Biological and biochemical analyses of the distinctive intracellular signals activated by interleukin-4

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ABSTRACT

Interleukin-4 (IL-4) is a type I cytokine which acts on multiple hemopoietic cells to promote an antibody-mediated response to infection. Dysregulated production or function of IL-4 can exacerbate diseases such as allergy, asthma and rheumatoid arthritis. In order to better understand the biochemical mechanisms by which IL-4 mediates its pleiotropic biological effects, I investigated two distinctive aspects of the intracellular signals activated by IL-4. First, IL-4 is different from most type I cytokines in its inability to activate Ras or Raf-1. IL-4 also fails to support cellular growth. I demonstrated that the signals provided by an active Ras or an inducibly active Raf-1 kinase could synergise with IL-4 to promote cell-cycle progression. Further investigation of the biochemical events associated with the stimulation of long-term growth showed that active Raf-1 not only synergised with IL-4 to stimulate growth, but also to increase levels of c-jun N-terminal kinase (JNK) activity. These observations raise the possibility that Raf-1 may be involved in regulating JNK activity, and that JNK may be involved in mediating certain effects of IL-4. Second, IL-4 and IL-13 are the only cytokines that activate the transcription factor STAT-6. I determined that activation of STAT-6 was required for IL-4-stimulated cell survival. However, I found evidence that this requirement for STAT-6 was indirect, and possibly related to STAT-6-dependent, IL-4-stimulated expression of the IL-4 receptor. I next investigated the hypothesis that STAT-6 was required for IL-4-mediated suppression of tumor necrosis factor α (TNFα) and interleukin-12 (IL-12) production in macrophages. When STAT-6 null macrophages were stimulated with lipopolysaccharide (LPS) and interferon γ (IFNγ), I continued to observe a significant inhibition of TNFα and IL-12 by IL-4, suggesting that IL-4 activates distinct, STAT-6 independent, inhibitory paths. IFNγ antagonizes many of the effects of IL-4, and I determined that IFNγ may regulate the activity of

STAT6 by altering expression of a STAT6 inhibitor, Bcl-6. Further investigation into the roles of JNK, Bcl-6 and novel, non-STAT-6-dependent pathways will be important for the design of strategies to therapeutically modulate the intracellular signals activated by IL-4.

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ABBREVIATIONS

2ME	2-mercaptoethanol
4HT	4-hydroxy tamoxifen
AP-1	Activating protein 1
ATCC	American Type Culture Collection
BAZF	Bcl-6-associated zinc finger protein
Bcl-6	B cell lymphoma 6
BCR	B cell receptor
BMMΦ	bone-marrow derived macrophage
bp	base pairs
BSA	bovine serum albumin
C-	carboxy-
C/EBP	CCAAT/enhancer binding protein
CD	Cluster of Differentiation
CM	conditioned medium
cpm	counts per minute
CSF-1	Colony Stimulating Factor-1
ECL	enhanced chemiluminescence
EF	Elongation Factor
EGF	Epidermal Growth Factor
EGFP	enhanced green fluorescent protein
ELISA	Enzyme linked immunosorbant assay
ER	estrogen receptor
ERK	Extracellular Regulated Kinase
FACS	fluorescence activated cell sorter
Fc	Fraction crystalisable
FCS	fetal calf serum
GapDH	Glyceraldehyde-3-phosphate dehydrogenase

GAS	Gamma Activating Sequence
GM-CSF	granulocyte-macrophage-colony stimulating factor
gpt	guanine/hypoxanthine phosphoribosyl transferase
GR	gene regulation
GST	Glutathione-S-transferase
HA	hemagglutinin
HAT	hypoxanthine, aminopterin, thymidine
HBSS	Hepes-buffered saline solution
HRP	horseradish peroxidase
I4R	insulin-IL-4 -receptor
IB	immunoblot
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IP	Immunoprecipitation
IRS	Insulin Receptor Substrate
ISRE	IFNα/β-stimulated response element
JAK	Janus Kinase
JNK	c-jun N-terminal kinase
KAB	kinase assay buffer
kDa	kilodalton
LCCM	L-cell conditioned medium
LPS	lipopolysaccharide
LT	lymphotoxin
MAP	Mitogen activated protein
MBP	myelin basic protein
MEK	MAP/ERK kinase 1
MEKK	MEK-kinase 1

MHC	Major histocompatability
MTT	3-[Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
N-	amino-
NF-AT	nuclear factor-activator of activated T cells
NF-κB	nuclear factor-kappa B
NK	Natural Killer
OD	Optical density
OP	Oligo precipitation
PCR	polymerase chain reaction
PDGF	Platelet derived growth factor
PDK-1	PIP_3-dependent kinase-1
PEC	peritoneal exudate cells
PH	Pleckstrin Homology
PI	Phosphatidyl inositol
PIAS	Protein Inhibitor of Activated STAT
PKB	protein kinase B
POZ	Pox viruses and Zinc fingers
PTB	Phosphotyrosine binding
r	recombinant
R	Receptor
RLU	relative light units
SCID	Severe combined immunodeficiency
SDF-1	Stromal Cell Derived Factor-1
SEM	standard error of the mean
SFM	serum free media
SH2	Src homology 2
SH3	Src-homology 3
SHP-2	Src homology phosphatase-2
SOCS	suppressor of cytokine signalling

STAT	signal transducer and activator of transcription
TBS	Tris-buffered saline
TCR	T cell receptor
TGFβ	transforming growth factor β
Th	T helper
TNF	tumor necrosis factor
TPA	phorbol ester
UTR	untranslated region
W3B	WEHI 3B conditioned medium

PREFACE

Thesis Format

This thesis includes 7 chapters. The first chapter beings with a general introduction to cytokines, and then focuses on the biological functions of IL-4 and the current state of knowledge about the intracellular signals that are activated by IL-4. Chapter 2 describes the materials and methods that were employed to perform the experiments described in Chapters 3-6. Chapters 3 and 5 focus on results contained in submitted manuscripts (see below). For Chapter 3, Darrell Bessette was a co-operative education student from Simon Fraser University, who worked under my direct supervision for 8 months. In collaboration, we generated Figures 3.2, 3.4, 3.6, and 3.7. Chapters 4 and 6 focus on unpublished data. Chapter 7 contains a summary of the general conclusions that can be drawn based on the work presented in this thesis, and a discussion of the future directions.

Publications obtained during the course of this thesis:

Welham, M. J. Bone, H. **Levings, M**. Learmonth, L. Wang, L. M. Leslie, K. B. Pierce, J. H. Schrader, J. W. 1997. Insulin receptor substrate-2 is the major 170-kDa protein phosphorylated on tyrosine in response to cytokines in murine lymphohemopoietic cells. *J. Biol. Chem.* 272: 1377-81.

Submitted manuscripts:

Levings, M.K., Bessette, D.C., Schrader, J.W. *Interleukin-4 synergizes with Raf-1 to promote long-term growth and activation of c-jun N-terminal kinase.*

Levings, M.K. and Schrader, J.W. *IL-4 inhibits the production of TNFα and IL-12 by STAT6 dependent and independent mechanisms.*

Orban, P.C., **Levings, M.K.**, Schrader, J.W. *Heterodimerization of the α and β chains of the Interleukin-3 (IL-3) receptor is necessary and sufficient for IL-3-stimulated proliferation.*

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CHAPTER 1. Introduction

1.1. General Overview

Cytokines are small polypeptides that facilitate communication between a wide variety of target cells. Unlike classical hormones, cytokines are usually produced by multiple cell types, and have pleiotropic effects. They control the development and differentiation of erythrocytes, leukocytes, embryos, organs and the growth and maintenance of body mass. Cytokines are never present in isolation, but rather function within a network, and it would be rare for a cell to encounter a single cytokine. Within this network, cytokines can have additive, synergistic, or antagonistic effects, which are mediated at the level of control of cytokine production and/or function (Vilcek, 1998).

Dysregulation of the cytokine network can have profound consequences, and may lead to severe immunodeficiencies, cancer, autoimmune diseases and chronic inflammatory diseases. However, the redundancy and complexity of the interactions between cytokines poses a major problem when designing treatments for diseases that result from dysregulated cytokine production and function. When the function or the production of one cytokine is altered, it is difficult to determine how many other cytokines may be inhibited or enhanced in parallel, and what the detrimental consequences of these indirect effects may be (Geoffrey et al., 1998).

Cytokines are divided into several families based on similarities in primary amino acid sequence and the predicted tertiary structures. Type I cytokines form the largest sub-family, which includes most of the interleukins (IL), as well as molecules such as erythropoietin, thrombopoietin, prolactin, growth hormone, leptin and leukemia-inhibitory factor. Although type I cytokines share very little primary sequence similarity, they are remarkably conserved at the three-dimensional level, with 4 alpha-helices in a spatially similar arrangement (Bazan,

1990). Type II cytokines include the interferons (IFN) and IL-10. Tumor necrosis factors (TNF) are a large family, which includes TNF-α and –β, lymphotoxins, CD40 and CD95 (FAS). Unlike type I and II cytokines, which act as monomers or dimers, TNF family members are biologically active as trimers. Others cytokine families include the IL-1, and transforming growth factor (TGF)-β families and the chemokines.

All cytokines act by binding to specific membrane-spanning receptors on the surface of responsive cells. With the exception of chemokines, ligand binding induces dimerisation and/or oligomerisation of these receptors. Cytokine receptors are distinct from other types of growth factor receptors, such as those for epidermal growth factor (EGF), or platelet derived growth factor (PDGF), in that they lack enzymatic activity. Rather, the receptors for type I and II cytokines require cytoplasmic tyrosine kinases to initiate the intracellular signalling cascades which ultimately lead to changes in gene expression and cellular functions (Vilcek, 1998).

In an attempt to understand how their pleiotropic effects are exerted, much research has been devoted to characterising the intracellular signalling cascades that are activated by cytokines. Surprisingly, it seems that most cytokines activate fundamentally similar intracellular signals, and there are few cases where specific biological effects can be attributed to a truly specific molecular signal. For example, almost all type I cytokines activate the same three signalling pathways: the Ras/mitogen activated protein (MAP) kinase pathway; the JAK/STAT pathway and the phosphatidyl-inositol (PI) 3' kinase pathway (Ihle et al., 1995). There are of course exceptions, and this thesis is centered on a type I cytokine known as interleukin-4 (IL-4), for which specific biological effects can be attributed to specific intracellular signals. Thus, in contrast to other type I cytokines, IL-4, and the highly related IL-

13, fail to activate Ras or MAP-family kinases, and they are the only cytokines that activate STAT-6.

IL-4 has a dual role in regulating the immune response. First, during an infection IL-4 acts on multiple cell-types to collectively promote an humoral immune response mediated by antibody production. In parallel, IL-4 inhibits cellular immunity mediated by phagocytic and cytotoxic cells. These dual functions of IL-4 are of particular interest in the pathology of diseases such as asthma and arthritis. IL-4 drives allergic reactions by promoting the formation of antibody-producing cells, the development of mast cells and the stimulation of eosinophil function. Through the inhibition of cell-mediated responses, IL-4 can have positive effects on the pathology of diseases, such as rheumatoid arthritis, which are exacerbated by the tissue damage resultant from cell-mediated immunity (Pascale et al., 1998).

When this thesis was initiated in September 1993, although the biological functions of IL-4 were well known, little was known about the molecular mechanisms that mediated these specifc effects. I therefore undertook an analyses of the biological functions of the unique aspects of the intracellular signals activated by IL-4.

1.2. Sources of IL-4

The production of IL-4 is tightly regulated and is normally undetectable in the absence of an infection. $CD4^+$ T helper (Th) cells are the best characterised source of IL-4; Th-1 cells produce IFNγ and very little IL-4, whereas Th-2 cells are a major source of IL-4 (Fernandez-Botran et al., 1988; Fiorentino et al., 1989). The mechanistic basis behind this Th-2-restricted production of IL-4 has been intensely studied. The regulation is complex, and controlled by multiple transcription factors which vary depending on the co-stimulatory signals. The promoter for IL-4

contains several well characterised transcription factor binding sites including those for nuclear factor-activator of activated T cells (NF-AT), CCAAT/enhancer-binding proteins (C/EBP), AP-1, NF-κB, STAT-6, and the proto-oncogene, *c-maf* (Li-Weber et al., 1997). Mice deficient for NF-ATc or NF-ATp have significantly reduced levels of IL-4, suggesting that activity of these transcription factors is essential for production of IL-4 (Hodge et al., 1996; Ranger et al., 1998; Yoshida et al., 1998). Expression of *c-maf* and GATA-3 may also be important in regulating Th-2-specific production of IL-4 (Ho et al., 1996; Zheng and Flavell, 1997). In contrast, deletion of the STAT-6 element in either the murine or human IL-4 promoter has no effect on T-cell receptor (TCR)-mediated stimulation of IL-4 production (Huang et al., 1997).

Recently, Kubo *et al.* (1997) reported the presence of a silencer element in the 3' untranslated region (UTR) of the IL-4 gene. This silencer element contains consensus binding sites for a variety of transcription factors, including STAT-6 (see 1.7). STAT-6 binds and inactivates this element specifically in Th-2 cells and not in Th-1 cells. Furthermore, if this STAT-6 binding site is deleted, expression of IL-4 mRNA is silenced even in Th-2 cells. Thus, the apparent inability of IL-4 to stimulate activation of STAT-6 in Th-1 cells (Huang and Paul, 1998; Kubo et al., 1997) may offer an explanation for the Th-2 restricted profile of IL-4 production.

IL-4 is the key developmental factor for Th-2 cells, but the initial source of IL-4 which promotes Th-2 commitment is unclear. Candidate cell-types include $NK1.1^+$ T cells, memory $CD4^+$ T cells, mast cells, and eosinophils (Bradley et al., 1991; Noben-Trauth et al., 1997; Yoshimoto and Paul, 1994). IL-4 is produced by mast cells and eosinophils following cross-linking of the receptors for IgE or IgG (Brown et al., 1987; Brunner et al., 1993; Plaut et al., 1989). Mast cells are capable of presenting antigens to T cells (Mecheri and David, 1997), and

in combination with IL-4, these cells may promote the initial development of Th-2 cells. $NK1.1^+$ T cells are capable of producing large amounts of IL-4 one hour after TCR stimulation *in vivo* (von der Weid et al., 1996; Yoshimoto and Paul, 1994). However CD1 deficient mice, which lack $NK1.1^+$ T cells, have Th-2 cells (Smiley et al., 1997), thus the precise role of $NK1.1^+$ T cells in mediating Th-2 development is unclear.

1.3. Biological Functions of IL-4

1.3.1. B cells

IL-4 is a growth co-factor for B lymphocytes, particularly in combination with ligation of CD40 (by CD40 Ligand) (Banchereau et al., 1991). IL-4 stimulates the expression of MHC class II, and CD23 (the low affinity receptor for IgE, FcεRII) (Defrance et al., 1987), and class switching to the IgE and IgG_1 isotopes (Shapira et al., 1992). Thus, although inactivation of the IL-4 gene in mice permits normal B cell development, there is a large reduction in IgE levels in response to parasitic infections (Kuhn et al., 1991). Stimulation with IL-4 also decreases expression of Fcγ receptors (O'Garra et al., 1987). IL-4, in combination with CD40 Ligand, induces morphological changes and cytoskeletal rearrangements in B cells which may be important for cell-cell interactions (Davey et al., 1998).

An important mechanism for ensuring that B cells that recognize self-antigen do not become activated is deletion of these cells by apoptosis. CD40 Ligand, which is expressed on activated Th cells, stimulates B cells to express FAS (CD95) and makes them susceptible to rapid cell death by FAS-mediated apoptosis (Nagata, 1997). Normally, B cells are rescued from this FAS-mediated apoptosis by co-stimulation of the B-cell receptor (BCR) by foreign antigen (Cornall et al., 1995). However, B cells that recognize self-antigen become "tolerant" during

their development and BCR stimulation by self antigen cannot rescue these cells from FAS-mediated apoptosis (Rathmell et al., 1995; Rathmell et al., 1996; Rothstein et al., 1995; Schattner et al., 1995). Interestingly, tolerant B cells that recognize self-antigen can be rescued by co-stimulation with IL-4, a phenomenon which may contribute to auto-antibody formation and promote auto-immune disease (Foote et al., 1996; Foote et al., 1998). Indeed, mice that express IL-4 under control of the MHC class I promoter show polyclonal B cell activation, increased production of autoantibodies, and autoimmune-like disorders (Erb et al., 1997).

1.3.2. T cells

The types of cytokines that are produced in the initial stages of an infection are dependent upon the type of infection, the dose and frequency of the infection, and the genetic background of the host. These early cytokines act on antigen-specific $CD4^+$ Th cells to promote the development of distinct subsets that are themselves distinguished by defined patterns of cytokine production (Figure 1.1) (O'Garra, 1998). Bacterial products, such as lipopolysaccharide (LPS), and IFNγ produced by natural killer (NK) cells, stimulate macrophages and dendritic cells to produce IL-12. IL-12 drives the development of Th-1 cells, which characteristically produce IFNγ and lymphotoxin α. The presence of IFNγ promotes cell-mediated immunity by stimulating phagocytic and cytotoxic cells to eliminate intracellular pathogens. In contrast, if IL-4 is present during the early stages of an immune response, Th-2 cells develop (Hu-Li et al., 1987; Mosmann et al., 1986; Spits et al., 1987). Th-2 cells characteristically produce IL-4, IL-5, IL-10 and IL-13 and promote humoral immunity.

The cytokines produced by these T cell subsets reciprocally regulate each other's development and function. For example, IL-4, IL-10, and IL-13 inhibit Th-1 development

indirectly by inhibiting the production of IL-12 by macrophages and dendritic cells (D'Andrea et al., 1995; Ohshima and Delespesse, 1997). IL-4 can also directly down-regulate expression of one of the receptor subunits for IL-12 (IL-12Rβ2) (Rogge et al., 1997). In contrast, IFNγ upregulates expression IL-12Rβ2 and the production of Th-1 cells (Szabo et al., 1997). The effects of IL-4 are dominant, and if levels of IL-4 predominate during an immune response, Th-2 cells will differentiate, leading to progressively higher levels of IL-4 (Seder and Paul, 1994).

1.3.3. Mast cells, basophils and eosinophils

In combination with IL-3, IL-4 induces the generation and proliferation of mast cells and basophils (Mosmann et al., 1986). By stimulating the production of IgE which can bind to Fcε receptors on mast cells, basophils and eosinophils, IL-4 can indirectly stimulate the release of histamine, leukotrienes, and other inflammatory mediators by these cells (Pascale et al., 1998). These stimulatory effects on mast cells and eosinophils are a major factor in the promotion of allergy, and in particular of asthma (Maggi, 1998).

1.3.4. Macrophages and monocytes

IL-4 inhibits the generation of monocytes and macrophages (Snoeck et al., 1993), and suppresses the production of many pro-inflammatory mediators such as IL-1, tumor necrosis factor α(TNFα), IL-6, IL-12, superoxide, prostaglandins and cyclooxygenase (Endo et al., 1996). This ability of IL-4 to inhibit macrophage-mediated inflammation can have a positive effect on chronic inflammatory diseases such as rheumatoid arthritis (Feldmann et al., 1996).

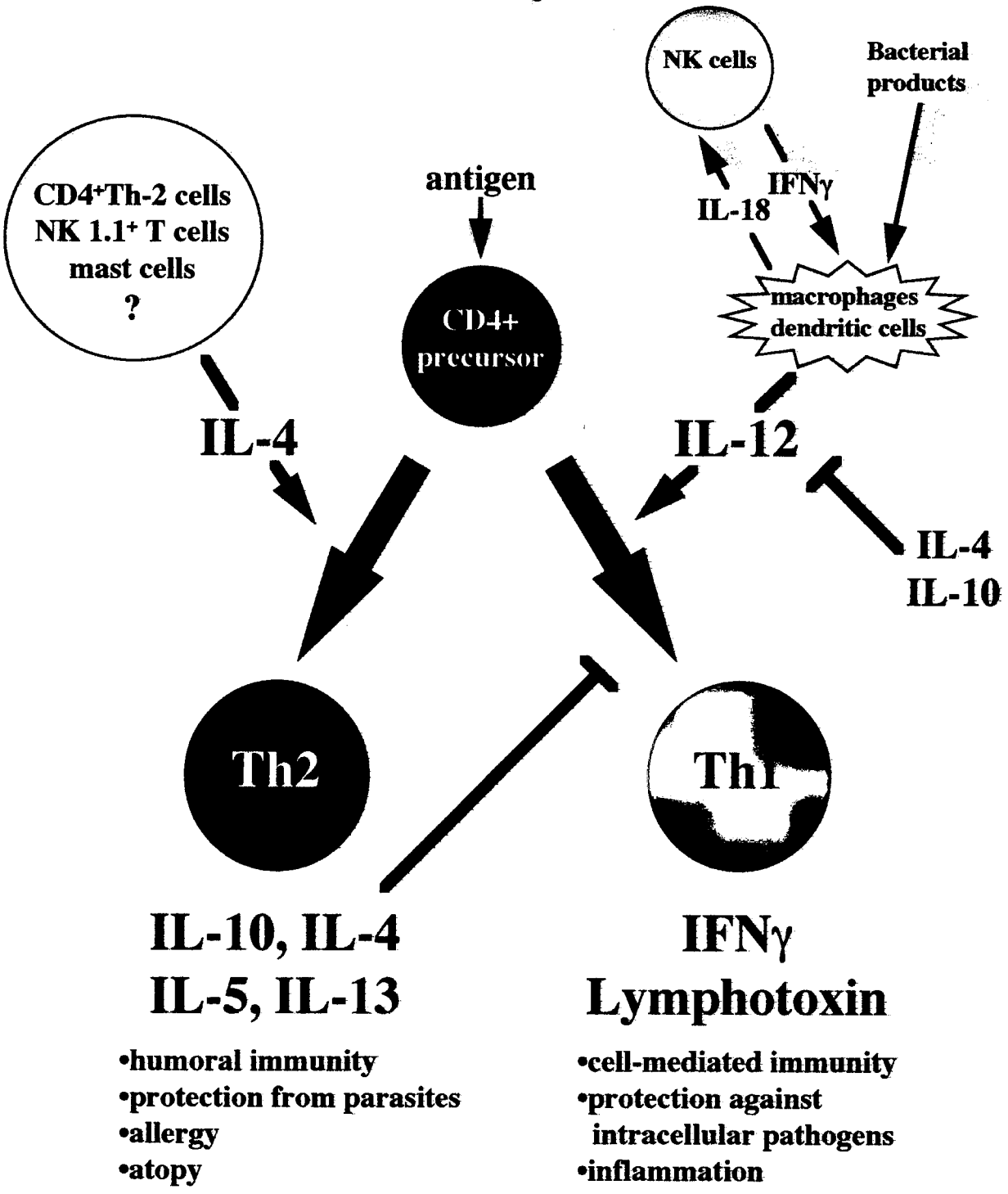

Figure 1.1. *Regulation of the development of the Th-1 and Th-2 cell subsets.* Naïve CD4+ T cells develop into Th-1 cells in the presence of IL-12 that is produced by macrophages and/or dendritic cells during an infection. Th-2 cells require IL-4 for their development. Differentiation of Th-1 cells can be inhibited by IL-4 and IL-10, by both indirect and direct mechanisms. Th-2 cytokines promote humoral immunity, and Th-1 cytokines promote cell-mediated immunity. Dysregulated production of either Th-2 or Th-1 cells can result in disease.

1.4. The IL-4 Receptor

1.4.1. IL-4Rα, γ_c and IL-13R

Receptors for IL-4 are expressed on most cell types (Park et al., 1997), and treatment with IL-4 further increases receptor expression (Galizzi et al., 1989). The high affinity IL-4 binding subunit is a 140 kDa transmembrane protein (IL-4Rα) (Ohara and Paul, 1987) which contains the hallmarks of the type I cytokine receptor family, namely four conserved cysteine residues and a WSXWS motif in the extracellular domain, a proline-rich domain in the membrane-proximal intracellular portion, and the lack of a tyrosine kinase activity (Miyajima et al., 1992). There are two distinct functional IL-4 receptors; the best characterised is a complex of IL-4Rα and the 65 kDa γ_c chain (Kondo et al., 1993; Russell et al., 1993). The γ_c subunit is also a component of the functional receptors for IL-2, -7, -9 and –15 (Kondo et al., 1993). A second functional IL-4R is composed of a complex of the IL-4Rα and the IL-13RI. This functional receptor also binds to IL-13 (Aman et al., 1996; Hilton et al., 1996). A second IL-13R, IL-13RII, can also associate with IL-4Rα to form a high affinity receptor for IL-4, however the biological significance of this receptor complex is unclear as IL-13RII only has 17 cytoplasmic amino acids (Caput et al., 1996; Murata et al., 1998). Several groups have shown that artificial homodimerization of the IL-4Rα, in the absence of γ_c or IL-13R, is sufficient for IL-4-mediated intracellular signalling and biological effects (Fujiwara et al., 1997; Kammer et al., 1996; Lai et al., 1996; Reichel et al., 1997). Homodimerisation of IL-4Rα can induce activation of intracellular signalling pathways, stimulate increased expression of CD23 and promote B-cell class switching to IgE. Thus the function(s) of the cytoplasmic domains of γ_c and the IL-13Rs remains unclear.

1.4.2. Functional domains in IL-4Rα

There are 3 distinct domains in the IL-4Rα, the box-1 and –2 domains, the I4R or "growth control" domain and the gene regulation domain (Keegan et al., 1994; Ryan et al., 1996; Wang et al., 1996) (see Figure 1.2). Each of these regions mediates the activation of a subset of the intracellular signals activated by IL-4. The proline rich regions in box-1 and/or -2 are conserved amongst all type I cytokine receptors and are required for activation of JAKs (see 1.5.1) (Harada et al., 1998; Tanner et al., 1995). In the absence of the box-1 and –2 domains, the IL-4Rα is non-functional (Deutsch et al., 1995). The growth control domain includes a tyrosine (Y497) residue which is surrounded by a sequence homologous to a region in the insulin receptor (the I4R motif). Tyrosine 497 acts as a docking site for phosphotyrosine-binding (PTB) domain containing proteins such as the insulin receptor substrates (IRS) -1 and –2 (see 1.6) (Harrison, 1996; Keegan et al., 1994; van der Geer and Pawson, 1995). Tyrosine 497 is required for IL-4-mediated protection from apoptosis (Wang et al., 1996). It can also regulate the activation of STAT-6 (Wang et al., 1998) and the phosphorylation of HMG-1(Y), a small non-histone chromosomal protein involved in the control of gene expression in hemopoietic cells (Wang et al., 1997).

The gene regulation domain (558-657) contains three tyrosine residues (Y575, 603 and 631) that are important for recruitment and activation of STAT-6. Truncation of the IL-4Rα at amino acid 557 results in a receptor that cannot stimulate STAT-6 DNA binding activity, but is able to prevent apoptosis (Pernis et al., 1995; Ryan et al., 1996). However, STAT-6 can also be activated in the absence of any tyrosine residues, and may in fact directly associate with JAK1. Hershey *et al.* have reported the presence of a novel allele of the IL-4Rα, which contains a Q576R point mutation, in patients with allergic inflammatory disorders such as atopy (Hershey

et al., 1997). This mutation is associated with an increase in IL-4-stimulated CD23 expression, which may result from a change in the subset of proteins interacting with this tyrosine residue due to the altered context of Y575.

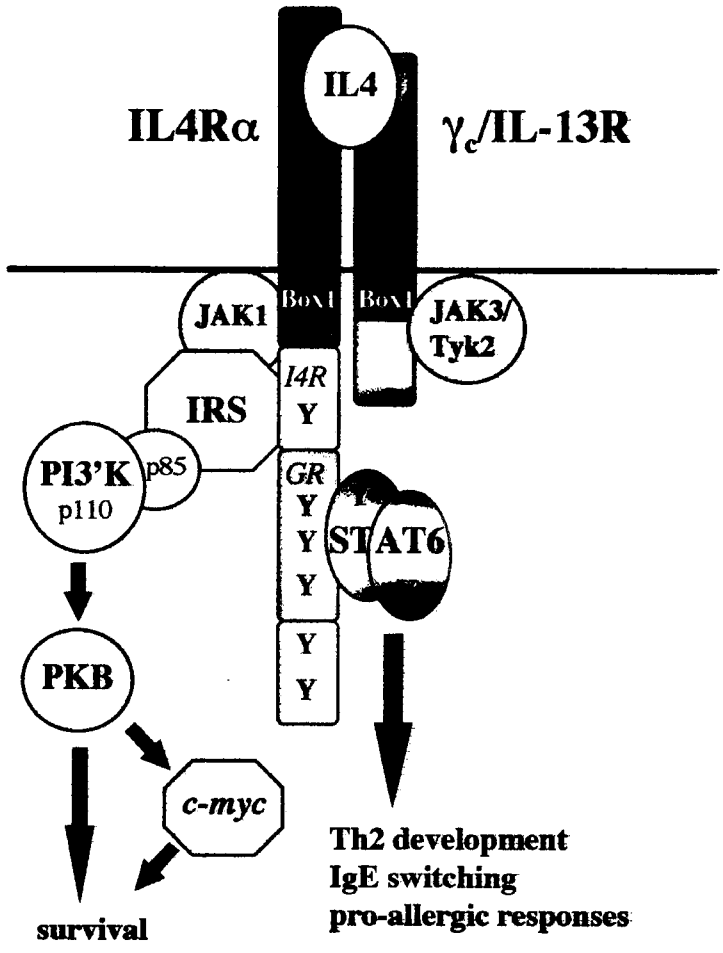

Figure 1.2. *Schematic diagram of the IL-4 Receptor and the known intracellular signalling pathways associated with distinct domains.* IL-4 binds to the IL-4Rα in conjunction with either γ_c or the IL-13RI. JAKs associate with the box-1 and -2 regions, and are activated following ligand-induced dimerisation or oligomerisation. The region with homology to the insulin R (***I4R***) recruits IRS-1/-2 and is thought to mediate activation of the PI3'kinase pathway. The gene regulation (***GR***) domain is important for activation of STAT-6.

1.5. Kinases activated by IL-4

1.5.1. JAKs

The Janus family of tyrosine kinases (JAKs) is characterised by two tandem kinase domains, one of which is inactive. The enzyme is therefore "two-faced", like the Roman god of gates and doorways, Janus. JAK has also been known to stand for "just another kinase", a reflection of the fact that these molecules were originally discovered by PCR or by low-stringency screens for new cDNAs with homology to other tyrosine kinases. It wasn't until George Stark, Ian Kerr and colleagues undertook a genetic approach to identify proteins in interferon signalling (see 1.7) that a function was attached to this family of cytoplasmic tyrosine kinases.

Through the use of JAK-deficient cell lines, dominant-negatives, and the recent generation of JAK-deficient mice, it is now recognized that JAKs are essential for the initiation of intracellular signals by type I and II cytokines (see Table 1.1). The current model for JAK activation assumes that the relevant JAKs are constitutively associated with the proline rich region of cytokine receptors, and that following ligand binding, and receptor homo- or hetero-dimerization, two or more JAKs are brought in close enough proximity to cross-phosphorylate and activate each other (Ihle et al., 1995). This model of kinase activation resembles that for receptor tyrosine kinases such as the EGFR or the insulin receptor, where the kinase domains within the receptor cross-phosphorylate and activate each other following ligand-induced homodimerization. The precise mechanism for activation of JAKs is unclear, as tyrosine phosphorylation can either have a positive or negative effect on the enzyme's activity depending on the site of phosphorylation (Zhou et al., 1997).

Depending on the cell-type, IL-4 has been shown to stimulate tyrosine phosphorylation of all members of the JAK family: JAK-1, -2, -3, and Tyk-2. However, it is important to note

that due to the difficulty in performing *in vitro* assays to quantitate levels of JAK activity, almost all biochemical analyses of JAK activation has relied on determining levels of tyrosine phosphorylation, which may or may not correlate with an increase in enzymatic activity (Zhou et al., 1997). Stimulation with IL-4 results in tyrosine phosphorylation of JAK1, which is constitutively associated with the IL-4Rα (Yin et al., 1994). Through the use of deficient cell-lines, it has been shown that JAK1 is essential for IL-4-mediated phosphorylation of IRS-1 and –2 (see 1.6), activation of the PI3'kinase pathway (see 1.5.3) and of STAT-6 (see 1.7) (Burfoot et al., 1997; Chen et al., 1997; Wang et al., 1997).

JAK3 is associated with γ_c, (Miyazaki et al., 1994; Russell et al., 1994), and when IL-4 signals through a complex of IL-4R α and γ_c, JAK3 is activated (Miyazaki et al., 1994; Oakes et al., 1996; Witthuhn et al., 1994). The fact that mice or humans lacking JAK3 display a remarkably similar phenotype to those lacking γ_c, suggests that a major function of γ_c is to recruit JAK3 (Macchi et al., 1995; Nosaka et al., 1995; Park et al., 1995; Russell et al., 1995; Thomis et al., 1995). Analyses of human B cells derived from X-linked SCID patients (which lack functional γ_c) have shown that expression of γ_c (and activation of JAK3) is required for maximal activation of IRS-1 and STAT-6 (Izuhara et al., 1996; Taylor et al., 1997).

When IL-4 signals through a complex of IL-4Rαand the IL-13RI, Tyk2 rather than JAK3 is activated (Orchansky et al., 1997; Welham et al., 1995). Analyses of JAK3 deficient mice have suggested that IL-4-mediated signals via Tyk2 may be less efficient than those through JAK3 (Oakes et al., 1996). In certain cell types, IL-4 can also stimulate phosphorylation of JAK2 (Murata et al., 1996; Welham et al., 1995), but it is unclear what the biological significance of this activation is.

1.5.2. Fes and Lsk

Stimulation with IL-4 results in tyrosine phosphorylation of the *c-fes* proto-oncogene product (Izuhara et al., 1994), and its association with IL-4Rα. Fes interacts with PI3'kinase via an interaction between the SH2 domain of the p85 subunit of PI3'kinase and the YXXM motif (where X is any amino acid) within Fes (Izuhara et al., 1996). This ability of Fes to recruit PI3'kinase to the IL-4R complex offers an alternate mechanism for activation of the PI3'kinase pathway in the absence of IRS proteins (see 1.6).

It has also been reported that IL-4 can activate Lsk. Lsk is a tyrosine kinase with homology to the COOH-terminal Src kinase, Csk (Chow et al., 1993), and levels of Lsk expression and activity are increased following addition of IL-4 (Musso et al., 1994). Based on homology with Csk, it is possible that Lsk is important for the negative regulation of Src family kinases. However the function of this kinase in IL-4-mediated signalling is currently unknown.

1.5.3. Phosphatidyl Inositol 3' kinase & PKB

IL-4 is a survival factor for most leukocytes, and this ability to inhibit apoptosis is at least partially mediated by the PI3'kinase pathway. PI3'K is a heterodimeric enzyme, consisting of an 85 kDa regulatory subunit (p85) and a 110 kDa enzymatic subunit (p110). Within the p85 subunit there are two SH2 domains (Wandless, 1996) which recruit the heterodimeric enzyme to the membrane by binding to the phosphorylated tyrosines within YXXM motifs of membrane-proximal proteins (e.g. cytokine receptors). The IL-4Rα, however, does not contain a YXXM motif, thus PI3'kinase is recruited to the active receptor complex via adapter molecules such as IRS-1 and –2, and Cbl (Ueno et al., 1998) or Fes (see 1.5.2.) (Izuhara et al., 1996), which do contain this YXXM motif. Recruitment to the membrane enables the p110 subunit to

phosphorylate its lipid substrates on the 3' position of the inositol ring, and results in the production of PI(3,4)P_2 and PI (3,4,5)P_3 (Vanhaesebroeck et al., 1997).

