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SHP-1 Differentially Regulates LPS-Mediated TNF-α and IL-10 Production in Murine Splenic Macrophages

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SHP-1 DIFFERENTIALLY REGULATES LPS-MEDIATED TNF-α AND IL-10 PRODUCTION IN MURINE SPLENIC MACROPHAGES

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ABSTRACT

Host-Pathogen interactions are characterized by inflammatory responses, aimed at eliminating invading pathogens. During inflammatory responses, immune cells secrete cytokines and chemokines, with a broad range of immuno-regulatory roles. The role of the signaling protein, SHP-1 in regulating signaling pathways activated by cytokine, antigen and growth factor receptors has been studied for some time. However, the involvement of SHP-1 in the regulation of Lipopolysaccharide (LPS)-activated signal transduction pathways leading to cytokine production is less well understood. Loss of SHP-1 leads to development of complex inflammatory disorder(s). To understand the role of SHP-1 during the inflammation process, the SHP-1-deficient moth-eaten (*me/me*) mouse model was used to investigate LPS-induced TNF-α and IL-10 production, representing pro and anti-inflammatory cytokines, respectively. Results show that *me/me* splenic macrophages, when challenged with LPS *in-vitro*, secreted lower levels of IL-10 and concomitantly elevated TNF-α levels, compared to normal healthy littermate controls. Deregulated LPS-induced TNF-α and IL-10 production in SHP-1 deficient splenic macrophages were observed irrespective of LPS dose and time of stimulation as demonstrated by ELISA. These findings were further confirmed by RT-PCR, SHP-1 antisense experiments in normal splenic macrophages and adenoviral reconstitution of SHP-1 expression in *me/me* macrophages, suggesting a dual role of SHP-1 as a negative regulator of LPS induced TNF-α and a positive regulator of IL-10.

To delineate the precise role of SHP-1 in regulating LPS-mediated IL-10

production, LPS-activated signaling proteins, which represent SHP-1 targets were examined. Results obtained established a role of p38 MAPK in LPS-mediated IL-10 secretion, but also demonstrate that P38 involvement is independent of SHP-1. Results of this study further reveals a role for tyrosine kinase Src and an adhesion molecule Pyk2 in SHP-1 dependent LPS-mediated IL-10 production and infer that optimal production of LPS-induced IL-10 requires an assembly of a signalosome, consisting of Src-Pyk2-SHP-1 proteins. In conclusion, we show for the first time that: SHP-1 is a positive regulator of LPS-induced IL-10 production in splenic macrophages and, LPS-mediated IL-10 production is governed by two distinct signaling pathways, only one of which is SHP-1 dependent.

DEDICATION

THIS THESIS IS DEDICATED TO MY PARENTS

MR G. E. OKENWA

AND

MRS C.P. OKENWA

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List of Abbreviations

α-	anti
Ab	antibody
AIA	Antigen-induced arthritis
AP1	Activating protein-1
APS	ammonium persulfate
ATP	adenosine triphosphate
BCR	B cell receptor
BMDMs	bone marrow derived macrophage cultures
bp	base pairs
BSA	bovine serum albumin
Btks	Bruton's tyrosine kinase
°C	degrees Celsius
cAMP	cyclic Adenosine monophosphate
CAPE	Caffeic acid phenethyl ester
cDNA	complementary DNA
CO_2	carbon dioxide
Cys	cysteine
DCs	dendritic cells
DD	death domain
DEPC	diethyl pyrocarbonate
DN	dominant negative
DNA	2'-deoxyribonucleotide triphosphate
DTT	dithiotreitol
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetra-acetic acid disodium salt
ELISA	Enzyme linked immuno-sorbent assay
EpoR	erythropoietin Receptor
ERK	extracellular-signal regulated kinase
FAK	Focal adhesion kinase
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FL	full length
fLMP	N-formyl-methionyl-leucyl-phenylalanine
FLS	fibroblast like synoviocyte
x g	force of gravity
GFP	green fluorescent protein
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte-macrophage colony stimulating factor
GPI	glycosylphosphatidylinositol
Grb2	growth factor receptor-bound protein 2
GSF	granulocyte-stimulating factor
GTP	guanosine triphosphate
Hcph	hematopoietic cell phosphatase

HCl	hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
h	hour
HRP	horseradish peroxidase
IBD	inflammatory bowel disease
IFN-αβ	interferon alpha beta receptor
IFN-γ	interferon-gamma
Ig	immunoglobulin(s)
IκB	inhibitor-kappa B
IL-	interleukin
IL-1Ra	IL-1 receptor antagonist
IP	immunoprecipitate/immunoprecipitation
IRAK	IL-1R associated kinase
ITIMs	immunoreceptor tyrosine-based inhibitory motif(s)
JAK	Janus associated kinase
JNK	c-Jun N-terminal kinase
JCA	juvenile chronic arthritis
Kb	kilobase
KCl	potassium chloride
kDa	kilodalton
LCM	L929 cell-condition medium
LBP	LPS binding protein
LIF	Leukemia inhibitory factor
LPS	Lipopolysaccharide
M	molar
mAb	monoclonal antibody/antibodies
MAPK	mitogen associated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPK Kinase kinase
MCP-1	Monocyte chemoattractant protein 1
M-CSF	macrophage colony-stimulating factor
Me/me mice	moth-eaten mice
Me^v/me^v mice	moth-eaten viable mice
2-ME	2-mercaptoethanol
MEK1	MAPK activating kinase
mg	milligram(s)
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulfate
min	minute(s)
MIP-2	macrophage inflammatory protein-2
mL	milliliter(s)
mM	millimolar
MKK3	MAP kinase kinase kinase
MOI	multiplicity of infection
mRNA	messenger Ribonucleic acid
MS	Multiple sclerosis

μg	microgram
μL	microlitre
μM	micromole
Na_2CO_3	sodium carbonate
NaCl	sodium chloride
NaF	sodium fluoride
$NaHCO_3$	sodium bicarbonate
NaN_3	sodium azide
NaOH	sodium hydroxide
$NaPO_4$	sodium phosphate
NIK	NF-κB inducible kinase
NF-κB	Nuclear factor kappa B
NK cells	Natural killer cells
nm	nanometer(s)
NP-40	nonidet P-40
NO	nitric oxide
NRTKs	nonreceptor tyrosine kinases
nt	nucleotides
ODNs	oligodeoxynucleotides
PAF	platelet activating factor
PBS	phosphate buffered saline
PCR	polymerized chain reaction
PE	phycoerythrin
PH	plekstrin homology
PHA	phytohaemagglutinin
PI3K	phosphotidyl inositol-3-kinase
PMSF	phenyl methyl sulfonyl fluride
PKB	protein kinase B
PTC	Peltier Thermal Cycler
PTEN	phosphatase and tensin homolog
PTK	protein tyrosine kinase
PTP	protein tyrosine phosphatase
Pyk2	proline-rich tyrosine kinase
RNA	ribonucleic acid
RT	room temperature
RF	Rheumatoid factor
RTKs	receptor tyrosine kinases
RT-PCR	Reverse transcriptase polymerase chain reaction
SAPK	stress activated protein kinase(s)
SD	standard deviation
SE	standard error
SFK	Src family kinases
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate poly acrylamide gel elctrophoresis
sec	seconds
Ser	serine

SH2	*Src*-homology domains 2
SHP-1	SH2 domain containing phosphatase-1
SHP-2	SH2 domain containing phosphatase-2
SLO	Streptolysin *O*
Sp1	Specificity protein 1
Src	Src member of the SFK discovered in Sarcoma virus.
SS	side scatter
SQ-RTPCR	Semi-quantitative (real time) RT-PCR
SRE	serum responsive element
STAT	signal transducers and activators of transcription
TAE	Tris acetate-EDTA buffer
TAK1	Transforming growth factor-β activated kinase 1
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
TLR	toll-like receptor
TNF-α	tumor necrosis factor-alpha
TRAF	TNF receptor associated factor
TRAM	TRIF related adaptor molecule
Tyr	tyrosine
U	unit(s)
UV	ultraviolet
V	volts
vs	versus
v/v	volume with respect to volume
WT	wild-type
w/v	weight with respect to volume
Y	Tyrosine
ZAP-70	zeta-chain associated protein-70

I. GENERAL INTRODUCTION

I.1 INNATE IMMUNITY AND INFLAMMATION

Innate immunity collectively describes a host's first line of defense against infection and other insults [1-3]. The innate immune system, although ancient, is a universal form of host defense whose recognition of pathogen(s) depends on germline encoded receptors [1, 2, 4-6]. Innate immune cells include macrophages, neutrophils, mast cells, dendritic cells, eosinophils and natural killer cells [1]. In contrast to the innate immune system, the adaptive immune system involves antibody producing B lymphocytes as well as T helper and cytototoxic lymphocytes [3, 7].

Inflammation is a complex but organized normal and beneficial response of innate immune cells to tissue damage, injury or infection [8]. The phenotypical hallmarks of inflammation include redness (*rubor*), heat (*calor*), swelling (*tumor*) and pain (*dolor*) and in pathological states, loss of function (*functio laesa*) [9, 10]. The entire innate immune inflammatory response process occurs in four basic but progressive stages. These are characterized by; identification or recognition of infection/injury, recruitment of innate immune cells to the site(s) of infection/injury, elimination of trigger by activated innate immune cells and finally resolution of inflammation [9, 11, 12]. The degree and duration of inflammation is largely dependent on the nature of the injury and/or microbe responsible for inflammatory trigger [13]. Although these triggers activate common innate immune receptor signaling pathways, their effects differ, at least in the specific set of genes they induce [14, 15]. Differential gene induction following a specific inflammatory trigger may

lead to escalation of inflammatory responses, abnormal or prolonged responses or the resolution of inflammation [16].

I.2 ACUTE AND CHRONIC INFLAMMATION:

Inflammation is a beneficial set of events induced by the host to heal itself. However in certain circumstances where inflammatory responses are prolonged, inflammation may develop into a pathological condition [17-21]. Inflammatory responses are therefore distinguished by the duration of inflammation and histopathological composition of the inflammation site [8, 22]. Based on these criteria inflammation is broadly divided into acute and chronic inflammation.

Acute inflammation is simply the early phase of normal inflammatory response and is characterized by vascular events and cellular (leukocyte) events [4, 8, 23]. It is also characterized by the cardinal signs of inflammation, which disappear when the inflammatory trigger is removed or resolved by inflammatory response [3, 4, 7]. Acute inflammation is generally of short duration, a time required to resolve the inflammatory trigger. If unresolved by the innate immune system, acute inflammation evolves to activate the adaptive immune system [22, 24].

In contrast, chronic inflammation occurs over an extended time, is predominantly observed in disease conditions and often arises due to the persistence of acute inflammatory responses or in certain cases can be attributed to unresolved and persistent microbial infection [18, 20, 25]. Antigenic mimicry by microbial pathogens or initiation of immune response to tissue originally containing pathogens give rise to autoantibody production [26] or chronic immune stimulation and may result in

secondary tissue damage [27-30]. This damage in turn elicits acute phase responses that likely continue unresolved due to recognition of self-antigens [31, 32]. In certain instances, prolonged and abnormal interactions between innate immune responses and cell-mediated or humoral adaptive immune responses underlie chronic inflammation [28, 33]. Autoimmune conditions such as rheumatoid arthritis and systemic lupus erythematosis (SLE) are classic examples of chronic inflammatory conditions where self-recognition elicits autoantibody production [34, 35]. The recognition and interaction of autoantibodies or their complexes with innate immune cells lead to sustained and unresolved inflammatory responses [34, 36]. It is therefore very important to distinguish chronic disease underlying inflammation from a normal and beneficial acute inflammation.

I.2.1 Initiation of Acute Inflammation:

The most important event in initiation of inflammation is the recognition of infection, tissue damage or injury [12]. Inflammatory responses usually occur in optimal and coordinated sequences of events starting out initially with a contraction of smooth muscles around large blood vessels, thus slowing the flow of blood at the site of trauma [37-39]. This is followed by a contraction of the endothelial cell lining on the walls of small blood vessels increasing vascular permeability and resulting in increased blood flow, observed as swelling, redness and heat [38, 40, 41]. The contraction of capillary endothelial cells leads to extravasations of water and plasma proteins resulting in swelling at the site of inflammation [40, 42]. Increased blood flow also enhances the infiltration of effector cells to sites of injury and surrounding tissue

[4, 43]. The cellular events include margination, where polymorphonuclear neutrophils exit vascular beds, get activated, and increase the expression of integrin molecules [4, 44]. Integrins facilitate attachment of neutrophils to endothelial walls allowing rolling by diapedesis into extravascular tissues [23, 44-47]. In the extravascular space, neutrophils and leukocytes respond by chemotaxis to a concentration gradient of chemokines secreted by mast cells and monocytes and sometimes degranulating cells resident at the site of trauma [48-50].

Polymorphonuclear neutrophils are the first set of cells to arrive *en masse* at the site(s) of trauma [51, 52]. Recruited polymorphonuclear neutrophils then secrete defensins and prostagladin E2 to recruit monocytes and lymphocytes such as NK cells [52-55]. Initial activation of these cells increases the surface expression of integrin molecules facilitating diapedesis and ultimately directing the influx of lymphocytes toward the inflammatory site [44, 47]. Arrival of lymphocytes results in increased cytokine production required to activate and induce the expansion of monocytes and macrophages [56, 57]. Upon arrival at the site of trauma, effector cells phagocytose foreign particles and some activated effector cells degranulate by secreting cytotoxic materials [51, 53, 58]. This is followed by a recruitment of antigen presenting cells (APCs) and the beginning of adaptive immune response [58]. The coordinated arrival of inflammatory cells is important for a successful inflammatory response, which is regulated by rapidly induced inflammatory mediators that coordinate and activate inflammatory cells [59, 60].

I.2.2 Inflammatory Mediators:

A coordinated recruitment of cells in conjunction with both direct and indirect cellular communication among innate immune cells is essential for the initiation, execution and resolution of inflammation [8, 61, 62]. Usually, communication between cells during inflammation is guided by signals induced by inflammatory mediators that are produced by innate immune cells [60]. Inflammatory mediators are principally subdivided into exogenous and endogenous mediators of inflammation [63]. Exogenous mediators of inflammation are components of invading microbial pathogens such as microbial endotoxin and other pathogen associated molecular motifs (PAMPs). Endogenous mediators of inflammation are usually produced by innate or adaptive immune cells and will be explored further.

Endogenous inflammatory mediators are soluble-diffusible molecules capable of acting both locally at the site of tissue damage and infection and at distant sites and can regulate cell activation, migration and recruitment, deactivation, apoptosis and other cellular activities that occur during inflammation [60, 63]. Inflammatory mediators can be classified into four categories; (a) lipid mediators of inflammation such as leukotrienes, lipoxins and prostaglandins, (b) protective enzyme and coagulation cascades, such as bradykinnin, serotonin in rodents and histamine in humans, (c) the complement system(s), especially the versatile complement cleavage products; C3a and C5a, and (d) cytokines and chemokines [63-65].

Lipid mediators of inflammation originate from the degradation of intracellular membrane phospholipids to arachidonic acid in activated cells [64, 66-68]. Arachidonic acid is then further degraded to leukotrienes, lipoxins and prostaglandins [66]. The lipid mediators of inflammation play roles in recruitment of cells

(leukotrienes), in vascular permeability and platelet activation (prostaglandins), in microcirculation and in vasoconstriction (lipoxins) [63]. Prostaglandins and other byproducts of arachidonic acid degradation also mediate other diverse functions in innate immune responses [63, 69].

The protective enzyme cascades are made up of proteins mainly involved with regulating vascular permeability, vasodilation, and pain [37, 63, 64, 66, 70-72]. A prominent member of this group, histamine, is stored in granules of platelets, tissue mast cells and basophils and is released upon cell activation [70, 73]. Histamine is also associated with local vascular permeability, chemotaxis of eosinophils, arteriolar dilation and arterial contraction [63, 74-76]. However, its precise function is dependent on the respective receptor with which it interacts and/or the receptor types expressed on a responding cell type [77, 78]. Coagulation cascades are made up of plasma enzymes produced by activated endothelial cells that trigger complex intrinsic and extrinsic pathways in response to damaged or disrupted blood vessels [71, 79-82]. These intrinsic and extrinsic pathways both converge at factor X, which plays a role in a common pathway that leads to thrombin production [80-82]. Thrombin is required for the conversion of factor XIII to factor XIIIa, essential for the formation of insoluble fibrin aggregates [83, 84]. Insoluble fibrin aggregates combined with platelets form clots and create physical barriers that prevent continuous bleeding and most importantly, restrict access of microbes [84-86]. This is one of the earliest steps directed at resolving extended inflammation.

The complement system is composed of several small proteins (~ >21 kDa) in the blood normally existing as inactive zymogens [4, 87]. Inflammatory triggers

activate proteases, which cleave specific complement proteins resulting in the activation and amplification of cleavage cascades [87]. There are three known biochemical cascades that activate the complement system, namely; the classical pathway, the alternative pathway and the mannose-binding lectin pathway [4, 87]. The end product of these cleavage cascades is the formation of the membrane-attack complex on the cell surface of intruding pathogens [87], resulting in the targeted killing of pathogens [88].

The key mediator of a given inflammatory response is largely dependent on the nature of the inflammatory trigger [25]. For example, inflammation triggered by injury predominantly elicits protective enzyme and coagulation cascades whereas inflammation triggered by infection initially evokes pro-inflammatory cytokine and chemokine responses. Despite these differences, the pro-inflammatory cytokine, tumor necrosis factor-alpha (TNF-α) is normally secreted very early during the acute phase in all types of inflammation, irrespective of inflammatory trigger [89-92]. Cytokines are essential components in the inflammatory response process. During inflammation, tissue macrophages migrate to the sites of injury following a gradient of chemokines secreted by resident cells at the site of injury [93-100].

1.2.3 Macrophages

Macrophages originate from the myeloid cell lineage and are released as monocytes from the bone marrow into the blood [101-104]. Monocytes migrate to respective tissues where they differentiate and become tissue-resident macrophages, for example, splenic macrophages, Kuppfer cells and alveoli macrophages are found

in the spleen, liver and lungs, respectively [105, 106]. Macrophages play a central role in the secretion of cytokines during inflammatory response because they are the predominant source of cytokines [107, 108].

