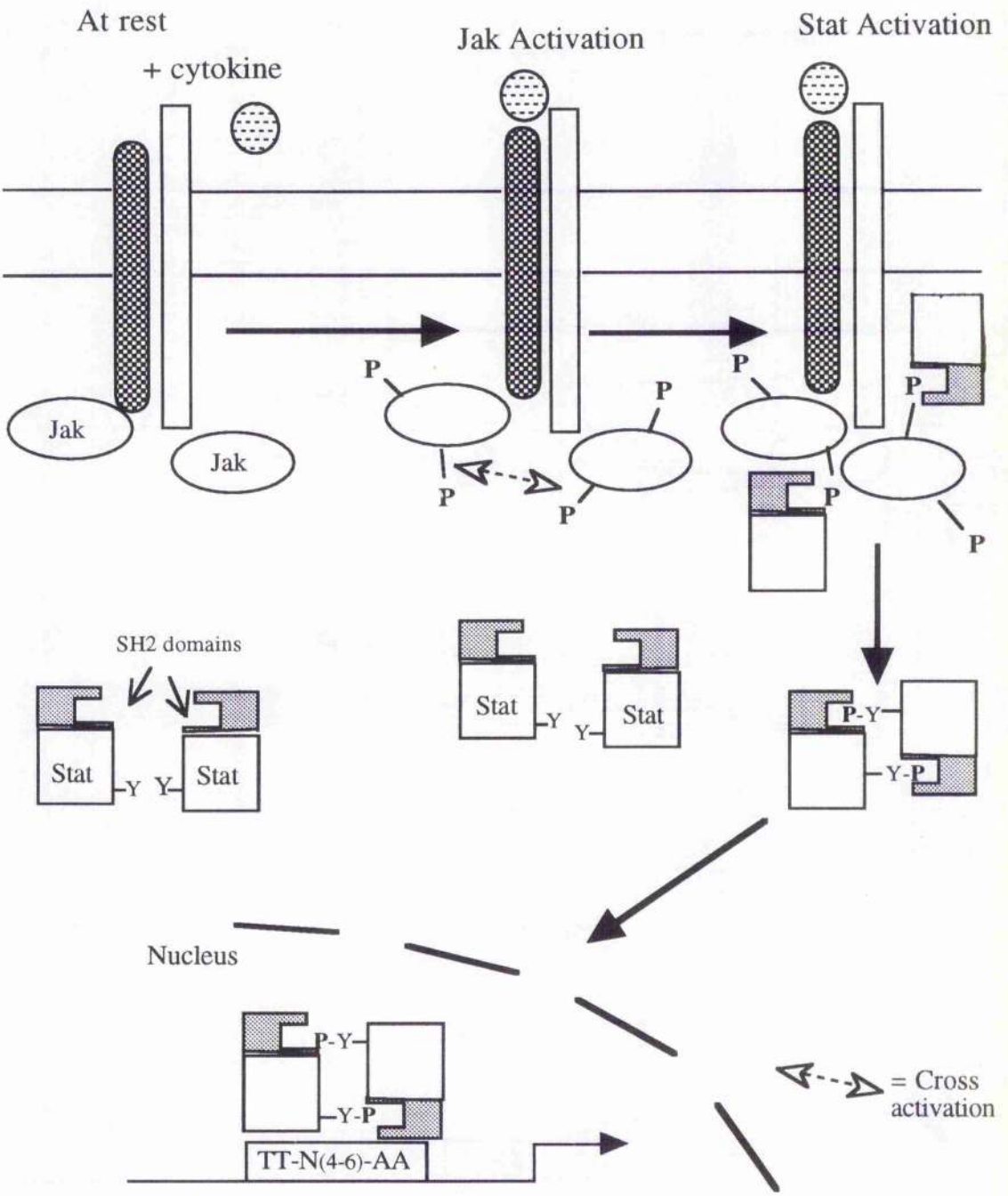
independent, intracellular protein tyrosine kinases with these receptors was the probable mechanism of activation. This hypothesis has been borne out by the discovery of the association with and activation of members of the *src* -proto-oncogene family by some cytokine receptors, e.g. IL-2 induces activation of $p56^{lck}$ [Hatakeyama *et al.*, 1991], and IL-4 and IL-9 both induce activation of IRS-2 [Wang *et al.*, 1993; Yin *et al.*, 1995]. Since the identification of Jak kinases it has been demonstrated that the vast majority of cytokines activate at least one, and usually two, members of this family. Jak kinases have a physical structure which distinguishes them from other groups of tyrosine kinases. Thus, they are characterized by the presence of two carboxyl terminal-kinase related domains and the absence of any SH2 or SH3 (*src* homology) domains, a feature common to the *src* family of tyrosine kinases and to certain plasma membrane receptors with intrinsic kinase activity in their cytoplasmic domains, e.g. the receptors for EGF and PDGF.

One of the major targets for activated Jak kinases are Stat proteins; a family of proteins with a carboxyl terminal SH2 domain which lies in close proximity to the target tyrosine residue for Jak kinase activity. Jak kinases activate Stat proteins by phosphorylation of specific tyrosine residues, whereupon monomeric Stat molecules form transcriptionally active homo- and heterodimers, via interactions between their SH2 domains and the newly phosphorylated tyrosines. The dimeric Stat complexes then translocate to the nucleus of the cell and bind specific DNA sequences within the promoter regions of specific cytokine activated genes and thereby regulate transcription.

### 1.4.1 The Janus family of Tyrosine Kinases

The Janus family consists of four members, Jak1, Jak2, Tyk2 and Jak3, which were originally identified by PCR and low stringency hybridization methods [Wilks, 1989]. Full length Jak1 and Jak2 cDNA clones were isolated using PCR amplification with probes characterizing their kinase catalytic domains [Wilks *et al.*, 1991]. Similar approaches were used to identify Tyk2 and, more recently, Jak3 [Cance *et al.*, 1993].

Figures 1.4: Mechanism of Jak-Stat Activation



Using kinase-specific antisera the association of various cytokine receptors with one or more members of the Jak kinase family have been defined (Table 1).

**Table 1.1 Cytokine Activation of Jak Tyrosine Kinases**

| Cytokine | Jak Kinase |
|---|---|
| IL-2, IL-4, IL-7, IL-9, IL-15 | Jak1, Jak3 |
| EPO | Jak2 |
| IL-3, GM-CSF, IL-5 | Jak1, Jak2 |
| IL-6, CNTF, LIF | Jak1, Jak2, Tyk2 |
| IFN$\alpha/\beta$ | Jak1, Tyk2 |
| IFN$\gamma$ | Jak1, Jak2 |

The importance of cytokine-induced association of multi-component receptors and concomitant association and aggregation of Jak molecules has been extensively studied. The accepted mechanism appears to be that in an inactive state, Jaks associate with the cytoplasmic domains of specific cytokine receptor components and ligand binding-induced receptor oligomerization brings different Jaks into close enough proximity to allow cross-phosphorylation at their autophosphorylation sites. The number of Jak molecules which are associated with each receptor is unknown, however, it has been hypothesized that the initial phosphorylation of one or two molecules allows the recruitment and subsequent activation of others thus, amplifying the signalling cascade. An excellent example of receptor association and co-operativity in Jak kinase activation is the IL-2 receptor. A number of independent studies have shown that IL-2 activates Jak1 and Jak3 in a variety of cell types, upon binding to its receptor [Beadling *et al.*, 1994; Johnston *et al.*, 1994; Miyazaki *et al.*, 1994; Tanaka *et al.*, 1994; Witthuhn *et al.*, 1994]. Use of chimeric receptors which have CD4 extracellular domains and both wild type and

mutant forms of the IL-2Rβ and IL-2Rγ intracellular domains, have demonstrated that Jak1 co-immunoprecipitates specifically with IL-2Rβ, and Jak3 with IL-2Rγ [Miyazaki *et al.*, 1994]. In addition, Jak1 appears to associate with the serine rich membrane proximal 'S' region of the cytoplasmic domain of IL-2Rβ and mutant receptors lacking this region are unable to activate not only Jak1 but also Jak3 [Miyazaki *et al.*, 1994]. This latter observation strongly suggests the requirement for receptor association in order for successful activation of a specific pattern of Jak kinases to occur. The ability of other cytokines which have γc as a receptor component (i.e., IL-4, IL-7, IL-9 and IL-15) to activate Jak3 [Johnston *et al.*, 1995; Witthuhn *et al.*, 1994; Yin *et al.*, 1995; Zeng *et al.*, 1994], demonstrates the extent of functional similarity between certain cytokine receptors. Indeed, the ability of almost all cytokines to activate at least one of the four Jak kinases, despite the fact that patterns of activation vary between cytokines, does beg the question of how a particular cytokine regulates the expression of a specific set of genes. One of the possible mechanisms underlying this specificity is the activation of specific patterns of Stat proteins.

### 1.4.2 Activation of Stat Proteins

The Stat family of proteins was initially identified in studies which dealt with interferon regulation of gene expression. IFNα/β activation of T cells was found to induce the formation of a DNA binding complex containing proteins of 91kDa, 84kDa, 113kDa and 48 kDa, a complex which is designated Interferon Stimulated Growth Factor-3γ (ISGF-3γ) [Fu, 1992a; Fu *et al.*, 1992b; Schindler *et al.*, 1992a]. The 91 and 84 kDa proteins have since been characterized as two splice variants of the same gene and have been designated Stat1α and Stat1β, respectively [Schindler *et al.*, 1992b]. p113 is Stat2 and p48 is a component which is specific to IFNα/β-induced DNA binding complexes. This oligomeric complex binds specific DNA elements in IFNα/β inducible genes. In contrast, IFNγ induces the formation of a Stat1 homodimer (STF-IFNγ) which interacts with IFNγ-activated sequences (GAS elements) in IFNγ-responsive genes [Shuai *et al.*, 1992; Shuai *et al.*, 1993]. There are now six identified and cloned members of the Stat

family and their activation by various cytokines is well characterized, though still not complete. Although the majority of Stat proteins are activated by more than one cytokine, the patterns of activation by particular cytokines seems to vary to a degree which, at least partially, accounts for the regulation of transcription of a specific set of genes. Thus, IL-2 and IL-4, both of which activate Jak1 and Jak3, differentially activate Stat proteins; IL-4 activates Stat6 and induces homodimerization to form the transcriptionally active STF-IL-4 [Hou *et al.*, 1994; Quelle *et al.*, 1995; Schindler *et al.*, 1994], whereas IL-2 activates Stat1, Stat3, Stat5 and two related Stat-like proteins, p94 and p95 [Frank *et al.*, 1995; Fuji *et al.*, 1995; Gaffen *et al.*, 1995; Johnston *et al.*, 1995]. IL-6 activates Stat1 and Stat3 and induces the formation of both Stat1 and Stat3 homodimers, as well as Stat1/3 heterodimers. IL-12, which also activates Jak1, is able to activate Stat4 [Bacon *et al.*, 1995] and IL-9 which, like IL-2, IL-4, IL-7 and IL-15 activates Jak1 and Jak3, induces activation of Stat3 [Yin *et al.*, 1995]. In addition, although Stat-binding DNA sequences have a high degree of homology with one another, the ability of Stat homo- and heterodimeric complexes to bind these elements is highly variable. Therefore, although almost all cytokines are capable of activating one of a limited number Jak kinases, their activation of Stat proteins appears to be more selective and it is possible that it is this latter observation, together with restriction in the DNA-binding capacity of specific Stat complexes to individual response elements, which defines the specificity of a particular cytokine response. Another factor which probably contributes to this specificity is the differential expression of Stats, depending on both the cell lineage (e.g. myeloid or lymphoid) and also the specific stage of development; e.g., Stat4 expression is restricted to early stages in myeloid development.

### 1.4.3 The Role of Jak/Stat Signalling Pathways in Haematopoietic Cell Development

The importance of the role of cytokines in developmental regulation of a number of cell lineages although being clearly in evidence, particularly in myeloid and lymphoid cells, is not entirely understood. Thus, the discovery that almost all cytokines activate signal

transduction pathways involving Jak kinases and Stat proteins suggested possible important implications for such pathways in cell development and mitogenesis. This remains an area where very little is understood, but is expanding rapidly, particularly with the production of a variety of gene knockout animals. Studies of IL-2-induced signal transduction in cells transfected with mutant IL-2Rβ, revealed that the serine rich 'S' region of the cytoplasmic domain, which is necessary for activation of Jak1 and Jak3, is also a requirement for mitogenesis [Witthuhn *et al.*, 1994]. In addition, over-expression of a Jak3-C terminal/PTK domain truncation mutant in F7 cells resulted in selective inhibition of IL-2 induced activation of Jak1 and Jak3 and inhibition of cell proliferation in response to IL-2 [Kawahara *et al.*, 1995]. Thus, it seems that Jak kinases do have a role in cell mitogenesis, e.g. Jak 1 and Jak3 are important in IL-2 induced proliferation of T cells. Over-expression of the Jak3 dominant negative mutant also inhibits IL-2 induction of expression of the proto-oncogenes c-*fos* and c-*myc*, but not of bcl-2 [Kawahara *et al.*, 1995]. In contrast, deletion of the C terminal region of IL-2Rβ, which is essential for IL-2 induced activation of Stat5, inhibits neither Jak1 and Jak3 activation, nor cell proliferation in response to IL-2 [Fuji *et al.*, 1995]. Similarly, IL-4 induced activation of Stat6 has been demonstrated to require the membrane distal region of the IL-4Rα chain [Quelle *et al.*, 1995], a region which is distinct from the acidic membrane proximal region reported to be critical for IL-4 induced cell growth and differentiation [Harada *et al.*, 1990; Harada *et al.*, 1992; Schultz *et al.*, 1995]. Therefore, whereas Jak kinases appear to have some role in cytokine induced proliferative responses, Stat proteins are not required for mitogenesis, despite their obviously important functions with respect to regulation of transcription. This is a hypothesis which so far seems to be supported by Jak/Stat gene knockout studies.

One usage of a Jak/Stat pathway is particularly interesting and demonstrates how selectivity of signal transduction pathways can profoundly influence cellular development and differentiation. IL-4 and IFNγ differentially regulate proliferation and differentiation of helper T cell (Th) clones; IFNγ inhibits the generation of Th2 but not Th1 clones

[Gajewski and Fitch, 1988]. IFNγ activates tyrosine phosphorylation of Jak 1 and Jak2, which in turn results in the phosphorylation of Stat1 and formation of Stat1 homodimers (STF-IFNγ) [Muller *et al.*, 1995; Shuai *et al.*, 1992; Watling *et al.*, 1993]. Stimulation of Th2 and Th1 clones with IFNγ results in activation of Jak1 and Jak2, phosphorylation of Stat1 and resultant STF-IFNγ formation and induction of IRF-1 mRNA transcripts in Th2 but not Th1 clones [Pernis *et al.*, 1995b]. Th1 clones produce IFNγ, however, addition of anti-IFNγ blocking antibodies to cell culture prior to assay of IFNγ activation of STF-IFNγ did not affect the response, ruling out the possibility that prolonged exposure to IFNγ results in down-regulation of the receptor. In addition, all the intracellular components of the pathway were both present and functional in both Th2 and Th1 clones [Pernis *et al.*, 1995b]. In fact, Th1 clones temper their responses to IFNγ by preventing the expression of the IFNγ-Rβ component of the receptor, thereby preventing activation of STF-IFNγ and transcription of a specific set of genes. Thus, selective use of a particular Jak/Stat pathway contributes to selective differentiation of T cells.

Recent findings have suggested there may be a degree of adaptability of cytokine receptors with respect to the signalling mechanisms they use. Expression of Jak3 is restricted to cell lineages which are lymphoid in origin, whereas the other Jak kinases are ubiquitously expressed. There is now evidence to suggest that IL-4 induces the tyrosine phosphorylation and activation of both Jak1, Jak2 and Tyk2 in epithelial cancer cells [Murata *et al.*, 1995]. Neither Jak3 nor the IL-2 receptor γc chain is expressed in these cells, however, a 70kDa protein which was distinct from γc was found to associate with the 140kDa IL-4Rα chain [Murata *et al.*, 1995]. Such observations indicate that cytokines may adapt their signal transduction pathways in different cell lineages depending on the available receptor components and associated signalling machinery.

## 1.5 Structural and Functional Similarities Between IL-4 and IL-13

IL-13 was first identified as a human cDNA homologue of a murine cDNA sequence (P600) in a human T cell cDNA library. The expressed proteins of both human and murine sequences were found to have a variety of immunoregulatory properties and were thus designated IL-13 [McKenzie *et al.*, 1993]. IL-13, synthesised and secreted by T cells, is an unglycosylated protein of ~10 kDa which is very closely related to IL-4. The genes for IL-4 and IL-13 are both located on chromosome 5 and the expressed proteins have approximately 30% sequence homology with one another and have a predominantly α-helical structure, as demonstrated by circular dichroism spectroscopy. In addition to sharing structural similarities, IL-4 and IL-13 exert many of the same functional effects upon target cells. Human IL-4 and IL-13 both elicit morphological and cell surface changes in human monocytes and promote the proliferation of B lymphocytes which have been pre-activated with anti-IgM antibodies or anti-CD40 (the latter presented to the B cells by a transfected murine Ltk$^-$ cell line) [McKenzie *et al.*, 1993]. IL-13 also stimulates human B cells to induce isotype switching and IgE production, up-regulation of MHC Class II and up-regulation of CD23 [Aversa *et al.*, 1993; Punnonen *et al.*, 1993]. As well as regulating monocyte and B cell activity, IL-13 and IL-4 also act on human mast cells in a functionally similar manner. Thus, both of these cytokines induce c-*fos* expression, decrease proliferation, increase expression of ICAM-1 and decrease expression of the Stem Cell Factor (SCF) receptor kit, on the HMC-1 cell line [Nilsson and Nilsson, 1995]. Interestingly in the case of the latter three responses, IL-4 is substantially more potent ($EC_{50}$ - 5ng/ml) than IL-13 ($EC_{50}$ - 100ng/ml), indicating the possibility of a degree of differential signalling between the two cytokines [Nilsson and Nilsson, 1995]. The major role of IL-4, not shared by IL-13, is activation of human T cells and in addition, IL-4, but not IL-13, is able to inhibit mononuclear cell/SCF induced differentiation of tryptase-positive mast cells [Nilsson and Nilsson, 1995].

The first evidence that IL-4 and IL-13 shared a receptor component was provided by the discovery that IL-13 competitively inhibits binding of IL-4 to IL-4 receptors on an IL-13 and IL-4 responsive myeloid cell line, TF-1 [Zurawski *et al.*, 1993]. In addition, investigation of tyrosine phosphorylation patterns of IL-4 and IL-13 demonstrated that both induce phosphorylation of the 140kDa subunit of the IL-4 receptor, IL-4Rα [Smerz-Bertling and Duschl, 1995]. The γc subunit of the IL-2 receptor was proposed as a candidate for an IL-4 and IL-13 common receptor subunit [Kishomoto *et al.*, 1994] (γc has been defined as the common receptor subunit of the lymphoid cytokine receptor subfamily and is a component of the IL-2, IL-4, IL-7, IL-9 and IL-15 receptors). However, there is little evidence in favour of γc being a component of the IL-13 receptor complex. Binding of hIL-4 to its receptor on a cell line which does not respond to IL-13, and also to a cloned IL-4 receptor expressed in heterologous cells, was not inhibited by IL-13 [Zurawski *et al.*, 1993]. In addition, there was a 100-fold difference in binding of hIL-4 to these two different cell types suggesting firstly, a form of the IL-4 receptor which was unable to bind IL-13 and secondly, that these functional IL-4 receptors contain a subunit which dramatically affects IL-4 binding affinity. IL-2 and IL-7, unlike IL-4 and IL-13, are unable to phosphorylate the 140kDa subunit of the IL-4 receptor [Smerz-Bertling and Duschl, 1995]; therefore, it is unlikely to be γc, a component of both IL-2 and IL-7 receptors, which mediates IL-4 induced phosphorylation of the IL-4Rα chain. Examination of IL-4- and IL-13-induced signal transduction demonstrates that while both induce phosphorylation of IRS-2 and Jak1 in myeloid TF-1 cells and monocyte U937 cells, only IL-4 induces phosphorylation of Jak3; a tyrosine kinase known to be associated with γc in IL-2, IL-4 and IL-7 receptor signalling [Keegan *et al.*, 1995; Schnyder *et al.*, 1996a; Wang *et al.*, 1995; Welham *et al.*, 1995]. X-SCID is useful as a model for naturally occurring mutant γc proteins. B lymphocytes from X-SCID patients do not proliferate in response to IL-2 and IL-15 but do proliferate and secrete IgE in response to IL-4 and IL-13 [Matthews *et al.*, 1995], suggesting that these IL-4-induced responses do not rely on γc and that the receptor for IL-13 probably lacks γc altogether. These differences in IL-4 and IL-2/IL-15 responses in X-SCID patients

may be partially explained by the finding that monoclonal antibodies to two distinct γc epitopes indicate that the regions which bind IL-7Rα and IL-2Rβ overlap, while the region of γc which binds IL-4Rα is distinct [He *et al.*, 1995b]. Thus, IL-13 signal transduction would seem to involve tyrosine phosphorylation of the IL-4Rα chain and IRS-2, but not Jak3. More recent studies have backed up such observations and also revealed that IL-13, like IL-4, is able to induce the association of phosphorylated IRS-2 with the 85kDa subunit of PI3-kinase [Wang *et al.*, 1995; Welham *et al.*, 1995]. In addition, there is also conflicting data concerning the ability of IL-13 to induce association of IRS-2 with Grb-2 [Wang *et al.*, 1995; Welham *et al.*, 1995] and more conclusive evidence against IL-13 being able to activate erk-1 and erk-2 and mediate IRS-2-Shc association [Welham *et al.*, 1995].

There is now plentiful evidence in favour of IL-4Rα, as opposed to γc, being the receptor component shared by IL-4 and IL-13 receptors and it seems probable that signalling pathways activated via IL-4Rα, upon IL-4 stimulation, are also integral in IL-13 induced signal transduction. However, more recent studies point towards the presence of a third, novel receptor component that may be part of both IL-4 and IL-13 receptor complexes. IL-4 affinity cross-linking studies in colorectal carcinoma cells indicate the presence of a novel 65kDa component [Schnyder *et al.*, 1996a], as well as the expected IL-4Rα chain and the γc subunits, in the IL-4 receptor complex and in epithelial cancer cells the IL-4 receptor appears to consist of the IL-4Rα chain and a novel 70kDa receptor component [Murata *et al*, 1995]. The novel 65kda subunit is also able to bind $^{125}$I-IL-13 and in epithelial cancer cells [Schnyder *et al.*, 1996a] and IL-13 inhibits the binding of $^{125}$I-IL-4 to both 140- and 70kDa receptor components [Murata *et al.*, 1995]. Further radiolabelled-IL-4 cross-linking studies, followed by immunoprecipitation with an anti-IL-4Rα monoclonal antibody (S697), showed IL-4 specific binding to IL-4Rα and to a lesser extent to a 65kDa band in endothelial cells [Schnyder *et al.*, 1996b]. In contrast, IL-13 bound this 65kDa subunit strongly, but no cross-linked receptor was precipitated by S697 and although IL-13 competed for binding

of the 65kDa subunit with IL-4, it could not completely displace IL-4 from the IL-4Rα chain [Schnyder *et al.*, 1996b]. Interestingly, in these endothelial cells anti-γc monoclonal antibodies failed to precipitate cross-linked IL-4 receptors, indicating that in these cells γc is not a functional component of the IL-4 receptor. More recently, cloning and characterization of a murine protein (NR4) has revealed it to be the alpha subunit for the IL-13 receptor, which induces a proliferative response to IL-13 when it is expressed in CTLL-2 cells and leads to cross-reactivity in the binding of IL-4 and IL-13 in these cells [Hilton *et al.*, 1996]. Together, these studies suggest the presence of a receptor component which, as well as being part of the IL-13 receptor, may also form weaker associations with the IL-4 receptor complex. Interestingly, these data suggest that the components which constitute the IL-4 receptor may vary depending upon cell lineage; e.g., in lymphoid and myeloid cells the IL-4 receptor almost certainly contains the γc subunit of the IL-2 receptor, whereas in endothelial cells it appears it does not. Such observations may explain the apparent ability of IL-4 to activate different members of the Jak kinase family [Murata *et al.*, 1995] as mentioned in the previous section, a phenomenon which also appears to be dependent upon cell lineage. However, there is also evidence that IL-4 induces low levels of tyrosine phosphorylation of Tyk2, as well as activation of Jak1 and Jak3, in lymphoid cells [Welham *et al.*, 1995] and that IL-13 induces tyrosine phosphorylation of Tyk2 and Jak1 in these cells. It may be, therefore, that the pattern of Jak kinases activated by IL-4 is dependent on the components which constitute the receptor to which it is bound and that the expression of different IL-4 receptors is not necessarily restricted to distinct cell lineages; i.e., there is a possibility that two different, functionally active receptor complexes may be expressed on a single cell type.

## 1.6 CD40 Regulation of B Cell Development

As mentioned earlier, B cell activation and development is regulated by T cell-B cell surface interactions, events which are mediated by cell surface adhesion molecules acting

in concert with stimulation through the B cell antigen receptor. One such interaction between adhesion molecules is the binding of the B cell antigen CD40 to its ligand, which is expressed on the surface of activated T cells. This interaction appears to participate in all T cell-dependent immune responses throughout the latter stages of B cell development.

### 1.6.1 CD40: Structure and Function

CD40, a 277 amino acid, type I glycoprotein with two N-glycosylation sites [Paulie *et al.*, 1989], is a member of a family of cell surface glycoproteins which includes the low affinity nerve growth factor receptor, two tumour necrosis factor α (TNFα) receptors, *fas*, CD27 and CD30 and has a high degree of homology to these proteins [Stamekovic *et al.*, 1989]. CD40 expression is observed fairly early in B cell development, following CD19 and CD10 expression and preceding the expression of CD21, CD22, CD24 and membrane IgM. As well as being expressed on B lymphocytes, CD40 is also found in monocytes, follicular dendritic cells, thymic epithelial cells and certain carcinoma cells [Clark *et al.*, 1989; Paulie *et al.*, 1989]. CD40 expression varies between different stages of B cell developement, e.g. lower levels are observed on peripheral blood B cells than on tonsillar B cells [Ledbetter *et al.*, 1987]. Stimuli which regulate expression of CD40 include anti-μ antibodies and IL-4, but not IL-2, although IL-2 can induce CD40 expression when administered as a co-stimulus with IL-4 [Ledbetter *et al.*, 1987; Valle *et al.*, 1989]. Activation via CD40 on the B cell surface has been studied using both the ligand for CD40 and anti-CD40 monoclonal antibodies.

Stimulation of resting, tonsillar B cells or B cell lines with anti-CD40 does not induce B cell proliferation, but promotes enlargement of resting tonsillar B cells, a feature which is indicative of partial activation. When used as a co-stimulant with either anti-μ antibodies or IL-4, anti-CD40 promotes proliferation of B lymphocytes [Ledbetter *et al.*, 1987; Paulie *et al.*, 1989]. This co-stimulant activity of anti-CD40 is unique to these two situations, as anti-CD40 does not stimulate proliferation when applied together with

either IL-1 or IL-2. Early studies on anti-CD40-stimulated signal transduction demonstrated that it did not induce an increase in c-*myc* expression or intracellular $Ca^{2+}$ levels, indicating that synergy with B cell receptor signalling does not rely on either of these pathways [Clark *et al.*, 1989]. B-CLL cells have a phenotype of mature, partially activated B cells and culture of these cells with IL-4 and anti-CD40 results in proliferation [Crawford and Catovsky, 1993], suggesting that while either of these two stimuli alone are insufficient to promote DNA synthesis and cell division, they are involved in B cell activation. Similarly, treatment of B cells with *E. coli* lipopeptide enhances proliferative responses to IL-4 and CD40 [Edinger *et al.*, 1994]. However, it should be noted that in both of these cases, the presence of feeder cells expressing FcγRII molecules were necessary to implement this proliferative response, indicating that cross-linking of anti-CD40 may be a requirement for proliferation. In addition to promoting a proliferative response, anti-CD40 co-stimulates B cells to induce isotype switching [Splawski *et al.*, 1993]; co-stimulation with IL-4 induces IgE secretion, whereas co-stimulation with IL-10 induces IgG and IgA secretion. Anti-CD40-induced isotype switching is enhanced further by treatment with the polyclonal activator, *Staphylococcus aureus* Cowan I (SAC). Indeed SA/anti-CD40/IL-4 induced isotype switching demonstrates a distinct distribution of Ig heavy chain expression i.e., IgE , as compared with SA/IL-2-induced switching, which promotes $IgG_4$ and IgA production [Splawski *et al.*, 1993]. Co-stimulation of B cells with anti-CD40 and IL-2 does not induce Ig production, indicating that anti-CD40 will only synergize with specific activation signals. Due to the ability of anti-CD40 to induce proliferation and isotype switching when used as a co-stimulant, CD40 and interaction with its ligand is now considered to be the major mediator of cell contact-dependent activation signals delivered to B cells during T cell-B cell interaction.

### 1.6.2 CD40 Ligand: Structure and Function

The ligand for murine CD40 (CD40L) was cloned and found to be a 260 amino acid, type II transmembrane glycoprotein, related in structure to TNFα [Armitage *et al.*, 1992]; the 33kDa human CD40L has very similar structural characteristics. CD40L is expressed

only on activated T cells and appears shortly after activation of both Th1 and Th2 clones and is therefore not exclusive to a particular helper T cell subpopulation. CV1/EBNA cells transformed with murine CD40L, induce proliferation of both murine and human B lymphocytes in the absence of any other co-stimulation [Armitage *et al.*, 1992]. This latter finding is consistent with the observation that cell association supports anti-CD40 stimulation of proliferation and suggests that the degree of cross-linking of CD40 is a major factor in implementing B cell activation. As well as proliferation, CV1/EBNA cells in combination with IL-4, also induce B lymphocytes to secrete IgE [Armitage *et al.*, 1992]. The difference in co-factors required for anti-CD40 induced proliferation and isotype switching emphasizes the potential difference in intracellular signals required for both of these cellular effects. Pre-activated T cells induce B cells to grow and secrete Ig in response to IL-2 [Blanchard *et al.*, 1994]. This is in direct contrast with the effects of anti-CD40/IL-2 co-stimulation and indicates that the CD40-CD40L interaction may play a role in IL-2 mediated B cell responses. Antibodies to CD40L and soluble CD40 are both able to block T cell-dependent B cell activation [Nishioka and Lipsky, 1994]. However, CD40L deficient T cell clones were found to be still capable of inducing initial B cell activation and Ig secretion [Nishioka and Lipsky, 1994], suggesting that T cell-B cell interactions other than CD40-CD40L are involved in regulation of B cell development. There appears to be an optimal balance of CD40-CD40L binding for induction of B cell activation: 40-100% binding of CD40 results in aggregation and apoptosis of B cells, whereas intermediate CD40 binding promotes B cell proliferation and Ig secretion [Bergman *et al.*, 1996]. Untreated B cells do not respond to $CD40^{Low}$ T cells unless accompanied by co-stimulation with anti-Ig [Poudrier and Owens, 1994], indicating that in a physiological situation, activation via the B cell antigen receptor is necessary to synergize with CD40-CD40L binding. The importance of CD40-CD40L interactions, with respect to isotype switching, is demonstrated in X-linked hyper IgM syndrome (HIgM-XL), a condition characterised by the excessive production of IgM but almost total absence of IgG, IgA and IgE secretion. Recently the HIgM-XL locus and the gene for CD40L have been localized to the long arm of the X chromosome at Xq26.3-27.2,

indicating that mutations in the gene for CD40L are responsible for development of this syndrome [Graf *et al.*, 1992].

### 1.6.3 Signal Transduction via CD40

CD40 is a transmembrane molecule with a short cytoplasmic tail of 65 amino acid residues. Patterns of protein phosphorylation demonstrate that anti-CD40 induces the dephosphorylation of proteins in a 50-110 kDa range and transient phosphorylation of a 28 kDa protein, in human B cell lines [Faris *et al.*, 1994]. In resting tonsillar B cells phosphorylation of this 28 kDa protein is more sustained [Faris *et al.*, 1994]. This pattern of phosphorylation is distinct from either anti-IgM or IL-4 stimulated phosphorylation and can be inhibited by both protein tyrosine phosphatase and protein tyrosine kinase inhibitors. Thus, at least one CD40 signalling pathway involves an array of tyrosine kinase and phosphatase activation. However, the cytoplasmic domain of CD40 contains no intrinsic tyrosine kinase or phosphatase activity [Paulie *et al.*, 1989] and it is likely, therefore that association with independent, cytoplasmic effector molecules is involved in CD40-mediated signal transduction. Mutagenesis studies suggest that threonine$^{234}$ in the cytoplasmic domain of CD40 is essential for activation of intracellular signalling pathways [Hu *et al.*, 1994]. In addition to protein phosphorylation, a $Ca^{2+}$ dependent signalling pathway has been implicated in CD40 activation of B cells, a result which is in conflict with previous studies [Clark *et al.*, 1989]. Anti-CD40 and IL-4 co-stimulation of murine B cell proliferation is abrogated by FK506 (an inhibitor of $Ca^{2+}$ dependent signalling pathways in T cells), as is anti-IgM stimulated proliferation [Klaus *et al.*, 1994]. However, the former stimulus becomes drug resistant following 24 hours whereas anti-IgM stimulation takes longer to acquire this resistance. Ligation of either CD40 or mIgM in the presence of IL-4 results in activation of the transcription factor NF-AT, via a FK506 sensitive pathway, indicating that CD40, anti-IgM and IL-4 activation of B cells may all occur through a $Ca^{2+}$ sensitive- NF-AT activating pathway, but that this pathway may only be functional in the early stages of B cell activation [Klaus *et al.*, 1994]. In addition to induction of NF-AT,

anti-CD40 also induces activation of NF-κB in resting tonsillar B cells [Berberich *et al.*, 1994]. Activation is rapid and occurs via a tyrosine kinase dependent pathway. DNA mobility shift assay allowed the isolation of a NF-κB binding complex which contains p50, p65 (Rel-A) and c-*rel* gene product proteins. Transient transfection assays in the Daudi B cell line demonstrated that cross-linking of CD40 promotes NF-κB-dependent gene expression [Berberich *et al.*, 1994]. In addition to this, a CD40 binding protein was cloned using the yeast-two hybrid system. This protein contains an N-terminal RING finger motif found in a number of DNA binding proteins (including the VDJ recombination activating gene product - RAG1) and a central coiled-coil segment which may promote homo- and hetero-oligomerization, suggesting that one mechanism of CD40 signalling may involve the direct activation of a DNA binding factor [Hu *et al.*, 1994].

## 1.7 Adenylyl Cyclase and the Generation of Cyclic AMP

Cyclic Adenosine- 3', 5'-monophosphate (cyclic AMP or cAMP) is a ubiquitous regulatory molecule in both prokaryotic and eukaryotic cells. In the latter it functions principally as a second messenger in signal transduction pathways, which ultimately result in regulation of gene expression. In mammalian systems cAMP generation is initiated by a vast range of stimuli in the majority of cell types and it is likely that it is downstream signalling events, particularly regulation of gene expression, which define the specificity of the stimulus which initiates this early signalling cascade. Intracellular levels of cAMP are controlled by a process of dual regulation; thus, cAMP is generated from cytosolic ATP by the enzyme adenylyl cyclase and degraded to 5'AMP by phosphodiesterase enzymes. Phosphodiesterases are required to define both the length and potency of a cAMP response and do so by degrading the second messenger at the correct time. In addition, the activity of phosphodiesterase enzymes is now known to play a very important regulatory role in a variety of signalling cascades by suppressing

levels of cAMP [Reviewed by Nicholson *et al.*, 1991]. However, it is the initial and often extensive generation of cAMP by adenylyl cyclase, which defines the importance of the second messenger as a signalling molecule.

Initial studies revealed that activation of adenylyl cyclase was hormone-sensitive and could be activated by a range of molecules including β-adrenergic amines, proteins, polypeptides and some prostaglandins [Chiba *et al.*, 1989; Johnson *et al.*, 1978; Mosishita *et al.*, 1990]. Following this, it was also noted that adenylyl cyclase was subject to inhibitory control by opiates, α-adrenergic amines, adenosine and acetyl choline [Reviewed by Combest *et al.*, 1988; Ross, 1980]. These hormones act upon a particular cell type by binding to membrane receptors which are linked by membrane-associated G protein molecules, to adenylyl cyclase [Reviewed by Neer, 1994; Segre, 1993]. The classical nature of G-protein linked receptors is a protein structure bearing seven hydrophobic transmembrane domains with an extracellular amino terminal domain, an intracellular carboxyl terminal domain and a large, third intracellular loop. The structure and function of G proteins is now well characterized and initial identification of a Gs (stimulatory) G protein revealed a structure of three distinct subunits, α, β and γ [Simon *et al.*, 1991]. In the inactive state, GDP is bound to the α subunit of Gs and is exchanged for GTP upon activation by a G protein-linked receptor, with resulting dissociation of the α subunit from βγ. GTP-bound Gsα then activates adenylyl cyclase. Hormone receptors which inhibit adenylate cyclase are also linked by an inhibitory G protein (Gi) and in this instance it is the Giα subunit which inhibits adenylate cyclase. Since the initial discovery of G proteins, a large and complex family has been identified and characterized, which demonstrates vast evolutionary diversity. Gs and Gi are now designated subfamilies of G proteins and in addition to these, two other sub-families, Gq and G12, have been identified [Reviewed by Simon *et al.*, 1991; Spiegel, 1992]. Each of these families has a number of distinct G proteins within them and, to complicate matters further, different isoforms of the α, β and γ subunits are being identified within these groupings [Strathmann and Simon, 1990; Strathmann and Simon, 1991;

Strathmann *et al.*, 1989; Tsukamoto *et al.*, 1991]. Thus, regulation of activation of adenylyl cyclase is dependent upon the particular hormone receptor which is bound by its ligand and also the specific G protein which is involved. Molecular cloning and characterization of adenylyl cyclase has been undertaken relatively recently and has revealed that there are a number of distinct isoforms of the enzyme. In addition, it has been demonstrated that regulation of adenylate cyclase activity is not confined to the alpha subunits of G proteins, as different isoforms are also regulated by the βγ G protein subunits, $Ca^{2+}$/Calmodulin and certain protein kinases.

### 1.7.1 Adenylyl Cyclase Isoforms

There are now eight recognized, distinct isoforms of adenylyl cyclase; full length cDNA sequences for types I-VIII have been cloned, sequenced and characterized. [Cali *et al.*, 1994; Defer *et al.*, 1994; Hellcuvo *et al.*, 1995; Katsushika *et al.*, 1992; Krupinski *et al.*, 1989; Watson *et al.*, 1994; Yoshimura and Cooper, 1992]. The first mammalian adenylyl cyclase isoform to be cloned was type I, a $Ca^{2+}$/Calmodulin sensitive form cloned from bovine brain [Krupinski *et al.*, 1989]. The structure, which has been predicted from all the available sequences, is a membrane protein with twelve transmembrane domains (two sets of six hydrophobic regions separated by a large central cytoplasmic loop) and intracellular N- and C-termini, the latter occurring at the end of a long cytoplasmic tail [Krupinski *et al.*, 1989]. The central cytoplasmic loop and N- and C-termini are highly conserved between the different isoforms of adenylyl cyclase and it is thought that these regions of high homology form part of the catalytic site. Indeed, using a baculovirus expression system in Sf9 cells, it has been demonstrated that there is no adenylyl cyclase activity upon expression of either of the two six transmembrane-spanning region domains alone. However, co-expression of both domains results in considerable enzyme actvity, suggesting that a physical interaction between the two is required for catalytic activity of adenylyl cyclase [Tang *et al.*, 1991a]. The tissue distribution of adenylyl cyclase is variable depending on the isoform: some are highly specific, such as type I, which seems to be confined to neuronal tissues; others like types

II, IV, V and VI are widely distributed throughout neuronal and non-neuronal tissues. While all isoforms are expressed to some degree in the brain, there is regional distribution of types I-II, V and VIII.

### 1.7.2 Regulation of Adenylyl Cyclase Activity

*(i) G - protein Regulation*

All mammalian isoforms of adenylyl cyclase cloned so far are stimulated by Gsα and forskolin, whereas Giα inhibits only types II, III and VI. Type I is inhibited by the βγ subunits of G-proteins [Katada *et al.*, 1987; Tang *et al.*, 1991a]. In contrast, types II and IV are stimulated by βγ in the presence of activated Gsα [Tang and Gilman, 1991b], whereas types III, V and VI are unaffected by βγ. There is evidence that adenylyl cyclase isoforms are capable of integrating a number of G protein-related signals at one time. In HEK cells transiently transfected with the Type II isoform, adenylyl cyclase activation by a Gs agonist is augmented by Gi- and Gq-activating agonists, via the Gi βγ subunits and PKC, respectively [Lustig *et al.*, 1993]. Down-regulation of PKC activity results in prevention of stimulation of Type II adenylyl cyclase by both PMA and Gq-linked receptor agonists [Lustig *et al.*, 1993].

*(ii) $Ca^{2+}$ Regulation*

Types I, III and VIII are activated by $Ca^{2+}$/Calmodulin; for type I this activation occurs independently of Gsα, whereas $Ca^{2+}$/Calmodulin activation of type III is inhibited by Gsα and activation of type VIII is synergistically increased in response to $Ca^{2+}$/Calmodulin and Gsα [Cali *et al.*, 1994; Choi *et al.*, 1992; Cooper *et al.*, 1988; Krupinski *et al.*, 1989; Tang *et al.*, 1991a]. Types V and VI are inhibited by low (micromolar) concentrations of $Ca^{2+}$ [Debernardi *et al.*, 1993; Yoshimura and Cooper, 1992]. Type V and VI have a high degree of sequence homology, even in the transmembrane regions, where generally the greatest degree of divergence between isoforms exisits. As such, types V and VI have been classified as a distinct adenylyl cyclase sub-group [Katsushika *et al.*, 1992]. Isoforms II, IV and VII are independent of any $Ca^{2+}$ mediated regulation [Watson *et al.*, 1994].

*(iii) Protein Kinase regulation*

It has been demonstrated that type II adenylate cyclase is the major target for PKC, as it can be activated by both PMA and $PBu_2$ but not the inactive PMA isomer, 4αPMA [Yoshimura and Cooper, 1993]. Additionally, these effects are inhibited by the PKC inhibitor staurosporine [Yoshimura and Cooper, 1993]. This same study also observed that PKC was not involved in the activation of types I or III adenylyl cyclase. However, other reports have demonstrated weaker regulatory effects of PKC activators on types I and III. Thus, pre-treatment with PMA augments $Ca^{2+}$/Calmodulin activation [Jacobwitz *et al.*, 1993] and forskolin activation [Choi *et al.*, 1992] of types I and III, respectively, in transiently transfected kidney 293 cells. PKC activation of type II does not require Gsα and produces nearly a 10-fold increase in intracellular cAMP levels in certain intact cells. Interestingly, PKC treatment abolishes Giα-induced inhibition of the type II enzyme but does not affect Giα inhibition of types III and VI. Recently, both rat and human forms of type VII have been shown to be activated in response to phorbol ester treatment [Helleuvo *et al.*, 1995; Watson *et al.*, 1994], indicating possible regulation by PKC. Pre-treatment with pertussis toxin (an inhibitor of Gi) does not affect this sensitivity to phorbol ester, suggesting that this activity is independent of modification by Gi [Watson *et al.*, 1994]. cAMP-dependent decreases in adenylyl cyclase activity have been observed in hepatocytes and S49 $kin^-$ lymphoma cells. Treatment with 8-bromo-cAMP results in a decrease in fluoride activation of adenylyl cyclase in chicken hepatocytes and addition of the catalitic subunit of PKA to S49 $kin^-$ lymphoma membranes, inhibits both fluoride and forskolin-stimulated adenylyl cyclase activity [Premont *et al.*, 1992]. Type VI adenylyl cyclase is common to both of these cell types and it has been suggested that PKA initiates a negative feedback loop to regulate activity of this isoform

The implications of this diversity in regulation of adenylyl cyclase isoforms is, that any individual type may be able to integrate a variety of signals in order to give a very measured and specific response. In addition, recent studies have revealed that there are

three splice variants of type VIII (VIII-A, VIII-B and VIII-C). VIII-A is the initially characterized type VIII, whereas, VIII-B and VIII-C differ by deletions of 90 and 198 base pair exons encoding a 30 amino acid extracellular domain with two N-glycosylation sites and a 66 amino acid cytoplasmic domain, respectively. VIII-A and VIII-C are modified by N-glycosylation and are sensitive to N-glycosidase, whereas VIII-B which lacks the relevant extracellular domain is not [Cali *et al.*, 1996]. Thus, it appears that differential regulation of splice variants of single isoforms of adenylyl cyclase may exist, hinting at additional regulatory controls.

### 1.7.3 cAMP Regulated Gene Expression

In eukaryotic cells the major target for cAMP action is a serine/threonine-specific cAMP-dependent protein kinase, protein kinase A (PKA). The inactive holoenzyme of PKA is composed of two regulatory (R) subunits and two catalytic subunits (C), $R_2C_2$. The enzyme is activated upon cAMP binding to the dimeric $R_2$ regulatory subunit, an event which induces the release and activation of the two catalytic subunits; thus, cAMP acts as an allosteric effector of PKA. The mechanism of cAMP activation of gene expression in prokaryotic cells has been well understood for a number of years, but it is only in the last ten years that the mechanism behind cAMP-dependent regulation of gene transcription in eukaryotes has come to light [Reviewed by Nigg, 1990]. There are two proposed mechanisms of regulation of cAMP-inducible genes by PKA in eukaryotic cells. Firstly, that the active catalytic subunit acts upon transcription factors which bind elements in the promoter sequence of cAMP-inducible genes, either by translocating to the nucleus and phosphorylating a transcription factor directly or by acting on an intermediate nuclear factor, or alternatively, by remaining in the cytosolic fraction and phosphorylating an intermediate activator which in turn translocates to the nucleus to initiate gene expression [Meinkoth *et al.*, 1990; Montminy and Bilezikjan, 1987; Sato-Bigbee *et al.*, 1994; Short *et al.*, 1986]. The second, more controversial, hypothesis is that the regulatory subunits of PKA, once bound by cAMP and released from the catalytic subunits, translocate to the

nucleus and regulate transcriptional activation directly by binding to specific sequences upstream of cAMP-inducible genes [Constantinou *et al.*, 1985; Nagamine and Reich, 1985].

### 1.7.3.a Activation of CREB

Initial research demonstrated that PKA affected gene expression when it was observed that PKA-deficient cell lines were unable to stimulate transcription in response to cAMP. In addition, the role of the C subunit in particular was identified when it was demonstrated that micro-injection of the C subunit directly into cells specifically activated transcription of cAMP inducible genes [Montminy and Bilezikjan, 1987]. In the case of one particular cAMP responsive gene, somatostatin, deletion mutants of gene promoter-CAT constructs, transfected into PC12 cells, defined a 30 nucleotide sequence which was essential for cAMP responsive transcription [Montminy *et al.*, 1986]. This 30 base pair region contains a short palindromic sequence motif, 5'- TGA CGT CA -3', which is very highly conserved in the promoter regions of a large number of cAMP inducible genes, including the c-*fos* proto-oncogene, somatostatin, tyrosine hydroxylase and many others [Montminy *et al.*, 1986]. This region was designated the cAMP responsive element (CRE) and shows classical enhancer sequence characteristics e.g., stimulating transcription from a distance and displaying functional activity irrespective of orientation. In addition, this sequence induces cAMP responsiveness when introduced into non-cAMP inducible genes. Footprinting analysis of PC12 extracts demonstrated the presence of a single footprint, covering 23 base pairs, which mapped to the site of the somatostatin CRE sequence [Montminy and Bilezikjan, 1987]. Using this particular sequence, a CRE binding protein (CREB) was purified by affinity chromatography and a 43kDa protein, which turned out to be both specific for CRE binding and which was phosphorylated by purified PKA catalytic subunits, was isolated [Montminy and Bilezikjan, 1987]. Other CRE binding proteins which are homologous to CREB [Habener, 1990; Short *et al.*, 1986] have been identified, building up a recognized family of CREB proteins, e.g., a 45kDa protein in oligodendroglial cells [Sato-Bigbee *et*

*al.*, 1994]. Gel retardation assays demonstrated two major CRE-binding, mobility shift bands - a monomeric and a dimeric form of which only the dimeric form appears to be transcriptionally active [Yamamoto *et al.*, 1988]. Cloning and sequencing of the full length cDNA has predicted a three domain structure: a transactivation domain with a series of PKA, PKC and casein kinase II phosphorylation sites, of which serine 133 seems to be critical for transcriptional activation [Gonzalez and Montminy, 1989]; a DNA binding domain, containing a series of basic amino acid residues; a leucine 'zipper' dimerization domain, critical for formation of the transcriptionally active dimeric form of CREB [Montminy *et al.*, 1990]. All this evidence points to CREB being a major substrate for PKA and the principal mediator of cAMP induced gene expression.

