SHP-1/ Src complex is a master regulator of the IL-12/IL-23 pro- and IL-10/IL-27 anti-inflammatory axis in TLR4-activated signaling pathways in human monocytes and macrophages

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ABSTRACT:

Although the etiology surrounding many autoimmune diseases remains unknown, the underlying characteristic of many of these diseases is a disruption in the balance of pro- and anti- inflammatory cytokines It is well established that the dysregulation of the IL-12 family of cytokines, an increase in IL-12/IL-23 and a decrease in IL-27 production has been implicated in these conditions. We used ELISA, RT-PCR, Immunofluorescence and Western immunoblotting in conjunction with pharmalogical inhibitors and siRNA to demonstrate the role of SHP-1/Src in the regulation of IL-12, IL-23, IL-27 and IL-10 in LPS-stimulated human THP-1 cells, monocytes and MDMs. My results show for the first time that Src kinase activity relies on SHP-1 activity, and together this complex functions in TLR4-mediated MyD88 and TRIF pathways. Furthermore Src exhibits a dual role as a positive regulator for anti-inflammatory IL-10/IL-27 and as a negative regulator of pro-inflammatory IL-12/IL-23 downstream of TLR4. Moreover, the involvement of PI3K and JNK MAPK, dependent on SHP-1/Src complex, in the regulation of IL-12 family and IL-10 downstream of TLR4 was shown.

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TABLE OF CONTENTS

ABSTRACT....ii

ACKNOWLEDGEMENTS....iii

TABLE OF CONTENTS....iv

LIST OF ABBREVIATIONS....vii

LIST OF FIGURES....xi

I. INTRODUCTION.... Error! Bookmark not defined.

I.I Inflammation.... **Error! Bookmark not defined.**

I.II Inflammatory Mediators.... 2

I.III Cellular Mechanisms of Inflammation.... 3

I.IV Cytokines and Inflammation.... 5

I.IV.A IL-12.... 5

I.IV.B IL-23.... 7

I.IV.C IL-27.... 9

I.IV.D IL-10.... 11

I.V Autoimmune Disease.... 13

I.VI Toll-Like receptor Signalling.... 14

I.VII TLR4 signaling pathways MAPKs and PI3Ks.... 18

I.VIII Tyrosine Phosphorylation of Proteins.... 21

I.IX Protein Tyrosine Phosphatases and SHP-1.... 22

I.X Protein Tyrosine Kinases and Src family.... 26

I.XI Rationale.... 29

I.XII Hypothesis.... 30

I.XIII Objectives.... 31

II. MATERIALS AND METHODS 32
II.I Reagents 32
II.II Cell Culture 32
I.II.A THP-1 cells 32
I.II.B Primary Human Monocytes 33
I.II.C Human Monocyte-derived Macrophages (MDMs) 33
II.III Treatment of cells with Pharmacological Inhibitors 34
II.IV Transfection of THP-1 cells and MDMs with siRNA 34
II.V RT PCR 35
II.VI IL-12p40 Protein Measurement by ELISA 37
II.VII IL-23p19/p40 Protein Measurement by ELISA 38
II.VIII IL-27 Protein Measurement by ELISA 39
II.IX IL-10 Protein Measurement by ELISA 39
II.X Western Immunoblot Analysis 40
II.X.A Preparation of Cell Lysates 40
II.X.B Bradford Assay and Sample Preparation 41
II.X.C Polyacrylamide Gel Electrophoresis (PAGE) 41
II.X.D Electrophoretic Transfer 42
II.X.E Immunoblotting 42
II.XI Immunoprecipitation 43
II.XII Phosphatase Assay 44
II.XIII Immunofluorescence 44
II.XIV Statistical Methods 45
II.XV Ethics Statement 46

III. RESULTS 47
III.I Application of the THP-1 pro-monocytic cell line as a model system to study the regulation of LPS-induced IL-12, IL-23 and IL-27 47
III.I.I The role of SHP-1 in the production of IL-12 family of cytokines 47
III.I.II The role of PTKs in the production of IL-12 family of cytokines 53
III.I.III The PTK involved in the production of IL-12 family of cytokines 56
III.I.IV Downstream signaling molecules in the regulation of the IL-12 family of cytokines 65

III.I.V SHP-1 and Src physically interact and this complex is involved in the activation of PI3K and JNK 75
III.II Applying the findings obtained using a THP-1 cell model to primary human monocytes.81
III.II.I The role of SHP-1 in the production of IL-12 family of cytokines in LPS-stimulated human monocytes 81
III.II.II The role of Src in the production of IL-12 family of cytokines in human monocytes 84
III.II.III The role of PI3K and JNK in the production of IL-12 family of cytokines in human monocytes 87
III.II.IV SHP-1 and Src regulate LPS/TLR4-activated PI3K and JNK pathways in human monocytes 92

III.III Examining the regulation of a pro- and anti- inflammatory axis in LPS-stimulated monocyte-derived macrophages (MDMs) 95
III.III.I The role of SHP-1 in the production of IL-12, IL-23, IL-27 and IL-10 in MDMs95
III.III.II The role of Src in the production of IL-12, IL-23, IL-27 and IL-10 in MDMs101
III.III.III The role of PI3K and JNK MAPK in the production of IL-12, IL-23, IL-27 and IL-10 in MDMs 105
III.III.IV The nature of the SHP-1 and Src protein complex in MDMs 114

IV. DISCUSSION.. 122
IV. Future Directions, Significance and Concluding Remarks............................. 133

V. REFERENCES.. 138

LIST OF ABBREVIATIONS

α-	anti
Ab	antibody
AP1	Activating protein-1
APC	antigen presenting cell
APS	ammonium persulfate
BCR	B cell receptor
BMDMs	bone marrow derived macrophage cultures
BMK-1	big MAPK-1
bp	base pairs
BSA	bovine serum albumin
°C	degrees Celsius
CCR2	CC-chemokine receptor 2
CD	cell differentiation marker
cDNA	complementary DNA
C/EBP	CCAAT/enhancer binding protein transcription factor
CLRs	C-type lectin like receptors
CO_2	carbon dioxide
c-Rel	transcription factor
CSF-1	colony stimulating factor 1
CX3CR1	CX3C chemokine receptor 1
Cys	cysteine
DCs	dendritic cell
DMSO	dimethyl sulphoxide
DNA	2'-deoxyribonucleotide triphosphate
DTT	dithiotreitol
EBI3	Epstein Bar Virus induced gene 3
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetra-acetic acid disodium salt
ELISA	Enzyme linked immuno-sorbent assay
Elk1	transcription factor
ERK	extracellular-signal regulated kinase
FAK	focal adhesion kinase
FBS	fetal bovine serum
FERM	4.1 protein, ezrin, radixin and moesin domain
FITC	fluorescein isothiocyanate
x g	force of gravity
GATA-1	transcription factor, GATA refers to DNA sequence
gp130	IL-27 receptor chain
GTP	guanosine triphosphate
HCl	hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hr	hour
HRP	horseradish peroxidase
IBD	inflammatory bowel disease

IFN-γ	interferon-gamma
Ig	immunoglobulin(s)
IκB	inhibitor-kappa B
IL-	interleukin
IL-10R	interleukin 10 receptor
IL-12R	interleukin 12 receptor
IL-23R	interleukin 23 receptor
IP	immunoprecipitate/immunoprecipitation
IRAK	IL-1R associated kinase
IRF-	interferon regulatory factor
ITIMs	immunoreceptor tyrosine-based inhibitory motif(s)
JAK	Janus associated kinase
JNK	c-Jun N-terminal kinase
Kb	kilobase
KCl	potassium chloride
kDa	kilodalton
LBP	LPS binding protein
LPS	Lipopolysaccharide
M	molar
mAb	monoclonal antibody/antibodies
MAPK	mitogen associated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPK Kinase kinase
M-CSF	macrophage colony-stimulating factor
MDMs	monocyte-derived macrophages
Me/me	moth-eaten mice
mg	milligram(s)
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulfate
MHC II	major histocompatibility complex II
min	minute(s)
mL	milliliter(s)
mM	millimolar
mRNA	messenger Ribonucleic acid
MS	Multiple sclerosis
MyD88	myeloid differentiation factor 88
μg	microgram
μL	microlitre
μM	micromole
Na_2CO_3	sodium carbonate
NaCl	sodium chloride
NaF	sodium fluoride
$NaHCO_3$	sodium bicarbonate
$NaPO_4$	sodium phosphate
NF-κB	Nuclear factor kappa B
NK cells	Natural killer cells

NLRS	Nod Like receptors
nm	nanometer(s)
NO	nitric oxide
nt	nucleotides
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerized chain reaction
PDZ	post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (zo-1)
PFA	paraformaldehyde
pg	picogram(s)
PI3K	phosphotidyl inositol-3-kinase
PRRs	pattern recognition receptors
PTK	protein tyrosine kinase
PTP	protein tyrosine phosphatase
PU1	transcription factor, Binds to purine rich region 5'-GAGGAA-3'
PVDF	polyvinylidene difluoride
RA	rheumatoid arthritis
RLRs	Retinoic acid-inducible gene 1 like receptors
RNA	ribonucleic acid
RORγ	Retinoic acid receptor- related orphan receptor gamma
RPM	Revolutions per minute
RT	room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
SD	standard deviation
SE	standard error
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate poly acrylamide gel elctrophoresis
Ser	serine
SH2	*Src*-homology domains 2
SHP-1	SH2 domain containing phosphatase-1
SHP-2	SH2 domain containing phosphatase-2
SP-	specificity protein
STAT	signal transducers and activators of transcription
TBE	Tris boric acid –EDTA
TBS	Tris buffered saline
TBST	Tris buffered saline and Tween-20
TCR	T cell receptor
TEMED	N,N,N’,N’-tetramethylethylenediamine
TGF-β	transforming growth factor-β
Th cells	T-helper cells
TIR	Toll/IL-1 receptor
TIRAP	Toll-interleukin 1 recetor adaptor protein
TLR	toll-like receptor
TNF-α	tumor necrosis factor-alpha
TRAF	TNF receptor associated factor

TRAM	TRIF related adaptor molecule
TRIF	TIR-domain-containing adapter-inducing interferon-β
T reg	T regulatory cell
Tyr	tyrosine
U	unit(s)
UTR	un-translated region
UV	ultraviolet
V	volts
v/v	volume with respect to volume
WT	wild-type
w/v	weight with respect to volume
WSX-1	IL-27 receptor chain
Y	Tyrosine

LIST OF FIGURES:

I.I TLR Receptors and their ligands
I.II Classical PTPs
I.III Src Kinase Structure
III.1 Sodium Stibogluconate inhibits production of IL-12, IL-23 and IL-27 in LPS-stimulated THP-1 cells
III.2 SHP-1 siRNA silences the expression of SHP-1 resulting in a decrease of IL-12, IL-23 and IL-27 cytokine production in LPS –stimulated THP-1 cells
III.3 Herbimycin A inhibits production of IL-12, IL-23 and IL-27 in LPS-stimulated THP-1 cells
III.4 PP2 inhibits production of IL-12, IL-23 and IL-27 in LPS-stimulated THP-1 cells
III.5 SU6656 inhibits production of IL-12, IL-23 and IL-27 in LPS-stimulated THP-1 cells
III.6 C-src siRNA silences the expression of Src resulting in a decrease of IL-12, IL-23 and IL-27 cytokine production in LPS –stimulated THP-1 cells
III.7 LY294002 inhibits production of IL-12, IL-23 and IL-27 in LPS-stimulated THP-1 cells
III.8 PD09856 has no effect on the production of IL-12, IL-23 and IL-27 in LPS-stimulated THP-1 cells
III.9 SB203580 has no effect on the production of IL-12, IL-23 and IL-27 in LPS-stimulated THP-1 cells
III.10 SP600125 inhibits production of IL-12, IL-23 and IL-27 in LPS-stimulated THP-1 cells
III.11 SHP-1 and Src physically interact upon LPS stimulation
III.12 SHP-1 and Src signalosome promote phosphorylation of Akt and JNK
III.13 Sodium Stibogluconate inhibits production of IL-12, IL-23 and IL-27 in LPS-stimulated Human Monocytes
III.14 SU6656 inhibits production of IL-27 and promotes production of IL-12 and IL-23 in LPS-stimulated Human Monocytes
III.15 LY294002 inhibits production of IL-12, IL-23 and IL-27 in LPS-stimulated Human Monocytes
III.16 SP600125 inhibits production IL-27 and promotes production of IL-12 and IL-23 in LPS-stimulated Human Monocytes
III.17 SHP-1 and Src signalosome promote phosphorylation of Akt and JNK
III.18 Sodium Stibogluconate inhibits production of IL-12, IL-23, IL-10 and IL-27 in LPS-stimulated MDMs
III.19 SHP-1 siRNA inhibits production of IL-12, IL-23, IL-10, and IL-27 in LPS-stimulated MDMs
III.20 SU6656 promotes production of IL-12, IL-23 and inhibits IL-10 and IL-27 production in LPS-stimulated MDMs
III.21 C-Src siRNA promotes production of IL-12 and IL-23 and inhibits production of IL-10 and IL-27 in LPS-stimulated MDMs
III.22 LY294002 inhibits production of IL-12, IL-23, IL-10 and IL-27 in LPS-stimulated MDMs

III.23 SP600125 promotes production of IL-12, IL-23 and inhibits IL-10 and IL-27 production in LPS-stimulated MDMs
III.24 SHP-1 and Src physically interact in LPS-stimulated MDMs
III.25 SHP-1 and Src physically interact with MyD88 in LPS-stimulated MDMs
III.26 SHP-1 and Src physically interact with TRIF in LPS-stimulated MDMs
I.IV.I Model for the SHP-1/Src complex dual regulation of IL-12/IL-23 and IL-10/IL-27 in LPS-stimulated monocytes and MDMs

I. INTRODUCTION

I.I Inflammation

Inflammation is a natural and beneficial response by the body designed to resolve infection, injury and repair tissue damage within a host system, ultimately returning it to a state of homeostasis.[1] It is a complex yet highly ordered and tightly controlled homeostatic process. Intrinsic to such a homeostatic process is the ability to mount an appropriate response, which balances rapid clearance of foreign stimuli, irritants or invading microorganisms while limiting the internal damage due to inflammatory mediators.[2] Clinically, inflammation is characterized by four cardinal symptoms: redness, edema, pain and heat. These signs are attributes of the initial acute inflammatory response.[3] The molecular basis underlying development of these symptoms is a widespread cellular signalling network, with the primary event being vasodilation at the site of infection allowing for the massive and coordinated infiltration of immune cells, an event that initiates the activation of the first line of defence which is the innate immune response.[3] Cells of the innate immune response include macrophages, neutrophils, mast cells, dendritic cells, eosinophils and natural killer cells.[4] The coordinated recruitment of these cells to the site of injury is crucial for a successful inflammatory response. This process is driven by a rapid induction of genes encoding inflammatory mediators that are responsible for activation and orderly influx of immune cells.[5-7] The signals produced by these mediators alter the local environment profile creating a chemotactic gradient that is mandatory for the recruitment of immune cells to the site of injury. Neutrophils are the first inflammatory cells recruited to the site of injury, followed by mononuclear cells, such

as monocytes, specific subsets of T and B lymphocytes and mast cells.[1,6] One of the critical events taking place during the innate immune response is a differentiation of monocytes into effector macrophages that are able to present antigen to antibody producing B lymphocytes as well as T helper and cytototoxic lymphocyte thereby activating the adaptive immune system.[8] This switch from innate to adaptive immunity is necessary for the proper resolution of the inflammation because it marks a change in the molecular signature of the inflammatory microenvironment. [8] This is because each cell involved in immunity has a unique chemokine and cytokine signature, and these molecules are either pro- or anti-inflammatory agents. [2,8] While acute inflammation is beneficial, dysregulation of the inflammatory switch may result in prolonged inflammation by retention of an activated mononuclear cell population. [8] These activated mononuclear cell populations have a pro-inflammatory chemokine and cytokine signature which results in tissue destruction, and present as clinical disorders such as autoimmune disease. [9]

I.II Inflammatory mediators

The inflammatory response is initiated, maintained and resolved by employing a plethora of mediators, which are secreted by the immune cells present at the site of injury.[1] For example, upregulated expression of some adhesion molecules by pro-inflammatory peptides of the Complement system enhances chemotaxis of neutrophils and monocytes, and activates phagocytes and local mast cells.[10] Other components of the Complement system such as vasoactive peptide

bradykinin and coagulation factor XII, increase vascular permeability that promotes the influx of plasma proteins/fluid into the extravascular spaces and promotes formation of fibrin clots that prevent the spread of infection and influx of microorganisms, respectively.[11] Most importantly, macrophages secrete inflammatory lipid metabolites such as platelet activating factor, prostaglandins, leukotriens, lipoxins, nitric oxide (NO), a potent endogenous vasodilator; and a group of cell-derived polypeptides, known as cytokines and chemokines.[7,12] Although precise mechanisms controlling the inflammatory processes are not fully understood, macrophages are critical in the inflammatory episode.

I.III Cellular mechanisms of Inflammation

Monocytes develop in the bone marrow from dividing common myeloid progenitors and are subsequently released into the blood as nondividing cells.[13] Monocytes have a very short half-life, about 3 days in humans, which suggests that they are a general reservoir of myeloid precursors repopulating tissue macrophages.[14] This is enforced by the demonstration that peripheral blood mononuclear cells (PBMCs) from the bone marrow upregulate expression of CCR2 in order to be homed to the tissues.[14] However, the population of blood monocytes is heterogenous, suggesting that they undergo further differentiation in the blood.[13,15] Indeed, based on surface expression of the Ly-6C marker two distinct monocytic populations have been described in the mouse model.[15] Ly-6C^{hi} monocytes express high levels of CCR2, which mediates their active accumulation in tissues in

response to damage and foreign stimuli. These monocytes differentiate into macrophages, which expand inflammation.[16] In contrast Ly-6C^{lo} monocytes express fractalkine receptor, CX3CR1, and are thought to be involved in the resolution of inflammation since they migrate to tissues with an inflammatory profile much later then their Ly-6C^{hi} counterparts.[15,16] In humans two equivalent populations have been described based on differential expression of CCR2 and CX3CR1 expression: CD14^{hi}CD16^{-} and CD14^{+}CD16^{+}, which refer to classical and non-classical monocytes respectively.[15] Like their monocytic precursors, upon differentiation, macrophages can also be sub-classified into two distinct populations based on their inflammatory profiles: M1 and M2.[15] M1 macrophages, otherwise distinguished as classically activated, display a pro-inflammatory character and are designated as effector macrophages.[17] Classical activation requires two signals, Interferon gamma (IFNγ) priming and tumour-necrosis factor (TNF) which result in the development of a highly microbicidal and tumoricidal population capable of secreting a high level of pro-inflammatory cytokines and mediators.[18] Recently, it has been described that toll-like receptor (TLR) agonists can act as the second signal required, in the place of TNF, for classical activation.[17] The necessary IFNγ is transiently produced by NK cells and therefore to adequately sustain a population of M1 macrophages the adaptive immune response, specifically the T-helper cells (Th1 cells) response is necessary.[18] M2 macrophages, alternatively activated, alleviate type 1 inflammatory responses and adaptive immunity and promote tissue repair.[19] Alternative activation is dependent on the presence of interleukin-4 (IL-4) and IL-13.[20] Like M1, adaptive immune responses are necessary to perpetuate the M2 population, specifically Th2

cells are required due to their abundant production of IL-4 and IL-13.[19,20] Therefore it is evident that macrophage activation leads to activation of various branches of adaptive immunity and this is underlined by the cytokine profile of the inflammatory environment.

I.IV Cytokines and Inflammation

I.IV.A IL-12

M1 macrophages are characterized by the ability to secrete high levels of pro-inflammatory cytokines.[1,21] Cytokines are small cell-signalling molecules secreted by numerous cells and used to transmit signals between different cell types to elicit appropriate cellular responses.[1] Cytokines can be broadly characterized into two groups, interleukins and interferons.[1] Amongst the pro-inflammatory cytokines, IL-12 assumes a primary role.[22] The main function of IL-12 is to create a bridge between innate and adaptive immunity by promoting a Th1 response.[23] Initiating a Th1 response perpetuates the IFNγ signal, which maintains the M1 phenotype. IL-12 is a heterodimeric cytokine composed of two subunits: IL-12p40 and IL-12p35 which are covalently linked through a disulfide bridge.[24] The genes encoding IL-12p35 and IL-12p40 proteins are found on two separate chromosomes in humans, 3 and 5 respectively, and therefore their expression is regulated independently.[24] IL-12p35 is ubiquitously expressed at low levels in all cell types.[25] Despite constitutive synthesis of p35 transcripts in unstimulated cells, an inhibitory ATG sequence in the 5'UTR prevents protein synthesis because it interferes with translational machinery.[25]

However, when cells become stimulated with bacterial ligands such as a cell wall component, Lipoolysaccharide (LPS) transcription of the p35 transcript is initiated in a different spot, through the induction of different transcription factors by LPS, which prevents the inclusion of the inhibitory ATG sequence and thus protein synthesis can resume because the translational machinery can now bind to the transcripts.[25] By contrast, IL-12p40 expression is limited to immune cells that secrete IL-12p70 heterodimer.[26] The p40 gene is regulated at the transcriptional level, through binding of NF-κB, IRF-1 and c-Rel, and is highly inducible by bacterial ligands.[26]

The biological activity of IL-12 can be broadly divided into two main functions: promoting a pro-inflammatory environment through the induction of IFNγ from activated T-cells and the differentiation of naive CD4+ T cells into Th1 helper cells.[27] IL-12 mediates its signal through the IL-12 receptor (IL-12R).[28] The IL-12R is a heterodimeric receptor composed of two chains: IL-12Rβ1 and IL-12Rβ2, co-expression is required for high-affinity binding of IL-12p70.[28] The IL-12p40 binds the IL-12Rβ1 chain, and IL-12p35 binds the IL-12Rβ2 chain, which is the component of the IL-12R responsible for signal transduction through the phosphorylation of tyrosine residues present in this chain.[28] IL-12R is expressed on both activated and naïve T cells, natural killer (NK) cells and dendritic cells (DCs).[29] However, on activated T cells IL-12R is differentially expressed based on the cytokine milieu present, IL-12Rβ1 and IL-12Rβ2 are co-expressed on Th1 cells because IFNγ promotes the expression of IL-12Rβ2. On the other hand, Th2 cells lack IL-12Rβ2 because IL-4 inhibits it.[29] IL-12 binding to IL-12R on NK cells and Th1 helper cells induces IFNγ by synergy with either IL-2 or IL-18.[30] Synergy with IL-2 induces post-

transcriptional modifications of IFNγ transcripts to increase their stability and nuclear mobilization.[30] Synergy with IL-18 promotes further binding of transcription factors STAT4 and AP1 in the promoter region of the IFNγ gene, thereby increasing IFNγ transcript levels.[30] As previously mentioned, another major role of IL-12 is the differentiation of naive T cells into Th1 helper cells. T cell differentiation is determined shortly after infection by the balance of IL-12 and IL-4, which favour Th1 and Th2 responses respectively.[31] As described, IL-12 induces IFNγ production, together IL-12 and IFNγ induce clonal expansion of naive T cells into Th1 helper cells by priming them for expression of cytokines such as IFNγ and deleting of IL-4 producing cells in the milieu.[31] Therefore, since IL-12 is necessary in promoting a Th1-mediated pro-inflammatory environment while inhibiting the Th2-mediated responses, which include anti-inflammatory signals, its expression must be tightly regulated. This is especially important because Th2 immunity is responsible for wound healing, which is necessary for repairing the tissue injury sustained during the inflammatory episode.[31]

I.IV.B IL-23

IL-23, discovered through homology searches against IL-12p35, is a member of the IL-12 family of cytokines.[32] IL-23 is also a heterodimeric cytokine composed of two subunits linked by a disulfide bridge: IL-12p40 and IL-23p19, which is 70% homologous with IL-12p35.[33] Unlike IL-12p35, IL-23p19 is not ubiquitously expressed but rather is only expressed by immune cells such as antigen presenting cells (APCs) and T cells.[33] The IL-23p19 gene is encoded on chromosome 19 and thus is under different regulation from its binding partner IL-12p40. The biologically

active IL-23 is formed by the association of both subunits present in the same cell.[33] Like IL-12p40, IL-23p19 is also inducible upon bacterial ligands' engagement of TLR3 or TLR4 and this is through the induction of transcription factor IRF3 that binds to the IL-23p19 promoter region.[34]

Due to the structural similarity between IL-12 and IL-23, it was thought that the roles of the two cytokines would be redundant.[32] However, emerging research suggests a unique role of IL-23 in memory T cell differentiation.[32] IL-23 binding requires two receptor chains, IL-12Rβ1, which is constitutively expressed on CD4+ T cells and IL-23R, which is absent from naive and effector T cells but highly expressed on memory T cells.[35] This implies that IL-23 does not induce Th1 differentiation in the same manner as IL-12, but instead has a unique role in the engagement of adaptive immunity, in addition to its involvement in the expansion of memory T cells, IL-23 is also necessary for the maintenance of a highly-inflammatory T cell subset known as the Th17 response. [36] In addition to IL-23R expression on memory T cells, it is also present on antigen presenting cells of innate immunity such as DCs, NK cells, monocytes and macrophages.[35] Therefore IL-23 can induce expression of pro-inflammatory cytokines such as, TNFα and IL-1β, which are required to maintain the Th17 population of cells.[37] Like the Th1 response, the Th17 response is also pro-inflammatory in nature and is characterized by the production of IL-17, IL-21, IL-22 and IL-26.[36] IL-17 induces chemoattractant CXCL8, which recruits neutrophils to the site of infection to promote further innate immune responses, and CCL20, which recruits Th17 cells to the site of infection.[38] IL-21 activates an alternative pathway to amplify the Th17 response.[39] IL-22 and IL-26 are

critical for induction of dermal and intestinal inflammation respectively.[40,41] Therefore like IL-12, IL-23 can be characterized as a pro-inflammatory cytokine due to its unique ability to activate the pro-inflammatory Th17 cellular response.

I.IV.C IL-27

Another member of the IL-12 family of cytokines is IL-27. IL-27, was also discovered through homology searches against IL-12p35.[42] The IL-27 cytokine is also heterodimeric composed of two subunits: EBI3, which is homologous to IL-12p40 and IL-27p28, which is homologous to IL-12p35.[42] EBI3, like IL-12p40, is expressed only in immune cells particularly in APCs, such as DCs and macrophages.[43] EBI3 is inducible upon TLR-2,-4 and -9 engagement by bacterial ligands and this induction requires the activation of transcription factors NFκB and PU1 on the EBI3 promoter region through the TLR-2,-4 or-9 associated Myd-88 signal transduction pathway. [43] Similarly to IL-23p19, despite its homology to IL-12p35, IL-27p28 is inducible in immune cells.[44] IL-27p28 induction requires activation of TLR4 by LPS and engagement of LPS adapter protein TRIF resulting in activation of transcription factors IRF3 and cRel on the p28 promoter region. [44] The two subunits are therefore regulated differentially, however to secrete biologically active IL-27 both proteins need to be produced in the same cell.[42]

The biological activity of IL-27 remains controversial as to its role as a predominantly pro- or anti-inflammatory cytokine in the immune response.[42] IL-27 signals through the IL-27 receptor, which is composed of two subunits: WSX-1 and gp130.[45] A recent analysis of WSX-1 expression revealed that naive T cells have

much lower surface expression of WSX-1 compared to activated T cells.[45] The pro-inflammatory properties of IL-27 are limited to its ability to enhance Th1 differentiation from naive CD4+ T cells by up-regulating their surface expression of IL-12Rβ2. Enhanced expression of IL-12Rβ2, increases the IFNγ levels within the inflammatory environment.[46] However, since surface expression of WSX-1 is very low on naive CD4+ T cells it is possible that this effect is redundant due to IL-27 similarity to IL-12 and therefore negligible in the inflammatory system. Moreover, studies using WSX-1 knockout mice demonstrated that the initially lower levels of IFNγ from antigen-experienced T cells were restored to wild-type levels shortly after infection of WSX-1 knockout mice with with Leishmania Major.[46] In addition, another study also has shown that stimulation with IL-27 was incapable of inducing IFNγ from naive CD4+ T cells [47] These observations therefore suggest that a unique role for IL-27 lies within its abundant anti-inflammatory properties. To build on this notion, a number of studies were conducted with WSX-1 knockout mice challenged with intracellular microbes. These mice failed to down-regulate the adaptive immune response and suffered from a lethal CD4+ T cell dependent inflammatory disease.[48] Characterized by a highly active population of T -cells over-expressing pro-inflammatory cytokines such as IFNγ, IL-6 and TNFα.[48] In addition to over-activated T cell populations, IL-27 is necessary to inhibit the Th17 response by activating STAT1, which down-regulates the transcription factor RORγ within these cells to prevent secretion of Th17 cytokines such as IL-17.[49] Therefore the biological activity of IL-27 is in direct opposition to the biological activity of IL-23. Moreover, IL-27 has most recently been described as necessary to induce IL-10, which is known as the

most immunosuppresive cytokine.[50] Interestingly this induction of IL-10 can only be achieved in Th17 free environment suggesting that IL-27 acts as an anti-inflammatory agent to resolve existing pro-inflammatory responses.[51] Based on these findings the dual role of IL-27 may be envisaged as a timely response. During the initial pro-inflammatory response, IL-27 induces T cell differentiation to induce itself through an autocrine feedback mechanism that ultimately, once IL-27 reaches higher levels, serves to shut down the excessive pro-inflammatory responses such as the Th17 response.

I.IV.D IL-10

The IL-10 cytokine does not belong to the IL-12 family of cytokines, however IL-10 is intimately connected with the members of the IL-12 family.[52] IL-10 inhibits production of IL-12 and IL-23 and is induced by IL-27.[52] The production of IL-10 is tightly regulated at three levels: epigenetic regulation, transcriptional control and post-transcriptional modifications.[53] IL-10 is a homodimer, two monomers approximately 17 kD linked by two disulfide bridges and produced by a large variety of immune cells including monocytes, macrophages, Th2 cells, T regulatory (T reg) cells and CD8+ T cells. IL-10 is also produced by non-immune cells including epithelial cells and tumour cells.[54,55] Because of the wide expression of IL-10 by different cell types and because of its potent biological activity, IL-10 is also regulated by cellular signals to determine which cells are expressing the cytokine at any given time.[53] The IL-10 gene locus is located on chromosome 1. [56] Among the innate immune cells, macrophages produce the highest amount of IL-10 upon stimulation of TLR2 or TLR4.[57] Upon binding of bacterial products by TLR2 or TLR4

both the MyD88 and TRIF-dependent pathways are engaged to induce a plethora of transcription factors.[57] The transcription factors described in the induction of the IL-10 promoter include: SP1, SP3, IRF3, STAT3 and NFκB.[56,58]

The biology of IL-10 is centred around its broad anti-inflammatory properties which can be classified into three general categories: altering the secretion of immune mediators, inhibiting antigen presentation and increasing phagocytosis.[55] IL-10 exerts its effects through binding to the IL-10R, which is composed of two chains IL-10R1 and IL-10R2.[59] IL-10 binds with high affinity to IL-10R1 and this induces conformational changes within the IL-10 molecule exposing an epitope for which IL-10R2 can bind.[59,60] IL-10R2 is broadly expressed on many cell types, and is able to dimerize with other receptor chains to provide functional receptors for other ligands.[61] In contrast, IL-10R1 is expressed only on immune cells and is most highly expressed on innate immune cells such as monocytes and macrophages.[62] This suggests that IL-10 exerts its effects primarily by attenuating the innate inflammatory response. Firstly, IL-10 modulates the secretion of immune mediators by binding to its receptor on innate immune cells and inhibiting the release of pro-inflammatory cytokines such as TNFα and IL-6 and enhances the release of anti-inflammatory mediators such as IL-1R**α**.[63,64] Interestingly, IL-10 directly inhibits the synthesis of IL-12, which switches off Th1 immunity.[65] Moreover, IL-10 acts on activated CD4+ T cells to limit the production of IFNγ.[65] Recently, IL-10 has been implicated in inhibition of IL-23 in murine bone marrow derived macrophages and this led to impaired Th17 immunity [66] Therefore IL-10 has an important role in limiting production of pro-inflammatory IL-12 cytokine family members. Secondly, IL-10 is

involved in inhibiting antigen presentation by innate immune cells by down-regulating surface expression of major histocompatibility complex II (MHC II) and co-stimulatory molecules such as CD86.[67] Finally, IL-10 inhibits the inflammatory response by increasing the phagocytic ability of monocytes and macrophages by increasing expression of various receptors, such as CD64, that are responsible for uptake of opsonized and non-opsonized micro-organisms.[68] Hence, IL-10 promotes expansion of a macrophage population exhibiting an inhibitory phenotype. This macrophage population is responsible for clearing the inflammatory episode.[68] Collectively, IL-10 is a broadly acting anti-inflammatory cytokine that can be used as the basis for studying the regulation of anti-inflammatory properties.

