# DIVERGENT MECHANISMS UTILIZED BY SOCS3 TO MEDIATE IL-10 INHIBITION OF TNF-α AND NITRIC OXIDE PRODUCTION BY MACROPHAGES

by

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## Abstract

The cytokine, interleukin-10 (IL-10), inhibits activation of macrophages by activators such as lipopolysacchride (LPS). However the mechanism by which IL-10 interferes with LPS signalling is still unclear. One well-characterized signalling pathway activated by IL-10 is that of Stat3 (Signal transducer and activator of transcription), which is essential for the anti-proliferative and anti-inflammatory actions of IL-10 on macrophages. In our search for IL-10-induced, Stat3-regulated genes, we found a candidate belonging to SOCS (suppressor of cytokine signalling) family of negative regulators of cytokine signalling. Using mutant IL-10 receptor and dominant negative Stat3, we show that IL-10 induces SOCS3 message and protein in a Stat3-dependent manner. However mere expression of SOCS3 protein in macrophages was not sufficient to inhibit TNF-$\alpha$ protein production in response to LPS, suggesting that additional IL-10-induced signals are required. And indeed we find IL-10 stimulation induces phosphorylation of tyrosine 204 of SOCS3 protein. In order to determine the role of SOCS3 in IL-10 inhibition of macrophage activation, we derived cell lines from SOCS3$^{-/-}$ and SOCS3$^{+/-}$ mice. SOCS3$^{-/-}$ macrophages respond to LPS in a manner similar to wild-type cells, but IL-10 is less effective in inhibiting LPS-induced TNF-$\alpha$ and NO production in the SOCS3$^{-/-}$ cells as compared to SOCS3$^{+/-}$ macrophages. Reconstitution of SOCS3$^{-/-}$ cells with a wild-type SOCS3 cDNA restored IL-10 responsiveness. In order to determine which SOCS3 domain is important in IL-10 signalling, SOCS3$^{-/-}$ macrophages were reconstituted with various SOCS3 domain mutants. IL-10 required all domains of SOCS3 protein for the inhibition of TNF-$\alpha$ protein expression. However, for inhibition of TNF-$\alpha$ mRNA expression, IL-10 does not seem to require the KIR domain of SOCS3 protein. In contrast, only the two tyrosine residues, 204 and 221, located in the SOCS-box domain are required for IL-10 inhibition of LPS-induced iNOS

protein expression and subsequent NO production. These studies demonstrate the importance of SOCS3 protein in the anti-inflammatory action of IL-10 and that inhibition of NO and TNF-$\alpha$ by IL-10 depends on different domains of SOCS3. Characterization of SOCS3 signalling domains and its immediate downstream targets will allow development of therapeutic strategies, which replicate the beneficial anti- inflammatory action of IL-10.

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| | |
|---|---|
| AAMΦ | Alternatively activated macrophage |
| Akt | V-Akt murine viral oncogene homology 1 |
| AP-1 | Activator Protein-1 |
| APC | Antigen presenting cell |
| ATP | Adenosine triphosphate |
| Ca | Calcium |
| CD14 | Monocyte differentiation antigen CD14 |
| CD80/86 | Co-stimulatory molecule CD80/86 |
| c-Jun | c-Jun mitogen activated kinase |
| CAMΦ | Classically activated macrophage |
| CREB | cyclic AMP responsive element binding protein |
| DNA | Deoxyribonucleic acid |
| ECL | Enhanced Chemiluminescence |
| EDTA | Ethylene-diamine-tetraacetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| ERK | Extracellular-signal regulated kinase |
| FACS | Fluorescence-activated cell sorting/cell-scanning |
| GFP | Green fluorescence protein |
| GM-CSF | Granulocyte macrophage colony stimulating factor |
| GP130 | Glycoprotein 130 (IL-6 receptor) |
| HO-1 | Heme-oxygenase-1 |
| ICAM | Intercellular adhesion molecule |
| IFN-$\lambda$ | Interferon-$\lambda$ |
| I$\kappa$B | I$\kappa$B protein |
| IKK | I$\kappa$B protein kinase |

| | |
|---|---|
| IL-1 | Interleukin-1 |
| IL-6 | Interleukin-6 |
| IL-8 | Interleukin-8 |
| IL-10 | Interleukin-10 |
| IL-12 | Interleukin-12 |
| ILK | Integrin-linked kinase |
| iNOS | inducible nitric oxide |
| JAK | Janus kinase |
| JNK | c-Jun-N-terminal kinase |
| KIR | Kinase inhibitory region |
| KO | Knock-out |
| LPS | Lipopolysaccharide |
| LIF | Leukemia inhibitory factor |
| MΦ | Macrophage |
| MHC | Major histocompatability complex |
| MAPK | Mitogen activated protein kinase |
| MD-2 | MD-2 protein |
| MIP | Macrophage inflammatory protein |
| MKK | MAPK kinase |
| MyD88 | Myeloid differentiation primary response gene 88 |
| NF-κB | Nuclear factor-κB |
| NMDA | N-methyl-D-aspartate |
| NO | Nitric oxide |
| p38 MAPK | p38 mitogen activated protein kinase |
| PBS | Phosphate buffered saline |
| PGE2 | Prostaglandin E2 |
| PI3-K | Phosphotidylinositol 3-kinase |

| | |
|---|---|
| PKA | Protein kinase A |
| PKC | Protein kinase C |
| PMSF | Phenylmethanosulfonly fluoride |
| RNA | Ribonucleic acid |
| ROO- | Reactive oxygen intermediates |
| SAPK | Stress activated protein kinase |
| SDS | Sodium dodecyl sulphate |
| SH2 | Src homology 2 |
| SOCS | Suppressor of cytokine signalling |
| SR | Scavenger receptor |
| STAT | Signal transducer and activator of transcription |
| TCF/LEF-1 | T-cell/lymphoid enhancer factor-1 |
| Thr | Threonine |
| TIR | Toll-interleukin-1 receptor |
| TIRAP | TIR domain-containing adaptor protein |
| TLR4 | Toll-like receptor-4 |
| TNF-$\alpha$ | Tumor necrosis factor-$\alpha$ |
| TNFR | TNF receptor |
| TRAF6 | TNF-receptor associated factor-6 |
| Tyr | Tyrosine |
| ng/mL | Nanagrams per milliliter |
| $\mu$g/mL | Micrograms per millilitre |
| U/mL | Enzymatic unit per millilitre |
| WT | Wild-type |
| Y204 | Tyrosine 204 residue |
| Y221 | Tyrosine 221 residue |

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# CHAPTER 1: Introduction

## 1.1 Macrophages: Functions

The immune system is composed of many interdependent cell types that have

specialized functions and collectively protect the body from bacterial, parasitic, viral infections

and growth of tumour cells. Among these, the macrophage is one of the most pleiotropic,

exhibiting a broad range of biological functions, including pro- and/or anti-inflammatory

activities and phagocytosis [4]. They also engulf and ingest foreign particles (innate immune

response) and present these antigens to T-cells (acquired immune response) and thus are often

referred to as antigen-presenting cells (APC) [5]. This is an important first step in the initiation

of an acquired immune response, which they regulate through a variety of cytokines and co-

stimulatory molecules [6]. Macrophage differentiation and activation states are greatly

influenced by environmental signals including, microbial antigens and cytokines [5, 7, 8]. Thus

the nature and consequence of macrophage activation is different among different macrophage

populations (e.g. bone marrow-derived, human blood monocytes, tissue macrophages from

different sites, and macrophages isolated from inflammatory lesions or wounds) and depends on

which cytokines are present [4].

## 1.1.1 Macrophages: Types

Over the past years distinct macrophage subsets have been characterized. These subsets

include classically activated macrophages (CAM$\phi$), alternatively activated macrophages

(AAM$\phi$), and Type-2 activated macrophages [9-11]. The best-studied are the CAM$\phi$, which are

induced by pro-inflammatory microbial molecules such as lipopolysaccharide (LPS) in a Th1 T-

cell (cell-mediated immune response) cytokine environment (e.g. Interferon-$\gamma$ (IFN-$\gamma$), tumour

necrosis factor-$\alpha$ (TNF-$\alpha$)) and release inflammatory and/or microbicidal products [9, 12].

These cells are distinguished by their ability to produce nitric oxide (NO) in addition to their

increased expression of major histocompatibility complex (MHC) class II and CD86, and their

enhanced antigen-presenting capacity [13, 14]. By increasing oxidative burst and NO release,

CAM$\phi$ play an important role in protection against intracellular pathogens [12]. They also exert

anti-proliferative and cytotoxic activities, partly due to their ability to secrete NO and pro-

inflammatory cytokines (TNF-$\alpha$, interleukin-1 (IL-1), IL-6) [9, 12, 15].

The observation that the development of CAM$\phi$ is inhibited by Th2 T-cell cytokines,

lead to characterization of alternatively activated macrophages (AAM$\phi$), which have a different

physiological function [16]. They do not produce IL-12, but secrete IL-10 and drive

preferentially a Th2 T-cell-like (antibody-mediated) immune response [10, 11, 17]. They also

express arginase activity, an enzyme that competes with inducible nitric oxide synthase (iNOS)

for L-arginine [10]. Arginase converts L-arginine to urea and L-ornithine thus shunting the

substrate away from the NO production pathway (Fig. 1). AAM$\phi$ has been shown to influence

the differentiation of T-cells, and determine whether immuno-stimulation versus immuno-

suppression or protective immune responses versus immuno-pathology responses occur [12, 15,

18, 19].

Co-stimulation of LPS activated macrophages through the Fc$\gamma$ receptor results in

inhibition of IL-12 synthesis and increased amounts of IL-10 as compared to LPS stimulation

alone [10, 20]. Furthermore, these cells switch from triggering a Th1 T-cell response dominated

by IFN-$\gamma$ secretion, to the induction of a Th2 T-cell response typified by IL-4 secretion and

increased antibody responses, mainly of the IgG1 isotype [10, 21]. These cells are called Type

2-activated macrophages. They exhibit functional similarities to CAMφ and AAMφ. Like CAMφ, they produce TNF-α, IL-1 and IL-6, but unlike CAMφ, Type 2-activated macrophages do not produce IL-12, but secrete IL-10 and drive preferentially a Th2 T-cell-like immune response [10, 11, 17]. This phenotype is similar to AAMφ, with the only difference being that Type 2-activated cells do not express arginase activity [10]; suggesting that macrophage populations with overlapping phenotypes and/or functions exist. Thus the classification of the distinct macrophage populations is still operational due to absence of definitive molecular markers.

## 1.1.2 Effectors and regulatory products of macrophages

Activation of macrophages by IFN-γ or bacterial cell products such as LPS induces a number of immunologic responses such as production of pro-inflammatory mediators (e.g. IL-1, IL-6, TNF-α, chemokines, myeloperoxidase etc). Activated macrophages also induce microbiocidal activity (e.g. release of reactive oxygen and nitrogen intermediates), lymphocyte activation (antigen processing and presentation) and tissue remodelling (e.g. production of elastase/collegenase/hyalruronidase enzymes) [11]. These responses are important for macrophages to exert their functions in killing bacteria, parasites, viral-infected cells and tumour cells and providing T-cell help.

### (i)  Interleukin-1 (IL-1)

IL-1 is produced primarily by activated macrophages, but can also be made by other cells. IL-1 has a number of physiological actions including inducing production of tissue factor thus triggering the blood-clotting cascade, decreasing blood pressure and inducing fever. But the predominant immune function of IL-1 is to enhance the activation of T-cells in response to antigens and thus initiate an adaptive immune response [22]. There is an increase in production

of IL-2 and its receptor by T-cells in response to IL-1, which in turn augments the activation of the T-cells in an autocrine manner [22]. IL-1 also induces expression of IFN-$\gamma$ by T-cells. This effect on T-cell activation by IL-1 is mimicked by TNF-$\alpha$ which is another cytokine secreted by activated macrophages.

(ii)    **Interleukin-6 (IL-6)**

IL-6 is a cytokine in the hematopoietin family [23] produced by activated macrophages, but also by fibroblasts, endothelial cells and activated T-helper cells [24]. Its functions range from key roles in acute-phase protein induction to B and T-cell growth and differentiation [25, 26]. Unlike IL-1, IL-2 and TNF-$\alpha$, IL-6 does not induce cytokine expression, but rather augments the responses of immune cells to other cytokines [27]. IL-6 acts in synergy with IL-1 and TNF-$\alpha$ in many immune responses, including T-cell activation [28]. IL-6 induces transcription of various proteins through the three major signal transduction pathways; protein kinase C, cAMP/protein kinase A, and the calcium release pathway [29]. IL-6 stimulates the acute-phase reaction, which alerts the innate immune system and protects against tissue damage [30]. This results in the increases synthesis of the two major acute-phase proteins, C-reactive protein (CRP), which increases the rate of phagocytosis of bacteria, and serum amyloid A (SAA). Similarly, it also increases the synthesis of fibrinogen, an important clotting agent. The local acute phase reaction leads to a systemic reaction, which includes: fever, increased erythrocyte sedimentation rate, increased secretion of glucocorticoids, and the activation of the complement and clotting cascades. [31] Dysregulation in IL-6 production has been shown to lead to septic shock and pathogenesis of many diseases and autoimmune disorders, such as liver autoimmune disease [32-34].

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(iii)    **Chemokines**

Chemokines are among the most abundant proteins produced by an activated macrophage. Interleukin-8 (IL-8), monocyte chemotactic protein-1 (MCP-1) and macrophage inflammatory protein-1$\alpha$ (MIP-1$\alpha$) are the major leukocyte chemoattractants produced during a bacterial infection by recruiting neutrophils, T-cells and more macrophages to the site [35]. IL-8 also stimulates neutrophils to degranulate.

(iv)    **Tumor Necrosis Factor-$\alpha$ (TNF-$\alpha$)**

TNF-$\alpha$ is a pleiotropic inflammatory cytokine produced by several types of cells, but primarily by activated monocytes/macrophages [36]. It is an acute phase protein, which initiates a cascade of cytokines and increases vascular permeability, thereby recruiting macrophage and neutrophils to a site of infection [37]. Biological effects of this molecule include induction of apoptosis, cytolysis of tumor cells, activation of polymorphonuclear (PMN) leukocytes, antiviral activity and induction of IL-1 [38, 39]. It possesses both growth stimulating and growth inhibitory properties. For instance, during inflammation, TNF-$\alpha$ induces neutrophil proliferation, but upon binding to the TNFR-55 receptor it causes neutrophil apoptosis [40]. Stimulated macrophages produce membrane-bound 27 kd TNF-$\alpha$, which can either bind directly to TNFR-55 and TNFR-75 receptors through cell-to-cell contact, but the majority of TNF-$\alpha$ protein undergoes cleavage and binds in its soluble form [41]. TNF-$\alpha$ secreted by the macrophage causes production of tissue factor resulting in blood clotting which serves to contain the infection [42, 43]. In the absence of TNF-$\alpha$, mice infected with gram-negative bacteria are susceptible to sepsis [44]. But high levels of TNF-$\alpha$ correlate with increased risk of mortality [37] and TNF-$\alpha$ also seems to be a central mediator in various pathologies [43]. A few such examples include: septic shock, cancer, AIDS, transplantation rejection, multiple

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sclerosis, diabetes, rheumatoid arthritis, Crohn's Disease, trauma, malaria, meningitis, ischemia-reperfusion injury, and adult respiratory distress syndrome.

(v)    **Nitric Oxide (NO)**

Macrophages use the cytotoxic properties of NO to eliminate parasites, bacteria and other potentially infectious particles [45]. In the vasculature, NO reacts with iron in the active site of the enzyme guanylyl cyclase (GC), stimulating it to produce the intracellular mediator cyclic GMP (cGMP) that in turn enhances the release of neurotransmitters resulting in smooth muscle relaxation and vasodilation [45]. NO toxicity is linked to its ability to combine with superoxide anions ($O^{2-}$) to form peroxynitrite (ONOO⁻), an oxidizing free radical that can cause DNA fragmentation and lipid oxidation [45]. NO is also a mediator in inflammatory diseases such as rheumatism and arthritis. The enzyme, inducible nitric oxide synthase (iNOS), catalyzes the production of NO from L-arginine (Fig. 1). Within macrophages, L-arginine can be metabolized by two different pathways that result in the production of: (i) L-citrulline and NO by iNOS; and (ii) ureum and L-ornithine by arginase. The regulation of iNOS–arginase balance by Th1 T-cell immune mediators (IFN-γ or LPS) and Th2 T-cell cytokines (IL-4 and IL-10) in distinct macrophage populations reflects their polarization to either classically or alternatively activated macrophages. Studies have shown that low blood pressure induced by septic shock, as well as inflammation associated with the development of arthritis and kidney disease is reversed with L-NMMA (an iNOS inhibitor) treatment [46]. Transgenic mice unable to generate NO during the immune responses display a reduced inflammatory response [47]. IL-10 inhibits NO production by either inducing upregulation of arginase expression, thus shunting away the L-arginine substrate from the iNOS pathway, or by inhibiting expression of iNOS protein itself.

