Regulation of Suppresor of Cytokine Signalling – 3 expression by Cyclic – AMP in Pro-myeloid cells

Laura Catherine Mullen BSc (Hons)

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Faculty of Biomedical and Life Sciences
University of Glasgow

Abbreviations

AC	Adenylyl cyclase
BSA	Bovine serum albumin
CREB	Cyclic AMP response element binding protein
ECL	Enhanced chemiluminescence
GPCR	G-protein-coupled receptor
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte macrophage-colony stimulating factor
HEK 293	Human embryonic kidney 293
JAK	Janus kinase
MAPK	Mitogen-activated protein kinase
NFκB	Nuclear Factor κB
SH	Scr homology
SOCs	Suppressors of cytokine signalling
STAT	Signal transducer and activator of transcription
TEMED	N,N,N',N'-tetramethylethylenediamine

Pharmacological Names

8P-CPT	8-(4-Chlorophenylthio)-2'-O-methyl-cAMP
SB 203580	4-(4 Fluorophenyl)-2-(4-methylsulfinylohenyl)-5-(-4-pyridyl)1H-imidazole
UO126	1,4-Diamo-2,3-dicyano-1,4-*bis*-(2-aminophenylthio) butadiene

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Abstract

"Suppressors of cytokine signalling" (SOCS) protein induction *via* the "Janus kinase-signal transducer and activator of transcription" (JAK-STAT) pathway has been shown to be a critical negative feedback mechanism that prevents inappropriately sustained signalling from activated cytokine receptors. It also provides a mechanism by which other signalling modules could potentially regulate the JAK-STAT pathway. In this study, I have demonstrated that elevation of the prototypical second messenger cyclic AMP (cAMP) is capable of promoting the time- and concentration-dependent accumulation of the SOCS-3 isoform in U937 human promyeloid cells and HL60 human promyoblast cells. Experiments with MG132 demonstrated that cAMP specifically promoted SOCS-3 synthesis rather than blocking its degradation by the proteasome. The accumulation of SOCS-3 in U937 cells correlated with a reduced ability of the granulocyte-colony stimulating factor (G-CSF) receptor, which is a *bona fide* target for SOCS-3 *in vivo*, to promote the Tyr phosphorylation of STAT3 and the dual Thr/Tyr phosphorylation of ERK, suggesting that the cAMP-mediated accumulation of SOCS-3 is sufficient to suppress JAK-STAT pathway activation by this receptor. Further characterisation of the response demonstrated that SOCS-3 induction could not be blocked by H89, an inhibitor of cAMP-dependent protein kinase (PKA), suggesting that a PKA-independent mechanism was responsible. Consistent with this hypothesis, selective activation of PKA with the selective cAMP analogue 6Be-cAMP failed to promote SOCS-3 accumulation. Surprisingly, selective activation of "Exchange protein activated by cAMP" (Epac), a recently identified PKA-independent intracellular sensor of cAMP, using the cAMP analogue 8pCPT-2OMe-cAMP also failed to promote SOCS-3 accumulation. Moreover, the inability of both 6Be-cAMP and 8pCPT-2OMe-cAMP to promote SOCS-3 accumulation was

not due to a lack of biological activity, since both were able to stimulate the phosphorylation of ERK in U937 cells at the concentrations used to assess SOCS-3 induction. Finally, preliminary experiments employing inhibitors of various signalling pathways revealed that cAMP-mediated SOCS-3 accumulation occurred *via* a JAK-, p38- and ERK-independent mechanism. Thus, it is proposed that cAMP elevation may promote the accumulation of SOCS-3 in U937 cells *via* a novel pathway leading to increased SOCS-3 synthesis that is independent of the known cAMP sensors PKA and Epac, and which may thus involve a currently unknown intracellular sensor of cAMP.

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CHAPTER 1

Introduction

1.1 Inflammation

Inflammation is a beneficial host reaction in response to foreign challenge or tissue injury and leads ultimately to the restoration of tissue structure and function (*Lawrence et al., 2002*). Several mechanisms have evolved to render this process an effective, intricately controlled self-limiting event. However, imbalance in this system often occurs resulting in pathological damage.

In the preliminary stages of the inflammatory response, there is sequential release of mediators, particularly inflammatory cytokines, and subsequent recruitment of circulating leukocytes (*Park et al., 2004*). Under normal physiological conditions, the inflammatory response is resolved by accumulation of intracellular negative regulatory factors. However with certain disease states, attenuation of inflammatory events does not occur (*Lawrence et al., 2002*). This is presumably due to failure of negative regulatory pathways, resulting in persistent accumulation and activation of leukocytes, a symptom of chronic inflammation.

Current treatment strategies for chronic inflammation focus on inhibition of pro-inflammatory mediators, thereby suppressing initiation and maintenance of the inflammatory response. Naturally, elucidation of the signalling events involved in endogenous negative regulatory pathways of the inflammatory response would lead to a greater pool of targets for pharmacological intervention.

Transduction pathways of well-characterised pro-inflammatory cytokines such as interleukin –1 (IL-1), tumour necrosis factor- α (TNFα), IL-12, IL-18, granulocyte-

macrophage-colony stimulating factor (GM-CSF) and granulocyte-colony stimulating factor (G-CSF) have been extensively studied. This has shown that many cytokines are able to exert pluripotent biological effects through activation of Janus kinases (JAKs) and signal transducer and activator of transcription (STAT) proteins (*Kisseleva et al., 2002*).

Until recently, relatively little work has been done to establish a detailed evaluation of the negative-feedback pathways of inflammation. Suppressors of cytokine signalling (SOCS) proteins function as inhibitors of cytokine receptor signalling by inhibiting the JAK/STAT signal transduction pathway (*Suzuki et al., 2001*). It has now emerged that SOCS are instrumental in the negative regulation of cytokine responses and furthermore may provide a clinical target for therapeutic intervention in the disease states associated with excessive inflammation (*Alexander., 2002*).

This study will consider the possible pathways involved in regulation of SOCS3 expression in two pro-monocytic cell lines under varying conditions.

1.2 Haematopoeisis

Every 60 seconds a human being generates about 120 million granulocytes and 150 million erythrocytes, as well as numerous mononuclear cells and platelets (*Rang, et, al., 2000).* The cells responsible for this remarkable productivity are derived from a relatively small number of self-renewing pluripotent stem cells laid down during embryogenesis (*Kuijpers et al., 2001*). The process is regulated by a complex array of haematopoietic growth factors, which act to direct the division, maturation and functional activity of the end-stage cells (*Kuijpers et al., 2001*).

1.2.1 Cytokine regulation of haematopoeisis

Proliferation, differentiation and functional activities of haematopoietic cells are regulated by a diverse group of protein factors, referred to collectively as the 'colony-stimulating factors'. They include the macrophage colony stimulating factor (M-CSF), granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF) and the multi-colony stimulating factor also know as interleukin-3 (Multi-CSF or IL-3) (*Smithgall., 1998*).

This study focuses on the effects of G-CSF and GM-CSF, which act synergistically to promote maximal outgrowth of various lingeages of cells. Indeed, G-CSF is the primary extracellular regulator of granulopoiesis, affecting the proliferation, survival and differentiation of all cells within the granulocytic lineage, from haematopoietic stem cells through to mature neutrophils (*Lieschke et.al., 1994*)

1.3 Common cytokine signalling pathways

1.3.1 JAK/STAT pathway

The JAK/STAT pathway is used to transduce a multitude of signals for development and homeostasis. Indeed, it forms the principal signalling mechanism for a wide variety a cytokines and growth factors, such as interleukin-6 (IL-6), via the gp130 subunit of the IL-6 receptor complex and growth hormone (GH) (*Ilangumaran, et. al., 2004*).

In mammals, the JAK family comprises four members: JAK1, JAK2, JAK3 and Tyk2. JAKs share seven regions of high homology, JH1–JH7 and are distinctive because they contain tandem kinase-homologous domains at the C-terminus (*Kisseleva et al.,*

2002) ***Figure 1.1***. JH1 encodes the functional kinase domain and is known to possess classical features of a tyrosine kinase receptor (*Hubbard and Till, 2000*). These include conserved tyrosines that form a critical component of the activation loop; Y1038/Y1039 in JAK1, Y1007/Y1008 in JAK2, Y980/Y981 in JAK3, Y1054/Y1055 in Tyk2 (*Leonard et al., 1998*). JH2 represents a pseudokinase domain or kinase-like (KL) domain which shares all the structural features of a tyrosine kinase, except catalytic activity. The KL domain appears to regulate JH1 catalytic activity (*Yeh et al,. 2000*) and is essential for proper JAK function. For example, deletion studies have found that Jak2 becomes hyperactivated in the absence of the KL domain (*Leonard et al,.1998*). The amino terminus (JH3-JH7) spans 550 amino acids and has been functionally implicated in receptor association, and binding specificity. However, the role of the JH7 region in this interaction remains undefined (*Kisseleva et al., 2002)*.

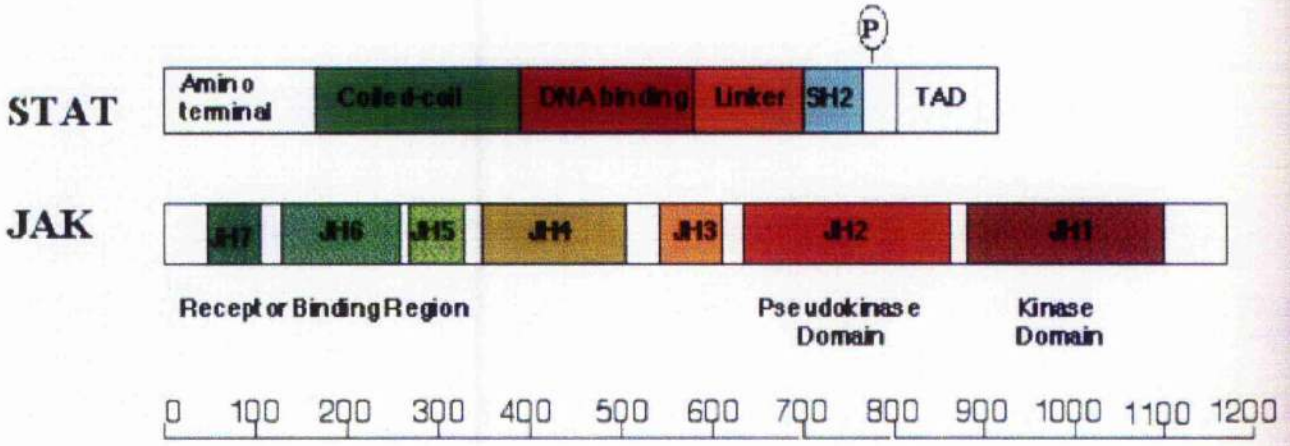

Figure 1.1 *Diagram of JAK and STAT structure. (Kisseleva, et.al.,2002)*

Seven STAT proteins have been identified in mammals; Stat1, Stat2, Stat3, Stat4, Stat5a, Stat5b and Stat6 (*Kisseleva et al., 2002*). The members of this family range in size from 750 to 850 amino acids and share structurally and functionally conserved domains including an amino-terminal domain (NH_2), a coiled- coiled domain (CCD),

the DNA binding domain (DBD), the linker domain and the SH2/tyrosine activation domain (*Kisseleva et al., 2002*). The SH2 domains are the most highly conserved STAT domain as it is linked to their role in receptor signalling where they are required to bind specific phosphotyrosine motifs (*Kawata et al 1997*). This high level specificity is important for three of the main events involved in STAT signalling. These include 1) recruitment of STAT to the cytokine receptor, 2) association of STAT with the activating JAK (*Barahmand-Pour et al., 1998*), and 3) the process of STAT dimerisation (*Gupta et al 1996*). The C-terminal region contains a transcriptional activation domain (TAD) which varies between members of the STAT family thereby contributing to STAT specificity (*Kisseleva et al., 2002*). Significant specificity is achieved in part, due to the fact that receptors capable of signal transduction via STATs are divided into five structurally and functionally related families. Tissue specific patterns of expression of ligands and receptors, as well as activation of additional signalling pathways by these receptors also influence the specificity of STAT activity.

The JAK/STAT pathway is initiated when ligand binding induces the multimerization of specific receptor subunits. In order for signal propagation to occur, the cytoplasmic domains of two receptor subunits must be associated with JAK tyrosine kinases (*Rawlings et al 2004*). Following ligand binding, multimerization results in two JAKs being brought into close proximity thereby allowing trans-phosphorylation. The activated JAKs subsequently phosphorylate additional targets, including both receptors and STATs (*Rawlings et al 2004*).

STATs are thought to reside latent in the cytoplasm until activated by JAKs via phosphorylation of a conserved tyrosine residue near their C-terminus. It is known that complete activation of STAT1 requires phosphorylation at both Y701 and a conserved PMS(727)P sequence, while STAT5 is tyrosine phosphorylated at Y694 (*Rane et al., 2002*) and STAT3 requires phosphorylation of Y705. The phosphotyrosine allows STAT dimerisation to occur through interaction with a conserved SH2 domain. The phosphorylated STAT dimers then enter the nucleus where they bind specific regulatory sequences to activate or repress transcription of target genes. Essentially the JAK/STAT pathway is a mechanism by which an extracellular signal can be translated into a transcriptional response (*Rawlings et al., 2004*).

Predictably, mutations that constitutively activate or fail to regulate JAK/STAT signalling properly are the cause of inflammatory disease, erythrocytosis, gigantism and a range of leukemias (*Touw, et al., 2000*).

1.3.2 Ras/Raf/Mitogen activated Protein Kinase (ERK)Pathway

Ras is an essential downstream component of most tyrosine kinase signal transduction pathways. Receptor activation leads to rapid conversion of inactive, GDP-bound Ras to the active GTP-bound state. This process is positively controlled by protein factors, known as guanine nucleotide exchange factors (GEFs) that promote GDP-GTP exchange and negatively regulated by GTPase-activating proteins (GAPs) which accelerate the intrinsic GTPase activity of Ras thereby promoting termination of the Ras signal (*Rang et al.,2000*). These regulatory proteins provide a biochemical link between Ras activation and tyrosine kinases via their SH2 domains. For example

autophosphorylated growth factor receptors interact directly with a GEF complex comprising growth factor receptor binder/son of sevenless (Grb2/SOS), consisting of a Ras GEF (SOS) coupled to an SH2 containing adaptor protein (Grb2) (*Cobb et al., 1996*). The SH2 domain of Grb2 serves to bring the complex into direct contact with specific autophosphorylated tyrosines on the activated growth factor receptor. The net effect of the process is to relocate the exchange activity form the cytoplasm to the plasma membrane where it will be brought into proximity to Ras to promote the guanine nucleotide exchange interaction (*Schlessinger,. 1993*). ***Figure 1.2***

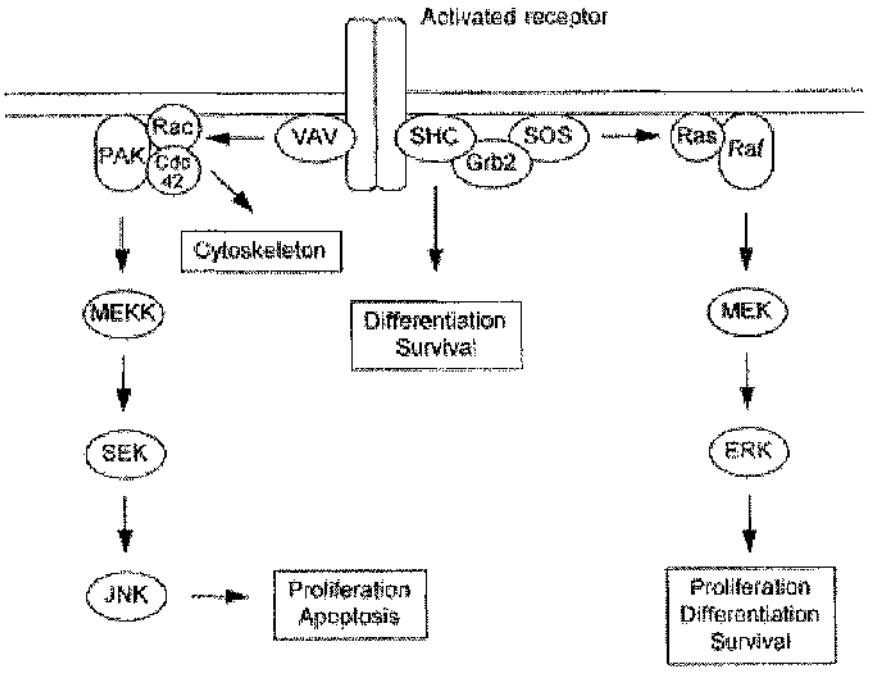

Figure 1.2 *Cytokine receptor activation of small G-protein/MAPK pathways*

The GTP-bound Ras undergoes a conformational change enabling activation of downstream target proteins, the best characterised protein being Raf1 (*Morrison et al., 1997*). In the case of cytokine receptors, the tyrosine phosphorylation of Raf may be mediated by Jak2 (*Xia et al., 1996*) or possibly a member of the Src kinase family

(*Fabian et al., 1993*). Once bound to Ras-GTP, Raf1 is relocated from cytoplasm to the plasma membrane. Raf1 then goes onto activate the dual specificity MAP kinase kinases, often referred to as MEKs, which inturn activate the MAP kinases, ERK-1 by phosphorylating Thr 183 and Tyr 185 and ERK-2 via phosphorylation of Thr 202 and Tyr 204 (*www.embbiosciences.com*). Activated ERKs are able to translocate into the nucleus where they are able to phosphorylate a variety of transcription factors, these include SMK-1, c-myc (Thr58/Ser62), ELK-1 (Ser838) and STAT3 (*www.embbiosciences.com*). STAT3 is of particular interest as it transcribes the suppressor of cytokine signalling (SOCS) molecule SOCS3, this is discussed in greater detail in section 1.6.

1.4 G-CSF and GM-CSF receptors

Hematopoeitic cytokines bind to members of the cytokine receptor superfamily, a large group of transmembrane proteins that share several structural features. Most cytokine receptors are characterised by a 200 amino acid region with 4 positionally conserved Cys residues and a signature WSXWS motif (where W=Trp, S=Ser and X=any amino acid) in the extracellular domain (*Wells et al., 1996*). The cytoplasmic domains of the receptor subunits are involved in signal transduction and exhibit limited homology in the membrane-proximal region. This region is often referred to as the Box-1/Box-2 motif and is essential for mitogenic signalling (*Smithgall., 1998*).

1.4.1 G-CSF receptor – structure

The G-CSFR is a member of the type I cytokine receptor subfamily and binds its ligand with high affinity. The G-CSFR does not have intrinsic tyrosine kinase activity and therefore relies on cytoplasmic enzymes for the stimulation of downstream

signalling events. The G-CSFR can be found on a variety of cells including monocytes, mature platelets and various non-hematopoietic cells and tissues (*Barreda et al., 2004*). However, it is primarily expressed in neutrophilic progenitors and mature neutrophils where it functions first and foremost to transmit signals for proliferation, differentiation and survival of these cells (*Barreda et al., 2004*).