The generation of these lipids results in activation of a kinase cascade. The N-terminal pleckstrin homology (PH) domain of protein kinase B (PKB) can bind to lipid products of PI3'kinase, and recruit PKB to the plasma membrane (Andjelkovic et al., 1997, Downward, 1998). Here, PKB is in proximity to another serine/threonine kinase, PDK1 (PIP_3-dependent kinase) (Alessi et al., 1997; Alessi et al., 1997). PDK1, which is also regulated by the lipid products of PI3'kinase, can then phosphorylate PKB at threonine 308. A second residue in PKB, serine 473, is also phosphorylated *in vivo*, but the kinase responsible for this modification has yet to be identified. Phosphorylation at both position 308 and 473 is necessary for PKB activity. The downstream targets of PKB are unclear and to date, glycogen synthase kinase (GSK-3) is the only direct substrate identified *in vivo* (Cross et al., 1995). There is evidence that the pro-apoptotic protein BAD may also be a target for PKB, but this is not a general mechanism as although IL-4 can activate PKB, it does not stimulate phosphorylation of BAD (Scheid and Duronio, 1998).

The PI3'kinase pathway can regulate cell-survival. Expression of constitutively active mutants of PI3'kinase or artificial targeting of PKB to the membrane is sufficient to protect many cell types from apoptosis induced by growth-factor withdrawal (Kulik et al., 1997; Songyang et al., 1997). Furthermore, inhibitors of the PI3'kinase pathway such as wortmannin or LY294002, block the survival of cells in the presence of factors such as IL-4 (Scheid et al., 1995).

1.6. Insulin Receptor Substrate 1 & 2

Although IRS-1 and –2 were first recognized as insulin receptor substrates (Yenush and White, 1997), it is now known that they are tyrosine phosphorylated following activation of many receptors, including those for IL-4 (Welham et al., 1997; Welham et al., 1995). In fact, IRS-2 is the most prominent tyrosine-phosphorylated protein in the whole-cell lysates of IL-4-stimulated cells (Figure 1.3). Surprisingly, the pattern of whole-cell tyrosine phosphorylation stimulated by IL-4 is more similar to that seen following stimulation by insulin (a classic hormone) rather than other members of the type I cytokine family. This observation emphasizes the distinctiveness of the intracellular signals activated by IL-4 compared to other type I cytokines. The IRS proteins are large 170-190 kDa molecules with an N-terminal pleckstrin homology (PH) domain, a phosphotyrosine binding (PTB) domain, and multiple tyrosine residues. The PH domain is important for interactions with membrane lipids (Voliovitch et al., 1995), and the PTB domain is important for association with tyrosine phosphorylated receptors (Harrison, 1996). Many of the tyrosine residues in IRSs occur within SH2-domain binding motifs, and mediate interactions with other signalling molecules such as PI3' kinase, Grb-2 and SHP-2 (Src homology phosphatase-2). The combined functions of these domains allows IRS molecules to serve as docking proteins, or links between activated receptors and cytoplasmic signalling molecules.

It is unclear what role the association of molecules such as Grb-2 or SHP-2 with IRS has in activating downstream pathways. Thus, although stimulation with IL-4 clearly results in phosphorylation of the tyrosine residues in IRS-2 that are capable of binding to Grb-2, the Ras pathway is not activated (Pruett et al., 1995; Welham et al., 1997; Welham et al., 1994).

Similarly, although stimulation with IL-4 results can result in recruitment of SHP-2 to IRS-2 *in vitro*, there is no increase in SHP-2 phosphatase activity (Welham et al., 1994)

Although IRS-1 and –2 are major substrates for tyrosine phosphorylation following stimulation of cells with IL-4 (Figure 1.3), it is unclear if they are necessary for IL-4-mediated biological effects. In an IL-3 dependent cell line, 32D, it has been shown that expression of IRS-1 restores IL-4-mediated activation of PI3'kinase (Myers et al., 1994) and mitogenesis (Wang et al., 1993). In contrast, I have shown that two cell types which are biologically responsive to IL-4, the CT.4S cell-line and primary bone-marrow derived mast cells, do not express detectable levels of IRS-1 or –2 (Welham et al., 1997). In murine cells it appears that IRS-2, rather than IRS-1, is the major substrate for tyrosine phosphorylation following stimulation with IL-4 (Welham et al., 1997). Mice deficient in IRS-2 have recently been generated (Withers et al., 1998) and it will be interesting to learn if these mice have any defects in IL-4-mediated biological effects.

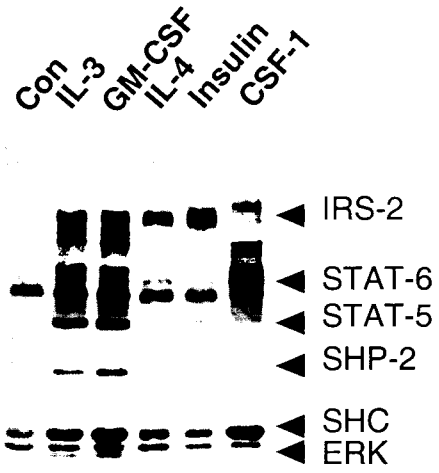

Figure 1.3. *Comparison of whole-cell tyrosine phosphorylation patterns stimulated by hemopoietins.* Factor-deprived FD5 cells were stimulated with the indicated factors. Whole-cell lysates were resolved by SDS-PAGE and immunoblotted (IB) with 4G10 (αPY) to compare patterns of tyrosine phosphorylation. Phosphorylated proteins that have been previously identified are marked. Note that the pattern of IL-4-stimulated whole-cell tyrosine phosphorylation most closely resembles that stimulated by insulin (which signals via a receptor tyrosine kinase) rather than by other type I cytokines (IL-3 and GM-CSF), or by colony stimulating factor (CSF)-1, which also signals through a receptor tyrosine kinase.

1.7. STATs

1.7.1 Historical perspective, how STATs were discovered

In 1993, when this thesis was initiated, through a combination of biochemical and genetic techniques, a novel signalling pathway that was activated in response to interferons (type II cytokines) was recognized (reviewed in Leonard and O'Shea, 1998). The promoters for genes induced by IFNα/β contain a conserved element knows as the ISRE (IFNα/β-stimulated response element) and those induced by IFNγ contain a GAS (gamma activating sequence) element. Early studies demonstrated that a complex of 3 proteins (known as ISGF-3) bound to the ISRE (Fu et al., 1990), and that at least one component of ISGF-3 was rapidly and specifically

activated in the cytoplasm. Following molecular cloning, it was revealed that the proteins that bound to the IRSE and GAS sequences constituted a novel family of transcriptional activators, the "signal transducers and activators of transcription" (STATs) (Fu et al., 1992; Schindler et al., 1992; Veals et al., 1992).

At the same that the ISGF-3 proteins were characterised, George Stark and Ian Kerr undertook a genetic approach to try and identify novel genes that were required for transcriptional activation by IFNα/β. In 1989, Pellegrini et al. (1989) reported the generation of a human fibrosarcoma cell-line that was transfected with a plasmid encoding the bacterial *gpt* (guanine/hypoxanthine phosphoribosyl transferase) gene from *E. coli* under control of an IFN α/β inducible promoter. The *gpt* gene allowed for positive selection of cells that expressed *gpt* in response to IFNα/β in HAT medium (containing hypoxanthine, aminopterin and thymidine) or for negative selection of cells that could no longer grow in IFN when the media contained 6-thioguanine (6-TG), a purine analog that is converted to a toxic nucleotide by *gpt*. This cell line was treated with the frame-shift mutagen ICR 191, and several clones which were no longer able to express *gpt* in the presence of IFNα/β were selected. Mutants in 6 complementation groups (U1-U6) were generated, and surprisingly, mutants in the U2-U4 complementation groups were not only defective in their response to IFNα/β, but also to IFNγ. In the following 4 years, it was revealed that 5 of the 6 mutants were defective in a member of the JAK/STAT pathway. Thus U1 mutants were complemented by expression of Tyk2 (Velazquez et al., 1992), U2 mutants by p48 (Bluyssen et al., 1995), U3 mutants by STAT-1 (Muller et al., 1993), U4 mutants by JAK1 (Muller et al., 1993), and U6 mutants by STAT2 (Leung et al., 1995).

This pioneering work on the activation of the JAK/STAT pathway in response to IFNα/β and IFNγ led to the establishment of a new paradigm for the transfer of signals from the

membrane to the nucleus. The importance of the JAK/STAT pathway in the interferon response led many investigators to analyze whether this pathway was also important in response to cytokines. Indeed, in the last 5 years is has been demonstrated that the JAK/STAT pathway is activated by all type I and type II cytokines. Through the use of dominant-negative molecules and analyses of mice with targeted deletions of JAKs or STATs, it has become clear that this pathway is essential for many of the biological effects of cytokines (Table 1.1).

There are currently 7 known mammalian members of the STAT family, STATs 1-6, with two genes for STAT5 (STAT5 a and b). Following a relatively non-conserved N-terminal region, STATs contain a DNA-binding domain (Horvath et al., 1995), a linker region (which was originally thought to encode an SH3 domain), an SH2 domain, a critical tyrosine residue and a C-terminal transactivation domain. In addition to the mammalian family members there is also a *Drosophila* STAT (STAT92E) which can regulate the expression of *eve*, an important transcriptional repressor involved in segment formation (Hou et al., 1996; Hou and Perrimon, 1997; Yan et al., 1996). Furthermore, a STAT-like protein has been discovered in *Dictyostelium* (Kawata et al., 1997), suggesting that this pathway is a very old intracellular signalling mechanism.

Table 1.1. *Summary of phenotypes associated with deficiencies in JAKs or STATs*

JAKs	**Phenotype**
JAK1	perinatal lethality, reduced B&T cells during embryogenesis (Rodig et al., 1998)
JAK2	embryonic lethal due to lack of erythropoiesis, defective signalling in a group of cytokine receptors (Parganas et al., 1998)
JAK3	Severe combined immunodeficiency similar to γ_c knock out (Park et al., 1995; Thomis et al., 1995)
Tyk2	not yet reported
STATs	
STAT-1	Defective innate immunity dues to absence of IFN $\alpha/\beta/\gamma$ responses (Durbin et al., 1996; Meraz et al., 1996)
STAT-2	Embryonic lethal (Chen et al., 1998)
STAT-3	Embryonic lethal (Takeda et al., 1997)
STAT-4	Defect in innate immunity due to insufficient Th-1 development and IFNγ production (Thierfelder et al., 1996)
STAT-5 a & b	Required for functions of growth hormone and prolactin (Teglund et al., 1998; Udy et al., 1997); defective GM-CSF signalling (Feldman et al., 1997) and CD25 (IL-2Rα) expression (Nakajima et al., 1997)
STAT-6	Defective humoral immunity due to insufficient Th2 development (Kaplan et al., 1996; Takeda et al., 1996)

Given that there are many more cytokines than STATs, each cytokine cannot activate a unique STAT. Two important exceptions to this are IL-12, which is the only known activator of STAT4, and IL-4 and IL-13 which are the only known activators of STAT-6. Although it has also been shown that STAT-6 can be activated by IL-3 (Quelle et al., 1995), PDGF (Patel et al., 1996), the B cell receptor (Karras et al., 1996), or CD40 (Karras et al., 1997), this has only been reported by a single group in each case, and I have been unable to reproduce activation of STAT-6 by any stimuli except for IL-4 or IL-13 (data not shown). The subset of STATs activated by cytokines can be directly correlated with the composition of the cytokine receptors. Thus cytokines which bind to highly homologous, or identical, receptors activate the same STATs (Leonard and O'Shea, 1998). Somewhat surprisingly, despite the fact that many cytokines activate STAT-1 and –5, analyses of mice deficient for these STATs have demonstrated an unexpected degree of specificity. The predominant phenotypes of STAT-1 null mice are restricted to defects in IFN $\alpha/\beta/\gamma$ signalling (Table 1.1; Durbin et al., 1996; Meraz et al., 1996). Similarly, although STAT5 is activated by most type I cytokines, the predominant phenotypes of mice lacking STAT 5a, 5b or both, are the result of defects in growth hormone or prolactin signalling (Table 1.1; Teglund et al., 1998; Udy et al., 1997).

1.7.2 STATs are recruited to the active receptor complex

The ability of a cytokine to recruit and activate particular STATs is dependent on the sequence of the receptor subunit(s). STATs are recruited to functional receptor complexes via SH2-phosphotyrosine interactions, and the SH2 domains of different STATs differ sufficiently that they recognize distinct phosphorylated motifs. For example, the SH2 domain in STAT3 prefers to bind to the sequence YXXQ (where X is any amino acid) (Stahl et al., 1995), whereas the

SH2 domain of STAT-6 prefers the sequence GY(K/Q)XF (Ryan et al., 1998). However, STATs may also be recruited to the active receptor complexes by direct association with JAKs (Fujitani et al., 1997; Migone et al., 1995). Moreover, in response to IFNα/β, STAT-1 docks on STAT2 which is in turn associated with the receptor (Li et al., 1997).

1.7.3 Phosphorylation of STATs

After STATs are recruited to the active receptor complex, they are in close proximity to active JAKs, which can phosphorylate the STAT C-terminal tyrosine. However, STATs are not exclusively tyrosine phosphorylated by JAKs as other cytoplasmic kinases such as *v-src* (Yu et al., 1995) or *v-abl* (Danial et al., 1995), and receptor tyrosine kinases such as EGFR (Park et al., 1996) and PDGFR (Vignais et al., 1996) can also mediate their phosphorylation. Furthermore, activation of STAT3 is necessary for transformation by *v-src* (Bromberg et al., 1998; Turkson et al., 1998). For STAT-1 and -3 it has been shown that in addition to tyrosine phosphorylation, a serine residue in the C-terminus must also be phosphorylated for optimal transactivation function (Wen et al., 1995; Zhang et al., 1995; Ng and Cantrell, 1997). There is evidence that the kinase responsible for serine phosphorylation of STAT-1 in response to IFNα/β may be the extracellular regulated kinase (ERK) (David et al., 1995). However, ERK is unlikely to be the only relevant kinase since it is not activated by IFNγ (Zhu et al., 1997). STAT4 (Cho et al., 1996), and STAT5 (Beadling et al., 1996) are also serine phosphorylated, but the relevant kinase has not yet been elucidated. STAT-6 lacks the conserved serine residue present in STATs 1-5.

1.7.4 Dimerization of STATs

Functional cytokine receptors often contain more than one STAT binding site, allowing for two (or more) STATs to be activated in close proximity. Following tyrosine phosphorylation, STAT monomers form high avidity homo- or hetero-dimers by reciprocal SH2-phosphotyrosine interactions (Figure 1.4). This bivalent interaction leads to a complex with a much higher affinity than the monovalent STAT-receptor interaction (Greenlund et al., 1995). STAT dimers also have a higher affinity for the palindromic target DNA sequence (see 1.7.5). Analyses of the crystal structure of STAT-1 (Chen et al., 1998) has confirmed that this reciprocal-SH2 interaction forms, and revealed that the short linker between the SH2 domain and the C-terminal tyrosine stretches the required 18Å distance to allow this interaction to occur (Figure 1.5). Furthermore, the crystal structure of STAT-1 has confirmed the prediction that the interactions between the SH2 domain and the phosphotyrosine are primarily restricted to residues C-terminal to the tyrosine (Greenlund et al., 1995).

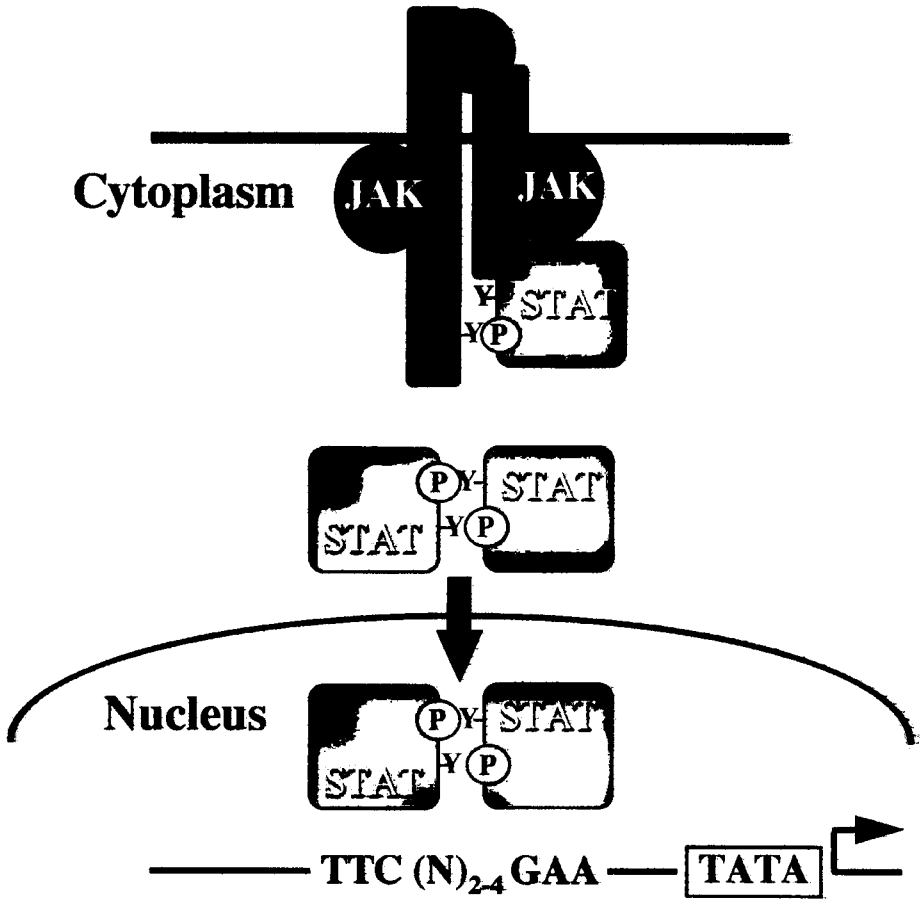

Figure 1.4. *Schematic model of STAT activation.* Following ligand-induced receptor dimerization and JAK activation, STATs are recruited to the active receptor complex. Here, STATs themselves are tyrosine phosphorylated by JAKs, and reciprocal dimers are formed. The dimers translocate to the nucleus, and co-operate with other transcription factors to transactivate promoters which contain the appropriate consensus binding sequences.

1.7.5 Nuclear translocation, DNA binding and transactivation of STATs

The mechanistic basis for the nuclear translocation of STAT dimers is currently unclear. STATs lack canonical nuclear localization sequence (NLS). However, there is evidence that STAT nuclear transport requires the activity of Ran, a small G protein that is required for the nuclear transport of proteins which do contain canonical NLS sequences (Sekimoto et al., 1996). Once in the nucleus, STATs bind to target DNA sequences, $TTCN_3GAA$ for STATs 1-5, and $TTCN_4GAA$ for STAT-6 (Seidel et al., 1995). In response to IFNα/β, a heterodimer of STAT-1 and STAT-2 forms, and interacts with a member of the interferon regulatory family (IRF) of transcription factors, ISGF-3γ (p48) (Bluyssen et al., 1995). This trimeric complex then binds to the ISRE consensus sequence (AGTTTNCNTTTCC). Analyses of the crystal structure of STAT-1 has revealed the nature of the interactions between STAT dimers and DNA (Chen et al., 1998). The general architecture of the DNA binding domain resembles an immunoglobulin fold, and is remarkably similar to the tertiary structure of NF-κB and p53. STAT-1 dimers interact with DNA over a 15 bp region, but with relatively few direct contacts (Figure 1.5), which may provide an explanation for the fact that STATs do not show sharply defined consensus binding sequences.

The STAT-DNA interaction has a relatively low affinity (nM range) and a short half-life (Vinkemeier et al., 1996), suggesting that interactions between STATs and other proteins may be important for forming a high-affinity protein/DNA complex. Indeed, there are often multiple GAS elements in a single promoter (Xu et al., 1996), and STAT N-terminal domains can mediate the formation of tetrameric complexes which have not only a higher affinity for DNA but also a higher transactivation potential (Vinkemeier et al., 1996). Competition experiments have shown that the off rate for a single STAT-1 homodimer is less than 30 seconds, compared

to 15-30 minutes for a STAT-1 tetramer (Vinkemeier et al., 1996). The crystal structure of the N-terminal domain of STAT-4 structure revealed that this domain forms a hook that mediates cooperative binding between STAT dimers (Vinkemeier et al., 1998).

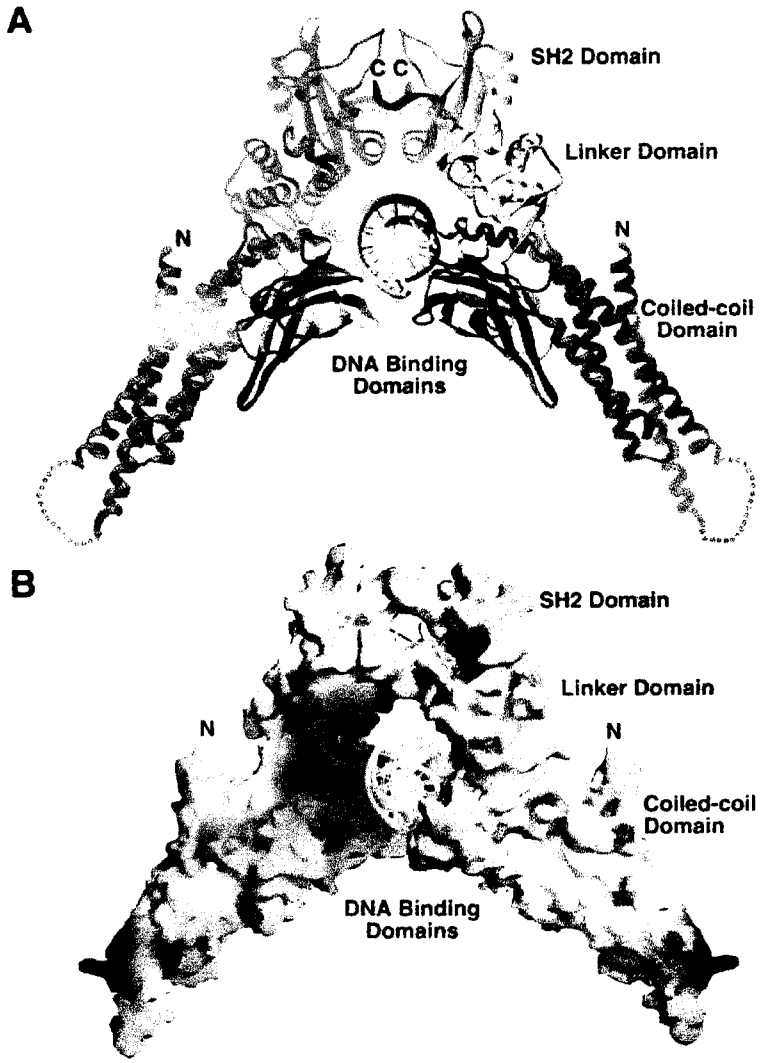

Figure 1.5. *X-Ray crystal structure of the STAT-1-DNA complex, from* (Chen et al., 1998). **A.** Ribbon diagram of the STAT-1 dimer bound to DNA. The red ribbons indicate the DNA-binding domain, and the blue ribbons indicate the SH2 domain. **B.** Molecular surface of the STAT-1 dimer bound to DNA. The surface is coloured according to electrostatic potential, with blue representing positive and red representing negative potential.

In addition to the intrinsic activity of STATs, there is often a requirement for interaction with other DNA-binding proteins for full transactivating activity. For example, STAT-6 cooperates with C/EBPβ (Mikita et al., 1998), a constitutively active transcription factor that appears to stabilize and prolong the ability of STAT-6 to bind to target sequences. Without this interaction, STAT-6 alone in unable to transactivate the promoter for IgE or CD23. Similarly, STAT-5 interacts with the glucocorticoid receptor (Stocklin et al., 1996) and STAT-1 and STAT-2 can interact with the p300 cAMP-response element binding protein (CBP) (Bhattacharya et al., 1996; Zhang et al., 1996).

1.7.6 Negative Regulation of STATs

There are several mechanisms that exist to turn STATs off. For example phosphatases can regulate STAT activity by de-phosphorylating the critical tyrosine, and/or serine residues (David et al., 1995; Haspel et al., 1996) and the N-terminal domain of STAT-1 has been shown to interact with a phosphatase (Shuai et al., 1996). STAT-1 can also be negatively regulated by ubiquitin/proteasome-mediated degradation (Kim and Maniatis, 1996). In addition, truncated forms of STATs are generated by proteolytic cleavage or by alternate splicing (Azam et al., 1997; Caldenhoven et al., 1996; Wang et al., 1996). These truncated STATs lack the C-terminal transcriptional activation domain, and function as natural dominant-negatives (see Chapter 4).

A novel family of inhibitors of cytokine signalling has recently been identified by several groups and named CIS, SOCS, JAB or SSI (hereafter referred to as the suppressor of cytokine signalling (SOCS) family). There are currently eight members of this family which appear to be a negative feedback pathway that regulates cytokine signal transduction (Hilton et al., 1998). SOCS family members contain a relatively non-conserved N-terminal region, an

SH2 domain, and a domain that has been termed the "SOCS box". SOCS-1 inhibits certain cytokines by binding to and inhibiting JAK activity (Endo et al., 1997; Naka et al., 1997; Starr et al., 1997). In contrast, CIS inhibits cytokine signalling by competing with signalling molecules, including STATs themselves, for binding to phosphorylated tyrosines on cytokine receptors (Yoshimura et al., 1995).

Another family of inhibitors of cytokine signalling are the protein inhibitors of activated STATs (PIAS) (Chung et al., 1997; Liu et al., 1998). PIAS family members bind to tyrosine phosphorylated STATs and inhibit their activity either by masking the DNA-binding domain or by preventing dimerization. To date, PIAS-1 has been shown to bind and inhibit STAT-1 (Liu et al., 1998), and PIAS-3 to bind and inhibit STAT-3 (Chung et al., 1997). However, there are at least two other uncharacterised members of the family, raising the possibility that there will be a PIAS specific for each STAT.

1.8. Ras and MAP-family kinases

Ras super-family members are membrane-bound small GTPases which enter the active GTP-bound state following treatment of cells with a variety of extracellular signals, including most cytokines (Lowy and Willumsen, 1993). The Ras super-family is divided into families based on primary sequence homology. The "classical" Ras family members include H-, N- and Ki-Ras, which are themselves implicated in mediating the activation of the Rho family of small GTPases. Activation of the Ras super-family is controlled by three classes of regulatory proteins: the guanine nucleotide exchange factors which active Ras by promoting the exchange of GDP for GTP; the GTPase-activating proteins which inactivate Ras by stimulating the hydrolysis of GTP; and the guanine nucleotide dissociation inhibitors, which lock Ras in either

the active or inactive conformation (Boguski and McCormick, 1993). Through biochemical and genetic analyses, Ras proteins have been implicated in cell proliferation and differentiation, and apoptosis (Marshall, 1995; Moodie and Wolfman, 1994). These diverse biological functions can be attributed to the ability of Ras super-family members to regulate multiple effector pathways, including the MAP-family kinases, the PI3'kinase pathway, and the activation of NF-kB (Downward, 1998).

The best characterised effector for classical Ras is the Raf family of serine/threonine kinases. Active Ras interacts with Raf, and promotes its translocation to the plasma membrane where further events occur to stimulate kinase activity. Active Raf phosphorylates and activates MEK (MAP/ERK kinase 1), which in turn activates the extracellular-regulated kinases (ERK). Classical Ras can also associate with and activate members of the Rho family, including Rac and CDC42 (Van Aelst and D'Souza-Schorey, 1997). Rac and CDC42 mediate the activation of the c-Jun-N-terminal kinases (JNK) and the p38 MAP-family kinases. Although JNK and p38 MAP-kinase were originally characterised as "stress"-activated kinases, it is now known that most stimuli (including cytokines) that activate ERK, also active JNK and p38 MAP-kinase (Minden and Karin, 1997).

IL-4 is distinct amongst type I cytokines in that it fail to activate Ras (Duronio et al., 1992; Satoh et al., 1991), or any of the MAP-family kinase cascades. Thus, IL-4 fails to activate ERK (Pruett et al., 1995; Welham et al., 1994; Welham et al., 1992; Welham et al., 1994), JNK (Foltz et al., 1998; Foltz and Schrader, 1997) or p38 MAP kinase (Foltz et al., 1997; Salmon et al., 1997).

1.9. Thesis Objectives

In 1993, when the projects described in this thesis were initiated, much was known about the biological effects of IL-4, but the molecular basis of these specific effects was unclear. It was known that IL-4 did not activate the Ras/Raf/Erk pathway (Welham et al., 1994; Welham et al., 1992; Welham et al., 1994), but did appear to activate JAKs (Welham et al., 1995), and a protein with functional similarities to STAT-1 (Kotanides and Reich, 1993; Schindler et al., 1994). During the course of this thesis, the STAT-1-like molecule that was activated by IL-4 was cloned, and designated STAT-6 (Hou et al., 1994).

Given the importance of IL-4 in regulating the immune response, I was interested in further characterising the biological functions of two distinctive aspects of the intracellular signals activated by IL-4. First, I tested the hypothesis that the inability of IL-4 to stimulate cell-cycle progression was directly related to the unique inability of IL-4 to activate the Ras/Raf/ERK pathway. Second, I determined if activation of STAT-6 was required for IL-4-mediated cell survival and/or the inhibition of the production of pro-inflammatory cytokines. Finally, I performed experiments designed to determine if IFNγ can inhibit the actions of IL-4 by regulating the expression of a newly characterised antagonist of STAT-6, Bcl-6.

CHAPTER 2. Materials and Methods

2.1. For Chapter 1

2.1.1. CELL STIMULATION AND LYSIS. FD5 cells were grown at 37°C in humidified incubators gassed with 5% CO_2 in RPMI 1640 (Gibco BRL, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) (Intergen, Purchase, NY), 50μM 2-mercaptoethanol (2ME), and 3% (v/v) media conditioned by AgXO63 cells that had been transfected with the murine IL-4 cDNA (IL-4 CM) (Karasuyama and Melchers, 1988). Cells were starved overnight in 0.3% IL-4, and then washed in Hepes-buffered saline solution (HBSS) to remove FCS and the remaining IL-4. Cells were deprived of serum and IL-4 for one hour in RPMI supplemented with 20mM Hepes (SFM) and then stimulated with synthetic IL-4 (20μg/mL), synthetic IL-3 (10μg/mL), or synthetic GM-CSF (10μg/mL) for 10 minutes, or recombinant porcine insulin (15μg/mL) (Sigma, Oakville, ON) or rCSF-1 (500ng/mL) for 3 minutes, or with medium without factor as a control. All synthetic factors were provided by Ian Clark-Lewis (The Biomedical Research Centre, Vancouver). Cells were lysed in lysis buffer (20mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 1μM phenylmethylsulfonyl fluoride, 2 μg/mL leupeptin, 0.7 μg/mL pepstatin, 10 μg/mL aprotinin, 10 μg/mL soybean trypsin inhibitor) that was supplemented with phosphatase inhibitors (200μM sodium orthovanadate, 1mM sodium molybdate, 10mM sodium fluoride). Insoluble material was removed following centrifugation, Laemmli's SDS-sample buffer (10% SDS, 50% glycerol, 200mM Tris, pH 6.8, bromophenol-blue) was added to the lysate, and the samples were boiled for 2 minutes.

2.1.2. SDS-PAGE AND IMMUNOBLOTTING The proteins from approximately 2-3 x 10^5 cells from each sample were resolved by SDS-PAGE. SDS-PAGE gels were run in 1X running buffer

(25mM Tris, 192mM Glycine, 0.1% SDS) at 80 volts for 30 minutes, then at 150 volts until the bromophenol-blue dye front had run off. Gels were transferred to nitrocellulose membranes (0.45μM pore size, Schleicher & Schull, Germany) at a constant amperage of 0.8 mA/cm^2 on a semi-dry transblotter (Pharmacia, Uppsala, Sweden) in 1X transfer buffer (39mM Glycine, 48mM Tris, 0.0375%(w/v) SDS, 20%(v/v) methanol). The membranes were stained with Ponceau S (Sigma, Oakville, ON) to elucidate molecular weight markers (BioRad), and blocked in 5% BSA (Boeringer Mannheim, Laval, QU); 1% Ovalbumin (Sigma) in 1X Tris-buffered Saline (20mM Tris, 150mM NaCl; TBS) for 30 minutes or overnight. 4G10 (Upstate Biotechnology Inc., Lake Placid, NY) was diluted to 1μg/mL in 1% BSA, 0.02% ovalbumin in TBS and incubated for 3 hours at room-temperature, or overnight at 4°C. Following three washes with TBSN (TBS, 0.05% NP-40), secondary antibody coupled to horseradish peroxidase (HRP) (Dako, Denmark), was added at a 1:10 000 dilution in TBSN. The membranes were incubated for one hour at room-temperature, and washed three times with TBSN. HRP was detected using the Amersham enhanced chemiluminesense (ECL) reagent according to manufacturers instructions (Amersham, Oakville, ON).

2.2. FOR CHAPTER 3

2.2.1. PKH26 CELL LABELING Ba/F3 cells were washed to remove serum, and incubated for 5 minutes in 4μM of the lipophilic dye PKH26 (Sigma, Mississauga, ON), which was diluted in buffer supplied by the manufacturer (Diluent C, Sigma). The labelling reaction was stopped by addition of RPMI 1640 supplemented with 10% FCS. Cells were washed extensively to remove all unincorporated dye. Immediately following washes, an aliquot of cells was analyzed by flow-cytometry (time 0), and the remaining cells were incubated at 37°C in media supplemented

with 2% (v/v) 10X concentrated WEHI-3B (W3B), or 3% (v/v) IL-4 CM. Aliquots of cells were analyzed by flow-cytometry at 24, 48 and 72 hours after addition of cytokine.

2.2.2. RECOMBINANT PLASMIDS. The cDNA for ^{Q61K}N-Ras was a gift from Dr. Rob Kay (Terry Fox Labs, Vancouver) and was cloned into the vector pcHA (Giulio Superti-Furga, EMBL, Heidelberg). PCR mutagenesis was used to replace the N-Ras ATG with a BamHI site to allow for in-frame fusion to a sequence encoding the hemagglutinin (HA) tag downstream of the CMV promoter in pcHA. The fidelity of the cDNA was confirmed by sequencing. The cDNAs encoding ΔRaf-1:ER or ΔRaf-1:ER K70W in pBABEpuro (Woods et al., 1997) were a gift from Dr. Martin McMahon (UCSF Cancer Centre, San Francisco, CA). The ΔRaf-1:ER protein produced consists of the enhanced green fluorescent protein (EGFP) at the N-terminus, fused to the kinase domain of human c-Raf-1, fused in turn to the hormone binding domain of the human estrogen receptor. The ΔRaf-1:ER K70W protein consists of the kinase domain of human c-Raf-1, which lacks the lysine residue critical for enzymatic activity, fused to the hormone binding domain of the estrogen receptor. The GST-JNK1 cDNA in pEFBOS was a gift from Dr. Leonard Zon (Sanchez et al., 1994).