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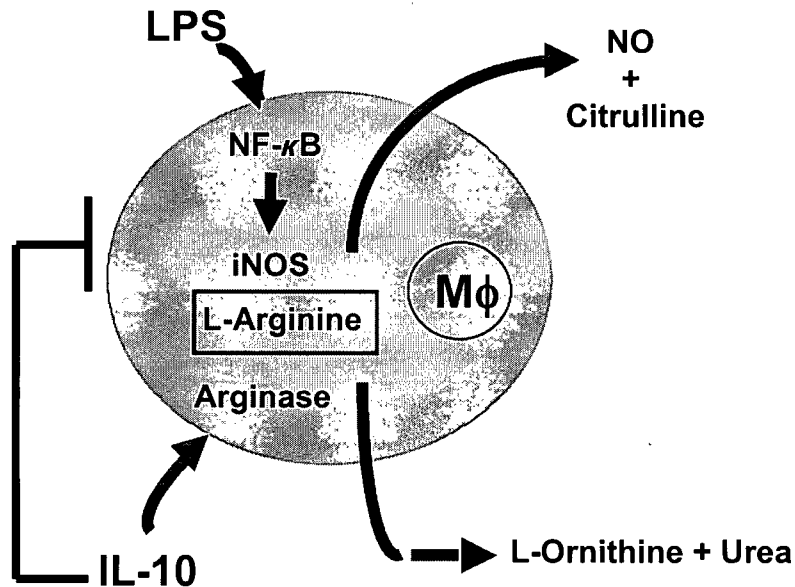
**Figure 1. Schematic representation of regulation of iNOS protein by LPS and IL-10 in macrophages.** LPS activates NFκB pathway which leads to induction of iNOS protein. iNOS protein uses L-arginine as a substrate to synthesize nitric oxide and carline, on the other hand the enzyme arginase, induced by IL-10, shunts the L-arginine away from the iNOS pathway and degrades it into products such as L-ornithine and urea. IL-10 can also inhibit iNOS protein directly, either by inhibiting its message or protein expression.

vi)     **Myeoloperoxidase (MPO)**

MPO, an iron-containing protein, is found in the azurophilic granules of neutrophilic polymorphonuclear leukocytes (PMNs) and in the lysosomes of monocytes in humans [11]. Its major role is to aid in microbial killing. Monocytes lose their MPO activity during conversion to tissue macrophages, therefore microbicidal and cytotoxic activity of macrophages is dependent mainly on reactive oxygen intermediates (ROI), NO and other substances which are similar to those in neutrophils [48-51]. However, macrophages may acquire MPO from their environment

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(e.g. from ingested neutrophils) to participate in their cytotoxic mechanisms. MPO may have a role in atherosclerosis, carcinogenesis, and degenerative neurological diseases.

## (vii) Prostanoids

Upon phagocytosis, macrophages release arachidonic acid from esterified glycerol phospholipids of the cell membranes [52, 53]. It is immediately metabolized into pro-inflammatory agents called prostanoids [especially prostaglandin E2 (PGE) and prostacyclin (PGI)]. These factors induce vasodilatation, act synergistically with complement component C5a and LTB, mediate fever and myalgia in response to IL-1, in the combination with bradykinin and histamine they contribute to erythema, edema, and pain induction.

## (viii) Extracellular Proteases

Macrophages not only secrete cytotoxic and inflammatory mediators, but they also release substances participating in tissue reorganization, which include enzymes, such as hyaluronidase, elastase, and collagenase. Hyaluronidase reduces tissue viscosity and allows for greater spreading of material in tissue spaces by destroying hyaluronic acid, an important component of connective tissue. Similarly, elastase and collagenase enzymes are involved in degradation of collagen and elastin, the basic components of connective tissue, resulting in disorganization of extracellular matrix, which is important for the integrity of the cells.

## 1.2 Macrophage Activation through LPS Signalling Pathway

Lipopolysaccharide (LPS) is an integral component of gram-negative bacterial cell wall, which elicits a broad spectrum of biological activities [4]. LPS causes activation of monocytes/macrophages, which are then able to recognize and kill foreign bacterial microorganisms [54]. LPS also induces production of endogenous mediators such as TNF-α, IL-1, IL-6, IL-8, NO, superoxide anions, and lipid mediators [55, 56]. However, excessive amounts of pro-inflammatory cytokines may result in fatal septic/endotoxic shock. When cytokine production increases to such an extent that it escapes the local infection, or when infection enters the bloodstream, sepsis develops [56, 57]. Systematic edema results in low blood volume, hypo-proteinanemia, neutropenia and then neutrophilia [44]. Victims of septic shock experience fever, falling blood pressure, myocardial suppression, dehydration, acute renal failure, tissue damage and then respiratory arrest [42]. Tissue damage is brought on by the loss of blood flow, which in turn increases the production of NO and leading to a further fall in blood pressure. Fatality due to organ failure may occur. Therefore, in order to understand how to maintain regulated macrophage function during inflammatory responses, it is important to study the molecular mechanisms that both underlie and limit LPS signalling.

### 1.2.1 LPS Signalling

LPS consists of four regions that are structurally and functionally distinct: the O-specific chain, the outer core, the inner core and the lipid A moiety [4]. The conserved lipid A region is responsible for the majority of biological activities of LPS [58]. The three molecules expressed on the surface of macrophages that are known to bind to lipid A moiety of LPS include: CD14, the macrophage scavenger receptor (SR), and the β2 (CD11/CD18) leukocyte

integrins [59]. However, the signalling receptor for LPS consists of toll-like receptor-4 (TLR4) and two other extracellular subunits, the glycosylphosphatidylinositol-linked CD14 and soluble MD2 protein, which increases the affinity of LPS binding [60-63] (Fig. 2). The serum protein, LPS binding protein (LBP), accelerates the high affinity binding of LPS to CD14 because it catalytically transfers LPS monomers from aggregates (including bacterial membranes) to CD14 [64-66]. The importance of CD14 was shown by experiments using blocking monoclonal antibodies to CD14 resulting in inhibition in the ability of LPS to stimulate phagocytes [59]. A soluble fragment of CD14 (sCD14) facilitates the activation of cells that do not express membrane CD14, such as endothelial cells [67-69]. Mice deficient in CD14 are resistant to endotoxin shock after LPS challenge [70, 71]. It is generally accepted that the interaction between lipid A and CD14 is significant in cellular activation of LPS, but since CD14 lacks a transmembrane and cytoplasmic domain, it must associate with another transmembrane molecule to transduce signals into the cell [57].

The transmembrane signalling subunit for the LPS receptor complex is TLR4 and its discovery has greatly contributed to the understanding of the molecular basis of LPS recognition and LPS-induced signalling events [72]. The involvement of TLR4 in LPS-induced signalling events was demonstrated using LPS-hyporesponsive mice, which have either a point mutation in TLR4 [73, 74] or are TLR4-deficient [62]. A third extracellular component of the LPS receptor is MD-2. Expression of MD-2, as well as TLR4, is required for reconstituting LPS responsiveness in the non-macrophage, Ba/F3, cell [75], and also human embryonic kidney (HEK) 293 cells [76]. Down-regulation of cell surface expression of TLR4/MD2 complex in mouse peritoneal macrophages correlates with endotoxin tolerance, a state of LPS unresponsiveness [77].

After LPS binds its receptor complex, TLR4 associates with the intracellular adaptor proteins, Myd88 and TIRAP proteins [22]. Myd88 recruits the IRAK kinase which associates with the adaptor protein TRAF6, leading to activation of the MAP3-kinases Tak1 and MEKK1 which phosphorylates and activates the IκB kinase (IKK) complex [78, 79]. IKK phosphorylation of IκB on serine[23] targets IκB for recognition and ubiquitination by the E3 ubiquitin ligase complex, E3RS [IkB] [80] and degradation by 26S proteasome. IκB regulates the activation of the Nuclear factor κB (NFκB), which is a transcription factor sequestered in the cytoplasm by IκB protein [79]. IκB phosphorylation by IKK results in release of NFκB from the NFκB/IκB complex. This phosphorylation leads to the exposure of the nuclear localization signals (NLS) on the NFκB subunits and the subsequent translocation of the molecule to the nucleus [81].
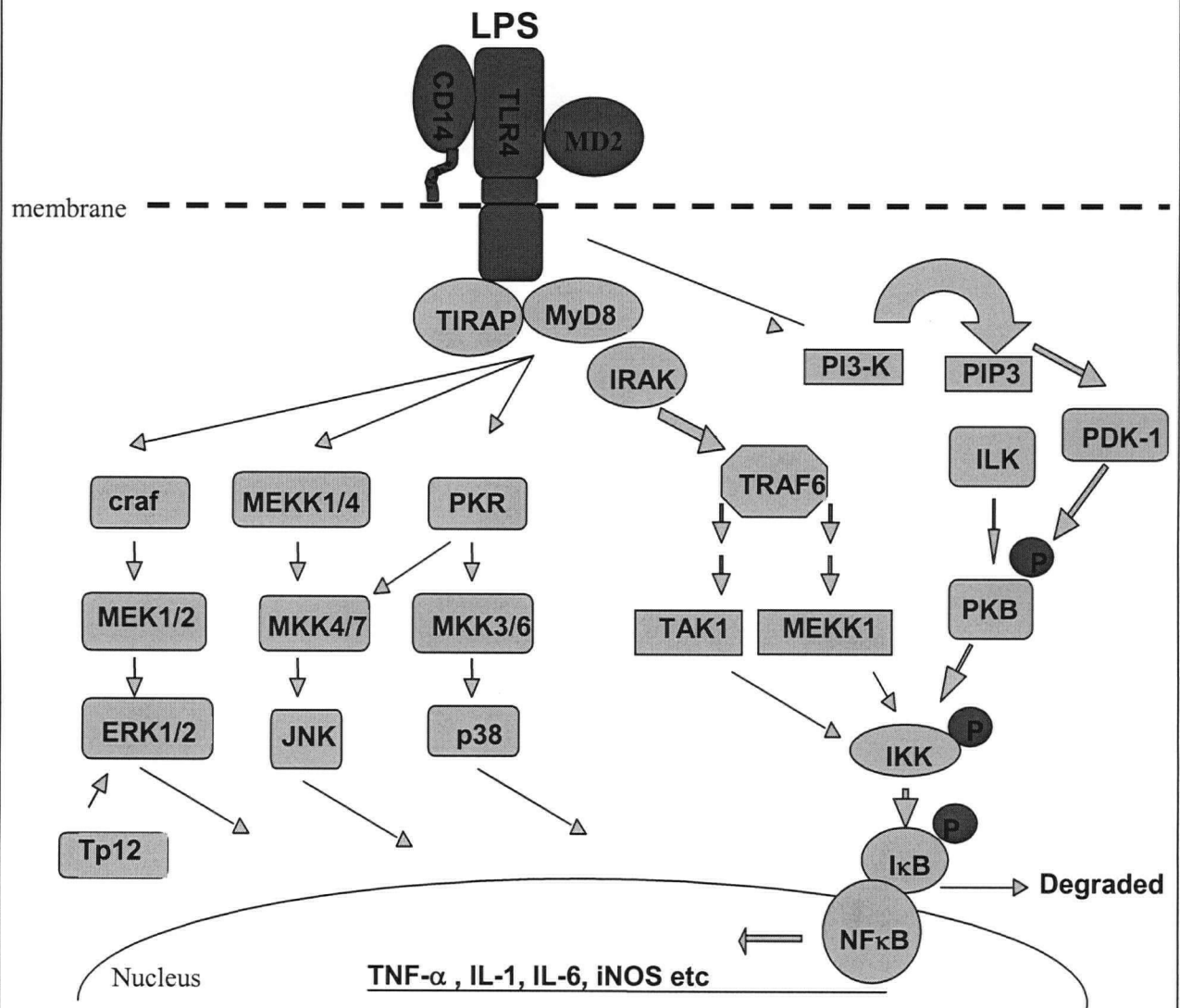
**Figure 2.   Schematic representation of LPS signalling.**
After LPS binds its receptor complex, TLR4 associates with the intracellular adaptor proteins, Myd88 and TIRAP proteins.  Myd88 recruits the IRAK kinase which associates with the adaptor protein TRAF6, leading to activation of the MAP3-kinases Tak1 and MEKK1/4, PKR, craf and PI3-kinase. IκB phosphorylation by IKK results in release of NFκB from the NFκB/IκB complex. This phosphorylation leads to the exposure of the nuclear localization signals (NLS) on the NFκB subunits and the subsequent translocation of the molecule to the nucleus, where transcription of regulated genes is induced.

### 1.2.2 Targets of LPS Signalling

### a) NFκB activation

There are five NFκB subunit family members that form dimers: RelA (p65), p50, RelB, c-Rel, and p52; and they dimerize in various combinations which have differing DNA binding affinity and transactivation potential [82]. The most common and best-characterized form of NFκB is the p65/p50 heterodimer, which binds to a consensus sequence (5' GGGACTTTCC-3') in the promoter of various genes and activates their transcription [82]. For instance, NFκB induces the transcription of various interleukins (e.g. IL-1), cytokines (e.g. TNF-α) and inducible nitric oxide synthase (iNOS) [83]. NFκB plays an important role in the regulation of immune responses, inflammation, cell-cycle progression, cell apoptosis, oncogenesis and various autoimmune diseases [84]. The activation of NFκB is thought to be part of a stress response as it is activated by a variety of stimuli that include TNF-α, IL-1, lymphokines, and UV [85]. Pyrollidine dithiocarbamate (an inhibitor of NFκB DNA binding activity) completely inhibits cytokine production, showing that activation of NFκB is essential for proinflammatory mediator production [39].

### b) PKB activation

LPS stimulation of protein kinase B (PKB) can also activate IKK complex by phosphorylation of IKK at threonine 23 [86, 87]. Additionally, it has been shown that PKB stimulates the transactivation activity of the RelA/p65 through induction of IKK and p38 MAPK [14]. Suppression of IKK activity by IL-10 has been shown to lead to inactivation of LPS and TNF-α induced NFκB expression [88].

## c)    ILK activation

LPS also activates integrin-linked kinase (ILK), an ankyrin-repeat containing serine/threonine protein kinase, whose activity is regulated by cellular levels of phosphatidylinositol-3,4,5-trisphosphate (PIP3) in a phosphatidylinositol-3'-kinase (PI3-K) dependent manner [89]. ILK induces phosphorylation and activation of protein kinase B (PKB) and IκB, leading to activation of NFκB, which then activates iNOS [90]. Besides regulation of NFκB in response to LPS, ILK also regulates the activity of transcription factors such as β-catenine-TCF/LEF-1 (T-cell/lymphoid enhancer factor), AP-1 (adaptor protein-1) [91] and CREB (cAMP responsive element binding protein) [92].

### 1.2.3   Activation of mitogen-activated kinases (MAPKs)

Other important events in LPS signalling include activation of the MAP kinases: JNK [85], ERK1/2 [93] and p38 MAPK [94]. The contribution of these pathways to expression of the prototypic proinflammatory cytokine TNF-$\alpha$ has been well studied.

### (i)    JNK

TNF-$\alpha$ transcription is regulated by the c-Jun-N-terminal kinase (JNK). Activation of JNK is necessary for phosphorylation of activating transcription factor 2 (ATF-2) and c-Jun (c-Jun mitogen activated kinase) which complexes with the transcription factor CREB to support transcription of TNF-$\alpha$ [85]. Functional analysis of TNF-$\alpha$ promoter, showed enhancer elements in the region containing CREB and NF-κB sites to be required for optimal transcription of the TNF-$\alpha$ gene in response to LPS [85]. But concerted participation of c-Jun complexes and p50/p65 are required for the maximal LPS induction of the TNF-$\alpha$ promoter [85].

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(ii)         **ERK1/2**

LPS activates ERK through the serine/threonine kinase, Tp12, and the ERK pathway is required for cytoplasmic transport of TNF-α mRNA [93]. The 3'-untranslated region (UTR) of the TNF-α transcript contains a well-characterized type II AU-rich element (ARE) involved in both translational control and mRNA stability [94, 95]. LPS-induced Tpl2/ERK-transduced signals target the AU-rich element in the 3'UTR of the TNF-α RNA and controls its cytoplasmic mRNA transport [93]. Tpl2$^{-/-}$ mice also demonstrate resistance to LPS/D-Galactosamine-induced shock due to post-transcriptional defect in the induction of TNF-α by LPS [93].

(iii)       **p38 MAPK**

p38 MAPK regulates translation of TNF-α protein [94]. LPS activation of p38 MAPK is essential for removing the TNF-α mRNA translational block and allowing association of TNFα mRNA with polyribosomes, the site of active protein translation [94]. This regulation is dependent on the presence of the ARE in the 3'UTR.

## 1.3 Biological Activities of IL-10

As discussed previously, the persistence of inflammatory processes often results in tissue damage, or even fatality; resulting in disorders such as: rheumatoid arthritis, Crohn's disease, and septic shock [96]. Thus the immune system is faced with a permanent challenge: how to control infection while limiting tissue damage. Hence, anti-inflammatory mechanisms are mandatory for host survival. Among the factors that limit inflammatory responses, the anti-inflammatory cytokine, IL-10, is one of the most important. IL-10 is a key regulator of both innate and acquired immunity. Produced by activated B-cells, keratinocytes, monocytes and macrophages, IL-10 was initially detected as a Th2 T-cell product that inhibited the proliferation, development and function of Th1 T-cells. The molecular cloning of IL-10 and subsequent studies utilizing recombinant cytokine revealed that although IL-10 exerted direct effects on T-cells [97, 98], its major site of action was the activated macrophage [99].

Activation of macrophages by IFN-$\gamma$ or LPS induces a number of immune responses, many of which are inhibited by IL-10 [100]. IL-10 suppresses production of proinflammatory mediators: cytokines, including TNF-$\alpha$ [101], IL-1 [102] IL-6; and chemokines such as MIP-1$\alpha$ [103], MCP-1and IL-8 [104]. It also inhibits phagocytosis [3] by inhibiting reactive oxygen and nitrogen intermediate production in monocytes/macrophages and neutrophils [101]. In addition to limiting and terminating inflammatory responses, IL-10 also regulates growth and/or differentiation of B cells, NK cells, cytotoxic and helper T-cells, mast cells, granulocytes, dendritic cells, keratinocytes, and endothelial cells [100]. By inhibiting expression of co-stimulatory molecules such as CD80/86, MHC II and ICAM-1, IL-10 makes macrophages a poor APC [105-108]. IL-10 also inhibits the production of IL-12, which together with the inhibition of other co-stimulatory molecules suppresses primary alloantigen-specific T-cell

responses [20, 109]. IL-10 induces a long lasting state of non-responsiveness (anergy) in T-cells, which cannot be reversed by IL-2 or by stimulation with anti-CD3 and anti-CD28 [110]. These inhibitory effects of IL-10 are not due to non-specific, global suppression of cellular metabolism, since production of the anti-inflammatory mediators such as IL-1R antagonist and soluble TNF-$\alpha$ receptor are enhanced by IL-10.