### 1.7.3.b Regulation of Gene Expression by the Regulatory Subunit of PKA

The idea that the $R_2$ subunit, when bound by cAMP, acts directly as a regulator of transcription by binding to consensus DNA sequences in cAMP responsive genes, is controversial and the evidence supporting this hypothesis is tentative. The type-2 regulatory subunit of PKA appears to possess intrinsic topoisomerase activity and it has been suggested that by altering the chromatin structure of genes the R subunit may subsequently regulate gene expression [Constantinou *et al.*, 1985]. An additional hypothesis is that the R subunit acts in a manner that is analogous to the bacterial CAP protein [Nagamine and Reich, 1985]. In prokaryotic cells cAMP binds to the catabolite repressor protein (crp) or the cAMP binding protein (CAP) and the complex binds to specific promoter sequences which influence gene transcription. A 29 base pair sequence in the 5' flanking region of a urokinase form of the plasminogen activator gene has been proposed as an R subunit binding site; 5' GAA AGG GTG AGA AAG AGC TGA TTG AGG GG 3' [Nagamine and Reich, 1985]. This sequence is highly homologous to sequences in other cAMP-inducible genes, bears no resemblance to the CRE element and is homologous with the *E. coli gal* operon sequence and other bacterial cAMP-CAP controlled gene promoter sequences. In addition, certain regions of the R subunit of

bovine cardiac PKA share characteristics with DNA binding regions of the bacterial CAP proteins. However, despite this, no direct evidence in favour of R2 acting as a transcriptional activator has been published.

### 1.7.3.c Other cAMP Responsive Elements

cAMP-responsive transcription factors and promoter elements, other than CREB and CRE, have also been identified. AP2, a 50kDa protein which interacts with regulatory elements of a number of genes is cAMP responsive [Comb *et al.*, 1986]. However, this responsiveness seems to be dependent upon the proximity of CREs, as gene expression is only noted when multiple copies of AP2 are present upstream of the transcriptional initiation site, whereas a single AP2 site will not respond to cAMP unless CRE is also present in the promoter region [Comb *et al.*, 1986]. Another element, placental tissue specific element (TSE), is usually found adjacent to two tandem CRE sites and is cAMP responsive only in the presence of CREB binding [Delegeane *et al.*, 1987]. However, recently the human tryptophan hydroxylase gene promoter has been shown to be cAMP-responsive and the critical cAMP-inducible element of this promoter is a motif which is entirely distinct from CRE [Boularand *et al.*, 1995]. Thus, it seems cAMP-induced regulation of gene expression can potentially be mediated via multiple signalling pathways and transcription factors. Recently, a second co-activator of transcription (CBP/p300) which associates with CREB has been identified. CBP or p300 interacts with CREB and phosphorylation of Serine133 on CREB appears to be a requirement for this association. CBP can also bind to the basal transcription factor TFIIB, thereby acting as a bridging molecule between CREB and the machinery which initiates transcription. CREB also undergoes PKA-independent phosphorylation by PKC upon activation of the T cell receptor [Brindle *et al.*, 1995], an event which does not lead to association with CBP. However, co-stimulation with a sub-optimal dose of a cAMP agonist results in CREB-CBP association in the absence of CREB phosphorylation, suggesting that PKA-induced phosphorylation of a second factor, possibly CBP itself, is required for co-activation of transcription. Injection of anti-CBP antibodies into cells

inhibits cAMP-stimulated gene activation, [Chakravarti *et al.*, 1996; Kamei *et al.*, 1996] and nuclear receptors for steroids, thyroids and retinoids also interact with CBP upon binding of the requisite hormone [Kamei *et al.*, 1996]. The HTLV-1 viral protein Tax can form a transcriptionally active complex with CREB and CBP and increases the binding of CREB to viral CRE sequences independently of CREB phosphorylation. In contrast, Tax induced binding to host cell CREs requires CREB phosphorylation [Kwok *et al.*, 1996].

### 1.7.4 cAMP Generation in Lymphocytes

The role of cAMP generation in lymphocytes remains equivocal and the available information demonstrates a dichotomy with respect to stimulatory and inhibitory effects; indeed there appears to be a great deal of variability dependent upon cell type, species and developmental stage. Cytokines which elevate intracellular cAMP in lymphoid cells include, IL-1 in murine thymocytes 70/Z3 pre-B cells, YT human natural killer-like cells and CESS human B lymphocytes [Shirakawa *et al.*, 1988], and IL-4 in human B lymphocytes [Finney *et al.*, 1990]. In addition, other cAMP generating stimuli which act upon lymphoid cells are $PGE_2$ and, interestingly, ligation of MHC class II in resting murine B cells [Andre *et al.*, 1994]. In T lymphocytes, elevation of cAMP levels exerts a variety of effects including inhibition of IL-2 production [Tsuruta *et al.*, 1995] and inhibition of expression of the β and α subunits of the IL-2 receptor complex [Krause and Deutsch, 1991], while having no effect upon IL-4 production. Elevation of cAMP also synergizes with $Ca^{2+}$ to regulate expression of CD7, a marker of T cell activation. VDJ recombination activity in lymphoid cells, an event which is associated with development and differentiation, is induced by agents which elevate cAMP [Menetski and Gellert, 1990]. In addition, cAMP activation of PKA acts to stabilize ionomycin-induced transcription of the IFN-γ gene in cytotoxic T cells, i.e., it stabilizes ionomycin-induced mRNA transcripts of the gene and promotes IFN-γ expression and secretion [Kaldy and Schmitt-Verhulst, 1995]. As well as positively regulating post-transcriptional events, cAMP has a role in transcriptional regulation in lymphoid cells. cAMP elevating drugs

and cAMP analogues are able to mimic IL-1's ability to induce biological responses such as κ-light chain expression in 70/Z3 pre-B cells and IL-2Rα chain expression in YT natural killer cells [Shirakawa *et al.*, 1989a; Shirakawa *et al.*, 1986]. IL-1 and cAMP generation are both able to induce transcriptional activation of κ-immunoglobulin-CAT constructs in 70/Z3 cells, and also activate cytosolic NF-κB and induce its translocation to the nucleus [Shirakawa and Mizel, 1989b]. All of these effects are inhibited by H8, an inhibitor of PKA and PKC, but not by PKC-specific inhibitors, suggesting that IL-1 acts upon NF-κB via a cAMP generating/PKA activating pathway. In addition IL-1, 8-bromo-cAMP and forskolin all induce activation of NF-κB in YT cells and the promoter of the IL-2Rα chain gene, which is expressed in response to IL-1 and cAMP in these cells, contains a binding site for NF-κB. IL-1 and 8-bromo-cAMP promote NF-κB binding to its specific promoter element in these cells and again, these effects are inhibited by H8 [Shirakawa and Mizel, 1989b]. Thus, it was concluded that IL-1 activates expression of IL-2Rα and κ-immunoglobulin genes, at least partially, by cAMP induced activation of NF-κB.

The role of cAMP in B lymphocyte proliferation seems particularly variable. In murine B lymphocytes, $PGE_2$ and other cAMP elevating agents inhibit IgM production, proliferation and enlargement of B cells, MHC Class II hyper-expression and CD23 expression [Roper *et al.*, 1994]. In addition, ligation of MHC class II in resting murine B cells induces cAMP generation [Andre *et al.*, 1994], a response which appears to have an anti-proliferative effect and induces apoptosis. In contrast, there is no observed generation of cAMP upon ligation of MHC class II in activated murine B cells. In human tonsillar B cells, the effects of cholera toxin and other cAMP elevating agents, such as forskolin and dibutyryl-cAMP, are dependent on the presence of other stimuli. Thus, cholera toxin, forskolin etc., enhance proliferation of activated B cells stimulated with anti-IgM in a dose-dependent fashion and they also enhance IL-4-induced DNA synthesis in anti-IgM-activated B cells [Garrone and Banchereau, 1993]. In contrast, cholera toxin, forskolin etc. inhibit IL-2 induced DNA synthesis in anti-IgM-activated B cells, an

effect which has been partially attributed to cholera toxin induced reduction in CD25 expression [Garrone and Banchereau, 1993]. However, this latter effect is unconvincing as levels of CD25 in these cells remains greatly elevated above basal and any observed decrease appears to be minimal. Thus, in murine B cells the effects of cAMP appear to be profoundly negative, whereas in human B cells elevated cAMP, in the majority of cases, seems to positively regulate proliferation. This is extremely interesting when one considers the ability of IL-4, which is a co-stimulant of B cell activation, to generate cAMP production in human B cells but not in murine B cells [Garrone and Banchereau, 1993].

## 1.8 The CD25 Promoter

As has been outlined in previous sections, the regulation of development of lymphoid cells is mediated by exposure to antigen, cytokines and adhesion molecules and is marked by the transient appearance of cell surface proteins which act as receptors for such stimuli. The activity of IL-2, with respect to T and B lymphocyte development, is dependent upon the presence of the high affinity ($\alpha\beta\gamma$) form of the IL-2 receptor complex at the cell surface. In both resting human T and B cells, the expression of CD25 (IL-2R$\alpha$ chain) is minimal or non-existent and regulation of expression is key to the production of high affinity IL-2 receptors and subsequent maximal activation responses to IL-2. Stimuli which regulate the expression of CD25 in T and B cells are markedly different and the signalling pathways employed in up-regulation of CD25 are poorly understood. In human B cells, the expression of CD25 in response to IL-4 does not appear until 24 hours after initiation of culture, suggesting that *de novo* synthesis of CD25 is required. Thus, in order to understand regulation of CD25 expression, it is necessary to examine the regulation of transcriptional activation, the pattern of DNA-protein binding to various enhancer elements in the 5' promoter region of the CD25 gene and the intracellular signalling pathways involved in regulating binding of transcription factors. The CD25 promoter has been well studied and is known to contain a number of common nucleotide

sequences which, by association with their appropriate transcription factors, are involved in regulation of a wide series of genes. These motifs include sites for NF-κB family proteins, Sp1 and serum response element (SRE) binding proteins. In addition, a number of other nucleotide sequences which define more lineage specific transcription factor binding elements have been identified within the CD25 promoter. These include motifs which bind two negatively acting factors, the retinoic acid response element (RARE) and a negative regulatory element (NRE), and a positive regulatory element, the IL-4 response element (IL-4RE). More recently, a Stat6 homodimer (STF-IL-4) binding site has been identified in the CD25 promoter. These various transcription factor binding elements and their spatial positions within the CD25 promoter are outlined in Figure 1.5.

### 1.8.1 Positive Regulatory Elements of the CD25 Promoter

#### 1.8.1.a NF-κB/SRE/Sp1 Binding Sites

While little is known of transcriptional regulation of CD25 in human B cells, there is considerably better understanding of the activation of transcription of CD25 in T cells. Resting T cells do not express CD25, but can be induced to do so through the activation of T cells by antigenic and mitogenic stimuli. In contrast, HTLV-1 infected T cells constitutively express high levels of CD25. HTLV-1 (human T cell lymphotropic virus-1) is a retrovirus considered to be responsible for the development of an aggressive form of adult T cell leukaemia (ATL). The expression of CD25 protein and mRNA transcripts in the HTLV-1 infected T cell lines MT2 and HUT102 provided a model for initial studies on transcriptional activation of the CD25 gene [Cross *et al.*, 1987]. Transfection of MT2 cells with CD25 promoter-deletion mutant-CAT constructs, indicated that progressive deletion from the 5' end of the promoter resulted in increases in reporter gene activity and a negative regulatory element lying 5' of -369bp from the transcription initiation site was proposed [Cross *et al.*, 1987]. Reporter gene activity fell from elevated levels when -253 and -244 deletion mutant-constructs were transfected into MT2 cells, and no detectable activity was observed after deletion beyond -177. Unlike HTLV-1 infected cells, normal Jurkat and YT-1 T cell lines show very low levels of basal

promoter-reporter gene activity, but can be readily induced by mitogenic stimulation with PMA or PHA [Cross et al, 1987; Lowenthal et al, 1988]. PMA induced a 4- to 12-fold increase in CAT activity in Jurkat and YT-1 cells transfected with -471 and -317 deletion mutants, whereas only a 2-fold increase was observed with -271 mutants, suggesting a 46bp PMA-inducible region located between nucleotides -317 and -271 [Lowenthal *et al.*, 1988]. Certain 5' deletion mutants which possess high activity in HTLV-1 infected cells (i.e., Δ-267 and Δ-253 mutants) are not transcriptionally active in Jurkat cells [Cross *et al.*, 1987], therefore, a region from -327 to -253 was defined as a potential site for HTLV-1 or HTLV-1 induced-host cell gene products. Tat-1 is the HTLV-1 gene product which transcriptionally activates HTLV-1-long terminal repeats (LTR) and co-transfection of Tat-1 cDNA with a CD25 promoter-CAT construct into Jurkat cells, reproduced some of the regulatory patterns observed in HTLV-1 infected T cell lines; thus, in the presence of Tat-1, PMA was no longer required to induce CD25 promoter activity. In region -293 to -241 of the CD25 promoter two imperfect direct repeats (IDR) occur. In DNA mobility shift assays, using extracts from PMA-activated T cells, oligonucleotides encompassing both of these IDRs or the downstream IDR alone, but not the 5' upstream IDR, are able to form DNA-protein binding complexes. Similarly the first two of these oligonucleotides, but not the third, are able to compete with each other for DNA-protein binding activity [Lowenthal *et al.*, 1988]. Thus, a positive regulatory element in the CD25 promoter occurs between base pairs -217 and -253 of the CD25 promoter and it appears that while the 3' end of this region is a potential site for HTLV-1-Tat-1 binding, sequences 5' from this were a requirement for PMA inducibilty in non-infected cells. A region of the CD25 promoter (-267 to -258) which binds members of the c-*rel* proto-oncogene family has been identified [Leung and Nabel, 1988]. In addition, overlapping sequences defining a CArG box (-253 to -244) and a GC box (-245 to -240), which bind SRF and Sp1 respectively, were identified in a region 3' of this site [Pomerantz *et al.*, 1989; Toledando *et al.*, 1990]. The c-*rel* proto-oncogene family includes; c-*rel* NF-κB (p50), Rel A (p65), NF-κB2 (p49 and p50B) and Rel B (p68) [Reviewed by Baldwin Jr., 1996]. Homo- and heterodimeric complexes of c-*rel* family

members regulate genes containing κB binding sequences in their 5' flanking promoter regions. In T cells, a constitutive occupancy of the κB site in the CD25 promoter was demonstrated: in unstimulated cells NF-κB-p50 homodimers bind to the promoter and inhibit transcription, possibly by prevention of SRF binding to the CArG box. Upon stimulation the p50 homodimer is exchanged for a NF-κB p50-p65 heterodimer, an event thought to facilitate SRF binding to SRE [Algarte *et al.*, 1995]. In Cos cells expressing low levels of c-*rel* family proteins, transfection with c-*rel* or p50 has been shown to co-operate with SRF to induce transcriptional activation and enhanced transcription is observed in the presence of c-*rel*, p50 and SRF [Pierce *et al.*, 1995]. Thus, in human T cells the major means of activating transcription of the CD25 gene appears to be binding of positive regulatory transcription factors to the κB and SRE sites. In contrast, in human B cells, binding of transcription factors to κB, SRE and Sp1 sites remains relatively unaltered in response to stimulation with IL-4, the major CD25 inducing stimulus for human B cells. It seems therefore, that mechanisms of transcriptional activation of the CD25 gene may be differentially regulated depending upon cell lineage.

### 1.8.1.b IL-4 Response Elements in the CD25 Promoter

The IL-4 response element (IL-4RE) in the CD25 promoter was identified by its sequence homology to an IL-4 response element in the promoter region of the IL-4-inducible CD23b (Fcε receptor II) gene [Kohler and Rieber, 1993]. Point mutations within a 9 base pair sequence (TTC TAA GAA) in this element showed it to be essential for IL-4-induced activation of transcription and the transcription factor which binds this element (NFIL-4) is not activated by $Ca^{2+}$ ionophore, phorbol ester or cAMP [Kohler and Rieber, 1993]. Other similar IL-4 response elements have also been identified in an enhancer element of the Iε gene [Ichiki *et al.*, 1993; Kohler and Rieber, 1993] and the promoter regions of a number of Class II MHC genes [Boothby *et al.*, 1988; Kretsovali and Papamatheakis, 1995]. A region (NF-BRE) upstream of the murine Class II MHC Aα gene, when cloned into an SV40 promoter of a pA10CAT plasmid and transfected into Class II MHC positive B lymphoma M12.A2 cells, shows a 70-fold increase in

reporter gene activity in response to IL-4 [Boothby *et al.*, 1988]. Similarly, nuclear extracts from IL-4 stimulated M12.A2 cells formed DNA-protein binding complexes with an oligonucleotide bearing the DNA sequence of this region, as did extracts from both normal and athymic spleen cells [Boothby *et al.*, 1988]. A DNA sequence described in the promoter of the Class II MHC Eα gene in murine M12 cells, has been described as an IL-4 response sequence (ILRS). IL-4 activates a nuclear factor (NFIL-4), which binds a novel element within ILRS. In addition, similar IL-4 inducible complexes are formed between nuclear extracts from IL-4 treated M12 cells and both the IFNγ response element (GRR) of the FcγRI gene and the acute phase response element (APRE) of the α2 macroglobulin gene promoter region. This complex, designated NFIL-4, also recognizes certain GAS-like sequences. Indeed, both GRR and APRE sites have sequence homology to GAS elements [Kretsovali and Papamatheakis, 1995]. A palindromic GGAA motif present in the response elements of the Eα, IFNβ, FcγR and α2M promoters has been highlighted as the possible underlying common motif which facilitates NFIL-4 binding to all these response elements. Thus NFIL-4, defined as a protein with a molecular weight of 75kDa, is able to successfully bind a range of cytokine response elements. However, this DNA-protein complex formation is not universal, as the IL-4RE of Class II MHC Aα gene [Boothby *et al.*, 1988], an IFNγ-stimulated element (a prototype GAS site) in the GBP gene and an IFNα response element (ISRE of the ISG54 gene) could not compete for NFIL-4 binding. Monoclonal antibodies to Stat1 (p91), which binds both GRR and APRE sites, do not disrupt the formation of an NF-IL-4-Eα gene-ILRS sequence complex, suggesting that IL-4 activates a unique transcription factor protein which is distinct from any of the Stat family proteins [Kretsovali and Papamatheakis, 1995]. Indeed, the molecular weight of NFIL-4 (75kDa) does not correlate with any of the known Stat proteins, e.g. Stat6 (~100kDa) [Hou *et al.*, 1994; Quelle *et al.*, 1995] which is the recognized member of the Stat family, activated by IL-4. Another distinction is, that NFIL-4 activation by IL-4 is not disrupted by pre-treatment of B cells with the protein tyrosine kinase inhibitor genistein, whereas high concentrations of staurosporine (500nM) abolish IL-4 induced NFIL-4 activation.

The recognized mechanism of Stat activation by cytokines is via phosphorylation by a member of the Janus family of protein tyrosine kinases. However, although inhibition of PTK activity does not affect the ability of IL-4 to activate NFIL-4, this activity is disrupted by the addition of anti-phosphotyrosine antisera [Kretsovali and Papamatheakis, 1995]. This suggests that there is perhaps a requirement for phosphotyrosine in NFIL-4-DNA complex formation and the inability of genistein to affect this activity may either be a result of NFIL-4 being tyrosine phosphorylated prior to activation, or that IL-4 activates a protein tyrosine kinase whose activity is unaffected by genistein.

IL-4 was found to induce a novel transcription factor which bound GAS sequences with a distinct mobility shift from IFNγ-induced p91 homodimers [Schindler *et al.*, 1994]. This IL-4 induced activity could be successfully competed for by both an IRF-1-GAS sequence and by RR1/2 oligonucleotide, an enhancer sequence from the germline Iε gene, which is homologous to other Stat binding sites. Nuclear extracts from the pre-B cell line 18.81A20, the mature B cell line M12.4.1, fresh murine splenocytes and human peripheral blood leukocytes all contained this IL-4-inducible DNA binding activity, designated STF-IL-4 [Schindler *et al.*, 1994]. IL-4 was found not to phosphorylate p91-Stat1, nor did anti-Stat1 antisera affect RR1/2-STF-IL-4 complex formation [Schindler *et al.*, 1994]. Thus, it appeared that IL-4 induced activation of a novel member of the Stat family, later isolated, characterized and cloned as Stat6; a protein with a molecular weight of 100kDa [Hou *et al.*, 1994; Quelle *et al.*, 1995]. The available data suggest that IL-4 induces activation of two distinct transcription factors; the 75kDa NFIL-4 and Stat6 (~100kDa), the latter forming a transcriptionally active homodimer STF-IL-4. Interestingly, the putative IL-4 response element in the CD25 promoter, identified by sequence homology, is similar to both the NFIL-4 binding site of the Class II MHC Eα gene promoter [Kretsovali and Papamatheakis, 1995], to Stat6 homodimer binding sites and also to GAS sequences. Despite this homolgy to Stat6 binding sequences, the putative CD25 promoter IL-4 response element does not contain the consensus Stat

binding motif (TT-$N_{(4-6)}$-AA). However, the implication of the sequence homology of this element in the CD25 promoter, with respect to transcription factor binding and IL-4 regulation of transcriptional activation in human B cells, remains to be determined. As well as a putative IL-4 response element, a Stat6 binding site has been identified in the CD25 promoter more recently, in a position downstream of the putative IL-4 response element, close to the transcription initiation site. The presence of two potential IL-4-regulated positive transcription factor sites is curious and the role of these will possibly provide a better understanding of IL-4 induced CD25 expression.

### 1.8.2 Negative Regulatory Elements of the CD25 Promoter

#### 1.8.2.a The Negative Regulatory Element (NRE)

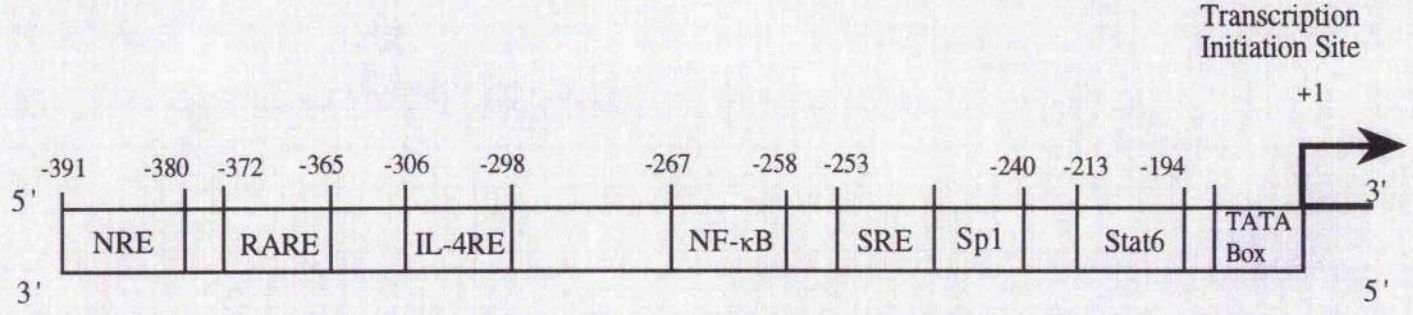
Studies of 5' CD25 promoter deletion mutant-CAT constructs transfected into HTLV-1 infected MT2 cells revealed, that deletion of 5' sequences of the CD25 promoter resulted in a 10-15-fold increase in reporter gene activity [Cross *et al.*, 1987], suggesting the presence of a Negative Regulatory Element (NRE) in the region 5' of a site -368 base pairs from the transcriptional initiation site. Similar studies in Jurkat and YT-1 T cell lines revealed, that in the latter a -317 deletion mutant was four times more transcriptionally active than a -471 deletion mutant, suggesting the presence of an NRE upstream of the PMA-inducible region of the promoter [Lowenthal *et al.*, 1988]. Interestingly, transfection studies in Jurkat cells demonstrated no noticeable difference between the ability of -471 and -317 mutants to initiate transcription in response to PMA and forskolin. Thus, it seems that the importance of this potential NRE and its binding by a transcription factor has different properties, dependent upon the developmental stage of the T cell; i.e., in cells which have an immature T cell phenotype (YT-1) the presence of NRE negatively regulates transcription, whereas in cells with a mature T cell phenotype (Jurkat cells) NRE does not appear to have a significant role with respect to CD25 expression. More specific deletion studies of the CD25 promoter [Smith and Greene, 1989] defined a 31 base pair region between nucleotides -400 and -368, as a candidate for the NRE sequence. Within this region there is an 11 base pair core region

which shares significant sequence homology with a region of the HIV-long terminal repeat (LTR) [Smith and Greene, 1989]. DNA mobility shift assay revealed the formation of three DNA-protein complexes which were successfully competed by HIV-NRE region, whereas mutant competitor oligonucleotides with five base pair substitutions within the 11 base pair core were unable to compete. Interestingly, in both YT-1 and MT2 cells the presence of NRE inhibits both basal and PMA inducible transcription, indicating that in these cells NRE binding proteins are constitutively expressed [Smith and Greene, 1989]. DNA crosslinking studies using a 27 base pair photoreactive probe containing part of the NRE sequence (-399 to -373) demonstrated the formation of a crosslinked species migrating at ~50kDa. This activity has been designated SP50, 'silencer protein'-50 [Smith and Greene, 1989]; no further published data, concerning the nature or identity of SP50, exists. The question of a similar silencer protein being present in human B cells, and if so the regulation of its DNA-binding activity for elements in CD25 promoter (in particular any possible regulatory effects of IL-4), is of considerable interest.

### 1.8.2.b The Retinoic Acid Response Element

Retinoic acid (RA), a natural derivative of retinol (Vitamin A), has profound effects upon cell growth and differentiation. These effects are mediated by two families of nuclear RA receptors: the RA receptor gene family, comprising RARα, RARβ and RARγ; the RXR family, RXRα and RXRβ, which only bind the 9-*cis* stereo-isomer of RA. RA affects transcription of CD25 by down-regulating transcription factor binding to a retinoic acid response element (RARE) in the promoter region of the gene. Transfection of human B cell lines IE8.6 and SKW6.4 with a 1.6kb CD25 promoter-CAT construct demonstrated increased reporter gene activity in response to RA. A -471 5' deletion mutant had low initial reporter activity, which increased in response to RA. In contrast, a -317 deletion mutant abolished transcriptional activation in response to RA [Bhatti and Sidell, 1994]. Internal deletion mutants lacking the NRE region, showed increased basal promoter activity and lost responsiveness to RA [Bhatti and Sidell, 1994]. The implications of this

Figure 1.5: *Cis* Elements of the CD25 Promoter (Not drawn to scale)




NRE - Negative Regulatory Element

RARE - Retinoic Acid Response Element

IL-4RE - IL-4 Response Element

Stat6 - STF-IL-4 Binding Site

SRE - Serum Response Element (CArG Box)

Sp-1 - Sp1 Binding Element (GC Box)

NF-κB - NF-κB Binding Element

were that a RARE site exists in the CD25 promoter, which binds a negatively regulating transcription factor which is released in response to RA. Also, it seems that this RARE site is either very closely associated with, or is possibly even indistinguishable from NRE.

## 1.9 Research Aims

There exists the potential for IL-4 to regulate CD25 gene expression in human B cells through a number of transcription factor binding sites. In T cells, mitogenic stimulation by agents such as PMA appears to involve the exchange of transcriptionally repressive DNA-binding proteins which occupy the κB site for a complex which activates transcription, along with concomitant binding of SRF and Sp1. In human B cells IL-4 is the only cytokine capable of inducing CD25 expression. Thus, the main aims of this thesis have been to establish how IL-4 regulates transcription factor binding to the CD25 promoter and the signal transduction pathways required to mediate such regulation. In particular, identifying the importance of regulation of NRE binding with respect to CD25 expression in human B cells and the potential for IL-4 to regulate transcription factor binding to this element, as a key to understanding IL-4 induced CD25 expression in these cells, forms a major part of the work presented here. Thus, we have examined the ability of IL-4 to elevate intracellular levels of cAMP and the effects of signalling pathways, subsequently activated by this second messenger, upon both the regulation of transcription factor binding to various elements within the CD25 promoter and upon transcriptional activation itself.

The presence of two putative IL-4 response elements in the CD25 promoter i.e., a Stat6 binding site and the IL-4RE, are potential targets for positive transcriptional activity of IL-4. The presence of two potential IL-4 response elements in the same promoter and the similarity in their sequence homology is curious. Therefore, determining the effects of IL-4 upon transcription factor binding to these regions and any potential relationship they

may have with each other may prove crucial in establishing the ability of IL-4 to up-regulate CD25 in human B cells. Thus, an attempt to establish the potential of the IL-4 response element site to act as a Stat6 binding site and also, using activators and inhibitors of intracellular signalling pathways, to gauge potential mechanisms of IL-4-induced regulation of the DNA binding activity of this element, have been made.

Finally, the potential of cytokines which have receptor components in common with the IL-4 receptor, to regulate CD25 expression or transcription factor binding to various elements within the CD25 promoter has been analysed. It is possible that the degree of redundancy that is observed between cytokine receptors and related signalling pathways results in the ability of other cytokines to mimic the ability of IL-4 to regulate, if not CD25 expression itself, then at least some of the individual regulatory components of transcription which are involved in expression of the gene.

# Chapter 2

# Materials & Methods

## 2.1 Materials

### 2.1.1 Cell Culture materials

RPMI-1640 medium, penicillin, streptomycin and glutamine were all obtained from Life Sciences Ltd., Paisley, U.K. Foetal Calf Serum was purchased from Sigma, Poole, Dorset, U.K. Tissue culture flasks were obtained from Costar Biochemicals Ltd, Northumbria, U.K. and additional sterile plastics were purchased from Sterilin Ltd., Feltham, U.K.

### 2.1.2 Materials for Isolation of Primary B Cells

Ficoll, as Histo-paque or Ficoll-Hypaque, was obtained from either Sigma, Poole, Dorset, U.K. or Calbiochem-Novabiochem Corporation, Nottingham, U.K., respectively, and Percoll was obtained from Sigma, Poole, Dorset, U.K. Sheep erythrocytes in Alsever's solution were provided by the Scottish Antibody Production Unit (SAPU), Carluke, U.K. Tonsils were obtained from the ENT surgical unit of Yorkhill Hospital for Sick Children, Glasgow, U.K.

### 2.1.3 Cytokines and Antibodies

IL-4 was a gift from the Immunex Corporation, Seattle, USA. IL-13 and IL-7 were obtained from R&D Systems, Europe Ltd., Abingdon, U.K. and IL-2 was donated by the Glaxo Institute for Molecular Biology (Geneva, Switzerland). Mouse anti-CD40 monoclonal antibody was obtained from Sero-tec, Abingdon, U.K. and goat anti-IgM antibody was from Sigma, Poole, Dorset, U.K. FITC conjugated anti-CD25 and PE conjugated anti-CD19 antibodies were from DAKO, Denmark; biotin conjugated anti-CD23 antibody was from The Binding Site Ltd., Birmingham, U.K. and Streptavadin-Quantum Red was from Sigma, Poole, Dorset, U.K. PY20 (mouse anti-phosphotyrosine antibody) and S20 (rabbit anti-human Stat6 antibody) monoclonal antibodies were obtained from Santa Cruz Biotechnology Inc., USA. HRP-conjugated

anti-mouse IgG and anti-rabbit IgG monoclonal antibodies were from Amersham International Plc., Amersham, U.K.

### 2.1.4 Fine Chemicals

Forskolin, Cholera Toxin, Ionomycin, Phorbol 12-myristate 13-acetate (PMA), Phorbol dibutyrate ($PBu_2$), Leupeptin, Aprotinin, Phenylmethylsulfonyl Fluoride (PMSF), dithiothreitol (DTT), acetyl co-enzyme A and poly-(dI-dC)-(dI-dC) were all obtained from Sigma, Poole, Dorset, U.K. H89 (N-[-2-(p-Bromocinnamylamino)ethyl]-5), Genistein, BAPTA-Acetoxymethyl ester [1,2-bis(o-Aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetra(acetoxymethyl) ester] and cyclicAMP were from Calbiochem-Novabiochem Corporation, Nottingham, U.K. Bisindolylmaleimide was from Boehringer Mannheim, U.K.

### 2.1.5 Materials for Gel Electrophoresis and Western Blotting

Acrylamide/bis-acrylamide mixture was from Biorad Laboratories, U.K. N,N,N',N'-tetramethylenediamine (TEMED), (Tween 20) and the 205kDa-29kDa molecular weight markers were from Sigma, Poole, Dorset, U.K. Nitro-cellulose and Enhanced Chemiluminescence (ECL) kits were from Amersham International Plc., Amersham, U.K. FUJI Medical X-ray film was from Genetic Research Instrumentation, Essex, U.K.

### 2.1.6 Radiochemicals

All radiochemicals were from Amersham International Plc., Amersham, U.K.

### 2.1.7 Oligonucleotides, CAT Reporter Gene Construct and Plasmid Vector

CREB, AP2 and Oct 1 sequences were from Promega, Southhampton, UK. IL-4RE, NRE and Stat6 double stranded oligonucleotide sequences were synthesized at the CRC Beatson Institute, Glasgow, U.K. T4 polynucleotide kinase and T4 polynucleotide

kinase buffer were a kit from Promega, Southampton, UK. A 590bp EcoRI-HindIII promoter-containing fragment from pIL-2α-CAT was a gift from Drs. W.C. Greene and D. W. Ballard, Howard Hughes Medical Institute, Duke University Medical Centre, Durham, NC, USA. The pGEM-7Zf(+) cloning vector was from Promega, Southampton, U.K.

## 2.2 Methods

### 2.2.1 Preparation and Culture of Cells

#### 2.2.1.a Isolation of High Density Tonsillar B Lymphocytes

Human B lymphocytes were prepared from a single cell suspension derived from tonsils, obtained from routine surgery, as described previously [Butcher and Cushley, 1990]. Cells were spilled into 20ml of RPMI-1640 medium supplemented with $10^2$ units/ml penicillin and 100µg/ml streptomycin (incomplete medium). The total cell suspension was layered, in 10ml aliquots, onto 10ml of Ficoll-Hypaque and centrifuged at 350 x g for 15 minutes. Mononuclear cells were harvested from the ficoll interface, washed twice and pelleted with AET-Sheep red blood cells for 5 minutes at 90 xg. 1 ml of FCS was layered onto the pellet and following a 30 minute incubation on ice the suspension was centrifuged through a ficoll cushion for 20 minutes at 350 xg. Non-rosetted cells were collected from the interface and washed twice in incomplete medium. This B cell enriched population was centrifuged through a five-step discontinuous percoll gradient at 1000 xg for 30 minutes [Butcher *et al*, 1991]. High density, quiescent B cells were removed from the 1.09 g/ml - 1.08 g/ml interface and washed three times in incomplete medium.

#### 2.2.1.b Culture of Primary B Lymphocytes and Cell lines

Following isolation primary B lymphocytes were cultured in RPMI-1640 medium supplemented with $10^2$ units/ml, 100µg/ml streptomycin, 2mM glutamine and 10% FCS

(complete medium), at 37°C in a 5% $CO_2$ humid atmosphere, unless otherwise stated. The EBV transformed EDR and Daudi human B cell lines and the human monocyte cell line U937 were cultured in complete RPMI-1640 medium (as described above).

## 2.2.2 Flow Cytometry

### 2.2.2.a Treatment of Resting B Cells

#### 2.2.2.a-1 Treatment of Resting B Cells With Pharmacological Agents

High density tonsillar B cells were suspended in complete medium at $1x10^6$ cells/ml. Cells were pulsed with the following stimuli for 30 second or 2 minute periods: 10nM PMA; 2nM $PBu_2$; 1μM Ionomycin; 100μM Forskolin. Reactions were terminated by dilution in a 20-fold volume of PBS, followed by three washes. Cells were also stimulated with a combination of $PBu_2$ and ionomycin and, in addition, PMA, $PBu_2$, ionomycin and $PBu_2$/ionomycin treated cells were stimulated, after approximately 15 minutes, with a 20 minute pulse with 100μM forskolin. Cells were then washed three times in PBS, resuspended in complete medium and incubated for 24 hours at 37°C.

#### 2.2.2.a-2 Chronic Phorbol Ester Treatment

High density tonsillar B cells were cultured at $1x10^6$ cell/ml in complete medium supplemented with 10nM PMA in $75cm^2$ flasks for 48 hours. Following chronic PMA treatment the cells were harvested, washed extensively to remove the excess phorbol ester then re-cultured for 24 hours in the presence of a range of concentrations of IL-4, anti-IgM antibody or anti-CD40 antibody.

#### 2.2.2.a-3 Chelation of Intracellular Calcium

In order to chelate intracellular calcium high density tonsillar B cells ($1 \times 10^6$ cells/ml) were loaded with 5μM BAPTA-acetoxymethylester (final concentration 1nM) as a complex with 0.1% (w/v) Pluronic-F127 detergent; both reagents were dissolved in dimethyl sulphoxide and the final concentration of DMSO in tissue culture experiments

was 0.1% (v/v). After 30 minutes the cells were washed to remove excess BAPTA-AM from the culture medium and then stimulated with IL-4.

### 2.2.2.a-4 Treatment With Cytokines and Monoclonal Antibodies

High density human B cells were suspended in complete medium at 1 x $10^6$ cells/ml. Cells were cultured for 24, 48, and 72 hours with a range of IL-4 concentrations. In addition, cells were also cultured with a range of concentrations of IL-2, IL-7, IL-13, anti-IgM antibody and anti-CD40 antibody for 24 hours. The concentrations of individual stimuli are given in the figure legends (see results).

### 2.2.2.b Fluorescent Antibody Labelling of Cells

Following 24 hour culture with the indicated stimuli (see results sections), both control and stimulated cells were harvested and washed three times with PBS. Cells were resuspended in PBS ($10^6$cells/100µl) and simultaneously stained with FITC-anti-CD25, PE-anti-CD19 and biotin-anti-CD23 monoclonal antibodies. Cells were then incubated on ice for 1 hour in the presence of antibody, before being washed twice in PBS. Cells labelled with biotinylated antibodies were incubated for a further 30 minutes in the presence of Streptavadin-Quantum Red in order to visualise biotin-conjugated antibodies. Cells were subjected to a final wash in PBS and fluorescence intensity in three fluorescence channels FL-1 530nm (FITC), FL-2 585nm (PE) and FL-3 > 650nm (Quantum-Red) were measured on a Becton-Dickinson FACScan flow cytometer, using an argon ion laser tuned to 488nm. For each individual experiment a negative control, consisting of B cells incubated in the absence of antibody, was prepared and used to determine levels of auto-fluorescence. In experiments where cells were labelled only with FITC-anti-CD25 and PE-anti-CD19 monoclonal antibodies, 10µl of Propidium Iodide (10µg/ml)was added immediately prior to data acquisition to allow gating of populations of dead or damaged cells. PI was not included in samples labelled with biotin conjugated antibodies as it is detected in the FL-3 channel and interferes with the detection of Quantum-Red labelling. In addition, cells were gated on forward and side

scatter during data acquisition to allow the identification of discreet lymphocyte populations.

### 2.2.2.c Flow Cytometric Analysis

Simultaneous labelling of B cells with three fluorescently conjugated antibodies allowed analysis of expression of three distinct cell surface proteins, i.e., CD19, CD23 and CD25. For analysis of expression of a single surface protein, results were determined as the Mean Channel Fluorescence or Mean Fluorescence Intensity of a histogram plot. Where analysis of expression of multiple cell surface markers was used, B cell populations were identified by labelling with PE-anti-CD19 monoclonal antibody, CD19 being a pan B cell surface marker from an early stage in B cell development. It is possible by 'two colour' dot-plot analysis, to identify total B cell populations expressing either CD23 or CD25. CD23 and CD25 positive B cell populations are calculated as a percentage of the total B cell population using the following equation:

$$\%CD25^{+}\ \text{B cells} = \frac{CD25^{+}/CD19^{+}\ \text{cells}}{CD25^{-}/CD19^{+}\ \text{cells} + CD25^{+}/CD19^{+}\ \text{cells}}$$

'Paint-A-Gate' software provides an programme that allows 'multi-colour' analyses of flow cytometry data files. This facility allows the breakdown of a total cell population, simultaneously stained with three distinct antibodies into discreet cell subpopulations of single-, double- and triple-marker protein positive cells. In this instance $CD19^{+}/CD23^{-}/CD25^{-}$, $CD19^{+}/CD23^{+}/CD25^{-}$, $CD19^{+}/CD23^{-}/CD25^{+}$ and $CD19^{+}/CD23^{+}/CD25^{+}$ cells.

## 2.2.3 Measurement of cAMP Generation

### 2.2.3.a Stimulation of Tonsillar B Cells and B Cell Lines

High density tonsillar B cells were isolated and cultured (5 x $10^6$ cells/ml) for 1 hour at 37°C, prior to stimulation. 100µl aliquots of cells were added to wells of a 96 well microtitre plate containing the required stimuli (see results - Chapter 4). Reaction were terminated at the indicated time points by addition of an equal volume of 4% perchloric acid (final concentration of PCA was 2%(v/v)). All reactions were carried out at 37°C and following termination of reactions, all samples were stored at 4°C for 1 hour prior to analysis. Samples were diluted 1:5 or 1:10 in assay buffer (50mM Tris/HCl, 4mM EDTA, (pH 7.4)) prior to analysis in order to ensure that measurement of the samples fell within the range of the standard curve. Each experimental determination was carried out in triplicate.

### 2.2.3.b Measurement of cAMP Generation.

Determination of cAMP production was measured according to the method described by Houslay *et al.*, (1976) based on the cAMP binding protein saturation assay of Brown *et al.*, (1972). The basis of this assay is the competitive binding of cAMP and tritiated cAMP to a cAMP-binding protein (the regulatory subunit of cAMP-dependent protein kinase), purified from bovine heart. The cAMP-binding protein was a gift from the laboratory of Prof. M.D. Houslay and prepared as described by Spence (1990).

50µl aliquots of stimulated and control samples were incubated with 100µl cAMP-binding protein, 100µl [$^3$H]-3', 5'-cAMP (20 000 - 30 000cpm/ml) and 50µl of 50mM Tris-HCl/4mM EDTA (pH 7.4) assay buffer. Assay mixtures were mixed by vortexing and incubated for a minimum of 2 hours to allow equilibration of radiolabelled cAMP and cAMP content of B cell samples. Unbound cAMP was removed by addition of 250µl of a solution of charcoal/BSA (2g/ml charcoal/ 1g/ml BSA); charcoal adsorbs free cyclic nucleotide. The charcoal suspension was removed by centrifugation in a microfuge for 5

minutes at 13 000rpm, 4°C. 400µl aliquots of the supernatant were removed and analysed by liquid scintillation counting, in 3 ml Ecoscint. A standard displacement curve was constructed for each assay to allow determination of cAMP content of the cell lysates. Tritiated cAMP was incubated with cAMP-binding protein in the absence of any competing, unlabelled cAMP in order to measure total cAMP binding. Non-specific binding was measured by incubation of tritiated cAMP in the absence of any cAMP-binding protein or competing, unlabelled cAMP. The standard curve was created by making a series of doubling dilutions of a 320nM solution of unlabelled cAMP in Tris/EDTA assay buffer. 50µl of each standard was incubated with 100µl cAMP-binding protein, 100µl [$^3$H]-3' 5'-cAMP and 50µl of 50mM Tris-HCl/4mM EDTA (pH 7.4). Thus, the concentration of the highest standard was 16pmol/assay tube and the lowest 0.0625pmol/assay. All conditions from this point were identical to those used for the test samples.

The samples were counted in an LKB scintillation counter which utilized an RIA curve fitting programme to allow automatic calculation of cAMP content of each sample from the standard curve. Results were re-calculated as cAMP produced/$10^6$ cells, taking into consideration the initial dilution factors employed in order to fit into the range of the standard curve.

## 2.2.4 Measurement of PKA Activity

### 2.2.4.a Preparation of Cell Samples for PKA Assay

High density tonsillar B cells ($1x10^7$/ml) were stimulated as indicated (see results sections). Following stimulation, cells were washed in ice-cold PBS and resuspended in extraction buffer ( 25mM Tris-HCl-pH 7.4, 0.5mM EGTA, 0.5mM EDTA, 10mM β-mercaptoethanol, 1µg/ml aprotinin, 0.5mM DTT, 1% NP40) and homogenised using a Dounce homogenizer, pre-cooled to 4°C. Lysates were centrifuged for 5 minutes at 4°C, at high speed in a microfuge to pellet cellular debris. Supernatant was removed and kept

on ice until use, or stored at -20°C for long term use.

### 2.2.4.b PKA Assay

Reactions were initiated by addition of 10µl of lysate to a reaction mix of PKA assay buffer (200mM Tris-HCl, pH 7.4, 100mM $MgCl_2$, 0.5mg/ml BSA), 25µM cAMP, 0.5mM PKA biotinylated peptide substrate (Kemptide - LRRASLG) and [$\gamma$-$^{32}$P]-ATP mix (100-200 cpm/pmol ATP). The reaction mix was pre-incubated at 30°C for 1 minute before addition of cell lysate. Control samples for each assay were made by addition of 10 µl of 0.1mg/ml BSA to reaction mix. Total reaction mixtures were then incubated for 15 minutes at 30°C and reactions terminated by addition of 7.5M guanidine-HCl termination buffer. A 25µl aliquot was removed from each sample and spotted onto Streptavadin-coated discs, and washed eight times with 1M NaCl, followed by two washes with distilled $H_2O$. Discs were allowed to dry at room temperature for 30-60 minutes. In order to calculate the specific activity of [$\gamma$-$^{32}$P]-ATP, 5µl aliquots (standard samples) from three separate reaction tubes were spotted onto Whatmann 3MM filters and dried immediately without washing. The dried discs were transferred to scintillation vials with 3ml of scintillation fluid and counted. The specific activity of [$\gamma$-$^{32}$P]-ATP and PKA activity were calculated using the following equations:

$$\text{(1) Specific activity of } [\gamma\text{-}^{32}\text{P}]\text{-ATP in cpm/pmol of ATP} = \frac{(75/5)\,(X)}{5000}$$

(2) Enzyme activity in pmol [$\gamma$-$^{32}$P]-ATP/minute/mg protein =

$$\frac{(\text{cpm}_{\text{reaction with enzyme}} - \text{cpm}_{\text{reaction without enzyme}}) \times (75)}{(25) \times (\text{time}_{\text{min}}) \times (\text{protein in reaction}_{\text{mg}}) \times (\text{specific activity of } [\gamma\text{-}^{32}\text{P}]\text{-ATP})}$$

Where 75 = sum of reaction volume (50µl) + volume of termination buffer (25µl)

5 = volume in µl of the standard sample

X = average cpm of the 5µl standard samples

5000 = number of pmol of ATP in the reaction

## 2.2.5 DNA Mobility Shift Assay

### 2.2.5.a Preparation of Nuclear and Cytosolic Extracts

High density, tonsil B cells were cultured for 16 hours in complete medium prior to treatment. Human B cell lines were isolated from culture and used one day after addition of fresh medium. Treatment of both primary human B cells and human B cell lines is as indicated (see results sections). $5x10^6$ cells were isolated at hourly intervals following initiation of treatment and washed in PBS. Cytosolic extracts were prepared on ice by incubating in extraction buffer (10mm HEPES-KOH, pH 7.5, 2mM $MgCl_2$, 0.1mM EGTA, 0.1mM EDTA) supplemented with 2µg/ml aprotinin, 2µg/ml leupeptin, 1mM DTT and 0.5mM PMSF. After 15 minutes NP-40 was added to a final concentration of 0.5% (w/v) and the mixture was immediately subjected to high speed centrifugation to isolate nuclei. The cytoplasmic supernatant was removed and stabilised by addition of 10% (w/v) glycerol. The isolated nuclei were incubated for a further 30 minutes at 4°C, in high salt buffer extraction (20mM Hepes-pH 7.8, 450mM NaCl) supplemented with 2µg/ml aprotinin, 2µg/ml leupeptin, 1mM DTT and 0.5mM PMSF and 25% (w/v) glycerol. Nuclear extracts were prepared by centrifugation at 10 000 x g to remove membranes and DNA. Nuclear and cytosolic extracts were stored at -70°C.