2.2.3. CELL CULTURE AND TRANSFECTIONS Ba/F3 cells and transfectants were grown at 37°C in humidified incubators gassed with 5% CO_2. Cells were routinely passaged in RPMI 1640 supplemented with 10% FCS, 50μM 2ME and 2% (v/v) of a 10X concentrate of medium conditioned by WEHI-3B (W3B) as a source of IL-3. Cells expressing ΔRaf-1:ER or ΔRaf-1:ER K70W were maintained as above, but in phenol-red free RPMI 1640 (Gibco BRL). To generate stable clones, cells were washed twice in PBS and $1x10^7$ cells were resuspended in 800μl of

transfection buffer (25mM Hepes, 0.75mM Na_2HPO_4, 140mM KCl, 5mM NaCl, 2mM $MgCl_2$, 0.5% Ficoll 400). For each transfection, cells were mixed with 15 μg of linearized vector, and subjected to electroporation using a Bio-Rad gene pulser at 960 μF and 270 V. After transfection, the cells were cultured in the appropriate media for 48 hours and then transferred to selection media in 96-well plates.

2.2.4. SCREENING FOR POSITIVE TRANSFECTANTS Individual clones of neomycin-resistant (for ^{Q61K}N-Ras and GST-JNK1) or puromycin-resistant (for ΔRaf-1:ER or ΔRaf-1:ER K70W) clones were tested for expression of the exogenous cDNA of interest. The drug-resistant clones were expanded, and approximately lysed in of lysis buffer. Lysates were either subjected to immunoprecipitation or analysed as whole cell lysates. Whole cells lysates or eluates from immune-complexes were resolved by SDS-PAGE (2.1.2). Expression of ^{Q61K}N-Ras was evaluated by immunoblotting whole cell lysates with antibodies against the HA tag (12CA5, Boehringer Mannheim, Laval, QU). Expression of ΔRaf-1:ER K70W was determined by immunoprecipitation with anti-estrogen receptor antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, catalogue #sc543) and immunoblotting with an anti-Raf-1 antibody (Pharmingen, San Diego, CA). Expression of GST-JNK1 was determined by immunoprecipitation with an anti-JNK1 antibody (Santa Cruz Biotech.) and immunoblotting with an anti-GST antibody (Molecular Probes). Expression of ΔRaf-1:ER was determined by flow-cytometric quantitation of levels of EGFP. Several positive clones with similar levels of expression of the exogenous cDNA were derived in each case.

2.2.6. CELL PROLIFERATION ASSAYS. Proliferation and viability of cells was assessed by cell counting, or [^{3}H]-thymidine incorporation into *de novo* synthesized DNA. For both assays, cells were washed three times with RPMI supplemented with 2% (v/v) FCS to remove IL-3. For cell counting experiments, the cells were incubated at 1 x 10^5 cells/mL at 37°C in complete media supplemented as indicated with 2% (v/v) 10X W3B, 2μg/mL synthetic IL-4, or 3% (v/v) IL-4 CM, and/or 100nM 4-hydroxy-tamoxifen (4HT) (Research Biochemicals, Natick, MA). The 4HT was stored as a 100μM stock in 100% ethanol at –20°C. An equivalent volume of ethanol was added to one set of cultures to rule out solvent effects. Cells that excluded trypan blue were counted at the indicated times, and cultures were diluted as appropriate to maintain a consistent density. For [^{3}H]-thymidine uptake assays, cells were plated at 250 cells/well in a Terasaki microtitre plate, incubated for 40 hours at 37°C in the indicated conditions, and then pulsed with 15μCi/ml of [^{3}H]-thymidine for a further 8 hours. Cells were harvested, and counted in a scintillation counter. Chemically synthesized IL-3 was used at a concentration of 1μg/mL shown to stimulate maximal proliferation of Ba/F3 cells. Synthetic IL-4 was used as indicated at a saturating dose (2μg/mL).

2.2.7. IMMUNOPRECIPITATIONS AND KINASE ASSAYS. To assess biochemical parameters under conditions in which cells were normally grown, cells were washed 3 times in RMPI supplemented with 2% FCS, and incubated at 37°C with 5% CO2 for sixteen hours at an initial density of 2 x 10^5 cells/mL in a 40 mL volume of complete media supplemented as indicated with IL-3 (2% v/v 10X W3B), IL-4 (2μg/mL synthetic IL-4 or 3% v/v IL-4 CM), and/or 4HT (100nM 4HT). Cells were harvested by centrifugation, washed once in RPMI, and lysed in lysis buffer which was supplemented with phosphatase inhibitors and 50mM β-glycerol phosphate.

The total amount of protein in each lysate was determined by the BCA protein assay (Pierce, Rockford, IL) and normalized amounts of protein were subjected to immunoprecipitation. Beads with immune-complexes were washed 3 times with lysis buffer, and once with kinase assay buffer (KAB). The *in vitro* kinase assays were initiated by addition of [γ-^{32}P]ATP and substrate.

ΔRaf-1:ER was immunoprecipitated with an anti-estrogen receptor antibody (Santa Cruz Biotechnology, catalogue #sc543) coupled to protein A-sepharose. To assay Raf activity, the KAB consisted of 25mM HEPES, 10mM $MgCl_2$, 1mM $MnCl_2$, and 1mM DTT. The reaction was initiated by addition of 0.5μg GST-MEK1 (Upstate Biotechnology Inc.), 1μM cold ATP and 10μCi of [γ-^{32}P]ATP, and incubated for 30 minutes at 30°C.

ERK-1 and -2 were immunoprecipitated with agarose-conjugated antibodies (Santa Cruz Biotechnology, catalogue #sc154). To assay Erk activity, the KAB consisted of 20mM HEPES, 5mM $MgCl_2$, 5mM EGTA, 50mM β-glycerol phosphate, 2mM sodium vanadate, and 5mM 2-ME. The reaction was initiated by addition of 15μg Myelin Basic Protein (MBP) (Sigma) and 10μCi of [γ-^{32}P]ATP, and incubated for 10 minutes at 30°C.

JNK-1 was immunoprecipitated with agarose-conjugated antibodies (Santa Cruz Biotechnology, catalogue # sc474). To assay JNK activity, the KAB consisted of 25mM HEPES, 25mM $MgCl_2$, 2mM DTT, 50mM β-glycerol phosphate, and 0.5 mM sodium vanadate. The reaction was initiated by addition of 1μg GST-cJun and 10μCi of [γ-^{32}P]ATP, and incubated for 20 minutes at 30°C. GST-cJun [1-169] was expressed in *E. coli* (UT5600 strain) and was purified by affinity chromatography on glutathione-sepharose beads (Pharmacia Biotech Inc., CA).

Reactions were stopped by addition of SDS sample buffer. The eluate was subjected to SDS-PAGE, and phosphorylated proteins were detected by autoradiography. To assess equivalency of loading, membranes were immunoblotted with either an anti-ERK-2 antibody (Santa Cruz Biotechnologies, catalogue #sc94), an anti-JNK-1 antibody (Santa Cruz Biotechnologies, #sc1648), an anti-GST antibody (Molecular Probes) or an anti-GFP antibody (Clontech).

2.3. For Chapter 4.

2.3.1. CLONING STRATEGY I received the cDNA encoding human STAT-6 in pBluescript from Ulrike Schindler (Tularik, San Francisco, CA). To avoid PCR-amplification of the entire 2.3kb cDNA, the mutagenesis was done in small sections and the cDNA was reconstructed making use of unique internal restriction sites. PCR mutagenesis was used to generate an in frame *BamHI* site to facilitate fusion to the sequence encoding two repeats of the myc tag. The 5' end of human STAT-6 was PCR amplified with VENT polymerase (New England Biolabs, Beverly, MA) using 5'STAT-6 as the sense primer (5' GC GGA TCC ATG TCT CTG TGG GGT CTG 3') and 3' STAT-6 as the antisense primer (5' TGC CCG GGT CTT GGG CTC AAG 3'). This PCR product was ligated into *BamHI* and *SmaI* cut pSSBSNmyc to fuse the 5' portion of STAT-6 to the double myc epitope to generate pSSmycSTAT-6#. The resultant plasmid was sequenced in both directions to insure there were no PCR-induced errors. Full length STAT-6 was regenerated by ligating a *BglII* blunt-*SmaI* fragment from pSSmycSTAT-6# into pBS STAT-6 that had been digested with *SmaI*. The resulting plasmid is referred to as pBSmycSTAT-6.

The 3' portion of STAT-6 was PCR amplified using S6B2 as the sense primer (5' GCC CAG AAG ATC TTC AAT GAC 3') and CSH2S6 as the antisense primer (5' TAC GGC CGT CAG TGG CTC CGG AA 3'). The product generates a stop codon at the end of the SH2 domain of STAT-6 (at amino acid # 623), followed by addition of an *EagI* site. The PCR product was ligated in *EcoRV* cut pSSBS to generate the plasmid pSSSTAT-6cterm SH2. The plasmid was sequenced to ensure there were no PCR-induced errors. To fuse the 3' truncated portion to the remainder or STAT-6, pSSSTAT-6cterm SH2 was digested with *BglII* and *EagI* and ligated to *BglII* and *EagI* digested pBSmycSTAT-6. The resultant plasmid was pBSmycSTAT-6Δ. The cDNA inserts from pBSmycSTAT-6 or pBSmycSTAT-6Δ were removed as *EcoRI-NotI* or *EcoRI-EagI* fragments and ligated into pEFBOSΔR1 (Mizushima and Nagata, 1990) that had been digested with *EcoRI* and *NotI*. All molecular biology methods used were according to methods described in Sambrook et al. (1989).

2.3.2. LUCIFERASE ASSAYS Ba/F3 cells were transiently transfected with 15μg of pEFBOS or pEFBOS mycSTAT-6Δ and 10μg of the pcεGL2 reporter plasmid (Alice Mui, DNAX, Palo Alto). The cells were washed twice in RPMI without serum, and resuspended with 1 x 10^7 cells in 400μL of RPMI that had been supplemented with 10μg/mL DEAE-Dextran. The cells were transferred to an electroporation cuvette (8 mm gap) (BioRad, Mississauga, ON), the DNA was added and the cells were electroporated at 270V, 960μF, with a resultant time constant of approximately 20 milliseconds. The cells were returned to complete media with IL-3 and allowed to recover. Sixteen hours later, the cells were washed 3 times in RMPI, 2% FCS to remove IL-3, and resuspended in RPMI, 10% FCS. Each stimulation condition was performed in a 5mL volume in a 37°C humidified incubator for 6 hours. The IL-4 stimulations were

performed with 5μg/mL of synthetic IL-4. Following stimulation, cells were washed once in room-temperature PBS, and lysed in 100μL of reporter lysis buffer (Promega, Madison, WI). The lysate was then either frozen at –70°C, or used immediately in a luciferase assay.

To measure luciferase activity, 20μL of cell lysate from each sample was placed in the well of an opaque 96-well plate. Next, 50μL of luciferase assay substrate (Promega) was added, and the plate was read immediately on a Dynex luminometer. The photons of light emitted from each well were measured as relative light units (RLU) and averaged over a 10 second period, at intervals of a 100^{th} of a second. The amount of protein in each sample was quantitated using the BCA assay (Peirce, Rockford, IL), and the RLU were normalized based on these results.

2.3.3. FACS ANALYSES Approximately 1 x 10^6 cells were washed in PBS; 2%FCS and incubated on ice with the appropriate concentration of the primary antibody for 15-30 minutes. The cells were washed in PBS; 2%FCS and incubated with the appropriate secondary antibody for 15-30 minutes on ice. The cells were washed once more, resuspended in PBS; 2%FCS and analysed by flow-cytometry with a Becton-Dickson FACScan. The mouse anti-human IL-4Rα and the rat anti-murine IL-4Rα were purchased from Genzyme (Cambridge, MA). and used at a concentration of 1μg/mL. The secondary goat anti-mouse Ig coupled to fluorescein isothiocyanate (FITC) and goat anti-rat Ig coupled to FITC were used at a dilution of 1:100 (Sigma).

2.3.4. CELL CULTURE AND TRANSFECTIONS Ba/F3 and FD5 cells and transfected clones were maintained in RPMI 1640 medium, supplemented with 10% FCS, 50μM 2ME, and 2% (v/v) W3B. Ba/F3 and FD5 cells were transfected as described in 2.2.3. For each transfection, cells

were mixed with 1 μg of linearized pPGKNeoR or pPGKPuroR together with 15 μg of the linearized expression vector of interest. For Ba/F3 cells G418 was used at 1mg/mL (Gibco BRL), and puromycin was used at 2μg/mL. For FD5 cells, G418 was used at 100μg/mL. Clones expressing the exogenous cDNA of interest were determined by immunoblotting whole cell lysates with 9E10 as described in 2.2.4.

2.3.5. Cell Stimulations, Immunoprecipitations, and Immunoblotting Cells were incubated in RPMI, 10% FCS, 0.2% W3B for 16 hours, washed three times with SFM and incubated at a density of $1x10^7$ cells/mL in SFM at 37°C for a further 1 hour. Cells were stimulated by addition of synthetic IL-4 (10 μg/mL) or IL-3 (5μg/mL). Following stimulation, the cells were lysed in lysis buffer that had been supplemented with phosphatase inhibitors as described in 2.1.1. Ten μg of rabbit polyclonal antibodies raised against STAT-6 (M20, Santa Cruz Inc., CA) were added and lysates were incubated for 30 minutes on ice, followed by the addition of Protein A coupled to sepharose (Pharmacia). Samples were rotated at 4°C for a further 60 minutes. The immune-complexes were washed three times with lysis buffer, and eluted by addition of Laemmli's SDS-Sample Buffer and boiling. Membranes were immunoblotted as described in 2.1.2. Anti-STAT-6 antibodies were used at a dilution of 1:1000 (Santa Cruz Biotechnology) and 4G10 (Upstate Biotechnology Inc.) was used at 1μg/mL. The hybridoma producing the 9E10 monoclonal antibody was obtained from the American Type Culture Collection (ATCC, Rockville, MD), and injected into the peritoneal cavities of 3 mice, that had previously been pristane primed. The acsites was collected 5 weeks later, and was used at a dilution of 1:2000. When necessary, blots were stripped of antibody by incubation in 1X

Stripping solution (62.5 mM Tris, pH 8.8, 2% SDS, 100mM 2ME) at 55°C for one hour. The membrane was thoroughly rinsed, and re-blocked before addition of a new primary antibody.

2.3.6. OLIGO PRECIPITATIONS. To analyse the ability of STAT-6 and STAT-6Δ to bind to DNA, complementary oligos containing a STAT-6 consensus binding site (in bold) (sense: 5' GAT CCT CGA C**TT CCC AAG AA**C AGC A 3'; antisense 5' TGC TG**T TCT TGG GAA** GTC GAG GAT C-biotin 3') with a biotin at the 3' end of the anti-sense oligo were made. The oligos were annealed by heating to 90°C, and slow cooling in the water bath. 10μL of the double stranded stock (100μM) was bound to streptavidin-conjugated sepharose (Sigma) by rotation at room temperature for 30 minutes, in 1M NaCl. Unbound oligo was removed by extensive washing in TE (10mM Tris, 1mM EDTA, pH 8.0); 1M NaCl. The oligo/sepharose slurry (20μL) was added to cell lysates, and samples were rotated for 1-2 hours at 4°C. Unbound proteins were removed by washing three times in lysis buffer, and bound proteins were eluted by addition of 5X Lamelli's SDS-Sample Buffer and boiling.

2.3.7. CELL VIABILITY AND PROLIFERATION ASSAYS Proliferation of cells was assessed by either [^{3}H]-thymidine incorporation into *de novo* synthesized DNA or 3-[Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) incorporation. Cells were washed three time with HBSS supplemented with 2% (v/v) FCS. Cells were plated at 1000 cells/well in a Terasaki microtitre plate to analyse [^{3}H]-thymidine incorporation or at 10 000 cells/well in a 96 well plate for MTT assays. Chemically synthesized IL-3 was used at 1μg/mL to assess maximal proliferation of the cells. Chemically synthesized IL-4 was added in two fold dilutions, at an initial concentration of either 10 or 4μg/mL. All points were repeated in triplicate. Cells were incubated at 37°C for

40 hours, and pulsed for a further 8 hours with either [^{3}H]-thymidine at a final concentration of 15 μCi/mL, or 0.75 mg/mL MTT (Sigma). [^{3}H]-thymidine assays were harvested, and counted on a scintillation counter. MTT assays were solubilised with 100μL of 10% SDS, 50% DMF, pH 4.5, and read at OD_{550} on a BioTek plate reader.

2.4. For Chapter 5

2.4.1. Isolation and Culture of Macrophages. STAT-6 null mice were obtained from Dr. Michael Grusby (Harvard Medical School) and wild-type Balb/C mice were obtained from Jackson Labs. Age- and sex-matched Balb/c or STAT-6 null mice were injected with 2mL of 2% (w/v) thioglycollate (Difco, Detroit MI) into the peritoneal cavity. Five days later, peritoneal exudate cells were harvested by flushing peritoneal cavities with PBS. Cells were plated in a 48 well plate (Corning, Cambridge, MA) at an initial density of 2 x 10^6 cells/mL in a 500μL volume of RPMI 1640 (Gibco BRL, Grand Island, NY), supplemented with 10% FCS (Intergen, Purchase NY) and 50μM 2-ME. Sixteen hours later the wells were washed to remove all non-adherent cells and 200μL of fresh media was added together with LPS and cytokines. The femurs from the same mice were harvested, and flushed with 5 mL of PBS. Bone-marrow cells were cultured in media that was supplemented with 20% L-cell conditioned media (LCCM) as a prominent source of CSF-1. Thirty-six to forty-eight hours later, before the macrophages differentiated and become adherent, the non-adherent cells were harvested and plated at an initial density of 2 x 10^5 cells/mL in a 1mL volume in 24 well plates (Nunc, Denmark). Day five *ex vivo*, when the bottoms of the wells were covered with confluent layers of macrophages, non-adherent cells were removed by washing and 500μL of fresh media was

added together with LPS and cytokines. All cells were cultured in humidified incubators at 37°C, with 5% CO_2.

2.4.2. STIMULATIONS. All stimulations were performed in triplicate as follows. LPS (*E.coli* strain 0111:B4, Difco, Detroit, MI) was used 15μg/mL, recombinant murine IFNγ (Genzyme, Cambridge, MA) was used at 100U/mL, recombinant murine IL-4 (R&D Systems, Minneapolis, MN) was used at 20ng/mL, and recombinant murine IL-10 (R&D Systems) was used at 10ng/mL. The supernatants were harvested 24 hours later. Results with peritoneal macrophages are representative of three independent experiments, and with bone-marrow derived macrophages of five independent experiments.

2.4.3. CAPTURE ELISAS. 96 well plates (MaxiSorp, Nunc) were coated overnight at 4°C with capture antibodies. Following washing, plates were blocked for 2 hours at room temperature with PBS, 3% BSA (blocking buffer). Standard dilutions of cytokines were prepared (2000 pg/mL to 31.25 pg/mL) and added to wells in parallel with supernatants. Plates were incubated overnight at 4°C with shaking and were washed four times. Biotinylated detection antibody was added, followed by incubated for 1 hour at 4°C with shaking. Plates were washed four times, and Streptavidin-HRP (Genzyme) was added for 15 minutes at 4°C with shaking. Plates were washed four times, developed with tetramethylbenzadine (TMB) (Sigma, St.Louis, MO), and read at 370nM. All washes were done with PBS, 0.05% Tween 20 using a pressurized garden sprayer. Supernatants, standards, detection antibodies and Streptavidin-HRP were diluted in blocking buffer. Capture and detection antibodies were purchased from Pharmingen (San Diego, CA): anti-mIL-12 p40 (C15.6), anti-mIL-12 p40 biotin (C17.8), anti-mTNFα (G281-

2626), anti-mTNFα biotin (MP6-XT3). Standards of recombinant murine TNFα and recombinant murine IL-12 were purchased from R&D Systems.

2.4.4. BIOCHEMICAL ANALYSES. Bone-marrow derived macrophages were derived from Balb/c or STAT-6 null mice by culture in media supplemented with 20% LCCM on 10cm dishes (Nunc) for 5-7 days. When the bottoms of the dishes were almost completely covered with adherent macrophages, non-adherent cells were removed by washing. Adherent macrophages were incubated overnight in RPMI with 10% FSC with a reduced concentration of CSF-1 (2% LCCM). Cells were washed three times with RPMI without FCS, and incubated in 3mL of RPMI without FCS for a further hour at 37°C in a humidified, gassed incubator. Cells were stimulated as indicated with addition of synthetic mIL-4 (20μg/mL), synthetic mIL-13 (20μg/mL), recombinant porcine insulin (15μg/mL), rmIFNγ (1000 U/mL), rmIL-10 (50ng/mL) or left unstimulated as a control. The cells were lysed in lysis buffer that was supplemented with phosphatase inhibitors. The amount of protein in each lysate was normalized based on total protein content as determined by the BCA protein assay (Pierce, Rockford, IL). IRS-2, STAT-1, STAT-3 or STAT-6 were immunoprecipitated from lysates by incubation with the appropriate antibodies followed by adsorption to Protein A sepharose (Pharmacia, Uppsala). Anti-IRS-2 antibodies were purchased from Upstate Biotechnology Inc., and anti-STAT-1, -3, and -6 from Santa Cruz Biotechnology (Santa Cruz, CA). The eluates were subjected to SDS-PAGE, and immunoblotting. Membranes were blotted first with 4G10 (Upstate Biotechnology Inc, Lake Placid, NY) to determine the amount of tyrosine phosphorylation and were subsequently stripped and re-blotted with antibodies to IRS-2, STAT-1 or STAT-6.

2.5. For Chapter 6

2.5.1. CELL STIMULATIONS, IMMUNOPRECIPITATIONS AND IMMUNOBLOTTING Macrophages were derived from the bone marrow of C57Bl/6 mice as described in 2.4.1. Biochemical stimulations were performed as described in 2.4.4. Cells were stimulated with IFNγ (1000 or 2000 U/mL, Genzyme) for 10 minutes, or with 50ng/mL rCSF-1 (R&D) for 3 minutes. SDS-PAGE and immunoblotting was performed as described in 2.1.2. Polyclonal anti-phospho-ERK-1/-2 antibodies (New England Biolabs, Beverly, MA) were used at a 1:1000 dilution. STAT-1 was immunoprecipitated by addition of 4μL of polyclonal anti-STAT-1 (Upstate Biotechnology Inc.), and immunoblotted with a monoclonal anti-STAT-1 antibody (Santa Cruz Biotechnology).

2.5.2. LUCIFERASE ASSAYS Transient transfections and assay for luciferase activity were performed as described in 2.3.2. Ba/F3 cells were stimulated with IFNγ (200U/mL, Genzyme), 2μg/mL synthetic IL-4, both together, or left unstimulated as a control.

2.5.3. EXPRESSION OF CD23 $CD19^+$ B cells (see 2.5.4) were cultured in RPMI, 10% FCS, 50μM 2ME, anti-IgM (5μg/mL) (Jackson Immunoresearch, West Grove, PA), and anti-CD40 (2.5μg/mL) (IC10, Maureen Howard, Anergen, Palo Alto) at an initial density of 8×10^5 cells/mL, in a 250μL volume. Cells were stimulated with IL-4 (3% IL-4CM), rIFNγ (200U/mL, Genzyme), or both together. Thirty-six hours later, the FcγRII/III receptors were blocked by addition of 2.4G2 (ATCC, anti-FcγRII/III, CD16/CD32), and cells were stained with FITC-coupled anti-CD23 (Pharmingen), and analysed by flow-cytometry.

2.5.4. PURIFICATION, CULTURE, AND STIMULATION OF CELLS $CD4^+$ T cells or $CD19^+$ B cells were purified by positive selection using magnetic bead sorting. T cells were isolated from inguinal and mesenteric lymph nodes from 3-4 month old C57Bl/6 x CBA (F1) mice. B cells were isolated from the spleens of F1 mice. The tissues were passed through a sieve to generate a single cell suspension. Erythrocytes were removed from spleen samples by addition of red cell removal buffer (150mM ammonium chloride, 1mM potassium carbonate, 0.1mM EDTA, pH 7.3) for 4 minutes at 37°C. Lysis was stopped by addition of an equivalent volume of complete media. Lymph node and spleen cells were washed with RPMI supplemented with 2% FCS prior to addition of antibodies. Biotinylated antibodies against murine CD4 or CD19 (Pharmingen) were added at a concentration of $1\mu g/10^6$ predicted target cells, and the cells were incubated on ice for 15 minutes. Cells were washed twice in MiniMac buffer (PBS pH 7.2, 0,5% BSA, 2mM EDTA) followed by addition of streptavidin-coated micro beads (Miltenyl Biotec, Gladbach, Germany). Cells were incubated with the microbeads for 15 minutes at 4°C, and unbound beads were removed by washing in MiniMac buffer. Cells were passed through a MS^+ column in the magnetic field of a MACS separator (Miltenyl Biotech), and bound $CD4^+$ or $CD19^+$ cells were eluted in MiniMac buffer upon removal from the magnetic field. After elution, the purity of the cell population was analysed by flow-cytometry. $CD4^+$ cells were stained with phycoerythrin conjugated anti-CD4 antibodies and FITC-conjugated anti-CD8 antibodies, and CD19+ cells were stained with phycoerythrin-conjugated anti-B220 antibodies. All antibodies were purchased from Pharmingen. Typically, cells were 97% pure.

T cells were activated by stimulation with an immobilized anti-T cell receptor antibody (2C11, ATCC) at a density of 1-3 x 10^6 cells/ mL in complete media supplemented with 2% medium conditioned by AgXO63 cells that had been transfected with the cDNA for murine IL-2

(IL-2CM) (Karasuyama and Melchers, 1988). After 48 hours, when cells had started to proliferate, they were transferred to a fresh dish, without 2C11, and expanded in media with 2% IL-2CM for 10-14 days.

To generate primary mast cells, bone marrow was harvested from the femurs of F1 mice and cultured for 4-6 weeks in complete media supplemented with 2% W3B and 3% IL-4CM. Jurkat cells were cultured in complete media, in the absence of additional factors.

For all stimulations, cells were first deprived of serum and factors for 4 hours, and then stimulated with: human rIFNγ (1000 U/mL, Genzyme), murine rIFNγ (1000 U/mL, Genzyme), synthetic IL-4 (5μg/mL), or TPA (5ng/mL) and ionomycin (250ng/mL) as indicated. Stimulations were performed at 37°C, in humidified incubators, gassed with 5% CO_2, and were timed such that all samples, including controls, were harvested simultaneously.

2.5.5. GENERATION OF BCL-6 ANTISENSE TEMPLATES I obtained an expressed sequence tag (EST) clone (#402286) encoding a fragment of human Bcl-6 from ATCC. The clone was sequenced, and a *HindIII/SacI* fragment encoding 320 bp at the 3' end of the cDNA was cloned into pTRIamp18 (Ambion, Austin, TX) in the antisense orientation. To generate a template for synthesis of antisense RNA for murine Bcl-6, total RNA was isolated from Ba/F3 cells (see 2.5.3) and cDNA was generated using random hexamers and Superscript Reverse Transcriptase (Gibco BRL). A 343 bp fragment from the 3' end of murine Bcl-6 was PCR amplified using the PFU polymerase (Stratagene) (sense primer 5' GC GGA TCC CAC TCT GGA GAA AAG CCC TAC 3'; antisense primer 5' AT TCA GCA GGC TTT GGG GAG CTC CGG AGG CAG 3'). After two rounds of PCR amplification, the fragment of the correct size was ligated into pSSBS. Antisense RNA was generated with T3 polymerase, after the plasmid had been

linearized with *BamHI.*

2.5.6. ISOLATION OF TOTAL RNA. Total RNA was isolated from cells with TRIZOL (Gibco BRL). Approximately 1 x 10^7 cells were resuspended in 1mL of TRIZOL, with 200μL of choloroform. The mixture was vortexed for 1 minute, and the aqueous and organic layers were separated by centrifugation at 14 000 rpm for 10 minutes at 4°C. The upper aqueous phase was transferred to a new tube, and the RNA was precipitated upon addition of isopropanol. The pellet was washed with 70% ethanol, air dried, resuspended in distilled water, and quantitated spectrophotometrically.

2.5.7. PROBE SYNTHESIS Antisense RNA for Bcl-6 was synthesised from the T7 promoter in pTRIamp18 hBcl6, or from the T3 promoter in pSSBS mBcl-6. Antisense RNAs for murine and human GAPDH were synthesized by T7 polymerase from linearized templates purchased from Pharmingen. Synthesis of antisense RNA was performed in total volume of 20μL with 1μL of template, 1X transcription buffer (Gibco BRL), 1μL RNAsin (Gibco BRL), 500μM each rGTP, rATP and rUTP (Pharmacia), 5μM rCTP (Pharmacia), 50μCi [αP^{32}]CTP (3000mCi/mmol) (NEN), and 1.5μL of T3 or T7 polymerase (Gibco BRL). The reaction was incubated at 37°C for 1.5 hours, and 2μL of DNAseI (5000 U/mL) was added for an additional 30 minutes.

Probes were purified on a 6% denaturing, polyacrylamide gel. The area of the gel corresponding to the full-length probe was excised and the probe was eluted in probe elution buffer (0.5M NH_4oAc, 1mM EDTA, 0.2% SDS, (Ambion)) overnight, with rotation at 45°C.

Bcl-6 probes typically had in 2-300 000 counts per minute (cpm) /μL, and GapDH probes 3-40 00 counts/μL, in a total volume of approximately 200μL.

2.5.8. HYBRIDIZATION AND DIGESTION Ten μg of total RNA for each sample was mixed with 500 000 cpm of Bcl-6 probe and 100 000 cpm of GapDH probe, along with 0.5M NH_4oAc, and three volumes of 100% ethanol. The RNA/probe mixture was precipitated and resuspended in hybridization buffer (80% deionized formamide, 100mM sodium citrate pH 6.4, 300mM NaoAc pH 6.4, 1mM EDTA, Ambion), heated to 95°C, and allowed to hybridize overnight at 45°C. Single stranded RNA was digested by addition of RNase digestion buffer (Ambion, patent pending,), 1 unit of RNase A (approximately 2μg) and 40 units of RNase T1 per sample. The digestion was allowed to proceed for 1 hour at 37°C and was stopped by addition of RNase Inactivation mixture (Ambion, patent pending), 5μg of yeast tRNA, and 100% ethanol. The double-stranded RNA was precipitated, allowed to air dry, and resuspended in 15μL of gel loading buffer (95% formamide, 0.025% xylene cyanol, 0.025% bromophenol blue, 0.5mM EDTA, 0.025% SDS, Ambion).. After heating to 95°C, the samples were loaded onto a 7% denaturing polyacrylamide gel, which was run in 0.5X TBE for approximately three hours at 200 volts. The gel was dried onto whatmann 3mm, and exposed to film at –70°C. Typically, the GapDH signal could be seen after a 30 minute exposure, whereas Bcl-6 was visible only after an overnight exposure.

CHAPTER 3. Interleukin-4 synergises with Raf-1 to promote long-term proliferation and activation of c-jun N-terminal kinase (JNK).

3.1. Introduction

IL-4 was originally known as B-cell growth factor or B-cell stimulating factor, by virtue of its ability to enhance the proliferation of B-cells, and to cause them to secrete IgG_1 and IgE (Paul, 1991). Subsequently, IL-4 was shown to have similar growth enhancing effects on multiple cell types, including T-cells, and mast cells (Lee et al., 1986; Mosmann et al., 1986; Yokota et al., 1986; Spits et al., 1987). Although IL-4 was originally classified as a B-cell growth factor, there is in fact little evidence that it can itself stimulate growth. IL-4 is unable to induce resting B-cells to leave G_0 and enter the G_1 phase of the cell cycle (Oliver et al., 1985; Rabin et al., 1985; Defrance et al., 1987; Clark et al., 1989). Furthermore the growth enhancing effects on primary T-cells or mast cells were detected in short-term assays, which measured [^{3}H]-thymidine incorporation, mitochondrial activity or cell numbers at 24-48 hours (Mosmann et al., 1986; Spits et al., 1987; Welham et al., 1992; Minshall et al., 1997).

Activation of the Ras super-family and the MAP-family cascades are, in general, strongly associated with progression through the cell cycle (Avruch et al., 1994). For example, it has been shown that activation of ERK is an absolute requirement for both hematopoietic cells and fibroblasts to progress from G_0 to S phase (Marshall, 1995; Muszynski et al., 1995; Perkins et al., 1996), and over-expression of a constitutively active mutant of the upstream activator of ERK, MEK1, promotes cell cycle entry (Brunet et al., 1994; Cowley et al., 1994; Mansour et al., 1994). I therefore tested the hypothesis that the failure of IL-4 to promote cell-cycle entry and long-term growth relates to its inability to activate the Ras/Raf/ERK pathway.

3.2. Results

3.2.1. IL-4 is not a true growth factor

The addition of IL-4 to cultures of primary mast cells, T-cells, B-cells or factor-dependent hemopoietic cell-lines over a period of 24-48 hours results in increased survival and incorporation of [^{3}H]-thymidine (Mosmann et al., 1986; Defrance et al., 1987; Spits et al., 1987). However, there is no sustained increase in cell numbers and the cells will eventually die. These observations are compatible with the notion that either IL-4 promotes short-term survival and cells that had previously entered the cell-cycle are permitted to complete DNA synthesis, or alternatively, that IL-4 was indeed able to stimulate cell-cycle progression and growth in a fraction of cells, but failed to support their long-term survival.

To distinguish between these possibilities, I exploited a technique based upon labelling the membranes of cells with a fluorescent dye (PKH26) and monitoring its dilution to determine the number of cell-divisions undergone by individual cells. The fluorescent dye cannot be transferred to neighboring cells but can be detected at a relatively lower level of fluorescence in daughter cells (Slezak and Horan, 1989). The membranes of an IL-3 dependent hemopoietic cell line, Ba/F3, were labelled with PKH26, and flow cytometric analysis of labelled Ba/F3 cells that were cultured in IL-3 showed a series of peaks corresponding to dilution of the dye after cell division (Figure 3.1A). In contrast, analysis of labeled Ba/F3 cells that were cultured for 3 days in IL-4 showed a peak (66%, Figure 3.1B) corresponding to cells that had completed one cell division, and no peaks corresponding to 2 or more cell divisions. These data suggest that IL-4 is unable to stimulate the repeated entry of cells into cycle.