Various animal model studies have substantiated the *in vivo* importance of an anti-inflammatory role for IL-10. Targeted disruption of the IL-10 gene results in mice characterized by inflammatory bowel disease [111] and exaggerated immune reactions when challenged with antigen or LPS [112]. Administration of IL-10, on the other hand, ameliorates disease in such models of endotoxemia [113], transplantation [114] and autoimmunity [115]. In humans, the presence of elevated endogenous IL-10 is a positive prognostic variable in autoimmune disease [116] and allogeneic transplant patients [117]. IL-10 has recently entered clinical trials for the treatment of human inflammatory bowel disease [118].

### 1.3.1 IL-10 Signalling Pathways in Macrophages

As shown in Figure 3, the IL-10R consists of at least two subunits [119-122]. Both belong to the type II cytokine receptor super-family, which include all the IFN receptor proteins [123] and both are necessary for IL-10 signal transduction. The primary ligand-binding component designated IL-10R$\alpha$, binds IL-10 with high affinity and in the presence of IL-10 associates with an accessory subunit called IL-10R$\beta$ (CRF2-4). Like other members of the class II cytokine receptor family, both IL-10R$\alpha$ and IL-10R$\beta$ possess a juxtamembrane box 1 motif important for interaction with members of the Janus family kinases (Jak1/Tyk2) [123]. Ligand-induced hetero-dimerization of the receptor chains results in activation of IL-10R$\alpha$-bound Jak1

17

and IL-10Rβ-bound Tyk2. Activated Jak kinases phosphorylate IL-10Rα on two cytoplasmic tyrosine residues (Y427/477 of mIL-10R1; Y446/496 of hIL-10R1) [123] and create docking sites for latent cytoplasmic transcription factors of the Stat family [124]. In the case of the IL-10R, Stat3 is recruited to these phosphotyrosyl residues [125] and becomes phosphorylated by receptor-bound Jak kinases. Stat1 also becomes tyrosine phosphorylated, but not to the same extent as Stat3 and the mechanism of receptor recruitment is not clear.

Upon phosphorylation, Stat3 and Stat1 hetero- and/or homodimerize, translocate into the nucleus, bind specific sequences in the promoters of target genes and stimulate transcription [125, 126]. Studies using mutant IL-10R1 lacking the tyrosine necessary for Stat3 activation, dominant-interfering Stat3 and a coumermycin dimerizable Stat3, have shown that the Stat3 pathway is essential for the anti-proliferative action of IL-10 on macrophage cell growth [127]. The absolute requirement of Stat3 in mediating the anti-inflammatory effects of IL-10 has been demonstrated [128]. Stat3 pathway is also essential for the ability of IL-10 to inhibit LPS-induced production of the proinflammatory cytokine TNF-α [129].

### 1.3.2 Genes regulated by IL-10 through Stat3 pathway

IL-10 has been shown to induce expression of certain genes in a Stat3-dependent manner. These genes include the cell cycle inhibitors, $p19^{INK4d}$ [130], heme-oxygenase-1 (HO-1) [131], nuclear protein Bcl3 [132],which is a member of IκB protein family harboring ankyrin repeat domains and suppressor of cytokine signalling 3 (SOCS3), which will be discussed in more detail on page 31.
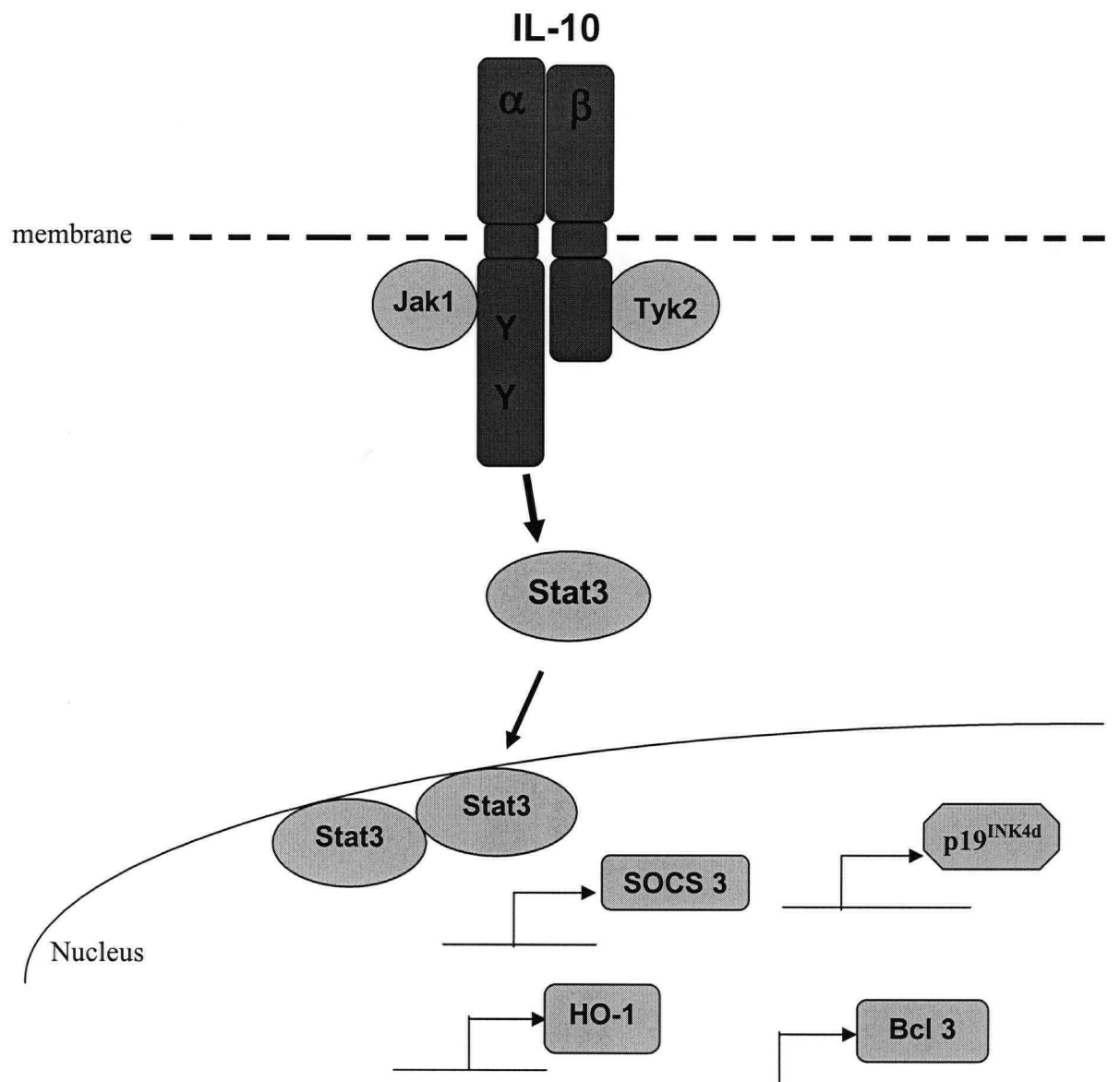
**Figure 3. Schematic representation of IL-10 signaling.**
IL-10 receptor contains two subunits, $\alpha$ and $\beta$, which interact with members of the Janus family kinases (Jak1/Tyk2). Ligand-induced heterodimerization of the receptor chains results in activation of Jak1 and Tyk2, which then phosphorylate IL-10R$\alpha$ on two cytoplasmic tyrosine residues (Y427/477 of mIL-10R$\alpha$; Y446/496 of hIL-10R$\alpha$) and create docking sites for Stat3 transcription factor, which is recruited to these phosphotyrosyl residues and becomes phosphorylated by receptor-bound Jak kinases. Upon phosphorylation, Stat3 homodimerizes, translocate into the nucleus, bind specific sequences in the promoters of target genes and stimulate transcription

### 1.3.3 Targets of IL-10 in Inhibition of LPS Signalling

**a) Inhibition of NFκB activation**

Although several studies have reported inhibitory action of IL-10 on either the NFκB [22, 78-80, 107, 133-135] or MAP kinase pathways [39], the mechanisms governing this process is still unclear. IL-10 pretreatment inhibits LPS and TNF-α induced NFκB DNA-binding by specifically disrupting the p65/p50 heterodimer complex in human PBMC [133] and murine macrophages [135]. TNF-α gene transcription is suppressed by IL-10 [136, 137], possibly by interfering with activation of NFκB [88, 133, 138]. In alveolar macrophages, IL-10 stabilizes IκB protein by delaying its LPS-mediated degradation and resulting in delayed nuclear translocation of the p65 subunit [139]. TNF-α induced IL-8 production is regulated through an NFκB-dependent mechanism [140], which is also inhibited by IL-10 through inhibition of NFκB transcriptional activity [104]. Therefore, NFκB-dependent transcriptional regulation is a target for the anti-inflammatory actions of IL-10 [135]. However, in contrast, other studies have demonstrated that IL-10 is unable to suppress LPS-induced activation of NFκB DNA binding in human monocytes [141]. These conflicting results may be due to different cell types used in the studies (human PBMC and murine macrophages *versus* human monocytes).

**b) Inhibiting production of other pro-inflammatory mediators**

IL-10 interferes with LPS-induced production of pro-inflammatory mediators at the level of transcription or mRNA stability, protein translation or protein stability [137-139, 142, 143]. Incubation of alveolar macrophages with IL-10 results in the steady state mRNA levels of TNF-α, IL-1α and IL-1β and inhibition of their gene expression [139]. In murine macrophages, IL-10 acts primarily through post-transcriptional mechanisms by destabilizing TNF-α mRNA

[142, 144] or by inhibiting gene translation via suppressing the activation of p38 MAPK [94]. Of particular note, IL-10 was recently shown to suppress TNF-α translation by inhibiting the association of TNF-α mRNA with polyribosomes in a mechanism also dependent on the presence of an intact ARE in the 3'-UTR [94].

Studies have also shown that some of the intracellular mechanisms by which IL-10 inhibits proinflammatory cytokine production by LPS-activated macrophages are dependent on de novo protein synthesis [3, 104, 142]. For instance, treatment with cycloheximide, which blocks protein synthesis, antagonized IL-10-dependent inhibition of TNF-α, IL-1β and IL-6 mRNA expression and cytokine release, suggesting an involvement of newly synthesized proteins in IL-10 action [88, 133, 142].

c)      **Inhibition of receptor expression**

Signalling pathways are also inhibited by suppression of receptor expression, thus the possibility that IL-10 inhibits LPS signalling by inhibiting expression of TLR4 has been examined in monocytes and polymorphonuclear leukocytes, where IL-10 has been shown to inhibit upregulation of TLR4 mRNA in response to LPS [145].

## 1.4 Suppressor of Cytokine Signalling 3 (SOCS3)

In our search for IL-10 induced, Stat3-regulated genes, we found a candidate belonging to the SOCS (Suppressors of Cytokine Signalling) family of negative regulators of cytokine signaling (Fig. 4). The SOCS proteins are a family of Stat-inducible genes variously referred to as CIS (cytokine-inducible SH2 protein) [146-148], SOCS [149] or SSI (Stat-induced Stat-inhibitor) [150, 151]. The first member of SOCS, CIS1, was cloned as an early gene induced by various cytokines, acting via the conserved intracellular region of their respective receptors. The encoded protein had a domain that differed in amino acid sequence from all the known SH2 domains. CIS plays an important role in inhibition of erythropoietin and IL-3 receptor signalling [152]. The second member of the family was cloned by three independent research groups and the protein was given three different names: 1) Endo et al, isolated a single positive clone from a cDNA library, expressing a Jak-binding protein, **JAB**, capable of interacting with the Jak kinase domain [147]; 2) Naka et al, discovered Stat3-induced Stat3 inhibitor (**SSI**) by using monoclonal antibodies against a sequence motif found in the SH2 domain of Stat3 [151]; and 3) Starr et al, discovered a protein capable of suppressing cytokine signal transduction, **SOCS1** [40]. SOCS1 and SOCS3 have been shown to be essential for proper regulation of interferon-γ and IL-6 responses, respectively [152 1999].
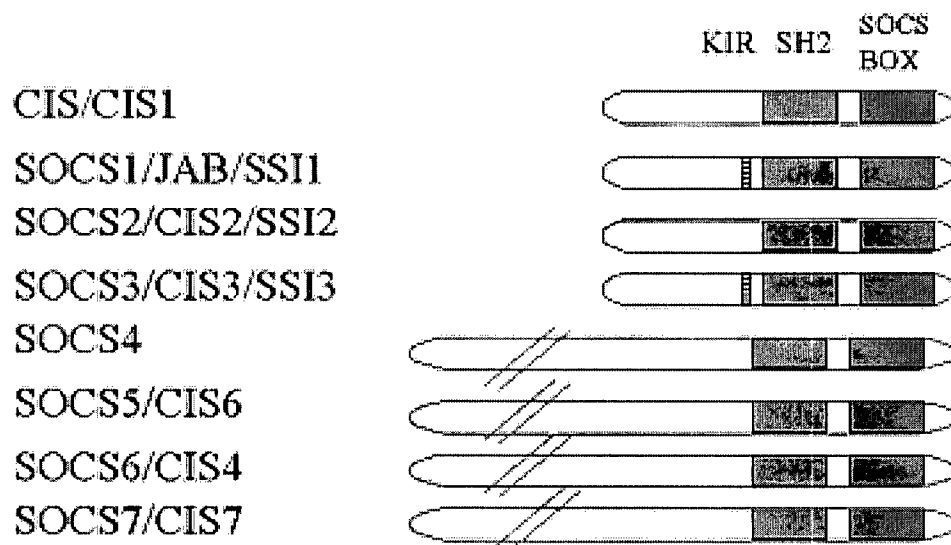
**Figure 4.** The alternate names and domain structures of various members of the SOCS family.

The kinase inhibitory region (KIR) of SOCS1 and SOCS3 is dashed. This figure is adapted from Larsen, L. et al, 2002 [2].

### 1.4.1 Conserved Domains of SOCS Proteins

#### a) KIR and SH2 domains

SOCS proteins are a family of seven proteins that possess a C-terminal, 70 amino acid region of homology referred to as the SOCS box as well as an SH2 domain [153] as shown in Figure 4. However, SOCS1 and SOCS3 are different from the rest of their family members since they not only have no introns, but also possess a 12 amino acid extended SH2 domain that constitute a kinase inhibitory region (KIR) [153]. SOCS proteins have also been found to inhibit the catalytic activity of Jak and non-Jak family kinases [154] and may act on other types of signalling molecules [155] through their KIR domains. SOCS1 and SOCS3 co-precipitate with Jaks upon cytokine stimulation, and are able to inhibit the kinase activity of these, although with differing affinity and kinetics [147, 152]. Figure 5 shows a schematic representation of the structure and model of kinase inhibition by SOCS1 and SOCS3. SOCS3 has weaker affinity for binding to the Y1007 of Jak2, residing inside an activation loop of the kinase domain, JH1 [152]. It is suggested that the primary binding for SOCS3 may not be Jak, but other molecules within the signalling cascade, such as the phosphorylated cytokine receptor or Stat proteins [148]. SOCS3 has been shown to interact through its SH2 domain with phospho-tyrosine sites on the receptors for the cytokine that induced it. These include the receptors for IL-6 (Y757/Y759) [156], insulin [157], erythropoietin (Y401) [158] and leptin (Y985) [159].
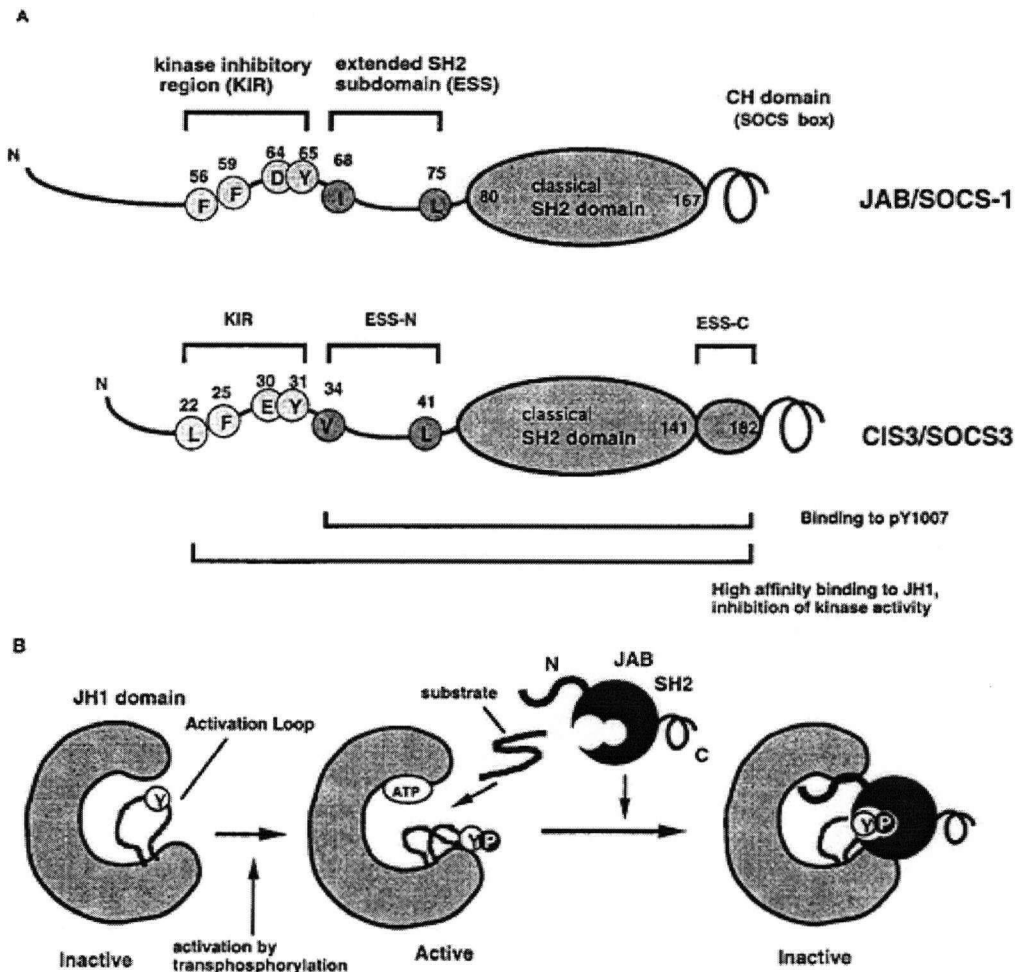
**Figure 5. Structure and model of kinase inhibition by JAB/SOCS1 and CIS3/SOCS3.**
(A) Schematic model of the functions of JAB and CIS3 domains. The bold highlighted circles represent the essential amino acids in the kinase inhibitory region (KIR) and the extended SH2 subdomain. (B) The model of JH1 activation and inhibition by JAB/SOCS-1. Binding of JAB to the activation loop prevents the access of substrates and/or ATP to the catalytic pocket This figure is adapted from Yasukawa, H. et al, 2000 [1].

b)     **SOCS-box domain**

The conservation of the SOCS-box domain in all SOCS proteins indicates that it plays

an important role in the physiological action of these proteins. All SOCS proteins have been

found to associate through their SOCS-box with Elongins B and C [153]. The Elongin BC

complex was initially identified as components of the mammalian transcription factor SIII,

which when interacting with a third protein (Elongin A), can increase the overall elongation rate

of RNA polymerase II in vitro [160]. Elongin B is ubiquitin-like protein and Elongin C is

similar in sequence to Skp1 protein, which through protein-protein interaction regulates cellular

processes, such as cell cycle, transcription and development [161]. Elongin A stabilizes the

Elongin BC complex and the motif by which Elongin A recognizes Elongin C has amino acid

similarity to the SOCS-box. This Elongins BC complex recruits Cullin-2 (Cul-2), Rbx1, and the

E2 ubiquitin-conjugating enzyme and this forms the E3 ubiquitin-ligase [162]. The proximity

of this ubiquitin ligase to SOCS-recruited signalling molecules can result in the latter's

ubiquitination and degradation [163, 164]. Figure 6 shows a schematic representation of several

mechanisms used by SOCS-box to target proteins for degradation.