Five forms of G-CSFR exists generated through alternative splicing (class I-V), class I being the most predominant (*Kasper et al., 1999*). Structurally, this receptor consists of a single chain type I transmembrane molecule which contains 604 amino acids in the extracellular region, 26 amino acids in the transmembrane region, and 183 amino acids in the intracellular region (*Kasper et al., 1999*) The extracellular portion of the receptor contains 6 structural domains which are extensively glycosylated at nine potential N-glycosylation sites. It also contains 19 conserved cysteine residues that appear to mediate the formation of 8 disulphide bonds (*Barreda et al., 2004*). From the amino terminus, there is an immunoglobulin-like (Ig-like) domain, a cytokine receptor homology (CRH) module with four conserved cysteine residues and a WSXWS motif that are essential for ligand binding, and three fibronectin type III (FN III) domains (*Anaguchi et al., 1995*). The cytoplasmic domain contains two closely conserved Box 1 and Box 2 regions of sequence homology with other cytokine receptors. This region appears to be essential for signal transduction of mitogenic and proliferative signals (*Barreda et al., 2004*). Additionally, a membrane distal domain, Box 3, contains a STAT3 binding site and appears to be essential for signal transduction of cellular differentiation signals (*Ziegler et al., 1993*). All five splice variants share identical extracellular domains but differ in their C-terminal sequence (*Akbarzadeh et al., 2001*).***Figure 1.3***

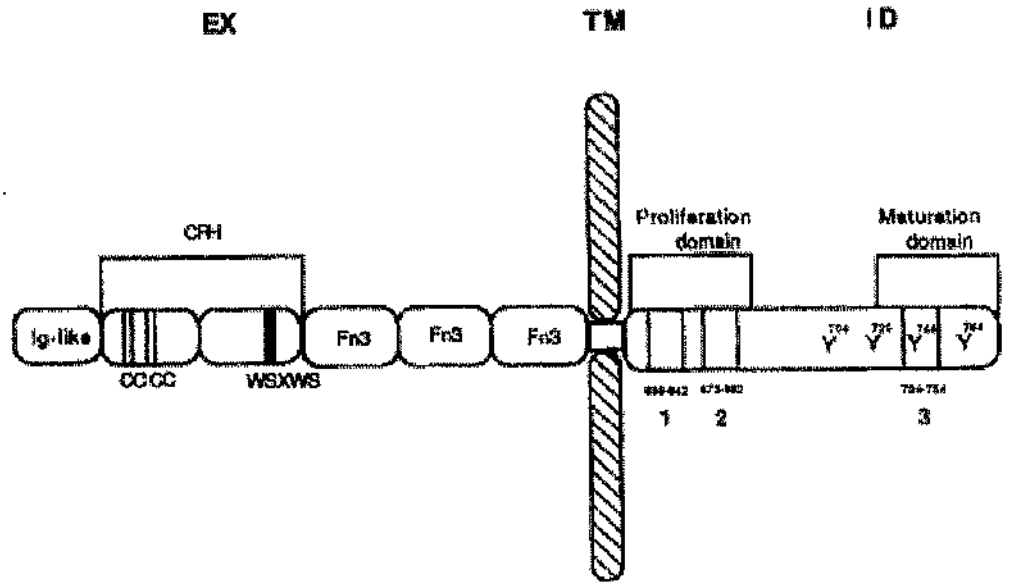

Figure 1.3 *Schematic diagram of the G-CSFR.* ***(Avalos., 1996)***

1.4.2 G-CSF - signal transduction

Following G-CSF binding, there is homodimeristion of the receptor chains resulting in the formation of a tetrameric complex containing two ligand and two receptor molecules. Oligomerization of the receptor chains leads to rapid phosphorylation of four tyrosines in the cytoplasmic region of the human G-CSFR (Y704, Y729, Y744 and Y764) (*Fukunaga et al., 1991*). These phosphorylated residues subsequently serve as docking sites for SH2 or phosphotyrosine binding (PTB) domains found in a variety of intracellular proteins (*Nicholson et al., 1994*). Specifically, JAK1, JAK2, Tyk2 and the Src kinases $p55^{lyn}$ and $p56/59^{hck}$ are recruited to the receptor where they become activated. This in turn, leads to phosphorylation and activation of STAT1, STAT3, and STAT5 as well as the $p21^{ras}$/Raf/ERK pathway *(Akbarzadeh et al., 2001).*

1.4.3 GM-CSFR - structure

GM-CSFR's are expressed in low numbers (~50-500/cell) in human and murine macrophage, neutrophil and eosinophil lineages (*Metcalf et al,. 1994*), as well as erythrocyte and megakaryocyte precursors, B and T foetal lymphocytes, vascular endothelial cells, fibroblasts, osteoblast-like cells and uterine cells (*Jubinsky et al., 1994*). Receptor activation leads to proliferation, differentiation and activation of the heamatopoietic cells, an effect most predominant in the neutrophil and macrophage lineage (*Rasko et al., 1997*).

The GM-CSFR is a member of the gp140 family of type I cytokine receptor group (*Barreda et al., 2004*) and is composed of two distinct chains, α and β. Splice variants exist for both the α- and β- chains (*Smithgall et al.,1998*). The most predominant α-chain consists of a 298 amino acid extracellular domain, 26 amino acid transmembrane domain, and a short cytoplasmic domain of 54 amino acids (*Nicola., 1991*) The extracellular domain contains a 100 amino acid N-terminal region followed by a cytokine receptor domain containing four conserved cysteine residues and a WSXWS motif common to other members of the receptor family (*Nicola., 1991*). The extracellular domain also contains 11 potential N-glycosylation sites which are necessary for both ligand binding and signal pathway initiation (*Ding et al., 1995*).

Two isoforms of the human βc-chain have been identified, the most predominant being a full length transcript 880 amino acids long, possessing three potential N-glycosidation sites (*Hayashida et al 1990*). The βc- chain can be subdivided into a 200 amino acid extracellular domain that contains four highly conserved cysteine

residues in the membrane-distal portion of the domain and a WSXWS motif in the membrane-proximal region. A single transmembrane domain links the extracellular portion to the 432 amino acid sequence of the cytoplasmic domain (*Rasko et al,.1997*).

The membrane proximal domain of the βc chain binds members of the JAK family and is essential for the induction of STAT5, cmyc, pim-1, and *cis* (*Sato et al., 1993*). The membrane distal region domains appear to be responsible for the major tyrosine phosphorylation of proteins that ultimately promotes c-fos and c-jun transcription, the induction of the Shc-Ras-ERK and phosphatidylinositol 3-kinase (PI3K) pathways, and prevention of apoptosis (*Sakamaki et al., 1992*). Membrane distal regions have also been implicated in the induction of differentiation and the negative regulation of receptor activation (*Nicola., 1991*).

The α-chain constitutes the main chain responsible for initially binding GM-CSF but does do with low affinity (*Shanafelt et al., 1992*). Although the βc chain is not involved directly with GM-CSF binding, it plays an essential role in mediating high affinity binding of GM-CSF to the receptor by forming a complex with the α-chain. (*Park et al., 1992*). ***Figure 1.4***

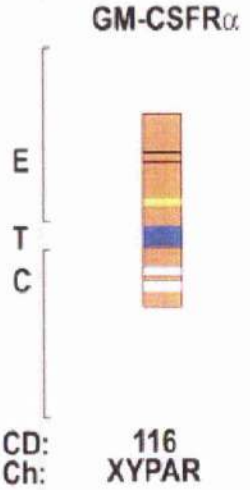

Figure 1.4 Schematic diagram of the GM-CSFR. *The extracellular domain (E) contains a membrane proximal WSXWS motif (yellow) and a homology module containing 2 fibronectin type III domains with paired cysteine residues (solid black lines). The βcE domain is similar but larger, with 2 homology modules and 2 pairs of cysteine residues. The transmembrane domain of each receptor is blue. The white boxes represent Box1 and Box2 JAK binding sites.* ***(Shearer et al. 2003)***

1.4.4 GM-CSFR - signal transduction

As has been discussed, GM-CSF initially binds the with low affinity to the α chain of the GM-CSFR which then leads to a high affinity binding state following formation of the α-/βc-subunit complex. When this complex is formed, a conformational change occurs resulting in activation of the receptor (*Woodcock et al., 1999*).

The α- or β- subunit cytoplasmic domains do not possess any intrinsic enzymatic activity therefore all downstream signalling events require the association of cytoplasmic proteins with tyrosine kinase activity, including JAKs (*D'Andrea et al., 2000*). The exact mechanism by which JAKs are activated following receptor oligomerization is undefined. However, it is speculated that JAKs constitutively associate with the Box-1 membrane-proximal region of the receptors cytoplasmic

domain resulting in trans-phosphorylation, a process augmented following formation of the ligand receptor complex (*D'Andrea et al., 2000*).

The activated JAKs, predominantly JAK2, proceed to initiate tyrosine phosphorylation of specific receptor cytoplasmic domain residues. This provides docking sites for a variety of Src homology (SH2) domain-containing proteins, including the cytoplasmic signalling proteins Shc and STATs activate further downstream signalling cascades (*Itoh et al., 1998*).

1.5 Negative regulation of G-CSF and GM-CSF signalling

Current literature focuses mainly on the negative regulatory pathways involved in G-CSF receptor binding and it appears relatively little research has focused on specific mechanisms involved in attenuation of the GM-CSF receptor signal. However, common to events involved in down-regulation of signalling from both receptors is involvement of a family of signalling proteins named "suppressors of cytokine signalling" or "SOCS".

1.6 SOCS

SOCS proteins are STAT-sensitive gene products which are induced as part of the cellular response to cytokine stimulation and are thought to act within a classical negative feedback loop to both inhibit activation of signalling pathways and target signalling components for proteasomal degradation (*Zhang, et al., 1999*). The classical inhibitory feedback mechanism of SOCS protein function is represented in ***Figure 1.5***, which shows that cytokine signalling induces the expression of SOCS proteins through the JAK–STAT signalling pathway (*Hanada et al., 2003*).

SOCS proteins comprise a family of at least eight members which include CIS (cytokine inducedSH2 containing protein) and SOCS1-SOCS7 (*Alexander., 2002*). All SOCS proteins have a central SH2 domain and relatively well-conserved amino acid sequences that form the SOCS box or CIS homology (CH) domain. SOCS1 and SOCS3 also have unique 30 amino acid domains at the N-terminal side of the SH2 domain, named the kinase inhibitory region (KIR) (*Kubo et al., 2003*). SOCS4, SOCS6 and SOCS7 are less well studied and are not discussed in detail here.

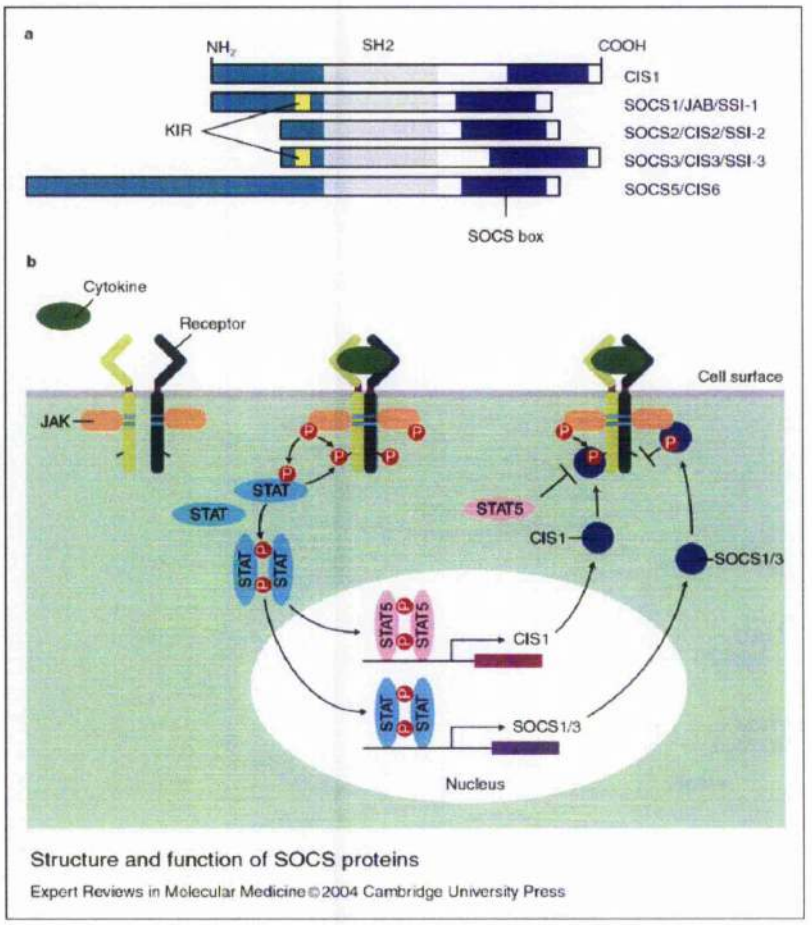

Figure 1.5 *Structure and function of SOCS proteins. Domain organisation of SOCS family members.*

1.6.1 Induction of SOCS proteins

In inactivated cells SOCS proteins exist at very low, almost undetectable levels (*Alexander., 2002*). Their induction is dependent on cellular stimulation resulting in activation of the JAK/STAT pathway (*Kubo et al., 2003*). Many cytokines are capable of SOCS induction, such as G-CSF and GM-CSF described above. However, certain cytokines that do not signal via JAKs and a number of non-cytokine ligands can also induce SOCS gene expression such as TNF and LPS signalling via NF-κB and MAP kinase pathways (*Ilangumaran et al., 2004*). Thus, SOCS induction may provide a mechanism by which multiple stimuli can regulate cytokine receptor signalling.

It has become apparent that the STAT family of transcription factors contribute significantly to the transcriptional upregulation of the CIS, SOCS1 and SOCS3 genes (*Krebs et al., 2001*). The promoter of CIS contains four STAT5-binding sites, all of which are required for erythropoietin (Epo)-dependent activation of the CIS promoter in reporter assays (*Matsumoto, et al., 1997*). Also, the SOCS1 promoter contains putative binding sites for STAT1, STAT3 and STAT6-binding sites (*Krebs et al., 2001*). Expression of SOCS3 is also STAT-regulated, the SOCS3 promoter containing a single STAT1/STAT3 binding element (*Krebs et al., 2002*).

SOCS3 expression was also found to be increased in human leukocytes following treatment with a combination of interleukin-10 (IL-10) and cAMP-elevating agonists, including prostaglandin E_2 (PGE_2), PGE_1, forskolin, dibutryl cAMP (dbcAMP) and cholera toxin (*Gasperini et al., 2002*). PGE_2 and dbcAMP prolonged the stability of SOCS3 mRNA isloated from human leukocytes and it was shown that inhibitors of cAMP-dependent protein kinase A (PKA) inhibitors (H89, KT5720 and St-Ht31

peptide) did not influence the action of PGE_2, dbcAMP and or IL-10 in the SOCS3 signal. This implied that SOCS3 is regulated via a PKA-independent route in these cells (*Gasperini et al., 2002*). This shows cAMP-elevating agents are capable of SOCS3 induction and furthermore, SOCS3 may be involved in the anti-inflammatory effects of cAMP-elevating drugs.

1.6.2 SOCS inhibition of cytokine signaling

SOCS proteins can inhibit signalling via multiple mechanisms. For example, they may comparatively inhibit the binding molecules to a Tyr phosphorylated receptor protein, thereby blocking interaction with downstream signalling proteins (*Ram, et al., 2000*). Alternatively, SOCS1 and SOCS3 can directly inhibit JAK kinase activity using their extended SH2 subdomains and their kinase inhibitory regions (KIR), two functional domains lacking in other family members (*Sasaki, et al., 1999*). SOCS1 has a high affinity for directly binding to Tyr phosphorylated JAKs, whereas SOCS3 binds preferentially to receptor phosphor-Tyr's (*Eyckerman, et al., 2000*). Another proposed mechanism of inhibition by SOCS involves interaction of the SOCS box with elongins B and C of the E3 ubiquitin ligase complex, which targets bound protein for proteosomal degradation (*Kamura, et al., 2000*). In support of this, the SOCS box of SOCS-1 has been shown to accelerate the ubiquitin-dependent proteolysis of TEL-JAK2 (*Kamizono et al., 2001*). It is believed that elongin-C links SOCS proteins and E3 ligase activity, thus targeting degradation by the proteosome (*Johnstone et al., 2004*). This has been supported by evidence that shows mutations or post-translational modifications of SOCS1 that disrupt interaction with elongin-C stabilise the protein (*Kamura et al., 1998*).

A dual function for SOCS has been identified in cytokine receptor signalling based on the interaction of SOCS3 with the Ras inhibitor p120rasGAP (*Cacalano et al., 2001*). The result is sustained ERK signalling which inhibits cell proliferation without hindering cell survival (*Ilanguraman et al., 2004*).

1.6.3 SOCS3

With respect to G-CSF, current literature suggests that SOCS3 is the most likely candidate as a physiological regulator of cellular responses to this cytokine. Evidence in support of this includes studies that have shown SOCS3 is induced in primary myeloid cells stimulated with G-CSF (*Hortner, et al., 2002*).

G-CSF stimulation results in the specific phosphorylation of STAT3, and less commonly STAT5 and STAT1 (*Tian, et al., 1994*). Recently, hematopoietic stem cells extracted from STAT3-deficient mice were found to contain trace levels of SOCS3 and did not show up-regulation of SOCS when stimulated with G-CSF (*Kamezaki, et al., 2005*). The same study found STAT3-null bone marrow cells displayed a significant activation of ERK1/ERK2 phosphorylation under basal conditions and this level was greatly enhanced following treatment with G-CSF. Furthermore, treatment with a MEK inhibitor resulted in a marked decrease in the proliferation of STAT3-null cells stimulated with G-CSF (*Kamezaki, et al., 2005).* It has therefore been concluded that STAT3 functions as a negative regulator of G-CSF receptor signalling by inducing SOCS3 expression and that ERK activation is the main factor responsible for inducing the proliferative response of hematopoietic cells to G-CSF (*Kamezaki, et al., 2005).*

SOCS3 binds selectively to phosphorylated tyrosine 729 (Y729) of the human G-CSFR (*Hortner et al., 2002*) and mutational studies have shown that Y729 regulation of STAT signalling is important for normal G-CSFR function in primary cells (*Hermans et al., 2002*).

1.7 SOCS-independent mechanisms of inhibition of cytokine signalling independent of SOCS

At least two classes of inhibitors other than SOCS are known to contribute to the negative regulation of cytokine receptor signalling. These include protein tyrosine phosphatases such as SH2-domain-Tyr-phosphatase (SHP) proteins, and protein inhibitors of activated STATs (PIAS).