I.V Autoimmune Disease

The expression levels of various pro- and anti-inflammatory cytokines is critical to the development and resolution of the inflammatory response. When the balance between pro- and anti- inflammatory cytokines is disrupted by yet undefined factors, disease ensues. In particular, autoimmune disease is characterized by the chronic activation of the immune system against self components.[69] Common autoimmune diseases include psoriasis, multiple sclerosis (MS), Crohn's disease, inflammatory bowel disease (IBD) and rheumatoid arthritis (RA).[69] These diseases are primarily T cell-mediated, recruiting monocytes and neutrophils to induce tissue-specific inflammation.[70] The IL-12 family of cytokines has been highly implicated in autoimmune diseases.[24] Psoriasis is a Th1-mediated skin condition in which skin lesions develop due to elevated levels of IL-12 and IL-23.[71] In MS, increased production of IL-12p40 and decreased production of IL-10 have been observed in

severe relapsing conditions.[72] In RA, IL-23 appears to be critical in the development of late-stage joint inflammation.[73] While, IL-12 and IL-23 have been implicated in the pathogenesis of inflammatory diseases IL-27 has been implicated as a possible therapy due to its anti-inflammatory properties.[42] Particularly in autoimmune disease where IL-23 is critical in disease progression, IL-27 therapy can be proposed due to its direct inhibition of the Th17 immune response activated by IL-23.[49] Therefore it is of interest to understand the molecular mechanisms behind the induction of the IL-12 family of cytokines to generate future therapies for autoimmune disease affecting a large number of people.

I.VI Toll-Like Receptor Signalling

Cytokine production requires the activation of signalling pathways within the cell that culminate in activation of transcription factors promoting induction of cytokine encoding genes.[74] Activation of signaling pathways is initiated by interaction of extracellular ligands with their cognate cell membrane receptors.[75] Immune cells express transmembrane molecules collectively known as pattern recognition receptors (PRRs).[74] The PRRs comprise of four families of receptors: Toll-like receptors (TLRs), C-type lectin like receptors (CLRs), RIG-1 like receptors (RLRs) and Nod Like receptors (NLRs).[75] TLRs play a critical role in the induction of both acquired immunity and in innate immunity. [74] Currently, there are 13 human TLRs that have been identified and expressed on a variety of cell types, including dendritic cells, macrophages, endothelial cells, lymphocytes, and epithelial cells.[75,76] Most

TLRs are expressed on the cell membrane (TLR1,2,4,5,6,10 and 11) whereas the remaining TLRs are expressed on intracellular membranes such as those present on endosomal compartments (TLR3,7,8, and 9).[75] TLRs are expressed as homodimers or heterodimers and each receptor pair is responsible for recognition of a different microbial ligand.[75] For example, TLRs can detect certain components of the bacterial cell wall such as lipoolysaccharide (LPS) from the cell wall of gram-negative bacteria (TLR4), peptidoglycan from gram-positive bacteria (TLR2), flagellin (TLR5) and genomic DNA (TLR9).[77-79] (Figure I.I) In contrast TLR3 and TLR7/8 are involved in viral recognition, and bind dsRNA and ssRNA respectively.[80,81] Engagement of TLRs by microbial ligands induces a broad range of pro- and anti-inflammatory cytokines.[75] Cytokines, in turn, interact with their cognate receptors on target cells and initiate inflammation and activate both the complement pathways and coagulation pathway[75]

Each member of the TLR family has a conserved trimodular structure composed of a central transmembrane domain, a C-terminus responsible for ligand binding and a N terminal Toll/IL-1 receptor (TIR domain).[2] The TIR domain is required for signal transduction through interaction with TLR adaptor proteins.[2] TLR adaptor proteins include myeloid differentiation factor 88 (MyD88), Toll-interleukin 1 receptor adaptor protein (TIRAP), TIR-domain-containing adapter-inducing interferon-β (TRIF) and TRIF-related adaptor molecule (TRAM) and the interaction is mediated by TIR-TIR binding.[82]

Figure I.I Schematic representation of Toll-Like Receptor Family. Engagement of LPS/TLR4 signalling cascade demonstrating the involvement of MyD88 and TRIF adaptor molecules in the activation of known signalling pathways such as NFκB, MAPK and IRF pathways resulting in the production of pro-inflammatory cytokines

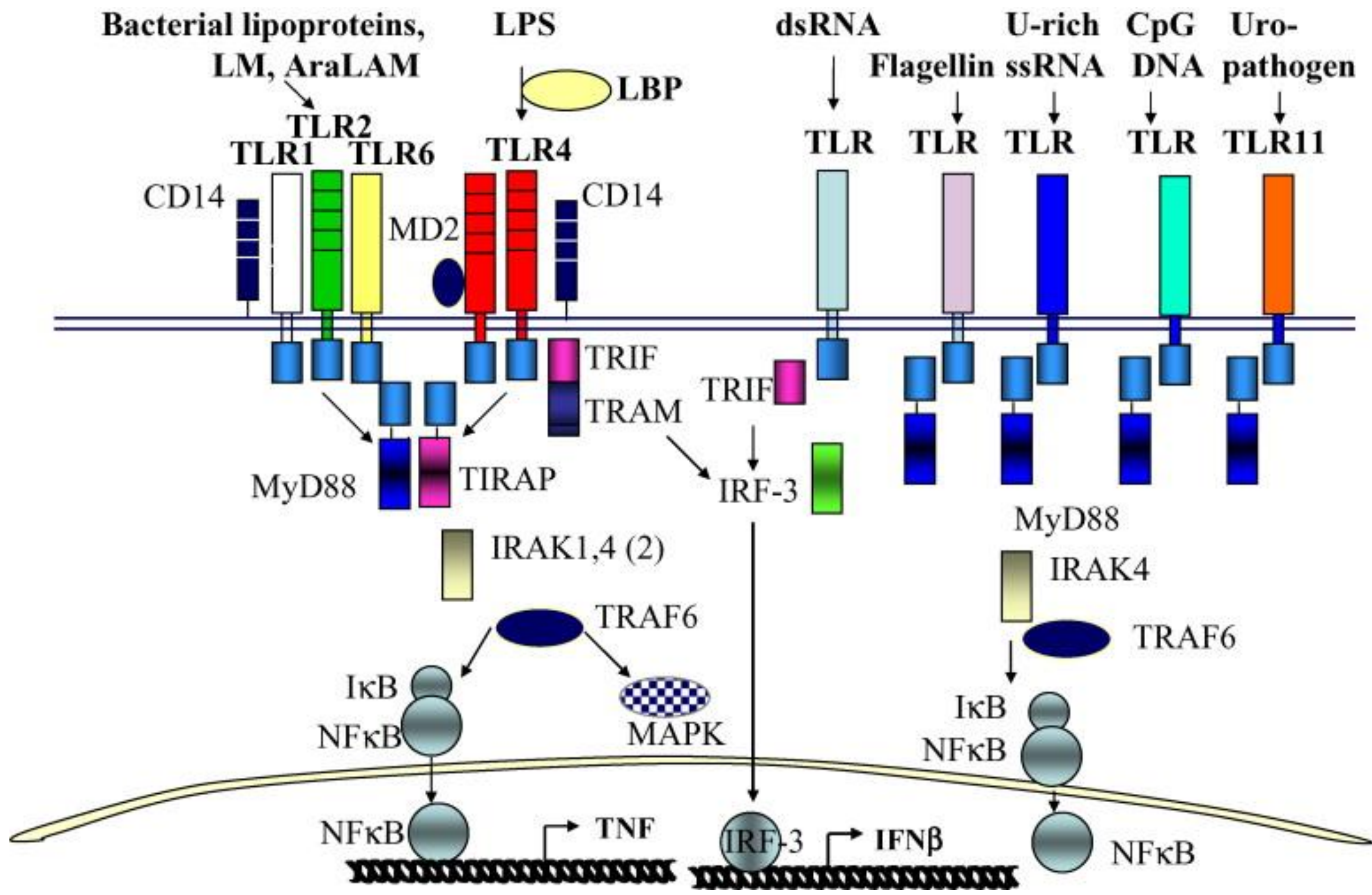


Fig. I.I (Quesniaux et al. Science. 2004)

TIR-TIR binding between TLR and adaptor proteins results in the recruitment of Interleukin-1 receptor associated kinase (IRAK) family proteins and TNF receptor associated factor 6 (TRAF6) to the complex, which further activates downstream pathways. Each TLR-signaling mechanism recruits a specific adaptor protein to activate signaling for specific transcription factors.[83] All TLRs recruit MyD88-dependent signaling except TLR3, which signals through a MyD88-independent pathway recruiting TRIF.[83] TLR4 recruits both MyD88- and TRIF-dependent pathways.[84] TLR4-MyD88 dependent signaling leads to the activation of MAPKs known to activate transcription factors such as AP-1 and NFκB important for the induction of pro-inflammatory cytokines such as IL-12 and IL-23 and for the induction of anti-inflammatory cytokines such as IL-27 ad IL-10.[66] In contrast, TLR4-TRIF dependent signaling activates IRF3 transcription factor to induce type-1 interferon genes.[84] This bimodal-signaling of TLR4 has potential to activate distinct signaling pathways, culminating in the induction of a high number of inflammatory mediators.

I.VII TLR4 signaling pathways MAPKs and PI3Ks

The interaction of LPS with CD14 promotes dimerization of the TLR4 and subsequent activation of a broad range of signaling pathways ultimately leading to the induction of genes including the genes encoding pro-inflammatory cytokines.[84] Among others, two major pathways activated by TLR4 are the phosphoinositide-3-kinase (PI3K) and the mitogen activated protein kinase (MAPK) pathways.[85] The

PI3K family of lipid kinases can be sub-divided into 3 classes: I, II and III based on structure and substrate specificity.[86] Class I can be classified into IA and IB depending on the regulatory system employed, the IA class of PI3Ks, are critical in immune cell function.[86] The IA PI3Ks consist of p110α, p110β and p110δ catalytic subunits that are either regulated by p85α or p85β regulatory subunits.[87] Activation of class IA PI3Ks is mediated by tyrosine phosphorylation, the regulatory subunit p85 becomes phosphorylated at its tyrosine residue followed by recruitment of the catalytic p110 subunit.[87] Activated PI3K phosphorylates AKT kinase, which acts on various targets downstream, one such suggested target is NFκB, thereby inducing pro-inflammatory cytokines.[87] This is reinforced by recent studies that demonstrate effective use of inhibitors for PI3K as therapeutics in the treatment of osteoarthritis and other autoimmune diseases.[88] Recently the link between TLRs and PI3K activation has also been explored. It has been shown that MyD88 contains a YXXM motif within its TIR domain that has been found to associate via tyrosine phosphorylation with p85 and MyD88 deficient macrophages were found to have defective Akt-phosphorylation.[89] However, it has also been described that TRIF signals mediating PI3K activation were required for Akt phosphorylation downstream of TLR4.[90] Together these data suggest that TLR4 adaptor proteins recruit an unknown protein(s) in the activation of PI3K.

The MAPK family regulates cell growth, differentiation, cell survival, neuronal response and the immune response.[91] The canonical activating pathway for MAPKs is a triple kinase module in which a MAPK kinase kinase (MKKK) phoshorylates a MAPK kinase (MKK) which activates a terminal MAPK through a dual phoshorylation

mechanism on a Tyrosine and Threonine residue.[92] Upstream of this canonical pathway it has been demonstrated that Ras and Rho families of GTPases mediate the signal transduction from receptor complexes at the membrane.[92] There are four major MAPK pathways: extracellular-signal-regulated kinase 5 (ERK-5; also known as Big MAPK-1 (BMK-1)), extracellular-signal-regulated kinase (ERK), c-Jun-N-terminal Kinase (JNK) and p38 pathways.[92,93] Additionally, each of the MAPKs is expressed as different isoforms: ERK1 and ERK2, JNK1 JNK2 and JNK3, and p38α p38β p38γ and p38δ.[92] Although the terminal MAPKs share an activating mechanism, their biological functions differ. Upon stimulation ERK1/2 molecules detach from anchor proteins and translocate to various organelles, 50-70% translocate to the nucleus.[94] ERK1/2 is most notable for its activation of Elk1 transcription factor involved in the induction of immediate early genes important for proliferation and differentiation.[94] Similarly to ERK1/2, p38 is also critical to proliferation and differentiation including that of hemapoietic cells.[95] p38α activates transcription factors C/EBP and GATA-1, and it has been shown that p38 is critical to maintain neutrophils at the site of infection.[96] In other myeloid cells p38 mediates activin-A-mediated differentiation.[96] Thus both ERK1/2 and p38 are important in cell survival and differentiation, where the latter specifically maintains immune cells. Interestingly, JNK specifically has been implicated in the production of many pro-inflammatory cytokines.[97] For example, the TNFα gene contains JNK-responsive promoter elements, including the AP-1 site known to bind ATF2 and c-Jun.[97] JNK is known to activate c-Jun and ATF2, and JNK-knockout cells exhibit severely reduced

levels of TNF.[97] Collectively, the MAPKs become activated upon TLR ligation and have been implicated in cytokine production.

I.VIII Tyrosine Phoshorylation of Proteins

Phosphorylation alters protein activity thus providing a simple, quick and reversible change in function of proteins.[98] Particularly, the regulation of tyrosine phosphorylation is important in the control of normal cellular processes, such as cell growth, cell cycle regulation, differentiation, cytoskeletal function, and of pathological events such as cellular transformation.[98] Recently, the role of tyrosine phosphorylation has been receiving increasing attention in TLR-mediated signal transduction in immune cells.[98] For example, it has been shown that binding of LPS to TLR4 triggers tyrosine phosphorylation of different signaling molecules within the target cells and activates protein tyrosine kinases (PTKs).[99] Activation of PTKs has been shown to be required for the induction of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) in response to LPS in murine macrophages.[100] Although tyrosine phosphorylation of proteins regulates a number of cellular processes its precise role in regulating LPS induced signaling pathways has not been well defined. Regulation of tyrosine phosphorylation of signaling molecules is controlled by competing activities of both PTKs and protein tyrosine phosphatases (PTPs).[101] PTKs catalyze the phosphorylation of tyrosine residues, and conversely, PTPs dephosphorylate the phosphotyrosine residues thus keeping the tyrosine phosphorylation level at a dynamic equilibrium in biological systems.[101] Any deviation from this balance

(generally associated with increased PTK signaling) can promote the intracellular accumulation of tyrosine phosphorylated proteins causing abnormal cell proliferation and differentiation and thereby resulting in diseases.[99] Similarly, deviation from this equilibrium also can be induced by decreased activity of PTPs. Mutations or deletions in genes encoding PTPs often lead to an increase in tyrosine phosphorylated proteins in cells and also result in disease state.[102]

I.IX Protein Tyrosine Phoshatases and SHP-1

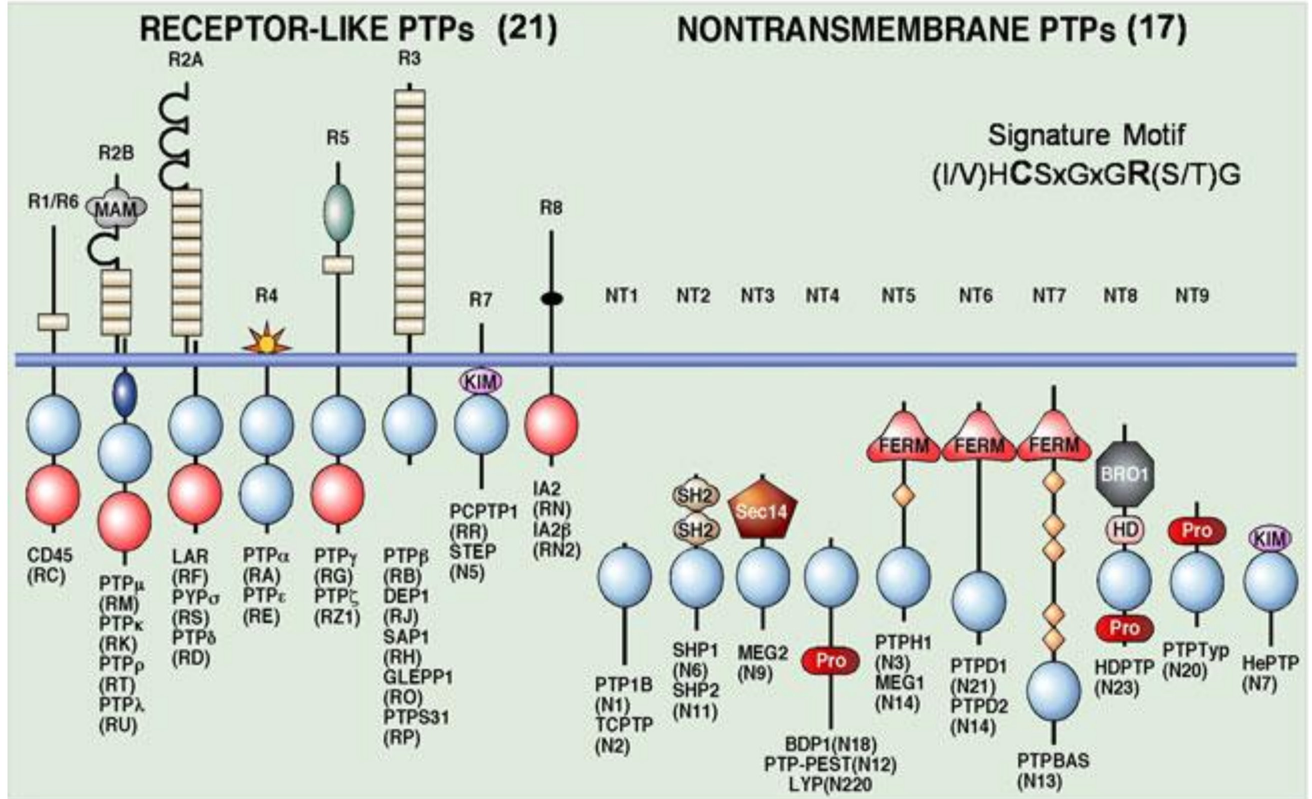
Homology searches based on known catalytic domains revealed 107 genes encoding PTPs, of which 81 are thought to be catalytically active, which is similar in number to the 85 catalytically active PTKs known.[103] PTPs can be classified into four families based on the structural similarity of their catalytic domains: Class I-cysteine based, Class II-cysteine based, Class III-cysteine based and Class IV-aspartate based.[103] The Class I PTPs are further classified into classical PTPs by the V/I-H-C-S-X-G motif in their catalytic domain and dual-specific phosphatases which can also recognize Ser/Thr sites.[103] (Figure I.II) The Classical PTPs contain two families, those that contain transmembrane receptors (rPTPs) and those that are cytosolic (nrPTPs).[103] Cytosolic PTPs are multi-functional due to their multi-domain structure, in which accessory domains such as PDZ, FERM, Pro-rich and SH2 have been added to their catalytic domain and these domains are presumed to play an important role in targeting to their appropriate substrates.[104] Only two cytosolic PTPs have been identified to contain tandem SH2 domains: SHP-1 and SHP-2.[104] SHP-1

is characterized by two tandem N-terminal SH2 domains (N-SH2 and C-SH2), a single catalytic PTPase domain and a short C-terminal tail with two tyrosine and one serine phosphorylation sites.[104] There are 4 isoforms of SHP-1, one of which is a haematopoietic cell specific isoform, which uses an alternative initiation site in the N-terminus.[105] The activation of SHP-1 is predominantly through its SH2 domains that can interact with a variety of tyrosine-phosphorylated molecules particularly receptors bearing immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in thier cytoplasmic domain.[106]

SHP-1 is primarily recognized as a negative regulator of signal transduction pathways in hematopoietic cells.[107] For example, SHP-1 negatively regulates the signaling molecules activated by antigen (TCR, BCR) and cytokine receptors (CSF-1, IFN-γ, EPO, c-kit, IL-10).[107] Recent data demonstrated a crucial and positive role for SHP-1 in LPS-mediated IL-6 production in SHP-1 null bone marrow-derived macrophages (BMDMs) from Motheaten mice (*me/me*).[100] Loss of SHP-1 function results in enhanced production of TNF-α contributing to profound inflammatory disorders in *me/me* mice.[100,102] SHP-1 has also been implicated as a positive regulator of IL-12p40 in murine macrophages.[108] However, the involvement of SHP-1 and PTKs in regulation of IL-12 family of cytokines production in human monocytic cells is not known.

Figure I.II Classification of Receptor and Non-Receptor Protein Tyrosine Phosphatases

‘Classical’ PTPs



Adapted from Tonks (2006) *Nat Rev Mol Cell Biol* **7**, 833-846

I.X Protein Tyrosine Kinases and Src family

PTPs and PTKs work cooperatively in signal transduction mechanisms resulting in the induction of various cytokines. Currently there are 90 PTKs identified in the human genome, which can be further classified into non-receptor tyrosine kinases and receptor tyrosine kinases.[109] The non-receptor tyrosine kinases contain 10 families: ABL, ACK, CSK, FAK, FES, FRK, JAK, SRC, TEC and SYK.[109,110] Of these families the Src family is the largest comprising of 9 kinases: Fgr, Frk, Fyn, Src, Yes, Blk, Hck, Lck and Lyn.[111] Each of these proteins has a molecular weight of about 60kD consisting of a conserved structure: N-terminal unique domain SH3, SH2 and tyrosine kinase domain.[111] Many Src family members, including Src, Fyn, Lyn and Hck, are expressed as two proteins due to alternative splicing.[111,112] Although the precise function of each variant is not fully understood, the differential splicing may play a role in subcellular localization.[112] Interestingly, under basal conditions the Src kinases are present in a restrictive state, where Tyr527 in the C-terminus is phosphorylated and binds intramolecularly with the Src SH2 domain.[113] (Figure I.III) This molecular interaction hides the catalytic domain and holds the kinase in an inactive state.[113] Therefore it requires a PTP, such as SHP-1, to activate it and promote downstream phosphorylation events. The Src family proteins are differentially expressed in different cell types.[111] Those specific to cells of the innate immune system include: Src, Fyn, Fgr, Lyn and Hck.[111] Furthermore, it has been shown that Src family kinase activation is inducible by inflammatory stimulations, such as LPS, in monocytes and macrophages.[114]

Figure I.III Schematic Representation of the Structure and Activation of Src Family Kinases Src Kinases are composed of SH2 and SH3 domains at the N-terminus and a kinase domain at the C-terminus. (bottom) Under basal conditions the Src kinases are present in a restrictive state, where Tyr527 in the C-terminus is phosphorylated and binds intramolecularly with the Src SH2 domain. This molecular interaction hides the catalytic domain and holds the kinase in an inactive state. It requires a PTP, such as SHP-1, to activate it through the dephosphorylation of Tyr527, and promote autophosphorylation of Tyr416 within the kinase domain. (top)

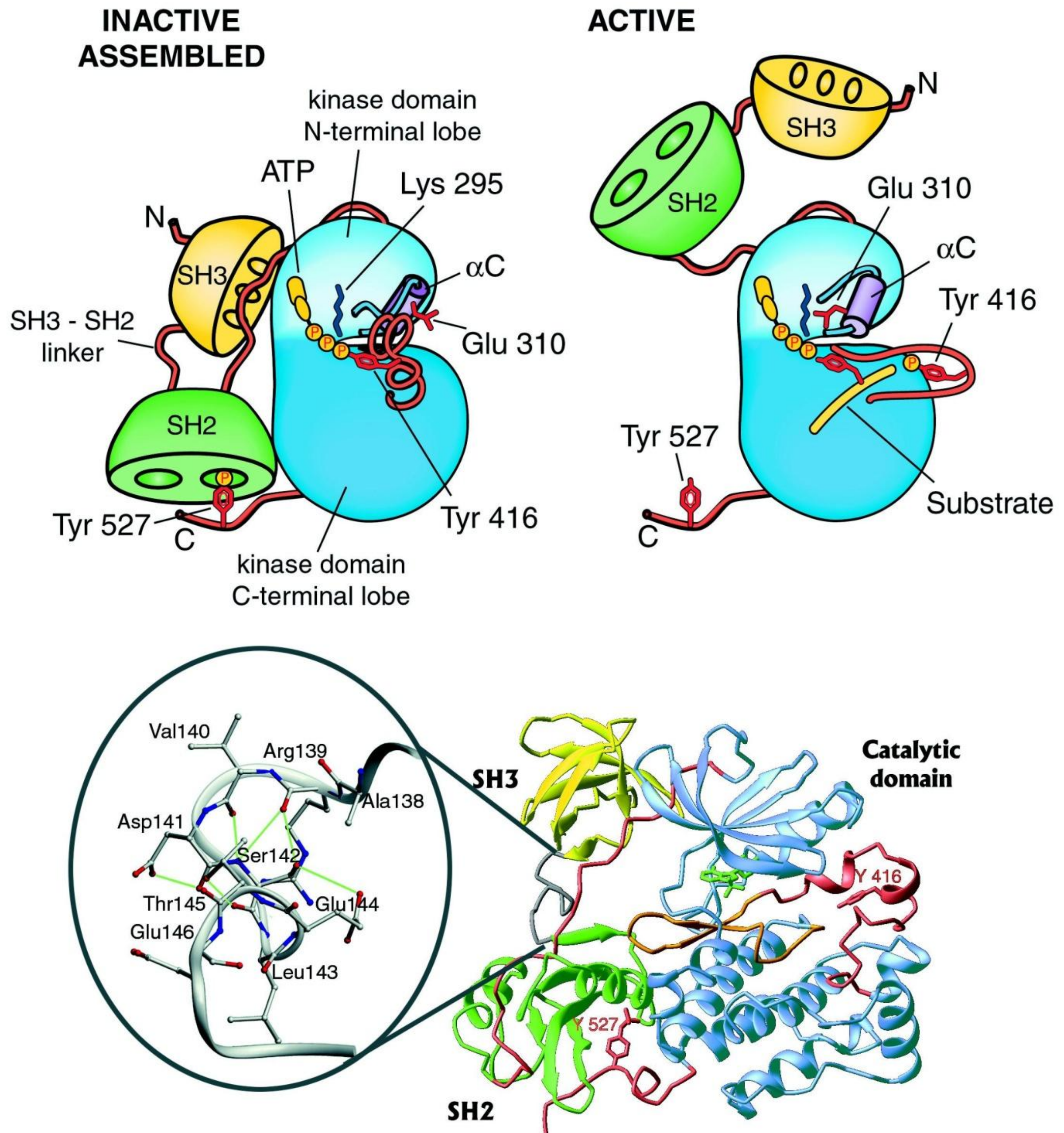


Fig. I.III (Young et al. Biochem J. 2007)

It has also been demonstrated in human monocytes, that Lyn activation results in PI3K activation upon LPS stimulation.[115] Furthermore, blocking of all Src family members has been shown to significantly reduce TNF production in murine macrophages.[116] Moreover, the activation of Src kinases is gradual involving many steps in a sequential process and full activity is only achieved when all activating stimuli are present.[117] Based on these findings it appears that Src activity is switch-like.[111,117] Recently, Src has been implicated in differential regulation of IL-6/TNFα pro- and anti-inflammatory axes in murine macrophages.[118] Therefore Src kinases provide an excellent target for SHP-1 phosphatase in the regulation of cytokine production downstream of TLRs.

I.XI Rationale

Innate immune cells respond to infection or injury by producing inflammatory mediators, which promote production of pro- and anti-inflammatory cytokines required for resolution of inflammatory episodes. Dysregulated balance of pro- and anti-inflammatory cytokines underlies autoimmune disease. In particular, dysregulated expression of IL-12 family of cytokines has direct implications in the progression of autoimmune diseases such as MS, IBD and RA.[119] Despite the importance of these cytokines in autoimmunity, regulation of their production by cells of the immune system such as monocytes and macrophages is not well understood. PTKs are critical regulators of many signaling pathways leading to the augmentation of cell proliferation, phagocytic activities and cytokine production.[99] Particularly, the

Src family kinases have been implicated in the production of important inflammatory mediators such as TNFα and IL-6.[118] The activity of the Src kinases is tightly regulated by reversible tyrosine phosphorylation.[110] Sequestration of the Src tyrosine phosphorylated tail by its SH2 domain maintains an inactive state of the kinase.[113] Src has been shown to be activated by SHP-1 through dephosphorylation of the inhibitory phosphotyrosine.[102] SHP-1 is a critical regulator of a large number of tyrosine phosphorylated molecules in hemapoietic cells and plays a role in LPS-induced cytokine production.[120] Lack of SHP-1 expression or function, as demonstrated by *me/me* mice, leads to hemapoietic abnormalities associated with profound autoimmunity. [118] Hence, SHP-1/Src may be involved in the regulation of the IL-12 family of cytokines.

Therefore, the purpose of this study was to identify and delineate the role of SHP-1 and Src phosphotyrosine proteins in regulating the LPS/TLR4 activated signal transduction pathways culminating in the induction of pro-inflammatory cytokines, IL-12/IL-23 and anti-inflammatory cytokines IL-27/IL-10 using THP-1 monocytic cells, primary monocytes and monocyte derived macrophages.

I.XII Hypothesis

SHP-1/Src signalosome plays a pivotal role in the regulation of IL-12,IL-23 and IL-27 production in LPS-stimulated human monocytic THP-1 cells, monocytes and MDMs.

I.XIII Objectives

(A) **Application of the THP-1 pro-monocytic cell line as a model system to study the regulation of LPS-induced IL-12, IL-23 and IL-27**

(i) Determine the role of SHP-1 in the production of IL-12 family of cytokines
(ii) Determine the role of PTKs in the production of IL-12 family of cytokines
(iii) Determine which PTK is involved in the production of IL-12 family of Cytokines
(iv) Determine downstream signaling molecules in the regulation of the IL-12 family of cytokines
(v) Determine if SHP-1 and Src physically interact and if this complex is involved in the activation of PI3K and JNK

(B) **Applying the findings obtained using a THP-1 cell model to primary human monocytes**

(i) Confirm the role of SHP-1 in the production of IL-12 family of cytokines
(ii) Confirm the role of Src in the production of IL-12 family of cytokines
(iii) Confirm the role of PI3K and JNK in the production of the IL-12 family of cytokines
(iv) Confirm if SHP-1/Src complex is involved in the activation of PI3K and JNK

(C) **Examining the regulation of a pro- and anti-inflammatory axis in LPS-stimulated monocyte-derived macrophages**

(i) Elucidating the role of SHP-1 in the production of IL-12, IL-23, IL-27 and IL-10
(ii) Elucidating the role of Src in the production of IL-12, IL-23, IL-27 and IL-10
(iii) Confirm the role of PI3K and JNK MAPK in the production of IL-12, IL-23, IL-27 and IL-10
(iv) Determine the nature of the SHP-1 and Src protein complex

II. MATERIALS AND METHODS

II.I Reagents

LPS (Lipopolysaccharide) was obtained from E.coli 0111:B4. (Sigma, St.Louis, MO) Recombinant IFNγ was obtained from E.coli (Thermoscientific, Ottawa, ON) The PTK inhibitor (Herbimycin A) was obtained from Tocris, Bristol, UK. The SHP-1 inhibitor (sodium stibogluconate), Src inhibitor (SU6656), ERK inhibitor (PD98523), p38 inhibitor (SB203580), JNK inhibitor (SP600125) and PI3K inhibitor (LY294002) were all obtained from Calbiochem, La Jolla, CA.