I.3.0 CYTOKINES AND INFLAMMATION:

Cytokines are a collection of small, water soluble, signal-inducing proteins or glycoproteins produced by cells to function as chemical messengers in regulating the activities of the same cell or different cells within the innate or adaptive immune system [109-112]. Cytokines are low molecular weight (8 to 30kDa) polypeptides, which are structurally stabilized by *N*-linked and/or *O*-linked glycosylation and intramolecular disulphide bridges [113, 114]. Cytokines play significant roles in intercellular communication by binding to cell surface receptors and initiating signal transduction cascades that result in the induction of genes whose products are directed towards specific cellular activities [60, 65, 113, 114]. The activation of specific signaling cascades arising from interaction of cytokines with their cognate receptors is dependent on; (a) the type of cytokine, (b) the cytokine receptor, (c) the cell type/lineage and (d) the microenvironment [115]. As a general rule, most cytokines tend to be pleiotropic in nature exhibiting multiple biological roles such as; growth and differentiation, migration, survival, and activation of effector cells in the innate and adaptive immune systems [114]. Other functions include induction of apoptosis and deactivation of effector cells [116]. Pleiotropic properties of cytokines can be ascribed to the availability of more than one receptor type for a cytokine or the expression of the same cytokine receptor on multiple cell types [115]. This may lead

to the activation of multiple, yet distinct, signaling pathways by the same cytokine, consequently triggering divergent cellular responses [115]. The pleiotropic nature of cytokines may also be attributed to the redundancy of cytokines, with two or more cytokines eliciting very similar biological responses. For example, the cytokines IL-2 and IL-4, secreted by T cells, both act as growth factors for activated T cells [117]. Although two cytokines may have a common function, the magnitude of the response induced may be further modulated by factors such as the differentiation stage of the responding cell and signal transduction cascade(s) engaged by the individual cytokine receptor in the target cell [115]. Cytokines can exert their effects in an autocrine manner by acting on the cytokine-secreting cell or can act on nearby cells in a paracrine manner or cells located distant from the sites of secretion in an endocrine manner [116]. In inflammation, cytokines can be identified by their ability to exert pro-inflammatory and/or anti-inflammatory effects important in the development and resolution of inflammation, respectively [60].

I.3.1 Pro-inflammatory cytokines

Pro-inflammatory cytokines are produced and secreted predominantly by activated innate immune cells such as monocytes and macrophages and promote the progress of acute inflammation [25, 118]. The pro-inflammatory cytokines activate signals that induce secondary messengers, including other cytokines [65]. Although the activation of cytokine genes in response to external stimuli during inflammation is short-lived, the effects induced by cytokines secreted by these cells can be long lasting through the amplification of early and initial signals induced by inflammatory triggers

[119, 120]. For example, amplification of initial trigger signals is essential to induce sufficient amounts of anti-microbial mediators required to clear microbes [121]. Pro-inflammatory cytokines include; TNF-α, IL-1α and IL-1β, IL-6, IL-8, IL-12, IL-17 and IL-18 [118, 122, 123]. TNF-α and IL-1 are the well-studied pro-inflammatory cytokines that mediate acute inflammation as observed in septic shock models of inflammation [89, 91, 121]. Both TNF-α and IL-1 induce fever, prostaglandins and acute phase proteins (from the liver) during the acute phase of inflammation [65, 123]. If unregulated, the continued production of pro-inflammatory cytokines usually leads to chronic/persistent inflammation associated with cell and tissue destruction and most often, results in chronic inflammatory diseased state(s) [118].

I.3.2 Anti-inflammatory cytokines

Anti-inflammatory cytokines are biological response modifiers with immuno-regulatory functions that can modulate inflammatory responses by regulating the unwarranted production and activity of pro-inflammatory cytokines [115, 118, 124]. Anti-inflammatory activity can be induced not only by anti-inflammatory cytokines such as IL-1Ra (Interleukin-1 receptor antagonist), IL-4, IL-10, IL-13 and TGF-β, but also by soluble cytokine receptors with antagonistic properties such as soluble TNF receptor p55 (sTNF-R1) or antibodies to pro-inflammatory cytokines such as anti-TNF-α [122]. IL-10 is one of the most prominent anti-inflammatory cytokine known to inhibit the production of pro-inflammatory cytokines by macrophages, neutrophil and Th-1 lymphocytes [125-127]. The inhibition of pro-inflammatory cytokine production by IL-10 indirectly attenuates the progress of inflammation and directs the

response toward resolution of the inflammatory episode [128, 129]. Most cytokines with anti-inflammatory activity are produced in the late phase of acute inflammatory responses and are often produced in high levels at the sites of inflammation [64, 66, 130]. In certain instances, anti-inflammatory cytokine production can be induced by pro-inflammatory cytokines using a regulatory feedback mechanism [64]. However, the induction of anti-inflammatory cytokines by pro-inflammatory cytokines can be specific to a cell type and/or differentiation state of the cell. For example, *ex-vivo* treatment with large amounts of TNF-α induces IL-10 production in dendritic cells but not in macrophages, although both cell types are of myeloid origins [131].

Among other functions of anti-inflammatory cytokines, the deactivation of innate immune cells triggering the resolution of inflammation appears to be their most important function [56, 120, 124]. The net effect of an inflammatory response is largely dependent on the balance between pro-inflammatory and anti-inflammatory cytokines [122]. Therefore, a deregulation in the production or activity of one set of cytokines may upset the delicate balance required for the initiation and/or resolution of an inflammatory episode and ultimately, elimination of inflammatory triggers.

I.4.0 TUMOUR NECROSIS FACTOR- ALPHA (TNF-α)

I.4.1 Regulation of Inflammation by TNF-α

TNF-α belongs to the TNF superfamily of cytokines produced in response to inflammatory stimuli including, bacterial lipopolyssacharide (LPS) [89, 91, 114]. TNF-α is secreted early in the acute phase of microbe-induced inflammation, where its production by mast cells and macrophages elicits the subsequent production of IL-

1, IL-6 and IL-8 (MIP-2 in mice) [65]. TNF-α-induced synthesis of these chemical messengers amplifies and extends inflammatory responses by evoking a cascade of cytokines with overlapping functions [31, 120, 132]. Complex synergistic interactions between TNF-α and IL-1 or IL-6 on vascular endothelial cells induce the expression of adhesion receptors [65, 118, 133]. Adhesion receptors expressed on vascular endothelial cells interact with integrin molecules expressed on blood leukocytes, tethering them on the surface of blood vessels and initiating a rolling motion (diapedesis) along the surface of blood vessels [4, 25, 63, 134]. Diapedesis culminates in the recruitment of cells along a chemokine gradient to the site(s) of injury [134-137]. TNF-α induction of IL-8, a chemotactic signal for neutrophils, directs the influx of neutrophils to the sites of inflammation where they are activated by secondary inflammatory mediators [138, 139]. In sepsis, endotoxin-induced production and release of large amounts of TNF-α by innate immune cells has been associated with systemic effects such as fever and hypotension, the hallmarks of septic shock [115, 118, 123]. In chronic inflammatory conditions, the perpetual production of TNF-α has been linked to cachexia and the induction of certain types of cancers [140-143]. Excessive and uncontrolled secretion of TNF-α has been implicated in autoimmune conditions such as rheumatoid arthritis, psoriatic arthritis and Crohn's disease[132, 144].

I.4.2 Cellular sources of TNF-α (Induction and Production)

TNF-α is generally not stored in cells but is synthesized following cell stimulation and activation [113, 114, 145-147]. TNF-α is synthesized by a number of

different cell types including; monocytes and macrophages, natural killer cells, lymphocytes, $CD4^+$ T cells, glomerular mesangial cells, astrocytes, fibroblasts, smooth muscle cells, microglia and Kupffer cells [114]. The induction of TNF-α is mediated by numerous ligands such as endotoxins, lipid A, immune complexes, C5a anaphylatoxin, IL-1, IL-2, GM-CSF, interferons and TNF-α itself [113, 148, 149]. Other inducers of TNF-α include; phorbol esters, phytohemagglutinin (PHA), inhibitors of cyclooxygenase, platelet activating factor (PAF) and bradykinins [146, 147]. Inihbitors of TNF-α induction include; cyclosporine A, vitamin D3, PAF antagonists, TGF-β, Prostaglandin E2, dexamethasone and IL-10 [124, 142, 146, 147].

I.4.3 Protein characteristics of TNF-α

Human TNF-α exists as a non-covalently linked trimeric molecule [150, 151]. Each subunit is a 157 amino acid, single polypeptide protein with a single intra-molecular disulphide bridge and a molecular weight of 17 kDa [152, 153]. Human TNF-α is not glycosylated, contrasting with the *N*-glycosylated murine TNF-α, however, human TNF-α retains 76% homology to murine TNF-α [145, 154]. TNF-α is produced as a pro-peptide of 223 amino acids (26 kDa). This pro-peptide is activated by cleavage of the 76 amino acid signal peptide sequence, producing a secreted soluble 17 kDa TNF-α polypeptide [145, 150, 153]. The 26 kDa pro-peptide is a transmembrane or cell-associated form of TNF-α expressed in macrophages and plays a significant role in cell-mediated cytotoxicity to TNF-α sensitive targets [132, 145, 152, 154]. TNF-α is 30% homologous to TNF-β and both are capable of forming trimers, an interaction required for efficient signaling through their respective cognate

receptors [149]. Structural analysis of the TNF-α monomer at 0.26-nm resolution using X-ray crystallography shows that TNF-α forms an elongated, anti-parallel β-pleated sheet sandwich with a "jelly roll" topology [155]. Three TNF-α monomers associate symmetrically to form a compact bell shaped trimer and the base of this trimer appears to be the site for interaction with TNF receptors [156] (See Fig I.1A). Two well-characterized receptors exist for TNF-α; TNFR1 (p55) and TNFR2 (p75) with a molecular weight of 55 kDa and 75 kDa, respectively. TNFR1 and TNFR2 have been recently renamed CD120a and CD120b, respectively. Both receptors bind and mediate TNF-α and TNF-β signals [145, 151-153, 156]. TNFR1 and TNFR2 receptors are part of a 29-member family of nerve growth factor/TNF-receptor gene family and are expressed at high densities (approx. 500-10,000 receptors per cell) on all somatic cells except erythrocytes [145, 152]. Like most receptors, the cellular response to TNF-α is regulated by cell surface expression of TNF-receptors. TNF-receptors are increased in cells treated with IFN-γ and their cell surface expression decreases following binding of TNF-α [157]. TNFR1 and TNFR2 are characterized by extracellular NH2 termini and cysteine-rich subdomains [145]. TNFR1 has an extracellular domain with 182 residues containing 24 cysteine sites, a transmembrane and an intracellular proline-rich region with 21 and 223 residues, respectively. TNFR2 has a larger extracellular domain with 235 amino acids including 22 cysteine residues followed by an extracellular proline-rich region consisting of 39 amino acids, a possible transmembrane domain with 21 amino acids and an intracellular region that spans 220 amino acids [145] (see Figure 1.1B). There is no sequence homology

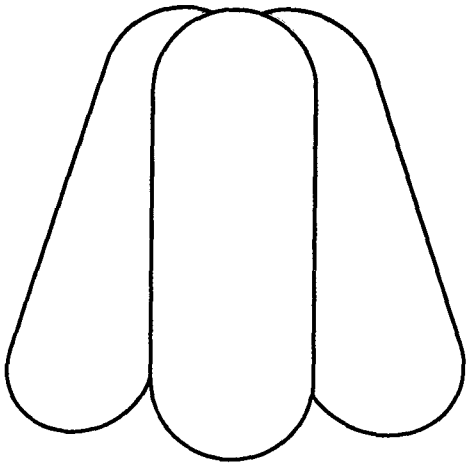
Fig I.1 Model showing a schematic representation of TNF-α and a simplified representation of the TNFR1 and TNFR2 receptors

(A) Functional TNF trimer showing The "Jelly roll" topology consisting of three TNF-α monomers.

(B) Diagramatic representation of TNFR1 and TNFR2 receptors highlighting the differences in their structural organization. Numbers beside each diagrammatic representation denote amino acid numbering and positioning.

Note the significant differences in the positioning of the transmemberane region between receptors.

(A)



(B)

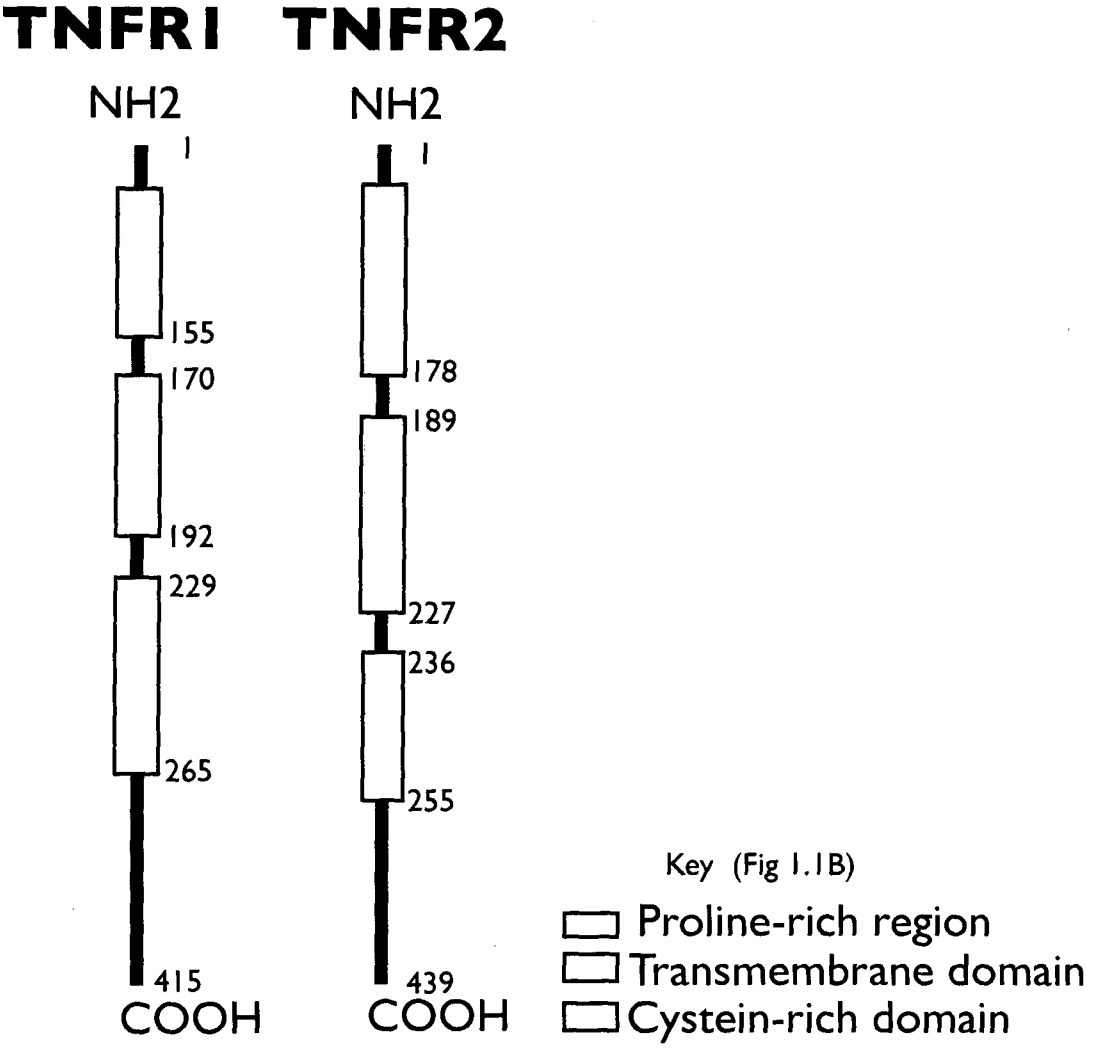


Fig 1.B Modified from Cammussi G., et al. Eur. J. Biochem (1991) with permission from Wiley-Blackwell Publishing. (See Appendix)

between the intracellular domains of TNFR1 and TNFR2, however both receptors activate signal transduction pathways by recruiting cytosolic proteins [145]. The presence of high concentrations of TNF-α directs signaling through TNFR1 [158, 159]. TNFR1 has been shown to mediate TNF-α signals that result in tissue injury, apoptosis and pro-inflammatory activities [159-161]. In contrast, the outcomes of TNFR2 signaling are not fully known, however, a few findings suggest that TNFR2 mediate signaling cascades that are responsible for tissue repair and angiogenesis [133, 146, 147, 152, 154].

I.4.4 Biological activities of TNF-α

TNF-α was originally discovered because of its cytotoxic effect on tumor cells and is now known to play multiple roles in diverse biological events such as cell proliferation, differentiation and apoptosis [152-154, 157]. These biological events are mediated by two TNF receptors that initiate differential signaling cascades in response to TNF-α binding. The biological effects exerted by TNF-α are dependent on; the relative concentration of TNF-α, the exposure interval and synergistic properties with other mediators (such as IL-1) within a defined microenvironment [152, 157]. TNF-α primarily modulates the production of IL-1, IL-6, IL-8 (and IL-10 in dendritic cells) and is central in regulating inflammation, septic shock, tissue remodeling, infection and immunity, cytotoxicity and cachexia [132, 133, 140, 146, 147, 152].

TNF-α induced necrotic cell lysis is typified by, distended cytoplasmic compartments, disintegration of organelles, nuclear chromatin clumping and disruption of cell membranes [162, 163]. These cytotoxic activities have been

described during the acute phase of innate immune response [164]. In conjunction with interferons, moderate amounts of TNF-α contribute to the resistance to viral infections by enhancing defenses of uninfected cells and by initiating selective cytotoxicity toward infected cells [157, 161]. TNF-α also induces lipolysis and glycogenolysis in adipocytes and skeletal myocytes [165, 166]. Hepatocytes express a modified third TNF-receptor and treatment of hepatocytes with TNF-α upregulates the production of acute phase proteins and enhances glucagon-induced amino acid uptake [145, 159, 160]. In human diploid fibroblasts, TNF-α functions as a growth factor by promoting the production of prostaglandin E2 and collagenase [167]. In endothelium, TNF-α in combination with IL-1 induces significant changes such as promoting thrombocytic processes and inhibiting anti-coagulatory activities [168]. In addition to mediating the above listed effects and, based on numerous studies on the role of TNF-α, it is noteworthy to mention that TNF-α mediated effects are largely dependent on cell lineage, level of TNF-α receptor expression and signaling proteins expressed in a given cell activated by TNF-α[152]. Since TNF-α has pleiotropic effects on a number of different cells, this dissertation will focus on its hematological and innate immune functions.