SHP proteins are constitutively expressed and are able to attenuate cytokine signal transduction by dephosphorylating signalling intermediates such as JAKs, STATs and cytokine receptors (*Rakesh et al 2005*). There are two members of the mammalian SHP family, SHP-1 and SHP-2, both of which bind phosphotyrosine residues by their SH2 domains on a variety of cytokine receptors to inhibit signal transduction. For example, SHP-1 can negatively regulate cytokine signalling by dephosphorylating signalling components such as the interleukin-4 (IL-4) receptor (*Kashiwada et al., 2001*), the stem cell factor receptor c-kit (*Yi et al., 1993*) the erythropoeitin receptor (*Klingmuller et al., 1995*) and JAK2 (*Yetter et al., 1995*). SHP-1 is also capable of an SH2-independent interaction with the insulin receptor (*Uchida et al,. 1994*) and with JAK2 (*Jiao et al., 1996*). In contrast SHP-2 acts mainly as a positive regulator of signalling (*Neel et al., 2003*) although some evidence argues for the existence of SHP-

2-dependent inhibition of cytokine signalling via interaction with the gp130 receptor for IL-6-family cytokines (*Symes et al., 1997*).

Members of the PIAS family are also constitutively expressed and attenuate signal transduction by suppressing STAT activity. The PIAS family contains five members, PIAS1, PIAS3, PIASy, PIASxα and PIASxβ (*Chen et al., 2004*). Each member of the PIAS family has a unique method of inhibiting STAT signalling. PIAS1 and PIAS3 are known to interact directly with STAT1 (*Chung et al., 1997*) and STAT3 (*Liu et al., 2001*) respectively, thereby inhibiting STAT association with DNA. PIASx or PIASy do not prevent their target STAT associating with DNA and therefore must act by another mechanism, although this remains undefined. However, experiments carried out in pursuit of this mechanism have suggested that PIAS proteins may function as E3 SUMO ligase proteins which function in a manner analogous to the role of E3 ubiquitin ligase in ubiquitination (*Johnson et al., 2001*). E3 ligase activity has been demonstrated for all members of the PIAS family and STATs have been identified as a target for this activity (*Johnson et al., 2001*). PIAS1, PIAS3 and PIASx all sumoylate STAT1 on Lys-703, close to the JAK phosphorylation site (Tyr 701), thereby targeting STAT1 for ubiquitination and terminating STAT signalling (*Rogers et al 2003*).

1.8 cAMP signalling and inflammation

Acute inflammation is defined as a short-lived inflammatory response that is localised to the site of tissue invasion or trauma. In contrast, inflammation becomes pathogenic when it occurs at an inappropriate site, or is excessive in extent or duration. Under

conditions of pathological stimulation, PMN bactericidal effector functions are non-specifically directed against the host's healthy tissue resulting in extensive collateral damage (*Lawrence et al., 2002*). It was postulated that under conditions of excessive collateral damage there may be accumulation of intracellular intermediates that act to down-regulate the inflammatory response. It was further assumed that such extracellularly accumulated intermediates might trigger increased intracellular levels of the immunosuppressive second messenger cyclic adenosine 3',5'-monophosphate (cAMP), leading to inhibition of overactive immune cells (*Torgersen et al.*, 2002).

The A_{2A} adenosine receptor ($A_{2A}AR$) has been established as a negative regulatory receptor of inflammation, an effect thought to be mediated via elevation of cAMP levels. Studies point to existence of a physiological immunosuppressive loop in which disturbance of local tissue by inflammatory stimuli results in local hypoxia and the accumulation of extracellular adenosine. This in turn, acts on the $A_{2A}AR$ leading to cAMP accumulation and suppression of the immune response (*Lukashev et al, 2004*).

A vast body of literature supports the anti-inflammatory effects of cAMP and as such there is huge interest in the pathways of induction and degradation of this nucleotide.

1.8.1 cAMP signalling systems

cAMP is a ubiquitous second messenger produced in cells in response to a variety of stimuli by the conversion of ATP by a family of membrane proteins called adenylyl cyclases (AC) (*Rang et al.,2002*). cAMP is inactivated by hydrolysis to 5'-AMP by

the action of a group of enzymes called phosphodiesterases (PDEs), which serve an important role in terminating cAMP signalling in cells (*Houslay et al., 2003*).

cAMP is produced downstream of activated G-protein coupled receptors (GPCRs), a family of receptors which consist of seven transmembrane α-helical domains. GPCRs transduce signals by activating heterotrimeric G-proteins, which consist of three subunits, α, β, γ (*Rang et al., 2001*). The G-protein produces effects by cycling between inactive GDP- and active GTP-bound states. In the inactive state the G-protein is anchored to the membrane where it exists as an αβγ complex, with GDP occupying the site on the α-subunit (*Rang et al., 2001*). Following receptor activation, a conformational change occurs involving the cytoplasmic domain of the receptor causing it to acquire high affinity for the trimeric G-protein. Association of αβγ with the activated receptor promotes GDP dissociation, allowing GTP to bind (*Rang et al., 2001*). This causes disruption of the trimer to release α-GTP and βγ-subunits; these are the active forms of the G-protein, which can associate with a multitude of effector proteins. GDP dissociation is the rate-limiting step of G-protein activation, thus activation is terminated when the hydrolysis of GTP to GDP occurs through an intrinsic GTPase activity of the α-subunit. The GDP bound α-subunit dissociates from associated effects and reforms the trimeric complex with βγ to complete the cycle (*Rang et al., 2001*).

G-proteins regulate the activity of various membrane enzymes and ion channels. G-proteins may be stimulatory (G_s) or inhibitory (G_i). G_s-stimulated AC activity is

responsible for production of cAMP, which in turn initiates activation of a variety of cellular proteins.

1.9 Intracellular cAMP sensors

cAMP has been traditionally thought to signal exclusively through cAMP-dependent protein kinase (PKA). However, PKA-independent actions of cAMP have been recognised in various experimental systems including cyclic nucleotide-gated ion channel activation and the exchange proteins directly activated by cAMP (Epacs1 and 2) (*de Rooij, et al., 1998*). All these proteins bind cAMP through conserved cAMP binding domains (*Dremier et al., 2003*).

1.9.1 PKA

For many years, PKA was thought to be the only intracellular target of cAMP. PKAs are present in most cells as two isoenzymes, PKAI and PKAII, which are tetramers compose of two catalytic (C) and two regulatory (RIα,β or RII α,β) subunits (*Kopperud et al., 2003*). The R subunit of PKA modulates its kinase activity indirectly by binding to the A-kinase anchor protein (AKAP) family (section 1.9.2). AKAPs are known to bind to the RII regulatory subunit to orchestrate activation of PKA (*Michel et al., 2002*). Each R subunit has two cAMP-binding domains (site A and site B). Upon binding of two cAMP molecules on both R subunits, the inactive tetramer is dissociated into one dimer of R subunits and two active C subunit monomers which can then phosphorylate various cytoplasmic and nuclear target proteins such as cAMP response element binding protein (CREB) (*Kopperud et al., 2003*). ***Figure 1.6***

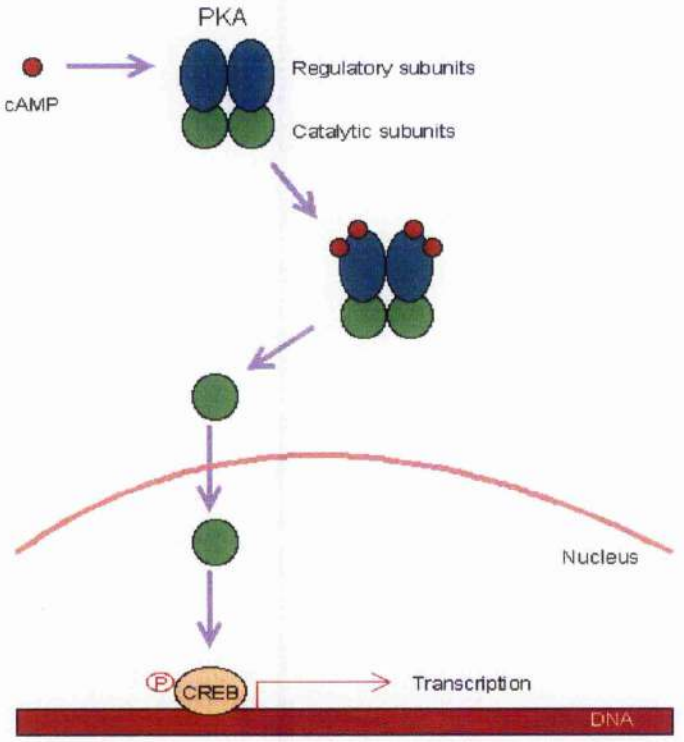

Figure 1.6 *PKA/cAMP signaling pathway: The active C subunit phosphorylates CREB protein resulting in transcription.*

1.9.2 AKAPs

GPCRs are capable of stimulating a variety of intracellular transduction pathways. However, its essential fidelity is maintained between each pathway in order to produce the correct physiological response *(Michel et al., 2002)*. This requires the accurate selection of effector molecules for regulated activation and deactivation and a principle strategy in achieving this selection specificity is compartmentalization of signalling enzymes (*Michel et al., 2002*).

AKAPs (A-kinase anchoring proteins) provide compartmentalisation of multivalent signalling processes (*Malbon et al., 2004*). These proteins bind to membrane bound GPCRs to orchestrate the interactions of a variety of proteins such as protein kinases

and protein phosphatases. For example, AKAPs bind to the regulatory subunit of PKA to direct the kinase to discrete intracellular locations (*Kopperund et al., 2003*).

Recently, functional studies aimed at disrupting AKAP–PKA complexes have demonstrated a role for anchored PKA in various cellular processes, including gene transcription, hormone-mediated insulin secretion and ion-channel modulation *(Alto et al., 2002)*. By binding to additional signalling molecules, AKAPs might function to coordinate multiple components of signal-transduction pathways (*Malbon et al., 2004*).

1.9.3 Epacs

In 1998, two new cAMP receptors were identified and named Epac1 and Epac2 (exchange protein directly activated by cAMP). Epac proteins function as GEFs which, upon cAMP binding, specifically activate the small G-proteins Rap1 and Rap2. Epac1 and Epac2 have one and two cAMP-binding sites respectively, which are located in the N-terminal part of the proteins. Epac1 is ubiquitously expressed and Epac2 is found predominantly in the brain and adrenal glands (*Kawasaki, et al.*, 1998).

Insights gained from structural modelling studies have lead to development of an Epac-selective cAMP analogue called 8-(4-chloro-phenylthio)-2'-O-methyladenosine-3', 5'cyclic monophosphatase or 8CPT-2-O-Me-cAMP (*Enserink et al., 2002*). This agent has allowed differentiation between Epac-dependent and PKA-dependent cAMP effects. Use of this selective activator has revealed that Epac's participate in various aspects of cell function. For example, Epac-1 has been found to play a functional role in integrin-mediated cell adhesion in non-myloid cell lines (*Enserink*

et al., 2004), while Epac-2 appears to be important in pancreatic β-cell insulin granule exocytosis (*Holz., 2004*). Both Epac-1 and Epac-2 have been implicated in the regulation of vascular endothelial barrier function (*Cullere et al., 2005*).

1.10 Experimental Hypothesis

Increased levels of cAMP are able to attenuate cytokine signalling in endothelial cells (*Sands et al., submitted for publication*). Furthermore, it has been established that an increase in cAMP is coupled with an increase in levels of SOCS proteins, in particular SOCS3. What has yet to be elucidated, is whether this response occurs in other cell types, this aim forms the basis of this project where it is also hoped to establish the mechanism by which SOCS3 is induced.

CHAPTER 2

Materials and Methods

2.1 Chemicals and Suppliers

All reagents were of the highest grade commercially available and obtained by the following suppliers:

Abcam plc.(UK)

phosphoCREB antibody (#ab3419) stock 1μg/ml

BDH biochemical, Poole, England (UK)

HEPES

Biolog Life Science Institute

8-(4-Clorophenythio)-2'-O-methyl-cAMP

Calbiochem-Novabiochem (UK)

Forskolin, H89, U0126, SB 203580, Jak Inhibitor 1, MG132

Cell Signaling technology

Stat1 Antibody (#9172), Phospho-Stat1 Antibody (#9171), Stat3 Antibody (#9132), Phospho-Stat3 Antibody (#9138), p44/42 MAP Kinase Antibody (#9102), Phospho-p44/42 MAPK Antibody (#9106), Rabbit anti-phosphoThr180/Tyr182-p38 Anitbody

CN Biosciences, Merck Biosciences Ltd., Beeston, Nottingham,UK

Genejuice transfection reagent

Fisher Scientific, Loughborough, Leicestershire,UK

Sodium dodecyl sulphate (SDS), methanol, ethanol, sodium flouride, concentrated hydrochloric acid

GIBCO BRL Life Technologies, Paisley, UK

Optimem

Invitrogen, Inchinnan Business Park, Paisley, UK

Rainbow marker

Inverclyde Biologicals, strathclyde Business park, Bellshill, Lanarkshire, UK

Protran nitrocellulose (Schleicher & Schuell), pore size: 0.2μm

LGC Promochem, Middlesex, UK

HL60 cells, U937 cells

Pierce, Rockford, IL, U.S.A

Western Blot stripping solution

Perkin Elmer, Wellesley, MA, U.S.A

Enhanced chemiluminescence (ECL) solutions

Roche Molecular Biochemicals/ Boehringer-Mannheim, Mannheim, Germany

Tris [hydroxymethyl]aminomethane;(Tris)

R&D Systems, Minneapolis, MN, U.S.A

G-CSF, GM-CSF

Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A

SOCS-3 antibody (#sc-7009) stock 200μg/ml

Sigma-Aldrich Company Ltd., Poole Dorset

Soybean trypsin inhibitor, benzamidine, bovine serum albumin, thimerosal, bromophenol blue, N,N,N',N'-tetramethylethylenediamine (TEMED), RPMI, Dulbecco's modified Eagle's medium (DMEM), trypsin, penicillin/streptomycin, L-glutamine, FBS, Anti-rabbit antibody (#A5420), Anti-goat antibody (#A5420), Anti-mouse (#A4416), acrylamide/bisacrylamide, PMSF, Triton X-100, Tween-20

Whatman international Ltd., Maidstone, Kent

Filter paper (Schleicher & Schuell), Ref: 10382658

SOCS3 positive control pcDNA/myc epitope-tagged human SOCS3 constructs were initially prepared in-house at Queens University, Belfast U.K., by Professor Jim Johnson.

2.2 Cell culture

2.2.1 Cell Maintenance

Cells were grown at 37°C in a humidified atmosphere containing 5% (v/v) CO_2.

Human promyelocytic HL60 and pre-monocytic U937 cells were maintained in RPMI medium supplemented with 10% (v/v) FBS, 1mM L-glutamine, 100 units/ml penicillin and 100μg/ml streptomycin. Cells were grown in suspension culture and maintained by replacement of medium every two to three days. Cells were cultured in T-1000 flasks, each flask containg 25ml of cell suspension. Cells were split by removing 20ml of cell suspension and replacing this with 20 ml fresh RPMI.

Human embryonic kidney 293 cells (HEK 293) cells were maintained in DMEM supplimented with 10% (v/v) FBS, 1mM L-glutamine, 100 units/ml penicillin and 100μg/ml streptomycin. Cells reached confluence every two to three days. Confluent monolayers of cells were washed thoroughly with 5ml PBS before being treated with 1ml trypsin. Cells were exposed to trypsin for up to 30 seconds before the flask was shaken to lift the monolayer of adherent cells from the base of the flask. 9ml of fresh DMEM was added to the flask to neutralise the trypsin. 9ml of this suspension was then removed and replaced with fresh trypsin resulting in a 1 in 10 dilution of cells.

2.3 Counting cells

HL60 and U937 cells were counted and plated onto a 6-well plate on the day of each stimulation. 1x 10^6 cells were required per well of a 6-well plate for each stimulation. Cells were counted using the haemocytometer method to estimate the cell population in each flask. The volume of suspension extracted from each flask varied daily

according to cell confluence. Once removed, the cell suspension was centrifuged at a speed of 100g for 2mins. The supernatent media was removed and replaced with 1ml fresh RPMI per $1x10^6$ cells. Cells were then plated on to a 6 well plate at 1ml per well.

2.4 Transient expression of SOCS3 in HEK 293 cells

Genejuice was used a the transfection agent to transfect pcDNA3/myc epitope-tagged human SOCS3 into HEK 293 cells. For a 6-well plate, DNA was transfected at a concentration of 2µg/well. 1.2ml Optimem was placed into a sterile microfuge tube with 7µl Gene Juice, vortexed and left to incubate for 5 minutes. 2µl of SOCS3 plasmid DNA (pCDNA/Flag-SOCS-3) was then added to the microfuge tube, the tube was vortexed briefly and left to incubate for 15min at room temperature. 200µl of the DNA-Genejuice mix was then added to each well containing 1ml of fresh medium and left to incubate overnight. Medium was replaced 24hr post-transfection and cells used after a further 24 hours.

2.5 Cell Stimulations

Undifferentiated U937 or HL60 cells were used for stimulations. Every stimulation experiment was carried out in a 6 well plate containing 1ml of cells at a concentration of $1x10^6$ per well. The agents used were as follows:

Figure 2.1 *Table of Treatments of monolytic cell lines*

AGENT	CONCENTRATION	INCUBATION PERIOD
Rolipram	10μM	6hr
Forskolin	10μM	6hr
H89	5μM	6hr or 15min
6Be	50μM	6hr or 15min
8P-CPT	0.1mM	6hr or 15 min
G-CSF	10ng/ml	30min
GM-CSF	10ng/ml	30min
SB	10mM	Pre-treat 30min + 6hr
UO126	1μM	Pre-treat 30min + 6hr
Jak inhibitor 1	0.5μM	Pre-treat 30min + 6hr
MG132	3μM	Pre-treat 30min

2.5.1 Preparation of cell lysates

At the end of the stimulation period, 6 well plates were placed on ice to terminate the reaction. The well contents were then transferred directly into cooled, sterile microfuge tubes and centrifuged at 8000g for 30secs. The excess media was removed and the pellet washed in ice-cold PBS before being centrifuged at 40,000g for 30secs,

this step was repeated to ensure all media was washed off. Following the second wash, the pellet was solubilised in 50µl RIPA (50mM sodium Hepes (pH7.5), 1% (v/v)Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 0.15M sodium chloride, 5mM EDTA, 10mM sodium flouride, 10mM sodium phosphate, 10µg/ml soybean trypsin inhibitor, 10µg/ml benzamidine, 0.1mM PMSF and 0.02% (w/v) protease inhibitor cocktail). The lysate was centrifuged at 40,000g for 15 min and the supernatent transferred into sterile microfuge tubes and frozen at -80°C. The samples were defrosted for protein normalisation and immunoblotting as described in section 2.5.1 and 2.5.2.