### 2.2.5.b DNA Mobility Shift Sense Oligonucleotide Sequences

DNA mobility shift assays were carried out using double stranded oligonucleotide with the sense sequences described below. CREB, OCT1 and AP2 oligonucleotides were bought from Promega, Southampton, U.K. NRE, IL-4RE and Stat6 sense and

antisense oligonucleotides were made at the CRC Beatson Institute, Glasgow, U.K. and annealed by mixing equimolar amounts of sense and antisense sequences, heating for 5 minutes in an 80°C water bath before being slowly cooled at room temperature.

| | |
|---|---|
| NRE | 5' TT CAT CCC AGG 3' |
| IL-4RE | 5' CAC TGC CAA GAA GTG CTT GCT 3' |
| STAT 6 | 5' GTA TTT CCA GAA AAG GAA C 3' |
| CREB | 5' AGA GAT TGC CTG ACG TCA GAG AGC TAG 3' |
| OCT 1 | 5' TGT CGA ATG CAA ATC ACT AGA 3' |
| AP2 | 5' GAT CGA ACT GAC CGC CCG CGG CCC 3' |

**2.2.5.c DNA Mobility Shift Assay**

The protein content of nuclear and cytosolic extracts was determined using the Lowry Protein Assay method. 10µl of B cell extracts were incubated with 1ml of reagent A (1 part Coppper/tartrate/carbonate solution [189mM $NaCO_3$, 4mM $CuSO_4$, 7.1mM KNa Tartrate], 2 parts 5% SDS and 1 part 0.8M NaOH) for 10 minutes at room temperature, followed by the addition of 0.5ml of reagent B (1 part Folin-Ciocalteau phenol reagent with 5 parts distilled $H_2O$). Samples were mixed thoroughly and allowed to stand at room temperature for a further 30 minutes before measurement of absorbance, at 750nm in an LKB Biochrom Ultrospec II, light spectrophotometer. Protein content of each sample was calculated from a BSA standard curve.

5µg of nuclear and cytosolic protein extracts were incubated with distilled $H_2O$ and binding buffer (20% glycerol, 5mM $MgCl_2$, 2.5mM EDTA, 2.5mM DTT, 250mM

NaCl, 50mM Tris-HCl pH7.5, 0.25 μg/ml poly(dI-dC)-poly(dI-dC)) for 5 minutes prior to addition of 0.175 pmole [$\gamma$-$^{32}$P]-end labelled, double stranded oligonucleotide. Samples were incubated for 20 minutes prior to loading onto a 7.5% (w/v) non-denaturing, polyacrylamide gel in 0.5x TBE (89mM Tris-HCl, 89mM Borate, 2mM EDTA-pH8.0) buffer. Gels were pre-run for 2 hours at 150V. Probe retardation was visualised by autoradiography with Fuji Medical X-ray film. Autoradiographs were developed using a Kodak X-omat Processor Model ME-3.

Labelling of oligonucleotide was carried out by incubating double stranded, annealed oligonucleotide (1.75pmol/μl) with T4 Polynucleotide Kinase, T4 Polynucleotide Kinase buffer (0.5M Tris/HCl-pH 7.6, 0.1M $MgCl_2$, 50mM DTT, 1mM spermidine, 1mM EDTA) and [$\gamma$-$^{32}$P]-ATP (3000Ci/mmol at 10mCi/ml) for 10 minutes at 37°C. The reaction was terminated by addition of 0.5M EDTA and TE (10mM Tris/HCl-pH 8.0, 1mM EDTA) buffer. Where unlabelled oligonucleotide (specific or non-specific) was used as a competitor for protein binding, the final concentration in the reaction was either 1.75pmole or 17.5pmole (i.e., a 10-fold or a 100-fold greater concentration than $^{32}$P-radiolabelled oligonucleotide).

## 2.2.6 Western Blotting

### 2.2.6.a Preparation of Whole Cell Lysates

High density tonsillar B cells were incubated for 16 hours in complete medium prior to treatment. The human EDR and U937 cell lines were isolated from culture and used one day after addition of fresh medium. Both primary human B cells and human cell lines ($1x10^7$/ml) were stimulated as indicated in the appropriate results sections. Following stimulation cells were washed in PBS, resuspended in 1ml RIPA buffer (50mM Tris-HCl, 1% NP40, 1mM Na Deoxycholate, 150mM NaCl, 1mM EGTA, 1mM $Na_3VO_4$, 1mM NaF, 1mM PMSF, 2 μg/ml leupeptin, 0.5mM DTT) and incubated on ice for 30 minutes. Following incubation, lysates were centrifuged at high speed in a microfuge to

remove cellular debris. Supernatant was removed and stored at -20°C until use. 10µg of protein together with 5µl of loading buffer (10% (w/v) glycerol) were boiled before addition to SDS-PAGE gel.

### 2.2.6.b Preparation of Immunoprecipitates

High density tonsillar B cells were incubated for 16 hours in complete medium prior to treatment. The human EDR and U937 cell lines were isolated from culture and used one day after addition of fresh medium. Both primary human B cells and human cell lines ($5x10^7$/ml) were stimulated as indicated in the appropriate results sections. Cells were centrifuged at 1500rpm for 5 minutes, resuspended in 1ml of RIPA buffer and incubated on ice for 10 minutes. Cell membranes were disrupted by repeated aspiration through a 21 gauge needle. Cellular debris was pelleted by centrifugation at 1000 x g at 4°C for 15 minutes. Supernatant was transferred to fresh tubes and the lysates pre-cleared by addition of 20µl of Protein G-agarose and immediate centrifugation at 275 x g, at 4°C for 5 minutes. 1ml of supernatant was transferred to microfuge tubes and 1 µg of S-20 (Rabbit-IgG anti-human-STAT-6 monoclonal antibody) added. Pre-cleared lysates were incubated in the presence of antibody for 1 hour at 4°C followed by addition of 20 µl agarose-protein G and placed on a rotator in a 4°C cold room overnight. Immunoprecipitates were collected by centrifugation at 3400 x g for 5 minutes at 4°C. Supernatant was discarded, immunoprecipitates washed three times in RIPA buffer and finally resuspended in 40µl of loading buffer (10% (w/v) glycerol). Lysates were stored in loading buffer at -20°C. Samples were boiled for three minutes and centrifuged at 3400 x g for 2 minutes to pellet Protein G-agarose. The supernatant was transferred to a fresh tube before addition to SDS-PAGE gel.

### 2.2.6.c SDS-PAGE Electrophoresis and Western Blotting

A 10% (w/v) acrylamide separating, gel was prepared using a Bio-rad mini rig. The gel was set with an isopropanol overlay after which a 5% (w/v) acrylamide stacking gel was poured. Both separating and stacking gels were made to the specifications outlined

below. Cell lysates and immunoprecipitates were added to the stacking gel and run at 100mA in electrophoresis buffer (25mMTris, 250mM glycine, 0.1% (w/v) SDS pH 8.3). When protein samples had reached the top of the separating gel the current was increased to 150V. Following separation of protein the stacking gel was removed and the separating gel transferred to a Bio-rad mini-wet blotting apparatus. Protein was transferred to a nitrocellulose membrane in protein transfer buffer (48mM Tris-HCl, 39mM glycine, 1.3mM SDS, 20% methanol - pH 9.2) at 77V.

**Table 2.1 SDS Electrophoresis Gel Constituents**

| | Final Concentration | |
|---|---|---|
| Reagent | Stacking Gel | Separating Gel |
| Acrylamide (40% w/v)/ bis acrylamide (3.3% w/v) | 5% (w/v) | 10% (w/v) |
| Tris -HCl-pH8.8 | - | 0.375M |
| Tris -HCl-pH6.8 | 0.13M | - |
| Ammonium Persulphate | 0.1% (w/v) | 0.05% (w/v) |
| SDS | 0.1% (w/v) | 0.1% (w/v) |
| TEMED | 0.07% (w/v) | 0.03% (w/v) |

### 2.2.6.d Immunoblotting

Nitrocellulose membranes were probed with either PY20 (Mouse-IgG anti-phospho-tyrosine monoclonal antibody) or S-20 (Rabbit-IgG anti-human-STAT-6 monoclonal antibody) as indicated in the appropriate results sections. Membranes probed with PY20 were blocked overnight with 5% (v/v) Tween/PBS at 4°C. Following blocking, membranes were rinsed with PBS and incubated at room temperature with a 1:2000 dilution (0.1µg/ml) of PY20 in 0.1% Tween/PBS for 90 minutes. Following incubation

with primary antibody, membranes were washed extensively with 1% (v/v) Tween/PBS for 1 hour, after which a secondary HRP-anti Mouse IgG antibody (1:1000 dilution in 0.1% (v/v) Tween/PBS) was added and incubated for a further 90 minutes. This secondary antibody incubation was again followed by an extensive washing with 1% (v/v) Tween/PBS for 1.5 hours. Membranes probed with S-20 were blocked overnight with 10% (w/v) Marvel/0.05% (v/v) Tween/0.1% (w/v) Thiomersol/PBS at 4°C. Following blocking membranes were rinsed with PBS and incubated with a 1:1000 dilution (0.1µg/ml) of S-20 in 0.5% (w/v) Marvel/0.05% (v/v) Tween/0.1% (w/v) Thiomersol/PBS for 45 minutes at room temperature. Membranes were then washed twice in 0.1% Tween/PBS followed by three 7 minute washes in PBS. A secondary HRP-anti-IgG antibody (1:1000 dilution in 0.5% (w/v) Marvel, 0.05% (v/v) Tween/0.1% (w/v) Thiomersol/PBS) was added and incubated for 1 hour followed by three 5 minute washes in 0.1% (v/v) Tween/PBS and three 5 minute washes in PBS.

**2.2.6.e ECL Detection system**

All membranes were developed using the ECL (*Enhanced Chemiluminescence* ) detection system. Horseradish Peroxidase, bound to the immobilized secondary antibody, catalyses the conversion of periacid to $H_2O$, $O_2^{2-}$, luminol and an enhancer complex. Luminol emits small quantities of light which are specifically enhanced up to 100-fold, allowing detection using light sensitive Fuji Medical X-ray film. Equal volumes of the two ECL solutions were mixed in the darkroom and added to the membrane. ECL solution was poured off after 30 seconds and the membranes blotted with tissue to remove excess liquid, wrapped in cling film and exposed to photographic film for varying time periods depending on the intensity of the signal.

## 2.2.7. CAT Reporter Gene Assay

### 2.2.7.a. Transfection and Stimulation of B Cells

EDR B Cells were cultured in fresh medium overnight to ensure they were in log phase growth immediately prior to transfection. Cells were harvested and washed and resuspended in serum free medium at a density of 6.25 x $10^6$ cells/ml. 0.8ml aliquots of cells (5 x $10^6$ cells/sample) were added to a Biorad Gene Pulser Cuvette (0.4cm gap) along with 40µg of plasmid in TE buffer. Cells were electroporated immediately after addition of plasmid at 0.35kV/960µFD capacitance. After electroporation the contents of the cuvette were added to 3ml of complete RPMI-1640 medium in a 6 well plate and cultured for two hours prior to stimulation. After 2 hours incubation the contents of each well were split into two 1.5ml aliquots which were then cultured with or without stimulant for a further 48 hours (this means that each stimulated sample has a corresponding control consisting of cells from the same batch of electroporated cells - this technique reduces the margin of error between samples due to different efficiencies of electroporation).

### 2.2.7.b Preparation of Cell Lysates and Chloramphenicol Acetyl Transferase (CAT) Assay

Transfected and stimulated cells were harvested from culture plates, washed once in sterile PBS and transferred to sterile eppendorf tubes in 1ml of PBS. The cells were centrifuged at 275 x g for 5 minutes after which the PBS was poured off, the cells re-centrifuged and any remnants of PBS were pipetted off. The cells were resuspended in 100µl of 250mM Tris-HCl pH 7.8 and either stored at 70°C until further use or immediately subjected to three cycles of freeze-thawing, in order to lyse the cells, by immersing in dry ice-ethanol a bath for 5 minutes followed by 5 minutes in a 37°C water bath. Following lysis, the extracts were centrifuged at 15 600 x g for 10 minutes and the supernatant retained and heat inactivated at 65°C for a further 10 minutes (to inactivate cellular acetylases). Samples were then centrifuged at 15 600 x g to pellet any precipitate

and the supernatant was removed and retained. Acetyl CoA (final concentration 5mM) and [$^{14}$C]-chloramphenicol (0.025 μCi) were added to 50μl aliquots of each extract and samples were incubated overnight at 37°C. Following incubation the assay was terminated by addition of 600μl of ethyl acetate and samples were vortexed for 30 seconds and centrifuged at 15 600 x g for 5 minutes. The upper phase of the ethyl acetate was removed and transferred to fresh sterile eppendorfs, followed by lyophilisation in a Jouan RC 10.22. centrifugal evaporator. Once samples were fully lyophilised they were resuspended in 20 μl of ethyl acetate and spotted onto silica Thin Layer Chromatography (TLC) plates. The TLC plates were run in in a solvent tank in 95% Chloroform/5% methanol for 1 hour, dried and placed on a FUJI phosphorimager screen for a minimum of 1 hour. Phosphorimager screens were analysed on a FUJIX Bas 1000 Bio-imaging Analyser and the generated images were analysed using MacBas software. 10μl aliquots of each sample were analysed for protein content using the Lowry protein assay method following the freeze-thawing extraction stage and values obtained by phosphorimage analysis were normalised to account for the protein content of each sample. In addition TLC plates were developed by autoradiography.

# Chapter 3

# Induction of Surface Expression of CD25 in Human B Lymphocytes

## 3.1 Introduction

CD25 expression in human B lymphocytes is an absolute requirement for expression of high affinity IL-2 receptors. Mature, quiescent B cells do not express CD25 but can be induced to do so by stimulation with IL-4 [Burlinson *et al.*, 1995; Butcher *et al.*, 1990; McKay and Cushley, 1995; Zola *et al.*, 1991]. To date, IL-4 is the only cytokine found to be capable of up-regulating CD25 in primary human B cells [Burlinson *et al.*, 1995]; however, the underlying signalling mechanisms responsible for this event have remained relatively unknown. IL-4 is thought to be able to bind two different receptors, a 140kDa IL-4Rα chain with or without a second γc subunit (forming high and low affinity receptors, respectively) [Russell *et al.*, 1993]. Intracellular signals induced by IL-4 in human B lymphocytes include a transient increase in $IP_3$ production and consequent $Ca^{2+}$ mobilization, followed after a lag period by an increase in levels of intracellular cAMP [Finney *et al.*, 1990]. It is possible to induce one of the effects of IL-4, namely soluble CD23 production, by stimulating B cells with a combination of calcium ionophore, phorbol ester and dibutyryl cAMP [Finney *et al.*, 1990]. It was of interest therefore, to determine whether similar reagents could be utilized to induce CD25 expression in human B cells, with a view to determining possible mechanisms of action of IL-4. In addition, IL-4 also induces tyrosine phosphorylation and resultant activation of both Jak1 and Jak3 kinases and of Stat6 [Hou *et al.*, 1994; Quelle *et al.*, 1995; Witthuhn *et al.*, 1994]. Phosphorylation of the latter results in formation of a transcriptionally active homodimeric complex. IL-4 also induces the activation of IRS-2 [Keegan *et al*, 1994], an event thought to be linked with the activation of PI-3 kinase, GRB-2/Shc association and $p70^{syk}$ activation [Myers *et al*, 1994].

IL-4 is a recognized co-stimulator of B cell activation when it is applied with simultaneous stimulation of B cells via the B cell receptor complex [Callard *et al.*, 1991; Paul, 1991]. Another T cell-dependent, mechanism of B cell activation involves stimulation via the B cell surface adhesion molecule CD40, either by activation with

CD40 ligand or with anti-CD40 monoclonal antibodies [Armitage *et al.*, 1992; Paulie *et al.*, 1989]. Therefore, the ability of stimuli associated with B cell activation, other than IL-4, to induce CD25 expression was of paramount interest. A feature of many cytokine receptors is the presence of commonly shared subunits in their structures. Thus, the IL-4 receptor complex contains γc, a subunit common to IL-2, IL-7, IL-9 and IL-15 receptors [Grabstein *et al.*, 1994; Kondo *et al.*, 1993; Russell *et al.*, 1993]. In addition, IL-13 is reported to have a number of cellular functions in common with IL-4 [McKenzie *et al.*, 1993] and it has been suggested that this is a result of a common receptor subunit [Kishomoto *et al.*, 1994]. However, the nature of this purported common component remains equivocal. It was, therefore, necessary to define whether or not cytokines which share γc as a feature of their receptors and also IL-13, which does not, were capable of inducing CD25 expression in human B cells.

## 3.2 Up-regulation of CD23 and CD25 in Resting B Lymphocytes

### 3.2.1 IL-4 Induces Dose Dependent Up-regulation of CD23 and CD25

IL-4 acts upon resting tonsillar B lymphocytes to elevate cell surface levels of both CD23 and CD25 in a dose dependent manner (Fig 3.1). Analysis of the concentrations of IL-4 necessary for half-maximal expression ($EC_{50}$) of these two markers in B cells, stained simultaneously for both, demonstrates the $EC_{50}$ for each to be different; with half-maximal induction of CD23 occurring at ~7 units/ml (approximately 35pM) and CD25 at 30 units/ml (0.15nM). These data could suggest that IL-4 is promoting CD23 and CD25 expression via distinct receptors, which are either of different affinities for the cytokine, or which are linked to distinct signal transduction pathways. The affinity of the IL-4 receptor for ligand is influenced by the presence or absence of the γc subunit in such a way that the affinity for ligand is increased some three-fold in the presence of γc [Russell *et al.*, 1993]; the difference in terms of $EC_{50}$ values observed here would be broadly consistent with responses being delivered via these two different receptors.

Simultaneous three colour analyses of the proportion of B cells expressing either CD23 and/or CD25 over a range of IL-4 concentrations was performed (Fig 3.2). The data illustrate that the proportion of $CD19^+/CD23^-/CD25^-$ B lymphocytes drops as the concentration of IL-4 increases, and that $CD19^+/CD23^+/CD25^-$ double-positive cells and $CD19^+/CD23^+/CD25^+$ triple-positive cells rise under the same conditions. Interestingly, IL-4 induced an increase in a population of $CD19^+/CD23^+/CD25^-$ cells at low concentrations (1-10 units/ml), whereas larger concentrations of IL-4 were required to induce CD25 expression. A minor population of $CD19^+/CD23^-/CD25^+$ double positive cells remained relatively constant over the applied concentration range of IL-4; this equlibrium could either be indicative of a constant turnover of CD25 on the cell surface of this population or a lack of response to IL-4. Thus, IL-4 is only shown to elevate CD25 on B cells which also express CD23 thereby increasing the triple-positive population. The proportion of B cells positive for CD23 appears to plateau, or drop slightly, at high concentrations of IL-4, an effect which could reflect either a maximal response, or alternatively loss, of CD23 as soluble CD23 from the B cell surface. However, it may be noted that this plateau effect occurs at a concentration of IL-4 which corresponds to the observed increase in the triple positive population. Therefore, this 'plateau' phenomenon may be due to a switch of cell phenotype from being double positive to triple positive; i.e, CD23 continues to be up-regulated in response to IL-4, but since CD25 is also being up-regulated, further increases in the $CD19^+/CD23^+/CD25^-$ pool are no longer observed. These data are again consistent with IL-4 inducing CD23 and CD25 expression via receptors of different affinity and also indicates the possibility that IL-4 up-regulates these two surface markers on a single B cell population.

### 3.2.2 IL-2, IL-7 and IL-13 Do Not Upregulate CD25 in Resting B Lymphocytes

The capacity of IL-4 to up-regulate CD25 on resting tonsillar B cells is clear and has been reported previously [Burlinson *et al.*, 1995; Butcher *et al.*, 1990; Zola *et al.*, 1991]. The

data described here are consistent with the interpretation that this response may be induced via a low affinity form of the IL-4 receptor, i.e., the IL-4R α chain minus the γc subunit. It was, therefore, of interest to determine whether other cytokines which contain γc as a component of their receptors had the capacity to induce CD25 expression on B cells. Stimulation with a wide range of concentrations of IL-2 and IL-7 ( both of which have γc in their receptors) failed to induce CD25 expression in resting B cells (Figs 3.3.A & 3.3.B). In addition, it has been reported that IL-13 shares certain cellular functions with IL-4 [Keegan *et al.*, 1995; Nilsson and Nilsson, 1995; Smerz-Bertling and Duschl, 1995] and that the receptors for IL-4 and IL-13 contain a shared component required for signal transduction [Zurawski *et al*, 1993]. The nature of this shared component has remained equivocal for some time, but it now seems likely that it is the IL-4Rα chain and not γc [He and Malek, 1995b], as once mooted. However IL-13, like IL-2 and IL-7, fails to elevate levels of surface CD25 in resting B cells (Fig 3.3.C). The failure of any of these cytokines to induce CD25 expression suggests a requirement for both the IL-4Rα chain and the γc subunit (a structure unique to the high affinity form of the IL-4 receptor) for elevation of this surface protein. Such an interpretation, however, appears at odds with data from the previous section which suggests that IL-4 induces CD25 via a low affinity form of the IL-4 receptor.

### 3.2.3 Anti-Immunoglobulin and Anti-CD40 Induce CD25 Up-regulation in Resting B Lymphocytes

To date, IL-4 is the only cytokine reported to be capable of up-regulating CD25 expression on human B lymphocytes [Burlinson *et al.*, 1995; Butcher *et al.*, 1990; Zola *et al.*, 1991], a fact that is supported by the data presented above. However, resting human B cells stimulated with anti-Immunoglobulin and anti-CD40 monoclonal antibodies elevate cell surface levels of CD25 in a dose-dependent manner (Figs 3.4 A & B) and elevation of CD25 is also observed in response to soluble CD40 ligand stimulation [Burlinson *et al.*, 1995]. These three stimuli, along with IL-4, appear to be the only reagents capable of inducing CD25 expression in resting human B cells and of

the three IL-4 appears to be the most potent, with an $EC_{50}$ of 0.15nM compared with 1.3-2nM and ~13nM for anti-immunoglobulin and anti-CD40, respectively. It is interesting that three such apparently diverse stimuli should have this cellular effect in common; however, if one considers that anti-Ig (as a substitute for antigen) [DeFranco *et al.*, 1982; Jelinek and Lipsky, 1987], anti-CD40 [Splawski *et al.*, 1993; Valle *et al.*, 1989] and IL-4 [Callard, 1991] are all signals for B cell activation, it seems logical that the induction of CD25 falls under the control of these cellular stimuli. CD25 is an absolute requirement for high affinity IL-2 receptors, the expression of which are necessary for IL-2 induced proliferation and differentiation in human B cells. Thus, stimuli which induce CD25 in these cells play an important regulatory role in B cell development.

## 3.3 Signalling Pathways Which Induce CD23 and CD25 in B Lymphocytes

### 3.3.1 IL-4 Mediated Induction of CD23 and CD25 expression can be Mimicked by the Same Pharmacological Agents

In view of the possibility that two different forms of the IL-4 receptor are responsible for CD23 and CD25 induction, it was of interest to determine whether early signal transduction events responsible for CD23 and CD25 up-regulation differed in any way. Thus, it has previously been shown that IL-4 induces $IP_3$ production and subsequent $Ca^{2+}$ mobilization followed, after a lag period, by a prolonged cAMP accumulation [Finney *et al.*, 1990]. In addition, it is possible to mimic one of the longer term effects of IL-4, namely soluble CD23 expression, using a combination of pharmacological agents which activate cellular signalling pathways [Finney *et al.*, 1990]. Figure 3.5 shows the mean channel number (or mean channel fluorescence) for expression of surface CD23 and CD25, following treatment of high density B lymphocytes with PMA and forskolin, either singly or in combination. For CD23 expression (Fig 3.5.A), comparatively modest increases in mean channel number, from 45 in the controls to a

maximum of 60, are observed in response to either PMA or forskolin as single stimuli; whereas a combination of these two stimuli resulted in a mean channel number of 82 (i.e.,~double the response). More striking increases in mean channel number were observed for CD25 levels following treatment with forskolin (from 13 in the control samples to 24 in the challenged cells) or PMA (a maximum of 23). However, stimulation with both PMA and forskolin resulted in an increase in mean channel fluorescence to between 31 and 35, approximately two-fold above baseline (Fig 3.5.B).

The effects of combining other pharmacological agents to stimulate multiple cellular signalling pathways were also investigated (Fig 3.6). Short pulses with forskolin, a direct activator of adenylyl cyclase, resulted in a modest up-regulation of CD23 and a more striking increase in surface CD25 (as above). However, stimulation with $PBu_2$ and/or ionomycin, followed by a more sustained challenge with forskolin caused a greater up-regulation of both CD23 (30-80% above baseline) and CD25 (90-160%) compared to unstimulated controls. These data are consistent with the interpretation that activation of adenylyl cyclase is important in up-regulation of CD23 and CD25, but that other stimuli, such as PKC activation and $Ca^{2+}$ mobilization, are also important in the response. Stimulation with either $PBu_2$ or ionomycin alone induced surface CD23 and CD25 to a modest degree (20-40% above basal); however, when these stimuli were administered in combination an increase in CD23 up-regulation was observed compared with the response to single stimuli, whereas the combination of these two stimuli appeared not to promote any further increases in CD25 expression. In contrast, either of these stimuli singly or in combination, together with a sustained exposure to forskolin, resulted in a more substantial increase in levels of CD25 than those of CD23. This is an interesting phenomenon and suggests, potentially, that generation of cAMP may play a more significant role in induction of CD25 than of CD23.

### 3.3.2 Chronic Phorbol Ester Treatment Abrogates Induction of CD23 and CD25 Expression in Response to IL-4

The involvement of PKC in IL-4 signal transduction pathways in human B cells was addressed by exposing resting tonsillar B cells to chronic stimulation with 10nM PMA for 48 hours, in an attempt to down-regulate intracellular PKC, prior to treatment with IL-4 for 24 hours and assay of CD23 and CD25 induction. The data presented in figures 3.7 A & B demonstrate that following chronic PMA treatment, IL-4 is unable to drive expression of either CD23 or CD25, in resting tonsillar B cells, regardless of the dose employed. These data suggest that one or more isoforms of PKC may be involved in some elements of IL-4 signal transduction in resting B lymphocytes. In addition, monoclonal antibodies to IgM and CD40 are able to promote increased expression of CD25 in resting human B cells (Figure 3.4), an effect which is also abrogated by chronic phorbol ester treatment (Fig 3.8). These latter data are consistent with the idea that activation of intracellular PKC is necessary to drive expression of the CD25 gene not only by IL-4, but also by the other stimuli known to promote expression of CD25 at the surface of human B cells.

### 3.3.3 Chelation of Intracellular Calcium Inhibits IL-4-Driven Upregulation of CD25

Calcium levels in the cytoplasm can be elevated in response to ligand stimulation either by an influx of the cation from the extracellular matrix or by release of $Ca^{2+}$ from intracellular organellar stores. Previous studies have indicated that IL-4 acts upon B cells to produce a transient increase in $IP_3$ production and consequent $Ca^{2+}$ mobilization. Therefore, in order to establish whether mobilization of intracellular $Ca^{2+}$ stores was involved in IL-4-driven CD25 expression in human B cells, resting tonsillar B cells were loaded with 5μM BAPTA-AM, a $Ca^{2+}$ chelating agent, and stimulated with IL-4 (Fig 3.9). The data illustrate that control B cells respond to IL-4 in a normal dose-dependent manner; however, the capacity of IL-4 to drive CD25 expression was severely impaired in BAPTA-loaded cells. Moreover, in BAPTA-loaded cells, CD25 expression in

response to stimulation with 15μg/ml anti-IgM was decreased by almost 33% compared to normal (data not shown). These results suggest that chelation of intracellular $Ca^{2+}$ can attenuate certain long term effects of IL-4 and are consistent with the interpretation that $Ca^{2+}$ release from intracellular stores is a central element in signal transduction leading to CD25 expression. All experiments described in this chapter were subjected to a minimium of three repeats.

## 3.4 Discussion

The data presented in this chapter illustrate the ability of IL-4 to up-regulate CD25 in quiescent, human B cells and also show that this induction is dose dependent, with an $EC_{50}$ which differs in magnitude from IL-4-induced expression of CD23. Although the difference in $EC_{50}$ values of these two surface markers is small, the values are in good agreement with the reported affinities of two IL-4 receptor populations on human B cells [Russell *et al.*, 1993]. Simultaneous 'three colour' analyses of cell surface expression of CD23 and CD25 on human B cells (CD19 positive cells), support these findings and allow the interpretation that IL-4 potentially up-regulates CD23 and CD25 on a single B cell population via receptors of two different ligand binding affinities. Since the dose response data indicated that CD23 and CD25 expression may be mediated through two distinct receptor populations, we attempted to establish whether different intracellular signals were responsible for inducing expression of the two cell surface markers. Maximal expression of both CD23 and CD25 occurred when cells were pulsed with either PMA or $PBu_2$ and/or ionomycin for short time periods, followed by a more sustained stimulus with forskolin - suggesting that PKC activation, $Ca^{2+}$ mobilization and elevation of intracellular cAMP may be important in IL-4 induced signal transduction. However, it was consistently noted that brief pulses with forskolin resulted in significant increases in CD25 expression ( typically a two-fold increase above basal). This fact, coupled with the observation that forskolin greatly enhanced CD25 expression when used in combination with other stimuli, suggests that cAMP may be a

necessary event in up-regulation of CD25 expression. Mechanistically, it is possible that either activated PKC and/or increased intracellular concentrations of $Ca^{2+}$ directly activate adenylyl cyclase type II and VII, or types I, III and VIII respectively [Cooper *et al.*, 1988; Krupinski *et al.*, 1989; Yoshimura and Cooper, 1993]. Alternatively, second messengers such as $Ca^{2+}$ and PKC activation may influence the activity of other signalling effectors which in turn activate adenylyl cyclase. However, it must also be considered that PKC activation and $Ca^{2+}$ mobilization, as well as functioning to activate adenylyl cyclase, may also act as independent signalling pathways which affect IL-4 target genes.

The observation that higher concentrations of IL-4 were required to elevate CD25 expression (compared with those required for CD23 expression), coupled with the observation that other cytokines such as IL-2 and IL-7 which share γc as part of their receptor structure are unable to induce CD25 expression, suggests that a low affinity form of the IL-4 receptor (i.e., the form which lacks γc) is responsible for up-regulating CD25. However, IL-13 a cytokine which shares a number of cellular functions with IL-4, is unable to up-regulate CD25. Although the nature of a shared component in IL-4 and IL-13 receptors is still under debate, it seems likely that it is the IL-4Rα chain and not, as was initially suggested, the γc subunit. If this is indeed the case, then it is possible that intracellular signals activated via both the γc and IL-4Rα, a feature unique to the high affinity IL-4 receptor, are required for induction of CD25 expression. The activation of Jak1 and Jak3 kinases in response to IL-4, although designated as being associated with the IL-4Rα chain and γc, respectively, appears to be reliant upon the physical association of the two individual receptor components. It has been suggested that ligand-induced receptor dimerization brings Jak1 and Jak3 into close spatial proximity, thus, allowing mutual cross-phosphorylation and subsequent activation of the two tyrosine kinases [Miyazaki *et al.*, 1994]. In addition, despite some reports suggesting that IL-4-induced activation of Jak1 and IRS-2 occur independently of one another [Kotiandes *et al.*, 1995], it appears that removal of the critical growth signal

transduction region of the IL-4Rα chain cytoplasmic domain [Harada *et al.*, 1990] abolishes both of these pathways [Pernis *et al.*, 1995c]. This 41 amino acid critical signal transduction domain has previously been demonstrated to be essential for both IRS-2 activation and physical association of the two receptor components [Keegan *et al.*, 1994]. The discovery that it is also crucial for Jak1 and Jak3 activation links the majority of known IL-4-induced signalling pathways to this region and suggests that IL-4 mediated receptor dimerization is a crucial event for successful signal transduction via the IL-4/IL-4 receptor complex.

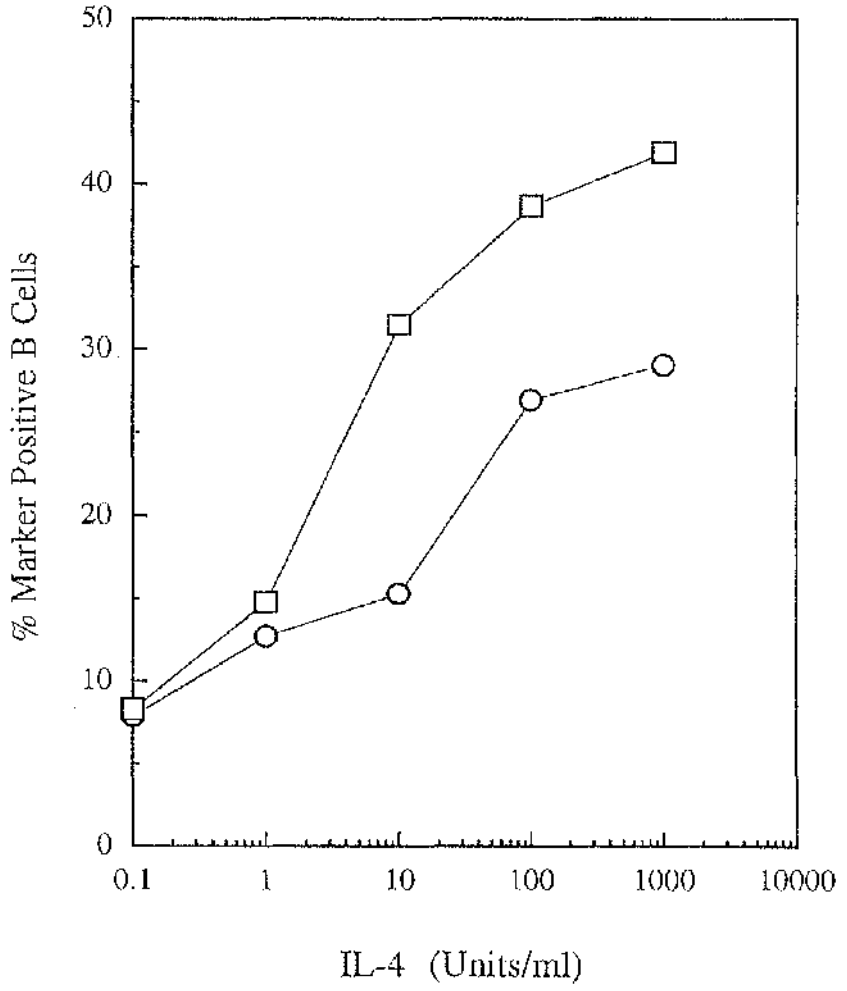
Interestingly the only stimuli, other than IL-4, capable of up-regulating CD25 appear to be activation of the B cell through the B cell receptor itself or through CD40. These three stimuli are all in some way involved in activation of mature human B cells. High concentrations of anti-IgM antibodies are able to induce DNA synthesis and cell division of resting human B cells and, although unable to promote proliferation themselves, both IL-4 and anti-CD40 antibodies act as co-stimulants with low, physiological concentrations of anti-IgM to promote cell division and activation. IL-2 acts to promote proliferation and differentiation of previously activated B cells and requires the presence of high affinity IL-2 receptors in order to mount such a response. Therefore, it seems logical that these three stimuli which are involved to some degree in B cell activation, should all have the capacity to induce CD25 expression in resting human B cells. Chronic phorbol ester treatment of B cells, in an attempt to down-regulate PKC, results in the inhibition of CD25 expression in response to IL-4, anti-IgM and anti-CD40, suggesting that the activation of PKC is necessary for regulation of induction CD25 expression in response to all three stimuli. Although there are no data reporting PKC activation by anti-CD40 stimulation it is well known that stimulation via the B cell receptor involves PKC activation.

## 3.1 IL-4 Induces Upregulation of CD23 and CD25 in a Dose Dependent Manner

High density tonsillar B cells were cultured with the indicated concentrations of IL-4 for 24 hours at 37°C in a 5% $CO_2$, humid atmosphere. Following incubation with IL-4 cells were simultaneously stained with FITC-anti-CD25, PE-anti-CD19 and biotinyl-anti-CD23 monoclonal antibodies (biotin conjugated antibodies were visualised using Streptavadin-Quantum Red) and analysed by flow cytometry. Results are expressed as % Marker positive B cells: CD23-open squares; CD25- open circles. IL-4 induces dose-dependent up-regulation of both CD23 and CD25 on the surface of human B cells.

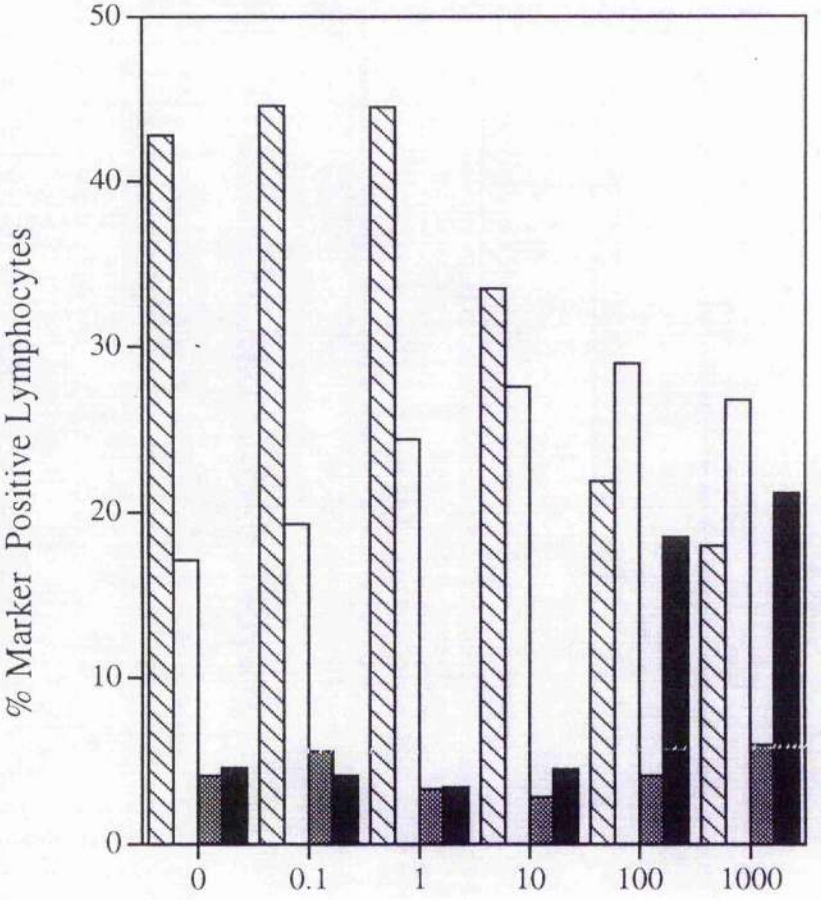
$EC_{50}$: CD23~35pM

CD25~150pM


50
40
30
20
10
0
% Marker Positive B Cells
0.1
1
10
100
1000
10000
IL-4 (Units/ml)

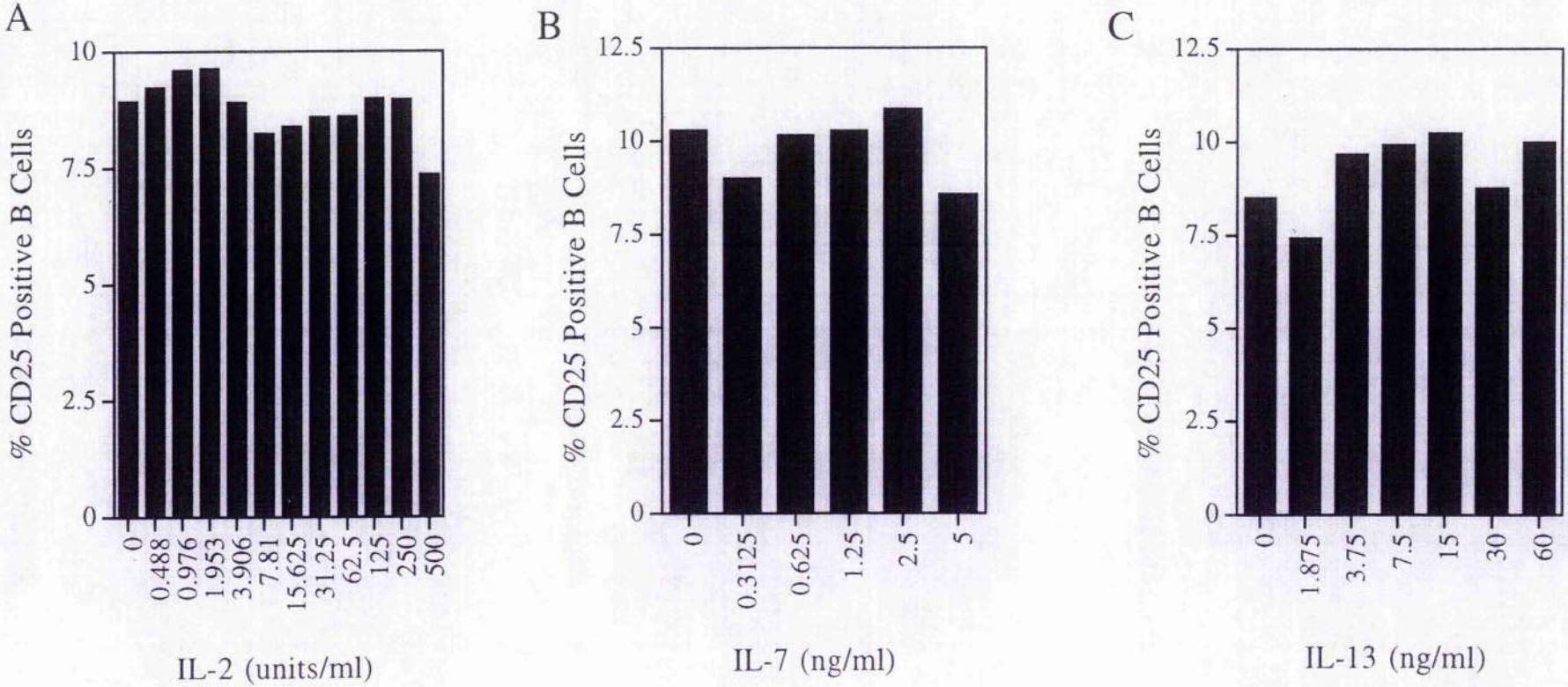
## 3.2 IL-4 Induces Up-regulation of CD23 and CD25 on the Same BCell Population via Two Different Affinity Receptors

High density tonsillar B cells were cultured with the indicated concentrations of IL-4 for 24 hours at 37°C. Following incubation with IL-4 cells were simultaneously stained with FITC-anti-CD25, PE-anti-CD19 and biotinyl-anti-CD23 monoclonal antibodies and analysed by flow cytometry. Analysis using the 'Paint a Gate' software allowed visualisation of single, double and triple positive subpopulations of cells: $CD19^+/CD23^-/CD25^-$ - striped bars; $CD19^+/CD23^+/CD25^-$ - white bars; $CD19^+/CD23^-/CD25^+$ - grey bars; $CD19^+/CD23^+/CD25^+$ - black bars.


50
40
30
20
10
0
% Marker Positive Lymphocytes
0
0.1
1
10
100
1000
IL-4 (Units/ml)

### 3.3 IL-2, IL-7 and IL-13 Do Not Up-regulate CD25 in Resting Human B Cells

High density tonsillar B cells were cultured with the indicated concentrations of (A) IL-2, (B) IL-7 and (C) IL-13 for 24 hours at 37°C. Following incubation with cytokines, cells were simultaneously stained with FITC-anti-CD25 and PE-anti-CD19 monoclonal antibodies and analysed by flow cytometry. Results are expressed as % CD25 positive B cells.


A
% CD25 Positive B Cells
10
7.5
5
2.5
0
0
0.488
0.976
1.953
3.906
7.81
15.625
31.25
62.5
125
250
500
IL-2 (units/ml)
B
% CD25 Positive B Cells
12.5
10
7.5
5
2.5
0
0
0.3125
0.625
1.25
2.5
5
IL-7 (ng/ml)
C
% CD25 Positive B Cells
12.5
10
7.5
5
2.5
0
0
1.875
3.75
7.5
15
30
60
IL-13 (ng/ml)

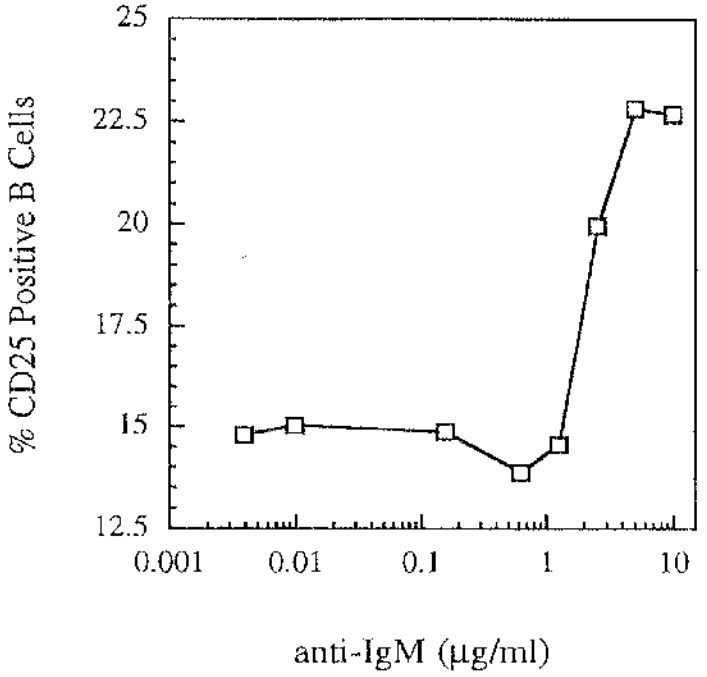
## 3.4 CD25 is Up-regulated in High Density Tonsillar B Cells in Response to Anti-IgM and Anti-CD40

High density tonsillar B cells were cultured for 24 hours at 37°C in the presence of the indicated concentrations of (A) anti-IgM and (B) anti-CD40 monoclonal antibodies. Following incubation with cytokines, cells were simultaneously stained with FITC-anti-CD25 and PE-anti-CD19 monoclonal antibodies and analysed by flow cytometry. Results are expressed as % CD25 positive B cells. Both anti-IgM and anti-CD40 induce dose dependent up-regulation of CD25 on the surface of human B cells.