II.II Cell Culture

II.II.A THP-1 Monocytes

The THP-1 Human monocytic leukemia cells (ATCC, Manassas, VA) were obtained from a stock that was reconstituted by the lab technician. The cells were cultured in a humidified environment at 37°C, 6% CO_2/air mixture, in Iscove's Modified DMEM 1X media (Wisent, St. Bruno, Quebec) supplemented with 10% v/v fetal bovine serum (FBS) (PAA, Etobicoke, Ontario), 10 units/mL of penicillin/gentamicin (Gibco-Invitrogen). Flasks were kept in an Ultratech WJ301T incubator (Baxter Scientific Products, Deerfield, IL). The media was changed every 2 days.

II.II.B Primary Human Monocytes

Human blood was extracted from healthy donors with informed consent and ficol (GE Healthcare, Uppsala, Sweden) was added to fractionate the blood by creating a density gradient following centrifugation at 1600 rpm for 45min at room temperature in Allegra X-12R centrifuge (Beckman Coulter, Mississauga, Ontario). The layer containing the peripheral blood mononuclear cells (PBMCs) was extracted and diluted with phosphate buffered saline (PBS) (Wisent, St. Bruno, Quebec). Cells were collected by spinning down the PBMCs at 1600 rpm for 10 min at 4 degrees. The cell pellet was resuspended in 10 ml of PBS and subjected to negative selection using the Miltenyi monocyte negative selection kit II. (Miltenyi Biotech, Germany). Negatively selected monocytes were purified using Automacs sorting. Purified monocytes were resuspended in Iscove's Modified DMEM 1X media (Wisent, St. Bruno, Quebec) supplemented with 10% v/v FBS at 1 million cells/ml. Cells were seeded at 1 ml/well in 12 well plates (Thermoscientific, Ottawa, ON) and left overnight before stimulation. Blood was drawn with informed consent and the protocol used was reviewed and approved by the ethics board of the Ottawa Hospital.

II.II.C Human Monocyte-derived Macrophages (MDMs)

The PBMC cell pellet was resuspended in Iscove's Modified DMEM 1X media at 4 million cells/ml. Cells were seeded at 1 ml/well in 12 well plates (Thermoscientific, Ottawa, ON) and allowed to adhere for 3 hr. The cells were cultured in a humidified environment at 37°C, 6% CO_2/air mixture, in Iscove's

Modified DMEM 1X media (Wisent, St. Bruno, Quebec) supplemented with 10% v/v FBS (PAA, Etobicoke, Ontario), 10 units/mL of penicillin/gentamicin (Gibco-Invitrogen) and 1 μg/ml of recombinant MCSF (R &D systems Minneapolis, MN) . Plates were kept in an Ultratech WJ301T incubator (Baxter Scientific Products, Deerfield, IL) for 6 days. The media was changed every 2 days.

II.III Treatment of cells with pharmacological inhibitors

To determine the effect of p38, ERK1/2, JNK, PI3K, PTK, SHP-1 and Src on LPS induced cytokine production, inhibitors specific to these signalling molecules were used. Cells were left either untreated or treated with various concentrations of PD98059, SB202190, SP600125, LY294002, Herbimycin A, sodium stibogluconate or SU6656 for 2 hr followed by stimulation with LPS (1 μg/mL) for 15 min, 4 hr or 24 hr and the cells were then analyzed by Western, ELISA or RT-PCR respectively for LPS-induced cytokine production. The viability of cells treated with Herbymicin A, Sodium stiboglugonate and SU6656 was tested by Flow cytometry. Prior to use, the inhibitors were diluted in either water or dimethyl sulphoxide (DMSO) and appropriate vehicle controls were included in all experiments.

II.IV Transfection of THP-1 Human Monocytes and MDMs with siRNA

THP-1 cells (2.5×10^5 cells/well) or MDMs (1×10^6 cells/well) were seeded in 250uL of antibiotic-free media. The transfection reagent (4μM) (Santa Cruz) was incubated with siRNA specific to SHP-1 (4μM) and c-Src (4μM) (Santa Cruz) for 30 min. This mixture was added to cells to make a final ratio of 40:4 of transfection reagent to siRNA followed by incubation for 5 hr. Iscove's Modified DMEM 1X media

(Wisent, St. Bruno, Quebec) supplemented with 10% v/v FBS (PAA, Etobicoke, Ontario) and 100 units/mL of penicillin/gentamicin (Gibco-Invitrogen) was added to the cells to stop transfection.

II.V RT-PCR

THP-1 cells (1×10^6 cells/well), Monocytes (1×10^6 cells/well) or MDMs(1×10^6 cells/well) were stimulated with 1 μg/mL of LPS for 4 hr. Cell pellets were collected by spinning down the cell suspensions at 1600 rpm for 5min in Allegra X-12R centrifuge. (Beckman Coulter, Mississauga, Ontario) RNA was extracted using RNeasy kit (Qiagen, Mississauga, Ontario) according to the manufacturer's protocol. Purified RNA concentration was measured using a spectrophotometer Nanodrop 2000C (Nanodrop Products, Wilmington, DE). For RT-PCR a mastermix was prepared with 7.5μl 10X Reverse Transcriptase (RT) Buffer, 3μl dNTP, 7.5μl Random Primers (RP), 3.75μl RT, 53.25μl ddH_2O. 25μl of mastermix was added to each sample to 50ng/μl of purified RNA and RT-PCR was carried out for 2 hr in a GeneAmp PCR System 2700 amplifier (Applied Biosystems, Carlsbad, CA) to yield cDNA. The PCR Program was as follows: initial incubation for 2 min at 50°C and 10 min at 95°C, cycles of denaturation at 95°C for 15 s were followed by annealing and elongation for 2 min at 60°C. 40 to 50 cycles were applied. cDNA was then used in Real-time PCR reactions. For this, samples were prepared with 12.5 μl Taqman DNA polymerases, 1.25 μl primer pairs (for each of the cytokine subunits) and 8.75μl ddH_2O. Real-time PCR was carried out for 2 hr in 7500 Real Time PCR System. The expression levels of the transcripts were shown as the ratio compared

to β-Actin by calculation of cycle threshold (Ct) values in amplification plots. (Applied Biosystems, Carlsbad, CA) Primer pairs were as follows (Applied Biosystems, (UK):

IL-23 p19 primers:

5`: AGCGGGACATATGAATCTACTAAGAGA

3`: GTCCTAGTAGGGAGGTGTGAAGTTG

IL-12 p35 primer:

5`: TACTAGAGAGACTTCTTCCACAACAAGAG

3`: TCTGGTACATCTTCAAGTCCTCATAGA,

IL-12 and 23 p40 primer:

5`: GACCATCACTGTCAAAGAGTTTCTAGAT

3`: AGGAAAGTCTTGTTTTTGAAATTTTTTAA

IL-27EBI3 primer:

5`: AGCAGCAGCCTCCTAGCCT,

3`: ACGCCTTCCGGAGGGTC

IL-27p28 primer :

5`: GGCCAGGYGACAGGAGACC,

3`: CAGCTTGTACCAGAAGCAAGGG

IL-10 primer:

5`: CTGTGAAAACAAGAGCAAGGC

3`: GAAGCTTCTGTTGGCTCCC

II.VI IL-12p40 protein measurement by ELISA (Enzyme-Linked Immunosorbent Assay)

1×10^6 cells were seeded in each well of a 12 well plate. The cells were then treated with an indicated concentration of either Herbimycin A, sodium stobogluconate, SU6656, PD98053, SB203580, SP600125, LY294002 for 2 hr or SHP-1 siRNA or Src siRNA for 5 hr after which 1 μg/mL of LPS was added to each well and the cells were cultured for another 24 hr. Supernatants were then collected by spinning down the cell suspensions at 1600 rpm for 5min in Allegra X-12R centrifuge (Beckman Coulter, Mississauga, Ontario) and removing the cell pellets. Supernatants were used in an ELISA assay to determine the secretion of IL-12p40. A 96-well plate was coated with 100 μl/well of anti-human IL-12 1°Ab (2μl/ml) (Invitrogen, Camarillo, CA) in coating buffer and incubated overnight at 4°C. The coating buffer was prepared by adding 4.24g of Na_2CO_3 (BDH, West Chester, PA) and 5.04g of $NaHCO_3$ (BDH, West Chester PA) to 1L of distilled water and adjusting the pH to 9.6. Following overnight incubation, the plates were washed six times with 300μL of wash buffer [0.05% Tween 20 (Sigma) in PBS, pH=7.2-7.4] using Elx50 ELISA washer (Biotek, Winooski, VT). Following the wash the plates were blocked with 200μl of 10% FBS in 1 X PBS per well at room temperature for 2 hr and the plates were washed again as described. An IL-12 standard (R&D systems, Minneapolis, MN) was prepared by serially diluting the stock (2000 pg/ml). Standards and samples were added at 100 μl/well and incubated overnight at 4°C. A total of 2 wells were used per condition. Following overnight incubation, the plates were washed as previously described and 100 μl/well of IL-12 2°Ab (0.4μl/ml) (Invitrogen, Camarillo, CA) in 10% FBS in 1 X PBS was then added and left to

incubate for 2 hr at room temperature. Following six washes, 100 μl/well of Streptavidin Peroxidase (1 μl/ml) (Jackson ImmunoResearch, West Grove, PA) in 10% FBS in 1 X PBS was added and plates were left to incubate for 30min at room temperature. Plates were then washed again and 100 μl/well of substrate solution was added to each well. The substrate solution was prepared by dissolving an o-phenylenediamine dihydrochloride (OPD) tablet (Sigma, St. Louis, MO) in 4μl of hydrogen peroxide (Sigma, St. Louis, MO) and 10 ml of citrate phosphate buffer (Sigma, St. Louis, MO). The reaction was stopped by adding 50μl/well of 1N HCl (BDH, West Chester, PA). The plates were read at 490nm using iMark Microplate reader (Biorad, Missisauga, Ontario). The sensitivity of ELISA for IL-12p40 was ≥ 2 pg/ml.

II.VII IL-23p19/p40 protein measurement by ELISA (Enzyme-Linked Immunosorbent Assay)

To measure secretion of IL-23p19/p40 following LPS stimulation and treatment with inhibitors or siRNA a protocol similar to the one described for IL-12p40 was followed except of using anti-human IL-23 1°Ab (4μl/ml) and IL-23 2°Ab (4μl/ml) both purchased from eBioscience, San Diego, CA. Following the binding of IL-23 2°Ab, 100 μl/well of Avidin Horse Radish Peroxidase (4μl/ml) (eBioscience, San Diego, CA) in 10% FBS in 1XPBS was added and plates were left to incubate for 30min at room temperature. Plates were washed four times as previously described and 100 μl/well of substrate solution was added to each well. (BioFX Labs, Owing Mills, MD) The reaction was stopped using 50μl/well of Stop Solution

(BioFX Labs, Owing Mills, MD). An IL-23 standard (R&D systems, Minneapolis, MN) was prepared by performing a serial dilution on the stock (10000 pg/ml). The sensitivity for IL-23 ELISA is ≥ 15 pg/ml

II.VIII IL-27 protein measurement by ELISA (Enzyme-Linked Immunosorbent Assay)

To measure secretion of IL-27 following LPS stimulation and treatment with inhibitors or siRNA a protocol similar to the one described for IL-12p40 was followed except for using anti- human IL-27 1°Ab (5.55μl/ml) and IL-27 2°Ab (5.55μl/ml) both purchased from R&D systems, Minneapolis, MN. Following the binding of IL-27 2°Ab, 100 μl/well of Streptavidin Horse Radish Peroxidase (5μl/ml) (R&D systems, Minneapolis, MN) in 1% BSA in 1XPBS was added and plates were left to incubate for 30min at room temperature. Plates were washed three times as previously described and 100 μl/well of substrate solution was added to each well. (BioFX Labs, Owing Mills, MD) The reaction was stopped using 50μl/well of Stop Solution (BioFX Labs, Owing Mills, MD). An IL-27 standard (R&D systems, Minneapolis, MN) was prepared by performing a serial dilution on the stock (10000 pg/ml). Plates were blocked and all Reagents were dissolved in 1% BSA in 1XPBS rather than in 10% FBS solution. The sensitivity for IL-27 ELISA is ≥ 15 pg/ml

II.IX IL-10 protein measurement by ELISA (Enzyme-Linked Immunosorbent Assay)

To measure secretion of IL-10 following LPS stimulation and treatment with inhibitors or siRNA a protocol similar to the one described for IL-12p40 was followed

except for using anti- human IL-10 1°Ab (4ul/ml) and IL-10 2°Ab (4ul/ml) both purchased from BD biosciences, Mississauga, ON. However, IL-10 2°Ab and Streptavidin Horse Radish Peroxidase (5μl/ml) (BD biosciences, Mississauga, ON) in 10% FCS in 1XPBS were added simultaneously and plates were left to incubate for 1 hr at room temperature. Plates were washed three times as previously described and 100 μl/well of substrate solution was added to each well. (BioFX Labs, Owing Mills, MD) The reaction was stopped using 50μl/well of Stop Solution (BioFX Labs, Owing Mills, MD). An IL-10 standard (BD biosciences, Mississauga, ON) was prepared by performing a serial dilution on the stock (2000 pg/ml). The sensitivity for IL-10 ELISA is ≥ 10 pg/ml

II.X Western ImmunoBlot Analysis

II.X.A Preparation of cell lysates

1×10^6 cells were seeded in each well of a 12 well plate. Each well was treated with a specific concentration of an inhibitor for 2 hr (Herbimycin A, sodium stibogluconate, SU6656,PD98053, SB203580, SP600125 or LY294002) or siRNA (SHP-1 or Src) for 24 hr. Following this, 1 μg/mL of LPS was added to each well. Plates were held in an incubator at a temperature of 37°C and a CO_2/air mixture of 6% for 15 min. Cell pellets were collected by spinning down the cell suspensions at 1600 rpm for 5min in an Allegra X-12R centrifuge (Beckman Coulter, Mississauga, Ontario) and removing the supernatant. Cell pellets were lysed by adding 30μl of complete lysis buffer and left to incubate for 1 hr on ice. Complete lysis buffer consists of complete protease inhibitor tablet (Roche, Mississauga, Ontario), 20mM

Tris HCl (Sigma, St. Louis, MO), 150 mM NaCl (BDH, West Chester, PA) and 1 mM sodium orthovanadate (Sigma, St. Louis, MO). Lysates were spun down at 20 000 g for 20min at 4°C.

II.X.B Bradford Assay & Sample Preparation

A standard was prepared by serial dilution of stock BSA (2mg/ml) (Calbiochem, La Jolla, CA). 2μl of each lysate was added to 198 μl of 1XPBS. 200μl of standards and samples were added to a 96 well plate together with 50μl of Protein Assay Dye Reagent Concentrate (Biorad, Mississauga, Ontario). Plates were read at 595nm using an iMark Microplate reader. (Biorad, Mississauga, Ontario) 10 μl of loading buffer (Biorad, Mississauga, Ontario) was added to 50ug of protein lysate. Samples were boiled for 5min.

II.X.C Polyacrylamide Gel Electrophoresis (PAGE)

Proteins were resolved on 10% SDS-PAGE resolving gel. The resolving gel was composed of 5.9ml distilled water, 5ml 30% Bis-Acrylamide solution (Biorad, Mississauga, Ontario), 3.8ml resolving buffer, 150 μl of 10% SDS (Calbiochem, La Jolla, CA), 150 μl of 10% APS (Ammonium Persulfate, Biorad, Mississauga, Ontario) and 6μl TEMED (Biorad, Mississauga, Ontario). The resolving buffer was prepared by adding 91 g of Tris Base and 2g of SDS (both purchased from Calbiochem, La Jolla, CA) to 300ml distilled water, adjusting the PH to 8.81, and bringing the final volume to 500 ml with distilled water. The 12% stacking gel was composed of: 3.4ml distilled water, 830μl 30% Bis-Acrylamide solution (Biorad, Mississauga, Ontario), 630μl stacking buffer, 50μl of 10% SDS (Calbiochem, La

Jolla, CA), 50μl of 10% APS (Ammonium Persulfate, Biorad, Mississauga, Ontario) and 5μl TEMED (Biorad, Mississauga, Ontario). The stacking buffer was prepared by adding 60.6g Tris Base (Calbiochem, La Jolla, CA) in 300ml distilled water, bringing the PH to 6.8, bringing the final volume to 500 ml with distilled water. Pre-stained broad range protein markers (Bio-Rad, Mississauga, Ontario) were used to estimate molecular mass of proteins. Electrophoresis was carried out in 1X running buffer at 107 volt for approximately 1.5 hr. The running buffer was prepared by a 1/5 dilution of 151 g Tris Base (Calbiochem, La Jolla, CA) and 72g Glycine (Biorad, Mississauga, Ontario) in 1L of distilled water.

II.X.D Electrophoretic Transfer

Resolved proteins were transferred from the gel to a PVDF membrane (Biorad, Mississauga, Ontario) at 100 volts for 1 hr, using 1X Transfer buffer. The transfer buffer was prepared by a 1/10 dilution of 60g Tris Base (Calbiochem, La Jolla, CA) and 72g Glycine (Biorad, Mississauga, Ontario) in 1L of distilled water.

II.X.E Immunoblotting

The membrane containing transferred proteins was incubated with 1°Ab specific to pERK, pp38, pJNK, pPI3K, pAkt, pSrc, SHP-1, gapDH or β-actin (all from Cell Signalling, Boston, MA) overnight at 4°C. The 1°Ab solution was prepared by dissolving 8μl of 1°Ab in 8ml of 2.5% BSA solution. The membrane was washed for 10 min three times in 1XTBST buffer. The TBST buffer was prepared by a 1/10 dilution of 24.2g Tris HCl (Sigma, St. Louis, MO) and 80g NaCl (Sigma, St. Louis, MO) with 10ml Tween 20. (Sigma, St. Louis, MO) Then 8μl of 2° Anti-rabbit Ab

(Biorad, Mississauga, Ontario) was placed in 8ml of 2.5% BSA solution and this solution was placed on the membrane for 1 hr at room temperature. The membrane was washed for 10 min three times in 1XTBST buffer. The membrane was then placed in an ECL solution (GE Healthcare, Buckinghamshire, UK) and visualized using HEMNI GENIUS^2 Bioimaging system. (Syngene, Frederick, MD). Membranes were stripped at 50°C for 20min in Stripping buffer. The Stripping buffer was prepared by adding 47.2g Tris HCl (Sigma, St. Louis, MO) and 10 g SDS (Calbiochem, La Jolla, CA) in 500ml distilled water and adding 3.65ml of B-mercaptoethanol. (Sigma, St. Louis, MO) Stripped membranes were sequentially re-probed with antibodies to different phosphorylated signalling molecules. Alternatively, and to ensure equal loading of proteins the membranes were stripped and reprobed with antibodies corresponding to GAPDH or β actin.

II.XI Immunoprecipitation

THP-1 cells ($1{\times}10^7$ cells/well) were stimulated with LPS (1 μg/mL) for 15 min. Cell lysates were collected by treating cell pellets with lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl2, 100 mM NaF, 100 mM sodium orthovanadate, and 1 mM EGTA pH 7.7) for 1 hr on ice. 20μL of Protein A Slurry (Invitrogen) was then added to lysate to prevent non-specific binding and the mixture was incubated for 2 hr at 4°C. The beads were removed and Anti-SHP-1 antibody was added and incubated overnight at 4°C to pull down all proteins attached to SHP-1. Following overnight incubation, immunoprecipitated proteins were collected and resuspended in loading dye then separated by SDS-PAGE

II.XII Phosphatase Assay

5ug of PTPN6 Recombinant Human SHP-1 phosphatase (Cedarlane, Burlington, Ontario) was incubated with various concentrations of Sodium Stibogluconate for 1 hr at 37°C. Release of pyrophosphate was determined by colorimetrically using MG Reagent as compared to a prepared phosphate standard from the kit. (Anaspec, Fremont, CA) The absorbance was measured using a spectrophotmeter.

II.XIII Immunofluorescence

MDMs (1×10^6 cells/well) were cultured in 12-well plates containing microscope slides. MDMs were either stimulated with LPS for 15 min or treated with Sodium stibogluconate or SU6656 for 2 hr prior to LPS stimulation for 15 min. Cells were fixed by adding 4% PFA (Sigma, St.Louis, MO) in PBS (Wisent, St. Bruno, Quebec) for 30min at 37°C. Cells were washed 3 times in PBS(Wisent, St. Bruno, Quebec). To quench the PFA cells were incubated in 50mM NH_4Cl (Sigma, St.Louis, MO) in PBS (Wisent, St. Bruno, Quebec) for 10 min at room temperature. Cells were washed 3 times in PBS (Wisent, St. Bruno, Quebec). Cells were then permeabilized by incubating in 0.1% Triton (Sigma, St.Louis, MO) in PBS (Wisent, St. Bruno, Quebec) for 10 min at room temperature. Cells were incubated with anti-SHP-1 antibody (generated in lab) or with anti-Src primary antibody (Cell Signalling, Boston, MA) overnight at 37°C. The antibodies was diluted at a concentration of 1:1000 in 2% FCS in PBS. Cells were washed 3 times in 5% FBS (PAA, Etobicoke, Ontario) in PBS (Wisent, St. Bruno, Quebec). Cells were incubated with a second primary

antibody: SHP-1 (generated in lab) or Src, TRIF, MyD88 primary antibody (Cell Signalling, Boston, MA) overnight at 37°C. Cells were washed 3 times in 5% FBS (PAA, Etobicoke, Ontario) in PBS (Wisent, St. Bruno, Quebec). Cells were then incubated with a secondary Alexafluor antibody red (Alexa Fluor 680 Donkey anti-rabbit IgG) to detect the first protein of interest (Molecular probes, Burlington, ON) for 1 hr at room temperature on a shaker. Cells were washed 3 times in 5% FBS (PAA, Etobicoke, Ontario) in PBS (Wisent, St. Bruno, Quebec). Cells were then incubated with a second secondary Alexafluor antibody green (Alexa Fluor 350 Donkey anti-rabbit IgG) (Molecular probes, Burlington, ON) for 1 hr at room temperature on a shaker to detect the second protein of interest. Cells were washed 3 times in in PBS (Wisent, St. Bruno, Quebec). Slides were then immersed in mounting medium containing DAPI (Molecular probes, Burlington, ON) placed face down on a slide and incubated at 37°C for 1 hr. Slides were then visualized by fluorescent microscopy.

II.XIV Statistical Methods

Data were plotted using Graphpad Prism 5. Significance was determined using a One-Way Anova test. A p-value of less than 0.05 was considered significant as denoted by an asterisk (*$p<0.05$, **$p<0.001$, ***$p<0.0001$) Unless otherwise specified, plotted data represent the mean ± SEM of at least three experiments.

II.XV Ethics Statement

Blood was obtained from healthy donors according to a protocol approved by the ethics review committee of the Ottawa General Hospital. Donors gave written informed consent.

III. RESULTS

III.I OBJECTIVE 1: Application of the human THP-1 pro-monocytic cell line as a model system to study the regulation of LPS-induced IL-12, IL-23 and IL-27

III.I.I Determine the role of SHP-1 in the production of IL-12 family of cytokines

It is well established that PTP SHP-1 regulates a large number of signal transduction pathways in hemapoietic cells.[101] Moreover, loss of SHP-1 function leads to profound inflammation and autoimmunity in mice, suggesting that SHP-1 plays an important role in the regulation of the IL-12 family of pro-inflammatory cytokines.[118] To explore the role of SHP-1 in the regulation of the IL-12 family of cytokines, human pro-monocytic THP-1 cells were employed as an experimental model. THP-1 cells were treated for 2 hr with sodium stibogluconate, a specific SHP-1 inhibitor, followed by stimulation with 1 µg/ml of LPS for 24 hr and the supernatants were assessed for IL-12p40, IL-23p19 and IL-27EBI3/p28 secretion by ELISA. LPS stimulation of THP-1 cells induced high levels of IL-12p40, IL-23 and IL-27 ranging from 2000 to 4000 pg/ml respectively. (Fig. III.1A) Treatment of THP-1 cells with sodium stibogluconate resulted in more than 80% suppression of IL-12p40, IL-23p19 and IL-27EBI3/p28 in a dose dependent manner. (Fig. III.1A) This data therefore suggests that SHP-1 function is critical for LPS-induced production of IL-12p40, IL-23 and IL-27. The biological activity of sodium stibogluconate was confirmed using an in-vitro phosphatase assay. (Fig. III.1B) Treatment with sodium stibogluconate inhibited the release of pyrophosphate by SHP-1 in a dose-dependent manner. (Fig. III.1B) The IL-12 family of cytokines are heterodimeric, it was therefore of interest to examine if SHP-1 also regulates the transcription of the

Figure III.1 Sodium Stibogluconate inhibits production of IL-12, IL-23 and IL-27 in LPS-stimulated THP-1 cells. THP-1 cells (1×10^6 cells) were treated with indicated concentrations of Sodium Stibogluconate for 2 hr followed by LPS stimulation for A) 24 hr and C) 4 hr. A) Secretion of IL-12, IL-23 and IL-27 protein as measured by ELISA. B) In vitro phosphatase activity assay of Recombinant Human SHP-1 phosphatase incubated with indicated concentrations of sodium stibogluconate for 1 hour. C) Expression of IL-12, IL-23 and IL-27 mRNA as measured by qRT-PCR. Bars represent mean ±SEM, *$p\leq0.05$, **$p\leq0.005$, ***$p\leq0.0005$ N=3

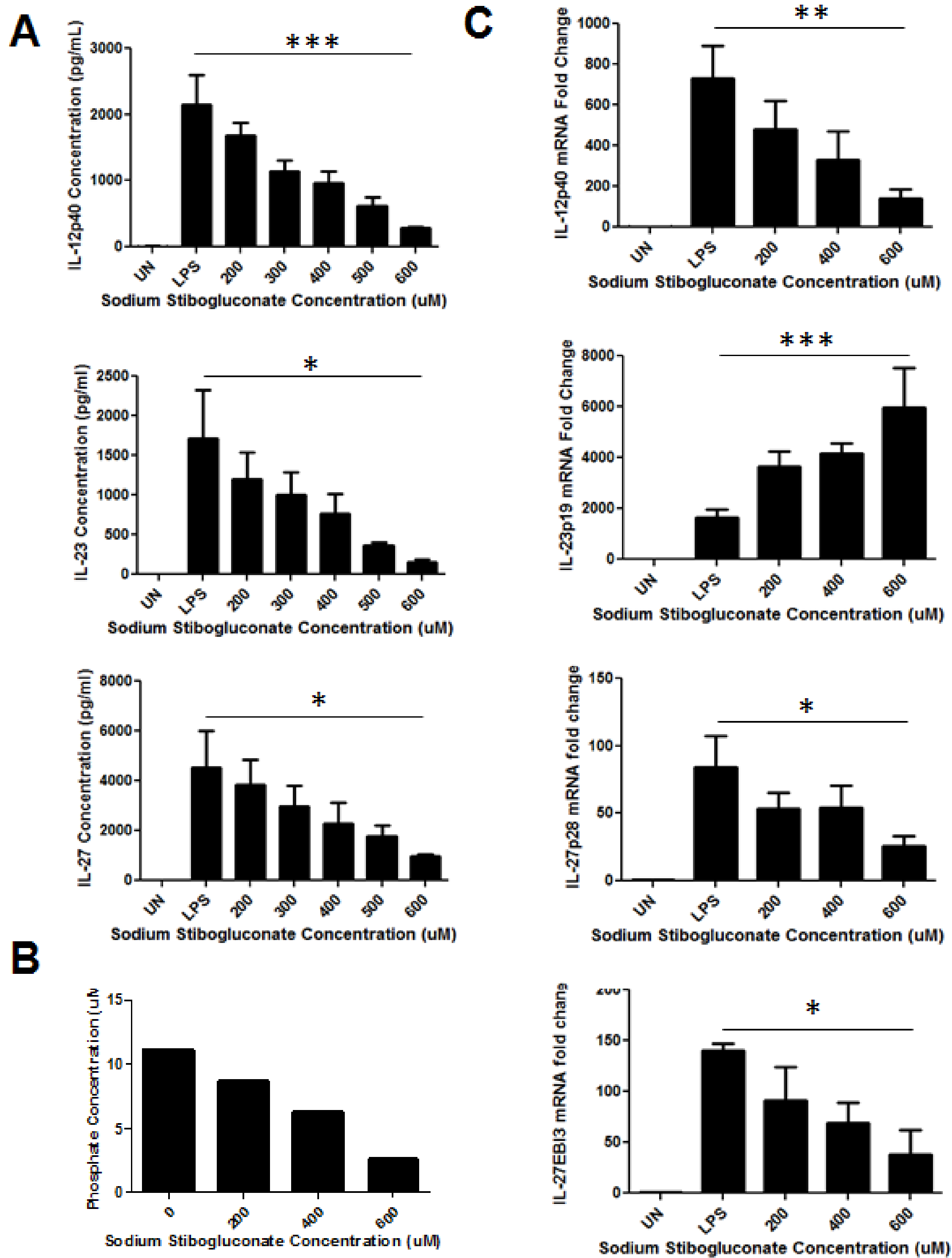


Fig. III.1

subunits that comprise the IL-12 family of cytokines.[121] To examine transcript levels of IL-12p40, IL-23p19, IL-27p28 and IL-27EBI3, THP-1 cells were treated for 2 hr with sodium stibogluconate followed by stimulation with 1 μg/ml of LPS for 4 hr and the RNA was purified from cell pellets and analyzed by RT-PCR. The results show that following inhibition of SHP-1, IL-12p40 transcription was suppressed by 80%. (Fig. III.1C panel 1) Similarly, following SHP-1 inhibition, transcript levels of both subunits of IL-27, p28 and EBI3, were also significantly reduced by approximately 60% compared to LPS alone. (Fig. III.1C panel 3-4) Interestingly, following inhibition of SHP-1 function, IL-23p19 transcripts levels were significantly increased by approximately 50% compare to LPS alone. (Fig. III.1C panel 2) The critical role of SHP-1 in the regulation of IL-12p40, IL-23p19, IL-27p28 and IL-27EBI3 gene induction was further confirmed by employing siRNA specific to SHP-1. THP-1 cells were transfected with SHP-1 siRNA for 5 hr followed by stimulation with 1 μg/ml of LPS for 24 hr and the supernatants were assessed for IL-12p40, IL-23p19 and IL-27EBI3/p28 secretion by ELISA. The results confirmed earlier observations, and show that the levels of IL-12p40, IL-23 and IL-27 were significantly decreased following transfection with SHP-1 siRNA as compared to transfection with a control siRNA. (Fig. III.2A) Thus, SHP-1 is a positive regulator of LPS-induced production of IL-12 family cytokines in THP-1 cells. Efficiency of SHP-1 knockdown was confirmed using Western blot analysis, which demonstrated that levels of SHP-1 protein were reduced following transfection with SHP-1 siRNA as compared to transfection with control siRNA. (Fig. III.2B) To determine that SHP-1 is also involved in transcriptional regulation of IL-12p40, IL-23p19, IL-27p28/EBI3, THP-1 cells were