2.5.2 Protein normalisation by Bicinchoninic acid (BCA) protein assay

Duplicate 0.01ml samples of known BSA standards in the range of 0-2 mg/ml, along with unknown protein samples were loaded onto 96-well plate. 0.2ml BCA solution (1% (w/v) 4,4 dicarboxy-2,2 biquinolone disodium salt, 2% (w/v) sodium carbonate, 0.16% (w/v) sodium potassium tartrate, 0.4% (w/v) copper (II) sulphate) were added to each well. A protein concentration-dependent reduction of Cu^{2+} to Cu^{1+} was indicated by a colour change from green to purple. This is quantified by measuring absorbance at 492nm. The colour was left to develop for 15 min before absorbance of the standards was measured and used to plot a best-fit straight line from which the unknown protein samples were calculated using Revelation software run by a PC linked to a platereader.

2.6 Laboratory techniques

2.6.1 SDS-PAGE and immunoblotting

Samples prepared for SDS-PAGE were equalised for protein using the bicinchoninic acid assay described in Section 2.5.3. Pre-stained protein markers (Invitrogen Rainbow Markers, range 6.5-175kDa) in sample buffer (50mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, 12% (w/v) SDS, 0.0001% (w/v) bromophenol blue, 1mM DTT) were also prepared in electrophoresis sample buffer to estimate protein molecular mass.

40µl of sample was equalised for protein content and subsequently subjected to discontinuous SDS-PAGE using a 6 cm 10% (w/v) polyacrylamide resolving gel (10% (w/v) acrylamide, 0.3% (w/v) bisacrylamide, 0.4M Tris (pH8.8), 0.1% (w/v) SDS, 3% (v/v) glycerol, 0.01% (w/v) ammonium persulphate and 0.001% (v/v) TEMED) and a 2cm 3% (w/v) stacking gel (3% (v/v) acrylamide, 0.1% (v/v) bisacrylamide, 0.1M Tris (pH 6.8), 0.1% (w/v) SDS, 0.01% (w/v) ammonium persulphate and 0.0001% (v/v) TEMED). Electrophoresis was carried out using Biorad Mini Protean II or III gel electrophoresis systems in running buffer (27.4mM Tris, 0.10M glycine, 0.1% (w/v) SDS) at 150V until the bromophenol blue dye reached the end of the gel. Proteins electrophoretically transferred from the gel onto a nitrocellulose membrane at 400mA for 45mins in transfer buffer (24.7mM Tris, 0.19M glycine in 20% (v/v) methanol). The nitrocellulose membrane was then neatly trimmed and rinsed briefly in PBS before blocking for 1hr at room temperature in Blotto (5% (w/v) skimmed milk, 0.2% (v/v) Tween in PBS). The membrane was then washed again in PBS before being placed into a plastic envelope with 2ml Blotto containing a 1:1000 dilution of primary antibody. Depending on the primary used, the blot was either be incubated overnight at 4°C in the cold room, or at room temp for

1hr on a rotating platform ***Table 2.2***. The membrane was then rinsed briefly in PBS and washed three times, at 10 min intervals in Blotto. After the final Blotto wash, the membrane was rinsed in PBS and transferred into an envelope containing 2ml Blotto and a 1:1000 dilution of the relevant HRP-conjugated secondary antibody. This was placed on a rotating platform for 1hr at room temperature. The membrane was then removed form the envelope, rinsed in PBS and washed for two 10min washed in Blotto, followed by two 10 min washed in PBS. Membranes were then exposed to an enhanced chemiluminescence (ECL) development procedure. HRP-specific oxidative degeneration of luminal caused emission of light at 428nm, detected by Kodak X-OMAT Blue X-ray film, thereby allowing visualisation of immunoreactive proteins.

Table 2.2 Antibody incubation conditions

PROTEIN	INCUBATION CONDITIONS		BLOTTO	
	1°AB	2°AB	1°AB	2°AB
SOCS3	SOCS3 1:1000 1hr, room temp	Anti-Goat HRP 1:1000 1hr, room temp	5% skimmed milk PBS TWEEN	PBS TWEEN
P-CREB	P-CREB 1:1000 Overnight, cold room	Anti-Rabbbit HRP 1:1000 1hr, room temp	1% BSA TBST	5%MILK TBST
ERK	ERK 1:1000 Overnight, cold room	Anti-Rabbbit HRP 1:1000 1hr, room temp	1% BSA TBST	5%MILK TBST
P-ERK	P-ERK 1:1000 Overnight, cold room	Anti-mouse HRP 1:1000 1hr, room temp	1% BSA TBST	5%MILK TBST
STAT1	STAT1 1:1000 Overnight, cold room	Anti-Rabbbit HRP 1:1000 1hr, room temp	1% BSA TBST	5%MILK TBST
P-STAT1	P-STAT1 1:1000 Overnight, cold room	Anti-Rabbbit HRP 1:1000 1hr, room temp	1% BSA TBST	5%MILK TBST
STAT3	STAT3 1:1000 Overnight, cold room	Anti-Rabbbit HRP 1:1000 1hr, room temp	1% BSA TBST	5%MILK TBST
P-STAT3	P-STAT3 1:1000 Overnight, cold room	Anti-mouse HRP 1:1000 1hr, room temp	1% BSA TBST	5%MILK TBST

2.6.2 Stripping nitrocellulose membranes for re-probing

The membrane was rinsed in PBS to remove traces of ECL solution, then placed in a plastic envelope containing 2ml Western Blot stripping solution. This was placed on a rotating platform and incubated for 30min at room temperature. At the end of this incubation, the membrane was removed from the enveloped, rinsed in PBS and developed using the ECL method described in 2.5.2. To ensure the membrane was stripped of all immunoreactive antibodies. The membrane was then rinsed in PBS and blocked in Blotto for 1hr. After this time, the membrane was rinsed in PBS and placed in an envelope containing 2ml Blotto and a 1:1000 dilution of the relevant primary antibody as described in 2.5.2.

Statistical Analysis

Non-saturating exposures of all immunoblots were analysed using Totallab v2.0 imaging analysis software (purchased from Phoretix, UK).

Statistical analyisis was performed using GraphPad Prism V4.0. One-sample t-tests were performed to determine whether there was a statistically significant difference between mean densitometry readings under various experimental conditions. Mean densitometry was calculated for n=3 for each experimental parameter compared Eg. Average of n=3 densitometry reading for treated Vs. Average of n=3 densitometry reading for untreated cells.

CHAPTER 3

RESULTS

3.1 Introduction

SOCS3 induction can occur via activation if several signalling mechanisms, the most extensively documented route being the JAK/STAT pathway (section 1.6.1). However, a correlation has also been found between elevated levels of cAMP and increased SOCS3 expression. Recent work has shown a link exists between increased levels of cAMP and SOCS3 in vascular endothelial cells leading to a decrease in cytokine signalling (*Sands et al., submitted for publication*). Since cAMP is a potent anti-inflammatory second messenger in many cells, it is desirable to investigate whether this phenomenon is manifested in other cell types. Thus, the main aim of this project is to establish whether cAMP elevation can induce SOCS3 expression in two myeloid cell lines and examine the possible mechanism(s) responsible for this induction as well as the functional consequences for cytokine signalling.

3.2 Results

In order to achieve a more detailed insight into the relationship between increased cAMP and SOCS3, the effects of forskolin (Fsk), an efficacious activator of adenylate cyclase catalytic units, on the activation of SOCS3 protein were investigated. Based on previous literature, Fsk is capable of SOCS3 induction in human neutrophils and PBMC when used in conjunction with IL-10 (section 1.6.1). However, the ability of Fsk alone to induce SOCS3 in myeloid cell lines has yet to be ascertained. To investigate this further time courses and concentration dependence were examined to establish the optimal effects of Fsk on naïve U937 histocytic lymphocytes and HL60 promyelocytic leukaemia cells. Rolipram, a Phosphodiesterase 4 inhibitor, was added to prevent breakdown of cAMP (**Figures 3.1-3.2**)

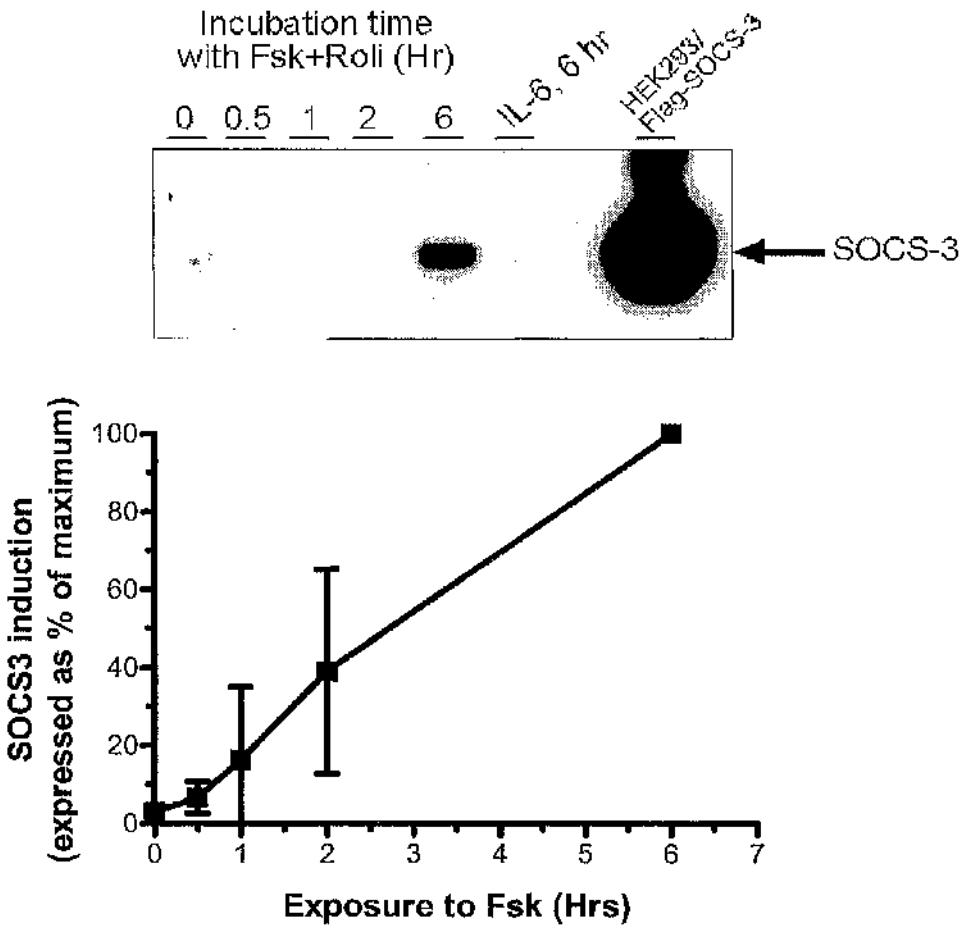

Figure 3.1 **Time course of Fsk induction of SOCS3 expression in U937 cells**

U937 cells were treated with Fsk [10μM] at a range of time points (n=3). Cell lysate was extracted and prepared according to section 2.4.1 and the protein content equalised prior to SDS-PAGE fractionation. The proteins were subsequently transferred onto nitro-cellulose and probed using a SOCS3 antibody. The results show there is a time-dependent increase in SOCS3 expression in U937 cells and that maximum expression occurs at 6hrs. Lysate from IL-6 [100ng/ml] stimulated cells was loaded as a comparison although no SOCS3 protein was expressed. Extract from HEK 293 cells expressing Flag-SOCS3 was used as a positive control for the blot.

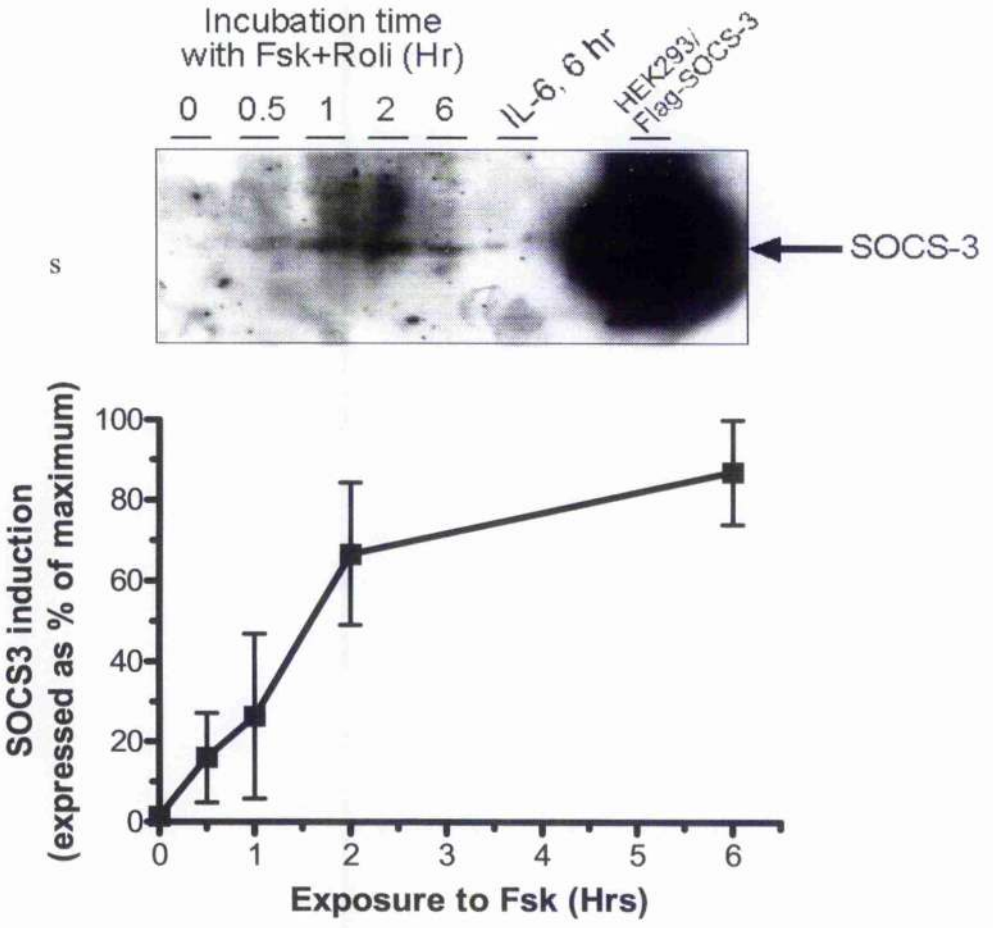

Figure 3.2 **Time course of Fsk induction of SOCS3 expression in HL60 cells**

HL60 cells were treated with Fsk [10μM] at a range of time points (n=3). Cell lysate was extracted and prepared according to section 2.4.1 and the protein content equalised prior to SDS-PAGE fractionation. The proteins were subsequently transferred onto nitro-cellulose and probed using a SOCS3 antibody. The results show there is a time dependent increase in SOCS3 expression in HL60 cells and that maximum expression occurs at 6hrs. Lysate from IL-6 [100ng/ml]-stimulated cells was loaded as a comparison although no SOCS3 protein was expressed. Extract from HEK 293 cells expressing Flag-SOCS3 was used as a positive control for the blot.

Figure 3.1 and 3.2 shows that SOCS3 expression does not plateaux at this 6hr time point in either U937 or HL60 cells. This suggests that maximal expression may occur beyond the 6hr stimulation. It has been shown that both HL60 and U937 cells express SOCS3 in response to a 6hr treatment with Fsk [10μM]. It has also been demonstrated that SOCS3 protein was not detected in cell extract treated from U937 and HL60 cells treated with IL-6 for 6hr (scan densitometry Figure 3.1-3.2). This may be due to the fact that IL-6 induction of SOCS3 occurs transiently at a lower time point, or that SOCS3 induction by IL-6 occurs sometime after the 6hr incubation. HEK293 extract expressing Flag SOCS3 acted as an effective positive control, as huge level of SOCS3 protein was detected. A 6hr Fsk exposure time was used for subsequent concentration dependence analysis of SOCS3 induction in U937 and HL60 cells.

For the purpose of this investigation, it was essential to establish the time point at which SOCS3 is significantly induced. This was determined by observing concentration dependence of Fsk-induced SOCS3 expression in U937 and HL60 cells (**Figures 3.3-3.4**).

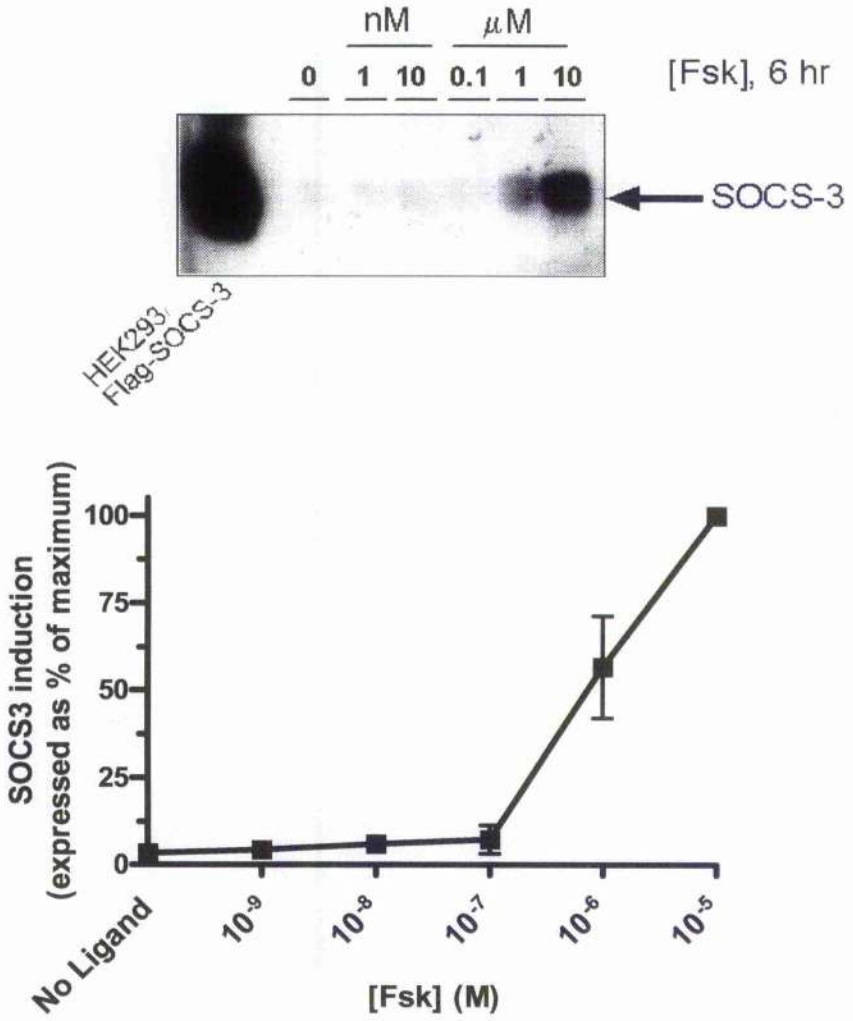

Figure 3.3 **Concentration dependence of Fsk-induced SOCS3 expression in U937 cells.** Undifferentiated U937 cells in a 6-well plate were treated with a range of Fsk concentrations for 6hrs (n=3). Cell lysate was extracted and prepared according to section 2.4.1 and the protein content equalised prior to SDS-PAGE fractionation. The proteins were subsequently transferred onto nitro-cellulose and probed using a SOCS3 antibody. The graph shows there was a concentration-dependent increase in SOCS3 expression when cells are exposed to Fsk for 6hrs and maximum induction of SOCS3 was achieved at 10μM.