I.4.5 Mechanisms of TNF-α association with disease

During normal inflammatory responses, TNF-α is produced as a primary mediator of inflammation and is responsible for inducing secondary mediators and initiating secondary events that promote inflammation [132, 140]. Persistent and unregulated production of TNF-α by macrophages and/or anomalous cells results in

incessant stimulation and constitutive activation of immune cells [132, 142, 146, 147]. Chronic TNF-α production is associated with continuous production of pro-inflammatory mediators, nonspecific destruction of self-cells by necrosis, activation of apoptotic cascades and sometimes, cachexia [35, 65, 120, 140, 141, 152]. These events are hallmarks of chronic inflammatory disorders and are generally observed in conditions such as septic shock and autoimmune diseases such as; rheumatoid arthritis (RA), psoriasis, multiple sclerosis and Crohn's disease [35, 45, 132, 142].

Septic Shock: Septic shock is a condition resulting from pathogenic microbe(s) entering the bloodstream (sepsis) [169]. Bacterial infection of the bloodstream results in a drastic drop in blood pressure consequently leading to multi-organ failure as a result of poor blood supply and in severe cases may be fatal [169-172]. Other hallmarks of septic shock include increased pulse, irregular heart rhythm and fever induced as a result of toxins and cytokines produced upon interaction of innate immune cells with the pathogen [170-172]. TNF-α represents one of the earliest cytokines secreted into the blood stream signaling the presence of pathogenic Gram-negative bacteria, and has been found in the blood streams of a majority of patients with acute forms of septic shock [18, 30, 123, 132, 171, 172]. TNF-α has also been demonstrated in many animal model studies to mediate the key symptoms of septic shock [123]. For example, the infusion of recombinant TNF-α in canine models triggered cardiovascular changes similar to those observed in human septic shock [173]. TNF-α mediates other effects observed in septic shock such as; hypotension, metabolic acidosis, headache, myalgia, nausea and multi-organ failure [118, 123, 171, 172]. The role of TNF-α in septic shock was further supported by studies showing that

treatment with anti-TNF-α monoclonal antibodies protected animals from shock, organ failure and death [174].

Rheumatoid Arthritis: Rheumatoid arthritis is a chronic inflammatory autoimmune disorder of the joints [34, 175-177]. It is characterized by hyper-proliferation and inflammation of the synovial tissue, triggering an infiltration of immune cells to synovial tissue and consequently resulting in a cumulative erosion of the cartilage and bone [34, 175, 176]. Animal models of arthritis show the initiation and progression of rheumatoid arthritis is dependent on the ability of TNF-α to transform resident synoviocytes into invasive fibroblast-like synoviocytes [34, 175, 176, 178]. The plasticity of synovial fibroblasts allows their transformation by TNF-α, possibly resulting in increased proliferation and deregulated invasion of cartilage tissues and bones [132, 176, 179-181].

Although chronic TNF-α production is central to the pathogenesis of these diseases, the suggestion that chronic TNF-α production is the main aetiology is, in-fact, erroneous [132, 142, 175, 179, 180, 182]. However, it is noteworthy to mention that certain anti-TNF-α therapies such as soluble TNF-receptors and TNF-α antibodies show remarkable efficacy in ameliorating septic shock, RA, Crohn's disease and psoriasis, suggesting that TNF-α is essential for disease progression [142, 182, 183]. Some reports suggest that the likely aetiologies of these chronic inflammatory conditions are self immune-complexes whose constitutive interaction with innate immune receptors elicits persistent TNF-α production [26-30, 35, 175]. Persistent TNF-α production induces irrepressible production of other pro-inflammatory mediators resulting in cell and tissue damage [28, 34, 35, 154, 179]. Due

to the nature of the autoimmune triggers (for example, self-immune complexes) it is difficult for the innate immune system to resolve the inflammatory episode [26, 27, 29, 30, 35]. Often, the failure to eliminate the aetiology underlying chronic inflammation engages the cell-mediated and humoral adaptive immune responses[18, 65, 108, 166], consequently complexing the mechanism(s) of the disease.

I.5.0 INTERLEUKIN 10 (IL-10)

Interleukin-10 (IL-10) is an anti-inflammatory cytokine that normally regulates the high levels of TNF-α secreted during inflammation [124-126, 129, 184]. IL-10 was first discovered as an unknown substance, secreted by Th2 cells, with inhibitory properties toward Th1 cell cytokine production and subsequently named cytokine synthesis inhibitory factor [185-187]. Later, IL-10 was shown to exhibit inhibitory activity toward a broad spectrum of myeloid cell functions including; cytokine production, NO production and macrophage/monocyte cell-mediated activation [186]. IL-10 is produced by monocytes and macrophages relatively late during the acute phase of inflammation signaling the beginning of the resolution of inflammation [64, 124, 129, 130, 188]. Viruses also express IL-10. For example, Epstein-Barr virus contains a related form of the IL-10 gene, BCRF1 and expresses viral IL-10 (vIL-10), a related variant of human IL-10 (hIL-10) [189, 190]. vIL-10 is active on human cells and its production may be used to increase host cell susceptibility to infection [191]. Also, increased expression of IL-10 has been observed in certain cancers such as ovarian [192], melanoma [193] and lymphoma [194] where its function(s) is/are not fully understood.

1.5.1 Cellular sources of IL-10

IL-10 is predominantly produced by Th2 T helper cells, $CD5^+$ B cells, and macrophages in mice [116]. In humans, IL-10 is produced by $CD8^+$ peripheral blood cells and $CD4^+$ helper T cells following antigen-specific and polyclonal activation [184, 195]. hIL-10 is also produced by monocytes treated with LPS, and B cells [184, 195]. IL-10 is produced during innate immune inflammatory responses predominantly by tissue macrophages and monocytes and can be inhibited either by IL-4 or by IL-10 itself [126]. In addition, certain B cells in diseased states such as Burkitt's lymphoma [196] or B cells derived from HIV-infected patients [194] show elevated and constitutive levels of IL-10. Among non-hematopoetic cells, IL-10 is produced by mast cells and keratinocytes [184, 195].

I.5.2 Protein characteristics of IL-10

IL-10 structurally belongs to the interferon and hematopoetic cytokine family and is characterized by an α-helical bundle structure [197, 198]. hIL-10 is 73% identical to mIL-10 and 84% identical to vIL-10. The IL-10 gene encodes a secreted protein of approximately 178 amino acids with a molecular weight of ~17-18kDa [190]. hIL-10 and vIL-10 are not *N*-glycosylated and contrast with an *N*-terminus *N*-glycosylated mIL-10 [113, 114, 116, 198, 199]. There are no reports on the effect of glycosylation on mIL-10 biological activity. hIL-10 is able to activate signaling cascades via IL-10R on mouse and human cells but mIL-10 only affects mouse cells [114, 195]. IL-10 is an acid-sensitive, noncovalently bound homodimer with two

interpenetrating polypeptide chains [198]. Although IL-10 is a dimeric molecule, attempts to alter its structure to a monomer generated a molecule that retained IL-10 biological activity albeit at reduced levels [200, 201]. Currently, it is not clear if such modified IL-10 molecules are capable of inducing intracellular signals of similar quality as the IL-10 homodimer or if they can promote the induction of IL-10. IL-10 mediates its function through binding to its cognate cell surface receptor [195, 202].

The IL-10 receptor (IL-10R) is composed of two subunits, IL-10R1 and IL-10R2, and belongs to the interferon receptor family [203, 204]. IL-10R1 is the ligand-binding subunit while IL-10R2 is a signaling subunit [205-207]. Both are predominantly expressed in hematopoietic cells [208] but IL-10R1 is inducibly expressed on non-hematopoetic cells [208]. In cells such as fibroblasts, epidermal cells or keratinocytes, IL-10R1 can be upregulated by treatment with LPS, glucorcorticoids and dihydroxy-vitamin D3, respectively [209-211].

hIL-10R1 has an estimated molecular weight of 90 to 120kDa and is *N*-glycosylated. hIL-10R1 is 60% homologous to mIL-10R1 but is unable to bind mIL-10 whereas mIL-10R1 binds and mediates signals induced by hIL-10 [195]. Comparative studies suggest that the binding of IL-10 to IL-10R1 is similar to that observed in IFNγ/sIFNγ-R1 [197, 198] and is likely mediated by the *N*-terminus, helix A, part of helix B, the AB loop and the C-terminal helices E/F [212]. This is also supported by evidence showing that the altered sequence and structure observed in the *N*-terminus of vIL-10 is responsible for low affinity interactions of vIL-10 with IL-10R1 [213]. Although IL-10R1 is expressed on different cell types the outcome of IL-10 interaction with IL-10R1 may differ markedly. For instance, mIL-10R1 expressed

on mast cells and macrophages, although identical, elicits different responses to cIL-10 and vIL-10 [214]. The underlying mechanisms governing the differences in cellular responses to cIL-10 and vIL-10 remain unknown [202, 214].

IL-10R2 is constitutively expressed in most cells and tissues and its regulation may not be activation-dependent [203, 207, 208]. IL-10R2 is considered a signaling component of the IL-10 receptor complex, where it associates with the IL-10R1 [215]. Although IL-10R2 does not bind IL-10, it is required to mediate IL-10 signals [207]. Evidence suggests that the key function of IL-10R2 is the recruitment the Jak family kinase, Tyk2 [195, 215, 216]. It has also been shown that IL-10R1 recruits Jak1 [22]. Tyk2 and Jak1 kinases activate the STAT3 (Signal transducer and activator of transcription 3) transcription factor. STAT3 molecules dimerize following their phosphorylation on tyrosine residues by Tyk2 or Jak1 [217]. STAT3 dimers rapidly translocate to the nucleus where they activate IL-10 dependent genes [216, 218]. IL-10R2 deficient mice are unresponsive to IL-10 and develop chronic severe enterocholitis similar to that observed in IL-10 deficient mice [215]. Monoclonal antibodies to hIL-10R2 block IL-10 responses, suggesting that they are required in IL-10 mediated signaling [195].

I.5.3 Biological activities of IL-10

The principal function of IL-10 is to modulate the expression of cytokines, cell surface receptors and soluble mediators [124, 125, 127, 184]. IL-10 exerts these effects principally on cells of myeloid origin, especially macrophages [219] and, to a lesser extent, monocytes, T cells, B cells and other cell types [195]. IL-10 is known to

inhibit the production of IL-1α and β, IL-2, IL-6, IL-8 (or MIP-2), IL-12, IL-18, IFN-γ GM-CSF, G-CSF, M-CSF, TNF, LIF and PAF [219-221]. IL-10-mediated inhibition of TNF-α and IL-1 in monocytes and macrophages ameliorates inflammatory responses, an effect representing a key mechanism by which IL-10 exerts its anti-inflammatory effects [124, 126, 184, 186, 220, 222]. In addition, IL-10 enhances the expression of certain anti-inflammatory mediators such as IL-1Ra (IL-1 receptor antagonist) [223] and soluble TNFR1 and TNFR2 [224] in activated monocytes and by doing so, suppresses the possible pro-inflammatory effects that may be exerted by TNF-α and IL-1. IL-10 also inhibits the inducible production of the CC and CXC classes of chemokines [225]. In monocytes, IL-10 upregulates the CCR1, CCR2, CCR5 and fMLP receptors, resulting in increased responsiveness of these cells to a wide number of chemokines [226-228], hence providing recruitment signals for the migration of leukocytes. In addition to inhibiting cytokine production, IL-10, in association with IL-3 and IL-4, acts as a co-stimulator for mast cell proliferation[229, 230]. IL-10 is pleiotropic in nature, playing multiple and diverse roles within the immune system. It is therefore important to note that the biologic activities of IL-10 are dependent on, the responding cell type and receptor repertoire, the composition of the microenvironment and the host's pathological state. Therefore, an understanding of the extensive biologic roles of IL-10 remains far from complete.

I.5.4 Mechanism of IL-10 involvement in diseases

The principal role of IL-10 is to potently regulate the synthesis of cytokines, especially pro-inflammatory cytokines. It is therefore conceivable that a loss of IL-10

may result in increased, prolonged and unresolved inflammatory episodes that are related to poor regulation of pro-inflammatory cytokine production. IL-10 knockout mice (IL-10-/-) are characterized by the development of severe forms of enterocolitis [231, 232] with a striking resemblance to Crohn's disease observed in humans [233, 234] and represent a murine model for evaluating inflammatory bowel disease (IBD) [235, 236]. Independent reports have also demonstrated that treatment with recombinant IL-10 ameliorates the symptoms of IBD, further stressing a role for IL-10 in IBD [235]. However, the exact mechanisms governing the initiation of Crohn's disease or its pathogenesis remain sketchy [237-239]. Inflammatory bowel disease observed in IL-10 deficient mice is typified by infiltration of $CD4^+$ Th-1 cells and macrophages as well as an increased production of IFN-γ [235, 240, 241]. These activities in IL-10-/- mice suggest that IL-10 is central to the modulation of $CD4^+$ Th-1 T cell responses. It therefore appears that, in the absence of IL-10, poor regulation of $CD4^+$ Th-1 T cell responses result in incessant production of INF-γ, which may subsequently activate gut-resident macrophages [236, 239, 242]. Activation of resident macrophages by INF-γ may result in increased cytokine production and lysosome activity [240, 241, 243, 244]. Activation of resident macrophages triggers the production of pro-inflammatory cytokines which further complicate inflammatory bowel disease pathogenesis [243]. Taken together, it appears that the lack of IL-10 favors a $CD4^+$ Th-1 type response in the gut and the lack of regulation of this Th-1 response drives the progression of inflammatory bowel disease, at least in mice. Other related conditions that may be observed in IL-10-/- mice include amplified airway inflammation and chronic relapsing experimental autoimmune encephalitis [184, 245].

Although the lack of IL-10 has been implicated in the above chronic inflammatory pathologies, excessive IL-10 production can be detrimental. For example, excess IL-10 production appears to increase CCR5 chemokine receptor expression, enhancing HIV entry in monocytes [226]. Furthermore, B cells and monocytes isolated from patients with Systemic Lupus Erythematosus (SLE) express high levels of IL-10 [246, 247]. This increased level of IL-10 is associated with an increase in autoantibody production by B cells as well as the immune complex pathology [247]. The role of excess IL-10 production in the SLE pathology was substantiated by studies showing that treatment of SLE patients with IL-10 monoclonal Ab resulted in an improvement of SLE pathogenesis [248]. Other diseases associated with increased production of IL-10 include rheumatoid arthritis [175, 181] and cancers such as ovarian, as well as carcinomas such as lymphoma/myeloma [192, 193]. The precise role of excess IL-10 production in the pathogenesis of cancer and rheumatoid arthritis remains the subject of intense studies [192-195, 202, 247].

There are many other diseases variably associated with IL-10 production and in some instances these diseases are species-dependent [235]. However, a consensus emerges that IL-10 association with different diseases appears to be complex and directly related to the pleiotropic effects of IL-10 [195, 202].

I.6.0 INNATE IMMUNE CELL RECEPTORS AND CELL SIGNALING

I.6.1 Innate Immune cell receptors and LPS

During microbial invasion, scavenging receptors or signal-inducing PRRs (pathogen recognition receptors) expressed on the innate immune cell surfaces bind microbial molecules leading to cellular activation and the synthesis and secretion of immuno-modulatory cytokines [6, 7, 24, 43]. The secretion of pro-inflammatory cytokines, chemokines and other inflammatory mediators in turn, enhance the recruitment of effector cells such as leukocytes and neutrophils to the site of trauma [59, 119, 120].

Innate immune cells are mainly characterized by the expression of pathogen recognition receptors such as; Nod-like receptors (NLRs) and Toll-like receptors (TLRs) [5, 11]. Immune cells detect and signal the presence of intruding microbes by interacting with conserved products of microbial metabolism, specifically produced by microbial pathogens [5, 11, 15]. The pathogen-associated molecular patterns (PAMPs) expressed in microbes interact with pattern recognition receptors (PRRs) expressed either on innate immune cell surfaces, located in intracellular compartments or secreted into the blood stream or tissue fluids [1, 2, 4, 7]. The main functions of pattern recognition receptors include the activation of pro-inflammatory signaling pathways, phagocytosis and, in certain conditions, the induction of apoptosis [2, 4]. PRR interaction with conserved molecular patterns on microbes enables the detection of a wide range of invading microbes, thus making it difficult for most microbes to escape immune surveillance [1]. Actual recognition of microbial pathogen-associated molecular motifs (PAMPs), such as lipopolysacharide, by TLRs initiates signal

transduction cascades in innate immune cells that trigger the onset of inflammation [2].

1.6.2 Toll like receptors

TLRs are type 1 transmembrane receptors characterized by an extracellular leucine-rich repeat (LRR) domain (LRRs are protein modules with 20 to 29 amino acids), a short transmembrane region (approximately 22 uncharged amino acids) and a cytoplasmic domain that is conserved and homologous to the cytoplasmic domain of IL-1 receptor family [5, 249-251]. TLRs and IL-1R utilize a common cytoplasmic signaling domain, Toll/IL-1R (TIR) domain, which spans approximately 200 amino acids [252, 253]. The main function of the TIR domain(s) is to bind adaptor proteins such as; myeloid differentiation primary response protein 88 (MyD88) and Toll/IL-1R associated protein (TIRAP) [253]. MyD88-TIRAP interaction is essential for the recruitment of an assembly of molecules required to further mediate and define downstream TLR signals [254] (See Fig I.2). TLR signaling via these molecules directs the induction of genes that control the progress of inflammation including cytokine, chemokine and receptor genes [253, 255]. TLRs are capable of recognizing a wide range of microbes enabling an efficient detection of pathogens. For instance, TLR4 recognizes LPS whereas TLR2 recognizes peptidogylcan, a component of Gram-positive bacteria cell wall [250, 252, 256]. In humans, a total of 10 TLRs (TLR 1 to 10) have been identified, of which TLR4 is the most widely studied among TLR family of receptors [250, 257].