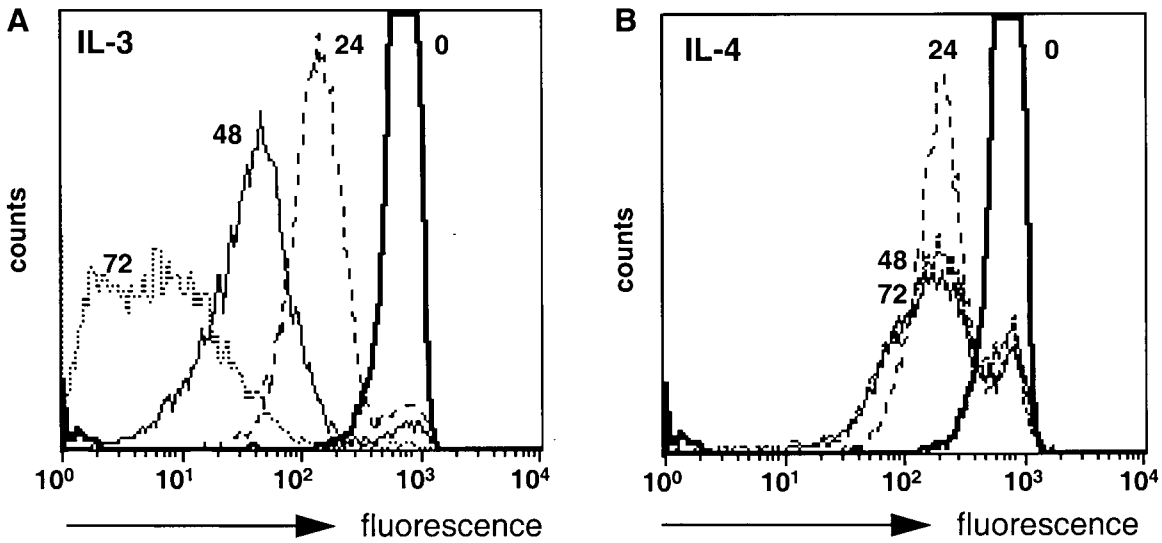

Figure 3.1. *IL-4 is not a growth factor.* Ba/F3 cells were labeled with the fluorescent dye PKH26. Immediately after labeling, incorporation of the dye was analyzed by flow-cytometry (time 0). Cells were cultured in complete media supplemented with IL-3 (**A**) or IL-4 (**B**) and aliquots of cells were analyzed by flow cytometry at 24, 48 and 72 hours to determine the amount of dye dilution.

3.2.2. IL-4 synergises with Ras to stimulate long-term proliferation

To test the hypothesis that the failure of IL-4 to promote the long-term growth of hematopoietic cells rests solely in its inability to stimulate Ras activity, stable clones of Ba/F3 cells that expressed an activated mutant of N-Ras (^{Q61K}N-Ras), fused to an N-terminal HA epitope-tag were generated. Ba/F3 cells transfected with ^{Q61K}N-Ras were selected on the basis of neomycin resistance. Several independent drug-resistant clones were obtained by limit dilution.

Expression of ^{Q61K}N-Ras was confirmed by immunoblotting of whole cell lysates using antibodies against the HA-tag. All the clones derived expressed similar levels of ^{Q61K}N-Ras.

The ability of parental Ba/F3 cells (Figure 3.2A) and Ba/F3 cells expressing ^{Q61K}N-Ras (^{Q61K}N-Ras cells) to proliferate in the presence or absence of IL-4 was compared by counting cells over a period of 5 days. ^{Q61K}N-Ras cells failed to grow in the absence of IL-3 (Figure 3.2B) although unlike parental Ba/F3 cells (which were all dead by 96 hours), they did not undergo apoptosis in the absence of factor over the five day duration of the experiment. In the presence of IL-3, no effect of ^{Q61K}N-Ras was seen. In striking contrast to parental Ba/F3, in the presence of IL-4, ^{Q61K}N-Ras cells grew exponentially at a rate comparable with that of cells in IL-3 (Figure 3.2B).

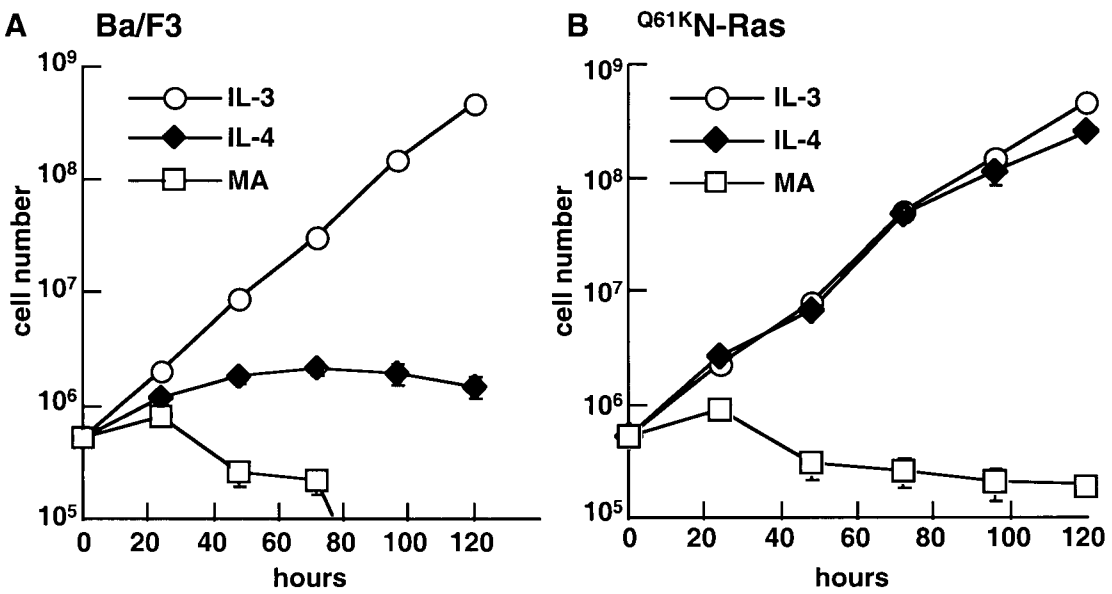

Figure 3.2. *Activated ^{Q61K}N-Ras in combination with IL-4 stimulates the long-term growth of Ba/F3 cells.* **A.** Ba/F3 or **B.** ^{Q61K}N-Ras cells were washed free of IL-3 and incubated in IL-3, IL-4 or media alone with no factor (MA) at a density of $1x10^5$ cells/mL. Cells were counted in triplicate at the indicated times, and diluted as appropriate to maintain a consistent density of cells. The results are representative of two independent experiments, with two independent clones. Error bars represent the SEM of triplicate samples.

3.2.3. IL-4 synergises with Raf-1 to promote long-term proliferation

There is evidence that Ras is upstream of the three MAP-kinase family cascades (Cano and Mahadevan, 1995), as well as the PI3'-kinase pathway (Rodriguez-Viciana et al., 1997; Rodriguez-Viciana et al., 1996) . Since IL-4 activates PI3'-kinase (Gold et al., 1994), but, unlike cytokines such as IL-3 that stimulate growth of hematopoietic cells, fails to activate Raf-1 or any of the three MAP-family kinases, ERK, JNK or p38 (Welham et al., 1992; Welham et al., 1994; Foltz et al., 1997; Foltz and Schrader, 1997), I focussed on the MAP-family cascades.

As activation of the Raf/ERK pathway has been strongly correlated with growth, I first investigated whether the synergistic effect of activated Ras on signals provided by IL-4 could be replaced by expression of activated Raf-1 protein. I obtained a form of human Raf-1 that was fused to the enhanced green fluorescent protein (EGFP) and the hormone-binding domain of the estrogen receptor, so that its kinase activity could be induced by addition of estradiol or an analog, 4-hydroxy-tamoxifen (4HT). When expressed in NIH-3T3 cells and activated by 4HT this protein results in rapid activation of MEK1 and ERK and transformation (Samuels et al., 1993). This cDNA was expressed in Ba/F3 cells and individual clones (hereafter referred to as ΔRaf-1:ER cells) were obtained and screened for the presence of EGFP by flow cytometry. Consistent with observations in NIH-3T3 cells by Pritchard *et al.* (1995), the level of expression of ΔRaf-1:ER was greatly increased by overnight incubation in 100nM 4HT (Figure 3.3) most likely due to a stabilization of the estrogen receptor in the presence of its ligand. As documented below, enhanced expression of ΔRaf1:ER was also accompanied by elevated Raf-1 activity.

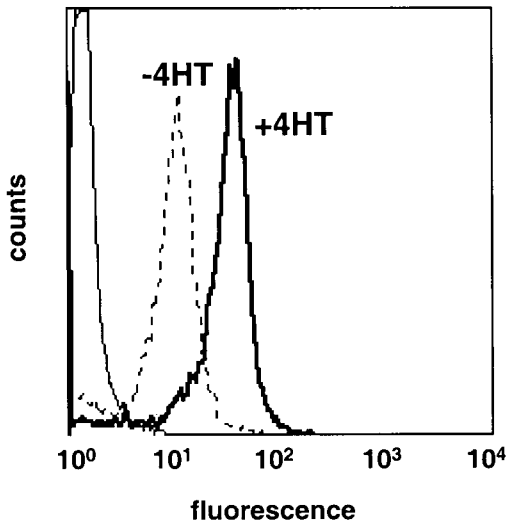

Figure 3.3. *Incubation with 4HT increases expression of ΔRaf-1:ER.* ΔRaf-1:ER cells were incubated in complete media, supplemented with IL-3, with (thick lines) or without (thin line) 100nM 4HT for 16 hours. Cells were then analysed for the presence of EGFP by flow-cytometry .

[^{3}H]-thymidine uptake assays were performed to analyze the effects of activation of ΔRaf-1:ER by increasing concentrations of 4HT on cellular proliferation (Figure 3.4A). Even the highest concentrations of 4HT resulted in only a small amount of DNA synthesis. In contrast, in the presence of IL-4, the addition of 4HT resulted in a dose-dependent, synergistic stimulation of DNA synthesis. The maximal biological response to 4HT was observed at 100nM, and this concentration was used for all subsequent experiments. Parental Ba/F3 cells failed to respond to 4HT alone or in combination with IL-4 (Figure 3.4A)

I next investigated whether Raf-1 activity and IL-4 synergised to support long-term proliferation of ΔRaf-1:ER cells by counting cells over a period of 4 days. As shown in Figure 3.4B, ΔRaf-1:ER cells cultured in media alone rapidly declined in number, and this decline could be delayed by the presence of IL-4. The induction of Raf-1 activity by the presence of

4HT also prevented the decline in cell numbers seen in media alone, and the number of cells increased very slowly. In contrast, the combination of 4HT and IL-4 resulted in a dramatic, synergistic stimulation of cell growth that closely resembled that seen with the combination of IL-4 and activated Ras (Figure 3.4B). To confirm that the synergistic proliferation in the presence of active Raf-1 and IL-4 was dependent on Raf-1 kinase activity, the experiment was repeated with stable clones of Ba/F3 cells that expressed a kinase inactive form of ΔRaf-1:ER (ΔRaf-1:ER K70W cells). ΔRaf-1:ER K70W cells in the absence of factor, or stimulated with 4HT, died at a similar rate, and the survival effects of IL-4 were not altered in the presence of 4HT (Figure 3.4C). Thus, the synergistic proliferation induced by active Raf-1 and IL-4 was dependent on Raf-1 kinase activity. These results suggest that the relevant pathway downstream of Ras that synergised with IL-4 was indeed the Raf/MEK/ERK pathway.

3.2.4. Stimulation of ΔRaf-1:ER activity leads to ERK activation

To confirm this notion at a biochemical level, the activity of Raf and other downstream kinases in ΔRaf-1:ER cells following addition of 4HT was determined. Maximal activation of ΔRaf-1:ER in an *in vitro* kinase assay, using MEK1 as a substrate, was not seen until 12-16 hours following addition of 4HT (Figure 3.5A). At longer time points, autophosphorylation of ΔRaf-1:ER was also detected. However, a significant increase in ERK activity was detected 2 hours after addition of 4HT (Figure 3.5B). This ability to detect ERK activity prior to Raf-1 activity is probably due to the inherent amplification of the enzyme cascade, and the higher sensitivity of the ERK kinase assay. All subsequent stimulations were performed for 16 hours to ensure maximal Raf kinase activity. Since the viability of the parental Ba/F3 in the absence of IL-3 could only be maintained for sixteen hours if IL-4 was present, it was necessary to perform the

4HT stimulations in the presence of IL-4.

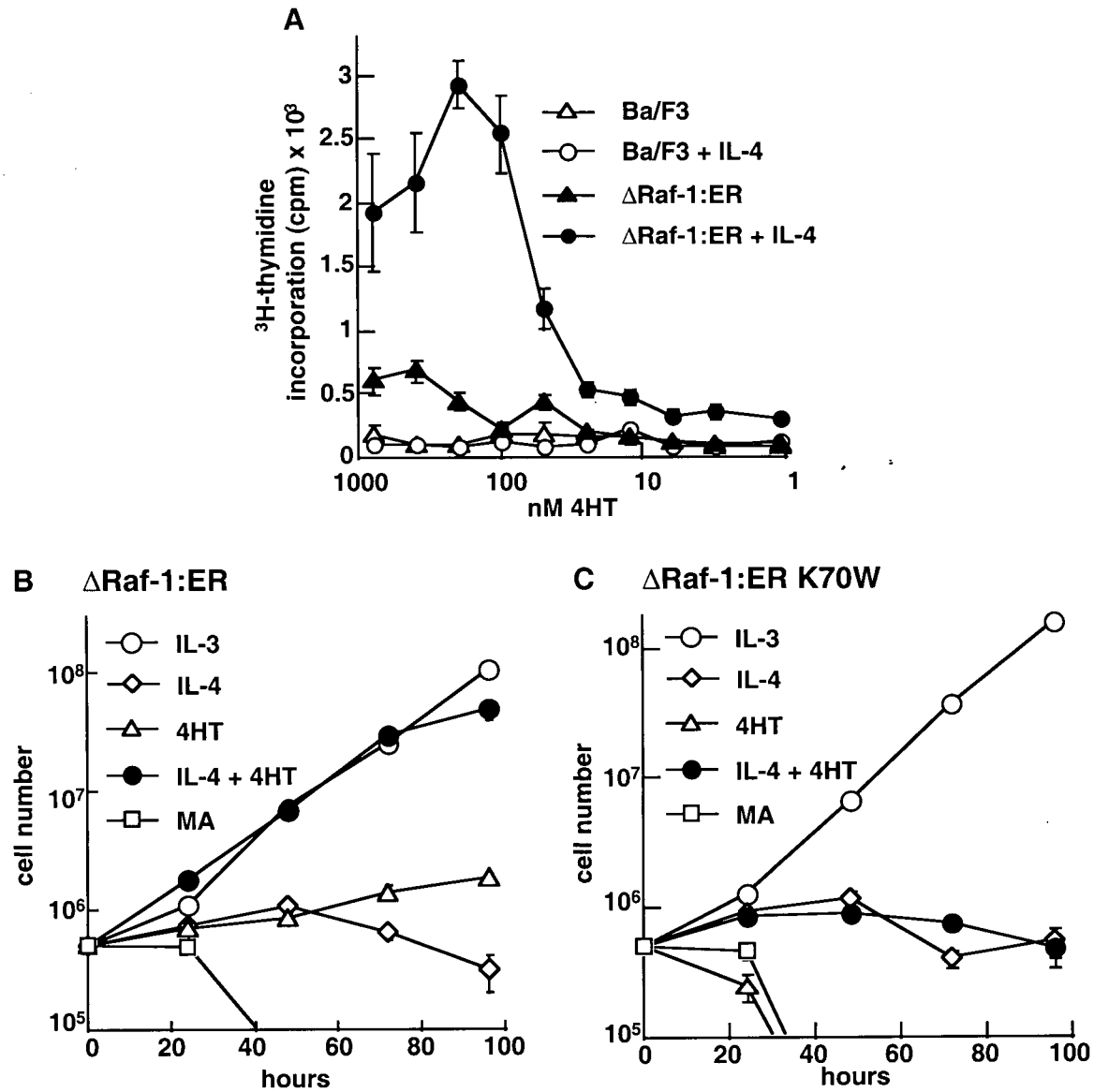

Figure 3.4. *Activation of ΔRaf-1:ER in combination with IL-4 stimulates DNA synthesis and the long-term growth of Ba/F3 cells.* **A.** Ba/F3 or ΔRaf-1:ER cells were washed free of IL-3 and plated at 250 cells/well in a Terasaki microtitre plate in medium alone or IL-4, with increasing concentrations of 4HT. After 40 hours the cells were pulsed with [^{3}H]-thymidine for an additional 8 hours, harvested, and counted in a scintillation counter. **B.** ΔRaf-1:ER or **C.** ΔRaf-1:ER K70W cells were washed free of IL-3 and incubated in IL-3, IL-4, 100nM 4HT, IL-4 plus 100nM 4HT or without factor (MA) at a density of $1x10^5$ cells/mL. Cells were counted in triplicate at the indicated times, and diluted as appropriate to maintain a consistent density. The results are representative of several independent experiments, with two independent clones. Error bars represent the SEM of triplicate samples.

Stimulation of ERK activity in ΔRaf-1:ER cells by addition of 4HT was dose dependent (Figure 3.6), and was seen at concentrations of 4HT above 25nM. Parental Ba/F3, or ΔRaf-1:ER K70W cells did not show any increase in ERK activity following stimulation with 4HT (data not shown). Ba/F3 or ΔRaf-1:ER cells that had been stimulated with IL-3 or IL-4 for 16 hours showed no detectable levels of ERK activity (Figure 3.6 and data not shown).

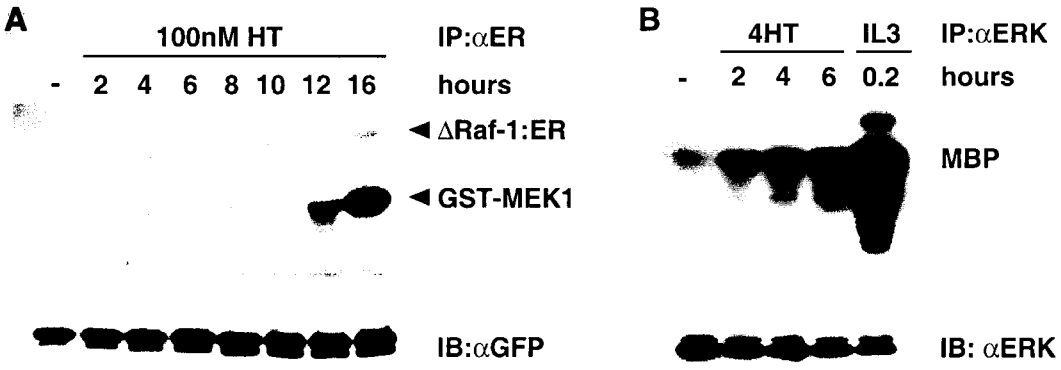

Figure 3.5. *Addition of 4HT activates ΔRaf-1:ER and ERK kinase activity.* ΔRaf-1:ER cells were stimulated at 37°C in RPMI,10% FCS and 100nM 4HT for the indicated time. The cells were lysed and analyzed for **A**. Raf-1 activity by immunoprecipitation with an anti-estrogen receptor antibody followed by an *in vitro* kinase assay using GST-MEK1 as a substrate, or **B.** ERK activity by immunoprecipitation with an anti-ERK antibody, followed by an *in vitro* kinase assay using MBP as a substrate. Positive control cells were stimulated with IL-3 for 10 minutes. Phosphorylated proteins were visualized after SDS-PAGE and autoradiography. The quantity of immunoprecipitated protein in each lane was assessed by immunoblotting (IB) with antibodies against GFP (αGFP) or ERK (αERK).

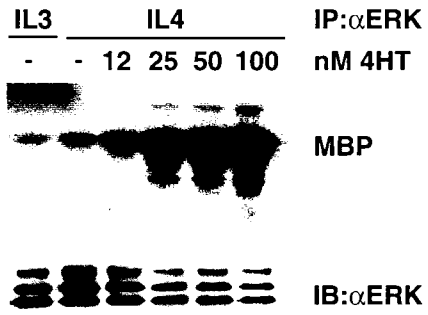

Figure 3.6. *Dose-dependent activation of ERK in ΔRaf-1:ER cells following stimulation with 4HT for 16 hours.* ΔRaf-1:ER cells were stimulated at 37°C for 16 hours at 2 x 10^5 cells/mL in RPMI, 10% FCS plus IL-3, IL-4, or IL-4 plus increasing concentrations of 4HT. ERK was immunoprecipitated from the cell lysate, and kinase activity was determined *in vitro* using MBP as a substrate. Phosphorylated proteins were visualized after SDS-PAGE and autoradiography. The quantity of immunoprecipitated protein in each lane was assessed by immunoblotting (IB) with antibodies against ERK (αERK).

3.2.5. Stimulation of ΔRaf-1:ER activity leads to JNK activation

Growth stimuli like IL-3 or Steel Locus factor induce activation of JNK, while IL-4 does not (Foltz and Schrader, 1997), thus correlating the ability of a growth factor to stimulate JNK with its ability to stimulate growth. I therefore also investigated the activity of JNK in ΔRaf-1:ER cells. Surprisingly, stimulation of ΔRaf-1:ER cells with 4HT for 16 hours in the presence of IL-4, resulted in dose-dependent activation of JNK (Figure 3.7A). This observation that expression of active Raf-1 correlated with activation of JNK was unexpected as many investigators have shown JNK to be downstream of MEK-kinase 1 (MEKK1), but not of Raf-1 (Minden et al., 1994; Robinson et al., 1996), and IL-4 does not activate JNK (Foltz and Schrader, 1997). JNK was originally identified as a stress-activated kinase (Kyriakis and Avruch, 1996), and I therefore needed to rule out the possibility that activation of JNK resulted from stress induced

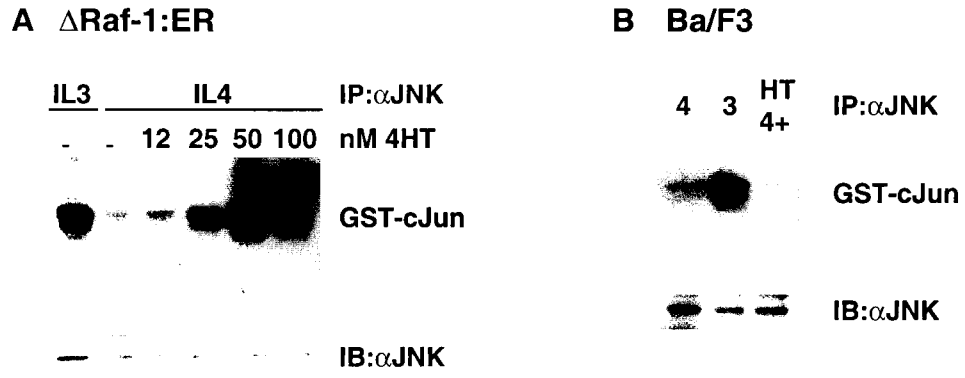

Figure 3.7. *Dose-dependent activation of JNK in ΔRaf-1:ER but not Ba/F3 cells following stimulation with 4HT for 16 hours.* **A.** ΔRaf-1:ER cells were stimulated at 37°C for 16 hours at 2 x 10^5 cells/mL in RPMI, 10% FCS plus IL-3, IL-4, or IL-4 plus increasing concentrations of 4HT. **B.** Ba/F3 cells were stimulated for 16 hours at 37°C in RPMI supplemented with 10% FCS and IL-3 (**3**), IL-4 (**4**) or IL-4 plus 100nM 4HT (**4+HT**). JNK was immunoprecipitated from the cell lysate, and kinase activity was determined *in vitro* using GST-cJun as a substrate. Phosphorylated proteins were visualized after SDS-PAGE and autoradiography. The quantity of immunoprecipitated protein in each lane was assessed by immunoblotting (IB) with antibodies against JNK (αJNK).

by addition of 4HT. The response to 4HT in parental Ba/F3 cells was examined, and in three independent experiments under the same conditions (ie 16 hour incubations), there was no induction of JNK activity with 4HT and IL4 (Figure 3.7B).

3.2.6. Activation of JNK is not due to the production of an autocrine factor

A similar activation of JNK following ΔRaf-1:ER activation was seen in NIH-3T3 cells, and was due to an autocrine production of heparin-binding epidermal growth factor (HB-EGF), which activated JNK following ligation of the EGF receptor (Minden et al., 1994; McCarthy et al., 1995; McCarthy et al., 1997). Ba/F3 cells do not express receptors for EGF (Collins et al.,

1988), but I was interested to know whether the activation of JNK we observed was due to another autocrine pathway. To address this question, medium was collected from ΔRaf-1:ER cells that had been stimulated with 4HT for 48 hours and concentrated 10 fold. Ba/F3 cells were incubated with this medium, starting with a concentration of 50%, however it had no ability to suppress apoptosis or promote growth of Ba/F3 cells either alone or in combination with IL-4. Moreover, incubation of Ba/F3 cells for 16 hours in this conditioned medium failed to result in activation of JNK (data not shown).

However, the approach used above does not exclude the possibility of an autocrine factor that was cell-surface bound or extremely labile. To address this possibility, stable clones of Ba/F3 cells that expressed JNK-1 that was tagged with GST at the N-terminus were made (Ba/F3 GST-JNK cells). The GST tag allowed the exogenous protein to be distinguished from the endogenous JNK in Ba/F3 cells. 1 x 10^6 of these Ba/F3 GST-JNK-1 cells were mixed together with 1x 10^7 ΔRaf-1:ER cells and incubated for 16 hours with IL-3, IL-4 or IL-4 plus 4HT. A 10:1 ratio of ΔRaf-1:ER cells to Ba/F3 GST-JNK1 cells was used to increase the possibility of detecting an autocrine factor. The mixture of cells was lysed and using glutathione sepharose beads, GST-JNK was specifically precipitated from Ba/F3 GST-JNK-1 cells. As shown in Figure 3.8A, GST-JNK-1 was not activated in mixed cultures that had been stimulated with IL-4 plus 4HT. In contrast, analysis of GST-JNK-1 from control mixed cultures that had been stimulated with IL-3 demonstrated activation of GST-JNK-1. After depletion of GST-JNK-1 from the lysates, the activity of the total endogenous JNK-1 in the mixed lysate was determined by immunoprecipitation with an anti-JNK-1 antibody. These immunoprecipitates contained JNK from both ΔRaf-1:ER and Ba/F3 GST-JNK-1 cells, but since there was a great excess of ΔRaf-1:ER cells, the signal from ΔRaf-1:ER cells predominated. As shown in Figure

3.8B, endogenous JNK was activated in the mixed cultures that were stimulated with IL4 and 4HT, reflecting the activation of JNK-1 in ΔRaf-1:ER cells I had previously observed (Figure 3.7A). The data presented in Figure 3.8 thus provide strong evidence that the activation of JNK induced in ΔRaf-1:ER cells cultured with IL-4 and 4HT was not secondary to the production of an autocrine factor.

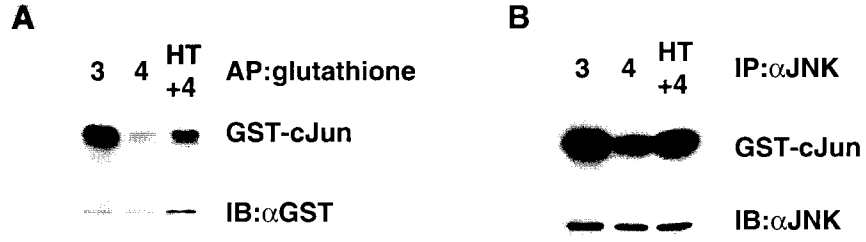

Figure 3.8. *Failure to detect an autocrine factor that can activate JNK in a cell mixing experiment.* 1 x 10^6 Ba/F3 cells expressing GST-JNK1 were mixed together with 1 x 10^7 ΔRaf-1:ER cells, and stimulated for 16 hours at 37°C in RMPI, 10% FCS with either IL-3 (**3**), IL-4 (**4**) or IL-4 plus 100nM 4HT (**4+HT**). **A**. GST-JNK1 was affinity purified from the mixed cell lysate with glutathione Sepharose beads, and the activity was determined *in vitro* assay using GST-cJun as a substrate. Following SDS-PAGE and autoradiography, the membrane was immunoblotted (IB) with antibodies against GST to assess equivalency of loading (αGST). **B.** After the lysate had been cleared with glutathione Sepharose, endogenous JNK was immunoprecipitated with anti-JNK1 antibodies, and the kinase activity was determined *in vitro* using GST-cJun as a substrate. Following SDS-PAGE and autoradiography, the membrane was immunoblotted (IB) with antibodies against JNK1 (αJNK) to assess equivalency of loading.

3.2.7. IL-4 and Raf-1 synergise to activate JNK

In the experiments described above, IL-4 was always included as in its absence the parental Ba/F3 cells died over the 16 hour period of the experiment. However as shown in Figure 3.4B, ΔRaf-1:ER cells are viable when cultured in 4HT alone. Therefore I was able to ask whether

4HT-induced Raf-1 activity alone was sufficient to induce JNK activity or whether addition of IL-4 was also necessary. To my surprise, although incubation of ΔRaf-1:ER cells for 16 hours with 4HT alone induced maximal activity of Raf-1 and ERK (Figure 3.5), JNK was only activated when both IL-4 and Raf-1 were present (Figure 3.9A). To confirm that the stimulation of JNK activity was dependent on Raf-1 kinase activity, I repeated the experiment in ΔRaf-1:ER K70W cells. The results demonstrated that like the synergistic proliferation, the synergistic activation of JNK by Raf-1 and IL-4 was dependent on Raf-1 kinase activity (Figure 3.9B). To investigate the kinetics of this synergy, I stimulated ΔRaf-1:ER cells with 4HT for 16 hours to ensure maximal levels of Raf-1 protein and activity, and then acutely stimulated the cells for 10 minutes with IL-3 or IL-4 (Figure 3.10A). Stimulation of these cells with IL-4 for 10 minutes failed to induce JNK activity, whereas control cells stimulated with IL-3 for 10 minutes exhibited activation of JNK (Figure 3.10B).

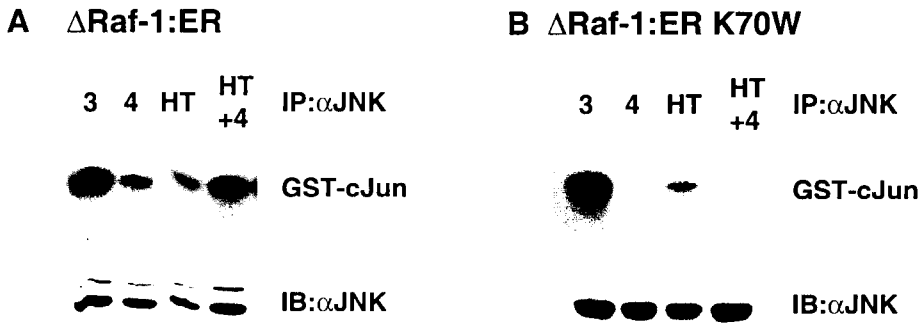

Figure 3.9. *ΔRaf-1:ER and IL-4 synergise to activate JNK.* **A.** ΔRaf-1:ER or **B.** ΔRaf-1:ER K70W cells were stimulated for 16 hours at 37°C in RPMI, 10% FCS supplemented with IL-3 (**3**), IL-4 (**4**), 100nM 4HT (**4HT**) or both IL-4 and 100nM 4HT (**4+HT**). The amount of JNK activity was determined *in vitro* using GST-cJun as a substrate. Phosphorylated proteins were visualized after SDS-PAGE and autoradiography. The membranes were immunoblotted (IB) with anti-JNK antibodies to assess equivalency of loading (αJNK).

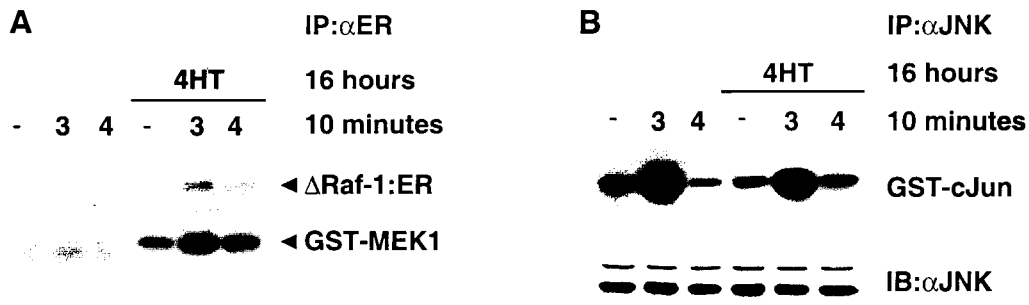

Figure 3.10. *Stimulation with IL-4 for 10 minutes in the presence of activated ΔRaf-1:ER is not sufficient to activate JNK.* ΔRaf-1:ER cells were stimulated for 16 hours at 37°C in RPMI, 10% FCS with or without 100nM 4HT. The cells were then stimulated for 10 minutes with IL-3 (**3**) or IL-4 (**4**) or left untreated as a control (**-**). **A.** Raf-1 was immunoprecipitated with anti-estrogen receptor antibodies and kinase activity was determined *in vitro* using GST-MEK1 as a substrate. **B**. JNK was immunoprecipitated with anti-JNK1 antibodies, and kinase activity was determined *in vitro* using GST-cJun as a substrate. Phosphorylated proteins were visualized after SDS-PAGE and autoradiography. The membrane was subsequently immunoblotted (IB) with an anti-JNK1 antibody to determine equivalency of loading.

3.3. Discussion

Here I show that IL-4 is not a true growth factor (Figure 3.1) and present evidence that this is due to its inability to activate the Ras pathway and in particular the Raf-1 kinase. Whereas neither activated Ras (Figure 3.2B) nor Raf-1 (Figure 3.4A&B) alone can stimulate proliferation, either can synergise with IL-4 and support long-term growth (Figure 3.2B, 3.4). These data support the notion that activation of Ras and Raf are necessary, but not sufficient, for cell-cycle progression (Marshall, 1995) and are consistent with other reports that expression of activated Ras or Raf did not lead to factor independent growth of hemopoietic cells, but are able

to inhibit apoptosis induced by withdrawal of IL-3 (Cleveland et al., 1994; Okuda et al., 1994; Kinoshita et al., 1995).

The Raf/MEK/ERK pathway is a key regulator not only of proliferation, but also of differentiation (Morrison, 1990; Avruch et al., 1994). Recently, it has become clear that the intensity of the Raf signal is one determinant whether a cell will divide or arrest (Marshall, 1995; Sewing et al., 1997; Woods et al., 1997). My results in hematopoietic cells are consistent with this concept as activation of ΔRaf-1:ER is clearly necessary for cell division (Figure 3.4). Moreover when a more highly active version of ΔRaf-1:ER (ΔRaf-1:ER Y340D, Y341D, Woods et al., 1997), which led to a higher level of ERK activity, was expressed, Ba/F3 cells underwent cell cycle arrest in the presence of IL-3 or IL-4 (Levings, Bessette and Schrader, data not shown).

The role of JNK in cell-cycle progression is less clear, as it can be activated not only by growth stimuli, but also by stress (Su et al., 1994; Kyriakis and Avruch, 1996; Foltz and Schrader, 1997; Rausch and Marshall, 1997). In Ba/F3 cells, activation of JNK was certainly not sufficient for cell-cycle progression as expression of an activated ^{V12}Rac, which activates JNK in Ba/F3 cells (Foltz et al., 1998), did not synergise with IL-4 to support the long-term growth of Ba/F3 cells (Levings, Salmon, Quin, Schrader, data not shown).