Figure III.2 SHP-1 siRNA silences the expression of SHP-1 resulting in a decrease of IL-12, IL-23 and IL-27 cytokine production in LPS –stimulated THP-1 cells. THP-1 cells (5×10^5 cells) were transfected with indicated concentrations of transfection reagent:siRNA specific to SHP-1 or control for 5 hours followed by LPS stimulation for A) 24 hr and B) 15 min C) 4 hr. A) Secretion of IL-12, IL-23 and IL-27 protein as measured by ELISA. B) Knockdown of SHP-1 protein expression was confirmed by Western immunoblotting C) Expression of IL-12, IL-23 and IL-27 mRNA as measured by qRT-PCR. Bars represent mean ±SEM, *p≤0.05, **p≤0.005, ***p≤0.0005 N=3

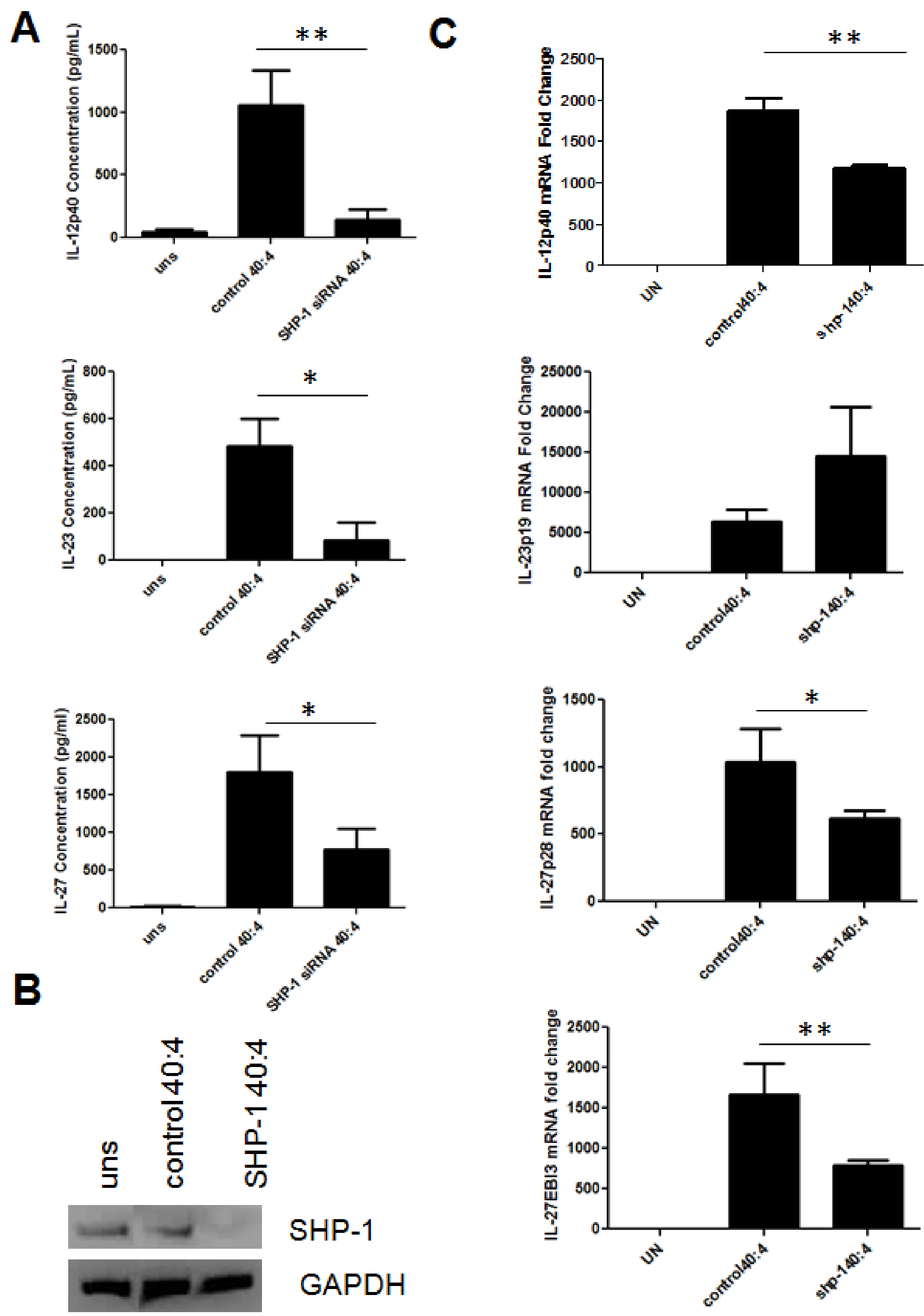


Fig. III.2

transfected with SHP-1 siRNA for 5 hr followed by stimulation with 1 μg/ml of LPS for 4 hr and the RNA was analyzed by RT-PCR. The results confirm earlier data obtained using sodium stibogluconate, and show that IL-12p40, IL-27p28 and IL-27EBI3 transcript levels were significantly reduced following SHP-1 knockdown. (Fig. III.2C panel 1,3,4) In contrast, IL-23p19 mRNA expression was trending upwards following transfection, as observed earlier using sodium stibogluconate. (Fig. III.2C panel 2) Collectively, these data strongly suggest that SHP-1 is a positive regulator for LPS-induced IL-12, IL-23 and IL-27 production through regulation of IL-12p40 and IL-27p28/EBI3 subunits in human monocytic THP-1 cells.

III.I.II Determine the role of PTKs in the production of IL-12 family of cytokines

It is well known that PTPs and PTKs are partners that regulate a large number of signaling pathways.[122] I have demonstrated that SHP-1 plays a role in the induction of the IL-12 family of cytokines. Moreover, PTKs are critical regulators of a large number of signal transduction pathways including those involved in the induction of cytokine genes.[116] However, their role in TLR4-mediated signaling is not well defined.[98] In addition, PTKs represent well recognized targets of SHP-1.[98] It was therefore of interest to examine if PTKs also play a role in the induction of the IL-12 family of cytokines downstream of TLR4. THP-1 cells were treated for 2 hr with broad PTK inhibitor Herbimycin A followed by stimulation with 1 μg/ml of LPS for 24 hr and the supernatants were examined for IL-12p40, IL-23p19 and IL-27EBI3/p28 production by ELISA. The data demonstrated that THP-1 cells following inhibition of PTKs with Herbimycin A exhibited an almost total suppression of IL-12p40, IL-23 and IL-27 production. (Fig. III.3A) Next, I analyzed if PTKs are

Figure III.3 Herbimycin A inhibits production of IL-12, IL-23 and IL-27 in LPS-stimulated THP-1 cells. THP-1 cells (1×10^6 cells) were treated with indicated concentrations of Herbimycin A for 2 hr followed by LPS stimulation for A) 24 hr B) 4 hr. A) Secretion of IL-12, IL-23 and IL-27 protein as measured by ELISA. B) Expression of IL-12, IL-23 and IL-27 mRNA as measured by qRT-PCR. Bars represent mean ±SEM, *p≤0.05, **p ≤0.005 ***p≤0.0005 N=3

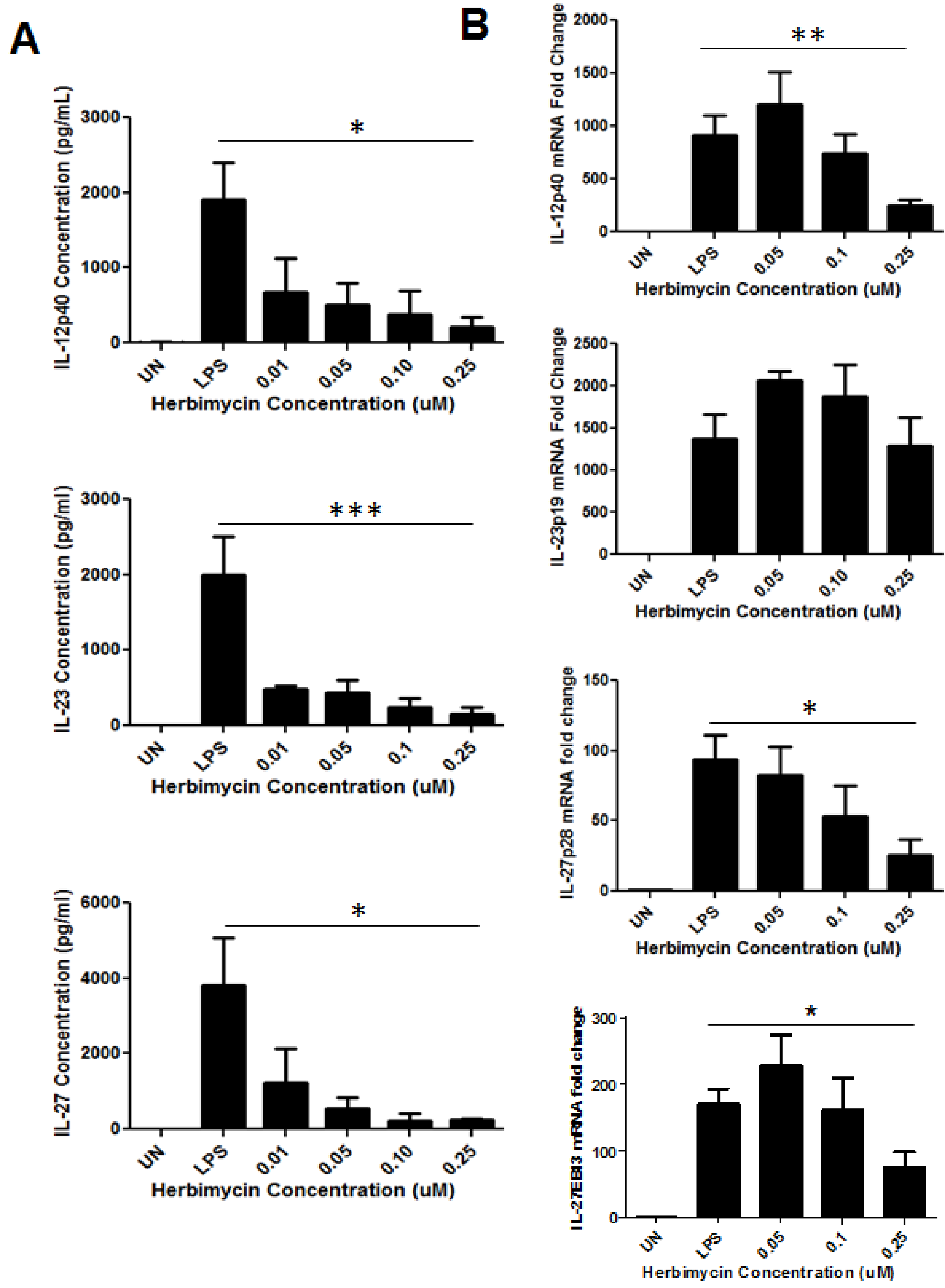


Fig. III.3

also involved in the transcriptional regulation of the genes encoding the subunits that comprise the IL-12 family of cytokines. To examine transcript levels of IL-12p40, IL-23p19, IL-27p28 and IL-27EBI3, THP-1 cells were treated for 2 hr with Herbimycin A followed by stimulation with 1 µg/ml of LPS for 4 hr and the RNA was analyzed by RT-PCR. The results show that, similar to SHP-1 inhibition, inhibition of PTKs, at 0.25 µM Herbimycin A, resulted in significantly reduced transcript levels of IL-12p40, IL-27p28 and IL-27EBI3 whereas levels of IL-23p19 transcript remained unchanged. (Fig. III.3B) This suggests that PTKs are also critically involved in LPS-induced IL-12 family cytokine production in THP-1 cells.

III.I.III Determine which PTK is involved in the production of IL-12 family of cytokines

The largest family of PTKs is the Src family comprising 9 members.[110] There are contradictory reports in the literature suggesting the involvement of Src family kinases in LPS/TLR4 signal transduction pathways.[114] Moreover Src family kinases have been implicated in the regulation of pro-inflammatory cytokines. [116] Therefore, I next examined the role of the Src family kinases in the production of the IL-12 family of cytokines. THP-1 cells were treated for 2 hr with Src family inhibitor, PP2, followed by stimulation with 1 µg/ml of LPS for 24 hr and the supernatants were examined for IL-12p40, IL-23p19 and IL-27EBI3/p28 production by ELISA. The results demonstrate that following Src family inhibition the secretion of IL-12p40, IL-23 and IL-27 were significantly reduced by more than 80% compared to cells stimulated with LPS alone. (Fig. III.4A) I next determined if the inhibition of IL-12, IL-23 and IL-27 cytokines by Src family kinase inhibitor, PP2, was mediated through inhibition of the transcriptional regulation of their subunits.

Figure III.4 PP2 inhibits production of IL-12, IL-23 and IL-27 in LPS-stimulated THP-1 cells. THP-1 cells (1×10^6 cells) were treated with indicated concentrations of PP2 for 2 hr followed by LPS stimulation for A) 24 hr and B) 4hr. A) Secretion of IL-12, IL-23 and IL-27 protein as measured by ELISA. B) Expression of IL-12, IL-23 and IL-27 mRNA as measured by qRT-PCR. Bars represent mean ±SEM, $^*p \leq 0.05$, $^{**}p \leq 0.005$ $^{***}p \leq 0.0005$ N=3

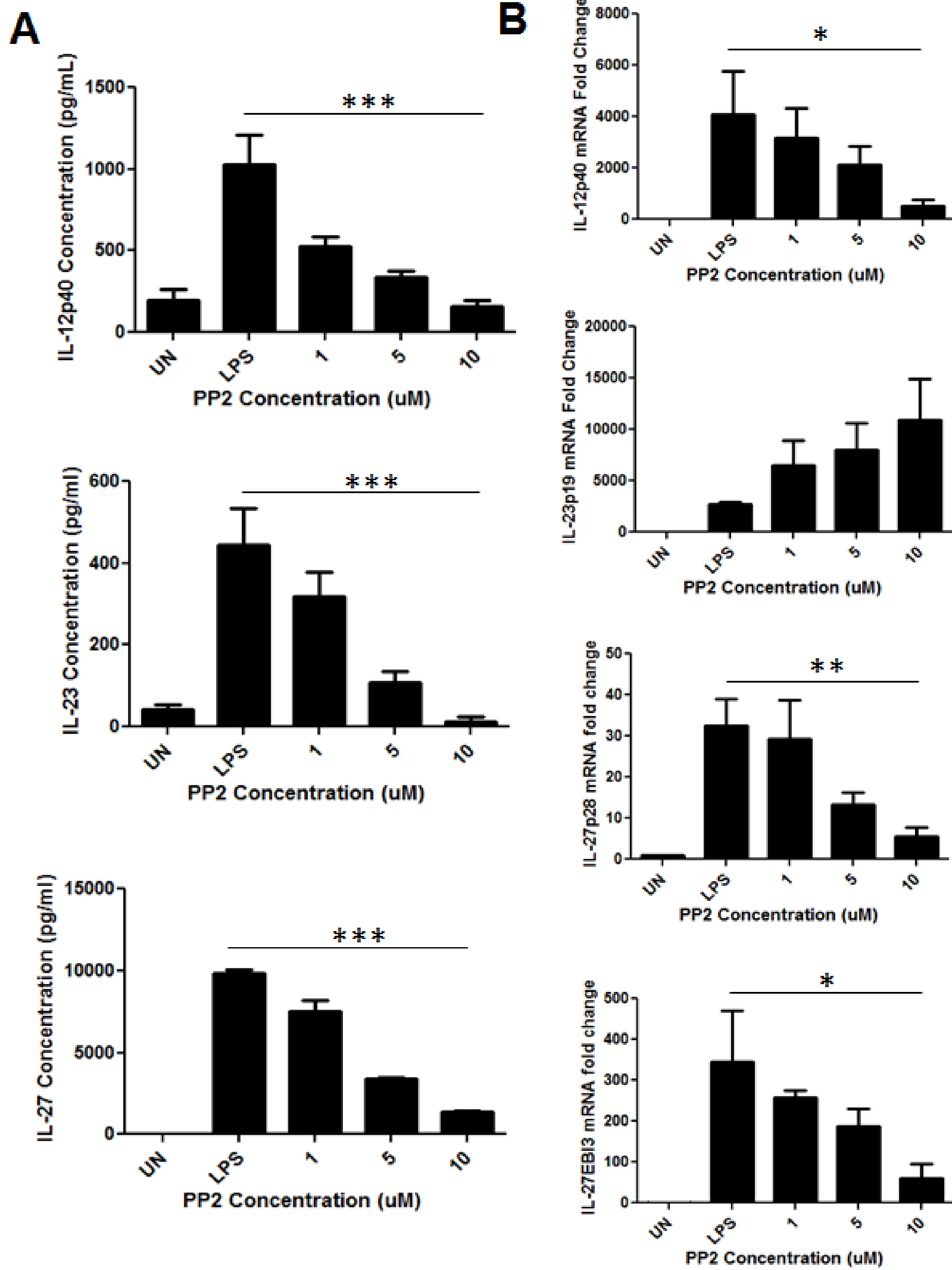


Fig. III.4

To examine transcript levels of IL-12p40, IL-23p19, IL-27p28 and IL-27EBI3, THP-1 cells were treated for 2 hr with PP2 followed by stimulation with 1 μg/ml of LPS for 4 hr and the RNA was analyzed by RT-PCR. Similar to previous results, the inhibition of Src family kinases by PP2 resulted in a significant reduction of IL-12p40, IL-27p28 and IL-27EBI3 transcript levels whereas IL-23p19 transcript levels were trending upwards. (Fig. III.4B) Therefore Src family kinases regulate LPS-induced production of the IL-12 family of cytokines.

In hemapoietic cells, such as monocytes, Lyn, Hck and Src are predominantly expressed.[111] There are reports in the literature demonstrating interaction of SHP-1 with Src in human neutrophils. [123] Moreover, Src has been implicated in differential regulation of IL-6/TNFα pro- and anti-inflammatory axis in murine macrophages.[118] Therefore, since I have shown that SHP-1 and Src family kinases are involved in the regulation of the IL-12 family of cytokines, I examined the role of Src in the regulation of the IL-12 family of cytokines. THP-1 cells were treated for 2 hr with a Src specific inhibitor, SU6656, followed by stimulation with 1 μg/ml of LPS for 24 hr and the supernatants were examined for IL-12p40, IL-23p19 and IL-27EBI3/p28 production by ELISA. The results demonstrate that following Src inhibition, the secretion of IL-12p40, IL-23 and IL-27 were significantly reduced by almost 90% compared to the robust induction of each cytokine by LPS. (Fig. III.5A) The biological activity of the inhibitor, SU6656, was confirmed using Western blot analysis, demonstrating that SU6656 effectively prevented phosphorylation of Src. (Fig. III.5B) Next, I determined if Src also regulates the transcription of the subunits that comprise the IL-12 family of cytokines.

Figure III.5 SU6656 inhibits production of IL-12, IL-23 and IL-27 in LPS-stimulated THP-1 cells. THP-1 cells (1×10^6 cells) were treated with indicated concentrations of SU6656 for 2 hr followed by LPS stimulation for A) 24 hr and C) 4 hr. A) Secretion of IL-12, IL-23 and IL-27 protein as measured by ELISA. B) Biological activity assay of SU6656 measured by Western immunoblotting. C) Expression of IL-12, IL-23 and IL-27 mRNA as measured by qRT-PCR. Bars represent mean ±SEM, *$p \leq 0.05$, **$p \leq 0.005$, ***$p \leq 0.0005$ N=3

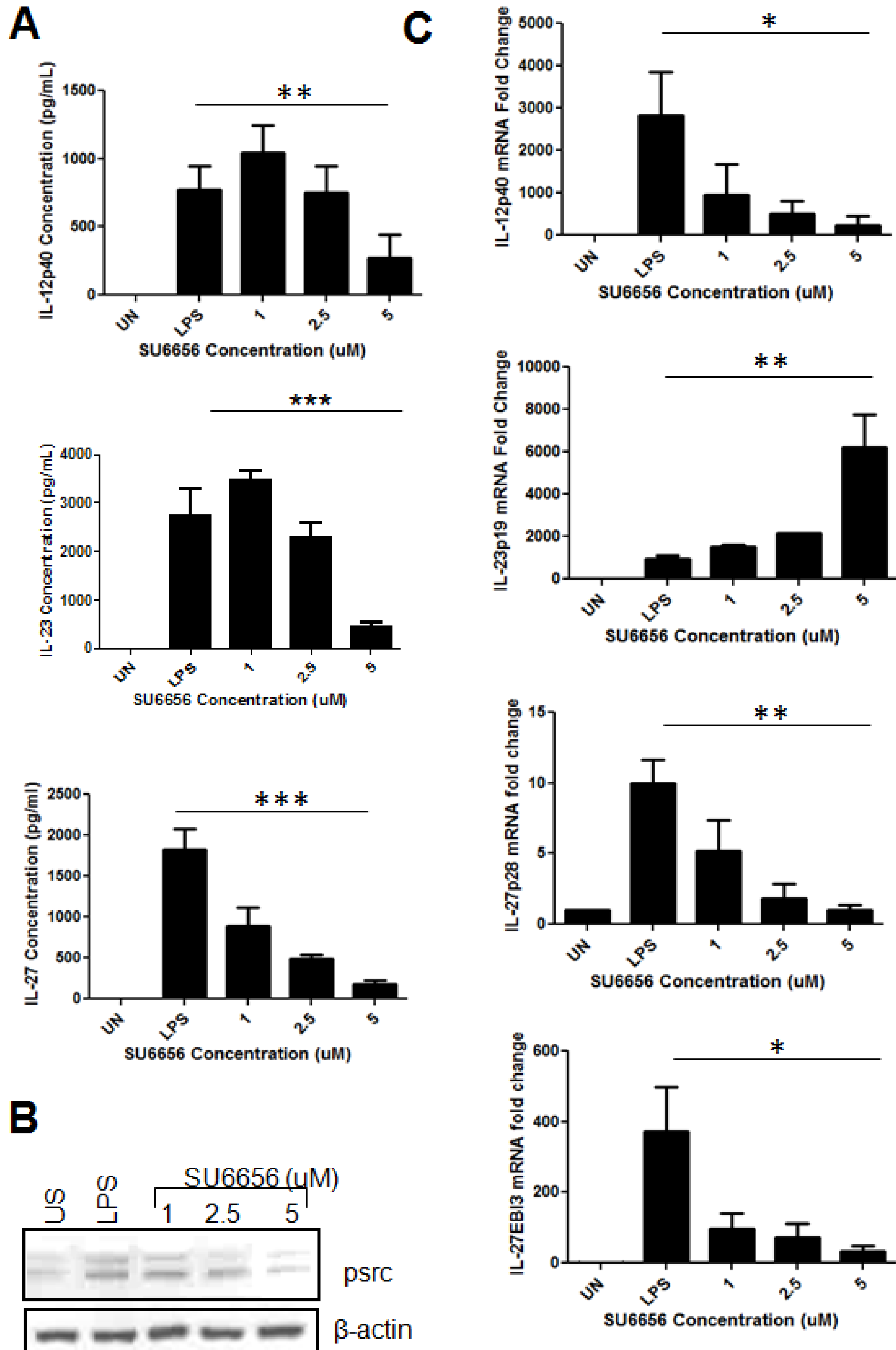
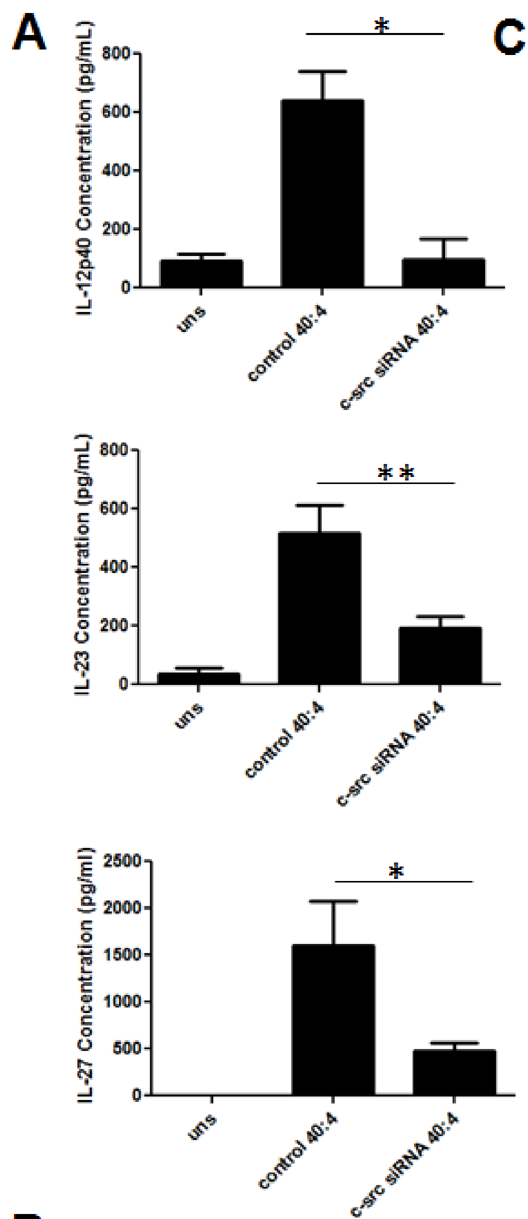
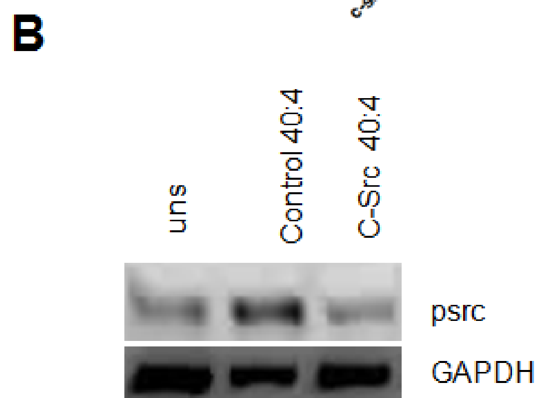


Fig. III.5

To examine transcript levels of IL-12p40, IL-23p19, IL-27p28 and IL-27EBI3, THP-1 cells were treated for 2 hr with SU6656 followed by stimulation with 1 μg/ml of LPS for 4 hr and the RNA was analyzed by RT-PCR. In accordance with previous results, the inhibition of Src resulted in the significant reduction of IL-12p40, IL-27p28 and IL-27EBI3 transcript levels while IL-23p19 transcript levels were significantly increased. (Fig. III.5C) The requirement of Src in the production of IL-12 family of cytokines, through the induction of IL-12p40, IL-27p28 and IL-27EBI3 subunits in LPS-stimulated THP-1 cells was further confirmed using c-Src siRNA. THP-1 cells were transfected with c-Src siRNA for 5 hr followed by stimulation with 1 μg/ml of LPS for 24 hr and the supernatants were assessed for IL-12p40, IL-23p19 and IL-27EBI3/p28 production by ELISA. Results show that the levels of IL-12p40, IL-23 and IL-27 were significantly decreased following transfection with c-Src siRNA as compared to transfection with a control siRNA. (Fig. III.6A) Thus, these results confirmed the requirement for Src activity in the production of IL-12 family cytokines in LPS-stimulated THP-1 cells. The efficiency of Src specific siRNA in inhibiting Src expression was ensured by Western blot analysis demonstrating decreased levels of pSrc compared to LPS-stimulated cells treated with control siRNA or c-Src siRNA. (Fig. III.6B) To confirm Src involvement in transcriptional regulation of IL-12p40, IL-23p19, IL-27p28 and IL-27EBI3, THP-1 cells were transfected with c-Src siRNA for 5 hr followed by stimulation with 1 μg/ml of LPS for 4 hr and the RNA was analyzed by RT-PCR. Following, Src silencing using c-Src siRNA, IL-12p40, IL-27p28 and IL-27EBI3 transcript levels were reduced while IL-23p19 expression was significantly increased following transfection. (Fig. III.6C)

Figure III.6 C-src siRNA silences the expression of Src resulting in a decrease of IL-12, IL-23 and IL-27 cytokine production in LPS –stimulated THP-1 cells. THP-1 cells (5×10^5 cells) were transfected with indicated concentrations of transfection reagent: siRNA specific to Src or control for 5 hours followed by LPS stimulation for A) 24 hr B) 15 min and C) 4 hr. A) Secretion of IL-12, IL-23 and IL-27 protein as measured by ELISA. B) Confirmation of Src silencing was done by Western immunoblotting C) Expression of IL-12, IL-23 and IL-27 mRNA as measured by qRT-PCR. Bars represent mean ±SEM, *$p \leq 0.05$, **$p \leq 0.005$, N=3





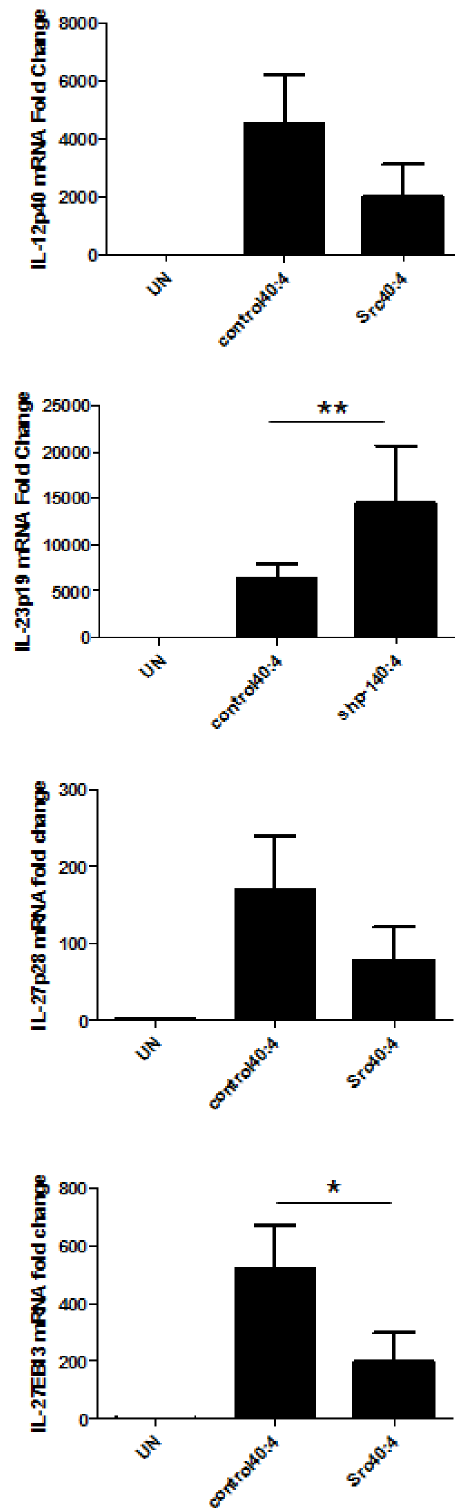


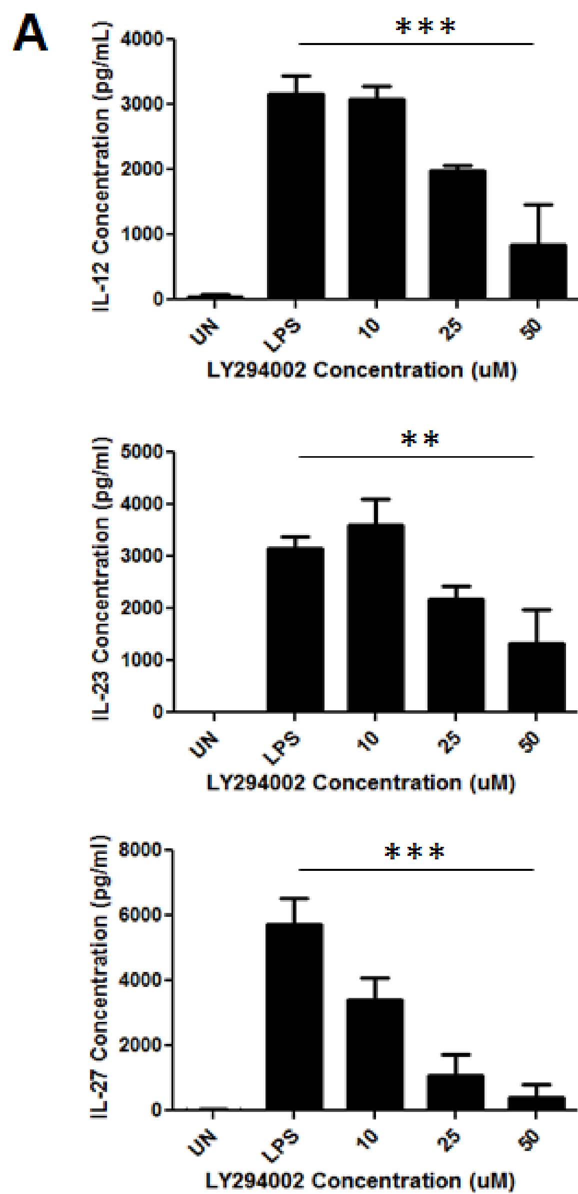
Fig. III.6

Therefore, collectively these results show that Src is a positive regulator for LPS-induced IL-12, IL-23 and IL-27 production through the induction of IL-12p40, IL-27p28 and IL-27EBI3 in LPS-stimulated THP-1 cells.