I.6.3 Lipopolysaccharide (LPS)

LPS is a significant component of the outer membrane of Gram-negative bacteria and is required for the structural integrity, rigidity and the protection of bacterial cell wall [258, 259]. LPS is composed of Lipid A (a disaccharide with multi fatty acid tails), the core oligosaccharide (which is attached to Lipid A) and the *O*-antigen also known as the polysaccharide side chain [258, 260-262]. The *O*-antigen is the most variable portion of LPS and confers its antigenic specificity [259]. *O*-antigens are *O*-side chains that extend from the core of the polysaccharide chain [263]. The length of the *O*-side chains renders LPS rough (short *O*-chains) or smooth (long *O*-chains) [259, 264]. Bacteria with rough LPS are more hydrophobic and are more readily penetrated by hydrophobic antibiotics [265].

LPS potently activates cells that express its cognate receptor and these cell types include but are not limited to: (a) myeloid cells such as monocytes, macrophages and mononuclear phagocytes, (b) lympocytes such as B cells and T cells (c) other cell types such as neutrophils, mast cells and fibroblasts, depending on the anatomic site [266, 267]. In macrophages, LPS is responsible for the induction (> 3 fold) of 259 diverse genes, which include; cytokine and chemokine genes, cell surface receptors, signaling/survival and transcription factor proteins and other miscellaneous proteins [219, 268]. The inflammatory effects of LPS are mediated by signaling through specific TLRs.

1.6.4 LPS/TLR4-mediated signal transduction

TLR4 has been experimentally identified as the key cellular component essential for LPS signaling [257]. Mutations in the TLR4 gene as discovered in the

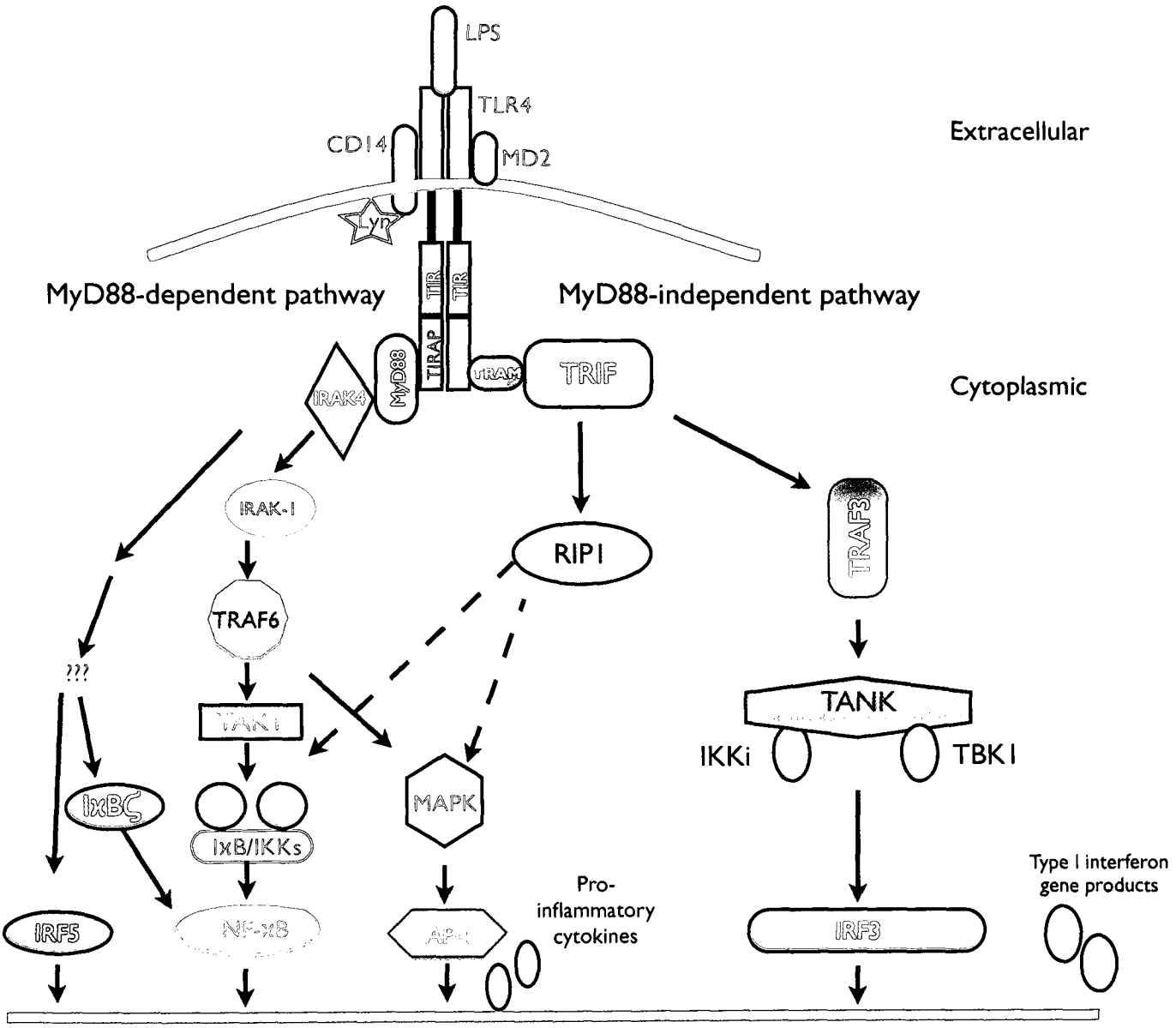
C3H/HeJ and *C57BL/10ScCr* murine strains render these mice non-responsive to LPS treatment [269-271].

LPS signaling through TLR4 occurs in progressive sequences that involve diverse proteins. LPS signaling begins upon association of LPS with LPS binding protein (LBP) [268, 272]. LBP is a soluble shuttle protein, which enhances LPS interaction with CD14 molecule [254]. CD14, a membrane-associated glycosylphosphatidylinositol-attached protein that lacks a cytoplasmic tail, enables the transfer of the LPS-LBP complex to the TLR4/MD2 complex (FIG I.2). MD2 is a soluble protein that associates with TLR4 in a non-covalent manner and is capable of independently associating with LPS [273]. Recognition of LPS by TLR4 promotes the oligomerization of TLR4 leading to the recruitment of downstream adaptor proteins that interact with the TIR domains of TLR4 [252, 253, 255]. To date there are five known TIR-binding adaptor proteins, namely; MyD88, MyD88-adapter-like/TIR-domain-containing Adaptor Protein (Mal/TIRAP), TIR-domain-containing adaptor protein inducing IFN-β (TRIF), TRIF-Related Adaptor Molecule (TRAM) and Sterile-Alpha and HEAT-Armadillo Motifs-containing protein (SARM) [253-255]. These five adaptor proteins define specific downstream signaling cascades in TLR signaling and only TLR4, among all TLRs, utilizes all five in mediating diverse signaling cascades and responses [250, 252, 254, 257].

Signaling pathways triggered by TLR4 are broadly classified into the conventional and non-conventional pathways. Conventional TLR4 mediated pathways are further subdivided into the classical MyD88-dependent and non-classical MyD88-independent pathways [253-255, 257].

Fig I.2 Classical LPS signaling pathway(s)

A schematic representation of the LPS signaling cascade highlighting similarities and differences between the MyD88 dependent and MyD88-independent signaling pathways.



Modified from Y.-C. Lu et al. / Cytokine 42 (2008) 145–151 with permission from Dr. Pamela Ohashi (see appendix)

CONVENTIONAL TLR4 SIGNAL TRANSDUCTION PATHWAYS

I.6.5 MyD88 dependent pathway

The MyD88-dependent pathway is utilized by all TLRs except TLR3 [274]. For TLR4 mediated signaling, MyD88 is recruited to the TLR4 receptor by binding the TIR domain with its C-terminal toll homology domain [274, 275]. The presence of a death domain on MyD88 facilitates the recruitment and subsequent autophosphorylation of IL-1R Associated Kinases (IRAKs), a serine-threonine kinase family that is characterized by the presence of a death domain and a kinase domain [275]. The phosphorylation of IRAK-1 or IRAK-4 provides a binding platform and phosphorylates TNF-receptor Associated Factor 6 (TRAF6) complex, consequently activating Transforming growth factor-β Activating Kinase 1 (TAK-1) [254, 275]. TAK-1 in turn activates Inhibitor of KappaB Kinase (IKK) and Mitogen Activated Protein Kinases (MAPKs) ultimately resulting in the activation and translocation of Nuclear Factor-κB (NF-κB) and Activating Protein-1 (AP-1) transcription factors, respectively (See Fig I.2). Activation of both NF-κB and AP-1 is critical for TLR4-mediated transcription of pro-inflammatory cytokine genes, especially, TNF-α, IL-1α /β genes [15, 255, 274, 275]. Sometimes, although in a not so clearly understood manner, MyD88 may directly mediate the activation of the transcription factor, Interferon Regulatory Factor 5 (IRF5) resulting in the transcription of Type 1 interferon genes [274].

I.6.6 MyD88-independent pathway

The MyD88-independent pathway begins with TRAM binding to the TIR domain of TLR4 and facilitation of the recruitment of TRIF [276]. The C-terminal region of TRIF contains a Rip interaction motif providing a platform for binding Receptor Interacting Protein 1 (RIP1), a serine/threonine kinase that mediates the activation of MAPKs and IKKs, culminating in activation of AP-1 and NF-κB transcription factors, respectively [277]. This pathway is similar to the MyD88-dependent cascade. In addition to RIP1 recruitment, TRIF also recruits TNF-receptor Associated Factor 3 (TRAF3), which associates with other proteins, namely; TRAF family member-Associated NF-κB Kinase (TANK), TANK Binding Kinase 1 (TBK1) and Inhibitor of Kappa B Kinase Epsilon (IKKi) [277-279]. The assembly and cross-activation of TBK1 and IKKi ultimately results in the activation, dimerization and translocation of the Interferon Regulatory Factor 3 (IRF3) transcription factor [276, 278] (Fig I.2). A combination of IRF3 and NF-κB activities results in the transcription of Type 1 interferon target genes [280].

In addition to these MyD88-dependent and independent signal transduction pathways there are other non-conventional pathways that are triggered by LPS/TLR4 interaction [281-283], namely; the MAPK pathway and Protein Tyrosine kinase (PTK)-dependent pathways [284].

NON-CONVENTIONAL TLR4-MEDIATED SIGNALING PATHWAYS

1.6.7 Mitogen Activated Protein Kinase (MAPK) Pathways

Significant roles for MAPK signal transduction pathways have been described in diverse cellular processes such as cell proliferation, differentiation, migration, inflammation and apoptosis [285, 286]. To date, three classes of proteins have been identified as indigenous members of the MAPK family of proteins and include; the c-Jun *N*-terminal kinases 1/2/3 (JNK1/2/3), the extracellular signal-regulated kinases 1/2 (ERK1/2) and the stress activated protein kinase, (p38 MAPK). Activation of the MAPK signaling cascade starts with the activation of Raf-1 [287-289], a conserved serine/threonine protein kinase that is also known as MAP kinase kinase kinase (MAPKKK or MEKK). The phosphorylation and activation of MAPKKK subsequently activates the dual specificity MAP kinase kinase, (MAPKK or MEK) which in turn phosphorylates the specific MAPKs at threonine and tyrosine residues located at ThrXyzTyr (where Xyz can be different amino acids) motifs [290, 291]. The central amino acid represented by Xyz in this motif can be used to distinguish particular MAPK family members. Glutamic acid represents ERK1/2, proline identifies the JNK family members and glycine distinguishes p38 MAPK [292].

Specific members of the MAPK family are activated in response to different sets of stimuli; the particular member(s) activated is/are largely dependent on the type and source of stimuli [286, 288, 293]. For instance, mitogen and growth factor stimuli have been shown to trigger the activation of ERK1/2 MAPK [293-295], while JNK and p38 MAPKs respond to environmental and cellular stress such as LPS, UV irradiation and other forms of mechanical stress [285, 286]. The key function of

activated MAPKs, is to modulate the activation of specific transcription factors such as NF-κB, SP-1 and AP-1 [286, 288]. Activation of these respective transcription factors initiates the cellular transcription of target genes that mediate diverse responses such as cytokine production, cell migration, cell proliferation and apoptosis [286, 288, 289, 291].

In addition to the MAPK cascade and within the LPS-TLR4-CD14-MD-2 signaling complex, PTKs have also been reported to control the activation of signaling proteins, which are essential for the activation and transcription of diverse target genes [282, 296].

I.6.8 Protein Tyrosine Kinases (PTKs)

PTKs are signaling proteins with kinase catalytic domains capable of transferring a phosphate group from an ATP molecule to a tyrosine residue on a protein. Cell signals that are directed toward cell growth, adhesion, motility, differentiation and apoptosis are routinely transmitted via tyrosine kinases [297]. PTKs are broadly divided into two large classes namely, receptor-type tyrosine kinases (RTK) and non-receptor protein tyrosine kinases (NRTK) [298]. In the human genome, a total of 90 PTKs have been identified, of which 58 are RTKs, allotted among 20 subfamilies and 32 are NRTKs distributed across 10 subfamilies [297-299]. RTKs are cell surface receptors that possess intrinsic tyrosine kinase activity and mediate most growth factor, hormone and differentiation signals [300-302]. On the other hand, NRTKs are cytoplasmic proteins that transmit signals originating from diverse cell surface receptors such as antigen receptors to downstream intermediates along pathways that mediate diverse cellular activities [299]. In LPS/TLR4 signaling,

the involvement of different NRTKs has been reported in very few studies [284, 303]. The precise function of these NRTKs in LPS/TLR4 signaling remains largely unexplored.

One of the most studied subfamily of NRTKs is the Src subfamily. Src family kinases have conserved domains that include a myristolated *N*-terminal tail, SH3 and SH2 domains, a tyrosine kinase domain and a short *C*-terminal tail [304-306]. The Src family of NRTKs is comprised of nine members namely; Src, Fyn, Yes, Yrk, Blk, Fgr, Hck, Lck, and Lyn [307]. The first four members are ubiquitously expressed, whereas the last five members are expressed in specific cell lineages. For example, Lck is expressed in T cells, where it modulates signals originating from the T cell receptor (TCR) whereas Hck is expressed predominantly in myeloid lineage cells [307]. Some reports show that the Src family kinase member, Lyn, is associated with CD14 and upon LPS treatment in primary macrophages, Lyn is recruited and activated [282]. Similarly, it has been shown that cells treated with antisense oligonucleotides to Hck, manifest limited LPS-mediated responses [308, 309]. In addition, longterm exposure of macrophages to LPS upregulates the synthesis of Hck and Lyn, and both kinases are essential in priming macrophages prior to respiratory burst [310]. However, data obtained from mutant mice with triple knockouts of Hck/Fgr/Lyn proteins show that, in LPS treated macrophages, Hck, Fgr and Lyn did not play significant roles in LPS-meidated responses [311]. Therefore, the specific functions of Hck, Fgr and Lyn in the LPS/TLR4 cascade still remain unclear.

In addition to Src familiy kinases, Brutons tyrosine kinase (Btk), a member of the Tec family, has also been implicated in TLR4 signaling [303]. Btk associates with

the TIR domain of TLR4 and is phosphorylated upon LPS treatment [312]. Based on limited evidence, it is presumed that Btk modulates signals leading to NF-κB activation and cytokine production in macrophages [312].

The focal adhesion kinase (FAK) family member, Pyk2 (proline-rich tyrosine kinase 2) also plays a role in LPS-mediated signaling and is involved in cytoskeletal reorganization [313]. Williams L.M. *et al.* described LPS mediated tyrosine phosphorylation of Pyk2 and a cytoskeletal protein, paxillin and their roles in actin reorganization in both monocytes and macrophages [314]. The LPS mediated tyrosine phosphorylation of Pyk2 did not occur through either MyD88-dependent or MyD88-independent pathway(s) but rather was dependent on the activity of Src kinase [315]. Very recently, LPS mediated Pyk2 activation has also been linked to IL-8 production in human endothelial cells [316], suggesting multiple roles for Pyk2 in the LPS signaling cascade. Elaborate mechanisms underlying Pyk2 activities and their integration into LPS classical cascades still remain vague.

I.6.9 Regulation of protein tyrosine phosphorylation and PTKs activity

Tyrosine phosphorylation is essential for the control of diverse cellular processes ranging from normal cell cycle regulation, differentiation, migration and cell growth to pathological events such as chronic inflammation and cell transformation [298, 304, 306]. Cell signaling cascades initiated by ligand binding to cognate receptors trigger tyrosine phosphorylation of diverse signaling proteins that mediate downstream signals, which in turn, play a role in the upregulation of specific genes [297, 299, 304, 306]. Tyrosine phosphorylation of proteins, including PTKs, results in

a reversible alteration of protein activity and sometimes, protein structure and function [298, 302].

Regulation of tyrosine phosphorylation of signaling molecules is controlled by reversible and competing activities of PTKs and protein tyrosine phosphatases (PTPs) [299, 305, 306]. The kinase domains on PTKs catalyze the phosphorylation of tyrosine residues on target proteins and the phosphatase domains of PTPs conversely dephosphorylate the phosphotyrosine residues. Phosphorylation and dephosphorylation of proteins, especially on tyrosine residues, are essential for maintaining dynamic equilibrium in biological systems. Poor tyrosine phosphatase activity due to funtional mutations in genes encoding PTPs may promote the accrual of tyrosine phosphorylated proteins, resulting in constitutively active pathways that subsequently cause abnormal cell responses [317, 318].