It is possible that activation of both the PI3'K pathway and the JAK-STAT pathway by IL-4 contribute to the mitogenic synergy with Raf-1. STAT-6 is dispensable for the inhibition of apoptosis by IL-4 in many cell types. However, the role of STAT-6 in mitogenesis is controversial. Kaplan et al. (1998) have recently shown that STAT-6 is required for the down-regulation of the cell-cycle inhibitor, $p27^{Kip1}$. Thus, one of the normal functions of STAT-6 is to promote cell cycle progression by inhibiting expression of cell-cycle inhibitors. However, in

Chapter 4, I present data that suggest the proliferative defect in STAT-6 null cells may also be due to an indirect effect caused by dysregulated IL-4Rα expression. The PI3'kinase pathway is likely to play a key role in the IL-4-mediated inhibition of apoptosis (Figure 3.2A) since blockade of PI3'kinase inhibits the ability of IL-4 to protect cells from apoptosis (Scheid et al., 1995). Ras itself is also capable of activating the PI3' kinase pathway in some cells (Khwaja et al., 1997; Rodriguez-Viciana et al., 1997; Rodriguez-Viciana et al., 1996), however this has not been demonstrated in hematopoietic cells, and it is unclear if expression of ^{Q61K}N-Ras in Ba/F3 cells would lead to PI3'kinase activation. Thus, both the anti-apoptotic signals (via PI3' kinase) and the inhibition of $p27^{Kip1}$ (via STAT-6), are candidates for the growth promoting signals downstream of IL-4 that synergise with activated Raf-1 to promote long-term growth.

I was surprised to find that whereas ERK activity was not detectable above control levels in cells growing in IL-3 (Figure 3.6), these cells exhibited levels of JNK activity equivalent to ΔRaf-1:ER cells that had been stimulated with IL-4 plus 4HT (Figures 3.7-3.9). These data demonstrate that, at least in Ba/F3 cells growing in mitogenic amounts of IL-3, high levels of JNK activity do not inhibit cell cycle progression or induce apoptosis. The high level of JNK activity observed in parental Ba/F3 cells, however, is not a general phenomenon, as under similar conditions other IL-3 dependent cell lines, IL-3 dependent primary mast cells, or IL-2 dependent primary T cells have undetectable levels of JNK activity. One potential explanation for the discrepancy in levels of JNK activity may be that Ba/F3 cells do not express a critical phosphatase such as M3/6 that normally downregulates JNK activity (Smith et al., 1997).

The results on the activation of ERK and JNK by ΔRaf-1:ER in Ba/F3 cells are quite distinct from those seen in NIH-3T3 cells (Samuels et al., 1993; McCarthy et al., 1995). First, ΔRaf-1:ER was not immediately activated (ie within 1 minute) following addition of 4HT

(Figure 3.5A). A possible explanation for this delayed activation in Ba/F3 cells is that in these cells the ΔRaf-1:ER protein is either unstable in the absence of steroid or expressed at lower levels, and only following addition of 4HT is ΔRaf-1:ER protein expressed at levels sufficient to activate ERK. It is also possible that in Ba/F3 cells there is a requirement for synthesis of a new protein (other than ΔRaf-1:ER) for maximal Raf kinase activity. Second, in contrast to results in NIH-3T3 cells, there was no increase in JNK activity when Raf-1 activity was induced by addition of 4HT (Figure 3.9); in Ba/F3 cells induction of JNK-1 activity required both Raf-1 activity and IL-4. This difference might be accounted for by the fact that in NIH-3T3 cells Raf-1 activity resulted in the secretion of HB-EGF which in turn activated JNK through an autocrine mechanism (McCarthy et al., 1995).

The activation of JNK following stimulation of Raf-1 activity and addition of IL-4 was unexpected as neither Raf nor IL-4 alone are able to activate upstream activators of JNK (Minden et al., 1994; Foltz and Schrader, 1997; Foltz et al., 1998). There was no evidence to suggest that activation of JNK was due to stress or the production of an autocrine factor (Figures 3.7B&3.8). An autocrine factor could not be detected in media conditioned by ΔRaf-1:ER cells that had been activated with 4HT in either a biological assay or a JNK kinase assay. Furthermore, the results of cell-mixing experiments shown in Figure 3.8 argue strongly against an indirect mechanism of activation of JNK through induction of an autocrine factor or cell-surface bound molecule. There remains however, the formal possibility that the activation of JNK is due to the production of an autocrine factor that acts on a receptor that is only expressed in cells expressing activated Raf-1.

Current evidence favors the view that ERK and JNK are activated by distinct, non overlapping mechanisms. These data suggest that this may not always be the case, since, in the

cells here studied, activation of Raf-1 is clearly able to influence JNK activity (Figures 3.8-3.10). The level at which the combination of Raf-1 and IL-4 influences the JNK and/or the upstream activators of JNK, MKK4&7 and/or MEKK1, remains to be determined. Nor is it clear whether it is Raf-1 itself or a downstream kinase (i.e. MEK or ERK) which can synergise with IL-4 to activate JNK (see Figure 7.1). The observation that the synergistic effect of IL-4 and Raf-1 on JNK activity was not evident after exposure to IL-4 for 10 minutes (Figure 3.10) argues against an acute effect of IL-4 and suggests that there may be a requirement for the synthesis of a new protein such as a kinase or another regulator of kinase cascades. Finally, it is also conceivable that the observed increase in JNK activity does not result directly from the activity of Raf and IL-4, but is rather a consequence of cell-cycle progression.

IL-4-stimulated increases in the lipid products of PI3'kinase could be involved in activation of Rac through the pleckstrin homology domains in Rho family exchange factors (Han et al., 1998). These phospholipid products alone, however, are not sufficient to activate JNK, or induce Rac-mediated transcription factor pathways (Reif et al., 1996). Although Frost et al. (1997) did not find any evidence for synergy between Raf and ^{V12}Rac or ^{V12}CDC42 in the activation of JNK, it is possible that the activated mutants provided a saturating signal that masked any potential cooperative effect with Raf-1. It would be interesting to see if co-expression of an activated PI3'kinase and activated Raf would lead to activation of JNK, and what effects addition of inhibitors of the PI3'kinase pathway would have on JNK activity in this system.

In conclusion I have demonstrated that the failure of IL-4 to support long-term cellular growth can be complemented by expression of activated Raf-1. These data support the notion that Raf-1 activity is necessary but not sufficient for cell-cycle progression. My observations

that IL-4 and Raf-1 synergise to induce activation of JNK raises the possibility that JNK activity is also necessary for cell-cycle progression and that it may be partially regulated downstream of Raf-1. The activation of JNK did not appear to involve early events in IL-4 signal transduction and there was no evidence for the involvement of autocrine mechanisms. Thus, further study to determine which pathway(s) downstream of IL-4 is involved in cross-talk with the Raf kinase cascade to activate JNK could provide new insight into the mechanisms that regulate JNK activity and proliferation.

CHAPTER 4. Expression of a dominant-negative STAT-6 inhibits IL-4-mediated cell-survival

4.1. Introduction

In 1994, the diverse biological functions of IL-4 were widely recognized. However, very little was known about the intracellular signals activated by IL-4 that mediated these biological effects. At that time, there was increasing evidence that activation of the JAK/STAT pathway was not only important for signalling by interferons, but also for signalling by type I cytokines (see 1.7). The recognition of this pathway offered a new paradigm for signalling from the membrane directly to the nucleus, and also presented a possible mechanism for the achievement of cytokine-specific effects.

Previous work on STAT-1 (Muller et al., 1993) and STAT-5 (Mui et al., 1996) had shown that deletion of the C-terminal domain of these STATs resulted in the generation of a dominant-negative molecule that, when over-expressed, blocked the ability of the endogenous STAT to transactivate STAT-responsive genes. Expression of these dominant-negative mutants in cell lines resulted in biological phenotypes that suggested that some, but not all, of the relevant cytokine's functions were dependent on the STAT pathway. In September 1994, Hou et al. (1994) reported the molecular cloning of an IL-4-Stat (STAT-6). Although the C-terminal domain of STAT-6 shared little homology with other STATs, in an attempt to generate a dominant-negative molecule I took an analogous approach, and introduced a stop codon at the end of the SH2 domain of STAT-6. I over-expressed this protein in a factor-dependent hemopoietic cell-line and analysed whether activation of STAT-6 was required for IL-4-mediated cell survival.

4.2. Results

4.2.1. Generation of mammalian expression vectors for STAT6 and STAT6Δ

I obtained the human STAT-6 cDNA, and in order to distinguish it from endogenous STAT-6, I tagged the N-terminus with a sequence encoding the epitope recognized by the monoclonal antibody 9E10. This sequence was originally identified in the proto-oncogene *c-myc*, and is thus referred to as the "myc" tag (Chan et al., 1987). I also generated a truncated version of the "myc-tagged" STAT-6 cDNA which had a stop codon introduced at amino acid 663 (STAT-6Δ). By truncating the molecule at this position, the DNA-binding domain and the SH2 domain remained intact, but the critical tyrosine and the transactivation domain were removed. Following subcloning and sequencing, the cDNAs encoding full-length STAT-6 (mSTAT-6) or STAT-6Δ were placed under control of the elongation factor 1α (EF1α) promoter in pEFBOS (Figure 4.1).

4.2.2. STAT-6Δ inhibits IL-4-induced transcription from a STAT-6 reporter gene

In order to confirm that expression of STAT-6Δ inhibited IL-4-stimulated transcription from a STAT-6 reporter gene I obtained the plasmid pCεGL2. This reporter gene encodes three tandem repeats of the STAT-6 target sequence from the germline C epsilon gene, followed by a minimal SV40 promoter, and controls the expression of firefly luciferase. Ba/F3 cells were transiently transfected with 10μg of pCεGL2 together with 15μg of pEFBOS or pEFBOS STAT-6Δ. The cells were allowed to recover overnight, and stimulated for 6 hours in the presence or absence of IL-4. The cells were lysed, and following incubation with the substrate for luciferase, luciferase activity was determined by luminometry. Cells transfected with empty vector (EFBOS) exhibited a 7-8 fold increase in luciferase activity in response to IL-4 (Figure 4.2). In contrast,

IL-4-stimulated STAT-6 activity was abrogated in cells that were co-transfected with STAT-6Δ. Thus as predicted, STAT-6Δ acted as a dominant-negative.

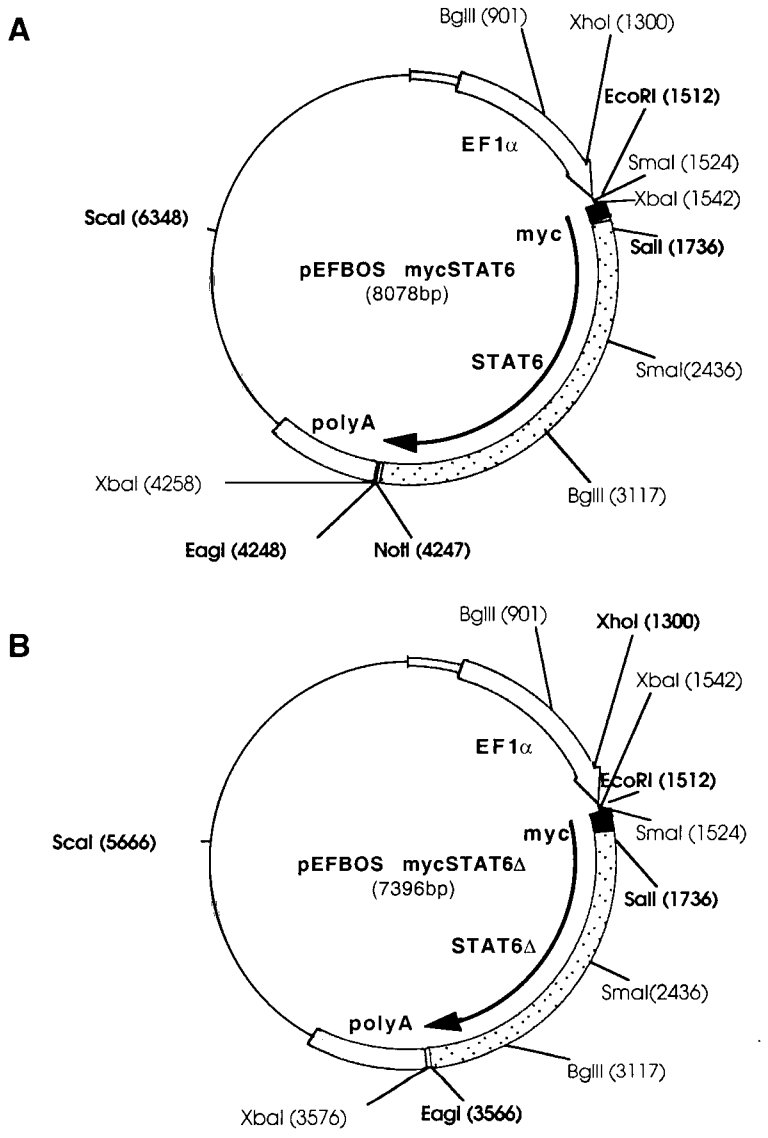

Figure 4.1. *Mammalian expression vectors encoding mSTAT-6 and STAT-6Δ.* **A**. The cDNA encoding full length STAT-6, tagged at the N-terminus with the epitope recognized by 9E10 (**myc**), was positioned downstream of the elongation factor 1α (**EF1α**) promoter using *EcoRI* and *NotI* as the cloning sites. **B**. The cDNA encoding the truncated, myc-tagged STAT-6 (STAT-6Δ) was positioned downstream of the EF1α promoter, using *EcoRI* and *EagI* as the cloning sites. The structure of the expression vectors was confirmed by digestion with *BglII* and *SmaI*. Inner arrows represent the portion of the vector which encodes protein sequence. **polyA** represents the region containing the rabbit β–globin polyadenylation sequence. Unique restriction enzyme sites are marked in bold.

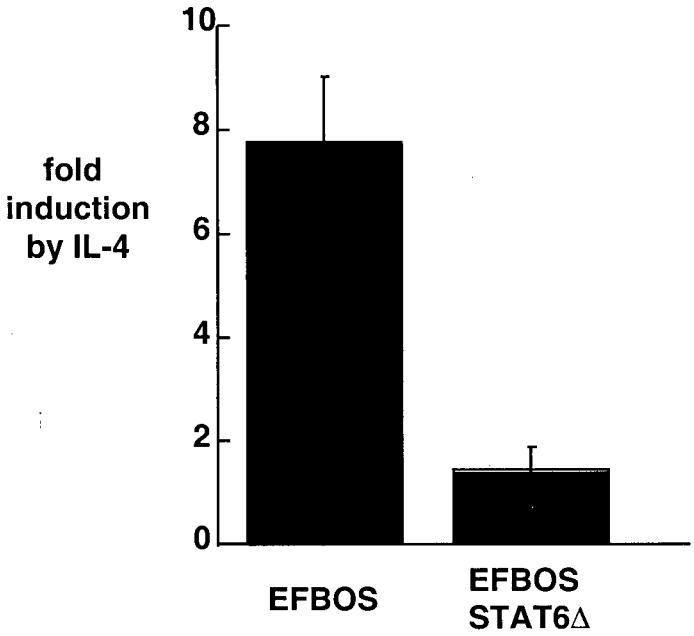

Figure 4.2. *Expression of STAT-6Δ inhibits IL-4 induced transcription of a STAT-6 reporter gene.* Ba/F3 cells were transiently transfected with pcεGL2 and pEFBOS or pEFBOS STAT-6Δ. After stimulation with or without IL-4 for six hours, the cells were lysed, and the amount of luciferase activity was determined by luminometry. The amount of luciferase activity was normalized to the total protein content in the sample, as determined by the BCA Pierce assay. Data are expressed as fold induction by IL-4 compared to the unstimulated sample from the same transfection. The error bars represent the SEM, taking into account the error of both the duplicate relative light units and the triplicate OD readings from the BCA assay.

4.2.3. Generation of stable cell-lines that express STAT-6Δ

I next determined the effect of STAT-6Δ on IL-4-induced tyrosine phosphorylation and DNA binding activity of endogenous STAT-6. Ba/F3 cells were transfected with pEFBOS mSTAT-6 or pEFBOS STAT-6Δ, and clones were selected on the basis of neomycin-resistance. Expression of the exogenous cDNA of interest was monitored by immunoblotting whole-cell lysates with the monoclonal antibody that recognizes the myc epitope, 9E10. Eight of the 10 drug-resistant clones transfected with mSTAT-6 expressed a protein at the approximate predicted molecular weight (99 kDa) which reacted with 9E10 (Figure 4.3A). All 8 drug-resistant clones transfected with STAT-6Δ expressed a protein that bound 9E10 and migrated at

the predicted molecular weight of 73kDa (Figure 4.3B). Four positive clones were saved from each transfection, and two clones were kept in culture for further biological and biochemical analyses.

mSTAT-6 and STAT-6Δ migrated at distinct molecular weights compared to endogenous STAT-6. Therefore, I was able to compare the expression levels of the exogenous cDNA and endogenous STAT-6 by immunoblotting cell-lysates with an anti-STAT-6 antibody. It is important to note that the anti-STAT-6 antibody was polyclonal, and raised against full length human STAT-6. Furthermore, the truncated STAT-6Δ is potentially missing a number of reactive epitopes. Nevertheless, in two independent clones, STAT-6Δ appeared to be expressed at lower levels than endogenous STAT-6, and mSTAT-6 appears to be expressed at significantly lower levels than endogenous STAT-6 (Figure 4.4).

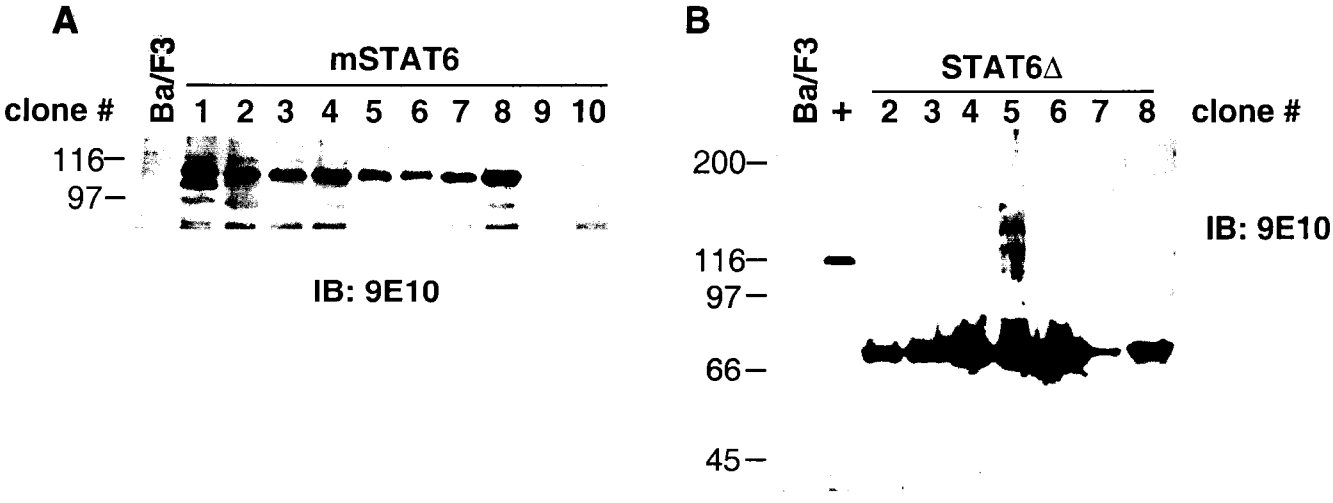

Figure 4.3. *Stable expression of mSTAT-6 and STAT-6Δ in Ba/F3 cells.* Lysates from untransfected Ba/F3 cells, positive control lysate (+) or drug-resistant Ba/F3 cells that had been transfected with **A**. pEFBOS mSTAT-6 or **B**. pEFBOS STAT-6Δ, were analysed for the presence of the exogenous protein by immunoblotting (IB) with 9E10. STAT-6Δ migrated at the predicted molecular weight of 73 kDa, and mSTAT-6 migrated slightly more slowly than the predicted molecular weight of 99 kDa.

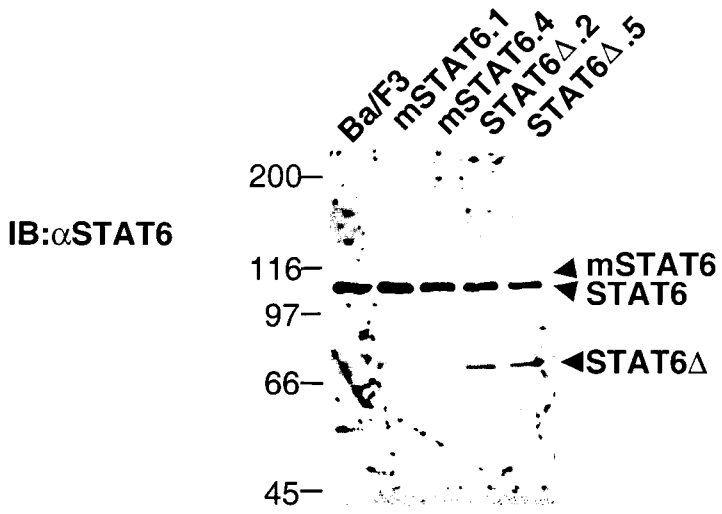

Figure 4.4. *Comparison of expression levels between endogenous STAT-6 and exogenous mSTAT-6 or STAT-6Δ.* Lysates from untransfected Ba/F3 cells, two mSTAT-6 clones (1&4) and two STAT-6Δ clones (2&5) were subjected to SDS-PAGE and immunoblotting (IB) with anti-STAT-6 antibodies to compare the levels of expression of the endogenous and exogenous proteins.

4.2.4. Biochemical Effects of STAT-6Δ

I next determined if STAT-6Δ effected IL-4-induced tyrosine phosphorylation or DNA-binding activity of endogenous STAT-6. Factor deprived Ba/F3 cells or Ba/F3 STAT-6Δ cells were stimulated with IL-4 or left unstimulated as a control. The cells were lysed, and lysates were subjected to immunoprecipitation with anti-STAT-6 antibodies. The precipitated proteins were resolved by SDS-PAGE and the membrane was immunoblotted to determine the amount of tyrosine phosphorylation (Figure 4.5). Expression of STAT-6Δ had no effect on IL-4-stimulated tyrosine phosphorylation of endogenous STAT-6.

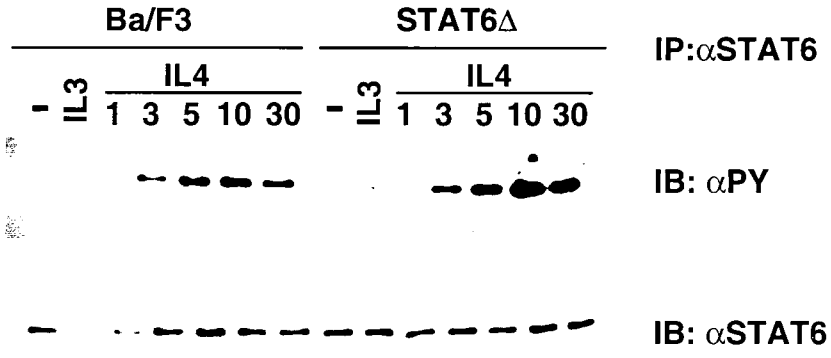

Figure 4.5. *Expression of STAT-6Δ does not affect IL-4-induced tyrosine phosphorylation of endogenous STAT-6.* Ba/F3 or STAT-6Δ cells were stimulated with IL-3 for 10 minutes (**IL3**), IL-4 for the indicated times (in minutes), or left unstimulated as a control (**-**). The lysates were subjected to immunoprecipitation (IP) with anti-STAT-6 antibodies, and the eluate was resolved by SDS-PAGE. The membrane was immunoblotted (IB) with 4G10 (α**PY**), and subsequently with anti-STAT-6 antibodies to determine equivalency of loading (α**STAT-6**).

To determine if STAT-6Δ effected the ability of endogenous STAT-6 to bind to DNA, I performed oligo precipitations in wild-type and STAT-6Δ cells. Ba/F3 or STAT-6Δ cells were stimulated with or without IL-4 and lysed. Cell lysates were incubated with a slurry of a double-stranded oligos (N4) which contained an optimal STAT-6 binding sequence, bound to streptavidin-conjugated sepharose. The bead eluates were subjected to SDS-PAGE, and immunoblotting with anti-STAT-6 antibodies (Figure 4.6A). Expression of STAT-6Δ did not affect IL-4-stimulated STAT-6 DNA-binding activity. The membrane was subsequently immunoblotted with 9E10 to determine if STAT-6Δ was also able to bind to the oligo. Indeed, as predicted, STAT-6Δ was also precipitated by the N4 oligo. Surprisingly, the ability of STAT-6Δ to bind target DNA sequences appeared to be independent of stimulation with IL-4 (Figure 4.6B).

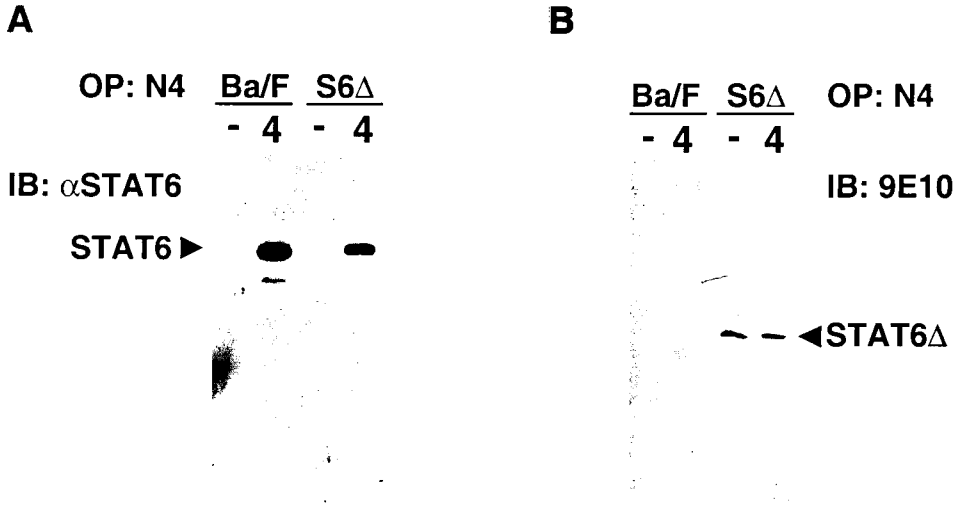

Figure 4.6. *Expression of STAT-6Δ does not effect IL-4-induced DNA binding of endogenous STAT-6.* Lysates from Ba/F3 or STAT-6Δ (**S6Δ**) cells that had been stimulated with IL-4 (**4**), or left unstimulated as a control (**-**) were subjected to oligo-precipitation (OP) by incubation with a slurry of N4 oligo bound to sepharose. The eluates were subjected to SDS-PAGE, and membranes were immunoblotted (IB) with **A**. anti-STAT-6 antibodies (αSTAT-6) or **B**. 9E10.

4.2.5. Biological effects of expression of STAT-6Δ

I examined the effects of STAT-6Δ on IL-4-stimulated cell-survival. I compared Ba/F3 mSTAT-6 and STAT-6Δ cells for the ability of IL-4 to prolong survival in the absence of IL-3. Expression of mSTAT-6 did not effect IL-4-stimulated cell viability (Figure 4.7). Strikingly, in two independent clones, there was a marked reduction in the ability of IL-4 to maintain the viability of Ba/F3 cells expressing STAT-6Δ.

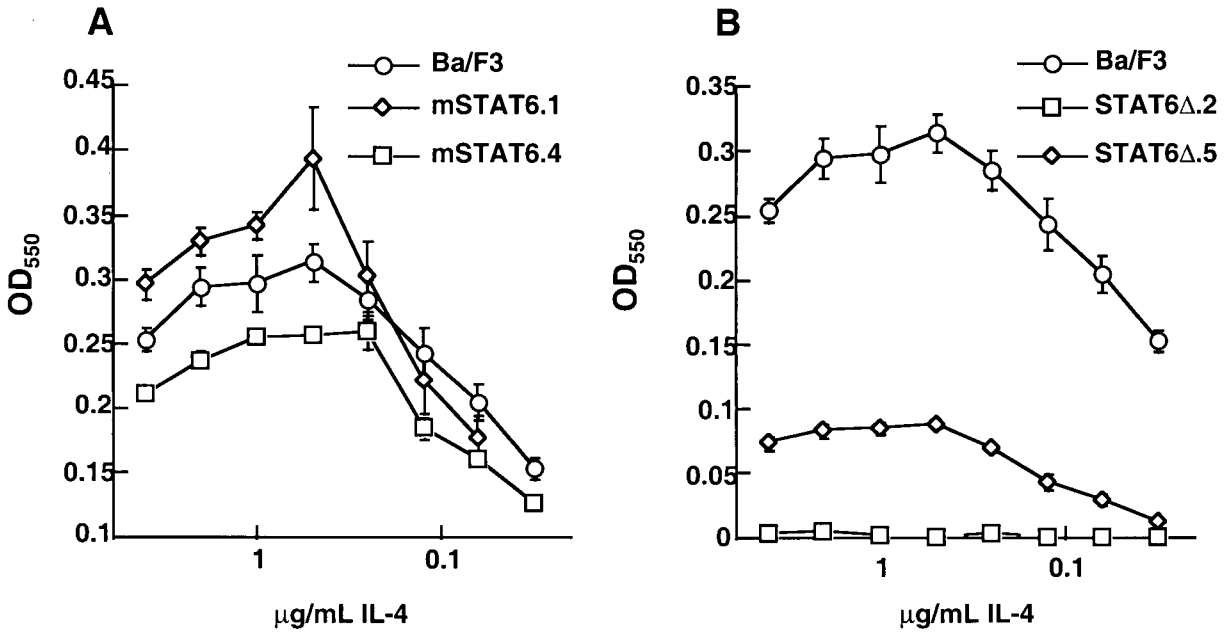

Figure 4.7. *Expression of STAT-6Δ inhibits the ability of IL-4 to maintain cell viability.* Ba/F3 cells and **A.** two independent clones expressing mSTAT-6 (1&4) or **B.** two independent clones expressing STAT-6Δ (2&5) were washed out of IL-3 and incubated with decreasing concentrations of IL-4. The ability of IL-4 to maintain cell-viability over a 48 hour period was determined by the amount of MTT reduction. Error bars represent the SEM of triplicate samples.

Ba/F3 cells respond to IL-4 transiently, and IL-4 does not stimulate cell-cycle progression in these cells (see Chapter 3, Figure 3.1). I therefore also examined the biological effects of STAT-6Δ in a mutant cell-line that proliferates in response to IL-4. FD5 cells are a subclone of FD-MAC II cells (a murine pre-myeloid cell line) which were selected for their ability to proliferate in response to IL-4 (Welham et al., 1994). I transfected FD5 cells with pEFBOS mSTAT-6 or pEFBOS STAT-6Δ, and expression of the exogenous cDNA was monitored by immunoblotting whole cell-lysates with 9E10 (Figure 4.8). Two independent clones from each transfection were analysed for their proliferative response to IL-4. Since IL-4 stimulated division of FD5 cells, [^{3}H]-thymidine uptake was used as a measure of cell-cycle

progression. Expression of STAT6Δ, but not mSTAT-6, inhibited the proliferative response to IL-4 (Figure 4.9).

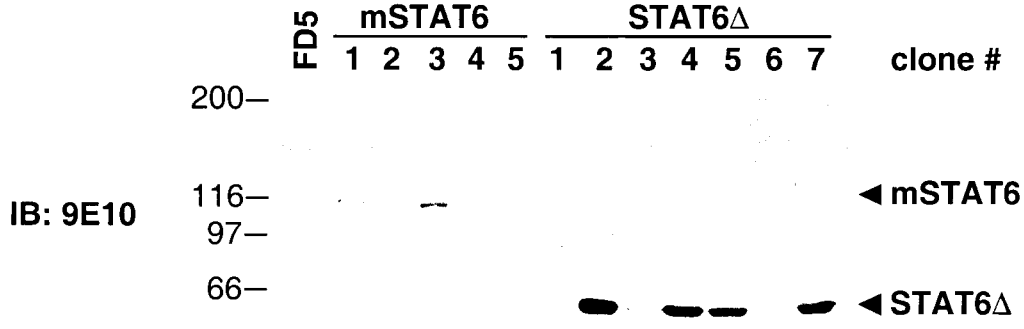

Figure 4.8. *Expression of mSTAT-6 and STAT-6Δ in FD5 cells.* Whole-cell lysates from untransfected FD5 cells (FD5) or drug-resistant clones that had been transfected with mSTAT-6 or STAT-6Δ were subjected to SDS-PAGE and analysed for expression of the exogenous cDNA by immunoblotting (IB) with 9E10.

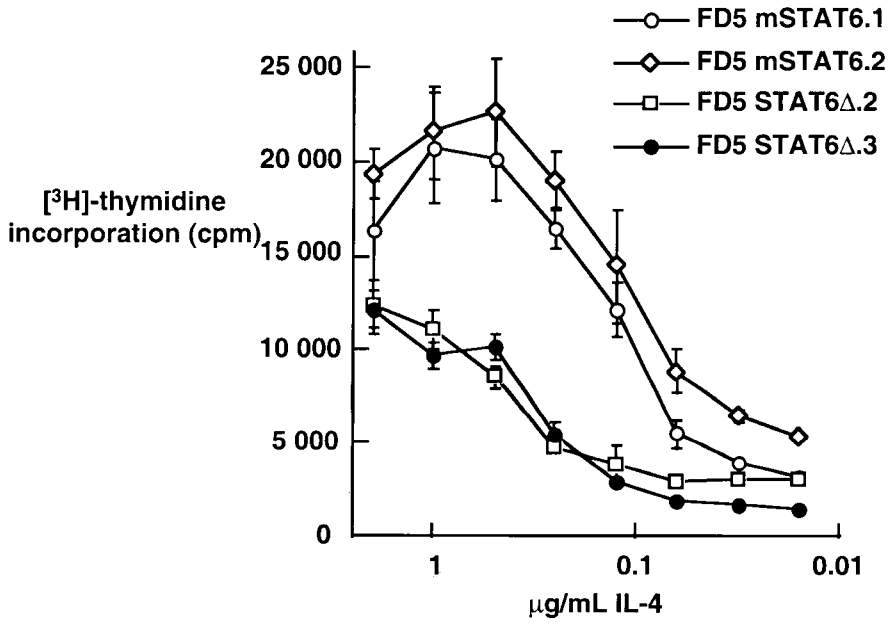

Figure 4.9. *Expression of STAT-6Δ in FD5 cells inhibits IL-4-stimulated proliferation.* Two independent clones of FD5 cells expressing mSTAT-6 (1&2) or STAT-6Δ (2&3) were washed out of IL-3, and incubated in decreasing concentrations of IL-4 for 48 hours in Terasaki microtitre plates. The cells were pulsed with [^{3}H]-thymidine for an additional 8 hours, harvested, and the amount of thymidine incorporation was determined by counting samples in a scintillation counter. Error bars represent the SEM of triplicate samples.

4.2.6. Basal levels of expression of IL-4Rα are not altered in STAT-6Δ cells

Stimulation with IL-4 is known to increase expression of the IL-4Rα (Paul, 1991), and there is a STAT-6 binding site in the promoter for the IL-4Rα gene (Kotanides and Reich, 1996). Thus, I investigated if the proliferative defect in Ba/F3 cells expressing STAT-6Δ could be due to dysregulated expression of IL-4Rα. I first compared the basal level of expression of IL-4Rα in Ba/F3 and STAT-6Δ cells. Flow-cytometric analyses demonstrated that the two cell lines expressed similar levels of IL-4Rα (Figure 4.10). I was unable to examine differences in the IL-4-stimulated increased in IL-4Rα expression as the receptor was internalized and could no longer be detected by flow cytometry (data not shown, Galizzi et al., 1989).

