ANALYSIS OF TRANSCRIPTIONAL REGULATORY ELEMENTS OF THE HUMAN CD23 GENE

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ABSTRACT:

CD23 ($Fc\varepsilon RII$), the low affinity receptor for IgE, is a single chain 45kDa Type II membrane glycoprotein member of the C- type lectin family. The CD23 molecule is a multifunctional receptor/ligand and cytokine, playing a role in antigen presentation, macrophage activation and cell adhesion. There are two distinct isoforms of human CD23, termed CD23a and CD23b, with the only difference being noted in a 6 or 7 amino acid region in the N-terminal cytoplasmic domain of the protein. These isoforms are generated from distinct transcription start sites and differential RNA splicing of the single CD23 gene, located on chromosome 19. CD23a is constitutively and cell type-specifically expressed on B cells, whereas CD23b can be expressed on B cells, monocytes and other cells of haematopoietic lineage when activated by stimuli including IL-4 and IL-13.

A large body of evidence suggests that CD23 plays a regulatory role in IgE production, and cross-linking of CD23 at the cell surface with IgE delivers a negative feed-back signal for IgE production and inhibits the release of soluble CD23 (sCD23). Cleaved sCD23 fragments larger than 25kDa are known to promote IgE production. Allergic disease is thought to be due to the dysregulation of CD23/IgE feed-back mechanisms, probably through increased cleavage of CD23 from the cell surface, leading to increased IgE production and release of inflammatory mediators. Soluble CD23 is considerably elevated in atopic and neoplastic individuals, and this sCD23 can originate from either the a or b isoform on B cells or just from the b isoform on other cells of haematopoietic origin.

The patterns of CD23 gene expression and functional diversity has led to the assumption that CD23a and b are involved in B cell function and IgE-mediated immunity, respectively, with divergence in the signalling pathways attributed to the N-terminal amino acid differences. It was of importance and interest, therefore, to

study and elucidate those events which lead to the expression of each CD23 isoform and to pin-point these to differences in promoter activation and regulation. In this research, the activation of the promoter region of each CD23 isoform was studied in great detail, and reporter vector construct studies have shown that each isoform promoter is differentially activated by distinct stimuli. The CD23a promoter contains two IL-4 response elements (IL-4RE), an NFκB site, a glucocorticoid response element (GRE), a B-cell-specific activator protein (BSAP) or Pax-5 binding site, and is activated by IL-4 only. The CD23b promoter, on the other hand, contains two IL-4RE's separated by an NFκB site, two AP-1 binding sites and is stimulated by IL-4, anti-CD40, anti-μ and PMA. Reporter vector constructs containing truncated promoters revealed that IL-4 stimulation of the CD23a promoter requires the presence of the first IL-4RE/STAT-6 site, while successful IL-4 stimulation of the CD23b promoter is dependent on the second IL-4RE/STAT-6 site. The discovery of these differentially regulated isoform promoter regions confirms the theory that CD23a and CD23b are involved in functionally different roles, and it is hypothesised that these include the prevention of plasma cell formation in B cells and IgE negative regulation, respectively.

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ABBREVIATIONS:

ADCC	Antibody-dependent cell-mediated cytotoxicity
AET	Aminoethylisothiouronium
AIDS	Acquired immunodeficiency syndrome
AP	Activator protein
ASB	Ankyrin and SOCS box-containing protein
ATF	Activating transcription factor
ATP	Adenosine trisphosphate
BB	Binding buffer
B-CLL	B cell chronic lymphocytic leukaemia
BCR	B cell receptor
bH-L-H	Basic helix-loop-helix
BM	Bone marrow
BSA	Bovine serum albumin
BSAP	B cell specific activator protein
BTK	Bruton's tyrosine kinase
cAMP	Cyclic adenosine monophosphate
CAT	Chloramphenicol acetyl transferase
CD	Cluster of differentiation
CIS	Cytokine-inducible SH2 domain containing protein
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CR	Complement receptor
CSPD	Chemiluminescent substrate disodium 3-(4-methoxyspiro [1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1(3,7)]decan]-4-yl) phenyl phosphate
DMS	Dimethyl sulphate
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
EBF	Early B cell factor
EBNA	Epstein-Barr virus nuclear antigen
EBNA-LP	Epstein-Barr virus nuclear antigen latent protein
EBV	Epstein-Barr virus
EBVCS	Epstein-Barr virus cell surface
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbant assay

EMSA	Electrophoretic mobility shift assay
EPO	Erythropoietin
ERK	Extracellular signal-related kinase
FACS	Fluorescence activated cell sorter
FCS	Foetal calf serum
FDC	Follicular dendritic cells
FITC	Fluorocein isothiocyanate
Fn	Fibronectin
GC	Germinal centre
GDP	Guanosine diphosphate
GRE	Glucocorticoid response element
GTP	Guanosine trisphosphate
HMG-1	High mobility group protein-1
HRP	Horseradish peroxidase
HSV	Herpes simplex virus
Id	Inhibitor of development
IFN	Interferon
Ig	Immunoglobulin
IκB	Inhibitor of kappa B
IL	Interleukin
IL-4RE	Interleukin-4 response element
IP3	Inositol trisphosphate
IRS	Insulin receptor substrate
JAK	Janus kinase
JNK	Jun nuclear kinase
LM-PCR	Ligation-mediated polymerase chain reaction
MAPK	Mitogen activated protein kinase
MCS	Multiple cloning site
MHC	Major histocompatability complex
mRNA	Messenger ribonucleic acid
NF-AT	Nuclear factor-AT
NFκB	Nuclear factor kappa B
NRE	Negative response element
NRE-BP	Negative response element-binding protein
NRR	Negative regulatory region
OSM	A complex beta polypeptide cytokine
PAGE	Polyacrylamide gel electrophoresis
pBCR	Pre B cell receptor
PCR	Polymerase chain reaction

PBL	Peripheral blood lymphocytes
PBS	Phosphate buffered saline
PE	Phycoerythrin
PI-3-K	Phosphoinositide-3-kinase
PIAS	Protein inhibitors of activated STATs
PIP	Phosphotidylinositol-3-phosphatase
PKA	Protein kinase A
PKC	Protein kinase C
PMA	Phorbol myristoyl acetate
PRR	Positive regulatory region
PTB	Phosphotyrosine binding domain
PTPase	Protein tyrosine phosphatase
RA	Rheumatoid arthritis
RAG	Recombinase activating gene
RAR	Retinoic acid receptor
RE	Response element
RNA	Ribonucleic acid
RT	Room temperature
SCID	Severe combined immunodeficiency
SCR	Short concensus repeat
SDS	Sodium dodecyl sulphate
SEAP	Secreted alkaline phosphatase
SHIP	SH2-containing inositol-5-phosphatase
SHP	SH2-containing phosphatase
SLE	Systemic lupus erythematosus
SOCS	Suppressors of cytokine signalling
SRBC	Sheep red blood cells
SSB	SPRY domain and SOCS-box containing proteins
STAF	Stimulated trans-activating factor
STAT	Signal transducer and activator of transcription
SV-40	Simian virus-40
TB	Transcription blocker
TCA	Tricarboxylic acid
TCR	T cell receptor
TdT	Terminal deoxynucleotidyltransferase
TNF	Tumour necrosis factor
VCAM	Vascular cell adhesion molecule
Vn	Vitronectin
WSB	WD-40 repeat and SOCS box containing protein

CHAPTER 1

INTRODUCTION

CHAPTER 1: INTRODUCTION

1.1: The Immune System

The immune system is the host's mechanism of protecting itself against disease-causing attacks from environmental infectious agents such as viruses, bacteria, protozoa and parasites (Janeway & Travers, 1997). The immune system involves a variety of specific and non-specific cells, known collectively as leukocytes (Roitt, *et al.*, 1993), including B cells, T cells, monocytes, macrophages and granulocytes (Zucker-Franklin, *et al.*, 1980). The immune response depends on two things; the ability of a host to recognise antigens as foreign material and then, secondly, the means to mount a response to the invader and eliminate it.

There are two primary categories of immune response, innate (non-adaptive) and adaptive. Innate immunity is non-specific and is present prior to the presentation of infectious agents (Ulevitch, 2000). Phagocytic cells such as monocytes and macrophages, granulocytes and the complement serum proteins (Yefenof, 2000) are the main mediators of innate immunity - with the opsonisation of micro-organisms with complement proteins aiding their internalisation and elimination. Granulocytes are so called because of the densely staining granules in their cytoplasm, and include neutrophils, eosinophils and basophils (Janeway & Travers, 1997). Neutrophils constitute over 90% of polymorphonuclear granulocytes and migrate in response to chemotactic agents such as complement, fibrinolytic and bacterial products. Eosinophils are thought to be important chiefly in the defence against parasitic infections, and are attracted by the products released by T cells, mast cells and basophils. Degranulation of eosinophils can be triggered by the appropriate stimuli and, although not their primary function, they appear to be capable of phagocytosing and killing ingested micro-organisms. Basophils are found in very small numbers in the circulatory system, and make up less than 0.2% of leukocytes. The function of

basophils is probably similar to that of mast cells, which are believed to play a part in protecting the mucosal surfaces of the body and degranulate to release substances which affect vascular permeability.

The adaptive immune response is, in comparison, highly specific for individual pathogens, and is only induced by exposure to them (Male, *et al.*, 1993). This type of immune response improves with each successive encounter with the same antigens and, in effect, induces immunological memory (Zinkernagel, *et al.*, 1996). Lymphocytes make up about 20% of all blood leukocytes and are central to adaptive immune responses and the specific recognition of antigens. There are two basic categories of lymphocytes: B cells and T cells. B lymphocytes represent about 5-15% of the circulating lymphoid pool, and are defined by the presence of surface immunoglobulins that act as specific antigen receptors (Mellman, *et al.*, 1988). The majority of human B cells in peripheral blood express IgM and IgD on their cell surface. A few express IgG, IgA or IgE, although large numbers of B cells expressing these immunoglobulins can be found in specific locations, such as IgA bearing cells in the intestinal mucosa. B cells also express MHC Class II molecules, which are important for co-operative interactions with T cells, complement receptors and various receptors for a variety of signalling molecules. These receptors include those for the Fc regions of antibodies and for B cell growth and differentiation factors.

Activated B cells proliferate and mature under the auspices of T cell help and influence and, ultimately, differentiate into memory B cells (or plasma cells - the latter of which are almost exclusively devoted to the production of secreted antibodies, the mediators of humoral immunity) which bind to particular target antigens. These target antigens can either be a molecule on the surface of a pathogen particle, or a toxin that it produces. Antibodies are the soluble form of the B cell antigen receptor and all have the same basic structure, with diversity being in the

antigen binding region. While one part of an antibody binds antigen, other parts interact with several innate immune system elements; such as phagocytes or complement. This allows for widespread recognition of specific pathogens. The effects of antibodies include: the neutralisation of soluble antigens, the activation of the complement system, the opsonisation of pathogen particles to enhance phagocytosis and antibody dependent cell-mediated cytotoxicity (ADCC).

T lymphocytes develop and differentiate in the thymus prior to seeding in the secondary, peripheral lymphoid tissues (Jenkinson, *et al.*, 1989). They recognise antigen and MHC molecules via a receptor molecule distinct from, but related to, immunoglobulin. This receptor consists of an antigen binding portion formed by two different polymorphic chains associated with CD3, the universal T cell differentiation marker (Strominger, 1989). There are two main subpopulations of T cells, distinguished by their expression of either CD4 or CD8, which act as receptors for class II and class I MHC molecules, respectively, and contribute towards T cell immune recognition and cellular activation. $CD4^+$ T cells act primarily as helper T cells (TH), whereas $CD8^+$ T cells are associated with the cytotoxic destruction of virally-infected cells. There are also sub-populations of both $CD4^+$ and $CD8^+$ T cells, with $CD29^+CD4^+$ cells being classed as activated or memory T cells and $CD45R^+$ $CD4^+$ cells being generally naive or virgin T cells. Functionally, these subsets act as efficient helpers and inducers of suppression respectively.

Blood cells, therefore, all differ in function, morphology, number and life span. To maintain homeostasis of each of these populations, each cell type is generated at an appropriate rate from a self-renewing population of pluripotent stem cells, found in the bone marrow (BM) of adult mammals. A stem cell has the capacity for unlimited self-renewal, and can divide asymmetrically to generate progenitor cells that are irreversibly committed to one or other of the haematopoietic lineages. These lineage restricted clones mature into specialised cells, such as B or T lymphocytes.

1.2: B cell lymphopoiesis

B cell lymphopoiesis occurs throughout life in the intermedullary cavity of the BM in adult mammals, and the stages of development are defined by the expression pattern of several B cell-restricted markers, cell size, growth properties and the re-arrangement status of immunoglobulin (Ig) genes (Melchers & Rolink, 1998). An overview of B cell lymphopoiesis is shown on Figure 1.1, along with cell surface markers and gene expression patterns for transcriptional regulatory elements. B lymphocytes are generated from a lymphoid progenitor cell expressing CD34, B220 (CD45) and CD10, that is capable of producing B cells, T cells, natural killer and dendritic cells, but not erythroid or myeloid lineages (Galy, *et al.*, 1995). The first identifiable stage of B cell development is the pro-B cell, which has un-rearranged Ig heavy and light chains, expresses B220 (CD45) (as do all cells committed to B lymphopoiesis) and CD43 (Hardy, *et al.*, 1991; Li, *et al.*, 1996; Melchers, *et al.*, 1995). As progenitors progress through the pro-B cell stage, they express terminal deoxynucleotidyltransferase (TdT), RAG-1, RAG-2, the surrogate light chains λ5 and VpreB, CD19, Igα (mb-1) and Igβ (B29). The cells also rearrange their Ig heavy chain (*Igh*) genes.

The subsequent pre-B cell stage is marked by a decrease in CD43 expression, lack of TdT expression, successful rearrangement of the IgH locus and the appearance of the pre-B cell receptor complex (pBCR) components (Karasuyama, *et al.*, 1994). Early stage cycling of the pre-B cells is also marked by a decrease of RAG-1 and RAG-2 expression (Grawunder, *et al.*, 1995). As differentiation continues, Ig light chain gene rearrangement begins and completion of antigen independent B cell development is marked by successful light chain gene rearrangement and the expression of surface IgM. The cells then exit the BM and migrate to the periphery.

Functionally, B220 (CD45) is a phosphotyrosine phosphatase (PTPase) which appears to regulate B cell receptor signalling by dephosphorylating src family tyrosine kinases, such as lyn, and also Igα and Igβ (Satterthwaite & Witte, 1996). CD45 is also assumed to integrate signals during development, although it is not absolutely required for B lymphopoiesis (Li, *et al.*, 1996). CD19 is thought to be important for B cell signalling and interacts with a number of tyrosine kinases, although the actual role of this protein is unclear as mice lacking CD19 still generate B cells. Appropriate expression of the surrogate light chain proteins, μ, Igα and Igβ is essential for B cell differentiation, and it is these molecules that form the pBCR that appears on the surface of the cell during the latter pro-B cell stage of development (Melchers, *et al.*, 1994; Rajewsky, 1996). The pBCR associates with non-receptor tyrosine kinases and provides critical signals for progressing to the pre-B cell stage and establishing *IgH* allelic exclusion (Loffert, *et al.*, 1996). Gene targeting experiments have confirmed that individual components of the pBCR are required for normal B cell differentiation, with Igβ lacking mice being unable to progress past the pro-B cell stage (Gong & Nussenzweig, 1996). In addition, λ5 and μ negative mice are blocked at the early pre-B stage of differentiation (Kitamura, *et al.*, 1992) and mice with non-functional Igα have significant reductions in their numbers of peripheral B cells (Torres, *et al.*, 1996). The role of pBCR signalling in lymphopoiesis may reflect cell fate decisions at the pro/pre-B cell transition stage. Consistent with this hypothesis is the finding that bcl-2 and bcl-x, which are involved in protecting cells against apoptosis, are differentially expressed during B cell development (Grillot, *et al.*, 1996).

Additional molecules involved in signalling that have a role in B cell development include; JAK 3, in which knockout mice lack $B220^{+}/CD45^{+}$ progenitors; flk 2, a receptor tyrosine kinase that influences progenitor B cell development (Mackarehtschian, *et al.*, 1995); syk, a tyrosine kinase necessary for generating pre-B cells (Turner, *et al.*, 1995); the src-related kinase lyn, which may affect the

maturation of immature B cells (Hibbs, *et al.*, 1995) and BTK (Bruton's tyrosine kinase), mutations of which are linked to X-linked immunodeficiency in mice and human X-linked agammaglobulinaemia (XLA). Other proteins that display B cell restricted functions include; TdT, which randomly adds nucleotides at the coding joins during *IgH* recombination; the IL-7 receptor (IL-7R), which is required for pro/pre-B cell generation, and integrin proteins that are necessary for mediating cell-cell and cell-extracellular matrix interactions (Candeias, *et al.*, 1997; Li, *et al.*, 1993).

1.2.1: Expression and rearrangement of immunoglobulin genes

Proper expression of Ig heavy and light chain genes is critical for B cell lymphopoiesis. In general, Ig genes are regulated by a number of tissue specific cis-acting promoters and enhancers (Calame & Ghosh, 1995), and are also partly governed by their assembly during B cell differentiation. V(D)J recombination occurs at specific times and begins during the pro-B cell stage of differentiation with DJ_H recombination (Reth & Alt, 1984). Successful rearrangement of the V_H segment to the previously joined DJ_H chains results in the expression of cytoplasmic μ chain, which is a characteristic of pre-B cells. In the final stages of primary B cell development, the pre-B cell must also successfully rearrange the V and J elements at one of its light chain loci.

V(D)J recombination is regulated by at least two mechanisms that both depend on transcription, namely the expression of RAG genes and the accessibility of the Ig genes to recombinase activity. The V(D)J recombination machinery components RAG-1 and 2, along with the DNA-dependent protein kinase complex, recognise recombination signal sequences and mediate cleavage (Ramsden, *et al.*, 1997). Defects in any of these components leads to deficiencies in recombination (Mombaerts, *et al.*, 1992). RAG-1 and RAG-2 mRNA, which can first be detected

in pro-B cells, are down-regulated upon *IgH* rearrangement completion and then transcribed again in small pre-B cells to allow light chain re-arrangement (Grawunder, *et al.*, 1995). The precise mechanism by which Ig genes are targeted for recombination is not completely understood, though is thought to involve multiple mechanisms, including changes in chromatin structure and variations in methylation patterns (Sleckman, *et al.*, 1996).

1.2.2: Transcriptional regulation of B cell differentiation: The roles of transcription proteins

As stated, after commitment to the B cell lineage, a cell transits through multiple stages of differentiation defined by many phenotypic and functional changes such as sequential immunoglobulin heavy and light chain re-arrangement and the expression of various cell surface molecules. External signals regulate lineage- and stage-specific expression of genes, including extracellular growth factors and cell to cell contact. Intracellularly, these signals are integrated by transcription factors which execute a programme of differentiation by directly regulating gene expression. Some of these transcription factors have been identified through studies of cis-acting regulatory elements of genes specifically expressed in individual haematopoietic lineages, and some have also been discovered by examining genes that are translocated or abberantly activated in leukaemias. Transcription factors are known to perform specific functions in cellular differentiation, suggested by the fact that they are expressed in a cell type specific manner. *In vitro* studies have shed further light on the biochemical and functional properties of these transcription factors, and definitive evidence for their role *in vivo* has come from the analysis of mice carrying targeted mutations.

A subset of transcription factors expressed in haematopoietic stem cells, as well as in B cell precursors, have been implicated in B cell lineage commitment and these are discussed below.

PU.1

PU.1 (spi 1) is an Ets family transcription factor member that is expressed at high levels in immature and mature B cells and in myeloid cells. The importance of PU.1 in B cell generation has been demonstrated by the analysis of mice carrying a targeted PU.1 gene mutation, which display a profound loss of all lymphoid and myeloid cells. Stem cells were also reduced in number, and found to be unable to generate B lymphoid and myeloid cells.

PU.1 is postulated to regulate various B cell specific genes; including mb-1 (Igα), immunoglobulin J chain and immunoglobulin μ, κ and λ chains. In the context of the regulation of light chain genes, PU.1 has been shown to act by co-operating with other transcription factors such as PIP (LSIRF, IRF-4), c-jun and c-fos (Pongubala & Atchison, 1997). PIP has been shown to be recruited to transcriptional control regions, such as the Igλ chain enhancer, by PU.1 and together these proteins are thought to be important in controlling the transcriptional activity and recombinatorial activity of immunoglobulin light chain genes. PIP also appears to repress transcription mediated by interferon α or β in a PU.1-independent manner (Brass, *et al.*, 1996; Yamagata, *et al.*, 1996). A recent targeted mutation of the PIP gene revealed that it is not required for immunoglobulin κ transcription and early B cell differentiation (Mittrucker, *et al.*, 1997). PIP-deficient mice, however, lack germinal centres in peripheral lymphoid organs, are defective in B cell activation and display severe lymphadenopathy - which may be related to PIP's function as a repressor of interferon-induced transcription.

Ikaros

Committed lymphoid precursor generation is also regulated by Ikaros proteins, which belong to a family of zinc finger transcription factors that includes Aiolos and Helios (Georgopoulos, *et al.*, 1997; Hahm, *et al.*, 1998). Ikaros is expressed in both stem cells and mature lymphocytes, and Ikaros-like proteins recognise binding sites in many B lymphocyte specific genes; including TdT, λ5, VpreB and lck (Georgopoulos, 1997). Alternative splicing of the Ikaros gene results in the generation of various isoforms (Ik 1-6) which differ in their DNA binding, dimerization and nuclear localisation potential (Sun, *et al.*, 1996).

The DNA binding domains of Ikaros proteins are located at the amino terminus, consisting of four zinc fingers. Dimerization among Ikaros proteins and other family members is mediated by two individual zinc fingers at the carboxyl terminus (Hahm, *et al.*, 1998) and mice carrying a carboxyl terminal mutation have null mutant protein and no B cells in either foetus or adult. A different targeted mutation, removing the Ikaros gene DNA binding domain, enables the synthesis of a stable mutant protein (Georgopoulos, *et al.*, 1994) and leads to a complete lack of B and T lymphocytes at all developmental stages.

Following B cell lineage commitment, cells differentiate into pro-B cells that undergo Ig D-J_H rearrangement and express the cell surface markers B220, CD43 and heat stable antigen. The next stage of differentiation leads to the addition of expression of BP-1 and also V_H-DJ_H gene recombination and cells at both these stages express the immunoglobulin surrogate light chain genes, λ5 and VpreB. Following rearrangement of the light chain gene, light chains are expressed on the cell surface together with mb-1 (Igα) and B29 (Igβ) - which are involved in signal transduction.

NFκB

NFκB is proposed to regulate several genes important in B cell differentiation, such as the Ig light chain, and this role of mediating growth factor generated signals has also led to the suggestion that it may be involved in the prevention of precursor cell apoptosis. NFκB is a heterodimer of p50 and p65 subunits, whose activity is regulated by interaction with IκB inhibitory proteins (Barnes & Karin, 1997). Both subunits of NFκB belong to the Rel family of transcription factors, which include p52, Rel-B and c-Rel. Targeted mutations in Rel family member genes, and subsequent analysis of these in mice, demonstrated that they are essential for multiple functions in the later stages of B cell differentiation (Baldwin, 1996). Analysis of mice carrying mutations in more than one NFκB subunit gene has shown that NFκB proteins also play a critical function in early B cell development. P65 null mice die *in utero* (Doi, *et al.*, 1997), so B cell development can only be monitored in adoptive transfer experiments, which reveal a reduction in cell number at each stage of B cell development. Mutant B cells were also found to be deficient in proliferative responses to mitogens and in the secretion of IgG and IgA (Horwitz, *et al.*, 1997). In contrast, p50 null mice display normal B cell development, but show impaired mitogen-induced proliferation and antibody isotype production (Snapper, *et al.*, 1996). Mice lacking both subunits have more severe defects than either of the single mutants, failing to generate any B cells whatsoever.

Interactions of a similar nature have been observed between p50 and other NFκB family members; Rel-B null mice have virtually normal B cell development, but show functional defects such as multi-organ failure, myeloid hyperplasia and splenomegaly (Weih, *et al.*, 1995). In contrast, p50/Rel-B double knockout mice display a marked reduction of immature B cells in the bone marrow, as well as a reduction of mature IgM positive B cells in the bone marrow and spleen. Finally, while mutation of the p52 gene alone affects only peripheral B cell function,

inactivation of both p50 and p52 subunits results in a block in the development of IgM^+/IgD^+ mature B cells .

E2A

Early B cell development is also regulated by E47 and E12, members of the basic helix-loop-helix (bHLH) transcription factor family, which are generated by alternative splicing of RNA encoded by the E2A gene (Kadesch, 1992). These proteins recognise sequences in the transcriptional control regions of most lymphoid cell specific genes; either as homodimers or heterodimers with other proteins. While E12 and E47 are widely expressed, the homodimeric form of E47 is unique to B lymphocytes.

Products of E2A are thought to be involved in the transcriptional regulation of the μ, κ and Ig heavy chain genes, and mice carrying mutations in E12 and E47 proteins have no mature B cells (Bain, *et al.*, 1994; Zhuang, *et al.*, 1994). These mice contain $CD43^+/B220^+$ (CD45) B cell precursors, arrested prior to $D\text{-}J_H$ gene re-arrangement. Mice lacking E12 alone have defects at later developmental stages, suggesting E12 and E47 are partially overlapping - but not identical in their influence on B cell development (Bain, *et al.*, 1997). There is also a noted gene dose effect with E47 proteins, with $E47^{+/-}$ heterozygotes showing exactly half the number of mature B cells relative to control mice (Zhuang, *et al.*, 1994).
The E2A gene products may regulate targets in conjunction with other factors, such as p300, since the activation of the Ig heavy chain enhancer has been seen to be increased by the recruitment of p300 via interaction with the E47 DNA binding domain.

Besides its importance in regulating early B cell development, E2A may also play a role in later B cell differentiation. This is suggested by the fact that E2A is

upregulated in lymphoid germinal centre cells and that class switching to some isotypes is inhibited in B cell lines that over express Id (inhibitor of development), which implies a role for bHLH family factors in terminal differentiation (Goldfarb, *et al.*, 1996).

EBF

Early B cell factor (EBF) has been shown to regulate early events in B cell differentiation. It is expressed at all stages of differentiation, with the exception of plasma cells (Hagman, *et al.*, 1991), and appears to be critical in the progression of B cell progenitors to the later stages of development. Both EBF and E2A deficient mice have defects at a similar B cell developmental stage and, therefore, these proteins may act co-operatively to regulate a common set of genes. Functional synergy between EBF and E47 is supported by the finding that ectopic expression of EBF, together with E47 dimers, induced the expression of VpreB and λ5 B cell specific genes in *in vitro* studies (Sigvardsson, *et al.*, 1997).

BSAP/Pax-5

Differentiation stages that follow the early pro-B cell stage are regulated by BSAP (B cell specific activator protein), encoded by the pax-5 gene and expressed in all B cell types. Pax-5 is alternatively spliced into 4 different isoforms that are differentially expressed during B cell development (Zwollo, *et al.*, 1997). Most isoforms are down-regulated during terminal differentiation, apart from pax-5b which remains expressed through this stage, suggesting different pax-5 isoforms may play distinct roles in B cell development. Mice carrying pax-5 gene targeted mutations are deficient in the progression of pro-B to pre-B cells in the BM (Urbanek, *et al.*, 1994).

Pax-5 is thought to function early in the development of foetal liver to allow cells to respond to stimuli specific to their micro-environment. Many genes are proposed to be potential pax-5 targets, but only CD19 expression is extinguished in pax-5 deficient B cells. Besides its role in early B cell development, pax-5 appears important in late B cell differentiation as well. This is indicated by the fact that anti-sense oligos that reduce pax-5 expression levels block mature B cell proliferation in response to liposaccharide (Wakatsuki, *et al.*, 1994). Also, cell lines overexpressing pax-5 display inefficient plasma cell formation, suggesting that down-regulation of pax-5 may be a prerequisite for terminal differentiation.

LEF-1

Lymphoid enhancer binding factor (LEF-1) is expressed in both pro- and pre-B cells, but not on immature or mature B lymphocytes (Reya, *et al.*, 2000). In the absence of LEF-1, B cell differentiation appears normal apart from a drop in the development of pro- and pre-B lymphocytes, which is thought to be a result of a decrease in the proliferation and survival of early B lineage progenitors. LEF-deficient pro-B lymphocytes have an increased level of Fas and c-myc RNA, both of which lead to an increase in apoptosis (Reya, *et al.*, 2000). This paper also includes data which suggests that Wnt signalling is also important for the development of B lineage progenitors, with LEF-1 deficient pro-B cells showing a reduced response to Wnt-3. LEF-1 has been shown to be a target of Wnt signalling pathways (Behrens, *et al.*, 1996). In response to Wnt binding to its receptor (Frizzled) signal transduction leads to stabilisation of cytosolic β catenin, which then accumulates in the nucleus and functions as a co-activator for LEF-1 transcriptional responses.

Regulation of late B cell differentiation

Following surface IgM expression mature B cells migrate to the periphery, express IgD and become fully capable of responding to antigen. Encounter of antigens induces other cascades of differentiation events which result in proliferation, plasma cell formation and immunoglobulin secretion. A number of transcription factors that are important for such events have been recently identified, including; oct-2, OCA-B, NFκB and Blimp-1 (Corcoran, *et al.*, 1993; Schubert, *et al.*, 1996; Turner, *et al.*, 1994).

1.2.3: Antigen dependent B cell development

The very first encounter between naive B cells and antigen via the BCR activates proliferation leading to antigen uptake, processing, presentation and altered gene expression patterns. Molecules involved in T cell/B cell interactions are upregulated, including Class II MHC proteins, CD80 (B7-1), CD86 (B7-2) and CD40, which contacts CD40 ligand on T-cells (Clark & Lane, 1991; Glimcher & Kara, 1992).

Early B lineage cells face one type of choice during their maturation, to live or die. Their survival depends on the correct rearrangement of the antigen receptor gene segment at each stage of maturation (Rajewsky, 1996). Mature peripheral B cells face two types of choice during antigen-driven immune responses; firstly, to live or die and, secondly, to undergo terminal differentiation or to maintain their original identity. B cells have to make the choice between the plasma cell and the memory cell pathways upon activation. During early B cell activation, it is essential for some activated B cells to differentiate into plasma cells in order to produce the antibodies required for antigen fixation, uptake by macrophages and subsequent clearance. Mechanisms exist to prevent all cells from differentiating into plasma cells, in order

for some to undergo affinity maturation to function as memory B cells. Similarly, after somatic mutation and selection in germinal centres (GC), high affinity GC B cells are required to differentiate into both plasma cells (to secrete high affinity antibody) and into memory B cells (which make a robust secondary response).

Germinal centre B cells will die unless they receive a positive signal, and this is clearly illustrated by the rapid onset of spontaneous apoptosis in isolated cultured cells (Liu, *et al.*, 1989). This apoptosis-prone property correlates with a high expression of the apoptosis inducing genes; c-myc, p53, Bax and Fas and with the absence of the survival gene Bcl-2 (Martinez-Valdez, *et al.*, 1996). Two surface signals have been identified as being able to rescue B cells from apoptosis; anti-Ig and anti-CD40. A model for positive selection would therefore be as such: after somatic hypermutation, mutants with low affinity antigen receptors apoptose due to their failure to bind antigens on follicular dendritic cells (FDC's). Those mutants with a high affinity antigen receptor will bind antigen on FDC's, pick it up, process and present it to GC T cells, thus activating them to deliver CD40 ligand (Kosco, *et al.*, 1988; Liu, *et al.*, 1992; MacLennan & Gray, 1986).

The danger of somatic hypermutation in GCs is the potential generation of high affinity autoreactive mutants, making a negative selection mechanism essential. The question is how the responding cells interpret an antigenic signal as positive or negative, and several factors have been found to influence this interpretation. The first is the length of antigen-receptor engagement or the concentration of antigen, and the second is dependent on T cell signals such as CD40 and Fas ligand. The survival effect of anti-Ig on GC B cells can be strongly enhanced and prolonged by anti-CD40 antibodies. In germinal centre B cells CD40 ligand represents the most powerful signal for maintenance of survival (Holder, *et al.*, 1993), for promotion of proliferation (Banchereau & Rousset, 1991) and for inhibition of terminal differentiation of B cells (Callard, *et al.*, 1995). Administration of anti-CD40 ligand

antibodies or soluble CD40 to mice abolished their GCs and prevented memory B cell generation (Noelle, 1996). Germinal centre T cells not only express CD40 ligand (Casamayor-Palleja, *et al.*, 1995), but also Fas ligand. *In vitro* experiments demonstrated that anti-Fas antibodies could accelerate the rate of spontaneous apoptosis in GC B cells, and that CD40 ligand was not able to prevent this Fas-mediated apoptosis (Liu, *et al.*, 1995). In fact, CD40 ligand can even prime resting B cells to be sensitive to Fas-mediated apoptosis, although anti-Ig can inhibit it (Rothstein, *et al.*, 1995).

Thus, germinal centre B cells will die if their antigen receptor is triggered for a prolonged period, if they do not interact with T cells within a short time scale or if they interact with CD40 ligand and Fas ligand-expressing T cells in the absence of B cell receptor triggering. The ability of Fas ligand to kill CD40 ligand-activated, but not antigen-activated, B cells provides a mechanism to prevent the survival of low-affinity or autoreactive GC B cells.

The third factor involved in GC B-cell response to antigen is the signal from follicular dendritic cells (FDCs). FDCs not only provide the optimal density of antigenic epitopes on their surface to stimulate high affinity GC B cells, but may also act as nurse cells (isolated GC B-cells survive well when they are in FDC clusters). The complement fragment, C3d, may signal GC B cells through CD21 and enhance the survival and proliferation effects of antigens (Dempsey, *et al.*, 1996). CD23 might also be involved in this enhancing effect (Liu, *et al.*, 1991).

1.2.4: Regulation of germinal centre B cell differentiation:

Germinal centre B cells can directly differentiate into both memory B cells and plasma cells (MacLennan, 1994; Zinkernagel, *et al.*, 1996), the important question

being whether a positive factor pushes the cells into a particular pathway or if negative signals prevent it from entering another.

Following successive rounds of somatic hypermutation (SHM), receptor editing, isotype switch recombination (ISR) and selection, germinal centre B cells are signalled to proliferate, exit the GC and differentiate into either memory or plasma cells. These cells lose their apoptotic phenotype by down-regulating Fas and increasing bcl-2, bcl-xL, cdk-4 and cdk-6 expression (Choe, *et al.*, 1996; Ishida, *et al.*, 1995). They switch from an autocrine to a paracrine dependence on IL-6, by down-regulating IL-6 expression and up-regulating IL-6R expression. *In vitro* experimental evidence (Arpin, *et al.*, 1995) suggests that CD40 ligand directs the differentiation of GC B cells ($CD20^+/CD38^+$) towards the memory B cell pathway and, upon withdrawal of the CD40 ligand, their rapid differentiation into plasma cells. The inhibitory effect of CD40 ligand on B cell differentiation has been confirmed in other human B cell culture systems (Lane, *et al.*, 1995). *In vivo*, CD40 signalling induces a number of transcription factors, including; jun, NFκB and NF-AT (Choi, *et al.*, 1994; Li, *et al.*, 1996), and the timing and interplay of signals from the BCR, CD40, B7-1 and B7-2 and cytokines are also thought to determine cell fate (Urashima, *et al.*, 1996).

Cells exiting the germinal centre are still proliferating, but outside they rapidly become non-dividing plasma cells with a life-span of about one month. IL-6 protects the plasma cells from apoptosis, induces Ig expression and leads to the activation of the cyclin-dependent kinase inhibitor p18, blocking cell division (Morse, *et al.*, 1997). Plasma cells have huge levels of Ig and J chain mRNA, with the expression of many genes being decreased, including; CD23, CD22, CD19, Class-II MHC, c-myc, BSAP, EBF and CIITA.

1.3: T cell lymphopoiesis

B lymphocytes are extremely important in the development of immune responses. As discussed, they express immunoglobulin and are activated when they bind to specific antigens. The story would not be complete, however, without mentioning a little about T-cell responses in this literature overview, as they play both a cytotoxic and helper role in the destruction of pathogen infected cells.

T lymphocytes are derived from bone marrow stem cells that differentiate as they pass through the thymus. The mature thymus is an epithelial lymphoid organ made up of three distinct regions, each containing cells in different maturational stages in the T cell pathway (Shortman & Wu, 1996; van Ewijk, 1991). Late in foetal development, BM derived T cell precursors arrive in the subcapsular region of the outer thymic cortex. Here, they are induced to undergo substantial proliferation into large lymphoblasts. These cells then progress to the deep cortex and pass through a network of dendritic epithelial cells, with the resulting small-medium lymphocytes passing into the medulla. The T-cells then mature further, under the influence of a loose network of epithelial and interdigitating dendritic cells. Following completion of maturation in the thymus, T cells leave the medulla by passing between the endothelial cells of small venules. Then, by acquiring specific surface molecules, they are able to bind selectively to the walls of post-capillary high endothelial venules. This enables T lymphocytes to cycle continuously from blood through the lymph nodes and spleen and to exit into the lymphatics, whence they return to the general circulation via the thoracic duct.

As in B cells, the process of maturation of T cells is accompanied by the acquisition of new properties and differentiation markers that include enzymes, glycoproteins and specific receptors (Petrie, *et al.*, 1990). The most important event in T cell maturation in the thymus is the acquisition of the specific antigen-recognising

receptor. The T cell receptor (TCR) comprises an antigen recognising molecule (Ti) in tight association with a complex of invariant polypeptides (CD3). Two different types of Ti are found; the first is known as αβ and the second as γδ (Livak, *et al.*, 1995). An individual T cell can express either αβ or γδ as its receptor, but never both. The same fundamental principles of gene rearrangement, as were described in B cells, are applicable for the T cell receptor (Hardardottir, *et al.*, 1995). Recombinase and joining sequences are used to link up a VJ or VDJ unit to generate the variable region specificity of particular polypeptide chains. T cell receptor genes also show allelic exclusion, ensuring that any one T cell makes a receptor with only a single antigenic specificity.

Other cell types involved in the immune response are the monocyte-macrophage series and the granulocytes. These cell types do not exhibit antigenic specificity, and among their many functions is the ability to process and present antigen to T cells.

1.4: Interleukin-4 (IL-4)

1.4.1: Signalling and biological functions of IL-4

IL-4 is a pleiotropic type 1 cytokine produced by TH2 cells, basophils and mast cells in response to receptor-mediated activation events (Seder & Paul, 1994). Natural killer T cells (NK T cells), γδ T cells and eosinophils have also been shown to produce IL-4 to a lesser extent (Dubucquoi, *et al.*, 1994; Ferrick, *et al.*, 1995), and mice lacking these cells fail to develop IL-4 dependent airway hypersensitivity upon immunisation with ovalbumin in alum (Zuany-Amorim, *et al.*, 1998).

IL-4 plays a central role in regulating the differentiation of antigen-stimulated naive T cells, causing them to produce IL-4 and a series of other cytokines (including IL-5, IL-10 and IL-13). It also powerfully suppresses the appearance of interferon γ (IFN-

γ) producing $CD4^+$ T cells. Another function of major physiological importance is the role of IL-4 in the control of immunoglobulin class switching. IL-4 stimulation prompts human B cells to switch to the expression of IgE and IgG_4 (Gascan, *et al.*, 1991) and mouse B cells to IgE and IgG_1 (Vitetta, *et al.*, 1985). In IL-4 and IL-4 receptor knockout mice, as well as mice which lack STAT-6, IgE production is reduced by a factor of 100 or more (Akira & Kishimoto, 1997; Shimoda, *et al.*, 1996) and IL-4 has a wide variety of other effects in haematopoietic tissues. IL-4 increases the expression of class II MHC molecules in B cells (Noelle, *et al.*, 1984), enhances CD23 expression (Defrance, *et al.*, 1987), upregulates expression of the IL-4 receptor (Ohara & Paul, 1988) and, in association with lipopolysaccharide, allows B cells to express Thy 1 (Snapper, *et al.*, 1988). IL-4 also acts as a co-mitogen for B cell growth (Howard, *et al.*, 1982). Although not a growth factor by itself for resting lymphocytes, IL-4 can prolong the lives of T and B cells in culture (Hu-Li, *et al.*, 1987) and can prevent apoptosis by factor-dependent myeloid lines that express IL-4 receptors (Boise, *et al.*, 1995; Minshall, *et al.*, 1997; Zamorano, *et al.*, 1996). IL-4 has an important role in inflammation and adhesion, acting with tumour necrosis factor (TNF) to induce vascular cell adhesion molecule-1 (VCAM-1) expression on vascular endothelial cells (Thornhill, *et al.*, 1991). This is thought to favour the recruitment of T cells and eosinophils, rather than granulocytes, into a site of inflammation.

1.4.2: The IL-4 receptor complex

IL-4 receptors are present in haematopoietic, endothelial, epithelial, muscle, fibroblast, hepatocyte and brain tissues, which is in keeping with the broad range of action of this cytokine (Lowenthal, *et al.*, 1988). The receptor consists of a 140kDa IL-4Rα chain that binds IL-4 with high affinity, and heterodimerizes with a second chain to generate biochemical signals within the cell (Fujiwara, *et al.*, 1997). This second chain is known as the gamma common (γc) chain, was first identified as a

component of the IL-2 receptor (Leonard, *et al.*, 1994; Letzelter, *et al.*, 1998) and is thought to be the dominant chain involved in this heterodimerization.

The IL-4Rα chain also functions as a component of the IL-13 receptor (IL-13R) (Obiri, *et al.*, 1997), which appears not to utilise the γc chains but employs IL-13Rα' chains in their place (Aman, *et al.*, 1996). A number of cell lines lacking γc are still IL-4 responsive, raising the possibility that IL-13Rα' functions with IL-4Rα as a component of an IL-4R complex (Murata, *et al.*, 1998). IL-4Rα is a member of the haematopoietin receptor superfamily, in which members share structural motifs in the extracellular region - consisting of type III fibronectin domains (Miyajima, *et al.*, 1992). These motifs include conserved paired Cys residues and a WSXWS motif, which is required for maintaining the receptor in a conformation favourable to cytokine binding (Livnah, *et al.*, 1996). Structural alterations in the extracellular region of IL-4Rα results in altered receptor signalling capabilities. In fact, a variant of the human IL-4Rα chain containing an Ile 50 Val (I50V) substitution has been isolated from atopic individuals, and has been shown to enhance signal transduction and increase production of IgE (Mitsuyasu, *et al.*, 1998).

1.4.3: Activation of signal transduction by the IL-4R

The presence of IL-4 causes heterodimerization of the IL-4Rα chain with the γc chain, leading to the activation of IL-4R signalling pathways (Kammer, *et al.*, 1996). This ligand-induced dimerization results in the activation of tyrosine kinases that phosphorylate cellular substrates and initiate signalling cascades (Miyajima, *et al.*, 1992). Since neither the IL-4Rα nor the γc chain has endogenous kinase activity, the IL-4R requires receptor-associated kinases for the initiation of signal transduction - a role which is filled by the Janus-family (JAK) tyrosine kinases (Ihle, 1995; Taniguchi, 1995).

JAK 1, 2 and 3 have been demonstrated to be activated in response to IL-4R engagement and to associate with components of the IL-4R complex (Murata, *et al.*, 1996; Witthuhn, *et al.*, 1994). JAK 1 and JAK-2 have been proposed to associate with the IL-4Rα chain, while JAK-3 associates with the γc chain (Miyazaki, *et al.*, 1994; Russell, *et al.*, 1994). IL-4 engagement of the IL-4Rα chain results in the tyrosine phosphorylation of JAK-1 and JAK-3, and studies have shown that it is the membrane proximal region containing the box 1 motif and the acidic region that are important for IL-4-mediated responses (Deutch, *et al.*, 1995). The importance of this region may reflect the fact that it is a potential site for the interaction of JAK 1. In addition to the JAK family kinases, the Src family kinase Fes has also been reported to associate with the IL-4Rα and be activated in response to IL-4 stimulation (Izuhara, *et al.*, 1994). Activation of the IL-4R-associated kinases leads to the tyrosine phosphorylation of the IL-4Rα chain itself, presumably on the 5 conserved Tyr residues in the cytoplasmic region. The identification of tyrosine residues critical for activation of signalling pathways and the analysis of molecules that interact with these residues has led to the biochemical characterisation of pathways activated by IL-4R engagement. Truncation and deletion mutants of the human IL-4Rα chain were studied, with mutant receptors unable to transmit signals that normally result in phosphorylation (Keegan, *et al.*, 1994; Koettnitz & Kalthoff, 1993; Seldin & Leder, 1994).

Although the IL-4Rα C-terminal region does not appear to be essential for IL-4-stimulated proliferative responses, further analysis of deletion mutants have indicated that this region is important for the induction of IL-4 responsive genes (Ryan, *et al.*, 1996). Thus, the IL-4Rα chain cytoplasmic region appears to have 3 functionally distinct domains - one that acts as an interaction site for the Janus kinases, one required for activation of proliferative pathways and a third involved in pathways leading to the induction of gene expression. An overview of IL-4 induced signalling pathways is given in Figure 1.2, and these are described below.

1.4.4: The insulin receptor substrate (IRS) signalling pathway

IL-4R activation was found to lead to the specific phosphorylation of a 170kDa phosphoprotein, initially termed the IL-4 phosphorylation substrate (4PS). This 4PS was subsequently shown to be related to insulin receptor substrate-1 (IRS-1), and was renamed IRS-2. The importance of IRS-1 and IRS-2 in response to IL-4 has been demonstrated using the factor-dependent myeloid progenitor cell line 32D, which does not express detectable levels of IRS-1 and IRS-2 (Wang, *et al.*, 1993). These cells do not proliferate in response to IL-4 stimulation, but once stably transfected with IRS-1 and IRS-2 they phosphorylate the IRS substrate they express when stimulated with IL-4 and show IL-4-dependent cell growth. These observations led to the conclusion that IRS-1/2 molecules link the IL-4R to signalling pathways involved in cellular proliferation. IRS-1 and IRS-2 associate with IL-4Rα chain following activation and γc chain recruitment, and the tyrosine phosphorylation of IRS-1 and 2 is thought to involve the JAK kinases (Yin, *et al.*, 1995). In proof, JAKs 1, 2 and 3 have been shown to directly phosphorylate IRS-1 and experiments on JAK-deficient human fibrocarcinoma cells show that JAK-1 is essential for IL-4-driven IRS-1 stimulation (Yin, *et al.*, 1995). The fact that the γc chain is required, suggests that other cytokines sharing this subunit may also use IRS activation to promote cellular proliferation (Johnston, *et al.*, 1995). The primary pathway linked to IRS-1 and IRS-2 phosphorylation is the PI-3 kinase pathway.

1.4.5: The phosphoinositide-3-kinase (PI-3-K) pathway

The principal form of PI-3-K activated by IL-4 is a complex of two subunits, an 85kDa regulatory (p85) and a 110kDa catalytic (p110) subunit. The p85 subunit contains tandem SH2 domains in the C-terminus and an N-terminal SH3 domain (Kapeller & Cantley, 1994; Kapeller, *et al.*, 1994). These SH2 domains flank a 104

amino acid sequence that mediates the interaction with the p110 catalytic subunit (Dhand, *et al.*, 1994); thus the p85 subunit acts as an adapter molecule linking the p110 subunit to tyrosine-phosphorylated molecules. IL-4 stimulation leads to the binding of the p85 subunit of the PI-3-K to phosphorylated IRS-1/2 molecules, with IRS-1 and 2 having 4 and 10 potential sites of p85 subunit binding, respectively (Sun, *et al.*, 1993). Interaction of the p85 subunit with phosphorylated IRS-1/2 results in a conformational change in the PI-3-K complex, leading to the activation of the p110 subunit (Dhand, *et al.*, 1994). Once activated, the p110 subunit is capable of phosphorylating membrane lipids as well as Ser/Thr residues of proteins, and the lipid kinase activity mediates the transfer of phosphate from ATP to the D3 position of inositol in the cellular membrane (Stephens, *et al.*, 1993; Toker & Cantly, 1997). The most biologically important forms of phosphorylated phosphatidylinositol appear to be phosphatidylinositol-(3,4,5)-trisphosphate and phosphatidylinositol-(3,4)-bisphosphate, which are produced within seconds of stimulation (Stephens, *et al.*, 1993) and are thought to act as second messenger molecules for IL-4 function. Phosphoinositides have also been implicated in the activation of a number of downstream kinases, including protein kinase C (PKC) and protein kinase B (PKB), that play a key role in cell survival (Franke, *et al.*, 1997). It can be hypothesised that the activation of the PI-3-K pathway by IL-4 enhances cell survival through the production of phosphoinositides and the subsequent activation of kinases critical for cell survival, with inhibitors of PI-3-K (e.g. Wortmannin) blocking the ability of IL-4 to prevent apoptosis (Zamorano, *et al.*, 1996). It is thought that activation of the PI-3-K Ser/Thr kinase activity may result in a negative feedback loop that contributes to the regulation of the IRS-1/2 signalling pathway.