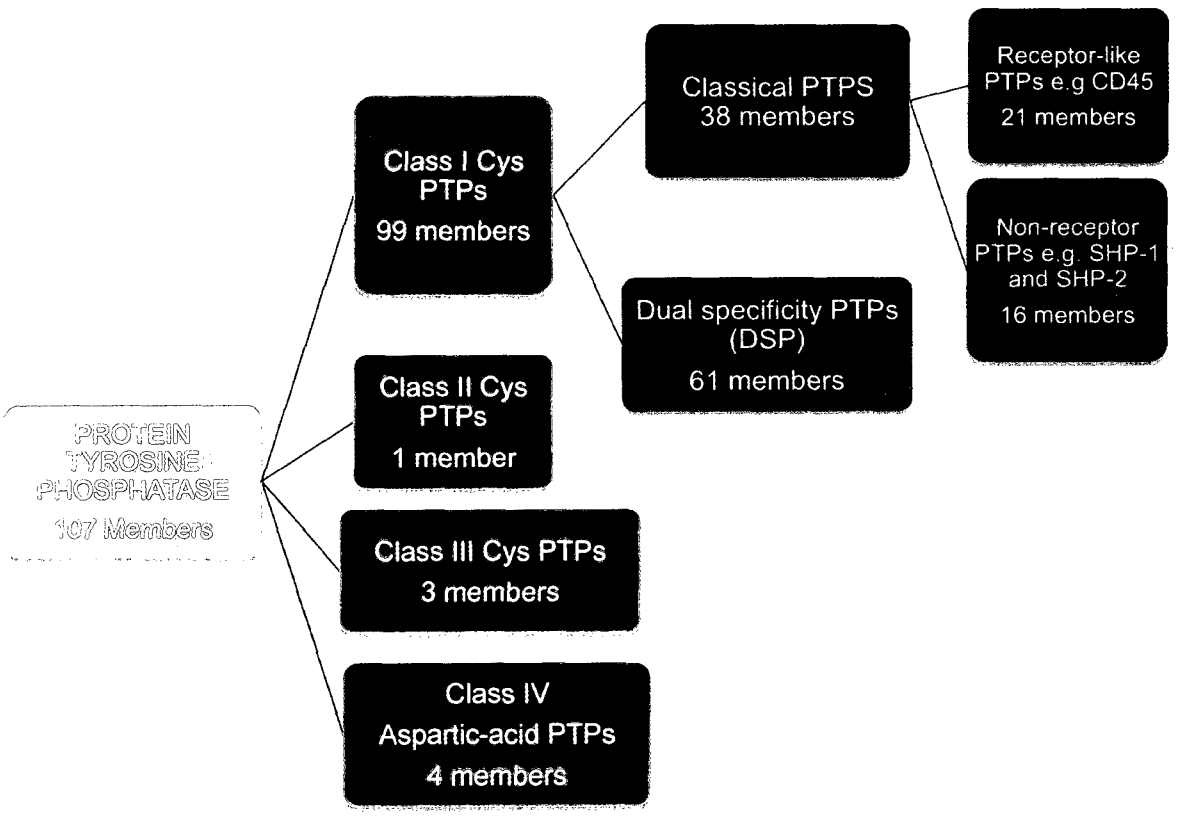
In addition to controlling target proteins that are phosphorylated on tyrosine residues by PTKs, PTPs also regulate PTKs. For example, PTPs can positively regulate PTKs by dephosphorylating inhibitory tyrosine motifs on PTKs [305]. Conversely, PTPs negatively regulate PTKs by dephosphorylating activation-dependent tyrosine residues on PTKs [317]. PTPs can therefore regulate intracellular tyrosine phosphorylation either by acting on the target proteins directly or by targeting PTKs that phosphorylate respective target proteins, making PTPs critical regulators of cellular processes.

I.7.0 PROTEIN TYROSINE PHOSPHATASES (PTP)

In the human genome, there are four established PTP families encoded by 107 genes [318]. These four PTP family groups are divided based on the amino-acid composition and similarity of their catalytic phosphatase domain. The largest class I cysteine-based PTPs include 99 members, whereas the class II and class III cysteine-based PTPs include 1 and 3 members, respectively [318, 319]. In addition, there are 4 members in the aspartic acid-based class of PTPs (Fig I.3). The class I cysteine-based PTPs are further subdivided into a 38 member classical PTP subclass and the 61-member dual specificity (Tyrosine and Serine/Threonine) PTPs (DSPs)[318, 319]. The classical PTPs are strictly phosphotyrosine-specific with similar catalytic domains containing the consensus V/I-H-C-S-X-G motif and are further subdivided into two subfamilies based on domain architecture [318]. These include the 21-member transmembrane receptor-like PTP (RPTPs) subfamily and the 16-member cytosolic or non-receptor PTP (NRPTPs) subfamily (See Fig I.3). The RPTPs contain a variable extracellular region with structural domains, a short transmembrane region and a long cytoplasmic region that typically contains two catalytic domains with only one of the PTP domains being catalytically active [320-322]. In contrast, cytosolic PTPs have no transmemberane or extracellular region but have one catalytic phosphatase domain, with variable add-on modules such as SH2 (Src Homology 2) and Pro-rich (proline-rich) domains. The add-on modules are critical for binding target proteins, localization and directing the catalytic domains to substrates sites [318].

Among the classical non-receptor type phosphatases, there are only two phosphatases that contain tandem SH2 domains, SHP-1 (SH2-domain containing

FigI.3 A simple function-based classification of Protein Tyrosine Phosphatases


PROTEIN TYROSINE PHOSPHATASE
107 Members
Class I Cys PTPs
99 members
Class II Cys PTPs
1 member
Class III Cys PTPs
3 members
Class IV Aspartic-acid PTPs
4 members
Classical PTPS
38 members
Dual specificity PTPs (DSP)
61 members
Receptor-like PTPs e.g CD45
21 members
Non-receptor PTPs e.g. SHP-1 and SHP-2
16 members

Phosphatase 1) and SHP-2 (SH2-domain containing Phosphatase 2) [317, 319, 320, 323, 324]. SH2 domains are conserved modular protein interaction domains of about 100 amino acids that bind phosphorylated tyrosine residues [325]. Among many functions, the key roles of SH2 domains include localizing proteins to specific sub-cellular compartments, controlling enzymatic activities and integration of proteins into larger protein complexes [325]. The two SH2 domains found on SHP-1 and SHP-2 are capable of binding a vast number of proteins phosphorylated on tyrosine residues including PTKs [323, 324]. Although both SHP-1 and SHP-2 are structurally similar, they exhibit diverse cellular functions and expression patterns. For example, SHP-1 is predominantly expressed in hematopoietic cells whereas SHP-2 is ubiquitously expressed [317, 321, 323, 324]. Studies show that SHP-1 predominantly exhibits negative signaling functions manifested through dephosphorylation of activation-dependent tyrosine sites whereas SHP-2 effects positive signaling functions (activation) frequently relaying signals originating from RTKs and cytokine receptors with PTK function [323, 326, 327]. The phosphorylation of Serine 591 and expression of a NLC (nuclear localization sequence) at the *C*-terminal of SHP-1 is presumed to be responsible for the significant differences in activity between SHP-1 and SHP-2 [326-329]. However, the exact biochemical mechanisms underlying the regulation of signal transduction pathways by both SHP-1 and SHP-2 are subjects of intense investigation. Recent studies have further demonstrated the involvement of both SHP-1 and SHP-2 in divergent pathological conditions [321-323, 330]. This dissertation, however, focuses on SHP-1 and its roles within the hematopoietic innate immune system.

I.7.1 Structure and function of SHP-1

SHP-1 is a 68 to 71 kDa cytosolic classical PTP 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 [320, 323, 331-333] (See Fig I.4A). There are 4 identified isoforms of SHP-1, 3 in hematopoietic cells and 1 existing in non-hematopoietic cells [334]. SHP-1 isoforms arise as a result of alternative initiation or splicing sites [333, 334]. For example, a variant of SHP-1, SHP-1L has also been identified in the human cells. The SHP-1L isoform is significantly longer than other SHP-1 isoforms, differing by the addition of 66 amino acids at the C-terminus. SHP-1L originates by an alternate splicing of SHP-1 transcript resulting in a reading frame shift [333]. SHP-1L is also characterized by the loss of a tyrosine phosphorylation site (Y564) normally found on SHP-1. Instead it is replaced with a proline-rich motif, which represents a binding site for SH3 domains [323, 324]. SHP-1L similar to SHP-2, lacks the nuclear localization sequence (NLS) denoted by the KRK (Lysine-Arginine-Lysine) sequence on the C-terminal of both proteins, enabling them to reside strictly within the cytosol and target cytosolic proteins [323, 324, 333]. Therefore, the variations observed among SHP-1 isoforms can result in a modification of SHP-1 activity and function.

SHP-1 activity is regulated by intramolecular interactions between the *N*-terminal SH2 domain and the catalytic phosphatase domain [323, 324, 335]. This interaction is a charge-charge interaction that results in the *N*-terminal SH2 domain making contact with the charged residues around the catalytic cleft, leading to insertion of some parts (NXGDY/F motif) of the *N*-terminal SH2 domain into the catalytic cleft [324], rendering the catalytic phosphatase site inaccessible (Fig I.4B).

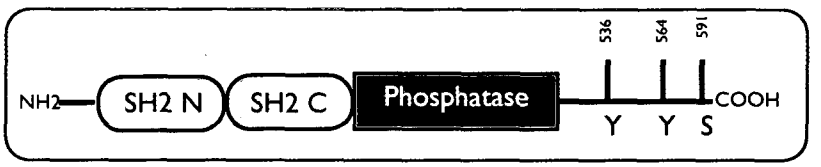
Fig I.4 Schematic representation of SHP-1

(A) Full length SHP-1 in an active conformation depicting the tandem SH2 domains, a phosphatase domain and two tyrosine residues at the *C*-terminal.

(B) Full length SHP-1 in an inactive conformation showing the NXGDY/F motif of *N*-terminal SH2 (*N*-SH2) binding the catalytic cleft on the phosphatase domain (a contact-based charge-charge interaction) resulting in sequestration of phosphatase domain. The small yellow coloured box represents tyrosine binding motifs on the N-SH2 domain

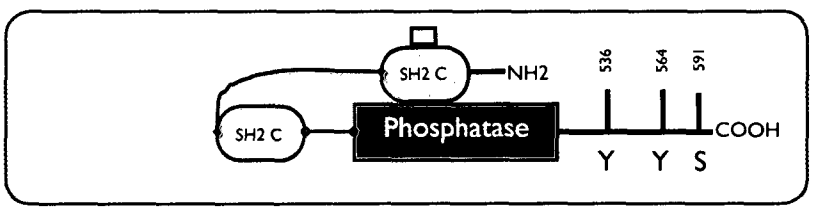
(C) Full length SHP-1 in an inactive conformation showing the *N*-terminal SH2 domain binding Y564 in the *C*-terminal resulting in an occlusion of the phosphatase domain.

(A)

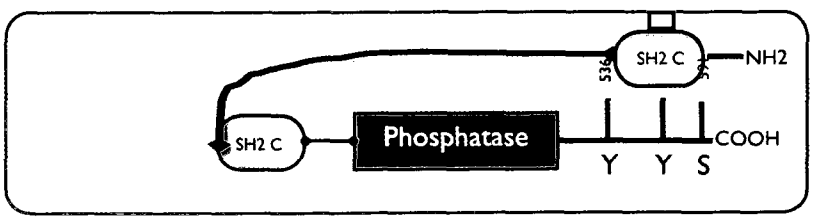


SHP-1 Active conformation

(B)



SHP-1 Inactive conformation



SHP-1 Inactive conformation

Upon binding of a phosphotyrosine peptide to the *N*-terminal SH2 domain, a conformational change is initiated that disengages the interaction between the SH2 domain and the catalytic cleft, resulting in an exposure of the catalytic phosphatase domain to substrates [335]. In other studies, the tyrosine residue, Y564, located at the C-terminal tail of SHP-1 also participiates in regulation of SHP-1 in a not so clearly understood manner [336]. Zhang *et al.* further speculate that the *N*-terminal SH2 domain of SHP-1 binds Y564, consequently restricting substrate access to the catalytic phosphatase domain [336] (Fig I.4C). This hypothesis is supported by the finding that SHP-1L, which lacks Y564, is constitutively active *in vivo* [333], suggesting a possible requirement for Y564 in regulating the activity of SHP-1. In contrast to the *N*-SH2 domain, the C-SH2 domain does not bind the phosphatase domain and consequently is not involved in regulating SHP-1 phosphatase activity [324]. The C-SH2 domain instead scans the cell for phosphotyrosine residues on proteins and PTKs to which SHP-1 can bind [324].

1.7.2 SHP-1 Targets

Numerous studies have demonstrated crucial roles for SHP-1 in negatively regulating a wide range of cytokine, antigen and growth factor receptors with intrinsic tyrosine kinase capabililties. For example, SHP-1 has been implicated in negative regulation of growth factor receptors EGFR, c-kit and CSF-1 [337-341], cytokine receptors (IL-3, IL-2, IL-4, Epo-R) [342-346], antigen receptor signaling (TCR and BCR) [297, 328, 347-350] and immune receptors containing tyrosine inhibitory motifs (ITIM) [351]. SHP-1 predominantly regulates most signaling cascades using two

distinct mechanisms which include binding directly to the tyrosine residues of activated RTKs or binding and dephosphorylating tyrosine residues of downstream proteins consequently terminating signals initiated by growth factor, cytokine or antigen receptors [323]. For example, SHP-1 binds and dephosphorylates the tyrosine residue 569 in the juxtamembrane region of the c-kit receptor, initiating a negative regulation sequence that terminates signals originating from the c-kit receptor [340]. In addition, SHP-1 downregulates signals emanating from the TCR by dephosphorylating components of the TCR such as Lck and ZAP-70 [352-354]. The number of intracellular SHP-1 protein targets continues to expand and, to-date, about 92 interacting partners have been listed for SHP-1 in the Human Protein Reference Database (www.hprd.org) [355].

The expression of SHP-1 is mostly, but not exclusively, limited to hematopoietic cells where SHP-1 plays critical roles in regulating the development and function of cells within the myeloid lineage subdivision of the hematopoietic cell system [331, 356]. These roles are highlighted by evidence that supports SHP-1's association with c-kit and CSF-1 receptors and further demonstrated by the abnormal and vast myeloid cell expansion observed in SHP-1-deficient moth-eaten (*me/me*) and moth-eaten viable (me^v/me^v) mice [357].

1.7.3 Moth-eaten (*me/me*) mice

The motheaten phenotype is the result of spontaneous mutations in *C57BL/6J* mice [358]. These mutations were mapped to the distal end of chromosome 6 in mice, homologous to chromosome 12p12-p13 in humans [358]. Both *me/me* and the related

me^{v}/*me*^{v} mice are characterized by recessive allelic mutations in the gene encoding SHP-1, also known as hematopoietic cell phosphatase gene (*Hcph*) [359] and the current nomenclature for the mouse SHP-1 gene is *PTPN6*. Both mutations occur as a result of an altered splice site sequence but at different locations of the *Hcph* gene. The *me/me* mutation occurs in the region coding for the *N*-terminal SH2 domain of SHP-1 whereas the *me*^{v}/*me*^{v} mutation occurs within the catalytic domain of SHP-1 [360].

The *me* mutation is the result of a deletion of a single cytidine residue at nucleotide position 228 which generates a novel splice donor site in the *N*-terminal SH2 domain. This abnormal splicing results in 101bp deletion and a frame shift with premature truncation within SHP-1 mRNA, ultimately producing a null mutation [358-360]. The resulting truncated protein is about 99 amino acids compared to 595 amino acids observed in the wild type protein.

The *me*^{v} mutation is caused by a thymidine to adenine transversion that disrupts a splice donor site, which in turn activates cryptic splice sites resulting in two possible mutations, an in-frame insertion of 69 nucleotides or a deletion of 15 nucleotides in the phosphatase catalytic domain [361, 362]. The loss of the highly conserved splice donor consensus results in the production of two alternative splice patterns or two transcripts with products that are 67 and 71 kDa in size and retain approximately 20% phosphatase activity when compared to wild-type SHP-1 [363].

Me/me and *me*^{v}/*me*^{v} mutations cause multiple hematologic abnormalities in mice that are attributed to abnormal development of myeloid cells and other hematopoietic cells. These abnormalities manifest as severe combined

immunodeficiency and systemic autoimmunity characterized by high titers of autoantibody [364], hyperagammaglobulinemia, overexpression of B cell lymphokines and deposition of immune complexes in numerous tissues and organs [365-368].

1.7.4 Phenotypic and Immunologic defects in *me/me* mice

Me homozygosity confers a distinct phenotype characterized by severe patchy dermatitis or alopecia that is visible as early as 3 to 4 days postnatal [359, 365, 367]. In time, *me/me* homozygous mice become readily distinguishable by their relatively reduced size, limb necrosis, feeble movement and progressive arthritis [358-360]. Alopecia observed in *me/me* mice arises as a result of accumulation of neutrophils and macrophages in the dermal and subdermal tissues of the skin consequently distorting normal skin architecture [361, 365, 369]. The distortion of normal skin architecture leads to englargement of foci that displaces hair follicles resulting in patchy, sparse and abnormal hairs, subsequently giving the fur a distinct "moth-eaten" appearance [360, 361, 365, 369]. *Me/me* mice suffer very high mortality and die at a mean age of 2 to 3 weeks. Mortality is attributed to hemorrhagic pneumonitis, a consequence of accumulation of macrophages and granulocytes in the aveoli of the lungs [360, 361, 365].

Many complex immunologic defects and autoimmune conditions have been observed in *me/me* mice. Among these, is the observation of abnormally high titres of autoantibodies that are directly linked to aberrant and significant expansion of a minor B cell population, distinguished by the expression of CD5, a pan-T cell surface glycoprotein also known as Ly-1 [361, 366, 368-371]. In contrast to normal mice,

where $CD5^+$ B cells are localized within the peritoneal cavity, $CD5^+$ B cells in *me/me* mice constitute the majority of peripheral B cells and are characterized by production of antibodies that predominantly use λ light chain and IgM and IgG3 classes of immunoglobulin [331, 360, 364, 365, 368]. The production of these autoantibodies underlies, in part, the aetiology of the complex immunopathologic autoimmune conditions observed in *me/me* mice. Other immunologic defects or complications include over expression of neutrophil chemokines and other B-cell lymphokines in resting cells, hyperagammaglobulinemia and glomerulonephritis [360, 361, 365, 369].

1.7.5 Hematopoietic cell defects in *me/me* mice

Me/me mice exhibit impaired myeloid lineage development, which is characterized by aberrant enormous expansion of myeloid cells and their localization in different tissues and organs [366, 368]. For example, splenomegaly, which is an enlargement of the spleen observed in *me/me* and me^v/me^v mice, is a consequence of a considerable overgrowth of macrophage-like cells and erythroid progenitor cells in the spleen [365, 366, 368, 369]. Furthermore, macrophages and other myeloid cell types, such as neutrophils and granulocytes, migrate to the skin, lungs and kidney where they deposit immune complexes that generate chronic inflammatory responses, further complicating the moth-eaten condition [367].