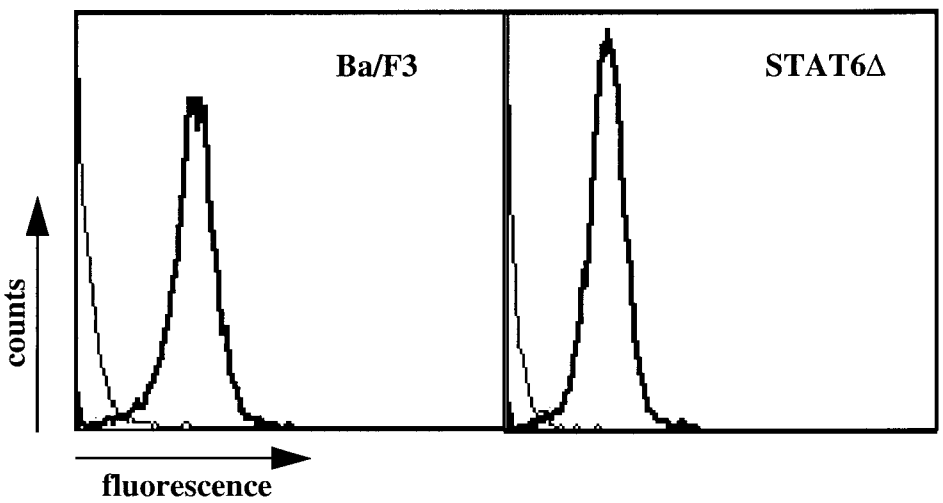

Figure 4.10. *Ba/F3 and STAT-6Δ cells express similar levels of IL-4Rα.* Ba/F3 or STAT-6Δ cells were incubated with secondary antibody (goat anti-rat Ig coupled to FITC) alone (thin line) or with an anti-IL-4Rα antibody, followed by addition of secondary antibody (thick line). Amount of FITC staining was determined by flow-cytometry.

4.2.7. Generation of BaF/3 cells which over-express the human IL-4Rα

I reasoned that if there was a defect in IL-4Rα expression in STAT-6Δ cells, it should be complemented by exogenous expression of IL-4Rα. Murine IL-4 is only able to bind to the murine IL4Rα in combination with murine γ_c. In contrast, human IL-4 can bind to the human IL-4Rα, in combination with either murine or the human γ_c. I therefore transfected Ba/F3 cells with an expression vector encoding human IL-4Rα to generate cells which expressed IL-4Rα at a constitutively high level that would not be affected by addition of IL-4. Positive clones were identified by flow-cytometry, and results from a representative clone are shown in Figure 4.11.

I confirmed that human IL-4 could activate the endogenous STAT-6 pathway in hIL-4Rα cells. hIL-4R cells were stimulated with murine IL-4, human IL-4 or left unstimulated as a control. As shown in Figure 4.12, human IL-4 (signalling through the exogenous hIL-4α) was stimulated tyrosine phosphorylation of endogenous STAT-6. Parental Ba/F3 cells were unresponsive to treatment with human IL-4 (data not shown).

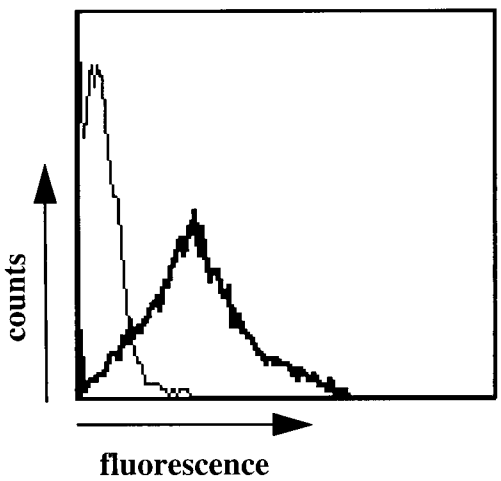

Figure 4.11. *Expression of hIL-4Rα in Ba/F3 cells.* A representative drug-resistant clone that had been transfected with the hIL-4Rα was incubated with secondary antibody (goat anti-mouse Ig coupled to FITC) alone (thin line) or with an anti-human IL-4Rα antibody followed by addition of secondary antibody (thick line). Amount of FITC staining was determined by flow-cytometry.

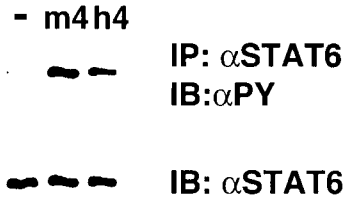

Figure 4.12. *Murine or human IL-4 stimulates tyrosine phosphorylation of STAT-6 in hIL-4R cells.* Cells were washed out of IL-3, and stimulated at 37°C for 10 minutes with murine IL-4 (**m4**), human IL-4 (**h4**), or left unstimulated as a control (-). The lysates were subjected to immunoprecipitation (IP) with anti-STAT-6 antibodies, and the elutes were resolved by SDS-PAGE. The membrane was immunoblotted (IB) with 4G10 (αPY), and subsequently immunoblotted with anti-STAT-6 (αSTAT-6) antibodies to asses equivalency of loading.

4.2.8. Expression of hIL-4Rα complements the IL-4-mediated survival defect in STAT-6Δ cells.

hIL-4R cells were transfected with pEFBOS STAT-6Δ and expression was monitored by immunoblotting whole-cell lysates with 9E10 (Figure 4.13). All hIL-4Rα STAT-6Δ clones expressed similar levels of endogenous murine IL-4Rα. (Figure 4.14). I compared the expression levels of STAT-6Δ between Ba/F3 STAT-6Δ and hIL-4Rα STAT-6Δ cells. Roughly equal amounts of STAT-6Δ was expressed in all cells examined (Figure 4.15).

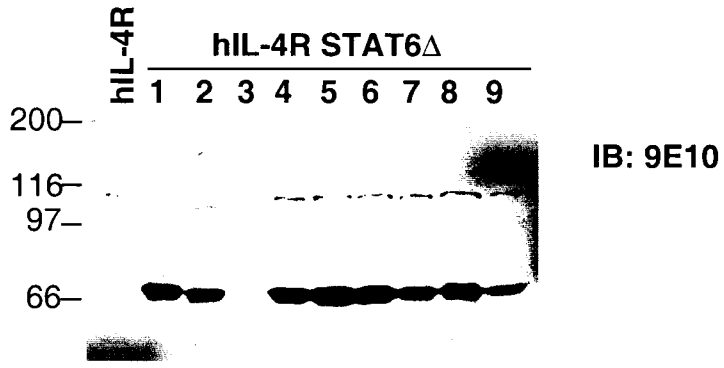

Figure 4.13. *Expression of STAT-6Δ in hIL-4R cells.* Whole cell-lysates from hIL-4R cells or hIL-4R cells that had been transfected with STAT-6Δ were separated by SDS-PAGE and analyzed for expression of the exogenous cDNA by immunoblotting (IB) with 9E10.

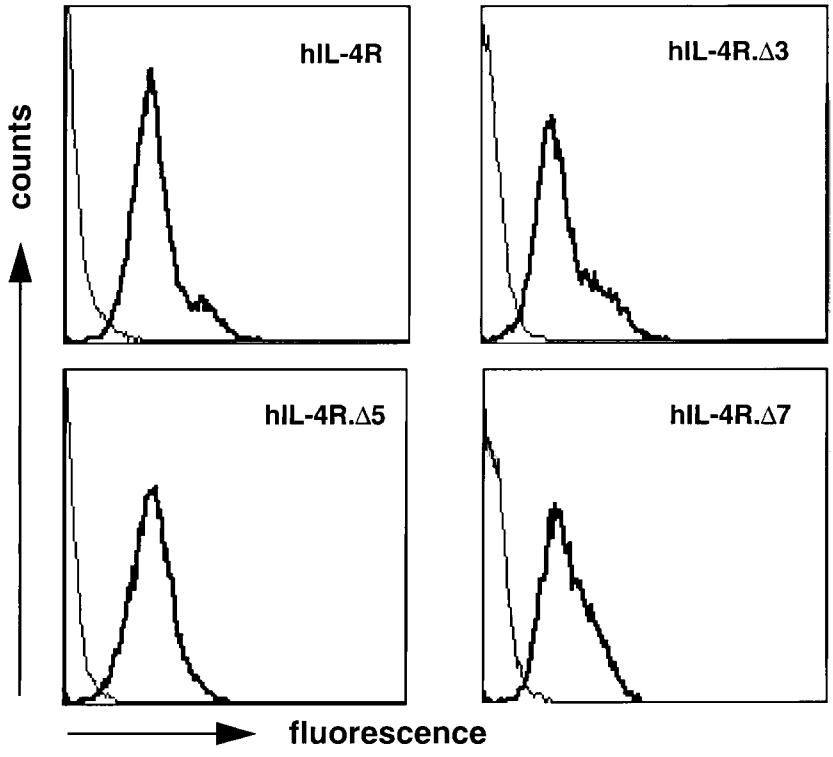

Figure 4.14. *hIL-4R and hIL-4R STAT-6Δ cells express similar levels of the murine IL-4Rα.* hIL-4R or three individual clones (3,5 &7) expressing STAT-6Δ were incubated with secondary antibody (goat anti-rat Ig coupled to FITC) alone (thin line) or an anti-murine IL-4Rα antibody followed by addition of secondary antibody (thick line). Amount of FITC staining was determined by flow-cytometry.

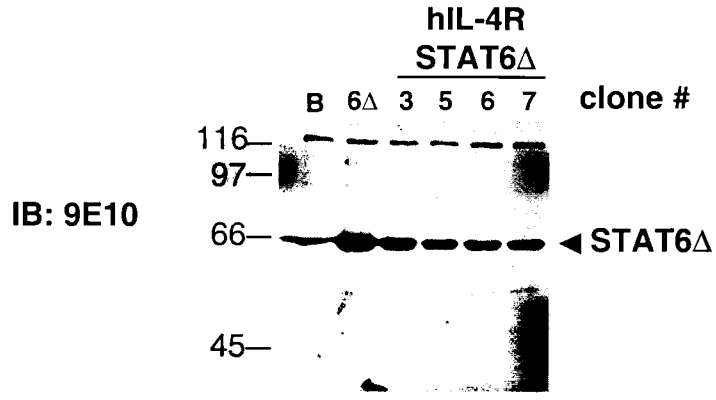

Figure 4.15. *STAT-6Δ and hIL-4R STAT-6Δ cells express similar levels of dominant-negative STAT-6.* Whole-cell lysates from Ba/F3 cells (**B**), STAT-6Δ cell (**6Δ**) or 4 individual clones of hIL-4R STAT-6Δ cells were resolved on by SDS-PAGE, and the membrane was immunoblotted (IB) with 9E10 to compare levels of expression of mSTAT-6 and STAT-6Δ. The smudge in lane 1 was due to spill-over from lane 2.

I compared murine and human IL-4-stimulated survival. Similar to my observations with Ba/F3 STAT-6Δ cells (Figure 4.7), the murine IL-4-stimulated survival in hIL-4Rα STAT-6Δ cells was reduced compared to hIL-4Rα cells (Figure 4.16A). In striking contrast, however, human IL-4 stimulated prolonged survival even in cells which expressed STAT-6Δ (Figure 4.16B). These data strongly suggest that a major component of the proliferative defect in STAT-6Δ cells is due to insufficient IL-4-stimulated expression of the endogenous IL-4Rα.

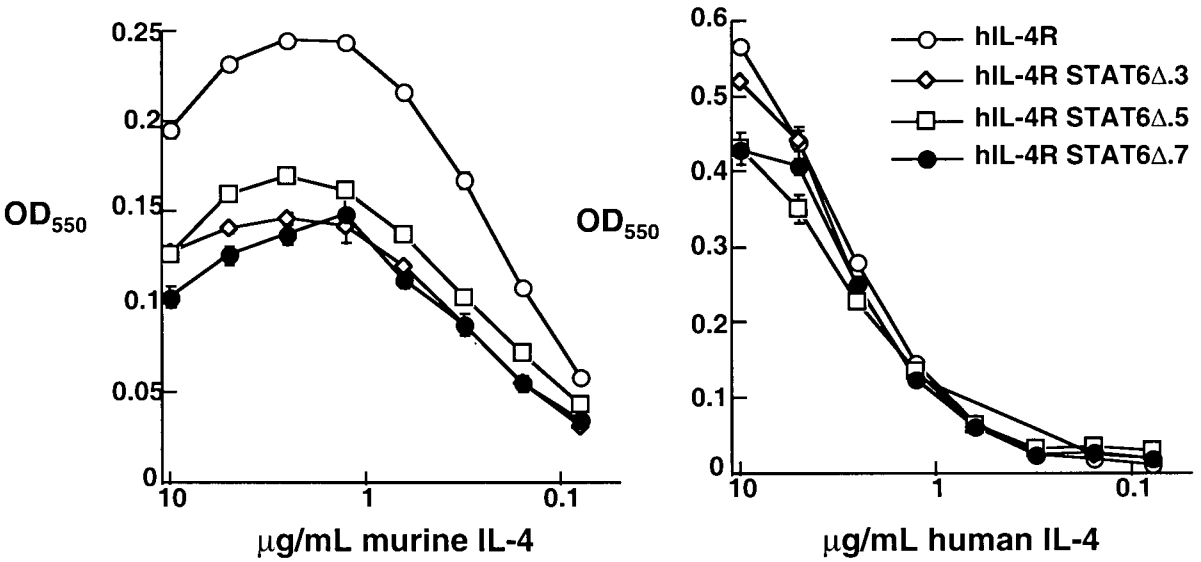

Figure 4.16. *Constitutive expression of hIL-4Rα complements the ability of STAT-6Δ cells to respond to IL-4.* Parental hIL-4R cells or three individual clones (3,5 & 7) expressing STAT-6Δ were washed out of IL-3 and incubated in increasing concentrations of **A**. murine or **B**. human IL-4. 48 hours later, the cells were pulsed with MTT and cell viability was assessed by amount MTT reduction. Error bars represent the SEM of triplicate samples.

4.3. Discussion

I have shown that deletion of the C-terminal transactivation domain of STAT-6 results in a dominant-negative protein. When this protein is exogenously expressed it inhibits IL-4-stimulated transcription from a STAT-6-dependent promoter (Figure 4.2). Whereas expression of full-length STAT-6 in Ba/F3 cells had no effect on the ability of IL-4 to maintain survival over a 48 hour period, expression of STAT-6Δ in Ba/F3 led to an 80-100% reduction in survival (Figure 4.7). Similarly, expression of STAT-6Δ, but not full-length STAT-6, in FD5 cells resulted in a reduction in IL-4-stimulated thymidine uptake (Figure 4.9). These results were surprising as there was good evidence that IRS-1 or –2, and not STAT-6, mediated IL-4-stimulated inhibition of apoptosis (Keegan et al., 1994; Zamorano and Keegan, 1998). Data that

suggested STAT-6 was required for regulation of the IL-4Rα (Kotanides and Reich, 1996) prompted me to examine if the survival-defect was related to a lack of sufficient IL-4Rα expression. Indeed, constitutive expression of the human IL-4Rα chain complemented the ability of human IL-4, but not murine IL-4, to support viability in Ba/F3 cells expressing STAT-6Δ (Figure 4.16)

The observation that deletion of the C-terminal domain of STATs results in a dominant-negative protein (Figure 4.2) is consistent with many other reports (Moriggl et al., 1996; Mui et al., 1996; Nakajima et al., 1996; Lu et al., 1997; Sasse et al., 1997). Recently, it has been formally shown that the C-terminal region of STAT-6 encodes a transcriptional activation domain that is capable of autonomous transactivation when fused to the heterologous DNA-binding domain of GAL4 (Moriggl et al., 1997). This GAL4-STAT-6 C-terminal fusion protein was a much more potent transactivator in lymphoid cells than in fibroblasts, suggesting that maximal STAT-6 activity may require additional cell-type specific co-factors such as C/EBPβ (Mikita et al., 1998).

The proposed mechanism of action for these truncated STATs is based on competition at the level of DNA binding. Expression of C-terminally truncated STATs does not interfere with cytokine-stimulated tyrosine phosphorylation of endogenous STATs (Figure 4.5; Mui et al., 1996). Truncated STATs are capable of binding to DNA (Figure 4.6; Mikita et al., 1998) and likely act as a decoys for full-length STATs. Thus truncated STATs could either form sterile homodimers and prevent wild-type dimers from binding to DNA, or form heterodimers with wild-type STATs which cannot efficiently transactivate promoters. Less efficient dominant-negative STATs can also be generated by mutation of the DNA-binding domain. These mutant

proteins can compete with wild-type molecules for binding to activated receptors and/or form sterile heterodimers (Walter et al., 1997).

The IL-4-mediated survival defects in Ba/F3 and FD5 cells expressing STAT-6Δ (Figure 4.7 and 4.9) are consistent with observations in STAT-6 null splenocytes and thymocytes which show a reduced ability to incorporate thymidine in response to IL-4 (Kaplan et al., 1996; Takeda et al., 1996). Kaplan et al. (1996) also reported a defect in IL-4-induced expression of IL-4Rα in STAT-6 null cells. The observation that constitutive expression of human IL-4Rα can rescue the survival-defect in STAT-6Δ cells (Figure 4.16) suggests that the proliferative defect in STAT-6 null cells may also be due to insufficient numbers of IL-4 receptors. A similar indirect proliferative effect was seen in STAT-5a null cells. Splenocytes derived from STAT-5a null mice displayed a markedly decreased proliferation in response to IL-2 which could be explained by a defect in IL-2-induced expression of the IL-2Rα (Nakajima et al., 1997).

However, Kaplan et al. (1998) argue that the proliferative defect in STAT-6 null lymphocytes is due to dysregulation of the cell-cycle inhibitor $p27^{Kip1}$. Treatment of wild-type, but not STAT6 null, lymphocytes with IL-4 led to a decreased expression of $p27^{Kip1}$, and a release of inhibition of cyclin dependent kinase-1 (cdk-1). Although Kaplan et al. (1998) showed that expression of IL-4Rα was equivalent in wild-type and STAT-6 null cells at the beginning of their assay for [^{3}H]-thymidine uptake, they did not rule out that expression of IL-4Rα had changed during the 48 hours the cells were cultured in IL-4.

Although human IL-4 appeared to signal normally for activation of endogenous STAT-6 (Figure 4.12), it is possible that the intracellular signals activated by the human IL-4Rα in these cells were qualitatively or quantitatively different from those activated by the endogenous

murine IL-4Rα. Differential signalling by human IL-4 could potentially overcome not only a defect in IL-4-mediated expression of IL-4Rα, but also in regulation of $p27^{Kip1}$.

In conclusion, I have shown that deletion of the C-terminal transactivation domain of STAT-6 results in a dominant-negative protein. The observation that expression of STAT-6Δ in Ba/F3 or FD5 cells led to a defect in IL-4-induced survival and proliferation, respectively, suggests that STAT-6 mediates these effects. However, the requirement for STAT-6 in cell survival appeared to be indirect, and secondary to a defect in STAT-6-dependent expression of IL-4Rα.

CHAPTER 5. IL-4 inhibits the production of TNFα and IL-12 by STAT-6 dependent and independent mechanisms.

5.1. Introduction

Key qualitative characteristics of immune responses are regulated by T cells subsets through their production of distinctive cytokines (O'Garra, 1998). Th-1 cells produce IFNγ and promote cell-mediated responses against bacteria by enhancing the ability of macrophages to kill intracellular pathogens and to release cytokines such as TNFα and IL-12. Th-2 cells produce a trio of cytokines, IL-4, IL-10 and IL-13, which antagonize these effects on macrophages and promote the production of IgE, and mast cells and eosinophils that protect against parasites. Moreover, the suppressive effects of IL-4 on the production of IL-12 by accessory cells appears to be a major mechanism that inhibits the generation of Th-1 cells (Macatonia et al., 1995). A better understanding of the molecular mechanisms through which cytokines such as IL-4 inhibit the production of TNFα and IL-12, may lead to new approaches to treat diseases like rheumatoid arthritis where the inflammatory responses may depend on TNFα and IL-12 (Brennan and Feldmann, 1996).

The anti-inflammatory properties of IL-4 appear to be mediated at multiple levels. IL-4 can directly suppress the production of pro-inflammatory cytokines at the levels of transcription (Hart et al., 1989) or message stability (Mijatovic et al., 1997; Wang et al., 1995), as well as antagonize the proinflammatory effects of IFNγ on superoxide production by macrophages (Abramson and Gallin, 1990), expression of cell surface antigens (te Velde et al., 1990) and cytokine production (Gautam et al., 1992; Skeen et al., 1996). However, the intracellular signals through which IL-4 exerts these direct and indirect effects are largely unknown.

Given its association with IL-4 effects, the STAT-6 pathway is a good candidate for the molecular mechanism that mediates the inhibitory effects of IL-4 on macrophages. Through the generation and analyses of mice that lack functional STAT-6 genes, it has been shown that STAT-6 plays an essential role in the production of Th-2 cells, the switching of B-cells to the production of IgE, the induction of antigen-dependent airway hyper-responsiveness, and in IL-4-mediated up-regulation of cell-surface molecules such as MHC class II and CD23 (Kaplan et al., 1996; Shimoda et al., 1996; Takeda et al., 1996; Akimoto et al., 1998; Kuperman et al., 1998). It is not clear, however, whether the suppressive effects of IL-4 on the production of TNFα and IL-12 by macrophages are also dependent on STAT-6.

TNFα is produced by macrophages when they are activated by stimuli such as LPS or parasites (Vassalli, 1992), and this production is enhanced by IFNγ (Hayes et al., 1995). The regulation of TNFα production is complex. It can occur at the level of transcription (Collart et al., 1990; Read et al., 1994) and message stability (Hayes et al., 1995; Mijatovic et al., 1997), and is suppressed by IL-4, IL-10 or IL-13 (Essner et al., 1989; Hart et al., 1989; Hart et al., 1991; de Waal Malefyt et al., 1993). Experiments using mice with genetic defects in TNFα production or response or with neutralizing mAbs have established that TNFα is a critical component in the host response to certain bacterial infections (Rothe et al., 1993) and of the pathogenesis diseases caused by uncontrolled inflammation (Brennan and Feldmann, 1996).

IL-12 likewise has protective and potentially pathogenic functions. Analyses of genetically manipulated mice unable to produce IL-12, or respond to IL-12, have established a critical role for IL-12 in the differentiation of Th-1 and NK cells (Kaplan et al., 1996; Magram et al., 1996), and protection from pathogens such as *Listeria* (Unanue, 1997). Biologically active IL-12 is a 70 kDa heterodimer composed of a 40 kDa (p40) and a 35 kDa (p35) chain,

and is produced by macrophages, dendritic cells and neutrophils upon stimulation with microbial products (Trinchieri, 1995). The p35 subunit is constitutively produced in most cell-types examined, whereas the p40 subunit is regulated at the level of transcription (D'Andrea et al., 1992). As is the case for TNFα, the production of IL-12 is greatly enhanced by IFNγ (Hayes et al., 1995), and suppressed by IL-4, IL-10 or IL-13 (Aste-Amezaga et al., 1998; D'Andrea et al., 1995). To better understand the mechanistic basis of the anti-inflammatory effects of IL-4, I investigated whether activation of STAT-6 was required for the IL-4-mediated inhibition of the release of TNFα and IL-12 from macrophages stimulated with LPS or LPS in presence of IFNγ.

5.2. Results

5.2.1. STAT-6 is required for IL-4-mediated inhibition of LPS-stimulated TNFα production

I compared the ability of IL-4 to inhibit the production of TNFα from macrophages derived from the bone-marrow of wild-type Balb/c or STAT-6 null mice (Kaplan et al., 1996). When bone-marrow cells were cultured for 5 days in medium supplemented with colony stimulating factor-1 (CSF-1), wells became confluent with adherent, morphologically differentiated macrophages. Wells were washed free of non-adherent cells and the remaining adherent macrophages were stimulated by addition of bacterial products (LPS), with or without IL-4. Supernatants were collected 24 hours later and analysed by capture ELISA for the presence of TNFα. Consistent with previous reports (Essner et al., 1989; Hart et al., 1989; te Velde et al., 1990; Hart et al., 1991), the presence of IL-4 resulted in a 50-65% reduction in the amount of TNFα produced by wild-type macrophages (Figure 5.1A). In contrast, the amount of TNFα produced by STAT-6 null macrophages was not reduced in the presence of IL-4.

IL-4 however, had relatively little effect on the production of TNFα by macrophages harvested from the peritoneal cavity after stimulation with thioglycollate (Figure 5.1B). In three independent experiments, the IL-4-mediated reduction in the amount of TNFα produced by wild-type cells ranged from 10-20%. In parallel cultures of STAT-6 null peritoneal macrophages stimulated with LPS, IL-4 exhibited no inhibitory effect (Figure 5.1B).

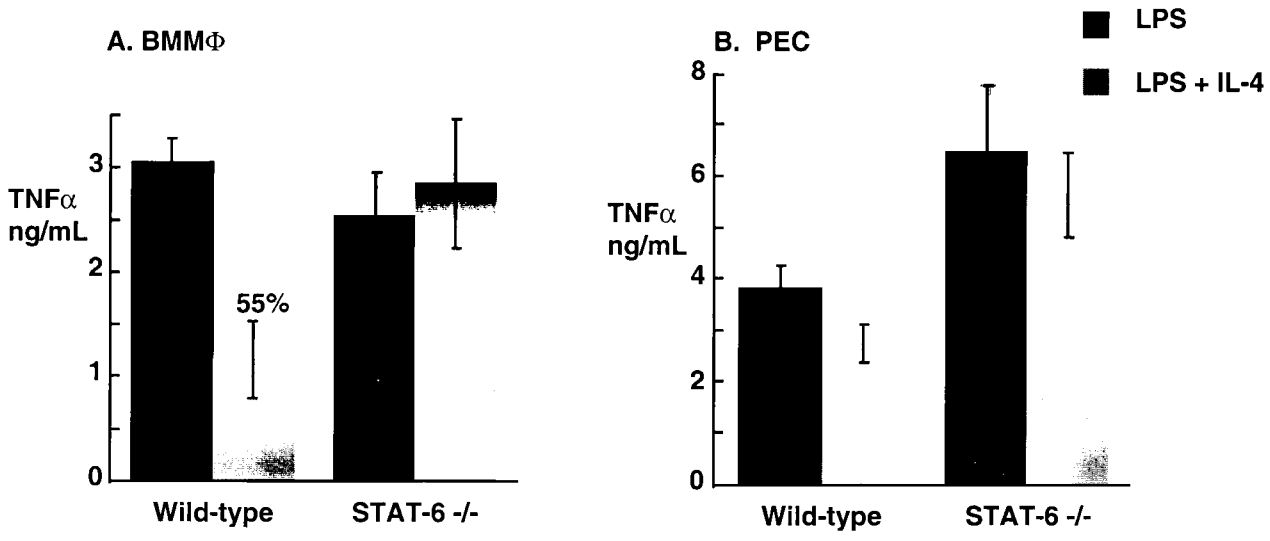

Figure 5.1. *STAT-6 is required for IL-4-mediated inhibition of TNFα production stimulated by LPS in macrophages.* Wild-type or STAT-6 null macrophages were **A.** generated *in vitro* by culture of bone-marrow in the presence of CSF-1 (**BMMΦ**) or **B.** were harvested from the peritoneal cavities after stimulation with thioglycollate (**PEC**). Macrophages were cultured in triplicate with LPS (15μg/mL), in the presence or absence of IL-4. After 24 hours, supernatants were harvested and assayed for TNFα by ELISA. TNFα was undetectable in supernatants of cultures containing medium alone or IL-4 alone (data not shown). Figures over bars indicate the % reduction in cytokine levels compares with culture in LPS alone. Error bars represent the SEM of triplicate samples.

5.2.2. A STAT-6 independent component of IL-4-mediated inhibition of TNFα production in the presence of IFNγ

IFNγ enhances the ability of macrophages to produce pro-inflammatory cytokines such as TNFα and IL-12 in response to LPS (Hayes et al., 1995a; Hayes et al., 1995b). Moreover, *in vivo*, bacterial infections are normally associated with the production of IFNγ from sources such as NK cells (Trinchieri, 1997) and except at the earliest times, macrophages will normally encounter LPS in the presence of IFNγ. Therefore, I examined the ability of IL-4 to inhibit the LPS-induced production of TNFα by macrophages in the presence of IFNγ. Addition of IFNγ resulted in a modest (2-5 fold) increase in the amount of TNFα produced in response to LPS in both wild-type and STAT-6 null macrophages (Figure 5.2). The presence of IL-4 resulted in a 72% reduction in the amount of TNFα released by wild-type bone-marrow derived macrophages in response to LPS (Figure 5.2A). However, in contrast to results seen with STAT-6 null bone-marrow macrophages stimulated with LPS alone (Figure 5.1A), in the presence of IFNγ the STAT-6 null macrophages were no longer refractory to the inhibitory effects of IL-4, which induced a significant reduction (43%) in TNFα production. Addition of IL-10, another potent inhibitor of the production of pro-inflammatory cytokines including TNFα (Wang et al., 1994), completely suppressed the production of TNFα in both wild-type and STAT-6 null cells (Figure 5.2A). Although in the experiment presented in Figure 5.2A, STAT-6 null macrophages produced slightly less TNFα than did their wild-type counterparts, in five independent experiments there were no consistent differences between cells derived from wild-type or STAT-6 null mice in the absolute amounts of TNFα produced by, with or without IFNγ.

These experiments were repeated with peritoneal macrophages, and as was the case with cells stimulated in the absence of IFNγ, IL-4 had little or no effect on TNFα production. Once

again IL-4 induced only a marginal (10-20%) reduction in TNFα production by wild-type and STAT-6 null peritoneal macrophages (Figure 5.2B). Addition of IL-10, however, resulted in the complete inhibition of the production of TNFα from both wild-type and STAT-6 null peritoneal-exudate cells (Figure 5.2B).

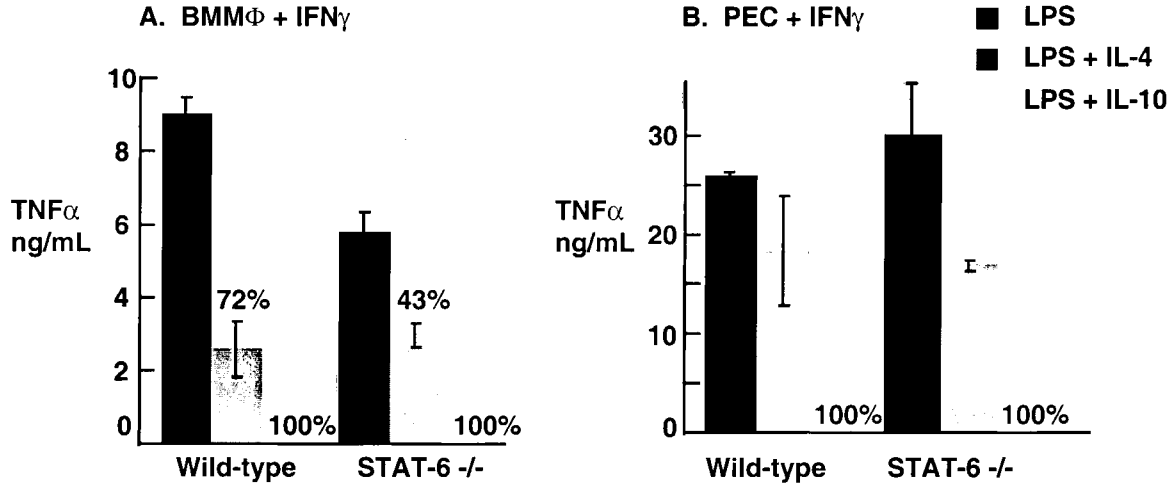

Figure 5.2. *STAT-6 independent IL-4-mediated inhibition of TNFα produced in response to stimulation with LPS and IFNγ.* **A.** BMMΦs or **B.** PECs were cultured as before and stimulated in triplicate with LPS (15μg/mL) and IFNγ (100U/mL) in the presence or absence of IL-4 for 24 hours. An additional group was cultured with LPS and IFNγ in the presence of IL-10 (10ng/mL). Supernatants were harvested and analyzed for TNFα by ELISA. TNFα was undetectable in supernatants of unstimulated cells or cells stimulated with IL-4 or IFNγ alone. Error bars represent the SEM of triplicate samples.

5.2.3. STAT-6 is required for IL-4-mediated inhibition of LPS-stimulated IL-12 production

I next evaluated the involvement of STAT-6 in IL-4-mediated suppression of IL-12 production. Wild-type or STAT-6 null bone-marrow derived macrophages were stimulated with LPS in the presence or absence of IL-4 for 24 hours and determined the amount of IL-12 (p40) in the supernatant by capture ELISA. In wild-type cells, IL-4 induced a marked (89%) inhibition of the LPS-stimulated production of IL-12 (Figure 5.3A). In keeping with my observations that IL-4 failed to inhibit the production of TNFα by bone-marrow derived macrophages from STAT-6 null mice (Figure 5.1A), IL-4 also failed to inhibit the production of IL-12 by bone-marrow derived macrophages from STAT-6 null mice (Figure 5.3A). In contrast to the marginal effects of IL-4 on the production of TNFα by wild-type peritoneal macrophages (Figure 5.1B), IL-4 induced a striking inhibition (94%) of LPS-stimulated production of IL-12 p40. This inhibitory effect of IL-4 on the production of IL-12 by peritoneal macrophages was abrogated in macrophages lacking STAT-6 (Figure 5.3B).

4.2.4. A STAT-6 independent component of IL-4-mediated inhibition of the enhanced production of IL-12 in the presence of IFNγ

I next examined the effects of IL-4 on IL-12 production in macrophages that had been stimulated with LPS in the presence of IFNγ. The presence of IFNγ in cultures of bone-marrow derived macrophages resulted in a much greater (5-10 fold) increase in the amount of IL-12 p40 secreted than it did in the amount of TNFα produced (2-5 fold). There was no consistent difference in the absolute amount of IL-12 produced by wild-type or STAT-6 null macrophages that had been stimulated with or without IFNγ. In wild-type bone-marrow derived macrophages,

IL-4 induced a marked reduction (80%) in the amount of IL-12 p40 released in response to LPS and IFNγ (Figure 5.4A). Moreover, the increased production of IL-12 p40 by STAT-6 null bone-marrow derived macrophages stimulated in the presence of IFNγ was significantly inhibited (53%) by IL-4 (Figure 5.4A). Virtually all of the increase in IL-12 p40 production stimulated by IFNγ was eliminated in the presence of IL-4.

Very similar results were obtained with macrophages from peritoneal exudates. IL-4 markedly inhibited the production of IL-12 p40 in response by both wild-type cells (78%), and STAT-6 null cells (56%) (Figure 5.4B). Addition of IL-10, which has previously been shown to inhibit the production of IL-12 (Aste-Amezaga et al., 1998), completely suppressed the release of IL-12 p40 by both cell types.