1.4.6: The Ras/MAPK pathway

Activation of the IRS-1/2 pathway is associated with the activation of the Ras/MAPK pathway in response to a number of cytokines. Phosphorylated IRS-1/2 has been proposed to interact with the SH2 domain of the adapter Grb2, which is constitutively complexed to the guanine nucleotide exchange protein Sos (Chardin, *et al.*, 1993). The primary function of Sos is to catalyse the exchange of GDP in inactive Ras for GTP, producing the active form of Ras (Downward, 1996).

The MAPK pathway is initiated by the Ser/Thr kinase Raf following its activation with Ras-GTP, with activated Raf initiating a cascade of kinase activation events resulting in the phosphorylation and activation of protein kinases ERK-1 and 2 (Denhardt, 1996; Marais & Marshall, 1996). Active ERK-1/2 translocates to the nucleus and activates genes, such as c-fos, by phosphorylating specific transactivating factors (Davis, 1995). Activation of Jun nuclear kinase (JNK) and other small GTPases results in the nuclear phosphorylation and activation of c-Jun as well as other transcription factors. Although IL-4 dramatically activates IRS-1/2 phosphorylation, IL-4 activation of the Ras/MAPK pathway is not consistently observed. In fact, stimulation of a number of cell lines with IL-4 failed to result in any detectable activation of components of the Ras/MAPK pathway (Welham, *et al.*, 1994; Welham, *et al.*, 1995), and led to the conclusion that IRS-1 phosphorylation and association with Grb2/Sos is not sufficient for the activation of cellular proliferation in certain cells and requires the activation of other molecules such as Shc (Pruett, *et al.*, 1995). Other studies, however, have shown that IL-4 stimulation does lead to the activation of the Ras/MAPK pathway in certain cell types, including B cells (Crowley, *et al.*, 1996; Wery, *et al.*, 1996).

The adaptor protein shc may play a pivotal role in the ability of certain cells to activate the Ras/MAPK pathway in response to IL-4, and it shares some structural

and functional characteristics with IRS-1/2. Shc contains 2 distinct domains capable of binding tyrosine-phosphorylated receptor sequences; The C-terminal region of the Shc protein contains an SH2 domain while the N-terminal region contains a PTB domain (Songyang, *et al.*, 1993). These domains mediate Shc's interaction with phosphorylated receptor molecules which leads to the phosphorylation of Shc itself at Tyr 317, serving as a docking site for the SH2 domain of Grb2 (Rozakis-Adcock, *et al.*, 1992; Salcini, *et al.*, 1994). Shc may link the Grb/Sos complex to phosphorylated receptors and thus catalyse Ras activation. Cb1, encoded by the proto-oncogene c-Cbl, is also an adaptor molecule and likewise plays a role in the activation of signalling pathways by IL-4 and is phosphorylated in response to IL-4 stimulation (Ueno, *et al.*, 1998). Cytokine-induced phosphorylation of Cb1 has been demonstrated to link Grb2/Sos to receptor complexes and thus may play a role in the activation of the Ras/MAPK pathway (Fukazawa, *et al.*, 1995; Panchamoorthy, *et al.*, 1996). IL-4-induced phosphorylation also leads to Cb1's association with the p85 subunit of PI-3-K, and Cb1 also contains a proline-rich sequence that can interact with SH3 domains of different molecules (Fukazawa, *et al.*, 1996).

1.4.7: The STAT-6 activation pathway

An important development in understanding the mechanism by which IL-4 and other cytokines rapidly activate gene expression has been the identification and characterisation of molecules termed signal transducers and activators of transcription (STAT's). Experiments have shown that JAK activation is required for STAT activation (Velazquez, *et al.*, 1992), thus, the STAT activation pathway is mostly referred to as the JAK-STAT pathway. STAT-6 is the principal STAT activated in response to IL-4 stimulation and is critical in the activation or enhanced expression of many IL-4 responsive genes, including class II MHC molecules, germline IgE and CD23 (Delphin & Stavnezer, 1995; Kaplan, *et al.*, 1996; Nakanishi, *et al.*, 1996; Shimoda, *et al.*, 1996). The mechanism for STAT-6

activation reflects the general model proposed for all STAT activation events, and is shown in Figure 1.3.

IL-4 engagement results in the activation of JAK1 and JAK3, and the phosphorylation of specific tyrosine residues in the receptor cytoplasmic region. STAT-6 then binds to the phosphorylated receptor through a highly conserved SH2 domain, enabling the activated kinases to phosphorylate STAT-6 at a C-terminal tyrosine residue (Mikita, *et al.*, 1996). Once phosphorylated, the STAT-6 molecule disengages from the IL-4R and forms a homodimer with a second STAT-6 molecule through interaction of its SH2 domain with the C-terminal phosphotyrosine residue of other STAT-6 molecules. Dimerized STAT-6 complexes then translocate to the nucleus where they bind to specific DNA motifs in the promoter regions of responsive genes, usually binding to the particular sequence TTC-N_4-GAA (Ihle, 1996; Schindler, *et al.*, 1995). The STAT-6 activation capability of different IL-4Rα mutants matched the ability of these receptors to stimulate IL-4-responsive gene expression, with mutants that lacked the gene regulation domain or had Tyr to Phe mutations in this region inducing little or STAT-6 phosphorylation and DNA binding (Ryan, *et al.*, 1996). This suggested that phosphorylation of Tyr residues in the gene regulation domain is critical for STAT-6 activation and that activation of STAT-6 is a critical step leading to the expression of IL-4-responsive genes. There are 3 conserved Tyr motifs in the gene regulation domain of IL-4Rα, and these are 27 amino acids away from each other. It is these motifs in the gene regulation domain that are proposed to be docking sites for the SH2 domain of STAT-6.

Deletion and mutational analysis of the STAT-6 protein itself have revealed and defined domains and residues within STAT-6 that are required for DNA binding and transcriptional activation. Deletions in the C-terminus blocked its ability to activate transcription (Mikita, *et al.*, 1996), reflecting the importance of this region and the presence of critical residues. Mutation of a Tyr residue (Y641) in this C-terminal

region also blocked DNA binding and STAT-6 function, and this residue is predicted to be the site of JAK phosphorylation and the target of SH2 domains of other STAT-6 molecules. Amino acid substitutions in the DNA binding domain also inhibited STAT-6 transcriptional activation, as did mutation of the conserved Arg residue in the SH2 domain (R562). The latter mutation is thought to prevent receptor interaction and dimerization of the mutated STAT-6 molecules. Naturally occurring deletion mutants of STAT-6, termed STAT-6b and STAT-6c, that result from alternative splicing have also been characterised (Patel, *et al.*, 1998). STAT-6b contains an N-terminal truncation that attenuates, but does not block, its function and co-expression of STAT-6b with full length STAT-6 did not alter the activation or function of the full length molecule (Patel, *et al.*, 1998). In contrast, STAT-6c contains a deletion in the SH2 domain and does not become phosphorylated in response to IL-4 stimulation.

The exact mechanism by which STATs activate transcription is still to be determined. They do, however, form complexes with other well characterised transcription factors such as c-Jun and SP1, and thus may activate transcription through co-operative interaction with these factors (Look, *et al.*, 1995; Schaefer, *et al.*, 1995). Co-operative action with the transcription factors C/EBPα and NFκB appears to be particularly important in the transcriptional activation of the immunoglobulin ε gene by STAT-6 (Delphin & Stavnezer, 1995; Shen & Stavnezer, 1998). Activation of the Ras/MAPK pathway is required for the full function of some STAT molecules (David, *et al.*, 1995), and it is likely that Ser/Thr phosphorylation has a function in regulating STAT function. A serine residue in the C-terminal region of STAT-1α, for example, has been shown to be phosphorylated in response to activation of the Ras/MAPK pathway (Wen, *et al.*, 1995).

The critical importance of STAT-6 activation *in vivo* has been demonstrated in STAT-6 knockout mice (Huang & Paul, 1998), in which no detectable serum level

of IgE or infection response can be found. These mice also fail to develop $CD4^+$ T cells of the TH2 type in response to *Nippostrongylus brasiliensis* (Nb) infection (Nakanishi, *et al.*, 1996). When the lymphocytes from STAT-6 knockout mice are studied *in vitro,* they fail to show switching to IgE and IgG1 in response to LPS and IL-4, and also fail to show IL-4-mediated enhancement in expression of CD23, class II MHC molecules and IL-4 receptors. The specific immunodeficiency of STAT-6 -/- mice is thought to result both from a block in IL-4-dependent Th2 cell development and from an inability of B cells to target the Cε gene for class switching, both because IL-4 is not being produced and because STAT-6 -/- cells would be insensitive even if IL-4 were being produced. The action of IL-4 as a co-mitogen for B and T cells is not ablated in STAT-6 deficient mice, although these cells can display a loss of IL-4-dependent lymphocyte DNA synthesis. The impairment of IL-4 mediated growth effects in the cells of STAT-6 knockout mice may be a consequence of the reduced expression of factors required for IL-4-induced proliferation, including reduced IL-4Rα chain and IRS-2 expression (Huang & Paul, 1998). A polymorphism in the human IL-4Rα chain has been reported in patients with hyper-IgE syndrome and atopic dermatitis: Q567R in the core STAT-6 binding sequence (Hershey, *et al.*, 1997). These patients responded to IL-4 with higher levels of expression of CD23 than patients with normal phenotype, implying that the IL-4α chain containing R567 have enhanced activity at the STAT-6 docking site and signal more vigorously upon IL-4 engagement.

1.4.8: Regulatory pathways of IL-4 receptor signalling

Regulatory pathways are important in the modulation of intracellular signals initiated by IL-4 and other cytokines. Just as the IRS-1/2 and JAK/STAT signalling pathways are activated through several different cytokine and growth factor receptors, certain negative regulatory pathways also appear to be involved in the regulation of signalling by different cytokine receptors.

Negative regulation of the JAK/STAT Pathway

The JAK/STAT pathway is one of the better characterised signal transduction pathways, and its activation is rapid and transient. There are several proposed different levels at which negative regulation can be achieved; these include down-regulation of the receptor ligand complex, degradation of signalling intermediates, inactivation of positive regulators by dephosphorylation and activation of specific suppressors.

Negative regulation of JAKs

Protein tyrosine phosphatases are obvious candidates for proteins that modulate tyrosine phosphorylation, and the phosphatase SHP-1 (also known as HCP, SH-PTP-1 and PTP1C) has been identified as a critical regulator (Fearson & Alexander, 1997). SHP-1 is an SH2 domain-containing tyrosine phosphatase expressed in haematopoietic cells, and its importance is clearly demonstrated in mice which lack SHP-1 expression (Shultz, *et al.*, 1997; Shultz, *et al.*, 1993). These mice display multiple haematopoietic abnormalities, including hyperproliferation and abnormal proliferation of granulocytes and macrophages. They also have an autoimmune-like phenotype. The defects present in these mice suggest that SHP-1 functions as a negative regulator of both lymphoid and myeloid lineages and, indeed, SHP-1 has been shown to directly associate with and dephosphorylate JAK 2 (Jiao, *et al.*, 1996). This association is independent of the SH2 domain.

In certain circumstances, SHP-1 has been noted to have a role in promoting signal transduction, with the expression of a catalytically inactive form in HeLa cells noted to suppress epidermal growth factor (EGF) and IFN-γ–induced STAT activation (You & Zhao, 1997). The related phosphatase SHP-2 is generally considered to be a

positive regulator of signalling (Fearson & Alexander, 1997), but has been shown to down regulate signalling through gp130 (Symes, *et al.*, 1997).

Suppressors of cytokine signalling (Socs)

Experiments to clone inhibitors of cytokine signalling have recently identified a new family of negative regulators, termed suppressors of cytokine signalling or SOCS. SOCS-1 was isolated by its ability to suppress macrophage differentiation of M1 cells in response to IL-6 (Starr, *et al.*, 1997), and has subsequently been cloned independently by several groups. These groups used either a two hybrid screen to find proteins interacting with the kinase domain of JAK-2 (Endo, *et al.*, 1997), or a screen to find proteins with homology to the SH2 domain of STATs (Naka, *et al.*, 1997). The SOCS family comprises at least 8 proteins (SOCS 1-7, CIS) that are all composed of similar structures (Hilton, *et al.*, 1998; Minamoto, *et al.*, 1997; Yoshimura, *et al.*, 1995). SOCS play a central role in determining the intensity and direction of cellular responses to many cytokines, and appear to switch off signals in at least 2 different ways. SOCS-1 functions by binding to activated JAK kinases and inhibiting their catalytic activity (Naka, *et al.*, 1997; Starr, *et al.*, 1997) while, in contrast, CIS appears to bind directly to phosphorylated receptors and compete with signalling intermediates for binding to the receptor (Matsumoto, *et al.*, 1997; Yoshimura, *et al.*, 1995).

All SOCS proteins share a similar structure, including a central SH2 domain, a variable N-terminal region (in both length and amino-acid sequence) and a region of homology at the C-terminus termed the SOCS box (Hilton, *et al.*, 1998). The SOCS box is a sequence of 40 amino-acids that is conserved throughout the SOCS family, and this bears no sequence homology to other known functional domains. Pairs of the proteins (CIS/SOCS-2, SOCS-1/SOCS-3, SOCS-4/SOCS-5, SOCS-6/SOCS-7) are more similar to each other than to the other SOCS proteins, suggesting they have

diverged to fulfil specific functions. The specific role of each domain of SOCS in suppressing cytokine-induced responses remains unclear. The SH2 domain of SOCS-1 appears to be involved in the association of SOCS-1 with the phosphorylated JH-1 domain of JAK-2, although this interaction is not sufficient for the inhibition of a biological response (Endo, *et al.*, 1997). Therefore, although the particular functions of the N-terminal and SOCS box regions have not been resolved, at least one of these domains appears to be critical for SOCS function.

Database searches have identified an additional 12 proteins that contain a C-terminal SOCS box (Hilton, *et al.*, 1998), and these differ from SOCS proteins by having protein motifs other than the SH2 domain N-terminal to the SOCS box. The WSB (WD-40/SOCS box) proteins (WSB1 and WSB2) contain WD-40 repeats, whereas others contain ankyrin repeats (ASB-1 to ASB-3) or a SPRY domain (SSB-1 to SSB-3) N-terminal to the SOCS box. RAR and RAR-like GTPases also contain a C-terminal SOCS box, and this conserved region in all 20 proteins is likely to play a similar and important role in each protein. However, whether this role is to inhibit kinase activity or to regulate other general functions such as protein stability or localisation remains to be determined - and it is the latter role that is considered most likely.

The expression of each of the SOCS genes is inducible in response to IL-6. Transcription of SOCS-1 and SOCS-3 is rapid and transient in response to IL-6, apparent within 20 minutes and declining in 2-4 hours. The kinetics of IL-6 induction of the other SOCS family members differ considerably, with transcription of SOCS-2 and CIS persisting up to 24 hours after cytokine exposure and induction of SOCS-5 expression being delayed and only detectable 8-12 hours after stimulation. Transcriptional regulation of the SOCS genes is mediated by STAT transcription factors, a proposal supported by several lines of evidence. Firstly, the expression of dominant negative STAT-3 mutants blocks the IL-6-induced

transcription of SOCS-1 in M1 cells (Naka, *et al.*, 1997) and, secondly, CIS expression is induced by IL-3 and Epo (which mainly signal through STAT-5) (Matsumoto, *et al.*, 1997). Since SOCS protein expression is induced by cytokine signal transduction pathways, and SOCS function to down-regulate these, they appear to form a negative feedback loop. Over time, expression of the SOCS genes is down-regulated to allow the cells to respond to future stimulation by cytokine. How SOCS inhibit JAK kinase activity is primarily unclear as, unlike SHP-1, the SOCS proteins do not appear to contain the motifs characteristic of known enzymes. The interaction of SOCS with the phosphotyrosine residues of JAKs may be sufficient to interfere with the function of JAK catalytic domains or, alternatively, SOCS may function by recruiting other molecules (such as phosphatases) to the JAKs. It appears likely that down-regulation of activated JAKs occurs either via dephosphorylation by phosphatases, or by specific degradation of phosphorylated JAKs.

Negative regulation of STATs

As well as the inactivation of receptors and JAK kinases, activated STAT dimers need to be down-regulated to switch off the signal in the nucleus. STAT activation is a transient event, and STAT molecules persist for, at most, a few hours after ligand stimulation. This inactivation can potentially be achieved in 3 ways: by specific inhibitors, by dephosphorylation by phosphatases or by proteolytic degradation.

Protein inhibitors of activated STATs (PIAS)

The PIAS family of negative regulators was originally identified using a two-hybrid screen for STAT-interacting proteins. PIAS-1 was identified as a specific interaction partner for STAT-1, and PIAS-3 was cloned on the basis of homology to PIAS-1

(Chung, *et al.*, 1997). PIAS-3 specifically associates with STAT-3 but not STAT-1, and appears to inhibit signal transduction by suppressing STAT activity. A putative zinc-binding motif is conserved in the PIAS family, but no other known motifs are present. PIAS-3 is expressed constitutively but associates with STAT-3 only upon stimulation of the cell with cytokines such as, IL-6, CNTF and OSM - suggesting that it specifically interacts with phosphorylated STAT molecules. The addition of PIAS-3 to IL-6-treated cell extracts completely suppressed the DNA binding activity of dimers containing STAT-3, although STAT-1 homo-dimers remained functional. It is notable that PIAS-3 is able to inhibit the DNA-binding activity of STAT-1/STAT-3 heterodimers and, considering that STAT-1 was not found in PIAS-3 immunoprecipitates, PIAS-3 appears likely to bind to activated STAT-3 monomers - functioning either by preventing dimer formation or by mediating the dissociation of activated STAT dimers.

Phosphorylation of STATs on a single tyrosine residue at the C-terminus is critical for STAT activation and function (Shuai, *et al.*, 1993). Phosphatase inhibitor studies have indicated a role for protein tyrosine phosphatases in down-regulating response to cytokines (Haque, *et al.*, 1995), and pulse-chase labelling experiments have shown that the half-life of activated STATs is short, but turn-over of the proteins themselves is considerably slower (Haspel, *et al.*, 1996; Lee, *et al.*, 1997). An alternative mechanism for inactivating STAT dimers is proteolytic degradation, with inhibitors of proteosome activity being shown to stabilise the activation of STATs. It is still unclear, though, whether the proteasomes directly target STATs (Kim & Maniatis, 1996) or regulate signalling intermediates upstream (Yu & Burakoff, 1997).

1.5: Interleukin-13 (IL-13)

In addition to IL-4, IL-13 is also a pleiotropic cytokine produced by T and B cells. IL-4R and IL-13R both share the IL-4Rα chain and promote STAT-6 activation, with mice deficient in either IL-4, IL-13, IL-4R or STAT-6 lacking IgE synthesis and TH-2 type reactions (Izuhara & Shirakawa, 1999). IL-4 and IL-13 genes are localised within a 25kb region on chromosome 5q31, a region which genome searches have identified as having a strong linkage with atopy and allergic asthma (Ober, 1998). As mentioned in the IL-4 section of this chapter (1.5.2) the IL-4Rα chain is an essential component of the IL-13R, along with one of either of two known component chains (IL-13Rα1 or IL-13Rα2). IL-13Rα1 has been shown to bind weakly to IL-13, but when joined in a heterodimer to IL-4Rα acts as highly functional IL-13 and IL-4 receptor. In contrast, IL-13Rα2 alone binds to IL-13 with high affinity. However a functional role for this latter interaction is unknown, as it is not known to be expressed on either B or T cells (Donaldson, *et al.*, 1998). A schematic representation of IL-4 and IL-13 signalling is shown in Figure 1.3. IL-4 binds and activates both receptor types through interacting with the IL-4Rα chain, whereas IL-13 only binds and activates the combined IL-4Rα-IL13Rα1 receptor (through the IL-13Rα1 chain). The IL-4Rα–γc and IL-4Rα-IL-13Rα-1 receptors have been named the Type-1 and Type-2 IL-4 receptors, respectively, with Type-1 being expressed on both T and B cells and Type-2 solely on B cells. Thus, IL-4 can stimulate both T and B cells but IL-13 can activate B cells only. Briefly, IL-4 and IL-13 signalling pathways lead to IgE and CD23 expression as follows. The IL-4Rα chain associates with JAK-1 and the γc chain with JAK-3, whereas the IL-13Rα1 is associated with Tyk-2. Both receptors lead to STAT-6 activation, with JAK-1 and STAT-6 but not JAK-3 being activated by IL-13. This implies that STAT-6 activation is due to the STAT-6 docking to the IL-4Rα chain.

1.6: B cell and T cell interaction, CD40 and CD40 ligand

CD40 is a 45-50kDa glycoprotein (Paulie & Rosen, 1989) expressed on B cells, T cells and FDCs (Banchereau, *et al.*, 1994). CD40 is a member of the tumour necrosis factor (TNF) receptor superfamily which includes, and has a high degree of homology with, the TNF-α receptor and Fas. The ligand for CD40 (CD40L) is a 39kDa member of the TNF family and is found on T cells, mast cells and basophils. Levels of CD40L expression on T cells increase as the cells mature (Nonoyama, *et al.*, 1995). As described in section 1.3.4 of this thesis, CD40L directs germinal B cell differentiation and the importance of CD40 ligation for B cell growth and survival is highlighted through the X-linked immunodeficiency disease, hyper-IgM syndrome (Kroczek, *et al.*, 1994). This syndrome is caused by mutations in, or the defective expression of, CD40L - where serum IgA, IgE and IgG levels are relatively low. In addition to its role in isotype switching, B cell CD40-T cell CD40L interaction is also involved in rescuing B cells from apoptosis (Holder, *et al.*, 1993).

The promotion of IgE synthesis and CD23 expression on uncommitted naive B cells requires a minimum of two signals, the first from IL-4 (or IL-13) and the second through CD40-CD40L interaction (Bonnefoy, *et al.*, 1995). Burlinson and colleagues reported that ligation of CD40 with either trimeric sCD40L or anti-CD40 monoclonal antibody induced both CD23 and CD25 expression on resting tonsillar B cells (Burlinson, *et al.*, 1996). This result contrasted with the data of Valle *et al* (Valle, *et al.*, 1989), who reported that anti-CD40 monoclonal antibody 89 (mAb89) stimulation did not lead to CD23 stimulation on tonsillar B cells. Work by Heath *et al* (Heath, *et al.*, 1994) confirmed the theory that anti-CD40 antibodies evoke different responses depending on which distinct epitope they recognise and bind to.

Early signalling events following B cell CD40 engagement have been investigated by a number of groups, the consensus being that protein tyrosine kinase (PTK)

activity is an important mediator (Kato, *et al.*, 1994; Ren, *et al.*, 1994). However, analysis of the cytoplasmic tail of CD40 revealed no intrinsic enzymatic activity, suggesting that the observed PTK activity is due to an association with soluble kinases (Ren, *et al.*, 1994). Extensive studies have revealed that the src-type kinases lyn, fyn and syk and PI-3 kinase were all phosphorylated and activated upon CD40 ligation (Faris, *et al.*, 1994; Ren, *et al.*, 1994). Engagement of CD40 also resulted in the phosphorylation of phospholipase Cγ2 (PLCγ2), but not PLCγ1, which is consistent with the increased production of inositol-(1, 4, 5)-trisphosphate (IP3). When CD40L-expressing T cells were used to stimulate B cells tyrosine-phosphorylation and increased cyclic AMP (cAMP) levels were also noted (Kato, *et al.*, 1994), along with a role for PKA.

Enhanced CD23 expression is noted when B cells are co-induced with both sCD40L and IL-4 (Burlinson, *et al.*, 1996). This increase in CD23 is thought to be due to the ability of IL-4 to elevate CD40 levels in B cells, thus increasing the number of receptors available for engaging sCD40L.

1.7: CD25 (IL-2Rα)

CD23 is the main topic of study for the research included in this thesis, although CD25 expression is analysed briefly in the LM-PCR experiments detailed in chapter 5. It is, therefore, necessary to briefly introduce CD25 at this stage.

CD25 is the unique, inducible α chain subunit of the receptor for interleukin 2 (IL-2R). It is 55kDa in size and is one of three IL-2R subunits, the others being the constitutively expressed 75kDa β and 64kDa γc chains - also termed CD122 and CD132, respectively. IL-2 is a multi-functional cytokine that modulates the growth, differentiation and apoptosis of many cells including B cells, T cells, and monocytes (Smith, 1988). CD25 is expressed at an early stage of lymphopoiesis and marks the

initiation of T cell receptor (TCR) rearrangement and commitment to the T cell lineage, prior to expression being lost until induction in mature resting T cells (Godfrey & Zlotnick, 1993; Rothenberg, 1992). In human mature B cells, CD25 is upregulated in response to B cell receptor (BCR) activation (Benschop & Cambier, 1999; Burlinson, *et al.*, 1996; Hewitt, *et al.*, 1997) and following IL-4 (Butcher, *et al.*, 1990), anti-CD40 antibody and anti-immunoglobulin stimulation of resting B cells (Hewitt *et al*, 1997; Burlinson *et al*, 1995).

Expression of human CD25 is regulated by three positive regulatory regions (PRR I-III) and two negative regulatory regions (NRR I-II) in the proximal region of the CD25 gene. PRR I contains binding sites for NFκB and serum response factor (SRF) (Pierce, *et al.*, 1995), and is responsive to mitogenic and antigenic signals resulting from TCR activation or stimulation. PRR II is a T cell specific promoter, contains binding sites for Ets family protein Elf-1 (Graves & Petersen, 1998) and the chromatin associated protein HMG-1 (Landsman & Bustin, 1993), and is involved in basal promoter activity (John, *et al.*, 1996). PRR III is similar to PRR II, with the addition of GATA and STAT-5 binding proteins, the STAT-5 site being activated in response to IL-2 ligation of the IL-2R.

The two negative regulatory regions of the CD25 promoter were identified using transient transfection studies (Hewitt, *et al.*, 1997; Smith & Greene, 1989), with a 31bp region of NRE I being involved in transcription regulation and referred to as the negative regulatory element (NRE). NRE is bound by a 50kDa binding protein (NRE-BP) which facilitates a repression in NRE activity, with the binding of NRE-BP being lost and CD25 expression being reduced upon stimulation of cells with IL-4 (Hewitt, *et al.*, 1997). The identity of NRE-BP remains undefined, although an interferon-regulated gene called stimulated trans-acting factor of 50kDa (Staf-50) has been suggested as a possible candidate for this protein (Tissot & Mechti, 1995; Tissot, *et al.*, 1996).

1.8: CD23 ($Fc_{\varepsilon}RII$)

1.8.1: Introduction

Human CD23 is a leukocyte cell surface antigen expressed on a wide range of cells of haematopoietic lineage, such as B cells, T cells and monocytes (Bonnefoy, *et al.*, 1997). CD23 has multiple functions, particularly in B lymphocytes where it is proposed to have roles in the regulation of cell growth and development, IgE regulation, antigen presentation, cell adhesion and in the prevention of apoptosis (Yokota, *et al.*, 1988). It is termed the low affinity receptor for IgE ($Fc_{\varepsilon}RII$) and is known to exist in both membrane bound and soluble forms.

1.8.2: Discovery and distribution

The low affinity receptor for IgE (CD23) has long been a subject of debate, since its initial identification by Gonzalez-Molina and Spiegelberg in 1977 (Gonzalez-Molina & Spiegelberg, 1977). In fact, it was not until 1987 that Yukawa and colleagues (Yukawa, *et al.*, 1987) brought together two separate avenues of immunological research to identify it unequivocally.

Gonzalez-Molina and Spiegelberg (1977) discovered a molecule with low affinity binding to IgE in human B lymphocytes that was clearly distinct from the high affinity IgE receptor found on mast cells and basophils. In 1981, Kinter and Sugden described a panel of monoclonal antibodies that precipitated out a 45kDa glycoprotein from the surface of Epstein-Barr virus (EBV) and termed it EBVCS, for EBV cell surface antigen (Kintner & Sugden, 1981). However this EBVCS was also found to be expressed on normal and, in particular, activated B cells and in 1986 Thorley-Lawson *et al* detected that it had a rapid turnover from the cell surface of

transformed B cells. This turnover was determined to be as a result of proteolysis of the EBVCS antigen, which was now being termed Blast-2 (Thorley-Lawson, *et al.*, 1986). Finally in 1987, Yukawa *et al* showed that antibodies within the CD23 cluster (including Blast-2) reacted with, and specifically precipitated, the 45kDa product of cDNA encoding for the low affinity IgE receptor ($Fc_{\varepsilon}RII$) (Yukawa, *et al.*, 1987). Thus, the low affinity receptor for IgE, EBVCS and Blast-2 were one and the same entity, and emerged as CD23; a multi-functional receptor with cytokine effects.

1.8.3: Structure

Human CD23 is a single chain 45kDa glycoprotein, containing 1 chain of N-linked complex carbohydrates, several O-linked carbohydrates and sialic acid residues. Features of this glycoprotein are structurally and functionally unique compared to other immunoglobulin (Ig) receptors and indeed, unlike other Fc receptors, CD23 does not belong to the Ig gene superfamily - being a novel type II membrane glycoprotein. In type II membrane proteins, their orientation is in the reverse configuration to the Ig superfamily, such that the amino terminus lies inside the cell and the carboxy terminus outside. The short intra-cytoplasmic tail consists of 23 residues, the single transmembrane domain of 20 residues and a large C-terminal extracellular region of 277 residues. CD23 is also a member of the C-type lectin family (Ludin, *et al.*, 1987), a family of proteins which bind carbohydrates in a calcium dependent manner (Drickamer, 1988). The region homologous to the C-type lectins spans from cysteine 163 to 282, contains 4 highly conserved cysteines (positions 191, 259, 273 and 282) and 2 partially conserved cysteines (positions 163 and 174) (Kikutani, *et al.*, 1986; Ludin, *et al.*, 1987). The lectin domain of CD23 has been subjected to mutational analysis and found to contain the IgE binding site (Bettler, *et al.*, 1992).

CD23 is an unique differentiation antigen for B cells, being restricted to certain stages of B cell development. It is expressed on μ+/δ+ mature B cells, but not immature bone marrow cells, and is thought to be involved in the regulation of growth and differentiation of B cells. The presence of CD23 has also been found on certain populations of eosinophils and monocytes, and is thought to be an important mediator in allergic reactions and in the immune response to parasitic infection (Capron & Dessaint, 1986). The binding of IgE to CD23 is calcium dependent (Richards & Katz, 1990) and can be inhibited by fucose-1-phosphate (Delespesse, *et al.*, 1992). In addition, this interaction also appears to involve protein-protein interactions, as de-glycosylation of IgE does not eliminate binding (Vercelli, *et al.*, 1989). The cytokine effects of CD23 are mediated by an epitope distinct from the IgE binding site (Mossalayi, *et al.*, 1992), and will be fully discussed later.

The extracellular portion of CD23, as stated, has a domain homologous to the C-type lectins and an extended C-terminal tail containing a reverse RGD (Arg, Gly, Asp) sequence that may act in cell adhesion. Another feature of CD23 structure is the presence of 3 short consensus repeats of 21 amino acids, located between the N-glycosylation site and the lectin domain. This repeated region contains 5 heptadic repeats of leucine or isoleucine, forming a leucine zipper motif. This region of CD23 adopts an alpha-helical coiled-coil structure, representing a "stalk" structure separating the lectin heads from the cell membrane. It is believed that this stalk region mediates the formation of protein dimers or trimers at the cell surface (Beavil, *et al.*, 1992) and more recent studies have found that, when subjected to protein-protein chemical cross-linking, CD23 forms trimers on the surface of cells (Beavil, *et al.*, 1995)

Similar to other Fc receptors, soluble CD23 (sCD23) fragments are cleaved from full length CD23 and released into the extracellular fluids. Five major sCD23 fragments of molecular masses 37, 33, 29, 25 and 16kDa have been identified, and are all

derived from cleavage sites situated in the 45kDa CD23 stalk region. The soluble components are first released as the 37kDa oligomeric form, which is subsequently cleaved to the other forms, of which the 25kDa form is the most stable (Letellier, *et al.*, 1989). All soluble forms apart from the 16kDa species contain the complete lectin domain and significant lengths of coiled-coil, and thus retain the ability to bind IgE. The 16kDa sCD23 binds IgE with much lower affinity, a property believed to be due to the monomeric nature of this soluble form (Bettler, *et al.*, 1989). All fragments bind to IgE, with those larger than and including 25kDa promoting IgE synthesis - and the 16kDa fragment thought to inhibit it. All forms of sCD23 are also thought to possess other important cytokine activities (Beavil, *et al.*, 1995). The rate of cleavage of CD23 is significantly reduced by IgE and anti-CD23 antibodies, and is increased after the use of agents that prevent CD23 glycosylation. This indicates that the carbohydrate chain exerts a stabilising effect on CD23 (Delespesse, *et al.*, 1989). Early studies suggested that CD23 was cleaved autocatalytically (Letellier, *et al.*, 1990), although CD23 does not resemble any known proteases. Marolewski and colleagues demonstrated in 1998 that, in several human cell types, the initial release of CD23 from the cell surface is mediated by a membrane bound metalloprotease (Marolewski, *et al.*, 1998). This process can be blocked by specific protease inhibitors. This group subsequently suggested that the proposal of auto-proteolysis is a result of the co-purification of the metalloprotease with CD23, and speculate that there may be a whole family of these metalloproteases which mediate sCD23 fragment cleavage (Marolewski, *et al.*, 1998).

1.8.4: Cytokine effects of sCD23

Human CD23 and its soluble forms display various biological activities in addition to their IgE binding function. These effects include: the promotion of myeloid colony generation, induction of CD3 expressing human thymocytes (Mossalayi, *et al.*, 1990), facilitation of cell adhesion and antigen presentation by B cells and

monocytes to T cells (Bertho, *et al.*, 1991; Gordon, *et al.*, 1989). In order to ascertain whether the cytokine effects of CD23 and its binding to IgE were mediated by the same epitope, Mossalayi *et al* (Mossalayi, *et al.*, 1992) prepared CD23 and sCD23 constructs and assayed their ability to bind IgE, sustain haematopoietic cell differentiation and to regulate IgE secretion in B cells. The IgE binding domain was subsequently mapped to residues running from Cys163 to Cys 282, with the cytokine activities seeming to require both this domain and Cys288. The assumption was made, therefore, that the CD23 domain necessary for cytokine activities is over-lapping but distinct from the IgE binding domain (Mossalayi, *et al.*, 1990).

1.8.5: Ligands for CD23 other than IgE

In addition to IgE binding, CD23 and sCD23 have been implicated in a number of other activities (1.8.4). There are several reported receptors for CD23, including: CD21, CD11b-CD18, CD11c-CD18 (Lecoanet-Henchoz, *et al.*, 1995) and the recently characterised αvβ3 (Hermann, *et al.*, 1999) and αvβ5 (Matheson, *et al.*, 2001) vitronectin receptors. The latter receptor was first suggested to exist in 1997 as a receptor in the pre-B leukaemic cell line SMS-SB (White, *et al.*, 1997).

CD21

Human CD21 is 145kDa membrane glycoprotein expressed on various cell populations including B cells, T cells and follicular dendritic cells (FDCs). CD21 has been identified as a receptor for the gp350/220 envelope protein of EBV (Tanner, *et al.*, 1987) and interferon-α (Declayre, *et al.*, 1991), and is also known as the complement receptor-2 (CR-2) (Weis, *et al.*, 1984). CD21 is present on the membrane of B cells within a molecular complex, in association with CD19, Leu 13 and TAPA-1 (Bradbury, *et al.*, 1992). Structurally, CD21 is composed of an extracellular domain, of approximately 16 short consensus repeats (SCRs) of 60 to

75 amino acids, followed by a transmembrane domain and an intracytoplasmic domain consisting of 34 amino acids (Weis, *et al.*, 1988). Aubry and colleagues in 1992 revealed that CD21 was a ligand for CD23 by the binding of CD23 liposomes to cells transfected with recombinant CD21 and, subsequently, in 1994 determined the sites of CD23 interaction on CD21 as the SCRs 5 to 8 (Aubry, *et al.*, 1994; Aubry, *et al.*, 1992). This was accomplished by the use of CD21 mutants bearing SCR deletions and the binding was found to involve a lectin interaction, as tunicamycin treatment inhibited CD21/CD23 binding in this region. Tunicamycin inhibits N-linked glycosylation of proteins during their biosynthesis (Sarfati, *et al.*, 1992). Short consensus repeats 1-2 were also found to be involved in CD21 to CD23 binding, but via a protein-protein interaction (Aubry, *et al.*, 1994).

CD11b-CD18/CD11c-CD18: β2 integrins

CD11b (17kDa) and CD11c (15kDa) represent the α chains of β2 integrin adhesion molecules which are known to participate in many cell-cell and cell-matrix interactions. These α chains exist as heterodimers with the common β subunit known as CD18, to form the CD11b-CD18 (Mac-1) and CD11c-CD18 (p150, 95) glycoprotein receptors (Corbi, *et al.*, 1988; Kurzinger & Springer, 1982).

Lecoanet-Henchoz and colleagues proved that CD23 bound to CD11b and CD11c on human monocytes using CD23 containing liposomes bound to Cos 7 cells transfected with CD11b-CD18 and CD11c-CD18 (Lecoanet-Henchoz, *et al.*, 1995). They suggested that the interaction involved both lectin and protein-protein interactions, as with CD21, as IgE binding to the lectin domain of CD23 inhibits the binding of monocytes. CD23 binding to monocytes via CD11b and CD11c leads to a marked increase in nitric oxide levels and the release of pro-inflammatory cytokines such as IL-1α and β, IL-6, IFN-γ and TNF-α (Lecoanet-Henchoz, *et al.*, 1995).

αvβ3

The αvβ3 integrin is an ubiquitous receptor that interacts with several ligands, such as vitronectin (Vn), fibronectin (Fn), osteopontin and metalloproteinase MMP-2 (Felding-Habermann & Cheresh, 1993). αvβ3 plays an important role in tumour cell invasion, angiogenesis and in the phagocytosis of apoptotic cells (Gladson & Cheresh, 1994), and is usually found physically and functionally associated with a member of the multispan transmembrane receptor family, CD47. Hermann and colleagues concluded that αvβ3 and its associated CD47 molecule may function as a receptor for sCD23 to mediate pro-inflammatory activity and, thus, may be involved in inflammatory processes (Hermann, *et al.*, 1999).

αvβ5

αvβ5 has recently been described in this laboratory as a further receptor for CD23. The presence of a receptor other than CD21 or the β2 integrins CD11b and CD11c was originally noted on the human pre-B-like leukaemic cell line SMS-SB (White, *et al.*, 1997). This receptor was found to be involved in the rescue of SMS-SB cells from apoptosis, possibly by influencing the expression of Bcl-2 (White, *et al.*, 1997). Affinity isolation experiments by Matheson and colleagues (Matheson, *et al.*, 2001) reported that human pre-B like cell lines, including SMS-SB, bind CD23 via the αvβ5 vitronectin receptor (VnR) complex.

1.8.6: Isoforms of CD23

Several lines of evidence suggest that CD23 has two main functions, playing a role in B cell growth and differentiation (Gordon, *et al.*, 1987; Kolb, *et al.*, 1990) and having a major part in the effector phases of IgE-mediated immunity and allergy

(Kehry & Yamashita, 1989; Luo, *et al.*, 1991). The existence of two CD23 isoforms was proposed and established by Yokota *et al* (Yokota, *et al.*, 1988), with the difference between the isoforms being solely in 6 or 7 amino-acids in the N-terminal cytoplasmic region (see Figure 1.4). The isoforms, termed CD23a and CD23b, are generated using different transcription initiation sites and differential RNA splicing of transcripts of the single CD23 gene (see Figure 1.5), located on chromosome 19 (Suter, *et al.*, 1987; Wendel-Hansen, *et al.*, 1990). The human CD23 gene (EMBL accession number: M30447) has been specifically mapped to chromosome 19p13.3, spanning approximately 13kb and consisting of 11 exons (Ludin, *et al.*, 1987). The promoter regions of each CD23 isoform will be studied in detail in chapter 4 of this thesis and they have been shown to possess distinct response elements. The promoter region for CD23a shows two IL-4 response elements (IL-4RE), followed by an NFκB site, a glucocorticoid response element (GRE) and a B cell activator protein (BSAP) or Pax-5 binding site. By contrast, the CD23b promoter region comprises two IL-4RE sites surrounding an NFκB site, and followed by two activated protein 1 (AP-1) binding sites. CD23a is found constitutively and cell type-specifically in B cells, whereas CD23b is expressed on several cell types of haematopoietic lineage following activation with various stimulants (IL-4, IL-13, anti-CD40, anti-μ chain, PMA). The presence of both cell type-specific and stimulant-specific expression of the two CD23 variants suggests that each isoform has a different cellular function, despite having identical extracellular domains.

Three species of FcεRII mRNA have been identified which differ only within their 5' regions. B cells are known to express all three types, with other cells such as monocytes and T cells having only one. Two mRNA's code for CD23a and their sequences differ in the 5' untranslated region by 4 nucleotides (Kikutani, *et al.*, 1986), which is thought to be due to differential splicing. The third mRNA codes for CD23b and is completely different from the other two in the 5' upstream region, in a position that corresponds to the exon 2-3 boundary of FcεRIIa mRNA. Cloning and

sequencing have demonstrated that the difference is explained by a novel exon specific for FcεRIIb located between exons 2 and 3 (see figure 1.5).

The regulated manner of CD23 gene expression suggests that CD23a and CD23b are involved in B cell function and IgE mediated immunity, respectively. Yokota *et al* in 1992 (Yokota, *et al.*, 1992) elucidated a molecular explanation for the functional difference between these two isoforms, and demonstrated that endocytosis was mediated only through CD23a and that phagocytosis is mediated through CD23b. As CD23a and CD23b differ only in 6 or 7 amino acid residues at their N-terminal cytoplasmic domain, the suggestion is that these regions must contain critical sequences to account for these functional differences. It is thought that it is the aromatic amino-acids in the cytoplasmic region that are required for the endocytosis of various receptors. This is exemplified by the fact that the FcεRIIa contains a tyrosine residue at position 6, whereas FcεRIIb has a serine residue at the equivalent position (Yokota, *et al.*, 1992). In a similar way, the two amino-acids asparagine and proline at positions 2 and 3 of FcεRIIb are essential for phagocytosis. It is notable that mutations in these important positions have an effect on whether endocytosis or phagocytosis of each individual receptor occurs, in fact mutant FcεRIIb which contains both amino acid residues required for endocytosis and phagocytosis can mediate both activities (Yokota, *et al.*, 1992).

1.8.7: Epstein-Barr virus (EBV) regulation of CD23 expression

Epstein-Barr virus (EBV)-transformed B cells show increased levels of cell surface CD23 expression. EBV is associated with a number of human B cell malignancies, including Burkitt's lymphoma, post-transplant lymphoma and central nervous system (CNS) lymphoma in AIDS. A proportion of Hodgkin's lymphoma is also EBV associated (Filipovich, *et al.*, 1990; Herbst, *et al.*, 1991). EBV infection of primary

B cells leads to continually proliferating lymphoblastoid cell lines, and the changes in B cell growth are likely to play a significant role in lymphomagenesis.

The first genes expressed during EBV infection are EBNA-LP and EBNA-2 (Alfieri, *et al.*, 1991; Allday, *et al.*, 1989). Initially the latency W promoter (Wp) is used and followed by the latency C promoter (Cp), which leads to the expression of EBNA-3A, -3B, -3C and EBNA-1 (Bodescot, *et al.*, 1987). In addition to its role in the regulation of EBV latency gene expression, EBNA-2 also alters the expression of cellular genes. CD23 is upregulated by EBNA-2, as is CD21 (Calender, *et al.*, 1987; Cordier, *et al.*, 1990), with EBV transformed cells highly expressing CD23 being immortal (Azim & Crawford, 1988). This transformed cell immortality is suggested to be related to the anti-apoptotic properties ascribed to CD23 which are discussed by Liu *et al* (Liu, *et al.*, 1991). EBV transformed B cells express both CD23 isoforms (a and b) (Cordier, *et al.*, 1990). Ling and co-workers demonstrated that EBNA-2 upregulation of the EBV latency promoter Cp and the cellular CD23 promoter both utilise a common targeting intermediate known as CBF-1 (Ling, *et al.*, 1994).

1.8.8: CD23 and IgE production

As the low affinity receptor for IgE, CD23 has been implicated in IgE regulation after the demonstration that anti-CD23 specific antibodies block the spontaneous release of IgE by peripheral blood lymphocytes (PBL's) (Sarfati, *et al.*, 1988), as well as IgE production by IL-4-treated normal cells. The cytokines IL-4 and IL-13, which induce CD23 expression, have also been found to induce IgE production by normal B cells through IgE isotype switching (Punnonen, *et al.*, 1993). The evidence for this is that IL-4-induced IgE synthesis has been found to be blocked by interferons (IFN) γ, α and prostaglandin E2, which also inhibit the induction of CD23 by IL-4 on B cells. Bonnefoy *et al* in 1990 demonstrated that this inhibition

is restricted to certain CD23 epitopes involved in IgE binding, suggesting that anti-CD23 antibodies bind surface CD23 and interfere with T and B cell interaction, which is necessary for IgE production (Bonnefoy, *et al.*, 1990). Further evidence for this model is that IgE production is increased on engagement of CD21 by recombinant soluble CD23 or anti-CD21 antibody (Aubry, *et al.*, 1992). The above data suggest a central role for CD23 in IgE production, and recent investigations with CD23 knockout mice have further characterised a negative feedback mechanism (see 1.9.10).

1.8.9: CD23 expression in disease

In humans the expression of CD23 is strikingly increased in many atopic (Yanagihara, *et al.*, 1992; Yanagihara, *et al.*, 1990), autoimmune (Bansal, *et al.*, 1992; Delespesse, *et al.*, 1991) and leukaemic (Sarfati, *et al.*, 1988) disorders in the form of both membrane expression on B cells and monocytes and, more noticeably, in terms of soluble CD23 (sCD23) production (Gagro & Rabatic, 1994).

Allergy and atopy

Allergy can be defined as an inappropriate response of the immune system to an otherwise harmless challenge from environmental antigens such as pollen or house dust mite (Robinson, *et al.*, 1997). Several epidemiological studies indicate that the development of atopic disease is linked to circulating levels of IgE (Burrows, *et al.*, 1989; Naclerio, *et al.*, 1993; Sears, *et al.*, 1991) as in healthy individuals the quantity of IgE in the serum is low, but in atopy these levels are greatly increased. IgE binds to mast cells and basophils via the high affinity IgE receptor ($Fc\varepsilon RI$), and subsequent crosslinking of the $Fc\varepsilon RI$-bound IgE molecules by antigen leads to the release of the pharmacologically-active mediators responsible for allergic tissue damage (Mudde, *et al.*, 1990). Estimates of the numbers of people suffering from allergic disorders in

the western world range from 5-15%, and are on the increase. Reasons for this are unclear, although studies are currently being conducted on environmental pollution, housing patterns and lifestyle.

Autoimmune diseases - Rheumatoid arthritis

Increased levels of CD23 have been reported in various chronic inflammatory diseases including systemic lupus erythematosus (SLE) (Bansal, *et al.*, 1992), inflammatory bowel disease (Kaiserlian, *et al.*, 1993), glomerulonephritis (Yano, *et al.*, 1992) and rheumatoid arthritis (Hellen, *et al.*, 1991). Rheumatoid arthritis (RA) is a long term inflammatory disease of the joints, resulting in severe swelling over the affected joints and extreme pain. Pro-inflammatory cytokines are known to be particularly important in RA, and a central role for TNF-α and IL-1β in the destruction of arthritic joints has been postulated (Elliot, *et al.*, 1993), with these cytokines also being present in the synovium of RA patients (Brennan, *et al.*, 1989). Neutralisation of TNF-α and IL-1β *in vivo* has led to a marked reduction in disease severity and tissue destruction (Williams, *et al.*, 1992).

Ikizawa and colleagues (Ikizawa, *et al.*, 1993) studied the cellular and molecular mechanisms for the elevated serum sCD23 in RA patients. It was postulated that an increase in $CD5^+$ B cells in these individuals had an important role in the increase of CD23 expression and subsequent release of sCD23 into the serum, although the exact mechanisms by which this process led to increased CD23 was not studied. A new role for CD23 in inflammation and arthritis was presented by Bonnefoy *et al* (Bonnefoy, *et al.*, 1996). CD23 expression is increased in RA but IgE is not implicated, leading to the conclusion that CD23 interacts with other ligands to produce pro-inflammatory mediators. As described in section 1.8.5, CD23 binds to CD11b and CD11c on monocytes (Lecoanet-Henchoz, *et al.*, 1995) and this interaction leads to the production of nitric oxide and pro-inflammatory cytokines,

suggesting CD23 is an inflammatory mediator. Recent evidence also suggests that the binding of sCD23 to $\alpha v\beta 3$ is involved in pro-inflammatory immune processes (Hermann, *et al.*, 1999).