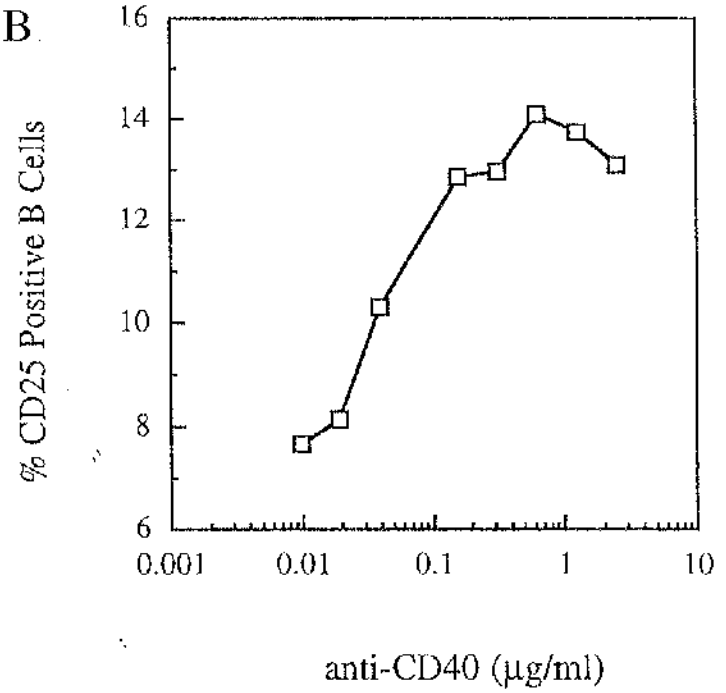
$EC_{50}$: Anti-IgM - ~13nM

Anti-CD40 - 1.3 - 2nM

A

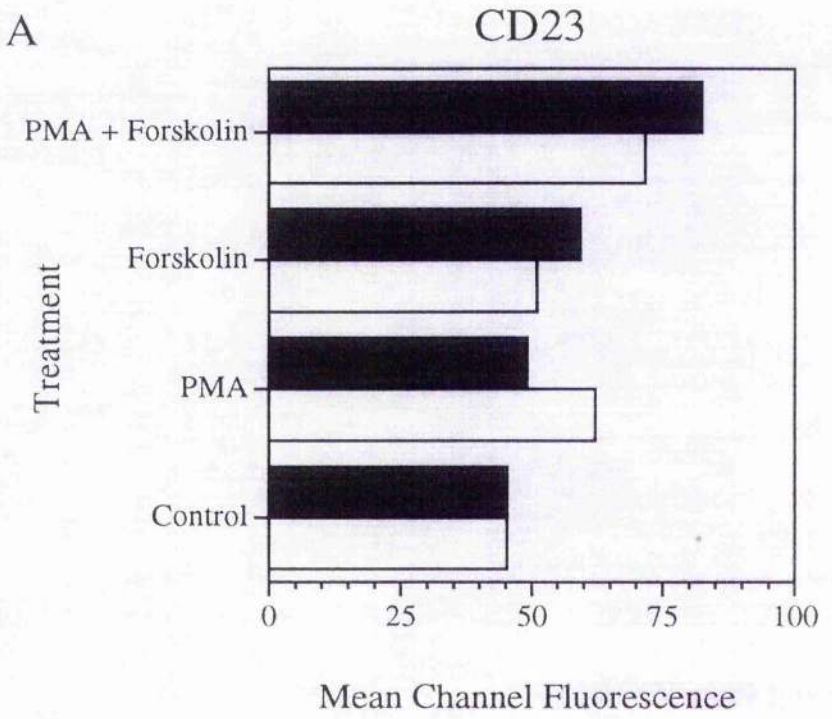

25
22.5
20
17.5
15
12.5
% CD25 Positive B Cells
0.001
0.01
0.1
1
10
anti-IgM (μg/ml)

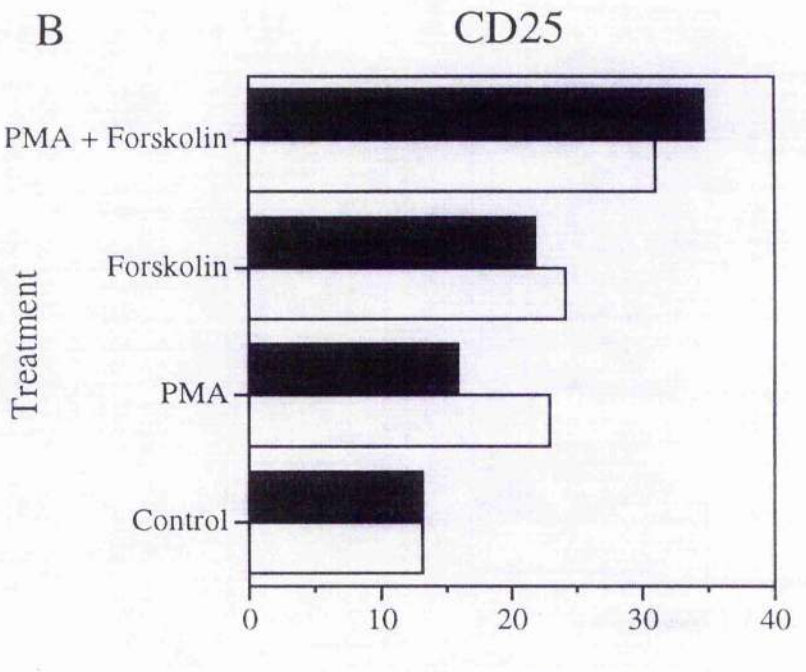
B


16
14
12
10
8
6
% CD25 Positive B Cells
0.001
0.01
0.1
1
10
anti-CD40 (μg/ml)

### 3.5 Stimulation of Resting Tonsillar B Cells with Phorbol Ester and Forskolin Induces CD23 and CD25 Expression

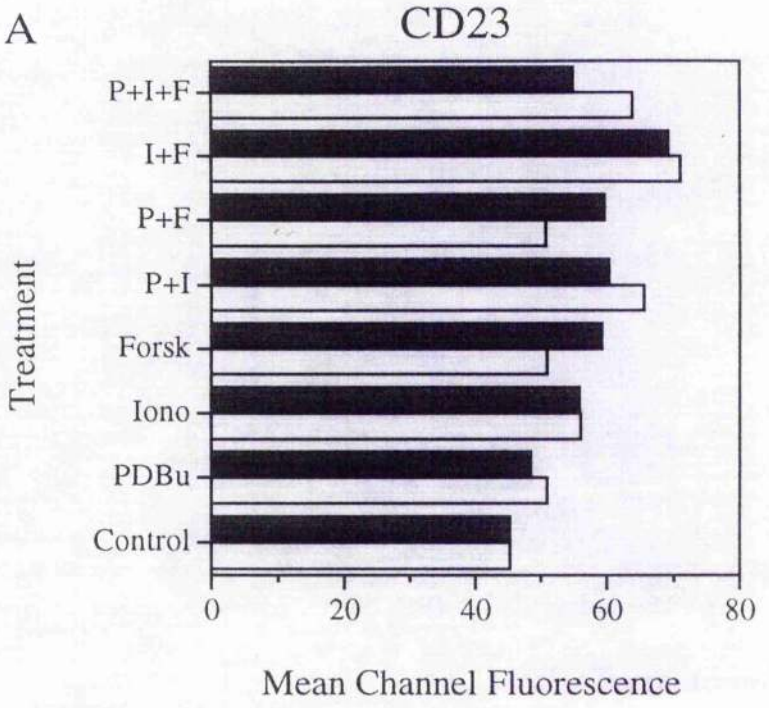
High density tonsillar B cells were stimulated with 100μM forskolin and 10nM PMA for 30 second and 2 minute time periods. In addition, PMA treated cells were further stimulated with 100μM forskolin for a sustained 20 minute period. Treated and control cells were cultured for 24 hours at 37°C. Following stimulation, cells were simultaneously stained with FITC-anti-CD25, PE-anti-CD19 and biotinyl-anti-CD23 monoclonal antibodies and analysed by flow cytometry. Results are expressed as mean channel fluorescence of (A) CD23 and (B) CD25 expression: 30 second pulse - black bars; 2 minute pulse - white bars.

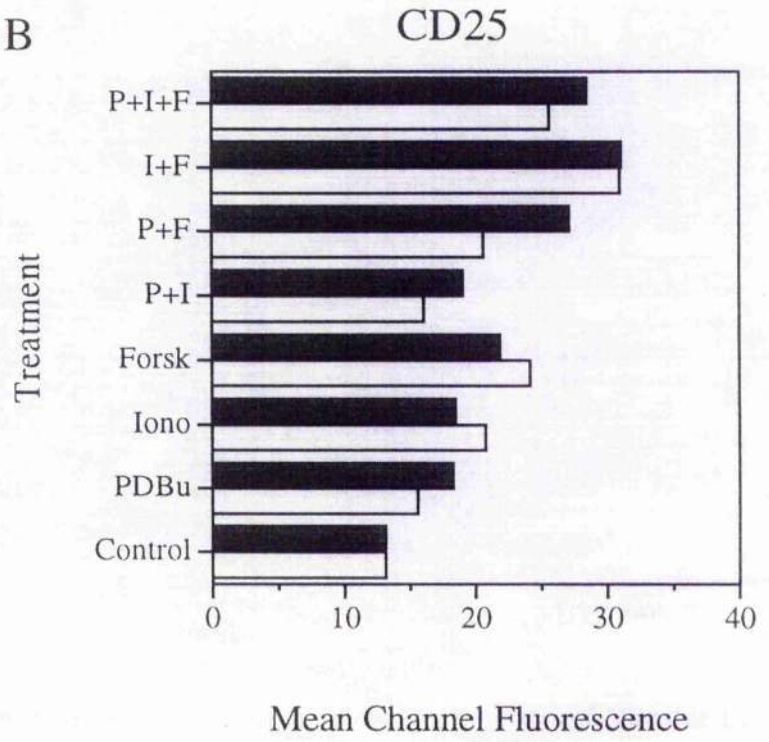

A
CD23
PMA + Forskolin
Forskolin
PMA
Control
Treatment
0
25
50
75
100
Mean Channel Fluorescence


B
CD25
PMA + Forskolin
Forskolin
PMA
Control
Treatment
0
10
20
30
40
Mean Channel Fluorescence

### 3.6 Stimulation of Resting Tonsillar B Cells with Phorbol Dibutyrate, Ionomycin and Forskolin Induces CD23 and CD25 Expression

High density tonsillar B cells were stimulated with 2nM $PBu_2$, 1μM ionomycin singly or in combination and 100μM forskolin for 30 second and 2 minute periods followed by 24 hour culture at 37°C. In addition, B cells pulsed for 30 seconds and 2 minutes with $PBu_2$ and ionomycin, both as single and as a combined stimulus, were further treated with 100μM forskolin for a 20 minute period. Cells were washed and cultured for 24 hours at 37°C. Following stimulation, cells were simultaneously stained with FITC-anti-CD25, PE-anti-CD19 and biotinyl-anti-CD23 monoclonal antibodies and analysed by flow cytometry. Results are expressed as mean channel fluorescence of (A) CD23 and (B) CD25 expression: 30 second pulse - black bars; 2 minute pulse - white bars.

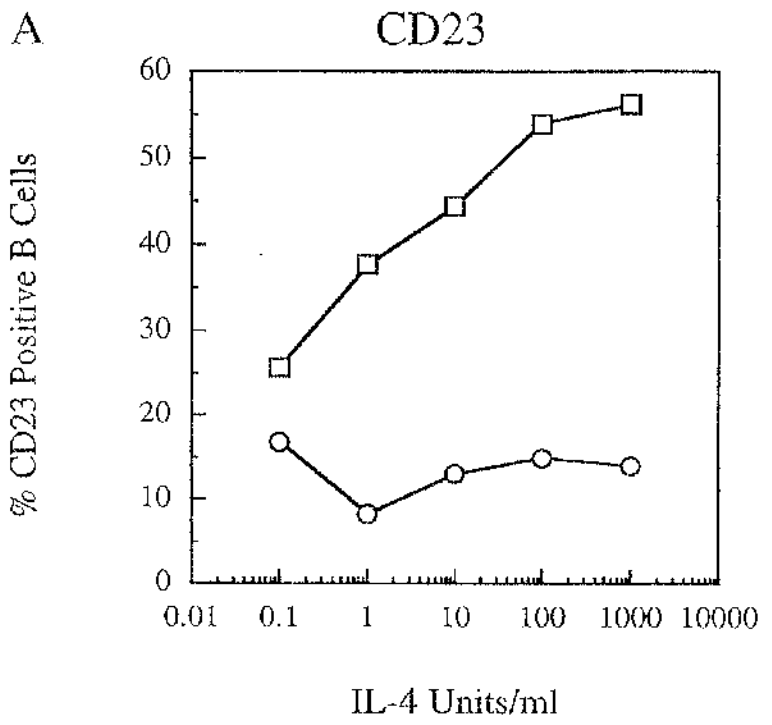

A
CD23
P+I+F
I+F
P+F
P+I
Forsk
Iono
PDBu
Control
Treatment
0
20
40
60
80
Mean Channel Fluorescence


B
CD25
P+I+F
I+F
P+F
P+I
Forsk
Iono
PDBu
Control
Treatment
0
10
20
30
40
Mean Channel Fluorescence

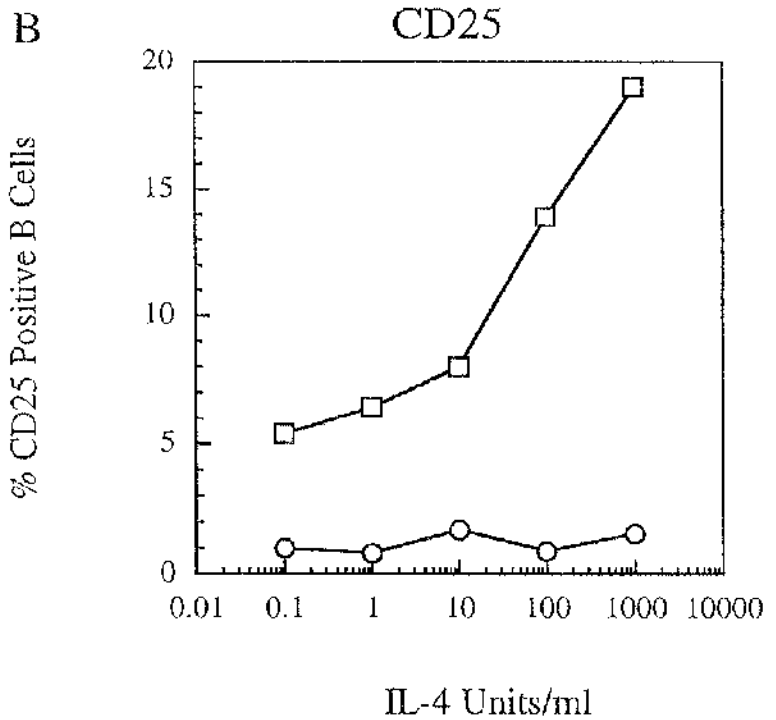
### 3.5 Stimulation of Resting Tonsillar B Cells with Phorbol Ester and Forskolin Induces CD23 and CD25 Expression

High density tonsillar B cells were stimulated with 100μM forskolin and 10nM PMA for 30 second and 2 minute time periods. In addition, PMA treated cells were further stimulated with 100μM forskolin for a sustained 20 minute period. Treated and control cells were cultured for 24 hours at 37°C. Following stimulation, cells were simultaneously stained with FITC-anti-CD25, PE-anti-CD19 and biotinyl-anti-CD23 monoclonal antibodies and analysed by flow cytometry. Results are expressed as mean channel fluorescence of (A) CD23 and (B) CD25 expression: 30 second pulse - black bars; 2 minute pulse - white bars.

A

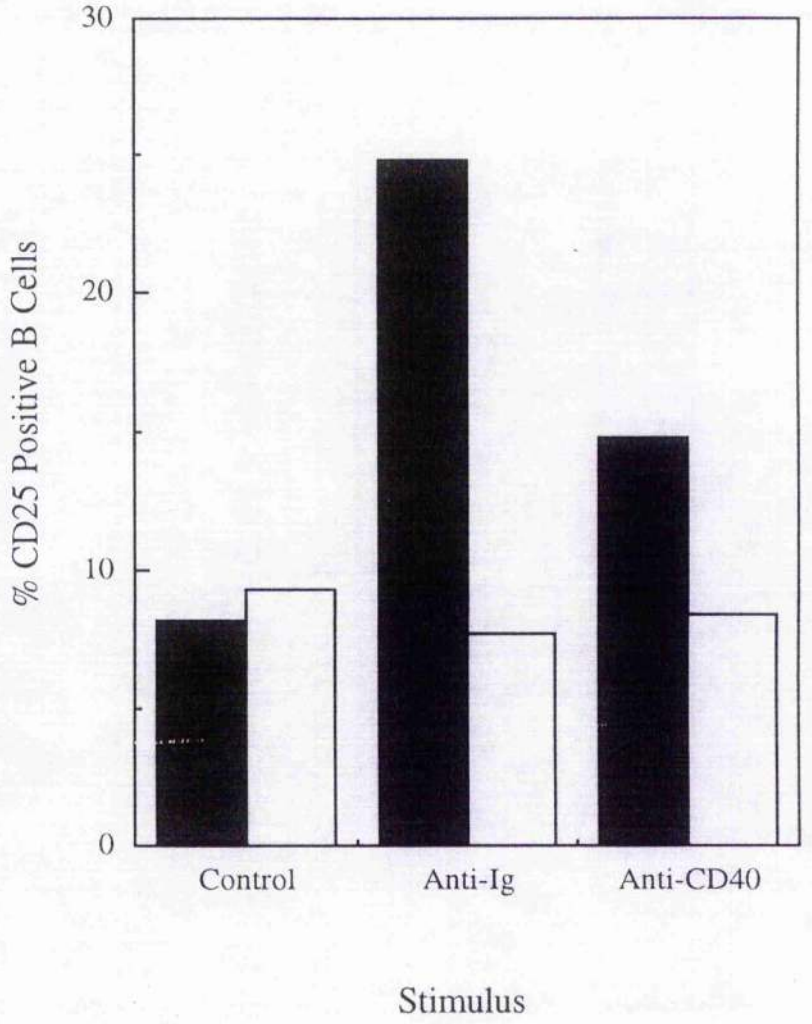

CD23
% CD23 Positive B Cells
60
50
40
30
20
10
0
0.01
0.1
1
10
100
1000
10000
IL-4 Units/ml

B


CD25
% CD25 Positive B Cells
20
15
10
5
0
0.01
0.1
1
10
100
1000
10000
IL-4 Units/ml

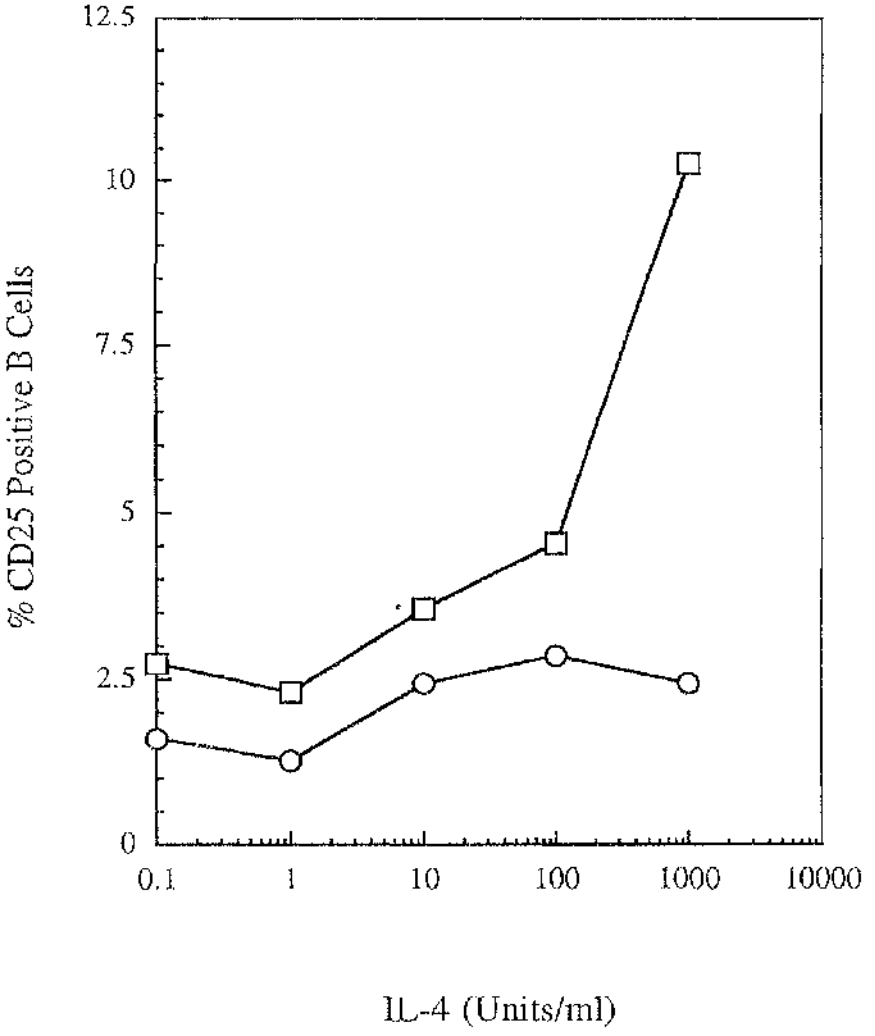
### 3.8 Anti-IgM and Anti-CD40 Antibody Induced Up-regulation of CD25 is Abolished by Chronic PMA Treatment

High density tonsillar B cells were treated with 10nM PMA for 48 hours, following which both treated and untreated cells were cultured with 25μg/ml anti-IgM and 5μg/ml anti-CD40 monoclonal antibodies for 24 hours at 37°C. Following incubation, cells were simultaneously stained with FITC-anti-CD25, PE-anti-CD19 and biotinyl-anti-CD23 monoclonal antibodies and analysed by flow cytometry. Results are expressed as % CD25 positive B cells: untreated cells - black bars; chronically PMA treated cells - white bars.


30
20
10
0
% CD25 Positive B Cells
Control
Anti-Ig
Anti-CD40
Stimulus

### 3.9 Chelation of Intracellular Calcium Abrogates the Ability of IL-4 to Up-regulate CD25

High density tonsillar B cells were loaded with 1nM BAPTA-acetoxymethylester (in the presence of 0.15% (w/v) Pluronic F127) to chelate intracellular $Ca^{2+}$. B cells were then treated with the indicated concentrations of IL-4 for 24 hours at 37°C. Following incubation with IL-4, cells were simultaneously stained with FITC-anti-CD25, PE-anti-CD19 and analysed by flow cytometry. Results are expressed as % CD25 positive B cells: untreated cells - open squares; BAPTA loaded cells - open circles.


12.5
10
7.5
5
2.5
0
% CD25 Positive B Cells
0.1
1
10
100
1000
10000
IL-4 (Units/ml)

# Chapter 4

# A Negative Regulatory Element in the CD25 Promoter is Regulated by a cAMP Generating Pathway

## 4.1 Introduction

It has been demonstrated previously that IL-4 is able to induce generation of intracellular cAMP in human B cells. However, the answers to a number of pertinent questions remain to be determined: the full nature of the kinetics of this second messenger generation; the dose dependency (if any) of IL-4 induced cAMP production; and most importantly, the consequences of activation of this pathway with respect to downstream signalling events and gene expression. Another issue which has not been addressed is which form of the IL-4 receptor is involved in cAMP production and whether other cytokines, in particular those with γc as part of their receptor complexes and also IL-13, share this capacity to elevate intracellular cAMP.

The promoter region of the CD25 gene contains a number of nucleotide sequences corresponding to motifs which bind common transcription factors including NFkB, Sp1 and the serum response factor (SRF) [Algarte *et al.*, 1995; Pierce *et al.*, 1995]. In addition to these, the promoter sequence also contains a number of motifs which are more lineage specific. These include two negative regulatory elements and a positive regulatory element: a retinoic acid response element (RARE) [Bhatti and Sidell, 1994] and the negative regulatory element (NRE) [Cross *et al.*, 1987; Smith and Greene, 1989] both of which potentially repress transcription when occupied; a putative IL-4 response element (IL-4RE), which could potentially promote transcription when bound. In addition, and more recently, a STAT 6 binding element, occurring downstream of the IL-4RE, has been identified. In T cells an 11 base pair, core element in the NRE has been shown to bind a 50kDa 'silencer protein' [Smith and Greene, 1989] and deletion studies show that removal of NRE from the promoter region results in elevation of both basal and PMA-inducible promoter activity [Lowenthal *et al.*, 1988; Smith and Greene, 1989]. However, the questions of whether the NRE in the CD25 promoter in B cells displayed similar transcription factor binding activity and, if so, how binding of this transcription factor is regulated, have not been addressed until now. The ability of IL-4

to regulate transcription factor binding to one or more of these regulatory elements is of particular interest due to its unique ability as the sole cytokine capable of up-regulating CD25 expression. In addition to regulation of transcription factor binding to individual elements in the CD25 promoter, it is necessary to examine the regulation of transcriptional activation in order to define the pattern of transcriptional regulatory protein binding which results in activation of the gene.

## 4.2 Generation of cAMP in Resting B Lymphocytes

### 4.2.1 IL-4 Induces cAMP Generation in Resting B Lymphocytes

IL-4 stimulation of resting tonsillar B lymphocytes consistently elevated intracellular levels of cAMP (Fig 4.1) a finding consistent with previous reports [Finney *et al.*, 1990]. However, contrary to previous findings the data presented here demonstrate that IL-4 induced cAMP generation over a period of 20 minutes is biphasic, with a short, transient peak occurring between 2-5 minutes which returns to baseline prior to the onset of a larger, more sustained peak arising approximately 10 minutes after ligand binding. The magnitude of this secondary peak was found to vary from one tonsil preparation to another within a range of approximately 65-120 pmol /$10^6$ cells. The reason for this variation in response is unclear; however, as the cells were used only one hour following preparation, it could possibly be attributed to the degree of prior, *in vivo* activation which varies from one tonsil preparation to another. If one examines cAMP accumulation in response to IL-4 over a longer time course, i.e., 1 hour following ligand binding, it can be observed that the secondary peak is sustained, until between 20 and 30 minutes, before it begins to decline back to basal levels (Fig 4.2)

A dose response titration for IL-4-stimulated cAMP accumulation at both 5 and 20 minutes (Fig 4.3.A & B) demonstrates an $EC_{50}$ of 1-10 units/ml (5 - 50pM) for both. Interestingly these values are consistent with cAMP being generated via a high affinity IL-4 receptor, i.e., the IL-4R $\alpha$ chain and $\gamma$c chain. In addition, the data presented in

Figures 3.1 and 3.2 suggest that IL-4 induced up-regulation of CD23, but not CD25, is mediated via a high affinity IL-4 receptor, with the $EC_{50}$ values for CD23 expression corresponding to the $EC_{50}$ value for IL-4 induced cAMP generation. In contrast, IL-4-induced CD25 up-regulation has a higher $EC_{50}$, corresponding to IL-4 mediating its effects via a low affinity form of the receptor, i.e. the IL-4R $\alpha$ chain alone. These results are interesting if one considers the apparent ability of forskolin both as a single stimuli and also in combination with phorbol esters and calcium ionophores, to increase levels of cell surface CD25. These latter data would suggest that the generation of cAMP does have an important role in inducing expression of CD25 in human B cells. However, the ability of a low affinity IL-4 receptor being able to successfully mediate IL-4-induced signal transduction now seems to be questionable.

### 4.2.2 IL-2 and IL-7 Induce cAMP Generation in Resting B Lymphocytes

Considering the possibility that IL-4 promotes cAMP production in resting B cells via the high affinity form of its receptor (i.e. the $\gamma$c containing form), it was of interest to determine whether or not other cytokines which share $\gamma$c as a component of their receptors, were capable of inducing cAMP production. Upon stimulation of tonsillar B cells with IL-2 and IL-7, an increase in intracellular cAMP levels in resting B cells was observed. Again the magnitude of this response varied from one cell preparation to another, although the general pattern appears to suggest a greater cAMP response to IL-2 as compared with IL-4 (Fig 4.4), whereas the response to IL-7 appeared to equal that of IL-4 (Fig 4.5). Thus, it appears that cAMP generation is a feature of the lymphoid, $\gamma$c-containing subfamily of cytokine receptors and these data reinforce the idea that IL-4 promotes cAMP production via a high affinity form of the receptor. In addition, it also seems likely that it is the $\gamma$c subunit itself which is responsible. IL-2 and IL-7, as described previously (Chapter 3), were found to be incapable of inducing CD25 expression in resting, human B cells. Therefore, if the premise is that cAMP generation is linked to $\gamma$c then, while it is necessary for induction of CD25, the generation of cAMP alone is insufficient for up-regulating the expression of CD25.

### 4.2.3 IL-13 Does Not Induce cAMP Production in Resting B Cells

It has been reported that IL-4 and IL-13 share certain common cellular functions [Keegan *et al.*, 1995; Nilsson and Nilsson, 1995; Smerz-Bertling and Duschl, 1995] and a common receptor subunit [Zurawski *et al.*, 1993], initially proposed to be γc, but which increasing evidence suggests is the IL-4Rα chain. Unlike IL-4, IL-13 is unable to generate cAMP production in resting tonsillar B cells (Fig 4.6). This appears to promote the idea that γc is not a component of the IL-13R complex and provides further evidence that the ability of IL-4 to induce an increase in cAMP levels in B cells is at least partly a function of the γc subunit of the receptor. It is known that certain signal transduction pathways induced by IL-4 and which are a feature of the IL-4Rα chain, are also common to IL-13-induced signal transduction [Keegan *et al.*, 1995]. If the hypothesis is that γc is responsible for generating cAMP and IL-4 induces CD25 expression via the low affinity receptor, the question then arises, how is artificially generating cAMP by stimulating B cells with forskolin involved in increasing expression of the CD25 gene (Figures 3.5 and 3.6)? As IL-13 is unable to induce an increase in surface CD25, it would appear that signalling pathways shared by IL-4 and IL-13 are insufficient to promote increased expression of particular genes and it is possible that a combination of signalling pathways generated by both γc and the IL-4Rα chain are required for induction of CD25. If this latter suggestion is the case then it would account for the ability of elevated cAMP levels to up-regulate CD25 at the cell surface.

### 4.2.4 IL-4 Induces PKA Activation in Resting B Lymphocytes

The data reported above give a strong indication that IL-4 regulates transcription factor binding to NRE in the CD25 promoter in human B cells via a cAMP generating pathway and the activation of PKA. To determine directly that IL-4 activates PKA, a target peptide phosphorylation assay was carried out and preliminary results revealed that PKA activity is indeed activated by IL-4 stimulation. The data presented here demonstrate

PKA activity in extracts made from B cells stimulated for 5 and 25 minutes, with and without IL-4, where stimulation with IL-4 shows a marked increase in PKA activity compared with unstimulated controls (Fig 4.7). These data are in good accordance with the biphasic cAMP production observed in response to IL-4 stimulation, with PKA activation being evident at time points where cAMP generation is clear. However, this preliminary data, while interesting, will need to be backed up by an extensive 60 minute time course.

## 4.3 Transcription Factor Binding to the Negative Regulatory Element of the CD25 Promoter

### 4.3.1 IL-4 Induces a Decrease in NRE Transcription Factor Binding Activity

An 11 base pair core region of the CD25 promoter is known to profoundly negatively regulate CD25 expression in human T cells [Ballard *et al.*, 1989; Lowenthal *et al.*, 1988; Smith and Greene, 1989]. Transfection of Jurkat cells with CD25 promoter-CAT constructs demonstrated that a deletion mutant lacking NRE (pΔNRE) induced a 6-fold increase in basal reporter activity, compared with the full length CD25 promoter, and a 12-fold increase following stimulation with PMA [Burlinson *et al.*, 1996]. The presence of protein transcription factors capable of binding NRE in resting human B cells and their sensitivity to modulation by IL-4 was assessed. As shown in figure 4.8., resting tonsillar B lymphocytes possess binding activity for the NRE oligonucleotide and the capacity of this protein (or proteins) to bind the oligonucleotide is attenuated by IL-4 stimulation, over a four hour period. There are two mobility shift bands appearing on the EMSA autoradiograph which, as the nature of NRE binding protein(s) remains uncharacterized, could possibly be attributed to the formation of homo- or heterodimeric complexes. However, the data presented suggest that it is the slowest of the two mobility shift bands which is the most sensitive to the effects of IL-4, with almost total attenuation of the DNA binding activity after 4 hours of cytokine stimulation. The

specificity of binding is demonstrated in figure 4.9 where unlabelled NRE oligonucleotide acted as a specific competitor for NRE binding, with particularly pronounced affects on the slowest mobility shift band. In contrast, an excess amount of unlabelled Oct 1 oligonucleotide was unable to compete with NRE for protein binding. The ability of IL-4 to attenuate the activity of protein(s) binding to the NRE site might partially explain the ability of IL-4 to up-regulate CD25 expression in human B lymphocytes; thus, this effect in combination with increased binding at positive regulatory elements, such as the IL-4 -Response Element, could facilitate expression of CD25.

### 4.3.2 Forskolin and Cholera Toxin Induce a Decrease in NRE Binding Activity

Stimulation of B cells with agents which activate intracellular signalling pathways can induce expression of the CD23 and CD25 proteins, in a manner analogous to IL-4 itself. In particular forskolin, which acts directly on the catalytic subunit of adenylyl cyclase thereby activating the enzyme and inducing an increase of intracellular cAMP [Seamon and Daly, 1981; Whetton *et al.*, 1983], is able to induce modest levels of CD25 expression at the B cell surface [McKay and Cushley, 1995]. However, this apparent relationship between cAMP generation and CD25 up-regulation appears at odds with the data suggesting that cAMP production is linked to γc and that increased CD25 expression is mediated via a low affinity (γc lacking) form of the IL-4 receptor. It was therefore of considerable interest to try and establish what relationship, if any, might exist between increasing intracellular cAMP levels and expression of the CD25 gene, particularly with respect to regulation of transcription factor binding. The effects of forskolin and cholera toxin upon NRE binding activity in B cells was tested. Protein extracts prepared from cells after four hours incubation in medium alone showed no significant decrease in NRE binding activity (Fig 4.10.A), while those exposed to IL-4 over the same period of time showed the expected loss of activity (4.10 B). Treatment of resting human B cells with forskolin resulted in a similar decrease in protein binding activity for the NRE

oligonucleotide over a four hour time course (Fig 4.10 C). This proposal is supported by the observation that cholera toxin (CT), the A subunit of which ADP ribosylates the Gsα protein (thereby activating adenylyl cyclase leading to an increase in intracellular cAMP) caused a similar decrease in NRE binding activity in B cells over a period of four hours (Fig 4.10 D). These data suggest that an elevation of intracellular cAMP levels results in decreased binding of a transcription factor at the negative regulatory element of the CD25 promoter, an idea which supports the observations that artificially inducing cAMP production by stimulation with forskolin results in elevation of CD25 upon the surface of the cell.

### 4.3.3 Inhibition of PKA and Tyrosine kinases but not PKC Prevents IL-4-Induced Attenuation of NRE Binding Activity

The accepted paradigm for the mechanism of action of cAMP in any cell type is its ability to activate a cAMP-dependent protein kinase (PKA). The observations that IL-4 promotes both cAMP generation and PKA activation and that artificially inducing cAMP production mimics the ability of IL-4 to reduce transcription factor binding to the NRE in the CD25 promoter, suggest that IL-4 is acting upon this element via a cAMP generating pathway. Therefore, to try and establish direct links between IL-4 binding to its receptor and the evident decrease in transcription factor binding to NRE, protein extracts were made from IL-4-treated resting B cells which had been pre-treated with a range of of protein kinase inhibitors (Fig 4.11). As shown previously, untreated controls (Fig 4.11 A) show no decrease in NRE binding activity over a four hour period compared with IL-4 treated extracts, which demonstrate a striking loss in binding activity (Fig 4.11 B). However, pre-treatment of B cells with H89, a potent and selective inhibitor of PKA with a Ki of 0.048μM [Chijiwa *et al.*, 1990; Geilen *et al.*, 1992], blocks the ability of IL-4 to cause attenuation of NRE binding activity over a 4 hour period (Fig 4.5.C). This observation provides further evidence in favour of the hypothesis that IL-4, by inducing cAMP production (probably by activating adenylate cyclase) and in turn activating PKA, exerts its effects upon NRE via a cAMP-dependent pathway. The activated catalytic

subunit of PKA may then either function by acting directly upon the transcription factor which binds NRE, or by phosphorylating some intermediate protein component in the pathway which ultimately influences NRE binding activity.

It has been observed that PKC has an involvement in the up-regulation of CD25 in human B cells, with respect both to the ability of phorbol ester treatment to elevate cell surface levels of CD25 and the attenuation of IL-4's ability to up-regulate CD25 following down-regulation of PKC (Chapter 3). In addition, it has been shown that IL-4, upon binding to its receptor, activates the protein tyrosine kinases Jak1 [Russell *et al.*, 1993; Witthuhn *et al.*, 1994] and Jak3 [Yin *et al.*, 1994]. There is the question, therefore, of the role that these second messengers play in IL-4 induced expression of CD25. Are they involved in regulating the cAMP/PKA pathway or do they act purely as independent signalling pathways which affect the CD25 promoter? IL-4 is known to activate STAT 6 via the IL-4Rα chain and phosphorylation of Jak1 [Hou *et al.*, 1994; Yin *et al.*, 1994] and the importance of this with respect to the CD25 promoter will be addressed in chapter 5. However, in an attempt to establish the involvement of PKC and potential protein tyrosine kinase activity in the regulation of transcription factor binding to NRE, resting B cells were pre-treated with PKC and protein tyrosine kinase inhibitors prior to treatment with IL-4. DNA mobility shift assay with extracts from these cells demonstrated that B cells pre-treated with the PKC inhibitor *bis*-indolylmaleimide (BIM) retained the capacity to attenuate NRE binding activity in response to IL-4 (Fig 4.11 D). In contrast, B cells pre-treated with the tyrosine kinase inhibitor genistein lost the capacity to decrease NRE binding activity in response to IL-4 (Fig 4.11 E). These data indicate that while PKC is not involved in IL-4 regulation of NRE binding activity, tyrosine kinase activity plays some role in this pathway, but what this latter activity is remains to be elucidated. However, it may be assumed that while the activity of PKC obviously has a role in IL-4 signal transduction and CD25 expression (Figures 3.5, 3.6, 3.7 & 3.8), it is independent from IL-4 regulation of NRE protein binding activity.

### 4.3.4 The NRE Element in the CD25 Promoter is not a Classical cAMP Responsive Element

A number of genes are sensitive to regulation by changing cAMP levels through a cis-acting DNA sequence known as the cAMP response element (CRE) [Macchi *et al.*, 1995; Montminy and Bilezikjan, 1987; Montminy *et al.*, 1990]. There are a number of different regulatory proteins which bind to CRE; however, the CREB family in particular appears to be regulated by phosphorylation by PKA [Habener, 1990]. The pattern of regulation of CRE by this family of proteins is usually one of transcriptional activation, i.e., they are positive regulatory elements whose binding to CRE is up-regulated by PKA induced phosphorylation. On the other hand, the situation with respect to NRE is one of cAMP/PKA inducing transcriptional activation by removing a negatively regulating factor from the promoter region. A competition assay, using an excess amount of unlabelled CRE oligonucleotide, demonstrated that this particular sequence was unable to compete for protein binding to NRE in human B cell extracts (Fig 4.12). This indicates that NRE is not regulated by a member of the CREB family of transcription factors. Indeed, if one examines the sequence of NRE (shown below) in the CD25 Promoter it bears little homology to the classical CRE sequence motif (CRE core element is underlined).

NRE 5' TT CAT CCC GGG 3'

CREB 5' AGA GAT TGC CTG ACG TCA GAG AGC TAG 3'

This occurrence of a cAMP/PKA-regulated transcription factor which is not a member of the CREB family is not extraordinary, as a new motif (a 9 base pair imperfect direct repeat, 5'-CCG CTG CCC-3', and an inverted CCAAT box, 5'-ATTGG-3') distinct from CRE, which is transcriptionally regulated by cAMP, has recently been reported in the promoter of the human tryptophan hydroxylase gene [Boularand *et al.*, 1995].

### 4.3.5 IL-2, IL-7 and IL-13 Do Not Induce a Decrease in Transcription Factor Binding to NRE

The apparent regulation of transcription factor binding to NRE in the CD25 promoter in human B cells by elevation of intracellular cAMP levels and PKA activation, raises the question of whether other lymphoid sub-family cytokines may have similar effects in human B cells. Neither IL-2, IL-7 nor IL-13 elevate CD25 expression in B cells; however, IL-2 and IL-7 do induce cAMP generation and their receptors do share the γc subunit with the IL-4 receptor. Therefore, it was a possibility that these two cytokines were able to regulate protein binding to NRE, but that this signal alone was insufficient to drive CD25 expression and that a decreased binding of NRE, together with other intracellular signals generated uniquely by IL-4, were required to drive transcription. Also, it was necessary to ask whether the inability of IL-13 to induce cAMP production, and the lack of γc in its receptor, was an impediment to its regulation of transcription factor binding to NRE. The results show that none of these cytokines are able to induce a decrease in DNA-protein interaction at NRE over a four hour period (Fig 4.13 B, C & D): this is in contrast with IL-4 which diminishes transcription factor binding to NRE almost entirely (Fig 4.13 A). These data are interesting in that they suggest that IL-4 alone is able to regulate DNA-protein binding activity at NRE, despite the capacity of IL-2 and IL-7 to generate cAMP in human B cells.

### 4.3.6 IL-4-Induced cAMP Generation is Regulated by Adenylyl Cyclase Activation rather than by PDE Inhibition

It is recognized that elevation of intracellular cAMP levels can be induced either by activation of adenylyl cyclase activity or by inhibition of phosphodiesterase (PDE) activity; cAMP is degraded to 5' AMP by phosphodiesterase enzymes. There are eight recognized PDE isoforms which have distinct sequences and are subject to different mechanisms of regulation. PDE inhibitor studies were used in order to determine whether the IL-4-induced cAMP generation which attenuates transcription factor binding to NRE is regulated by activation of adenylyl cyclase activity or by inhibition of PDE

activity. IBMX is an general inhibitor of most PDE isoenzymes; thus resting tonsillar B cells were treated with either IBMX or IL-4 for four hour periods and nuclear extracts, made at two hourly intervals, were examined for NRE binding activity. As observed previously, IL-4 induces a decrease in NRE binding activity over a period of four hours (Fig 4.14 A), whereas IBMX was unable to induce a similar decrease transcription factor binding to NRE oligonucleotide (Fig. 4.14 B). These data indicate that inhibition of PDE activity in human B cells is unable to promote sufficient cAMP generation to induce a decrease in NRE binding activity, therefore, it seems likely that IL-4-induced cAMP generation is regulated by activation of one or more isoforms of adenylyl cyclase.

## 4.4 Regulation of NRE Protein Binding Activity in EDR B Cells

### 4.4.1 Induction of CD25 Expression in EDR B Cells

Despite the ability of IL-4 to induce CD25 expression in primary, resting human B cells, the contrary has been true for most of the human B cell lines tested. In particular, the mature EBV transformed B cell line EDR was found to be incapable of responding to IL-4 by up-regulating CD25 at the cell surface. It was considered a possibility that certain elements of the intracellular signalling machinery were inactive, perhaps as a result of the transformation process; whether such lesions are at the level of receptor coupling or transcriptional regulation remains equivocal. However, co-stimulation of EDR B cells with IL-4 and forskolin revealed that when these cells are stimulated with a single concentration of IL-4 in conjunction with increasing levels of forskolin they respond by increasing cell surface expression of CD25 in a dose dependent manner (Fig 4.15 A). Similarly when these cells are stimulated with a high concentration of forskolin the response to IL-4 becomes dose dependent (Fig 4.15 B).

### 4.4.2 IL-4 Induced cAMP Production in EDR B Cells

Taking the above results into account, it seemed possible that the inability of IL-4 to induce CD25 might be coupled to a defect in cAMP production in EDR B cells. In fact IL-4 does induce cAMP generation in the EDR B cell line, but this response is extremely low, approximately 10-fold less (Fig 4.16), in comparison to the response observed in primary human B cells (Fig. 4.1). The lack of an IL-4-induced cAMP response of the magnitude seen in primary cells could account for the inability of IL-4 to up-regulate CD25 at the cell surface of EDR B cells; this interpretation is in agreement with the capacity of forskolin to restore induction of CD25 expression when used as a co-stimulant with IL-4.

### 4.4.3 Regulation of Transcription Factor Binding to NRE in EDR B Cells

In primary B cells, IL-4 is able to induce CD25 expression and a reduction in transcription factor binding to the Negative Regulatory Element (NRE) in the CD25 promoter. It seems likely that a decrease in DNA-protein interactions at NRE is regulated via a cAMP/PKA pathway and that this phenomenon is at least partially responsible for the ability of IL-4 to induce CD25 expression. In contrast, CD25 up-regulation is not observed in the EDR B cell line upon stimulation with IL-4 and IL-4-induced cAMP generation is severely diminished in this cell line, as compared with primary B cell responses. It is possible, therefore, that these two factors are linked by a lack of IL-4-induced reduction of NRE transcription factor binding in EDR B cells. Indeed, the DNA mobility shift data indicate that IL-4 is unable to inhibit DNA-protein interactions at NRE over a four hour period in EDR B cells (Fig 4.17.A), whereas in primary B cells such stimulation leads to an almost total abrogation of transcription factor binding to NRE (Fig 4.8). The converse is true for forskolin treatment of these cells; as with the primary B cells, forskolin treatment leads to almost total loss of protein binding to this element after four hours. Therefore, it would appear that the reduced capacity of IL-4 to induce cAMP production in EDR B cells is responsible for the loss of ability to down-regulate protein binding to the NRE in the CD25 promoter - an effect which prevents IL-4 up-regulation

of CD25 - and that this discrepancy can be overcome by artificially elevating intracellular cAMP using forskolin (Fig 4.17 B).

### 4.4.4 Transcriptional Activation of the CD25 Promoter

The data presented so far support the hypothesis that transcription factor binding to a negative regulatory element in the CD25 promoter prevents expression of the gene. In human B cells IL-4 is responsible for control of this binding activity by down-regulating it via a cAMP-generating pathway. However, the question of the significance of this event with respect to transcriptional activation has remains unaddressed. Is the removal of this negative influence sufficient to drive transcriptional activation or is it one of a number of factors required for successful expression of the CD25 gene? In an attempt to address these questions it was necessary to look directly at activation of transcription, using a CD25 promoter-CAT reporter gene construct. Due to the difficulties of transfecting primary, resting human B cells, the EDR B cell line was chosen as a model system. Additionally, as this cell line only gives a cellular response to IL-4 (i.e., cell surface CD25 expression) when co-stimulated with forskolin, it provides an ideal system for studying the importance of elevating intracellular cAMP with respect to transcriptional activation. Transfected cells which had been stimulated with IL-4 for 48 hours showed no up-regulation of CD25 at the plasma membrane, as judged by flow cytometry; in contrast, forskolin stimulation of transfected cells showed increased CD25 expression and this response was enhanced when transfected cells were co-stimulated with IL-4 and forskolin (Fig 4.18 A). Measurement of CAT reporter gene activity demonstrated that, although there was no real difference between cells treated with and without IL-4, the forskolin stimulated cells showed considerably increased reporter activity, compared with unstimulated transfected cells. Cells which had been co-stimulated with IL-4 and forskolin showed enhanced reporter activity, a response which appears to be synergistic (Fig 4.18 B & C). These latter data are in very good agreement with the cell surface data of Figure 4.18 A and suggest that while cAMP-induced down-regulation of protein binding at NRE is able to drive some expression of the CD25 gene, this response, in

combination with other unknown signalling pathways also activated by IL-4, is necessary for maximal transcriptional activation. In other words, IL-4-induced cAMP generation is a necessary but insufficient signal required to drive CD25 gene expression.

## 4.5 Discussion

The results presented in this chapter demonstrate that IL-4 is able to induce a decrease in DNA-protein binding activity at the negative regulatory element in the CD25 promoter, and that this effect occurs over a four hour time period, a result which is consistent with the initial appearance of CD25 at the plasma membrane [Butcher *et al.*, 1990]. In addition, it is possible to mimic this decrease in transcription factor binding by stimulating the cells with forskolin or cholera toxin, indicating that an increase in intracellular cAMP is able to regulate binding to NRE. This finding was of particular interest, as IL-4 has been shown to induce cAMP production in human B cells, an observation which suggests a link between IL-4-induced cAMP generation and inhibition of protein binding to NRE. Using a PKA inhibitor, H89, it was possible to demonstrate this link more directly, as PKA inhibition results in total abrogation of the ability of IL-4 to reduce DNA-protein interactions at NRE. Conversely, inhibition of PKC had no such inhibitory effects, suggesting that if PKC is involved in IL-4 signal transduction then it exerts its effects through an entirely independent pathway. These results are in very good accordance with the ability of IL-4 to elevate PKA activity in resting human B cells; however, the question of how PKA acts upon NRE binding proteins remains to be elucidated. CREB is known to be one of the major cellular targets for PKA and its activation by phosphorylation of the amino acid Ser133 renders it transcriptionally active [Habener, 1990; Montminy and Bilezikjan, 1987]. The inability of CRE DNA sequences to compete for NRE binding activity in human B cells, would appear to eliminate CREB as a candidate for an NRE binding transcription factor. However, other putative means of PKA regulating transcription have been put forward, including the action of the

regulatory (RII) subunit of the enzyme acting directly as a regulator of transcription [Constantinou *et al.*, 1985; Nagamine and Reich, 1985] and also the identification of a new cAMP responsive element in the tryptophan hydroxylase gene promoter [Boularand *et al.*, 1995]. Mutational analyses of two short sequences within this element, a 9 base pair imperfect direct repeat (5'-CCG CTG CCC-3') and an inverted CCAAT box (5'-ATTGG-3'), revealed that point mutations within both abrogated gene transcription in response to cAMP. Both of these sequences are distinct from recognized CRE elements (5'- TG ACG TCA -3'), identifying the promoter region of this gene as a novel cAMP responsive element. The newly identified cAMP responsive element in the tryptophan hydroxylase gene promoter has a sequence which is distinct from the sequence of NRE (5'-TT CAT CCC AGG -3') thus, it would seem unlikely that the two were related; however, this does not discount the possibility of the existence of other unidentified cAMP/PKA-responsive transcription factors, which are structurally distinct from members of the CREB family. There is little information about the regulation of transcription factor binding to NRE in T cells. However, there exists an apparent dichotomy in the importance of this promoter element, with respect to CD25 expression, which seems to be dependent upon the developmental stage of T cells. Thus, in a cell line with an immature T cell phenotype (YT-1 cells) the removal of this element profoundly enhances both basal and PMA-inducible expression of CD25, whereas in a phenotypically more mature T cell line (Jurkat cells) a similar deletion has relatively little effect upon transcriptional activation [Lowenthal *et al.*, 1988]. The implications of these observations may be due to differences in developmental activation of T cells; e.g., the availability of a fully functional signalling pathway at a particular developmental stage or the differential expression of transcription factor proteins at each phase of development. Either way a more comprehensive knowledge of the underlying mechanisms which account for this diversity and its relevance to NRE binding, may assist in the understanding of regulation of an NRE-binding transcription factor. The importance of cAMP regulation of CD25 expression has been reported elsewhere, in as much that IL-1-induced cAMP generation in natural killer cells is responsible for transcriptional

regulation of the CD25 gene [Shirakawa and Mizel, 1989b]. However, there are two very important differences in this system compared with the data presented here. Firstly, IL-1-induced elevation of cAMP in these cells results in increased binding of a positive regulatory factor to the CD25 promoter [Shirakawa and Mizel, 1989b], whereas cAMP regulation of transcription factor binding to NRE seems to involve the removal of a negative regulatory factor. Secondly, the nature of the DNA binding factors themselves is profoundly different; IL-1 induced generation of cAMP results increased binding activity to an NF-κB site in the CD25 promoter [Shirakawa and Mizel, 1989b], an element which is both structurally distinct and spatially removed from NRE. However, it is a possibility that a common signalling pathway such as cAMP generation/PKA activation, may utilize different effector molecules, i.e., two entirely different transcription factors, in order to achieve a common function. The need for this diversity may arise from cell lineage-specific expression of, and therefore the availability of, specific transcription factors.