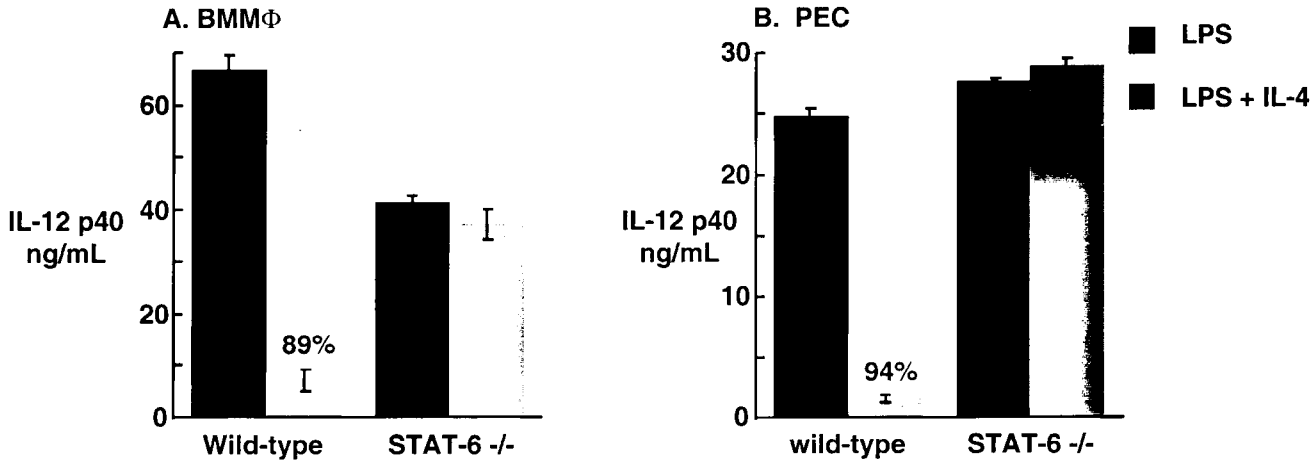

Figure 5.3. *STAT-6 is required for IL-4-mediated inhibition of IL-12 production stimulated by LPS in macrophages.* **A.** BMMΦs or **B.** PECs were cultured as before and stimulated in triplicate with LPS (15μg/mL) in the presence or absence of IL-4 for 24 hours. Supernatants were harvested and analyzed for the p40 subunit of IL-12 by ELISA. IL-12 p40 was undetectable in the supernatants of unstimulated cells, or cells stimulated with IL-4 alone. Error bars represent the SEM of triplicate samples.

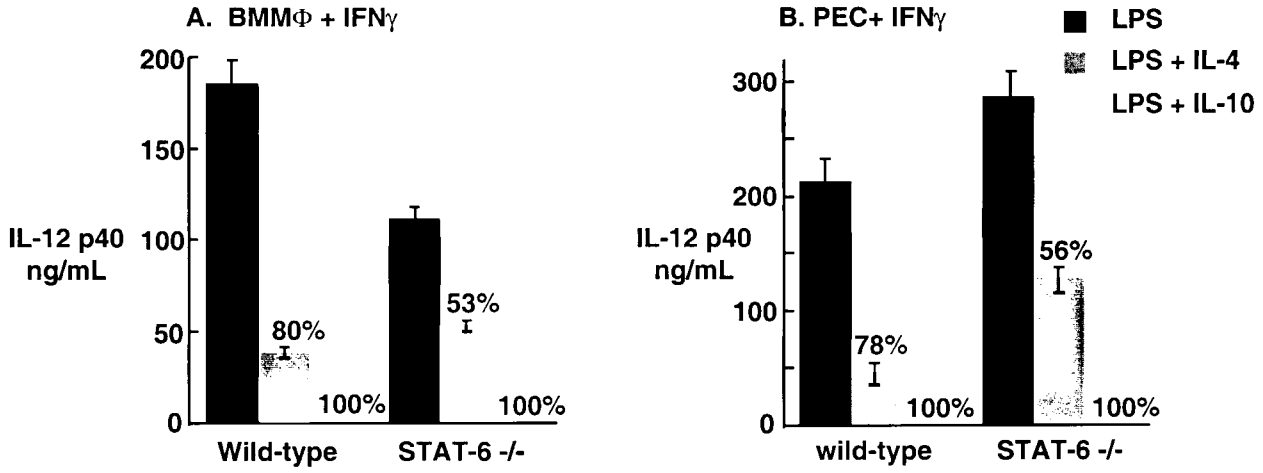

Figure 5.4. *STAT-6 independent IL-4-mediated inhibition of IL-12 produced in response to stimulation with LPS and IFNγ.* **A.** BMMΦs or **B.** PECs were cultured as before and stimulated in triplicate with LPS (15μg/mL) and IFNγ (100U/mL) in the presence or absence of IL-4 for 24 hours. An additional group was cultured with LPS and IFNγ in the presence of IL-10 (10ng/mL). Supernatants were harvested and analyzed for the p40 subunit of IL-12 by ELISA. IL-12 p40 was undetectable in unstimulated cells and cells stimulated with IL-4 or IFNγ alone. Error bars represent the SEM of triplicate samples.

5.2.5. IL-4-induced signalling in STAT-6 null cells

In that the promoter for the IL-4Rα gene contains a STAT-6-consensus binding site (Kotanides and Reich, 1996), I wanted to determine whether the defect in IL-4-mediated inhibition of cytokine production in STAT-6 null cells simply reflected a lack of expression of IL-4Rα. I therefore examined the ability of IL-4 to induce tyrosine phosphorylation of IRS-2 in bone-marrow derived macrophages. Cells were starved of CSF-1 and left unstimulated as a control, or stimulated with insulin, IL-4 or IL-13, three factors which have previously shown stimulate phosphorylation of IRS-2 in this cell-type (Welham et al., 1997). IL-4 and IL-13 both signal through the IL-4Rα, whereas insulin signalling should be independent of any effects on expression of the IL-4Rα that result from the lack of STAT-6. As shown in Figure 5.5, wild-

type and STAT-6 null bone-marrow derived macrophages responded equivalently to insulin, IL-4 or IL-13 in terms of stimulation of phosphorylation of IRS-2. These results are consistent with those of Kaplan et al. (1998) in lymphocytes. Thus, the defect in IL-4 signalling in STAT-6 null macrophages does not reflect a deficiency in the basal levels of IL-4Rα on STAT-6 null macrophages.

5.2.6. Pre-treatment with IL-4 does not effect the ability of IFNγ to stimulate tyrosine phosphorylation of STAT-1

One of the potential mechanisms by which IL-4 could antagonize the production of pro-inflammatory cytokines by macrophages stimulated with LPS and IFNγ, is by affecting the activation of STAT-1 by IFNγ. Ohmori and Hamilton (1997) observed that pre-treatment of a murine macrophage cell-line with IL-4 did not inhibit the ability of IFNγ to stimulate phosphorylation of STAT-1; nor did IFNγ affect IL-4-stimulated phosphorylation of STAT-6. However, Dickensheets and Donnelly (1997) observed a marked reduction in the IL-4-induced tyrosine phosphorylation, nuclear translocation and DNA-binding activity of STAT-6 in monocytes that had been pre-treated with IFNγ. I wished to evaluate the effects of pre-treatment with IL-4 or IFNγ on phosphorylation of STAT-1 or STAT-6 respectively in this model system, bone-marrow derived macrophages. Pretreatment for 1 hour with IFNγ failed to effect the IL-4-stimulated tyrosine phosphorylation of STAT-6 (Figure 5.6). Similarly, pre-treatment with IL-4 for one hour did not effect the ability of IFNγ to stimulate tyrosine phosphorylation of STAT1.

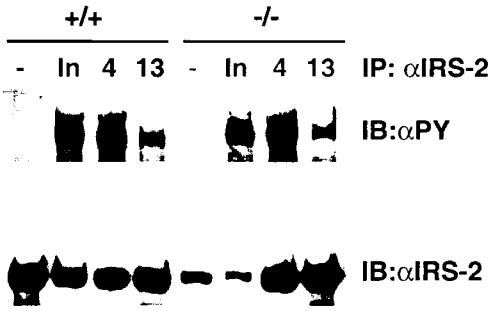

Figure 5.5. *STAT-6 null macrophages exhibit normal tyrosine phosphorylation of IRS-2 in response to IL-4.* Wild-type or STAT-6 null bone-marrow derived macrophages were stimulated for 10 minutes with insulin (**In**), IL-4 (**4**), IL-13 (**13**) or with medium alone as a control (**-**). Lysates were immunoprecipitated (IP) with anti-IRS-2 antibodies, and eluate from immune-complexed beads was subjected to SDS-PAGE. The membrane was immunoblotted (IB) with 4G10 (α**PY**) to determine the level of tyrosine phosphorylation, and subsequently with anti-IRS-2 antibodies to determine equivalency of loading (α**IRS-2**).

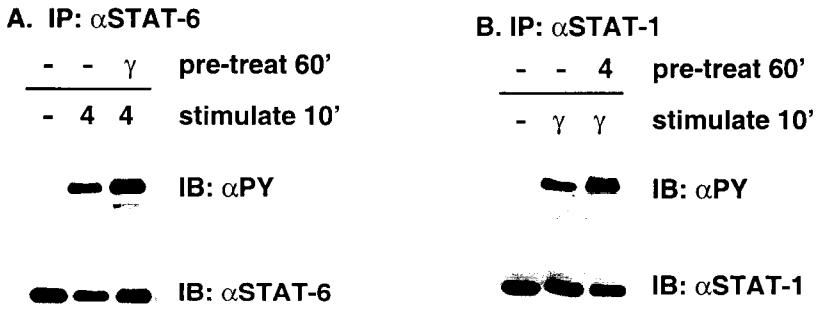

Figure 5.6. *Pre-treatment with IL-4 does not effect IFNγ-stimulated STAT1 phosphorylation.* Bone marrow derived macrophages were starved of CSF-1, and **A**. stimulated with IL-4 (4) for 10 minutes, or pretreated with IFNγ (γ) for 60 minutes prior to stimulation with IL-4 for 10 minutes or left unstimulated as a control (**-**). Lysates were subjected to immunoprecipitation with anti-STAT-6 antibodies. **B**. Cells were stimulated with IFNγ for 10 minutes or pretreated with IL-4 for 60 minutes prior to stimulation with IFNγ for 10 minutes or left unstimulated as a control. Lysates were subjected to immunoprecipitation (IP) with anti-STAT1 antibodies. Eluate from immune-complexed beads were subjected to SDS-PAGE and membranes were immunoblotted with 4G10 (αPY) to assess amount of tyrosine phosphorylation and subsequently with anti-STAT-6 or anti-STAT1 antibodies to assess equivalency of loading.

5.2.7. IL-4 induces tyrosine phosphorylation of STAT-3

Recent work in the laboratory has shown that after stimulation with IL-4, not only STAT-6, but also STAT-3 is tyrosine phosphorylated (Orchansky & Schrader, manuscript in preparation). Based on these observations, I was interested to know if IL-4 could also stimulate phosphorylation of STAT-3 in macrophages. Factor deprived bone-marrow derived macrophages from wild-type and STAT-6 null mice were stimulated with media alone as a control, IL-4 or IL-10, which has previously been shown to activate STAT-3 (O'Farrell *et al.*, 1998). Although IL-4 stimulated an increase in tyrosine phosphorylation of STAT-3, it was modest compared to that induced by IL-10 in wild-type cells (Figure 5.7). Furthermore, the shift in mobility that is associated with serine phosphorylation of STAT-3 (Chung *et al.*, 1997) was only observed in samples that had been stimulated with IL-10.

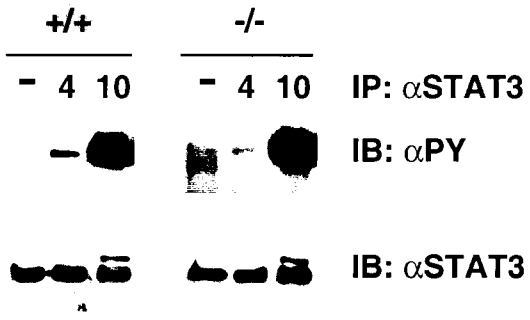

Figure 5.7. *IL-4 stimulates tyrosine phosphorylation of STAT-3.* Wild-type or STAT-6 null bone-marrow derived macrophages were stimulated for 10 minutes with IL-4 (**4**), IL-10 (**10**) or with medium alone as a control (**-**). Lysates were immunoprecipitated (IP) with anti-STAT-3 antibodies, and eluate from immune-complexed beads was subjected to SDS-PAGE. The membrane was immunoblotted (IB) with 4G10 (αPY) to determine the level of tyrosine phosphorylation, and subsequently with anti-STAT-3 antibodies to determine equivalency of loading (αSTAT-3).

5.3. Discussion

I have shown that there are STAT-6 dependent and independent mechanisms for IL-4-mediated inhibition of the production of TNFα and IL-12 by murine macrophages. When bone-marrow derived macrophages were stimulated with LPS alone, there was an absolute requirement for the presence of STAT-6 for IL-4 to inhibit the production of either TNFα or IL-12 (Figures 5.1A & 5.3A). In contrast, when IFNγ was present, STAT-6 was not required for IL-4 to significantly inhibit the production of TNFα or IL-12 (Figure 5.2A & 5.4A). On average, the IL-4-mediated inhibition of the production of TNFα or IL-12 in bone-marrow macrophages stimulated in the presence of IFNγ was slightly less (43-53%) in STAT-6 null cells than in wild-type cells (72-80%), indicating that a minor part of the inhibitory effect of IL-4 depended on STAT-6 even in IFNγ treated cells.

Macrophages from peritoneal cavity exudates similarly exhibited STAT-6 dependent and independent mechanisms of IL-4-mediated inhibition of IL-12 production. In the absence of IFNγ, IL-4 almost completely suppressed the production of IL-12 by wild-type cells (94%), but had no effect on the production of IL-12 by STAT-6 null peritoneal cells (Figure 5.3B). The presence of IFNγ resulted in a large increase in the LPS-stimulated production of IL-12 (10X), that was suppressed by IL-4 in both wild-type (78%) and STAT-6 null cells (56%) (Figure 5.4B).

In contrast to the results in bone-marrow derived macrophages, in peritoneal macrophages the effects of IL-4 on TNFα production did not parallel those on production of IL-12. Thus, IL-4 had little or no inhibitory effect on the amount of TNFα produced by wild-type or STAT-6 null cells in response to LPS whether or not IFNγ was present (Figures 5.1B &

5.2B), despite the significant inhibitory effects on IL-4 the production of IL-12 in the same cultures (Figures 5.3B & 5.4B). This failure of IL-4 to significantly inhibit TNFα production in thioglycollate-elicited peritoneal macrophages is consistent with the observations of Oswald *et al.* (1992), and suggests that different mechanisms regulate TNFα production in bone-marrow derived and peritoneal macrophages. It is possible that peritoneal cells do not express sufficient levels of γ_c, which has recently been shown to be required for IL-4-mediated inhibition of TNFα production in human monocytes (Bonder et al., 1998). Collectively, these data indicate that IL-4 inhibits the production of IL-12 and TNFα through distinct mechanisms.

In the absence of IFNγ, IL-4-mediated inhibition of TNFα and IL-12 was absolutely dependent on STAT-6 (Figures 5.1 & 5.2). STAT-6 could potentially influence the production of pro-inflammatory cytokines at several levels. One possibility is that STAT-6 could directly antagonize the action of transcription factors necessary for production the of pro-inflammatory cytokines. For example, NF-κB is essential for upregulation of both TNFα and IL-12 p40 mRNA (Ma et al., 1996; Murphy et al., 1995) and IL-4 can inhibit NF-κB DNA-binding activity (Donnelly et al., 1993). Recently, Bennett et al. (1997) have shown that STAT-6 can compete with NF-κB for binding to the E-Selectin promoter, raising the possibility that one mechanism of IL-4 mediated inhibition of TNFα or IL-12 p40 transcription may be at the level of competition between STAT-6 and NF-κB. However, this is certainly not a general mechanism as others saw cooperation between STAT-6 and NF-κB for induction of IgE transcription (Messner et al., 1997; Shen and Stavnezer, 1998).

There is also evidence that STAT-6 can indirectly inhibit the production of pro-inflammatory cytokines. For example, STAT-6 is necessary for IL-4-induced expression of the IL-1 Receptor antagonist (Ohmori et al., 1996). The synthesis of other proteins that regulate

cytokine levels could also be STAT-6 dependent. There is evidence that IL-4-mediated suppression of TNFα mRNA levels requires synthesis of new proteins (Gautam et al., 1992; Rhoades et al., 1995; Standiford et al., 1992), and IL-4 can also induce translational repression of TNFα, mediated by the 3' AU-rich sequence in the transcript (Mijatovic et al., 1997), conceivably through the activity of a newly synthesized STAT-6-dependent protein. Likewise, the IL-4-induced down-regulation of the receptor for LPS (CD14) mRNA expression (Lauener et al., 1990), may also depend on STAT-6.

Although the IL-4Rα chain is constitutively expressed on most hemopoietic cells, expression is increased following stimulation by IL-4 (Paul, 1991). There is a STAT-6 binding site in the promoter for IL-4Rα (Kotanides and Reich, 1996), but the constitutive level of IL-4Rα expression is not dependent on expression of STAT-6 (Kaplan et al., 1996) and there was no defect in the acute biochemical responses to IL-4 in STAT-6 null cells, as judged by phosphorylation of IRS-2 (Figure 5.5) or the induction of *c-myc* (Kaplan et al., 1998). However observations in STAT-6 null mice (Kaplan et al., 1996), and my data from cells expressing a dominant-negative STAT-6 (Chapter 4), suggest that the increase in IL-4Rα expression that occurs following stimulation with IL-4 is dependent on STAT-6. In it conceivable that the IL-4-mediated inhibition of production of TNFα and IL-12 by LPS-stimulated macrophages requires a STAT-6 dependent up-regulation of IL-4Rα expression in order to generate the necessary strength or duration of this signal, and that the defective inhibition by IL-4 observed in STAT-6 null macrophages was due to the absence of a STAT-6 dependent up-regulation of IL-4Rα.

IL-4 induces an increased production of IL-10 (Kambayashi et al., 1996), and it is possible that at least part of the anti-inflammatory effects of IL-4 are secondary to the production of IL-10. However, in this system, IL-4 induced only a modest enhancement of IL-

10 production in STAT-6 null macrophages (data not shown). Thus, differences between wild-type and STAT-6 macrophages in IL-4-mediated suppression of TNFα and IL-12 were not correlated with the effects of IL-4 on IL-10 production.

The presence of IFNγ resulted in a large increase in the LPS-stimulated production of IL-12 (5-10X) but only a modest increase (2-5X) in the production of TNFα in the same cultures. Seventy-100% of this IFNγ-induced increase in the LPS-stimulated production of TNFα or IL-12 was inhibited by IL-4 via a mechanism that did not require STAT-6 (Figures 5.2A & 5.4). *In vivo*, bacterial products directly induce the production of IL-18 and IL-12 resulting in the early production of IFNγ from NK cells (Takeda et al., 1998; Trinchieri, 1997). Thus, the STAT-6 independent mechanism of IL-4-mediated inhibition of TNFα and IL-12 production when IFNγ is added to cultures is likely to be physiologically significant. The data suggest that the inhibitory effects of IL-4 on the production of pro-inflammatory cytokines during infections, where IFNγ is present, will be largely independent of STAT-6.

The mechanism of the STAT-6 independent suppression of IFNγ-enhanced production of TNFα and IL-12 is unclear. I observed that in both wild-type or STAT-6-null bone-marrow derived macrophages, stimulation with IL-4 lead to a modest increase in tyrosine phosphorylation of STAT-3 (Figure 5.7). In that STAT-3 requires both serine and tyrosine phosphorylation for optimal activity (Wen et al., 1995), it is unclear whether stimulation with IL-4 would also lead to an increase in STAT-3-dependent transcription. O'Farrell et al. (1998) have shown that activation of STAT-3 by IL-10 is dispensable for IL-10 mediated inhibition of TNFα production in a murine macrophage cell line. However, it is conceivable that the STAT-6-independent inhibition of the production of pro-inflammatory cytokines by IL-4 is mediated by STAT-3.

IL-4 also activates the PI3'kinase pathway (Gold et al., 1994). In that most cytokines, including IFNγ (Argetsinger et al., 1996), and LPS (Herrera-Velit and Reiner, 1996) stimulate the PI3'kinase pathway, activation of this pathway is unlikely to inhibit the production of pro-inflammatory cytokines. It therefore seems likely that IL-4 activates other as yet uncharacterised pathways that inhibition the enhanced production of TNFα and IL-12 seen in the presence of IFNγ.

Although the greater part of IL-4-mediated inhibition of the production of TNFα and IL-12 that was stimulated by LPS and IFNγ was not dependent on STAT6, there also appeared to be a STAT-6 dependent component. The IL-4-mediated inhibition of IFNγ-enhanced production of TNFα or IL-12 was approximately 20% lower in STAT-6 null cells than in wild-type cells (compare 72% vs 43%, Figure 5.2A; 80% vs 53% Figure 5.4A; and 78% vs 56% Figure 5.4B). IL-4 opposes the activities of IFNγ in many systems. Many of the effects of IFNγ are mediated through activation of STAT-1 (Durbin et al., 1996; Meraz et al., 1996). Acute treatment with IL-4 did not inhibit the tyrosine phosphorylation of STAT-1 induced by IFNγ in bone-marrow derived macrophages (Figure 5.6). However, through a STAT-6-dependent mechanism, IL-4 might influence the production of a member of the PIAS family (Liu et al., 1998), or the SOCS family (Nicholson and Hilton, 1998) that could inhibit activation of STAT-1. It is also possible that STAT-6 may compete with STAT-1 for binding to STAT-1 sites (Ohmori and Hamilton, 1997) as STAT-6 can bind to the TTC(N_3)GAA sequence recognized by STAT-1 (Seidel et al., 1995).

In conclusion, IL-4 can inhibit the production of TNFα and IL-12 through STAT-6 dependent and independent mechanisms. The STAT-6 independent mechanisms only operated on the enhanced production of TNFα and IL-12 induced by IFNγ. LPS stimulates the

production of IFNγ, and thus this STAT-6 independent pathway is likely to be important for the inhibitory effects of IL-4 on the production of pro-inflammatory cytokines *in vivo*. Characterisation of this new pathway will be an important step towards understanding the mechanisms through which IL-4 suppresses the production of cytokines that promote the generation of Th-1 cells and inflammation.

CHAPTER 6. IFNγ regulates the expression of a STAT-6 antagonist

6.1. Introduction

IFNγ antagonizes many of the actions of IL-4. In B-cells, IFNγ inhibits the IL-4-stimulated synthesis of IgG_1 and IgE transcripts (Coffman and Carty, 1986; Snapper, 1987; Berton et al., 1989; Pene et al., 1988), and the expression of the low affinity receptor for IgE, CD23 (Galizzi et al., 1988; te Velde et al., 1990). By enhancing the production of IL-12, IFNγ indirectly inhibits the generation of Th-2 cells (Ma et al., 1996). IFNγ also antagonizes IL-4-induced expression of molecules such as CD30 on the surface of T cells (Nakamura et al., 1997) and the receptor for SDF-1 (CXCR4) on cultured Langerhans cells (Zoeteweij et al., 1998). Collectively, these inhibitory effects tend to antagonize humoral immune responses that are necessary for the defense against parasites, but that can also lead to autoimmune diseases.

Despite the fact that IFNγ and IL-4 have opposing effects, they activate similar intracellular signalling pathways. Like IL-4, IFNγ mediates the majority of its biological effects through the activation of the JAK/STAT pathway. Analyses of genetically altered mice which lack STAT-1, has shown that activation of STAT-1 is required for many, if not all, IFNγ-mediated effects (Durbin et al., 1996; Meraz et al., 1996).

Given the central role for STAT-6 in IL-4-mediated effects (with the notable exception of inhibition of cytokine production, Chapter 5), inhibition of STAT-6 is a good candidate mechanism for IFNγ-mediated inhibition of IL-4. I have shown that IFNγ does not inhibit the ability of IL-4 to stimulate the tyrosine phosphorylation of STAT-6 (Figure 5.6). Furthermore, STAT-1 dimers are unable to bind to the $TTCN_4GAA$ consensus sequence for STAT-6 (Seidel et al., 1995), ruling out competition between STAT-1 and STAT-6 for binding to DNA as a potential inhibitory mechanism. There is, however, evidence that IFNγ-mediated inhibition of

IL-4-induced CD23 expression requires new protein synthesis, suggesting that IFNγ may induce the expression of an antagonist(s) of IL-4 signalling (Lee et al., 1993). I reasoned that a potential mechanism by which IFNγcould antagonize IL-4 is by increasing the expression of a newly characterised inhibitor of STAT-6, Bcl-6.

B-cell lymphoma-6 (Bcl-6) was recognized in 1993 as a gene that was translocated in 30-40% of non-Hodgkin's lymphomas (Ye et al., 1993). These translocations, which usually occur in the first intron, result in fusion of the gene to heterologous promoters (often to the immunoglobin promoter) and dysregulated expression (Baron et al., 1993; Kerckaert et al., 1993). In fact, mutations of the Bcl-6 promoter region are the most frequent genetic alterations in human B-cell malignancies (Niu et al., 1998). Bcl-6 is a 95 kDa transcription factor that contains 6 C-terminal Krüppel-type zinc-fingers which mediate DNA-binding, and a BTB/POZ (bric-à-brac tramtrack broad complex / pox viruses and zinc fingers) domain at the N-terminus. The POZ domain is likely to mediate protein-protein interactions with molecules such as the transcriptional co-repressor SMRT (silencing mediator or identified retinoid and thyroid receptor), which may be involved in recruiting histone deacetylases to the protein/DNA complex (Dhordain et al., 1995; Dhordain et al., 1997). The POZ domain also contains an autonomous transcriptional repressing activity (Chang et al., 1996; Seyfert et al., 1996), and thus unlike most transcriptions factors, Bcl-6 binds to promoters and inhibits transcription. Recently, a Bcl-6 homolog, BAZF, has been identified, and preliminary observations suggest that this protein may share overlapping functions with Bcl-6 (Okabe et al., 1998).

Data on the expression pattern of Bcl-6 suggested that it is most highly expressed in germinal centre B-cells and $CD4^+$ T-cells (Cattoretti et al., 1995). However, its biological function was essentially unknown until three independent groups reported the phenotype of

mice deficient in Bcl-6 (Dent et al., 1997; Fukuda et al., 1997; Ye et al., 1997). These mice were unable to form germinal centres or to mount a humoral immune-response. Accordingly, the mice had a tendency to succumb to bacterial infections. They also dramatically overproduced Th-2 cells, and consequently also IL-4, IL-5 and IL-10. Elevated levels of IL-5 and IgE led to infiltrates of eosinophils in multiple organs.

Although the preferred DNA-binding site for Bcl-6 had been previously characterised, it was only recently recognised that it was in fact the same sequence recognized by STAT-6. Moreover, over-expression of Bcl-6 can inhibit IL-4-stimulated gene expression (Dent et al., 1997). These data suggested that regulation of Bcl-6 expression could be a key mechanism for regulating STAT-6 activity, and that the overproduction of Th-2 cells in Bcl-6 deficient mice could be due to unchecked IL-4 production and/or signalling. I noted that there was a consensus binding site for STAT-1 present in the sequence of the Bcl-6 promoter (Ohashi et al., 1995), and I was interested to know whether IFNγ could modulate the expression of Bcl-6, and thereby inhibit STAT-6 and antagonize the actions of IL-4.

6.2. Results

6.2.1. IFNγ and IL-4 activate similar intracellular signalling pathways.

It has been reported that STAT-1 must be phosphorylated not only on tyrosine, but also on serine for maximal transactivation activity (Wen et al., 1995) and IFNγ-mediated anti-viral effects (Horvath and Darnell, 1996). ERK may be the relevant kinase that serine phosphorylates STAT-1 in response to IFNα/β (David et al., 1995). However, the ability of IFNγ stimulate an increase in ERK activity is controversial (Zhu et al., 1997; Sakatsume et al., 1998; Stancato et al., 1998). Most reports that have investigated the ability of IFNγ to stimulate ERK activity

have used adherent cells lines, such as NIH-3T3 cells, which are not likely to be the most biologically relevant targets for IFNγ. I therefore examined whether IFNγ could stimulate an increase in ERK activity in bone-marrow derived macrophages, a biologically important target for IFNγ-mediated promotion of pro-inflammatory cytokine production (Chapter 5).

Factor-deprived macrophages were left untreated as a control or treated with IFNγ, or CSF-1. A portion of the whole-cell lysate was immunoblotted with antibodies specific for the tyrosine phosphorylated and kinase active forms of ERK-1 and –2. The remaining cell lysate was subjected to immunoprecipitation with anti-STAT-1 antibodies. Stimulation with CSF-1 lead to an increase in the amount of tyrosine phosphorylated ERK-1 and ERK-2. In contrast, stimulation with two different doses of IFNγ had no effect (Figure 6.1A). The failure of IFNγ to stimulate ERK activity was not because the cells were refractory to IFNγ, as in the same cell lysates IFNγ stimulated tyrosine phosphorylation of STAT-1 (Figure 6.1B). These data suggest that IFNγ, like IL-4, does not active ERK, and that ERK is not responsible for IFNγ-induced serine phosphorylation of STAT-1.

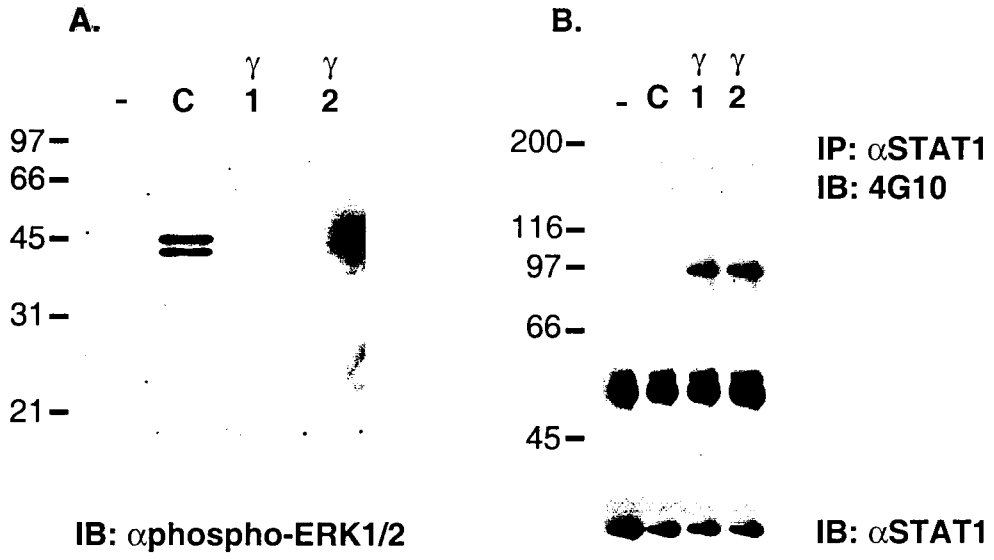

Figure 6.1. *IFNγ fails to activate ERK.* Factor-deprived bone-marrow derived macrophages were stimulated with 1000U/mL (**1**) or 2000 U/mL (**2**) of rIFNγ (γ) for 10 minutes, or with CSF-1 (**C**) for 3 minutes, or left unstimulated as a control (**-**) **A**. Whole cell lysates were subjected to SDS-PAGE and immunoblotting with polyclonal antibodies specific for tyrosine phosphorylated, kinase active ERK (αphospo-ERK). **B**. The same cell lysates were subjected to immunoprecipitation (IP) with anti-STAT-1 antibodies, and eluates were resolved by SDS-PAGE. The membranes was immunoblotted with 4G10 (αPY) to determine the amount of tyrosine phosphorylation, and subsequently stripped and re-blotted with anti-STAT-1 antibodies to determine equivalency of loading.

6.2.2. IFNγ inhibits STAT-6-dependent transcription

To determine if IFNγ can inhibit STAT-6-dependent events, I examined the ability of IFNγ to inhibit induction of the pCεGL2 reporter. I have previously shown that IL-4-mediated induction of this reporter gene is blocked by expression of a dominant-negative STAT-6 (Figure 4.2). Ba/F3 cells were transiently transfected with pCεGL2 and left unstimulated as a control, or stimulated with IL-4, IFNγ, or both together. In accordance with the results presented in Figure

4.2, stimulation with IL-4 resulted in a 7-8 fold induction of luciferase activity (Figure 6.2A). Addition of IFNγ alone had no effect. However, in the presence of IFNγ, IL-4-stimulated transcription was reduced by approximately 50%. Similar results were obtained when I examined the STAT-6-dependent, IL-4-induced increase of CD23 expression (Takeda et al., 1996). $CD19^+$ B cells were purified from spleen cells and stimulated with IL-4, IFNγ, or both together for 24 hours. The cells were then analysed for CD23 expression by flow cytometry. Consistent with other reports (Galizzi et al., 1988; te Velde et al., 1990), addition of IFNγ inhibited the IL-4-stimulated increase in CD23 expression (Figure 6.2B).

6.2.3. Generation of templates for the production of anti-sense Bcl-6 RNA

To determine if IFNγ can regulate the expression of the STAT-6 antagonist, Bcl-6, I generated plasmids from which antisense RNA for human or mouse Bcl-6 could be synthesised *in vitro* and used in an RNAse protection assay (Figure 6.3).