The role of Elongins BC in SOCS function however is not entirely clear. Various reports

suggest that association of SOCS protein with Elongins BC serves to protect [162, 165] rather

than accelerate proteasome-mediated degradation [160]. Interaction of SOCS-box with Elongin

BC complex has been shown to markedly increase the stability of the SOCS1 protein and

protect it from proteasomal degradation [162, 166]. A study by Hanada et al, has shown that

relative to SOCS3 wild type controls, T-cells expressing SOCS3 protein with a mutant SOCS-

box domain, express significantly lower levels of SOCS3 protein due to destabilization of the

protein, which is reversed with over-expression of Elongin BC [165]. This dichotomy reflects

variable abilities of different SOCS/Elongins complexes to recruit an ubiquitin-ligase activity.
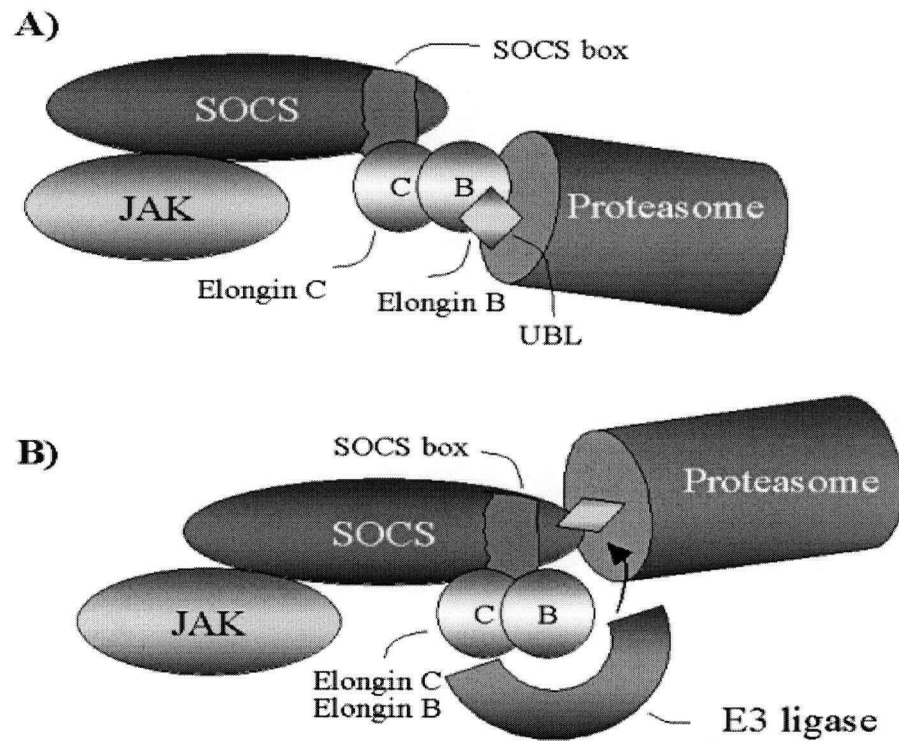


**Figure 6. The SOCS-box targets proteins for proteasomal degradation by several mechanisms.**
(A) After SOCS binding to JAK, elongin B and C are recruited to the SOCS-box, and the ubiquitin-homology domain on SOCS-box is recoginzed by the proteasome. (B) Elongins B and C bind to the SOCS-box and recruit an E3 ligase, transferring ubiquitin to the SOCS protein and targeting the complex for proteasomal degradation. UBL, ubiquitin like protein. This figure is adapted from Larsen, L. et al, 2002 [2].

### 1.4.2 Tyrosine Phosphorylation of SOCS3 Protein

Jaks and receptor tyrosine kinases can phosphorylate SOCS3 at two tyrosine residues, 204 and 221, within the conserved SOCS-box domains which then allows it to bind to the SH2 domain of the Ras inhibitor p120 RasGAP leading to inhibition of STAT5 activation but maintaining ERK activation [167, 168]. Since SOCS proteins inhibit growth factor responses; tyrosine phosphorylation of SOCS3 can ensure cell survival and cell cycle progression (proliferation) through Ras pathway. Recently, the same group have shown that tyrosine phosphorylation of SOCS3 at Y204/Y221 allows it to interact with adaptor proteins Nck-1 and Crk-L, and thus modulate their downstream signaling [174]. Another study has shown that SOCS3 tyrosine phosphorylation regulates protein stability and Elongin BC interaction [169]. Tyrosine phosphorylation decreased SOCS3 protein half-life by disrupting the interaction between SOCS3 and Elongin BC, while SOCS3/SOCS1 chimera (3/1/3), which bound much more strongly to Elongin BC, was significantly more stable than wild-type SOCS3 [169]. Thus a phospho-SOCS3 protein may serve different functions depending on the stimuli, time and location in the cell.

### 1.4.3 Role of SOCS3 in Inflammatory Diseases

In case of inflammatory diseases such as rheumatoid arthritis, ulcerative colitis and Crohn's disease, SOCS3 is highly expressed in affected tissues [165]. In patients with Crohn's disease, there is constitutive activation of Stat3 in T-cells from colon mucosa due to inability of SOCS3 to inhibit IL-6 induced phosphorylation of Stat3 [170]. The mechanism underlying the inability of SOCS3 to inhibit IL-6-induced Stat3 activation in these patients is not known. Transgenic mice expressing a dominant interfering SOCS3 protein exhibit a more potent Stat3

activation and a more severe colitis than compared to the wild-type littermates [165]. These

results suggest that SOCS3 acts as a negative feedback regulator in inflammatory diseases, and

therefore SOCS3 may be utilized by IL-10 to exert its anti-inflammatory actions.

**The focus of this thesis**

**Hypothesis:** SOCS3 mediates IL-10 deactivation of macrophages by targeting signalling proteins activated by LPS.

The overall objective of this proposal is to define at a molecular level the mechanism by which IL-10-induced signalling events interfere with those of macrophage activators and characterize the role they play in mediating IL-10's anti-inflammatory action. The importance of the Stat3 pathway in the anti-inflammatory action of IL-10 has been established and some of the Stat3-regulated gene induction events involved in macrophage deactivation by IL-10 has been identified [132]. Preliminary experiments examining the IL-10 responsiveness of various candidate genes showed that SOCS3, a member of a family of negative regulators of cytokine signalling, is regulated in a Stat3-dependent manner and may be important in mediating IL-10's anti-inflammatory action.

In most other cytokine receptor systems, the Stat pathway interacts with and is modulated by the action of other pathways. Preliminary observations suggest that SOCS3 may be involved in the mechanism by which IL-10 antagonizes LPS action [3]. In this study, we set out to examine the modulation of LPS-induced cytokine production by determining the stage at which cytokine production (i.e. transcription, translation) is affected by SOCS3 expression. Mice disrupted for SOCS3 gene are embryonically lethal due to impaired placental development [22]. Analysis of these mice showed that during trophoblast giant cell differentiation, SOCS3 normally functions to modulate leukemia inhibitory factor (LIF) signalling. Similarly, tetraploid rescued SOCS3$^{-/-}$ mice die within 3 weeks of birth because of cardiac monocyte hypertrophy caused by a loss of SOCS3-mediated inhibition of LIF and CT-1 signalling [169]. Thus, in

order to study the effects of loss of SOCS3 protein on IL-10 signalling, we derived macrophage cell lines from SOCS3$^{-/-}$ fetal liver cells [152]. We have also investigated which domain of SOCS3 protein is important for inhibition of LPS signalling pathway in response to IL-10 by reconstituting SOCS3$^{-/-}$ macrophages with WT and mutant SOCS3. Therefore, determining the point at which SOCS3 interferes with LPS signalling pathways and identifying the specific SOCS3 region responsible for this action will give insights into the mechanism by which SOCS3 mediates inhibition of macrophage activation and define new targets for SOCS3 action.

## CHAPTER 2: Material/Methods

### 2.1 Reagents

All reagents were obtained from Sigma (Oakville, Canada) unless otherwise indicated. TNF-α ELISA kits were obtained from BD Pharminogen (Mississauga, Canada). Antibodies to SOCS3 protein and phospho-SOCS3 protein (phospho Tyr 204/221) were kindly supplied by Dr. Nicholas Cacalano (UCLA). Erk antibody was from Cell Signalling (Missassauga, Canada) and iNOS antibody was from Santa Cruz (Santa Cruz, USA). IL-10 was kindly supplied by Dr. Kevin Moore (DNAX Research Institute).

### 2.2 Cell Culture

J774.1 murine macrophage cell line (American Type Tissue Culture Collection) were cultured in Dubelcco's Modified Eagle's Medium (DMEM) supplemented with 9% (v/v) fetal calf serum (FCS) on tissue culture grade dishes. Macrophages were cell lines also derived from embryonic fetal liver hematopoetic progenitor cells to generate SOCS3$^{+/-}$ and SOCS3$^{-/-}$ immortalized macrophage cells (kindly provided by Dr. James Ihle, St. Jude Children's hospital, TN). These cells were grown in Iscoves Modified Dubelcco's Medium (IMDM) supplemented with 9% (v/v) FCS and 5% C127 conditioned media (contains Colony-Stimulating Factor-1). All cells were grown at 37°C and 5% $CO_2$ in a standard tissue culture incubator.

### 2.3 CaCl$_2$ Competent cells

E. coli cells, DH5α, were grown in 2 ml liquid broth (LB) medium overnight in a 37°C shaker. The next day, inoculated 100 ml LB-medium with 1 ml of overnight culture and grew until OD600=0.45 or a little less (usually takes about an hour, if OD did not reach then checked every 10 minutes). The culture was chilled in a flask on ice for about 15-30 minutes. The centrifuge tubes were prechilled on ice. The culture was spun down cold at 4000 rpm (2000g) for 10

minutes and the pellet was resuspended in 1/3 of original volume with 100 mM ice cold $CaCl_2$

and incubated on ice for 30 minutes. The tubes were spun cold at 3000 rpm (1500 g) for 10

minutes. The pellet was then resuspended in 1/25 of original volume with ice cold 100 mM

$CaCl_2$/15% glycerol. The bacterial culture was then incubated at 4°C for a few hours. Bacterial

cells were aliquoted into prechilled tubes (200 µl/tube) and stored at –80°C. (Molecular Cloning

Manual, Shambrook et al)

## 2.4    E. Coli Transformation

The $CaCl_2$ competent E. coli cells (DH5$\alpha$) were thawed on ice and used at 50µl per

transformation. 3-5 µl of plasmid was added to the cells and they were kept on ice for 1 hour.

Cells were put in heat bath set at 42°C for 2 minutes and then put on ice for 2 minutes, prior to

the addition of 2 ml of pre-warmed LB (37°C) and incubation at 37°C incubator for 1 hour to

allow the bacteria to recover. Tubes were then spun down at 12000 rpm and the supernatant was

aspirated off.  The bacterial pellet was resuspended in 50 µl of LB. Then LB+Ampicillin (100

µg/mL) agar plates were streaked with 50 µl of bacterial culture. Colonies were picked with

autoclaved toothpicks to inoculate 4 ml of LB+Ampicillin (100 µg/mL) solution.  The cultures

were grown in a 37°C shaker overnight for approximately 16 hours. (Molecular Cloning

Manual, Shambrook et al)

## 2.5    Plasmid DNA prep (miniprep)

DNA was isolated using Miniprep kit from Qiagen (Maryland, USA). The bacterial solution was

centrifuged in eppendorf tubes at 14000 rpm for a few seconds.  LB was aspirated off and

bacterial pellet was re-suspended in 200 µl P1 buffer (25 mM Tris pH 8.0, 10 mM EDTA pH

8.0, RNase also added) by mixing up and down. Then 200 µl of P2 buffer (0.2 N NaOH, 1%

SDS) was added to lyse the bacteria.  The tubes were mixed thoroughly and 350 µl of N3 buffer

(3 M KOAC, 3 M HOAC pH 4.8-5.5, ethanol was also added) was added. Each tube was immediately mixed gently to allow $K^+$ ion precipitate SDS with genomic DNA. The tubes were spun at 12000 rpm for 10 min and the supernatants (~750 µl) were transferred onto columns with collection tubes. The columns were spun at 12000 rpm for 1 min and 750 µl of 70% ethanol was added to the column. The columns were spun at 12000 rpm for 1 min and the fluid in the collection tubes were poured out so that columns could be spun for another 1 min to ensure all the residual ethanol has been spun out of the columns. The columns were transferred into eppendorf tubes and DNA was eluted from columns by addition of 50 µl of water. After 1 min, the columns were spun at 12000 rpm for 1 min. The DNA concentration was based on spectrophotometer measurements at 260 nm.

## 2.6    Retrovirus Infection

Myc-tagged SOCS3 constructs in pMX-IRES-EGFP (provided by Dr. Akihiko Yoshimura) were transfected into Plat-E viral packaging cell line, using liposomal-mediated transfection. Plat-E cells were plated at $3X10^6$ cells per plate. The next day, 0.9µl of 40 mM liposome were added to 200 µl of autoclaved water in one tube and 3 µg of DNA to 200 µl of water in another tube. The two solutions were mixed and incubated @ RT for 25 minutes and 2ml of DMEM was added to the mixture and added to the Plat-E cells for 6 hours. Then 2 ml of 9% DMEM was added to rescue the cells from starvation. The next day, media was aspirated off the Plat-E cells and 2 ml of 9% IMDM supplemented with 5% C127 conditioned media was added to the cultures. The next day, virus-containing supernatant was collected, centrifuged twice for 10 min at 15000 rpm and added to the SOCS3$^{-/-}$ cells ($2X10^6$ SOCS3$^{-/-}$ cells in 6 cm plates). Infected cells expressing GFP were detected with a fluorescence microscope. GFP expression was observable 1 day after infection, but was more evident on the second day after infection. These

cells were sorted based on GFP expression using a Fluorescence Activated Cell Sorter. After sorting, these cells were grown in 9% IMDM supplemented with 5% C127 conditioned media.

## 2.7 Determination of protein expression by Western Blot Analysis

Parental J774.1, SOCS3$^{+/-}$, and SOCS3$^{-/-}$ macrophages (5X10$^6$) were treated with mIL-10 (100 ng/mL), LPS (10 ng/mL), or IL-10+LPS at the concentrations indicated, washed with cold phosphate-buffered saline (PBS), and lysed in 200 µl of buffer (150 nM NaCl, 50 mM Tris-HCl, 2 mM EDTA, 1mM NaVO$_4$, 1mM NaF and 1%NP40) supplemented with Complete Protease Inhibitor Cocktail (Roche, Montreal, Canada). Cell lysates were collected following centrifugation at 15000 rpm for 10 minutes to remove the nuclei and the protein concentration was measured using BCA method. Aliquots of cell lysate containing 100 µg of protein were boiled in 1X sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and then subjected to SDS-PAGE. Proteins were subsequently electro-transferred onto a PVDF membrane (BioRad, Canada). Membranes were blocked with Tris-buffered saline (TBS) 3% BSA at room temperature for 1 hr, then incubated with the appropriate primary antibodies in TBS-3% BSA overnight on a shaker. The membranes were then washed several times with TBS-0.05% Tween buffer and incubated with fluorescently-labeled (Alexor Flour 680) secondary antibodies (Molecular Probes, Oregan, USA) for protein detection using the Licor Odyssey system. The following antibodies were used at 1:1000 dilution: anti-SOCS3 [168], phospho-specific antibodies to Y204/Y221 on SOCS3 [168], anti-iNOS and anti-ERK.