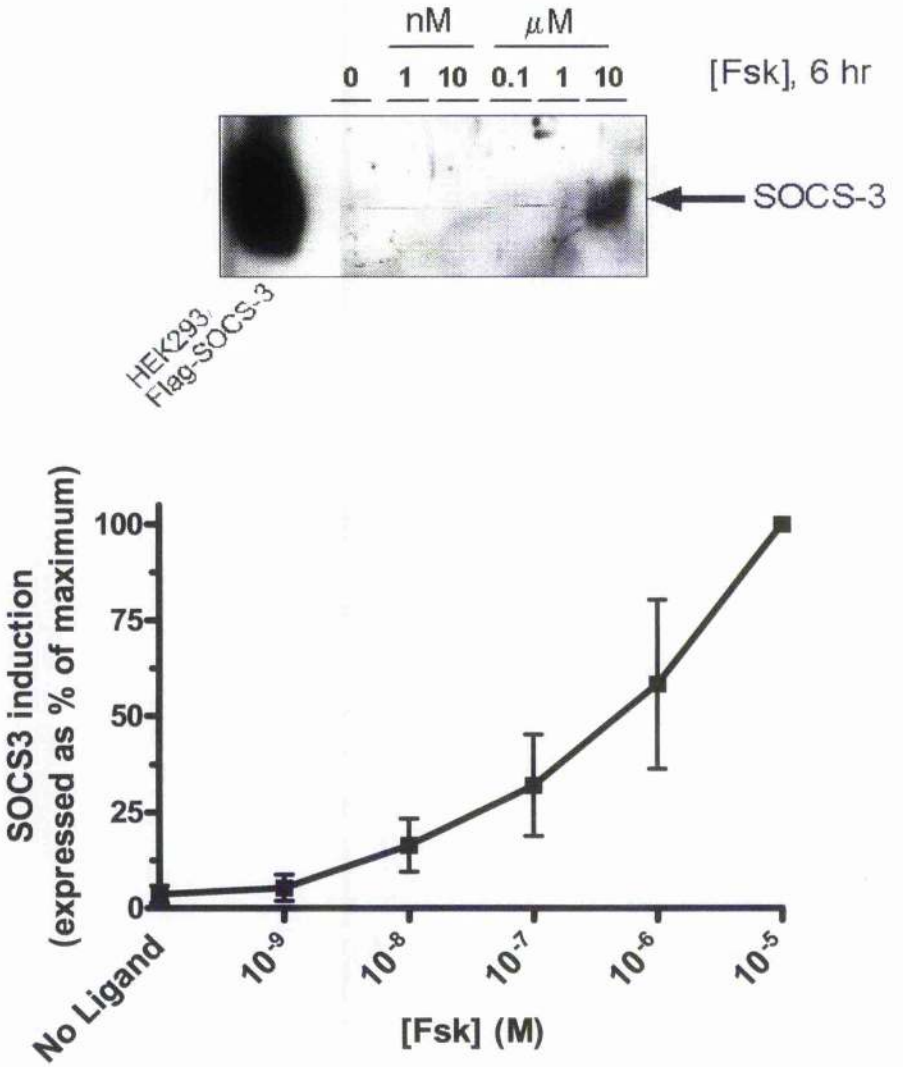

Figure 3.4 **Concentration dependence of Fsk-induced SOCS3 expression in HL60 cells.** Undifferentiated HL60 cells in a 6-well plate were treated with a range of concentrations of Fsk for 6hrs (n=3). Cell lysate was extracted and prepared according to section 2.4.1 and the protein content equalised prior to SDS-PAGE fractionation. The proteins were subsequently transferred onto nitro-cellulose and probed using a SOCS3 antibody. As with U937 cells, there was a concentration-dependent increase in SOCS3 expression. The maximum expression of SOCS3 was achieved at a concentration of 10μM.

SOCS3 protein is subject to rapid turnover in many cell types via proteolysis. This is attributed to the interaction of the conserved SOCS-box domain with Elongins B and C that form part of the ubiquitin ligase complex (*Zhang et al., 1999*) which ubiquitinates and targets associated proteins, and SOCS proteins themselves, for degradation (*Krebs et al., 2001*). It was therefore appropriate to ensure the SOCS3 signal was not subject to degradation. Both cell lines were treated with Fsk [10μM] in the presence of proteasome inhibitor (MG132) at a range of time points. MG132 reduces degradation of ubiquitin conjugated proteins by disrupting proteasomal interaction with elongin B/C of the SOCS3 protein. The results of these experiments are represented in Figures 3.5 and 3.6.

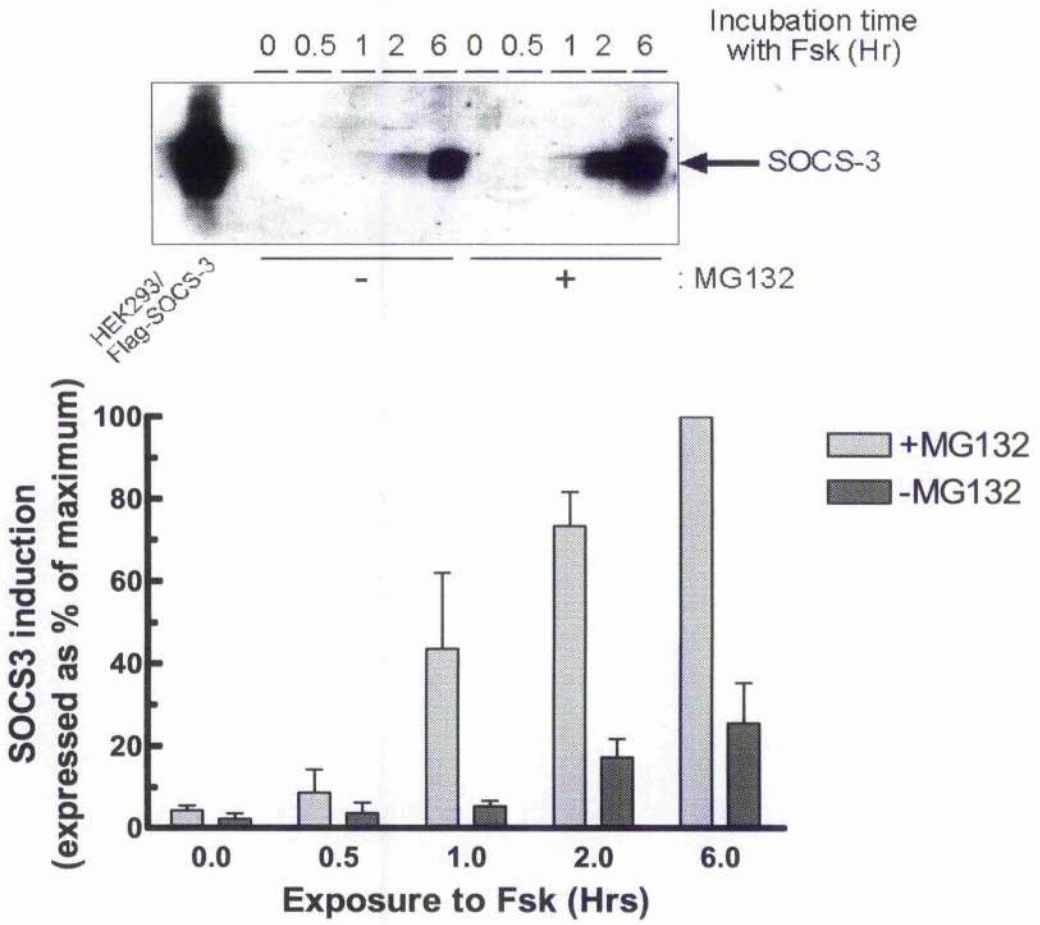

Figure 3.5 **Effect of proteosome inhibitor MG132 on Fsk-induced SOCS3 expression in U937 cells.** U937 cells in a 6-well plate were treated with Fsk [10μM] for 6hrs both in presence and absence of MG132 [3μM] (n=3). Cell lysate was extracted and prepared according to section 2.4.1 and the protein content equalised prior to SDS-PAGE fractionation. The proteins were subsequently transferred onto nitro-cellulose and probed using a SOCS3 antibody. Flag-SOCS3 was loaded as a positive control. The 6hr time point appeared to produce the strongest SOCS3 signal. When the average densitometry measurement of SOCS3 at 6hrs in cells treated with Fsk+MG-132 was compared to cells treated with Fsk alone a p value of 0.07 was obtained. Thus, treating U937 cells with MG132 does not significantly increase the level of SOCS3 induction.

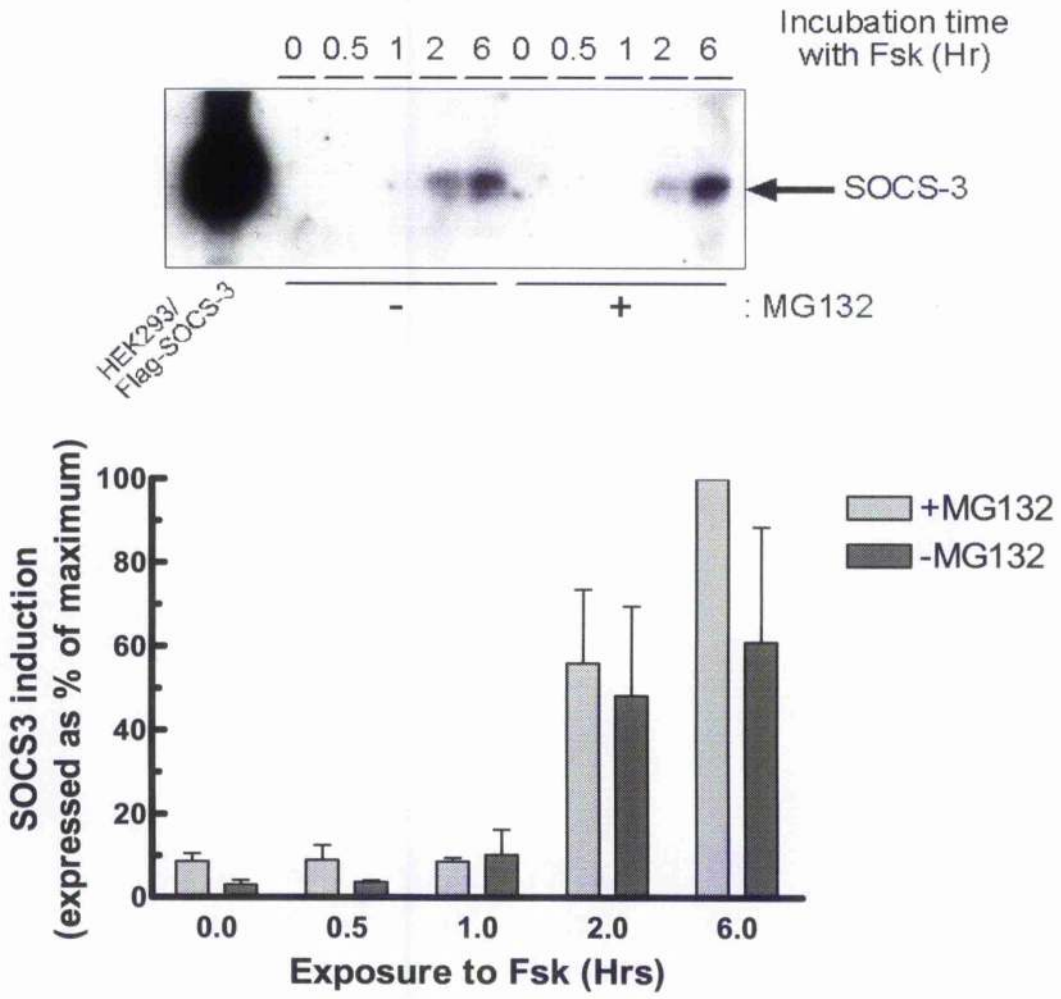

Figure 3.6 **Effect of proteasome inhibitor MG132 on Fsk-induced SOCS3 expression in HL60 cells.** HL60 cells in a 6-well were treated with Fsk [10μM] for 6hrs both in presence and absence of MG132 [3μM] (n=3). Cell lysate was extracted and prepared according to section 2.4.1 and the protein content equalised prior to SDS-PAGE fractionation. The proteins were subsequently transferred onto nitro-cellulose and probed using a SOCS3 antibody. The 6hr time point appeared to produce the strongest SOCS3 signal. When the average densitometry measurement of SOCS3 at 6hrs in cells treated with Fsk+MG-132 was compared to cells treated with Fsk alone a p value of 0.1912 was obtained. Thus, treating HL60 cells with MG132 does not significantly increase the level of SOCS3 induction.

MG132 did not significantly increase SOCS3 expression in U937 or HL60 cells. This suggests that at SOCS3 is not subject to proteasomal degradation in these cell lines following a 6hr treatment with Fsk. Based on these results, MG132 was not used in any other stimulations. Both cell types produced the same pattern of results for both time course and concentration response experiments, however, from this stage it was decided to proceed with U937 cells only. This decision was made based on the fact that U937 cells were capable of inducing SOCS3 expression to greater extent than HL60 cells, as determined by comparison to the SOCS3 positive control run on each gel.

SOCS proteins may be induced by multiple stimuli, not just activators of the JAK/STAT pathway. An efficacious inducer of SOCS3 is lipoplysaccharide (LPS), a component of the outer cell wall of Gram-negative bacteria (Bode et al., 2003). LPS stimulates the TLR4 signalling system to activate intracellular messengers such as NFκB and ERK (Bode et al., 2003). ERK, as has been discussed, is capable of indirect induction of SOCS3 by phosphorylating STAT3 (1.3.2). It is therefore important to demonstrate any positive SOCS3 response was due solely to the effects of forskolin and not bacterial contamination of stock solution. In order to eliminate the effects of LPS contamination on the level of SOCS3, cells treated with and without Fsk were placed in media containing filter-sterilised polymyxin-B as a control. Polymixin B is a bacteriopeptide that binds LPS, thus neutralising its biological effects. The results of this experiment are shown in **Figure 3.7.**

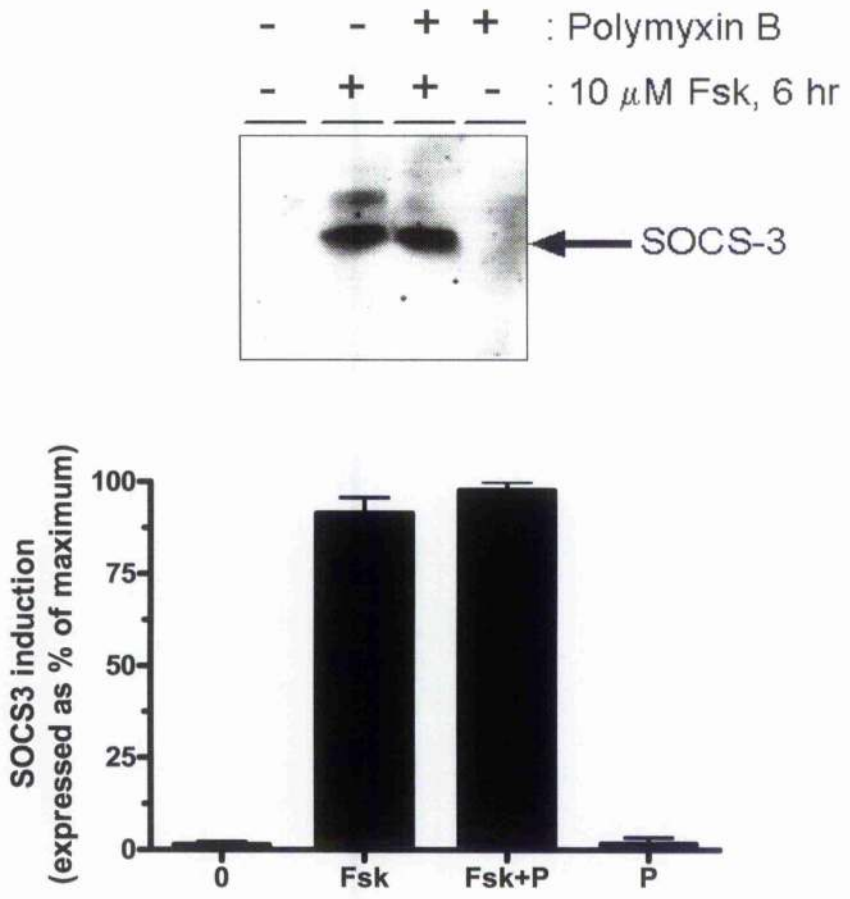

Figure 3.7 **Effect of polymyxin B on Fsk-induced SOCS3 expression.**

U937 cells in a 6-well plate were exposed to Fsk [10μM] for 6hrs both in the presence and absence of Polymyxin B (P) [1μg/ml] (n=3). Cell lysate was extracted and prepared according to section 2.4.1 and the protein content equalised prior to SDS-PAGE fractionation. The proteins were subsequently transferred onto nitro-cellulose and probed using a SOCS3 antibody. A one-sample t-test was performed to compare the level of SOCS3 in cells treated with Fsk Vs Fsk+Polymixin B. A p value of 0.2219 was obtained which confirmed there was no statistically significant difference in levels of SOCS3 induced in cells treated with Fsk + Polymixin B Vs. Fsk alone. Untreated cells and cells treated with Polymixin B alone were used as controls.

There was no statistically significant difference in the level of SOCS3 between cells bathed in Polymixin B media versus polymyxin B- free media. Thus, SOCS3 induction is due solely to Fsk treatment and not due to any bacterial contamination.

3.3 G-CSF and GM-CSF signalling pathways

G-CSF and GM-CSF receptor signalling pathways were chosen to test the functional significance of SOCS3 induction. G-CSFR interaction with SOCS3 has been well documented (*van de Geijin et al., 2004*) and due to functional similarities of the GM-CSFR, it was decided to investigate the functional consequences of SOCS3 induction of the signalling pathways of both receptors.

The literature states that the G-CSFR signals through STAT1, STAT3, STAT5 as well as the Ras/Raf/ERK pathway. In contrast, the GM-CSFR transduction pathway is less well documented. It was hypothesised that both the G-CSFR and GM-CSFR would be capable of SOCS3 induction via STAT activation and that in turn, SOCS3 induction would have a negative feedback mechanism to diminish the level of STAT phosphorylation by each receptor, since STAT activation by SOCS3-targeted receptors should be reduced.