B cell chronic lymphocytic leukaemia (B-CLL)

CD23 is also over-expressed and abnormally regulated in the most frequent adult leukaemic disorder in the western world, B cell chronic lymphocytic leukaemia (B-CLL). The hallmark of B-CLL is the accumulation of mature $CD5^+$ B cells, co-expressing sIgM, sIgD and CD23 antigens, which have escaped programmed cell death and have undergone cell cycle arrest in the G0 phase (Montserrat & Rozman, 1995; O'Brien, *et al.*, 1995). In Western Europe and north America 30% of reported leukaemia cases are B-CLL, and sera from patients has been reported to contain 3- to 500-fold more sCD23 than control sera (Sarfati, *et al.*, 1988). sCD23 levels have been shown to reliably reflect disease activity (Reinisch, *et al.*, 1994) and significantly correlate with tumour load (Beguin, *et al.*, 1993) and prognostic evaluation (Sarfati, *et al.*, 1996). The latter study showed that patients with serum sCD23 concentrations above the median value of patients in a studied group (574U/ml) had significantly shorter survival times. Recently, the effect of STAT-6 on CD23 expression in B-CLL cells has been studied (Kneitz, *et al.*, 2000) in order to investigate the possibility of constitutive STAT-6 activation. This was considered a possibility as both CD23a and CD23b isoform promoters contain STAT-6 binding sites and can be upregulated by IL-4 (Kohler & Rieber, 1993; Suter, *et al.*, 1989). However, experiments revealed no difference between the STAT-6 concentration of non-malignant B lymphocytes and B-CLL cells. This leads to the speculation that the dysregulation of, hitherto, unknown mechanisms may contribute to the generation of B-CLL. CD23a and CD23b promoter STAT-6 binding sites are studied and discussed in Chapter 4 of this thesis.

1.8.10: Studies with CD23 knockout mice

Gene knockout and transgenic technology has been attempted by several groups to determine the function of CD23 in relation to IgE synthesis, however the phenotype and results from these animals has been controversial. Yu *et al* developed CD23 knockout mice (Yu, *et al.*, 1994) and reported that immunisation of these animals with thymus dependent antigen resulted in an increased and sustained IgE level. Two other groups, however, also developed CD23 knockout mice but were unable to support these findings (Fujiwara, *et al.*, 1994; Stief, *et al.*, 1994), with the $CD23^{-/-}$ mice having similar IgE responses compared to those of littermate controls. Heymen *et al* have reported that IgE and antigen complexes could augment immune responses by interacting with CD23 (Heyman, *et al.*, 1993), and that this augmentation was not present in $CD23^{-/-}$ mice. Thus, there was considerable proof that CD23 acted as a negative regulator for IgE production.

A transgenic model which over-expressed CD23 by utilising the Thy1.1 promoter was developed by Texido and colleagues (Texido, *et al.*, 1994). These mice exhibited about 50% suppression of the IgE response following alum/antigen and anti-IgD injections and *Nippostrongylus brasiliensis* (Nb) infection. Expression of the transgene in this case was mainly in T lymphocytes, although back-crossing into the $CD23^{-/-}$ phenotype did demonstrate some expression on B cells. The dramatic suppression in IgE responses in these models lends further support to the concept that CD23 is a regulator of IgE levels. A recent paper (Haczku, *et al.*, 2000) has further determined the role of CD23 in IgE responses, by concluding that CD23 exhibits a negative regulatory effect on allergic sensitisation and airway hyper-responsiveness. Haczku and colleagues demonstrated the inhibitory role of CD23 by comparing the *in vivo* effects of gene deficiency and over-expression in transgenic mice with those of anti-CD23 treatment. The CD23 knockout mice showed increased responses to ovalbumin sensitisation and challenge, while the over-

expression of CD23 led to an inverse correlation with these changes. In addition, anti-CD23 treatment induced negative regulatory effects on both IgE and IgG_1, although the authors were unclear as to whether this was due to the activation of target cells or secondary to the blocking of CD23 binding to other effector molecules. This could also be due to anti-CD23 binding to IgE promoting sCD23 production.

Transgenic and knockout mouse models have provided new insight into understanding the immunological functions of CD23, although there are a number of limitations to these models. The clinical relevance of observations viewed in mice is difficult to extrapolate to humans as there are marked differences in the CD23 immunobiology between the two species. Firstly, at a molecular level, there is only 57% amino-acid sequence homology between human and mouse CD23 and, furthermore, the murine CD23 is naturally truncated at its carboxy terminus - omitting the RGD motif (Delespesse, *et al.*, 1991). In humans most of the regulatory effects of sCD23 are ascribed to its IgE binding capacity, whereas mouse sCD23 does not retain the same extent of IgE binding (Bartlett, *et al.*, 1995). In addition, the existence of isoforms of murine CD23 is controversial (Conrad, *et al.*, 1993; Richards, *et al.*, 1991) and the supposed single murine isoform (likened to hCD23a) is found expressed on mature B cells, T cells and FDCs. This is in direct contrast to the existence of the two human CD23 isoforms and constitutive CD23a expression only on B cells, with CD23b expression requiring activation on other haematopoietic lineages (see 1.9.6). Whether some effects ascribed to CD23 are unique to the human situation remains to be resolved but, as it would provoke obvious ethical problems to perform *in vivo* experiments on humans, murine models remain the most practical experimental tool. Models more relevant to humans would clearly be a great advantage and, on this note, the work of Mayer *et al* is interesting. Here, human-peripheral blood lymphocyte-reconstituted SCID (hu-PBL-SCID) mice were

used to show the inhibition of CD23 processing correlates with the inhibition of IL-4 stimulated IgE production (Mayer, *et al.*, 1999).

Altogether, a large body of evidence suggests that, in humans, CD23 plays a regulatory role in IgE regulation with antagonistic effects. Crosslinking of CD23 at the cell surface with IgE delivers a negative feedback for IgE production and inhibits the release of sCD23, whereas sCD23 fragments larger than 25kDa promote continuing IgE production. This positive effect upon IgE synthesis is thought to be mediated by at least two mechanisms, including sCD23 directly stimulating IgE production through CD21 triggering and the ability of sCD23 to trap IgE preventing negative feedback through membrane-bound CD23. Allergic disease, therefore, is thought to be due to dysregulation of the CD23/IgE feedback mechanisms - perhaps due to inappropriate cleavage of CD23 from cell membranes and subsequent increases in sCD23-mediated increases in IgE expression. Prime candidates for causing CD23 cleavage are a wide range of allergens that have enzymatic activity (Stewart & Thompson, 1996). The 25kDa Der p 1 cysteine protease is one of the most studied allergenic enzymes (Robinson, *et al.*, 1997; Thomas, 1993), found in the faeces of the house dust-mite *Dermatophagoides pteronyssinus*. Der p 1 is considered to be the most immunodominant allergen involved in the expression of IgE-mediated dust-mite hypersensitivity, and has been shown to cleave CD23 (and CD25) from cultured human B cells (Schulz, *et al.*, 1995) and also *in vivo* (Shakib, *et al.*, 1998).

Therapies for IgE dysregulation and atopy

Anti-IgE therapy is currently being tested in clinical trials and is successful in removing IgE from the body for at least three months, presumably via clearance of immune complexes by the reticuloendothelial system. (Heusser & Jardieu, 1997). However, repeat injections are necessary, because IgE synthesis levels are not

affected, and this can lead to serum sickness. An alternative therapy would be to develop protocols to elevate membrane CD23 levels prior to B cell activation (Payet, *et al.*, 1999). This would be a great benefit, providing techniques to carry out this task could be found that did not involve corresponding increases in sCD23 - such as the use of metalloprotease inhibitors (Christie, *et al.*, 1997). Recent clinical, epidemiological and serological data have indicated that specific immunotherapy against dust mite allergens in young children may prevent the development of new allergic reactions (Des Roches, *et al.*, 1997). Therefore targeting the enzyme active site of Der P 1, perhaps with a cysteine protease inhibitor, may provide an effective way of controlling IgE-mediated hypersensitivity to this allergen and allergic asthma.

1.9: Aims of research

The expression of CD23 has important implications in B cell development and IgE regulation in both normal human B cells and those from atopic or leukaemic individuals. The CD23a isoform is found constitutively expressed on B cells, whereas CD23b can be induced on a number of haematopoietic lineages. As CD23 has such far ranging clinical implications it was considered of major importance to study the expression of both these CD23 isoforms, on cells in both the naive and activated state.

Firstly in this research, cell lines and primary tonsillar B cells will be phenotypically analysed, and their CD23 expression both prior to and following activation will be noted. Immunoprecipitation studies will attempt to follow the activation of STAT-6, which is thought to be a major contributor in CD23 expression, and mobility shift assays will confirm these studies. In addition, mobility shift assays will also attempt to confirm NFκB involvement in the CD23 activation pathway. Secondly, the promoter regions of each CD23 isoform will be analysed in depth by reporter vector

construct studies. Full length and precisely truncated promoter region PCR products will be inserted into the pSEAP reporter vector, and reporter region activation quantified using SEAP (SEcreted Alkaline Phosphatase) assays. These experiments will include the activation of each vector construct with various potential stimuli and mitogens. Lastly, analysis of the CD23a and b promoter regions will be attempted at the level of protein-DNA interactions, using the ligation mediated PCR (LM-PCR) technique and *in vivo* footprinting analysis.

Together, this research will attempt to determine if the two isoforms of CD23 are subject to differential regulation and expression and, if so, what implications this could have on the role of CD23 both in B cell development and in allergic and neoplastic disorders.

Figure 1.1: Schematic representation and overview of B cell lymphopoiesis

B cell development occurs in two main stages; antigen-independent development, which occurs in the bone marrow (BM) and antigen-dependent, which takes place in the periphery. Antigen-independent B cell lymphopoiesis can be described as a progression between 4 primary cell types including the lymphoid progenitor, pro-, pre- and immature B cells. Different cell surface markers and proteins are expressed throughout differentiation and these are shown on the diagram, as are the specific IgH and IgL genes expressed at each stage. B cell fate with or without the presence of antigen is shown in the periphery phase of the scheme, as well as the progression from mature B cell to memory or plasma cell.

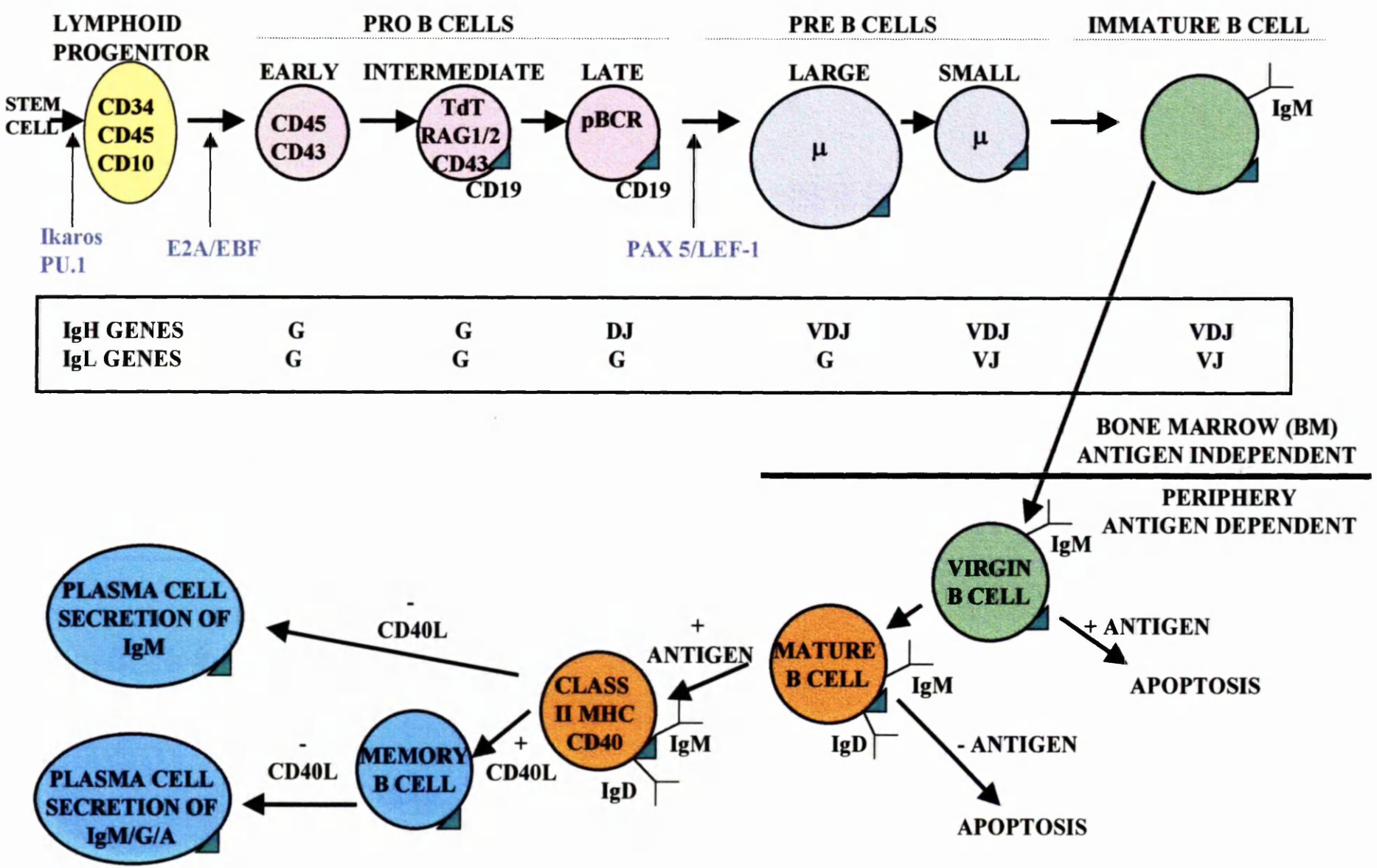

LYMPHOID PROGENITOR
PRO B CELLS
PRE B CELLS
IMMATURE B CELL
STEM CELL
CD34 CD45 CD10
EARLY
CD45 CD43
INTERMEDIATE
TdT RAG1/2 CD43
CD19
LATE
pBCR
CD19
LARGE
μ
SMALL
μ
IgM
Ikaros PU.1
E2A/EBF
PAX 5/LEF-1
IgH GENES G G DJ VDJ VDJ VDJ
IgL GENES G G G G VJ VJ
BONE MARROW (BM) ANTIGEN INDEPENDENT
PERIPHERY ANTIGEN DEPENDENT
VIRGIN B CELL
IgM
+ ANTIGEN
APOPTOSIS
MATURE B CELL
IgM
IgD
- ANTIGEN
APOPTOSIS
+ ANTIGEN
CLASS II MHC CD40
IgM
IgD
- CD40L
PLASMA CELL SECRETION OF IgM
+ CD40L
MEMORY B CELL
- CD40L
PLASMA CELL SECRETION OF IgM/G/A

Figure 1.2: IL-4 receptor signalling

The IL-4 receptor (IL-4R) is composed of two chains, the IL-4Rα chain and the common γ chain (γc). Upon IL-4 binding, members of the JAK family are activated via phosphorylation which, in turn, leads to the activation of IRS-1/2 and STAT-6. IRS-1/2 activation leads to PI-3-K and Ras/MAPK activation, and ultimately leads to either cell growth and differentiation, proliferation or cell survival via the shown pathways. STAT-6 signalling is discussed in detail separately.

IRS-1/2 (Insulin Receptor Substrate); PI-3-K (Phosphoinositide-3-Kinase, composed of p85 and p110 subunits); PKB (Protein Kinase B); PKC (Protein Kinase C)

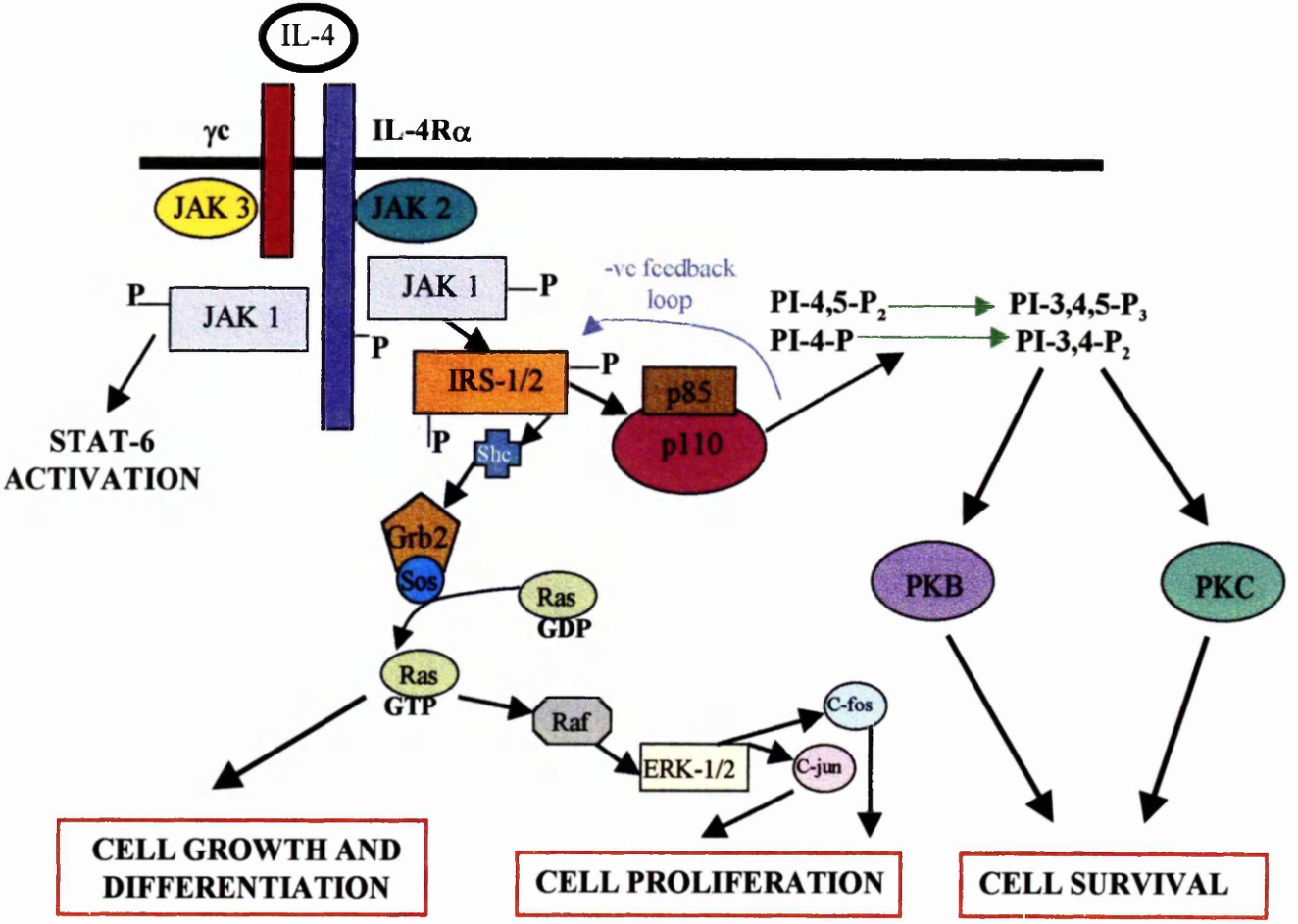

IL-4
γc
IL-4Rα
JAK 3
JAK 2
JAK 1
P
JAK 1
P
P
-ve feedback
loop
PI-4,5-P2
PI-3,4,5-P3
PI-4-P
PI-3,4-P2
IRS-1/2
P
p85
p110
P
Shc
STAT-6
ACTIVATION
Grb2
Sos
Ras
GDP
Ras
GTP
Raf
ERK-1/2
C-fos
C-jun
PKB
PKC
CELL GROWTH AND
DIFFERENTIATION
CELL PROLIFERATION
CELL SURVIVAL

Figure 1.3: IL-4 and IL-13 activation of STAT-6

The receptors for IL-4 and IL-13 share the IL-4Rα chain, with the IL-4 receptor also comprising the γc chain and the IL-13 receptor including the IL-13Rα chain. IL-4 can activate both receptors through utilisation of the IL-4Rα chain and, thus, the IL-4 and IL-13 receptors can be known as the Type I and Type II IL-4 receptors, respectively. Following IL-4 or IL-13 binding, JAK-1 is activated by phosphorylation via the IL-4Rα chain and this, in turn, activates STAT-6. Phosphorylated STAT-6 then dimerises, is translocated to the nucleus and leads to IgE and CD23 expression (in this case). The Type I IL-4 receptor is found on both B and T cells, whereas Type II is expressed on B cells only.

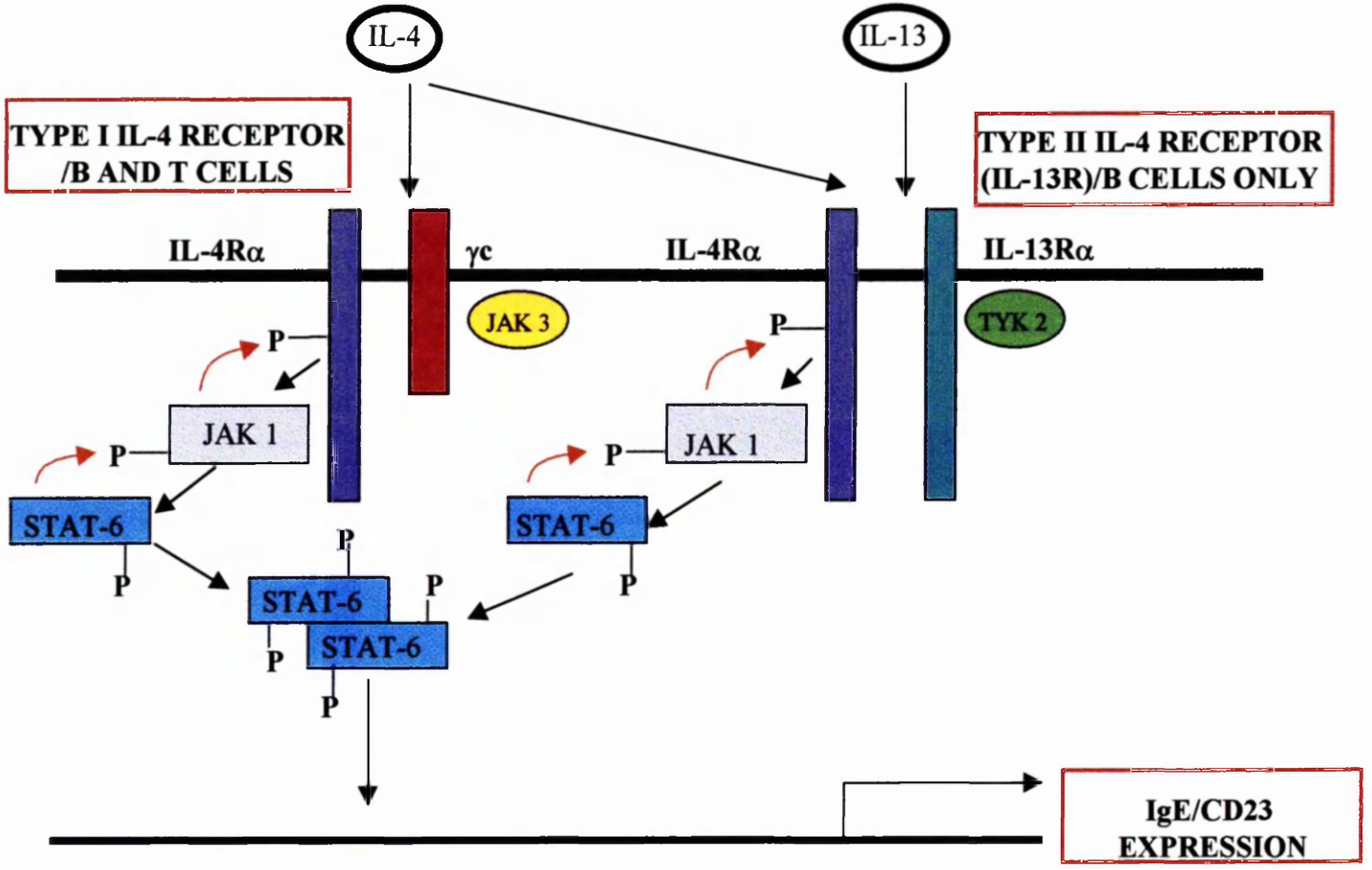

IL-4
IL-13
TYPE I IL-4 RECEPTOR /B AND T CELLS
TYPE II IL-4 RECEPTOR (IL-13R)/B CELLS ONLY
IL-4Rα
γc
IL-4Rα
IL-13Rα
JAK 3
TYK 2
P
JAK 1
P
JAK 1
P
STAT-6
STAT-6
P
P
P
STAT-6
P
STAT-6
P
P
IgE/CD23 EXPRESSION

Figure 1.4: Structure of CD23 isoforms (CD23a and CD23b)

The two isoforms of CD23, termed CD23a and CD23b, differ solely by the 7 (CD23a) or 6 (CD23b) amino acids in their N-terminal cytoplasmic region. The identity of each of these amino acids is shown, with S representing serine, Y representing tyrosine, Q representing glutamine, G representing glycine, E representing glutamic acid, M representing methionine, P representing proline and N representing asparagine. The entire cytoplasmic domain is representative of amino acids 1-23.

The hydrophobic transmembrane domain of CD23 is in the form of a helix and comprises of amino acids 23-44. The remainder of the protein is extracellular. The stalk region of the CD23 monomer (amino acids 82-157) consists of a leucine zipper domain and multiple heptad repeats. These imply that CD23 exists as a multimer on the cell surface, and evidence suggests that this is a trimer. The cleavage points for different sized soluble CD23 (sCD23) fragments are located within the leucine zipper domain and are indicated on the diagram. The lectin domain of CD23 consists of amino acids 163 to 321, and contains the site for IgE binding. Disulphide bonds are represented by S-S.

This figure was adapted from Marolewski *et al,* (1998)

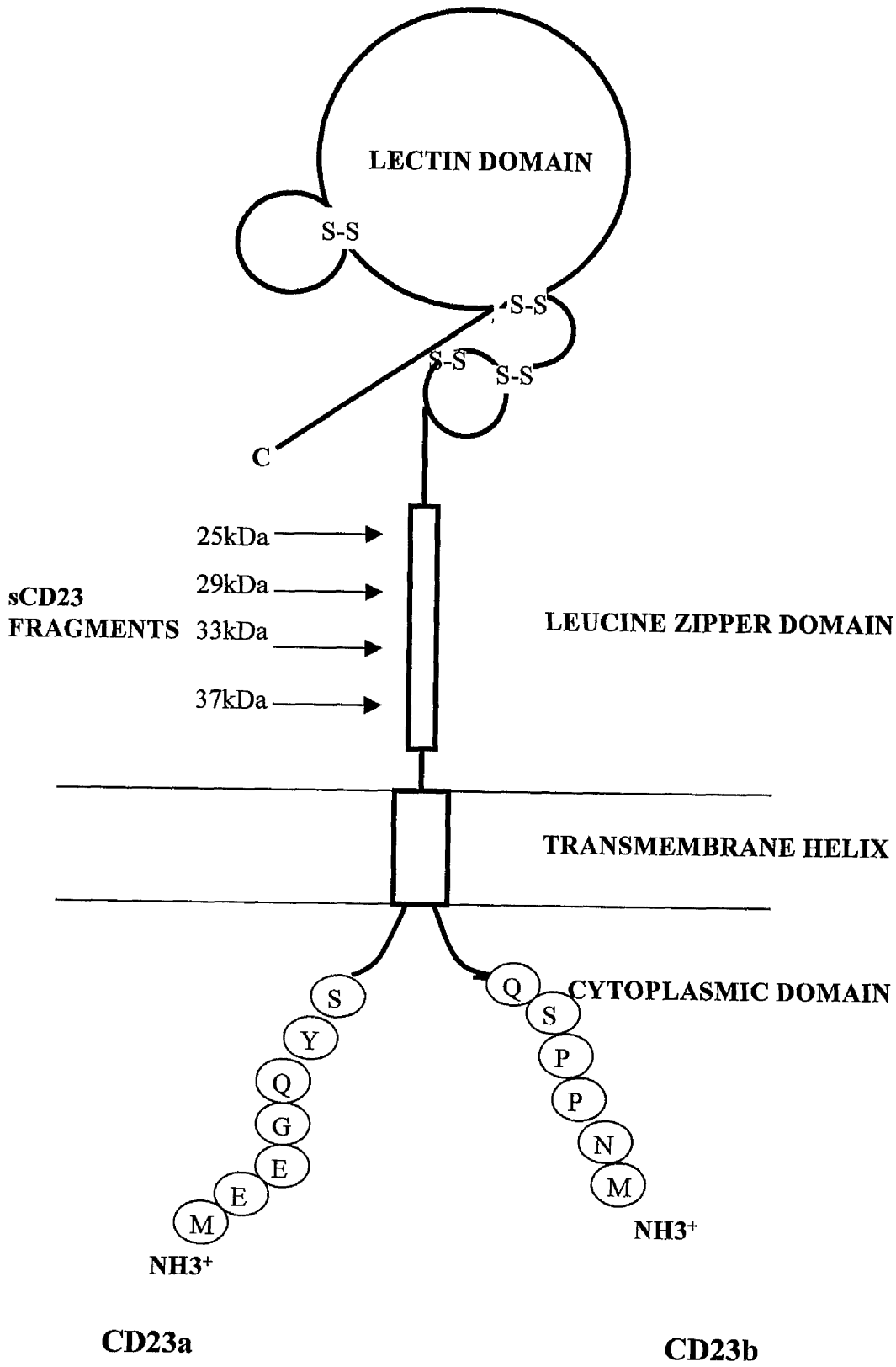
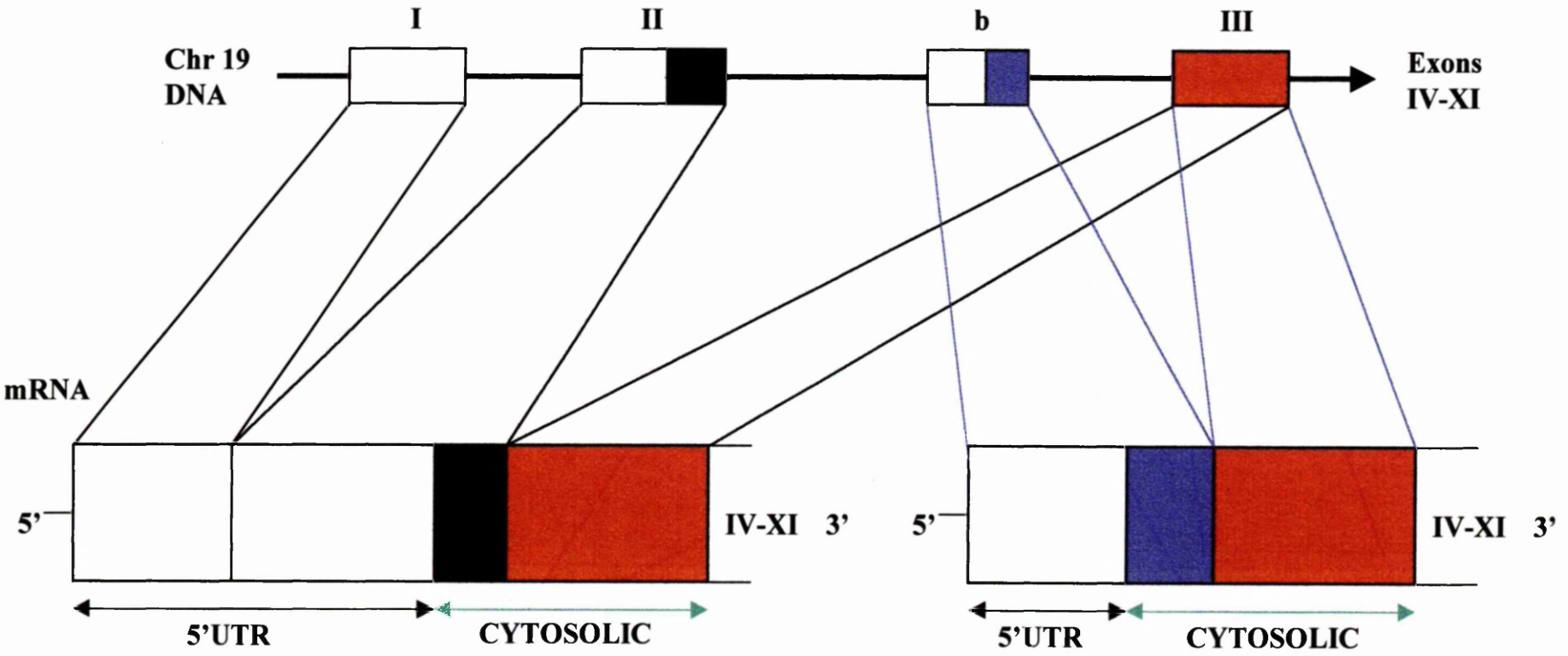

LECTIN DOMAIN
S-S
S-S
S-S
S-S
C
25kDa
29kDa
33kDa
37kDa
sCD23
FRAGMENTS
LEUCINE ZIPPER DOMAIN
TRANSMEMBRANE HELIX
CYTOPLASMIC DOMAIN
S
Y
Q
G
E
E
M
NH3+
Q
S
P
P
N
M
NH3+
CD23a
CD23b

Figure 1.5: Origins of CD23a and CD23b

The two isoforms of CD23 are generated using different transcription start sites and differential RNA splicing of the single CD23 gene, located on chromosome 19. CD23a mRNA comprises of exons I-XI, while CD23b mRNA omits exons I-II but includes the unique exon b. The 5' untranslated (UTR) region of each isoform is indicated by the white boxes, exon III is represented by red and the unique translated area for the CD23a or CD23b isoform is shown by the black and blue boxes, respectively.


I
II
b
III
Chr 19
DNA
Exons
IV-XI
mRNA
5'
IV-XI 3'
5'
IV-XI 3'
5'UTR
CYTOSOLIC
5'UTR
CYTOSOLIC

CHAPTER 2

MATERIALS AND METHODS

2.1: MATERIALS

2.1.1: GENERAL CHEMICALS AND MATERIALS

All general chemicals and reagents, unless otherwise stated below, were purchased from Sigma-Aldrich or BDH Chemicals, both Poole, Dorset, UK. All reagents were of 'AnalaR' grade.

CHEMICAL/REAGENT	SUPPLIER
γ-[^{32}P] ATP (3000Ci/mmol) [^{14}C] Chloramphenicol (50mCi/ mmol) Rainbow protein molecular weight markers. Hybond ^{TM} ECL nitrocellulose membrane	Amersham Life Science Ltd. Buckinghamshire, UK.
Bio-Rad Protein Assay 40% Acrylamide/Bis solution, 29:1	Bio-Rad Laboratories Ltd. Hertfordshire, UK.
Agarose MP (multi-purpose) DNA molecular weight marker X (0.07-12.2 kbp) DNA molecular weight marker XIV (100bp ladder) dNTP's (PCR grade)	Boehringer Mannheim Ltd. Sussex, UK.
Sheep red blood cells	Diagnostics Scotland. Lanarkshire, UK.
Long Ranger ^{TM} gel solution	Flowgen. Staffordshire, UK.

TRIzol ^{R} Reagent	Gibco BRL, Life Technologies Ltd. Paisley, UK.
Bacto-agar Bacto-tryptone Yeast Extract	Oxoid Ltd. Hampshire, UK.
Vent R^{R} DNA polymerase	New England BioLabs inc.
Restriction endonucleases and buffers Taq DNA polymerase T4 DNA ligase T4 Polynucleotide kinase	Promega UK Ltd. Southampton, UK.
RNase OUT ^{TM}	VH BIO Ltd. Newcastle, UK.

2.1.2: KITS

KIT	SUPPLIER
ECL Western Blotting detection system	Amersham Life Sciences Ltd. Buckinghamshire, UK.
Prime-a-Gene ^{R} Labelling System	Promega UK Ltd. Southampton, UK.
Great EscAPe SEAP Reporter System 2	Clontech Laboratories UK Ltd. Hampshire, UK.
Qiagen Plasmid Maxi Purification kit Qiagen QIAquick Gel Extraction kit	Qiagen Ltd. West Sussex, UK.

2.1.3: CELL AND TISSUE CULTURE REAGENTS AND MATERIALS

REAGENT/MATERIAL	SUPPLIER
Disposable plastic graduated pipettes	Bibby Sterilin Ltd. Staffordshire, UK.
Tissue culture Flasks 50ml centrifuge tubes 6 well plates	Corning Costar. Buckinghamshire, UK.
RPMI-1640 medium Foetal Calf Serum Penicillin/Streptomycin/Glutamine Cryovials	Gibco BRL, Life Technologies Ltd. Paisley, UK.
Haemocytometer	Phillip Harris Scientific. Lanarkshire, UK.
Trypan Blue Histopaque Percoll	Sigma-Aldrich. Dorset, UK.

2.1.4: CYTOKINES AND MITOGENS

CYTOKINE/MITOGEN	SUPPLIER
Recombinant human IL-4 Recombinant human IL-13	R & D Systems Europe, Ltd. Oxon, UK.
Mouse anti-human CD40 (LOB7/6) Isotype IgG1	Serotec. Oxford, UK.
Phorbol 12-Myristoyl 13-acetate (PMA)	Sigma-Aldrich. Dorset, UK.
Rabbit anti-human IgM (μ-chain specific)	Sigma-Immunochemicals. Dorset, UK.

2.1.5: ANTIBODIES

ANTIBODY	SUPPLIER
IL-4 STAT (STAT-6) rabbit polyclonal IgG p-Tyr (phosphotyrosine) mouse monoclonal IgG B Cell, CD47 (BRIC126) Isotype IgG2b	Autogen Bioclear. Wiltshire, UK.
Protein-A, with horseradish peroxidase (HRP) conjugate	Amersham Life Science Ltd. Buckinghamshire, UK.
B Cell, αvβ5/FITC (P1F6) Isotype IgG1	Chemicon International Inc. Harrow, UK.

B Cell, CD19/ R-phycoerythrin (RPE) (HD37) Isotype IgG1 B Cell, CD19/FITC (HD37) Isotype IgG1 IL-2 Receptor, CD25/FITC (ACT-1) Isotype IgG1 B Cell, CD23/FITC (MHM6) Isotype IgG1 B Cell, CD21/FITC (IF8) Isotype IgG1 B Cell, CD11b/FITC (2LPM19c) Isotype IgG1 B Cell, CD11c/FITC (KB90) Isotype IgG1 T Cell, CD3/FITC (UCHT1) Isotype IgG1	DAKO Ltd. Cambridgeshire, UK.
Anti-Rabbit IgG, with HRP conjugate Anti-Mouse IgG, with HRP conjugate Anti-human IgM (μ-chain specific)/(FITC)	Scottish Antibody Production Unit (SAPU). Lanarkshire, UK.

2.1.6: PLASMIDS AND TRANSFECTION REAGENTS

PLASMID/REAGENT	SUPPLIER
Gene Pulser Electroporation Cuvettes	Bio-Rad Laboratories Ltd. Hertfordshire, UK.
pSEAP Control vector pSEAP Basic vector pSEAP Enhancer vector	Clontech Laboratories UK Ltd. Hampshire, UK.
pLW2 Chloramphenicol acetyl -transferase reporter vector.	Gift from Professor B Ozanne, CRC Beatson Laboratories, Glasgow, UK.

2.1.7: CELL LINES

CELL LINE	SUPPLIER
EDR Mature human B lymphoblastoid cell line	Gift from Prof. C. Watts, University of Dundee, UK.
JIJOYE Mature human B lymphoblastoid cell line	Gift from Prof. C. Steel, Dept of Medical Biology, University of St. Andrews, UK.
P3HR1 Mature human Burkitt's Lymphoma B cell line	Gift from Dr J. Wilson, Dept of Genetics, University of Glasgow, UK.
RAMOS Mature human Burkitt's Lymphoma B cell line	Gift from Dr M.M. Harnett, Dept of Immunology, University of Glasgow, UK.
SMS-SB Pre-BII human ALL cell line	Gift from Prof. B. Ozanne, CRC Beatson Laboratories, Glasgow, UK.
NALM-6 Pre-BII human leukaemic cell line	Gift from Prof. R.E. Callard, Institute of Child Health, London, UK.

JURKAT Leukaemic T cell lymphoblast	Laboratory Stocks.

2.1.8: TONSILS

Tonsils were obtained from the paediatric ENT clinic at the Royal Hospital for Sick Children, Yorkhill, Glasgow, courtesy of consultant Mr Sadiq. All parents/guardians gave formal informed consent to the use of tissue, and the work was approved by the North Glasgow Universities NHS Trust Ethics Committee.

2.1.9: OLIGONUCLEOTIDES

All oligonucleotides, apart from NFκB, were synthesised and supplied by MWG-Biotech UK Ltd, Waterside house, Peartree Bridge, Milton Keynes, MK6 3BY. NFκB was supplied by Promega UK Ltd. Southampton, UK.

OLIGUNUCLEOTIDE NAME	OLIGONUCLEOTIDE SEQUENCE
EMSA Studies	
STAT-6	**5'** TTTGACGTGAAT **3'** **3'** AAACTGCACTTA **5'**
NFκB	**5'** AGTTGAGGGGACTTTCCCAGGC **3'** **3'** TCAACTCCCCTGAAAGGGTCCG **5'**

LM-PCR Studies (CD25)	
CD25 Prom (1)	**5'** CAGGTTATAAGCCTTGGGG **3'**
CD25 Prom (2)	**5'** ATTCTCAGGATCCTTCAGTTCGCCG **3'**
CD25 Prom (3)	**5'** GGATCCTTCAGTTCGCCGCATCCTTCTCC **3'**
Common linker	**5'** GCGGTGACCCGGGAGATCTGAATTC **3'** **3'** CTAGACTTAAG **5'**
LM-PCR Studies (CD23)	
CD23a Prom (1)	**5'** GCCTTCCGTGGCTCCCCAGGGC **3'**
CD23a Prom (2)	**5'** GCCCTCTGTGATCGGCCATAGTGGT **3'**
CD23a Prom (3)	**5'** CGGCCATAGTGGTATGATTCAGTGTG **3'**
CD23b Prom (1)	**5'** GTAGGAGGACTCTCTGGTTCTAACG **3'**
CD23b Prom (2)	**5'** CGTTGGCAGAAGCAATGACCCTTAGC **3'**
CD23b Prom (3)	**5'** CCTTAGCTACTCCTTTCACCCAGAAG **3'**
Common linker	**5'** GCGGTGACCCGGGAGATCTGAATT C **3'** **3'** CTAGACTTAAG **5'**
CD23a (Rev)	**5'** CAC TGA CAG CTT CTA TTT TCA TAG ACC **3'**
CD23b (Rev)	**5'** GCT AAA TTC TGC TTG TTC CAA GTT CC **3'**

Reporter vector construct studies (CD23a)	
A1: Full CD23a Promoter	**5'** GGCGGTACCAAACTGTCCATATTTGACC
A2: CD23a Prom/ΔS61	**5'** GGCGGTACCAGCCTATTTGCTCAATCATC
A3: CD23a Prom/ΔS62	**5'** GGCGGTACCTGGTGGGGTGTCTGCTGGTA
A4: CD23a Prom/ΔκB	**5'** GGCGGTACCAGTGGTATGATTCAGTGTGCAGT A**3'**
AR: CD23a Prom/Reverse	**5'** GCA GAT CTT GAC AGC TTC TAT TTT CAT**3'**
Reporter vector construct studies (CD23b)	
B1: Full CD23b Promoter	**5'** GGC GGT ACC ATG GAG GAA GGT CAA TAT TC **3'**
B2: CD23b Prom/ΔS61	**5'** GGC GGT ACC GAA GCG GGG CTT CCC AGT CCC **3'**
B3: CD23b Prom/ΔκB	**5'** GGC GGT ACC TGA ATT TCT AAG AAA GGG **3'**
B4: CD23b Prom/ΔS62	**5'** GGC GGT ACC TGG TGT GAGTAA GGA GGT GA **3'**
B5: CD23b Prom/ΔAP1(1)	**5'** GGC GGT ACC TTT TCT GAT TCA ACA CCC TC **3'**
B6: CD23b Prom/ΔAP1(2)	**5'** GGC GGT ACC GCA ATA GAG TCA GAG GCC AA **3'**
BR: CD23b Prom/Reverse	**5'** GCA GAT CTG CAC TCA CCC TGG CTT GG **3'**

2.2: METHODS

2.2.1a: CULTURE OF CELL LINES AND PRIMARY TONSILLAR B CELLS

Cell lines and tonsillar B cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated Foetal Calf Serum (FCS), 100μg/ml penicillin/streptomycin and 2mM glutamine. They were incubated at 37°C in a 5% CO_2 humidified incubator, and were sub-cultured every 2-3 days in accordance with experimental requirements and optimal growing concentrations. The cells were manipulated aseptically in a laminar flow hood.

2.2.1b: FREEZING DOWN AND THAWING OF CELL LINES

Frozen stocks of routinely used cell lines were stored and maintained in liquid nitrogen. To freeze, 10^7 logarithmically growing cells were centrifuged, resuspended in 1ml of chilled freezing media (90% (v/v) heat-inactivated FCS/10% (v/v) dimethylsulphoxide (DMSO)) and quickly transferred to cryovials. The cells were cooled at a rate of approximately 1°C per hour, being kept at -70°C for 3-4 days and then transferred to liquid nitrogen. Cells removed from liquid nitrogen were rapidly thawed and immediately washed in 10ml of incomplete medium (no FCS/penicillin, streptomycin, glutamine), in order to remove the DMSO. The cells were then resuspended in 5ml of complete medium and allowed to recover overnight prior to any further manipulations.

2.2.1c: QUANTITATION OF CULTURES AND CELL VIABILITY

Cell concentration and viability was determined using trypan blue staining, and a haemacytometer. Dead cells rapidly take up trypan blue as they have lost membrane integrity, and can therefore be readily distinguished from healthy viable cells. 20μl

of cell suspension was mixed with an equal volume of trypan blue, loaded onto a haemocytometer and counted. The number of live cells per ml of culture was estimated by multiplying the average live count of 4 x 16 square area grids by 2 (accounting for dilution), and then multiplying by 1 x 10^4. The number of dead cells was also determined in this fashion, and the percentage of viable cells calculated.

2.2.2a: PREPARATION OF TONSILLAR B CELLS

As adapted from the method used by Butcher *et al* (1990), freshly excised tonsils were rinsed twice in RPMI-1640 serum-free medium, supplemented with 100µg/ml penicillin/streptomycin and 2mM glutamine. The tonsils were then diced with a scalpel, mashed through a wire mesh, spilled into a 50ml Falcon tube with serum-free medium and the large clumps allowed to settle. The cell suspension was then transferred to a fresh tube, washed twice with serum-free medium and the cells pelleted at 350 x g for 5 mins at RT. Cells were resuspended in 20ml of medium, and 10ml aliquots layered on to two 10ml cushions of Histopaque. The tubes were then centrifuged at 350 x g for 15 min at RT, with the brake off, mononuclear cells harvested from the interface, pooled (if from the same donor), diluted into 50ml of medium and washed twice - in serum-free medium. Cells were resuspended in 10ml of medium, prior to the addition of 1ml AET-SRBC (see following protocol) and subsequently pelleted at 90 x g for 3 mins with the brake off. 1ml of FCS was added just above the pellet and the tube incubated on ice for 30 min, followed by gentle rocking and addition of the entire suspension to a 10ml Histopaque cushion. Centrifugation at 350 x g for 20 min, with brake off, was followed by harvesting cells from the interface and 3 wash steps as before. The B cells were then resuspended in 1.5ml of medium and layered carefully on to a 5 step discontinuous percoll gradient (see appendix), which was centrifuged for 30 min at 1400 x g. Resting B cells were harvested from the 1.08/1.09g/ml interface, washed twice in serum-free and complete medium and transferred to culture in complete medium.

2.2.2b: PREPARATION OF AET-SHEEP RED BLOOD CELLS

Sheep red blood cells (SRBC) were pelleted at 350 x g and washed twice with RPMI-1640 serum-free medium. 102mg of Aminoethylisothiouronium Bromide (AET) was then dissolved in 10ml of distilled water, the pH adjusted to 9 with NaOH and the solution filter-sterilised. 4ml of the prepared AET was then added to the washed SRBC, incubated at 37°C for 40 min, washed 5 times in serum-free medium and resuspended in 9ml of serum free medium.

2.2.3: FLOW CYTOMETRIC ANALYSIS OF CELL LINES AND PRIMARY TONSILLAR B CELLS

Cells were harvested from culture, washed twice in ice-cold PBS and resuspended at $1x10^6$ cells per 100µl of PBS in 12 x 75mm FACS tubes. 3µl of each FITC- or PE-labelled antibody was then added as required for experimental conditions, prior to incubation on ice for 1 hr. After incubation, tubes were centrifuged at 350 x g for 5 min and washed twice in ice-cold PBS. Labelled cells were then resuspended in 500µl of PBS and analysed on a Becton Dickinson FACScan flow cytometer.

2.2.4: WESTERN BLOTTING

2.2.4a: PREPARATION OF WHOLE CELL LYSATES

Stimulated or unstimulated cells were isolated from culture, washed twice with serum-free RPMI-1640 medium, re-suspended in ice-cold PBS at $5x10^6$ cells per ml and washed by centrifugation at 350 x g for 5 minutes. Cells were then resuspended in 500µl of RIPA buffer (see appendix) and lysed by pipetting. Cells were further lysed by incubation on ice for 20 min, and the cellular debris removed by

centrifugation at 16, 000 x g for 15 min at 4°C. The supernatant was retained and the protein concentration of each sample determined by Biorad protein assay, followed by storage of aliquoted samples at -70°C.