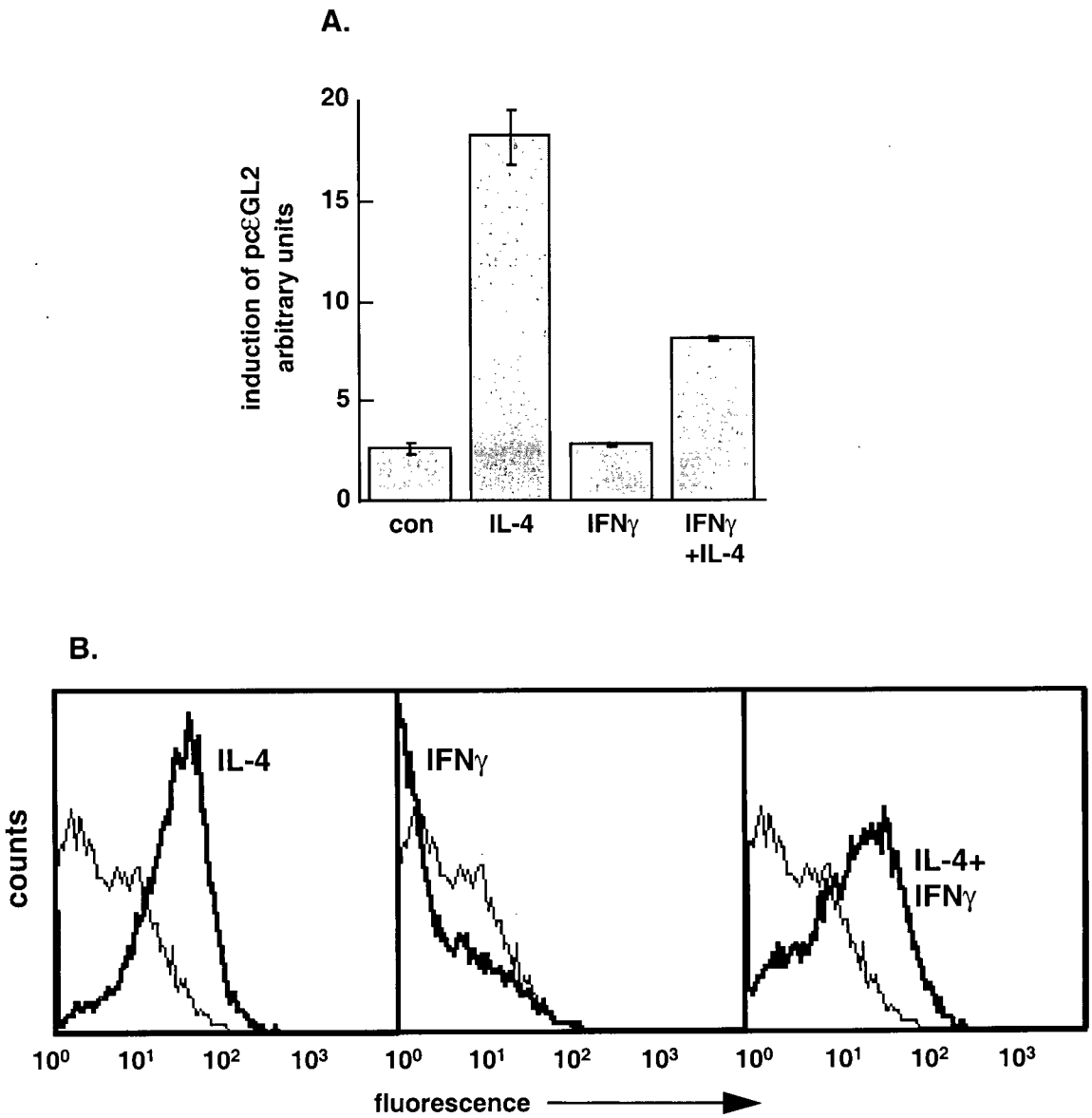

Figure 6.2. *IFNγ inhibits STAT-6 dependent gene expression.* **A.** Ba/F3 cells were transiently transfected with 15μg of pCεGL2, and allowed to recover overnight. The cells were washed to remove IL-3, and then stimulated for 6 hours with IL-4 (2μg/mL), IFNγ (200U/mL), both together, or with media alone (**con**) as a control. Cell lysates were analysed for luciferase activity by luminometry. Relative light units were normalized to the amount of protein in each lysate, as determined by the BCA assay. Error bars represent the SEM of duplicate relative light units, and triplicate OD readings of protein concentration. **B.** $CD19^+$ B cells were cultured for 36 hours in complete media with IL-4, IFNγ, both together, or in the absence of factor. Cells were stained with a FITC-coupled anti-CD23 antibody, and amount of FITC staining was determined by flow cytometry. The thin line histograms represent cells in the absence of factor, and thick line histograms represent cells that had been stimulated with the indicated factor.

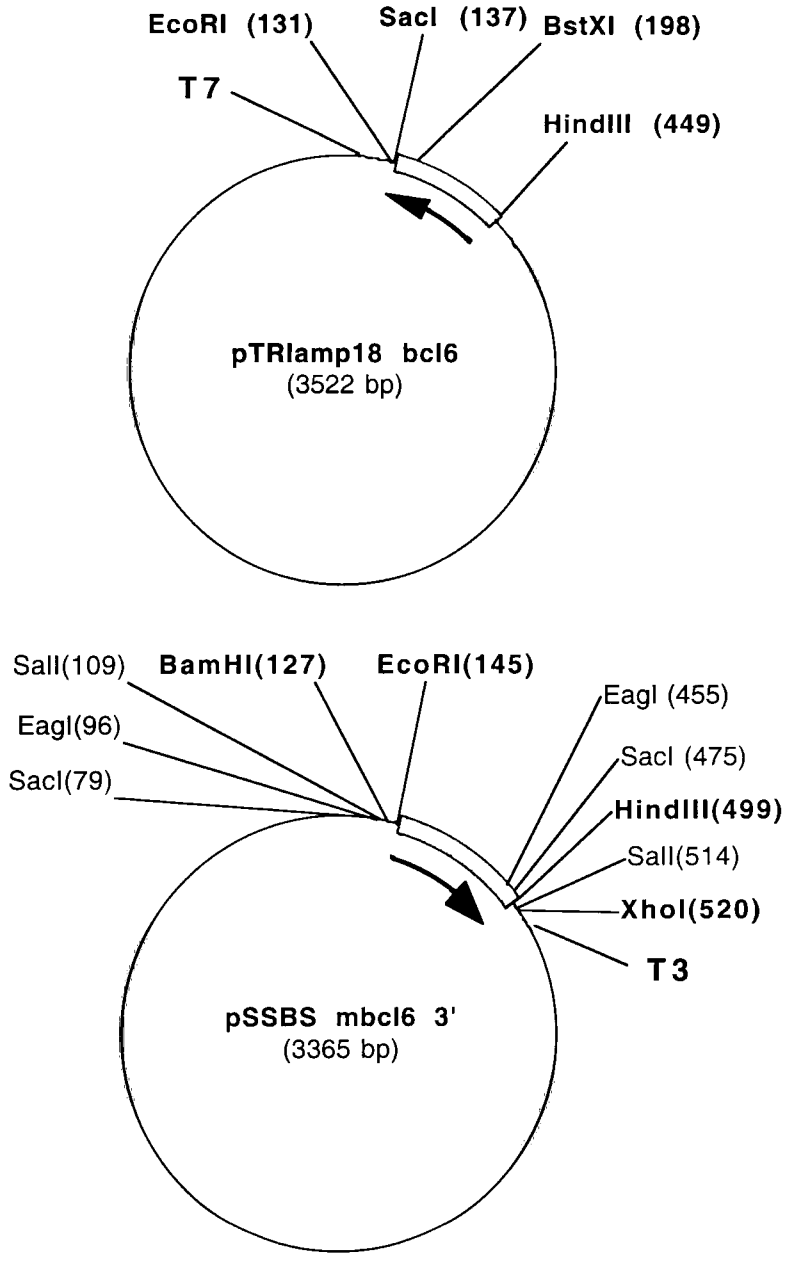

Figure 6.3. *Plasmids used to generate antisense RNA for human and mouse Bcl-6.* **A.** To generate a template for synthesis of antisense human Bcl-6 RNA, a 320 bp *HindIII/SacI* fragment from the 3' end of Bcl-6 was cloned into pTRIamp18 in the antisense orientation. After the plasmid had been linearized with *HindIII*, antisense RNA was synthesized by T7 polymerase (T7). **B.** To generate a template for synthesis of antisense RNA for murine Bcl-6, a 343 bp fragment from the 3' end of murine Bcl-6 was amplified by RT-PCR and cloned into pSSBS. After the plasmid had been linearized with *BamHI*, antisense RNA was generated by T3 polymerase (T3). Thin inner arrows represent the region encoding for antisense RNA. Unique restriction enzyme sites are marked in bold.

6.2.4. IFNγ stimulates an increase in Bcl-6 mRNA in Jurkat cells

Addition of phorbol esters and calcium ionophores (which mimics TCR stimulation) to $CD4^+$ T cells can increase mRNA levels Bcl-6 (Fukuda et al., 1995). Therefore, I first examined the ability of IFNγ to regulate Bcl-6 mRNA levels in a human, $CD4^+$ T-cell line, Jurkat. Serum-deprived Jurkat cells were stimulated with IFNγ or phorbol esters (TPA) and ionomycin for 30, 60 or 90 minutes. At the appropriate time, the cells were lysed, total RNA was isolated, and incubated with a mixture of [α-^{32}P]CTP-labelled antisense RNAs for human Bcl-6 and GapDH (glyceraldehyde-3-phosphate dehydrogenase). Following hybridization and digestion with RNAse, the protected fragments were resolved on a denaturing polyacrylamide gel. Stimulation of Jurkat cells with IFNγ resulted in the induction of Bcl-6 mRNA, which was maximal at 60 minutes, and did not diminish over the duration of this experiment (Figure 6.4). As predicted, stimulation with TPA and ionomycin also led to an increase in Bcl-6 mRNA. Interestingly, two distinctly sized protected fragments were protected, both of which were induced following stimulation. The upper band, marked as Bcl-6, migrated at the predicted position. The identity of the lower band is unclear, but it is possible that it is the result of an alternatively spliced variant.

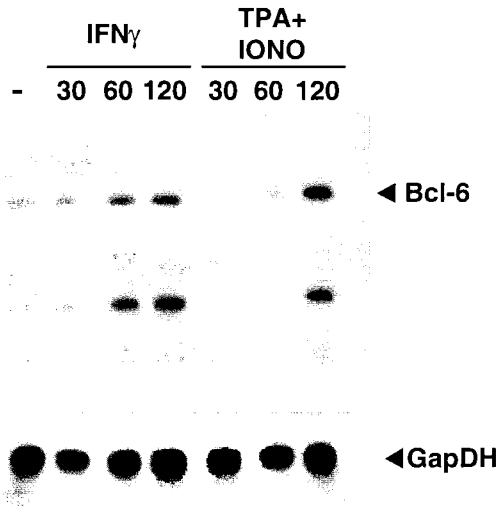

Figure 6.4. *IFNγ stimulates an increase in Bcl-6 mRNA in Jurkat cells.* Serum deprived Jurkat cells were treated with IFNγ (1000U/mL), TPA (5ng/mL) and ionomycin (250ng/mL) or left unstimulated as a control (-). At the appropriate time (30, 60 or 90 minutes) total RNA was isolated and 10 μg from each sample was hybridized to a mixture of [α-^{32}P]CTP-labelled antisense RNAs for human Bcl-6 or GapDH. After digestion with RNAse, protected fragments were separated on a denaturing polyacrylamide gel and visualized by autoradiography.

6.2.5. IFNγ stimulates an increase in Bcl-6 mRNA in activated CD4$^+$ T cells

It was important to determine if stimulation with IL-4 could also influence the expression of Bcl-6 mRNA. Jurkat cells do not express receptors for IL-4 (Wieler & Schrader, unpublished data), however primary CD4$^+$ T-cells do respond to IL-4 as judged by induction of *c-myc* mRNA (Wieler & Schrader, unpublished data) and tyrosine phosphorylation of STAT-6 (data not shown). Murine CD4$^+$ T cells were isolated from lymph nodes, activated by TCR stimulation, and expanded in IL-2 for 10-14 days. These proliferating T-cells were deprived of serum and IL-2, and left unstimulated as a control or stimulated with IFNγ, IL-4, IL-4 and IFNγ, or pre-treated with IL-4 for 4 hours prior to addition of IFNγ. Thirty, 60, or 90 minutes after addition of the stimulus, total RNA was isolated and hybridized with a mixture of [α-^{32}P]CTP-

labelled antisense RNAs for murine Bcl-6 and GapDH. Similar to the results in Jurkat cells (Figure 6.4), IFNγ induced an increase in Bcl-6 mRNA, which was maximal at 60 minutes (Figure 6.5). Importantly, stimulation with IL-4 had no effect on levels of Bcl-6 message. Co-stimulation, but not pre-treatment, with IL-4 abrogated the IFNγ-mediated increase in Bcl-6 mRNA. In murine cells only a single protected fragment of Bcl-6 was observed.

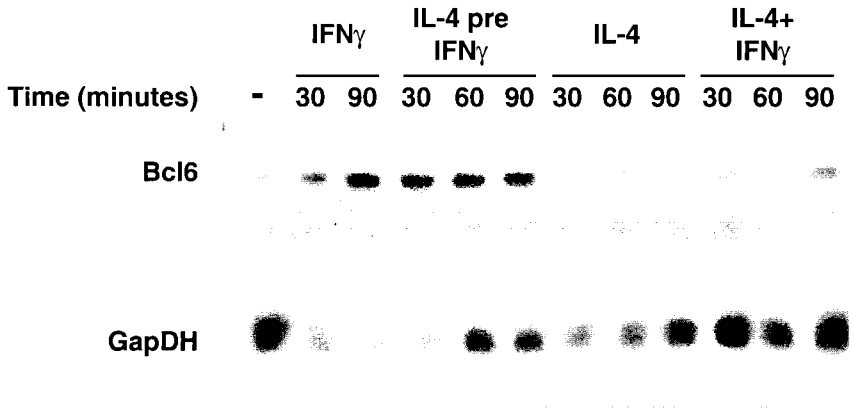

Figure 6.5. *IFNγ stimulates an increase in Bcl-6 mRNA in activated CD4⁺ T cells.* Serum and factor deprived CD4⁺ T cell blasts were stimulated with IFNγ, IL-4, IL-4 and IFNγ, pretreated with IL-4 for 4 hours (during the starvation) prior to stimulation with IFNγ, or left unstimulated as a control (-). At the appropriate time points (30, 60 or 90 minutes) total RNA was isolated, and10 μg from each sample was hybridized to [α-^{32}P]CTP-labelled antisense RNA for murine Bcl-6 or GAPDH. After digestion with RNAse, protected fragments were separated on a denaturing polyacrylamide gel and visualized by autoradiography.

6.2.6. IFNγ stimulates an increase in Bcl-6 mRNA in primary mast cells

IFNγ antagonizes the effects of IL-4 in multiple cells types, and I wished to determine if IFNγ could also stimulate an increase in Bcl-6 mRNA in primary mast cells. Mast cells were derived from bone-marrow by culture in medium supplemented with IL-3 and IL-4 for 4-8 weeks (Schrader and Nossal, 1980). Cells were deprived of factors, and stimulated with IFNγ, IL-4, TPA and ionomycin, or left unstimulated as a control. Total RNA was isolated and hybridized to a mixture of [α-^{32}P]CTP-labelled antisense RNAs for murine Bcl-6 and GapDH. Following digestion with RNAse, the protected fragments were resolved and visualised by autoradiography. Stimulation with either IFNγ or TPA and ionomycin resulted in an increase in Bcl-6 mRNA (Figure 6.6). As was the case in T cells, stimulation with IL-4 had no effect.

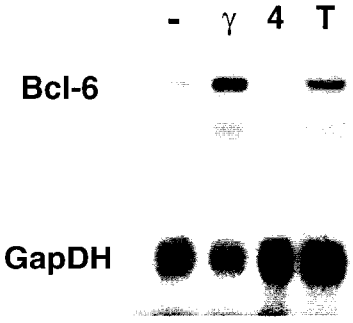

Figure 6.6. *IFNγ stimulates an increase in Bcl-6 mRNA in mast cells.* Bone-marrow derived mast cells were deprived of serum and cytokines for 4 hours and stimulated with IFNγ (γ), IL-4 (**4**), TPA plus ionomycin (**T**) or left unstimulated as a control (-) for 60 minutes. Total RNA was isolated and 10 μg from each sample was hybridized to a mixture of [α-^{32}P]CTP-labelled antisense RNAs for murine Bcl-6 or GapDH. After digestion with RNAse, protected fragments were separated on a denaturing polyacrylamide gel and visualized by autoradiography.

6.3. Discussion

Despite their opposing biological effects, the intracellular signals activated by IFNγ and IL-4 are remarkably similar. In both cases, activation of the JAK/STAT pathway mediates the majority of the biological effects, and unlike most other cytokines, neither can stimulate ERK activity (Figure 6.1; Welham et al., 1992). Many IL-4-mediated effects are dependent on activation of STAT-6, (Kaplan et al., 1996; Shimoda et al., 1996; Takeda et al., 1996), and IFNγ is clearly able to inhibit STAT-6 dependent events such as induction of the pCεGL2 reporter gene and CD23 (Figure 6.2). These data suggest the existence of a mechanism for IFNγ-mediated inhibition of STAT-6.

I have shown here that stimulation of human or murine $CD4^+$ T-cells (Figure 6.4 & 6.5) or murine mast cells (Figure 6.6) with IFNγ results in an increase in mRNA for the newly recognised inhibitor of STAT-6, Bcl-6. The IFNγ-induced increase in Bcl-6 mRNA appeared to be lower in primary murine $CD4^+$ cells (Figure 6.4) compared to Jurkat cells (Figure 6.3). This observation may be the due to the fact that proliferating T cells can themselves make large amounts of cytokines, including IFNγ, which could cause relatively high levels of Bcl-6 mRNA in the control samples. It is tempting to speculate that the ability of IFNγ to regulate levels of Bcl-6 mRNA is dependent upon activation of STAT-1 as there is a consensus binding sequence for STAT-1 at position –526 in the promoter for Bcl-6. It would be interesting to determine if IFNγ could also regulate the expression of members of the newly characterised families of cytokine-inhibitors, SOCS and PIAS (Chung et al., 1997; Hilton et al., 1998), that might be specific for STAT-6.

Whereas co-stimulation with IL-4 inhibited the ability of IFNγ to increase expression of Bcl-6 mRNA in $CD4^+$ T cells, pre-treatment with IL-4 for 4 hours had no effect (Figure 6.5).

The ability of IL-4 to rapidly inhibit (i.e. within 60 minutes and in the absence of new protein synthesis) IFNγ-mediated effects has previously been documented (Deng et al., 1994; Ohmori and Hamilton, 1997). Although IL-4 does not inhibit the ability of IFNγ to stimulate tyrosine phosphorylation of STAT-1 (Figure 5.6), there is evidence that STAT-6 could compete with STAT-1 for binding to DNA (Ohmori and Hamilton, 1997). The activity of STATs is regulated by classical negative-feedback mechanisms, which involves induction of SOCS and PIAS family proteins (Adams et al., 1998; Liu et al., 1998). It is possible that pre-treatment with IL-4 for 4 hours had no effect on the IFNγ -stimulated increase in Bcl-6 mRNA because that allowed sufficient time for the IL-4 signal to be down-regulated by a member of the SOCS and/or PIAS family.

It will be important to determine if treatment with IFNγ also leads to an increase in Bcl-6 protein expression. The regulation of Bcl-6 expression appears to be complex, and is controlled not only at the level of transcription but also by post-translational modification. Bcl-6 is phosphorylated on multiple serines by ERK (Moriyama et al., 1997), and Niu et al. (1998) have shown that, at least in response to B cell receptor (BCR) activation, this ERK-mediated phosphorylation targets the protein for degradation by the ubiqitin/proteasome pathway. Since expression of the Bcl-6 protein is restricted to B-cells within germinal centres, it is unclear how BCR signalling may be modified to allow for high levels of expression at this point in development. IFNγ does not activate ERK (Figure 6.1), thus if IFNγ does stimulate an increase in Bcl-6 protein expression, it is unlikely that it would be degraded by the ubiquitin/proteasome pathway. Furthermore, Xu et al. (1994) have shown that pre-treatment with IFNγ could actually inhibit the ability of PDGF or phorbol esters, to activate Raf-1 and ERK. An intriguing hypothesis is that co-stimulation with IFNγ could inhibit ERK-mediated degradation of Bcl-6.

Previous data have suggested that Bcl-6 was primarily expressed in germinal centre B cells (Cattoretti et al., 1995; Onizuka et al., 1995) and $CD4^+$ T cells (Fukuda et al., 1995). I have demonstrated that Bcl-6 mRNA is also expressed in mast cells (Figure 6.4). However, preliminary data suggest that the levels of Bcl-6 protein expression are significantly lower in murine mast cells at least compared to certain B cells lines (Levings, Lee, and Schrader, data not shown). It will be important to determine the functional significance of Bcl-6 in this cell type.

In conclusion, the data presented in Chapter 6 raise the possibility that one mechanism by which IFNγ could influence IL-4-stimulated effects is by modulating the level of Bcl-6 expression. It will be important to determine if levels of protein expression are also altered upon treatment with IFNγ. Further insight into the mechanisms that exist naturally to modulate IL-4 are important for the design of strategies to inhibit dysregulated IL-4 production and/or function.

CHAPTER 7. General Conclusions and Future Directions

An understanding of the molecular basis for the specific biological functions of IL-4 is an important step towards the design of treatments for diseases such as allergy, asthma and rheumatoid arthritis. In order to improve this understanding, the goal of this thesis was to test several hypothesis about the biological functions of the distinctive intracellular signals activated by IL-4.

The first hypothesis I examined was based on the observation that IL-4 and IL-13 are the only type I cytokines that fail to activate Ras, Raf and MAP-family kinases (Welham et al., 1992; Foltz et al., 1997; Foltz and Schrader, 1997). I demonstrated that IL-4 is not a true growth factor (Figure 3.1) and examined the hypothesis that this inability of IL-4 to activate Raf can be directly correlated with its inability to stimulate cell growth. Indeed, expression of activated Raf-1 complemented the ability of IL-4 to promote long-term growth (Figure 3.4). These observations correlate with the notion that activation of Raf-1 is generally necessary for cell-cycle progression (Marshall, 1995). Important evolutionary questions are raised by these data. Did IL-4 lose the ability, or did other type I cytokines gain the ability, to activate the Raf pathway? If IL-4 lost the ability to activate Raf, was it simply by chance, and apart from losing the ability to stimulate growth, were there any other biological consequences? It will be important to repeat the experiments described in Chapter 3 in primary cells where the effect of Raf-1 activity on IL-4-mediated effects such as induction of gene expression can be evaluated. Another informative approach to these questions would be to add a sequence that could mediate activation of Ras (e.g. a Grb-2 binding site) to the IL-4Rα, and to express this mutant receptor in primary cells, or in transgenic mice, and to analyse the effects on IL-4-dependent functions such as Ig class switching, Th-2 development, and inhibition of pro-inflammatory cytokines.

Surprisingly, IL-4 and Raf-1 synergised not only to stimulate growth, but also to activate JNK (Figure 3.9). It will be important to determine if IL-4 and Raf can also synergise to activate the other family of stress-activated kinases, the p38 MAP-kinases. Further work is also required to determine which IL-4-mediated signals are involved in this synergy, and at what level they "cross-talk" with signals downstream of Raf to result in activation of JNK (see Figure 7.1). Synergistic activation of JNK has been previously observed in B and T cells. Stimulation of the BCR or TCR by antigen leads to potent activation of ERK. However, only in combination with the co-stimulatory signals activated by CD19 or CD28, are JNK and p38 MAP-kinase activated (Su et al., 1994; Salmon et al., 1997; Tooze et al., 1997). Although IL-4 alone is not a growth factor, it is a potent co-stimulatory signal for cell cycle progression, particularly in B and T cells (Defrance et al., 1987; Spits et al., 1987). An intriguing hypothesis is that like CD19 and CD28, signals activated by IL-4 may synergise with those from the BCR and/or TCR to stimulate an increase in JNK activity.

When the STAT family was originally characterised, it was thought to be the long sought after signalling pathway that could mediate the specific effects of particular cytokines. However, in response to type I cytokines, only STAT-4 and STAT-6, appear to mediate truly cytokine specific effects. Thus STAT-4 appears to be exclusively activated by IL-12 (Bacon et al., 1995) and IL-4 and IL-13 are the only cytokines that activate STAT-6. Through the analyses of mice deficient for STAT-6, is has been shown that a majority of IL-4-mediated effects are dependent on activation of this pathway (Kaplan et al., 1996; Shimoda et al., 1996; Takeda et al., 1996).

I tested the hypothesis that STAT-6 was required for IL-4-mediated cells survival and inhibition of pro-inflammatory cytokines production. I have shown in Chapter 4 that although

STAT-6 is required for IL-4-stimulated cell survival, it functions via an indirect mechanism. The data presented in Chapter 4 also suggest that at least some of the phenotypes of STAT-6 null mice may also be secondary to defects in IL-4-stimulated upregulation of the IL-4Rα. One way to address this issue would be to constitutively express the IL-4Rα in the STAT-6 null cells (or in the STAT6 null mice themselves), at levels approximately equivalent to those seen upon stimulation with IL-4. Furthermore, since STAT-6 may also be essential for controlling the production of IL-4 itself, quantitative defects in the production of IL-4 may confound the analyses of STAT-6 null mice (Curiel et al., 1997).

I examined the role of STAT-6 in IL-4-mediated inhibition of pro-inflammatory cytokines. Since I continued to observe IL-4-mediated effects in STAT-6 null macrophages, any defect in IL-4Rα expression in the experimental system had a relatively minor effect. Indeed, it is possible that the reduced IL-4-mediated inhibition (approximately 20%) of TNFα and IL-12 production by STAT-6 null macrophages, that had been stimulated with LPS and IFNγ, was due to dysregulated IL-4Rα expression. The observation that IL-4 could inhibit TNFα and IL-12 production in the absence of STAT-6 was surprising and unexpected. These data suggest that there are other uncharacterised signalling pathways that are critical for IL-4-mediated inhibition of pro-inflammatory cytokine production (Figure 7.2).

Stimulation of bone-marrow derived macrophages with IL-4 resulted in tyrosine phosphorylation of STAT-3 (Figure 5.7), which was likely to be mediated by the IL-13RI (Orchansky & Schrader, manuscript in preparation). However, IL-4 failed to induce a shift in the mobility of STAT-3 that is typically associated with serine phosphorylation (Chung et al., 1997), suggesting that IL-4 may not stimulate STAT-3 transactivation activity. A mutant STAT-3 that lacks the critical serine is approximately 50% less efficient at stimulating IFNα-

induced transcription from a reporter gene (Wen et al., 1995). An intriguing hypothesis is that tyrosine (but not serine) phosphorylated STAT-3 could act as a dominant-negative by binding to target sequences, but not stimulating transcription efficiently. Furthermore, since STAT-3 is able to bind to STAT-1 sites (Seidel et al., 1995), tyrosine phosphorylated STAT-3 could also block IFNγ-mediated effects, such as promotion of TNFα and IL-12 production. Expression of a mutant STAT-3 that lacks the critical serine along with a STAT-1 reporter gene should resolve this issue. Mice lacking STAT-3 do not survive to birth (Takeda et al., 1997), thus experiments in STAT-3 null macrophages analogous to those described in Chapter 4 are not yet possible. However, genetically manipulated mice bearing a conditionally null mutant STAT-3 allele have recently been reported (Alonzi et al., 1998). It should be possible to generate bone-marrow derived macrophages from these mice, and to eliminate expression of STAT-3 *in vitro* by transient expression of the cre recombinase.

Despite their opposing biological effects, IFNγ and IL-4 share similarities in their intracellular signalling pathways; activation of the JAK/STAT pathway is necessary for the majority of IL-4- and IFNγ-mediated biological effects. Furthermore, both cytokines fail to stimulate increased ERK activity (Figure 6.1; Welham et al., 1992). The mechanisms by which IFNγ inhibits the effects of IL-4 are unknown, and to date, have not yet been addressed in the literature. It seems likely that such a mechanism may involve inhibition of STAT-6 activation, as IFNγ is clearly able to inhibit STAT-6-dependent events such as regulation of pCεGL2 and CD23 (Figure 6.2; Lee et al., 1993; Galizzi et al., 1988).

I provide evidence in Chapter 6 that IFNγ may regulate the expression of the STAT-6 antagonist, Bcl-6. IFNγ-mediated induction of Bcl-6 mRNA could be mediated by STAT-1, and further study with dominant-negatives and/or STAT-1 null cells will clarify this issue. It will be

important to determine if IFNγ can also stimulate an increase in Bcl-6 protein expression, and/or alter the ERK-mediated degradation of Bcl-6 (Niu et al., 1998). If regulation of Bcl-6 is an important inhibitory mechanism, IFNγ-mediated suppression of IL-4 should be diminished or abrogated in cells derived from genetically altered mice that lack Bcl-6. It will also be interesting to determine if IFNγ regulates the expression of the Bcl-6 homolog, BAFZ. IFNγ may also regulate the expression of a member of the SOCS or PIAS families that inhibits STAT-6. Further insight into the mechanisms that exist naturally to regulate the signals activated by IL-4 is important for the design of strategies to therapeutically regulate the effects of IL-4.

An understanding of how intracellular signals mediate the diverse biological effects of IL-4 is critical for the rational design of therapeutics that can specifically inhibit or augment the actions of IL-4. IL-4 is a particularly attractive cytokine to target as, to date, it is one of the only cytokines for which we can correlate specific intracellular signals with specific biological effects. Thus, STAT-6 appears to mediate a majority of the biological functions of IL-4. However, in this thesis I have reported several unexpected findings that suggest STAT-6 may not be the only important intracellular signal to consider. The observation that IL-4 and Raf-1 synergise to activate JNK raises the possibility that activation of JNK may also be important for some of the biological effects of IL-4. I have presented data that suggest much of the analyses of STAT-6 null mice must be reconsidered in the context of a possible indirect effect on IL-4 signalling due to dysregulated expression of the IL-4Rα. Finally I have provided evidence for a STAT-6 independent mechanism of IL-4-mediated inhibition of TNFα and IL-12 production. Further investigation into these aspects of IL-4-mediated intracellular signalling will ultimately lead to a better understanding of how IL-4 exerts its pleiotropic biological functions.

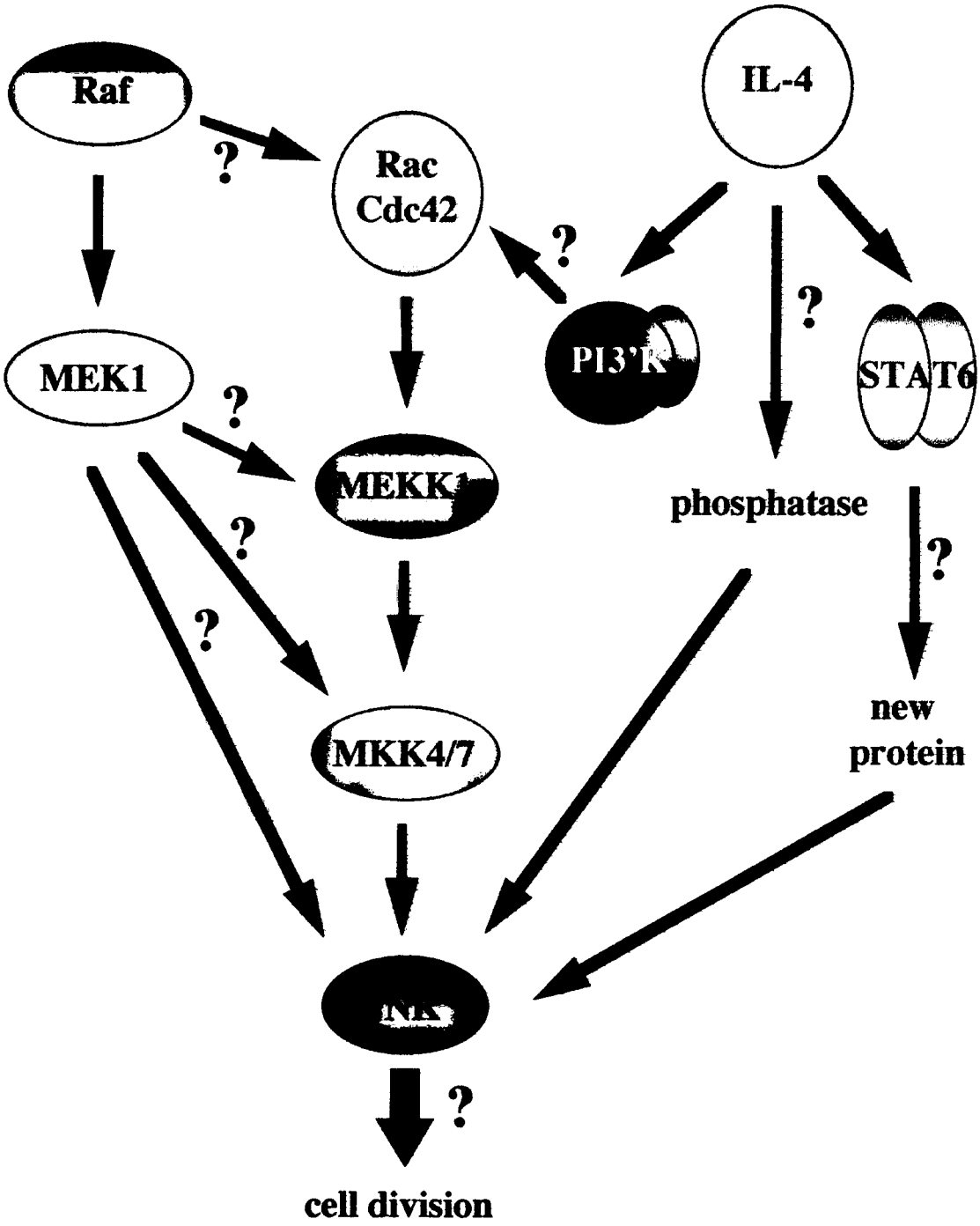

Figure 7.1. *Possible mechanisms for synergistic activation of JNK by IL-4 and Raf-1.*

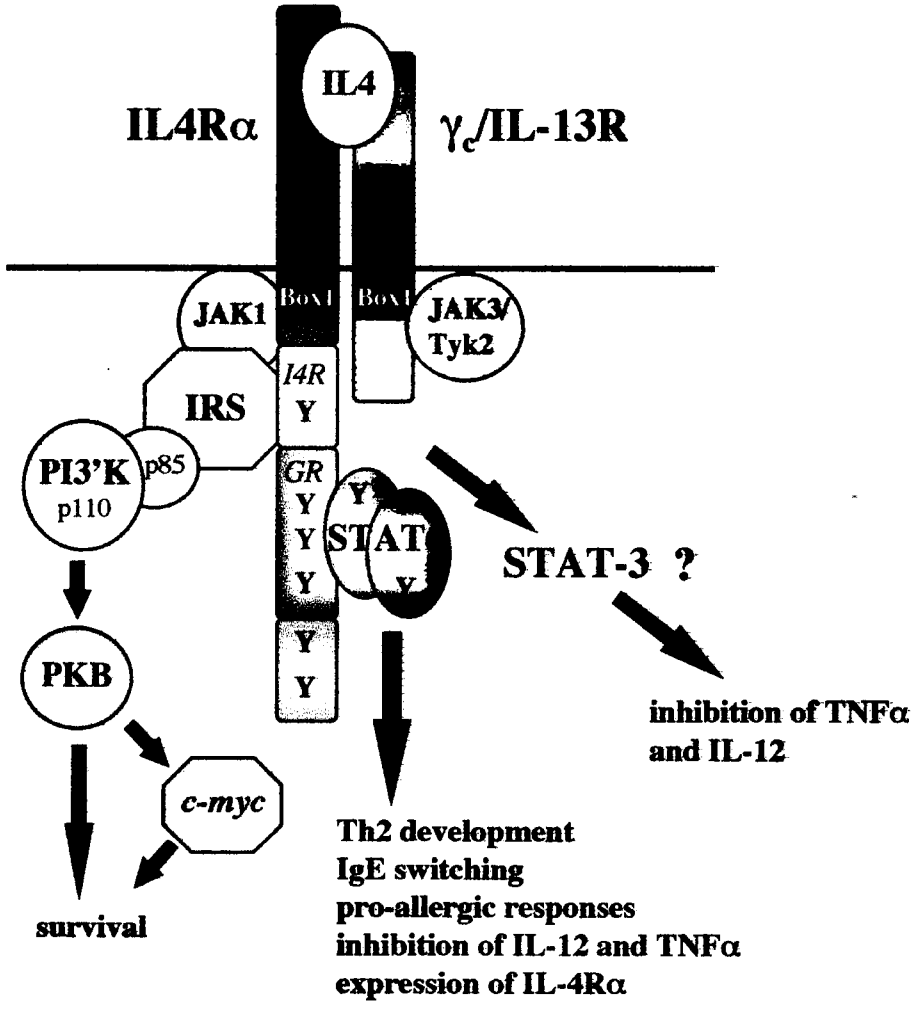

Figure 7.2. *Schematic model of the signalling pathways activated by IL-4, including results presented in this thesis.*

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