It is unlikely that IL-4-induced CD25 expression is determined solely by a cAMP generating pathway inducing a decrease in transcription factor binding to NRE. Rather, it is probable that this second messenger response is a necessary but insufficient signal for gene expression. The EDR B cell line, unlike primary B cells, does not up-regulate CD25 in response to IL-4. However, when these cells are co-stimulated with IL-4 and forskolin a substantial increase in cell surface expression of CD25 is observed. In addition, IL-4-induced cAMP generation in this cell line is greatly reduced in comparison to normal human B cells, an approximately 10-fold lower response, and DNA-protein binding interactions at NRE remain unaffected by stimulation of the cells by IL-4, but are abrogated by treatment with forskolin. The implication of these findings was that the cAMP response to IL-4 in EDR B cells was insufficient to induce a decrease in binding of transcription factors to NRE, but by artificially elevating cAMP levels it is possible to override this block in the signal transduction pathway and up-regulate CD25. Transient transfection of EDR B cells with a CD25 promoter-CAT reporter gene construct lends

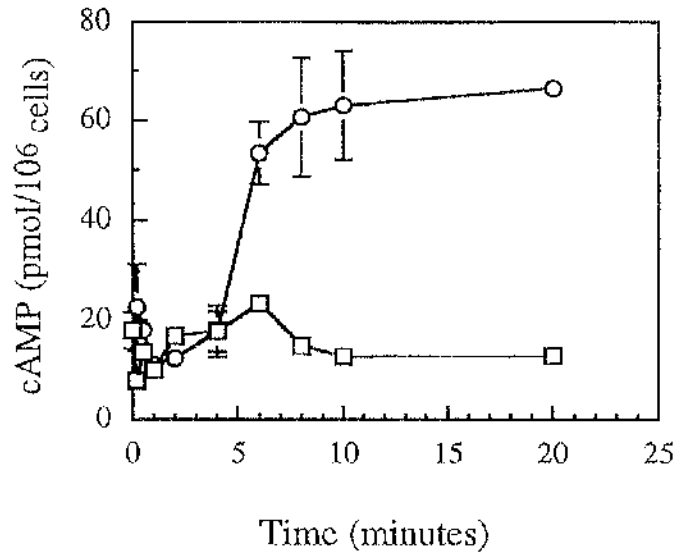
further support to this hypothesis, in that IL-4 is unable to drive transcription whereas a substantial increase in reporter activity is observed upon stimulation with forskolin and co-stimulation with IL-4 and forskolin results in an enhancement of this response. The apparently synergistic elevation in reporter activity in reponse to co-stimulation with IL-4 and forskolin, suggests the involvement of a second, or even third, IL-4-induced signalling pathway in increasing CD25 gene expression. There are a number of candidates for this secondary activation pathway, based on the presence of other known DNA binding elements in the CD25 promoter. In T cells, CD25 promoter deletion studies have highlighted a region required for transcriptional activation of the gene, which seems to be PMA-inducible. Recognized elements within this PMA-responsive region include an NF-κB site, an SRE and an Sp-1 binding site. In human B cells it has been demonstrated that there is no particular alteration in DNA binding activity to these elements in response to IL-4 as detected by DNA mobility shift assay [Burlinson, personal communication] cells involves the exchange of a negative regulatory complex (an NFκB p50 homodimer) for a positive regulatory complex (an NFκB p50-p60 heterodimer), at the NFκB binding site in the 5' promoter region of the gene [Algarte *et al.*, 1995; Pierce *et al.*, 1995]. In such a case a change in transcription factor binding may remain undetected by DNA mobility shift assay. Therefore, the possibility of the NFκB binding site of the CD25 promoter having a role in IL-4-induced gene transcription in human B cells remains equivocal. Finally, there are two putative IL-4 response elements in the CD25 promoter, one of them a Stat6 binding site, and these seem likely sites for IL-4-induced regulation of CD25 gene expression.

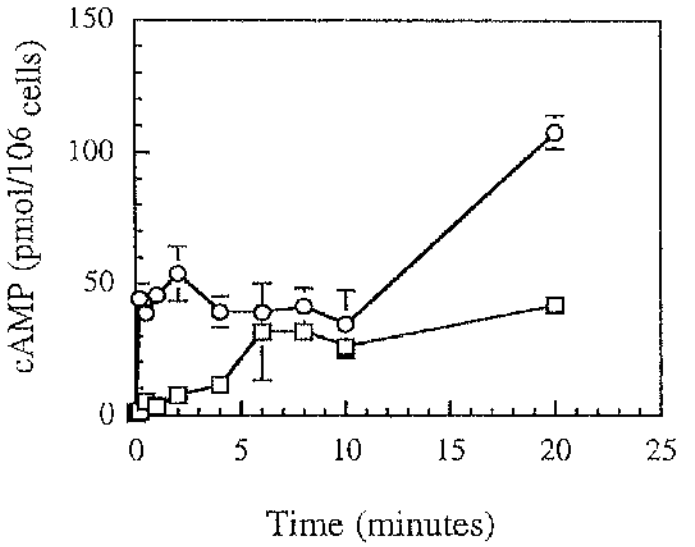
IL-2 and IL-7, but not IL-13, are able to induce cAMP generation in resting human B cells. Such a result, if one agrees with the hypothesis that IL-13 does not share γc as part of its receptor complex, suggests that cAMP generation is linked to the γc subunit of these lymphokine receptors. IL-2 and IL-7, unlike IL-4, are unable to induce a decrease in transcription factor binding to NRE, an event which appears to be regulated by a cAMP-dependent pathway. However, the inability of IL-2 and IL-7 to decrease NRE

binding, despite their ability to induce cAMP generation, is consistent with the inability of both these cytokines to up-regulate cell surface CD25 in human B cells. This result also highlights the ability of a particular cell stimulus to initially generate a ubiquitous second messenger pathway, which somehow translates into a highly specific response at a point much further downstream. This phenomenon is found throughout most known signalling events and remains relatively unexplained.

## 4.1 IL-4 Induces cAMP production in Resting tonsillar B Cells

High density tonsillar B cells (5 x $10^6$/ml) were incubated in complete medium for 1 hour prior to stimulation with IL-4 (100 units/ml) for the indicated time points, at 37 °C in a 5% $CO_2$, humid atmosphere. Reactions were stopped by the addition of 2% (v/v) PCA and 50µl aliquots were removed for analysis. Results are expressed as picomoles cAMP produced per $10^6$ cells: control cells - open squares; stimulated cells - open circles. Three independent repeats are shown.


80
60
40
20
0
cAMP (pmol/106 cells)
0
5
10
15
20
25
Time (minutes)


150
100
50
0
cAMP (pmol/106 cells)
0
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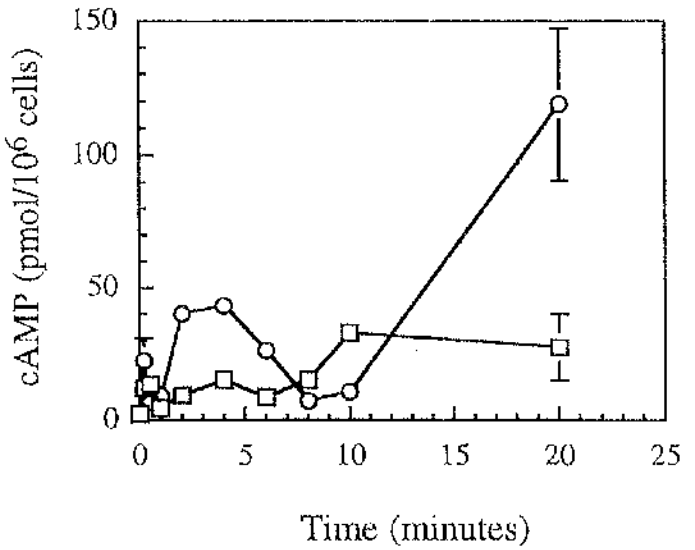
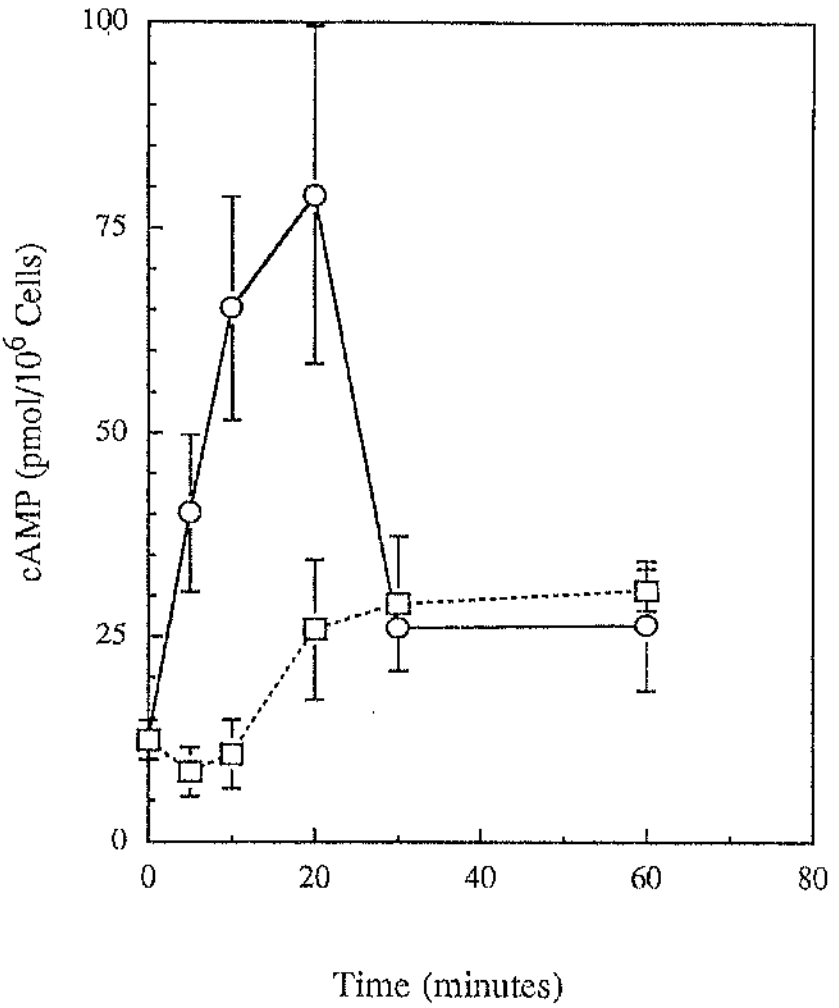
20
25
Time (minutes)


150
100
50
0
cAMP (pmol/10^6 cells)
0
5
10
15
20
25
Time (minutes)

## 4.2 IL-4 Induces a Sustained Production of cAMP over a 60 Minute Period

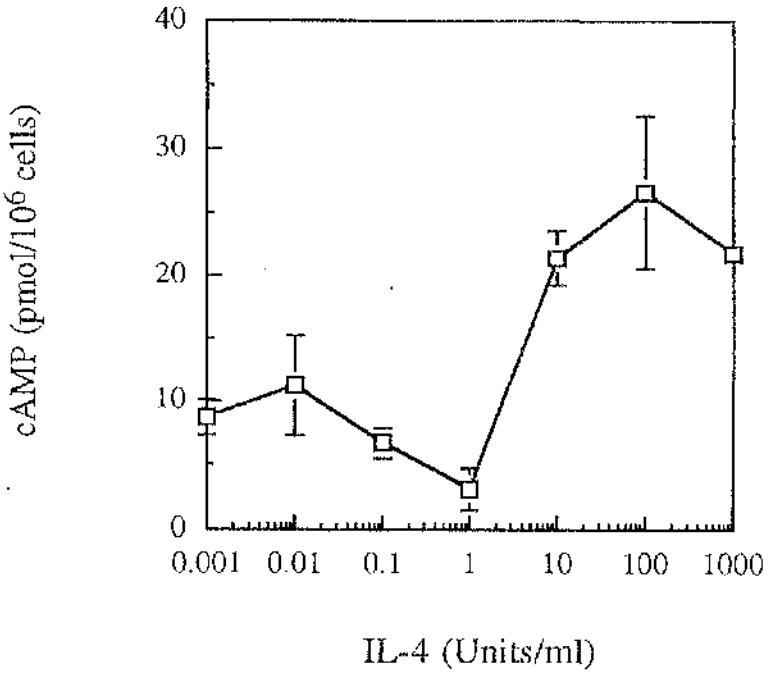
High density tonsillar B cells ($5 \times 10^6$/ml) were incubated in complete medium for 1 hour prior to stimulation with IL-4 (100 units/ml) for the indicated time points, at 37°C. Reactions were stopped by the addition of 2% (v/v) PCA and 50µl aliquots were removed for analysis. Results are expressed as picomoles cAMP produced per $10^6$ cells: control cells - open squares; stimulated cells - open circles.


100
75
50
25
0
cAMP (pmol/10^6 Cells)
0
20
40
60
80
Time (minutes)

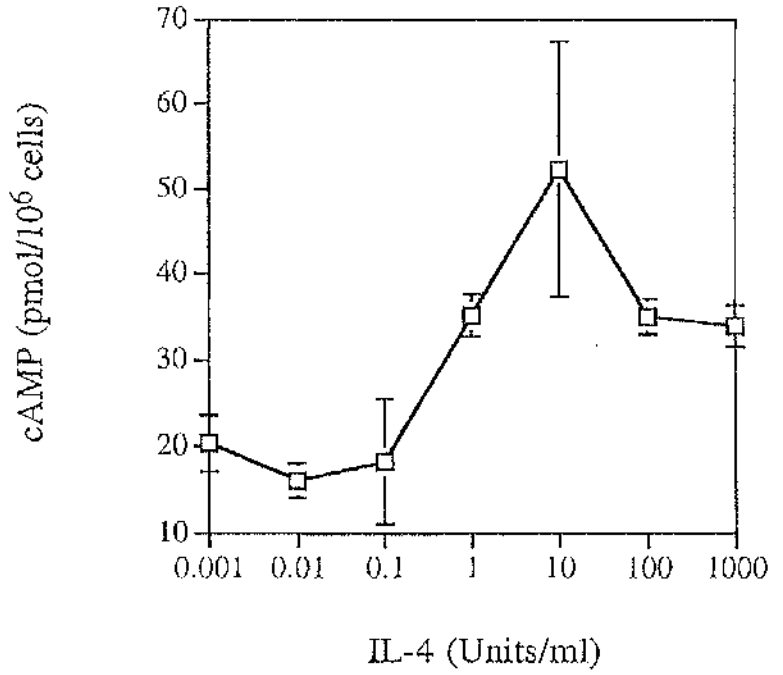
### 4.3 IL-4 Induced cAMP Production in Resting B Cells is Dose Dependent

High density tonsillar B cells (5 x $10^6$/ml) were incubated in complete medium for 1 hour prior to stimulation with the indicated concentrations of IL-4, for (A) 5 minutes and (B) 20 minutes, at 37°C. Reactions were stopped by the addition of 2% (v/v) PCA and 50µl aliquots were removed for analysis. Results are expressed as picomoles cAMP produced per $10^6$ cells.

A

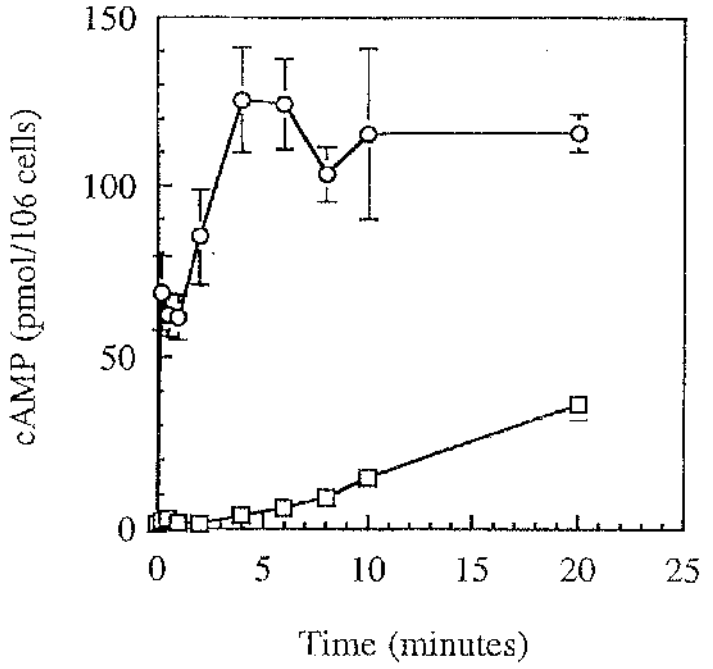

40
30
20
10
0
cAMP (pmol/10^6 cells)
0.001
0.01
0.1
1
10
100
1000
IL-4 (Units/ml)

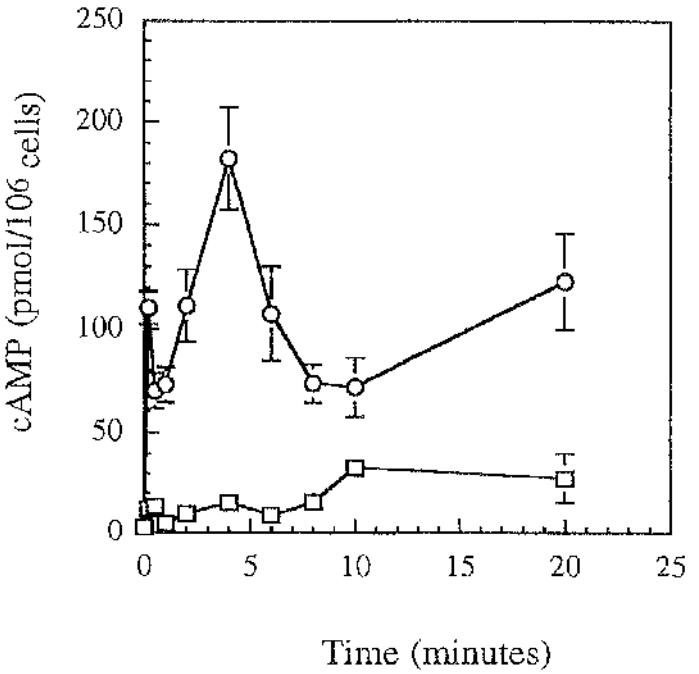
B


70
60
50
40
30
20
10
cAMP (pmol/10^6 cells)
0.001
0.01
0.1
1
10
100
1000
IL-4 (Units/ml)

## 4.4 IL-2 Induces cAMP Production in Resting Tonsillar B Cells

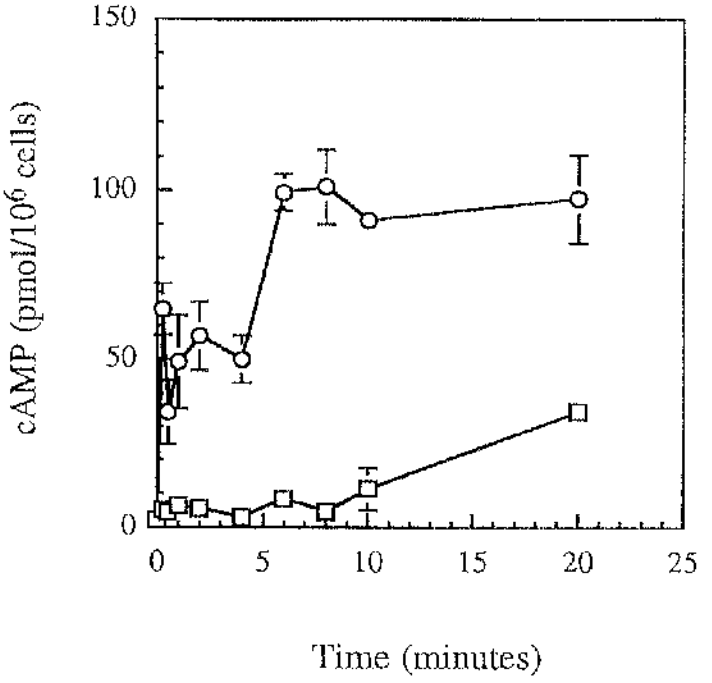
High density tonsillar B cells (5 x $10^6$/ml) were incubated in complete medium for 1 hour prior to stimulation with IL-2 (10ng/ml) for the indicated time points, at 37°C. Reactions were stopped by the addition of 2% (v/v) PCA and 50µl aliquots were removed for analysis. Results are expressed as picomoles cAMP produced per $10^6$ cells: control cells - open squares; stimulated cells - open circles. Two independent repeats are shown.

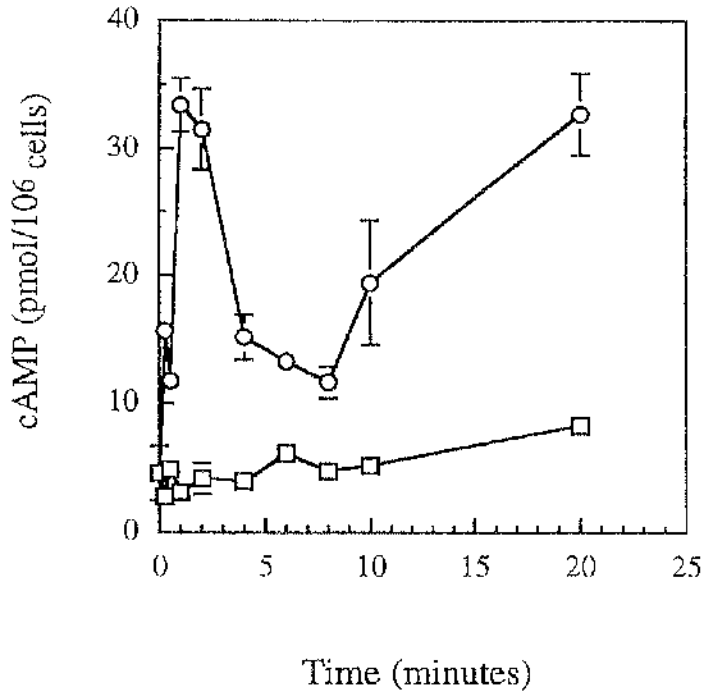

150
100
50
0
cAMP (pmol/106 cells)
0
5
10
15
20
25
Time (minutes)


250
200
150
100
50
0
cAMP (pmol/10^6 cells)
0
5
10
15
20
25
Time (minutes)

## 4.5 IL-7 Induces cAMP Production in Resting Tonsillar B Cells

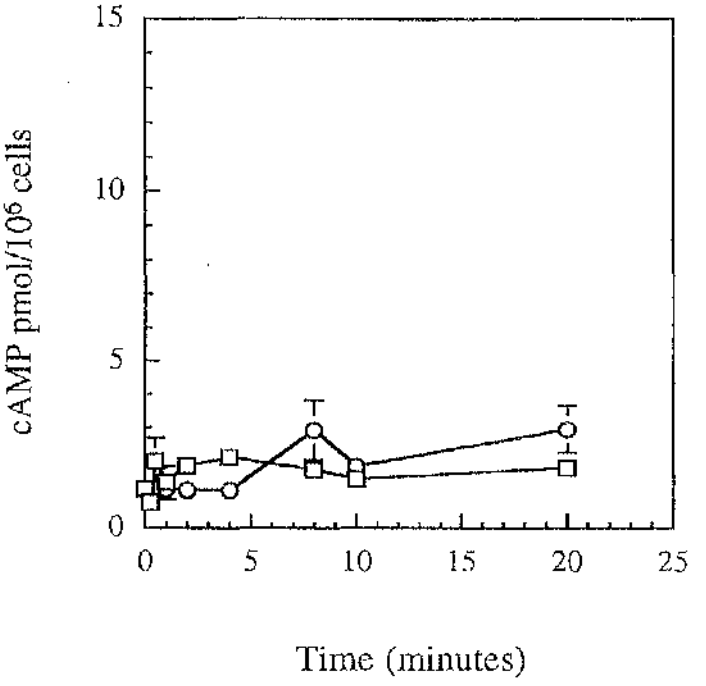
High density tonsillar B cells (5 x $10^6$/ml) were incubated in complete medium for 1 hour prior to stimulation with IL-7 (0.5ng/ml) for the indicated time points, at 37°C. Reactions were stopped by the addition of 2% (v/v) PCA and 50µl aliquots were removed for analysis. Results are expressed as picomoles cAMP produced per $10^6$ cells: control cells - open squares; stimulated cells - open circles. Two independent repeats are shown.

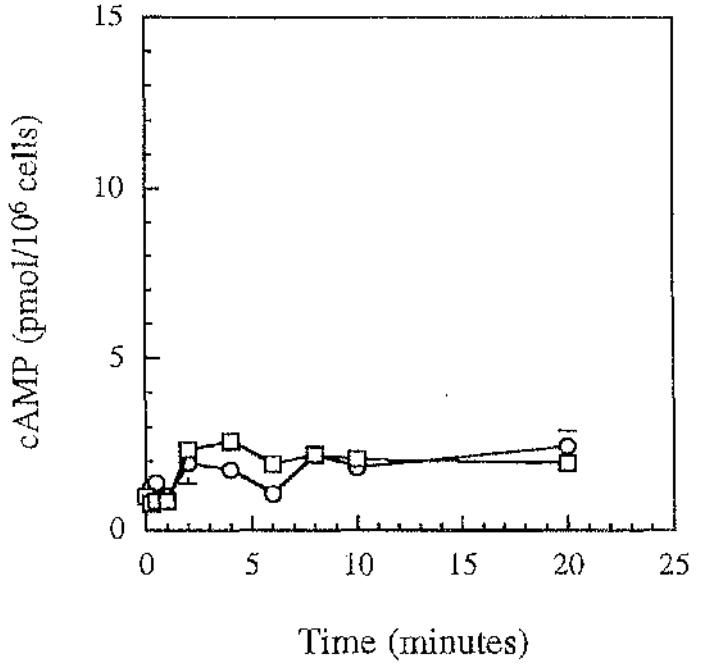

150
100
50
0
cAMP (pmol/10^6 cells)
0
5
10
15
20
25
Time (minutes)


40
30
20
10
0
cAMP (pmol/10^6 cells)
0
5
10
15
20
25
Time (minutes)

### 4.6 IL-13 Does Not Induce cAMP Production in Resting Tonsillar B Cells

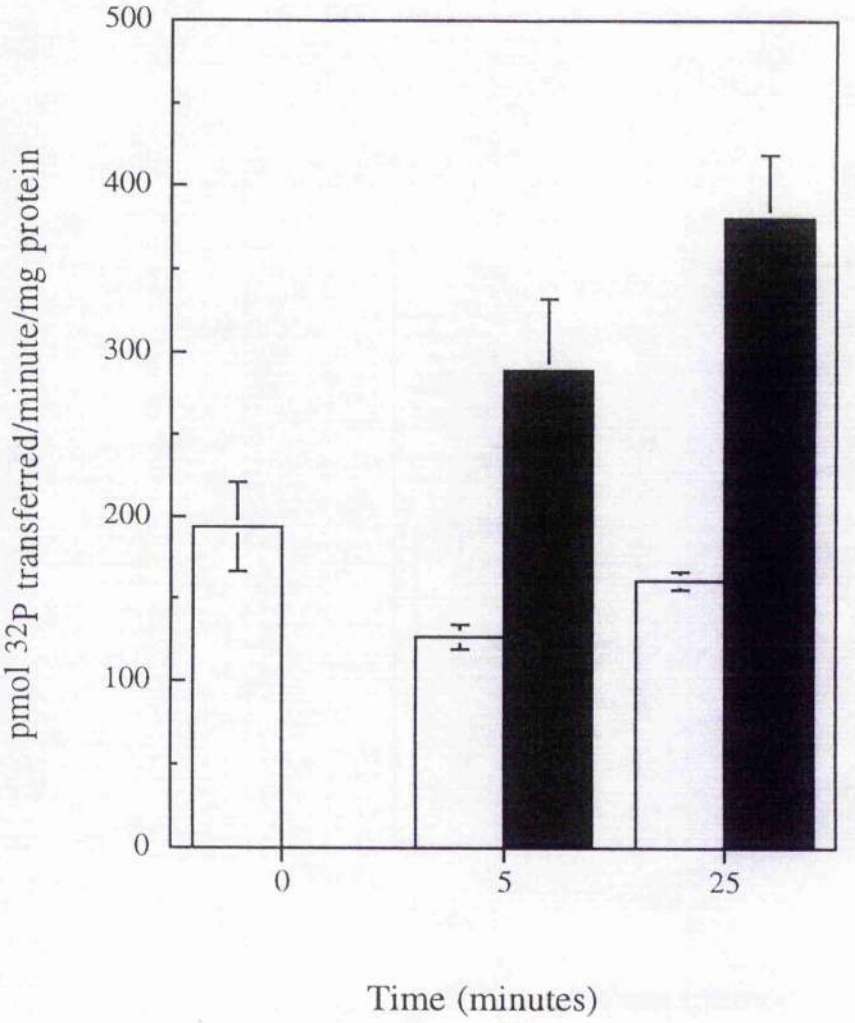
High density tonsillar B cells ($5 \times 10^6$/ml) were incubated in complete medium for 1 hour prior to stimulation with IL-13 (6ng/ml) for the indicated time points, at 37°C. Reactions were stopped by the addition of 2% PCA (v/v) and 50µl aliquots were removed for analysis. Results are expressed as picomoles cAMP produced per $10^6$ cells: control cells - open squares; stimulated cells - open circles. Two independent repeats are shown.


15
10
5
0
0
5
10
15
20
25
cAMP pmol/10⁶ cells
Time (minutes)


15
10
5
0
0
5
10
15
20
25
cAMP (pmol/10⁶ cells)
Time (minutes)

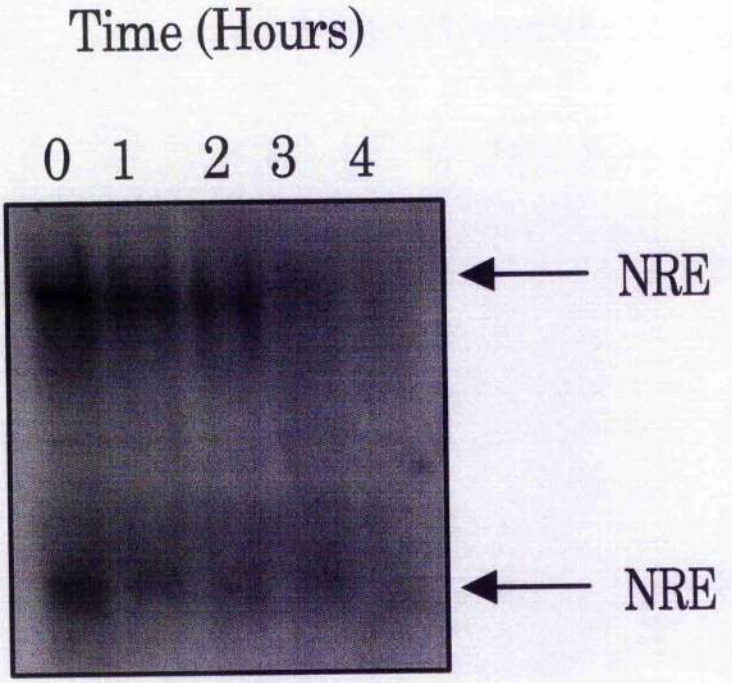
### 4.7 IL-4 Induces Activation of PKA in Resting Tonsillar B Cells

High density tonsillar B cells (5 x $10^6$/ml) were stimulated for 5 and 20 minute periods with IL-4 (100units/ml), at 37°C. Lysates were made at the appropriate time points from both treated and untreated cells and zero minutes, unstimulated cells. 25µl aliquots were incubated with 25µM cAMP, 100cpm/pmol $\gamma$-[$^{32}$P]-ATP/ATP mix, 0.5mM biotinylated PKA Peptide substrate and PKA assay buffer for 15 minutes. Reactions were terminated and aliquots transferred to streptavidin-coated discs, washed and counted by liquid scintillation spectrometry. Results are expressed as pmol $^{32}$P transferred/minute/mg protein: IL-4 treated B cells - black bars; unstimulated B cells - open bars.


500
400
300
200
100
0
pmol 32P transferred/minute/mg protein
0
5
25
Time (minutes)

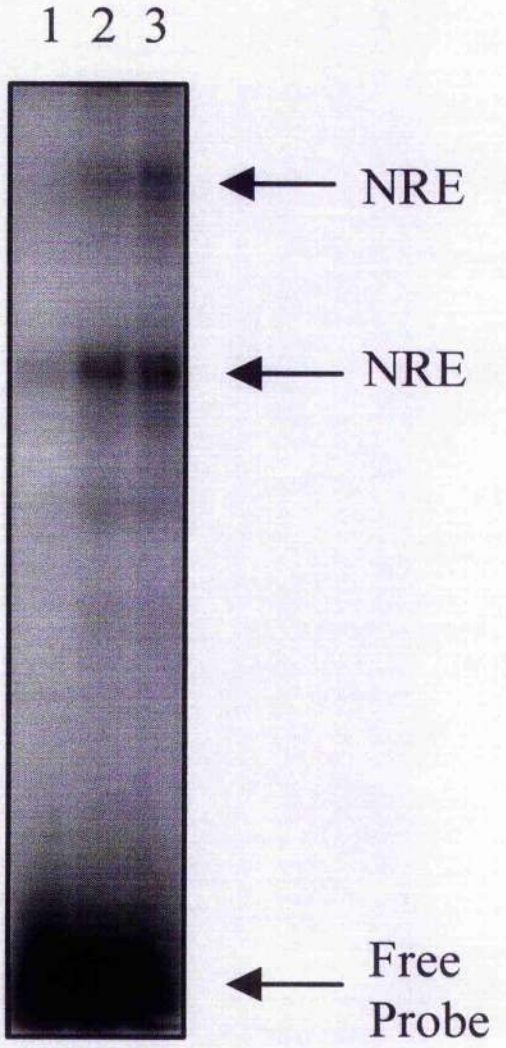
## 4.8 IL-4 Induces a Decrease in Transcription Factor Binding to NRE

High density tonsillar B cells (5 x $10^6$/ml) were incubated in complete medium at 37°C for 16 hours prior to stimulation with IL-4 (100 units/ml) for 4 hours. Nuclear extracts were made at hourly intervals and examined by DNA mobility shift, with 0.175pmol, $^{32}$P-labelled NRE oligonucleotide probe. The lane numbers at the top of the figure correspond to time of culture in the presence IL-4 in hours. Two distinct mobility shift bands are observed.


Time (Hours)
0 1 2 3 4
NRE
NRE

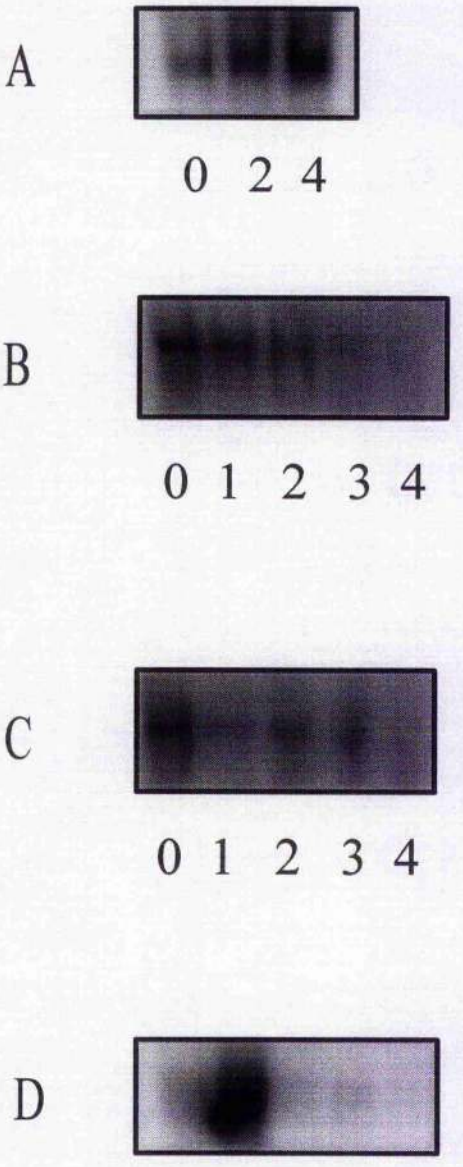
### 4.9 Specificity of NRE Binding Activities in Human B Cells

Nuclear extracts from resting tonsillar B cells were incubated with $^{32}$P-labelled NRE and excess, unlabelled NRE oligonucleotide (lane 1) or excess, unlabelled Oct 1 oligonucleotide (lane 2). Extracts were also incubated in the absence of any competing oligonucleotide (lane 3). Protein binding to NRE appears to be specific for both distinct mobility shift bands in human B cells.


1 2 3
NRE
NRE
Free
Probe

## 4.10 Forskolin and Cholera Toxin Mimic the Ability of IL-4 to Induce a Decrease in NRE Binding

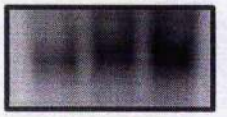
High density tonsillar B cells (5 x $10^6$/ml) were incubated in complete medium at 37 °C for 16 hours prior to incubation with (A) medium alone, (B) 100units/ml IL-4 (C) 100μM Forskolin or (D)1μg/ml Cholera Toxin for 4 hours. Nuclear extracts were made at hourly intervals and examined by DNA mobility shift, with 0.175pmol, $^{32}$P end labelled NRE oligonucleotide probe. The lane numbers at the bottom of the figure correspond to time (hours) in culture with or without stimulus.


A
0 2 4
B
0 1 2 3 4
C
0 1 2 3 4
D
0 1 2 3 4
Time (hours)

## 4.11 Inhibition of PKA and Protein Tyrosine Kinase activity, but not PKC Inhibits the Ability of IL-4 to Induce a Decrease in NRE Binding Activity

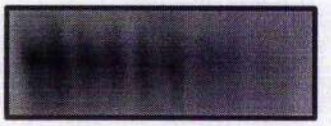
High density tonsillar B cells (5 x $10^6$/ml) were incubated in complete medium at 37°C prior to treatment. Cells were incubated with either (A) medium alone or (B) IL-4 (100 units/ml) for 4 hours, or pretreated (C) 50nM H89, (D) 100nM Bisindolylmaleimide or (E) 10μM Genistein for 30 minutes prior to stimulation with IL-4 (100 units/ml) for 4 hours. Nuclear extracts were made at hourly intervals and examined by DNA mobility shift, with 0.175pmol, $^{32}P$ labelled NRE oligonucleotide probe. The lane numbers at the bottom of the figure correspond to time of culture with or without IL-4 in hours.

A



0 2 4

B

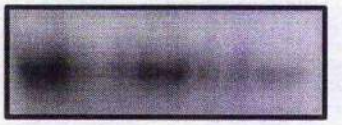


0 1 2 3 4

C

0 1 3 4

D



0 1 2 3 4

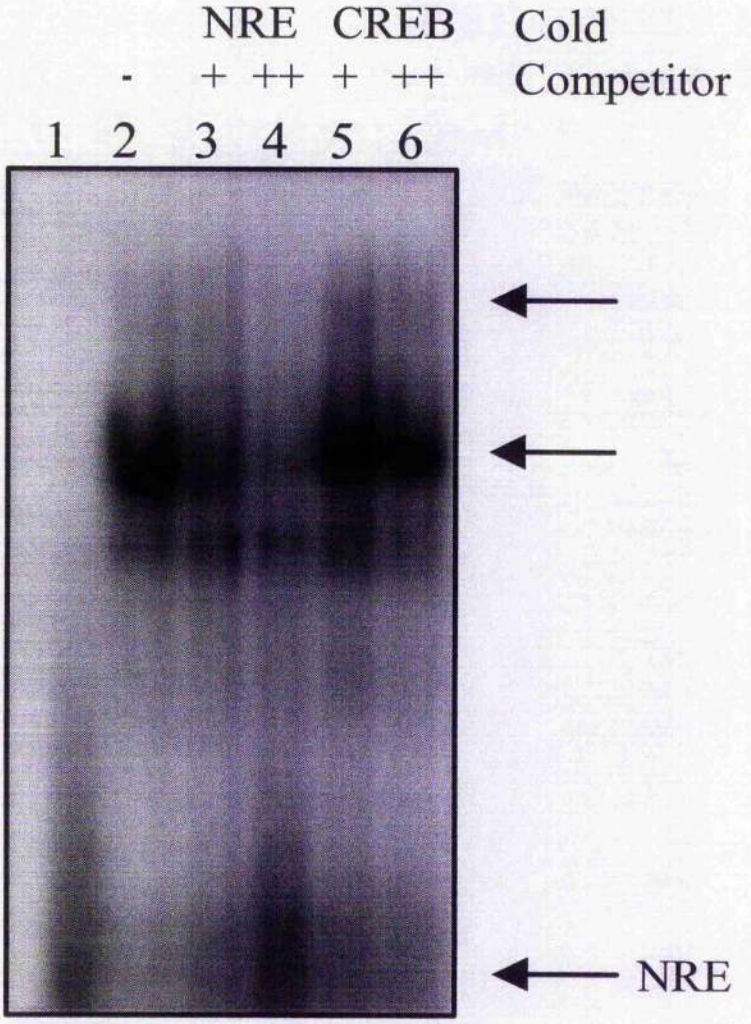
E

0 1 2 3 4

Time (hours)

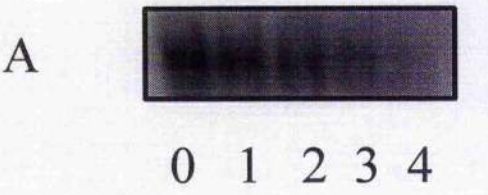
### 4.12 cAMP Response Element Does Not Compete for Binding With NRE

Nuclear extracts from high density tonsillar B cells were incubated with $^{32}$P-labelled NRE oligonucleotide and a 10-fold (+) or 100-fold (++) excess of either unlabelled NRE oligonucleotide (lanes 3 & 4) or unlabelled CREB oligonucleotide (lanes 5 & 6). Extracts were also incubated in the absence of any competing oligonucleotide (lane 2) and $^{32}$P-labelled NRE oligonucleotide was incubated in the absence of protein extracts (lane 1). Unlabelled NRE but not unlabelled CREB competes for protein binding with labelled NRE oligonucleotide.


NRE
CREB
Cold
Competitor
-
+
++
+
++
1
2
3
4
5
6
NRE

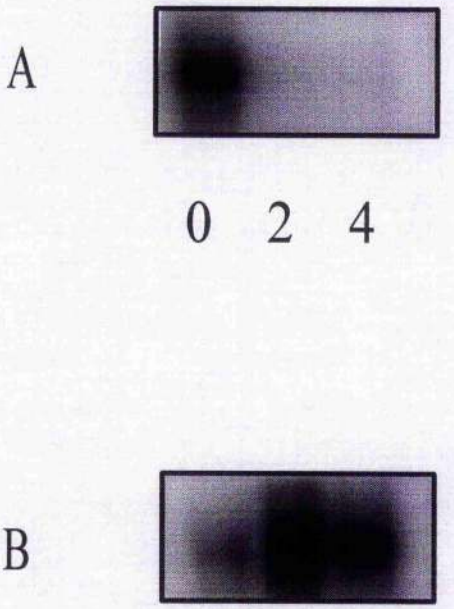
## 4.13 IL-2, IL-7 and IL-13 Do Not Affect Transcription Factor Binding to NRE

High density tonsillar B cells (5 x $10^6$/ml) were incubated in complete medium at 37°C for 16 hours prior to stimulation with (A) IL-4 (100units/ml), (B) IL-2 (10ng/ml), (C) IL-7 (0.5ng/ml) or (D) IL-13 (6ng/ml) for 4 hours. Nuclear extracts were made at hourly intervals and examined by DNA mobility shift, with 0.175pmol, $^{32}P$ labelled NRE oligonucleotide probe. The lane numbers at the bottom of the figure correspond to time of culture with each cytokine, in hours.


A
0 1 2 3 4
B
0 1 3 4
C
0 1 3 4
D
0 1 2 3 4
Time (hours)

### 4.14 Inhibition of PDE Activity Does Not Attenuate NRE Binding Activity

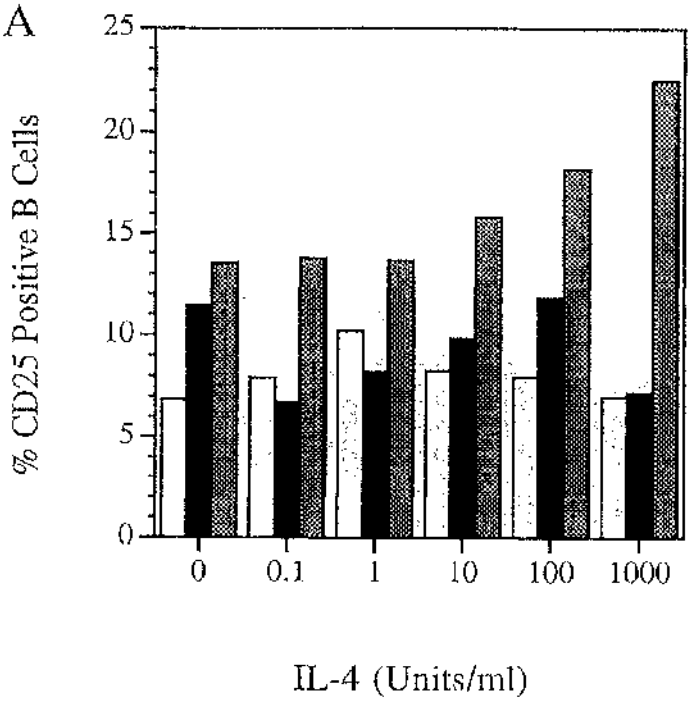
High density tonsillar B cells (5 x $10^6$/ml) were incubated in complete medium at 37°C for 16 hours prior to stimulation with (A) IL-4 (100units/ml), (B) 1μM IBMX for 4 hours. Nuclear extracts were made at two hourly intervals and examined by DNA mobility shift, with 0.175pmol, $^{32}P$ labelled NRE oligonucleotide probe. The lane numbers at the bottom of the figure correspond to time of culture with IL-4 in hours.