2.2.4b: PROTEIN CONCENTRATION ASSAY (BIORAD)

BSA standards were diluted to concentrations of 2mg/ml to 0.2mg/ml in dH_2O in descending 0.2mg/ml increments and the Biorad assay buffer diluted 1 in 5 with dH_2O. The solution was then filtered to remove any precipitates, and 1ml was added to each of the required number of labelled eppendorf tubes (i.e. enough for all standards and samples). 20µl of each standard was added to its designated tube, as was each protein sample (diluted 1 in 10 with dH_2O). All tubes were then vortexed, left for a minimum of 5 min at RT and their contents subsequently added to cuvettes for assay. Samples and standards were analysed at A_{595} on a Helios γ spectrophotometer, and the unknown protein concentrations each estimated in accordance with the resulting standard curve.

2.2.4c: PREPARATION OF IMMUNOPRECIPITATES

Cells were isolated from culture, washed twice in incomplete RPMI-1640 medium and resuspended at $1x10^7$ cells/ml in complete medium. They were then stimulated over a specific time-course, as indicated in the appropriate results section, and recovered by centrifugation for 5 min at 350 x g. Cells were subsequently lysed by the addition of 400µl of RIPA buffer (see appendix) and incubated on ice for 20 min, after which cell membranes were disrupted by repeated aspiration through a 21 gauge needle. Cellular debris was pelleted by centrifugation at 16, 000 x g at 4°C for 15 min, and the supernatant transferred into fresh tubes. In accordance with the method described by Harlow and Lane in 1988, the protein solution was then pre-cleared for 2 hr by rotation with 5µl of Protein-G agarose, prior to the addition of

1µg of appropriate antibody and subsequent incubation at 4°C for 4 hr on a rotating table. Samples were finally rotated overnight with 10µl of Protein-G agarose at 4°C, in order to allow the immunoprecipitates to be captured. Immunoprecipitates were then collected by centrifugation at 320 x g for 5 min, washed twice with RIPA buffer and re-suspended in 20µl of protein loading buffer. Each sample was boiled for 3 min to denature, centrifuged gently to pellet the Protein-G agarose and the protein loaded on to an appropriate percentage gel.

2.2.4d: SDS-PAGE ELECTROPHORESIS AND WESTERN BLOTTING

Appropriate percentage acrylamide separating gels were prepared as described by Laemmli (1970) and set with an isopropanol overlay, prior to the addition of a 5% (w/v) acrylamide stacking layer (Gel compositions are shown in the appendix). Samples were added to the gel and electrophoresed at 15mA in running buffer (see appendix) until through the stacking gel. When the samples entered the separating gel, the current was increased to 25mA and the gel run until completion. Following electrophoresis the glass gel plates were removed, the stacking gel cut off and the separating gel transferred to a Scotlab wet blotting apparatus. The protein was then transfered to a nitrocellulose membrane in protein transfer buffer (see appendix) at 100mA for 1 hour.

2.2.4e: IMMUNOBLOTTING

Following western blotting, the nitrocellulose membrane was incubated at 4°C overnight with either 10% (w/v) marvel or 1% (v/v) TWEEN 20 in order to block non-specific antibody binding. The membrane was then rinsed in PBS and incubated with primary antibody, which was prepared at 1:1000 with 0.5% (w/v) marvel/PBS, at RT for 3 hours. Three 10 min wash steps with PBS/0.1% (v/v) TWEEN were then carried out, followed by incubation for 3 hours with the

secondary antibody (prepared as with the primary), and another 3 subsequent wash steps. The membrane was developed using ECL, following closely the manufacturers instructions. Briefly, in the dark room, equal volumes of the two ECL solutions were mixed. This was then poured over the nitrocellulose, left for 1 min and the excess dabbed off with a paper towel. The membrane was then placed in an autoradiographic cassette, covered with a thin polythene sheet and exposed to film for the required amount of time before placing in X-omat developer.

2.2.5: ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

2.2.5a: OLIGONUCLEOTIDE PREPARATION

Complimentary oligonucleotides were annealed by mixing together equimolar amounts, heating to 80°C for 5 min and allowing them to slowly cool down to RT. The annealed oligonucleotide was then radiolabelled at the 5' end by incubating the following reaction mixture at 37°C for 20 min:

2µl of annealed oligonucleotide (1.75pmol/µl)

5µl of dH_2O

1µl of T4 Polynucleotide Kinase 5 x buffer

1µl of T4 Polynucleotide Kinase

1µl of [γ-^{32}P]ATP (3000Ci/mmol at 10mCi/ml)

The reaction was terminated by adding 1µl of 0.5M EDTA and 89µl of TE buffer (see appendix). Storage was at -70°C.

The EMSA protocol was adapted from the method of Schindler *et al*, 1994.

2.2.5b: EMSA

The following reaction mixtures were assembled in microfuge tubes:

NEGATIVE CONTROL

7µl of dH_2O

2µl of 5 x gel shift binding buffer (BB)

0µl of extract (RIPA lysate)

POSITIVE CONTROL

5µl of dH_2O

2µl of 5 x BB (appendix)

2µl of extract (5µg)

COMPETITOR CONTROL

4µl of dH_2O

2µl of 5 x BB

2µl of extract (5µg)

1µl of specific oligo (1.75pmol)

NON-COMPETITOR

4µl of dH_2O

2µl of 5 x BB

2µl of extract (5µg)

1µl non-spec.(1.75pmol)

The contents of each tube were briefly spun down and incubated at RT for 10 min, prior to the addition of 1µl of the previously [^{32}P]-labelled specific oligonucleotide. The mixture was then incubated at RT for a further 20 min, and loaded on to a non-denaturing polyacrylamide gel.

2.2.5c: RUNNING OF NON-DENATURING GELS AND AUTORADIOGRAPHY

Reactions were run on a 16 x 18cm^2 7% (w/v) acrylamide non-denaturing gel that was assembled as follows:

9ml of 40% Acrylamide:Bis Acrylamide (29:1)

2.3ml of 5 x TBE (appendix)

34.3ml of d H_2O

450µl of 10% Ammonium Persulphate

22.5µl of TEMED

The gel was pre-run in 0.5 x TBE buffer for at least 1hr at 150V, prior to the addition of samples. 10µl of sample loading buffer (250mM Tris-HCl pH7.5, 0.2% bromophenol blue, 0.2% xylene cyanol, 40% glycerol) was added to the empty wells, in order to visualise sample progression. The sample was electrophoresed at 150V for 2 and a half hours, dried onto filter paper and visualised by autoradiography.

2.2.6: pSEAP REPORTER VECTOR STUDIES

2:2:6a: POLYMERASE CHAIN REACTION (PCR)

In order to study the importance of specific transcription factor binding sites on both the CD23a and CD23b promoters, a series of synthetic oligonucleotides were designed using the published cDNA sequences of each (EMBL accession number: M30447). These oligonucleotides contained both a Kpn I and Bgl II restriction enzyme site in order to assist in subsequent cloning steps, and are described in the relevant materials and results sections. All PCR reactions were carried out in accordance with the method of Saiki *et al* (1985) in 0.5ml microfuge tubes and performed in a Techne Genius PCR machine. Optimal conditions and components were determined for each primer and gene amplification, including that the PCR annealing temperature was at least 5°C below the Tm of each primer. The reaction mixtures are described below, as is an example of the PCR cycle parameters.

REACTION MIXTURE:

Component	Final concentration
Template DNA	10ng/µl
Upstream Primer	50pmol
Downstream Primer	50pmol
dNTP mix	0.2mM
10 x Taq Reaction Buffer	1x
25mM magnesium	1.25mM
Taq DNA Polymerase	0.025u/µl
dH_20	to 40µl

COMMONLY USED CYCLE PARAMETERS:

95°C for 2 min
{95°C for 1 min
(Tm-5°C) for 1.5 min **X 35 Cycles**
72°C for 1.5 min}
72°C for 7 min

Following the PCR reaction, 2 x loading buffer (Appendix) was added to the samples and they were analysed on a 1% (w/v) agarose gel, purified, digested and cloned into the pSEAP enhancer vector.

2.2.6b: AGAROSE GEL ELECTROPHORESIS AND PURIFICATION OF DNA FROM EXCISED BANDS

A 1% (w/v) agarose gel was prepared by mixing 1g of multi-purpose agarose into a total volume of 100ml 1 x TAE buffer (see appendix), boiling and allowing to cool

to 55°C prior to the addition of 200µl of 0.5mg/ml ethidium bromide. The gel was then poured to 1cm thick and allowed to set at room temperature. PCR reaction products were subsequently loaded onto the prepared gel, separated by electrophoresis at 100V and the desired DNA band excised using a clean, sterile scalpel. The DNA was purified from the agarose by using a Wizard gel extraction kit, in accordance with manufacturers instructions. Briefly, the agarose slice was placed into a 1.5ml microfuge tube and melted by incubation at 70°C, prior to mixing with 1ml of Wizard gel extraction resin. The resin/DNA mix was then introduced to a Wizard minicolumn under vacuum, washed by pulling 2ml of isopropanol over the DNA/resin complex and dried by continuing to draw the vacuum for a further 30 seconds. Any residual isopropanol was removed by centrifugation at 10,000 x g for 2 min, followed by DNA elution with 50µl of water. DNA concentration was then estimated, as below, and the DNA aliquoted and stored at -20°C until required.

2.2.6c: DNA PURITY AND CONCENTRATION

DNA purity and concentration was measured using a Helios γ spectrophotometer. The A_{260}:A_{280} ratio setting allowed purity to be calculated, with a figure of 1.8 to 2.0 indicating an acceptably high level of purity. The concentration of DNA was calculated by, firstly, diluting the DNA sample 1 in 100 with the same buffer it was resuspended in and, secondly, manipulating the A_{260} reading as follows:

$$\frac{\mathbf{A260 \times 100 \times 50}}{\mathbf{1000}} = \mathbf{x\ mg/ml}$$

where 100 is the dilution factor and 50 is the DNA concentration multiplication factor.

2.2.6d: RESTRICTION ENZYME DIGESTS OF VECTORS AND INSERTS

The following digestion reaction mixture was assembled for each restriction enzyme, and incubated at 37°C for 2 hr:

10µl of DNA/vector
2µl of restriction enzyme
4µl of restriction digest buffer
24µl of dH2O
40µl

After the incubation, a phenol-chloroform extraction was performed, followed by a chloroform-only extraction and subsequent precipitation by incubation for 1 hr at -70°C with 20µl of ammonium acetate and 100µl of 100% ethanol. The digest was centrifuged at 12, 000x g for 5 min at 4°C, washed with 70% ethanol and allowed to air dry. This step removed restriction digest buffers. After which, the DNA could be cut with other restriction enzymes, resuspended in TE buffer (appendix) and separated on an agarose gel to verify sizes.

2.2.6e: LIGATION OF VECTORS AND INSERTS

Assuming that:

si = size of insert, sv = size of vector, riv = molar ratio of insert:vector, t = total amount of DNA (vector and insert), i = amount of insert required, v = amount of vector required and;

$$i = \frac{si \times t}{[(sv/riv) + si]} \qquad v = \frac{t \times sv}{sv + (si \times riv)}$$

The ligation reaction was assembled as such:

vμl of vector DNA(100ng/μl)

iμl of insert DNA (100ng/μl)

1μl of T4 DNA ligase

1μl of 10 x ligase buffer

dH_2O to 10μl

The mixture was incubated at 16°C overnight, and then used to transform competent cells.

2.2.6f: TRANSFORMATION OF COMPETENT CELLS

100μl of competent bacterial cells (JM109 or DH5α) were placed into a sterile pre-chilled eppendorf tube, and 1μl of purified control plasmid or 5μl of ligation reaction added. The suspension was gently mixed, without vortexing, prior to incubation on ice for 30 min. The cells were then heat shocked at 42°C for 40 seconds and returned to ice. Incubation with 100μl of SOC medium (see appendix) at 37°C for 1 hr followed, prior to spreading 100μl of the mixture onto an LB/Ampicillin agar plate. The plate was allowed to dry, inverted and incubated at 37°C overnight.

2.2.6g: SMALL SCALE PREPARATION OF PLASMID DNA (MINI-PREP)

A single bacterial colony was picked from an LB/Amp agar plate and grown up in 10ml of LB broth, supplemented with 0.05 mg/ml ampicillin, overnight at 37°C. 1.5ml of the cell suspension was then spun down at 350 x g for 5 min, and the cell pellet resuspended in 100ml of STET mixture (9 volumes of STET [see appendix] and 1 volume of 10mg/ml lysosyme). The preparation was then boiled for 2 min,

prior to centrifugation for 10 min at 12, 000 x g. The supernatant was transferred into a fresh tube, along with 400µl of 0.3M NaOAc and 500µl of isopropanol, and precipitated at -20°C for 1 hr. The precipitate was spun down at 12000 x g for 10 min at 4°C, washed with 70% ethanol, and resuspended in 30µl of TE buffer. 5µl of the prepared DNA was cut out and run on a 1% agarose gel.

2.2.6h: LARGE SCALE PREPARATION OF PLASMID DNA (MAXI-PREP)

After successful analysis of mini-prep DNA, large quantities of "purified" DNA for use in salt-sensitive applications - such as PCR or transfection - were required. This was prepared using a Qiagen Maxi Plasmid Kit in accordance with the manufacturer's instructions, and using the materials and reagents supplied.

Briefly, 200µl bacterial cultures in LB broth, supplemented with 0.05mg/ml ampicillin, were grown overnight and harvested by centrifugation at 6, 000 x g for 15 min at 4°C. The pellet was then resuspended in 10ml of supplied solution P1, with a further addition of 10ml of solution P2 prior to incubation at RT for 5 min. 7.5ml of chilled buffer P3 was then added and the resulting lysate mixed by inversion, incubated on ice for 20 min and centrifuged twice at 20, 000 x g for 30 min at 4°C. The supernatant was then loaded on to a pre-equilibrated Qiagen Maxi column and allowed to flow through under gravity, the DNA remaining in the column matrix. Three wash steps with buffer QC followed and then elution of the plasmid DNA was achieved using buffer QF. Eluate DNA was precipitated out by the addition of 10.5ml of isopropanol, centrifugation at 15, 000 x g for 30min at 4°C, followed by washing twice with 70% ethanol. After air drying the pellet, the DNA was resuspended in 300µl of TE buffer and the DNA concentration determined by spectrophotometry. Aliquoted DNA was stored at -20°C.

2.2.6i: ELECTROPORATION OF CELLS

Cells were harvested from culture by centrifuging at 350 x g for 5 min at RT, washed with serum-free RPMI-1640 medium, resuspended at a concentration of 6.25 x 10^6 cells/ml and chilled on ice for 10 min. Chilled electroporation cuvettes were prepared by adding 40µg of plasmid DNA (in TE buffer) to each, and then 0.8ml of chilled cell suspension. Cuvettes were transferred to a BIO-RAD electroporator, pulsed for 10 seconds at 0.3kV and 960µF and then placed on ice for a further 10 min. Cells were transferred to 4.2ml of complete, pre-warmed RPMI-1640 medium, rested for 1-2 hr prior to stimulation and then cultured for 48 or 72 hr (depending on assay conditions) at 37°C in a 5% CO_2 humidified environment.

2.2.6j: pSEAP REPORTER VECTOR ASSAY

The chemiluminescent SEAP assay (Clontech) was carried out as in the manufacturer's instructions, and using the reagents provided. Briefly, 125µl of cell culture was removed at the required time-point, and cells were pelleted by centrifugation at 12000 x g for 10 seconds. The supernatant was then transferred to a fresh microfuge tube and, if necessary, could be stored at -20°C until ready for use. Sufficient volumes of buffers were allowed to equilibrate to RT, and 1 x dilution buffer was prepared by diluting the supplied 5 x buffer with dH_20. Samples were thawed, 15µl added to each well of a 96 well opaque plate and subsequently diluted with 45µl of the prepared dilution buffer. The resulting mixtures were incubated at 65°C for 30 min to heat-inactivate their intracellular acetylases, allowed to cool to RT and then 60µl of assay buffer was added. The solutions were incubated at RT for 5 min, prior to the addition of 60µl of diluted CSPD substrate (1 in 20 with chemiluminescent enhancer). The plate was then incubated for at least 10 min at RT, and the results acquired on a plate luminometer. SEAP assay results were

normalised using pLW2 co-transfection and subsequent CAT assay, allowing transfection efficiency to be taken into account.

2.2.6k: CHLORAMPHENICOL ACETYL TRANSFERASE (CAT) ASSAY

As described by Ono *et al* in 1991, transfected cells were harvested from culture by centrifugation at 350 x g for 5 min and washed twice with 25ml of PBS. Cells were resuspended in 100µl of 250mM Tris-HCl (pH 7.8), and subjected to 3 freeze/thaw cycles by immersing in dry ice/ethanol and then a 37°C water bath at 5 min intervals. Samples were then centrifuged at 12, 000 x g for 10 min, the supernatant retained in fresh sterile eppendorfs and the cellular acetylases heat-inactivated at 65°C for 10 min. The protein content of each sample was determined at this point, and they were each adjusted to account for concentration and volume. Acetyl co-enzyme A was added to a final concentration of 5mM, followed by the addition of 1ml of [^{14}C]-chloramphenicol (0.025µCi) and incubation at 37°C for 3 hr. The assay was terminated by the addition of 600µl of ethyl acetate, vortexed for 30 sec and centrifuged at 12, 000 x g for 5 min. 520µl of the upper phase was transfered to a fresh tube and lyophilised in a speedivac for 30 min, followed by resuspension in 20µl of ethyl acetate. Each sample was dotted onto a silica thin layer chromatography plate, and separated using the 95% chloroform/5% methanol solvent system for 1 hr. The CAT assay was then visualised by exposing on a phosphorimaging screen overnight.

2.2.7: *IN VIVO* FOOTPRINTING UTILISING LIGATION-MEDIATED POLYMERASE CHAIN REACTION (LM-PCR)

2.2.7a: DNA PREPARATION - MAXAM AND GILBERT METHOD (Maxam and Gilbert, 1980)

10^7 cells were harvested from culture and resuspended in 1ml of 10% (v/v) incomplete RPMI-1640 medium in dH_2O, prior to the addition of 40µl of 0.5M HEPES (pH 7.3). For *in vivo* prepared DNA, 5µl of dimethyl sulphate (DMS) was added, left for 1 min and the reaction stopped by centrifugation for 5 min at 350 x g. The pelleted cells were then washed twice with ice-cold PBS containing 2% (v/v) mercaptoethanol, and lysed by rotating at 50°C overnight using the following lysis buffer:

20mM of Tris-HCl (pH7.5)

10mM of NaCl

10mM of EDTA

100µl of 10% (v/v) SDS

100µl of 10% (v/v) NP40

200µl of 10mg/ml Proteinase K

Following cell lysis, 1.2ml of 5M NaCl was added to the lysate and proteins removed by centrifugation at 5, 000 x g for 40 min. The DNA was then ethanol-precipitated from the lysate by incubation at -20°C for 2 hr, followed by centrifugation at 12, 000 x g for 15 min at 4°C. The DNA pellet was resuspended in 200µl of TE buffer and the DNA concentration determined at A_{260}. 20µl of 98% (v/v) piperidine was then added, prior to the DNA being cleaved by heating to 90°C for 30 min. The piperidine was evaporated off by lyophilising the sample in a

speedivac, prior to resuspension of the DNA with TE buffer to a concentration of 1μg/ml. To prepare naked DNA, cells were lysed prior to DMS treatment.

2.2.7b: LIGATION-MEDIATED PCR OF CD25 AND CD23 PROMOTER REGIONS

All of the recipes for solutions used in this technique are described in the appendix, and the primers utilised are listed in the relevant results section. An overview of the ligation mediated PCR technique is shown on Figure 5.1 (page 157), and was described by Algarte *et al* in 1995. Initially, a first strand synthesis reaction was performed, in order to ensure correct annealing of primer 1 to the DNA. This was done by assembling the following reaction mixture components:

2μg of Maxam and Gilbert technique prepared DNA
5μl of first strand buffer
5 nmol of each dNTP
0.3pmol of primer 1
1 unit of Vent DNA polymerase
sterile dH_2O to 25μl

This rection mixture was then transferred for one cycle of PCR at the following parameters:

95°C for 1 min, (Tm-5°C) for 30 min, 76°C for 10 min.

After which the samples were chilled on ice and ligated overnight at 17° following the introduction of the following solutions:

25µl first strand reaction (from above)

20µl dilution solution

10µl 2 x ligation solution

15µl ligation mix (100pmol unidirectional linker, 4.5u T4 DNA ligase, dH_2O)

After ligation, 9.4µl of precipitation solution was added along with 220µl of 100% ethanol and nucleic acid precipitation was achieved at -20°C for 2 hours. The resulting nucleic acid pellet was resuspended in 70µl of dH_2O and the following reaction mixture assembled and amplified by 18 PCR cycles as follows:

95°C for 1 min, (Tm-5°C) for 2 min, 76°C for 3min

with 5 seconds being added to each successive 76°C step in cycles 2-17, and the 76°C step being run for 10 min in cycle 18. Samples were chilled on ice for 5 min, prior to the addition of 5µl of labelling mix (^{32}P labelled primer prepared as for EMSA assay - 2.2.5a) 1 unit of Vent DNA polymerase and running of the following thermal cycle:

95°C for 3.5 min, (Tm-5°C) for 2 min, 76°C for 10 min, 95°C for 1 min, (Tm-5°C) for 2 min, 76°C for 10 min.

Therefore, two labelling cycles are completed. 300µl of ice-cold stop solution was then added and followed by a 400µl phenol chloroform extraction step. The aqueous phase was then split into 4 aliquots, ethanol precipitated at -20°C for 2 hours and the labelled DNA collected by centrifugation at 12, 000 x g for 15 min at 4°C. Washed DNA samples were then resuspended in 8µl of load dye, heated for two minutes at 75°C and run on a 6% "long ranger" sequencing gel.

2.2.7c: FOOTPRINTING GEL PREPARATION AND RUNNING

A 6% (w/v) DNA sequencing gel solution was prepared using the ingredients listed in the appendix to this thesis. The solution was then de-gassed by vacuum and poured into 40 x 20 x 0.4 cm^3 sequencing gel apparatus, prior to the insertion of a comb in order to form sample wells. Cling film was placed over the plates and the gel was then allowed to set for approximately two hours to allow efficient crosslinking of the acrylamide. Once set, the gel was pre-run for 10 minutes, and the wells washed out to remove any precipitated urea. 4µl of each LM-PCR sample was then placed in a corresponding well on the gel with fine duck-billed tips, and the gel electrophoresed at 55W for approximately 3 hours. It was important to maintain a gel temperature of 40-50°C to allow efficient running.

Following electrophoresis, the gel was placed on blotting paper and dried down for approximately 30 mins. This short drying time was sufficient, as the gel was so thin. The gel was then placed on autoradiographic film and the film developed on an X-omat developer after a 24 hour exposure.

CHAPTER 3

RESULTS

CHARACTERISATION OF CD23 PROTEIN EXPRESSION IN B LYMPHOCYTES AND B CELL LINES.

CHAPTER 3: CHARACTERISATION OF CD23 PROTEIN EXPRESSION IN B LYMPHOCYTES AND B CELL LINES.

3.1: INTRODUCTION

The functional properties of CD23, the low affinity receptor for IgE, have been reviewed and discussed in Chapter 1, as has the general cell biology of both the membrane-bound and soluble forms. Certain signalling mechanisms have also been proposed, including the STAT-6 and NFκB activation pathways. CD23 has been implicated in IgE negative feedback control mechanisms through knockout mouse studies (Haczku, *et al.*, 1997), with the soluble form in particular found to be elevated considerably in atopic and in some forms of leukaemia (Jung, *et al.*, 1995). As a result of CD23 being implicated in so many disorders it is a prime candidate for further study and, later in this thesis, the differential activation of expression of each CD23 isoform (a and b) will be studied in detail through reporter vector constructs and LM-PCR studies. This chapter, however, describes the initial studies that were required for the long term outcome of this research, and helped to determine which experimental techniques and cellular models were to be used to further the study.

It was considered paramount to phenotype and characterise the potential cell lines and primary cells that were to be utilised, and also to define candidate CD23 transcriptional activation pathways. The following pages, therefore, include flow cytometric analysis, immunoprecipitation and western blotting studies of IL-4 and IL-13 stimulated cells and electrophoretic mobility shift assays (EMSAs) carried out using transcription factors suspected to be involved in CD23 activation.

3.2: RESULTS

3.2.1: Phenotypic analysis of cell lines and primary tonsillar B cells

Cell lines and primary tonsillar B cells were phenotyped using flow cytometric analysis for the detection of Cluster of Differentiation (CD) markers expressed on their cell surfaces. Flow cytometry is a means of measuring certain physical and chemical characteristics of cells or particles as they travel in suspension, one by one, past a sensor. A typical flow cytometer is represented on Figure 3.1 and consists of a light source, collection optics, electronics and a computer to translate signals into data. A laser in the cytometer omits coherent light at a specified wavelength and scattered and emitted light is collected by two lenses, one set in front of the light source and the other set at right angles to it. Specific bands of fluorescence can be measured by a series of optics, beam splitters and filters This information was then used to define the cellular origin and maturation status of each cell type. The applications of flow cytometry are numerous and physical characteristics such as cell size, shape, internal cellular complexity, and, in this study, any cell component or function that can be detected by a fluorescent compound can be examined. Typical flow cytometric histograms are shown on Figure 3.2 for the EDR cell line and in relation to the unstained cell population, which is represented by the shaded part of the graph, the cell surface antigens which are expressed by these cells are clearly shifted to the right. Therefore, EDR cells are CD19 positive, CD3 negative, CD23 positive, CD25 negative, CD11b slightly positive, CD11c negative, $\alpha v\beta 5$ negative, CD21 positive and CD47 highly positive.

The phenotyping data for all the other cell lines and primary B cells used in the course of this thesis are shown on Table 3.1., with "+++" indicating high marker expression, "++" showing medium expression and "+" or "+/-" representing low or borderline results. As would be expected, "-" indicates no detectable marker

expression whatsoever. CD19 is the universal marker for lymphocytes of B cell lineage, with CD3 being the equivalent marker for T cells. CD23 is the low affinity receptor for IgE and CD25 is the α chain component of the IL-2 receptor (Benschop & Cambier, 1999; Hewitt, *et al.*, 1997), which can be induced on B cells, but is primarily involved in T-cell growth and development. CD21 is a membrane glycoprotein and cell adhesion molecule which binds CD23, as do CD11b and c, which represent the α chains of β2 integrin adhesion molecules (Lecoanet-Henchoz, *et al.*, 1995). The αvβ5 complex is a CD23 receptor expressed on the pre-B cell line SMS-SB, as demonstrated in this laboratory (Matheson, *et al.*, 2001) as well as on the mature human Burkitt's lymphoma cell line Ramos and on Jurkat T cell lymphoblasts. CD47 is a widely expressed cell marker, is thought to stabilise binding of the αvβ3 (and possibly αvβ5) vitronectin receptors to their ligands (Lindberg, *et al.*, 1993) and is a recently described thrombospondin receptor (Gao, *et al.*, 1996).

Much variation in marker expression was noted between the different cell lines, although a general trend was evident. In particular, the pre-B cells (e.g., SMS-SB) showed little significant expression of several tested markers, with the exception of CD19, αvβ5 and CD47. It was, indeed, the lack of the other CD23 binding molecules on SMS-SB that led to the identification of αvβ5 as a CD23 receptor (Matheson, *et al.*, 2001). The mature B cell line EDR, however, which is EBV transformed, showed high expression of CD19, CD23, CD21 and CD47. This was not entirely unexpected, as EBV transformation has been shown to constitutively switch on CD23 expression in the majority of B cell lines (Kintner & Sugden, 1981) although P3HR1 cells are also EBV transformed, and show little constitutive CD23 expression. Further experiments will determine if these cells can be successfully induced to express CD23 and CD25.

The phenotype of tonsillar B cells is also shown on Table 3.1, and dot plots of these data are provided on Figure 3.3, with CD19, CD21 and CD47 being significantly expressed. Primarily naive, unstimulated B cells are selected for culture during the tonsil cell preparation and purification process. The tabulated data for the tonsillar B cells is representative of the trend observed through many sets of tonsils, as each individual set showed minor variations in overall levels of markers. This is attributed to the fact that tonsils were obtained following excision from several different patients and, subsequently, each set was prone to minor physiological variations. Those patients harbouring some sort of bacterial infection, for example, would invariably have a higher amount of activated B cells in the cell preparation, potentially leading to the higher expression of certain markers upon FACS analysis (e.g., CD23 and CD25).

3.2.2: CD23 cell surface expression can be induced and increased by IL-4

Much of the work in this thesis was dependent on being able to stimulate CD23-negative cells to express the CD23 protein on their cell surface, in order that different stimuli, transcription factors and promoters could be studied. The next step, therefore, was to determine which cell lines would give a significant rise in CD23 expression upon stimulation with IL-4. Analysis was also carried out on primary tonsillar B cells. The technique used in this instance was, again, flow cytometry - which was used to detect CD23 expression on IL-4-treated and untreated cells.

Figure 3.4 shows a FACS histogram of CD23 expression on P3HR1 cells, with a clear increase after a 24 hour stimulation with IL-4. This data, and that for all the other cell lines (as well as tonsillar B cells), is shown in tabulated form on Table 3.2. The pre-B cell line SMS-SB shows no increase in CD23 expression whatsoever, which, as it is an immature cell line, is not a surprise. EDR cells show a high

expression of CD23 prior to stimulation, so no significant increase is observed on stimulation. P3HR1 cells on the other hand, which show little CD23 expression prior to IL-4 treatment but exhibited a great increase after challenge. Tonsillar B cells show a similar activation pattern to the P3HR1 cells, but with an even greater level of stimulated expression of CD23. This is due to the fact that tonsillar B cells are from primary tissue and are, therefore, more sensitive to activation than established and transformed cell lines. It was concluded from these data that future experiments would be carried out using the P3HR1 cell line and, wherever possible, that primary tonsillar B cells would be preferentially used. At the initiation of these experiments primary tonsillar tissue was available only periodically, making these preliminary experiments and temporary substitution necessary. The result for Jurkat T cells is included in order to demonstrate the fact that T cells can also be induced to express CD23, as well as B cells. CD23a is constitutively and cell type specifically expressed on B cells, whereas CD23b can be found on other haematopoietic cells when induced. Therefore the increase in CD23 expression on Jurkat T cells is due to CD23b, but the CD23 rise in B cells can be a result of an increase in expression of the a or b isoform.

3.2.3: Stimulation of P3HR1, SMS-SB and tonsillar B cells with IL-4, IL-13, anti-CD40, anti-μ chains and PMA

Throughout the course of this research, different stimuli will be used to activate the CD23a and CD23b promoters and the following FACS analysis was carried out in order to determine the effect of each of these agents on CD23 and CD25 cell surface marker expression. Primary tonsillar B cells, EBV-transformed P3HR1 mature B cells and SMS-SB pre-B cells were harvested following treatment with various potential mitogens and stimulants. The cells were analysed for both CD23 and CD25 expression and the reagents used were IL-4, IL-13, anti-CD40, anti-IgM (μ chain specific) and PMA (phorbol myristoyl acetate). Although the main content of

this research is carried out on CD23 it was necessary to investigate CD25 stimulation at this stage, as the CD25 promoter was used as a model in the footprinting work discussed in chapter 5.

3.2.3a: Stimulation of CD23 and CD25 on tonsillar B cells

Dot plots for the results for CD23 expression on stimulated primary tonsillar B cells are shown on Figure 3.5. CD23 expression is substantially increased by IL-4 and PMA, increased to a lesser extent by IL-13, but not at all with anti-CD40 and anti-μ. IL-4 and IL-13 are thought to activate CD23 expression through a STAT-6-dependent pathway, utilising the IL-4Rα chain present in both receptors, and PMA is known to activate cells through a PKC (protein kinase C) dependent pathway. Anti-CD40 generally stimulates CD23 expression through activation of NFκB transcription factors (Berberich, *et al.*, 1994) and, as both CD23 promoter regions are known to have these sites, it would be expected that anti-CD40 would be a more potent CD23 inducer than this result implies. An explanation for this lack of induction could be that the particular anti-CD40 monoclonal antibody used did not recognise the specific epitope required for proper binding and activation. Burlinson *et al* (Burlinson, *et al.*, 1996) touched on this subject when they compared their results with those of Valle *et al* (Valle, *et al.*, 1989) and concluded that anti-CD40 antibodies evoked different responses on tonsillar B cells depending on which specific epitope they recognised and bound. Anti-μ chain antibody is known to be a general B cell activator. It is a surprise, then, that CD23 expression does not appear to be stimulated following the addition of anti-μ. It was proposed that perhaps the cells were so highly stimulated that CD23 was being cleaved from the cell surface and was, therefore, undetectable through FACS analysis. This interpretation was tested by CD23 ELISA of conditioned cell culture medium from both control and anti-μ stimulated cells, with this result being shown on Figure 3.6. The concentration of soluble CD23 in the culture medium of stimulated cells was found

to be comparable with that of the control cells, therefore, the low expression of cell surface CD23 is not a result of enhanced cleavage of CD23 by cell surface proteins.

CD25 expression on tonsillar B cells was stimulated significantly with both IL-4 and PMA, with a small increase evident with IL-13 and anti-CD40 (Fig. 3.7). No apparent stimulation was observed following treatment with the anti-μ chains. These data are consistent with work carried out by Hewitt *et al* (Hewitt, *et al.*, 1997) and McKay *et al* (McKay, *et al.*, 2000), who investigated the IL-4 signalling pathways of tonsillar B cells in great detail. These included the JAK/STAT, cAMP/PKA, PKC and PI-3 kinase pathways, and it was concluded that IL-4 achieves CD25 up-regulation by removing NRE (Negative Response Element) binding activity via a cAMP/PKA-sensitive pathway. A small increase in CD25 activation is also apparent in the lower right hand quadrant of the plot. This CD25 expression is not on B cells, as there is no CD19 expression evident, and is thought to be due to very small numbers of T cells escaping the T cell rosetting stage of the tonsillar B cell preparation.

3.2.3b: Stimulation of CD23 and CD25 on P3HR1 and SMS-SB cells

P3HR1 cells show similar patterns of response to stimulation to those observed with the tonsillar B cells (Table 3.3), although expression of CD23 and CD25 are not activated to such a great extent by IL-4 and PMA in the former case. This variation may be due to the fact that naive cells are more readily affected by certain stimuli than continually passaged cell lines. The P3HR1 cells were thus further concluded to be a good candidate as a model system for tonsillar B cells, and were used whenever difficulties were encountered in obtaining primary tissue.

The SMS-SB, pre-B ALL cell line, does not express CD23 or CD25 at any time, even following stimulation with IL-4, IL-13, anti-CD40, anti-μ or PMA. SMS-SB

cells were therefore considered as a good negative control for CD23- and CD25-expressing cells throughout the course of this work.

3.2.4: Phosphorylation of STAT-6 is increased following IL-4 and IL-13 stimulation of tonsillar B cells

Flow cytometric analysis has demonstrated that IL-4 and IL-13 can lead to substantial increases in CD23 expression. As discussed, IL-4 and IL-13 are known to act through similar receptors and are both known to activate STAT-6. STAT-6, a signal transducer and activator of transcription, is a member of a family of cytoplasmic transcriptional activators (STAT's 1-4, 5a, 5b and STAT-6). All STATs contain a carboxy-terminal SH2 domain, an SH3 domain and a conserved DNA binding region and are linked to receptor ligation via phosphorylation with the Janus kinases (JAKS) (Wilks, *et al.*, 1991). Phosphorylated STAT-6 dimerises and is translocated to the cell nucleus, where it binds to a specific response element on the DNA and initiates gene expression (see "The STAT-6 Activation Pathway" - 1.4.7).

Experimental evidence was sought to determine if IL-4 and IL-13 were capable of stimulating CD23 expression through a STAT-6 dependent pathway in this instance. In order to do this, STAT-6 was immunoprecipitated from extracts of control or stimulated cells, run on an SDS-PAGE gel and immunoblotted with anti-p-Tyr to follow the extent of STAT-6 phosphorylation over a specific period of time. Figures 3.8 and 3.9 show the anti-p-Tyr immunoblots for IL-4- and IL-13- stimulated tonsillar B cells, respectively. A clear increase in STAT-6 phosphorylation was observed over the 30 minute time-course with both stimulants, although the result for IL-13 stimulation showed a more clear-cut distinction between the treated and control cells. It was considered that, perhaps, it was the actual amount of STAT-6 that was increasing over time, rather than its phosphorylation. Therefore an identical experiment was run in parallel, with the exception that blotting was for STAT-6

itself as opposed to p-Tyr. The results of the STAT-6 immunoblotting are also shown on Figures 3.8 and 3.9, and confirm that it is solely tyrosine phosphorylation status of STAT-6 that increases upon IL-4 and IL-13 activation, and not the STAT-6 concentration. It can be concluded through these immunoprecipitation experiments that IL-4 and IL-13 stimulate STAT-6 dependant pathways, and it can be hypothesised that the increase observed in CD23 expression upon IL-4 and IL-13 stimulation may occur as a result of this.

3.2.5: Electrophoretic Mobility Shift Assay (EMSA) shows increases in both STAT-6 and NFκB activation following stimulation

Flow cytometry has shown in section 3.2.3a that certain stimuli can drive cell surface expression of CD23 on tonsillar B cells. Mainly IL-4, IL-13, anti-CD40 and PMA were seen to elicit an increase in the presence of CD23, although FACS alone could not determine exactly how this was achieved or which transcription factors were involved in this process. A technique was required that would allow an insight into which DNA binding proteins/transcription factors were activated via each potential stimulator of CD23 expression.

The electrophoretic mobility shift or "gel-shift" assay is a method by which oligonucleotides, which bind specifically to certain cellular proteins, are radio-labelled and mixed with a protein extract. Upon recognition and binding of the specific protein, and subsequent loading on to a non-denaturing electrophoresis gel, the radiolabelled oligonucleotide/protein complex electrophoreses more slowly than the unbound radiolabelled oligo). Bands, representing specific binding species on the gel, can then be visualised by autoradiography and used to determine activation states.

EMSA was carried out for the specific transcription factors STAT-6 and NFκB, on cellular lysates prepared following treatment of tonsillar B cells with IL-4, IL-13, anti-CD40, anti-μ and PMA. The oligonucleotides used are listed in the Materials section of this thesis (2.1.8) Figure 3.10 shows the autoradiograph of a STAT-6 gel shift assay following a 24hr stimulation period with all of the potential stimulants. The specific STAT-6 binding species, labelled with an arrow, is seen to increase in intensity upon stimulation with IL-4 and IL-13, but not with anti-CD40 or PMA. Anti-μ, in fact, appears to cause a loss of band intensity. This work further supports the hypothesis that IL-4 and IL-13 stimulate the expression of CD23 via a STAT-6-phosphorylation dependent pathway. How STAT-6 or STATs in general are able to activate transcription is unclear, although it is believed that activated STAT's form complexes with other transcription factors in the nucleus - such as nuclear factor κB (NFκB) (Shen & Stavnezer, 1998).

NFκB, a widely expressed protein which regulates gene expression, is comprised of homodimers or heterodimers that belong to the Rel family of proteins (Baldwin, 1996). It is bound to inhibitor proteins called IκB, of which there are three isoforms; IκB-α, β and ε. When NFκB is induced, the IκB serine residues are phosphorylated and the IκB protein degraded, allowing NFκB to translocate to the nucleus where it is able to regulate gene expression (Di Donato, *et al.*, 1997; Maniatis, 1997).

NFκB EMSAs were carried out on whole cell extracts, following stimulation of tonsillar B cells with IL-4, IL-13, anti-CD40, anti-μ and PMA (Fig. 3.11). Increases in specific band intensity are clearly observed from the cell lysates following IL-13, anti-CD40 and anti-μ stimulation, although IL-4 treatment actually appears to reduce the binding to the NFκB oligonucleotide in relation to the non-stimulated cells. The latter could indicate that, upon IL-4 stimulation, some sort of negative regulatory process blocks NFκB activation. IL-13 is normally associated with the STAT-6 activation pathway, but the activation of NFκB in these experiments suggests an

additional role for the IL-13 cytokine in NFκB signalling pathways, perhaps through the activation of some intermediary step. PMA treatment causes no change in specific NFκB EMSA band intensity. To further investigate the effects of anti-CD40, a time-course of exposure to this stimulant was also analysed by NFκB EMSA (Fig. 3.12). The autoradiograph for this experiment shows an increase in specific band intensity (highlighted by arrow) following 45 minutes of anti-CD40 stimulation, followed by a further increase after 60 minutes of treatment. Anti-CD40 stimulation of NFκB is thought to be bi-phasic, however, with NF-κB activation increasing with an initial fast response and then a later, presumably more sustained, greater response. The fact that this bi-phasic pattern is not shown here could be as a result of no shorter time points being taken within the first 15 minute interval.

It should be borne in mind that these EMSA results are representative of the stimulation and preparation of the protein content of whole cells. These whole cell lysate (WCL) stimulations reflect the general results for STAT-6 and NFκB activation for the entire cell and not just in relation to the promotion of CD23 transcription. Also, these results are representative of a whole cell population, which may not respond uniformly to stimulation. Therefore, caution should be exercised when interpreting these results as in direct relation to CD23, or indeed any other specifically expressed protein.

3.2.6: Conclusion and discussion

The preceding experiments have provided valuable information on the cells which will be used in the bulk of the research in this thesis; information such as phenotype, CD23 expression, susceptibility to stimulation and activation of STAT-6 and NFκB. To re-cap, the main findings were as follows: Different B cell lines and tonsillar B cells all express varying degrees of particular cell surface markers, including the low affinity receptor for IgE, CD23. The EDR mature B cell line has constitutively high

levels of CD23, which is thought to reflect its EBV-transformed status, whereas the immature B cell lines do not. Additionally P3HR1 cells, which are also EBV transformed, and tonsillar B cells show low constitutive CD23 levels which are considerably elevated upon stimulation with IL-4 in particular. This similarity between P3HR1 cells and tonsillar B cells made the former a suitable substitute for the latter when tonsillar tissues was scarce. The immature B cell lines are not subject to stimulus-induced CD23 expression.

IL-4 and IL-13 stimulation were both shown to cause an increase in STAT-6 tyrosine phosphorylation, with no observable increase in actual STAT-6 concentration. These results led to the hypothesis that IL-4 and IL-13 could activate expression of CD23 through a STAT-6-dependent activation pathway. Although, it should be noted that from the evidence presented here alone (IL-4 and IL-13 stimulate both CD23 expression and STAT-6 phosphorylation), it is not possible to categorically define a link between the two events. Principally, this is due to the fact that the immunoprecipitation experiments were carried out using whole cell protein lysates, meaning that the results are representative of all the STAT-6 in the cells and not just that which is acting on the CD23 promoters. This fact is also worth noting where the EMSA results are concerned, as they are also carried out on proteins from whole cell lysates.

These EMSA results confirm that the activation of STAT-6 occurs upon cell stimulation with IL-4 and IL-13, with increases in band intensity evident on samples treated in such a fashion. They also show that NFκB stimulation is increased upon IL-13, anti-μ and anti-CD40 stimulation, with anti-CD40 stimulation of NFκB occurring as a bi-phasic response.

It is extremely important to note at this stage that none of the above B cell phenotyping or stimulation experiments give information concerning which isoform

of CD23 was specifically expressed in each case (although the stimulated T cell CD23 is likely to be CD23b only). B cells constitutively express CD23a and can be stimulated to upregulate the expression of both isoforms, although the monoclonal antibodies used for flow cytometry detect only the presence of CD23 and not the individual isoforms. This is attributed to the fact that the only observable difference between the two isoforms is intra-cytoplasmic and not on the extracellular domain. It was considered of great importance to use the information discussed in this chapter to design experiments which could hopefully help define the differential expression of each isoform, and the subsequent activation of each. This will be addressed in depth in the reporter vector construct studies in Chapter 4.

Figure 3.1: Overview of flow cytometer function

Flow cytometry is a means of measuring certain physical and chemical characteristics of cells or particles as they travel in suspension, one by one, past a sensing point. A typical flow cytometer is represented on this figure and consists of a light source (laser), collection optics, electronics and a computer to translate signals into data. Samples enter the system at the interrogation point shown on this diagram and light from the laser, which omits coherent light at a specified wavelength, is scattered and collected by two lenses, one set in front of the light source (towards the forward scatter detector) and the other set at right angles to it. Specific bands of fluorescence are measured by a series of optics, beam splitters and filters, with the side scatter detector being at right angles to the beam splitter and the fluorescence (FL) 1 and 2 detectors detecting light of 530nm (FITC) and 585nm (PE), respectively. The dichroic mirror allow light of less than 560nm to pass straight through to the FL1 detector but reflects light above this frequency towards the FL2 detector, hence the ability of the cytometer to separate the fluorescence emitted from both wavelengths (e.g.. both FITC and PE labelled cells). This information is then used to define the cellular origin and maturation status of each cell type. Emitted light from the cells, as well as fluorescent label, is also collected.

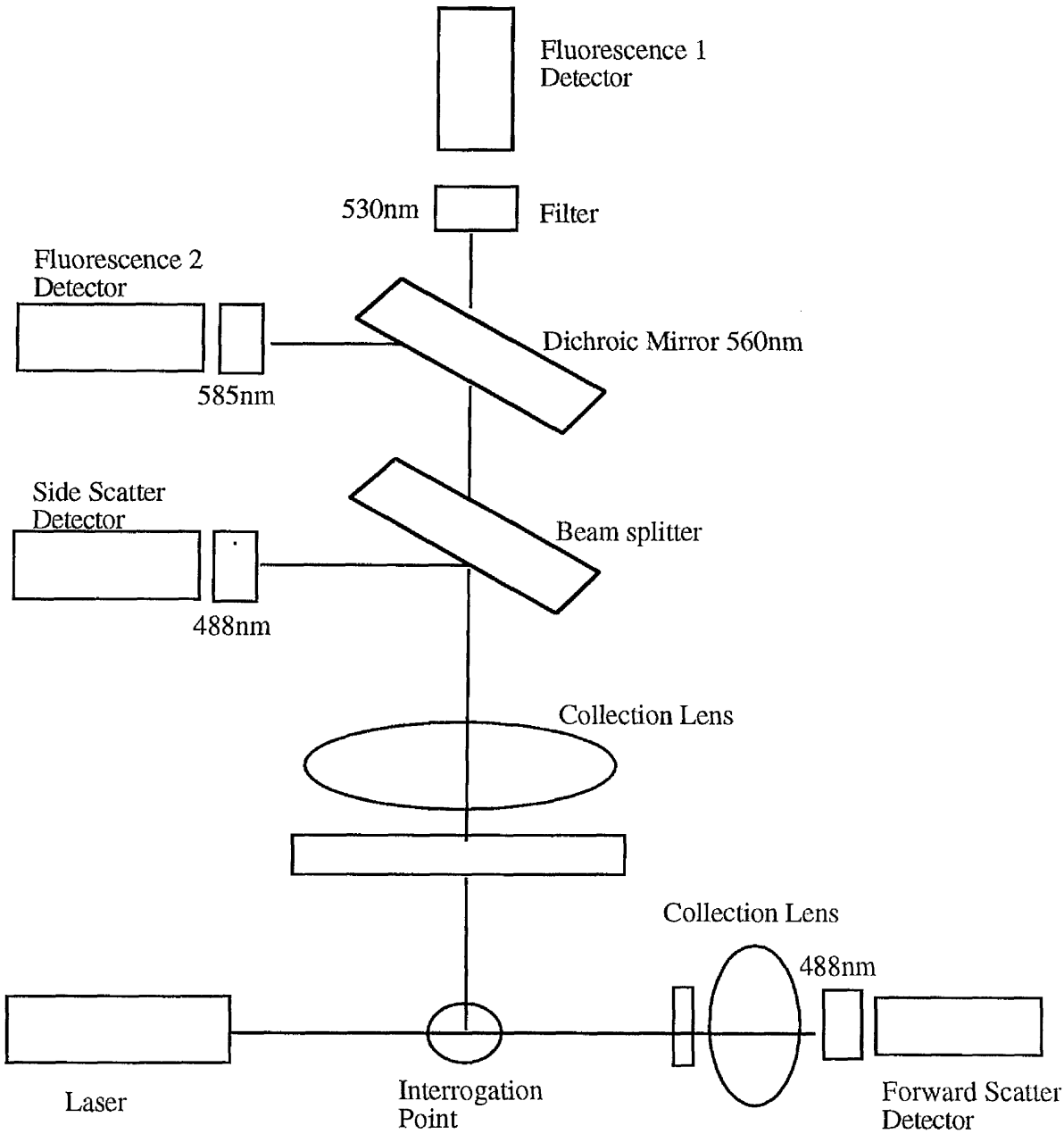
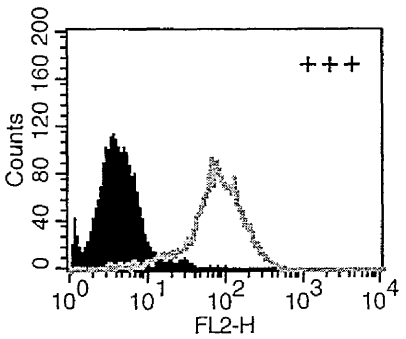

Fluorescence 1
Detector
530nm
Filter
Fluorescence 2
Detector
Dichroic Mirror 560nm
585nm
Side Scatter
Detector
Beam splitter
488nm
Collection Lens
Collection Lens
488nm
Laser
Interrogation
Point
Forward Scatter
Detector

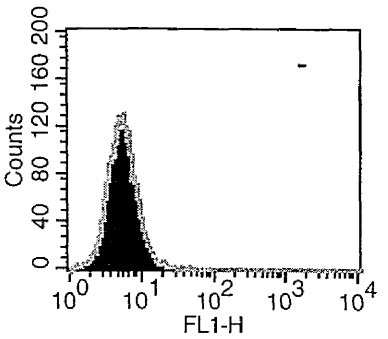
Figure 3.2: Flow cytometric phenotyping of EDR cells

EDR B cells were harvested from culture, washed twice in ice-cold PBS and resuspended at $1x10^6$ cells per 100µl of PBS. 3µl of each FITC- or PE- labelled antibody was then added as required to each sample, (all of the antibodies used here were FITC-labelled, apart from anti-CD19 which was PE-labelled), prior to incubation of the samples on ice for 1hr. Cells were then again washed twice with ice-cold PBS, resuspended in 500µl of PBS and analysed on a Becton Dickinson FACScan flow cytometer.