## 2.8 Determination of TNF-α protein levels by Enzyme-Linked Immuno-sorbent Assay (ELISA)

SOCS3$^{+/-}$ and SOCS3$^{-/-}$ macrophages were plated at 2X10$^5$ cell/well in IMDM containing 9% serum supplemented with 5% C127 conditioned media in 24 well tissue culture plates and

grown overnight. The next day, the media was changed to DMEM containing 9% serum and cells were stimulated with LPS (100 ng/mL) or LPS+mIL-10 (10 ng/mL) for 2 hours. The supernatants were collected and analyzed for the presence of TNF-$\alpha$ protein using mouse-TNF-$\alpha$ ELISA kit. The 96 well ELISA plates were coated overnight with 50 µl of TNF-$\alpha$ capture antibody diluted 1:250 in coating buffer (1mM $NaHCO_3$, 3mM $NaCO_3$). The wells were washed 5 times with PBS/tween-20 (0.05%) and incubated with 100 µl of assay diluent (10% FCS in 1XPBS) for 1 hour to block non-specific binding. 100 of samples were added to each well and incubated for 2 hours at RT. The wells were washed 5 times and incubated with 50 µl of biotin-conjugated detection antibody (1:250) for 1 hour. The wells were washed 5 times and incubated with 50 µl of avidin-conjugated HRP enzyme (1:250) for 30 minutes. Then wells are washed 7 times (30 sec each) and finally incubated with 100 µl of substrate. The substrate used was O-Phenylenediamine tablet (Sigma, St. Louis, USA) dissolved in phosphate-citrate buffer at final concentration of 0.4 mg ml, and activated by addition of 0.03% hydrogen peroxide. The ELISA plates were developed in the dark for 30 minutes. The reaction was stopped with 100 µl of 3M Sulfuric acid. Using an ELISA plate reader, the wells were read at 492 nm. The standard curve was obtained by using TNF-$\alpha$ (100 µg/mL) with the top concentration of 2000 pg/mL. Triplicate samples were assayed so that statistics could be applied to calculate p-values (p-value $\leq 0.01$ indicates significance).

## 2.9    Detection of Nitrite production

Nitrite production was measured by the Greiss method [127]. SOCS3$^{+/-}$ and SOCS3$^{-/-}$ macrophages were plated at $2X10^5$ cell/mL in IMDM containing 9% serum supplemented with 5% C127 conditioned media in 24 well tissue culture plates and grown overnight. The next day, the media was changed to DMEM containing 9% serum and cells were stimulated with either

LPS (100ng ml) or LPS+IL-10 (10 ng/mL) for 24 hours. The supernatants were collected and analyzed for the presence of nitrite using the Greiss assay. Seventy-five μl of the culture supernatant was combined with 75 μl of greiss reagent: 1% (v/v) sulfanilamide, 0.1% (v/v) naphthylethylenediamine hydrochloride, 2.5% (v/v) phosphoric acid, and water, and incubated at room temperature for 5 min. Absorbance at 550 nm was measured by an ELISA plate reader. Sodium Nitrite (1 mM) was used to generate a standard curve with top concentration of 250 nM. Triplicate samples were assayed so that statistics could be applied to calculate p-values (p-value $\leq 0.01$ indicates significance).

## 2.10 Determination of TNF-α RNA expression by Northern Blot Analysis

SOCS3$^{+/-}$ and SOCS3$^{-/-}$ cells were treated for 1-2 hours with LPS (100 ng/mL) or LPS+IL-10 (10 ng/mL). Cells were harvested in 1 ml of TriZol. Chloroform (400 μl) was added, and then samples were shaken vigorously for 30 seconds and incubated for 15 minutes at room temperature (RT). The samples were then centrifuged (14000 rpm) for 10 minutes (4 °C) and the top aqueous phase was transferred into RNase free 1.5 ml eppendorff tubes. The samples were vortexed after addition of 500 μl of cold isopropanol and incubated for 15 min (RT) and centrifuged (14000 rpm) to precipitate the RNA. The supernatant was aspirated off and 1 ml of cold 75% ethanol was added to wash the pellet. Samples were centrifuged at 11500 rpm for 10 min. The supernatant was aspirated off and the pellet was left at RT for 10 min to air dry. Once the pellet was dry, it was resuspended in 10 μl of RNase-free water. Formaldehyde-gel loading buffer (formaldehyde, formamide, MOPS, 50% glycerol, 1mM EDTA, 0.25% bromophenol blue, .25% xylene cyanol FF) was added and RNA denatured by heating for 10 minutes at 55°C. Equivalent amounts of RNA were resolved by electrophoresis on 1% agarose gel containing 37% formaldehyde, blotted onto a nylon membrane, and cross-linked by exposure to UV light.

The membranes were then prehybridized, hybridized, and washed according to standard procedures (Molecular Cloning Manual, Shambrook et al). The TNF-$\alpha$ and GAPDH probes were radiolabeled with [a-$^{32}$P] dCTP by the random priming method.

## 2.11 Statistical Analysis

Data are expressed as the mean $\pm$ SE. JMPIN4 software program was used to perform One-way ANOVA analysis and p-values $< 0.01$ were considered significant.

# CHAPTER 3: Results

## 3.1  Induction of SOCS3 message by IL-10 in a Stat3-dependent manner.

In a preliminary survey of SOCS family members, we found that IL-10 treatment of

J774.1 cells induces SOCS3 mRNA expression. SOCS3 mRNA is induced by IL-10 as early as

30 min and this expression is sustained for as long as 10 hours post stimulation (Fig. 7a). In

order to investigate the role of the Stat pathway in IL-10 regulation of SOCS3, a dominant

inhibitory form (lacking the C-terminal transactivation domain, ΔStat) was constructed and

retrovirally transduced into J774.1 cells. Expression of ΔStat3 inhibited the ability of IL-10 to

induce SOCS3 mRNA (Fig. 7b). In contrast, ΔStat1 did not inhibit IL-10 induction of SOCS3 in

these cells (Fig. 7e) indicating that SOCS3 mRNA expression is Stat3-dependent. Next we

wanted to determine whether the two tyrosines (Y427/477 for mIL-10R; Y446/496 for hIL-10R)

in the cytoplasmic domain of the IL-10 receptor (IL-10R) were required for SOCS3 induction.

We expressed either the wild-type (WT) or hIL-10R: Tyr$^{FF}$ mutant (lacking both Y446 and

Y496) in J774 cells. Cells were treated with human IL-10 in the presence of a blocking anti-

mIL-10R antibody to prevent hIL-10 stimulation of the endogenous mouse IL-10R. As shown

in Fig. 7c, although the WT IL-10R signaling was able to induce SOCS3 expression, the

tyrosine mutant was not (Fig. 7d).
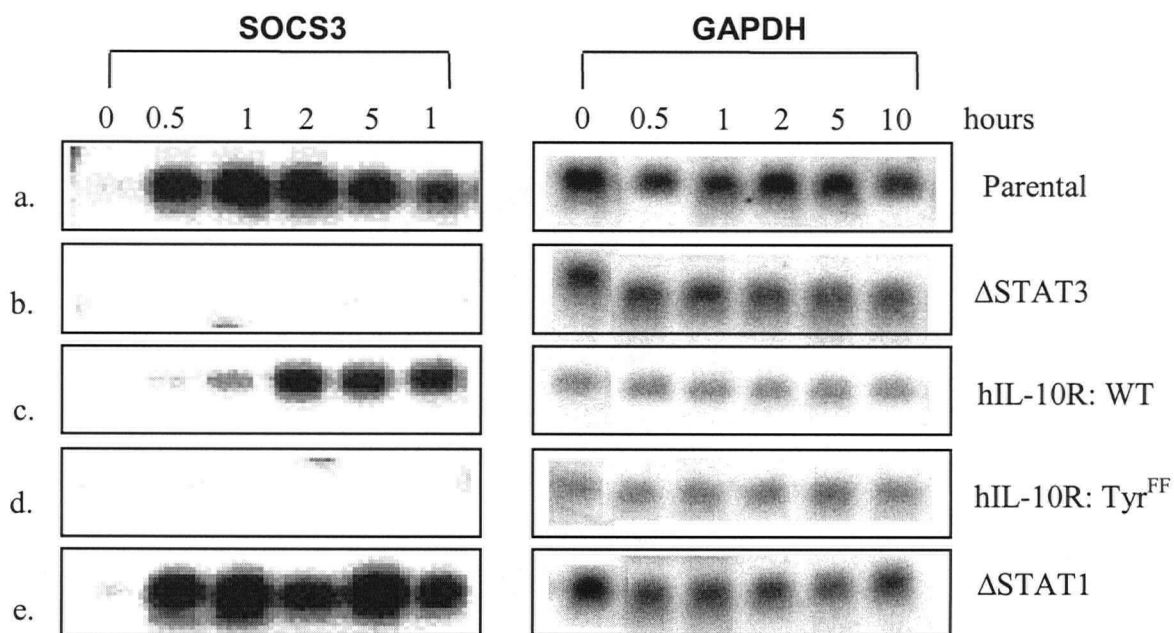
**Figure 7. Induction of SOCS3 message by IL-10 in a Stat3-dependent manner.**
Parental J774.1 cells or cells expressing a dominant Stat1 (ΔStat1), dominant negative
Stat3 (ΔStat3), wild-type hIL-10 (hIL10R:WT) or tyr-null (hIL10R:TyrFF) were
treated for the indicated times with LPS or LPS + IL-10. (Cell lines described in
O'Farrell et al EMBO J 17:1006, 1998 [3]). The RNA blot was analyzed by Northern
analysis for SOCS3 mRNA expression and GAPDH mRNA to confirm equal RNA
loading in each sample.

**3.2    Induction of SOCS3 protein by IL-10.**

We then determined whether IL-10 induces SOCS3 protein expression, since mRNA

expression does not always correlate with protein levels.  SOCS3 protein is known to be very

unstable, so to prevent SOCS3 protein degradation via the 26S proteasome, J774.1 cells were

pretreated with 3 nM of MG132 (a proteasome inhibitor) for 30 minutes.  These cells then were

stimulated with LPS±IL-10 or IL-10 at various times (Fig. 8).  SOCS3 protein expression was

observed in response to IL-10 by 1 hour (Fig. 8b).  Interestingly, LPS+IL-10 treatment induces

SOCS3 protein even more rapidly (Fig. 8c) compared to IL-10 alone.  LPS alone was not able to

induce SOCS3 protein at any time point (Fig. 8a).

**Figure 8.  Induction of SOCS3 protein by IL-10.**
J774.1 cells were treated with control buffer or (a) 10 ng/mL LPS, (b) 100 ng/mL IL-10
or (c) 10 ng/mL LPS with 100 ng/mL IL-10 for the indicated length of time. Lysates
were made 1X in SDS-PAGE sample buffer, resolved by SDS-PAGE and subjected to
immunoblot analysis with antibody to SOCS3 protein. The top bands are non-specific
showing equivalent protein levels in each sample. These are results from 3 independent
experiments.

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**3.3   Ectopic expression of SOCS3 protein is not sufficient to completely inhibit TNF-α production in response to LPS.**

Once it was determined that SOCS3 mRNA was induced by IL-10 in a Stat3-dependent manner, we ectopically expressed SOCS3 protein in J774.1 murine cells. Both constitutively expressing SOCS3 and parental J774 cells were stimulated with LPS± IL-10 for 10 hours and their supernatants were collected for TNF-α determination by ELISA. Figure 9 shows that J774.1 cells constitutively expressing SOCS3 protein make less TNF-α protein in response to LPS as compared to the control group. Notably however, complete inhibition of TNF-α production required addition of IL-10.
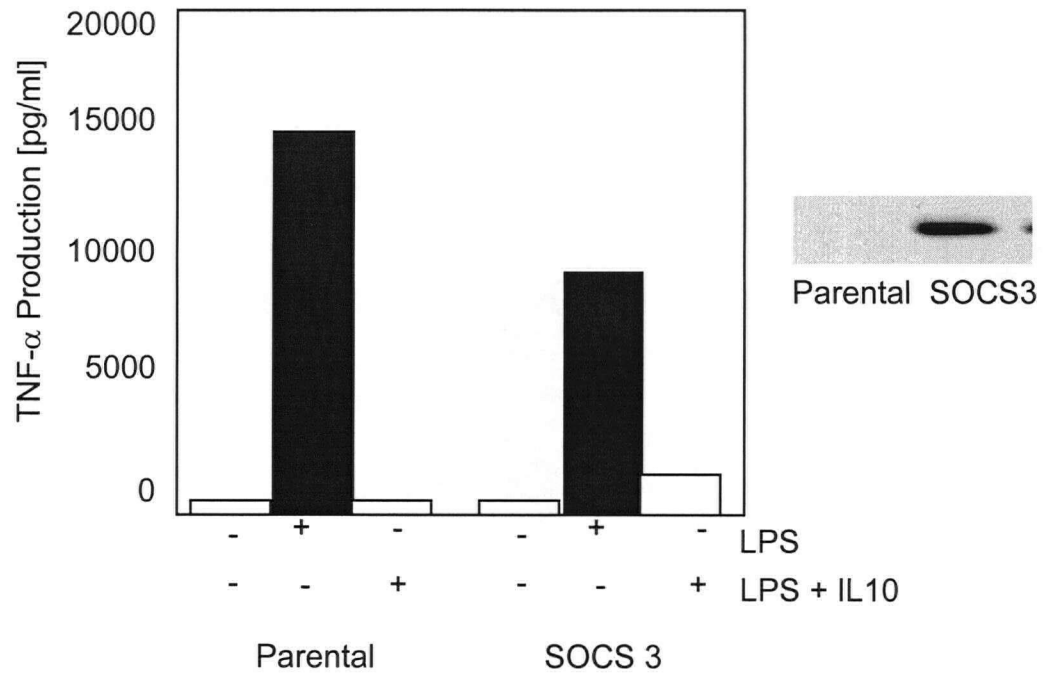
**Figure 9. Ectopic expression of SOCS3 protein is not sufficient to completely inhibit TNF-α production in response to LPS.**
We ectopically expressed SOCS3 protein in J774.1 murine cells. These cells were stimulated with LPS± IL-10 and their supernatant was tested for TNF-α levels after 10 hrs via an ELISA. (This ELISA assay was carried out by Dr. Alice Mui)

### 3.4 IL-10 induces phosphorylation of SOCS3 protein at tyrosine 204 in the SOCS-box domain.

Since mere expression of SOCS3 protein in macrophages was not sufficient to inhibit TNF-$\alpha$ protein production in response to LPS, this suggests that additional IL-10 signals are required. It has been shown that SOCS3 protein is phosphorylated in response to cytokines, growth factors and by several families of kinases, including Jaks and receptor tyrosine kinases [168]. J774.1 cells were pretreated with 10 µM NaVO$_3$ and 3 µM MG132 (a proteasome inhibitor) for 30 minutes prior to stimulation with 100 ng/mL of LPS$\pm$IL-10 (100 ng/mL) or IL-10 alone. IL-10 was able to induce phosphorylation of SOCS3 protein at Y204 (Fig. 10a) at 2 hours post stimulation. The blot was re-probed for protein SOCS3 antibody (Fig. 10c), and later for Erk 1/2 protein (Fig. 10d) to confirm equal protein loading. Phospho-specific antibody raised against Y204 was tested for its specificity. The phospho-specific Y204 antibody detected SOCS3 protein in cells expressing WT or SOCS3$^{Y221F}$ protein, but not in SOCS3$^{-/-}$ or SOCS3$^{Y204F}$ macrophages (Fig. 10e). The same blot was reprobed with anti-SOCS3 antibody to confirm the presence of SOCS3 protein in these samples (Fig. 10g). Another blot was probed for phospho-Y221, and there were bands in IL-10 treated samples (Fig. 10b). However, the phospho-specific Y221 antibody did not show specificity (Fig. 10f).
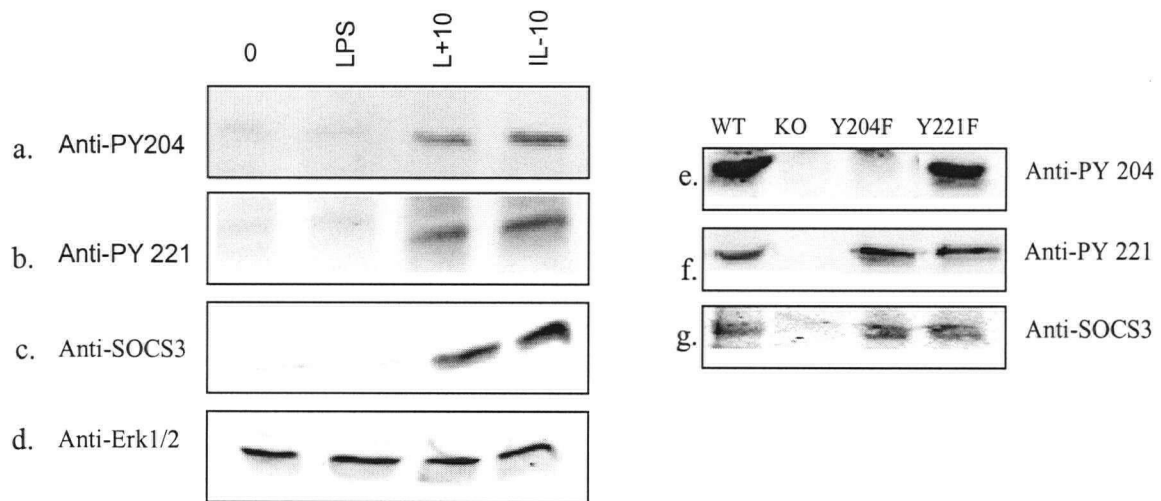
**Figure 10. IL-10 induces phosphorylation of SOCS3 protein at tyrosine 204 in the SOCS-box domain.**

J774.1 cells were pretreated with 10 $\mu$M NaVO$_3$ and 5 $\mu$M MG132 proteosome inhibitor for 1 hour at 37°C. Then the samples were treated with control buffer or 100 ng/mL LPS $\pm$ 100 ng/ml IL-10 or 100 ng/mL IL-10 alone for 2 hrs before preparation of cell lysates. Lysates were made 1X in SDS-PAGE sample buffer, resolved by SDS-PAGE and subjected to immunoblot analysis with antibodies to phospho-tyr 204 SOCS3 (PY204), phospho-tyr 221 SOCS3 (PY221) or SOCS3 protein. The same blot was reprobed for Erk protein to confirm equal protein loading in each sample. SOCS$^{-/-}$ cells and SOCS$^{-/-}$ reconstituted with cDNA for SOCS3 (WT), Y204F or Y221F mutants of SOCS3 were treated LPS+IL-10 for 2 hrs and samples were treated as above. The samples were subjected to immunoblot analysis with antibodies to PY204 (10e) and PY221 (10f). The blots were then reprobed with anti-SOCS3 antibody to confirm presence of SOCS3 protein (10g). This experiment was carried out 3 independent times.