Impaired lymphocyte development and function contribute to lymphocyte abnormalities such as compromised migration of prothymocytes to the thymus, leading to premature thymic involution and culminating in impaired T cell maturation and development [372]. In addition, defects in lymphocyte development observed in

me/me and *me^v/me^v* mice extend to B cell development, which has been linked to a reduction in the number of bone marrow B cell precursors resulting in a skewed B cell population ($CD5^+$ B cells) [368, 371-374].

The suppression of myeloid lineage development and lymphopoesis is mediated by adherent bone marrow cells in *me^v* mice, as demonstrated by Hayashi *et al.* [375] and is attributed to cellular interactions rather than the bioactivity of a soluble factor. It is therefore presumed that the impairment of lymphopoesis and myeloid lineage development occurs as a result of altered but preferential growth of a stromal cell type that may act directly by inhibiting hematopoiesis [375].

Taken together, all phenotypes and defects observed in *me/me* mice are directly linked to the lack of SHP-1, a PTP that is present in normal wild type animals. It is therefore legitimate to suggest that the lack of SHP-1 expression, activity and function are central to the aberrant conditions observed in both the *me/me* and the related *me^v/me^v* mice.

1.8.0 PURPOSE OF STUDY

Innate immune cells respond to infection and insults by producing inflammatory mediators that influence cell migration, cell proliferation, cytokine production, and resolution of inflammatory episodes [1, 2, 6, 52, 55]. Key inflammatory mediators such as IL-1α/β and TNF-α regulate the progress of inflammation by inducing the synthesis and secretion of secondary cytokines and mediators such as, IL-8, IL-6, and inducible nitric oxide synthase (iNOS) [56, 60, 119, 121, 152]. These secondary mediators in turn effect physiologic and immune

responses that extend the inflammatory episode. TNF-α is central in mediating various biologic roles, especially during innate immune responses and inflammation. If unregulated, the persistent production of TNF-α can lead to a pathologic state [132, 140, 143, 180, 243]. Previous studies implicated excessive levels of TNF-α in numerous immunopathological and chronic inflammatory conditions. For example, high levels of TNF-α are found in Crohn's disease, rheumatoid arthritis, renal diseases (glomerulonephritis), experimental autoimmune encephalitis and psoriasis [109, 132, 142, 179, 180]. The anti-inflammatory cytokine IL-10 regulates pro-inflammatory cytokine (TNF-α) production and thus is able to control the magnitude and duration of inflammatory episodes [56, 124, 125, 187]. IL-10 is one of the most potent anti-inflammatory inhibitors of TNF-α and also inhibits several other pro-inflammatory mediators [126, 195, 225]. Thus far, it appears that a balance between TNF-α and IL-10 is required to effectively initiate and resolve inflammatory episodes.

Chronic inflammation attributed to increased levels of TNF-α in the sera has been well documented in SHP-1 deficient *me/me* mice [360, 365]. In these mice, increased and persistent TNF-α levels have been associated with migration of neutrophils to the skin, arthritis, limb necrosis and infiltration of myeloid cells to the lungs and kidneys [360, 361, 365, 366, 368, 376]. The direct association of TNF-α with the above symptoms was supported by evidence pointing to ameliorated symptoms and longevity of *me/me* mice that were treated with anti-TNF-antibody [376]. *Me/me* mice develop chronic inflammation and show symptoms related to inflammatory bowel disease (IBD) [365], a condition also observed in IL-$10^{-/-}$ mice. Therefore, it remains unclear if persistent and elevated levels of TNF-α observed in

me/me mice are a consequence of distorted anti-inflammatory cytokine production. Since IL-10 plays a critical role in controlling inflammation by down regulating TNF-α production, the characterization of signal transduction events that depend on SHP-1 and lead to IL-10 production is pivotal in understanding the mechanism(s) underlying chronic inflammatory disorders.

Therefore, the purpose of this study was to identify the role of SHP-1 in chronic inflammatory disorders by examining the role of SHP-1 in regulating antigen and LPS-induced cytokine production and cytokine networks, in particular that of TNF-α and IL-10 using both normal and *me/me* splenic macrophages as experimental models.

RATIONALE AND HYPOTHESIS

1.8.1 Rationale

1. *Me/me* mice lack SHP-1 function and are characterized by abnormal activation patterns, hyper-proliferation and expansion of cells of the myeloid lineage. This leads to complex chronic inflammatory conditions characterized by infiltration of myeloid cells to the spleen, lungs and skin and resulting in splenomegaly, pneumonitis, alopecia and ultimately, premature death of moth-eaten mice.
2. Loss of SHP-1 function in *me/me* mice skews myeloid cell lineage development, resulting in anomalous expansion of macrophages. Since macrophages are principal sources of cytokine and chemokine production it is

reasonable to postulate that the development of chronic inflammation is invariably related to altered cytokine and chemokine production.

3. Chronic Inflammation developed in *me/me* mice is a direct consequence of loss of the signal transduction protein SHP-1, which may be involved in the regulation of antigen-induced signal transdution pathways that govern the prodution of both pro-inflammatory and anti-inflammatory cytokines.

Collectively, these observations justify the use of *me/me* mice as a tool and model for the investigation of SHP-1's role in regulating antigen-induced signal transuction pathways governing cytokine production.

1.8.2 Hypothesis

SHP-1 is a critical regulator of LPS and antigen-induced signal transduction pathways regulating the production of pro-inflammatory (TNF-α) and regulatory (IL-10) cytokines in murine splenic macrophages.

1.9.0 OBJECTIVES

(A) Establish pure cultures of splenic macrophage derived from spleens of normal and *me/me* mice.

(B) Profile the levels of pro-inflammatory cytokines TNF-α, IL-1β, and MIP-2 and the anti-inflammatory cytokine, IL-10 and;

(i) Determine if the high levels of TNF-α produced by *me/me* splenic macrophages can be modulated by exogenous recombinant murine IL-10.

(ii) Evaluate the role of SHP-1 in the regulation of LPS-induced TNF-α and IL-10 production in splenic macrophages by:

a. Using SHP-1 anti-sense oligonucleotides to interfere with SHP-1 expression in normal splenic macrophages.

b. Reconstituting SHP-1 expression in *me/me* splenic macrophages using adenovirus vector expressing the intact SHP-1 gene.

(C) Analyze LPS activated signal transduction pathway(s) involved in the regulation of IL-10 expression and determine the role of SHP-1 in LPS-induced signal transduction pathway(s) resulting in IL-10 production using normal and *me/me* splenic macrophages.

(i) Determine the role of the MAPK pathway in LPS-mediated IL-10 production.

(ii) Evaluate whether SHP-1 is involved in the regulation of LPS-induced MAPK pathway leading to IL-10 production

(iii) Identify and evaluate PTKs family members that participate in the regulation of LPS-induced IL-10 production.

(iv) Analyze the role of SHP-1 in regulating PTKs in LPS-induced IL-10 production.

II. MATERIALS AND METHODS

II.1 REAGENTS AND ANTIBODIES:

Recombinant murine IL-10 and TNF-α were purchased from R&D systems. (Minneapolis MN, USA). LPS (lipopolysaccharide) from *Escherichia Coli* serotype 055:B5 and Streptolysin O (SLO) from *Streptococcus Pyogenes* were purchased from Sigma-Aldrich Canada Ltd (Oakville, ON). Antisense SHP-1, SHP-2, CD4 and scrambled oligonucleotides, were made by University Core DNA services at the University of Calgary, Alberta. Specific pharmacological inhibitors for SHP-1 (PTP-I, PTP-II, PTP-III), ERK (PD98059), P38 MAPK (SB202190, SB203580) Src family tyrosine kinases (PP2, PP1, SU6656), Protein Tyrosine Kinases (Herbimycin A and Genistein) were all purchased from Calbiochem (LaJolla, CA).

CD11b (Mac-1)-phycoerythrin (PE) conjugated, CD19-fluorescein isothiocyanate (FITC) conjugated and CD-3 FITC conjugated monoclonal antibodies were obtained from BD Biosciences (Mississauga, ON). F4/80 FITC conjugated monoclonal antibody was obtained from Serotec (Raleigh, NC). Monoclonal anti-SHP-1, anti-phospho BTK, anti-Hck, anti-Fyn, anti-FAK and anti-Pyk2 antibodies were purchased from BD Biosciences (Mississauga, ON). Polyclonal anti-Pyk2 and monoclonal anti-phospho Src (Y416) antibodies were purchased from Sigma-Aldrich Canada Ltd (Oakville, ON). Polyclonal anti-phospho Src antibodies, (Y416) and (Y527), were purchased from Cell Signaling Technology (Danvers, MA). Trulight Src kinase assay kits were purchased from EMD-Calbiochem (LaJolla, CA).

Fetal Bovine Serum (FBS) was purchased from Cansera (Burlington, Ontario), Penicillin and Streptomycin were purchased from GibcoBRL, now Invitrogen Canada (Burlington, ON). Opti-MEM®I media was purchased from Invitrogen Canada (Burlington, ON).

II.1.1 Cell Lines:

EL-4, murine thymoma cells and CCL1-L929, murine connective tissue fibroblast cell line, was obtained from American Type Culture Collection (ATCC) (Manasass, VA). CCL1-L929 culture supernatant was used as a source of murine M-CSF.

II.2.0 MICE:

Mice used for experiments were all from the *C3H/HeJFeLe* strain (Jackson Laboratory, Bar Habour, Maine.). To obtain *me/me* (SHP-1 homozygote mutant) mice, heterozygote (*+/me*) pairs were crossed. For the purpose of crossing, offsprings of crossed heterozygotes were genotyped from genomic DNA obtained from tail tissue of mice using DNeasy tissue kit (Qiagen, Mississauga, ON) as described below. For the purposes of this study, homozygous *me/me* mice were selected based on an evident phenotype and compared with healthy littermate controls. *Me/me* mice are distinct and can be recognized by 4 days of age, when several small abscesses appear on the surface of the skin. Healthy littermate controls were termed "normal". The animals used were between 10 and 12 days old at the time of sacrifice. Mice were bred and raised under pathogen-free conditions and used according to approved practice guidelines of the Animal Care Committee, Protocol #2006-029, Animal Resource Division, Health Canada (Ottawa, ON).

GENOTYPING

Following the isolation of genomic DNA from mice tails, two methods were used for genotyping animals: genotyping by cleavage using Taq I enzyme and genotyping by bi-directional PCR amplification of specific alleles (Bi-PASA).

II.2.1 Genotyping by cleavage using Taq I enzyme:

A 426 base pair (bp) sequence of genomic DNA encompassing the site of the *me/me* mutation was amplified by PCR and used in genotyping animals. For the PCR reaction, the forward primer 5′-CGCTGCTGCTCATGTATCTC-3′ and the reverse primer 5′-GCCTACAGAGGGAAGAGGCT-3′ (Core-DNA Services, University of Calgary) were used. The PCR reaction was prepared as described below:

Genomic DNA sample (20μL), 10X PCR buffer (5μL), dNTPs (1μL), 5′ forward primer (5μL), 3′ reverse primer (5μL) (diluted 1:110), Taq DNA polymerase (1μL) and nuclease free water (19μL) for a final volume of 50μL. Two control reactions were included: (a) no genomic-DNA template, to ensure that PCR products were not the result of primer dimers, (b) no Taq polymerase to ensure Taq specific amplification. The reactions were carried out in a PTC-200 Peltier Thermal cycler (MJ Research, Woburn, MA). The PCR program used for this experiment was as follows: denaturation at 95°C for 2 min, annealing at 58°C for 1 min, extension at 72°C for 1min for a total of 30 cycles. Samples were subjected to a final denaturation at 95°C and elongation at 72°C for 7 min. The amplicons were purified using the Qiagen PCR purification kit (Qiagen, Mississauga, ON) as per manufacturer's protocol. The purified PCR products were digested using the restriction digest enzyme, Taq I. For this, PCR product (24 μL), Taq I (2 μL), TaqI Reaction Buffer, 10X Buffer B (3 μL)

at 65°C for 6 h. Digests were resolved by electrophoresis in 12% Polyacrylamide gels (PAGE). The PAGE gel ran at 100V for 1 h. Gels were stained with 10μL (10mg/mL) ethidium bromide in 15mL 1X TBE (Bio-Rad Mississauga, ON) for 10 min and DNA bands were visualized under UV light. Wildtype animals were distinguished from heterozygotes by the presence of two bands (238 and 257bp) demonstrating complete digestion of PCR products while heterozygotes had three bands (238, 166 and 91bp) indicative of digestion of only one wildtype allele (Data not shown).

II.2.2 Genotyping by duplex allele-specific PCR and melting curve analysis

The absence of SHP-1 protein and the resulting *me/me* phenotype results from homozygosity of a frameshift deletion (cytosine) at position 709 of the *Ptpn6* gene (Genbank Accession Number NC_000072.5). PCR primers pairs were designed to specifically amplify either the wild type or *me/me* sequence. This was achieved by ensuring a 3' mismatch of the *wt* forward primer with the *me/me* genomic sequence and a 3' mismatch of the *me/me* reverse primer with the *wt* sequence. Primers were obtained from (Applied Biosystems, Mississauga, ON). The primer pairs for wildtype and *me/me* sequences amplified products with a size of 257 bp and 120 bp, respectively. Reactions containing both primer pairs were run on a real-time PCR instrument (LightCycler, Roche Applied Science, Mannheim, Germany) using SYBR Green to detect double stranded amplification products (Roche Applied Science, Mannheim, Germany). The 10 μL PCR reaction was assembled as described below; 2μL of LightCycler FastStart DNA Master^{PLUS} Sybr Green I mix, 0.5μM of each oligonucleotide primer and 1μL of genomic DNA (1/10 dilution of the eluate from a spin-column preparation of mouse tail genomic DNA, corresponding to approximately

10 ng). Touchdown PCR conditions were used to ensure allele-specificity of the reactions and the minimal extension time was used to minimize the formation of the larger (333bp), nonspecific product of the outermost primers. PCR amplification products were differentiated based on their respective melting temperatures using the LightCycler melting curve analysis system (LightCycler Data Analysis v3.54, Roche Diagnostics, Mannheim, Germany). Samples could be unambiguously classified as *wt/wt, wt/me* or *me/me* (Data not shown).

II.3 CELL ISOLATION AND CELL CULTURE

II.3.1 Isolation and Propagation of Primary Splenic Macrophages

Spleens from both normal and *me/me* mice were obtained by dissecting sacrificed animals. For each experiment, between 2 and 4 spleens were pooled. Using frosted glass slides, spleens were teased into cell suspensions in conditioned Opti-MEM®I media containing 10% FBS (Cansera, Burlington ON), 20% CCL1-L929 culture conditioned medium (a source of murine M-CSF), 1% 10U/mL penicillin G and 10μg/mL streptomycin. Approximately 5 X 10^7 splenocytes were transferred to 75cm^2 Corning non-pyrogenic, polystrene flasks (Corning/Fisher, Nepean, ON) in conditioned medium. The cultures were maintained at 37°C, 5% CO_2 for up to 72 h to allow splenic macrophages to adhere to coated plastic surfaces. After 72 h, cells were washed with phosphate buffered saline (PBS) (Gibco-Invitrogen Canada, Burlington ON) and replenished with fresh growth media followed by further incubation for 24-72 h. The isolation procedures for splenic macrophages were developed and modified based on human monocyte isolation procedures [377, 378].

Six-day old cultures from normal and splenic macrophages in 96 well Nunc plates were observed under an upright Axioskop 40 light microscope using 10X and 20X objectives (Carl Zeiss Ltd, Toronto, ON). Digital images were acquired under bright-field using attached Axiocam 1.2 digital camera. Images were exported as JPEG files.

II.3.2 Harvesting Primary Splenic Macrophages

After four to six days in cultures, supernatants were aspirated and non-adherent cells were washed off with ice cold 1X PBS (Gibco-Invitrogen Canada, Burlington, ON). Adherent cells were gently detached using a cell scraper (Nalge Nunc International, Rochester, NY). Cells lifted off using the scraper were suspended in serum-free Opti-MEM®I media. Cells were concentrated by centrifuging at 800 x g for 5min at 4°C, and cell pellets were resuspended in 0% FBS Opti-MEM®I (Gibco-Invitrogen Canada, Burlington, ON). Cell suspensions were maintained at 37°C, 5% CO_2 for 4 h to allow them return to a quiescent state. Cells were then pelleted by centrifuging, at 800 x g for 5mins at 4°C. Cell pellets were stored at -80°C until ready to use for western immunoblotting, SQ-RTPCR, immunoprecipitation and kinase assays.

II.4 FACS ANALYSIS

II.4.1 Preparation of adherence-isolated splenic macrophage cultures for flow cytometric analysis

Cells from six-day old cultures were harvested as described above, counted using a haemocytometer and approximately 5 X 10^5 cells in 100μL of 1 X PBS

containing 0.1% NaN_3 were transferred into 6 mL polystrene tubes (Sarstedt, St. Laurent QC). First, cells were blocked for 10 min at RT in 100μL 1% BSA in PBS and subsequently mixed with Ab combinations and incubated in the dark for another 10 min. Combinations of antibodies used are summarized in Table II.1. The cells were subsequently washed for 5 min at RT, 200 x g with PBS then fixed in 500 μL of 2% paraformaldehyde. Cells were stored at 4°C overnight and analyzed using a FACScan flow cytometer (Becton Dickinson, San Diego, CA) and CellQuest software (Becton Dickinson).

II.4.2 Preparation of whole blood samples (FACS controls) for flow cytometric analysis

1-2 ml of whole blood obtained from cardiac punctures of normal mice were collected in EDTA coated polystyrene tubes (Sarstedt, St Laurent. QC). Whole blood volumes of 100 μL were aliquoted into 6 mL polystyrene tubes and the cells were stained with antibody combinations for 10 min at Room temperature (Table II.1). White blood cells (WBCs) were stabilized and Red blood cells (RBCs) were lysed using the 35 sec cycle on the Q-prep Workstation system (Beckman Coulter, Mississauga ON). Alternatively and as an option, cells were washed for 5 mins at RT, 200x g with PBA and resuspended with 2% paraformaldehyde to a final volume of 500 μL.