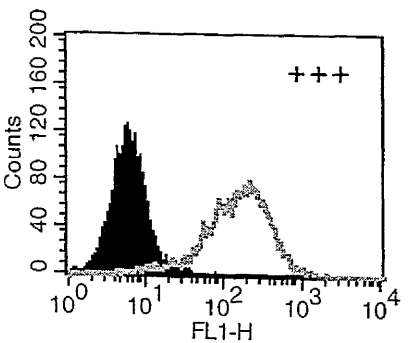
The y-axis is representative of the number of events counted, and the x-axis (FL-1 or FL-2) shows the intensity of signal at each specific wavelength.



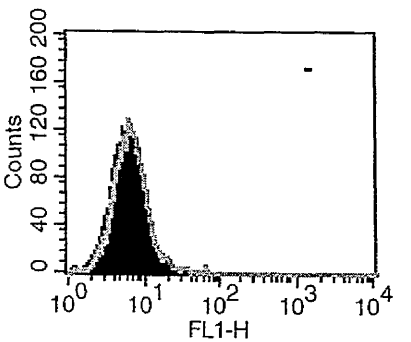
CD19



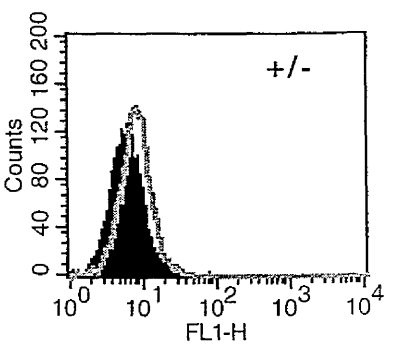
CD3



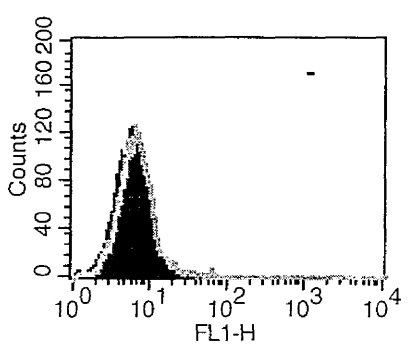
CD23



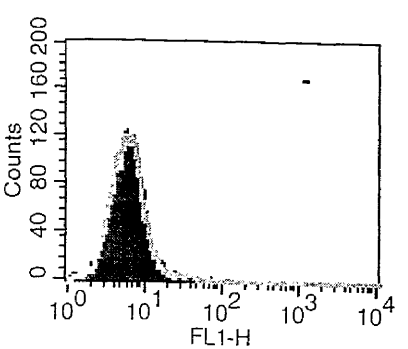
CD25



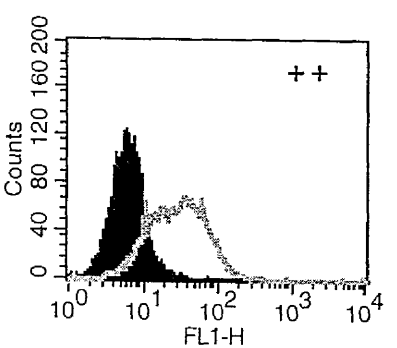
CD11b



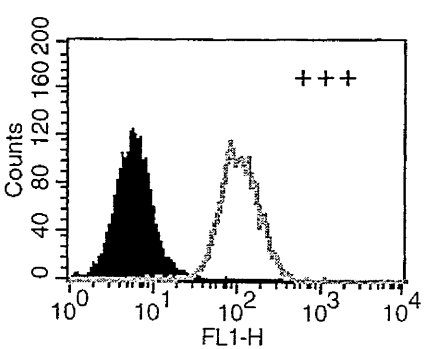
CD11c



αvβ5



CD21



CD47

Table 3.1: Phenotyping of cell lines and tonsillar B cells

B cell lines and primary tonsillar B cells were harvested from culture, washed twice in ice-cold PBS and resuspended at $1x10^6$ cells per 100µl of PBS. 3µl of FITC- or PE- labelled antibody was then added as required to each sample, (all of the antibodies used here were FITC-labelled, apart from anti-CD19 which was PE-labelled), prior to incubation of the samples on ice for 1hr. Cells were then again washed twice with ice-cold PBS, resuspended in 500µl of PBS and analysed on a Becton Dickinson FACScan flow cytometer. FITC-labelled cells were detected with FL1 and PE-labelled cells with FL2.

The phenotyping data was then translated and compiled as shown; with "+++" indicating high marker expression, "++" showing medium expression, "+" or "+/-" representing low or borderline results and "-" indicating no detectable cell surface marker presence.

CELL LINE	STATUS	ORIGINAL SOURCE	CD19	CD3	CD23	CD25	CD21	CD11 b	CD11 c	αvβ5	CD47
EDR	MATURE B CELL	HUMAN B LYMPHOBLASTOID CELL	+++	-	+++	-	++	+/-	-	-	+++
JIJOYE	MATURE B CELL	HUMAN B LYMPHOBLASTOID CELL	+++	-	+	+	+	N/D	N/D	N/D	+++
P3HRI	MATURE B CELL	HUMAN BURKITT'S LYMPHOMA/HIGH EBV	+++	-	-	-	-	+	+	-	+++
RAMOS	MATURE B CELL	HUMAN BURKITT'S LYMPHOMA	+++	-	+	++	+++	++	N/D	++	+++
SMS-SB	PRE-BII CELL	HUMAN PRE-B CELL ALL	+++	-	-	-	-	-	-	++	+++
NALM-6	PRE-BII CELL	HUMAN LEUKAEMIA B CELL	+++	-	-	-	+	-	-	-	+++
JURKAT	MATURE T CELL	HUMAN LEUKAEMIA T LYMPHOBLAST	--	+++	-	-	++	+/-	-	+	+++
TONSIL B CELLS	PRIMARY B CELL	PREPARED FROM PRIMARY TONSILLAR TISSUE	+++	-	-	+	+++	+	+/-	-	+++

Figure 3.3: **Flow cytometric phenotyping of tonsillar B cells**

Tonsillar B cells were prepared from primary tissue as indicated in the methods section of this thesis (2.2.2a), harvested from culture, washed twice in ice-cold PBS and resuspended at $1x10^6$ cells per 100µl of PBS. 3µl of each FITC- or PE- labelled antibody was then added as required to each sample, (all of the antibodies used here were FITC-labelled, apart from anti-CD19 which was PE-labelled), prior to incubation of the samples on ice for 1hr. Cells were then again washed twice with ice-cold PBS, resuspended in 500µl of PBS and analysed on a Becton Dickinson FACScan flow cytometer.

The lower left hand quadrant of the dot plot indicates the unstained cell population, the lower right shows FL1-only fluorescing cells (i.e.. FITC-labelled), the upper left indicates FL2 only fluorescing cells (i.e. PE-labelled, $CD19^+$ B cells) and the upper right hand quadrant is representative of cells which are doubly positive for both PE and FITC staining. Thus, the upper right hand quadrant distinguishes B cells which also show expression of the tested cell surface marker.

The numbers shown represent the percentage of CD19 expressing B cells which also express the tested cell surface marker.

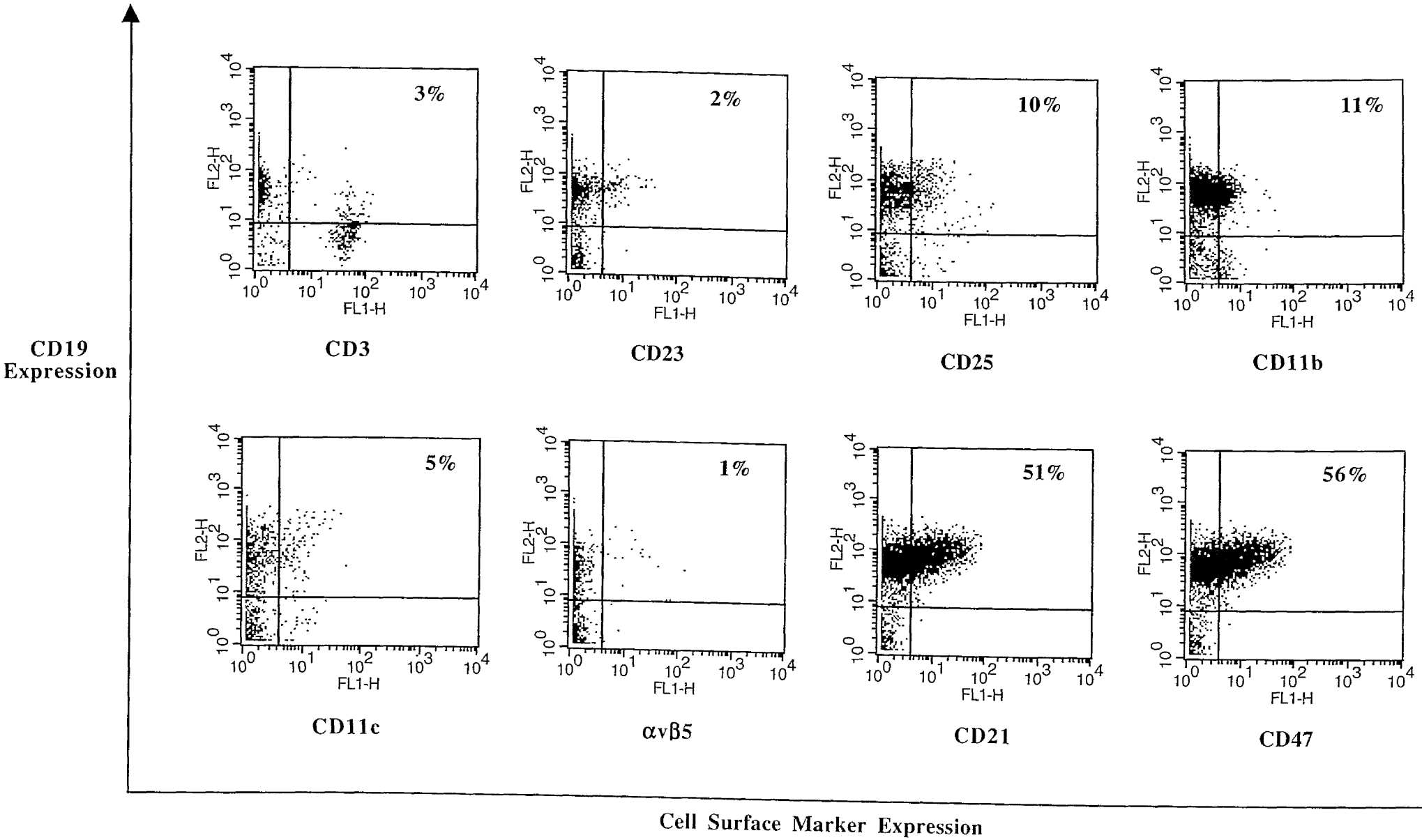

CD19 Expression
3%
CD3
2%
CD23
10%
CD25
11%
CD11b
5%
CD11c
1%
αvβ5
51%
CD21
56%
CD47
FL2-H
FL1-H
Cell Surface Marker Expression

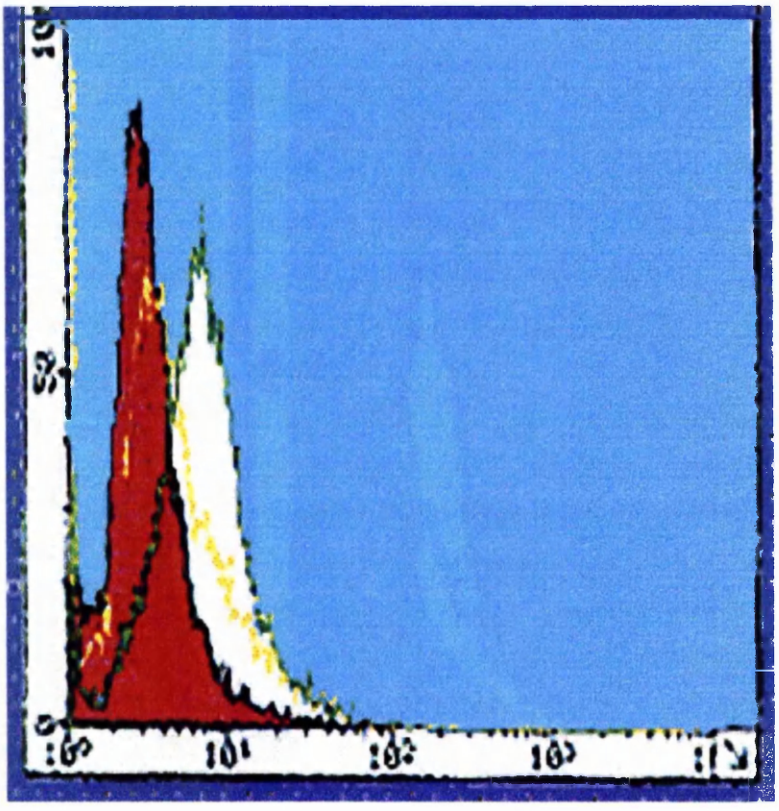
Figure 3.4: Flow cytometric analysis of IL-4-stimulated P3HRI B cells

Freshley sub-cultured P3HRI B cells were stimulated for 24hr with 1nM of IL-4, harvested from culture, washed twice in ice-cold PBS and resuspended at $1x10^6$ cells per 100µl of PBS. 3µl of FITC-labelled anti-CD23 antibody was then added prior to incubation of the samples on ice for 1hr. Cells were again washed twice with ice-cold PBS, resuspended in 500µl of PBS and analysed on a Becton Dickinson FACScan flow cytometer. FITC-labelled cells were detected with FL1.

The shaded red part of the histogram is representative of unstained cells, the yellow peak shows the base-line CD23 expression on unstimulated cells and the green peak is indicative of CD23 cell surface expression following IL-4 stimulation.

Table 3.2: CD23 expression on IL-4-stimulated and unstimulated cells

The indicated B cell lines and tonsillar B cells were prepared for flow cytometric analysis as above, and the resulting data was then translated and compiled as shown; with "+++" indicating high marker expression, "++" showing medium expression, "+" representing low results and "-" indicating no detectable cell surface marker presence.



CELL TYPE/LINE	CD23 EXPRESSION/- IL-4 STIMULATION	CD23EXPRESSION/+ IL-4STIMULATION
EDR	+++	+++
JIJOYE	+	++
P3HR1	-	+++
RAMOS	+	++
SMS-SB	-	-
NALM-6	-	-
JURKAT	-	++
TONSIL B CELLS	-	+++

Figure 3.5: CD23 expression on stimulated tonsillar B cells

Tonsillar B cells were stimulated for 24hr with each of 14ng/ml of IL-4, 6ng/ml IL-13, 1μg/ml anti-CD40, 1 μg/ml anti-μ and 6nM PMA. Cells were then harvested from culture, prepared for flow cytometric analysis as before (2.2.3) and analysed on a Becton Dickinson FACScan flow cytometer.

Anti-CD23-FITC-labelled $CD19^+$ B cells are indicated in the upper right hand quadrant of the dot plots.

The numbers shown represent the percentage of CD19 expressing B cells which also express CD23.

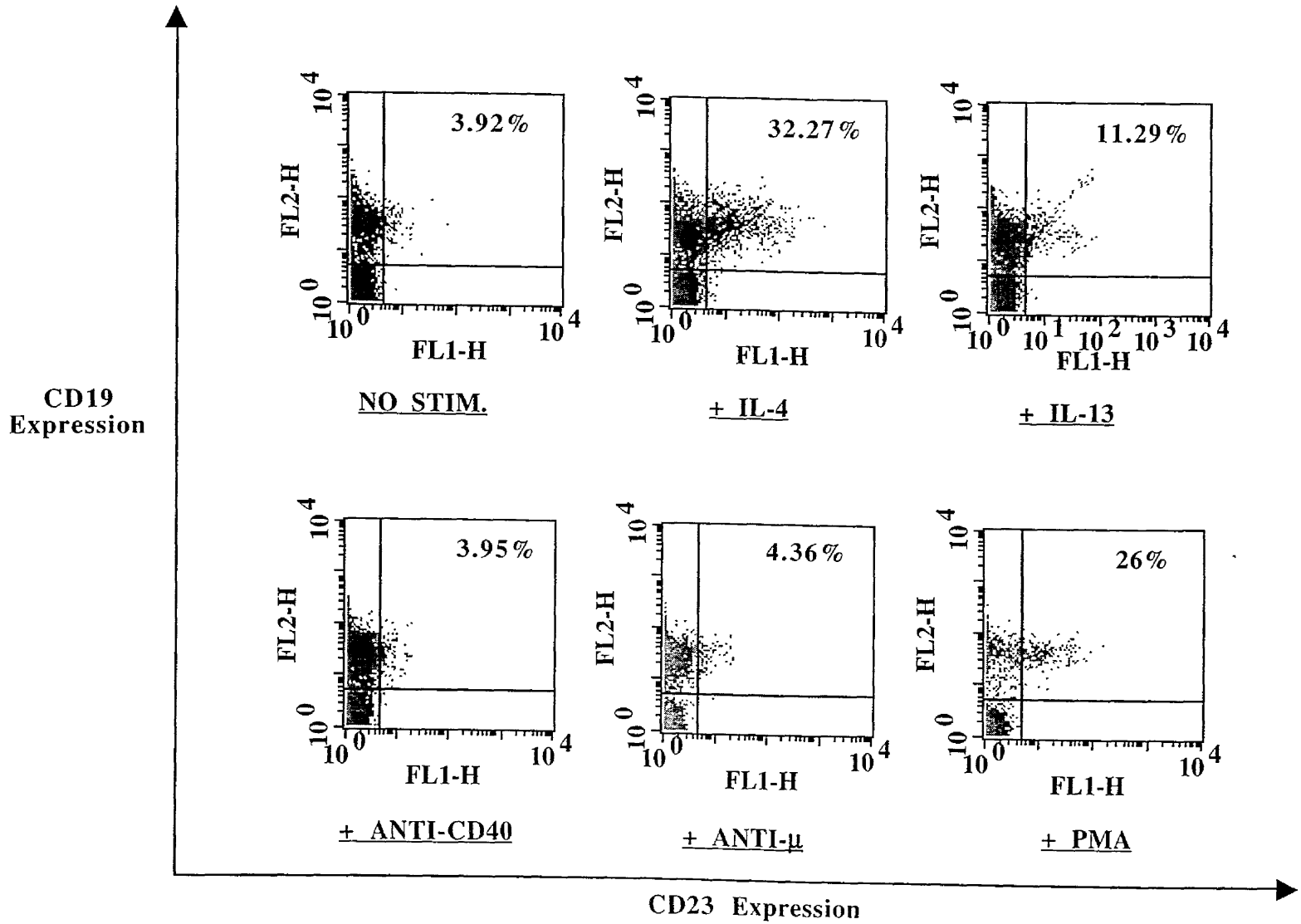
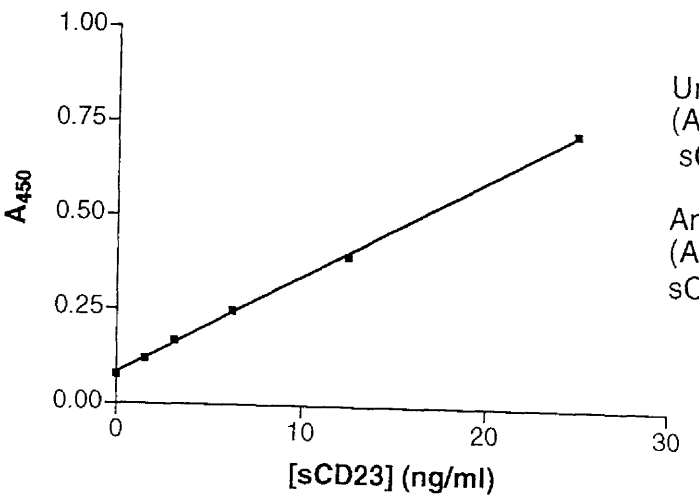

CD19 Expression
3.92%
FL2-H
FL1-H
NO STIM.
32.27%
+ IL-4
11.29%
+ IL-13
3.95%
+ ANTI-CD40
4.36%
+ ANTI-μ
26%
+ PMA
CD23 Expression

Figure 3.6: sCD23 ELISA of anti-μ stimulated tonsillar B cells

Tonsillar B cells were stimulated for 24hr with 1μg/ml of anti-μ, supernatant removed and analysed for sCD23 concentration by enzyme linked immunosorbant assay (ELISA). ELISA protocol provided and carried out by Dr Gillian Borland.

sCD23 ELISA standard curve



Untreated cells
(A_{450} = 0.371)
sCD23 = 11.12ng/ml

Anti-μstimulated cells
(A_{450} = 0.421)
sCD23 = 13.061ng/ml

Figure 3.7: CD25 expression on stimulated tonsillar B cells

Tonsillar B cells were stimulated for 24hr with each of 14ng/ml of IL-4, 6ng/ml IL-13, 1μg/ml anti-CD40, 1 μg/ml anti-μ and 6nM PMA. Cells were then harvested from culture, prepared for flow cytometric analysis as before (2.2.3) and analysed on a Becton Dickinson FACScan flow cytometer.

Anti-CD25-FITC-labelled $CD19^+$ B cells are indicated in the upper right hand quadrant of the dot plots, with the lower right hand quadrant showing CD25 expressing non-B cells.

The numbers shown represent the percentage of CD19 expressing B cells which also express CD25.

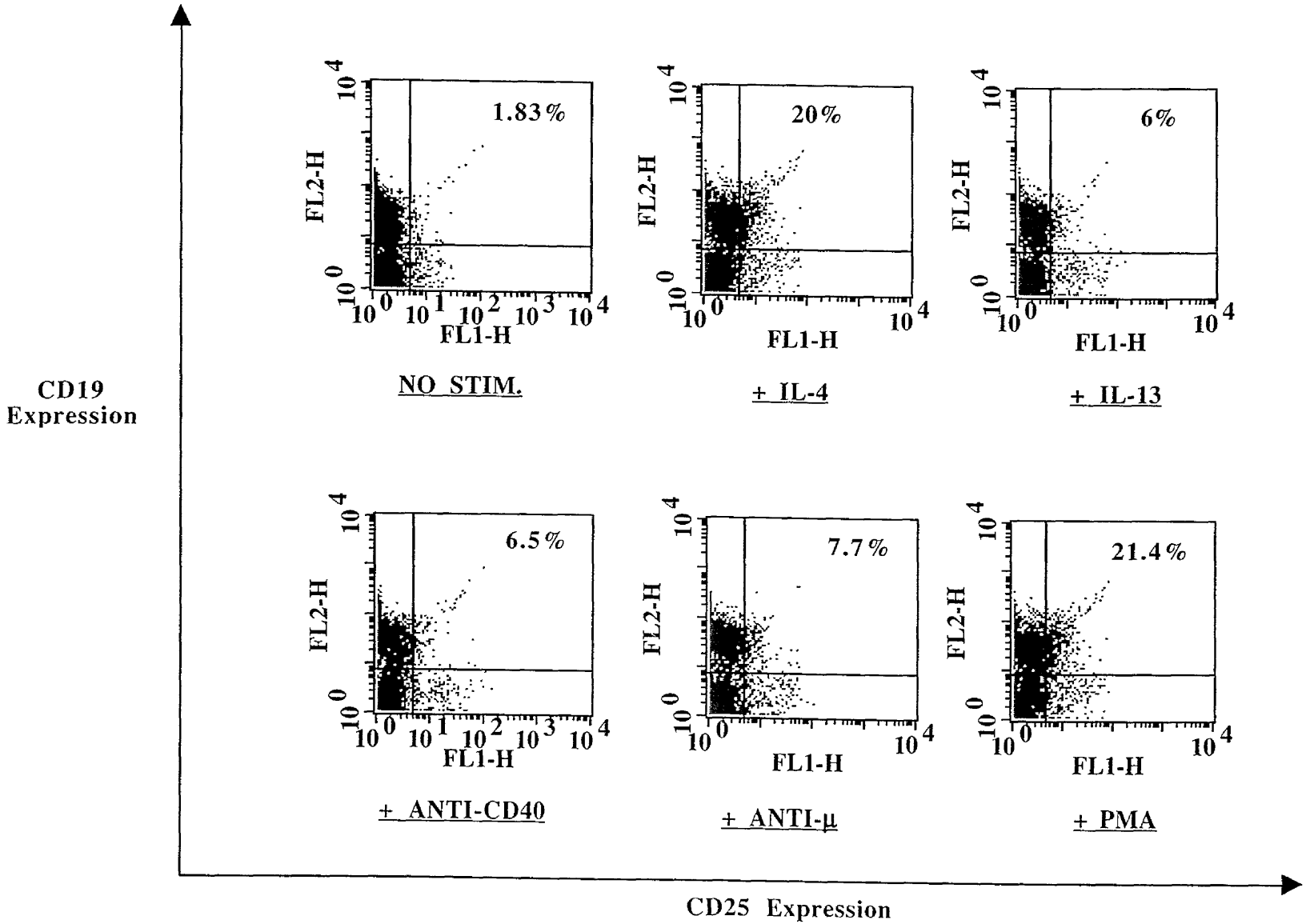

CD19
Expression
1.83%
FL2-H
FL1-H
NO STIM.
20%
+ IL-4
6%
+ IL-13
6.5%
+ ANTI-CD40
7.7%
+ ANTI-μ
21.4%
+ PMA
CD25 Expression

Table 3.3: CD23 and CD25 expression on stimulated tonsillar B cells and the B cell lines P3HRI and SMS-SB

P3HRI and SMS-SB cell lines and tonsillar B cells were stimulated separately for 24hr with each of 14ng/ml of IL-4, 6ng/ml IL-13, 1μg/ml anti-CD40, 1 μg/ml anti-μ and 6nM PMA. Cells were then harvested from culture, prepared for flow cytometric analysis as before (2.2.3) and analysed on a Becton Dickinson FACScan flow cytometer.

The resulting data was translated and compiled as shown; with "+++" indicating high marker expression, "++" showing medium expression, "+" or "+/-" representing low or borderline results and "-" indicating no detectable cell surface marker presence.

	CD23 Expression following stimulation with						CD25 Expression following stimulation with					
CELL TYPE	-	+ IL-4	+ IL-13	+ αCD40	+ αμ	+ PMA	-	+ IL-4	+ IL-13	+ αCD40	+ αμ	+ PMA
Tonsillar B cells	-	++	+	-	-	++	-	++	+	+	+	++
P3HRI	-	++	+/-	+	-	+	+/-	++	+/-	+/-	-	++
SMS-SB	-	-	-	-	-	-	-	-	-	-	-	-

Figure 3.8: STAT-6 phosphorylation and concentration upon IL-4 stimulation

$1x10^7$ tonsillar B cells per ml were prepared as described in section 2.2.4c. Briefly, cells were stimulated over a 30min time-course with 14ng/ml IL-4, lysed in RIPA buffer and incubated at 4°C for 4hr with 1μg of anti-STAT-6 antibody. 10μl of protein-G agarose were then added to the solution and samples rotated overnight at 4°C to allow the formation of immunoprecipitates. Immunoprecipitates were collected, washed in RIPA buffer, resuspended in loading buffer, denatured and run on a 10% (w/v) polyacrylamide gel. Protein was transferred to nitrocellulose via western blotting, as described in 2.2.4d, prior to immunoblotting with anti-p-Tyr and anti-STAT-6 antibodies (2.2.4e).

Unstimulated control samples are represented with " -" and IL-4-stimulated samples with "+".

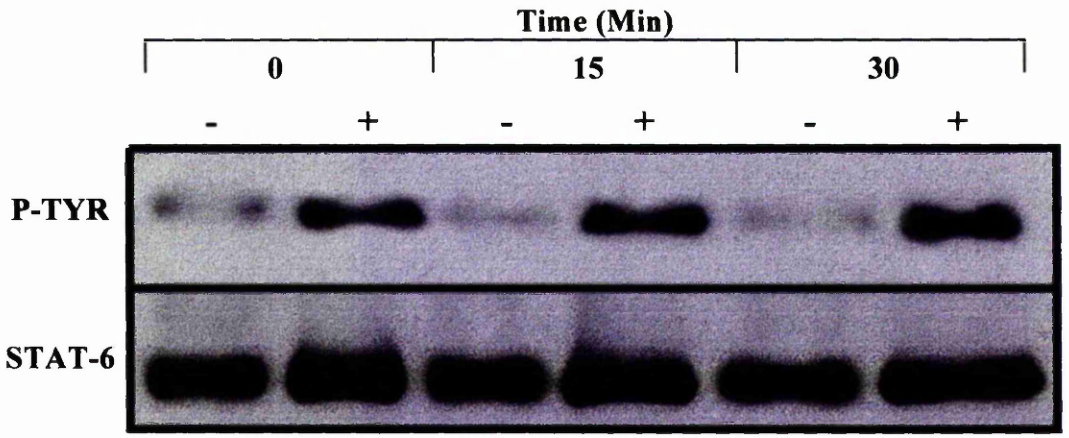

Time (Min)
0
15
30
-
+
-
+
-
+
P-TYR
STAT-6

Figure 3.9: STAT-6 phosphorylation upon IL-13 stimulation

$1x10^7$ tonsillar B cells per ml were prepared as described in section 2.2.4c. Briefly, cells were stimulated over a 30min time-course with 6ng/ml IL-13, lysed in RIPA buffer and incubated at 4°C for 4hr with 1µg of anti-STAT-6 antibody. 10µl of protein-G agarose were then added to the solution and samples rotated overnight at 4°C to allow the formation of immunoprecipitates. Immunoprecipitates were collected, washed in RIPA buffer, re-suspended in loading buffer, denatured and run on a 10% (w/v) polyacrylamide gel. Protein was transferred to nitrocellulose via western blotting, as described in 2.2.4d, prior to immunoblotting with anti-p-Tyr and anti-STAT-6 antibodies (2.2.4e).

Unstimulated control samples are represented with " -" and IL-13-stimulated samples with "+".

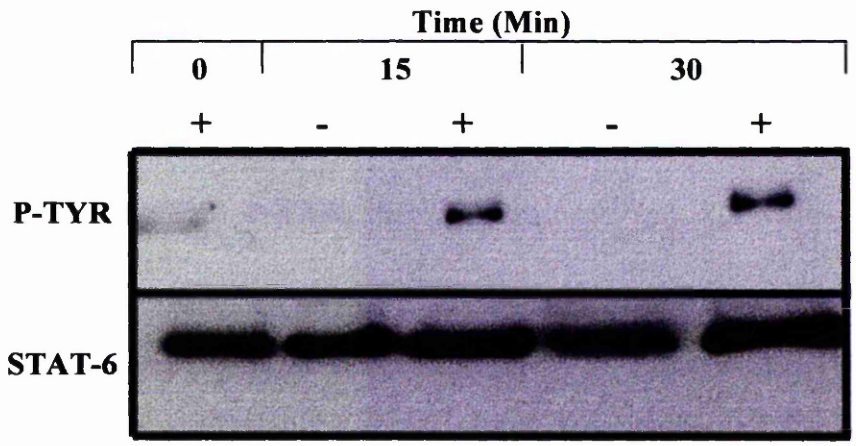
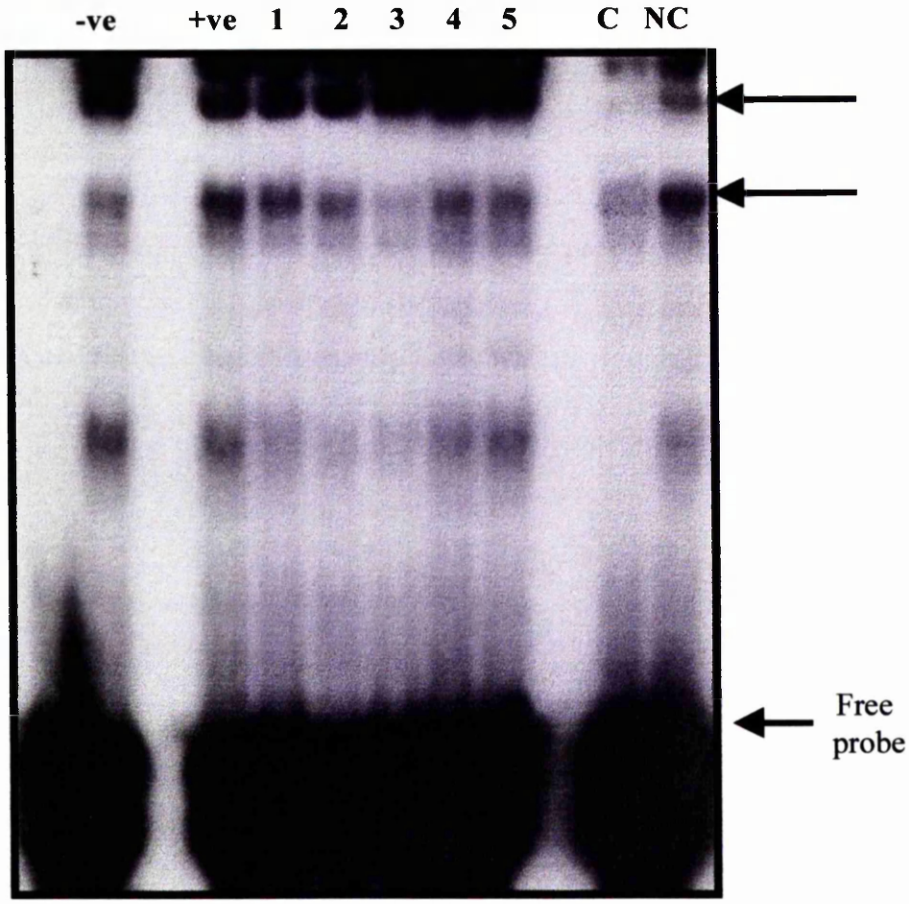

Time (Min)
0
15
30
+
-
+
-
+
P-TYR
STAT-6

Figure 3.10: STAT-6 EMSA of stimulated tonsillar B cell lysate

Tonsillar B cells were stimulated for 24hr with each of 14ng/ml of IL-4, 6ng/ml IL-13, 1μg/ml anti-CD40, 1 μg/ml anti-μ and 6nM PMA. Cells were then harvested from culture, lysed in RIPA buffer and 5μg of lysate added to each EMSA sample (2.2.5b). Briefly, 5μg of stimulated or unstimulated extract was added to a 10μl reaction mixture containing binding buffer (BB), dH_2O, and ^{32}P-labelled STAT-6 oligonucleotide (1μl of specific or non-specific oligonucleotide is added for competitor or non-competitor controls, respectively). The reaction mixtures were then incubated at room temperature for 20min prior to running on a 7% non-denaturing gel (section 2.2.5c), drying and autoradiography.

Specific STAT-6 binding species are highlighted by arrow, with the specific stimuli used noted at the top of the graph.



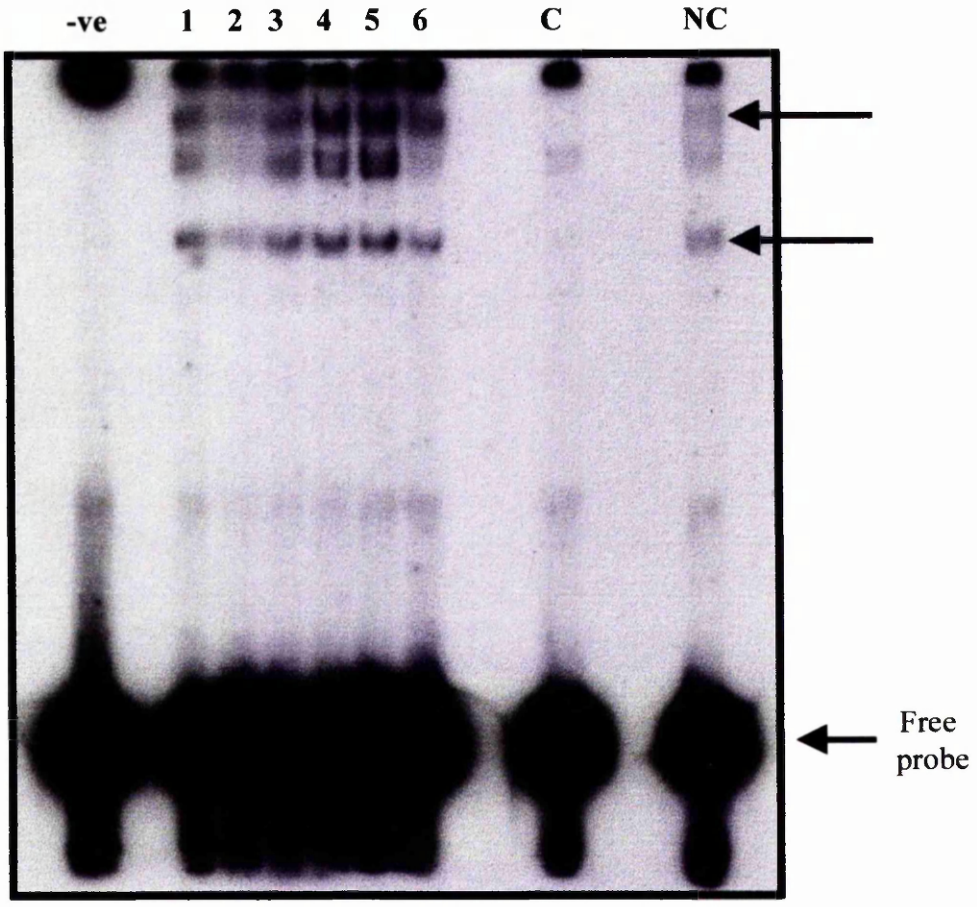
-ve = Negative control, +ve = Positive control
C = Competitor control, NC = Non-competitor control

1 = + IL-4
2 = + IL-13
3 = + anti-CD40
4 = + anti-μ
5 = +PMA

Figure 3.11: NFκB EMSA of stimulated tonsillar B cell lysate

Tonsillar B cells were stimulated for 24hr with each of 14ng/ml of IL-4, 6ng/ml IL-13, 1μg/ml anti-CD40, 1 μg/ml anti-μ and 6nM PMA. Cells were then harvested from culture, lysed in RIPA buffer and 5μg of lysate added to each EMSA sample (2.2.5b). Briefly, 5μg of stimulated or unstimulated extract was added to a 10μl reaction mixture containing binding buffer (BB), dH_2O, and ^{32}P-labelled NFκB oligonucleotide (1μl of specific or non-specific oligonucleotide is added for competitor or non-competitor controls, respectively). The reaction mixtures were then incubated at room temperature for 20min prior to running on a 7% non-denaturing gel (section 2.2.5c), drying and autoradiography.

Specific NFκB binding species are highlighted by arrow, with the specific stimuli used noted at the top of the graph.



-ve = Negative control, C = Competitor control, NC = Non-competitor control

1 = No Stimulation

2 = + IL-4

3 = + IL-13

4 = + anti-CD40

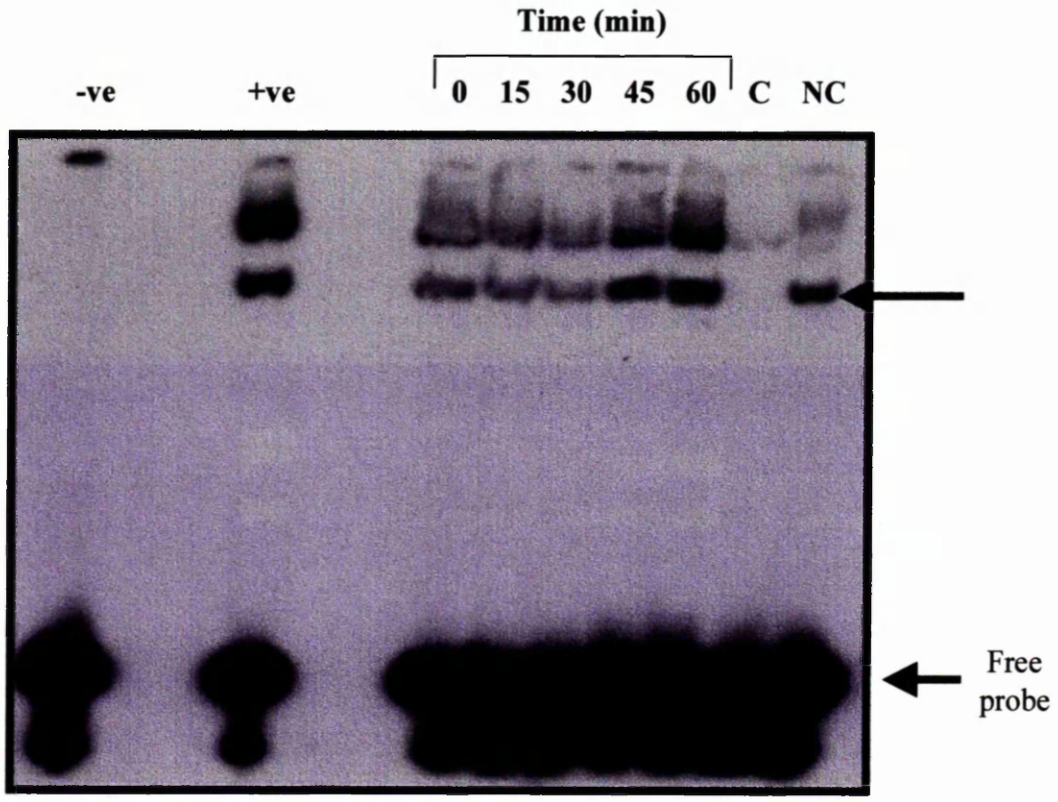
5 = + anti-μ

6 = +PMA

Figure 3.12: NFκB EMSA of anti-CD40 time-course stimulated tonsillar B cell lysate

Tonsillar B cells were stimulated over a 1hr time-course with 1μg/ml of anti-CD40 monoclonal antibody. Cells were harvested from culture at the appropriate time, lysed in RIPA buffer and 5μg of lysate added to each EMSA assay (2.2.5b). Briefly, 5μg of time-course stimulated or unstimulated extract was added to a 10μl reaction mixture containing binding buffer (BB), dH_2O, and ^{32}P-labelled NFκB oligonucleotide (1μl of specific or non-specific oligonucleotide is added for competitor or non-competitor controls, respectively). The reaction mixtures were then incubated at room temperature for 20min prior to running on a 7% non-denaturing gel (section 2.2.5c), drying and autoradiography.

Specific NFκB binding species are highlighted by arrow, and the time of each stimulation noted at the top of the graph.



-ve = Negative control, +ve = Positive control, C = Competitor control, NC = Non-competitor control

CHAPTER 4

RESULTS

REPORTER VECTOR CONSTRUCT STUDIES OF THE CD23a AND CD23b PROMOTER REGIONS

CHAPTER 4:

REPORTER VECTOR CONSTRUCT STUDIES OF THE CD23a AND CD23b PROMOTER REGIONS.

4.1: INTRODUCTION

CD23 expression can be influenced by the presence of IL-4, IL-13, anti-CD40 and PMA, and this has been demonstrated in the previous studies. Although STAT-6 and NFκB pathways were implicated in CD23 transcriptional activation through immunoprecipitation and EMSA studies, conclusive isoform (CD23a or b) expression patterns remained to be fully elucidated. In order to address the details of the separate isoform pathways in this chapter, the CD23a and b promoter regions were studied by designing reporter vector constructs for use in stimulation experiments. The reporter vector used in this study was one expressing a truncated secreted form of alkaline phosphatase (SEAP), which can be detected by chemiluminescent assay, and is termed pSEAP. There are several advantages to this system, including the fact it is extremely sensitive (detecting as little as 10^{-13}g of SEAP protein), is non-radioactive and that the cells need not be lysed for analysis (allowing for further manipulation).

A diagramatic representation of the pSEAP Enhancer vector is shown on Figure 4.1, with the significant regions on it highlighted and described: the multiple cloning site (MCS) contains 11 unique restriction enzyme digestion sites, is the region of insert insertion (Kpn I/Bgl II in this instance) and is located upstream of the SEAP gene; the SV40 late polyadenylation signal is inserted downstream of the SEAP coding sequence to ensure the proper and efficient processing of the transcript in eukaryotic cells; and the SV40 enhancer increases the transcription from inserted promoters (which, from previous experience, is known to be required). The vector contains an

f1 origin for single-stranded DNA production, a pUC19 origin of replication and an ampicillin resistance gene for propagation in *E.coli.* A synthetic transcription blocker (TB), composed of adjacent polyadenylation and transcription pause sites, reduces background transcription.

In this chapter, the methodology, results obtained and conclusions drawn when full and truncated CD23a and b promoter regions were inserted into the pSEAP reporter vector will be discussed, as will stimulation studies of these constructs.

4.2: RESULTS

4.2.1: The CD23a and CD23b promoter regions: splicing and amplification

In order to fully study the activation of each CD23 isoform, amplification of the promoter region of each was initially performed. The upstream elements of the CD23a and CD23b promoter regions are shown in diagramatic form in Figure 4.2a, along with the specific primer start sites for producing the full promoter and specific splice variants. The actual primer sequences are listed in Figure 4.2b. Both CD23 isoform promoter regions contain several upstream elements, with certain differences being noted between each. The CD23a promoter shows, from upstream to downstream, two IL-4 response elements (IL-4RE), an NFκB site, a glucocorticoid response element (GRE) and a B-cell-specific activator protein (BSAP) or Pax-5 binding site. The CD23b promoter also has two IL-4REs but, in contrast, these are separated by an NFκB site. The second IL-4RE is then closely followed by two AP-1 binding sites. IL-4REs are thought to be the binding sites for the signal transducer and activator of transcription STAT-6 (Delphin & Stavnezer, 1995) and NFκB sites are known to be activated by anti-CD40 antibodies or CD40L (Berberich, *et al.*, 1994). The B-cell-activator protein is discussed in the introduction of this thesis (1.3.2), and is known to a universal B cell activator

(Zwollo, *et al.*, 1997). AP1 has also been implicated in the activation of transcription following anti-CD40 stimulation (Hsing, *et al.*, 1997).

The different primer start sites shown in Figure 4.2a lead to PCR products being amplified which represented the CD23a or b promoter regions to their full extent, or in truncated shortened forms. The truncated variants allowed analysis of promoter region function upon stimulation when increasing numbers of response elements and protein binding sites were removed. The CD23b promoter region, for example, was progressively truncated to the extent that no known response elements were present in the PCR product amplified using the B6 and BR primers, allowing hopefully complete analysis of the important sites required for CD23b expression upon each type of stimulation. It is worth noting at this point that primers were originally designed to remove the GRE and BSAP sites on the CD23a promoter, as well as the two IL-4RE and NFκB sites as shown. Unfortunately these PCRs proved to be unsuccessful, even after several attempts and trial conditions, and this was thought to be perhaps down to the proximity of these sites to the transcription start site of CD23a and subsequently the reverse primer. Upon consideration, it was decided to continue assembling the vector constructs without these GRE and BSAP sites being removed. This allowed for data to be compiled when both IL-4RE sites and the NFκB site were removed from the CD23a promoter.

4.2.2: Construction of CD23 promoter reporter vector constructs.

Once amplified, the CD23 promoter region amplicons were ligated into the MCS of the pSEAP enhancer vector between Kpn I and Bgl II restriction digest sites prior to the transformation of DH5α cells. The reporter vector constructs were then purified and prepared from the cultured cells, and characterised by restriction digest mapping and DNA sequencing.