A
0 2 4
B
0 2 4
Time (hours)

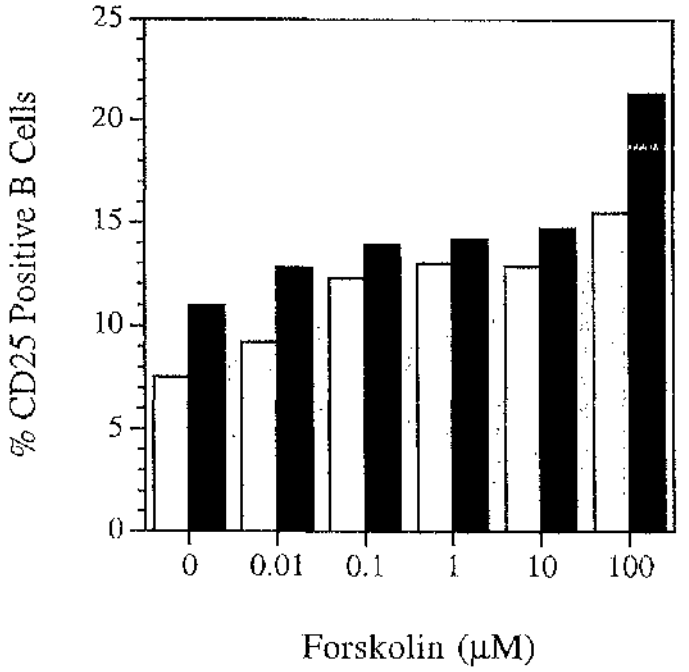
## 4.15 Costimulation of EDR B Cells with IL-4 and Forskolin Induces CD25 Expression

EDR B cells (1 x $10^6$ cells/ml) were resuspended in fresh, complete medium for 24 hours prior to stimulation with either (A) the indicated concentrations of IL-4 alone (white bars), or in the presence of 10μM (black bars) or 100μM (grey bars) forskolin or (B) the indicated concentrations of forskolin in the absence (white bars) or presence (black bars) of 400units/ml IL-4 , at 37°C, 5% $CO_2$ for 48 hours. Following incubation with IL-4 cells were simultaneously stained with FITC-anti-CD25 and PE-anti-CD19 monoclonal antibodies and analysed by flow cytometry. Results are expressed as % CD25 positive cells

A

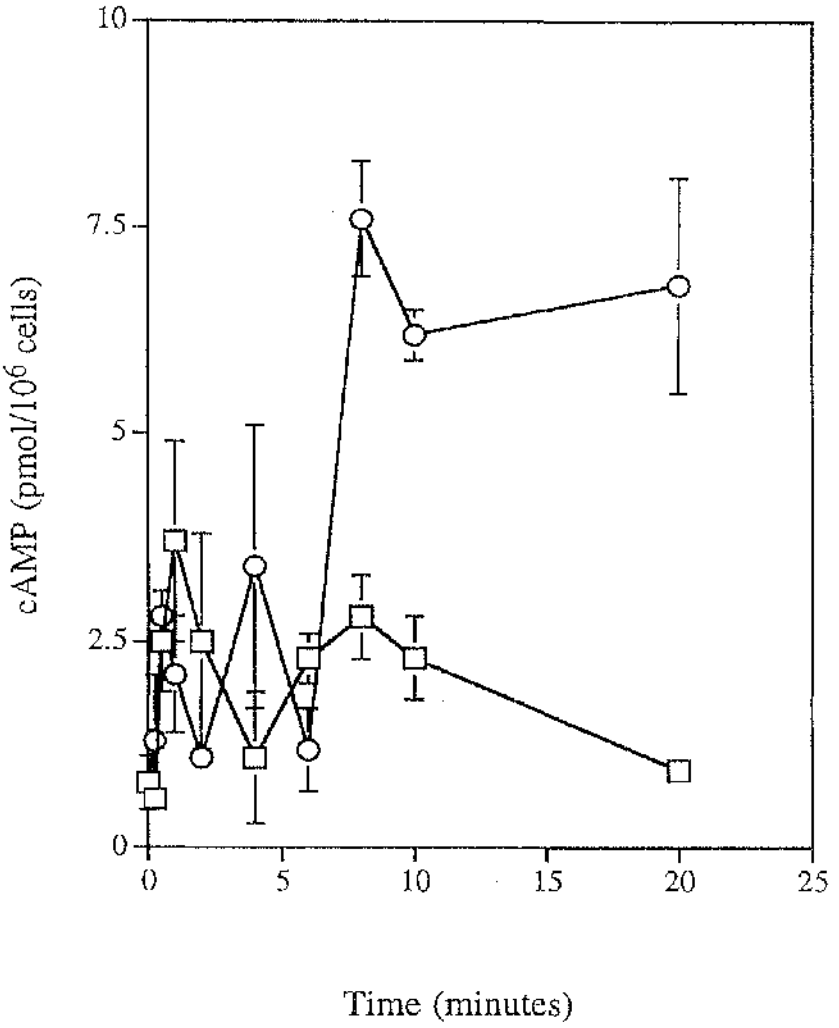

% CD25 Positive B Cells
25
20
15
10
5
0
0
0.1
1
10
100
1000
IL-4 (Units/ml)

B


% CD25 Positive B Cells
25
20
15
10
5
0
0
0.01
0.1
1
10
100
Forskolin (μM)

## 4.16 cAMP Production in EDR B Cells is Significantly Lower than in Primary B Cells

EDR B cells (5 x $10^6$ cells/ml) were resuspended in fresh, complete medium for 24 hours prior to stimulation with IL-4 (100 units/ml) for the indicated time points. Reactions were stopped by the addition of 2% (v/v) PCA and 50µl aliquots were removed for analysis. Results are expressed as picomoles cAMP produced per $10^6$ cells: control cells - open squares, stimulated cells - open circles.


10
7.5
5
2.5
0
cAMP (pmol/10^6 cells)
0
5
10
15
20
25
Time (minutes)

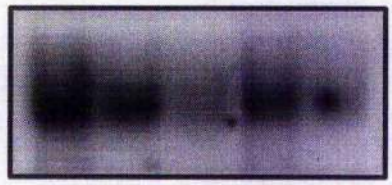
## 4.17 Forskolin but not IL-4, Induces a Decrease in Transcription Factor Binding to NRE in EDR B Cells

EDR B cells (5 x $10^6$ cells/ml) were resuspended in fresh, complete medium for 24 hours prior to stimulation with (A) IL-4 (100 units/ml) or (B) 100μM forskolin for 4 hours. Nuclear extracts were made at hourly intervals and examined by DNA mobility shift, with 0.175pmol, $^{32}$P-labelled NRE oligonucleotide probe. The lane numbers at the bottom of the figure correspond to time of culture with IL-4 or forskolin in hours.

A

0 1 2 3 4

B

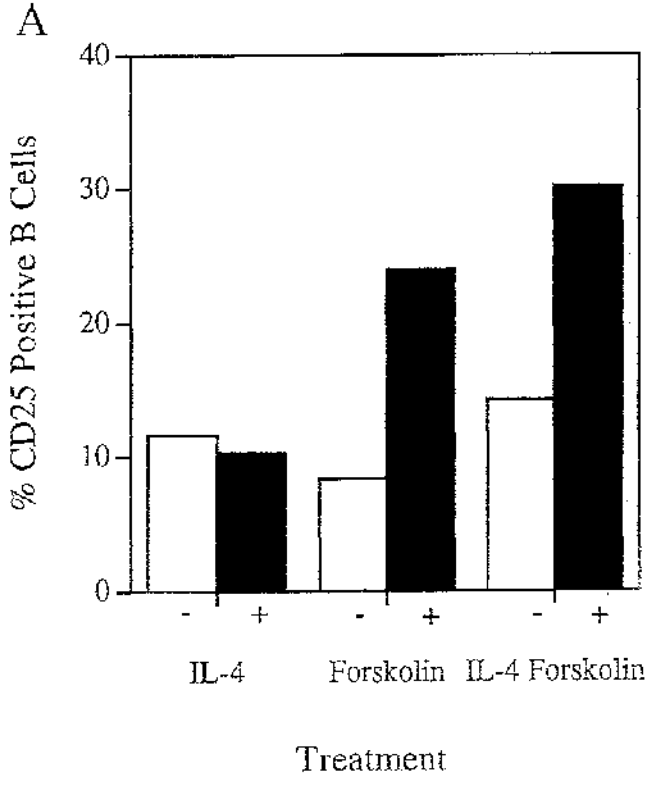


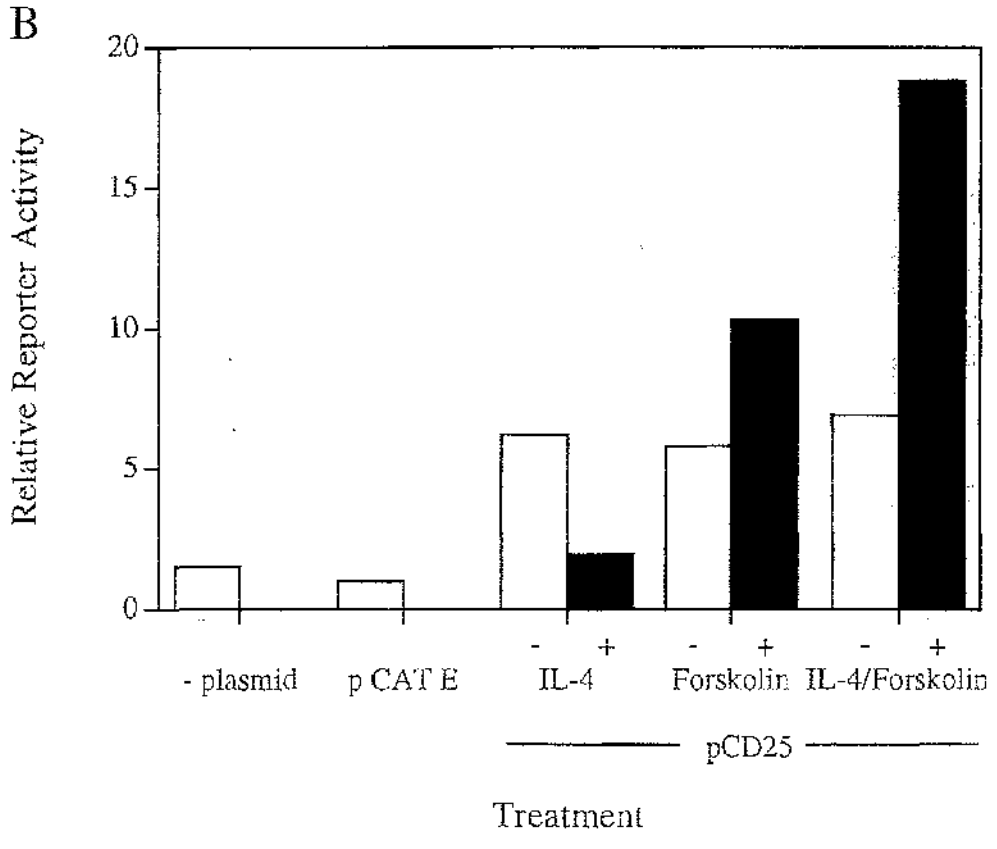
0 1 2 3 4

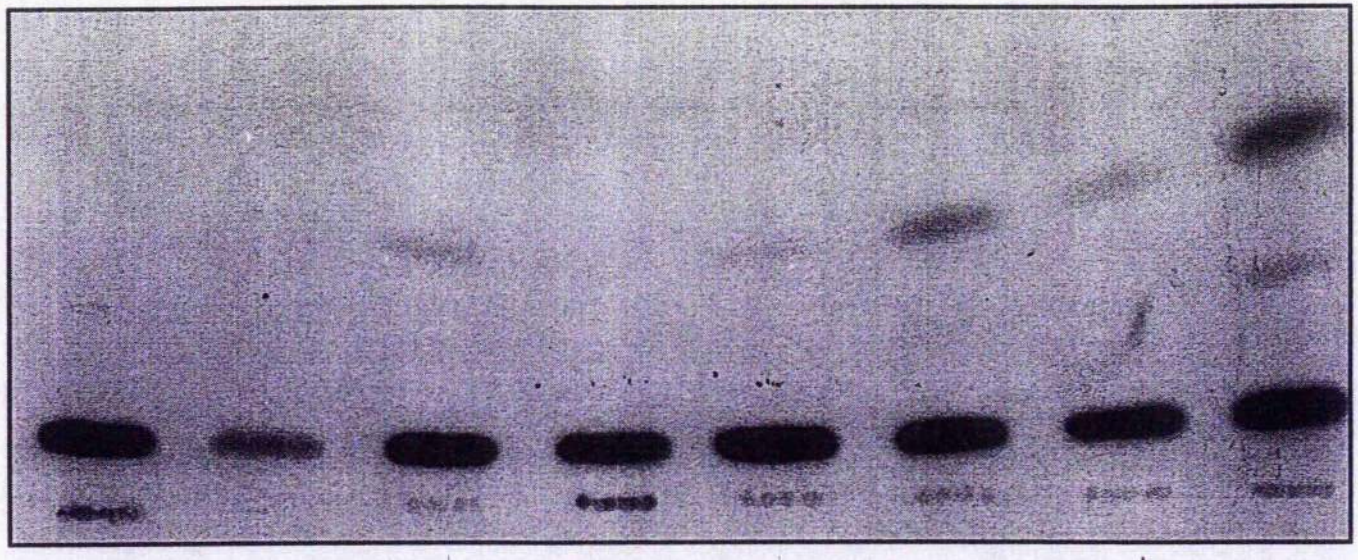
Time (hours)

## 4.18 IL-4 and Forskolin Induce Synergistic Transcriptional Activation of the CD25 gene

Mature EDR B cells (6.25 x $10^6$ cells/ml) were resuspended in fresh, complete medium for 24 hours prior to transfection with 40µg of the pGEM-7Zf(+) cloning vector containing a promoter fragment from pIL-2α-CAT. Cells were stimulated, 2 hours following transfection, with either 400 units/ml IL-4, 100µM forskolin or both these stimuli in combination and cultured at 37°C, for 48 hours. After 48 hours an aliqout of transfected cells were taken and simultaneously stained with FITC-anti-CD25 and PE-anti-CD19 monoclonal antibodies and analysed by flow cytometry (A). Cell lysates were made from remaining cells and transcriptional activation was analysed by CAT assay. Results are presented as Relative Reporter Activity from phosphorimager data (B) and as an autoradiograph of the TLC plate (C). The upper black arrow indicates mono-acetylated chloramphenicol (C).


A
% CD25 Positive B Cells
40
30
20
10
0
- + - + - +
IL-4
Forskolin
IL-4 Forskolin
Treatment


B
Relative Reporter Activity
20
15
10
5
0
- + - + - +
- plasmid
p CAT E
IL-4
Forskolin
IL-4/Forskolin
pCD25
Treatment


Origin
No Plasmid
pCAT-E
- IL-4
+ IL-4
- Forskolin
+ Forskolin
- IL-4/ Forskolin
+ IL-4/ Forskolin
pCD25

# Chapter 5

# IL-4-Induced Regulation of Transcription Factor Binding Activity to the IL-4 Response Element of the CD25 Promoter

## 5.1 Introduction

A number of elements which have been defined as binding motifs for a range of intracellular transcription factors, have been identified within the promoter region of the CD25 gene. Thus, there exists a group of positive regulatory elements - a κB binding site, a serum response element (SRE) and an Sp1 binding site - whose sequences are adjoining and in the case of SRE and Sp1, overlapping [Algarte *et al.*, 1995; Pierce *et al.*, 1995]. In T cells, activation of transcription of the CD25 gene appears to be predominantly regulated by the exchange of a negatively acting NF-κB p50-p50 homodimer for a transcriptionally active p50-p60 heterodimer at the κB site [Algarte *et al.*, 1995]. In addition, this exchange of a negative factor for a positively acting one appears to facilitate binding of SRF and Sp1 to their respective binding elements [Pierce *et al.*, 1995]. A sequence 5' of this region (NRE) which binds a 50kDa 'silencer' protein does not appear to play a large functional role in the induction of the CD25 gene in human T cells, despite the observed increase in both basal and PMA-inducible reporter activity of a deletion mutant-CAT construct lacking this particular element [Lowenthal *et al.*, 1988]. In contrast, the binding of a transcription factor to NRE seems to be one of the major regulatory components of CD25 transcription in human B cells. IL-4, which has been demonstrated to be one of the most potent inducers of CD25 in human B cells (Chapter 3, Fig 3.4), is able to induce a decrease in DNA-protein binding activity to NRE via a cAMP generating pathway (Chapter 4). This latter effect appears to be at least partially responsible for IL-4 induction of CD25 up-regulation in human B cells, assigning a more important role to NRE with respect to CD25 expression than is observed in T cells. In addition, yet another contrast between T and B lymphocytes, with respect to CD25 transcriptional activation, is the apparent inability of IL-4 to induce any alteration in transcription factor binding to NF-κB, SRE or Sp1 in human B cells [Burlinson, personal communication]. This data is, however, inconclusive and the question, which other signalling mechanisms might IL-4 employ in human B cells in order to elevate expression of CD25, arises. Two putative positive regulatory response

elements are present in the CD25 promoter in human B cells. The first of these is an IL-4 response element (IL-4RE), a sequence located upstream of the NF-κB binding site, which has sequence homology to IL-4 response elements located in the promoter regions of a number of IL-4 inducible genes [Boothby *et al.*, 1988; Kohler and Rieber, 1993; Kretsovali and Papamatheakis, 1995]; e.g., a sequence which binds a nuclear factor-IL-4 (NFIL-4) has been located in the C region of the Class II MHC Eα gene promoter [Kretsovali and Papamatheakis, 1995]. In addition, the IL-4RE of the CD25 promoter bears a degree of sequence homology with both the IFNγ-activated sequence (GAS) located in the FcγRI gene promoter and with the Stat6 sequence described in the human FcγRI gene [Hou *et al.*, 1994; Witthuhn *et al.*, 1994]. The second and more recently identified element is a Stat6 binding site. IL-4 is the predominant activator of Stat6 in the majority of lymphoid cells and is reported to mediate this activation via tyrosine phosphorylation by Jak1 following IL-4 receptor oligomerization, the association of Jak1 with the IL-4Rα chain and its subsequent phosphorylation [Quelle *et al.*, 1995]. It was, therefore, of interest to determine the regulatory effects of IL-4 upon transcription factor binding to the IL-4RE and also whether any relationship existed between this element and Stat6. Indeed, one or both of these positive regulatory elements may provide the key to IL-4 induced expression of CD25 in human B cells. In addition, the effects of cytokines which contain IL-4 receptor subunits in their own receptor complexes i.e., IL-13 (putatively the IL-4Rα chain) and IL-7 (γc), were of interest; neither is able to regulate NRE binding activity or induce CD25 expression, so the possibility of these cytokines sharing any signalling pathways with IL-4, with respect to CD25 expression, comes into question.

## 5.2 IL-4 Up-regulates Transcription Factor Binding to an IL-4 Response Element in the CD25 Promoter

Positive regulatory IL-4 responsive elements have been identified in a number of IL-4 inducible genes [Boothby *et al.*, 1988; Kretsovali and Papamatheakis, 1995; Schindler *et*

*al.*, 1994]. Stat6, identified by its ability to bind to GAS (interferon-γ activated sequences) elements, binds specific DNA sequences in its homodimeric (STF-IL-4) form [Schindler *et al.*, 1994]. An alternative IL-4 response element has also been identified in the promoter regions of the CD23 (low affinity IgE receptor- FcγRII) gene [Kohler and Rieber, 1993]. A similar element has been located in the CD25 promoter in human B cells based on these sequences. Considering the ability of IL-4 to up-regulate cell surface expression of CD25 in human B cells, it was of interest to determine whether IL-4 could regulate transcription factor binding to this element in these cells. The response to IL-4 in resting tonsillar B cells is variable; however, the consensus seems to be that IL-4 up-regulates the activity of a protein which binds this element over a four hour period of stimulation (Figure 5.1). The five independent repeats of the same experiment shown in Figure 5.1 demonstrate the variation in transcription factor binding to IL-4RE, in response to IL-4, which occurs from one tonsil preparation to another. The extent of IL-4's ability to induce this DNA -protein binding activity appears to be dependent upon the level of binding occurring at time zero, i.e., in extracts from unstimulated cells cultured in medium alone. The degree of DNA binding activity in unstimulated cells is variable between tonsil preparations and is more than likely a feature of differential states of *in vivo* activation of B cells in the tonsils. When binding of IL-4RE oligonucleotide is non-existent at time zero the ability of IL-4 to induce an increase is in some instances extensive, with maximal binding occurring one hour after stimulation (Fig. 5.1 A), while in others the response is less dramatic even after four hours (Fig. 5.1 B). In contrast, in preparations where transcription factor binding is apparent at time zero, i.e., prior to any exogenous stimulation, any observed increases in response to IL-4 are small (Fig. 5.1C) and in some instances there is no discernible increase in DNA binding over the four-hour stimulatory period (Fig. 5.1D). It could be predicted that if the degree of *in vivo* activation is high, the exposure of B cells to IL-4 will have induced activation of an IL-4RE binding factor to such an extent that exposure to exogenous IL-4 is unable to induce any further response, i.e., the binding protein has been maximally activated. Alternatively, exposure to endogenous IL-4 *in vivo* could result in desensitzation of the

IL-4 receptor.

Another factor which is variable between different preparations is the nature of the mobility shift band which is affected by IL-4. There are in all cases, two distinct mobility shift bands observed on the gel. In the majority of experiments carried out with IL-4RE oligonucleotide it is the upper band, i.e., the slowest mobility shift band, which is sensitive to IL-4 stimulation. However, in one or two instances it is the faster of the shift bands which is up-regulated in response to IL-4; an increase is observed over a four hour period of stimulation (Fig. 5.1E), whereas in one experiment an increase in DNA binding in the second mobility shift band is observed after one hour stimulation, subsequently dropping back to basal levels at the three hour time point (Fig. 5.1A). The presence of two mobility shift bands is probably due to the presence of monomeric and dimeric forms of the transcription factor which binds IL-4RE. The question of the variability in sensitivity of these bands to IL-4 activity is curious. Despite this, the obvious underlying mechanism is one of IL-4 inducing an increase in binding of a transcription factor to this IL-4 response element, i.e., IL-4 increases DNA-protein binding activity at a positive regulatory element in the CD25 promoter in human B cells. This effect is no doubt partially responsible for IL-4-induced transcriptional activation and expression of the CD25 gene in human B cells, possibly acting in parallel with the decreased binding to NRE.

## 5.3 A Stat6 Binding Site Competes with the IL-4 Response Element for DNA Binding Protein

One of the major signal transduction pathways activated by IL-4, which has been identified in recent years, is the activation of two members of the Janus tyrosine kinase family, Jak1 and Jak3, and the tyrosine phosphorylation and concomitant activation of Stat6 [Hou *et al.*, 1994; Quelle *et al.*, 1995; Witthuhn *et al.*, 1994]. The promoter region of the CD25 gene in human B cells, in addition to containing the IL-4RE, also contains a

putative binding site for Stat6, or rather its activated DNA binding homodimeric form (STFIL-4). Stat6 binding sites are present in an array of IL-4-inducible genes and the binding of Stat6 homodimers acts in a positive regulatory manner to activate gene expression [Hou *et al.*, 1994; Quelle *et al.*, 1995; Schindler *et al.*, 1994]. It is interesting that the CD25 promoter should contain two positive regulatory elements which are putative IL-4 response elements. IL-4RE bears a distinct sequence from that of the Stat6 site although there is a degree of homology. However, the consensus sequence for a Stat binding site is TT-$N_{(4-6)}$-AA, a sequence which is not found in the IL-4 response element (the underlined regions shown below demonstrate the absence of a consensus Stat binding site in the IL-4RE).

| | |
|---|---|
| Stat6 | 5' GTA TTT CCA GAA AGG GAA 3' |
| IL-4RE | 5' CAC TGC CAA GAA GTG CTT 3' |

A competition assay was carried out in an attempt to determine the potential of the IL-4 response element to act as a Stat6 binding site and at the same time determine the specificity of IL-4RE binding in human B cells. DNA-protein binding to $^{32}$P-labelled IL-4RE oligonucleotide in human B cell extracts is sensitive to competition by an excess of both unlabelled IL-4RE and Stat6 oligonucleotide (Fig. 5.2). It seems likely, therefore, that the IL-4 response element is a Stat6 binding site. What is particularly interesting is that unlabelled Stat6 oligonucleotide competes for transcription factor binding far more strongly than cold IL-4RE; i.e., at a 100-fold excess of unlabelled Stat6 there is no binding of radio-labelled oligonucleotide whereas there is still obvious radio-labelled oligonucleotide binding, although substantially diminished, in the presence of cold IL-4RE oligonucleotide. Thus, it would seem that the IL-4 response element is a weak Stat6 binding site and that its reduced capacity to bind Stat6 is probably due to the observed differences in DNA sequence, in particular, the absence of a consensus Stat binding site. A second feature of note is that unlabelled oligonucleotide bearing the sequence of an AP2 binding site cannot compete with radio-labelled IL-4RE oligonucleotide for DNA-protein binding activity in human B cells. Therefore transcription factor binding to IL-

4RE is specific in as much as only IL-4RE or Stat6-binding oligonucleotides are able to compete with radiolabelled probe for protein binding. The Stat6 competition studies demonstrate that Stat6 may be at least a component of the IL-4RE binding complex.

## 5.4 Forskolin and Cholera Toxin do not Affect Protein Binding Activity of the IL-4 Response Element

The data presented in the previous chapter indicate that an IL-4-induced cAMP generating/PKA activating pathway is a major factor in induction of CD25 expression in that it initiates a decrease in transcription factor binding to a negative regulatory element in the CD25 promoter. Removal of this negative influence is at least partially responsible for up-regulation of CD25 in human B cells. It was, therefore, of considerable interest to examine the effects of forskolin and cholera toxin, both of which elevate intracellular cAMP, on DNA-protein interactions at the IL-4 response element. Neither forskolin nor cholera toxin mimic the ability of IL-4 to elevate binding to IL-4RE in resting human B cells. Again the presence of DNA binding activity present in unstimulated, time zero extracts is problematic, indicating that prior activation of some B cells has occurred even in a high density, quiescent B cell fraction (Figure 5.3 A & B). There is a modest increase in IL-4RE binding at the 2 hour time point in response to stimulation with forskolin (Fig 5.3 A), whereas treatment with cholera toxin appears not to produce such a response (Fig 5.3 B). However, in B cell extracts where binding of IL-4RE oligonucleotide at time zero is low, no elevation of transcription factor binding to this element in response to forskolin or cholera toxin is observed over four hours (Figure 5.3 C & D). Thus, the general consensus from a number of independent repeats is that IL-4RE binding remains relatively unaffected by cAMP generating reagents. One feature which is of considerable interest is, that neither forskolin nor cholera toxin is able to diminish DNA-protein binding to IL-4RE to any significant degree. This particular result serves as a very good internal control for the NRE-binding response observed to both these stimuli (Chapter 4), indicating that abrogation of transcription factor binding to

NRE is a result of genuine, specific sensitivity of the NRE-binding protein to cAMP and not a general decrease in DNA protein interactions in response to gross stimulation of the B cell.

## 5.5 Tyrosine Kinase and PKC Inhibition Do Not Affect IL-4 Induced Transcription Factor Binding to the IL-4 Response Element

The data presented so far have demonstrated that IL-4 up-regulates a protein which binds to a putative positive regulatory element in the CD25 promoter in human B cells. In addition, evidence suggests that this protein may be Stat6 and that IL-4RE is a weak Stat6 binding site. Pre-treatment of resting tonsillar B cells with the tyrosine kinase inhibitor genistein prior to stimulation with IL-4 does not affect the ability of IL-4 to up-regulate transcription factor binding to IL-4RE. In the two independent repeats of this experiment which are shown, IL-4 strongly up-regulates IL-4RE binding activity over a four hour period when there are no apparent levels of binding at time zero (Fig. 5.4 A), and in the instance where levels of DNA-protein binding activity at time zero are high, genistein does not exert any negative influence over IL-4RE binding (Fig. 5.4 C). This is interesting as IL-4RE has been shown to act as a Stat6 binding site and the accepted mechanism of IL-4-induced activation of Stat6 is mediated via its tyrosine phosphorylation by Jak1. However, no available information exists on the ability of genistein to inhibit members of the Janus kinase family. It is a possibility, therefore, that this particular inhibitor is unable to affect the kinase activity of this family. Treatment of human B cells with a protein kinase C inhibitor prior to IL-4 treatment does not diminish transcription factor binding to IL-4RE (Fig. 5.4 B & D). Therefore, PKC activity, which was demonstrated to have a role in IL-4 induced CD25 expression (Chapter 3, Figs. 3.3 & 3.7), has no effect upon IL-4 induced regulation of Stat6 binding to the IL-4 response element in the CD25 promoter.

## 5.6 The Effects of IL-13 and IL-7 on Transcription Factor Binding to the IL-4 Response Element

The suggestion that IL-13 and IL-4 share a common receptor component and activate similar signal transduction pathways is commonly accepted [Kishomoto *et al.*, 1994], however, the identity of the receptor subunit and the nature of common signalling pathways remain equivocal. Evidence against γc being a component of the IL-13 receptor is strong and the suggestion that the IL-4Rα chain is the component which both the IL-4 and IL-13 receptors share seems more plausible [He *et al.*, 1995a; He and Malek, 1995b; Keegan *et al.*, 1995; Matthews *et al.*, 1995; Smerz-Bertling and Duschl, 1995; Zurawski *et al.*, 1993]. Considering the ability of IL-4 to activate Stat6, via the IL-4Rα chain and Jak1, and also that the IL-4 response element of the CD25 promoter is a potential Stat6 binding site, it was of interest to determine whether or not IL-13 was capable of regulating protein binding to IL-4RE. The data presented from two independent repeats of a 4 hour time course of IL-13 stimulated resting B cells are not particularly compelling. However, in one instance there does appear to be a slight increase in DNA-protein binding activity to IL-4RE over the 4 hour period (Fig. 5.5A). Again the problem of prior activation in the high density B cell fraction, resulting in high levels of IL-4RE binding at time zero, makes observation of any changes in IL-4RE binding difficult (Fig. 5.5 A & B). It has also been possible to observe the effects, or lack thereof, of IL-7 on elevation of transcription factor binding to IL-4RE. Thus, in two independent repeats it seems clear that IL-7 (Fig 5.6 A & C), unlike IL-4 (Fig 5.6 B & D), is unable to induce any increase in DNA-protein binding activity to IL-4RE. These data are consistent with the theory that Stat6 forms part of the IL-4RE binding complex, as many cytokines demonstrate a high degree of selectivity with respect to activation of Stat family members.

## 5.7 IL-4 Induces Tyrosine Phosphorylation of Stat6 in EDR B Cells

The data presented in this chapter indicate that IL-4 is capable of inducing an increase in transcription factor binding to an IL-4 response element in the CD25 promoter in resting, human B cells. In addition, competition data from section 5.5 strongly suggest that IL-4RE is a weak Stat6 binding site. It has been shown previously that IL-4 activates Stat6 by tyrosine phosphorylation in a variety of cell types [Hou *et al.*, 1994; Quelle *et al.*, 1995; Schindler *et al.*, 1994]. One of the major problems of determining the ability of IL-4 to up-regulate DNA-protein binding activity at a positive regulatory element such as IL-4RE in resting, tonsillar B cells, is the varying degree of prior activation of the B cells between one cell preparation and another. Thus, even in high density B cell fractions there will exist some proportion of previously activated cells. This is observed when one examines tyrosine phosphorylation of Stat6 in response to IL-4 in tonsillar B cells. Immunoprecipitates made, using a rabbit anti-Stat6 monoclonal antibody (S-20), from a control population of high density B cells demonstrate a degree of tyrosine phosphorylation of Stat6 which is essentially no different from IL-4 stimulated cells (Fig. 5.8). Western blot analysis of the Stat6 content of control and stimulated resting B cells and the EDR B cell line demonstrates that total Stat6 content of these cells is not altered by stimulation (Fig. 5.7). The monocyte cell line U937, which has been included as a control for IL-4 induced Stat6 activation, showed considerably less Stat6 content than either EDR or tonsillar B cells. The difference observed between control and stimulated U937 cells is probably due to a problem in gel loading, as opposed to a genuine difference in Stat6 levels in response to IL-4. In contrast to resting tonsillar B cells, a dramatic increase in tyrosine phosphorylation of Stat6, i.e., the level of Stat6 activation in response to IL-4 is observed in EDR B cells. The observation that IL-4 induces activation of Stat6 in EDR B cells does not necessarily mean that a Stat6 homodimer will regulate CD25 expression in these cells. However, CAT-reporter gene studies pCD25-CAT transfected EDR cells suggest that IL-4 activates a second signalling pathway which

is required, in combination with a cAMP-dependent pathway, for maximal transcriptional activation of the gene. The ability of IL-4 to activate Stat6 in these cells and the presence of a Stat6 binding site in the CD25 promoter, suggest STF-IL-4 formation as a possible candidate for this second IL-4-induced pathway.

## 5.8 Discussion

The major conclusions which can be drawn here are, firstly, that the putative IL-4 response element in the CD25 promoter has DNA-protein binding activity which is up-regulated in response to IL-4 and, secondly, competitive DNA binding studies revealed this site to be a weak Stat6 binding site. This latter observation is rather unusual if one considers the absence of a consensus Stat binding site in the IL-4RE. What is also unusual is that two potential Stat6 binding sites are present in the same promoter. One possible explanation for this is that the availability of two positive regulatory binding sites in the CD25 promoter may facilitate a greater response, with respect to expression of the gene. What has not been determined is whether the increased DNA binding activity which is observed in response to IL-4 (Figure 5.1) is actually the binding of STF-IL-4, a Stat6 homodimer. Reports of a second IL-4-responsive transcription factor, NFIL-4, define a 75kDa protein which is distinct from Stat6 [Kretsovali and Papamatheakis, 1995]. The IL-4RE in the CD25 promoter has a core sequence of 9 base pairs which shares a high degree of sequence homology with both the 9 base pair IL-4RE in the CD23b gene [Kohler and Rieber, 1993] and a region of the 42 base pair sequence believed to confer IL-4-inducibility on the MHC class II Eα promoter [Kretsovali and Papamatheakis, 1995] (the sense sequences for these three elements are shown below, the underlined bases signifying the regions of homology).

| | |
|---|---|
| IL-4RE CD25 | 5'- CAC <u>TGC CAA GAA</u> GTG -3' |
| IL-4RE CD23b | 5'- GAT <u>TTC TAA GAA</u> AGG -3' |
| MHC class II Eα | 5'- GGT <u>TCG AAG GAA</u> CCC -3' |

Does the similarity between CD25 IL-4RE and these NFIL-4 binding sequences, and

also the ability of a Stat6 site to compete for transcription factor binding with the CD25 IL-4RE, then suggest the presence of a multi-functional IL-4 response element capable of binding two distinct transcription factors? If this were so, might it be a function of differential expression of DNA binding proteins at different stages of B cell development? However, expression of Stat6 appears to be ubiquitous throughout IL-4-responsive cell types and also throughout most stages of B cell development, a fact that would negate the requirement for a second IL-4 responsive transcription factor. One observation which may provide a clue to the nature of the DNA-protein binding activity of IL-4RE in the CD25 promoter is the apparent insensitivity of this activity to treatment with the protein tyrosine kinase inhibitor genistein. IL-4-induced Eα binding activity by NFIL-4 is also unaffected by genistein, indicating that tyrosine kinase activity is not a requirement for activation of this binding protein [Kretsovali and Papamatheakis, 1995]. Treatment of extracts of human B cells with anti-phosphotyrosine antisera inhibits activation of NFIL-4 and it has been determined that anti-phosphotyrosine antibodies also abrogate the binding activity of the IL-4RE in the CD25 promoter [Burlinson, personal communication]. The accepted mechanism for Stat6 activation is tyrosine phosphorylation by Jak1 [Quelle *et al.*, 1995], however, as no information exists on the ability of genistein to inhibit phosphorylation of Stat6 the nature of the protein which binds the IL-4RE in the CD25 promoter remains very much equivocal.

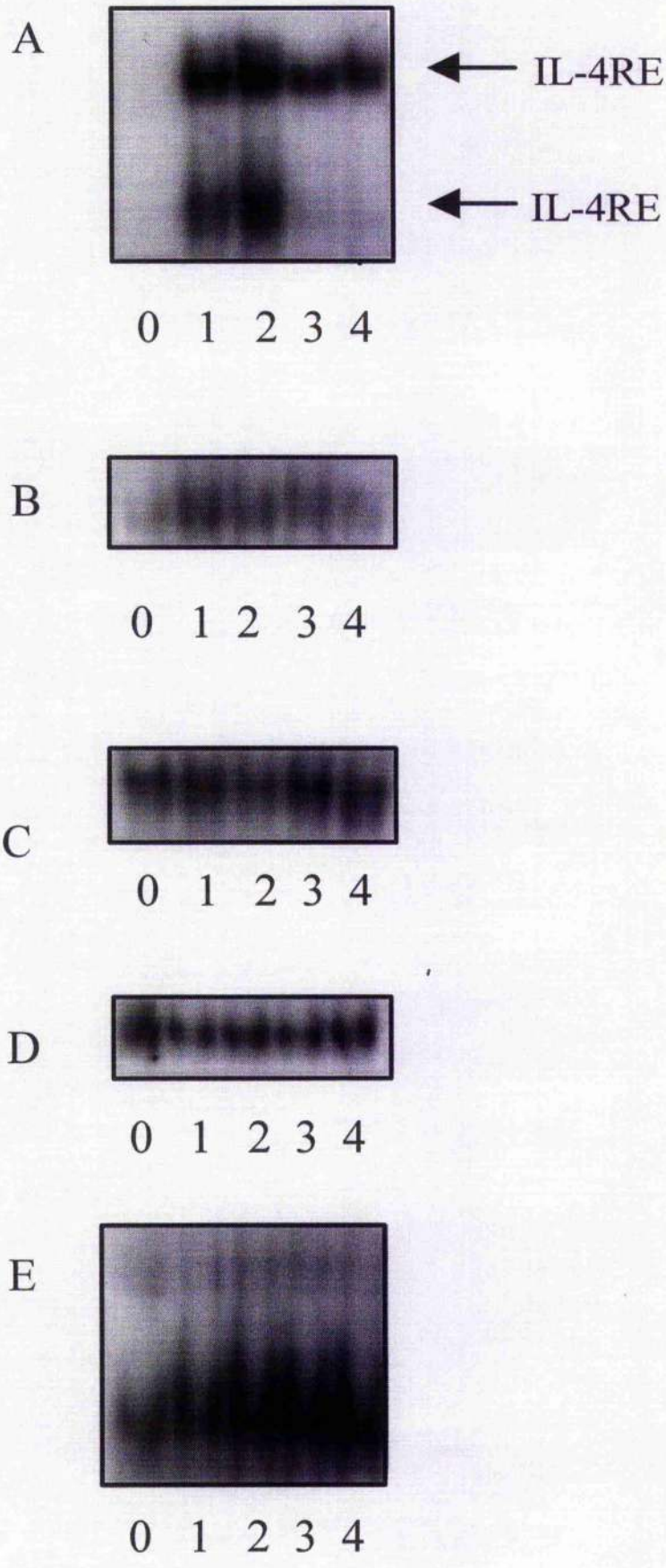
Another important observation concerns the effects of the cytokines IL-7 and IL-13 on DNA-protein binding activity to IL-4RE. IL-7, unlike IL-4, is unable to up-regulate transcription factor binding to IL-4RE over a 4 hour period. The receptor complexes for IL-4 and IL-7 share a common subunit, γc, identified as the third component of the IL-2 receptor complex. The inability of IL-7 to induce IL-4RE DNA binding activity strongly suggests that IL-4 mediates its effects on transcriptional regulation of this element via the IL-4Rα chain. There is a suggestion in Figure 5.5 that IL-13 up-regulates DNA-protein binding activity to IL-4RE, however, these data are not particularly compelling. IL-13 shares certain cellular functions with IL-4 and extensive studies have revealed that the

receptors for these two cytokines share a common component, the majority of evidence suggesting that this is the IL-4Rα chain. Thus, IL-13 inducing an increase in binding activity to IL-4RE further promotes the notion that IL-4 mediates this response via the IL-4Rα chain.

Analysis of the ability of IL-4 to activate Stat6 in EDR B cells revealed that Stat6 is tyrosine phosphorylated in response to a 15 minute stimulation with IL-4. In the previous chapter, transient transfection studies demonstrated that although IL-4 alone could not initiate transcription from the CD25 promoter, it did synergize with forskolin to induce transcriptional activation. The suggestion is that activation of a cAMP pathway, while necessary for facilitating transcription from the CD25 promoter, is insufficient for a complete response and that a second signalling pathway, activated by IL-4, is required to initiate maximal activation of transcription. The ability of IL-4 to induce tyrosine phosphorylation of Stat6 in EDR B cells and the presence of a Stat6 binding site in the CD25 promoter seems to provide a suitable candidate for this second pathway. However, the presence of the IL-4 response element in the CD25 promoter provides another possible target for IL-4 activation of CD25 transcription. Although there is some evidence to suggest that this IL-4RE is bound by an NFIL-4-like transcription factor, the nature of the IL-4RE binding protein remains equivocal. However, a suggested mechanism for IL-4-induced up-regulation of CD25 in human B cells is that the combination of a decrease in binding to a negative regulatory element, mediated via a cAMP-dependent pathway and an increase in binding to one or both of these positive regulatory elements, is sufficient to drive transcription of the CD25 gene. A final observation relating to the inability of IL-13 to up-regulate CD25 in human B cells is, that if both of these signalling pathways are required for transcriptional activation of the CD25 gene then the inability of IL-13 to generate cAMP or affect NRE binding activity, despite its possible potential to activate Stat6 or NFIL-4 and increase binding to IL-4RE, may account for the lack of induction of CD25 in reponse to IL-13.

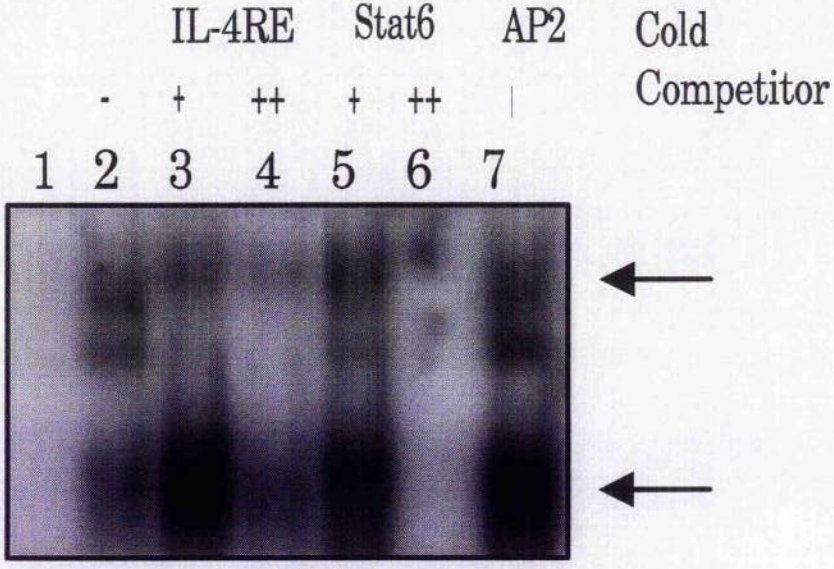
## 5.1 IL-4 Induces an Increase in Transcription Factor Binding to IL-4RE

High density tonsillar B cells were incubated in complete medium at 37°C, 5% $CO_2$ for 16 hours prior to stimulation with IL-4 (100 units/ml) for 4 hours. Nuclear extracts were made at hourly intervals and examined by DNA mobility shift assay, with 0.175pmol, $^{32}P$ labelled IL-4RE oligonucleotide probe. The lane numbers at the bottom of the figure correspond to time of culture with IL-4 in hours. One full length DNA mobility shift demonstrates two distinct mobility shift bands are observed (A). In addition four independent repeats are shown (the upper, i.e., the slowest, mobility shift band B, C, D and both mobility shift bands E).


A
IL-4RE
IL-4RE
0 1 2 3 4
B
0 1 2 3 4
C
0 1 2 3 4
D
0 1 2 3 4
E
0 1 2 3 4
Time (hours)

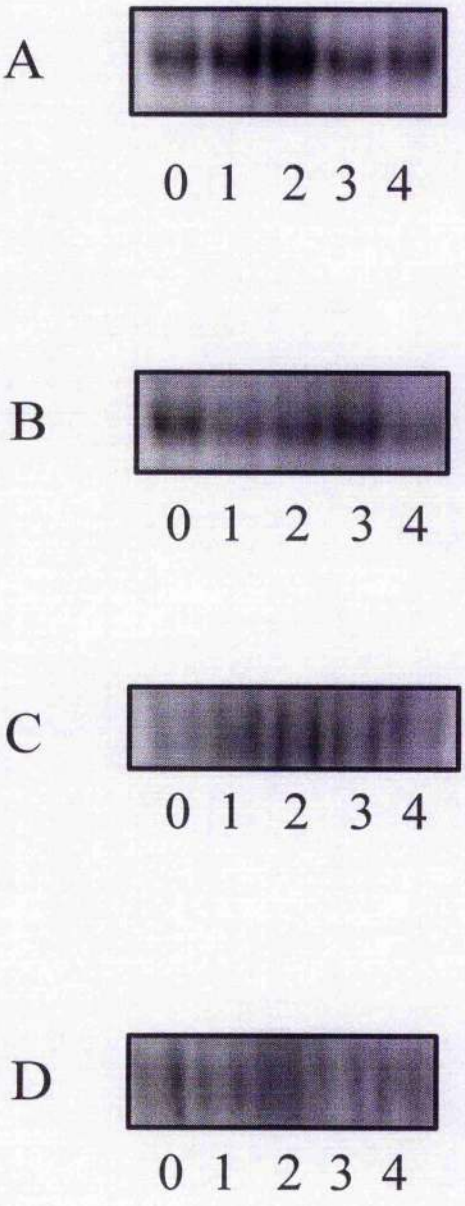
## 5.2 A Stat6 Binding Element but not an AP2 Binding Element Competes with the IL-4 Response Element for Transcription Factor Binding

Nuclear extracts from high density tonsillar B cells were incubated with $^{32}$P-labelled IL-4RE oligonucleotide and either a 10-fold (+) or 100-fold (++) excess of unlabelled IL-4RE oligonucleotide (lanes 3 & 4) or unlabelled Stat6 oligonucleotide (lanes 5 & 6), or a 100-fold excess of unlabelled AP2 (lane 7). Extracts were also incubated in the absence of any competing oligonucleotide (lane 2) and $^{32}$P-labelled IL-4RE oligonucleotide was incubated in the absence of protein extracts (lane 1). Unlabelled IL-4RE and unlabelled Stat6, but not unlabelled AP2, compete with radio-labelled IL-4RE oligonucleotide for protein binding.


IL-4RE
Stat6
AP2
Cold
Competitor
-
+
++
+
++
|
1 2 3 4 5 6 7

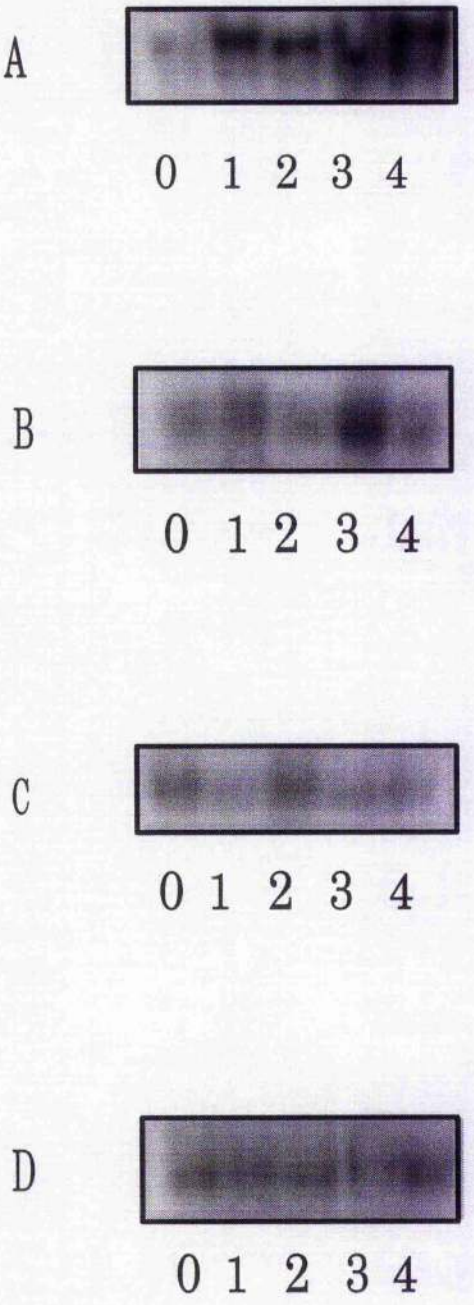
## 5.3 Forskolin and Cholera Toxin Do Not Affect Transcription Factor Binding Activity at IL-4RE

High density tonsillar B cells were incubated in complete medium at 37°C, 5% $CO_2$ for 16 hours prior to stimulation with 100μM Forskolin or 1μg/ml Cholera Toxin for 4 hours. Nuclear extracts were made at hourly intervals and examined by DNA mobility shift assay, with 0.175pmol, $^{32}P$ labelled IL-4RE oligonucleotide probe. The lane numbers at the bottom of the figure correspond to time of culture with IL-4 in hours. Two independent repeats of extracts from forskolin (A & C) and cholera toxin (B & D) treated B cells are shown.


A
0 1 2 3 4
B
0 1 2 3 4
C
0 1 2 3 4
D
0 1 2 3 4
Time (hours)

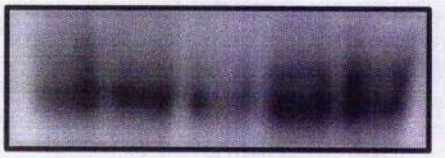
## 5.4 Inhibition of Protein Tyrosine Kinase Activity or PKC Activity, Does Not Inhibit the Ability of IL-4 to Induce an Increase in IL-4RE Binding Activity

High density tonsillar B cells were incubated in complete medium at 37°C, 5% $CO_2$ for 16 hours prior to treatment. Cells were pre-treated with 10μM Genistein or 100nM Bisindolylmaleimide for 30 minutes prior to stimulation with IL-4 (100 units/ml) for 4 hours. Nuclear extracts were made at hourly intervals and examined by DNA mobility shift assay, with 0.175pmol, $^{32}P$ labelled IL-4RE oligonucleotide probe. The lane numbers at the bottom of the figure correspond to time of culture with IL-4 in hours. Two independent repeats of extracts from genistein (A & C) and bisindolylmaleimide (B & D) pre-treated B cells are shown.