Initially it was important to verify that the JAK/STAT signalling pathway was engaged in U937 cells in response to treatment with G-CSF and GM-CSF. Furthermore, it was hoped to establish if elevated levels of cAMP were capable of diminishing the G-CSF/GM-CSF induced signal thereby inhibiting cytokine signalling.

U937 cells were exposed to G-CSF and levels of STAT 1, STAT 3 and ERK 1,2 were measured. The results are shown in **Figures 3.8-3.10.**

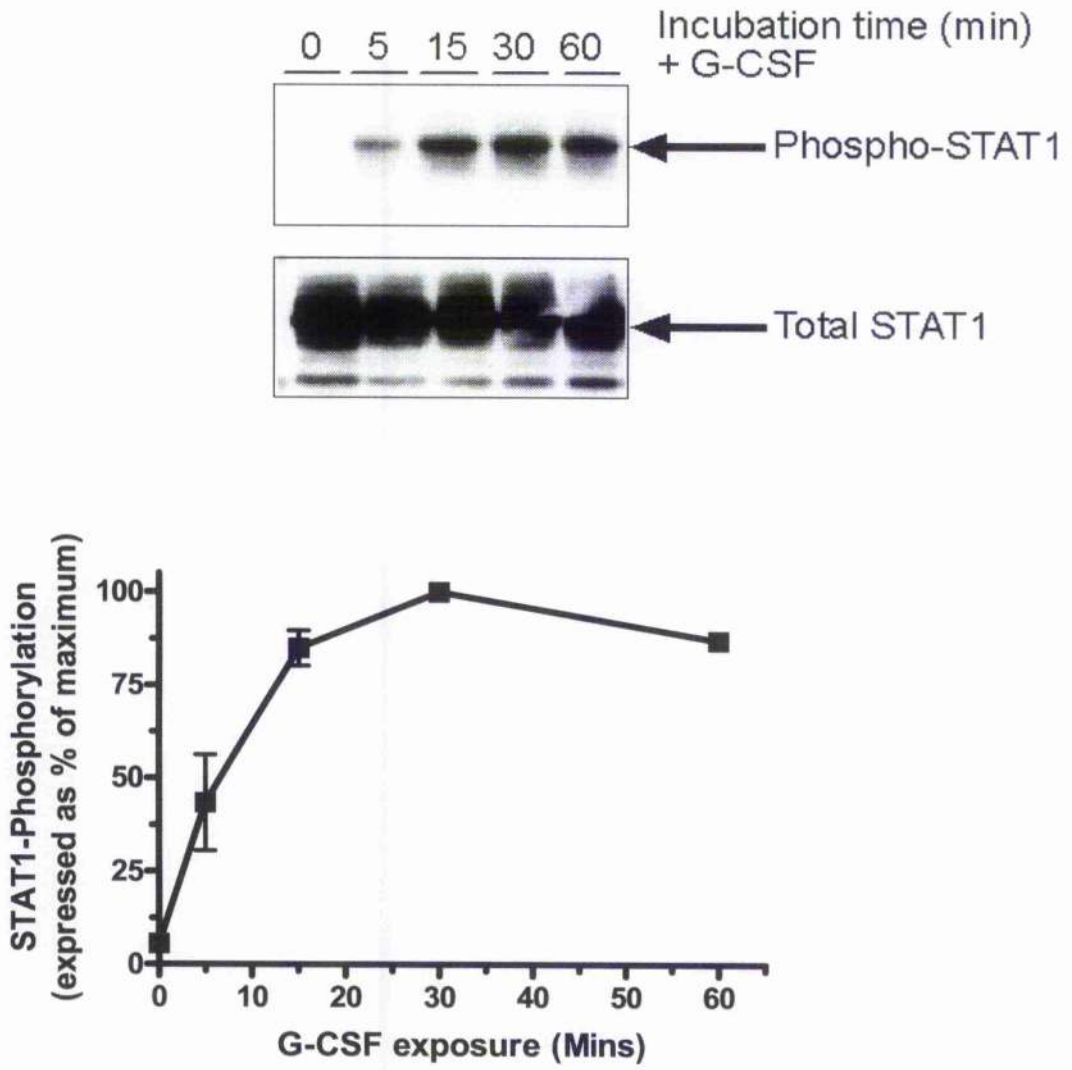

Figure 3.8 **Time course of G-CSF-induced STAT1 phosphorylation in U937 cells.** U937 cells were treated with G-CSF [10ng/ml] at a range of time points (n=3). Cell lysates were then prepared as described in section 2.4.1 before protein content of each sample was equalised prior to fractionation by SDS-PAGE. The proteins were then transferred to nitro-cellulose and subject to immunoblotting with total STAT1 and phospho-Tyr701- STAT1 antibodies. STAT 1 was first detectable after 5mins, maximal STAT1 phosphorylation is achieved after 15min and this response is sustained for the duration of the time course.

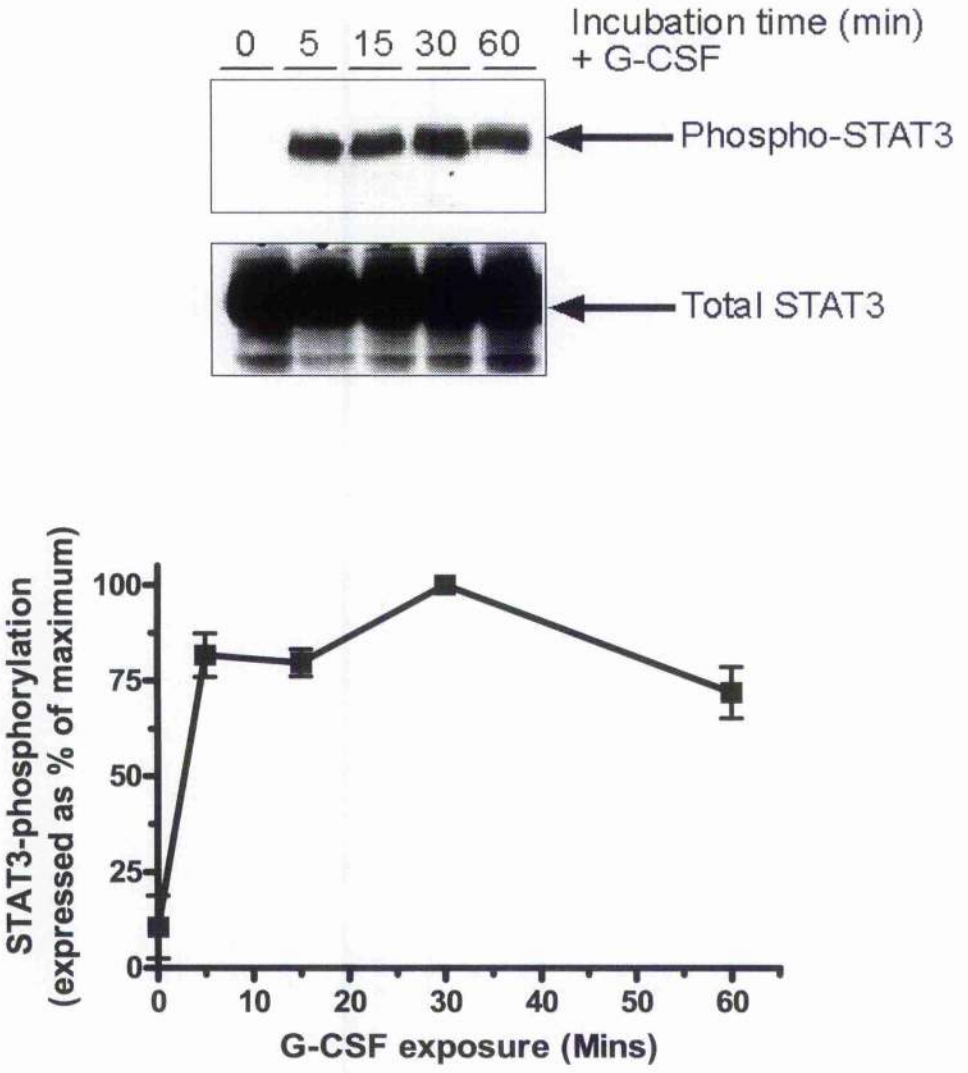

Figure 3.9 **Time course of G-CSF-induced STAT3 phosphorylation in U937 cells.** U937 cells were treated with G-CSF [10ng/ml] for a range of time points (n=3). Cell lysates were prepared according to section 2.4.1 and equalised for protein content before being subject to fractionation by SDS-PAGE. Proteins were then transferred to nitro-cellulose and immunoblotting was performed using total STAT3 and phospho-Tyr705-STAT3 antibodies. The blot and graph show that STAT3 is strongly phoshporylated after 5mins and this is sustained remaining time points.

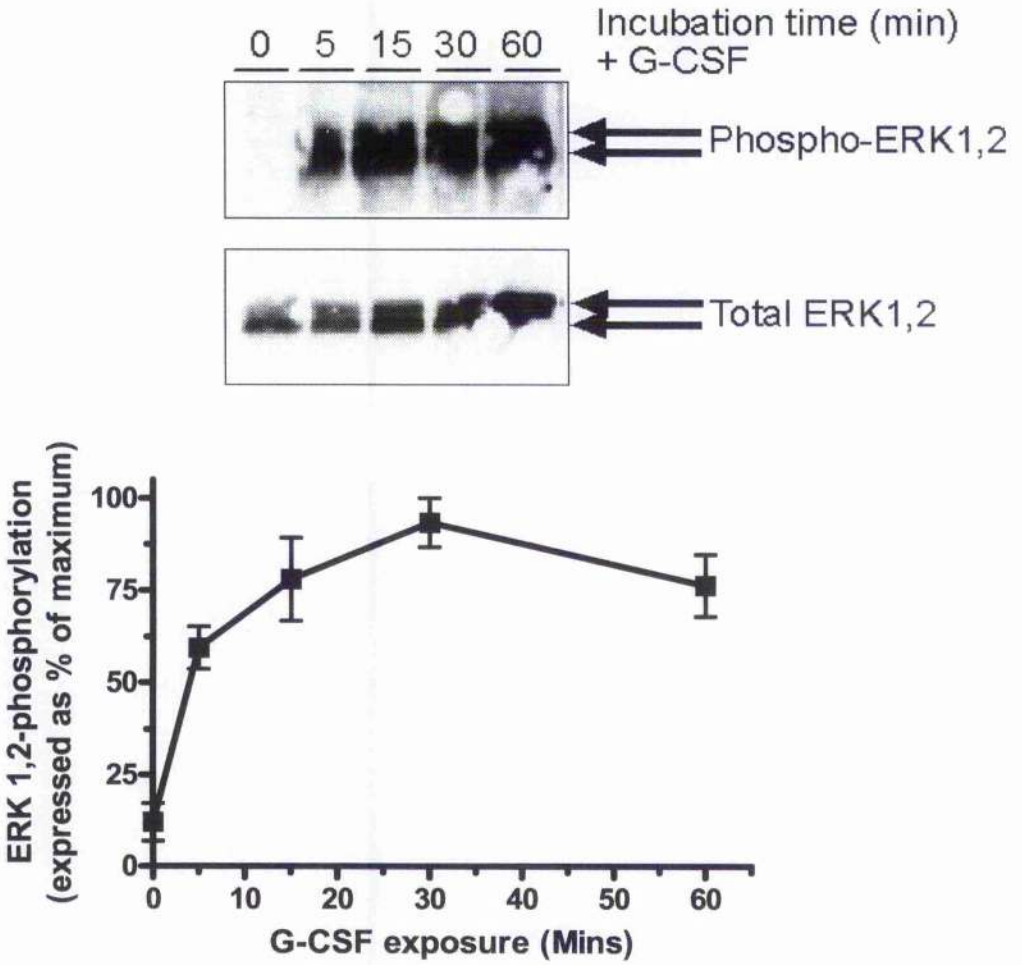

Figure 3.10 **Time course G-CSF-induced ERK1,2 phosphorylation in U937 cells.** This U937 cells were treated with G-CSF for a range of time points (n=3). Cell lysates were prepared according to section 2.4.1 and the protein content of each sample equalised before being subject to SDS-PAGE fractionation. The proteins were then transferred to nitro-cellulose and immunoblotting was performed using total ERK and phospho-Thr202,Tyr204-ERK1,2 antibodies. ERK phosphorylation was detectable at 5mins and maximal level of phosphorylation noted at 15min, this is sustained for the remainder of the time course.

In a second set of stimulations, U937 cells were exposed to GM-CSF. As with G-CSF, levels of STAT 1, STAT 3 and ERK 1,2 were measured. The results are shown in **Figures 3.11-3.13.**

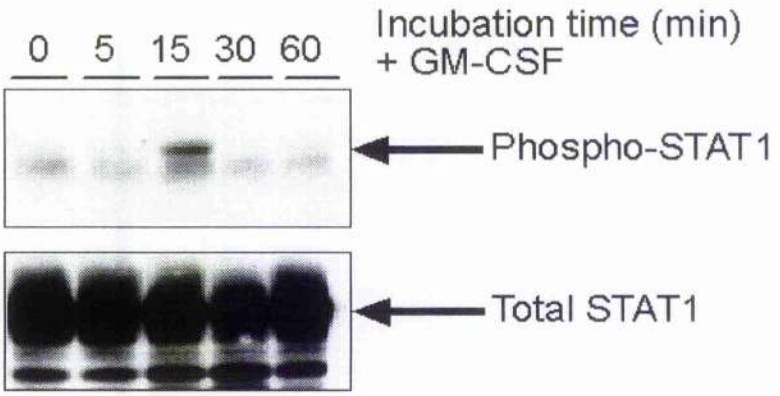

Figure 3.11 **Time course of GM-CSF-induced STAT1 phosphorylation in U937 cells.** U937 cells were treated with GM-CSF for a range of time points (n=3). Cells lysates were prepared as described in 2.4.1 and the protein content of each sample equalised prior to fractionation by SDS-PAGE. The proteins were transferred onto nitro-cellulose and probed for total STAT1 and phospho-Tyr701- STAT1 antibodies. STAT1 is transiently phosphoryated at 15min.

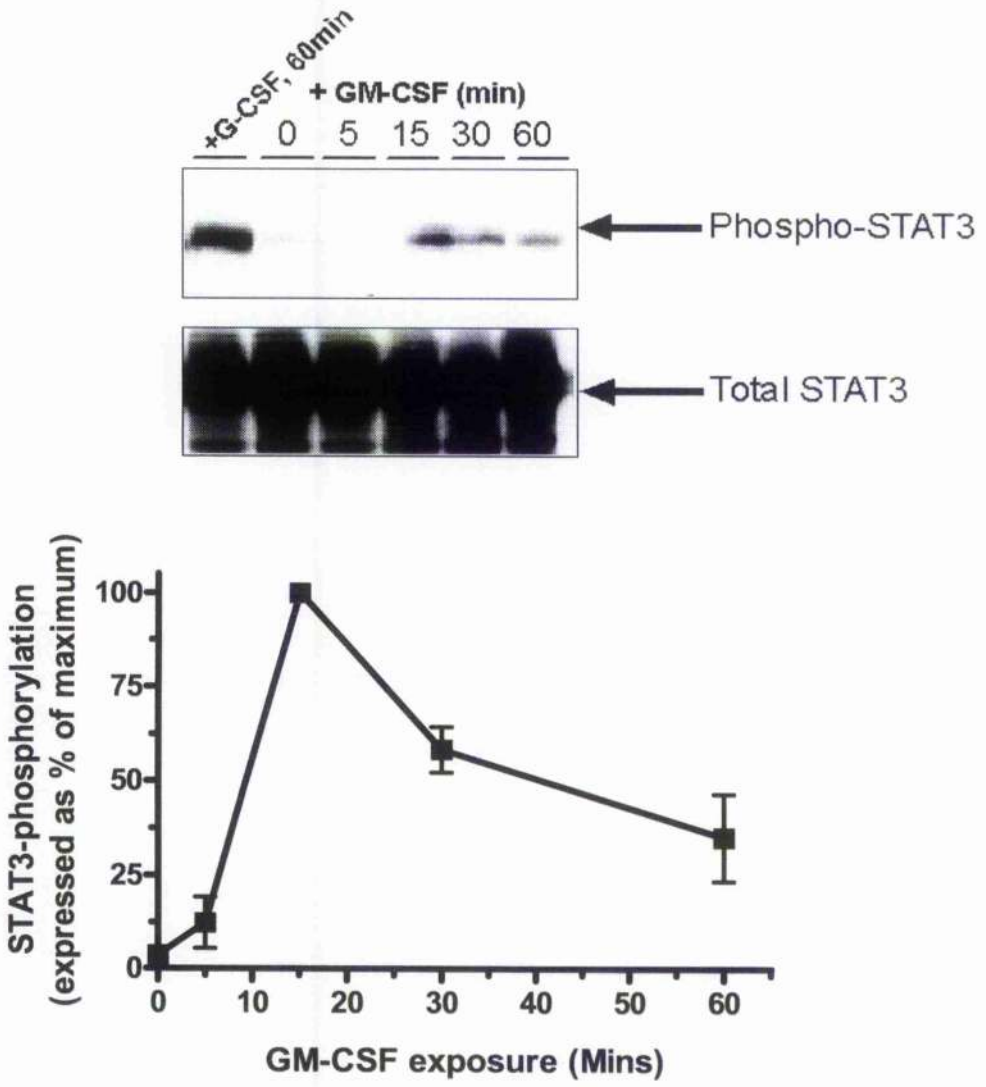

Figure 3.12 **Time course GM-CSF-induced STAT3 phosphorylation in U937 cells.** U937 cells were treated with GM-CSF [10ng/ml] for a range of time points (n=3). Cell lysates were prepared according to section 2.4.1. and equalised for protein content prior to fractionation by SDS-PAGE. Proteins were then transferred to nitro-cellulose followed by immunoblotting with total STAT3 and phospho-Tyr705-STAT3 antibodies. STAT3 phosphorylation is a transient event, maximal at 15min, at which point the level of STAT3 phosphorylation appears to diminish. G-CSF was loaded for comparison purposes, it shows that G-CSF stimulated cells produce a greater level of STAT3 phosphorylation than GM-CSF stimulated cells.