Figure 4.3 shows an agarose gel of the reporter vector constructs digested with Kpn I and Bgl II, indicating the separated pSEAP vector and cleaved CD23 promoter insert. The linearised vector shows as a band at approximately 4.9kbp and the cleaved inserts are shown as bands reducing in size as promoter region deletion progresses. This gel clearly shows that inserts of approximately the correct size have been inserted into the pSEAP vector, these being 577, 422, 385, 259bp respectively for the CD23a promoter truncations and 391, 322, 284, 264, 184, 111bp respectively for the CD23b promoter. Restriction digest maps also confirmed that these were the correct length, as well as in the proper orientation (not shown). Further and conclusive evidence for the successful construction of the reporter vector constructs was achieved through sequence analysis of the DNA, and an example of this for the CD23a Prom/ΔS61 is shown in Figure 4.4. There are seen to be two small changes in DNA sequence, which are underlined, and these are G^{545}-A^{545} and A^{579}-G^{579} transitions. It was not certain, upon looking at the graphic print-out of the sequence, as to whether these changed bases were genuinely modified in the PCR process or whether the sequencing readout is simply ambiguous at this point. In fact, these changes are not situated in any of the sequence that codes for the upstream elements being studied and do not lead to the encoding of any inappropriate start or stop codons, so they are considered inconsequential. All of the reporter vector constructs were thus considered ready for use in the following SEAP assays.

4.2.3: Transfection efficiency of B cells with pSEAP/CD23 promoter reporter vector constructs

Electroporation was chosen as the transfection method of choice in these experiments, as chemical and lipid transfection methods have been seen, in certain circumstances, to interfere with subsequent reporter assay studies (from Tom Carr, personal communication). Although a highly successful technique, electroporation can be particularly harsh on human B cells unless the electroporation voltage is

strictly controlled and optimised. In short, enough voltage has to be present to allow the vectors to enter the cells, but not so much as to kill them.

4.2.3a: Analysis of cell death in relation to electroporation voltage

A constitutively active pSEAP control vector was electroporated into tonsillar B cells at varying voltages, increasing from 0.05 to 0.4kV (voltages which had previously been used in T cell electroporations). Following 48hrs of culture, cells were stained with 40μg/ml of propidium iodide and analysed for cell death by flow cytometry. These results are shown in Figure 4.5 and indicate that cell death increases with electroporation voltage, with the biggest increase being noted with 0.4kV. This result does not indicate relatively high levels of cell death although, upon visual analysis under a microscope, there appears to be a considerable number of damaged cells. With hindsight, it would have been extremely informative to have analysed the cultures for apoptotic cells - e.g. with annexin-V staining, as well as staining for complete cell death. The electroporation voltage of 0.30kV was used in all of the following experiments, as this setting resulted in an acceptable level of both cell death and control SEAP assay results (not shown).

4.2.3b: Internal control for transfection efficiency / Chloramphenicol Acetyl Transferase (CAT) assay

As previously described, SEAP results are detected by chemiluminescence assay on culture medium. These are representative of the amount of SEAP being expressed by the cells, but give no indication of the transfection efficiency of each electroporated set - which could lead to incorrect comparison of results. Therefore, an internal control system was required to take account of tranfection efficiency, ensuring that results could be attributed to change in stimuli or reporter vector construct alone. In order to facilitate this, a pLW2 control plasmid was co-

transfected into cells with the pSEAP reporter vector. The plasmid pLW2 comprises a CAT gene under the control of the HSV (Herpes Simplex Virus) tk promoter, which is constitutively active. In each case, a CAT assay was performed as well as the SEAP assay to allow results to be correlated with the actual transfection efficiency. A representation of the pLW2/CAT reporter vector is shown in Figure 4.6, and a typical CAT autoradiograph is shown in Figure 4.7. The upper two bands of the autoradiograph represent the converted chloramphenicol, with the intensity of each band being measured by phosphorimaging analysis. The intensity of the two bands in each sample are then added together, and used to perform standardising calculations to correct the SEAP results for transfection efficiency. A worked example of this calculation is shown in Figure 4.8.

The results which will be shown are all representative of experiments carried out on several tonsillar B cell preparations, and it was therefore paramount to account for the variability of each such set. In these instances, each non-stimulated vector construct result was designated as "100%" and the subsequent stimulated results from the same vector correlated accordingly. As implied, each tonsillar set can be variable to differing degrees, mainly in cell number and base-line marker expression. Many cells were required for each experimental set, hence the necessity to use more than one set in each case.

4.2.4: The CD23a promoter is activated with IL-4, whereas the CD23b promoter can be triggered by IL-4, anti-CD40 and anti-μ chain

Initial studies were performed using only the full CD23a and b promoter constructs along with CAT vector transfections into P3HRI cells, which were chosen as they perform similarly to tonsillar B cells. As stated earlier, supply of primary tonsillar tissue was erratic and a robust and representative cell line model was required. The

results for the full CD23a and CD23b promoter vector construct stimulations are shown on figures 4.9a and b, and show the following trend.

Upon stimulation with anti-CD40 the CD23a promoter transfectant show no significant up-regulation of SEAP expression, while a considerable decrease in promoter activity was evident with anti-μ chain treatment. IL-4 stimulation, on the other hand, leads to a two-fold increase in CD23a promoter activity. These results were a little surprising, as it was expected that anti-CD40 would lead to activation of this promoter through the NFκB site that was present. Anti-μ treatment also led to interestingly negative results, as this antibody is known to be a dose-dependent universal activator of B cell transcription. Explanations for these observations will be discussed in due course.

Results for the CD23b promoter reporter vector construct studies (Fig. 4.9b) are strikingly different, as anti-CD40, anti-μ and IL-4 are all seen to stimulate the promoter activity. This suggests differential regulation of each CD23 promoter isoform. The above experiments were repeated (with the inclusion of PMA stimulation) in tonsillar B cells (Figures 4.10a and 4.10b) and tonsillar B cells were also used in all of the succeeding construct studies, utilising both full and truncated promoters and including the further addition of PMA as a potential stimulant.

4.2.5: Full and truncated CD23 promoter reporter vector construct studies indicate activation occurs through specific response elements

All of the CD23a and b promoter vector constructs were electroporated as before into the tonsillar B cells, and stimulated with IL-4, anti-CD40, anti-μ or PMA. Upon media and cell harvesting for SEAP and CAT assays, respectively, $5x10^5$ cells were also removed from each sample for analysis of CD23 expression by flow cytometry, in order to observe if they reacted normally upon stimulation and no adverse effects

resulted from the electroporation. Figures 4.11 and 4.12 show examples of the dot plots resulting from stimulation of cells transfected with the CD23a and b promoter reporter vector constructs respectively, with the results for the percentage of electroporated B cells expressing CD23 for all of the CD23 constructs and stimulation experiments being calculated and presented on Table 4.1. In experiments using the CD23a reporter constructs, for example, it was observed that, in each instance, IL-4 stimulation resulted in approximately 50-60% of B cells expressing CD23. It can be stated that electroporation and transfection of these tonsillar B cells does not appear to alter the pattern of CD23 expression by stimulated cells, in comparison with the non-electroporated cells discussed in Chapter 3. Unfortunately, the flow cytometric analysis in chapter 3 was conducted following only a 24hr stimulation period with each of the stimulants, whereas the results noted in this chapter were following 48hr simulations. This was because 48hr is the optimal time for SEAP protein expression, and secretion in to the culture medium, following electroporation with the reporter vector constructs. If time had permitted, comparable 48hr stimulation experiments of non-electroporated cells would have been accomplished.

4.2.5a: CD23a full-length and truncated promoter reporter vector stimulation studies

IL-4 is the primary stimulant involved in the activation of the CD23a promoter (Figs. 4.9a and 4.10a). It was hypothesised that, if IL-4 was activating CD23a expression through a STAT-6 dependent pathway, removal of the IL-4RE/STAT-6 binding sites would result in a loss of activation. The truncated CD23a promoter region results are shown in Figures 4.13a-d, and are arranged such that results from each stimulus are shown in separate graphs.

When analysed, it can be seen that IL-4 stimulation (Fig. 4.13a) leads to a 60% increase in activation of the full length CD23a promoter. However, upon removal of only the first IL-4RE, this activation is completely ablated and does not recover upon additional truncation. This implies that IL-4 leads to activation of CD23a mainly through a pathway which utilises the first IL-4RE on the CD23a promoter. As discussed in the introduction, the presence of IL-4 leads to phosphorylation and dimerization of STAT-6 which, in turn, binds to the IL-4RE and leads to transcriptional activation of CD23.

As anticipated from the initial experiments utilising only the full CD23a promoter (Figs. 4.9a, 4.10a), anti-CD40, anti-μ and PMA do not lead to an increase in reporter vector expression. In addition, no substantial increase in expression is noted following truncation of each promoter region prior to stimulation, although perhaps a small rise is evident following the removal of the second IL-4RE with anti-μ treatment. This increase in expression is of approximately 10% and it is difficult to interpret whether this is an artefact of experimental technique or, perhaps, down to the genuine removal of some unknown negative regulator.

4.2.5b: CD23b full-length and truncated promoter reporter vector stimulation studies

The results for the full-length CD23b promoter reporter vector construct and its truncated forms are extremely informative and interesting, and are shown on Figures 4.14 a-d.

The full CD23b promoter displays a 100% increase in SEAP expression following activation with IL-4 (Fig. 4.14a) and, in contrast to CD23a, there is no significant decrease in activation upon removal of the first IL-4RE. Further truncation leading to the additional removal of the following NFκB site also fails to induce a drop in

SEAP expression. Removal of the second IL-4RE, however, leads to the complete loss of increased promoter/SEAP activation and expression - which returns to baseline levels. This indicates that it is this second STAT-6 binding site that is paramount in the IL-4-stimulated induction of CD23b expression, as removal of the first STAT-6 site has no observable effect upon gene expression but removal of the latter completely abolishes it. No additional changes in promoter activation occurred upon the removal of the two AP1 sites.

Anti-CD40 stimulation of the CD23b promoter and its truncated forms is also extremely interesting (Fig. 4.14b). Anti-CD40 induces a modest increase in the activity of the full-length CD23b promoter of about 20%. However, this activation increases to around about 50% following removal of the first IL-4RE site, suggesting that an undefined negative regulator of anti-CD40 induced activation was potentially present in this deleted area. Further truncation and removal of the next NFκB site resulted, not surprisingly, in the promoter activity dropping to the original unstimulated level. This made sense, as it was expected that anti-CD40 treatment lead to activation of CD23b through a pathway involving the NFκB binding site of its promoter.

Treatment of full-length CD23b promoter reporter construct transformed cells with anti-μ leads to an increase in SEAP expression of approximately 50% (Fig. 4.14c). This trend continues right up until the removal of the second AP1 site, and thus removal of all the known response elements. This suggests that, either anti-μ can activate this promoter through all or some of these sites or that the second AP1 site is of the most importance.

PMA activation results, as shown in Figure 4.14d, were altogether more confusing than previous data sets. PMA induced an increase in SEAP expression of approximately 20% with the full-length CD23b promoter, although this appears to

decrease slightly upon the removal of the first IL-4RE and NFκB binding site. Stimulus induced expression from the CD23b promoter then proceeds to rise to approximately 20, 30 and 60% above unstimulated values upon each succesive deletion of IL-4RE(2), AP1(1) and AP1(2). This bi-phasic trend is unusual and may indicate that some sort of repressor element is being removed and that a previously unidentified site responsive to the mediators of PMA stimulation is present nearer to the transcription start site of the CD23b promoter. Incidentally, further visual analysis of the CD23b promoter region led to the identification of a third putative AP1 binding site approximately 70bp downstream of the second AP1 site. It is also possible that a decrease in insert size means that the vector construct was more easily inserted into the cells, and that the cells were less perturbed by the presence of a smaller vector. This is thought unlikely, however, as electroporation efficiency is accounted for by the insertion of the pLW2 control vector and CAT assay. Also, experiments with the CD23a promoter, which has even smaller truncated inserts, do not show this phenomenon.

4.3: Conclusion and discussion

The main conclusions of this chapter are that, firstly, the CD23a and CD23b promoter regions are differentially activated, as shown in reporter vector construct studies. The CD23a promoter shows an increase in activity with IL-4 stimulation only, whereas the CD23b promoter is susceptible to all of IL-4, anti-CD40, anti-μ and PMA induction. Secondly, truncation of the inserted CD23a and b promoter regions indicates that the CD23a promoter appears to utilise the first IL-4RE/STAT-6 binding site only, while the CD23b promoter is activated through its second available IL-4RE/STAT-6 site. Truncated CD23b promoter reporter vector constructs studies following anti-CD40, anti-μ and PMA stimulation revealed that removal of the NFκB binding site abolished the increase in promoter activity

following anti-CD40 stimulation, and that anti-μ and PMA appear to utilise all or some of the identified sites and presently undefined sites, respectively.

Flow cytometry of the electroporated cells following 48hr stimulation showed that the cells reacted much like un-electroporated tonsillar B cells and, thus, the actual transfection technique does not appear to be damaging the live cells. A small increase in cell death was, however, noted upon observation following electroporation, but cells surviving the initial shock were considered to show perfectly normal responses to stimulation.

If more time had permitted, further analysis using reporter vector construct studies would have been attempted. CD23a and b promoter point mutation studies would have been carried out to facilitate the removal of certain specific response elements, intermediately placed in the promoter region, in addition to the truncated promoter experiments - which are representative of successively cleaved promoters, rather than the removal of central sites and leaving surrounding ones. The removal of central and specific sequences would allow analysis of the possibility that perhaps some response elements work together or compensate for the loss of each other. This theory has been hinted upon by Delphin and Stavnezer (Delphin & Stavnezer, 1995), who hypothesised that STAT-6 and NFκB worked together to increase the expression of CD23. Also, if further time had been available, treatment of the transfected cells with more than one stimulant would have been undertaken. An example of this would have been IL-4 in association with anti-CD40, the combination of which has been implicated in IgE and CD23 superinduction (Clark & Ledbetter, 1994; Richards & Katz, 1997), and also treatment with anti-CD40 and anti-μ. These latter two experiments, in conjunction with the removal of certain response elements, would have been extremely enlightening in relation to the proposed co-operation of certain transcription factors. Also, data would have been

obtained as to whether compensation for missing response elements is achieved upon dual stimulation.

The fact that CD23a and CD23b are differentially stimulated and expressed, and that CD23b is also found on other cells of the immune system apart from B cells, supports the theory that they are involved in B cell function and IgE-mediated immunity, respectively (Yokota, *et al.*, 1988; Yokota, *et al.*, 1992). However, reporter vector construct studies are reliant on removing the promoter region of genes from its *in vivo* environment and placing it in a reporter vector prior to transformation. These results, therefore, are really a representation of the ability of the CD23a and CD23b promoter regions to stimulate the expression of SEAP in the pSEAP enhancer vector - and not for the actual expression of cell surface CD23. The CD23 promoter regions may also be subject to negative regulatory signals *in vivo* that may not be present in the reporter vector construct studies. A further experimental technique was therefore required to support these findings *in vivo* and a perfect candidate for this was considered to be *in vivo* footprinting, incorporating ligation-mediated PCR. *In vivo* footprinting allows for the analysis of the binding of transcription factors to specific cellular DNA intracellularly, and thus gives promoter activation information most closely related to the cells in their natural state.

Figure 4.1: Map of pSEAP Enhancer vector

The pSEAP enhancer vector is 4.9kb in size and comprises several functional regions, which are highlighted that can be described as follows: the multiple cloning site (MCS) contains 11 unique restriction enzyme digestion sites, is the region of insert insertion (Kpn I/Bgl II in this instance) and is located upstream of the SEAP gene; the SV40 late polyadenylation signal is inserted downstream of the SEAP coding sequence to ensure the proper and efficient processing of the transcript in eukaryotic cells; and the SV40 enhancer increases the transcription from inserted promoters (which, from previous experience, is known to be required). The vector contains an f1 origin for single-stranded DNA production, a pUC19 origin of replication and an ampicillin resistance gene for propagation in *E.coli*. A synthetic transcription blocker (TB), composed of adjacent polyadenylation and transcription pause sites, reduces background transcription.

CD23 promoter inserts were cloned in to the vector via the Kpn I/Bgl II digest sites in the MCS.

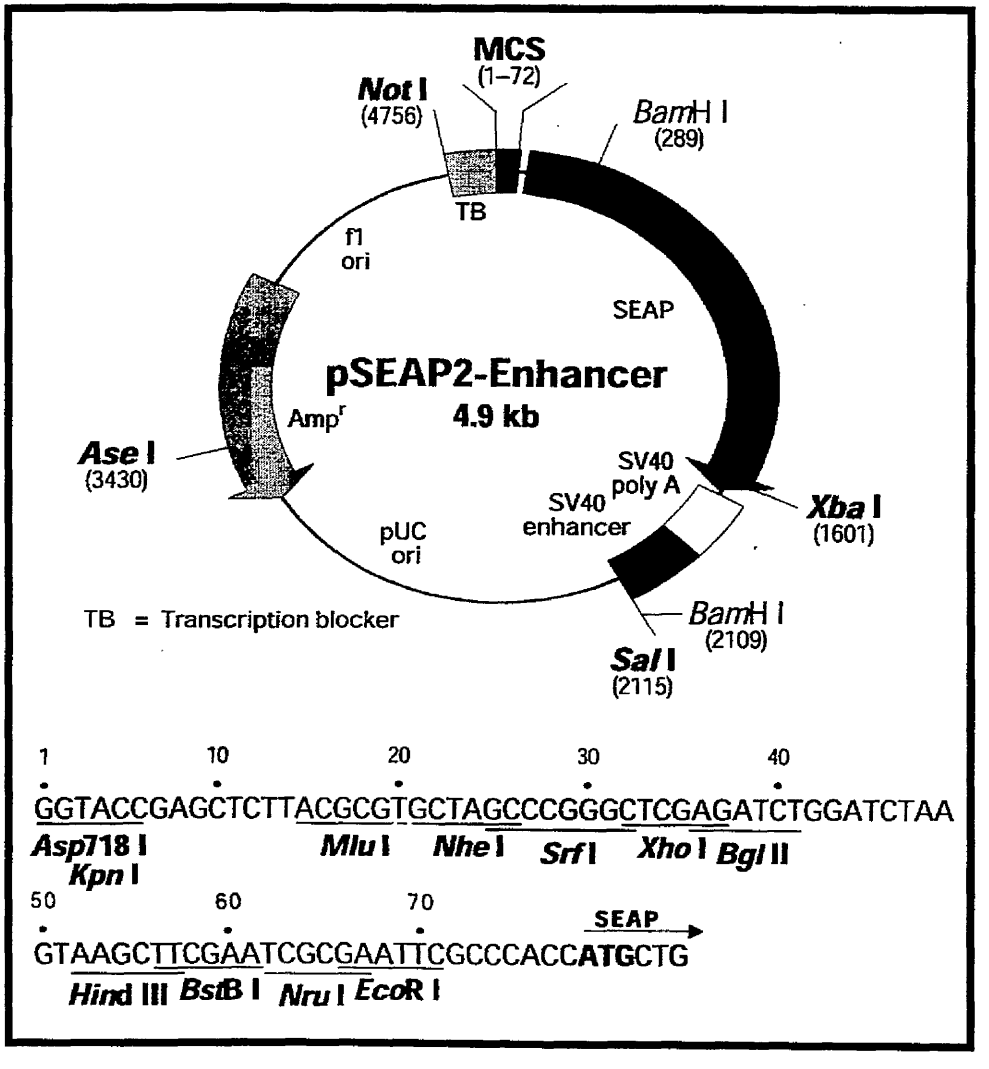

MCS
(1–72)
Not I
(4756)
BamH I
(289)
TB
f1
ori
SEAP
pSEAP2-Enhancer
4.9 kb
Amp^r
Ase I
(3430)
SV40
poly A
SV40
enhancer
Xba I
(1601)
pUC
ori
TB = Transcription blocker
BamH I
(2109)
Sal I
(2115)
1 10 20 30 40
GGTACCGAGCTCTTACGCGTGCTAGCCCGGGCTCGAGATCTGGATCTAA
Asp718 I
Kpn I
Mlu I
Nhe I
Srf I
Xho I
Bgl II
50 60 70
SEAP
GTAAGCTTCGAATCGCGAATTCGCCCACCATGCTG
Hind III
BstB I
Nru I
EcoR I

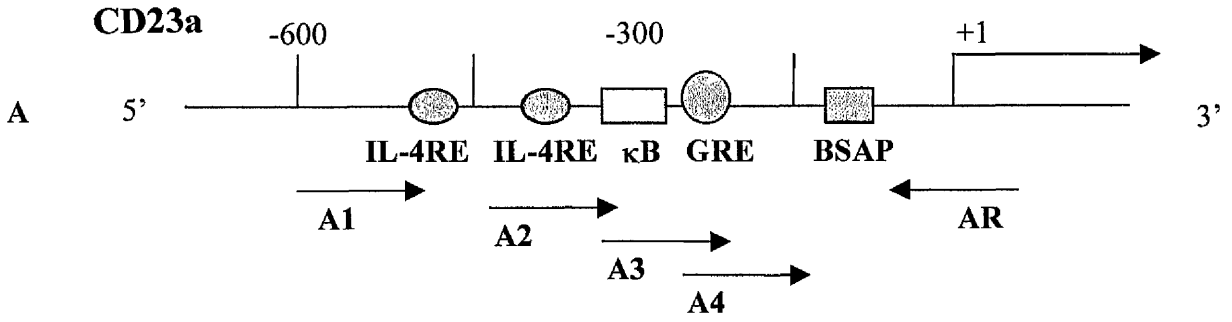
Figure 4.2a: Upstream elements of the CD23a and CD23b promoter regions

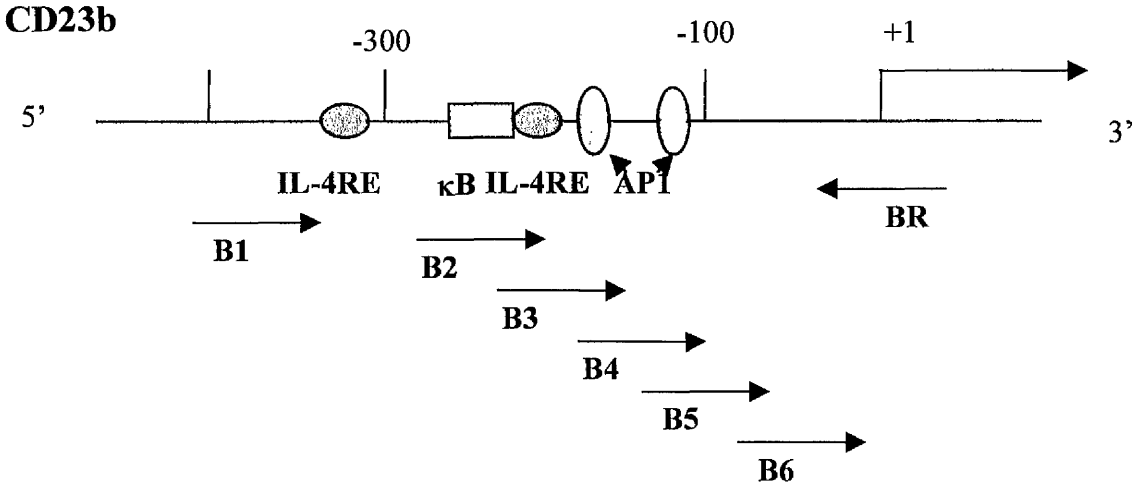
The CD23a and b promoter regions are approximately 600 and 400 base pairs in length, respectively. Both promoter regions are known to contain several upstream elements and putative protein binding sites based on work by Tinnell *et al* in 1998 and extensive searches on transcription factor binding site databases (ie TRANSFAC). A representation of these sites is shown here, and the specific primer start sites for producing the full promoter and specific splice variants are highlighted by the arrows, with the primer sequences detailed below .

Figure 4.2b: Primer sequences for amplification of the CD23a and CD23b promoter regions

Synthetic oligonucleotides of the CD23a and CD23b promoter regions, containing both a Kpn I and Bgl II restriction enzyme site, were designed. These primers were then used to amplify CD23 promoter regions of DNA by polymerase chain reaction (PCR), as described in section 2.2.6a of the materials and methods chapter.

A





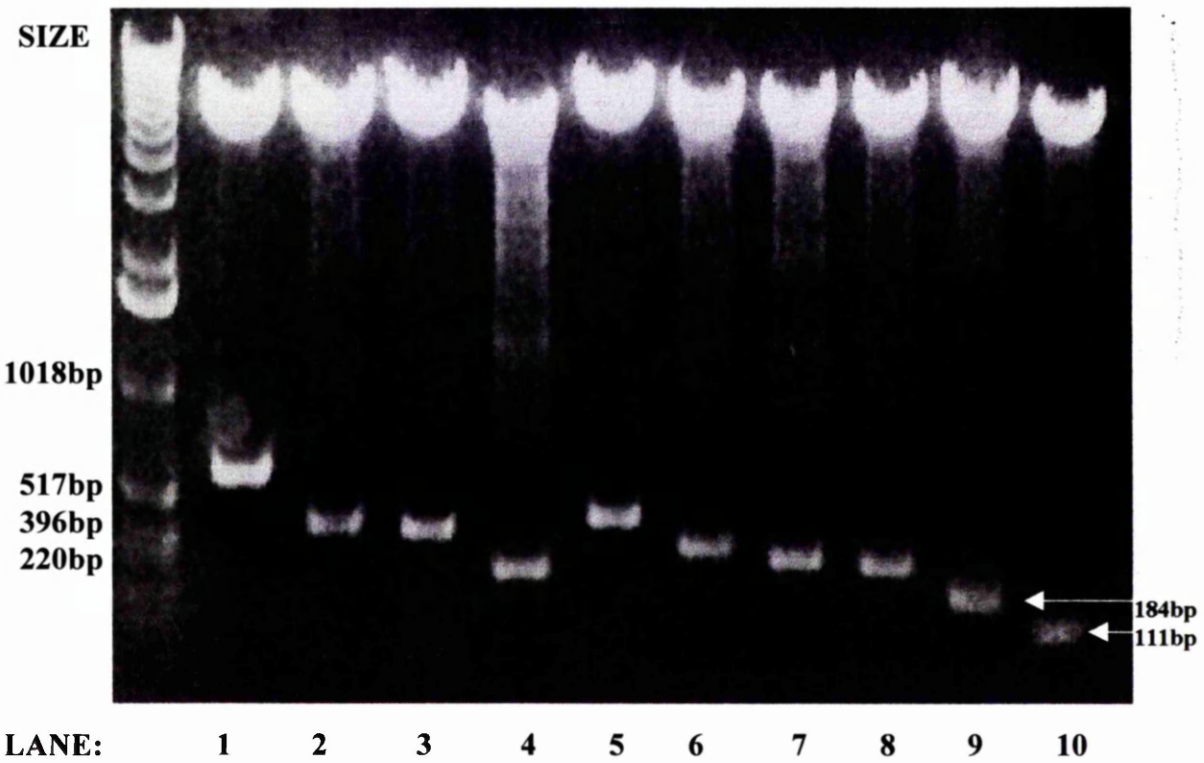
B

PRIMERS:

A1: Full CD23a Promoter - GGC GGT ACC AAA CTG TCC ATA TTT GAC C
A2: CD23a Prom/ΔS61 - GGC GGT ACC AGC CTA TTT GCT CAA TCA TC
A3: CD23a Prom/ΔS62 - GGC GGT ACC TGG TGG GGT GTC TGC TGG TA
A4: CD23a Prom/ΔκB - GGC GGT ACC AGT GGT ATG ATT CAG TGT GCA GTA
AR: CD23a Prom/Reverse - GCA GAT CTT GAC AGC TTC TAT TTT CAT
B1: Full CD23b Promoter - GGC GGT ACC ATG GAG GAA GGT CAA TAT TC
B2: CD23b Prom/ΔS61 - GGC GGT ACC GAA GCG GGG CTT CCC AGT CCC
B3: CD23b Prom/ΔκB - GGC GGT ACC TGA ATT TCT AAG AAA GGG
B4: CD23b Prom/ΔS62 - GGC GGT ACC TGG TGT GAG TAA GGA GGT GA
B5: CD23b Prom/ΔAP1(1) - GGC GGT ACC TTT TCT GAT TCA ACA CCC TC
B6: CD23b Prom/ΔAP1(2) - GGC GGT ACC GCA ATA GAG TCA GAG GCC AA
BR: CD23b Prom/Reverse - GCA GAT CTG CAC TCA CCC TGG CTT GG

Figure 4.3: Restriction digest of pSEAP Enhancer vector containing CD23 promoter inserts

Assembled pSEAP reporter vector constructs containing full and truncated CD23 promoter regions were digested with the restriction enzymes Kpn I and Bgl II, as described in section 2.2.6d. The resulting digests were electrophoresed on a 1% agarose/10mg/ml ethidium bromide gel, along with the indicated DNA size marker, and viewed under ultra-violet (UV) light.



1. CD23a
2. CD23aΔIL-4RE(1)
3. CD23aΔIL-4RE(2)
4. CD23aΔκB
5. CD23b
6. CD23bΔIL-4RE(1)
7. CD23bΔκB
8. CD23bΔIL-4RE(2)
9. CD23bΔAP1(1)
10. CD23bΔAP1(2)

Figure 4.4: Sequencing analysis of the pSEAP/CD23a Prom ΔS61/IL-4RE reporter vector construct

Sequencing analysis was carried out by the Institute of Biomedical and Life Sciences (IBLS) sequencing service on all of the assembled reporter vector constructs, and a representative sequence result is shown here for CD23a Prom ΔS61.

The sequences of enhancer elements are highlighted, and any changes noted from the published sequences are underlined.

CD23a Promoter ΔS61sequence:

3' GNNNGGTNTNNNATTGAANNCNTTNaATGGGCTTGCCaCCCAGATAACATT

BSAP

535 ACACGCATGGCCTCTCCTGGTGCTCCCCACACCCGGGTGAAGTCCGTGTTA 485

484 TTATTATCTCCATTTCTCAGGTAAGAGAATTGGGCGAGAACTAGAGATGTT 434

433 AAGCAGCAAGTTCCCACAGGGCTGAAGGAGGTGAGTGGTAGCGTCAAGAT 383

GRE

382 GTGGACCACTGTTACTGCACACTGAATCATACCACTATGGCCGATCACAGA 332

331 GGGCCCTGGGGAGCCACGGAAGGCTCTAGGCAGAGGCAGGGCCTCTTGGG 281

280 TTAGAAGGACGGCCTGGAGTTGGGATCCTGCCACCCGGCACTTACCAGCA 230

NFκB

S62

229 GACACCCCACCATGAGATTATGTCTCTTGATGATTGAGCAAATAGGCT 182 5'

Figure 4.5: Analysis of electroporated cell death by propidium iodide (PI) staining.

A constitutively active pSEAP control vector was electroporated into tonsillar B cells at varying voltages, increasing from 0.05 to 0.4kv (2.2.6i). Cells were cultured for 48hr, stained with 40μg/ml of propidium iodide and analysed for cell death by flow cytometry (2.2.3). The graph represents the percentage of PI-stained dead cells counted upon increasing electroporation voltage.

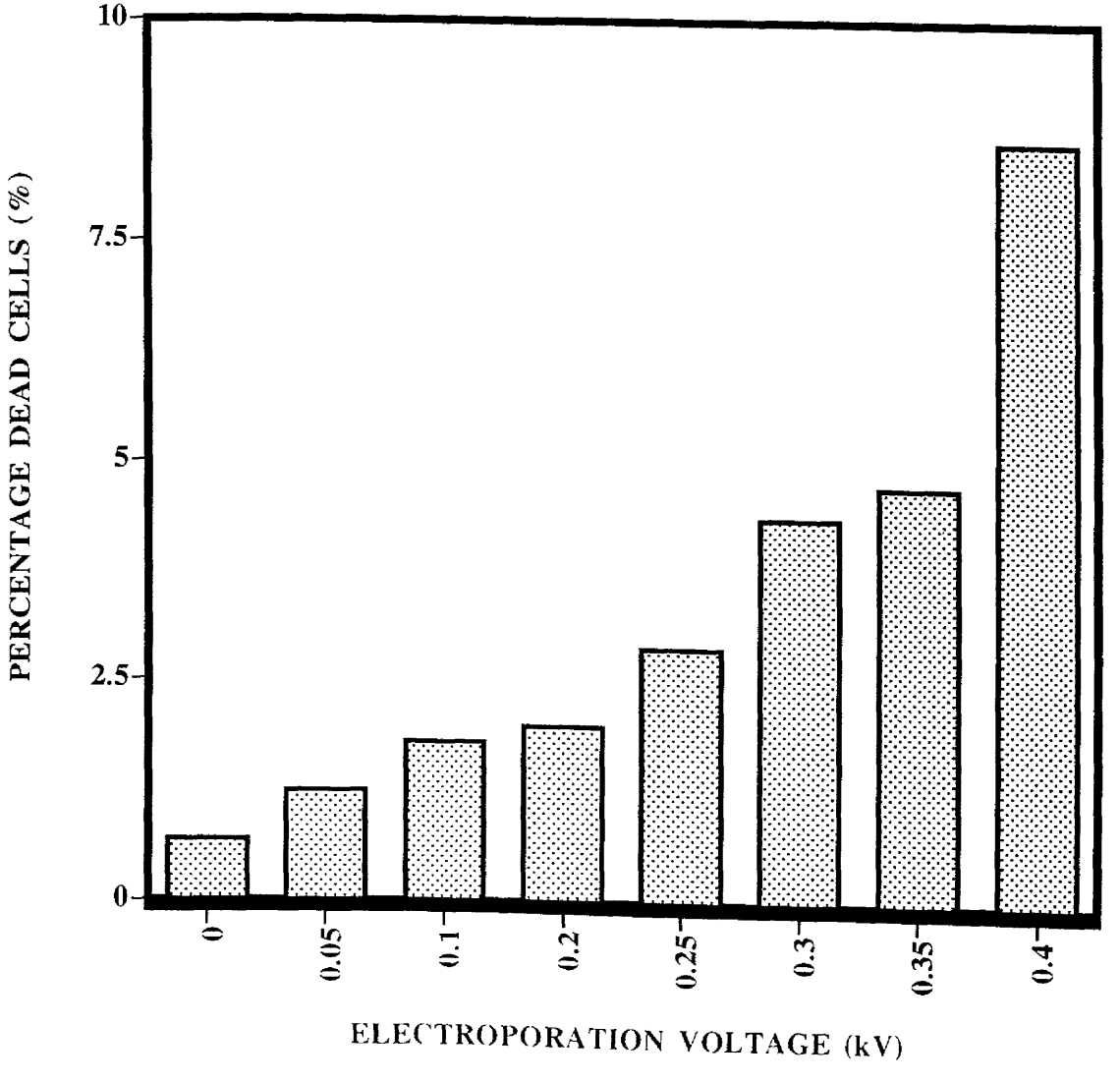

10
7.5
5
2.5
0
PERCENTAGE DEAD CELLS (%)
0
0.05
0.1
0.2
0.25
0.3
0.35
0.4
ELECTROPORATION VOLTAGE (kV)

Figure 4.6: Map of pLW2 reporter vector

pLW2 is a 3.77kb reporter vector comprising a CAT gene under the control of the HSV (Herpes Simplex Virus) tk promoter, which is constitutively active. Restriction enzyme digest sites are noted, as is the ampicillin resistance gene which allows for selection of transfectants upon transformation of competant cells.

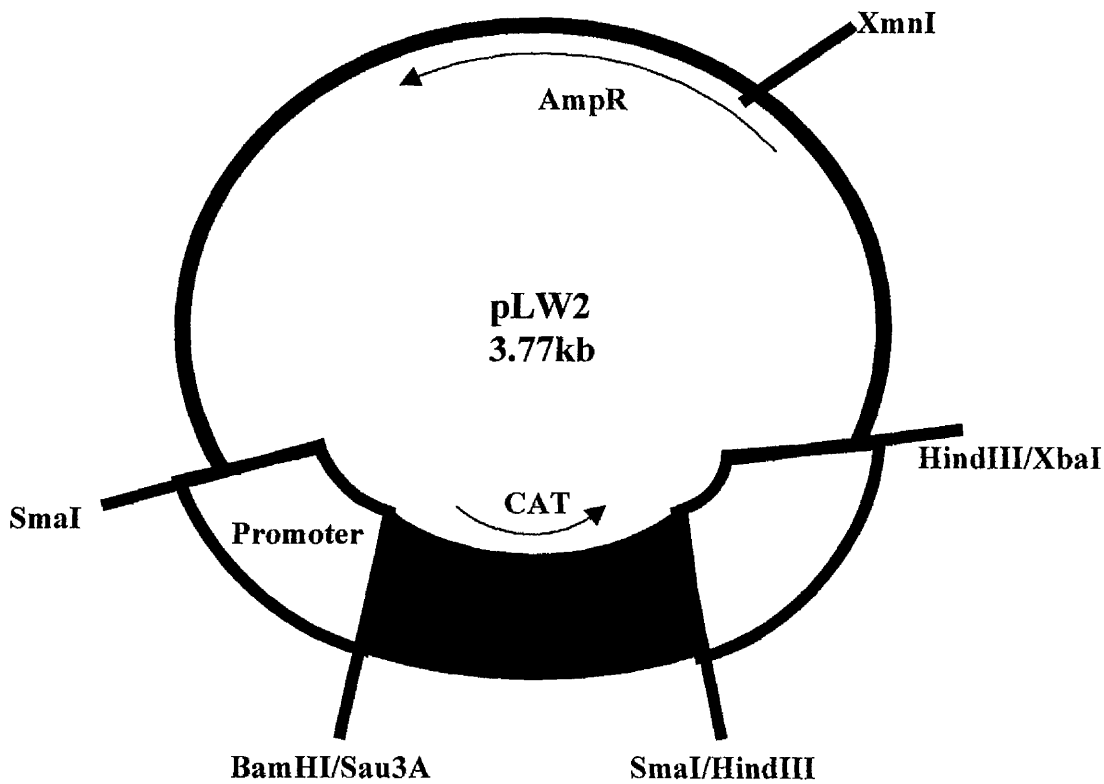

XmnI
AmpR
pLW2
3.77kb
HindIII/XbaI
SmaI
Promoter
CAT
BamHI/Sau3A
SmaI/HindIII

Figure 4.7: Chloramphenicol Acetyl Transferase (CAT) assay autoradiograph

20μg of pLW2 control plasmid was co-transfected (via electroporation) with 40μg of pSEAP reporter vector into $5x10^6$ B cells, as described in section 2.2.6i of the methods chapter of this thesis. Following culture for 48hr in complete RPMI-1640 medium, cells were harvested and washed twice with ice-cold PBS. The chloramphenicol acetyl transferase (CAT) assay was then performed in accordance with the protocol in section 2.2.6k. Briefly, cells were resuspended in 250mM Tris-HCl (pH 7.8), subjected to 3 freeze/thaw cycles and cellular acetylases heat-inactivated at 65°C. Acetyl co-enzyme A was then added to a final concentration of 5mM, followed by 1μl of ^{14}C-chloramphenicol and incubation at 37°C for 3 hr. The reaction was terminated by the addition of 600μl ethyl acetate and the top layer was removed and lyophilised, prior to resuspension in 20μl ethyl acetate and thin layer chromatography (TLC).

TLC plates were visualised as shown by phosphorimage analysis, with the highlighted bands representing converted chloramphenicol. The intensity of these bands was measured and used to perform transfection efficiency standardising calculations for the corresponding pSEAP assay.

Lanes A-F are representative of samples co-transfected with 20μg of pLW2 control plasmid along with 40μg of each CD23b promoter reporter construct.

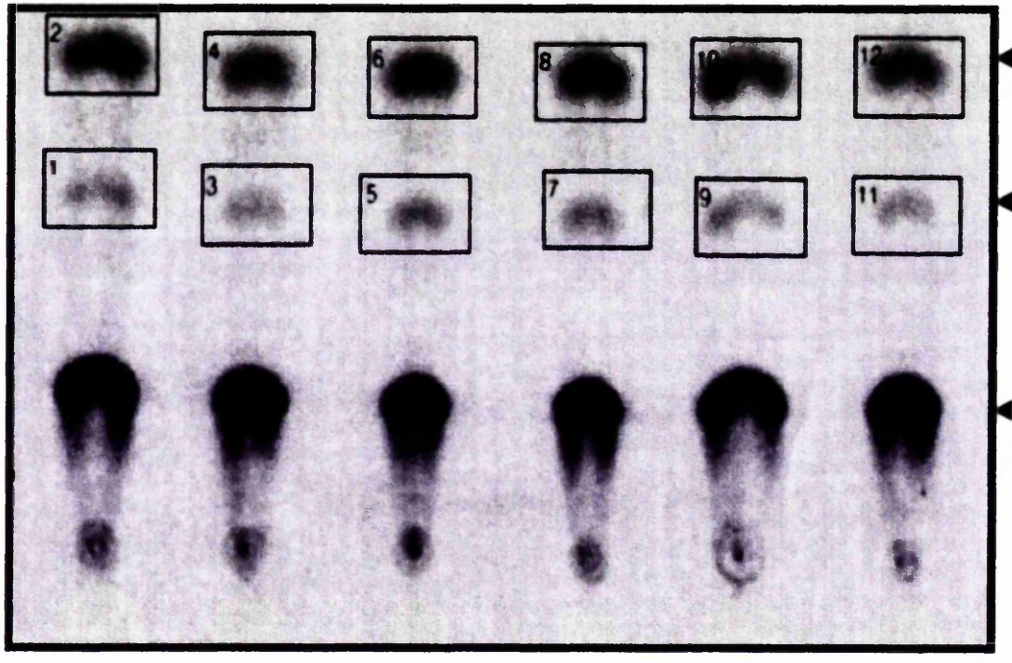

2
4
6
8
10
12
1
3
5
7
9
11
Converted Chloramphenicol
Unconverted Chloramphenicol
Sample:
A
B
C
D
E
F

Figure 4.8: Calculation of SEAP results in regards to transfection efficiency

Following CAT assay (2.2.6k) transfection efficiency was calculated by, firstly, adding together the two results for band intensity resulting from the pLW2 electroporated cells, defining the highest of these as 1 and standardising the other results accordingly. SEAP results were then adjusted according to the CAT assay result determined ratio, and thus were normalised for transfection efficiency.

A minimum of 3 experimental sets for each experiment were performed and, to standardise results in relation to variability in prepared primary tonsillar B cells, all results were represented in relation to unstimulated cells being termed as 100%. Resulting error bars are shown to indicate variability between results from different tonsil sets.

SAMPLE	BAND DENSITY	STANDARD -ISATION RATIO	pSEAP RESULT (RLUs)	ADJUSTED pSEAP RESULT (RLUs)
EXAMPLE	(Upper + Lower bands) X	(Fraction of largest x value) Y	Z	Z x $\frac{1}{Y}$
A	688.9	1	0.463	0.463
B	484.2	0.702	17.31	24.67
C	637.7	0.926	0.413	0.446
D	660.9	0.959	0.441	0.460
E	538	0.781	0.577	0.739
F	336.7	0.489	0.631	1.29

REPRESENTATION OF COMPILED pSEAP RESULTS:

NON-STIMULATED SAMPLES = 100%

STIMULATED SAMPLES

$$\frac{\text{STIMULATED pSEAP RESULTS}}{\text{NON-STIMULATED pSEAP RESULTS}} \times 100 = A\%$$

STANDARD DEVIATIONS AND ERROR BARS

$$S_A = \sqrt{\frac{\Sigma(A\text{-Amean})^2}{n-1}}$$

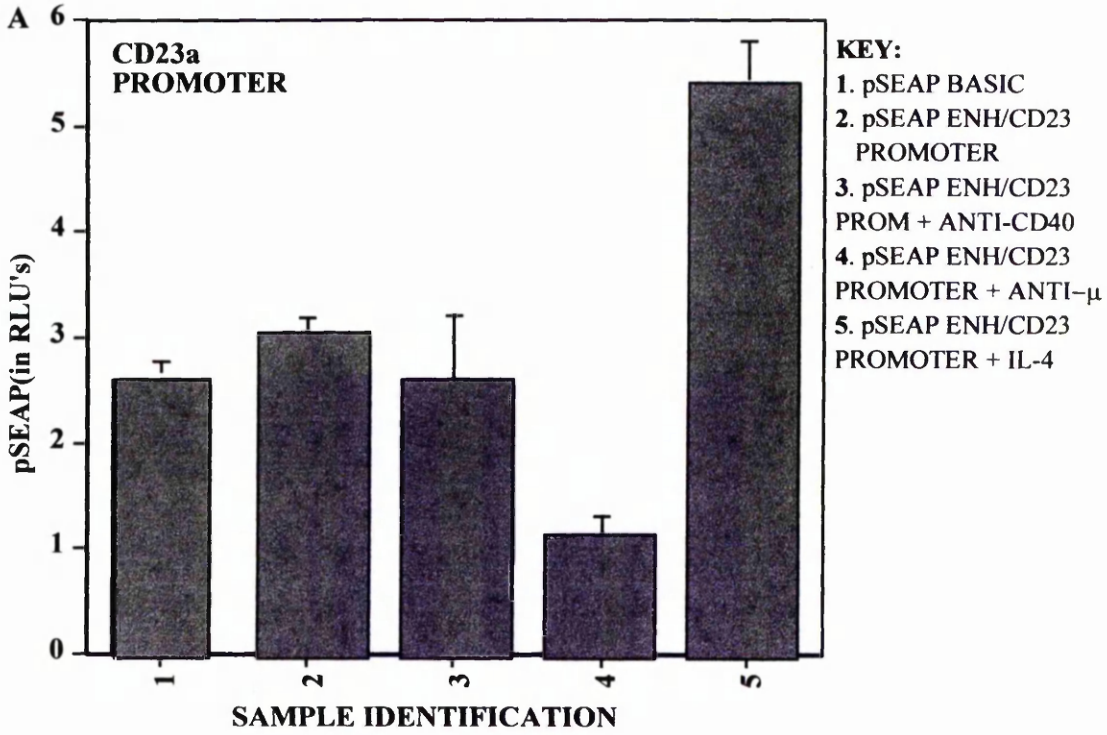
Figure 4.9: SEAP assay results for P3HRI cell pSEAP/CD23a and pSEAP/CD23b Promoter reporter vector construct transfections

40μg of each pSEAP reporter vector construct was electroporated into $5x10^6$ P3HRI B cells, as described in section 2.2.6i of the Methods chapter, along with 20μg/ml of pLW2. Cells were allowed to rest for 2hr, prior to stimulation with 1μg/ml anti-CD40, 1μg/ml anti-μ and 14ng/ml IL-4 and subsequent 48hr culture in complete RPMI-1640 medium. Cells were then harvested (for CAT assay or flow cytometry) and 125μl of medium conserved for SEAP assay, which was performed in accordance with section 2.2.6j. All reagents were supplied by Clontech in their Great EscAPe SEAP Reporter System 2 kit (see Materials 2.1.2 for details) Briefly, medium was centrifuged at 12, 000 x g, to remove any cells, and 15μl was added to the wells of a 96 well opaque plate and diluted with 45μl of dilution buffer. Intracellular acetylases were then heat inactivated at 65°C, prior to the addition of 60μl of assay buffer and 60μl of substrate. The reactions were then incubated for 15min at room temperature (RT), prior to interpretation of results on a plate luminometer.

The results on these graphs have been normalised for transfection efficiency by pLW2 transfection and CAT assay, and are represented in relative light units (RLUs). Error bars are indicative of the differences between triplicate SEAP assays on the same experimental cell set, as only one full set of SEAP electroporations were carried out with P3HRI cells prior to tonsils becoming available.