### 3.5 IL-10 inhibition of TNF-α protein expression requires SOCS3 during the early phase of signaling.

TNF-α is a primary mediator of numerous immunologic functions, including inflammation and regulation of immune proliferative and activation responses. We chose to study TNF-α as one of the readouts for an activated macrophage due to following reasons: 1) TNF-α production is a hallmark characteristic of macrophage activation, 2) TNF-α expression is regulated at multiple levels and 3) Signal transduction mechanisms involved in this regulation are well-characterized. In order to determine the potential role of SOCS3 protein in IL-10 inhibition of TNF-α protein expression, we examined production of TNF-α in response to LPS$\pm$ IL-10 after 2 hours, in the SOCS3$^{+/-}$ vs SOCS$^{-/-}$ macrophages. IL-10 was able to inhibit production of TNF-α in SOCS$^{+/-}$ cells and not in SOCS3$^{-/-}$ macrophages (Fig. 11, panel A). To confirm that the inhibition in these cells are truly due to the absence of SOCS3 protein, we examined the production of TNF-α in SOCS3$^{-/-}$ macrophages reconstituted with SOCS3 cDNA via retroviral transduction. SOCS3$^{-/-}$ cells reconstituted with SOCS3 cDNA regained their responsiveness to IL-10 and were able to inhibit TNF-α production similar to SOCS$^{+/-}$ macrophages (Fig. 11, panel A). But when SOCS3$^{+/-}$ and SOCS3$^{-/-}$ cells were treated with LPS$\pm$IL-10 for 4 hours, IL-10 was able to inhibit TNF-α production both in SOCS3$^{+/-}$ and SOCS3$^{-/-}$ cells (Fig. 11, panel B). These results suggest that the dependence of IL-10 on SOCS3 for inhibition of TNF-α production occurs only during the early phase of IL-10 signalling.

A.



| | LPS | L+10 |
|---|---|---|
| SOCS3 $^{+/-}$ | 362±4.9 | 147±1.4 |
| SOCS3 $^{-/-}$ | 350±6.0 | 282±1.4 |
| SOCS3 $^{-/-}$:WT | 372±4.2 | 128±2.3 |

B.



| | LPS | L+10 |
|---|---|---|
| SOCS3 $^{+/-}$ | 1847+1.3 | 249±1.4 |
| SOCS3 $^{-/-}$ | 1073±2.5 | 152±1.2 |
| SOCS3 $^{-/-}$:WT | 1306±1.9 | 57±1.0 |
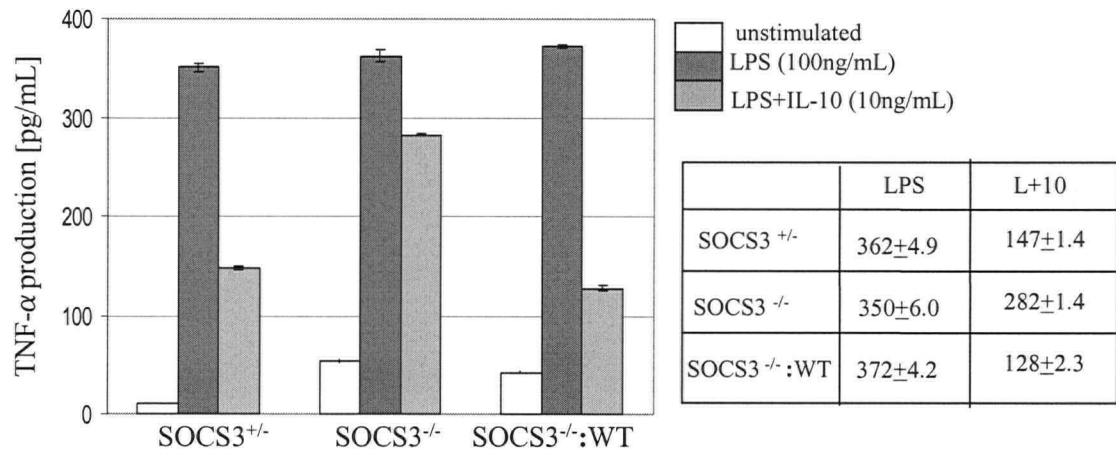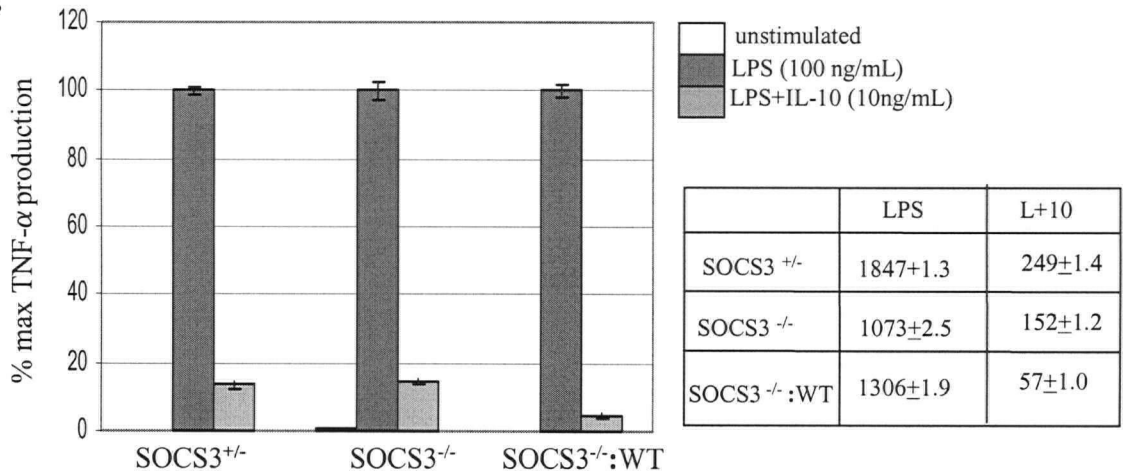
**Figure 11. IL-10 inhibition of TNF-α protein expression requires SOCS3 during the early phase of signaling.**
Macrophage cell lines were derived from wild-type or SOCS3$^{-/-}$ bone marrow derived macrophages. The reconstituted cells are the SOCS3$^{-/-}$ clone which has been transduced with the cDNA for SOCS3. Cells were stimulated with 100 ng/mL LPS ± 10 ng/mL IL-10 for (A) 2 hrs and (B) 4 hrs. TNF-α levels in the supernatants were measured by ELISA. The tables above contain the absolute levels of TNF-α protein (pg/mL) for each set of data. This data represents 1 of 4 experiments.

## 3.6 IL-10 inhibits expression of TNF-α mRNA in a SOCS3-dependent manner.

Since we observed inhibition of TNF-α protein production by IL-10 in a SOCS3-dependent manner (Fig. 11), we then examined the expression of TNF-α mRNA in response to LPS$\pm$IL-10 at 2 hours. IL-10 was able to inhibit TNF-α mRNA expression in the SOCS^{+/-} macrophages (Fig. 12a, panel A), but not in SOCS3^{-/-} macrophages (Fig. 12b, panel A). The same blot was reprobed for GAPDH to confirm equal loading (Fig. 12, panel B). Once again, IL-10 did not require SOCS3 protein to inhibit TNF-α mRNA expression at 4 hours (data not shown).



**Figure 12. IL-10 inhibits expression of TNF-α mRNA in a SOCS3-dependent manner.** SOCS3^{+/-} and SOCS3^{-/-} macrophages were treated with 100 ng/mL of LPS $\pm$ 100 ng/mL IL-10 for 1 and 2 hrs. Samples were collected in TriZol and RNA was extracted and resolved by 1% agarose gel and then subjected to RNA blot analysis with probes for TNF-α. Panel B shows the same blot was reprobed for GAPDH to confirm equal RNA in each sample. This experiment was performed 5 times.

### 3.7 Reconstitution of SOCS3$^{-/-}$ cells with WT and mutant SOCS3.

SOCS proteins have three conserved regions: SOCS-box, SH2 and KIR domains. We retrovirally transduced SOCS3$^{-/-}$ macrophage cell clones with wild type or mutant SOCS3 cDNAs (as described by Yoshimura, A et al in JBC 2000) using infection methods devised for high efficiency gene transfer into macrophage cells [127, 130]. Figure 13A shows a schematic representation of these various SOCS3 domain mutants. The cells were sorted based on GFP expression. Lysates prepared from these cells were examined via western analysis to confirm expression of SOCS3 protein (Fig. 13B, lanes 3-9). Samples then were also prepared from the parental SOCS3$^{+/-}$ and SOCS3$^{-/-}$ cells (Fig. 13B, lanes 1-2) treated with 100 ng/mL of IL-10 for 1 hour to induce expression of endogenous SOCS3 protein. The expression of each SOCS3 protein was confirmed to be similar to each other and similar to levels of endogenous SOCS3 protein induced by IL-10.
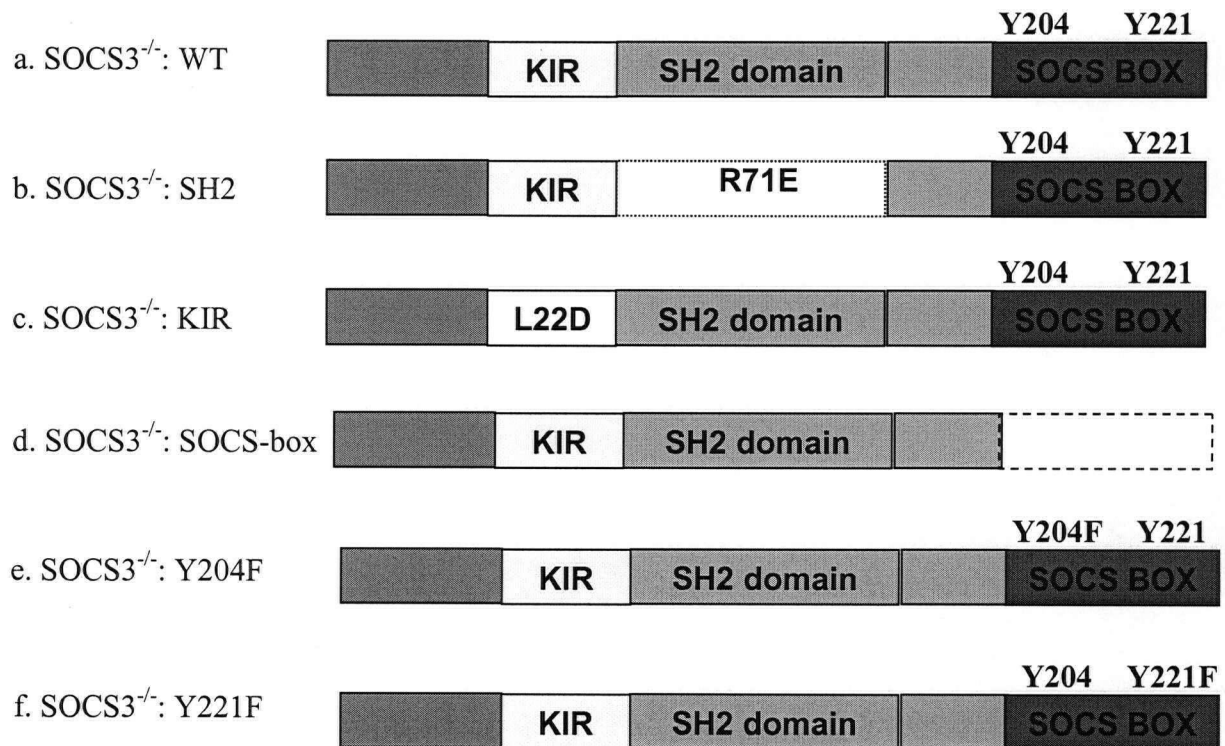
**Figure 13A. Schematic representation of various SOCS3 protein mutants.**
The SOCS3$^{-/-}$: SH2 and SOCS3$^{-/-}$: KIR have a pointtation in the domains, while the SOCS3$^{-/-}$: SOCS-box has a completely deleted domain. The SOCS3$^{-/-}$: Y204F and SOCS3$^{-/-}$: Y221F have a point mutation in one of the tyrosine residues in the SOCS-box domain.

**Figure 13B. Reconstitution of SOCS3$^{-/-}$ cells with WT and mutant SOCS3.**
SOCS3$^{+/-}$ cells (lane 1) and SOCS3$^{-/-}$ cells (lane 2) were treated with 100 ng/mL LPS+IL-10 for 1 hour to induce expression of endogenous SOCS3 protein. The reconstituted cells (lane 3-9) are the SOCS3$^{-/-}$ cells which have been transduced with an empty vector, cDNA for SOCS3, SH2 domain mutant (R71E), KIR domain mutant (L22D), SOCS-box deletion (DC40), Y204F or Y221F, which constitutively express the myc-tagged SOCS3 protein as described in the Material and Methods. Lysates were made 1X in SDS-PAGE sample buffer, resolved by SDS-PAGE and subjected to immunoblot analysis with antibody to SOCS3 protein. The same blot was reprobed with anti-erk antibody to show equal protein present in each sample. These results are representative of 3 independent experiments.

### 3.8    IL-10 requires all domains of SOCS3 protein for inhibition of TNF-α protein production.

Once it was confirmed that all the SOCS3^{-/-} macrophages reconstituted with various SOCS3 domain mutants express comparable levels of SOCS3 protein, experiments were conducted to determine which one of these domains are important in the inhibitory effects of IL-10 on TNF-α protein production. Cells were stimulated with 100 ng/mL of LPS±IL-10 (10 ng/mL) for 2 hours. The levels of TNF-α protein in culture supernatant were determined via an ELISA. Figure 14 shows that IL-10 required all domains of SOCS3 protein for inhibition of TNF-α protein production.

**Figure 14. IL-10 requires all domains of SOCS3 protein for inhibition of TNF-α protein production.**

SOCS3$^{+/-}$, SOCS3$^{-/-}$ and the SOCS3$^{-/-}$ reconstituted cells (as described in Fig. 13) were treated with 100 ng/mL LPS and 10 ng/mL IL-10 for 2 hours. The supernatants were collected to be analyzed for TNF-α secretion by ELISA. This experiment was carried out 7 independent times and p-values were calculated using one-way ANOVA analysis to determine the significance of the difference between each SOCS3$^{-/-}$ reconstituted cells relative to SOCS3$^{+/-}$ or SOCS3$^{-/-}$ cells. These p-values are listed in the table, a p-value $\leq$ 0.01 was considered significant.

| Cells | SOCS3$^{+/-}$ | SOCS3$^{-/-}$ | n |
|---|---|---|---|
| SOCS3$^{-/-}$ | 1x10$^{-6}$ | 1x10$^{-6}$ | 7 |
| Vector | 9x10$^{-7}$ | 0.80 | 7 |
| WT | 0.80 | 1x10$^{-6}$ | 7 |
| SH2 | 9x10$^{-5}$ | 0.06 | 7 |
| KIR | 4x10$^{-6}$ | 0.08 | 7 |
| SOCS-box | 6x10$^{-5}$ | 0.06 | 7 |
| Y204F | 3x10$^{-5}$ | 0.18 | 7 |
| Y221F | 1x10$^{-6}$ | 0.07 | 7 |