III.I.IV Determine Downstream signaling molecules in the regulation of the IL-12 family of cytokines

I have thus far demonstrated that SHP-1 and Src are both positive regulators of the IL-12 family of cytokines following LPS stimulation of TLR4. Although there is scarce information regarding involvement of SHP-1 and Src in TLR4 signalling, it is well established that both molecules function proximally to the signalling domains of receptor chains.[122] Therefore, they may regulate downstream pathways also involved in the regulation of the IL-12 family of cytokines. LPS stimulation activates PI3K and MAPK pathways, which have been implicated in the regulation of cytokine production in monocytic cells. [97] Thus, my next step was to examine whether MAPK and PI3K pathways are involved in the regulation of LPS-induced IL-12, IL-23 and IL-27 production. To first examine the role of PI3K, THP-1 cells were treated for 2 hr with PI3K inhibitor, LY294002, followed by stimulation with 1 µg/ml of LPS for 24 hr and the supernatants were examined for IL-12p40, IL-23p19 and IL-27EBI3/p28 production by ELISA. The data demonstrated that THP-1 cells following inhibition of PI3K exhibited significant reduction in IL-12p40, IL-23 and IL-27 production. (Fig. III.7A) The biological activity of the inhibitor was confirmed using Western blot analysis, demonstrating that 20 uM of LY294002 abrogated the phosphorylation of Akt a known target of PI3K.[124] (Fig. III.7B) This suggests that production of the IL-12 family of cytokines is dependent on PI3K activation.

Figure III.7 LY294002 inhibits production of IL-12, IL-23 and IL-27 in LPS-stimulated THP-1 cells. THP-1 cells (1×10^6 cells) were treated with indicated concentrations of LY294002 for 2 hr followed by LPS stimulation for A) 24 hr and B) 15min. A) Secretion of IL-12, IL-23 and IL-27 protein as measured by ELISA. B) Biological activity assay of LY294002 inhibitor measured by Western immunoblotting. Bars represent mean ±SEM, $**p\leq0.005$, $***p\leq0.0005$ N=3



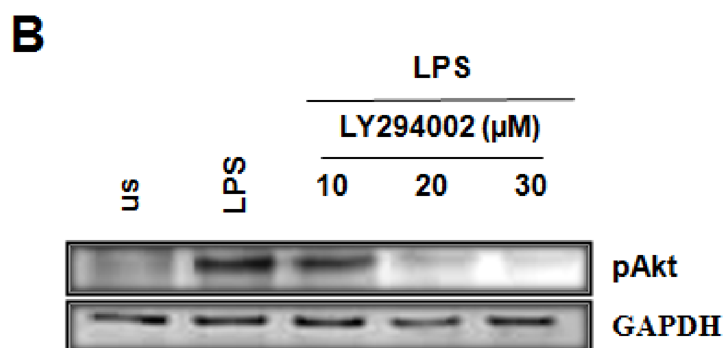


Fig. III.7

Next, I analyzed the involvement of the MAPK family in LPS-induced IL-12, IL-23 and IL-27 production. THP-1 cells were treated for 2 hr with ERK, p38 or JNK inhibitor, PD98059, SB203580 or SP600125, respectively, followed by stimulation with 1 µg/ml of LPS for 24 hr and the supernatants were examined for IL-12p40, IL-23p19 and IL-27EBI3/p28 production by ELISA. The data demonstrated that THP-1 cells following inhibition of ERK exhibited no change in IL-12p40, IL-23 and IL-27 production. (Fig. III.8A) The biological activity of PD98059 was confirmed using Western blot analysis, showing significant and dose-dependent reduction of pERK with PD98056. (Fig. III.8B) Therefore ERK MAPKs do not play a role in the regulation of the IL-12 family of cytokines following LPS stimulation. Similarly, there was no change in the secretion of IL-12p40, IL-23 and IL-27 following the inhibition of p38 MAPK compared with LPS stimulation alone. (Fig. III.9A) The biological activity of SB203580 was confirmed using Western blot analysis, which show decreased phosphorylation of pp38 by SB203580. (Fig. III.9B) Therefore, p38 MAPK also does not play a role in the regulation of the LPS-induced production of the IL-12 family of cytokines. In contrast, following the inhibition of JNK, there was a significant decrease in the secretion of IL-12p40, IL-23 and IL-27.(Fig. III.10A) The biological activity of SP600125 was confirmed using Western blot analysis, which shows a dose-dependent reduction of pJNK following treatment with SP600125. (Fig. III.10B) These data suggest that JNK is a positive regulator of the IL-12 family of cytokines in LPS-stimulated THP-1 cells. Collectively, the results show that PI3K and JNK MAPK are critical positive regulators in the regulation of the IL-12 family of cytokines and therefore may represent downstream targets of SHP-1 and Src.

Figure III.8 PD09856 has no effect on the production of IL-12, IL-23 and IL-27 in LPS-stimulated THP-1 cells. THP-1 cells (1×10^6 cells) were treated with indicated concentrations of PD09856 for 2 hr followed by LPS stimulation for A) 24 hr and B) 15 min. A) Secretion of IL-12, IL-23 and IL-27 protein as measured by ELISA. B) Biological activity assay of PD09856 measured by Western immunoblotting. Bars represent mean ±SEM

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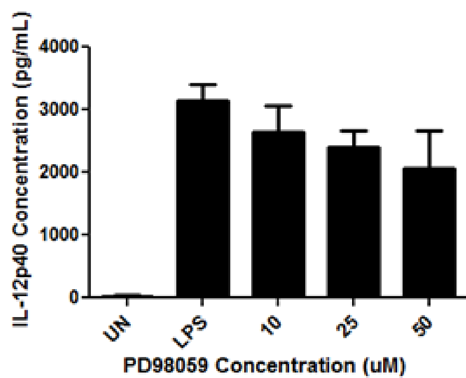

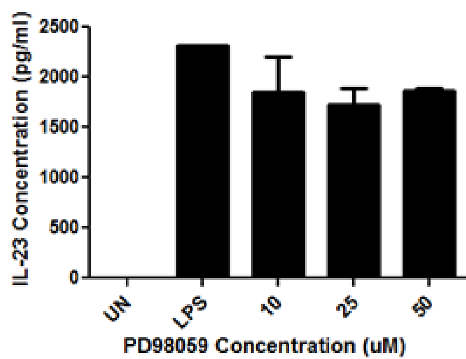

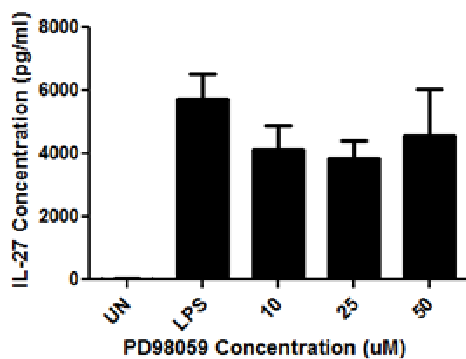

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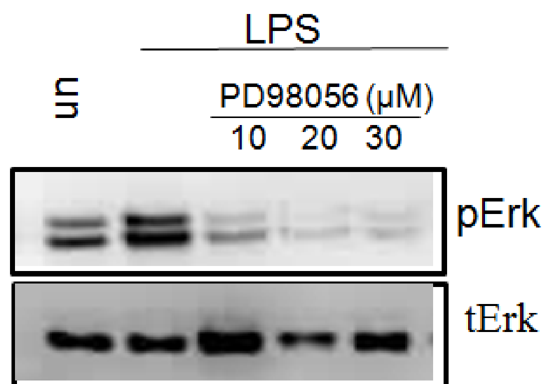

Fig. III.8

Figure III.9 SB203580 has no effect on the production of IL-12, IL-23 and IL-27 in LPS-stimulated THP-1 cells. THP-1 cells (1×10^6 cells) were treated with indicated concentrations of SB203580 for 2 hr followed by LPS stimulation for A) 24 hr and B) 15 mins. A) Secretion of IL-12, IL-23 and IL-27 protein as measured by ELISA. B) Biological activity assay of SB203580 measured by Western immunoblotting. Bars represent mean ±SEM, N=3

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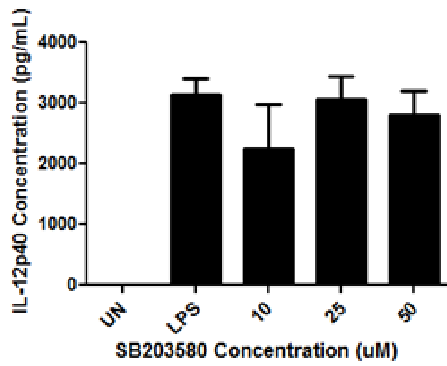

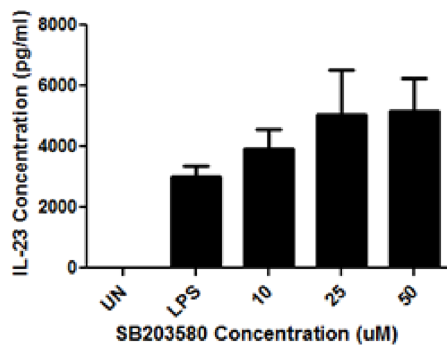

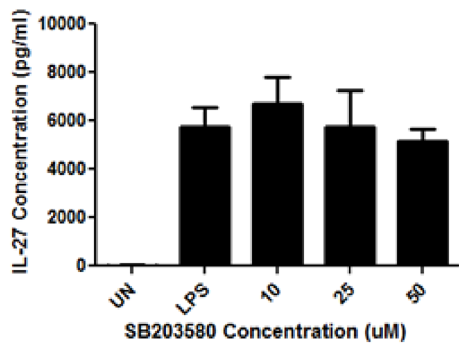

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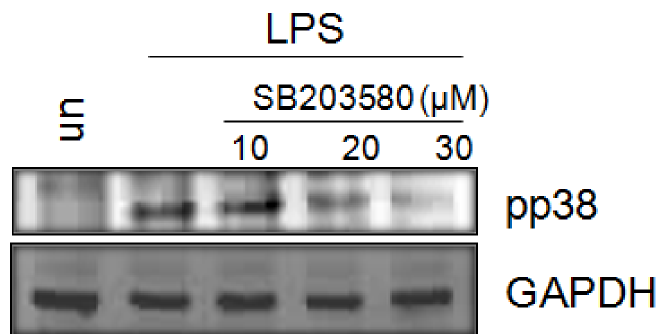
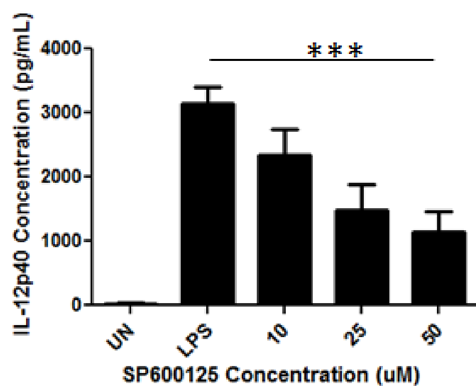
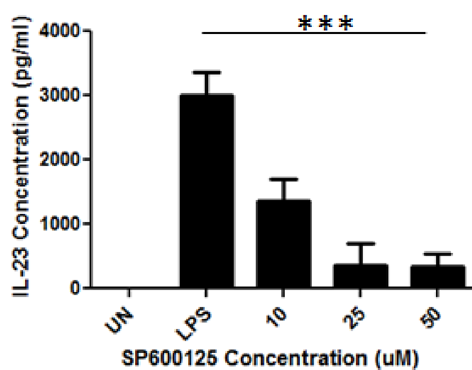
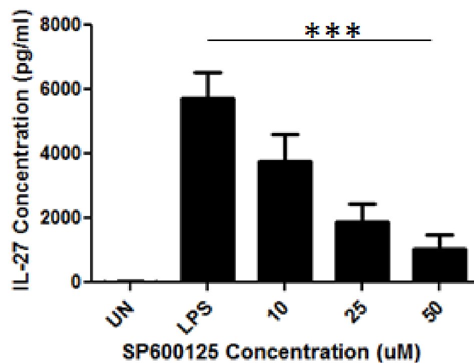


Fig. III.9

Figure III.10 SP600125 inhibits production of IL-12, IL-23 and IL-27 in LPS-stimulated THP-1 cells. THP-1 cells ($1×10^6$ cells) were treated with indicated concentrations of SP600125 for 2 hr followed by LPS stimulation for A) 24 hr B) 15 mins. A) Secretion of IL-12, IL-23 and IL-27 protein as measured by ELISA. B) Biological activity assay of SP600125 measured by Western immunoblotting. Bars represent mean ±SEM, ***$p \leq 0.0005$ N=3







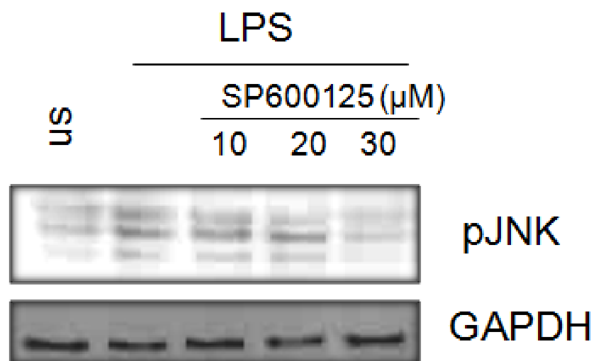


Fig. III.10

III.I.V Determine if SHP-1 and Src physically interact and if this complex is involved in the activation of PI3K and JNK

It is known that tyrosine phosphatases and tyrosine kinases work cooperatively to regulate signaling pathways.[122] Src tyrosine kinases are held in an inactive state at basal levels and require activation by particular tyrosine phosphatases.[113] I have demonstrated that SHP-1 and Src are both involved in the regulation of the IL-12 family of cytokines in LPS-stimulated THP-1 cells. Therefore, I wanted to investigate whether the activity of the two molecules is increased and if they physically interact upon LPS stimulation. THP-1 cells were stimulated with LPS for 15 min and cell lysates were immuno-precipitated (IP) using anti-SHP-1 antibody and these SHP-1 IP's were first analyzed for SHP-1 biological activity using an *in-vitro* phosphatase assay. The results indicate that the biological activity of SHP-1 is increased by 40% upon LPS stimulation. (Fig. III.11A) This suggests that SHP-1 is activated by TLR4 signaling. In parallel, to investigate the tyrosine phosphorylation status of Src upon TLR4 engagement by LPS, THP-1 cells were stimulated with LPS for 15 min and cell lysates were analyzed using Western immunoblotting to detect active Src, pSrc, levels. (Fig. III.11B) The results show that upon LPS stimulation the levels of phosphorylated Src increase within the cell. (Fig. III.11B) Thus, these results suggest that like SHP-1, Src is also activated via TLR4 signaling. Therefore, it was of interest to examine if SHP-1 and Src physically interact downstream of TLR4. To do this, THP-1 cells were stimulated with LPS for 15 min and cell lysates were extracted and Src proteins were immuno-precipitated

Figure III.11 SHP-1 and Src physically interact upon LPS stimulation. THP-1 cells ($1{\times}10^6$ cells) were stimulated with LPS for 15min. Cell pellets were lysed to extract proteins. A) SHP-1 was isolated by immunoprecipitation and lysates were assayed with MG reagent B) Western immunoblotting was done to detect levels of pSrc C) Immunoprecipitation was done to extract Src protein which was analyzed by Western immunoblotting to detect interaction with SHP-1

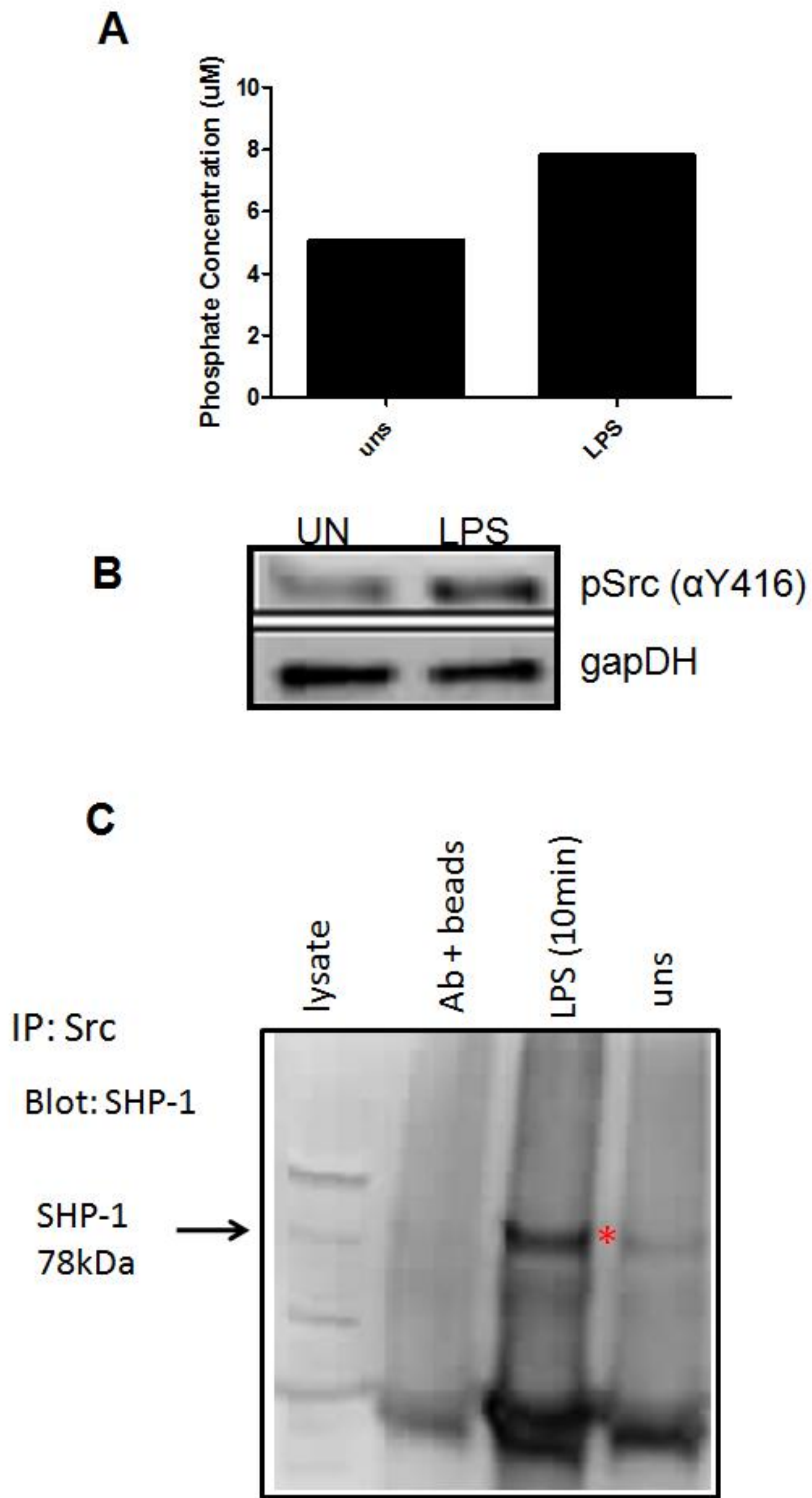


Fig. III.11

from unstimulated and LPS-stimulated cells and analyzed for SHP-1 by Western immunoblotting. The result demonstrates that there is a strong interaction between SHP-1 and Src following LPS stimulation. (Fig. III.11C) This suggests that SHP-1 and Src may act cooperatively to propagate signaling pathways downstream of TLR4.

I have demonstrated that PI3K and JNK pathways are required for the induction of the IL-12 family of cytokines. I have also demonstrated that SHP-1 and Src physically interact upon LPS stimulation and are positive regulators of IL-12p40, IL-23 and IL-27. Therefore the next step was to examine if the SHP-1/Src complex regulates PI3K and JNK activation. THP-1 cells were treated with either sodium stibogluconate or SU6656 for 2 hr or with SHP-1 or c-Src siRNA for 24 hr followed by stimulation with 1 μg/ml of LPS for 15 mins. Cell pellets were collected and subsequently lysed for each condition and the proteins were examined by Western immunoblotting. The membranes were incubated with either anti-pAkt, anti-pJNK, anti-pSrc, anti-GAPDH or anti-β-actin antibodies. The results suggest that following inhibition of SHP-1, with either sodium stibogluconate or SHP-1 siRNA, phosphorylation of Src, JNK and Akt is impaired downstream of TLR4. (Fig. III.12A, Fig. III.12B) Similarly following inhibition of Src, with either SU6656 or c-Src siRNA, levels of pJNK and pAkt were reduced downstream of TLR4. (Fig. III.12C, Fig. III,12D) Collectively these results show that the LPS-activated SHP-1/Src complex regulates PI3K and JNK MAPK pathways, culminating in the induction of IL-12, IL-23 and IL-27 production in THP-1 pro-monocytic cells, used as a model system.

Figure III.12 SHP-1 and Src signalosome promote phosphorylation of Akt and JNK. THP-1 cells ($1{\times}10^6$ cells) were treated with indicated concentrations of inhibitors specific to A) SHP-1 and B) Src for 2 hr followed by LPS stimulation for 15 min. THP-1 cells ($1{\times}10^5$) were transfected with C) SHP-1 siRNA and D) c-Src siRNA for 5 hr followed by LPS stimulation for 15 min. All Cell pellets were lysed to extract proteins which were anlysed by Western immunoblotting to detect phosphorylation levels of pSrc, pAkt and pJNK. N=4 representative blot is shown.

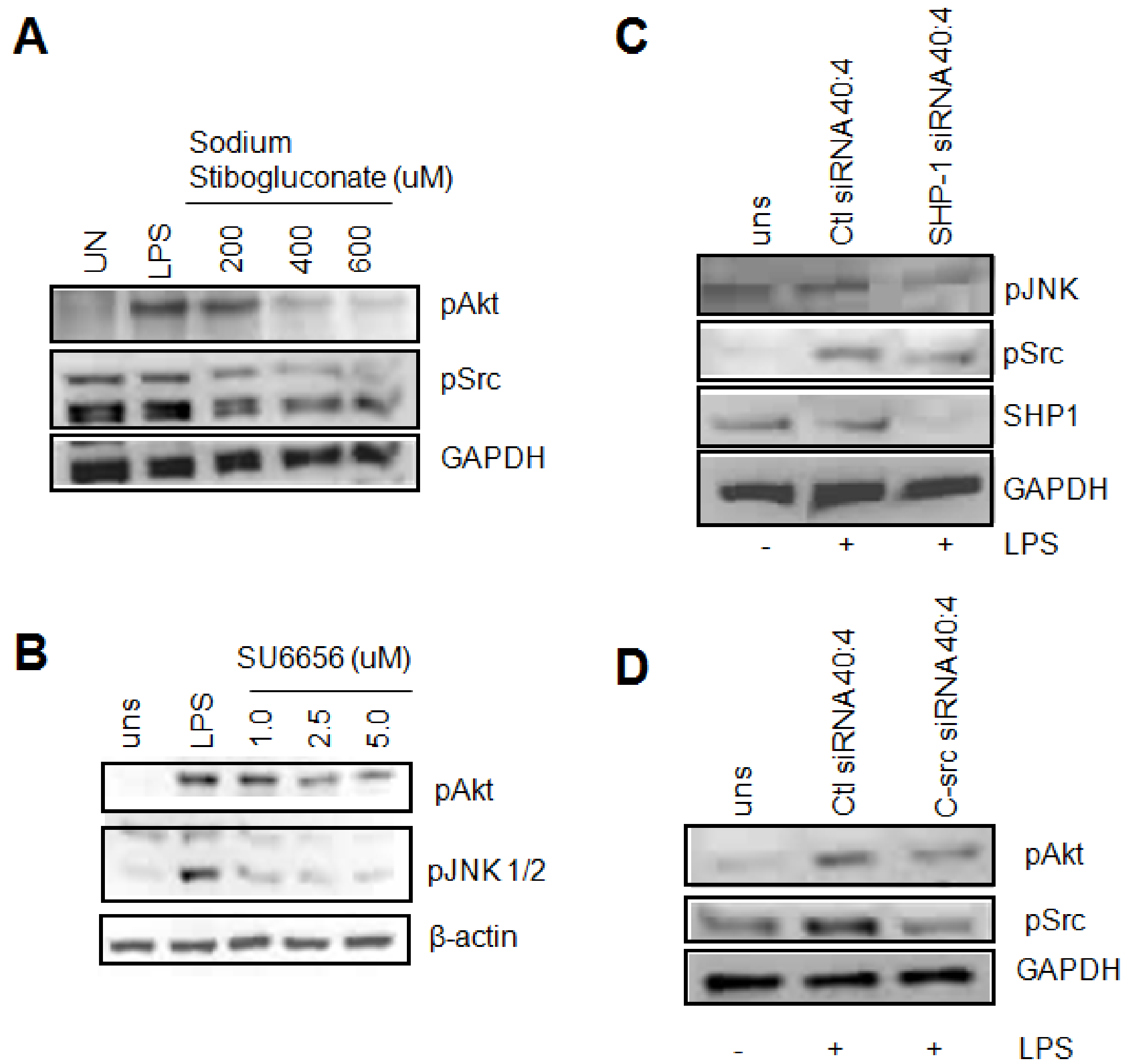


Fig. III.12

III.II OBJECTIVE 2: Applying the findings obtained using a THP-1 cell model to primary human monocytes

During inflammation, one of the first cells recruited are monocytes which migrate from the blood stream into tissues to establish a precursor population destined to differentiate into the effector antigen presenting cells such as macrophages and DCs.[125] My findings clearly demonstrate an important role for SHP-1/Src complex together with JNK and PI3K pathways in regulating the production of IL-12, IL-23 and IL-27 cytokines. It was therefore of interest to validate these novel findings using primary human monocytes isolated from the blood to derive therapeutic significance.

III.II.I Confirm the role of SHP-1 in the production of IL-12 family of cytokines in LPS-stimulated human monocytes

SHP-1 was shown to be a positive regulator of the production of IL-12, IL-23 and IL-27 in THP-1 cells. Therefore, it was of interest to examine if SHP-1 is also a positive regulator of these cytokines in primary human monocytes. Primary human monocytes were isolated from blood using negative selection and treated for 2 hr with sodium stibogluconate followed by stimulation with 1 μg/ml of LPS for 24 hr and the supernatants were assessed for IL-12p40, IL-23p19 and IL-27EBI3/p28 secretion by ELISA. Similarly to THP-1 cells, LPS robustly induced the production of all three cytokines ranging from 300 to 600 pg/ml and treatment of monocytes with sodium stibogluconate resulted in significant suppression, almost 70%, of IL-12p40, IL-23p19 and IL-27EBI3/p28 in a dose dependent manner. (Fig. III.13)

Figure III.13 Sodium Stibogluconate inhibits production of IL-12, IL-23 and IL-27 in LPS-stimulated Human Monocytes. Negatively selected human monocytes (1×10^6 cells) isolated from blood were treated with indicated concentrations of Sodium Stibogluconate for 2 hr followed by LPS stimulation for A) 24 hr. A) Secretion of IL-12, IL-23 and IL-27 protein as measured by ELISA. Bars represent mean ±SEM, *$p \leq 0.05$, **$p \leq 0.005$ N=3

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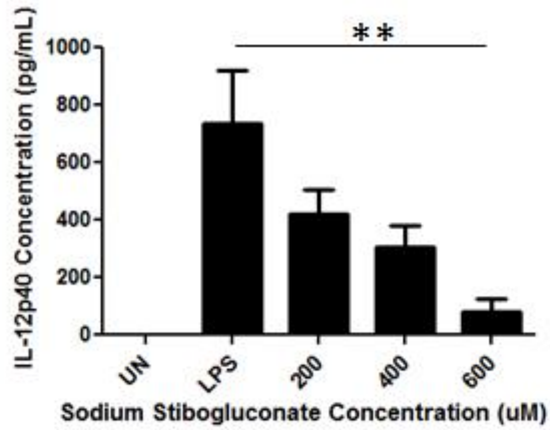
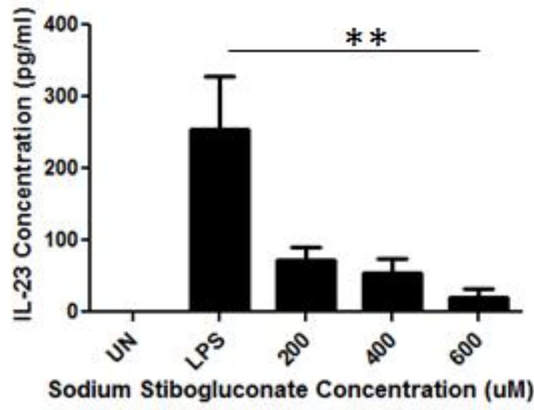




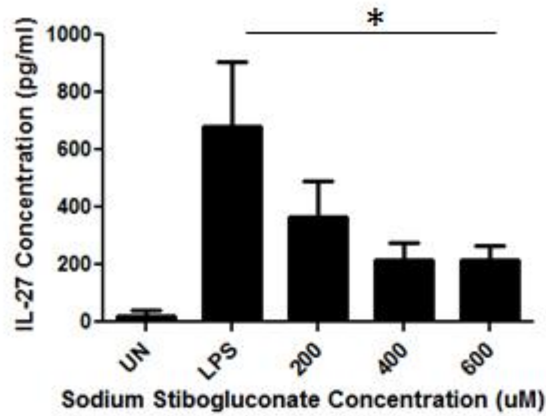


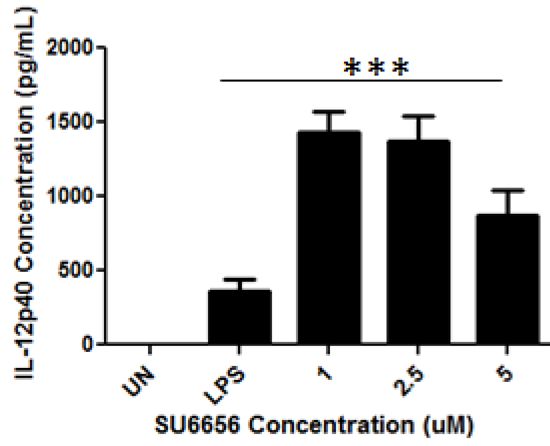
Fig. III.13

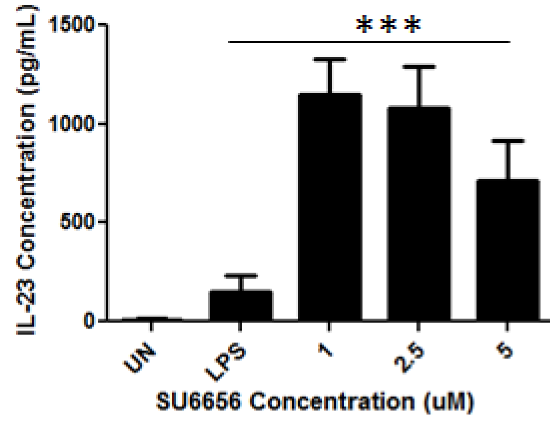
III.II.II Examine the role of Src in the production of IL-12 family of cytokines in human monocytes

Src was shown to be a positive regulator of the production of IL-12, IL-23 and IL-27 in LPS-stimulated THP-1 cells. Therefore, it was of interest to examine if Src is also a positive regulator of these cytokines in primary human monocytes. Primary human monocytes were isolated from blood using negative selection and treated for 2 hr with SU6656 followed by stimulation with 1 μg/ml of LPS for 24 hr and the supernatants were assessed for IL-12p40, IL-23p19 and IL-27EBI3/p28 secretion by ELISA. LPS-stimulated monocytes again produced levels of the three cytokines ranging from 300-1000 pg/ml. (Fig. III.14) Interestingly, treatment of monocytes with SU6656 resulted in a significant 3-fold increase of IL-12p40 and IL-23 which is in contrast with the results obtained using LPS-stimulated THP-1 cells. (Fig. III.14) However, in line with previously generated findings in THP-1 cells, IL-27 production was significantly reduced following Src inhibition. (Fig. III.14) These data therefore reveal that Src functions differently in LPS-stimulated THP-1 cells and LPS-stimulated monocytes. In THP-1 cells Src acts as a positive regulator for IL-12 and IL-23 production, however in primary human monocytes Src acts as a negative regulator for the production of IL-12 and IL-23. In both THP-1 cells and monocytes Src acts as a positive regulator for the production of IL-27. Therefore, in monocytes Src differentially regulates LPS/TLR4-induced IL-12 family of cytokines.