A = SEAP assay results for CD23a promoter reporter vector constructs

B = SEAP assay results for CD23b promoter reporter vector constructs


A
CD23a
PROMOTER
pSEAP(in RLU's)
0
1
2
3
4
5
6
1
2
3
4
5
SAMPLE IDENTIFICATION
KEY:
1. pSEAP BASIC
2. pSEAP ENH/CD23 PROMOTER
3. pSEAP ENH/CD23 PROM + ANTI-CD40
4. pSEAP ENH/CD23 PROMOTER + ANTI-μ
5. pSEAP ENH/CD23 PROMOTER + IL-4

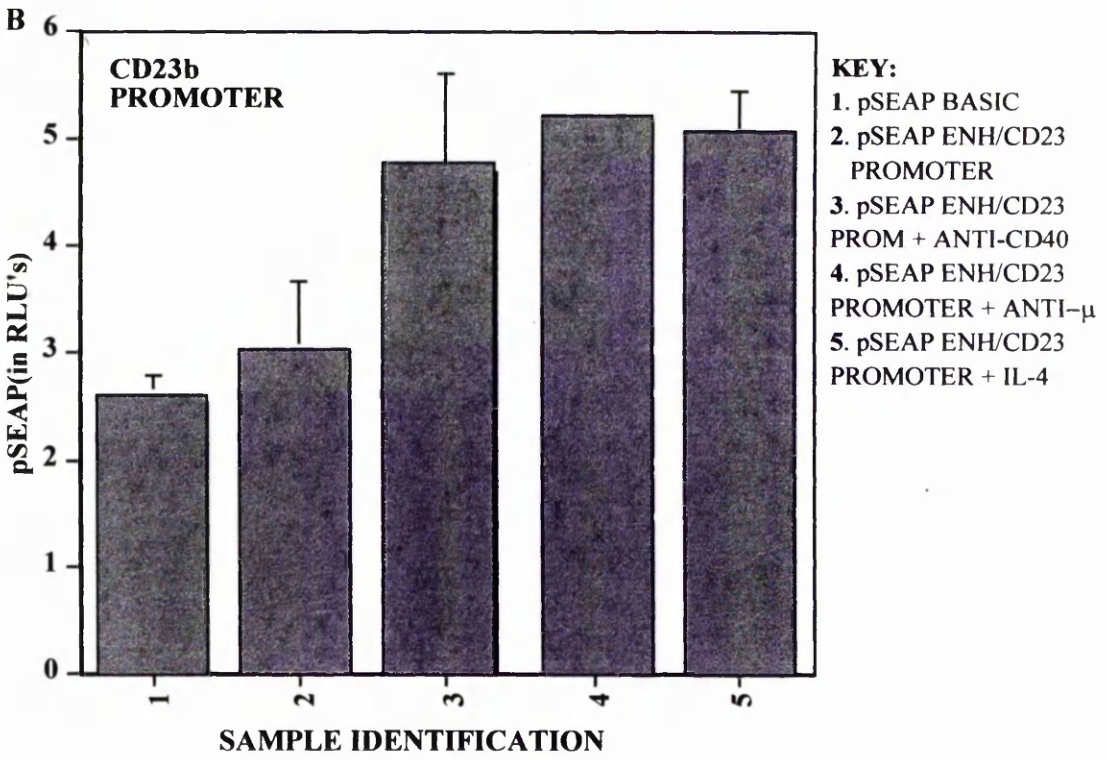

B
CD23b
PROMOTER
pSEAP(in RLU's)
0
1
2
3
4
5
6
1
2
3
4
5
SAMPLE IDENTIFICATION
KEY:
1. pSEAP BASIC
2. pSEAP ENH/CD23 PROMOTER
3. pSEAP ENH/CD23 PROM + ANTI-CD40
4. pSEAP ENH/CD23 PROMOTER + ANTI-μ
5. pSEAP ENH/CD23 PROMOTER + IL-4

Figure 4.10: SEAP assay results for tonsillar B cell pSEAP/CD23a and pSEAP/CD23b Promoter reporter vector construct transfections

40μg of each pSEAP reporter vector construct was electroporated into $5x10^6$ tonsillar B cells, as described in section 2.2.6i of the Methods chapter, along with 20μg/ml of pLW2. Cells were allowed to rest for 2hr, prior to stimulation with 1μg/ml anti-CD40, 1μg/ml anti-μ and 14ng/ml IL-4 and subsequent 48hr culture in complete RPMI-1640 medium. Cells were then harvested (for CAT assay or flow cytometry) and 125μl of medium conserved for SEAP assay, which was performed in accordance with section 2.2.6j. All reagents were supplied by Clontech in their Great EscAPe SEAP Reporter System 2 kit (see Materials 2.1.2 for details) Briefly, medium was centrifuged at 12, 000 x g, to remove any cells, and 15μl was added to the wells of a 96 well opaque plate and diluted with 45μl of dilution buffer. Intracellular acetylases were then heat inactivated at 65°C, prior to the addition of 60μl of assay buffer and 60μl of substrate. The reactions were then incubated for 15min at room temperature (RT), prior to interpretation of results on a plate luminometer.

The results were then normalised for transfection efficiency by pLW2 transfection and CAT assay, and a minimum of 3 experimental sets for each experiment were performed using different tonsil sets. To standardise results in relation to variability in prepared primary tonsillar B cells, all results were represented in relation to unstimulated cells being termed as 100%. Resulting error bars are shown to indicate variability between results from different tonsil sets.

A = SEAP assay results for CD23a promoter reporter vector constructs

B = SEAP assay results for CD23b promoter reporter vector constructs

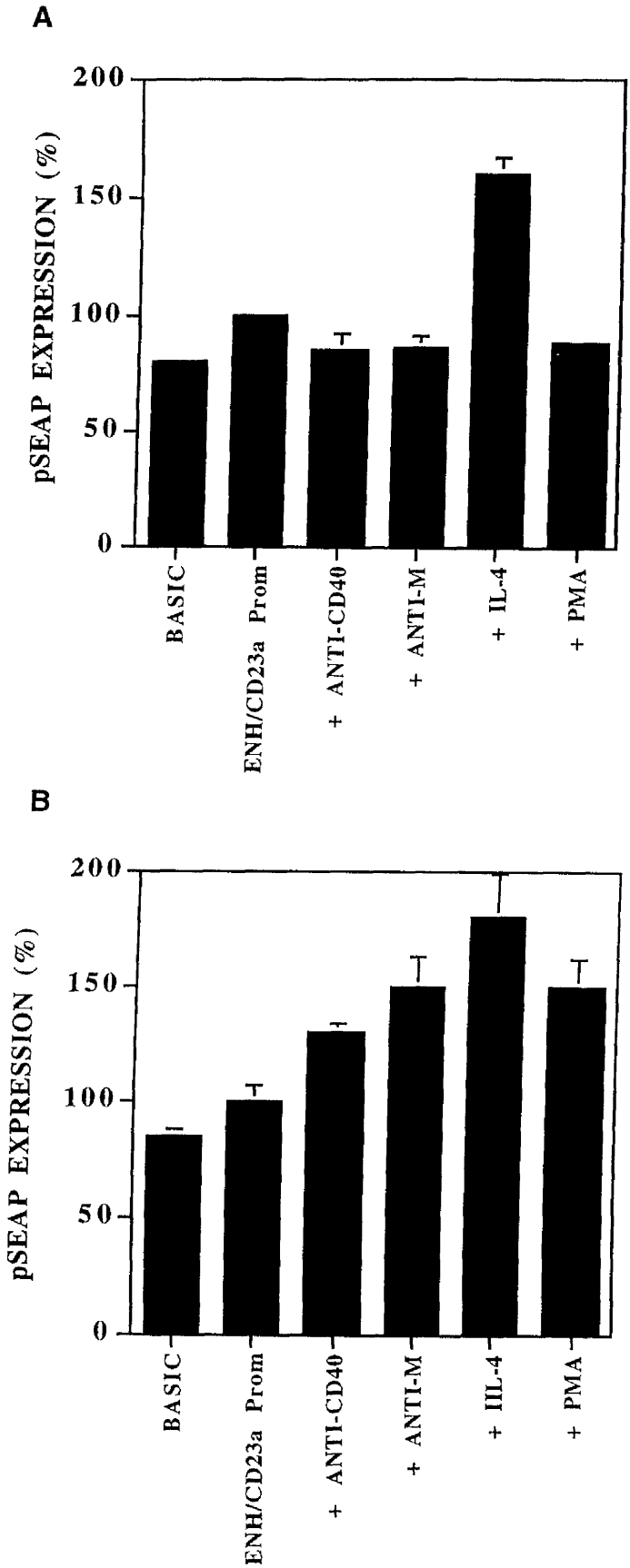
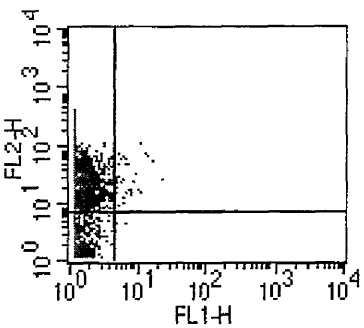

A
pSEAP EXPRESSION (%)
200
150
100
50
0
BASIC
ENH/CD23a Prom
+ ANTI-CD40
+ ANTI-M
+ IL-4
+ PMA
B
pSEAP EXPRESSION (%)
200
150
100
50
0
BASIC
ENH/CD23a Prom
+ ANTI-CD40
+ ANTI-M
+ IL-4
+ PMA

Figure 4.11: Flow cytometric analysis of stimulated CD23 Promoter construct transfected tonsillar B cells

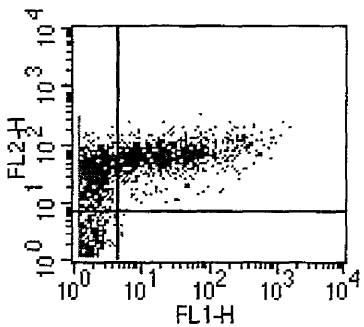
Tonsillar B cells were electroporated with CD23 full-length and truncated promoter reporter vector constructs (along with 20μg/ml of pLW2) as described in section 2.2.6i, allowed to rest for 2hr and then stimulated with 14ng/ml IL-4, 1μg/ml anti-CD40, 1μg/ml anti-μ and 6nmol PMA and cultured for 48hr in complete RPMI-1640 medium. Cells harvested from culture, washed twice in ice-cold PBS and resuspended at $1x10^6$ cells per 100μl of PBS. 3μl of PE-labelled anti-CD19 and 3μl of FITC-labelled anti-CD23 antibodies were then added prior to incubation of the samples on ice for 1hr. Cells were then again washed twice with ice-cold PBS, resuspended in 500μl of PBS and analysed on a Becton Dickinson FACScan flow cytometer.

A representative set of dot plots for both the CD23a and CD23b Promoter constructs are shown here, with anti-CD23 FITC labelled $CD19^+$ B cells being indicated in the upper right hand quadrant.

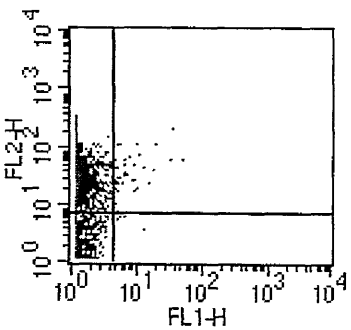
CD19
Expression



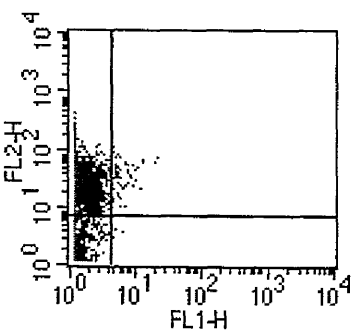
CD23a Prom/No Stim.



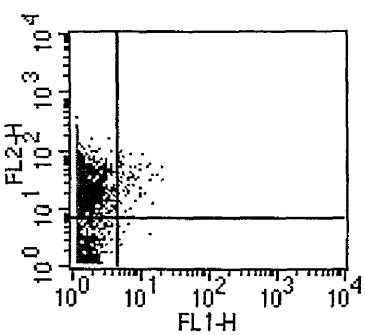
CD23a Prom/+IL-4



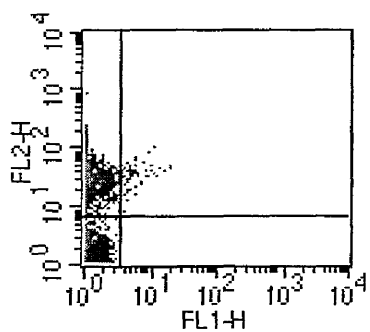
CD23a Prom/+anti-CD40



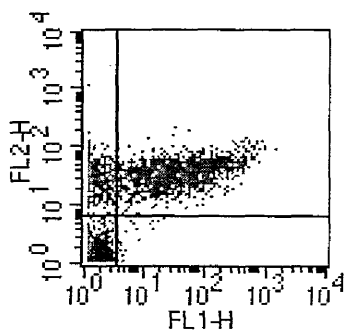
CD23a Prom/+anti-μ



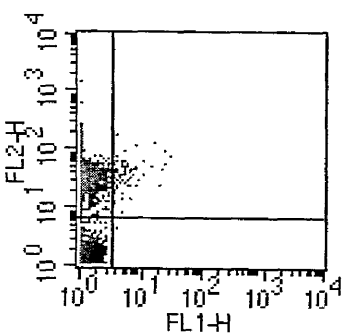
CD23a Prom/+PMA



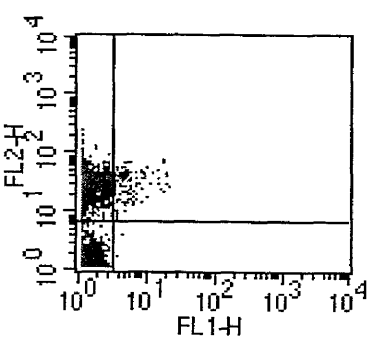
CD23b Prom/No Stim.



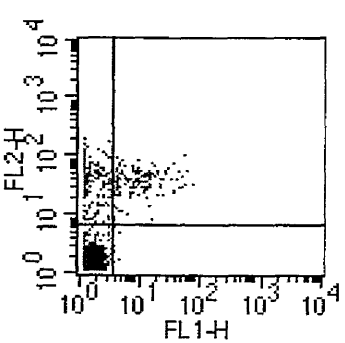
CD23b Prom/+IL-4



CD23bProm/+anti-CD40



CD23b Prom/+anti-μ



CD23b Prom/+PMA

CD23 Expression

Table 4.1: Percentage of CD23a and CD23b promoter reporter construct transfected tonsillar B cells expressing CD23 following stimulation

Tonsillar B cells were electroporated with CD23 full and truncated promoter reporter vector constructs and analysed as described in the legend for Figure 4.11.

The percentage of electroporated B cells expressing CD23 for all of the CD23 constructs and stimulations was calculated and is represented on this table.

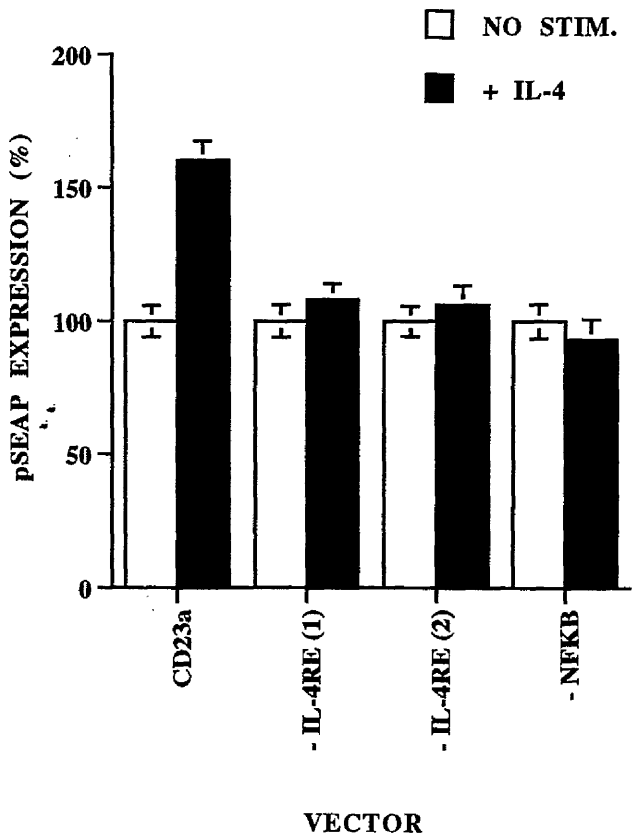
	Percentage of electroporated B cells expressing CD23 cell surface marker following 48hr stimulation as shown (%)				
Pseap Vector/ Insert	**No Stimulation**	**+ IL-4**	**+ Anti-CD40**	**+ Anti-μ**	**+ PMA**
CD23a Prom	0.52	53.00	0.75	0.90	1.64
CD23a Prom/ ΔS61	0.84	53.90	0.36	0.57	1.80
CD23a Prom/ΔS62	0.73	59.30	1.20	1.05	2.67
CD23a Prom/ ΔκB	0.75	60.00	1.12	1.33	1.53
CD23b Prom	2.30	69.00	2.59	4.60	26.2
CD23b Prom/ ΔS61	1.50	71.43	2.80	4.95	27.2
CD23b Prom/ΔκB	1.93	62.00	2.23	2.70	20.8
CD23b Prom/ ΔS62	2.80	65.20	2.80	3.00	20.00
CD23b Prom/ ΔAP1(1)	1.75	70.00	2.40	4.00	23.00
CD23b Prom/ ΔAP1(2)	2.70	66.00	2.50	3.60	22.40

Figure 4.13: CD23a full and truncated promoter reporter vector stimulation studies.

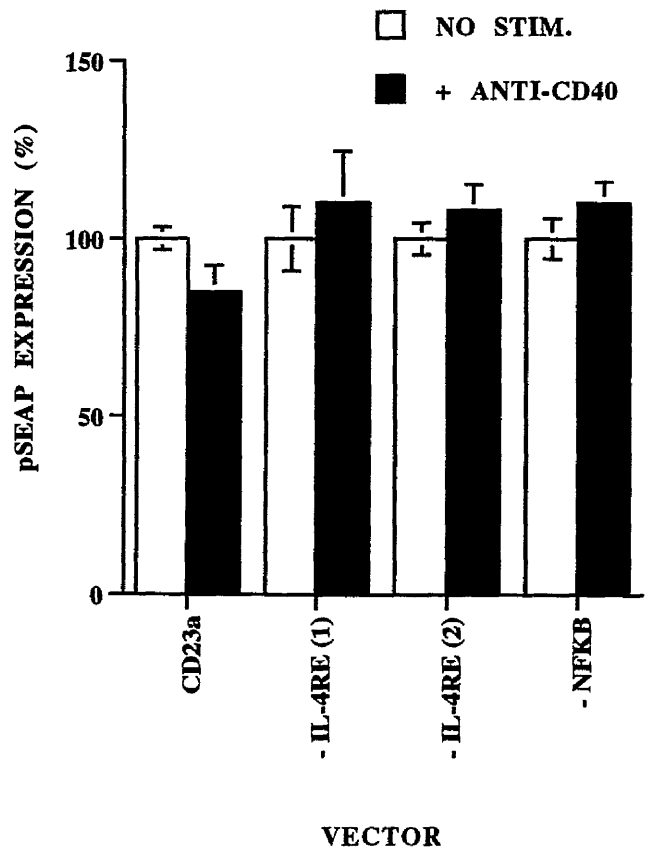
40μg of each CD23a pSEAP reporter vector construct was electroporated into $5x10^6$ tonsillar B cells, as described in section 2.2.6i of the methods chapter of this thesis, along with 20μg/ml of pLW2. Cells were allowed to rest for 2hr, prior to stimulation with (A) 14ng/ml IL-4, (B) 1μg/ml anti-CD40, (C) 1μg/ml anti-μ, (D) 6nmol PMA and subsequent 48hr culture in complete RPMI-1640 medium. Cells were then harvested (for CAT assay or flow cytometry) and 125μl of medium conserved for SEAP assay, which was performed in accordance with section 2.2.6j. All reagents were supplied by Clontech in their Great EscAPe SEAP Reporter System 2 kit (see Materials 2.1.2 for details) Briefly, culture medium was centrifuged at 12, 000 x g, to remove any cells, and 15μl was added to the wells of a 96 well opaque plate and diluted with 45μl of dilution buffer. Intracellular acetylases were then heat inactivated at 65°C, prior to the addition of 60μl of assay buffer and 60μl of substrate. The reactions were then incubated for 15min at room temperature (RT), prior to interpretation of results on a plate luminometer.

The results were then normalised for transfection efficiency by pLW2 transfection and CAT assay, and a minimum of 3 experimental sets for each experiment were performed using different tonsil sets. To standardise results in relation to variability in prepared primary tonsillar B cells, all results were represented in relation to unstimulated cells being termed as 100%. Error bars are shown to indicate variability between results from different tonsil sets, with those on non-stimulated 100% samples indicating variability between triplicate samples.

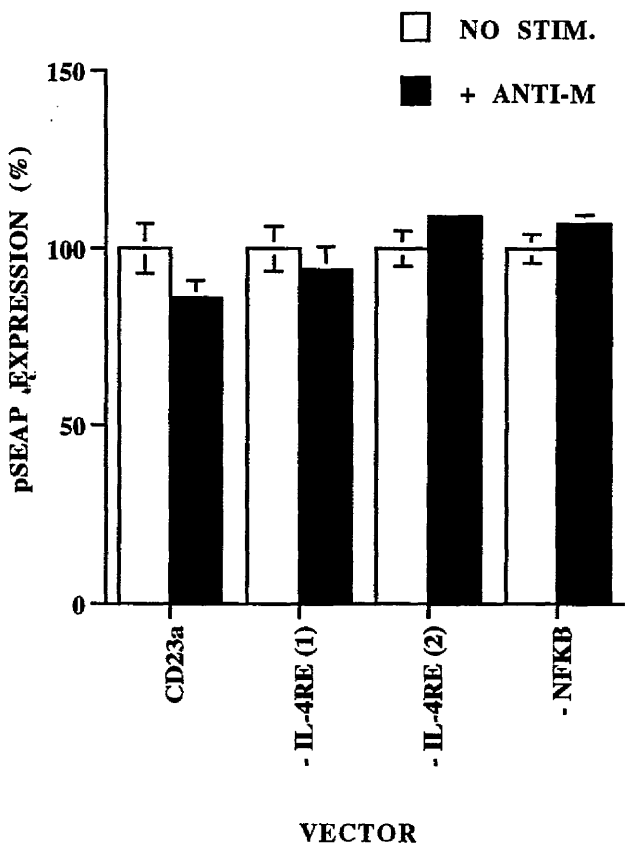
A


NO STIM.
+ IL-4
pSEAP EXPRESSION (%)
200
150
100
50
0
CD23a
- IL-4RE (1)
- IL-4RE (2)
- NFKB
VECTOR

B


NO STIM.
+ ANTI-CD40
pSEAP EXPRESSION (%)
150
100
50
0
CD23a
- IL-4RE (1)
- IL-4RE (2)
- NFKB
VECTOR

C


NO STIM.
+ ANTI-M
pSEAP EXPRESSION (%)
150
100
50
0
CD23a
- IL-4RE (1)
- IL-4RE (2)
- NFKB
VECTOR

D

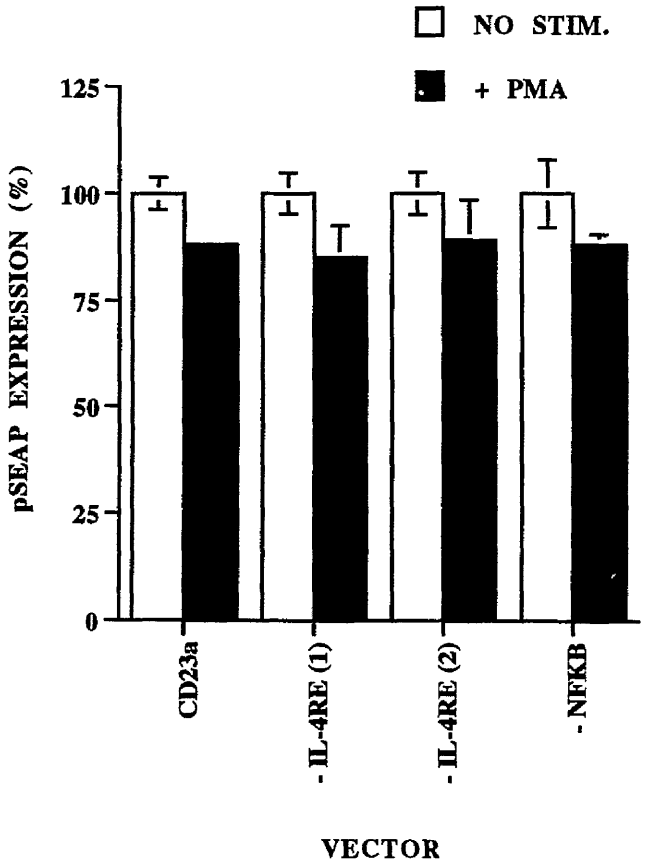
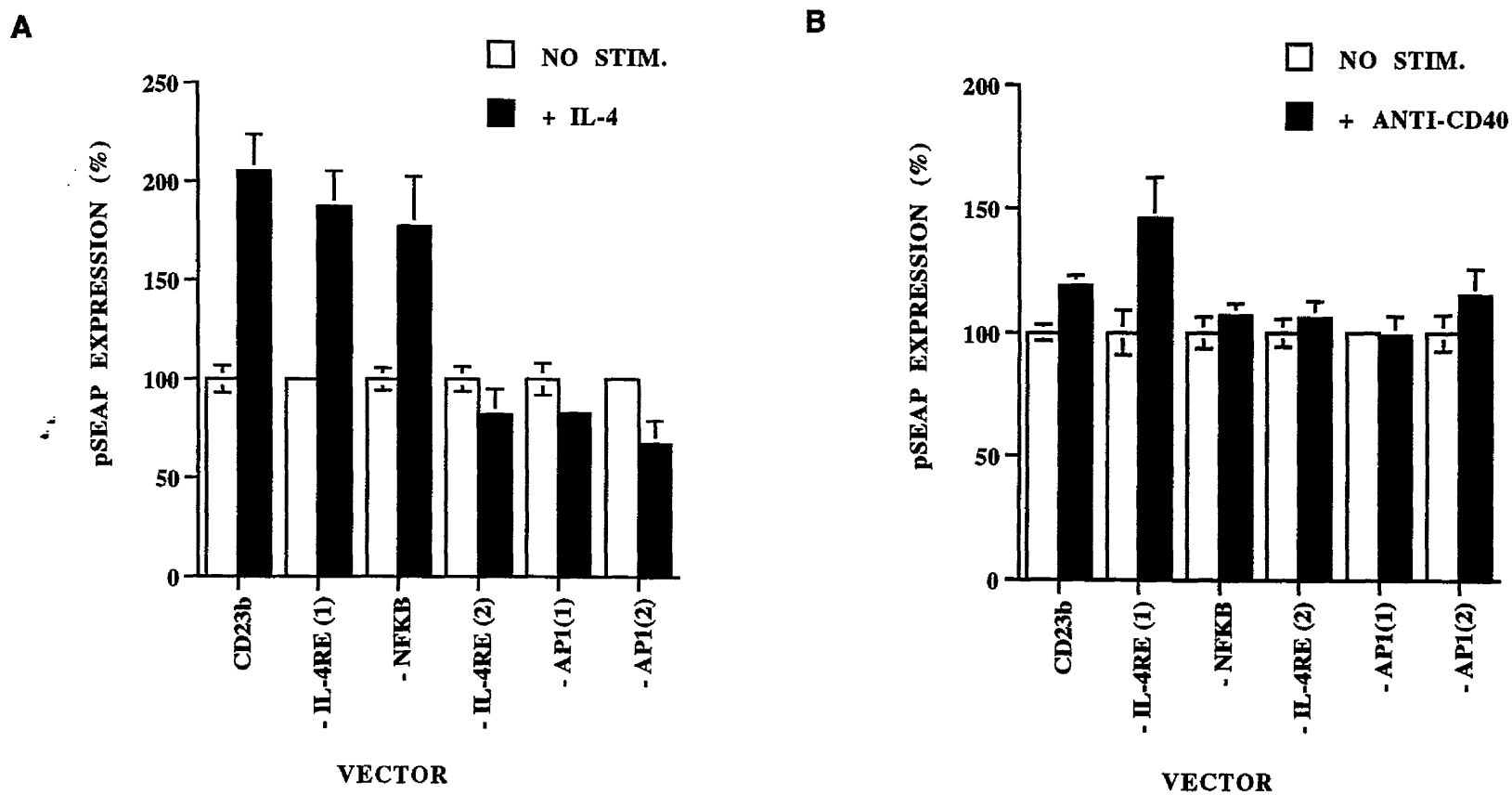
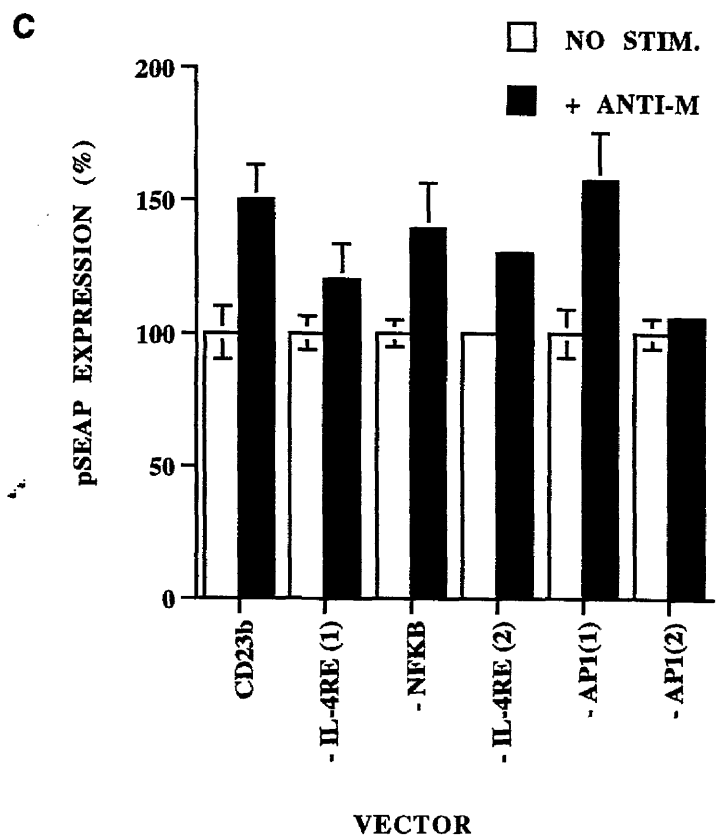

NO STIM.
+ PMA
pSEAP EXPRESSION (%)
125
100
75
50
25
0
CD23a
- IL-4RE (1)
- IL-4RE (2)
- NFKB
VECTOR

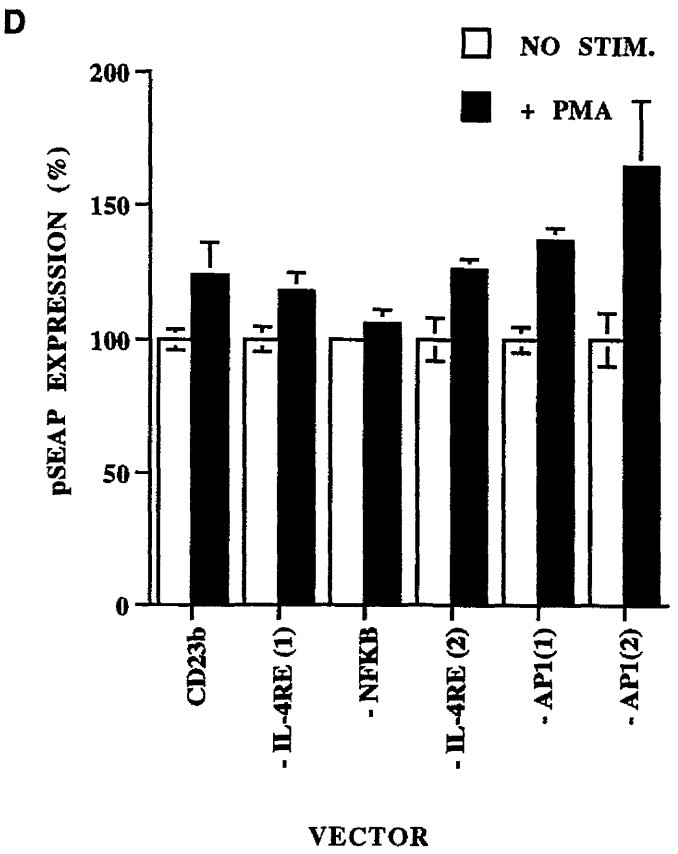
Figure 4.14: CD23b full and truncated promoter reporter vector stimulation studies.

40μg of each CD23b pSEAP reporter vector construct was electroporated into $5x10^6$ tonsillar B cells, as described in section 2.2.6i of the methods chapter of this thesis, along with 20μg/ml of pLW2. Cells were stimulated and analysed as described in the legend for Figure 4.13.

(A) + 14ng/ml IL-4, (B) + 1μg/ml anti-CD40, (C) + 1μg/ml anti-μ, (D) + 6nmol PMA


A
NO STIM.
+ IL-4
pSEAP EXPRESSION (%)
250
200
150
100
50
0
CD23b
- IL-4RE (1)
- NFKB
- IL-4RE (2)
- AP1(1)
- AP1(2)
VECTOR
B
NO STIM.
+ ANTI-CD40
pSEAP EXPRESSION (%)
200
150
100
50
0
CD23b
- IL-4RE (1)
- NFKB
- IL-4RE (2)
- AP1(1)
- AP1(2)
VECTOR


C
NO STIM.
+ ANTI-M
pSEAP EXPRESSION (%)
200
150
100
50
0
CD23b
- IL-4RE (1)
- NFKB
- IL-4RE (2)
- AP1(1)
- AP1(2)
VECTOR


D
NO STIM.
+ PMA
pSEAP EXPRESSION (%)
200
150
100
50
0
CD23b
- IL-4RE (1)
- NFKB
- IL-4RE (2)
- AP1(1)
- AP1(2)
VECTOR

CHAPTER 5

RESULTS

IN VIVO FOOTPRINTING OF THE CD23a AND CD23b PROMOTERS

CHAPTER 5: *IN VIVO* FOOTPRINTING OF THE CD23 AND CD25 PROMOTERS INCORPORATING LIGATION MEDIATED PCR (LM-PCR)

5.1: INTRODUCTION

CD23a and CD23b expression has been shown to be regulated by several mitogens and stimulants, with the promoter regions of each isoform being clearly, differentially and independently activated. The reporter vector studies in Chapter 4 show that the CD23a promoter is activated with IL-4 only, whereas the CD23b promoter is activated with all of IL-4, anti-CD40, anti-μ chain and PMA. It was a natural progression, therefore, to study these interactions at the level of promoter DNA, and the proteins binding thereto. The technique of *in vivo* footprinting, incorporating ligation-mediated PCR (LM-PCR) was considered ideal for this task.

In vivo footprinting, as a method, is utilised to study protein-DNA interactions in intact cells, and can yield interesting and useful results with respect to gene expression. However, special methods are required to visualise these results and the technique can prove to be extremely problematic. LM-PCR is known to substantially increase the absolute signal and signal-to-noise ratio obtained for genomic sequencing (Mueller & Wold, 1989). It does so by coupling PCR with genomic sequencing to provide specific amplification of a sequence "ladder", while preserving the identity and relative quantitative representation of each rung of the original cleaved genomic DNA preparation. The application of LM-PCR has made *in vivo* footprinting more readily accessible to organisms with large genomes, as the sequence and primers for only one end need be known and utilised.

As described in the appropriate Methods section, DNA was prepared according to Maxam and Gilbert (1980) (Maxam & Gilbert, 1980), where naked DNA (prepared following cell lysis) was cleaved at every G residue and *in vivo* alkylated DNA only

being cut where no proteins were bound. An overview of the basic LM-PCR protocol is shown on Figure 5.1, starting at the top of the page with a representation of Maxam and Gilbert prepared DNA and putative binding proteins. Primers 1-3 are nested and have overlapping start sites, the reason for which will become apparent. The first strand reaction was initiated by the annealing of primer 1 followed by the subsequent PCR of one strand, thus producing double stranded DNA with a specific start point for our promoter. Vent polymerase is used for all of the PCR steps in this technique, as it is more faithful and specific than Taq polymerase and ensures high fidelity copying of the template DNA. A common linker is then ligated on to the opposite end of the first strand product at 17°C overnight, in order to provide an end point for the succeeding amplification reactions. This step is then followed by 18 amplification steps with nested Primer 2 firstly from one end and then the linker primer from the other, with the linker primer being identical to the larger part of the common linker. The product of this amplification step is thus blunt ended, which ensures clear cut footprints upon completion. Following the amplification step, the products are then labelled by a two cycle PCR program with nested Primer 3, the two steps ensuring that each rung of the ladder is labelled at one end only. Each LM-PCR reaction will terminate wherever a G residue is cleaved, thus producing a DNA ladder. The resulting products are ran on a 6% sequencing gel which is read from the bottom up, as the smallest products are terminated closest to the radioactively labelled end.

While LM-PCR has been used successfully by a few investigators to obtain high quality *in vivo* footprint information (Algarte, *et al.*, 1995; Kara & Glimcher, 1991) there are several problems that can significantly compromise data quality. These anomalies will be discussed in detail in this chapter and include weak, missing or unclear genomic ladders, as well as the general lack of footprint quality. Modification and fine-tuning of this technique will be assessed, as will the experiments used to determine the correctness and specificity of each primer.

Limited, but hard earned, data showing CD25 and CD23 promoter footprints will also be extensively discussed.

5.2 RESULTS

5.2.1 THE CD25 PROMOTER CAN BE STUDIED THROUGH IN-VIVO FOOTPRINTING

Initial studies were carried out on the CD25 promoter, based on studies by Algarte et al (Algarte, *et al.*, 1995). This was pursued in order to ensure that the protocol could be effectively and correctly utilised for the purpose of characterising the CD23 promoter. The CD25 promoter has also been extensively studied and characterised in this laboratory, making it ideal for a control study. Figure 5.2a shows a diagram of the CD25 promoter, and also the start sites for the primers used in subsequent LM-PCR reactions. These primers are listed fully on figure 5.2b. The CD25 promoter LM-PCR and footprinting was relatively straight forward and is shown on Figure 5.3, with the transcription factor binding sites highlighted as shown. These areas are possible to determine by "reading" the number of G residues present, working from the bottom of the footprinting gel. Although there is an obvious problem with the general clarity of the banding on this footprint, which will be addressed accordingly later, visible footprints can be identified and are putatively thought to be at the AP1 and NRE sites. As well as the work of Algarte *et al* (Algarte, *et al.*, 1995), this banding pattern also backs up EMSA data resulting from the work of J. Curran and A.B. Allen, personal communication, suggesting that 3 protein species bind to these sites. The narrowness of the footprint lanes in the above CD25 promoter footprint made it difficult to define if any doublet bands were present, as the resulting ladders contained labelled dots for each rung as opposed to the concise straight bands required. This phenomenon was a result of the narrow sequencing comb used for the task of loading, and a wider welled gel was required to

rectify this problem. Further gels were therefore produced using larger 8mm wells, and a resulting naked DNA G ladder is shown on figure 5.4. This new well size clearly increases the clarity and sharpness on the banding pattern on the gel and following this successful footprinting attempt, it was considered appropriate to move to the study of the CD23a and CD23b promoters.

5.2.2: LM-PCR AND IN-VIVO FOOTPRINTING OF THE PROMOTER REGION OF CD23a

Following the moderately successful generation of the CD25 promoter results, footprinting of the CD23a and b promoter regions was attempted. Figure 5.5a illustrates the promoter regions and LM-PCR primer start sites for each promoter, and Figure 5.5b lists these primers in full. Firstly, in order to ensure banding could be visualised, G-ladders of the CD23a promoter regions of naked DNA from different cell lines was attempted. This, surprisingly, proved to be extremely difficult and time-consuming and, as unforeseen problems hampered the process, lead to very few results. Problems ranged from no visible banding, infrequent banding, indistinct or low resolution bands and various collaborations of all of the above. The LM-PCR and *in vivo* footprinting techniques subsequently required much trouble shooting, modification and refining to attempt to eliminate these uncertainties, and this took the form of two specific trains of thought: inaccuracies as a result of incorrect gel running or problems with the actual PCR.

Modification of footprinting gels and electrophoresis:

Several attempts at refining the pouring and running of footprinting gels was attempted, including the introduction of "Long Ranger" gel solution instead of using normal acrylamide:bis acrylamide. This purpose-made sequencing gel solution by Flowgen contains acrylamide and a non-disclosed polymer, which allows for longer

gel-read lengths and significantly more resolution of doublet and triplet bands. Great care was also taken to ensure the correct temperature of the gel for high quality gel running, and this was found to be at approximately 55W and 50°C. Gels were still not producing quality banding however and it was considered that perhaps gels were not setting consistently, leading to gel running anomalies. The gel solutions were subsequently "de-gassed" using a vacuum pump prior to pouring and the setting wells protected from the air by covering with plastic wrap. Once all of the above attempts had been exhausted and no real observable improvement in band clarity was achieved, changes and improvements were established to the actual LM-PCR procedure.

Refinement and modification of LM-PCR

Promising results were generally always observed following use of freshly prepared reaction buffers and labelled Primer 3. Therefore, buffers were always mixed directly prior to the immediate requirement and labelled primer of more than one half life in age discarded (^{32}P has a half-life of approximately 14 days). Newly excised and prepared Maxam and Gilbert DNA was also always used. Sufficient magnesium concentration in the LM-PCR solutions was also paramount, as magnesium is essential for primer extension. 50mM of $MgSO_4$ was thus added to the amplification solution to produce significantly better results. End-labelling reactions of Primer 3 for the labelling mix was also optimised to a 40 minute incubation time, with radioactive incorporation studies (TCA precipitation) revealing that this time span gives the best percentage of labelling (approximately 90%). The actual LM-PCR reaction was also subjected to modification in an attempt to improve band clarity, with variations in annealing temperature being tried. Optimum banding was observed at annealing temperatures of 5°C below the lowest melting temperature of the oligonucleotides used, even though lower annealing temperatures were tried

and tested. Annealing temperatures too low would have resulted in non-specific banding anyway.

Finally, it was considered that perhaps there was something wrong with the actual primers used in the CD23 promoter LM-PCR reactions. This was possible, as major problems only occurred when the transition was made from the CD25 to CD23 promoter studies. Primer sequences were re-checked in relation to their DNA binding sequences and were found to be correct, and PCR experiments were performed using tonsillar B cell DNA and amplifying with each primer set. The results of this are shown on Figure 5.6, with the expected band sizes being observed. Therefore, the primers appear to be correct and not to blame for the experimental failure.

5.2.3: CD23a promoter G ladder

Limited success was achieved with the LM-PCR technique, although actual *in vivo* footprints were elusive. Naked DNA G ladders were produced for the CD23a promoter from SMS-SB and P3HR1 cell lines, and clear banding pattern differences were observed between the two. These ladders are shown on Figures 5.7a and 5.7b, along with the known transcription factor binding sites which were determined with respect to the position of cleaved G residues. Footprints were attempted, but unfortunately not perfected - even after several attempts.

5.2.4: CD23a promoter footprint of stimulated tonsillar B cells

Stimulation experiments were attempted prior to the LM-PCR and attempted footprinting of the CD23a promoter region in tonsillar B cell DNA. These studies have revealed clear differences in, albeit incomplete, *in vivo* DNA ladders following IL-4, anti-CD40 and anti-μ stimulation, suggesting that proteins bind to the CD23a

promoter differentially and spatially following treatment with various stimuli. A representation of these data is shown on the scan in figure 5.8. Unfortunately in these instances the actual naked DNA G ladder and unstimulated *in vivo* DNA ladder did not work, the former meaning that footprinting comparisons between DNA with and without bound protein could not be made. However these results were promising and, perhaps if more time had been available, useful footprinting results would have forth-coming. The limited data reveal that upon IL-4 stimulation, 3 bands are lost in comparison with the anti-CD40 stimulated lane and one band is lost in the anti-μ stimulated lane. This is indicative of differential protein (transcription factor) binding, and it is suggested that this limited footprint area may be situated over the BSAP site in the CD23a promoter. However, as the naked DNA G ladder was missing, no further comparisons or assumptions can truly be made.

5.3: Conclusions and discussion

Although extremely time consuming and resource-draining, the effective utilisation of LM-PCR and *in vivo* footprinting to study the CD23 promoter has remained ever elusive. The technique was extensively refined, modified and theoretically improved in an attempt to achieve acceptable results for the CD23 promoter as had been generated for CD25, but banding patterns were never full established. Primary and optimistic results revealed that the naked DNA G ladders can be achieved in SMS-SB and P3HR1 cells, and there are observable differences between the two. It was considered that both promoters, although from different cell lines, should produce similar G ladders and the fact that they did not was surprising. It is suspected, though, that these cell lines had DNA with varying degrees of natural alkylation and thus, in addition to piperidine cleavage of the DMS (di-methyl sulphate) alkylated G residues, other pieces of DNA were also being cleaved. Hyper G cleavage sites have also been mentioned by Algarte *et al* (Algarte, *et al.*, 1995). Also, if piperidine is

not completely eliminated when preparing the DNA, it can lead to excess improper cleavage of the DNA.

The tonsillar B cell DNA results are very limited but further work on them may be extremely interesting, and would have provided a wealth of information to be used in conjunction with the reporter vector study results in chapter 3. They indicate that upon stimulation with IL-4, anti-CD40 and anti-μ differential protein binding to the CD23a promoter occurs. Further investigation and refining of this technique is obviously required, and if the technique had been fully operative during this research both the CD23a and CD23b promoters would have been fully studied. A further consideration is that perhaps the actual Maxam and Gilbert preparation of DNA has to be modified, although Algarte *et al*'s method was followed precisely. It is also possible that the technique is proving to be much more difficult with the CD23 promoter purely because we were trying to study it in B cells and not in T cells as were used by Algarte and colleagues. There are several reasons why this could be the case, including the fact that the B cells we use are particularly fragile, and the DNA could thus be subject to excess cleavage and disruption when the cells are lysed, or even when cleaved with the piperidine.

In conclusion, LM-PCR and *in vivo* footprinting are extremely enlightening techniques and, if they work, can provide substantial rewards in the form of results and information. Unfortunately in this case the footprinting data was not forthcoming, but considerable time and experience was spent on trying to refine the technique, making it too important to discount the results altogether.

Figure 5.1: Ligation Mediated-PCR (LM-PCR) protocol

2μg of Maxam and Gilbert cleaved DNA (2.2.7a) is represented at the top of this figure, with numbers 1-3 being representative of overlapping primer start sites, and letters A-E the sites of potential DNA cleavage. A potentially *in vivo* bound protein is represented over the B and C cleavage points, and would stop cleavage from taking place in the *in vivo* sample. All of the reagents required for this technique are listed in section 2.2.7b of the methods section, as are the actual PCR specifications. A first strand reaction mixture was initially performed using P1 and Vent DNA Polymerase, which is preferentially used because of its high fidelity. A common linker was then ligated on to the first strand at 17°C overnight using T4 DNA ligase, prior to 18 rounds of amplification with primer P2 (P1 and P2 start sites overlap) and the linker primer. The resulting amplicons were then labelled from one end only by PCR with ^{32}P labelled primer 3. Samples were run on a 6% "Long Ranger" sequencing gel, and the resulting autoradiographs analysed from the bottom up.

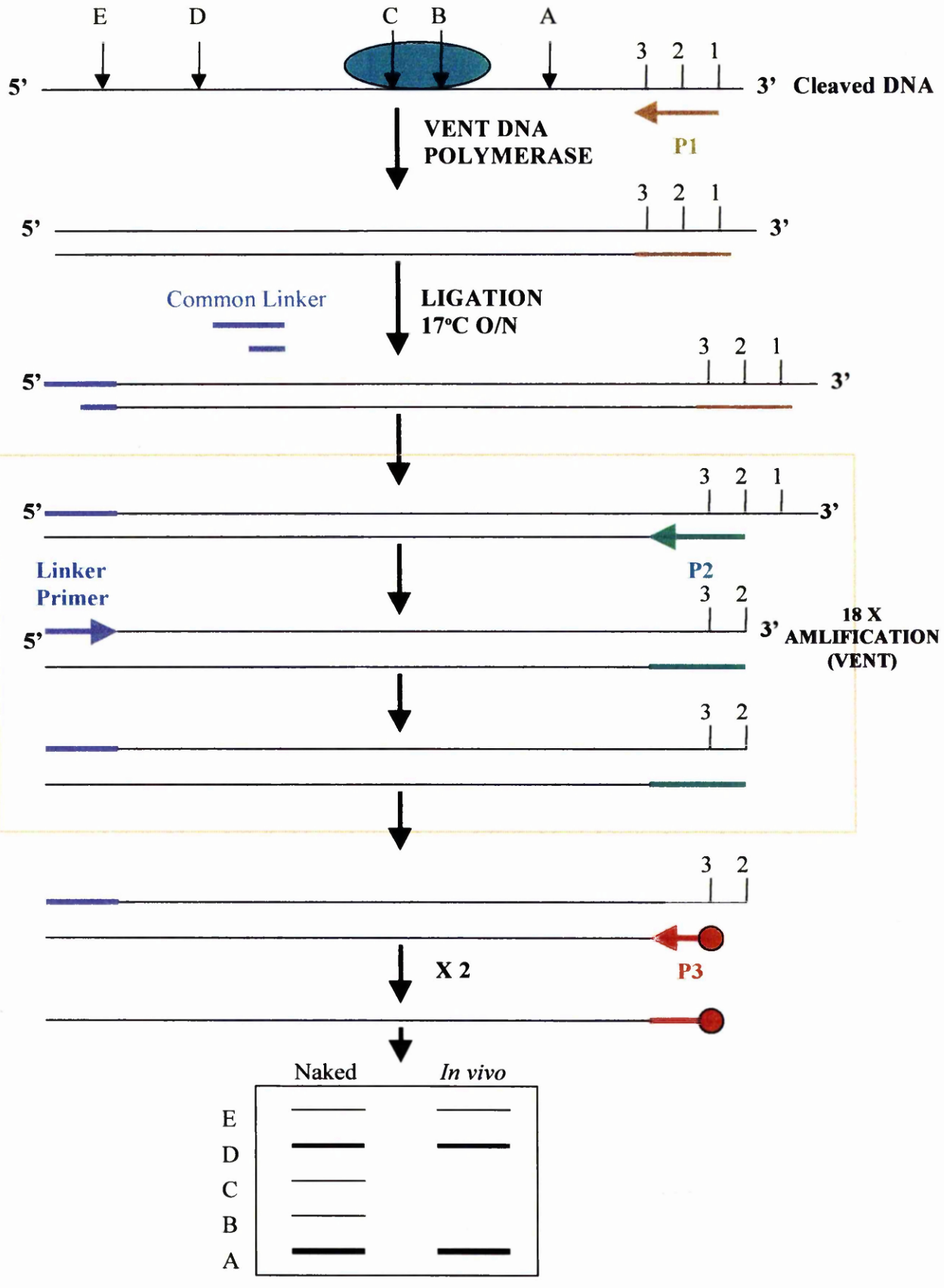

E
D
C
B
A
3 2 1
5'
3' Cleaved DNA
VENT DNA POLYMERASE
P1
Common Linker
LIGATION 17°C O/N
Linker Primer
P2
18 X AMLIFICATION (VENT)
X 2
P3
Naked
In vivo
E
D
C
B
A

Figure 5.2: The promoter region and upstream elements of CD25

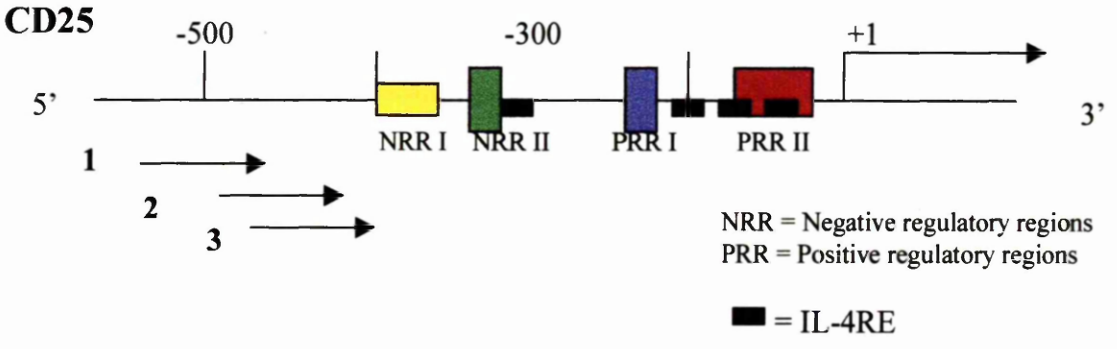
The CD25 promoter region is approximately 500 base pairs in length and contains several upstream elements as shown here (adapted from Algarte *et al*, 1995). There are two negative regulatory elements (NRR I-II) followed by two positive regulatory elements (PRR I-II), and putative IL-4 response elements (IL-4RE)/STAT-6 sites are shown as black boxes.