## 3.9 Excluding the KIR domain, IL-10 requires all domains of SOCS3 protein for inhibition of TNF-α mRNA expression.

In order to determine the domain of SOCS3 involved in IL-10 inhibition of TNF-α mRNA, SOCS3$^{+/-}$ and SOCS$^{-/-}$ macrophages, as well as all the SOCS3$^{-/-}$ macrophages reconstituted with various SOCS3 domain mutants were treated with 100 ng/mL of LPS+IL10 (10 ng/mL) for 2 hours and total RNA was prepared for a Northern analysis. SOCS3$^{-/-}$:WT-cells regained their responsiveness to IL-10 and were able to inhibit TNF-α mRNA expression, while the SOCS3$^{-/-}$:Vector behaved similar to parental SOCS3$^{-/-}$ cells (Fig. 15c, d, respectively). Figure 15 also shows that TNF-α mRNA expression is inhibited in SOCS3$^{-/-}$: KIR (15g), but not in SOCS3$^{-/-}$: SOCS-box (Fig. 15e), SOCS3$^{-/-}$: SH2 (Fig. 15f), SOCS3$^{-/-}$: Y204F (Fig. 15h), and SOCS3$^{-/-}$: Y221F (Fig. 15i) macrophages. Reprobing the northern blot for GAPDH mRNA confirmed equal RNA loading.
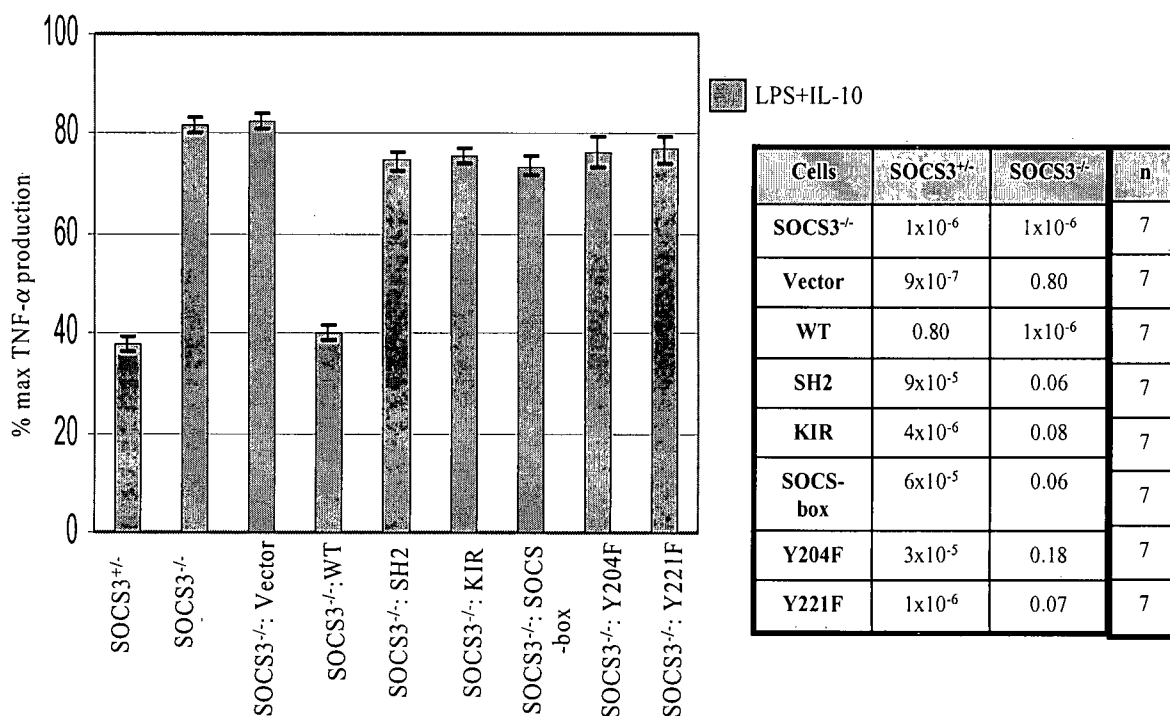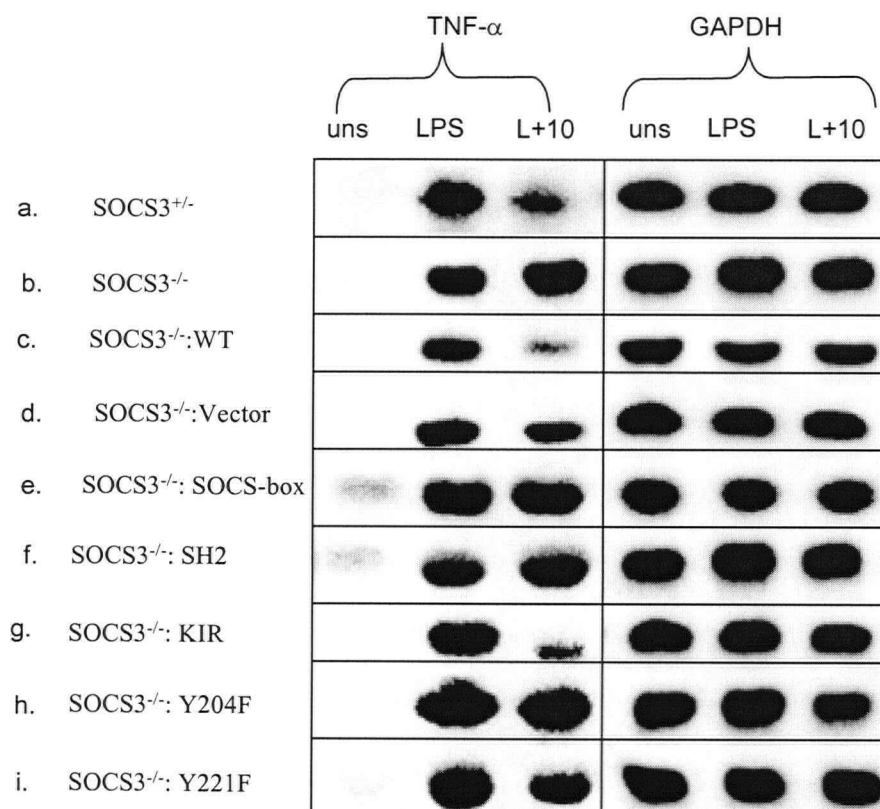
**Figure 15. Excluding KIR domain, IL-10 requires all domains of SOCS3 protein for inhibition of TNF-α mRNA expression.**
SOCS3$^{+/-}$, SOCS3$^{-/-}$ and the SOCS3-/- reconstituted cells (as described in Fig. 13) were treated with 100 ng/mL LPS and 10 ng/mL IL-10 for 2 hours. The RNA was collected in TriZol and samples were resolved by 1% agarose gel and subjected to RNA blot analysis with probe for TNF-α. The same blot was re-probed for GAPDH mRNA to confirm equal RNA in each sample. The bands were quantitated using a phosphoimager, and the ratios of TNF-α mRNA/GAPDH mRNA were plotted as histograms (right-hand panel). This experiment was carried out 4 times.

### 3.10 Y204/Y221 of SOCS3 protein is important for inhibition of NO production by IL-10.

We then examined whether inhibition of NO production by IL-10 is also SOCS3-dependent. Parental SOCS3$^{+/-}$ and SOCS3$^{-/-}$ macrophages were treated with 100 ng/mL of LPS+IL-10 (10 ng/mL) for 24 hours, and the levels of NO in the supernatants were determined using the Greiss assay. IL-10 was able to inhibit NO production in SOCS3$^{+/-}$ cells, but not SOCS3$^{-/-}$ cells. To ensure that this effect is due solely to the absence of SOCS3 protein, SOCS3$^{-/-}$ cells reconstituted with SOCS3 cDNA were tested as well and Figure 16 shows that reconstitution with wild-type SOCS3 restored responsiveness to IL-10, while the SOCS3$^{-/-}$ cells reconstituted with an empty vector behave like parental SOCS3$^{-/-}$ cells. Next, SOCS3$^{-/-}$ cells reconstituted with various SOCS3 domain mutants were examined in order to determine which of these regions are important in IL-10's ability to inhibit NO production. Only mutation of Y204 and Y221 in the SOCS-box domain abrogated the ability of IL-10 to inhibit NO production (Fig. 16).

| cells | SOCS3$^{+/-}$ | SOCS3$^{-/-}$ | N |
|---|---|---|---|
| SOCS3$^{-/-}$ | 2x10$^{-6}$ | 2x10$^{-6}$ | 7 |
| Vector | 6x10$^{-6}$ | 0.70 | 7 |
| WT | 0.70 | 2x10$^{-5}$ | 7 |
| SH2 | 0.60 | 5x10$^{-3}$ | 7 |
| KIR | 0.20 | 9x10$^{-5}$ | 7 |
| SOCS-box | 0.20 | 3x10$^{-4}$ | 7 |
| | | | 7 |
| Y204F | 4x10$^{-3}$ | 0.90 | 7 |
| Y221F | 1x10$^{-4}$ | 0.90 | 7 |
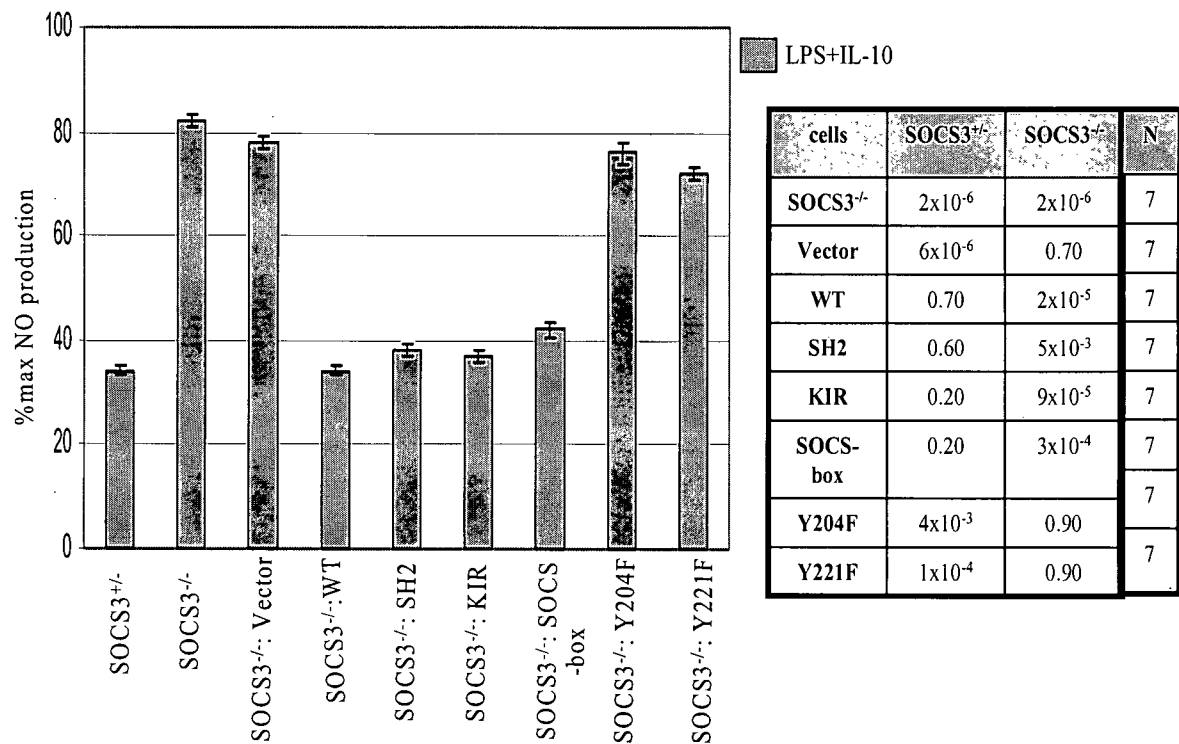
**Figure 16. Y204 and Y221 of SOCS3 protein is important for inhibition of NO production by IL-10.**

SOCS3$^{+/-}$, SOCS3$^{-/-}$ and the SOCS3$^{-/-}$ reconstituted cells (as described in Fig. 13) were treated with 100 ng/mL LPS and 10 ng/mL IL-10 for 24 hrs. The supernatants were collected to be analyzed for NO production via nitrite assay. This experiment was carried out 7 independent times and p-values were calculated using One-way ANOVA to determine the significance of the difference between SOCS3$^{-/-}$ reconstituted cells relative to SOCS3$^{+/-}$ or SOCS3$^{-/-}$ cells. These p-values are listed in the table, a p-value of $\leq 0.01$ was considered significant.

### 3.11 IL-10 requires Y204 and Y221 of SOCS3 for inhibition of iNOs protein expression.

Since we observed NO inhibition by IL-10 in a SOCS3-dependent manner (Fig. 16), we investigated whether the inhibition of NO by IL-10 is due to downregulation of inducible nitric oxide synthase (iNOS), which catalyzes the production of NO from L-arginine. Parental SOCS3$^{+/-}$ and SOCS3$^{-/-}$ cells, as well as the SOCS3$^{-/-}$ reconstituted with various SOCS3 constructs (as mentioned above) were treated with 100 ng/mL of LPS+ L+10 (10 ng/mL) for 24 hours and cell lysates were prepared for determination of iNOS protein by immunoblot analysis. Figure 17 shows that IL-10 was able to inhibit expression of iNOS protein in SOCS3$^{+/-}$ but not in SOCS3$^{-/-}$ cells and this inhibition required both Y204 and Y221 in the SOCS-box domain. The blots were re-probed with antibody against ERK1/2 to confirm equal amount of protein was loaded for each sample.
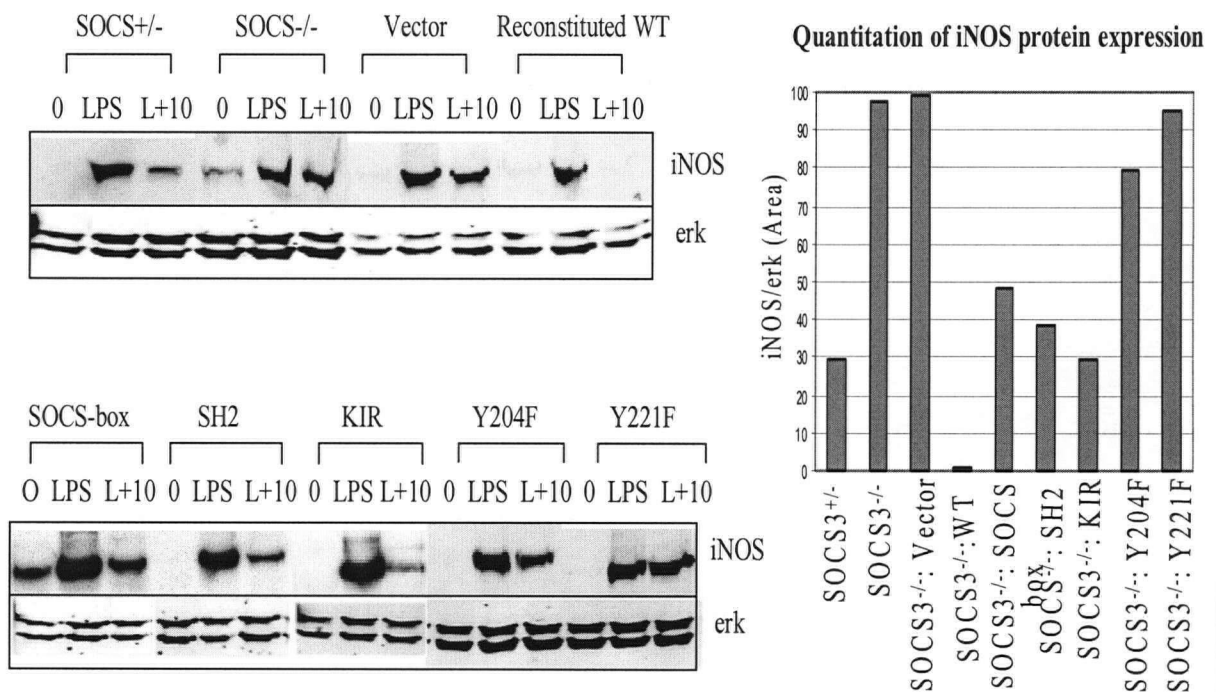
**Figure 17. IL-10 requires Y204 and Y221 of SOCS3 for inhibition of iNOs protein**

**expression.**

SOCS3$^{+/-}$, SOCS3$^{-/-}$ and the SOCS3$^{-/-}$ reconstituted cells (as described in Fig. 13) were treated with 100 ng/mL LPS and 10 ng/mL IL-10 for 24 hrs. Lysates were prepared in 1X in SDS-PAGE sample buffer, resolved by SDS-PAGE and subjected to immunoblot analysis with antibody to iNOS protein. The same blot was reprobed with antibody to erk protein to confirm equal protein loading in each sample. The bands were quantitated by densitometry and the ratios for iNOS protein/erk protein were plotted as histograms (right-hand panel). These are results from 3 independent experiments.

# CHAPTER 4: Discussion

The innate immune system initiates local and sometimes systemic inflammatory responses that alert the body to the presence of potential threats and guides the development of subsequent adaptive immune responses. Hence there are two faces to the inflammatory process; while on one hand inflammation is usually helpful and protective to the host, if left unchecked, the excessive amount of inflammatory cytokines could cause tissue destruction, physiological changes and complications such as septic shock. It is still poorly understood how an initial, beneficial host response to infection mediated by inflammatory mediators, can sometimes progress to a toxic systemic reaction. LPS is a potent activator of macrophages and this causes release of various pro-inflammatory mediators such as TNF-$\alpha$, NO, IL-1, IL-6 and IL-8, all of which are suppressed by IL-10 [100]. However, the mechanism by which IL-10 inhibits LPS signalling pathway is not defined as of yet. IL-10 induces expression of many proteins in a Stat3-dependent manner. One candidate protein whose regulation is by IL-10 in a Stat3-dependent manner is SOCS3. SOCS family of proteins are transcriptionally activated by a broad range of extracellular ligands and functions in a classical feedback loop to regulate signal transduction through multiple cytokine and growth factor receptors [157, 158, 171]. These proteins were initially cloned as cytokine-inducible immediate-early genes that could inhibit activation of Stat proteins and biological responses to several cytokines [40, 147, 151].

We and others have found that IL-10 rapidly induces SOCS3 mRNA in macrophages (Fig. 7a) [105] and in neutrophils [172]. This induction occurs in a Stat3 (Fig. 67b, 7c) and Y446/Y496 (human-IL-10R$\alpha$) dependent manner (Fig. 7d, 7e). SOCS3 protein is induced by IL-10 alone as early as 1 hour (Fig. 8b). However, stronger SOCS3 protein expression is observed in macrophages treated with LPS+IL-10 (Fig. 8c) and the same is true for SOCS3

mRNA (data not shown). This enhancement may be due to post-transcriptional stabilization of the mRNA or post-translational regulation of SOCS3 protein [173]. Other investigators have reported LPS induction of SOCS3 protein, however this induction was observed only after 3 hours post stimulation [173], at which time autocrine cytokines like IL-10 are being produced. In our hands however, LPS treatment did not result in induction of SOCS3 protein at any of the time points examined.

Since mere expression of SOCS3 protein in macrophages was not sufficient to inhibit TNF-$\alpha$ protein production in response to LPS, this suggests that additional IL-10 signals such as tyrosine phosphorylation, protein modification or activation of other proteins may be required. SOCS3 has been reported to be phosphorylated by activated JAKs, Src family kinases, and receptor tyrosine kinases (eg. EPOR, PDGFR) at Y204 and Y221 in the conserved SOCS-box motif. SOCS3 can bind RasGAP, an inhibitor of Ras, when phosphorylated by PDGF, resulting in ERK activation important for cell survival despite its inhibition of Stat5 phosphorylation due to inhibition of IL-2 signalling pathway [168]. Tyrosine phosphorylation of SOCS3 can ensure cell survival and cell cycle progression (proliferation) through the Ras pathway in this cell system.

IL-10 induced phosphorylation of SOCS3 protein at Y204 within 2 hours post stimulation of J774.1 macrophages (Fig. 10a and 10b, respectively). The specificity of the phospho-specific antibodies raised against Y204/Y221 residues were tested on SOCS3$^{-/-}$: Y204F and SOCS3$^{-/-}$: Y221F macrophages. Unfortunately, we were only able to confirm the specificity of the phospho-specific 204 antibody (Fig. 10e). The phospho-specific 221 antibody also reacted with SOCS3$^{-/-}$: Y221F cells (10f), so it either cross-reacts with the SOCS3 protein or SOCS3$^{PY204}$. By reconstituting SOCS3$^{-/-}$ macrophages with the SOCS3$^{Y204F/Y221F}$ double mutant,

we will be able to exclude the latter possibility. Therefore, by immunoblot analysis we cannot conclude whether IL-10 induces phosphorylation at tyrosine 221 of SOCS3 protein. However, our analysis with the SOCS3$^{-/-}$: Y221F macrophages suggest that this residue is indeed important for IL-10 inhibition of TNF-α and NO production.