Figure III.14 SU6656 inhibits production of IL-27 and promotes production of IL-12 and IL-23 in LPS-stimulated Human Monocytes. Negatively selected human monocytes (1×10^6 cells) isolated from blood were treated with indicated concentrations of SU6656 for 2 hr followed by LPS stimulation for A) 24 hr. A) Secretion of IL-12, IL-23 and IL-27 protein as measured by ELISA. Bars represent mean ±SEM, ***$p\leq0.0005$ N=3

A





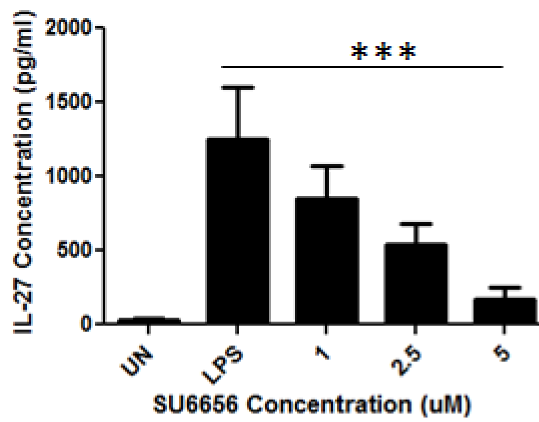


Fig. III.14

III.II.III Investigating the role of PI3K and JNK in the production of IL-12 family of cytokines in human monocytes

PI3K was shown to be a positive regulator of the production of IL-12, IL-23 and IL-27 in LPS-stimulated THP-1 cells. Therefore, it was of interest to examine if PI3K is also a positive regulator of these cytokines in LPS-stimulated primary human monocytes. Primary human monocytes were isolated from blood using negative selection and treated for 2 hr with LY294002 followed by stimulation with 1 μg/ml of LPS for 24 hr and the supernatants were assessed for IL-12p40, IL-23p19 and IL-27EBI3/p28 secretion by ELISA. Similarly to LPS-stimulated THP-1 cells, treatment of LPS-stimulated monocytes with LY294002 resulted in significant suppression of IL-12p40, IL-23p19 and IL-27EBI3/p28 in a dose dependent manner. (Fig. III.15) This data, therefore, supports the notion that PI3K is a positive regulator of the production of the IL-12 family of cytokines in primary human monocytic cells. JNK MAPK was also shown to be a positive regulator of the production of IL-12, IL-23 and IL-27 in THP-1 cells. Therefore it was also of interest to examine if JNK is a positive regulator of these cytokines in primary human monocytes. Primary human monocytes were isolated from blood using negative selection and treated for 2 hr with SP600125 followed by stimulation with 1 μg/ml of LPS for 24 hr and the supernatants were assessed for IL-12p40, IL-23p19 and IL-27EBI3/p28 secretion by ELISA. Interestingly, treatment of monocytes with SP600125 resulted in a significant increase of IL-12p40 and IL-23 which is in contrast to what I have observed using THP-1 cells. (Fig. III.16) However, IL-27 production was significantly reduced following JNK inhibition which confirms the result obtained in LPS-

Figure III.15 LY294002 inhibits production of IL-12, IL-23 and IL-27 in LPS-stimulated Human Monocytes. Negatively selected human monocytes (1×10^6 cells) isolated from blood were treated with indicated concentrations of LY294002 for 2 hr followed by LPS stimulation for A) 24 hr. A) Secretion of IL-12, IL-23 and IL-27 protein as measured by ELISA. Bars represent mean ±SEM, *$p \leq 0.05$, ***$p \leq 0.0005$ N=3

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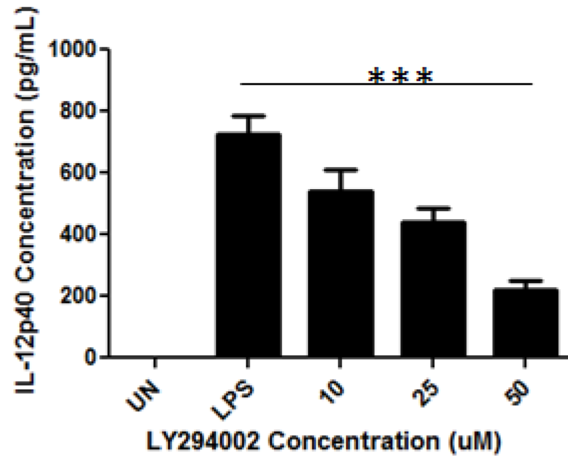
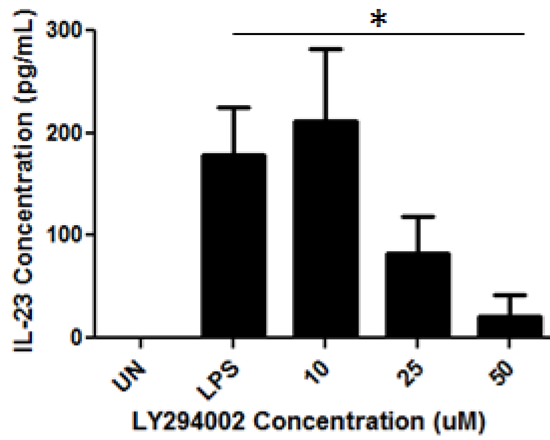




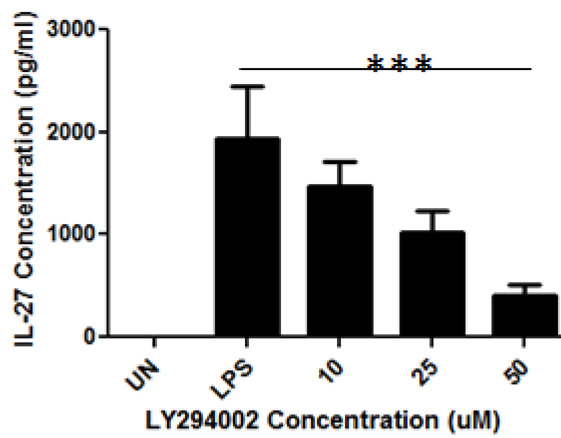


Fig. III.15

Figure III.16 SP600125 inhibits production IL-27 and promotes production of IL-12 and IL-23 in LPS-stimulated Human Monocytes. Negatively selected human monocytes (1×10^6 cells) isolated from blood were treated with indicated concentrations of LY294002 for 2 hr followed by LPS stimulation for A) 24 hr. A) Secretion of IL-12, IL-23 and IL-27 protein as measured by ELISA. Bars represent mean ±SEM, *$p \leq 0.05$, **$p \leq 0.005$, ***$p \leq 0.0005$ N=3

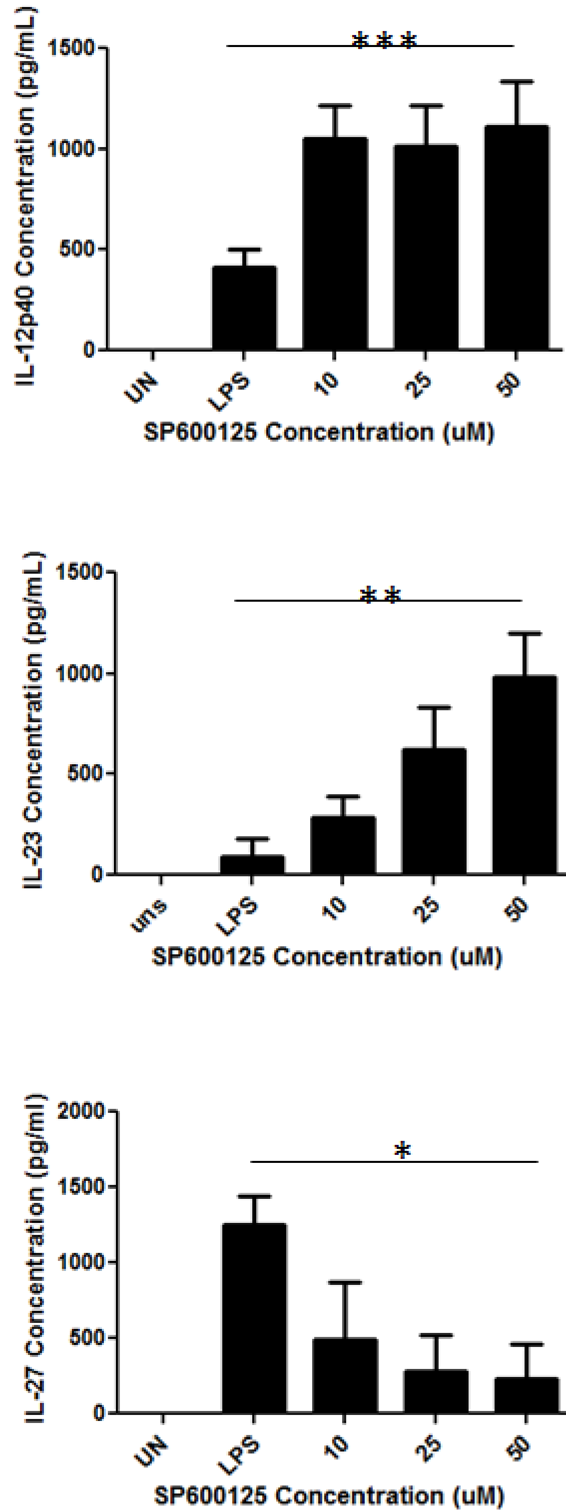


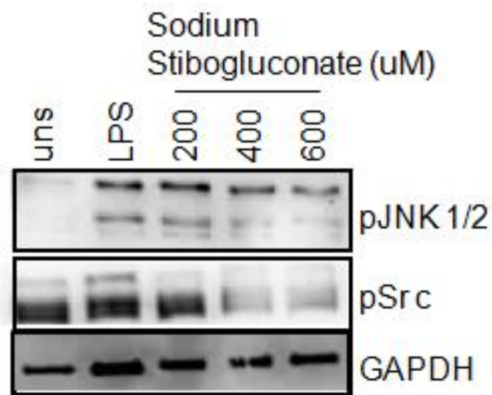
Fig. III.16

stimulated THP-1 cells. (Fig. III.16) These data therefore reveal that JNK functions in a different manner in LPS-stimulated THP-1 cells and LPS-stimulated monocytes. In LPS-stimulated THP-1 cells, JNK is a positive regulator of IL-12 and IL-23 production, however in primary human monocytes JNK appears to function as a negative regulator for the production of IL-12 and IL-23. In both LPS-stimulated, THP-1 cells and monocytes JNK acts as a positive regulator for the production of IL-27. Therefore JNK differentially regulates LPS-induced IL-12 family members in primary monocytes.

III.II.IV Analyze if SHP-1 and Src regulate LPS/TLR4-activated PI3K and JNK pathways in human monocytes

It has been demonstrated in THP-1 cells that SHP-1 and Src physically interact and that they regulate the activation of PI3K and JNK downstream of TLR4. Therefore, I next examined if these signaling pathways regulate production of the IL-12 family of cytokines in LPS-stimulated primary human monocytes. Primary monocytes were isolated from blood using negative selection and were treated with Sodium stibogluconate or SU6656 for 2 hr followed by stimulation with 1 μg/ml of LPS for 15 mins. Cell pellets were collected and subsequently lysed for each condition and the proteins were examined by Western immunoblotting. The membranes were incubated with either anti-pAkt, anti-pJNK, anti-pSrc, anti-GAPDH or anti-β-actin antibodies. Similarly to LPS-stimulated THP-1 cells, the results suggest that following inhibition of SHP-1, with sodium stibogluconate, phosphorylation of Src and JNK is impaired downstream of TLR4. (Fig. III.17A) Therefore SHP-1 regulates Src and JNK activation following LPS stimulation. Moreover, the results show that following inhibition of Src with SU6656,

Figure III.17 SHP-1 and Src signalosome promote phosphorylation of Akt and JNK. Negatively selected human monocytes isolated from the blood (1×10^6) were treated with indicated concentrations of inhibitors specific to A) SHP-1 and B) Src. All cell pellets were lysed to extract proteins which were analysed by Western immunoblotting to detect phosphorylation levels of pSrc, pAkt and pJNK. N=4 representative blot is shown.



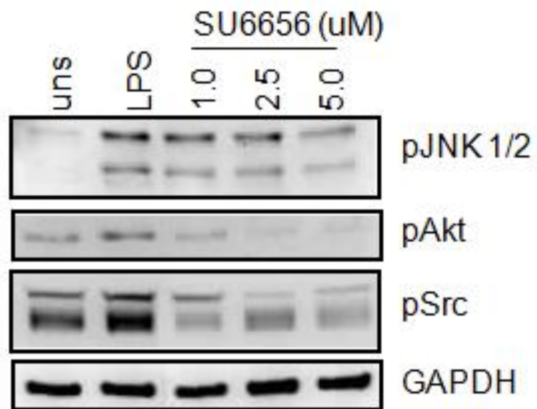


Fig. III.17

11 qq

phosphorylation of Akt and JNK is impaired downstream of TLR4. (Fig. III.17B) Therefore, Src regulates Akt and JNK activation following LPS stimulation. Thus, collectively it appears that SHP-1 and Src regulate JNK and Akt activation and this pathway is conserved between THP-1 cells and primary human monocytes.

III.III OBJECTIVE 3: Examining the regulation of a pro- and anti-inflammatory axis in LPS-stimulated monocyte-derived macrophages (MDMs)

III.III.I Elucidating the role of SHP-1 in the production of IL-12, IL-23, IL-27 and IL-10 in MDMs

Monocytes have a very short half-life in the blood and will eventually differentiate into macrophages.[14] Macrophages are effector cells of innate immunity, and as such secrete many inflammatory mediators, such as cytokines, that either promote or antagonize inflammation.[18] Within the IL-12 family, IL-12 and IL-23 exhibit predominantly pro-inflammatory characteristics, whereas IL-27 exhibits predominantly anti-inflammatory characteristics.[37,48] In view of the results obtained in primary human monocytes, it was of interest to determine if the SHP-1/Src signaling axis, differentially regulating the production of the IL-12 family of cytokines, is conserved in effector cells such as macrophages. Furthermore, the study was expanded to include IL-10, because IL-10 is a known anti-inflammatory cytokine and therefore, its regulation could be compared to that of IL-27.[63] Such a comparison would provide evidence for the role of the SHP-1/Src signaling pathway in differentially regulating the production of pro- (IL-12, IL-23) and anti- (IL-10, IL-27) inflammatory cytokines. MDMs were treated for 2 hr with sodium stibogluconate followed by stimulation with 1 μg/ml of LPS for 24 hr and the supernatants were

assessed for IL-12p40, IL-23p19, IL-10 and IL-27EBI3/p28 secretion by ELISA. The results support observations made in THP-1 cells and primary monocytes, and show that treatment of MDMs with sodium stibogluconate resulted in significant suppression of IL-12p40, IL-23p19, IL-10 and IL-27EBI3/p28 in a dose dependent manner. (Fig. III.18A) These data therefore suggest that SHP-1 is a critical positive regulator of cytokine production and that this role is conserved following differentiation of monocytes into macrophages. I next examined if SHP-1 also regulates the transcription of the subunits that comprise the IL-12 family of cytokines and IL-10. To examine transcript levels of IL-12p40, IL-23p19, IL-10, IL-27p28 and IL-27EBI3, MDMs were treated for 2 hr with sodium stibogluconate followed by stimulation with 1 μg/ml of LPS for 4 hr and the RNA was purified from cell pellets and analyzed by RT-PCR. The results show that following inhibition of SHP-1, transcript levels of all the subunits except EBI3 are significantly reduced. (Fig. III.18B) These results were further confirmed using siRNA specific to SHP-1. MDMs were transfected with SHP-1 siRNA for 5 hr followed by stimulation with 1 μg/ml of LPS for 24 hr and the supernatants were assessed for IL-12p40, IL-23p19, IL-10 and IL-27EBI3/p28 secretion by ELISA. Results confirm that the levels of IL-12p40, IL-23, IL-10 and IL-27 were significantly decreased following transfection with SHP-1 siRNA as compared to transfection with a control siRNA. (Fig. III.19A) Hence, SHP-1 function is required for the production of IL-12 family cytokines and IL-10 in LPS-stimulated MDMs. Efficiency of SHP-1 knockdown was ensured using Western blot analysis demonstrating the SHP-1 siRNA reduced SHP-1 expression to levels of untransfected cells. (Fig. III.19B) I have also confirmed that SHP-1 is involved in

Figure III.18 Sodium Stibogluconate inhibits production of IL-12, IL-23, IL-10 and IL-27 in LPS-stimulated MDMs. MDMs (1×10^6 cells) were treated with indicated concentrations of the inhibitor for SHP-1 followed by LPS stimulation for A) 24 hr and B) 4 hr. A) Production of IL-12, IL-23, IL-27 and IL-10 were measured by ELISA. B) Induction of IL-12, IL-23, IL-27 and IL-10 transcripts were measured by qRT-PCR. Bars represent mean + SEM N=3, *$p<0.05$, **$p<0.005$, ***$p<0.0005$ as measured by One-way Anova

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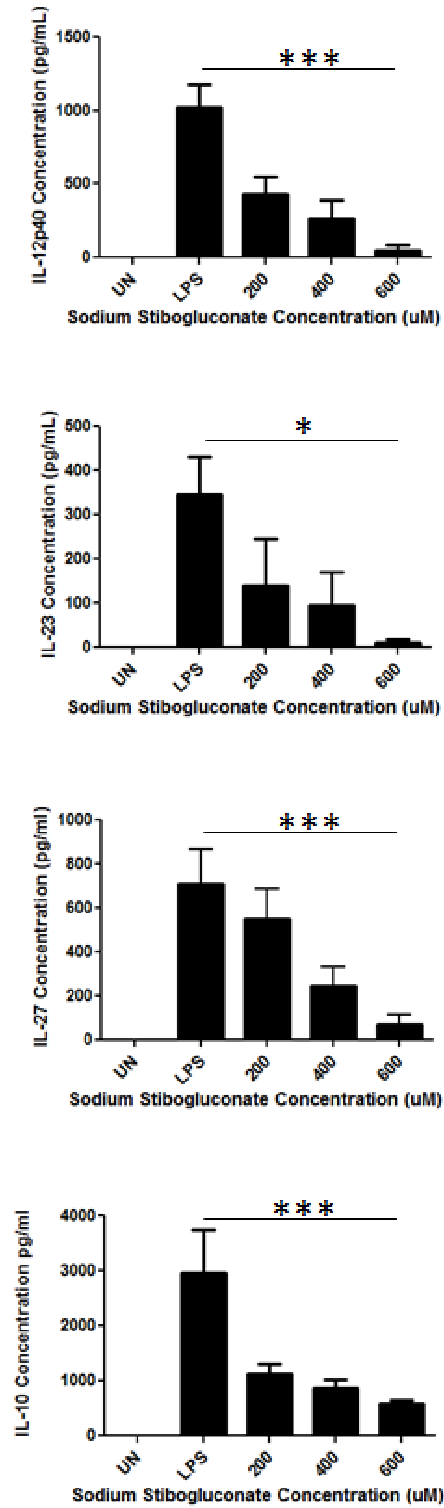

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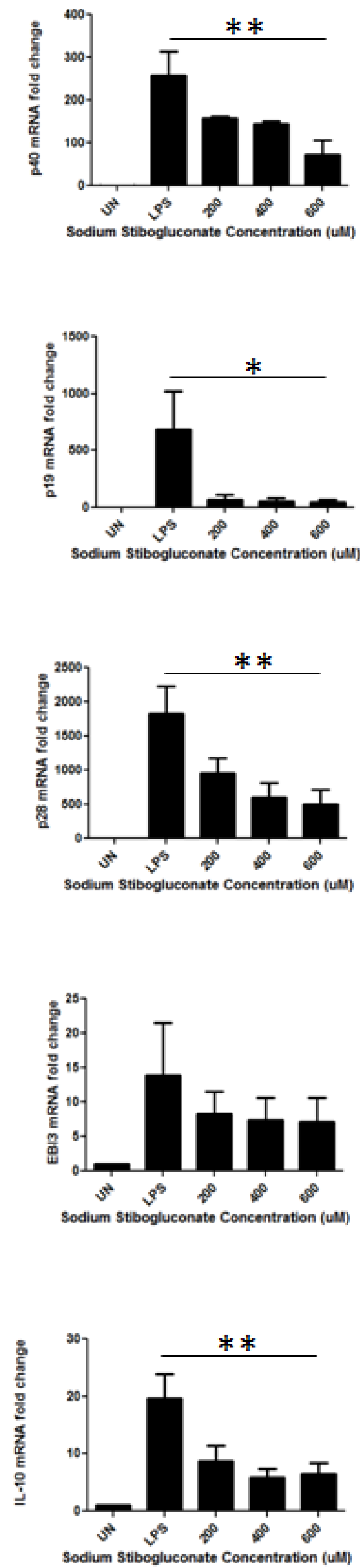

Fig. III.18

Figure

19. SHP-1 siRNA inhibits production of IL-12, IL-23, IL-10, and IL-27 in LPS-stimulated MDMs. MDMs (1×10^5 cells) were transfected with indicated concentrations of SHP-1 siRNA for 2 hr followed by LPS stimulation for A) 24 hr and B) 15 min and C) 4 hr. A) Production of IL-12, IL-23, IL-27 and IL-10 were measured by ELISA. B) Confirmation of SHP-1 silencing was done by Western immunoblotting C) Induction of IL-12, IL-23, IL-27 and IL-10 transcripts were measured by qRT-PCR. Bars represent mean + SEM N=3, *p<0.05, **p<0.005, ***p<0.0005 as measured by One-way Anova

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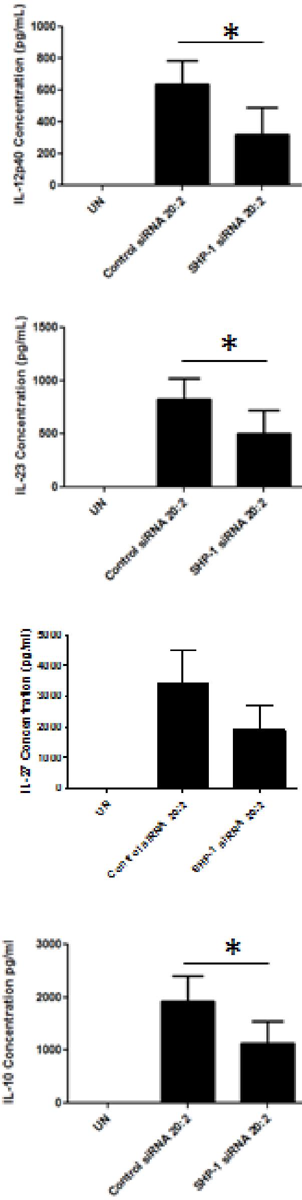

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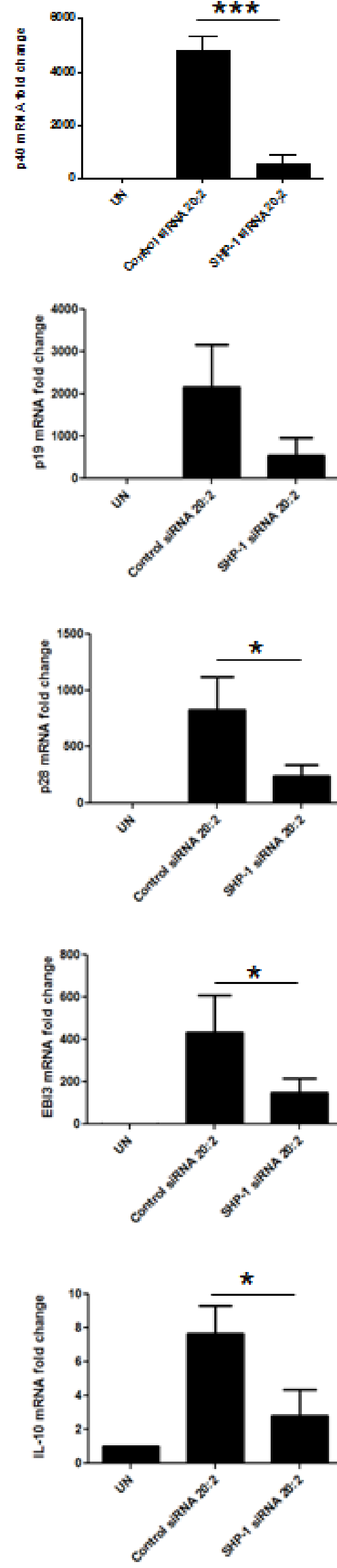

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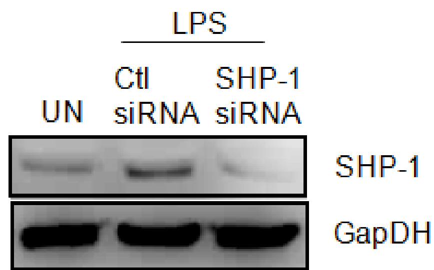


Fig. III.19

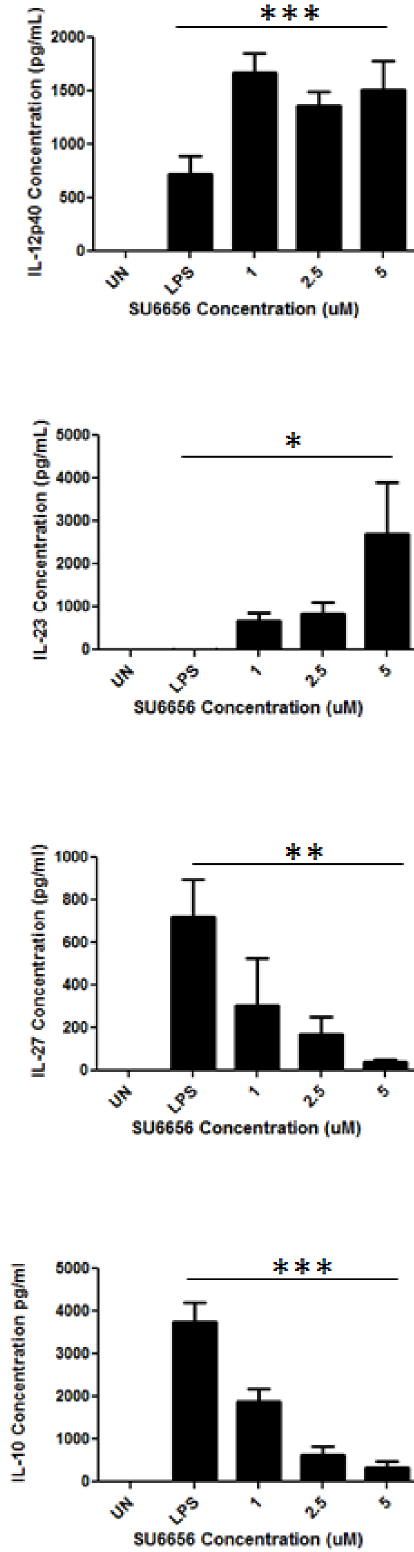
transcriptional regulation of IL-12p40, IL-23p19, IL-10, IL-27p28 and IL-27EBI3 using SHP-1 siRNA. MDMs were transfected with SHP-1 siRNA for 5 hr followed by stimulation with 1 μg/ml of LPS for 4 hr and the RNA was purified from cell pellets and analyzed by RT-PCR. The results confirm the results obtained using sodium stibogluconate, where IL-12p40, IL-27p28 and IL-27EBI3 transcript levels were significantly reduced following SHP-1 knockdown and IL-23p19 expression was trending down towards significance following transfection. (Fig. III.19C) In summary, SHP-1 is a positive regulator of LPS-induced production of IL-12, IL-23, IL-10 and IL-27 and this function is conserved following differentiation of monocytes into macrophages.