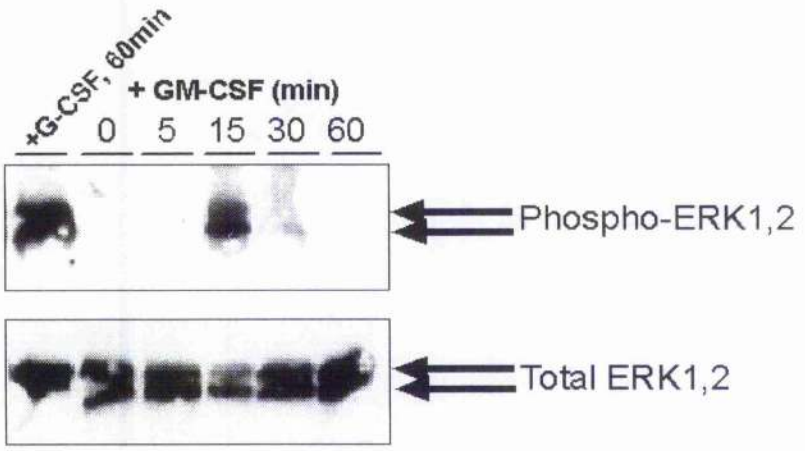

Figure 3.13 Time course of GM-CSF-induced ERK1,2 phosphorylation in U937 cells. U937 cells were treated with GM-CSF for a range of time points (n=3). Cell lysates were prepared according to section 2.4.1 and protein content equalised prior to fractionation by SDS-PAGE. The proteins were transferred to nitrocellulose followed by immunoblotting with total ERK and phospho-Thr202,Tyr204-ERK1,2 antibodies. The blot shows that ERK1,2 phosphorylation is a transient event peaking at 15min, it is also detected at 30min. G-CSF was loaded for comparison purposes, it shows that G-CSF stimulated cells produce a greater level of ERK phorsphorylation than GM-CSF stimulated cells.

From these experiments, it can be concluded that both G-CSF and GM-CSF activate the JAK/STAT pathways in U937 cells. However, there were several distinct aspects about the responses to each cytokine. For example, in U937 cells treated with G-CSF, phosphorylation of STAT1, STAT3 and ERK1,2 was achieved at 15min and that this level of phosphorylation was sustained for up to 1 hour. In contrast, in U937 cells treated with GM-CSF, STAT1 was phosphorylated markedly transient at 15min. Similarly, phoshporylation of STAT3 and ERK1,2 in response to GM-CSF peaked at 15min and diminished over the remainder of the time course. Therefore, U937 cells produce optimal phosphorylation of STAT1, STAT3 and ERK 1,2 following a 15min treatment with G-CSF or GM-CSF and this signal is sustained for up to 60mins.

3.5 Effects of elevated cAMP on G-CSF and GM-CSF signalling

It has been shown that elevated levels of cAMP are able to induce SOCS3. Previous literature states that SOCS proteins have a role in G-CSF and GM-CSF signalling (Barreda et al., 2004). More specifically, SOCS3 has been implicated in attenuation of the G-CSFR signalling pathway (*van de Geijin et al., 2004)*, although the specific SOCS protein(s) involved in GM-CSFR signalling have yet to be elucidated. Therefore, it was decided to test the effects of increased cAMP on SOCS3 induction in G-CSF-stimulated cells and compare these effects with GM-CSF-stimulated cells. To investigate the effects of elevated cAMP on G-CSF and GM-CSF signalling, U937 cells were treated with forskolin in combination with either G-CSF or GM-CSF for 30min before SOCS3 levels were measured. The results of these experiments are illustrated by **figures 3.14 and 3.15.**

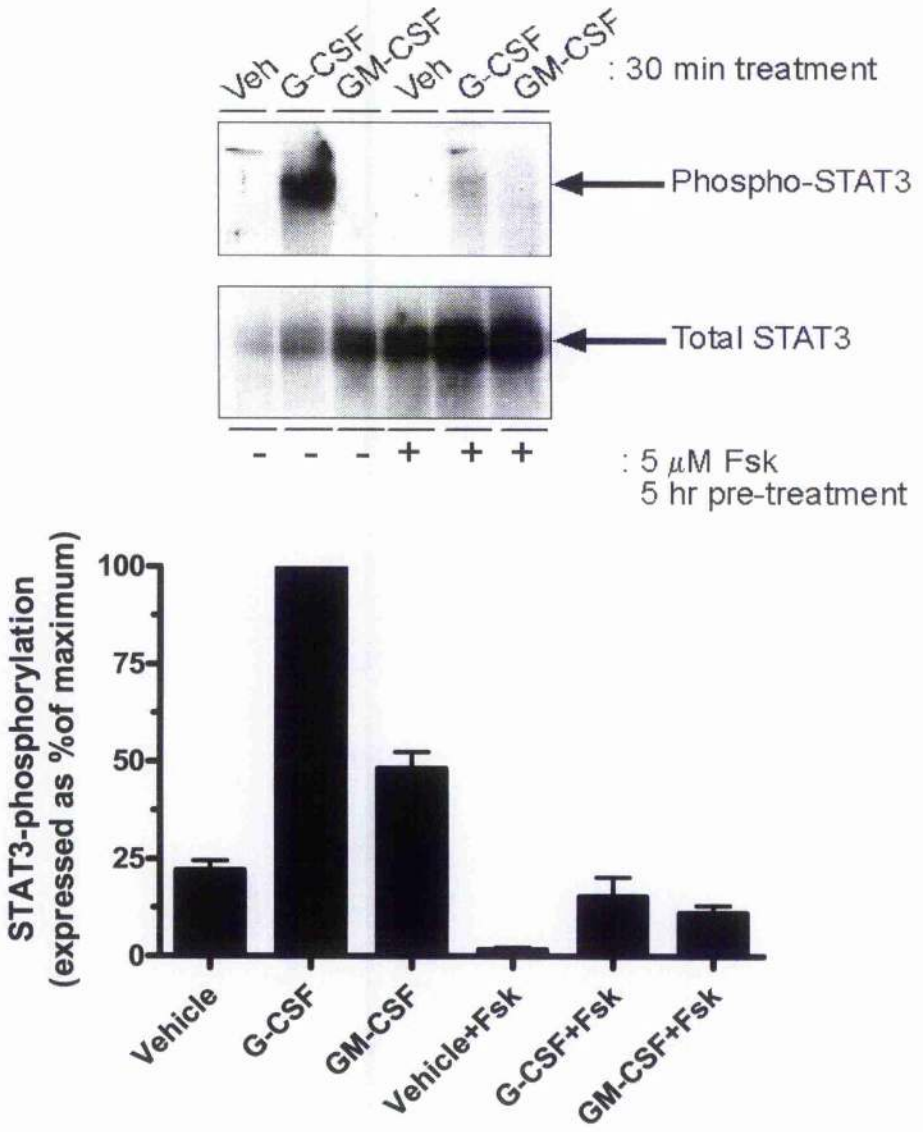

Figure 3.14 **Effect of Fsk-pre-treatment on G-CSF/GM-CSF induced STAT3 phosphorylation.** U937 cells were plated onto a 6-well dish as described in section 2.2. Three wells were pre-treated with forskolin [10μM] for 6hr before addition of G-CSF [10ng/ml] and GM-CSF [10ng/ml] to the respective well for 30min (n=3). Cell lysates were prepared as described in section 2.4.1. and protein content equalised prior to SDS-PAGE fractionation. The proteins were then transferred onto nitro-cellulose before immunoblotting with total STAT3 and phospho-Tyr705-STAT3 antibodies. Two one-sample t-tests were performed on the data presented in this graph. The first was used to compare average densitometry measurement of STAT3 in cells treated with G-CSF Vs. cells treated with G-CSF + Fsk. A p value of 0.0019

was obtained therefore pre-treatment with Fsk did not statistically increase G-CSF induced levels of STAT3. The second one sample t-test compared cells treated with GM-CSF Vs GM-CSF +Fsk a p-value of 0.0018 was obtained which shows that pre-treatment with Fsk did not statistically increase GM-CSF induced levels of STAT3.

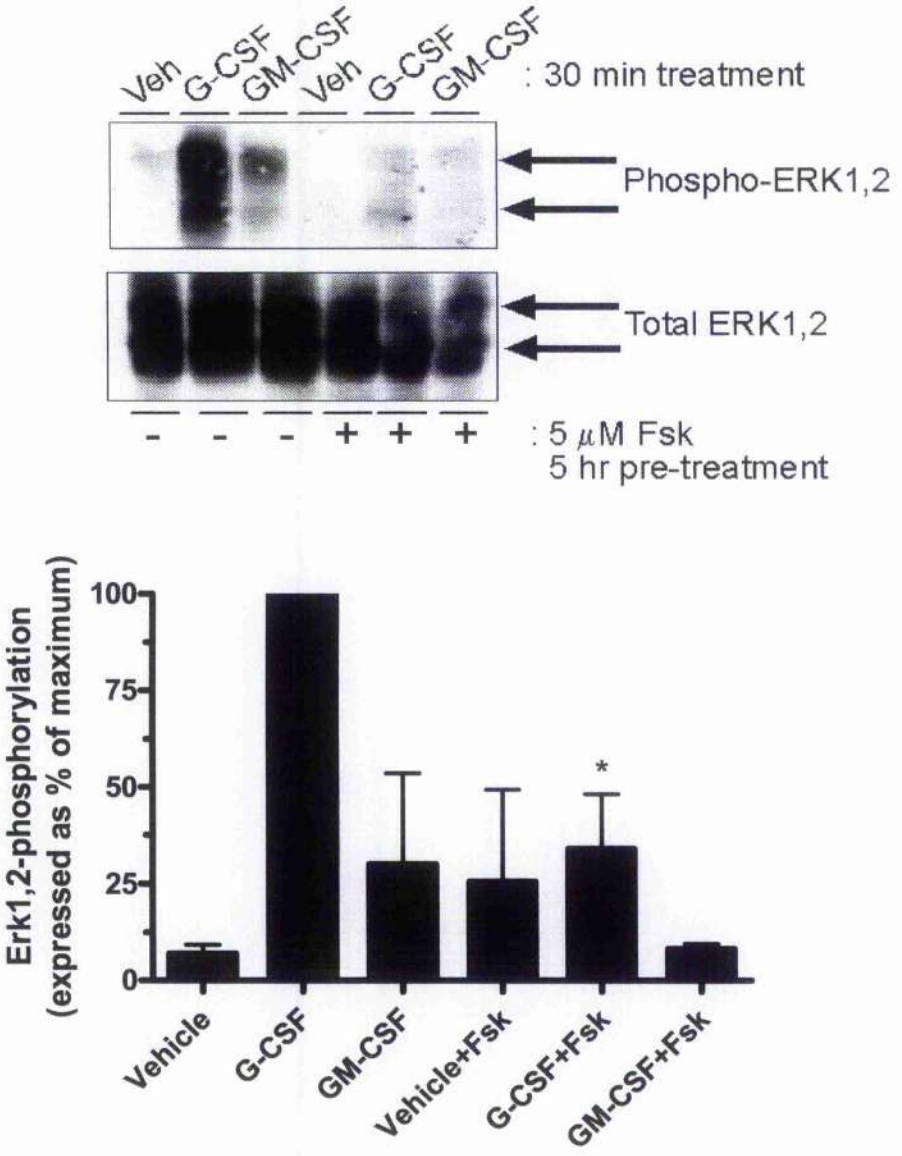

Figure 3.15 Effect of Fsk pre-treatment on G-CSF/GM-CSF induced ERK-1 phosphorylation. U937 cells were plated into a 6-well dish as described in section 2.2. Three wells were pre-treated with forskolin [10μM] for 6hrs before addition of G-CSF [10ng/ml] and GM-CSF [10ng/ml] to the respective well for 30min (n=3). Cell lysates were prepared as described in section 2.4.1. and the protein content equalised prior to SDS-PAGE fractionation. The proteins were then transferred onto nitro-cellulose before immunoblotting with the phospho-ERK1,2 and phospho-Thr202,Tyr204-ERK1,2 antibodies. The ERK-1 signal was stronger than that recorded for ERK-2 therefore, average densitometry was used to produce the graph presented above.

Average densitometry readings of ERK-1 were compared using a one-sample t-test. For G-CSF Vs G-CSF + Fsk p=0.010 therefore Fsk significantly reduces ERK-1 in U937 cells (*). A one sample t-test was also employed to compare average ERK-1 in cells treated with GM-CSF Vs. GM-CSF + Fsk to produce a p-value of 0.259 therefore Fsk does not significantly reduce levels of ERK-1 in U937 cells.

These results of these experiments prove that forskolin is capable of reducing levels of phoshpo-STAT3 and phospho-ERK1,2 in G-CSF stimulated cells and phospho-STAT3 in GM-CSF stimulated cells. This implies elevated levels of cAMP are able to reduce cytokine signalling in U937 cells. However, the mechanism by which cAMP is able to contribute to this response has yet to be ascertained.

3.6 Relationship between SOCS3 induction and cAMP

To this point, results have established that an increase in cAMP is linked to increased expression of SOCS3, and that elevated levels of cAMP are capable of diminishing cytokine signaling via the G-CSFR and GM-CSFR. However, the mechanism by which cAMP is able to induce SOCS3 remains unknown.

As has been discussed in section 1.8.1, cAMP signals via various downstream effectors, including Epac and PKA. In order to establish involvement of Epac or PKA in the induction of SOCS3, U937 cells were treated for 6hrs with a variety of agents These included a PKA inhibitor (H89), a selective PKA activator (6Be-cAMP), and a selective Epac activator (8-pCPT-2-O-Me-cAMP) (*Christensent et al., 2003*). Levels of induced SOCS3 were monitored as a direct assessment of the effect of each agent. The results of these experiments are displayed in **figure 3.16**.

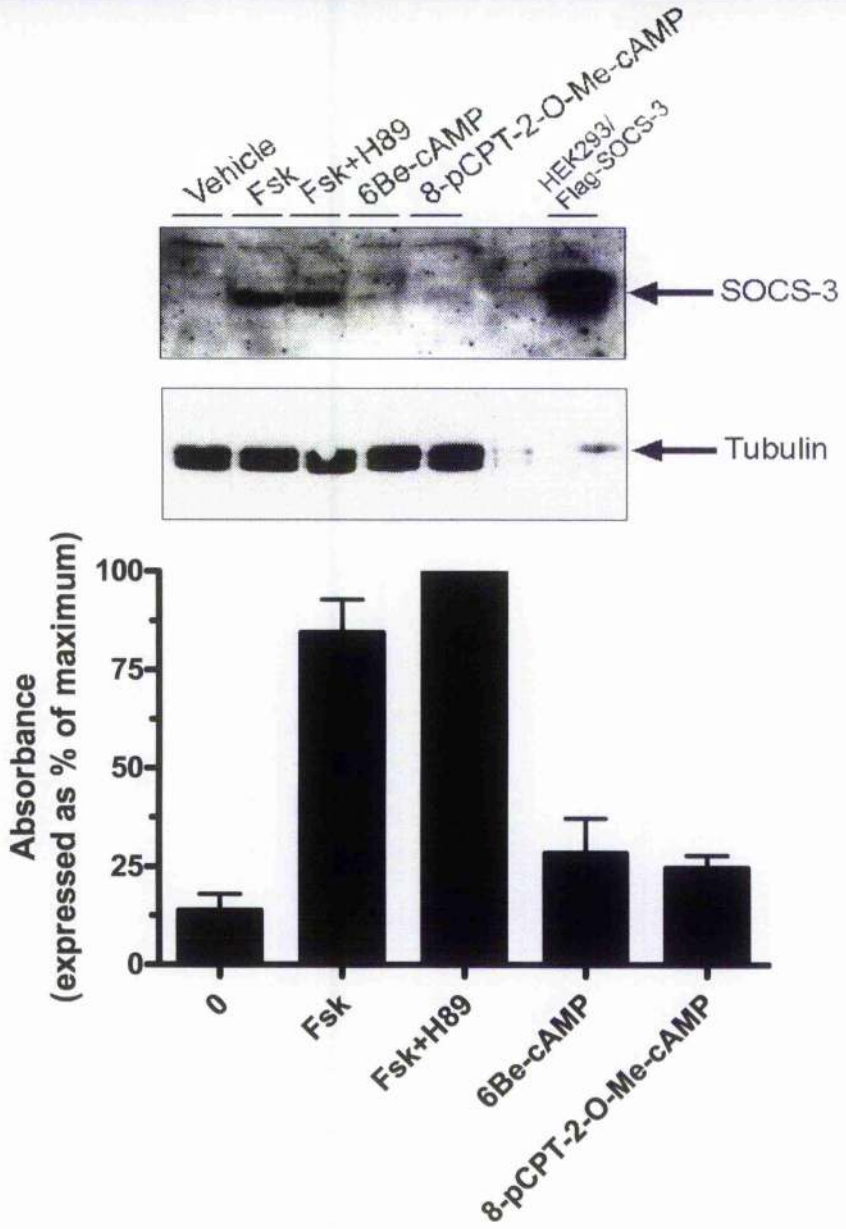

Figure 3.16 **Effects of cAMP signalling pathway activators on SOCS3 induction in U937 cells.** U937 cells split into a 6-well plate as described in section 2.2. Each well was treated with a combination of agents (0, Fsk[10μM], Fsk[10μM]+H89[5μM], 6Be-cAMP[50μM], 8-pCPT-2-O-Me-cAMP[0.1mM]), for 6hr (n=3). After this period, cell lysates were prepared according to section 2.4.1 and the protein content of each was normalised prior to fractionation by SDS-PAGE. The proteins were transferred onto nitro-cellulose and probed using a tubulin and SOCS3 antibodies. The graph shows that SOCS3 expression is significantly greater in Fsk and Fsk+H89 treated cells compared to cells treated with a6Be-cAMP and 8P-CPT.

These results suggest SOCS3 is induced by a cAMP dependent, PKA/ERK independent pathway. This implies involvement of a separate cAMP pathway. Based on these results it was decided to test the ability of 6Be-cAMP and 8-pCPT-O-Me-cAMP to activate the ERK pathway, to ensure that both compounds were active. The results of this experiment are displayed below in **figure 3.17.**

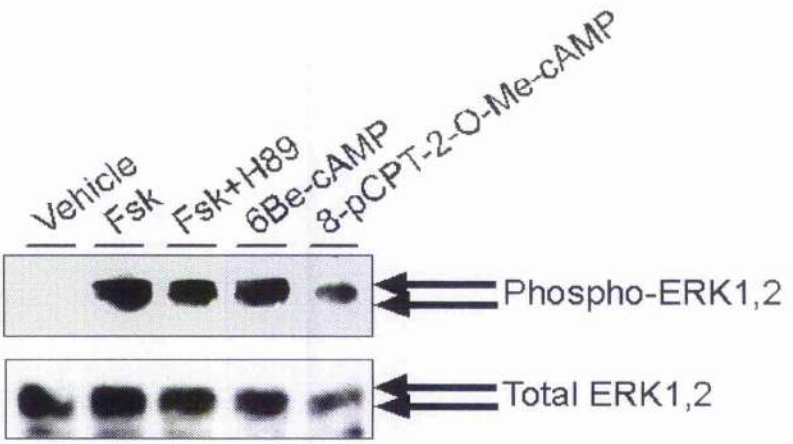

Figure 3.17 **Effects of H89, 6Be-cAMP and 8-pCPT-O-Me-cAMP on ERK activation in U937 cells.** U937 cells split into a 6-well plate as described in section 2.2. Each well was treated with a combination of agents (0, Fsk[10μM], Fsk[10μM]+H89[5μM], 6Be-cAMP[50μM], 8-pCPT-2-O-Me-cAMP[0.1mM]), for 6hr (n=3). After this period, cell lysates were prepared according to section 2.4.1 and the protein content of each was normalised prior to fractionation by SDS-PAGE. The proteins were transferred to nitrocellulose followed by immunoblotting with total ERK and phospho-Thr202,Tyr204-ERK1,2 antibodies. Both 6Be-cAMP and 8-pCPT-O-Me-cAMP caused increased phosphorylation of ERK thereby suggesting that both compounds were active.