Recently it has been shown that SOCS3 phosphorylation at Y221, allows SOCS3 to bind Nck and Crk-L, adaptor proteins, and recruit Nck to activated receptor tyrosine kinases and modulates Nck tyrosine phosphorylation in fibroblasts, thus regulating adaptor protein signal transduction [174]. Nck SH3 domains are involved in activating the JNK and p38 MAP kinase cascades [175], and Crk-L SH3 domains are involved in activating ERK and JNK pathway [176]. Therefore, phosphorylation of SOCS3 causes modulation in the regulation of proteins that interact with SOCS3 protein, and thus affecting the downstream events. Hence it is possible that IL-10 induces phosphorylation of SOCS3 protein, which allows SOCS3 protein to interact and inhibit proteins that effect transcription and translation of inflammatory mediators, such as TNF-α and NO.

Inhibition of TNF-α production by IL-10 has been attributed to effects on NFκB activation [88], MAPK signalling pathway [94], rate of transcription [177], mRNA stability [142], and translational efficiency [94]. We now show that TNF-α protein production and mRNA expression was inhibited by IL-10 in a SOCS3-dependent manner, but only up to 2 hours post stimulation (Fig. 11, panel A and Fig. 12, respectively). We did not see any requirement for SOCS3 protein at 4 hours post stimulation (Fig. 11, panel B), so we conclude that prior to 2 hours, IL-10 inhibits LPS signalling in SOCS3-dependent manner, but after 2 hours there may be other Stat3-dependent or independent pathways that come into play.

## 4.1. SH2 domain of SOCS3 protein is important in inhibition of TNF-α protein production and mRNA expression by IL-10.

IL-10 requires the SH2 domain of SOCS3 protein for inhibition of LPS-induced TNF-α protein production (Fig. 14) and mRNA expression (Fig. 15f). Possible mechanisms by which this may happen is discussed below. Although SOCS1 binds directly to the activation loop of Jaks through its SH2 domain, the SH2 domain of SOCS3 binds to cytokine receptor. SH2 domain of CIS stably associates with the tyrosine-phosphorylated cytoplasmic part of EPO and IL-3 receptors [178]. Y401 of EPO receptor is an essential tyrosine site for both SOCS3-SH2 domain binding and Stat5 activation, suggesting that SOCS3 inhibits activity of Stat5 through its SH2 domain [146]. Mutation in the SH2 domain enhances the LIF-dependent Stat3 and EPO-dependent Stat5 transcriptional activity [165]. The SH2 domain of SOCS3 has been shown to bind to Y757/759 of gp130, Y985 of the leptin receptor, and Y401 of the EPO receptor, some of which are the same binding sites for SH2-containing tyrosine phosphatase 2 (SHP2) [83, 158, 159, 179]. Since SOCS3 inhibits gp130 signalling and SHP2 promotes gp130 signalling through activation of mitogen-activated protein kinases, thus it is possible that SOCS3 suppresses aspects of gp130 signalling by competing with SHP2 for receptor binding. Hence, in SOCS3^{-/-} macrophages reconstituted with SH2 domain mutant, it is likely that IL-10 is unable to inhibit TNF-α protein (Fig. 14) and mRNA expression (Fig. 15f), because loss of SH2 domain of SOCS3 may allow for SH2 domain of SHP2 or some other SH2-containing protein to bind LPS signalling targets such as JNK, and promote transcription of TNF-α and subsequent TNF-α protein expression by targeting other MAPKs.

## 4.2. KIR domain of SOCS3 protein is important in inhibition of TNF-$\alpha$ protein production by IL-10.

The KIR domain of SOCS3 protein is important for inhibition of LPS-induced TNF-$\alpha$ protein production by IL-10 (Fig. 14). In other systems, SOCS3 has been shown to bind to the tyrosine-phosphorylated peptide Y759 of IL-6 receptor (gp130), through its SH2 domain, which brings its KIR domain in proximity to Jak2 and causes its inhibition. SOCS3 has been shown to specifically bind to tyrosine 1007 in the activation loop of Jak2, the phosphorylation of which is required for its activation. Thus, SOCS3 might inhibit Jak kinase activity through the pseudosubstrate, KIR, by recruiting and binding to a critical phospho-tyrosine at the intracellular part of a cytokine receptor. Mutation in the KIR domain enhances the LIF-dependent Stat3 and EPO-dependent Stat5 transcriptional activity [165]. A point mutation in the KIR domain overcomes the inhibitory effect of both SOCS3 and JAB protein and transgenic mice expressing SOCS3 mutated in KIR domain show more potent Stat3 activation and a more severe colitis induced with dextran sulphate sodium (DSS) [180]. It is possible that in SOCS3$^{-/-}$: WT macrophages, KIR domain of SOCS3 is able to induce a translational suppression of TNF-$\alpha$ protein by inhibiting an upstream kinase activity, but may be KIR domain's role is not extended to transcriptional regulation, which would explain why in SOCS$^{-/-}$: KIR, inhibition of TNF-$\alpha$ protein production (Fig. 14), but and not TNF-$\alpha$ mRNA expression (Fig. 15g) by IL-10 is disabled. Thus one potential target is the p38 MAPK pathway which has been shown to be responsible for translational control of TNF-$\alpha$ protein [93].

### 4.3. SOCS-box domain is important in inhibition of TNF-$\alpha$ protein production and mRNA expression by IL-10.

IL-10 requires SOCS-box domain of SOCS3 protein for inhibition of LPS-induced TNF-$\alpha$ protein production (Fig. 14) and mRNA expression (Fig. 15e). In addition to interference with kinase activity and binding to other signalling molecules, SOCS can also target associated proteins through interaction with the SOCS-box for proteasome-mediated degradation. The SOCS-box domain has no catalytic activity and mediates physiological effects of SOCS3 through protein-protein interactions [181]. A model is proposed where the target molecules containing phospho-tyrosine might become a substrate of the proteolytic machinery by binding to SOCS: after binding of SOCS, the SOCS-box acts as an adaptor molecule, bringing into its complex Elongin BC [160, 163]. The target protein is then ubiquitinated through recruitment of the E3 ligase. During the subsequent proteolytic association, the substrate and the associated SOCS proteins may be destroyed, and the cell is ready to respond once again if the stimuli are still present [163]. Evidence exists that Jaks can be immunoprecipitated in a complex containing SOCS1, Elongin B and Elongin C [162]. It has been shown that IL-6-induced expression of SOCS3 is sustained in the presence of the proteasome inhibitor, LLnL, implying that SOCS3 protein may be rapidly targeted for proteasomal degradation soon after its induction [163].

In support of these results, using anti-ubiquitin antibodies, it has been shown that a post-translational modified form of CIS protein (~37 kDa) exists in addition to the predicted in vitro translated protein size of CIS (~33 kDa). This 37 kDa form accumulates in the presence of the proteasome inhibitors, LLnL and lactacystin, but rapidly degrades when the protein synthesis is blocked by cyclohexamide [162, 178, 182]. SOCS protein expression can also inhibit EPO

receptor and Stat5 phosphorylation, which is not seen in the presence of the proteasome inhibitors indicating proteasome involvement in inactivation of both EPO-receptor and Stat5 [182]. Since SOCS-box has been shown to be involved in both stabilizing and degrading its interacting molecules, it is possible that it may serve to stabilize the IκB protein, thus inhibiting NFκB pathway. In alveolar macrophages, IL-10 stabilizes IκB protein by delaying its LPS-mediated degradation and resulting in delayed nuclear translocation of the p65 subunit [139]. It is also possible that SOCS-box may interact with other LPS signalling proteins such as p38 MAPK, JNK or IKK and cause their degradation in response to IL-10, which would also result in inhibition of NFκB pathway (involved in transcription of TNF-α and iNOS). This may explain the loss of inhibition at TNF-α protein production (Fig. 14) and TNF-α mRNA expression (Fig. 15e) in macrophages expressing SOCS-box mutant. But we do not observe inhibition of iNOS protein expression by IL-10 in macrophages expressing SOCS-box mutant, possibly due to different mechanisms coming into play at different times. Macrophages were stimulated with LPS+IL-10 for 2 hours for TNF-α experiments, while cells were stimulated for 24 hours for the iNOS protein immunoblot. For inhibition of TNF-α protein in response to LPS, the role of SOCS-box may be critical for suppression of the targets of IL-10, but for inhibition of iNOS protein expression, SOCS-box domain may only play a secondary role, which can be compensated for by the other domains when SOCS-box domain is inaccessible to IL-10.

## 4.4.    Y204 and Y221 of SOCS3 are important in inhibition of NO production and iNOS protein expression by IL-10.

NO mediates the ability of macrophages to kill or inhibit the growth of tumour cells, bacteria, fungi and parasites [45]. But this regulation must be tightly controlled since unregulated NO production causes fall in blood pressure (vasodilation), which potentially

results in injury to host tissue. Here we show that IL-10 inhibits NO production in SOCS3-dependent manner and only requires Y204 and Y221 of SOCS3 protein to be intact (Fig. 16). We further examined the effect of SOCS3 in response to IL-10 on iNOS, the enzyme that is responsible for production of NO. IL-10 inhibition of iNOS protein expression reflected the results obtained for NO production. iNOS protein expression was inhibited by IL-10 in SOCS3-dependent manner and only requiring the Y204 and Y221 of SOCS3 protein (Fig. 17). This observation is supported by another study carried out by O'Farrell et al, where they have demonstrated that IL-10 inhibits NO production and iNOS protein expression in a Stat3-dependent manner [127].

Interestingly IL-10 is able to inhibit NO production and iNOS protein expression when the entire SOCS-box domain is deleted (SOCS3$^{-/-}$: SOCS-box), but not when there is a point mutation in either of the two tyrosine residues, 204 (SOCS3$^{-/-}$: Y204F) and 221 (SOCS3$^{-/-}$: Y221F), located in the SOCS-box domain (Fig. 16 and Fig. 17). This interesting observation could be explained by the following model: when the SOCS-box domain is deleted, SOCS3 is not able to interact with Elongin B/C complex and target proteins for ubiquitination, but the other domains of SOCS3 (SH2/KIR domains) are still capable of carrying out inhibitory effects on protein **X** (in the LPS signaling pathway). For instance, SOCS3 protein can inhibit the association of protein **X** with other molecules by interacting with the phospho-tyrosines of protein **X** through its SH2 domain, or if protein X is a kinase, then SOCS3 can suppress its kinase activity via the KIR domain. However, in SOCS3$^{-/-}$: Y204F and SOCS3$^{-/-}$: Y221F macrophages, SOCS-box domain is intact and can still associate with Elongin B/C complex, but SOCS3 cannot be phosphorylated by IL-10 since there is point mutation in either Y204 or Y221. Thus when SOCS3/Elongin BC complex interacts with a target protein **X**, it may

enhance the stability of protein **X** rather than degrading it becauseY204/Y221 cannot be

phosphorylated by IL-10 and thus cannot be targeted for ubiquitination. Thus, when SOCS3

protein interacts with Elongin B/C and there is no "degradation signal" (ie. phosphorylation by

IL-10), then this interaction serves to stabilize the SOCS3 protein and its associated protein. As

well, this makes the other domains of SOCS3 protein unavailable and inaccessible for other

inhibitory mechanisms, since they are in complex with Elongin B/C. Therefore we observe

complete loss of inhibitory effects of IL-10 on NO production and iNOS protein expression in

SOCS3$^{-/-}$: Y204F and SOCS3$^{-/-}$: Y221F SOCS3, but not when SOCS-box domain is deleted.

## 4.5. Summary

In this study we have successfully demonstrated the importance of SOCS3 protein in mediating the anti-inflammatory action of IL-10, which is also supported by the SOCS3 over-expression study [3]. Our results also show that inhibition of TNF-$\alpha$ and NO production by IL-10 is mediated by different domains of SOCS3 protein, which suggests that there are different mechanisms that come into play in response to IL-10 (Fig. 18). Table 1 summarized the requirement for different SOCS3 domains in mediating IL-10 inhibition of TNF-$\alpha$ protein production and mRNA expression, as well as NO production and iNOS protein expression.

Table 1. Summary of the requirement for different SOCS3 domains in mediating IL-10 inhibition of various macrophage responses.

| SOCS3 domain | TNF-$\alpha$ protein production | TNF-$\alpha$ mRNA | NO production | iNOS protein expression |
|---|---|---|---|---|
| KIR | Yes | No | No | No |
| SH2 | Yes | Yes | No | No |
| SOCS-box | Yes | Yes | No | No |
| Y204 | Yes | Yes | Yes | Yes |
| Y221 | Yes | Yes | Yes | Yes |

**Figure 18. Schematic representation of the mechanism by which IL-10 may be inhibiting LPS signaling pathway.**

IL-10 induces SOCS3 protein expression, which then inhibits LPS signaling at multiple levels by different domains. IL-10 requires SH2 domain, SOCS-box domain, Y204 and Y221 of SOCS3 protein to inhibit TNF-$\alpha$ mRNA expression possibly by interfering with NF$\kappa$B protein activation. SOCS3 requires all its domains for inhibition of TNF-$\alpha$ protein release by IL-10, possibly by interfering either with the MAPKs, or I$\kappa$B protein stability or receptor tyrosine activity. IL-10 requires Y204 and Y221 of SOCS3 protein [for] inhibition of iNOS protein expression by IL-10, which is possibly either through inhibition of ILK pathway or NF$\kappa$B pathway, and ultimately inhibition of iNOS protein expression leads to inhibition of NO production.

## 4.6     Other Studies

Our hypothesis that SOCS3 is involved in the anti-inflammatory action of IL-10

disagrees with conclusions reached by Lee and Chau [131].  They observed no effect of SOCS3

deletion (through antisense oligonucleotides) on the ability of IL-10 to inhibit TNF-α

production.  However, their experimental system differs from ours in that they add IL-10 to cells

4 hours prior to LPS challenge. We show that IL-10 requires SOCS3 only in the early phase ($\leq 2$

hours) of inhibition of TNF-α protein production. In their system another IL-10-induced gene

called heme oxygenase-1 (HO-1) appears to be central to mediating macrophage deactivation.

HO-1 is not induced until 3 hours after IL-10 stimulation and maximal levels are not achieved

until 24 hours; which is why they chose to add IL-10 four hours prior to LPS stimulation. We

and others [3, 94] have chosen a system where IL-10 and LPS are added simultaneously since

the physiological target of IL-10 is the activated macrophage. The resting macrophage is

different from the LPS activated macrophage, and it might be reasonable to expect different

mechanisms to come into play under different circumstances.  In contrast, studies carried out by

Jung et al showed that although HO-1 gene is upregulated by IL-10 (in DNA array), a specific

HO-1 inhibitor zinc-protoporphyrin (ZnPP) was not able to abrogate IL-10 inhibition of LPS-

induced TNF-α protein production [183].  This suggests that other IL-10 regulated genes are

mediating the anti-inflammatory effects of IL-10.

Our demonstration of a role of SOCS3 in IL-10 inhibition of macrophage function is

also in apparent contrast to the results obtained by H. Yasukawa et al, where they have shown

that when SOCS3 gene is disrupted in macrophages, there is no affect on IL-10's ability to

inhibit TNF-α as compared to SOCS3 wild-type macrophages [173].  Once again, their system

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is very different, as they treated their cells with LPS+IL-10 for duration of 5 to 25 hours and then examined for affect on TNF-$\alpha$ production. As we have shown, IL-10 does not require SOCS3 to inhibit TNF-$\alpha$ production during the late phase of signaling (> 2 hours) (Fig. 11, panel B) and mRNA expression (data not shown). Thus SOCS3 protein is important during the early inhibitory action of IL-10 on TNF-$\alpha$ expression by a macrophage, while other regulatory mechanisms come into play at later times. However, in support of a role for SOCS3 in IL-10 inhibition of inflammation Suzuki *et al* have demonstrated that interference with SOCS3 function in a transgenic mouse enhanced the mouse's susceptibility to intestinal inflammation suggesting that the ablation of SOCS3 does indeed interfere with the normal negative regulatory action of IL-10 in control of colon inflammation [180].

# CHAPTER 5: Future Directions

One of the priorities in further understanding the mechanism by which SOCS3 mediates the anti-inflammatory action of IL-10 is to identify the proteins which interact with the various domains of SOCS3. The effect of SOCS3 could be examined in response to IL-10 on activity of LPS signalling molecules such as ILK, IKK, IκB, and PKB. Studies will be designed which will make use of glutathione-S-transferase (gst) fusion proteins containing the different regions of SOCS3 (for example, SOCS-box domain, SH2 domain and the N-terminal domain which includes the 22 amino acid KIR domain. In case of the KIR domain, the candidate protein could be a kinase, such as the src tyrosine kinase family member Lyn, which is activated by LPS and inhibited by IL-10 in monocytes [184].

As well, the tyrosine phosphorylation sites 204 and 221 located in the SOCS-box domain are found to be important in supporting IL-10 inhibition of TNF-α production and mRNA expression, as well as NO production and iNOS protein expression. Experiments will be designed to identify the proteins that associate with these phosphotyrosyl sites using biotinylated phosphopeptide corresponding to the relevant tyrosine phosphorylation site as the affinity reagent. The biotinylated phosphopeptide will be pulled down using streptavidin agarose and the associated proteins will be visualized by silver staining. If the bands cannot be identified through a known antibody, then proteins will be purified for microsequence analysis. Once identified, the interaction of these known or novel proteins with intact SOCS3 protein can be confirmed by co-immunoprecipitation studies and their role in IL-10 signalling tested by RNAi-mediated knock-down or over-expression studies. Finally using micro-array analysis, we

could identify other LPS-induced genes that are inhibited by IL-10 in a SOCS3-dependent

manner.

**Concluding remarks**

We have definitively shown the importance of SOCS3 protein in the anti-inflammatory action of IL-10 and also have shown that inhibition of NO and TNF-$\alpha$ by IL-10 is mediated by different domains of SOCS3 protein, which suggests that there are different mechanisms that come into play in response to IL-10. This is the first demonstration of alternate mechanisms of action of SOCS3 protein on divergent pathways activated by the same stimuli. A better understanding of these signalling pathways will allow us to discover therapeutic targets in hopes of a cure for many of the inflammatory diseases that affect millions of individuals in our society.

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