III.III.II Investigating the role of Src in the production of IL-12, IL-23, IL-27 and IL-10 in LPS-stimulated MDMs

In THP-1 monocytic cells, it was observed that Src was a positive regulator for the production of IL-12, IL-23 and IL-27. However, in primary human monocytes it was observed that Src differentially regulates IL-12p40/IL-23 and IL-27. Moreover, SHP-1 was shown to be a positive of regulator of IL-12p40, IL-23, IL-27 and IL-10 in LPS-stimulated MDMS. In view of the findings that SHP-1 and Src form a complex it was of interest to also determine the role of Src in effector macrophages. MDMs were treated for 2 hr with SU6656 followed by stimulation with 1 μg/ml of LPS for 24 hr and the supernatants were assessed for IL-12p40, IL-23p19, IL-10 and IL-27EBI3/p28 secretion by ELISA. The results show that similarly to primary monocytes, treatment of MDMs with SU6656 resulted in significant suppression of IL-27EBI3/p28 by 90% in a dose dependent manner, moreover IL-10 secretion was

also significantly decreased by 90% following Src inhibition. (Fig. III.20A panel 3,4) In contrast, as observed in primary monocytes, the production of IL-12p40 and IL-23 was significantly increased following Src inhibition. (Fig. III.20A panel 1,2) It should be noted that LPS induces only a very modest levels of IL-23 ranging between 0 and 400 pg/ml. This limited induction is greatly enhanced upon inhibition of Src, suggesting a negative role for Src in the regulation of pro-inflammatory cytokines in LPS-stimulated MDMs. Moreover, this data suggests that Src differentially regulates IL-12/IL-23 and IL-10/IL-27 and that this is conserved following differentiation of monocytes into macrophages. In view of these findings, I next determined if Src also differentially regulates the transcription of the subunits that comprise the IL-12 family of cytokines and IL-10. To examine transcript levels of IL-12p40, IL-23p19, IL-10, IL-27p28 and IL-27EBI3, MDMs were treated for 2 hr with SU6656 followed by stimulation with 1 μg/ml of LPS for 4 hr and the RNA was analyzed by RT-PCR. The results show that following inhibition of Src, transcript levels of IL-12p40 were significantly increased with transcript levels of IL-23p19 trending to significance, whereas levels of IL-10, IL-27p28 and IL-27EBI3 transcripts were significantly reduced following Src inhibition in a dose-dependent manner. (Fig. III.20B) These results were also confirmed using siRNA specific to Src. MDMs were transfected with Src siRNA for 5 hr followed by stimulation with 1 μg/ml of LPS for 24 hr and the supernatants were assessed for IL-12p40, IL-23p19, IL-10 and IL-27EBI3/p28 secretion by ELISA. Results are in agreement with results obtained using SU6656 and show that the levels of IL-12p40 and IL-23 were significantly increased whereas IL-10 and IL-27 were significantly decreased following transfection with Src siRNA

Figure III.20 SU6656 promotes production of IL-12, IL-23 and inhibits IL-10 and IL-27 production in LPS-stimulated MDMs. MDMs (1×10^6 cells) were treated with indicated concentrations of the inhibitor for Src followed by LPS stimulation for A) 24 hr and B) 4 hr. A) Production of IL-12, IL-23, IL-27 and IL-10 were measured by ELISA. B) Induction of IL-12, IL-23, IL-27 and IL-10 transcripts were measured by qRT-PCR. Bars represent mean + SEM N=3, *p<0.05, **p<0.005, ***p<0.0005 as measured by One-way Anova



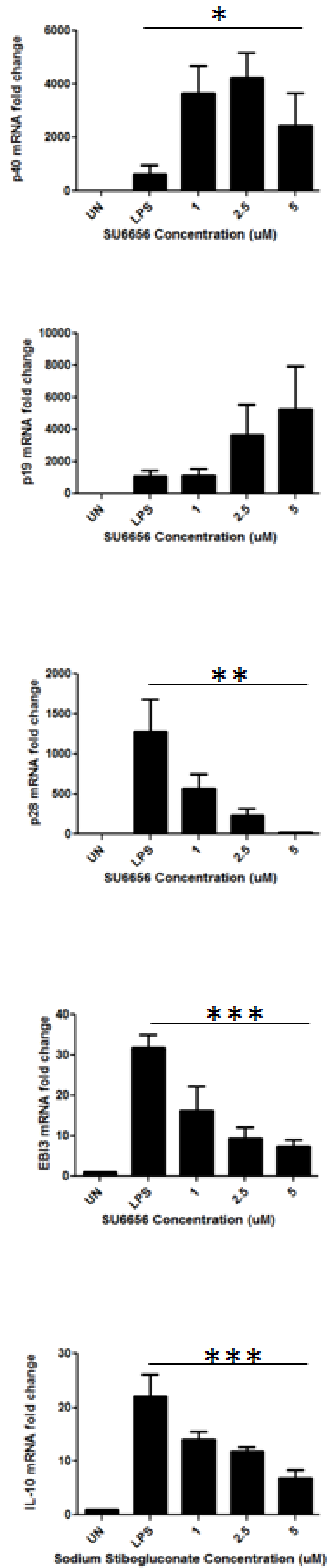


Fig. III.20

as

compared to transfection with a control siRNA in LPS-stimulated MDMs. (Fig. III.21A) These data suggest that Src is differentially regulating the LPS-induced production of IL-12/IL-23 and IL-10/IL-27. Efficiency of Src knockdown was ensured by Western blot analysis showing that treatment with Src siRNA reduced levels of Src protein compared to transfection with control siRNA in LPS-stimulated MDMs (Fig. III.21B) To examine the involvement of Src in transcriptional regulation of IL-12p40, IL-23p19, IL-10, IL-27p28 and IL-27EBI3, MDMs were transfected with Src siRNA for 5 hr followed by stimulation with 1 μg/ml of LPS for 4 hr and the RNA was analyzed by RT-PCR. The results confirm the data generated using SU6656, where IL-12p40 and IL-23 transcript levels were significantly increased whereas IL-10, IL-27p28 and IL-27EBI3 transcript levels were significantly reduced following Src knockdown (Fig. III.21C) Collectively, these data demonstrate that Src is a negative regulator of IL-12p40/IL-23 and a positive regulator for IL-27/IL-10 in LPS-stimulated MDMs and this function is conserved following differentiation of monocytes into macrophages. Moreover, it is also intriguing to speculate that Src functions as a switch to regulate the production of pro- and anti-inflammatory cytokines.

III.III.III Confirm the role of PI3K and JNK MAPK in the production of IL-12, IL-23, IL-27 and IL-10 in MDMs

I have thus far demonstrated that in monocytes and THP-1 cells, PI3K is positively regulating IL-12, IL-23 and IL-27. I have also demonstrated that JNK differentially regulates IL-12/IL-23 and IL-27 in LPS-stimulated monocytes. In view of these findings, it was of interest to determine if these two pathways are also involved in the regulation of the IL-12 family of cytokines and IL-10 after differentiation of monocytes into macrophages.

Figure III.21 C-Src siRNA promotes production of IL-12 and IL-23 and inhibits production of IL-10 and IL-27 in LPS-stimulated MDMs. MDMs (1×10^5 cells) were transfected with indicated concentrations of c-Src siRNA for 2 hr followed by LPS stimulation for A) 24 hr and B)15 min and C) 4 hr. A) Production of IL-12, IL-23, IL-27 and IL-10 were measured by ELISA. B) Confirmation of c-Src silencing was done by Western immunoblotting C) Induction of IL-12, IL-23, IL-27 and IL-10 transcripts were measured by qRT-PCR. Bars represent mean + SEM N=3, *p<0.05, **p<0.005, ***p<0.0005 as measured by One-way Anova

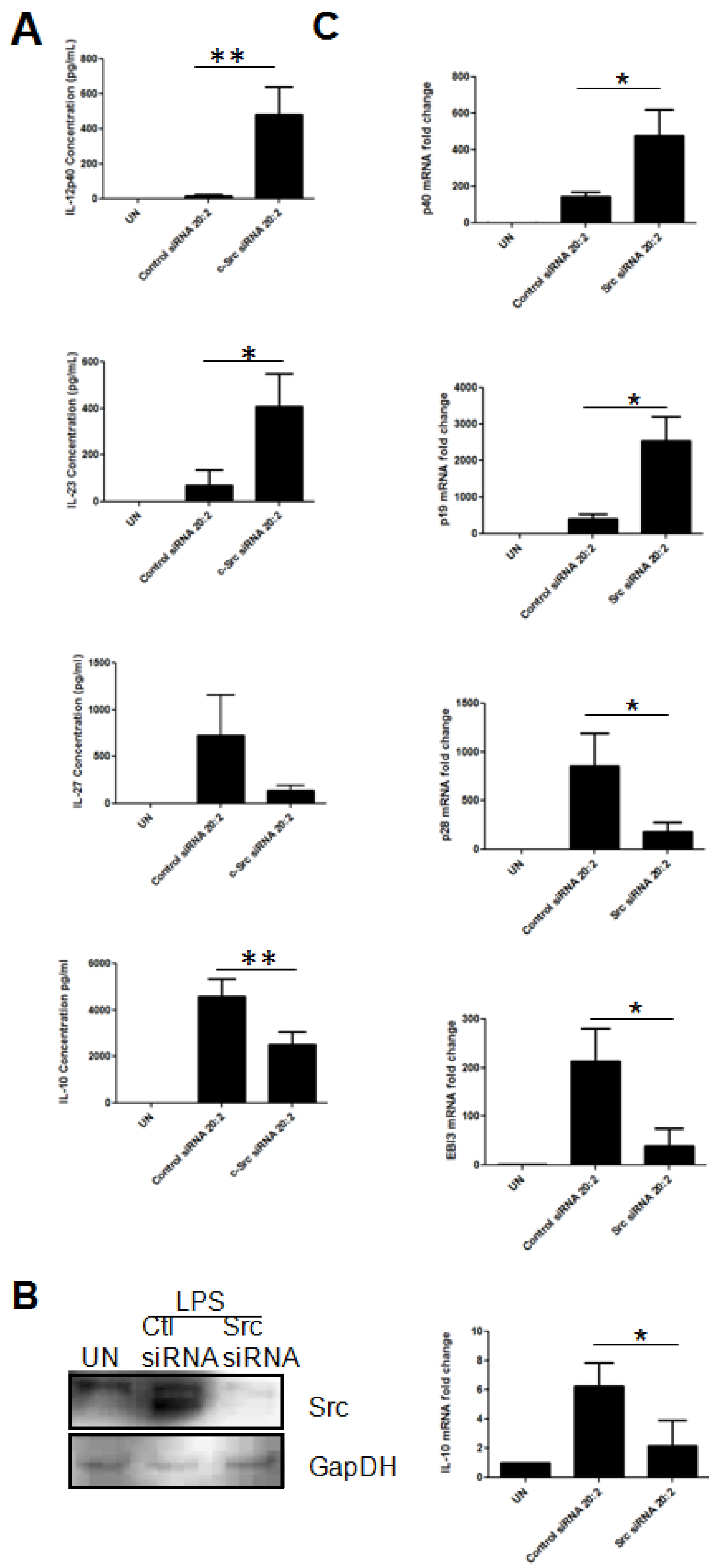
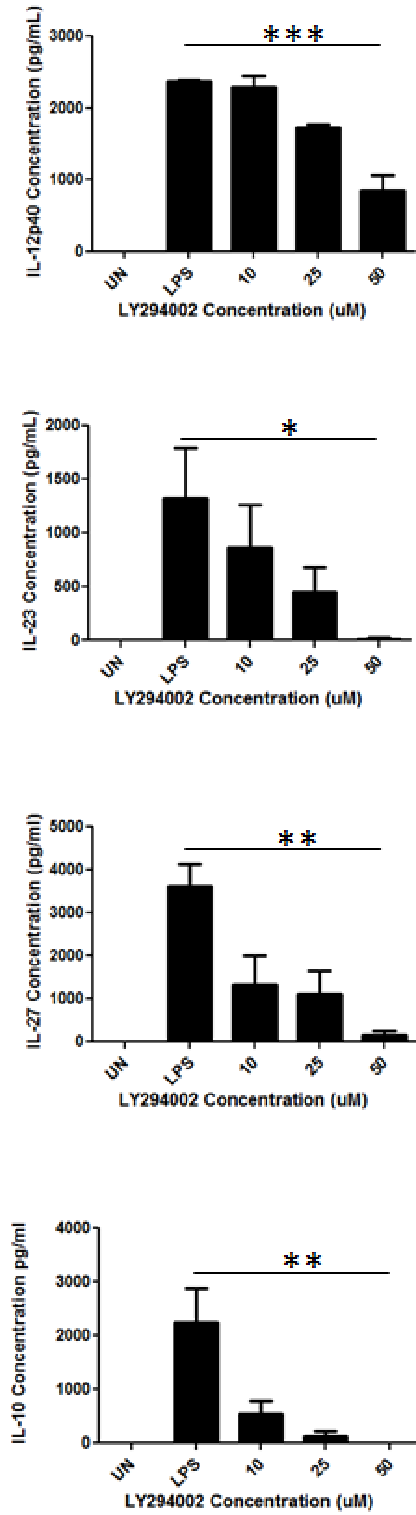


Fig. III.21

MDMs were treated for 2 hr with LY296002 followed by stimulation with 1 μg/ml of LPS for 24 hr and the supernatants were assessed for IL-12p40, IL-23p19, IL-10 and IL-27EBI3/p28 secretion by ELISA. The results show that similarly to THP-1 cells and primary monocytes, treatment of MDMs with LY294002 resulted in significant suppression of IL-12p40, IL-23p19, IL-10 and IL-27EBI3/p28 in a dose dependent manner. (Fig. III.22A) This data therefore suggests that activation of PI3K pathway is required for the production of IL-12, IL-23, IL-27 and IL-10 in LPS-stimulated MDMs and this function is conserved following differentiation of monocytes into macrophages. I next analyzed, if PI3K also regulates the expression of the subunits that comprise the IL-12 family of cytokines and IL-10 at the transcriptional level. To examine transcript levels of IL-12p40, IL-23p19, IL-10, IL-27p28 and IL-27EBI3, MDMs were treated for 2 hr with LY294002 followed by stimulation with 1 μg/ml of LPS for 4 hr and the RNA was purified from cell pellets and analyzed by RT-PCR. The results show that following inhibition of PI3K, transcript levels of all the subunits were significantly reduced. (Fig. III.22B) Therefore PI3K pathway is a positive regulator of the production of these cytokines and this pathway is conserved in monocytes and macrophages. To examine the role of JNK in pro- and anti-inflammatory cytokine production, MDMs were treated for 2 with SP600125 followed by stimulation with 1 μg/ml of LPS for 24 hr and the supernatants were assessed for IL-12p40, IL-23p19, IL-10 and IL-27EBI3/p28 secretion by ELISA. The results show that similarly to primary monocytes, treatment of MDMs with SP600125 resulted in significant suppression of IL-27EBI3/p28 in a

Figure III.22 LY294002 inhibits production of IL-12, IL-23, IL-10 and IL-27 in LPS-stimulated MDMs. MDMs (1×10^6 cells) were treated with indicated concentrations of the inhibitor for PI3K followed by LPS stimulation for A) 24 hr and B) 4 hr. A) Production of IL-12, IL-23, IL-27 and IL-10 were measured by ELISA. B) Induction of IL-12, IL-23, IL-27 and IL-10 transcripts were measured by qRT-PCR. Bars represent mean + SEM N=3, *$p<0.05$, **$p<0.005$, ***$p<0.0005$ as measured by One-way Anova



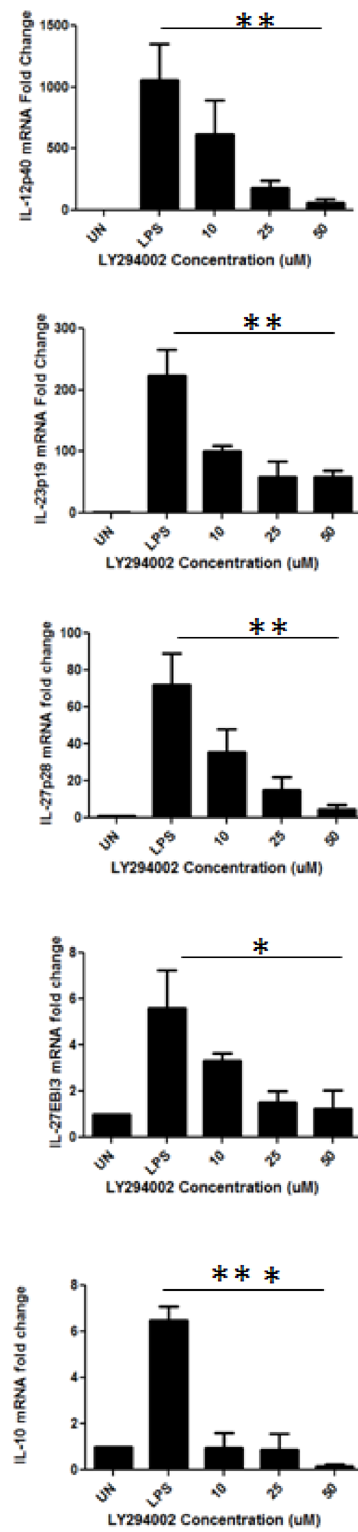
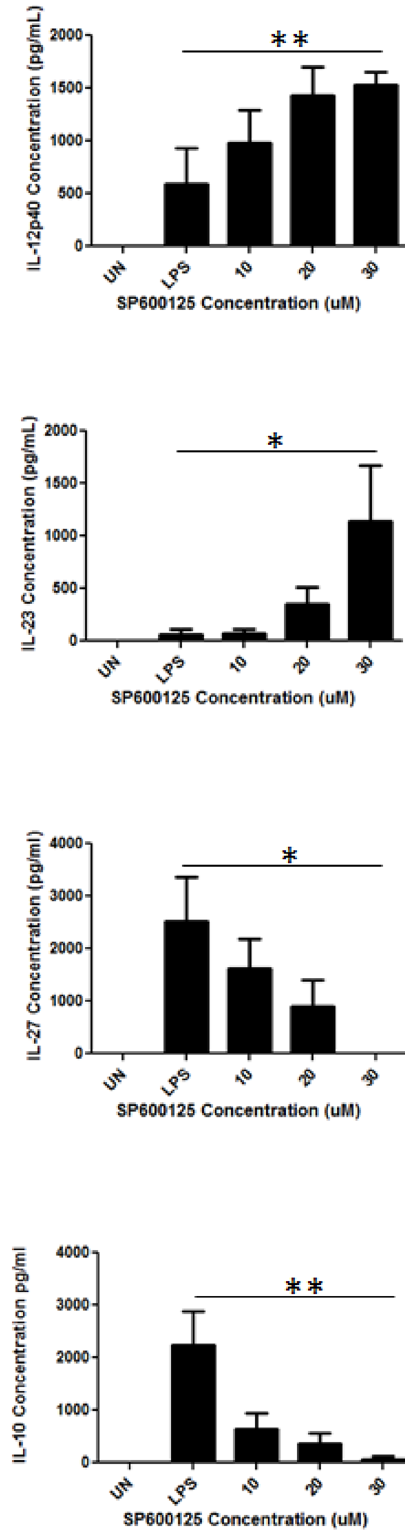


Fig. III.22

dose dependent manner, in addition IL-10 production was also significantly decreased following JNK inhibition. (Fig. III.23A panel 3,4) In contrast, and in accordance with observations made in primary monocytes, the production of IL-12p40 and IL-23 was significantly increased in dose dependent manner following the inhibition of JNK. (Fig. III.23A panel 1,2) This data, therefore, suggests that JNK differentially regulates IL-12/IL-23 and IL-10/IL-27 and that this is conserved following differentiation of monocytes into macrophages. Next, I examined if this differential regulation also extends to the expression of the subunits that comprise the IL-12 family of cytokines and IL-10. To examine transcript levels of IL-12p40, IL-23p19, IL-10, IL-27p28 and IL-27EBI3, MDMs were treated for 2 hr with SP600125 followed by stimulation with 1 μg/ml of LPS for 4 hr and the RNA was purified from cell pellets and analyzed by RT-PCR. The results show that following inhibition of JNK, transcript levels of IL-12p40 an IL-23p19 were significantly increased, whereas levels of IL-10 and IL-27p28 transcripts were significantly reduced with IL-27EBI3 transcript levels trending downwards following JNK inhibition. (Fig. III.23B) Therefore, like Src, JNK pathway appears to be involved in the differential regulation of IL-12/IL-23 and IL-10/IL-27 and this regulation is conserved in monocytes and macrophages. Activation of the Src/JNK axis is required for the induction of IL-27/IL-10 downstream of TLR4.

Figure III.23 SP600125 promotes production of IL-12, IL-23 and inhibits IL-10 and IL-27 production in LPS-stimulated MDMs. MDMs (1×10^6 cells) were treated with indicated concentrations of the inhibitor for JNK followed by LPS stimulation for A) 24 hr and B) 4 hr. A) Production of IL-12, IL-23, IL-27 and IL-10 were measured by ELISA. B) Induction of IL-12, IL-23, IL-27 and IL-10 transcripts were measured by qRT-PCR. Bars represent mean + SEM N=3, *p<0.05, **p<0.005, ***p<0.0005 as measured by One-way Anova

A



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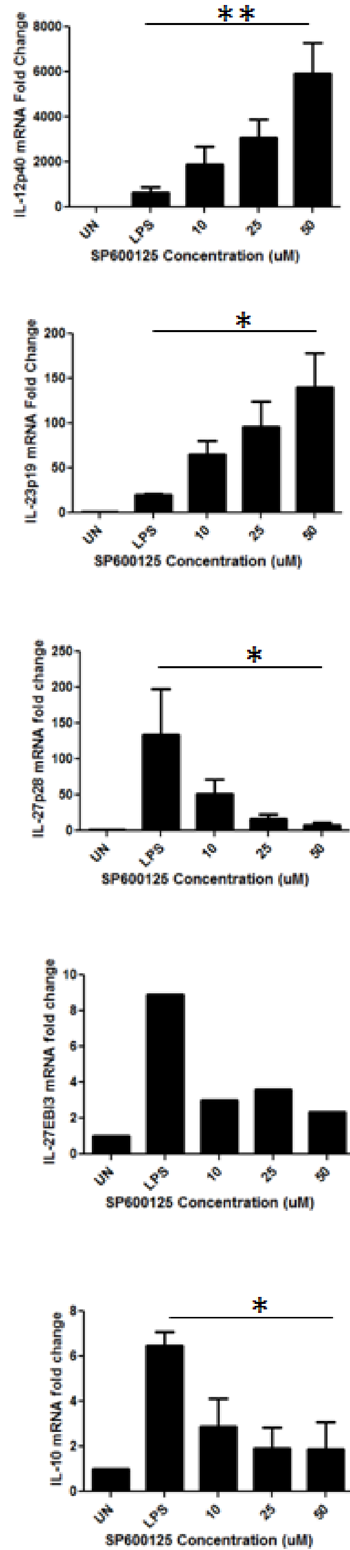


Fig. III.23

III.III.IV Determine the nature of the SHP-1 and Src protein complex in MDMs

I have demonstrated that tyrosine phosphatase, SHP-1 and Tyrosine kinase, Src are mandatory regulators of LPS/TLR4-activated signalling pathways culminating in the production of IL-12, IL-23, IL-10 and IL-27. Moreover, I have demonstrated that Src and JNK pathway exhibit differential regulation of pro- and anti-inflammatory cytokines. TLR4 signaling is unique among TLRs in that it comprises two distinct signaling mechanisms, MyD88-dependent and MyD88-independent (TRIF-dependent) and emloys them in an equal manner.[84,90] Therefore, it was of interest to determine if the SHP-1/Src protein complex functions downstream of MyD88-dependent or MyD88-independent pathway or both. Firstly, to demonstrate this, MDMs were stimulated with LPS for 15 min and were analyzed by immunofluorescence to confirm the observation in THP-1 cells showing that SHP-1 and Src physically interact upon TLR4 activation. The results visualize the SHP-1 and Src complex co-localized upon LPS stimulation of TLR4. (Fig. III.24) Next, I determined if SHP-1 and Src protein complex interacts with the adaptor protein, MyD88. MDMs were stimulated with LPS for 15 min and were analyzed by immunofluorescence to confirm that SHP-1/Src and MyD88 physically interact upon TLR4 activation. The results visualize that SHP-1 and MyD88 co-immunofluoresce indicating co-localization. (Fig. III.25A) Similarly, Src and MyD88 were also found to co-immunofluoresce. (Fig. III.25B) Collectively, these two observations show that SHP-1 and Src complex is co-localized with MyD88 upon stimulation of MDMs with LPS. Next, I determined if SHP-1 and Src protein complex also interact with TRIF adaptor protein following TLR4 activation. MDMs were stimulated with LPS for

Figure III.24 SHP-1 and Src physically interact in LPS-stimulated MDMs. MDMs (1×10^{6} cells) were untreated, stimulated with LPS for 15 min, or treated with indicated concentrations of Sodium stibogluconate followed by LPS stimulation 15 min. Wells were stained with anti-mouse SHP-1 antibody and anti-rabbit Src antibody and then analyzed using immunofluorescence

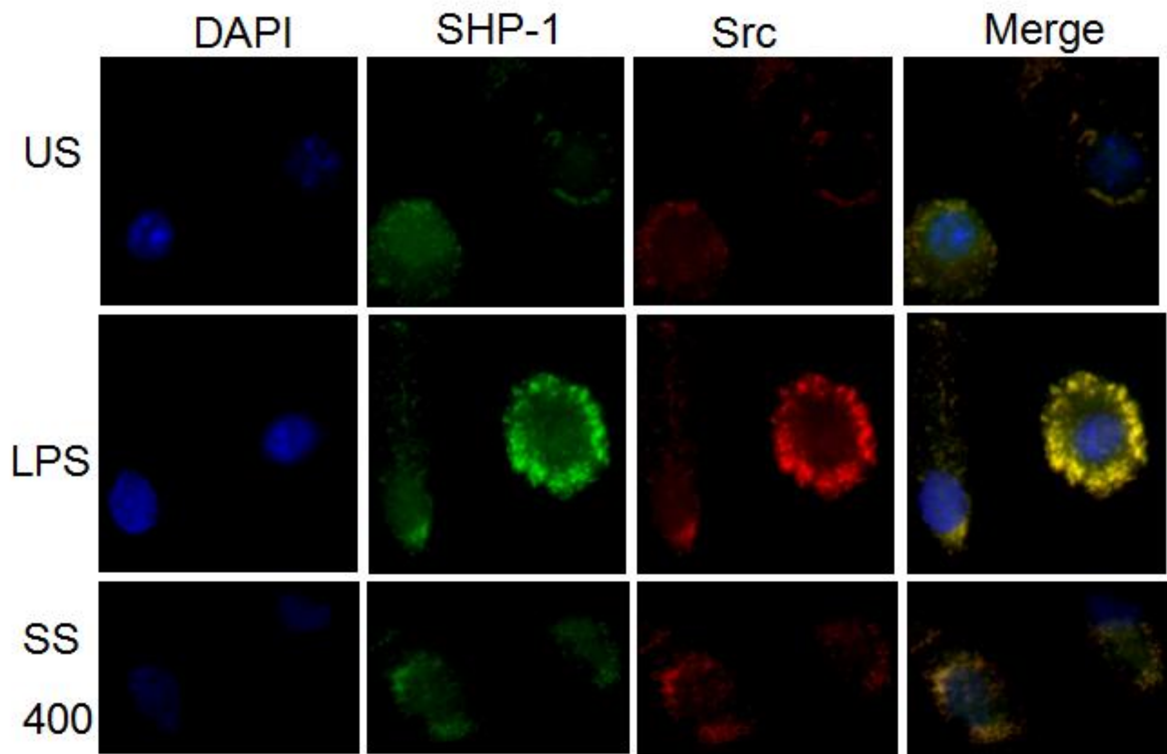
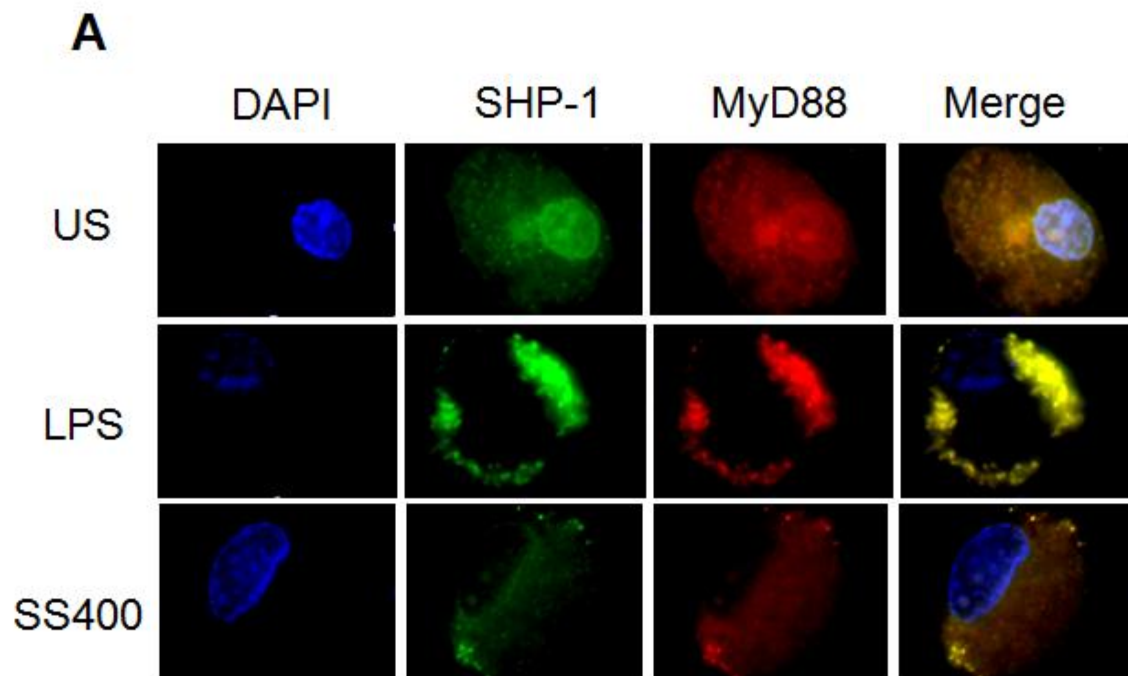


Fig. III.24

Figure III.25 SHP-1 and Src physically interact with MyD88 in LPS-stimulated MDMs. MDMs (1×10^6 cells) were untreated, stimulated with LPS for 15 min, or treated with indicated concentrations of Sodium stibogluconate followed by LPS stimulation for 15 min. A) Wells were stained with anti-mouse SHP-1 antibody and anti-rabbit MyD88 antibody or B) Wells were stained with anti-mouse Src antibody and anti-rabbit MyD88 antibody and then analyzed using immunofluorescence



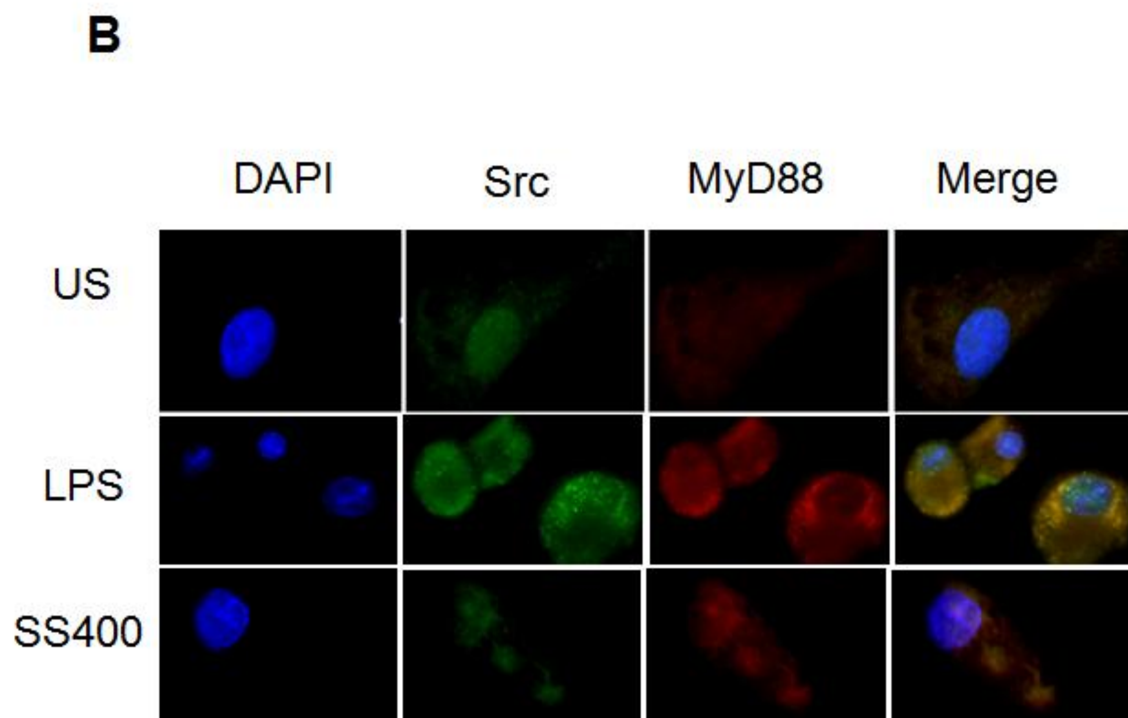
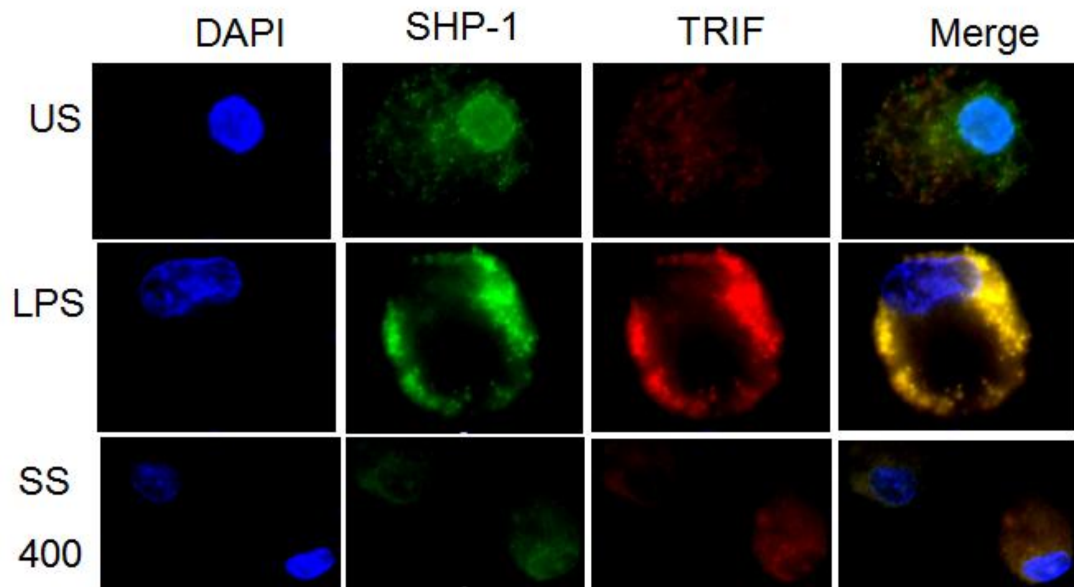


Fig. III.25

15 min and were analyzed by immunofluorescence. The results show that SHP-1 and TRIF co-immunofluoresce. (Fig. III.26A) Similarly Src and TRIF molecules also co-immunofluoresce, indicating co-localization. (Fig. III.26B) Collectively, these two observations show that SHP-1 and Src complex is co-localized with TRIF upon stimulation of MDMs with LPS. These results taken together, suggest that the SHP-1/Src protein complex employs both MyD88-dependent and MyD88-independent (TRIF) signaling.