SOCS3 induction is controlled by various signalling pathways, all capable of activating transcription of the SOCS3 gene. Ultimately, cAMP appears to be involved at some stage in this process. For example, previous literature has stated that cAMP is capable of direct activation of p38 (*Chio et al., 2004*). Furthermore, results from this study show that elevated levels of cAMP are able to activate components of the Ras/Raf/ERK pathway.

To further investigate the involvement of defined signalling pathways in cAMP-mediated induction of SOCS3, stimulation experiments were carried out using a set of signalling pathway inhibitors. An ERK inhibitor (UO126), a selective inhibitor of p38 (SB203580) and a JAK inhibitor were incubated with U937 cells for 6hr in the absence and presence of Fsk. The results of these experiments are recorded in **figure 3.17.**

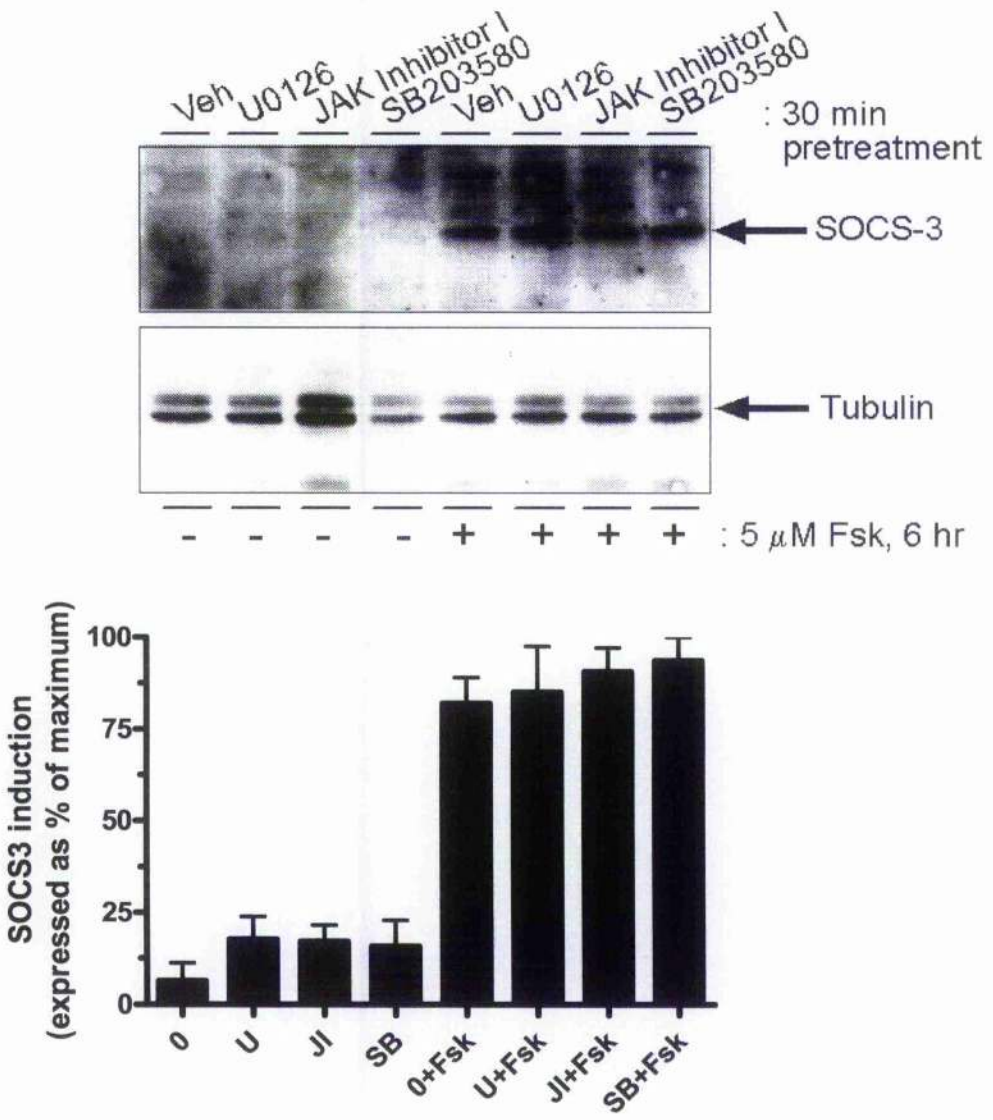

Figure 3.17 **Effects of signalling pathway inhibitors of Fsk-stimulated SOCS3 induction in U037 cells.** U937 cells were pre-treated with a each inhibitor (UO126[1μM], JAK inhibitor[0.5μM], SB203580[10mM]) for 30mins before addition of Fsk[10μM] for 6hrs (n=3). Cell lysates were prepared as described in section 2.4.1 and the protein content of each lysate was normalised prior to fractionation by SDS-PAGE. The proteins were transferred onto nitro-cellulose and probed using a SOCS3 antibody. SOCS3 expression is significantly greater in the cells treated with Fsk. The graph illustrates there is no significant difference in SOCS3 expression between each inhibitor treatment.

This experiment shows that none of the inhibitors diminished SOCS3 induction in response to Fsk. This suggests an alternative signalling pathway is utilised by cAMP to induce SOCS3.

Since initial submission of this thesis, my colleagues have performed efficacy tests on two of the inhibitors used in the previous experiment. I have included the results of here for prosperity.

U937 cells were treated with pre-treated with each inhibitor prior to stimulation of the cells with known ERK and p38 agonists. The results of each experiment are represented in **figures 3.18 and 3.19.**

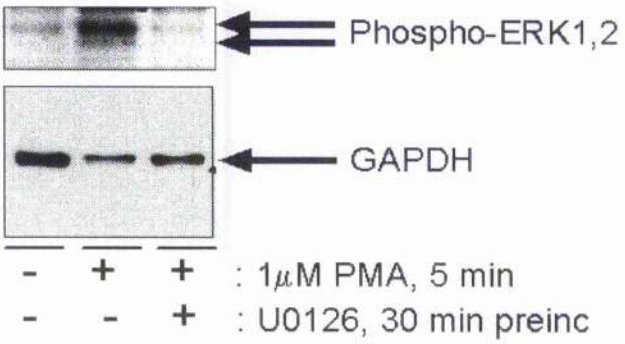

Figure 3.18 **Effects of U0126 on ERK activation in U937 cells.** U937 cells were pre-treated with U0126 for 30mins prior to a 5min incubation with PMA. Cell lysates were prepared as described in section 2.4.1 and the protein content of each lysate was normalised prior to fractionation by SDS-PAGE. The proteins were transferred to nitrocellulose followed by immunoblotting with phospho-Thr202, Tyr204-ERK1,2 antibodies. GAPDH was used as a loading control. U0126 was clearly active since it was capable of inhibiting the phospho-ERK signal observed in cells treated with PMA alone.

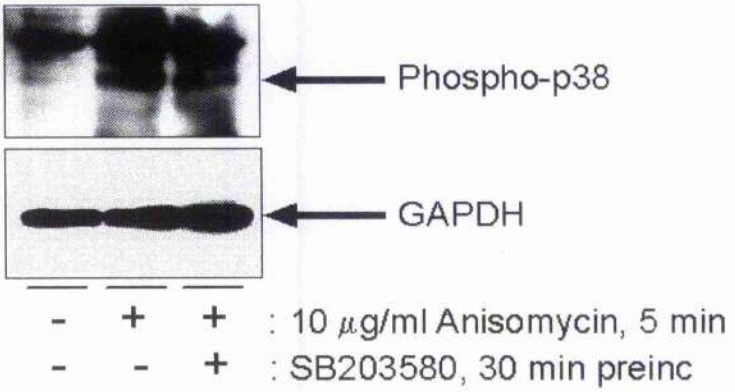

Figure 3.19 Effects SB203580 on Phospho-p38 activation in U937 cells. U937 cells were pre-treated with SB203580 for 30mins prior to a 5min incubation with Ansiomycin. Cell lysates were prepared as described in section 2.4.1 and the protein content of each lysate was normalised prior to fractionation by SDS-PAGE. The proteins were transferred to nitrocellulose followed by immunoblotting with Rabbit anti-phosphoThr180/Tyr182-p38 antibodies. GAPDH was used as a loading control. U0126 was clearly active since it was capable of inhibiting the phospho-ERK signal observed in cells treated with PMA alone.

No efficacy testing has been performed on the JAK inhibitor to date however, the concentration used is optimal as determined by abolition of IL-6-mediated STAT3 phosphorylation in vascular endothelial cells (*Palmer, personal communication*).

CHAPTER 4

DISCUSSION

4.1 Fsk/cAMP-mediated accumulation of SOCS3

Fsk induced SOCS3 in both U937 myeloid and HL60 promyoblast cells. The optimal conditions for Fsk-induced SOCS3 expression was a 6hr incubation period at a concentration of 10μM. Fsk elevates intracellular cAMP levels by binding and activating adenylyl cyclase. Therefore it was concluded that elevated cAMP is responsible for induction of SOCS3 in both cell lines. Treatment with polymyxin B had no effect on the maximal level of SOCS3 expression induced by Fsk. Polymyxin B eliminates LPS generated SOCS3 thus, it was concluded that contamination of the Fsk preparation with LPS does not contribute to the SOCS3 induction obtained in this study.

Interestingly, the proteasome inhibitor (MG132) had minimal effect on the accumulation of SOCS3 in both U937 and HL60 cells. SOCS3 turnover is a tightly regulated process (*Hanada et al., 2003*). The SOCS box region is responsible for regulating the stability of the protein, by mediating an intracellular interaction with an elongin C-containing complex (*Haan, S et al 2003*). Phosphorylation of SOCS3 at two tyrosine residues within the conserved SOCS box, Tyr204 and Tyr221, appears to inhibit the SOCS3-elongin C interaction and trigger proteasome-mediated SOCS3 degradation (*Haan, S et al 2003*). Therefore, the minimal effect of MG-132 in U937 cells in particular may suggest that increased cAMP has no effect on tyrosine phosphorylation of SOCS3. Tyrosine phosphorylation of SOCS3 is thought to be mediated predominantly by the Src family tyrosine kinases (*Sommer U, et al 2005*). However, there is no convincing evidence to link elevated cAMP with regulation of Src kinases, therefore the lack of effect of MG-132 is consistent with this hypothesis.

SOCS3 stability can also be increased by a separate phosphorylation event on the N-terminus of the protein by Pim-1which encodes an oncogenic serine/threonine kinase (*Peltola KJ et al 2004*). Pim-1 is a target gene of STAT5, which plays a critical role in cytokine-induced survival of heamatopoietic (*Peltola KJ et al 2004*). Pim-1 is a component of an inhibitory feedback pathway which serves to reduce STAT-5 activity. During this process, Pim-1 interacts directly with SOCS1 and SOCS3 to potentiate their inhibitory effects on STAT5. This is thought to occur via a phosphorylation-mediated stabilisation of the SOCS proteins (*Peltola KJ et al 2004*). Currently, there is no evidence to support cAMP regulation of Pim-1. However, cAMP may be linked to increased activity of a kinase capable of phosphorylating the same sites on SOCS3, thus stabilising the protein by preventing proteasomal degradation and providing resistance to MG-132.

To test if elevated cAMP produces a stabilising phosphorylation of SOCS3 thus accounting for the minimal effect of MG-132 on SOCS3 accumulation, future experiments may involve ^{32}P-radiolabelling U937 cells prior to treatment with Fsk. By performing subsequent immunoprecipitation of SOCS3, it would then be possible to determine whether increased cAMP causes an increase in Ser/Thr phosphorylation of SOCS3.

4.2 Effects of Fsk on cytokine signalling

Based on the results obtained in this study, it can be concluded that G-CSF and GM-CSF can induce phosphorylation of STAT1, STAT3 and ERK in U937 cells. This is consistent with previous studies which demonstrated that G-CSF signals via the JAK/STAT and Ras/Raf/ERK pathways (*Akbarzadeh et al., 2001*). Results also

showed that increased cAMP diminished G-CSF and GM-CSF-induced phosho-STAT3 and phospho-ERK1,2.

Elevated cAMP is coupled to an increase in SOCS3 expression and in turn, SOCS3 has been shown to selectively bind the G-CSF receptor to block signalling *in vivo* (*Hortner, et al., 2002*). It is therefore feasible to suggest that the Fsk-induced increase in SOCS3 is responsible for the decrease in phospho-STAT3 and phospho-ERK1,2. However, in order to definitively address a role for SOCS-3, one would need to determine the consequences of blocking SOCS-3 induction by Fsk on Fsk-mediated inhibition of G-CSF signalling. To block SOCS-3 accumulation and unequivocally assign a role for it in mediating cAMP's effect, one could use either siRNA or antisense RNA strategies. In this report, it is also important to remember that we cannot, at the moment, exclude other possible mechanisms for this response. For example, SHP-1, a tyrosine phosphatase induced by G-CSF in certain cells, is able to dephosphorylate both JAKs and cytokine receptors (*Haque et al 1998*), thus theoretically preventing subsequent phosphorylation of ERK proteins. It is also theoretically possible that the receptor could be down-regulated in response to increased levels of cAMP, thereby reducing the response to Fsk which is represented by diminished levels of phospho-ERK1,2 as observed here.

In order to ascertain the mechanism underlying the reduction in G-CSF receptor signalling following a cAMP-induced SOCS3 upregulation, it would be necessary to examine the existence/importance of the alternative mechanisms suggested above.

This could be achieved by examining the following issues:

1. Do tyrosine phosphatase inhibitors block the inhibitory effect of Fsk on inhibiting GCSF induced phosphorylation of STAT3 and ERK1,2? If this is not found to be true, presumably SHP-1 is not involved in mediating the effects of cAMP.
2. Does the inhibition of protein synthesis block the inhibiting effects of cAMP? If this is observed, in conjunction with a lack of effect of Tyr phosphatase inhibitors, it could suggest that cAMP-mediated induction of SOCS3 may be responsible for the cAMP-mediated decrease in levels of phospho-STAT3 and phospho-ERK1,2.

4.3 Analysis of the signalling pathway mediating Fsk induction of SOCS3

Fsk is capable of inducing SOCS3 and it has been suggested that Epac and PKA may be involved in this process *(de Rooj et al., 1998. Kopperund et al., 2003)*. This study investigated the potential role of Epac and PKA in Fsk-induced SOCS3 using selective activators and inhibitors. PKA and Epac activators (6Bc-cAMP, 8-pCPT-2-O-Me-cAMP respectively) did not induce SOCS3 in U937 cells. Furthermore, a PKA inhibitor (H89) did not inhibit Fsk-induced SOCS3. Thus, it was concluded that PKA and Epac are not involved in the Fsk mediated induction of SOCS3.

In further consideration of the PKA response (or lack there of), even though H89 did not inhibit Fsk stimulated SOCS3 in U937 cells, the concentration used *is* effective in inhibiting PKA function in human umbilical vein endothelial cells (HUVECs) at the concentration used here (*Sands et al., submitted for publication*), as judged by abolition of Fsk-stimulated Ser133 phosphorylation of cyclic AMP response element binding protein (CREB). It was not possible to reproduce this experiment using U937

cells as very high basal levels of phospho-CREB were recorded, probably due to the action of other CREB kinases (data not shown). However, the lack of reduction of Fsk-induced SOCS3 expression in response to the PKA-inhibitor (H89), coupled with the lack of effect of the selective PKA activator (6Be-cAMP) would suggest the involvement of a PKA-independent pathway. One possible mediator of the PKA-independent effects of cAMP is Epac. A selective Epac activator (8-pCPT-2-O-Me-cAMP) has been shown to induce SOCS3 in HUVECs (*Sands et al., submitted for publication*). However, this effect could not be reproduced in U937 cells using batches of 8pCPT-2-O-Me-cAMP that were active in HUVECs. Due to this lack of response, Epac is likely not responsible for cAMP-mediated induction of SOCS3 in U937 cells. In addition, while unable to induce SOCS-3, 6Be-cAMP and 8-pCPT-2-O-Me-cAMP were each able to stimulate the phosphorylation of ERK in U937 cells, confirming that both drugs were active under these conditions. In view of these results, the role of cAMP in SOCS3 induction is independent of PKA and Epac and as such, it is possible that an unknown and as yet uncharacterised sensor of cAMP is responsible.

To identify any new intracellular sensor of cAMP in these cells, cAMP-conjugated argarose could be used as an affinity purification probe for isolating cAMP-binding proteins. After releasing PKA and Epac by elution with 6Be-cAMP and 8-pCPT-2-O-Me-cAMP respectively, any remaining cAMP-binding proteins could be eluted and identified by mass spectometry and peptide mass fingerprinting.

Final experiments attempted to address the signalling pathway mediating SOCS3 induction via cAMP. A set of inhibitors were selected based on their ability to block

specific pathways that could be involved in cAMP induction of SOCS3. The MEK inhibitor (UO126) blocks Fsk-induced SOCS-3 in HUVECs (*Sands et al., submitted for publication*), SB203580 is a selective inhibitor of p38 stress activated kinase which is activated by cAMP in some cell types and a JAK inhibitor which would block the JAK/STAT pathway directly thereby preventing STAT-mediated SOCS3 transcription. Experiments in HUVECs and fibroblasts demonstrated that each inhibitor was active at the concentrations used. Results showed that none of the inhibitors were capable of attenuating Fsk induced SOCS3. This adds further support to the involvement of an independent and as yet undefined mechanism for cAMP related-SOCS3 induction.

As more questions evolve from the results of this study, it is important to consider the primary mechanisms by which cytokines induce SOCS3 transcription.

Cytokines induce SOCS3 via activation of the JAK-STAT pathway which leads to STAT-mediated transcription of the SOCS3 gene (*Hanada et al., 2003*). However, elevated cAMP does not appear to utilise this mechanism since the JAK inhibitor does not block SOCS3 induction. The combination of JAK inhibitor used here is effective when applied to HUVECs where an abolition of sIL-6Rα/IL-6-induced SOCS3 is observed (*Sands et al., submitted for publication*). This effect is consistent with very little evidence in the literature to suggest that increased cAMP can activate the JAK-STAT pathway.

Attenuation of ERK with MEK inhibitor (U0126) also has no effect on induction of SOCS3. This is consistent with the fact that Fsk, 6Be-cAMP and 8-pCPT-2-O-Me-cAMP can all increase levels of phosphorylated ERK in U937 cells, but only Fsk can

induce SOCS3. HUVECs exhibit a contrasting response, where U0126 abolishes cAMP-mediated SOCS3 induction (*Sands et al., submitted for publication*) Therefore, while the ability of elevated cAMP to induce SOCS3 appears to be conserved across different cell lines, the molecular mechanisms involved in this process may be distinct.

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