II.4.3 Staining cells for double or triple-colour flow cytometric analysis

Adherent splenocytes that had been harvested and counted were double or triple stained to evaluate the proportion of splenic macrophages in relation to other cell

Table II.1 Summary of various Ab panel used in defining the purity of macrophages population by FACS analysis.

	ANTIBODY	CLONE	COMPANY
AUTOFLUORESCENT CONTROL	No antibodies used		
WHITE CELLS	α-CD45 (LCA, Ly-5)	30-F11	Becton Dickinson
Macrophages	α-F4/80-FITC	A3-1	Serotec
	α-F4/80-PE	A3-1	Serotec
	α-MAC-1-FITC	M1/70	Becton Dickinson
	α-MAC-1-PE	M1/70.15	Cedarlane Labs
	Ab combinations used: Three colour: α-CD-45/α-F4/80/α-MAC-1		
T Cells	α-CD3-FITC	17A2	Becton Dickinson
	Ab combinations used: Three colour: α-CD45/α-CD-3/α-MAC-1		
B Cells	α-CD-19-PE	1D3	Becton Dickinson
	Ab combinations used: Three colour: α-CD45/α-CD-19/α-MAC-1		
Granulocytes	α-GR-1-PE	RB6-8C5	Becton Dickinson
	Ab combinations used: Three colour: α-CD-45/α-Gr-1/α-MAC-1, α-CD-45/α-Gr-1/α-F4/80		

types in six-day old splenocyte cultures. Autofluorescent controls and appropriate colour compensaton controls were included in the panel. All antibodies (Ab) were empirically titrated and optimal concentrations of Ab were used in subsequent experiments.

II.4.4 Flow Cytometric analysis of cells

Cells were gated initially using forward and side scatter profiles to determine cell size and granularity, respectively. The forward and side scatter profile was used to exclude dead cells and debris. A minimum of 10 000 events per condition was recorded, based on electronically gated $CD45^+$ (a pan white cell marker) population(s). The mean channel fluorescence values were used for comparison of CD45 expression levels. For whole blood controls containing heterogenous populations, gates were established individually for lymphocytes and monocytes and these cell subpopulations were individually analyzed. Significant changes in median fluorescence intensity were determined by overlaying histograms. The observation of a distinct population shift in log fluorescence intensity confirmed supposed changes in mean channel fluorescence. Data were acquired using the FACScan flow cytometer (BD Biosciences, San Jose, CA) equipped with an air-cooled argon-ion laser emitting 15mW at 488nM and CellQuest software (Macintosh version) was used for the analysis. After establishing the purity of six-day old splenic macrophage cultures, cells grown in a similar manner were used in other experiments including western immunoblotting, immunoprecipitation, RNA extraction, kinase assays and microscopy.

II.5 ELISA ASSAYS:

II.5.1 Cell Culture for ELISA analysis

Splenocytes derived from normal and *me/me* mice were plated in clear 96-well plates (Nunc, Mississauga, ON) at a density of 5 X 10^6 cells/mL in a 200 μL volume and incubated at 37°C, 5% CO_2 for 72 h. Cells were plated in quadruplicate for each treatment condition. Non-adherent cells were washed off after 72 h with 1 X PBS and the wells containing adherent cells were replenished with fresh growth medium. Cells were left untreated or treated with either LPS only at doses ranging from 0 to 50 μg/mL, inhibitors only or with inhibitors plus LPS for varying durations as dictated by the specific experiment.

After 72 h in culture, cells were washed using with 1 X PBS and cultures were replenished with fresh growth media. In most experiments cells were then treated with 10 μg/mL of LPS for 24 h. Cell supernatants from independent wells were harvested and stored at -85°C until analyzed by ELISA for IL-1β, IL-6, MIP-2, TNF-α and IL-10 production.

II.5.2 Cytokine Analysis

IL-10, TNF-α, IL-1β, IL-6 and MIP-2 levels were determined using commercially available, colourimetry-based ELISA kits (Biosource, Camarillo, CA). The protocol for measuring cytokine levels was provided by the manufacturer. The standard curve was constructed using a cytokine standard provided in each kit. The levels of detection for each cytokine (kit) was < 3 pg/mL. Samples were prepared in duplicates and absorbance was read at a wavelength of 450 nm using a colourimetric microplate reader (Dynex technologies, Chantilly, VA) running MRX Revelation

Software. Results were manually exported to Excel (Microsoft Corp. Redmond, WA) and analysed using Graph Prism and/or SAS statistical packages (SAS Institute, Toronto ON).

II.5.3 Pharmacological Inhibitors

Inhibitors to P38 (SB203580 and SB202190), ERK1/2 (PD98059), PTK (Herbimycin A and Genistein), SHP-1 (PTP I, PTP II and PTP III), Pyk2 (AG-17) and Src (PP2 and SU6656) were used to determine the involvement of these signalling proteins in LPS-induced IL-10 production in normal and *me/me* splenic macrophages. Splenic macrophages were either left untreated or treated with varying concentrations of respective inhibitors for 2 h followed by treatment with LPS (10 μg/mL) for 24 h. In control wells, the viability of cells following inhibitor treatment was tested using the trypan blue exclusion test. Culture supernatants were collected and analysed by ELISA for IL-10 production. The biological activities of pharmacological inhibitors were evaluated by western immunoblotting to determine changes in phosphorylation levels of respective target proteins.

II.6 CONVENTIONAL RT-PCR AND REAL TIME PCR QUANTITATION OF IL-10 AND TNF-α MESSAGE LEVELS

5 X 10^6 cells from six-day old cultures were starved in Opti-MEM media (Gibco-BRL) with 5% CO_2 at 37°C for 4 h. For time course experiments, harvested cells were either left untreated or treated with LPS at 10 μg/mL for 4, 5, 6 and 7 h. In some experiments, the cells were treated with pharmacological inhibitors specific to

distinct signalling proteins. In these experiments, cells were either left untreated or pretreated with PP2, an inhibitor to Src family kinases (Calbiochem,) or AG-17, an inhibitor to Pyk2 (Calbiochem,) for 1 to 2 h, followed by treatment with LPS (10μg/mL) for 7 h. Following various treatments the cells were pelleted by centrifugation at 300 x g for 5min at 4°C. To ensure RNA stability during extended storage, samples were resuspended in 250μL 1X PBS and 25μL of RNA Later (Ambion, Austin, TX, USA) and stored at -80°C until ready to use.

Frozen cell pellets were allowed to thaw at room temperature followed by a 1:1 (v/v) dilution with 1X PBS. Samples were then inverted three to five times to mix thoroughly and pelleted by centrifuging at 700g for 5 mins at room temperature. To ensure maximal disruption of cells, the mixture was passed through a QiaShredder column (Qiagen, Mississauga, ON) and centrifuged for 2 min at 20 000 x g. RNA was purified from these samples using the RNeasy system (Qiagen, Mississauga, ON) according to the manufacturer's protocol. 50 μl of nuclease-free water was used to elute total RNA. To ensure maximal recovery of Total RNA, the first eluate was re-used for a second elution. Co-purified DNA was removed by performing a DNase digest. Briefly, 40μl total RNA, 4 μl of 10X reaction buffer (Ambion) and 0.67μl of DNase 1 enzyme (2 U/μl) (Ambion) were combined on ice, then heated to 37°C for 30 min. Samples were treated with 2 μl 100mM EDTA and heated to 72°C for 15 min in a PTC200 thermal cycler (MJ research). A 10 X dilution of samples was used for RNA Quantitation (Helios Alpha Ultraspec, Thermo Scientific). RNA intergrity was assessed by 1% formaldehyde gel (See Appendix I) electrophoresis. RNA samples were stored at -80°C until ready to use.

II.6.1 cDNA Synthesis – Reverse Transcription

A high capacity cDNA archive kit (Applied biosystem, Missisauga ON) was used to prepare cDNA from total RNA. A total volume of 100μL was used for cDNA synthesis. Specifically, 50 μl of the RT master mix (See Appendix I) was combined with 30 μL nuclease-free water and 2-5μg (in 20 μL) of RNA and incubated at 25°C for 10 min and for an additional 2 h at 37°C. Samples were split into two 50 μL aliquots and stored at -20°C until ready to use.

II.7.4 Conventional semi-quantitative PCR

Semi-quantitative PCR was performed using 5 μl of cDNA, 10X PCR buffer (500mM KCl, 25mM $MgCl_2$, 100mM Tris-HCl, and 0.01% gelatin), 25mM of each dNTP (Invitrogen Life Technologies, Burlington, ON), 1 μM of each of the appropriate 5′ and 3′ primers, 5 U/μl of *Taq* DNA polymerase (Roche diagnostics, Mannheim, Germany) were added to each thin-walled reaction tube (Perkin Elmer) in a final volume of 50 μl. The oligonucleotide primer sequences for mouse TNF-α, IL-10 and β-actin (Stratagene, La Jolla, CA) are as follows: sense strand TNF-α primer, 5′-ATG AGC ACA GAA AGC ATG ATC-3′; antisense strand TNF-α primer, 5′-TAC AGG CTT GTC ACT CGA ATT-3′; sense strand IL-10 primer, 5′-GGA CAA CAT ACT GCT AAC CCC GAC-3′; antisense strand IL-10 primer 5′-AAA ATC ACT CTT CAC CTG CTC-3′. Appropriate positive (5 μl of cDNA provided by the company for each of the primers) and negative controls (5 μl of ddH_2O) were also included in each

PCR batch experiment. PCR was carried out in a PTC-100 programmable thermal controller (MJ Research Inc., Watertown, MA).

For PCR, 2 μl of reverse transcribed product~100ng cDNA was used for amplification. The conditions for amplification were as follows: TNF-α: denaturation at 94°C for 45 sec, annealing at 60°C for 45 sec, extension at 72°C for 1:30 min, 30 cycles and a final extension 72°C for 10 min; IL-10: denaturation at 94°C for 1 min, annealing at 60°C for 45 sec, extension at 72°C for 1:30 min, 30 cycles and a final extension at 72°C for 10 min.

The amplified products (5 μl) for TNF-α, IL-10 and β-actin were resolved by electrophoresis on 1.2% agarose gels at 95 volts (V) for 60 min. Gels were stained with ethidium bromide (1 μg/mL) in 1X TAE buffer (Appendix) for 10 min and visualized under UV light. The expected sizes of specific PCR products were 258, 226, and 514bp for IL-10, TNF-α and β-actin, respectively. Specific bands were verified by reference to a 1-kilobase DNA ladder (Gibco-BRL). To quantify the relative changes in the expression of cytokine mRNA levels following stimulation, the band intensity of the β–actin PCR product was compared to the band intensity of the cytokine PCR product band.

II.7.5 Real time semi-quantitative RT-PCR

Using commercially available gene expression assays (Assays on Demand, β-actin (Mm00607939), TNF-α (Mm0400443258) and IL-10 (Mm00439616), Applied Biosystems, Foster City, CA), reactions were prepared in 20 μL volumes containing 10 μL 2X Taqman Fast Universal PCR master mix, 1 μL of 20X gene specific primers

and probes, 5 μL cDNA from corresponding to 100ng of input total RNA. Reactions were processed on the ABI 7500 Fast Real time PCR system (Applied Biosystems, Foster City, CA). Standard cycling parameters include an initial incubation at 95°C for 20 sec followed by 95°C for 3 sec and 60°C for 30 sec. Each reaction was run in triplicate. Target specific IL-10 and TNF-α were run on the same plate with β-actin as endogenous controls. Samples were set up from a master mix to ensure reproducibility and to minimize pipetting error. Data were acquired in real time and results were analyzed using the relative quantitation algorithm of the SDS 3.5.3 software of the ABI 7500 Fast PCR system. Baselines and thresholds were set manually. Results were calibrated to the untreated control for that set of experiments. Wildtype results were calibrated to wildtype untreated controls and *me/me* results were calibrated to *me/me* untreated controls. Data were represented as fold change relative to the calibrator sample. Error bars represent the technical variation (maximum and minimum points within experiments) among replicates within a single experiment.

II.7 ANTISENSE EXPERIMENTS

II.7.1 Reversible cell permeabilization

The pore-forming toxin, Streptolysin O (SLO) was used to reversibly permeate cells for introduction of oligonucleotides into cells. In each well, 200 μL of splenocyte cell suspension at a density of 5 X 10^6 cells/mL were cultured in 96-well micro-titre plates. Cells were incubated at 37°C, 5% CO_2 for 72 hr. Non-adherent cells were aspirated and washed away using 1X PBS and cultures were replenished with fresh growth media. Cultures were then treated with 20 ng/mL of SLO (*Streptolysin O*) in

the absence of Ca^{2+} with or without oligonucleotides at varying concentrations for 10 to 15 min. Cells were resealed by treating with warm growth medium containing Ca^{2+} (10 mM $CaCl_2$) and 20% FBS for 2 h at 37°C. Viability of cells following reversible permeabilization was determined by adding trypan blue to cells and examining under an inverted microscope for trypan blue stained cells. Cells unable to reseal absorb trypan blue.

II.7.2 Interfering with expression of SHP-1 and Pyk2

Phosphorothioate modified antisense oligonucleotides corresponding to SHP-1, Pyk2 and SHP-2 sequences were used to interfere with the expression of SHP-1, Pyk2 and SHP-2, respectively. SHP-2 is a control for related but distinct SH-2-containing protein tyrosine phosphatase. Scrambled oligonucleotides consisting of random sequences of the same length as the test oligo were used to control for specificity. Oligonucleotides containing phosphorothioate backbone modifications were purchased from the University of Calgary core-DNA Services. The sequences were SHP-1 ODN (30bp), 5'-GAG GTC TCG GTG AAA CCA CCT CAC CAT CCT-3', scrambled ODN (30bp), 5'-GGC CAG GTC CCA TCG ACA CCG ATA CAT CTT-3' or SHP-1 (15bp) 5'-AAA CCA CCT CAC CAT-3'.

Alternatively, in separate but similar experiments not utilizing *Streptolysin O*, cells were treated with 15 μM and 30 μM SHP-1 oligonucleotide, 5'-AAA CCA CCT CAC CAT-3', 30 μM SHP-2 oligonucleotide 5'-TCT CCG CGA TGT CAT-3' and control, unrelated CD4 oligonucleotide, 5'-AGG GAC TCC CCG GTT CAT-3'. Cells were allowed 2 to 3 h for antisense target activity. Cells were then either left untreated

or treated with LPS for 24 h. Supernatants from individual wells were collected for IL-10 or TNF-α ELISA analysis. As a measure of antisense activity, SHP-1 or Pyk-2 protein expression was measured in cell lysates by Western immunoblotting.

II.8 ADENOVIRAL SHP-1 GENE RECONSTITUTION EXPERIMENTS

II.8.1 Preparation of splenic macrophages for adenovirus infection

Cells for adenoviral transfections were cultured as described previously. The total number of cells from four representative wells was counted using a haemocytometer and the average number of cells per well was used as an estimate for the number of cells per well.

II.8.2 Adenoviral Transduction of normal and *me/me* splenic macrophages

Adenoviruses and/or their constructs used in this study were kind gifts from Dr. Peter Liston (University of Ottawa/CHEO, Ottawa, ON). A total of four adenovirval constructs were used. Ad-GFP (Adenovirus containing Green Fluorescent Protein) was used as a negative control, Ad-SHP-1 (Adenovirus containing full length SHP-1) was used to reconstitute *me/me* splenic macrophages lacking SHP-1. Ad-AEG40A (Adenovirus containing substrate-trapping SHP-1 mutant, expressing only SH2 domains of SHP-1 but lacking the phosphatase domain of SHP-1) was used as a dominant negative mutant in normal splenic macrophages and Ad-ENI039 (Adenovirus containing phosphatase domain inactive mutant) was also used as a dominant negative mutant in normal splenic macrophages.

II.8.3 Adenovirus infection of splenic macrophages

Multiplicity of Infection (MOI) was determined based on the average cell count per well from 4 representative wells. Normal and/or *me/me* splenic macrophages were then washed and replenished with 150 μL, 1 X PBS and allowed to stabilize by incubating at 37°C, 5% CO_2 for 30 min. Adenoviruses were added to individual wells in quadruplicates and gently rocked every 15 min for a total of 90 min. To equilibrate the cells and encourage growth, 350 μL of growth media containing 20% FBS in Opti-MEM was added to each well for a total volume of 500 μL and cells were further incubated at 37°C, 5% CO_2 for 24 h followed by stimulation with LPS for another 24 h. Depending on the experiment, supernatants were harvested either by pooling the contents of four wells into 2 mL screw-cap tubes (Nunc, Mississauga, ON) or by individually transferring the contents of each well into new sterile 24-well plates. Samples were stored at -80°C until ready to use.

For western immunoblotting, wells were scraped very gently to detach splenic macrophages and collected in 500 μL volumes of 1 X PBS. Quadruplicate wells with identical treatments were pooled and pelleted by centrifuging at 800 x g for 5 min at 4°C. Supernatants were discarded and pelleted samples were lysed immediately by adding 25μL of protein sample buffer (50 μL β-Mercapto-Ethanol in 950 μL Laemlli buffer (BIORAD, Mississauga, ON)). Samples were completely lysed by sonicating at 37°C for 15 min and heating samples at 100°C for 5 min. Samples were stored at -80°C until ready to use.

II.9 WESTERN BLOT ANALYSIS AND IMMUNOPRECIPITATION

II.9.1 Western Immunoblotting

Following cell harvest of splenic macrophage cultures and subsequent treatment with specific inhibitor(s) and/or ligand(s), as determined by individual experiments, protein lysates were prepared by resuspending approximately 10^7 normal or *me/me* splenic macrophages in 100 µL of lysis buffer (1X PBS containing 1% triton X-100, 1% Tween 20, 1 mM sodium orthovanadate, 1 µg/mL aprotinin, 1 µg/mL leupeptin). Supernatants from cell pellets were clarified by centrifugation for 20 min at 21000g at 4°C. Protein concentrations of the supernatants were quantified by Bradford assay using Bradford reagent (Bio-Rad Laboratories, Mississauga ON). Samples were boiled for 5 min in Laemlli sample buffer containing SDS (Bio-Rad Laboratories) supplemented with 5% v/v ß-mercaptoethanol (Sigma-Aldrich) and subjected to electrophoresis and immunoblotting. Fifty to 100 µg of total protein, as specified, were electrophoresed through 8%, 10% or 12% SDS-polyacrylamide gels as specified. Gels were then electro-blotted into PVDF Immobilon-P membranes (Millipore, Mississauga, ON) for 1 h at 100 V using a wet Bio-Rad mini cell apparatus (Bio-Rad Laboratories, Mississauga, ON). The membranes were briefly rinsed with 1X TBST (Appendix I) and then blocked for 1 h with western blot blocking reagent (Roche Diagnostic, Laval, QC). Membranes were incubated with primary antibodies diluted in 1 X western blot blocking reagent (Roche Diagnotstic, Laval, QC). Membranes were then washed 3 times with 1X TBST at 5 min intervals. This was followed by treatment with corresponding secondary antibodies conjugated to HRP (Bio-Rad Laboratories, Mississauga, ON) followed by 3 washes with 1 X TBST at 5 min

intervals. Blots were then treated with ECL (enhanced chemiluminesence) reagents (GE Healthcare, formerly Amersharm, Mississauga, ON) and exposed to BioMax X-ray films (Kodak, New Haven, CT). X-ray films were developed using Kodak M35A XOMAT processor.