Figure III.26 SHP-1 and Src physically interact with TRIF in LPS-stimulated MDMs. MDMs (1×10^6 cells) were untreated , stimulated with LPS for 15 min, or treated with indicated concentrations of Sodium stibogluconate followed by LPS stimulation for 15 min. A) Wells were stained with anti-mouse SHP-1 antibody and anti-rabbit TRIF antibody or B) Wells were stained with anti-mouse Src antibody and anti-rabbit TRIF antibody and then analyzed using immunofluorescence



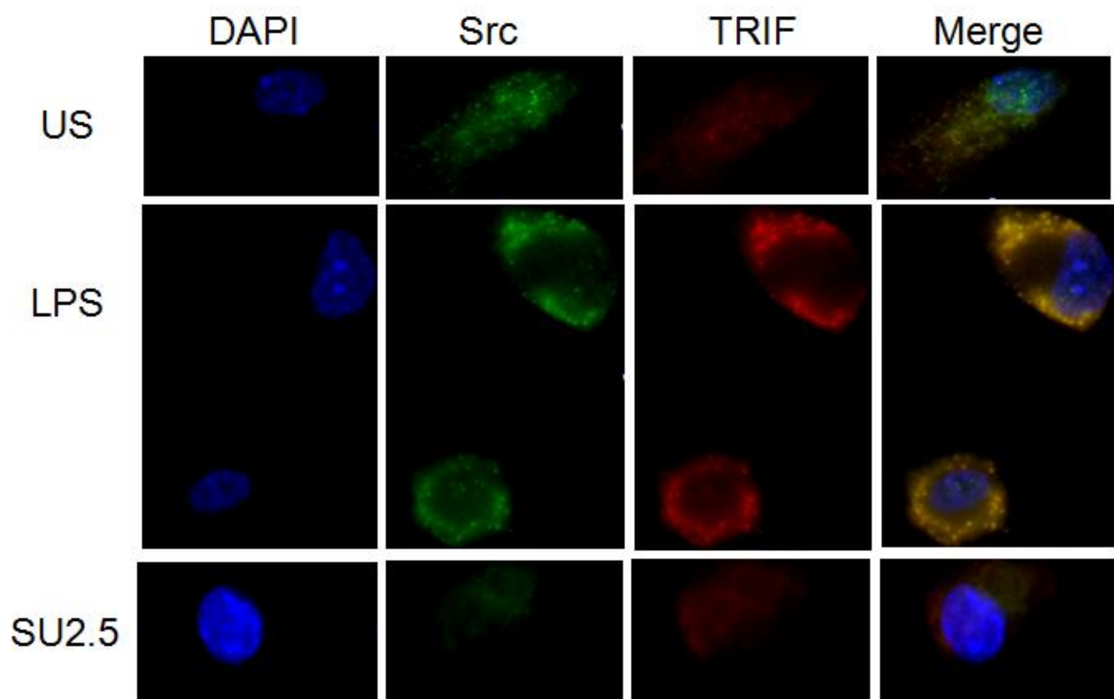


Fig. III.26

IV. DISCUSSION

Autoimmune disorders such as rheumatoid arthritis and multiple sclerosis are characterized by a sustained inflammatory response against host tissue.[69] A shift in the balance between pro- and anti- inflammatory cytokines towards the pro-inflammatory mediators is a characteristic of chronic inflammation. [1] In normal host systems, the detection of antigen by cells of the innate immune system such as macrophages results in mounting an effective inflammatory response followed by the subsequent attenuation of this response by anti-inflammatory mechanisms to return the system to homeostasis.[1] M1 macrophages promote the production of pro-inflammatory mediators such as IFNγ and IL-12, which are required for effective engagement of adaptive immunity through the differentiation of Th1 helper T cells.[17] Conversely, M2 macrophages secrete anti-inflammatory mediators such as IL-10, to attenuate the adaptive immune response.[17] Dysregulated production of the IL-12 family of cytokines: IL-12, IL-23 and IL-27 have been implicated in autoimmune diseases, with a hallmark of an enhanced IL-12/ IL-23 expression and suppression of IL-27.[119,121] However, the regulation of IL-12, IL-23 and IL-27 is not fully understood at the molecular level. Therefore, the focus of this study was to investigate the nature of the regulation of the IL-12 family of cytokines in cells of the innate immune system such as monocytes and macrophages. My results reveal for the first time a dual role of SHP-1 in the control of inflammation through initiation of a feedback mechanism. My results also demonstrate for the first time that monocytes and macrophages employ the SHP-1/ Src complex in the regulation of IL-12, IL-23, IL-27 and IL-10 downstream of TLR4. The tyrosine phosphatase SHP-1 through the

activation of the PI3K is a mandatory positive regulator of LPS-induced production of IL-12, IL-23, IL-10 and IL-27. Moreover, the Src tyrosine kinase function is dependent on SHP-1 expression and activation of the conserved Src/JNK complex. More importantly my study also demonstrates that SHP-1 function regulates the inflammatory episodes by maintaining the balance between pro-inflammatory and anti-inflammatory cytokines in LPS-stimulated monocytes and macrophages. This role of SHP-1 is manifested by its dual role in LPS/TLR4 activated cells. On one hand SHP-1 activation is required for the production of IL-12, IL-23, IL-27 and IL-10 through engagement of the PI3K pathway, on the other hand it also keeps the amount of pro-inflammatory cytokines, IL-12 and IL-23, in check through activation of Src. The SHP-1 dependent conserved Src/JNK complex inhibits the level of TLR4-induced pro-inflammatory IL-12 and IL-23 while also enhancing the levels of anti-inflammatory cytokines, IL-27 and IL-10. Furthermore, maintaining the balance between pro- and anti-inflammatory cytokines by SHP-1 requires recruitment of MyD88 and TRIF- dependent pathways downstream of TLR4

One mechanism contributing to signal transduction is tyrosine phosphorylation, which is the reversible phosphorylation of tyrosine residues found on key signaling proteins.[126] This balance is maintained through the counteractive properties of PTKs and PTPs.[126] PTP SHP-1, was shown to play a critical role in protection against autoimmunity in the murine model.[100] SHP-1 deficient *me/me* mice succumb to severe glomerulnephritis and pneumonitis, at an early age of 10 days.[127] Recently, employing the *me/me* mice, SHP-1 was shown to be a key regulator of IL-6 and IL-21 production in CD4+ T cells resulting in a dampened murine Th17

response.[128] In support of this notion, it was also demonstrated that SHP-1 is a positive regulator of LPS/TLR4-induced IL-6 production in murine bone-marrow derived macrophages.[100] Therefore, the murine studies highlight a critical role for SHP-1 in the production of inflammatory mediators and in the regulation of innate and adaptive immune responses. In view of the results indicating that loss of SHP-1 function results in profound autoimmunity in the murine model, it was of interest to examine if in human mononuclear cells, SHP-1 was also essential in the regulation of the IL-12 family of cytokines, critically implicated in human autoimmune diseases. SHP-1 has been predominantly described as a negative regulator of signal transduction pathways in hemapoietic cells.[107] However, the results of this study clearly demonstrate for the first time that SHP-1 is a critical positive regulator of the production of IL-12, IL-23 and IL-27 in LPS-stimulated human monocytes and THP-1 cells and this function is conserved following the differentiation of monocytes into macrophages. The positive role of SHP-1 in the production of the IL-12 family of cytokines is mediated through the transcriptional regulation of IL-12p40, IL-27p28 and IL-27EBI3 subunits in THP-1 cells. Interestingly SHP-1 was shown to negatively regulate IL-23p19 transcript levels in THP-1 cells, despite positively regulating IL-23 cytokine production. IL-23 is composed of IL-12p40 and IL-23p19, and to be biologically active both subunits must be present in equimolar concentrations in the same cell.[32,129] Therefore, it can be speculated that IL-12p40 may be a negative regulator of IL-23p19 to prevent excessive formation of a subunit that is non-functional without its binding partner. Thus when this negative pressure of IL-12p40 is removed, by the loss of SHP-1 positive regulation, the IL-23p19 subunit would

have increased in transcription as observed. Hence SHP-1 function maintains the stoichometric balance between these two subunits.

Following the recruitment of monocytes into tissues, the monocytes differentiate into two subclasses of macrophages M1 and M2.[17] Studies have shown that SHP-1 positively regulates cytokines that are predominantly produced by the pro-inflammatory M1 macrophages such as IL-6, and my results confirm that SHP-1 positively regulates pro-inflammatory IL-12 and IL-23 in LPS-stimulated MDMs. Interestingly, my results also demonstrate that SHP-1 positively regulates anti-inflammatory cytokines IL-10 and IL-27 in LPS-stimulated MDMs, which are predominantly secreted by M2 macrophage phenotype. Since the loss of SHP-1, as manifested by motheaten mice, leads to profound inflammation associated with systemic sutoimmunity, it is therefore conceivable that one of the function's of SHP-1 in immune cells is to maintain the balance between excessive production of either pro- or anti-inflammatory mediators. SHP-1 may fulfill this function by interacting with signal transduction molecules such as Src to inhibit production of IL-23 and IL-23 and enhance production of IL-27 and IL-10 as shown by this study.

PTPs and PTKs work intimately to regulate many signal transduction pathways.[122] PTKs, represent targets for SHP-1 activity, therefore it was of interest to determine a specific target for SHP-1 phosphatase activity in LPS/TLR4-induced IL-12, IL-23, IL-27 and IL-10 production in human monocytes and macrophages. Of the non-receptor protein tyrosine kinases, the Src family of kinases is the largest comprising of 9 members: Fgr, Fyn, Src, Yes, Blk, Hck, Lck, Lyn and Frk.[111] Of these, Src, Fyn, Fgr, Lyn and Hck are expressed in monocytes and macrophages.[111]

The role of these kinases in the regulation of cytokines has produced conflicting reports. For example, in human macrophages, Hck mediates TLR4-induced transcription of IL-6 and TNFα.[130] In addition, Src kinase was shown to induce TNFα and IL-1β in activated microglia.[131] In contrast, Meng et al. describe that Src family kinases do not play a significant role in LPS-induced signaling, since triple knockout Hck/Fgr/Lyn murine macrophages, responded normally to LPS stimulation.[132] Therefore, it is plausible that Src kinase plays a larger role in LPS-signaling in macrophages compared to its family members. Interestingly, under basal conditions, Src is present in a restrictive state, where Tyr527 in the C-terminus is phosphorylated and binds intramolecularly with the Src SH2 domain.[113] This molecular interaction sequesters the catalytic domain and holds the kinase in an inactive state and a PTP, such as SHP-1 can activate it to promote downstream phosphorylation events.[113] Abram et al. has demonstrated that SHP-1 dephosphorylates Tyr527 in c-terminus of c-Src in human neutrophils.[102] In view of my finding that SHP-1 is critically involved in the regulation of the IL-12 family of cytokines and IL-10, it was of interest to examine if Src is also involved in the regulation of these cytokines. Employing THP-1 cells as a model system, my results demonstrate that, similarly to SHP-1 function, Src also positively regulates the secretion of IL-12, IL-23 and IL-27 and as it has been observed for SHP-1, Src positively regulates the IL-12 family through the positive transcriptional regulation of IL-12p40, IL-27p28 and IL-27EBI3 and negative transcriptional regulation of IL-23p19. However, when the study was expanded by using primary human monocytes Src was shown to differentially regulate IL-12/IL-23 and IL-27. One possible

explanation for the discrepancy between THP-1 monocytes and primary human monocytes, is that THP-1 monocytic cells are generated from leukemia cells, and Src has been known to be highly activated in cancer cells.[133] Nevertheless, based on the involvement of SHP-1 and Src in the regulation of the IL-12 family of cytokine production these findings suggest an intimate relationship between SHP-1 and Src downstream of TLR4 receptor in THP-1 cells.

During an inflammatory episode primary monocytes serve as precursors for macrophage development.[1] In view of the differential role of Src in the regulation of IL-12/IL-23 and IL-27 in LPS-stimulated human monocytes, I have also examined if this differential role of Src is conserved following differentiation of monocytes into MDMs. The role of Src in the differential regulation of the IL-12 family of cytokines was found to be conserved between monocytes and MDMs. Src was shown to negatively regulate production of pro-inflammatory IL-12/IL-23 and positively regulate production of anti-inflammatory IL-27 in LPS-stimulated MDMs. This differential role of Src was mediated through the transcriptional regulation of the subunits. To this effect, Src was shown to inhibit the transcription of IL-12p40 and IL-23p19 while being necessary for the transcription of IL-27p28 and IL-27EBI3. It has been demonstrated that the activation of Src family kinases is gradual involving many activation steps in a sequential process and full activity is only achieved when all activating stimuli are present.[117] My results suggest that Src activity is switch-like.[117] The hallmark of autoimmune disorders is an imbalance of pro- and anti-inflammatory cytokines, more specifically elevated levels of IL-12/IL-23 and decreased levels of IL-27.[37,42] My study implicates that the differential regulation of

IL-12/IL-23 and IL-27 by SHP-1 activated Src was due to its switch-like regulation of pro- and anti-inflammatory axes, since IL-27 is characterized as predominantly an anti-inflammatory cytokine compared to IL-12 /IL-23. Therefore, I also examined the role of Src in the regulation of IL-10, a known anti-inflammatory cytokine. Interestingly, Src was also necessary for the transcription and production of IL-10 in LPS-stimulated MDMs. Therefore, it is plausible that one of the functions of SHP-1 is to balance the production of pro- and anti- inflammatory cytokines through Src which acts as a modulator of the level of IL-12/IL-23 and IL-27/IL-10 in LPS-stimulated MDMS.

SHP-1 and Src have been shown to be critically involved in the production of the IL-12 family of cytokines in THP-1 cells, primary monocytes and macrophages. In addition to this SHP-1 and Src were shown to be critically involved in the production of IL-10 as well. Since SHP-1 is known to interact with cytoplasmic tails of receptor chains and membrane associated proteins, including Src, activity of SHP-1 and Src appear to be proximal in signal transduction.[123] Therefore, it was of interest to determine downstream signal transduction pathways that may include SHP-1/Src activity in the production of the IL-12 family of cytokines. One such pathway known to be activated by TLR4, is the PI3K pathway which has been strongly implicated in inflammatory cytokine production. Activation of the PI3K pathway is required to positively regulate the production of pro-inflammatory IL-6 in airway smooth muscle tissue.[134] However, in various cells of the immune system there is controversy regarding whether PI3K is a negative or positive regulator of the production of IL-12, IL-23 and IL-27. For instance, it has been shown that activation

of the PI3K pathway is necessary for the production of IL-17 pro-inflammatory cytokine in T-cells.[135] Since IL-23 is a mandatory inducer of IL-17, this finding is suggestive of the positive involvement of PI3K in the regulation of IL-23.[37] To this effect, it has been shown that PI3K positively regulates IL-23 production downstream of CCR7 signaling.[136] However, in dendritic cells PI3K has been predominantly described as a negative regulator of IL-12.[137] Therefore, it was of interest to clarify the role of PI3K in the production of IL-12, IL-23 and IL-27 in human mononuclear cells of the immune system downstream of TLR4. This study demonstrates that the activation of PI3K pathway is critical for the production LPS-induced IL-12, IL-23 and IL-27 production in THP-1 cells and primary human monocytes. In addition this function of PI3K pathway was conserved following differentiation of monocytes into macrophages. Interestingly, the PI3K pathway was also shown to be a positive regulator of IL-10 production by LPS-stimulated macrophages. Therefore, like SHP-1, PI3K pathway appears to be critical for LPS-induced cytokine production and could therefore be under SHP-1 regulation in the same signaling axis. This is supported by the findings in THP-1 cells and primary human monocytes, where inhibition of SHP-1 results in decreased activation of Akt, ~~which is~~ a downstream target of PI3K.

MAPK pathways have also been implicated in the production of cytokines, the MAPK family comprises of: ERK, JNK and p38 kinases, which could pose potential targets for SHP-1/Src activity downstream of TLR4 in the induction of the IL-12 family of cytokines.[92] ERK MAPK, as previously described, is most importantly involved in cell survival and proliferation.[94] Specifically, ERK has been shown to

regulate cell death receptor signalling via cellular FLICE-like inhibitory protein (c-FLIP) in ulcerative colitis[138] Therefore, ERK is of interest in the study of molecular targets for cancer therapy. [139,140] The results demonstrate that in THP-1 cells ERK MAPK is not involved in the regulation of the IL-12 family of cytokines, thus the role of ERK MAPK was not further pursued in primary monocytes or MDMs. p38 MAPK has been implicated as a regulator of hemapoietic cell proliferation [95] However, since p38 is activated in certain immune cells, it has been linked with the regulation of some cytokines such as IL-10.[141,142] My results demonstrate that p38 is not involved in the regulation of IL-12, IL-23 and IL-27 in THP-1 cells. Thus the role of p38 MAPK in the regulation of the IL-12 family of cytokines was also not pursued in primary monocytes or MDMs.

In contrast to ERK and p38, JNK MAPKs have been predominantly described within the context of the regulation of pro-inflammatory cytokine production, such as TNFα.[143] The results of this study clearly demonstrate that JNK is a positive regulator for the production of IL-12, IL-23 and IL-27 in LPS-stimulated THP-1 cells. Interestingly, in primary monocytes and MDMs, JNK is differentially involved as a negative regulator for the production of IL-12 and IL-23 pro-inflammatory cytokines and a positive regulator of IL-27 and IL-10 anti-inflammatory cytokines. In accordance with my findings, JNK has been described as a negative regulator of IL-12p40 in LPS-stimulated RAW264.7 macrophage cell line.[144] Moreover, it has also been described that JNK1 and JNK2 differentially regulate IL-12 production in THP-1 cells.[145] It was shown that JNK1 plays a positive role and JNK2 plays a negative role in IL-12 production.[145] Hence, it is possible that THP-1

cells express higher levels of JNK1 and primary mononuclear cells such as monocytes and MDMs express higher levels of JNK2 and this would account for the discrepancy observed in the data. However, interestingly this differential regulation of IL-12/IL-23 and IL-27/IL-10 by JNK is similar to the differential regulation of these cytokines by Src kinase. Therefore, it is conceivable that SHP-1 activated Src and JNK act in the one signaling axis to modulate the amount of pro- and anti-inflammatory cytokines which have been induced through SHP-1/PI3K axis.

Tyrosine phosphorylation of proteins is a widely used signal transduction mechanism in immune cells.[98] In the literature, the interaction of SHP-1 and Src kinases has been described. It has been shown that SHP-1 and Lyn form a complex that regulates B cell receptor signalling.[146] In osteoblasts, SHP-1 was shown to be necessary in the activation of Src, and together SHP-1 and Src were critical for integrin signalling.[147] Furthermore, SHP-1 and Src were shown to complex in the rat hindbrain and this complex was essential for cerebellar development and neuronal differentiation.[148] Interestingly, in dendritic cells, it was demonstrated that SHP-1 and Src are involved in dendritic cell immunoreceptor signalling triggered by HIV-1.[149] Interaction of SHP-1 with Src assumes a conserved and a dominant role in signal transduction regulating many biological responses across many cell types. My results provide evidence for interaction of SHP-1 with Src in LPS-stimulated THP-1 cells and demonstrate that this interaction is increased upon the engagement of TLR4. Moreover, SHP-1 function appears to be required for Src activation, since inhibition of SHP-1 function results in lowered levels of Src phosphorylation in LPS-stimulated THP-1 cells and primary human monocytes. In addition, SHP-1 and Src

form complexes which co-immunofluoresce at the cell membrane in LPS-stimulated MDMs. These complexes can be disrupted by inhibiting SHP-1 or Src function. Taken together these results demonstrate for the first time, that monocytes and macrophages employ SHP-1 and the SHP-1/ Src complex in the induction and modulation of IL-12, IL-23, IL-27 and IL-10 downstream of TLR4.

The SHP-1/Src complex has been implicated in the regulation of multiple downstream signalling pathways.[107,114] One such signaling pathways is the PI3K pathway. In HEK-GHSR1a cells, it was demonstrated that overexpression of SHP-1 resulted in tyrosine phoshorylation of the PI3K regulatory subunit p85, leading to increased levels of pSrc and pPI3K which led to the activation of Akt.[150] Similarly a study in glioma cells confirmed that Akt is a downstream target of Src activity.[151] The results demonstrate that upon LPS-TLR4 engagement in THP-1 cells, SHP-1 and Src are required in the activation of PI3K and Akt. Furthermore, in primary monocytes, SHP-1 and Src were also shown to be necessary in the activation of Akt following LPS stimulation. In the future, this result should be confirmed in MDMs.

Another downstream signalling pathway shown to be regulated by the SHP-1/Src complex is the JNK MAPK pathway. SHP-1 was shown to be an intrinsic global regulator of DC function by modulating LPS signaling through inhibition of NFκB, AP-1, ERK and JNK activity.[152] Furthermore, SHP-1 has also been implicated in the inhibition of JNK activity downstream of TLR9 activation in mouse macrophages.[153] Src was also shown to activate JNK in apoptotic pathways in the mouse model.[154] The Src/JNK pathway has also been implicated in angiotensin II signalling in vascular smooth muscle cells.[155] Indeed, the results of this study

demonstrate that the Src/JNK axis is also conserved in monocytes. The results show that upon inhibition of SHP-1 and Src, levels of pJNK in THP-1 cells and monocytes are also reduced. This result should be confirmed in MDMs. Taken together these results reveal a feedback loop and a dual role of SHP-1, as a mandatory and positive regulator of IL-12, IL-23, IL-27 and IL-10 in addition to SHP-1's role as an activator of Src required to modulate the amount of pro- and anti-inflammatory cytokine production in monocytes and macrophages through the regulation of PI3K and JNK signal transduction pathways downstream of TLR4. (Figure IV.1)

IV.I Future Directions , Significance and Concluding Remarks

Recently, several studies have been directed at the characterization of inflammasomes.[156,157] Inflammasomes are multi-protein complexes of the innate immune system important in initiating inflammatory responses during infection.[158] It has been described that the nature and exact composition of these inflammasomes depends on the microbial activator.[158] This study has provided evidence for the formation of the SHP-1/Src complex which controls the levels of pro-inflammatory IL-12/IL-23 and anti-inflammatory IL-27/IL-10 cytokines. To further understand the nature of this complex, known TLR4-associated proteins were examined by immunoflourescence to determine which of them, together with SHP-1/Src, may constitute a large inflammasome activated by TLR4 leading to the activation of the IL-12/IL-10 axis. Preliminary data suggests that SHP-1 and Src interact with both MyD88 and TRIF. TLR4-MyD88 dependent signaling leads to the activation of MAPKs and NFκB, whereas TLR4-TRIF dependent signaling activates IRF3

Figure IV.I The Working Model for the regulation of the IL-12/IL-23 pro- and IL-10/IL-27 anti-inflammatory axis. Upon LPS stimulation of TLR4, SHP-1 activity is necessary for the activation of Src downstream of MyD88. Subsequently Src/JNK negatively regulate pro-inflammatory IL-12/IL-23 and positively regulate anti-inflammatory IL-10/IL-27. SHP-1/Src complex also interact with TRIF which suggests the activation of the interferon response. When IFNγ is present it inactivates the inhibitory Src/JNK complex to achieve full macrophage activation, an M1 phenotype. Therefore the SHP-1/Src complex may constitute an important switch in the shift of M1 to M2 phenotype.

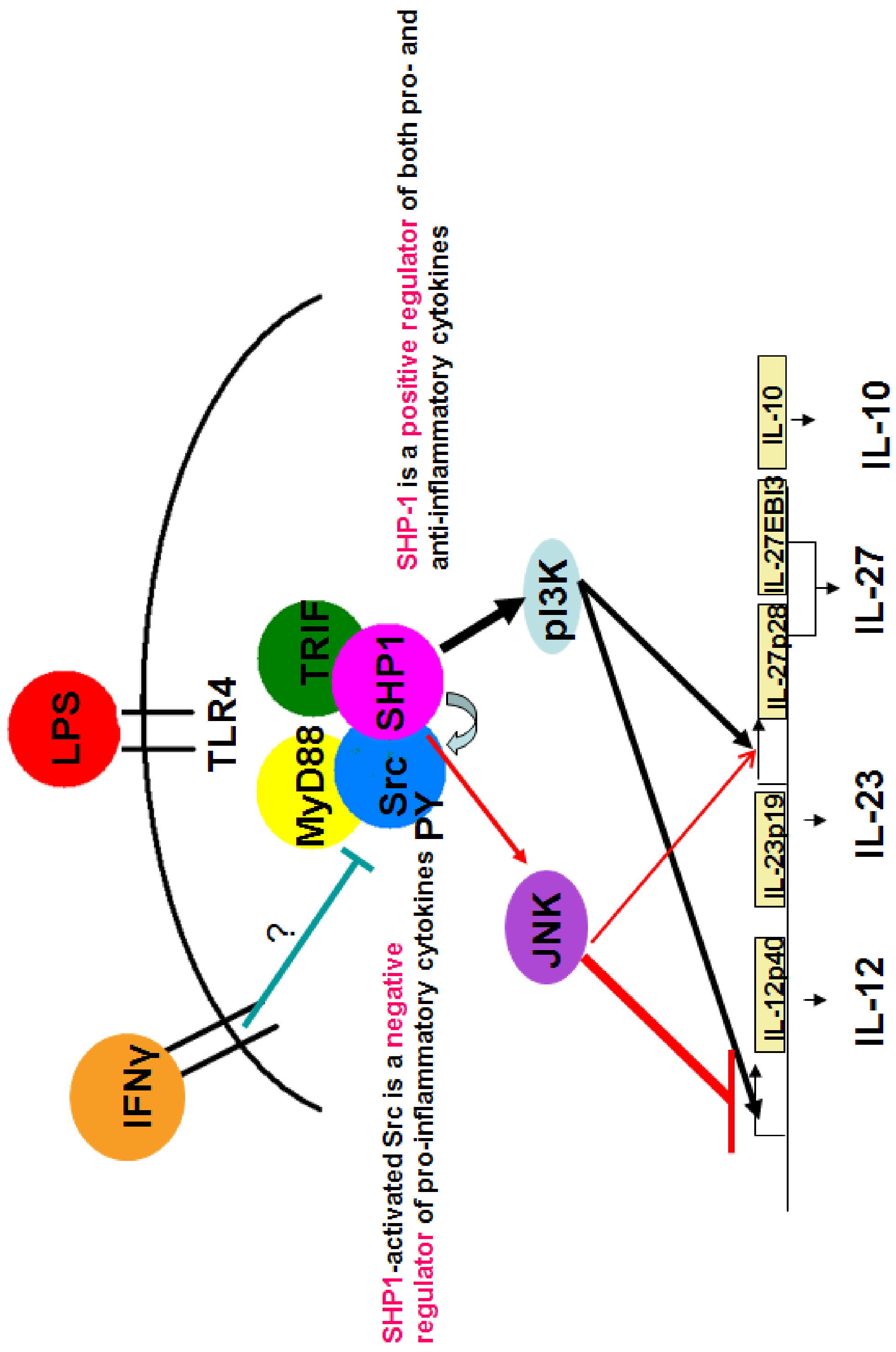


Fig. IV.1

transcription factor to induce type-1 interferon genes.[84] The results show that indeed SHP-1 and Src mediate activation of JNK MAPK. However, the observation that SHP-1/Src also employ TRIF signalling would suggest that SHP-1/Src protein complex also induces IFNγ signalling. This finding establishes a base for further study of the role of IFNγ signalling in the activation of the TLR4-induced Src/JNK signalling pathway, to explain the differential regulation of pro- and anti-inflammatory cytokines. Recently, Xu et al. described that full macrophage activation is driven by cytokine-mediated feedforward loops and excessive activation is prevented by feedback inhibition.[159] It was proposed that IFNγ signalling promotes full macrophage activation by inactivating feedback inhibitory mechanisms.[159] The Src/JNK pathway negatively regulates pro-inflammatory IL-12 and IL-23 while positively regulating IL-27 and IL-10. Therefore it is plausible the IFNγ signalling interferes with the Src/JNK pathway mediated feedback inhibitory mechanisms Further research should be directed at the effect of IFNγ signalling on the activity of Src and JNK. Furthermore, IFNγ has been shown to promote high IL-12 levels and an M1 pro-inflammatory macrophage phenotype.[17] In addition, IFNγ signalling induces IL-27, which in turn has been shown to shut off the Th17 response mediated by IL-23.[160] IL-27 also induces IL-10, which functions to shut down IL-12-mediated inflammation, such as the Th1 response and drives the M2 anti-inflammatory phenotype.[65,161] Therefore, for the first time the findings of this study initiate a base in with to investigate whether IFNγ signalling may constitute the switch between M1 and M2 phenotypes through the activation of the SHP-1/Src complex. Understanding this regulatory switch,

would gain insight into possible therapeutic interventions for the treatment of autoimmune diseases.

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