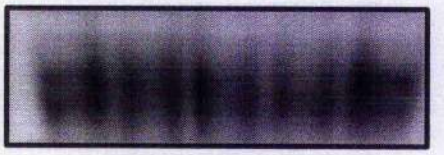

A
0 1 2 3 4
B
0 1 2 3 4
C
0 1 2 3 4
D
0 1 2 3 4
Time (hours)

## 5.5 IL-13 Induces a Slight Increase in Transcription Factor Binding to NRE

High density tonsillar B cells were incubated in complete medium at 37°C, 5% $CO_2$ for 16 hours prior to stimulation with IL-13 (6ng/ml) for 4 hours. Nuclear extracts were made at hourly intervals and examined by DNA mobility shift assay, with 0.175pmol, $^{32}P$ labelled IL-4RE oligonucleotide probe. The lane numbers at the bottom of the figure correspond to time of culture with IL-4 in hours. Two independent repeats of extracts from IL-13 treated B cells are shown.



0 1 2 3 4



0 1 2 3 4

Time (hours)

## 5.6 IL-7 Does Not Increase DNA-Protein Binding Activity to the IL-4 Response Element

High density tonsillar B cells were incubated in complete medium at 37°C, 5% $CO_2$ for 16 hours prior to stimulation with either IL-4 (100Units/ml) or IL-7 (0.5ng/ml) for 4 hours. Nuclear extracts were made at hourly intervals and examined by DNA mobility shift assay, with 0.175pmol, $^{32}P$ labelled IL-4RE oligonucleotide probe. The lane numbers at the bottom of the figure correspond to time of culture with each cytokine, in hours. Two independent repeats of extracts from IL-4 (A & C) and IL-7 (B&D) treated B cells are shown.


A
0 1 2 3 4
B
0 1 2 3 4
C
0 1 2 3 4
D
0 1 2 3 4
Time (hours)

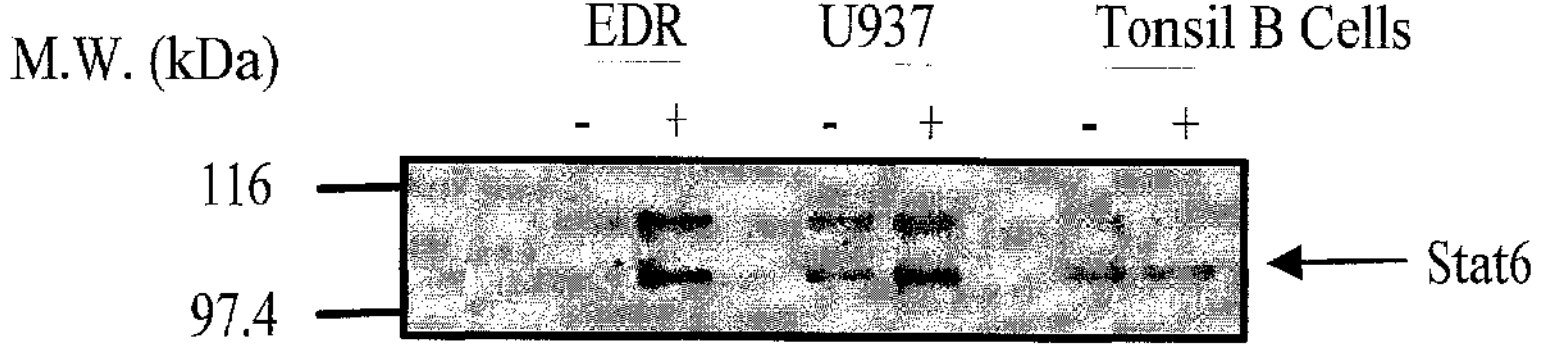
## 5.7 Stat6 is Expressed in Equal amounts in both Resting and IL-4 Treated Resting, Human B Cells and EDR B Cells

High density tonsillar B cells (1 x $10^7$ cells/ml) were incubated in complete medium at 37°C, 5% $CO_2$ for 16 hours and EDR B cells and U937 monocytes were isolated from culture 24 hours after addition of fresh medium, prior to stimulation with IL-4 (100Units/ml) for 20 minutes. 15µg of cell lysate protein for each sample were denatured by boiling in the presence of β-mercaptoethanol for two minutes and separated by SDS-PAGE. Western blots were probed with 0.1µg/ml S-20 monoclonal antibody and visualized by ECL. Equal quantities of Stat6 protein were detected in both untreated (-) and IL-4 stimulated (+) tonsillar B cells, U937 monocytes and EDR B cells.


M.W (kDa)
EDR
U937
Tonsil B Cells
-
+
-
+
-
+
116
97.4
Stat6

## 5.8 Stat6 is Tyrosine Phosphorylated in EDR B cells in Response to IL-4

High density tonsillar B cells (1 x $10^7$ cells/ml) were incubated in complete medium at 37°C, 5% $CO_2$ for 16 hours and EDR B cells and U937 monocytes were isolated from culture 24 hours after addition of fresh medium, prior to stimulation with IL-4 (100Units/ml) for 20 minutes. Unstimulated (-) and IL-4 (100 units/ml) treated B cells were lysed and immunoprecipitated with S-20 monoclonal antibody. 20µl aliquots of immunoprecipitates were denatured by boiling in the presence of β-mercaptoethanol for two minutes and separated by SDS-PAGE. Western blots were probed with 0.1µg/ml PY-20 monoclonal antibody and visualized by ECL. IL-4 induces tyrosine phosphorylation of Stat6 in EDR B cells.


M.W. (kDa)
EDR
U937
Tonsil B Cells
-
+
-
+
-
+
116
97.4
Stat6

# Chapter 6

# Discussion

## 6.1 Major Conclusions

It has been reported previously that IL-4 promotes $PIP_2$ hydrolysis, $IP_3$ production and consequent $Ca^{2+}$ mobilization, PKC activation and cAMP generation in resting human B cells [Arruda and Ho, 1992; Finney *et al.*, 1990]. The results presented here, further demonstrate the ability of IL-4 to induce cAMP generation in human B cells and also provide direct links between this second messenger production and the transcriptional activation of an IL-4-inducible gene, CD25. Thus, we have shown that IL-4-induced cAMP production is biphasic; with a small transient peak occurring 1-2 minutes after ligand stimulation followed by a larger, more sustained generation starting ~10 minutes after ligand binding and returning to basal levels within 35-40 minutes after initiation of stimulation. In addition, preliminary data demonstrate the ability of IL-4 to induce activation of the cAMP-dependent protein kinase, PKA. Thus, PKA is activated at time points which correspond to the generation of cAMP.

IL-4 promotes simultaneous expression of both CD23 and CD25 antigens in resting human B cells in a dose dependent manner. Simultaneous three-colour flow cytometry revealed that $CD19^+/CD23^+/CD25^+$ triple positive cells were derived from a $CD19^+/CD23^-/CD25^-$ pool, and that induction of CD23 required lower doses of IL-4 than did induction of CD25 ($EC_{50}$ values for IL-4 induced expression of CD23 and CD25 are 35pM and 150pM respectively). It was found that both CD23 and CD25 expression could be induced by stimulation of the B cells with the same combination of pharmacological reagents. Thus, maximal expression of CD23 and CD25 was obtained with a 30 or 120 second pulse with phorbol ester and/or ionomycin followed by a sustained treatment with forskolin. Use of BAPTA to chelate intracellular calcium suggested that IL-4-driven CD25 expression required mobilization of intracellular calcium. Additionally, the use of chronic phorbol ester treatment to down-regulate PKC in resting human B cells abolished the ability of IL-4 to elevate both CD23 and CD25 expression. Similarly, phorbol ester treatment abrogated the ability of anti-

Immunoglobulin and anti-CD40 monoclonal antibodies to induce CD25 expression in resting human B cells. These two stimuli are the only factors, other that IL-4, known to induce expression of CD25 in human B cells.

The importance of cAMP generation, with respect to IL-4 up-regulation of CD25 expression, is demonstrated in the ability of reagents which elevate intracellular cAMP, such as forskolin, to mimic the ability of IL-4 to decrease transcription factor binding to a negative regulatory element (NRE) in the CD25 promoter. The involvement of a cAMP-dependent protein kinase in this pathway is further demonstrated by the abrogation of IL-4-induced down-regulation of this DNA-protein binding activity following pre-treatment of resting human B cells with a selective PKA inhibitor, H89. Inhibition of protein tyrosine kinase activity also abrogates IL-4-induced attenuation of NRE binding whereas, in contrast, inhibition of PKC has no effect. The importance of the cAMP/PKA pathway is highlighted in the EDR B cell line, where IL-4 fails to up-regulate CD25. This phenomenon is possibly a result of the reduced capacity of IL-4 to induce cAMP generation in this cell line compared with the response observed in primary resting B cells, which is ~10-fold greater in magnitude. Indeed, IL-4 fails to promote a decrease in DNA binding activity to NRE in EDR B cells, whereas forskolin elicits similar responses to those observed in primary B cells. Studies of transcriptional activation, in EDR cells transfected with a full length CD25 promoter-CAT reporter gene construct, demonstrate that IL-4 fails to promote any increase in reporter activity, whereas forskolin induces CAT activity approximately twice that of basal levels. Stimulation of these cells with both IL-4 and forskolin promotes greater enhancement of reporter gene activity (three times basal levels). This latter observation suggests, that while IL-4-induced generation of cAMP and activation of PKA are necessary for induction of CD25 expression, this signal alone is insufficient to promote maximal transcriptional activation of the gene and a second or even third IL-4-induced signalling pathway is required. A potential candidate for such a pathway is the activation of Stat6 by IL-4; the CD25 promoter in human B cells contains a Stat6 binding site and also an

IL-4 response element which has been demonstrated as a weak Stat6 binding site. In addition, in EDR B cells, IL-4 has been shown to activate Stat6 by induction of tyrosine phosphorylation of this signal transducing molecule.

## 6.2 The IL-4 Receptor and Signal Transduction

It is generally accepted that receptors of the haematopoietin receptor superfamily are multi-component structures and that subfamilies within this superfamily can be grouped according to the common receptor subunits which feature in their structure [Cosman *et al.*, 1990; Foxwell *et al.*, 1992]. Thus, the lymphoid receptor family, including IL-2, IL-4, IL-7, IL-9 and IL-15, all share the common gamma chain ($\gamma$c) as a component of their receptor complexes [Grabstein *et al.*, 1994; Kondo *et al.*, 1993; Russell *et al.*, 1993]. This sharing of receptor subunits between cytokine receptors is believed to confer a degree of functional redundancy upon cytokines, with respect to early signal transduction pathways and occasionally to certain biological activities, e.g. IL-4 and IL-13 have a number of biological effects in common [Aversa *et al.*, 1993; McKenzie *et al.*, 1993; Punnonen *et al.*, 1993]. However, there remains a large degree of distinction between many of the cellular functions of cytokines; thus, they are able to activate or repress a specific set of genes, activate different types of target cells and promote distinct responses within a single cell type. The question of how one commonly utilized early signal transduction pathway is translated into a specific response, dependent upon the cytokine which initiates it, is an intriguing one and one which has arisen often with respect to a number of second messenger systems in a multitude of cell types. The answers to such questions remain to be determined; however, evidence from the study of cytokine signal transduction pathways suggests that the availability of multi-receptor components which are able to activate an array of second messengers, results in a specific pattern of activation which is unique to a single stimulus.

### 6.2.1 High and Low Affinity IL-4 Receptors

Evidence suggests that IL-4 is able to bind two receptors of different affinity; a high affinity receptor consisting of the 140kDa IL-4Rα chain and the 65kDa γc chain, and a low affinity receptor consisting of the 140kDa IL-4Rα chain [Russell *et al.*, 1993]. The differences in binding affinity of these two receptors are small and are 79pM and 266pM, respectively [Russell *et al.*, 1993]. However, the presence of two different affinity receptors, as described by ligand binding analyses, does not necessarily imply that both are functionally active. The accepted mechanism of cytokine activation is that ligand binding induces the association of individual receptor components and increasing evidence suggests that receptor oligomerization is a requirement for successful activation of intracellular signalling pathways [Miyazaki *et al.*, 1994; Pernis *et al.*, 1995a]. If this is the case, then binding of IL-4 to a low affinity form of the receptor may not result in the formation of an active ligand/receptor complex. Thus, the availability of the γc component may limit the number of fully functional IL-4 receptors which are formed and although the cytokine is able to bind to a single IL-4Rα chain, an inability to associate with γc may render it functionally inactive. Data reporting the potential for two biologically active IL-4 receptors of different affinities, describe the differential capacity of IL-4 to up-regulate CD23 and membrane Ig (mIg) in human B cells [Rigley *et al*, 1991]. IL-4-induced CD23 and mIg expression displayed EC50 values of approximately 40pM and 4pM respectively [Rigley *et al*, 1991]. Interestingly, these values are lower than both those described in ligand binding analyses [Russell *et al*, 1993]and those reported here, for IL-4-induced CD23 and CD25 expression. It has been accepted for some time that the receptors for both IL-4 and IL-13 share IL-4Rα as a common subunit. Recent studies have highlighted the presence of a third IL-4 receptor component which may constitute part of the low affinity IL-4 receptor. Use of biotinylated IL-4 in recetor binding studies revealed the presence of an IL-4 binding protein of 65-75 kDa on the membrane of a pre-B cell line [Fanslow *et al*, 1993]. Subsequent purification and characterisation of this subunit determined it to be novel and distinct from both the IL-4Rα and γc chains [Fanslow *et al*, 1993]. Additionally, recent studies have

demonstrated the presence of an IL-4 binding protein of 65-75 kDa on the membrane of endothelial cells and colorectal epithelial cancer cells [Murata *et al*, 1995; Schnyder *et al*, 1966a]. This as yet unidentified protein has a high binding affinity for IL-13 and a low affinity for IL-4, suggesting the formation of a low affinity IL-4 receptor between this protein and the IL-4Rα. These is also the possibility that this low affinity IL-4 receptor forms a tripartate complex with the IL-13 receptor [Hilton *et al*, 1996; Murata *et al*, 1995; Schnyder *et al*, 1966].

In resting human B cells IL-4 induces expression of both CD23 and CD25 at the plasma membrane. Dose response data for the induction of these two surface markers demonstrates a difference in the $EC_{50}$ values which is in accordance with the responses to IL-4 being mediated via two different, high and low, affinity IL-4 receptors. Additionally, simultaneous 'three colour' analyses of CD23 and CD25 expression on CD19 positive B cells, demonstrate that these two surface markers are up-regulated on the same sub-population of cells and again it appears that IL-4 mediates these two responses via receptors of two different affinities. Interestingly, these 'three colour' analyses highlight the presence of a small $CD19^+/CD23^-/CD25^+$ population which remains at equilibrium, i.e., CD25 levels remain constant over the IL-4 titration range. The underlying reasons for the apparent lack of responsiveness of this sub-population are unknown, either there is a constant turnover of CD25 at the B cell surface or the cells are unable to respond to IL-4. If the latter scenario is the case, a possible explanation may lie with the presence, or rather, absence of functional IL-4 receptors. Since the dose response data suggested that the effects of IL-4 upon CD23 and CD25 expression might be mediated by distinct receptors, we attempted to establish whether different intracellular signals were responsible for inducing CD23 and CD25 expression. Quiescent tonsillar B cells were stimulated with combinations of phorbol ester, calcium ionophore and the adenylate cyclase activator forskolin and, while the combinations of stimuli required for maximal up-regulation of the two markers were essentially identical, some interesting differences were evident. Thus, maximal expression of both CD23 and CD25 occurred

when cells were stimulated with PMA, $PBu_2$ or ionomycin alone, or $PBu_2$ and ionomycin combined, followed by a more sustained period of stimulation with forskolin; suggesting that PKC, $Ca^{2+}$ mobilization and elevation of intracellular cAMP levels are important for up-regulation of these two cell surface proteins. However, it was consistently noted that brief pulses with forskolin alone could promote a significant two-fold increase in CD25 expression, whereas CD23 levels were only increased by approximately one third over baseline. This fact, coupled with the observation that forskolin further enhanced CD25 expression when used in combination with other stimuli, suggests that cAMP generation may be necessary in the up-regulation of CD25.

### 6.2.2 The Receptor Paradox

The data presented here highlight an apparent paradox which is not easily explained. The dose response data for IL-4-induced cAMP generation give an $EC_{50}$ value which is in agreement with its generation being induced by a high affinity form of the IL-4 receptor. Artificially generating cAMP by stimulation with forskolin seems to more efficiently elevate CD25 levels than those of CD23. However, the dose response data for IL-4-induced CD25 expression are in line with a low affinity form of the receptor, whereas IL-4-induced CD23 expression and cAMP generation are in agreement with both each other and these responses being delivered via a high affinity receptor. The importance of IL-4-induced cAMP generation with respect to CD25 expression is apparent when one examines the data for regulation and activation of transcription of the CD25 gene and will be discussed in more detail in a future section. If the high and low affinity receptors for IL-4 are both functionally active and if, as the dose response data presented here suggests, IL-4-induced CD23 and CD25 expression are mediated by high and low affinity receptors respectively, it seems rather incongruous that by artificially activating similar second messenger systems one can induce both of these cell surface marker proteins. In addition, the apparent importance of cAMP regulating transcription factor binding to elements within the CD25 promoter conflicts with the suggestion that IL-4-induced cAMP elevation and CD25 expression are under control of the high and low

affinity IL-4 receptors, respectively. This apparent paradox is not one which is easily understood or explained. There is now much evidence in favour of the idea that cytokine oligomerization is a requirement for full activation of intracellular signalling pathways. Recent studies utilizing chimeric receptors containing wild type and mutant IL-2Rβ and IL-2Rγ cytoplasmic domains have demonstrated the importance of the association of individual receptor components [Miyazaki *et al.*, 1994]. Transfection of a T cell line with c-kit-IL-2 receptor chimeras demonstrated that a successful proliferative response to stem cell factor (the natural ligand for c-kit receptors) only occurred when c-kit-IL-2Rβ and c-kit-IL-2Rγ were co-expressed [Miyazaki *et al.*, 1994]. In addition, mutation of the cytoplasmic domain of one or other of the two IL-2 receptor components resulted in the abrogation of this proliferative response. Co-transfection of chimeric receptor molecules with wild-type IL-2Rβ and IL-2Rγ cytoplasmic domains bearing different extracellular domains also resulted in abrogation of this proliferative response, suggesting that association of the extracellular-ligand binding domains is a requirement for successful signal transduction and is likely to be responsible for the association of the cytoplasmic domains. Additionally, expression of mutant forms of the IL-4 receptor demonstrates that receptor association is also important for successful IL-4 signal transduction. Mutations within the critical growth signal transduction domain of the IL-4Rα chain result, not only in loss of proliferation and Jak1, IRS-2 and Stat6 activation in response to IL-4 (events all associated with the IL-4Rα) [Keegan *et al.*, 1994; Pernis *et al.*, 1995a], but also in the failure to activate Jak3. Activation of Jak3 is an event associated with the γc component of all cytokine receptors which share this particular subunit [Johnston *et al.*, 1994; Witthuhn *et al.*, 1994; Yin *et al.*, 1994; Zeng *et al.*, 1994]. In addition, it is not only a disruption in the signalling capacity of IL-2Rβ, IL-4α and IL-7α receptor chains which diminishes γc signal transduction, as disruption of γc associated signalling also inhibits cytokine responses. Over-expression of a γc truncation mutant into BA/F3 pro-B cells, co-transfected with either IL-2Rβ, IL-4Rα or IL-7Rα chains, results in inhibition of proliferation in response to all three of these cytokines [Kawahara *et al.*, 1994]. The dominant negative effect of this γc truncation mutant is also observed

with inhibition of IL-2-induced c-*myc* expression [Kawahara *et al.*, 1994]. The revelation that the mechanism of activation of Jak tyrosine kinases most likely involves cross-phosphorylation by two kinase molecules (previously associated with their respective inactive individual receptor components), an event which only occurs when they are brought into close spatial proximity by receptor subunit association, reinforces the apparent need for receptor oligomerization for successful functional receptor activity. In light of such findings, and if one accepts the idea that a single functional receptor type is capable of inducing all of the signals required for up-regulation of CD23 and CD25, it seems logical that the activation of similar signalling pathways mimics IL-4-induction of both these surface proteins. However, the difference in dose-response data remains unexplained. In order to try and define more clearly the relationship between the IL-4 receptor, cAMP generation and CD25 expression, the effects of three other cytokines on these cellular responses were examined. Neither IL-2, IL-7, both of which have γc in their receptor complexes, nor IL-13 which is known to have certain biological activities in common with IL-4, were capable of inducing CD25 expression in human B cells. In contrast, IL-2 and IL-7, but not IL-13, were able to increase levels of intracellular cAMP in these cells. This latter observation, together with the fact that IL-4 also induces cAMP generation, indicates the possibility that increases in this second messenger may be associated with γc. Such a proposition is supported by the inability of IL-13 to mediate such a response. IL-4 and IL-13 are thought to share a receptor component and, although γc was originally proposed as a candidate for this role [Kishomoto *et al.*, 1994], there is much evidence to suggest that this is not the case. The IL-4Rα chain is now favoured as the receptor component common to these two cytokines [Keegan *et al.*, 1995; Matthews *et al.*, 1995; Smerz-Bertling and Duschl, 1995; Zurawski *et al.*, 1993]. Reports which suggest the possibility of a low affinity IL-4 receptor which may be indistinguishable in structure from the IL-13 receptor, promote the idea of a functionally dimeric low affinity IL-4 receptor. It has also been hypothesized that the functional capacity of IL-13 depends upon the availability of IL-4Rα, which is often sequestered by γc in human B cells. Such an hypothesis might explain IL-13's inability to induce cAMP

generation if this particular response were linked to IL-4 stimulation via IL-4Rα. The data presented here are further in favour of cAMP generation being a function of the high affinity IL-4 receptor but do not resolve the conflict of this idea with the dose-response data for IL-4-induced CD25 expression. Indeed, the ability of IL-2 and IL-7 to induce cAMP production, but not up-regulation of CD25, seems to confuse the issue further. However, the relationship between cAMP generation and the high affinity IL-4 receptor seems clear, as does the relationship between cAMP production and transcriptional regulation of the CD25 gene (see section 6.3.2 ). The inability of IL-2, IL-7 and IL-13, all of which share one or other of the receptor components of the IL-4 receptor, to induce CD25 suggests that this response is unique to IL-4 and as such may require the presence of both IL-4 receptor subunits (i.e., the high affinity form) for successful completion. Furthermore, if the hypothesis that receptor oligomerization is necessary for complete activation of cytokine signal transduction is correct, then the importance of IL-4-induced cAMP generation in CD25 expression seems somewhat more logical. The apparent differences in $EC_{50}$ values for CD23 and CD25 expression are small and the higher $EC_{50}$ value for IL-4-induced CD25 expression may merely be a function of a requirement for total receptor occupation in order to initiate this response.

## 6.3 IL-4-Induced Transcriptional Regulation of CD25

The CD25 promoter has been well characterised and described, particularly in human T cells, to the extent that sequences which bind to well known transcription factor complexes have been identified along with more lineage restricted binding elements. Information regarding the regulation of transcription factor binding to these elements is varied, some being well characterised while others are less so. Thus, it is known that in Jurkat and YT-1 T cell lines, an NF-κB binding site is regulated by differential binding of dimeric complexes of the c-*rel* oncogene family and, in addition, is closely associated with and, to an extent, regulates the binding of a serum response element and an Sp-1 binding site [Algarte *et al.*, 1995; Pierce *et al.*, 1995]. Use of CD25 promoter-CAT

reporter gene constructs demonstrated that stimulation of transfected T cells with PMA increases reporter gene activity and deletion mutants lacking this NF-κB binding element lose the ability to respond to phorbol ester, indicating that this site is under the control of a protein kinase C regulated pathway [Cross *et al.*, 1987; Lowenthal *et al.*, 1988]. In contrast, analyses of deletion mutants of the CD25 promoter, lacking a region 5' of this NF-κB site, led to the discovery of an element which negatively regulates transcription [Cross *et al.*, 1987; Lowenthal *et al.*, 1988; Smith and Greene, 1989]. Although it is known that this element binds a 50kDa 'silencer' protein, which represses transcription when bound to the promoter [Smith and Greene, 1989], the nature of this protein and the signal transduction mechanisms which regulate its binding remain relatively unknown. Until recently, mechanisms of regulation of the DNA binding proteins involved in transcriptional activation of the CD25 gene in human B cells were less well understood. The discovery that IL-4 is the only cytokine capable of inducing CD25 expression in human B cells, led us to try and understand the mechanisms by which IL-4-induced signal transduction regulates transcription factor binding to the CD25 promoter. The identification of two putative IL-4 response elements in the promoter pointed towards an obvious pathway for IL-4-induced regulation of CD25 expression, i.e., the activation of the transcriptional regulator Stat6. However, the work presented in this thesis describes an IL-4 regulatory pathway which targets transcription factor binding to the NRE and demonstrates the importance of removal of a DNA binding protein(s), which binds this element, from the promoter.

### 6.3.1 Regulation of Negative Regulatory Element Binding Activity

The Negative Regulatory Element (NRE) consists of an 11 base pair core element lying within a region -400 to -368 base pairs upstream from the transcriptional initiation site. Transfection of 5' deletion mutants into YT-1 T cells and HTLV-1 transformed MT-2 T cells demonstrated that deletion of this region of the CD25 promoter resulted in a 4-5-fold increase in basal and PMA-inducible transcription of a CAT reporter gene [Cross *et al.*, 1987; Lowenthal *et al.*, 1988]. In the human EDR B cell line, deletion of NRE resulted

in a 5-fold increase in basal promoter activity and a 13-fold increase in PMA-inducible activity [Burlinson *et al.*, 1996]. The data presented here demonstrate that treatment of primary human B cell with IL-4 results in almost total attenuation of DNA-binding activity to this element over a period of four hours. Considering the ability of IL-4 to up-regulate CD25 in these cells, this result indicates that a potential mechanism of achieving such a response lies with the IL-4-induced removal of DNA binding factor which represses transcription. Interestingly, in human T cell lines, IL-2, but not IL-4, induces cell surface expression of CD25 [Jankovic *et al.*, 1989] and, in contrast to the situation in B cells, IL-4 promotes an increase in binding activity to NRE [Burlinson *et al.*, 1996]. The ability of forskolin, a direct activator of adenylyl cyclase, to induce a modest elevation of cell surface CD25 in human B cells and to act with phorbol ester and calcium ionophore to enhance this expression, points to the second messenger cAMP as a candidate for regulation of CD25 transcription. Indeed, treatment of primary human B cells with either forskolin or cholera toxin, which activates adenylyl cyclase by inducing ADP-ribosylation of Gsα, mimics the ability of IL-4 to reduce transcription factor binding to NRE. IL-4 has been shown, both here and by others, to elevate intracellular levels of cAMP and we have demonstrated here for the first time that IL-4 stimulation of primary human B cells leads to activation of the cAMP-dependent protein kinase, PKA. These data, along with the knowledge that IL-4 and adenylyl cyclase-activating agents abrogate DNA-binding activity to NRE, suggest that IL-4 mediates this latter response via a cAMP generating pathway. Indeed, the ability of a specific PKA inhibitor to abolish IL-4's capacity to reduce transcription factor binding to NRE provides a direct link between IL-4-induced cAMP generation/PKA activation and regulatory control by this cytokine of a DNA binding protein which binds to the CD25 promoter. These data, despite providing evidence that IL-4 is able to regulate DNA-protein binding activity to the CD25 promoter, do not demonstrate any direct regulation of transcription of the CD25 gene, nor do they provide evidence that a cAMP-dependent pathway contributes to transcriptional activation of the gene.

Using a full length CD25 promoter -CAT reporter gene construct (pCD25-CAT), it has been possible to demonstrate the importance of a cAMP-dependent signalling pathway on transcriptional activation of the CD25 gene. The EDR B cell line, a mature EBV transformed B cell line, was chosen for ease of transfection and also because the inability of IL-4 to drive CD25 expression in these cells provided a model system where some part of the endogenous signalling pathways were blocked. Initial studies of CD25 expression at the surface of these cells demonstrated that IL-4 was unable to promote CD25 expression over a wide concentration range. However, there was an increase in CD25 expression when EDR cells were co-stimulated with IL-4 and a high concentration of forskolin. Such observations hinted that adenylyl cyclase and downstream signalling machinery were intact and that by directly activating adenylyl cyclase it was possible to promote the ability of IL-4 to induce CD25 expression. Examination of cAMP production in EDR cells in response to IL-4 revealed that, while it was possible to promote cAMP generation in these cells the response was approximately 10-fold lower than that which is observed in primary human B cells. In addition, stimulation of EDR cells with IL-4 failed to promote a decrease in NRE binding over a period of four hours, whereas forskolin mimicked the response observed in primary B cells by inducing almost total attenuation of DNA binding activity to this element over the same time period. These data suggested that the inability of IL-4 to promote sufficient cAMP generation in these cells, or a resultant decrease in binding of a repressive transcription factor to NRE, was responsible for the inability of IL-4 to induce up-regulation of CD25 at the surface of these cells. Transfection of EDR cells with pCD25-CAT demonstrated that while IL-4 was unable to induce reporter gene activity, stimulation with forskolin doubled reporter activity compared with basal activity in unstimulated pCD25-CAT transfectants. Additionally, co-stimulation with IL-4 and forskolin enhanced this response (CAT activity was three times that in unstimulated cells), indicating that while the removal of transcription factor binding from NRE via a cAMP dependent pathway is necessary for transcriptional activation of the CD25 gene, it is insufficient for inducing a maximal response. It appears, therefore, that a second or even third IL-4-induced signalling

pathway may be involved in regulation of CD25 transcriptional activation and expression.

### 6.3.2 Regulation of IL-4 Response Element Binding Activity

There is now much evidence documenting the involvement of Jak kinases and a member of the Stat family of proteins (Stat6) in IL-4 signal transduction pathways [Hou *et al.*, 1994; Quelle *et al.*, 1995; Schindler *et al.*, 1994]. Thus, upon binding to the high affinity form of its receptor, IL-4 promotes tyrosine phosphorylation and activation of Jak1 and Jak3 kinases via the IL-4Rα chain and γc, respectively [Witthuhn *et al.*, 1994]. In addition, there is now evidence supporting the ability of IL-4 to activate Jak2 in epithelial cancer cells [Murata *et al.*, 1995] suggesting that in the absence of a particular kinase (i.e., Jak3) cytokines will adapt to their environment by using other, closely related, signalling molecules. IL-4 activates the transcription factor Stat6 by promoting its tyrosine phosphorylation by Jak1 and inducing consequent homodimerization, to form a transcriptionally active protein which translocates to the nucleus of the cell. The homodimeric form of Stat6 (STF-IL-4) binds specific GAS-related binding sequences in the promoter regions of IL-4-inducible genes [Schindler *et al.*, 1994].

The presence of a Stat6 binding site in the CD25 promoter along with a putative IL-4 response element (IL-4RE), identified due to its sequence similarity to the IL-4 response elements in the CD23 gene promoter [Kohler and Rieber, 1993], suggested two potential sites for IL-4 regulation of transcription of the CD25 gene. DNA mobility shift analysis revealed that IL-4 promotes an increase in binding activity to the IL-4-response element over a four hour period, demonstrating the ability of IL-4 to induce transcription factor binding to a putative positive regulatory element in the CD25 promoter. Interestingly, competition analyses determined that protein binding to this IL-4-response element could be competed for by a recognised Stat6 sequence and that this competition was stronger than that of unlabelled IL-4RE probe itself. These data suggest that either the IL-4-response element is a weak Stat6 binding site, or that the protein complex which binds to

IL-4RE contains Stat6. The presence of two Stat6 binding sites, with different transcription factor binding affinities, within the same promoter seems unusual. One possible explanation is that the availability of multiple binding sites for a single transcription factor allows for greater transcriptional activation of a gene. An alternative explanation centres around the identification of a second IL-4 responsive transcription factor of 75kDa, designated NFIL-4 (Nuclear Factor IL-4), which binds an IL-4-response element in the MHC class II Eα gene promoter [Kretsovali and Papamatheakis, 1995]. This particular promoter element is able to bind to IL-4 response elements of similar sequence in a number of other genes and also bears a high degree of sequence homology to the IL-4 response element in the CD25 promoter. The Eα IL-4 response element is similar in sequence to certain GAS elements and NFIL-4 is able to bind some Stat recognition sequences. However, super-shift data using anti-Stat antibodies suggest that NFIL-4 is distinct from Stat proteins [Kretsovali and Papamatheakis, 1995]. Thus, the IL-4 response element in the CD25 promoter could potentially bind two distinct transcription factors, i.e., the Stat6 homodimer STF-IL-4 or the 75kDa NFIL-4 described previously. The presence of an IL-4 response element with dual binding ability in the CD25 promoter could be a function of differential expression of specific transcription factor proteins in different lymphoid cell types and indeed in different developmental phases of lymphoid cells. However, such an hypothesis seems rather unlikely as reports suggest that Stat6 is expressed in the majority of cell lineages examined, therefore, making the presence of a second IL-4-inducible transcription factor somewhat superfluous. One piece of evidence which is in favour of the CD25 promoter IL-4RE binding a factor similar to NFIL-4, is the inability of the tyrosine kinase inhibitor genistein to inhibit IL-4-induced binding to this element or binding of NFIL-4 to Eα. As Stat6 activation relies on the Jak1 tyrosine kinase, it may be predicted that such activity would be inhibited by genistein. DNA mobility super-shift assays carried out in this laboratory, using anti-Stat6 antibodies, have demonstrated no super-shift of the IL-4RE oligonucleotide binding complex from human B cell extracts. However, anti-phosphotyrosine antibodies do prevent the formation of a CD25 promoter IL-4RE

complex with B cell extracts, an observation which also correlates with the activation of NFIL-4. Thus, there appears to be the potential for IL-4 to regulate the binding of two positive transcriptional activators to the CD25 promoter, but whether it is NFIL-4 or Stat6 which mediates IL-4-induced CD25 expression remains equivocal. In light of this, future studies which directly examine the nature of the transcription factor which binds to the CD25 promoter IL-4RE, in response to IL-4 in human B cells, will be important.

### 6.3.3 Other Transcriptional Binding Sites in the CD25 Promoter

The CD25 promoter has a number of other elements with sequences which are able to bind known transcription factors; including NF-κB, SRE and Sp1 binding sites, which bind members of the c-*rel* proto-oncogene family, serum response factor and Sp1, respectively [Leung and Nabel, 1988; Pomerantz *et al.*, 1989]. There is also a retinoic acid response element which is very closely associated with the NRE [Bhatti and Sidell, 1994]. It is now known that in unstimulated T cells the NF-κB site is constitutively bound by an NF-κB p50 homodimer which represses transcription and that, upon stimulation, this repressive binding complex is exchanged for an NF-κB p50-p60 heterodimer capable of activating transcription [Algarte *et al.*, 1995]. In addition, the region encompassing the NF-κB, SRE and Sp1 binding sites has been proposed as a PMA-inducible region of the CD25 promoter in T cells. Examination of transcription factor binding to the NF-κB and Sp1 binding sites in human B cells, by DNA mobility shift assay, demonstrated no changes in binding activity in response to IL-4 over a four hour period of stimulation [Burlinson, personal communication]. These observations led to the suggestion that IL-4 did not affect expression of the CD25 gene via transcriptional activation through these particular binding sites. In light of the evidence which suggests that it is the nature of the DNA-binding complex associated with the NF-κB site which changes upon stimulation and that this element is bound in both an inactive and active state, a lack of alteration in DNA binding activity, as observed by DNA mobility shift assay, does not necessarily indicate that IL-4 is unable to regulate transcription via these promoter elements. Chronic phorbol ester treatment of resting B cells attenuates CD25

up-regulation in response to IL-4 and both anti-IgM and anti-CD40 antibodies. These data, together with the observation that both PMA and $PBu_2$ in combination with other stimuli are able to induce CD25 in human B cells, suggest the potential involvement of PKC activation in IL-4-induced CD25 expression in human B cells and also the importance of PKC in the signalling pathways of other stimuli known to promote CD25 expression. Bearing such observations in mind, and with the knowledge that PMA regulates NF-κB binding activity in T cells, it may be that a PKC-activating pathway is utilized by IL-4 in order to regulate transcription factor binding to this element in human B cells. As the DNA mobility shift data concerning DNA binding activity to NF-κB in human B cells does not give a definitive answer about the capacity of IL-4 to regulate such activity, such a hypothesis cannot be ruled out. It will, therefore, be an important future step to define whether IL-4 is able to induce an exchange of a repressive transcriptional complex for an active one at the NF-κB site in human B cells and if so, to determine which signalling pathway(s) are responsible for regulating such an exchange. Interestingly, IL-1 has been shown to induce the up-regulation of CD25 by a cAMP-dependent pathway in a natural killer cell line, a signalling pathway which is believed to be linked to transcriptional activation through an NF-κB binding site. With the certain knowledge that IL-4 is able to promote cAMP generation and up-regulation of CD25 in human B cells, it will be of interest to determine whether IL-4 can mediate similar cAMP-dependent effects on NF-κB binding activity in these cells.

## 6.4 What is the NRE Binding Protein?

Little is known about the nature of the transcriptional repressor protein which binds to the negative regulatory element in the CD25 promoter. Information regarding this factor in human T cells tells us only that NRE is bound by a 50kDa 'silencer' protein [Smith and Greene, 1989], i.e., a protein which represses transcription of the CD25 gene when it is bound to the promoter. The lack of published data concerning regulation of binding of this 'silencer' protein to NRE in T cells has meant that, to date, speculation about the

nature of this factor has been minimal. The observations presented in this thesis regarding the regulation of protein binding to NRE via a cAMP-dependent pathway, have allowed the proposition of certain hypotheses as to what this DNA-binding protein may be. However, a more definitive statement may be made about what this protein is not, rather than what it is.

Our data have indicated that IL-4 regulates binding of a transcriptional repressor to the CD25 promoter via a cAMP-dependent pathway. The most commonly recognized means of cAMP and active PKA regulating transcription is by the serine/threonine phosphorylation and subsequent activation of CREB (cAMP-responsive element binding protein), which is followed by its binding of CRE sequences in cAMP-responsive genes [Reviewed by Montminy *et al.*, 1990]. In this instance, however, the evidence suggests that NRE binding protein is not a member of the CREB family; thus, the 11 base pair core sequence of NRE is distinct from a classic CRE sequence and, more definitively, data presented here demonstrate that a CRE sequence is unable to compete with NRE for DNA-protein binding activity. However, CRE sequences and CREB proteins are not the only reported cAMP-responsive transcriptional activators. There is a somewhat controversial hypothesis that while the catalytic subunit of PKA activates CREB family members by phosphorylation, the simultaneously released regulatory subunits are also capable of translocating to the nucleus and acting directly as transcriptional activators by binding to specific sequences in the promoters of certain cAMP-responsive genes [Nagamine and Reich, 1985]. Additionally, a recent study has identified novel motif within the promoter of the cAMP-responsive tryptophan hydroxylase gene [Boularand *et al.*, 1995]. This element contains two motifs which have been determined essential for cAMP-responsiveness, an inverted CCAAT box and a 9 base pair sequence 5'-CCG CTG CCC-3', which are entirely distinct from a classic CRE sequence. While neither of the sequences of this element bear any resemblance to the NRE sequence (Table 6.1), it highlights the potential for the existence of a variety of novel cAMP-responsive elements.

**Table 6.1 cAMP-Responsive Transcription Factor Binding-Promoter Elements**

| cAMP-responsive element | Sequence (5'-3') |
|---|---|
| CRE | AGA GAT TGC CTG ACG TCA GAG AGC TAG |
| NRE | TT CAT CCC GGG |
| RII Binding motif | GAA AGG GTG AGA AAG AGC TGA TTG AGG |
| Tryptophan hydroxylase: | |
| - inverted CCAAT motif | ATTGG |
| - 9 base pair motif | CCG CTG CCC |

Thus, it seems that the sequence of NRE defines a novel cAMP-response element and the question of the nature of the 50kDa binding protein remains unknown. It is, however, probably safe to surmise that CREB is not involved in this IL-4-induced cAMP/PKA dependent signalling pathway.

Efforts to identify DNA binding sites for other lymphoid expressed transcription factors which have a recognition sequence similar to that of NRE have revealed the existence of a zinc finger DNA binding protein, YY1, which binds a consensus sequence with a high degree of homology to NRE. Analyses of the binding sites for YY1 from a number of gene promoters revealed a consensus sequence consisting of a conserved 5'-CAT-3' core flanked by two variable regions with the consensus 5'- (C/g/a) (G/t) (G/t/a) CATN (T/a) (T/g/c)-3' [Hyde-DeRuyscher *et al.*, 1995]. If one examines the sense strand sequence for NRE (5'- TT CAT CCC AGG -3') it is possible to observe the sequence similarity between NRE and YY1 sites. Judging by the sequence similarity between the YY1 and NRE binding sites it seems possible that NRE may bind a YY1 complex. In addition to structural similarities between the two sequences, they have certain functional

characteristics in common. Thus, NRE binding protein very obviously acts as a repressor of transcription of the CD25 gene and it has been shown that YY1 has the capacity to act either as a repressor or activator of transcription, depending upon the gene which it regulates. An element within the GM-CSF promoter (sequence CATT(A/T)) which is required for promoter activity was found to bind YY1 [Ye *et al.*, 1994]. These studies were the first to link YY1 activity to regulation of cytokine gene expression. Sequences close to the initiator region of HIV-1 are also homologous to YY1 binding sequences and HIV long terminal repeat expression (i.e., an LTR-CAT gene construct) can be repressed by co-transfection with a YY1 expression vector [Margolis *et al.*, 1994]. Thus, YY1 is highly versatile and can act as both an activator or repressor of transcription. Considering the sequence similarity between YY1 binding sites and NRE, the possibility that the NRE binding protein contains YY1 as at least part of its structure is something which should be examined.

## 6.5 How does the IL-4 Receptor Couple to Adenylyl Cyclase?

There are now eight fully characterised isoforms of adenylyl cyclase all of which are under multiple differential mechanisms of regulation [Reviewed by Pieroni *et al.*, 1993; Taussig and Gilman, 1995]. Thus, all can be activated by the alpha subunit of the stimulatory G protein, Gs. However, other G protein subunits produce different, less ubiquitous, patterns of regulation such that some are activated by the βγ subunits of Gs while others are inhibited by Gi alpha [Tang and Gilman, 1991b]. In addition to regulation by G proteins, some adenylyl isoforms are activated by $Ca^{2+}$/Calmodulin [Krupinski *et al.*, 1989; Tang *et al.*, 1991a] and others are inhibited by increases in intracellular calcium [Debernardi *et al.*, 1993; Yoshimura and Cooper, 1992], while the activity of one or two isoforms are enhanced by protein kinase C activity [Yoshimura and Cooper, 1993]. It is now apparent that the activity of each individual isoform of adenylyl cyclase may be subject to regulation by more than one signalling pathway and that the mechanism of regulation is ultimately dependent upon the initial stimulus received at the

cell surface. Additionally, it is probable that certain isoforms are subject to more than one mechanism of regulation at any one time in response to specific stimuli and so the magnitude of the cAMP response which is subsequently generated is under very tight control. The duration and magnitude of a cAMP response, as well as being dependent on activation of adenylyl cyclase, is also regulated by the activity of phosphodiesterases, enzymes which degrade cAMP to inactive 5'-AMP. In addition to the attenuation of an adenylyl cyclase response by degrading cAMP, elevation of basal levels of cAMP by the inhibition of phosphodiesterase activity is now known to have a role in intracellular signalling in certain circumstances. Thus, a cAMP response can be generated either by activation of adenylyl cyclase or by inhibition of phosphodiesterase activity.

Data presented here, and previously, have demonstrated the ability of IL-4 to promote cAMP generation in primary human B cells. The question of how IL-4 induces this response, i.e., how the IL-4 receptor complex couples to either an adenylyl cyclase or a phosphodiesterase remains to be determined, as does the question of whether it is activation of an adenylyl cyclase or inhibition of a phosphodiesterase which is responsible for mediating IL-4-induced cAMP generation. We have shown that by stimulation of human B cells with certain pharmacological agents it is possible to induce modest increases in CD25 expression at the plasma membrane of these cells. Thus, stimulation with forskolin alone promotes some up-regulation of CD25 and co-stimulation of human B cells with either PMA and forskolin or with $PBu_2$ and/or ionomycin in combination with forskolin enhances this response. It is a possibility that activation of protein kinase C or elevating levels of intracellular calcium may enhance the effects of forskolin by directly activating specific adenylyl cyclase isoforms. PKC is able to activate types II and VII adenylyl cyclase and $Ca^{2+}$/Calmodulin activates types I, III and VIII. It cannot be ruled out however, that activation of PKC and mobilization of $Ca^{2+}$ in this way have a role in up-regulating CD25 via signalling pathways which are distinct from the cAMP-dependent pathway. The ability of IL-4 to decrease transcription factor binding to NRE in the CD25 promoter and the knowledge that this is most likely

mediated via a cAMP-dependent pathway, provides a model for examining other second messengers which may be responsible for IL-4 regulation of cAMP generation. The inability of the PKC inhibitor bisindolylmaleimide to abrogate IL-4-induced down-regulation of NRE binding activity, in the manner which the PKA inhibitor H89 does, strongly suggests that IL-4 does not regulate adenylyl cyclase activity via a PKC dependent pathway. The effects of tyrosine kinase inhibitor genistein on this binding activity are somewhat more ambiguous; in some instances the inhibitor abrogates IL-4-induced attenuation of NRE binding and in others it does not. The concept of tyrosine kinases being able to regulate adenylyl cyclase activity is a controversial one, although there is some evidence in favour of growth factor receptors with intrinsic tyrosine kinase activity being able to regulate adenylyl cyclase. It has been demonstrated that EGF, which is known to activate adenylyl cyclase via Gsα, can also activate this enzyme via the tyrosine kinase domain of the EGF receptor [Nair and Patel, 1993]. Thus, inhibition with tyrphostins and treatment with protein tyrosine phosphatases inhibited both EGF receptor phosphorylation and EGF-induced adenylyl cyclase activation without alteration of specific binding of the ligand [Nair and Patel, 1993]. Whether EGF directly activates adenylyl cyclase, by tyrosine phosphorylation of the latter, or whether it achieves activation via a mediator molecule, such as a switch kinase, is unknown. The possibility that chelation of intracellular calcium affecting this NRE binding activity is one which remains to be investigated and may give some more insight into how IL-4 regulates adenylate cyclase activity, as will examination of the effects of PKC, tyrosine kinase inhibitors etc. on the ability of IL-4 to generate cAMP directly. The question of whether it is IL-4-induced activation of adenylyl cyclase or inhibition of phosphodiesterase activity which is responsible for the cAMP-dependent pathway which regulates NRE binding activity, is more readily answered. Treatment of primary human B cells with the general phosphodiesterase inhibitor IBMX does not result in a decrease in DNA binding activity to NRE over a four hour period, a result which is in direct contrast with the effects of IL-4 on B cells isolated from the same source. It seems likely, therefore, that IL-4 generates cAMP in human B cells by activating adenylyl cyclase rather than by

inhibiting phosphodiesterase activity. The question of which isoform of adenylyl cyclase IL-4 activates remains unanswered, however, from the evidence presented here it does appear that it is unlikely to be activated by PKC. This does not rule out the possibility of the involvement of one of the isoforms which can be regulated by PKC because, as mentioned earlier, all eight isoforms are subject to more than one regulatory mechanism. Examination of the pattern of expression of adenylyl cyclase isoforms in human B cells, either by use of adenylyl cyclase isoform-specific antibodies or by PCR, may give at least some clues to which isoforms are available for signal transduction. Additionally, one plausible method of determining whether or not Jak3 was involved in generation of cAMP,would be the over expression of a Jak3 mutant with a kinase inactive domain in an IL-4-responsive B cell line. Similar studies have shown that such mutants can exert a dominant negative effect on Jak2 related signalling pathways [Guschin *et al.*, 1995].

Another factor in IL-4 regulation of adenylyl cyclase activity is which component of the receptor is involved in coupling the receptor to cAMP generation. Most evidence suggests that receptor subunit oligomerization is a requirement for functional activation of cytokine-induced signal transduction pathways. The high affinity form of the IL-4 receptor consists of the IL-4Rα chain and γc, however, there is now evidence of a second multi-component receptor that is able to bind IL-4 and possibly IL-13 as well. This contains the IL-4Rα chain and a novel protein of 65-70kDa molecular weight, which has also been proposed as the IL-13Rα chain, and is generally considered to be the structure of the IL-13 receptor complex. One defining characteristic of this IL-4/IL-13 receptor is the absence of the IL-2 receptor γc chain. We examined the ability of cytokines such as IL-2 and IL-7, both of which have γc in their structure, and IL-13, which is known to share a number of biological functions with IL-4, to generate cAMP in human B cells. Our data demonstrate that while both IL-2 and IL-7 promote the generation of cAMP, IL-13 does not. This would seem to suggest the possibility of γc being involved in the activation of cAMP. In contrast, neither IL-2, IL-7 or IL-13 were capable of promoting a decrease in transcription factor binding to NRE in the CD25

promoter. Although this lack of response is in agreement with the inability of all three of these cytokines to up-regulate CD25 in human B cells, it does seem at odds with the cAMP data and may be the function of a requirement for more than one signalling pathway to regulate NRE binding activity. As previously suggested the up-regulation of CD25 by cytokines may require a pattern of signal transduction pathways unique to IL-4 signalling through its unique high affinity receptor.