The specific primer start sites for producing the CD25 promoter in vivo footprints are highlighted by the arrows, with the primers identified below .

Figure 5.2b: Primer sequences for LM-PCR of the CD25 promoter region

The synthetic oligonucleotides shown here were designed and used to amplify the CD25 promoter region by LM-PCR, as described in section 2.2.7b of the materials and methods chapter.

A



B

PRIMERS:

CD25 Prom (1) - 5' CAG GTT ATA AGC CTT GGG G

CD25 Prom (2) - 5' ATT CTC AGG ATC CTT CAG TTC GCC G

CD25 Prom (3) - 5' GGA TCC TTC AGT TCG CCG CAT CCT TCT CC

Common linker - 5' GCG GTG ACC CGG GAG ATC TGA ATT C
3' C TAG ACT TAA G

Figure 5.3: *In vivo* footprinting of the CD25 promoter

Jurkat T cell DNA was prepared in accordance with the method of Maxam and Gilbert (Maxam and Gilbert, 1980), as described in section 2.2.7a. Briefly, DNA was alkylated on G residues by DMS, either inside the cell or following cell lysis (Intracellularly bound proteins protect G residues from alkylation). Piperidine was then used to cleave the DNA at the point of alkylation.. 2μg of this prepared DNA was then used to perform ligation mediated-PCR (LM-PCR) as described in 2.2.7b, along with the reagents required. Briefly, a first strand reaction was initially performed using P1 and Vent DNA Polymerase, which is preferentially used because of its high fidelity. A common linker was then ligated on to the first strand at 17°C overnight using T4 DNA ligase, prior to 18 rounds of amplification with primer P2 (P1 and P2 start sites overlap) and the linker primer. The resulting amplicons were then labelled from one end only by PCR with ^{32}P labelled primer 3. Samples were then run on a 6% "Long Ranger" sequencing gel, and the resulting autoradiographs analysed from the bottom up.

Proposed positions of upstream elements are highlighted next to the footprint.

CD25 Promoter
***in vivo* Footprint**

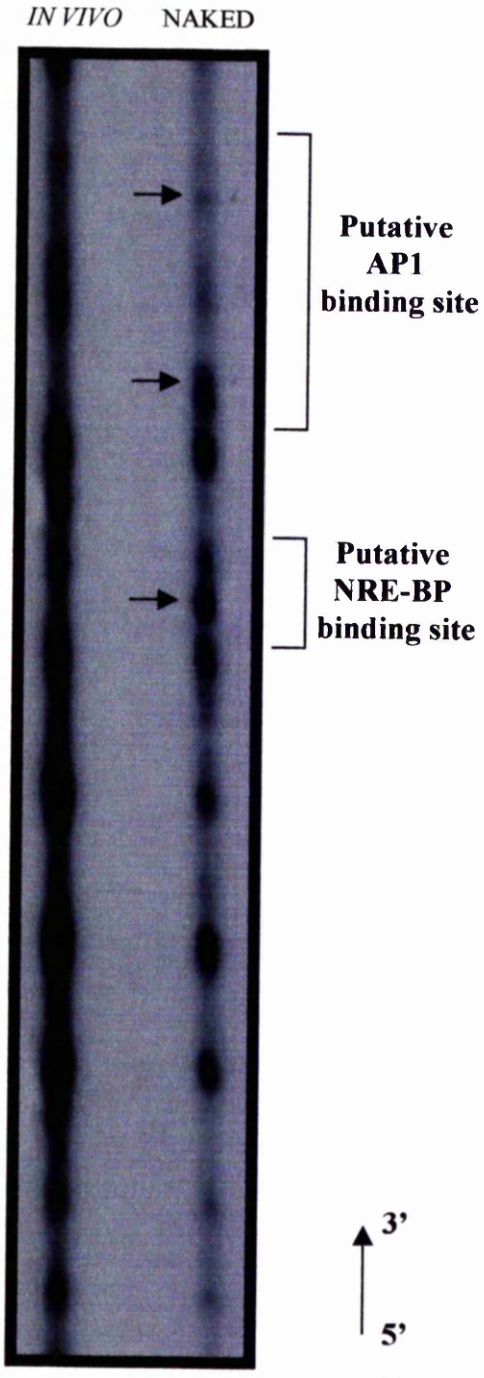


Figure 5.4: Naked DNA G ladder of the CD25 promoter

Jurkat T cell DNA was prepared in accordance with the method of Maxam and Gilbert (Maxam and Gilbert, 1980), as described in section 2.2.7a. 2μg of this prepared DNA is then used to perform ligation mediated-PCR (LM-PCR) as described in 2.2.7b, along with the reagents required. Samples were then run on a 6% "Long Ranger" sequencing gel, following formation of wells with a larger 8mm comb, and the resulting autoradiographs analysed from the bottom up.

CD25 Promoter
(Naked)

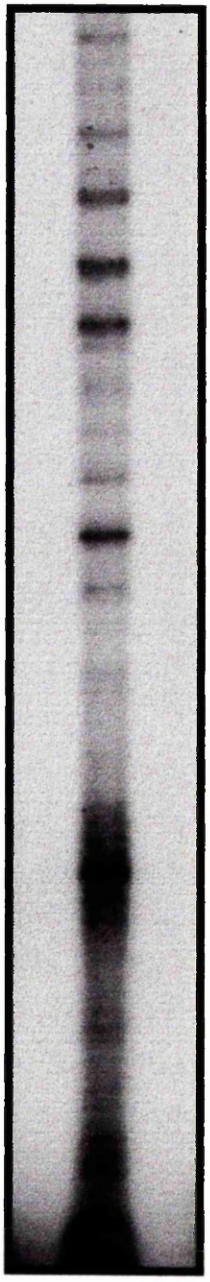


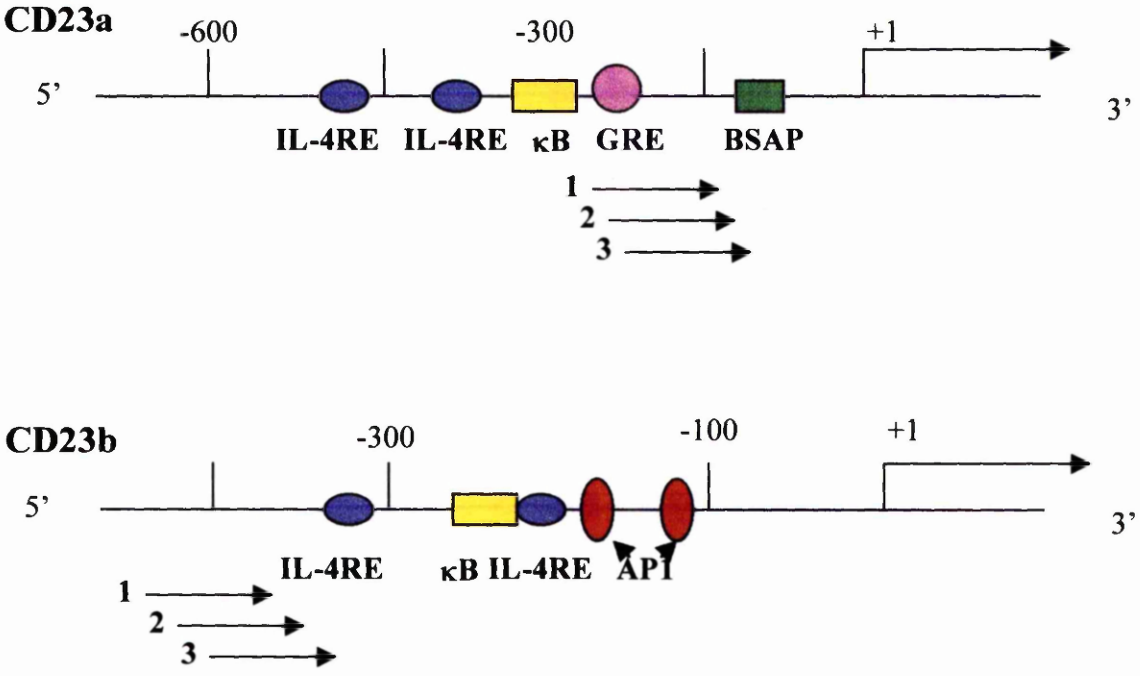
Figure 5.5a: The promoter regions and upstream elements of CD23a and CD23b

The CD23a and b promoter regions are approximately 600 and 400 base pairs in length, respectively, and both contain several upstream elements which are shown here. The CD23a promoter shows two IL-4 response elements (IL-4RE), an NFκB site, a glucocorticoid response element (GRE) and a B-cell-specific activator protein (BSAP) or Pax-5 binding site. The CD23b promoter also has two IL-4RE's but, in contrast, these are separated by a κB site. The second IL-4RE is then closely followed by two AP-1 binding sites. The specific primer start sites for LM-PCR of the CD23a and CD23b promoters are highlighted by the arrows, with the primers identified below .

Figure 5.5b: Primer sequences for LM-PCR of the CD23a and CD23b promoter regions

Oligonucleotides to facilitate LM-PCR of the CD23a and CD23b promoter regions were designed and are shown here. These primers were then used to amplify CD23 promoter regions of DNA by LM-PCR, as described in section 2.2.7b of the materials and methods chapter.

A



B

PRIMERS:

CD23a Prom (1) - 5' GCC TTC CGT GGC TCC CCA GGG C

CD23a Prom (2) - 5' GCC CTC TGT GAT CGG CCA TAG TGG T

CD23a Prom (3) - 5' CGG CCA TAG TGG TAT GAT TCA GTG TG

CD23b Prom (1) - 5' GTA GGA GGA CTC TCT GGT TCT AAC G

CD23b Prom (2) - 5' CGT TGG CAG AAG CAA TGA CCC TTA GC

CD23b Prom (3) - 5' CCT TAG CTA CTC CTT TCA CCC AGA AG

Common linker - 5' GCG GTG ACC CGG GAG ATC TGA ATT C
3' C TAG ACT TAA G

Figure 5.6: LM-PCR technique trouble-shooting - primer check

Viability of designed primers was checked by polymerase chain reaction (PCR) (2.2.6a) of unmodified cellular DNA. Each primer, 1-3, for each CD23 promoter isoform was placed in the reaction mixture with a known end point primer (CD23a Rev. or CD23b Rev.) and the expected band size calculated. The resulting amplicons were then electrophoresed on a 1% (w/v) agarose gel along with DNA size markers, and bands photographed and checked for size.

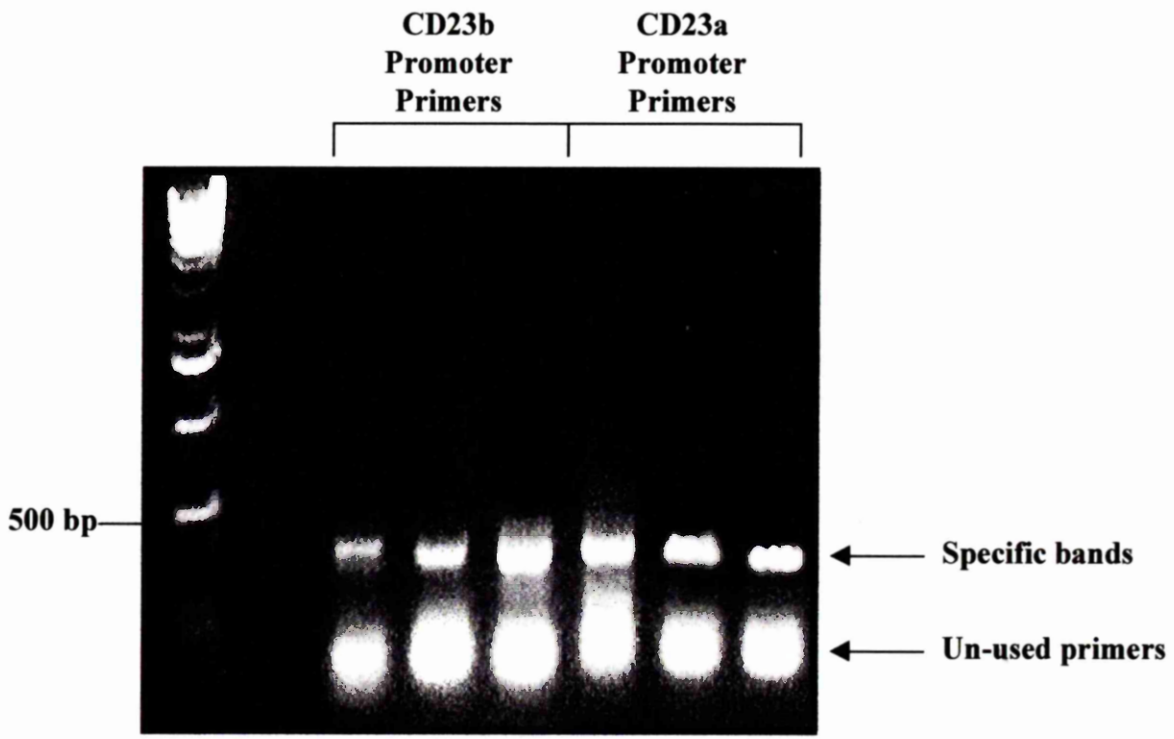

CD23b Promoter Primers
CD23a Promoter Primers
500 bp
Specific bands
Un-used primers

Figure 5.7: Naked DNA G ladders of the CD23a promoter from SMS-SB and P3HRI cells

SMS-SB and P3HRI cell DNA was prepared in accordance with the method of Maxam and Gilbert (Maxam and Gilbert, 1980), as described in section 2.2.7a. 2μg of extracellularly prepared DNA and the CD23a primers were then used to perform ligation mediated-PCR (LM-PCR) as described in 2.2.7b, along with the reagents required. Samples were then run on a 6% "Long Ranger" sequencing gel, and the resulting autoradiographs analysed from the bottom up.

Proposed positions of upstream elements are highlighted next to the gel banding.

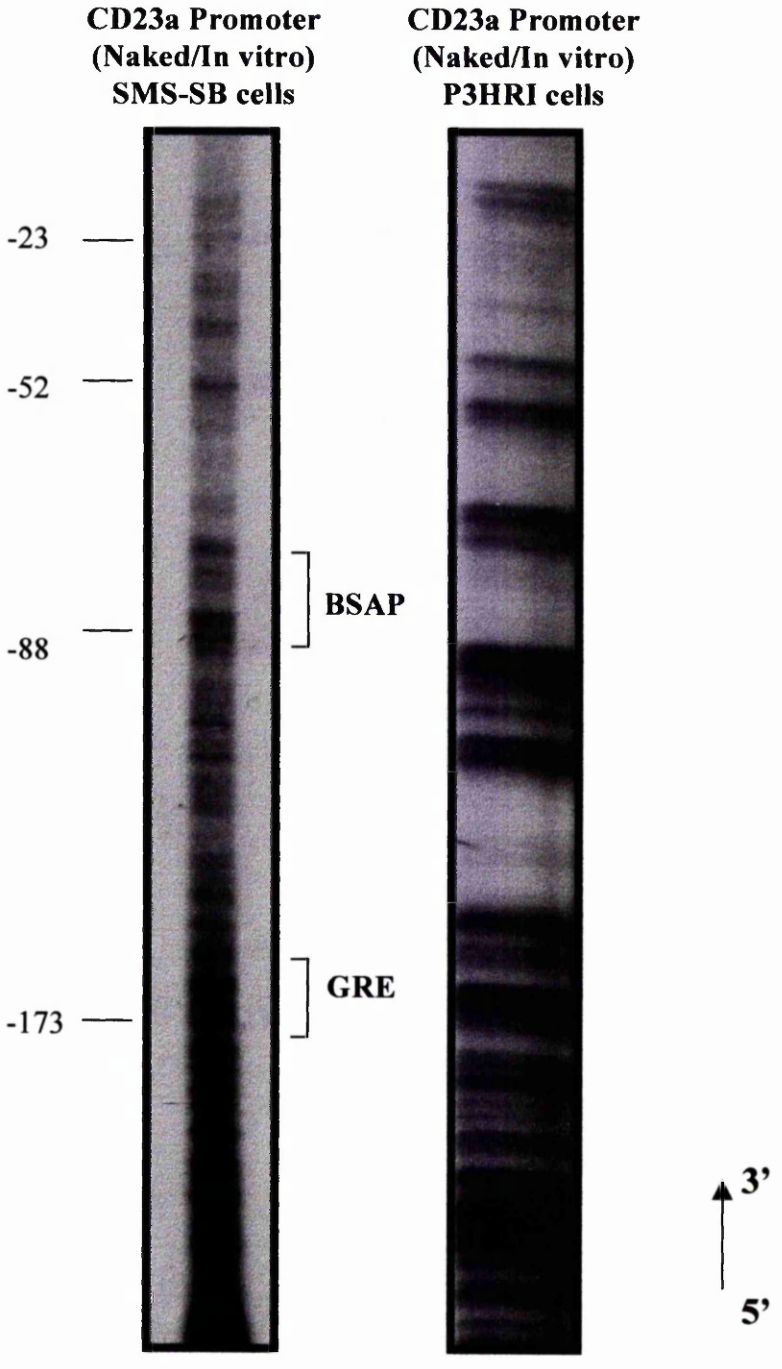
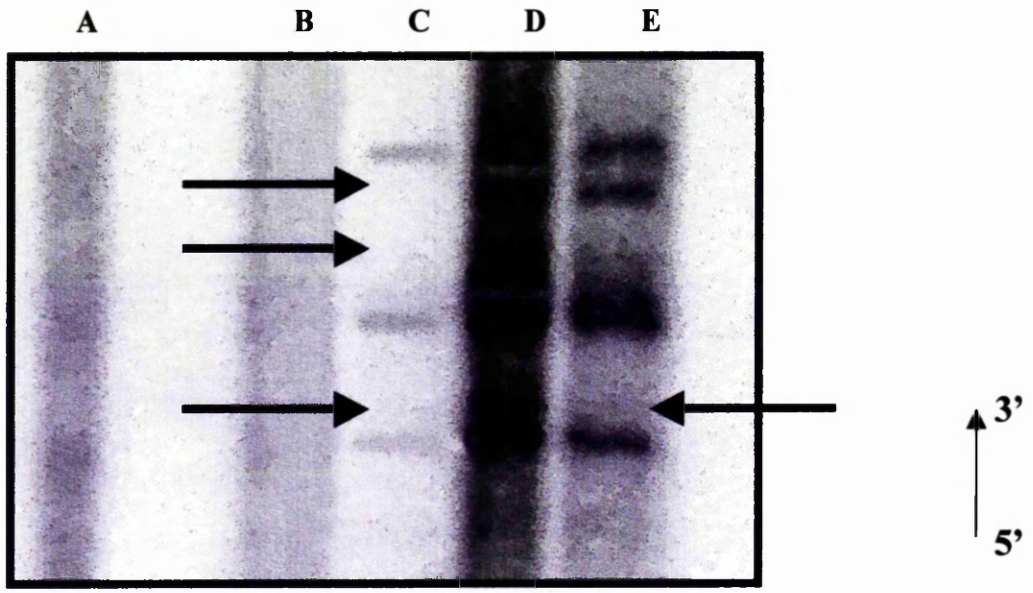

CD23a Promoter
(Naked/In vitro)
SMS-SB cells
CD23a Promoter
(Naked/In vitro)
P3HRI cells
-23
-52
-88
-173
BSAP
GRE
3'
5'

Figure 5.8: CD23a promoter footprinting of stimulated tonsillar B cells

1 x 10^7 tonsillar B cells/ml were stimulated for 24hr with 14ng/ml IL-4, 1μg/ml anti-CD40 and 1μg/ml anti-μ. Cells were then harvested, prepared in accordance with the method of Maxam and Gilbert (Maxam and Gilbert, 1980) (2.2.7a) and ligation mediated-PCR (LM-PCR) performed as described in 2.2.7b. Samples were then run on a 6% "Long Ranger" sequencing gel, and the resulting autoradiographs analysed from the bottom up.

Footprints are denoted by the presence and absence of a rung of the labelled DNA ladder.



A = Non-stimulated naked/ *in vitro* DNA, B = Non-stimulated *in vivo* DNA

C = IL-4 stimulated *in vivo* DNA, D = Anti-CD40 stimulated *in vivo* DNA, E = Anti-μ stimulated *in vivo* DNA

CHAPTER 6

GENERAL CONCLUSIONS AND DISCUSSION

CHAPTER 6: GENERAL CONCLUSIONS AND DISCUSSION

6.1: CD23 ($Fc_{\varepsilon}RII$) is a multifunctional lymphokine and regulator of IgE expression.

CD23 is the low affinity receptor for IgE and is expressed on various cells of haematopoietic lineage including B cells, T cells and monocytes. CD23/$Fc_{\varepsilon}RII$ is also a multifunctional receptor/ligand and cytokine, playing a role in antigen presentation, macrophage activation and cell adhesion (Bonnefoy, *et al.*, 1997). There are two CD23 isoforms, termed CD23a and CD23b, with the former being constitutively and cell type specifically expressed on B cells and the latter being expressed on B cells, T cells and monocytes following activation with various stimuli (Yokota, *et al.*, 1988). The only structural difference noted between the two CD23 isoforms is limited to 7 amino acids in the N-terminal cytoplasmic domain, and the molecules are generated using different transcription start sites and differential RNA splicing of the single CD23 gene located on chromosome 19 (Suter, *et al.*, 1987; Wendel-Hansen, *et al.*, 1990). It has been observed that the differential CD23a and CD23b expression could be involved in B cell function and IgE mediated immunity respectively (Yokota, *et al.*, 1988).

A large body of evidence suggests that CD23 plays a regulatory role in IgE production (Haczku, *et al.*, 2000; Texido, *et al.*, 1994; Yu, *et al.*, 1994), and cross-linking of CD23 at the cell surface with IgE delivers a negative feed-back mechanism for IgE production and inhibits the release of sCD23. Cleaved soluble CD23 fragments larger than 25kDa are known to promote IgE production. Allergic disease is thought to be due to the dysregulation of CD23/IgE feed-back mechanisms, probably through increased cleavage of CD23 from the cell surface, leading to increased IgE production and release of inflammatory mediators. Soluble CD23 is considerably elevated in atopic and neoplastic individuals, and this sCD23

can originate from either of the a or b isoforms on B cells or just from the b isoform on other cells of haematopoietic origin. In this research, the activation of the promoter region of each CD23 isoform was studied in great detail, and the conclusions discussed herewith.

6.2: Main conclusions

The main findings of this research were principally two fold. Firstly, CD23 cell surface expression can be increased considerably on cell lines and naive tonsillar B cells upon 24 or 48 stimulation with IL-4, IL-13 and PMA, but only very slightly with anti-CD40 and anti-μ. Immunoprecipitation and EMSA studies revealed that IL-4 and IL-13 stimulation utilises a STAT-6 phosphorylation dependent pathway, with the EMSA's revealing that anti-CD40 leads to NFκB activation.

Secondly, the promoter regions of the two isoforms of CD23 have different transcription factor binding sites on them, suggesting that they are differentially activated via various signalling pathways. Indeed, reporter vector construct studies have shown that these promoters are differentially activated by distinct stimuli, with the CD23a promoter being sensitive to IL-4 only, and the CD23b promoter being activated by IL-4, anti-CD40, anti-μ and PMA. Reporter vector constructs containing truncated promoters revealed that IL-4 stimulation of the CD23a promoter required the presence of the first IL-4RE/STAT-6 site, while successful IL-4 stimulation of the CD23b promoter was dependent on the second IL-4RE/STAT-6 site. This data is interesting as it suggests one of the IL-4RE present may be redundant in each case.

Limited in vivo footprinting studies have revealed that differential protein/transcription factor binding to the CD23a promoter DNA upon treatment with various stimuli, although actual promoter region areas could not be defined.

6.3: Cell surface CD23 expression is upregulated in tonsillar B cells by IL-4 and IL-13 and PMA, but not with anti-CD40 and anti-μ

As discussed, IL-4, IL-13 and PMA B cell stimulation results in an increase in cell surface CD23 expression, although with anti-CD40 and anti-μ no great increase in expression is observed (Table 3.3). Further immunoprecipitation (Figures 3.8, 3.9) and EMSA (Figures 3.10, 3.11, 3.12) studies revealed that IL-4 and IL-13 stimulation lead to phosphorylation and activation of STAT-6, and that anti-CD40 treatment caused an increase in overall cell NFκB activation. IL-4 and IL-13 stimulation leads to an increase in STAT-6 phosphorylation alone and not the total amount of STAT-6 (Figures 3.8, 3.9), although a report (Arinobu , *et al.*, 2000) has indicated that anti-CD40 and anti-μ stimulation leads to increases in actual STAT-6. These reports lead to the assumption that perhaps anti-CD40 and anti-μ stimulation primes cells for IL-4 and IL-13 activation, by increasing STAT-6 available for phosphorylation. This will be discussed further in due course. Reporter vector construct studies of the CD23a and CD23b promoter revealed that the CD23a promoter was switched on with IL-4 alone, but the CD23b promoter was stimulated by all of IL-4, anti-CD40, anti-μ and PMA. Flow cytometry analysis of cells stimulated in the same way, however, showed no increase in cell surface CD23 expression with anti-CD40 and anti-μ treatment. Explanations were therefore sought as to the source of this discrepancy between expressed and promoted CD23.

It has been demonstrated (Clark & Ledbetter, 1994; Richards & Katz, 1997) that the expression of CD23 and IgE are both upregulated on B cells stimulated with IL-4 in conjunction with anti-CD40 and, in contrast with IgE production, CD23 can be considerably upregulated by IL-4, and moderately so by lipo-polysaccharide or anti-CD40 alone (Armitage, *et al.*, 1992; Gordon, *et al.*, 1991). IL-4 has been shown to play a major role in B and T cell immune responses (Paul, 1991), with signalling

occurring through the two chain receptor consisting of the IL-4Rα and γc chain. The IL-13 receptor is very similar to the IL-4 receptor, with both sharing the IL-4Rα chain and IL-13 also including the IL-13Rα. Two general IL-4 signalling pathways have been described, the first involving IRS-1/2 (Pernis, *et al.*, 1995) and the second involving the STATs. Separate sites on the cytoplasmic domain of the α chain interact with members of the JAK-STAT pathway family and IRS-1/2 (Ihle & Kerr, 1995). It is believed that the IRS pathway is involved in lymphocyte proliferation and the STAT pathway central to the induction of specific genes such as CD23 and MHC-II (Major histocompatability complex class II).

CD40 stimulation has been shown to use signalling pathways that utilise the NFκB transcription factor (Berberich, *et al.*, 1994). The NFκB family is composed of five members including p50, p52, p65 (Rel A), c-rel and Rel-B, and these can all homo- and hetero-dimerize in various combinations (Verma, *et al.*, 1995). Single and double subunit knockout mice of the NFκB family have shown a variety of mild to severe immunological defects (Baeuerle & Baltimore, 1996). CD40 has also been shown to stimulate the activation of transcription factors other than NFκB, such as AP-1 (Fos/Jun), NF-AT, ATF-2 and the STATs (Hsing, *et al.*, 1997).

Maximum CD23 induction has been well documented to require a B cell activation signal (i.e. CD40L) in addition to the presence of IL-4 "Superinduction", with CD40L alone being shown to give a modest induction of cell surface CD23 expression. Analysis of STAT-6 -/- mice revealed that IL-4-induced expression was blocked (Shimoda, *et al.*, 1996). However Tinnell *et al* (Tinnell, *et al.*, 1998) noted that superinduction with CD40 ligand trimer (representative of native state) and IL-4 was only very marginally reduced in STAT-6 -/- mice in comparison to normal controls (Tinnell, *et al.*, 1998). This implied that, while STAT-6 enhances CD40-induced CD23 expression, it is not absolutely required for CD23 superinduction via CD40L trimer and IL-4. IL-4 can, therefore, still synergise with CD40 in the

absence of STAT-6. Likewise, IgE production can still be clearly induced by CD40L trimer and IL-4 in the absence of STAT-6 - although this IgE induction is strongly enhanced when STAT-6 is present. IgE production is, however, absolutely dependent on the presence of IL-4. An explanation for IL-4 and CD40L synergy in STAT-6 -/- mice is that an alternative STAT family member is induced, and strong EMSA and Western Blot evidence suggests that STAT-3 and STAT-5 are involved in IL-4 and CD40L superinduction in the absence of STAT-6 (Tinnell, *et al.*, 1998). This theory is also supported by the absence of these STATs in unstimulated controls. The involvement of STAT-3 has also been recently discussed by Arinobu *et al* (Arinobu , *et al.*, 2000), and is activated by IL-4 and IL-13 through the utilisation of IL-13Rα (IL-4 can work through both the IL-4 and IL-13 receptor).

Delphin and Stavnezer have also suggested that STAT-6 and NFkB interact with each other to produce CD23 expression (Delphin & Stavnezer, 1995). They propose that this explains the observed IL-4/CD40L synergy, and promoter analysis combined with linker scanning mutagenesis has confirmed this interaction. The mechanisms of CD40 signalling are less well understood than those for IL-4, and have been reviewed by Hsing *et al* in 1997 (Hsing, *et al.*, 1997). Binding motifs for the transcription factor NFκB, which is known to be induced by anti-CD40 (Berberich, *et al.*, 1994), are present on the CD23a and CD23b promoters and linker scanning mutagenesis has shown that NFκB is important for CD23 superinduction with IL-4 and CD40L trimer in mice(Richards & Katz, 1997). Messner *et al* have also shown that the NFκB sites and STAT-6 sites are co-operative in IL-4-induced protein expression (Messner, *et al.*, 1997). Regarding CD40 signalling, there is strong evidence to suggest that NFκB family members compensate for the lack of others - as do STAT family members. As mentioned, Hsing *et al* (Hsing, *et al.*, 1997) have reported that CD40-induced CD23 expression does not necessarily utilise NFκB family members, but can also utilise AP-1, NF-AT, ATF-2 and STAT sites. This is interesting as the CD23b promoter has two AP1 sites that could also be

involved in anti-CD40 stimulation. Reconciliation of the difference between studies which have shown the need or lack of need for NFκB in CD23 expression are difficult, and clearly more analysis is required in addition to the currently available data.

STAT-6 expression is also augmented in B cells stimulated with anti-IgM and anti-CD40, and STAT-3 expression is similarly augmented at transcription level. STAT-3 augmentation in turn lead to the upregulation of IL-4 and IL-13 signalling and activation of CD23 expression. In the research documented in this thesis, anti-μ or anti-CD40 stimulation alone did not appear to have a significant effect on CD23 expression, although the reporter vector construct studies showed that the CD23b promoter was activated considerably with both of these stimulants.

Reasons for this discrepancy were sought and, as hinted in the results section, one of these could be that the actual epitope recognised by the particular anti-CD40 antibody used in this experiment was not sufficient to elicit an expressional response. This disparity has been touched upon by Burlinson *et al* in 1996 and Challa *et al* in 1999, who noted epitope dependent synergism and antagonism between anti-CD40 antibodies and soluble CD40 ligand (Burlinson, *et al.*, 1996; Challa, *et al.*, 1999). However, because activation of the actual promoter was noted, and only CD23 cell surface expression was absent, this is not considered to be the case.

Anti-CD40 and anti-μ have been noted to work synergistically to produce up to 4 fold increases in STAT-6 activation (Arinobu , *et al.*, 2000) and, indeed, CD23 expression. The fact that low CD23 expression was noted in this research with either anti-CD40 or anti-μ treatment alone was found to be in accordance with the results of Arinobu, and perhaps more convincing CD23 stimulation would have been achieved if synergistic anti-CD40 and anti-μ treatment had been given. Overall, the

current data are consistent with the model that STAT-6 functions as an enhancer of the transcription of IL-4 sensitive genes, rather than being absolutely required. The situation in relation to NFκB is possibly likewise.

Information is still required as to why, while the promoter region of CD23b is activated by stimulation with either anti-CD40 and anti-μ, no observable cell surface CD23 increase is seen. It should be noted that, while the promoter constructs give valuable information in to the activation of specific response elements of the two CD23 isoforms, the gene *in vivo* will be subject to many other positive and negative regulatory effects. It is likely that the promoter regions of each isoform, and also the intervening and proximal regions, confer inhibitory and stimulatory effects on each other. In short, what happens *in vitro* may not necessarily be the case *in vivo*. The possibility of translational control has also been considered, with translational initiation perhaps being dependent on the attainment of a specific minimum value of promoter activation.

Translational control of protein expression

Induction of translation is usually the rate limiting step in protein synthesis, which makes it an attractive target for regulation. An example of this phenomenon is the kinetics of haemoglobin synthesis(Stryer, 1995). In the absence of haem, haemoglobin subunit protein synthesis halts because of the abrupt formation of a protein synthesis inhibitor. This inhibitor is a kinase which phosphorylates eIF2, thus blocking protein synthesis. A recent paper (Meyuhas, 2000) discusses the synthesis of mammalian proteins, and the associated synthesis of translational apparatus in relation to several mitogenic and nutritional stimuli. It is suggested that perhaps CD23b synthesis requires IL-4, or indeed some other stimuli, in addition to the presence of solely anti-CD40 or anti-μ to produce surface CD23 expression.

Thus, IL-4 can enhance the activation of, for example, the anti-CD40 stimulated CD23b promoter to such a level that increased cell surface expression is observed.

It should also be noted that, in the reporter vector construct studies, an SV40 enhancer is present in the vector to increase transcription of the SEAP protein. The high increases observed in promoter activity may, therefore, be slightly over estimated in relation to the actual *in vivo* increase in promoter activity.

The fact that the CD23a promoter does not appear to be switched on by either anti-CD40, anti-μ or PMA indicates that repressors may bind to the NFkB binding sites, thus blocking the binding of NFκB proteins to this promoter. The BSAP/Pax-5 site on the CD23a promoter supports the theory that CD23a is involved in B cell function and development, rather than IgE-mediated immune responses. It is possible, therefore, that a repressor is formed at some stage around the immature/mature B cell boundary that blocks CD23a activation (other than with IL-4), and leaves CD23b to be responsible for IgE-mediated inflammatory responses (i.e. with the mitogens tested in this research).

6.4: Differential regulation of the CD23a and CD23b promoter

It has been suggested that CD23a expression is involved in B cell function and that CD23b is involved in IgE-mediated immune responses (Yokota, *et al.*, 1988). As implied, the presence of a BSAP/Pax-5 site on the CD23a promoter lends support to this theory, as does the fact that CD23a is expressed solely on B cells. Pax-5 codes for the transcription factor BSAP, which is expressed throughout B cell development except in terminally-differentiated plasma cells (Adams, *et al.*, 1992). Mammalian Pax genes encode for developmental regulators which are defined by the presence of a highly conserved DNA binding motif of 128 amino acids (Stapleton, *et al.*, 1993; Treisman, *et al.*, 1991), known as the paired domain. Mice lacking pax-5 exhibit an

early block in B cell development, manifested by the absence of pre-B, B and plasma cells (Urbanek, *et al.*, 1994). Anti-sense oligonucleotide-mediated inhibition of BSAP synthesis has been used to show that BSAP is involved in the control of proliferation of mature B cells (Wakatsuki, *et al.*, 1994), but cell cycle regulation in pro-B cells lacking Pax-5 is normal (Nutt, *et al.*, 1997). Thus, B lymphocytes do not seem to require BSAP early in the developmental pathway, but become dependent on this transcription factor in the later stages of B cell differentiation.

Pax-5 is expressed up to and including the activated B cell stage Busslinger *et al* (Busslinger & Nutt, 1998) have shown that, in addition to being a transcriptional activator, BSAP can function as a repressor. The repressor and activator functions are dependent on the concentrations of BSAP and the context of the protein binding site. Sites that lead to the activation of gene expression were found to have a higher affinity for BSAP than repressive sites, leading to the conclusion that Pax-5 activator functions predominate at low concentrations of BSAP protein and Pax-5 will function only as a repressor at higher protein concentrations.

CD23 has been found to be expressed only at the mature B cell stage (Rajewsky, 1996; Waldschmidt, 1992), and there is some overlap in BSAP and CD23 expression (BSAP is expressed right up to the plasma cell stage). CD23a transcription could thus be primarily activated by the presence of BSAP in newly mature B cells, and this could be highly dependent on the BSAP concentration. This theory is supported by the fact that CD23 expression is absent in plasma cells, as is the expression of BSAP. It can be surmised, therefore, that the CD23 present in naive B cells will be CD23a only, rather than CD23b which is involved in the IgE-mediated response. It remains unclear as to what the function of CD23a is, and as to why it would be required on newly-developed B cells. Bearing in mind that the only difference in the two isoforms is on the 7 amino acid region of the N terminal cytoplasmic region, perhaps different intracellular mediators bind to the tail of each isoform and, in the

case of CD23a, may be involved in preventing B cells from differentiating into plasma cells. Upon the withdrawal of BSAP, and the resulting drop in CD23 expression, B cells would be free to differentiate into plasma cells.

The CD23b isoform can be expressed on B cells, T cells and other haematopoietic cells following stimulation with IL-4, anti-CD40, anti-μ and PMA. It is thus implied that it this isoform that is involved in the IgE mediated immune response and atopy, and not in B cell differentiation. It is possible that an intra-cellular protein binds to the unique tail region of CD23b to lead to increased proteolytic cleavage of the CD23 molecule to produce the elevated sCD23 levels that are indicative of atopy. To re-cap, membrane bound CD23 complexes to and is a negative regulator of IgE, but sCD23 actually leads to an increase in IgE expression. sCD23 has also been shown to rescue the human pre-B like leukaemic cell line SMS-SB from apoptosis (White, *et al.*, 1997), so can be involved in the maintenance of neoplastic cells as well.

6.5: Future Studies

Several lines of study have arisen and could be pursued in future following the research carried out in this thesis. Reporter vector construct studies were carried out using both full-length and truncated forms of the CD23a and CD23b promoter regions, and future experiments could be to build constructs with promoter deletion mutations as opposed to truncations. This would allow analysis into whether certain response elements, and the transcription factors binding to them, can interact with one another to compensate for the loss of distinct singular sites. It is possible, for example, that when the second IL-4RE/STAT-6 site is deleted on the CD23b promoter the first IL-4RE/STAT-6 site (or another unknown site around this area) comes into play. Also, further analysis is required on the putative negative

regulatory effect region observed and removed upon truncation of the CD23b promoter past the first IL-4RE/STAT-6 site.

Reporter vector construct studies of the CD23a and CD23b promoter revealed that the CD23a promoter was activated by IL-4 alone, but the CD23b promoter was initiated with all of IL-4, anti-CD40, anti-μ and PMA. The expression of CD23 is upregulated on B cells stimulated with IL-4 in conjunction with anti-CD40 (Clark & Ledbetter, 1994; Richards & Katz, 1997). It has also been implied that, while STAT-6 enhances CD40 induced CD23 expression, it is not absolutely required for CD23 superinduction via anti-CD40 and IL-4 (Tinnell, *et al.*, 1998). IL-4 can, therefore, still synergise with CD40 in the absence of STAT-6 and this suggests that an alternative STAT family member is induced. Follow-on experiments would be to co-stimulate the reporter vector construct transformed cells with both IL-4 and anti-CD40, and to remove or incapacitate the STAT-6 sites in order to analyse any effects on promoter activation. If other sites are involved in CD23 super-induction, and are present in the cloned promoter region, they will compensate for the loss of STAT-6 sites.

To further investigate the evidence by Hsing *et al* (Hsing, *et al.*, 1997) that CD40 induced CD23 expression does not necessarily utilise members of the NFkB family, but also AP-1, NF-AT, ATF-2 and STAT sites, further studies could also involve the anti-CD40 stimulation and IL-4 and anti-CD40 co-stimulation of reporter vector construct transformed cells, following the removal of NFκB binding sites. The CD23b promoter has two AP1 sites that could be involved in anti-CD40 stimulation, as well as two STAT-6 sites. The data from these experiments would allow the determination of the necessity of the NFκB site for CD40 signalling, and also uphold or banish the theory of AP-1 utilisation in this instance.

Isolation of the promoter regions of each isoform does, as stated, provide valuable information on the *in vitro* regulation of each. An interesting experiment would be to build a reporter vector construct containing both the CD23a and CD23b promoter, in order to determine if they convey negative and positive regulatory effects on each other. Ideally, this construct should contain the intervening DNA regions between the two promoters, so that any response elements and regulatory domains within these areas could be studied. Logistically, however, an insert of this size would be almost impossible to include within the pSEAP reporter vector, so only the minimal promoter regions would have to be ligated and inserted.

I have hypothesised that CD23a may be involved in the prevention of mature B cells differentiating into plasma cells, and that the expression of CD23a is dependent on levels of BSAP. The transformed plasma cell line RPMI-8866, interestingly, expresses large amounts of cell surface CD23 (J. Matheson, personal communication). It would be of major interest to discover if these cells also expressed high levels of BSAP. Alternatively, normal plasma cells could be transfected with Pax-5/BSAP and the resulting CD23 levels measured in order to analyse the proposed link between the expression of the two proteins.

In summary, the research in this thesis has provided valuable information into the regulatory mechanisms of each CD23 isoform and, as a result, has opened a wealth of avenues for future research.

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APPENDIX

APPENDIX:

SOLUTIONS AND BUFFERS:

SOLUTION	REAGENTS AND COMPOSITION
DNA LOADING DYE (5X)	0.25% (w/v) Bromophenol blue, 30% Glycerol
EMSA BINDING BUFFER (5X)	20% Glycerol, 5mM $MgCl_2$, 2.5mM EDTA, 2.5mM DTT, 250mM NaCl, 50mM Tris-HCl (pH 7.5), 0.25mg/ml poly dI-dC
LB AGAR	15g Bacto-Agar to 1L LB media. Autoclave, cool to 55°C, supplement with 0.05mg/ml Ampicillin
LB BROTH/MEDIA	10g Bacto-Tryptone, 5g Bacto-Yeast extract, 10g NaCl. pH to 7.5, dH_2O to 1L, supplement with 0.05mg/ml Ampicillin
LM-PCR AMPLIFICATION SOLN	400mM NaCl, 50mM $MgSO_4$, 1% (v/v) Triton-X-100, 0.1% (w/v) Geletin, 200mM Tris-HCl (pH 8.9)
LM-PCR DILUTION SOLN	18mM $MgCl_2$, 50mM DTT, 0.0125% (w/v) BSA, 110mM Tris-HCl (pH 7.5)
LM-PCR FIRST STRAND BUFFER (5X)	200mM NaCl, 25mM $MgSO_4$, 0.5% (w/v) Geletin, 50mM Tris-HCl (pH 8.9)
LM-PCR LABELLING MIX	1 x Amplification mix, 2mM each dNTP, 2.3pmol [^{32}P]- labelled Primer 3

LM-PCR LIGATION SOLN (2X)	20mM $MgCl_2$, 40mM DTT, 6mM ATP, 0.01% (w/v) BSA, 500mM Tris-HCl (pH 7.7)
LM-PCR LOADING DYE	98% de-ionised formamide, 10mM EDTA, 0.25% (w/v) xylene-cyanol, 0.25% (w/v) Bromophenol blue
LM-PCR PRECIPITATION SOLN	0.1% yeast transfer RNA, 2.7M Na Acetate (pH 7)
LM-PCR STOP SOLN	67µg/ml yeast transfer RNA, 4mM EDTA, 260mM Na Acetate (pH 7), 10mM Tris-HCl (pH 7.5)
MAXI-PREP (QIAGEN) P1	50mM Tris-HCl (pH 8), 10mM EDTA, 100µg/ml RNase A
MAXI-PREP (QIAGEN) P2	200mM NaOH, 1% (w/v) SDS
MAXI-PREP (QIAGEN) P3	3M K Acetate. pH to 4.8
MAXI-PREP (QIAGEN) QC	1M NaCl, 50mM MOPS (pH 7), 15% (v/v) Isopropanol
MAXI-PREP (QIAGEN) QF	1.25mM NaCl, 50mM Tris-HCl (pH 8.5), 15% (v/v) Isopropanol
PBS(PHOSPHATE BUFFERED SALINE) (10X)	1.37M NaCl, 26.8M KCl, 42mM Na_2HPO_4, 14.7mM KH_2PO_4. pH to 7.2
PROTEIN LOADING BUFFER (4X)	200mM Tris-HCl (pH 8), 8% (w/v) SDS, 0.4% (w/v) Bromophenol blue, 40% (v/v) Glycerol, 20% (v/v) β-Mercaptoethanol
PROTEIN TRANSFER BUFFER	48mM Tris, 39mM Glycine, 1.3mM SDS, 20% (v/v) Methanol

RIPA BUFFER	50mM Tris-HCl (pH 7.4), 1% (v/v) NP40, 1mM Na Deoxycholate, 150mM NaCl, 1mM EGTA, 1mM Na_3VO_4, 1mM NaF, 1mM PMSF, 2µg/ml Leupeptin, 0.5mM DTT
SDS-PAGE RUNNING BUFFER	25mM Tris-HCl (pH 8.3), 250mM Glycine, 0.1% (w/v) SDS
SOC MEDIUM	2g Bacto-tryptone, 0.5g Bacto-yeast, 0.01M NaCl, 0.01M $MgCl_2$, 0.01M $MgSO_4$, 0.02M Glucose
STET	8% (v/v) Sucrose, 2.5ml 2M Tris (pH 8), 40ml 250mM EDTA (pH 8), 10ml Triton-X-100, dH_2O to 200ml
TAE BUFFER (50X)	242g Tris, 18.6g EDTA, 57ml of Glacial Acetic acid, dH_20 to 1L
TBE BUFFER (10X)	0.9M Tris-HCl, 0.9M Boric acid, 2mM EDTA. pH to 8.3
TE BUFFER	10mM Tris-HCl (pH 8), 1mM EDTA

COMPOSITION OF GELS:

PERCENTAGE OF GEL	REAGENTS AND COMPOSITION
10% SDS-PAGE SEPARATION GEL	10% (w/v) Acrylamide/Bis Acrylamide, 0.375M Tris-HCl (pH 8.8), 0.1% (w/v) SDS, 0.05% (w/v) Ammonium Persulphate, 0.0003% (v/v) TEMED
12% SDS-PAGE SEPARATION GEL	12% (w/v) Acrylamide/Bis Acrylamide, 0.375M Tris-HCl (pH 8.8), 0.1% (w/v) SDS, 0.05% (w/v) Ammonium Persulphate, 0.0003% (v/v) TEMED
5% SDS-PAGE STACKING GEL	5% (w/v) Acrylamide/Bis Acrylamide, 0.13M Tris-HCl (pH 6.8), 0.1% (w/v) SDS, 0.1% (w/v) Ammonium Persulphate, 0.0007% (v/v) TEMED
6% 'LONG RANGER' SEQUENCING GEL FOR FOOTPRINTING	9.6ml 'Long Ranger' gel solution (50% v/v), 16ml of 5 X TBE, 40.4g Urea, 70mg Ammonium Persulphate, mix, dissolve, filter. Add 25µl TEMED.