## 6.6 Hypothetical Model for IL-4 Signal Transduction Pathways Which Regulate CD25 Expression

A partial hypothetical model of how IL-4 regulates both the binding of transcription factors to the CD25 promoter element and transcriptional activation of the gene can be considered (Figure 6.1). IL-4-induced elevation of intracellular levels of cAMP results in the activation of a cAMP-dependent protein kinase, PKA. This cAMP-generating/PKA-activating pathway is responsible for modulation of the DNA binding activity of a protein which constitutively binds to the negative regulatory element of the CD25 promoter in unstimulated cells. How PKA mediates this response is unknown, however, our evidence suggests that it does not involve the activation of CREB, an observation which is in accordance with the lack of sequence similarity between NRE and a consensus CREB binding element, CRE. There are two possible mechanisms for PKA regulating NRE binding protein(s); either PKA directly phosphorylates the binding protein(s) on serine/threonine residues or it activates an intermediary signalling molecule(s) which in turn regulates NRE binding activity. Until the identification of the NRE binding protein, there is no easy way of determining which of these mechanisms is employed. The data demonstrating the inability of phosphodiesterase inhibitor IBMX to mimic the ability of IL-4 to attenuate DNA binding activity to NRE suggests that IL-4-induced cAMP generation is a product of activation of adenylyl cyclase rather than inhibition of a phosphodiesterase. Additionally, the data presented here which demonstrate the ability

of IL-2 and IL-7, but not IL-13, to induce cAMP generation, point towards activation of adenylyl cyclase being regulated by the γc component of the IL-4 receptor. If this is the case it is possible that such a response is mediated by Jak3, the major signalling molecule associated with γc. There is no evidence demonstrating the tyrosine phosphorylation of adenylyl cyclase isoforms, however, it has been shown that inhibition of tyrosine phosphorylation does interfere with the ability of the EGF receptor to activate adenylyl cyclase. It is possible that if the intrinsic tyrosine kinase domain of the EGF receptor does not induce this activation directly, it may do so via an intermediate factor such as a 'switch' kinase.

CD25 promoter-CAT reporter transfection studies in the EDR B cell line demonstrate the importance of cAMP generation with respect to transcriptional activation of the CD25 gene. The ability of forskolin and IL-4 to synergistically increase reporter gene activity in these cells highlights the need for a second IL-4-induced signal transduction pathway to promote a maximal transcriptional response. One plausible candidate for a second IL-4-induced signalling pathway is the activation of Stat6 and the formation of a transcriptionally active homodimeric complex, STF-IL-4. IL-4 is able to induce the tyrosine phosphorylation and therefore activation of Stat6 in EDR B cells. In addition, there are two putative IL-4-response elements in the CD25 promoter; a Stat6 binding site and an IL-4-response element. The latter has sequence homology to other IL-4 response elements which have been identified binding to a second 75kDa IL-4 induced transcription factor, NFIL-4. Our data demonstrate the IL-4 response element in the CD25 promoter is a weak Stat6 binding site, although super-shift data suggest that the IL-4RE-binding complex in human B cells is not Stat6. The tyrosine kinase inhibition data also seem to favour NFIL-4 as the IL-4RE binding protein, however, the role of such a factor and Stat6 in regulation of CD25 expression remains equivocal.

With respect to other regulatory elements in the CD25 promoter; IL-4 promotes the attenuation of DNA binding activity to a retinoic acid response element (RARE) which,

when bound by its transcription factor(s), negatively regulates transcription of the gene. However, little is known about the signal transduction pathways which regulate RARE binding. Despite reports that IL-4 does not promote any alteration in DNA-protein complex binding to the NF-κB site in the CD25 promoter in human B cells, such observations remained undefined. The PMA-induced exchange of a repressive transcriptional complex for a positive one at the NF-κB site in T cells suggests a process of activation that may not necessarily be detectable by DNA mobility shift. This fact, together with the data demonstrating that a phorbol ester-sensitive PKC dependent pathway may be involved in IL-4 signal transduction, particularly in IL-4-induced up-regulation of CD25, suggests that IL-4 regulation of transcription factor binding to NF-κB should be re-investigated.

The work presented in this thesis has demonstrated a down-stream target for the previously identified IL-4-induced cAMP generation, namely, the as yet unidentified NRE-binidng protein(s). Additionally, there is strong evidence that this cAMP response occurs as a result of activation of adenylyl cyclase and that the effects of this second messenger upon NRE binding activity are mediated by PKA. IL-4-induced down-regulation of transcription factor binding to NRE via this cAMP dependent pathway, while being necessary for transcriptional activation, is an insufficient signal for producing maximal expression of the CD25 gene. The CD25 promoter is complex and there are a number of potential regulatory elements through which IL-4 may control transcription. Thus, determining the importance of IL-4-induced regulation of transcription factor binding to the IL-4 response element, the Stat6 site and the NF-κB site may be the key to fully understanding the ability of IL-4 to up-regulate CD25 expression in human B cells.

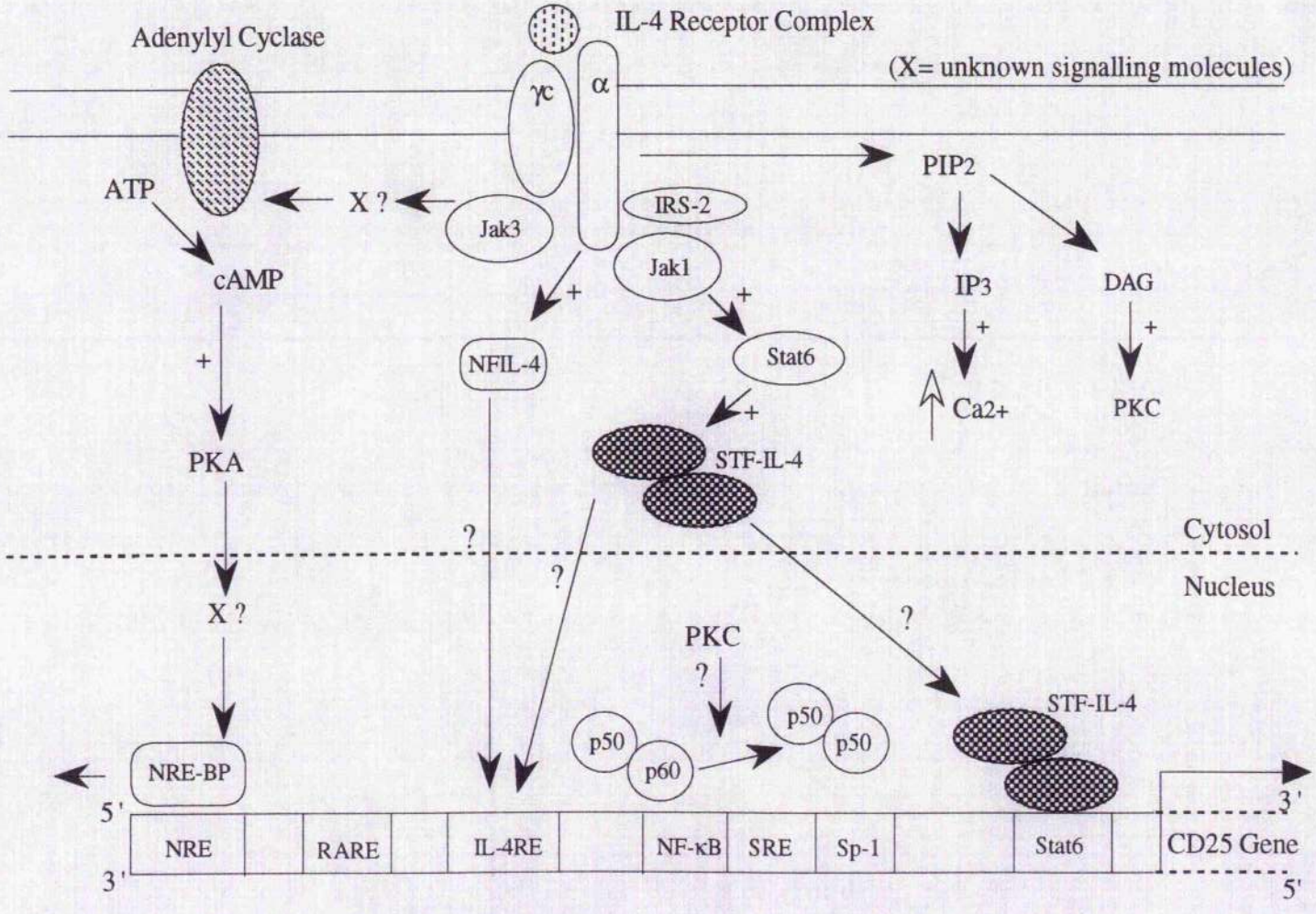



Figure 6.1: Hypothetical Model for IL-4-Mediated Regulation of CD25 Expression in Human B Lymphocytes

# References

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

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

F. W. Alt, E. M. Oltz, F. Young, J. Gorman, G. Taccioli and J. Chen (1992) *Immunol. Today*, **13**, 306-313.

J. Andersson, M. H. Schreier and F. Melchers (1980) *Proc. Natl. Acad. Sci. USA.*, **77**. 1612-1620

P. Andre, J. C. Cambier, T. K. Wade, T. Ratz and W. F. Wade (1994) *J. Exp. Med.*, **179**, 763-768.

R. J. Armitage, W. C. Fanslow, L. Strockbrine, T. A. Sato, K. N. Clifford, B. M. Macduff, D. M. Anderson, S. D. Gimpel, T. Davis-Smith, C. R. Maliszewski, E. A. Clark, C. A. Smith, K. H. Grabstein, D. Cosman and M. K. Spriggs (1992) *Nature*, **357**, 80-82.

S. Arruda and J. L. Ho (1992) *J. Immunol.*, **149**, 1258-1264.

G. Aversa, J. Punomen, G. B. Cocks, R. D. W. Maleyfyt, F. Vega Jr., S. M. Zurawski, G. Zurawski and J. E. de Vries (1993) *J. Exp. Med.*, **178**, 2213-2218.

C. M. Bacon, E. F. Petricoin III, J. R. Ortaldo, R. C. Rees, A. C. Larner, J. A. Johnston and J. J. O'Shea (1995) *Proc. Natl. Acad. Sci. USA*, **92**, 7307-7311.

A. S. Baldwin Jr. (1996) *Ann. Rev. Immunol.*, **14**, 649-681.

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

J. Banchereau, Y. J. Lui, F. Briere and F. Rousset (1994) *Stem Cells*, **12**, 278-288.

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

I. Berberich, G. L. Shu and E. A. Clark (1994) *J. Immunol.*, **153**, 4357-4366.

M. C. Bergman, J. F. Attrep, A. C. Grammer and P. E. Lipsky (1996) *J. Immunol.*, **156**, 3118-3132.

I. Bhatti and N. Sidell (1994) *Immunology*, **81**, 273-279.

D. Blanchard, C. Gailard, P. Herman and J. Banchereau (1994) *Eur. J. Immunol.*, **24**, 330-335.

J.-Y. Bonnefoy, J. Shields and J.-J. Mermod (1990) *Eur. J. Immunol.*, **20**, 139-144.

J.-Y. Bonnefoy, T. DeFrance, C. Peronne, C. Menetrier, F. Rousset, J. Pene, J. E. de Vries and J. Banchereau (1988) *Eur. J. Immunol.*, **18**, 117-122.

M. Boothby, E. Gravallesse, H.-C. Liou and L. H. Glimcher (1988) *Science*, **242**, 1559-1562.

J. Borst, G. S. Brouns, E. de Vries, M. C. M. Verschuren, D. Y. Mason, J. M> Van Dongen (1993) *Immunol. Rev.*, **132**, 49-84

M. C. Bosco, I. Epinoza-Delgadp, M. Schwabe, G. L. Gusella, D. L. Longo, K. Sugamura and L. Varesio (1994) *Blood*, **83**, 2995-3002.

G. W. Both, L. Taylor, J. W. Pollard and E. J. Steele (1990) *Mol. Cell Biol.*, **10**, 5187-5196.

S. Boularand, M. C. Darmon, P. Ravassard and J. Mallet (1995) *J. Biol. Chem.*, **270**, 3757-3764.

P. Brindle, T. Nakajima and M. Montminy (1995) *Proc. Natl. Acad. Sci. USA*, **92**, 10521-10525.

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

B. L. Brown, R. P. Ekins and J. D. M. Albano (1972) *Adv. Cyclic Nucleotide Res.*, **2**, 25-40.

E. L. Burlinson, B. W. Ozanne and W. Cushley (1996) *EMBO J. Submitted.*

E. L. Burlinson, H. M. Pringle, B. W. Ozanne and W. Cushley (1995) *Immunol. Letts.*, **45**, 93-98.

P. D. Burrows and M. D. Cooper (1993) *Curr. Opin. Immunol.*, **5**, 201-206.

R. D. J. Butcher and W. Cushley (1991) *Immunology*, **72**, 511-518.

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

J. J. Cali, R. S. Parekh and J. Krupinski (1996) *J. Biol. Chem.*, **271**, 1089-1095.

J. J. Cali, J. C. Zwaagastra, N. Mons, D. M. F. Cooper and J. Krupinski (1994) *J. Biol. Chem.*, **269**, 12190-12195.

R. E. Callard (1990) *Cytokines and B lymphocytes.* Academic Press, London.

R. E. Callard (1991) *Brit. J. of Haemat.*, **78**, 293-299.

R. E. Callard, S. H. Smith and E. Scott (1991) *Int. Immunol.*, **3**, 157-163.

W. G. Cance, R. J. Craven, T. M. Weiner and E. T. Lui (1993) *Int. J. Cancer*, **54**, 571-577.

D. Chakravarti, V. J. LaMorte, M. C. Nelson, T. Nakajima, I. G. Schulman, H. Jugulion, M. Montminy and R. M. Evans (1996) *Nature*, **383**, 99-103.

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

T. Chiba, A. Yamaguchi, T. Yamatani, A. Nakamura, T. Morishita, T. Inui, M. Fukase, T. Noda and T. Fujita (1989) *Am. J. Physiol.*, **256**, 331-335.

T. Chijiwa, A. Mishima, M. Hagiwarwa, M. Sano, K. Hayashi, T. Inoue, K. Naito, T. Toshioka and H. Hidaka (1990) *J. biol. Chem.*, **265**, 5267-5272.

E.-J. Choi, Z. Xia and D. R. Storm (1992) *Biochem.*, **31**, 6492-6498.

E. Clark, G. Shu, B. Luscher, K. Draves, J. Banchereau, J. Ledbetter and M. Valentine

(1989) *J. Immunol.*, **143**, 3873-3880.

H. C. Clevers, M. A. Oosterwegel and K. Georgopoulos (1993) *Immunol. Today*, **14**, 591-596.

M. Comb, N. C. Birnberg, A. Seasholtz, E. Herbert and H. M. Goodman (1986) *Nature*, **323**, 353-356.

W. L. Combest, T. J. Bloom and L. G. Gilbert (1988) *J. of Neurochem.*, **15**, 1581-1591.

A. I. Constantinou, S. P. Squinto and R. A. Jungmann (1985) *Cell*, **42**, 429-437.

S. J. Cook and M. J. O. Wakelam (1992) *Rev. Physiol. Biochem. Pharmacol.*, **119**, 13-58.

D. M. F. Cooper, M. K. Ahlijanian and E. Perez-Reyes (1988) *J. Cell. Biochem.*, **36**, 417-427.

D. Cosman, S. D. Lyman, R. L. Idzerda, M. P. Beckmann, L. S. Park, R. G. Goodwin and C. J. March (1990) *TIBS*, **15**, 265-269.

D. H. Crawford and D. Catovsky (1993) *Immunology*, **80**, 40-44.

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

W. Cushley and M. M. Harnett (1993) *Biochem. J.*, **292**, 313-332.

M. A. Debernardi, R. Munshi, M. Yoshimura, D. M. F. Cooper and G. Brooker (1993) *Biochem. J.*, **293**.

N. Defer, O. Marinx, D. Stengel, A. Danisova and V. Iourgenko (1994) *FEBS Lett.*, **351**, 109-113.

T. Defrance, B. Vanbervliet, I. Durand and J. Banchereau (1989) *Eur. J. Immunol.*, **19**, 293-299.

A. L. DeFranco, E. S. Raveche, R. Asofsky and W.E. Paul (1982) *J. Exp. Med.*, **155**, 1523-1527.

A. M. Delegeane, L. H. Ferland and P. L. Mellon (1987) *Moll. Cell. Biol.*, 7, 3994-4002.

H. H. J. Deutsch, K. Koettnitz, J. Chung and F. S. Kalthoff (1995) *J. Immunol.*, 3696-3703.

J. P. DiSanto, A. Dautry-Varsat, S. Certain, A. Fischer and G. de Saint Basile (1994) *Eur. J. Immunol.*, **24**, 475-479.

M. Edinger, W. G. Bessler and B. Kleine (1994) *Immunol.*, **81**, 205-210.

A. Ehlich, S. Schaal, H. Gu, D. Kitamura, W. Muller and K. Rajewsky (1993) *Cell*, **72**, 695-704.

P. Engel, L. J. Zhou, D. C. Ord, S. Sato, B. Koller and T. F. Tedder (1995) *Immunity*, **3**, 39-50.

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

M. Faris, F. Gaskin, J. T. Parsons and S. M. Fu (1994) *J. Exp. Med.*, **179**, 1923-1931.

D. T. Fearon and R. H. Carter (1995) *Ann. Rev. Immunol.*, **13**, 127-149.

M. J. Finney, G. Guy, R. H. Michell, J. Gordon, B. Dugas, K. P. Rigley and R. E. Callard (1990) *Eur. J. Immunol.*, **20**, 151-156.

B. M. J. Foxwell, K. Barrett and M. Feldman (1992) *Clin. Exp. Immunol.*, **90**, 161-169.

D. A. Frank, M. J. Robertson, A. Bonni, J. Ritz and M. E. Greenberg (1995) *Proc. Natl. Acad. Sci. USA*, **92**, 7779-7783.

A. S. Freedman, G. Freeman, J. Whitman, J. Segil, J. Daley, H. Levine and L. M. Nadler (1989) *Eur. J. Immunol.*, **19**, 849-855.

X. Y. Fu (1992a) *Cell*, **70**, 323-325.

X. Y. Fu, C. Schindler, T. Improta, R. Aebersold and J. E. J. Darnell (1992b) *Proc. Natl. Acad. Sci. USA*, **89**, 7840-7843.

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

S. L. Gaffen, S. Y. Lai, W. Xu, F. Gouilleux, B. Groner, M. A. Goldsmith and W. G. Greene (1995) *Proc. Natl. Acad. Sci. USA*, **92**, 7192-7196.

T. Gajewski and F. Fitch (1988) *J. Immunol.*, **140**, 42-45.

P. Garrone and J. Banchereau (1993) *Mol. Immunol.*, **30**, 627-635.

C. Geilen, M. Weiprecht, T. Wieder and R. Werner (1992) *FEBS Letts.*, **309**, 381-384.

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

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

M. A. Goldsmith, W. Xu, C. Amaral, E. Kuczek and W. C. greene (1994) *J. Biol. Chem.*, **269**, 14698-14704.

G. A. Gonzalez and M. R. Montminy (1989) *Cell*, **59**, 675-680.

K. H. Grabstein, J. Eisenman, K. Shanebeck, C. Rauch, S. Srinivasan, V. Fung, C. Beers, J. Richardson, M. A. Schoenborn, M. Ahdieh, L. Johnson, M. R. Alderson, J. D. Watson, D. M. Anderson and J. G. Giri (1994) *Science*, **264**, 965-968.

D. Graf, U. Korthauer, H. Mages, G. Senger and R. Kroczek (1992) *Eur. J. Immunol.*, **22**, 3191-3194.

D. Guschin, N. Rogers, J. Briscoe, B. A. Witthuhn, D. Watling, F. Horn, S. Pellegrini, K. Yasukawa, P. Heinrich, G. R. Stark, J. N. Ihle and I. M. Kerr (1995) *EMBO J.*, **14**, 1421-1429.

J. F. Habener (1990) *Mol. Endocrinil.*, **4**, 1087-1094.

N. Harada, B. E. Castle, D. M. Gorman, N. Itoh, Y. Schreurs, R. L. Barret, M. Howard and A. Miyajima (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 857-861.

N. Harada, G. Yang, A. Miyajima and M. Howard (1992) *J. Biol. Chem.*, **267**, 22752-22758.

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

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

G. Haughton, L. W. Arnold, A. C. Whitmore and S. H. Clarke (1993) *Immunol. Today*, **14**, 84-87.

Y.-W. He, R. B. Levy and T. R. Malek (1995a) *Proc. Natl. Acad. Sci. USA*, **92**, 5689-5693.

Y.-W. He and T. R. Malek (1995b) *J. Immunol.*, **155**, 9-12.

K. Helleuvo, M. Yoshimura, N. Mons, P. L. Hoffman, D. M. F. Cooper and B. Tabakoff (1995) *J. Biol. Chem.*, **270**, 11581-11589.

D. J. Hilton, J. G. Zhang, D. Metcalf, W. S. Alexander, N. A. Nicola and T. A. Willson (1996) *Proc. Natl. Acad. Sci. USA*, **93**, 497-501.

J. Hou, U. Schindler, W. J. Henzel, T. C. Ho, M. Brasseur and S. L. McKinght (1994) *Science*, **265**, 1701-1706.

M. D. Houslay, J. C. Metcalfe, G. B. Warren, T. R. Hesketh and G. A. Smith (1976) *Biochim. Biophys. Acta.*, **436**, 489-494.

M. Howard, J. Farrar, M. Hilfike, B. Johnson, K. Takatsu, T. Hamaoka and W. E. Paul (1982) *J.Exp. Med.*, **155**, 914-923.

H. M. Hu, K. O'Rourke, M. S. Boguski and V. M. Dixit (1994) *J. Biol. Chem.*, **269**, 30069-30072.

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

T. Ichiki, W. Takahashi and T. Watanabe (1993) *J. Immunol.*, **150**, 5408-5417.

R. L. Idzerda, C. L. March, B. Mosely, S. D. Lyman, T. Vandenbos, S. D. Gimpel, W. S. Din, K. H. Grabstein, M. B. Widmer, L. H. Park, D. Cosman and M. P. Backman (1990) *J.Exp. Med.*, **171**, 861-876.

J. N. Ihle, B. A. Witthuhn, F. W. Quelle, K. Yamamoto and O. Silvennoinen (1995) *Annu. Rev. Immunol.*, **13**, 369-398.

K. Izuhara, M. Sakai, R. Inaba, T. Imamura, M. Howard and N. Harada (1995)

*Cellular Immunol.*, **163**.

O. Jacobwitz, J. Chen, R. T. Premont and R. Iyenger (1993) *J. Biol. Chem.*, **268**, 3829-3832.

D. L. Jankovic, M. Gibert, D. Baran, J. Ohara, W. E. Paul and J. Theze (1989) *J. Immunol.*, **142**, 3113-3129.

D. F. Jelinek and P. E. Lipsky (1983) *J. Immunol.*, **130**, 2597-2604.

D. F. Jelinek and P. E. Lipsky (1987) *Adv. in Immunol.*, **40**, 1-59.

D. F. Jelinek and P. E. Lipsky (1988) *J. Immunol.*, **141**, 164-173.

G. L. Johnson, B. B. Wolfe, T. K. Harden, P. Molinoff and J. P. Perkins (1978) *J. Biol. Chem.*, **253**, 1472-1480.

J. A. Johnston, C. M. Bacon, D. S. Finbloom, R. C. Rees, D. Kaplan, K. Shibuya, J. R. Ortaldo, S. Gupta, Y. Q. Chen, J. D. Giri and J. J. O'Shea (1995) *Proc. Natl. Acad. Sci. USA*, **92**, 8705-8709.

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

J. Jongstra and V. Misener (1993) *Immunol. Rev.*, **132**, 107-123.

L. Justement, Z. Chen, L. Harris, J. Ransom, V. Sandoval, C. Smith, D. Renwick, N. Roehm and J. Cambier (1986) *J. Immunol.*, **137**, 3664-3670.

P. Kaldy and A.-M. Schmitt-Verhulst (1995) *Eur. J. Immunol.*, **25**, 889-895.

Y. Kamei, L. Xu, T. Heinzel, J. Torchia, R. Kurokawa, B. Gloss, S.-C. Lin, R. A. Heyman, D. W. Rose, C. K. Glass and M. G. Rosenfeld (1996) *Cell*, **85**, 403-414.

A. Kantor (1991) *Immunol. Today*, **12**, 389-391.

A. Kantor, A. Stall, S. Adems and L. Herzenberg (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 3320-3324.

H. Karasuyama, A. Kudo and F. Melchers (1990) *J. Exp. Med.*, **172**, 969-972.

W. Kasinrerk, O. Majdic, K. Praputpittaya and N. Sittisombut (1994) *Immunol.*, **83**, 33-37.

T. Katada, K. Kusakabe, M. Oinuma and M. Ui (1987) *J. Biol. Chem.*, **262**, 11897-11900.

S. Katsushika, L. Chen, J.-I. Kawabw, R. Nilakantan, N. J. Halnon, C. J. Homcy and Y. Ishikawa (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 8774-8778.

A. Kawahara, Y. Minami, T. Miyazaki, J. N. Ihle and T. Tanigucji (1995) *Proc. Natl. Acad. Sci. USA*, **92**, 8724-8728.

A. Kawahara, Y. Minami and T. Taniguchi (1994) *Mol. Cell Biol.*, **14**, 5433-5440.

B. L. Kee and C. J. Paige (1995) *Int. Rev. Cytol.*, **157**, 129-179.

A. D. Keegan, J. A. Johnston, P. J. Tortolani, L. J. McReynolds, C. Kinzer, J. J. O'Shea and W. E. Paul (1995) *Proc. Natl. Acad. Sci. USA*, **92**, 7681-7685.

A. D. Keegan, K. Nelms, M. White, L.-M. Wang and J. H. Pierce (1994) *Cell*, **78**, 810-820.

T. Kishomoto, T. Taga and S. Akira (1994) *Cell*, **76**, 253-262.

D. Kitamura, A. Kudo, S. Scaal, W. Muller, F. Melchers and K. Rajewsky (1992) *Cell*, **69**, 823-831.

G. G. B. Klaus, M. S. K. Choi and M. Holman (1994) *Eur. J. Immunol.*, **24**, 3229-3232.

G. G. B. Klaus and M. M. Harnatt (1990) *J. Immunol.*, **20**, 2301-2307.

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

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

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

H. Kotiandes, M. Moczygemba, M. F. White and N. C. Reich (1995) *J. Biol. Chem.*, **270**, 19481-19486.

K. Kotowicz and R. E. Callard (1993) *Eur. J. Immunol.*, **23**, 2550-2556.

D. S. Krause and C. Deutsch (1991) *J. Immunol.*, **146**, 2285-2294.

A. Kretsovali and J. Papamatheakis (1995) *Nuc. Acid Res.*, **23**, 2919-2928.

J. Krupinski, F. Coussen, H. A. Bakalyar, W.-J. Tang, P. G. Feinstein, K. Orth, C. Slaughter, R. R. Reed and A. G. Gilman (1989) *Science*, **244**, 1558-1564.

A. Kumar, J.-L. Moreau, D. Baran and J. Theze (1987) *J. Immunol.*, **138**, 1485-1493.

R. P. S. Kwok, M. E. Laurence, J. R. Lundblad, P. S. Goldman, H.-M. Shih, L. M. Connor, S. J. Marrioy and R. H. Goodman (1996) *Nature*, **380**, 642-646.

J. Ledbetter, G. Shu, M. Gallager and E. Clark (1987) *J. Immunol.*, **138**, 788-794.

S. Lederman, M. J. Yellin, L. R. Covey, A. M. Cleary, R. E. Callard and L. Chess (1993) *Curr. Opin. Immunol.*, **5**, 439-444.

K. Leung and G. J. Nabel (1988) *Nature*, **333**, 776-778.

P. E. Lipsky, S. Hirohata, D. F. Jelenik, L. McAnally and J. B. Splawski (1988) *Scand. J. Rheumatol. Suppl.*, **76**, 229-235.

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

K. D. Lustig, B. R. Conklin, P. Herzmark, H. R. Taussig and H. R. Bourne (1993) *J. Biol. Chem.*, **268**, 13900-13905.

S. Lutzker, P. Rothman, R. Pollock, R. Coffman and F. W. Alt (1988) *Cell*, **53**, 177-184.

P. Macchi, A. Villa, S. Giliani, M. G. Sacco, A. Frattini, F. Porta, A. G. Ugazio, J. A. Johnston, F. Candotti, J. J. O'Shea, P. Vezzoni and L. D. Notarangelo (1995) *Nature*, **377**, 65-68.

D. M. Margolis, M. Somasundaran and M. R. Green (1994) *J. Virol.*, **68**, 905-910.

D. J. Matthews, P. A. Clark, J. Herbert, G. Morgan, R. J. Armitage, C. Kinnon, A. Minty, K. H. Grabstein, D. Caput, P. Ferrara and R. E. Callard (1995) *Blood*, **85**, 38-42.

G. M. McGarvie and W. Cushley (1989a) *Cell. Signalling*, **1**, 447-460.

G. M. McGarvie and W. Cushley (1989b) *Immunol. Lett.*, **22**, 221-226.

C. E. McKay and W. Cushley (1996) *Cytokine*, **8**, 305-312.

A. N. J. McKenzie, J. A. Culpepper, R. de Waal Malefyt, F. Briere, J. Punnonen, G. Aversa, A. Sato, W. Dang, B. G. Cocks, S. Menon, J. E. de Vries, J. Banchereau and G. Zurawski (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 3735-3739.

J. L. Meinkoth, Y. Ji, S. S. Taylor and J. R. Feramisco (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 9595-9599.

F. Melchers, H. Karasuyama, D. Haasner, S. Bauer, A. Kudo, N. Sakaguchi, B. Jameson and A. Rolink (1993) *Immunol. Today*, **14**, 60-68.

J. P. Menetski and M. Gellert (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 9324-9328.

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

245-267.

T. Miyazaki, Z.-J. Liu, A. Kawahara, Y. Minami, K. Yamada, Y. Tsujimoto, E. L. Barsoumian, R. M. Perlmutter and T. Taniguchi (1995) *Cell*, **81**, 223-231.

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

J. Mizuguchi, M. A. Beaven, J. Ohara and W. E. Paul (1986) *J. Immunol.*, **137**, 2215-2219.

M. R. Montminy and L. M. Bilezikjan (1987) *Nature*, **328**, 175-178.

M. R. Montminy, G. A. Gonzalez and K. Yamamoto (1990) *Trends Neurosci.*, **13**, 184-188.

M. R. Montminy, K. A. Sevarino, J. A. Wagner, G. Mandel and R. H. Goodman (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 6682-6686.

T. Mosishita, A. Yamaguchi, T. Fujita and T. Chiba (1990) *Diabetes*, **39**, 875-877.

B. Mosley, M. P. Beckmann, C. J. March, R. L. Idzerda, S. D. Gimpel, T. VandenBos, D. Friend, A. Alpert, D. Anderson, J. Jackson, J. M. Wignall, C. Smith, B. Gallis, J. E. Sims, D. Urdal, M. B. Widmer, D. Cosman and L. S. Park (1989) *Cell*, **59**, 335-348.

C. W. Muller, F. A. Rey, M. Sodeoka, G. L. Verdine and S. C. Harrison (1995) *Nature*, **373**, 311-317.

M. Murakami, M. Narazaki, M. Hibi, H. Yawata, K. Yasukawa, R. M. Perlmutter and T. Taniguchi (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 11349-11355

T. Murata, P. D. Noguchi and R. K. Puri (1995) *J. Biol. Chem.*, **270**, 30829-30836.

M. G. Myers, T. C. Grammer, L.-m. Wang, X. J. Sun, J. H. Pierce, J. Blenis and M. F. White (1994) *J. Biol. Chem.*, **269**, 28783-28789.

Y. Nagamine and E. Reich (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 4606-4610.

B. G. Nair and T. B. Patel (1993) *Biochem. Pharmacol.*, **46**, 1239-1245.

T. Nakarai, M. J. Robertson, M. Streuli, Z. Wu, T. L. Ciardelli, K. A. Smith and J. Ritz (1994) .

E. J. Neer (1994) *Protein Sci.*, **3**, 3-14.

B. H. Nelson, J. D. Lord and P. D. Greenberg (1994) *Nature*, **369**, 333-336.

C. D. Nicholson, R. A. J. Challiss and M. Shahid (1991) *TIPS*, **121**, 19-27.

E. A. Nigg (1990) *Adv. Cancer Res.*, **55**, 271-310.

G. Nilsson and K. Nilsson (1995) *Eur. J. Immunol.*, **25**, 870-873.

Y. Nishioka and P. E. Lipsky (1994) *J. Immunol.*, **153**, 1027-2035.

R. J. Noelle, P. H. Krammer, J. Ohara, J. W. Uhr and E. S. Vitetta (1984) *Proc. Nat. Acad. Sci. USA*, **81**, 6149-6153.

M. Noguchi, H. Yi, H. M. Rosenblatt, A. H. Filipovich, S. Adelstein, W. S. Modi, O. W. McBride and W. J. Leonard (1993) *Cell*, **73**, 147-157.

A. O' Garra, K. P. Rigley, M. Holman, J. B. McLaughlin and G. G. B. Klaus (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 6254-6258.

A. O'Garra, G. Srapleton, V. Dhat, M. Pearce, J. Schumacher, H. Rugo, D. Barbis, A. Stall, J. Cupp, K. Moore, P. Vieira, T. Mosmann, A. Whitmore, L. Arnold, G. Haughton and M. Howard (1990) *Int. Immunol.*, **2**, 821-832.

K. D. O'Neal and L. Yu-Lee (1993) *Lymphokine Cytokine Res.*, **12**, 309.

Y. Okayama, P. Bradding, J. M. Tunondelara, S. T. Holgate and M. K. Church (1995) *Chem. Immunol.*, **61**, 114-134.

S. S. Patel, D. L. Theile and P. E. Lipsky (1992) *Cell. Immunol.*, **146**, 362-381.

W. E. Paul (1991) *Blood*, **77**, 1859-1870.

W. E. Paul and R. A. Seder (1994) *Cell*, **76**, 241-251.

S. Paulie, A. Rosen, B. Ehlin-Henriksson, S. Braesch-Anderson, E. Jakobson, H. Koho and P. Perlmann (1989) *J. Immunol.*, **142**, 590.

C. A. Pennel, K. M. Sheenan, P. H. Brodeur and S. H. Clarke (1989) *Eur. J. Immunol.*, **19**, 2115-2121.

A. Pernis, S. Gupta, K. J. Gollob, E. Garfien, R. L. Coffman, C. Schindler and P.

Rothman (1995b) *Science*, **269**, 245-247.

A. Pernis, B. A. Withuhn, A. D. Keegan, K. Nelms, E. Garfein, J. N. Ihle, W. E. Paul, J. H. Pierce and P. Rothman (1995a) *Proc. Natl. Acad. Sci. USA*, **92**, 7971-7975.

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

J. P. Pieroni, O. Jacobowitz, C. Jianquiang and R. Iyengar (1993) *Curr. Opin. in Neurobiol.*, **3**, 345-351.

C. M. Pleiman, D. D'Ambrosio and J. C. Cambier (1994) *Immunol. Today*, **15**, 393-399.

J. L. Pomerantz, F. Mauxion, M. Yoshida, W. C. Greene and R. Sen (1989) *J. Immunol.*, **143**, 4275-4281.

J. Poudrier and T. Owens (1994) *Eur. J. Immunol.*, **24**, 2993-2999.

R. T. Premont, O. Jacobowitz and R. Iyengar (1992) *Endocrinol.*, **131**, 2774-2783.

W. Pruett, Y. Yuan, E. Rose, A. G. Batzer, N. Harada and Y. Skolnik (1995) *Mol. & Cell Biol.*, **15**, 1778-1785.

J. Punnonen, G. Aversa, B. G. Cocks, A. N. J. McKenzie, S. Menon, G. Zurawski, R. De Waal Malefyt and J. E. De Vries (1993) *Proc. Natl. Acad. sci. USA*, **90**, 3730-3734.

F. W. Quelle, K. Shimoda, W. Thierfelder, C. Fischer, A. Kim, S. M. Ruben, J. L.

Cleveland, J. H. Pierce, A. D. Keegan, K. Nelms, W. E. Paul and J. N. Ihle (1995) *Mol. and Cell Biol.*, **15**, 3336-3343.

K. P. Rigley, S. M. Thurstan and R. E. Callard (1991) *Int. Immunol*, **3**, 197-203

R. J. Robb, W. C. Greene and C. M. Rusk (1984) *J. Exp. Med.*, **160**, 1126.

A. Rolink, U. Grawunder, T. H. Winkler, H. Karasuyama and F. Melchers (1994) *Int. Immunol.*, **6**, 1257-1264.

R. L. Roper, J. W. Ludlow and R. P. Phipps (1994) *Cell. Immunol.*, **154**, 296-308.

E. M. Ross (1980) *Annu. Rev. Biochem.*, **49**, 533-564.

P. Rothamn, S. Lutzker, W. Cook, R. Coffman and F. W. Alt (1988) *J. Exp. Med.*, **168**, 2385-2389.

P. Rothman, S. Lutzker, W. Cook, R. Pollock, R. Coffman and F. W. Alt (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 7704-7708.

S. M. Russell, A. D. Keegan, N. Harada, Y. Nakamura, M. Noguchi, P. Leland, M. C. Freidmann, A. Miyajima, R. K. Puri, W. E. Paul and W. J. Leonard (1993) *Science*, **262**, 1880-1883.

C. Sato-Bigbee, E. L. P. Chan and R. K. Yu (1994) *J. of Neurosci. Res.*, **38**, 621-628.

T. Satoh, M. Y., T. Kono, K. Yamada, A. Kawahara, T. Taniguchi and Y. Kaziro (1992) *J. Biol. Chem.*, **267**, 25423-25427.

C. Schindler, X. Y. Fu, T. Improta, R. Aebersold and J. E. J. Darnell (1992b) *Proc. Natl. Acad. Sci. USA*, **89**, 7836-7839.

C. Schindler, H. Kashleva, P. A., R. Pine and P. Rothman (1994) *EMBO J.*, **13**, 1350-1356.

C. Schindler, K. Shuai, V. R. Prezioso and J. E. J. Darnell (1992a) *Science*, **257**, 809-813.

B. Schnyder, H. Lahm, G. Woerly, N. Odartchenko, B. Ryffel and B. D. Car (1996a) *Biochem. J.*, **315**, 767-774.

B. Schnyder, S. Lugli, N. Feng, H. Etter, R. A. Lutz, B. Ryffel, K. Sugamura, H. Wunderli-Allenspach and R. Moser (1996b) *Blood*, **82**, 4286-4295.

C. Schultz, K. Izuhara, R. Coffman and N. Harada (1995) *Immunol. Lett.*, **46**, 215-219.

K. B. Seamon and J. W. Daly (1981) *J. Cyc. Nucleot. Res.*, **7**, 201-224.

G. V. Segre (1993) *Trends in Endocrinol. and Metabolism*, **4**, 309-314.

C. Seldin and P. Leder (1994) *Proc. Natl. Acad. Sci. USA*, **91**, 2140-2144.

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

J. G. Shields, R. J. Armitage, B. N. Jamieson, P. C. L. Beverley and R. E. Callard (1989) *Immunol.*, **66**, 224-227.

F. Shirakawa, M. Chedid, J. Suttles, B. A. Pollok and S. B. Mizel (1989a) *Mol. & Cell Biol.*, **9**, 959-964.

F. Shirakawa and S. B. Mizel (1989b) *Mol. & Cell. Biol.*, **9**, 2424-2430.

F. Shirakawa, Y. Tanaka, S. Eto, H. Suzuki, J. Yodoi and U. Yamashiya (1986) *J. Immunol.*, **137**, 551-556.

F. Shirakawa, U. Yamashita, M. Chedid and S. B. Mizel (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 8201-8205.

J. M. Short, A. Wynshaw-Boris, H. P. Short and R. W. Hanson (1986) *J. Biol. Chem.*, **261**, 9721-9726.

K. Shuai, C. Schindler, V. R. Prezioso and J. E. J. Darnell (1992) *Science*, **258**, 1808-1812.

K. Shuai, G. R. Stark, I. M. Kerr and J. E. J. Darnell (1993) *Science*, **261**, 1744-1746.

M. I. Simon, M. P. Strathmann and N. Gautam (1991) *Science*, **252**, 802-807.

C. Smerz-Bertling and A. Duschl (1995) *J. Immunol.*, **270**, 966-970.

M. R. Smith and W. C. Greene (1989) *Proc. Nat. Acad. Sci. USA*, **86**, 8526-8530.

A. M. Spiegel (1992) *Curr. Opin. Cell. Biol.*, **4**, 203-211.

J. B. Splawski, S. M. Fu and P. E. Lipsky (1993) *J. Immunol.*, **150**, 1276-1285.

J. B. Splawski, D. F. Jelinek and P. E. Lipsky (1989) *J. Immunol.*, **142**, 1569-1575.

I. Stamekovic, E. Clark and B. Seed (1989) *EMBO J.*, **8**, 1403.

M. Strathmann and M. I. Simon (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 9113-9117.

M. Strathmann and M. I. Simon (1991) *Proc. Natl. Acad. Sci. USA*, **88**.

M. Strathmann, T. M. Wilkie and M. I. Simon (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 7407-7409.

S. Swendeman and D. A. Thorley-Lawson (1987) *EMBO J.*, **5**, 1637-1642.

T. Taga and T. Kishimoto (1993) *FASEB*, 7, 3387-3396.

T. Takeshita, H. Asao, K. Ohtani, N. Ishii, S. Kumaki, N. Tanaka, H. Munakata, M. Nakamura and K. Sugamura (1992a) *Science*, **257**, 379-382.

T. Takeshita, K. Ohtani, H. Asao, S. Kumaki, M. Nakamura and K. Sugamura (1992b) *J. Immunol.*, **148**, 2154-2158.

N. Tanaka, H. Asao, K. Ohbo, N. Ishii, T. Takeshita, M. Nakamura, H. Sasaki and K. Sugamura (1994) *Proc. Natl. Acad. Sci. USA*, **91**, 7271-7275.

W.-J. Tang and A. G. Gilman (1991b) *Science*, **254**, 1500-1503.

W.-J. Tang, J. Krupinski and A. G. Gilman (1991a) *J. biol. Chem.*, **266**, 8595-8603.

R. Taussig and A. G. Gilman (1995) *J. Biol. Chem.*, **270**, 1-4.

K. M. Toellner, D. Scheel-Toellner, R. Sprenger, M. Duchrow, L. H. Trumper, M. Ernst, H. D. Flad and J. Gerdes (1995) *Cytokine*, **7**, 344-354.

S. Tohma and P. E. Lipsky (1991) *J. Immunol.*, **146**, 2544-2552.

M. B. Toledando, D. G. Roma, N. F. Halden, B. B. Lin and W. J. Leonard (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 1830-1834.

S. Tonegawa (1983) *Nature*, **305**, 575-581.

T. Torigoe, H. U. Saragovi and J. C. Reed (1992) *Proc. Natl. Acad. Sci. USA.*, **89**, 2674-2678.

U.-C. Tornberg and D. Holmberg (1995) *EMBO J.*, **14**, 1680-1689.

K. E. Truitt, G. B. Mills, C. W. Turck and J. B. Imboden (1994) *J. Biol. Chem.*, **269**, 5937-5943.

T. Tsubata, R. Tsubata and M. Reth (1992) *Int.Immunol.*, **4**, 637-641.

T. Tsukamoto, R. Toyma, H. Itoh, T. Kozasa, M. Matsuoka and Y. Kaziro (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 2974-2978.

L. Tsuruta, H.-J. Lee, E. S. Masuda, N. Koyano-Nakagawa, N. Arai, K.-I. Arai and T. Yokota (1995) *J Immunol.*

T. Uchiyama, S. Broder and T. A. Waldmann (1981) *J. Immunol*, **126**, 1393-1397

P. Urbanek, Z. Q. Wang, I. Fetka, E. F. Wagner and M. Busslinger (1994) *Cell*, **79**,

901-912.

A. Valle, C. E. Zuber, T. Defrance, O. Djosou, M. Die Rie and J. Banchereau (1989) *Eur. J. Immunol.*, **19**, 1463-1467.

T. Waldmann, C. K. Goldman, R. J. Robb, J. M. Depper, W. J. Leonard, S. O. Sharrow, K. F. Bongiovanni, S. J. Korsmeyer and W. G. Greene (1984) *J. Exp. Medicine*, **160**, 1450-1466.

T. J. Waldschmidt, F. G. M. Kroese, L. T. Tygrett, D. H. Conrad and R. Lynch, G. (1991) *Int. Immunol.*, **3**, 305-312.

L.-M. Wang, A. D. Keegan, J. S. Gutkind, W. E. Paul, M. A. Heidaran and J. H. Pierce (1992) *EMBO J.*, **11**, 4899-4908.

L.-M. Wang, A. D. Keegan, W. Li, G. E. Leinhard, S. Pacini, J. S. Gutkind, M. G. Myers, X.-J. Sun, M. F. White, S. A. Aaronson, W. E. Paul and J. H. Pierce (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 4032-4036.

Y. Wang, K. K. Morella, J. Ripperger, C.-F. Lai, S. P. Campos and H. Baumann (1995) *Blood*, **86**, 1671-1679.

D. Wading, D. Guschin, M. Muller, O. Silvennoinen, B. A. Witthuhn, F. W. Quelle, N. C. Rogers, C. Schindler, G. R. Stark, J. N. Ihle and I. M. Kerr (1993) *Nature*, **366**, 166-170.

P. A. Watson, J. Krupinski, A. M. Kempinski and C. D. Frankenfeild (1994) *J. Biol. Chem.*, **269**, 28893-28898.

M. J. Welham, L. Learmonth, H. Bone and J. W. Schrader (1995) *J. Biol. Chem.*, **270**, 12286-12296.

A. D. Whetton, L. Needham, N. J. F. Dodd, C. M. Heyworth and M. D. Houslay (1983) *Biochem. Pharmacol.*, **32**, 1601-1608.

L. J. White, P. Grabr, J.-P. Aubry, J.-Y. Bonnefoy, B. W. Ozanne and W. Cushley (1996) *Blood (submitted).*

A. F. Wilks (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 1603-1607.

A. F. Wilks, A. G. Harpur, R. R. Kurban, S. J. Ralph, G. Zurcher and A. Ziemiecki (1991) *Mol. and Cell Biol.*, **11**, 2057-2065.

B. A. Witthuhn, O. Silvenoinen, O. Miura, K.-S. Lal, C. Cwik, E. T. Liu and J. N. Ihle (1994) *Nature*, **370**, 153-157.

K. K. Yamamoto, G. A. Gonzalez, W. H. Biggs and M. R. Montminy (1988) *Nature*, **334**, 494-498.

J. Ye, H. A. Young, J. R. Ortaldo and P. Ghosh (1994) *Nucleic. Acids. Res.*, **22**, 5672-5678.

T. Yin, S. R. Keller, F. W. Quelle, B. A. Witthuhn, M. L.-S. Tsang, G. E. J. Lienhard, J.N. Ihle and Y.-C. Yang (1995) *J. Biol. Chem.*, **270**, 20497-20502.

T. Yin, M. L.-S. Tsang and Y.-C. Yang (1994) *J. Biol. Chemistry*, **269**, 26614-26617.

M. Yoshimura and D. M. J. Cooper (1992) *Proc. Nat. Acad. Sci. USA*, **89**, 6716-

6720.

M. Yoshimura and D. M. J. Cooper (1993) *J. biol. Chem.*, **268**, 4604-4607.

Y.-X. Zeng, H. Takahashi, M. Shibata and K. Hirokawa (1994) *FEBS Lett.*, **353**, 289-293.

Y. Zhuang, P. Soriano and H. Weintrub (1994) *Cell*, **79**.

H. Zola, H. Weedon, G. R. Thompson, M. C. Fung, E. Ingley and A. J. Hapel (1991) *Immunology*, **72**, 167-173.

S. M. Zurawski, F. Vega Jr, B. Huyghe and G. Zurawski (1993) *EMBO J.*, **12**, 2663-2670.