II.9.2 Preclearing Lysates for Immunoprecipitation (IP) Experiments

To prevent non-specific binding of ubiquitous cellular proteins to protein A beads, cell lysates were precleared using 50 μL of a 50% Protein A slurry (Protein A-coated Sepharose™ 6MB beads, (GE Healthcare, Mississauga, ON)) added to 1-2 mg of lysate from murine splenic macrophages. The final volume was increased to 1 mL using lysis buffer. The mixture was incubated on a rotating platform at 4°C for 45 min. Lysates were separated from protein A beads by centrifuging at 800 x g for 2 min. Cleared lysates were transferred to a fresh eppendorf tube.

Two methods were employed for immunoprecipitation: (a) Traditional immunoprecipitation and (b) Catch and Release reversible immunoprecipitation system (Millipore, Mississauga, ON). The catch and release method was employed to obtain purified enzyme samples for kinase assay.

II.9.3 Traditional Immunoprecipitation (SHP-1, FAK, Src, Pyk2 Immunoprecipitations)

For conventional IP, lysates were mixed with 1 to 2 μg of commercially purchased antibody corresponding to the respective target proteins (SHP-1, FAK, Src and Pyk2). Mixtures were incubated on a rotating platform at 4°C overnight followed

by addition of 50 μL protein A slurry for 2 hr at 4°C. Immune complexes were separated by centrifuging at 20,000 x g for 5 min at 4°C and purified by washing with 1 mL lysis buffer (for stringent conditions) or 1 X PBS (for less stringent washes). After washing, immune complexes were resuspended in 25 μL of Laemlli sample buffer (Bio-Rad, Mississauga, ON) boiled for 5 min then subjected to western immunoblotting procedures.

II.10.4 Catch and Release Immunoprecipitation (Src, Pyk2, Kinase assay)

For catch and release IPs, lysates were added to a spin-column containing washed resin slurry, the components were added in the following order; 1 X wash buffer (Millipore, Billerica, MA) (as per manufacturer instruction), 1 mg of precleared lysate, 1-4 μg of antibody and 10 μL of Antibody Capture Affinity Ligand (proprietary reagent, provided by manufacturer, (Millipore, Billerica, MA)). The final reaction volume was increased to 500 μL, spin columns were capped and incubated on a rotating platform at 4°C for 4 h or overnight. Immune complexes were purified by washing the column with 1 X wash buffer (provided by manufacturer(Millipore, Billerica, MA)) and centrifuging for 30 sec at 8000 x g. This step was repeated three times. Purified immune complexes were eluted from the column using 75 μL non-denaturing buffer (provided by manufacturer(Millipore, Billerica, MA)). For kinase assay applications, samples were used as eluted. For western immunoblotting applications, 30 μL Laemlli buffer was added to eluates. Samples were then boiled for 5 min and loaded unto SDS-PAGE gels.

II.11 KINASE ASSAY

II.11.1 Fluorescent superquenching-based kinase assay

Src or Pyk2 immunoprecipitates from untreated, LPS treated or inhibitor + LPS treated normal and *me/me* splenic macrophages were used for the kinase assay. The activity of Src was measured using Trulight Src kinase assay kits (EMD Biosciences, NJ) strictly adhering to manufacturer's protocol. In a white 96-well plate, these components were added in the following order; 5μL 800 μM ATP working solution, 10μL 4μM substrate working solution (AAAEEIYGEI peptide), 25 μL enzyme working solution (fresh immunoprecipitates). Each condition or sample type was prepared in triplicate. In certain preliminary experiments, Src calibrators (phosphorylated peptide; AAAEEIpYGEI) were included as controls. The contents of all wells were manually mixed and incubated at room temperature for 1 h. After incubating, 10 μL post reaction buffer (100 mM MES-based high salt buffer) was added to each well to stop the reaction. For reaction readout, 30 μL of 1 X sensor (microspheres coated with fluorescent polymers) were added to each well and incubated in the dark for 30 min at room temperature. Samples were read in triplicate and fluorescence was measured using Varioskan Fluorescent Microplate Reader (Thermo Scientific, Waltham, MA) version 3.00.7 (serial/model number 3001-45) with an excitation wavelength of 450 nm and an emission wavelength of 490 nm. The parameters for reading were set using Skanit Software version 2.2. Results were exported to Microsoft Excel for further analysis.

II.12 STATISTICAL ANALYSIS

All experiments were done at least three times and showed similar trends in each experiment. However, data from one representative experiment were shown in the results section of this dissertation. In all experiments samples were set up in technical duplicates (ELISA) or triplicates (Q-PCR). Data shown represent the mean values of technical replicates. Unless otherwise described, error bars represent the variation from the mean within the experiment (among technical replicates). Comparison between two groups with a single variable was made using Student t test. Comparisons between multiple groups (more than two) were made using one-way ANOVA or Multiple Comparisons test.

III. RESULTS

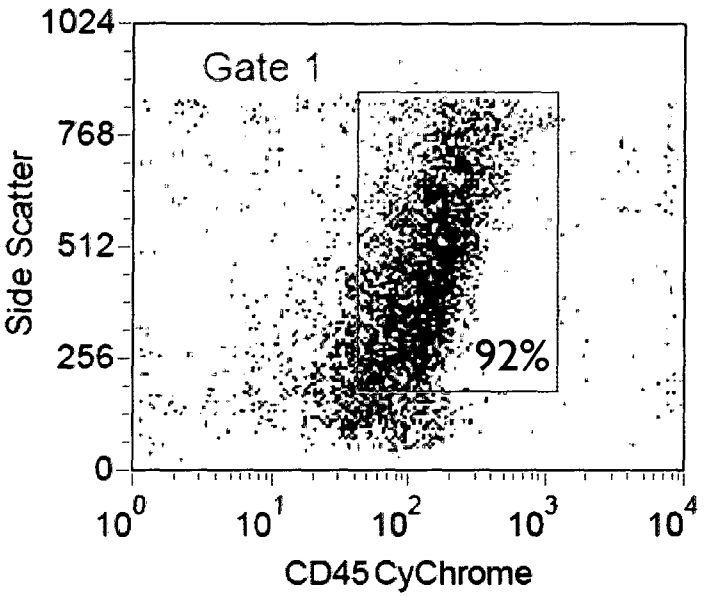
III.1 FLOW CYTOMETRIC ANALYSIS OF CELL SURFACE EXPRESSION OF MURINE MACROPHAGE SPECIFIC MARKERS IN NORMAL AND *ME/ME* SPLENIC MACROPHAGES.

All experiments performed in this study employed splenic macrophages. Since splenocytes represent a very heterogenous population of cells, the first objective of the study was to establish a pure population of splenic macrophages. Cell enrichment and adherence-based procedures were used in the isolation of splenic macrophages [377, 378]. Cultured splenocytes from normal and *me/me* mice were harvested after six-days of culture and analyzed by flow cytometry for expression of different cell surface markers. This was done to confirm that the cultures were largely composed of macrophages. A minimum of 10 000 events were recorded based on electronically gated $CD45^+$ cells, a pan-leukocyte marker [379]. Forward and side scatters were used to establish the cell size and cell complexity, respectively. Analysis of the cultured splenocyte populations using side scatter and CD45-Cychrome as parameters revealed 92% and 96% of $CD45^+$ normal and *me/me* splenocytes, respectively (Fig III.1A and III.1B). Further analysis of $CD45^+$ populations of normal and *me/me* splenocytes using MAC-1-PE (CD-11b) and F/480 FITC, specific markers of murine macrophages [380-382], showed that 94% and 96% of cells, respectively, were double positive for these two markers (Fig III.2A and III.2B). These results indicate that ~ 94% (normal) and 96% (*me/me*) of six-day old splenocyte cultures were predominantly macrophages. In two separate cultures of splenocytes the percentage of macrophages in both normal and *me/me* cultures exceeded 90%. Microscropic images of normal and *me/me* splenic

Fig. III.1 Analysis of side scatter and CD45 cychrome in six-day culture of normal and *me/me* splenocyte populations.

CD45 expression in six-day old culture of normal (A) and *me/me* (B) splenocytes. Splenocyte populations (Gate 1) were electronically gated based on their side scatter characteristics and CD45 expression. All experiments were repeated at least three times. Data from a representative experiment are depicted in the figure.

(A)


1024
768
512
256
0
Side Scatter
Gate 1
92%
10^0
10^1
10^2
10^3
10^4
CD45 CyChrome

(B)

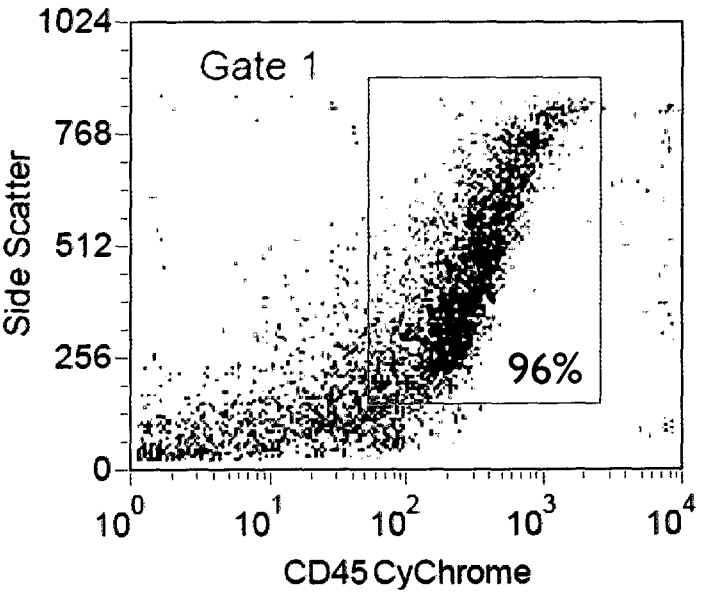
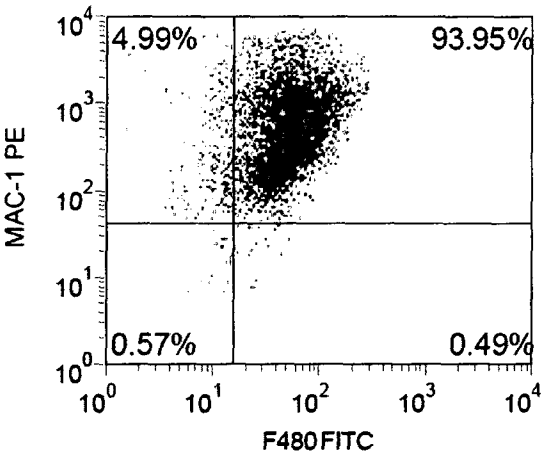

1024
768
512
256
0
Side Scatter
Gate 1
96%
10^0
10^1
10^2
10^3
10^4
CD45 CyChrome

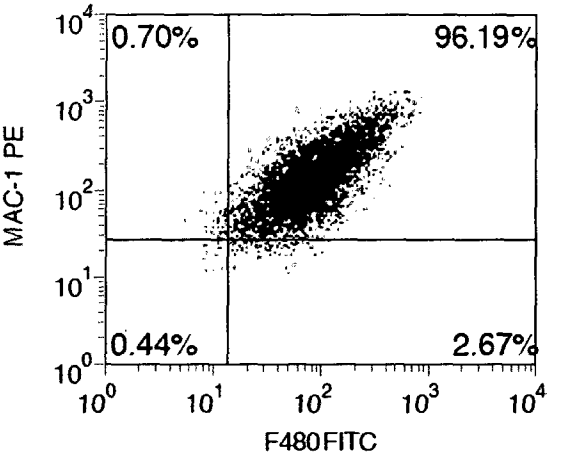
Fig. III.2 Assessment of splenic macrophage populations in six-day old cultures of normal and *me/me* splenocytes.

Six-day old cultures of normal (A) and *me/me* (B) splenocytes were triple-stained using α-MAC-1-PE, α-F4/80-FITC and α-CD45-cychrome monoclonal Abs. Monocytes were initially gated based on side-scatter and $CD45^+$ expression, followed by acquisition of MAC-1 $(CD\text{-}11b)^+$ and/or $F4/80^+$ populations. Microscope digital pictures of six-day old normal (C) and *me/me* (D) splenic macrophages under 10X bright field lighting. All experiments were repeated at least three times. Data from a representative experiment are depicted in the figure.

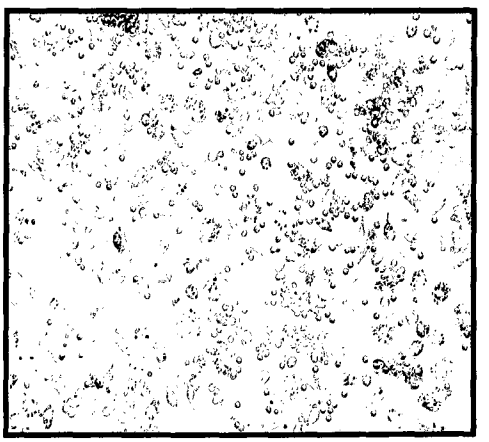
(A)



(B)

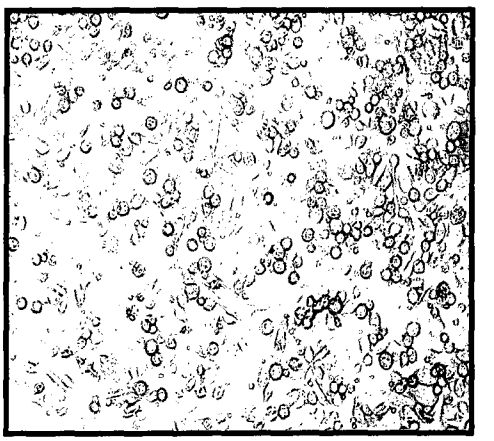


(C)



Normal
Untreated
(10X BF)

(D)

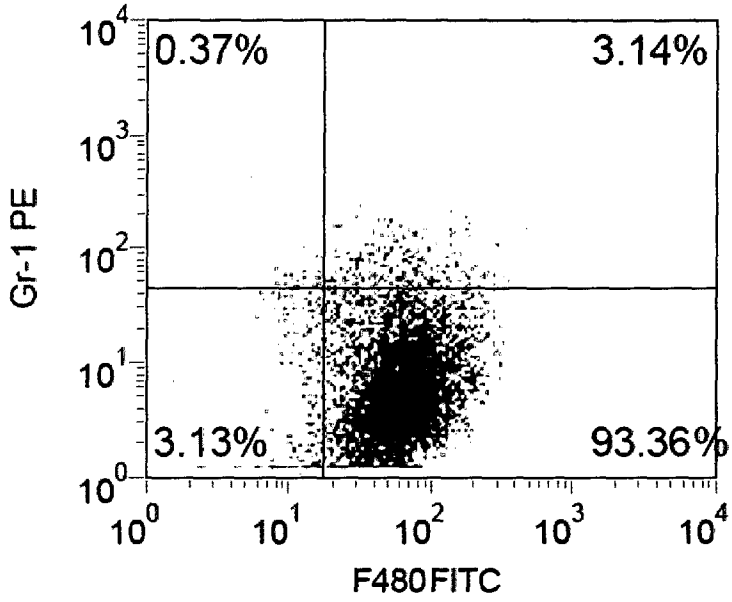


me/me
Untreated
(10X BF)

Fig. III.3 Assessment of granulocyte population in six-day old normal and *me/me* splenocyte populations

Six-day old cultures of normal (A) and *me/me* (B) splenocytes were triple-stained using α-Gr-1-PE, α-F4/80-FITC and α-CD45-cychrome monoclonal Abs. Splenocytes were initially gated based on side-scatter and $CD45^+$ expression, followed by acquisition of Gr-1-PE^+ and/or $F4/80^+$ populations. All experiments were repeated at least three times. Data from a representative experiment are depicted in the figure.

(A)


0.37%
3.14%
3.13%
93.36%
Gr-1 PE
F480FITC

(B)

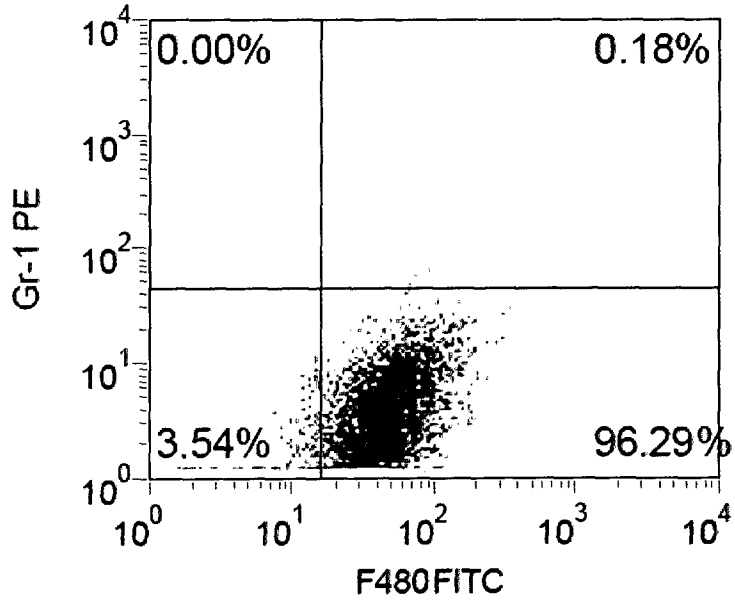
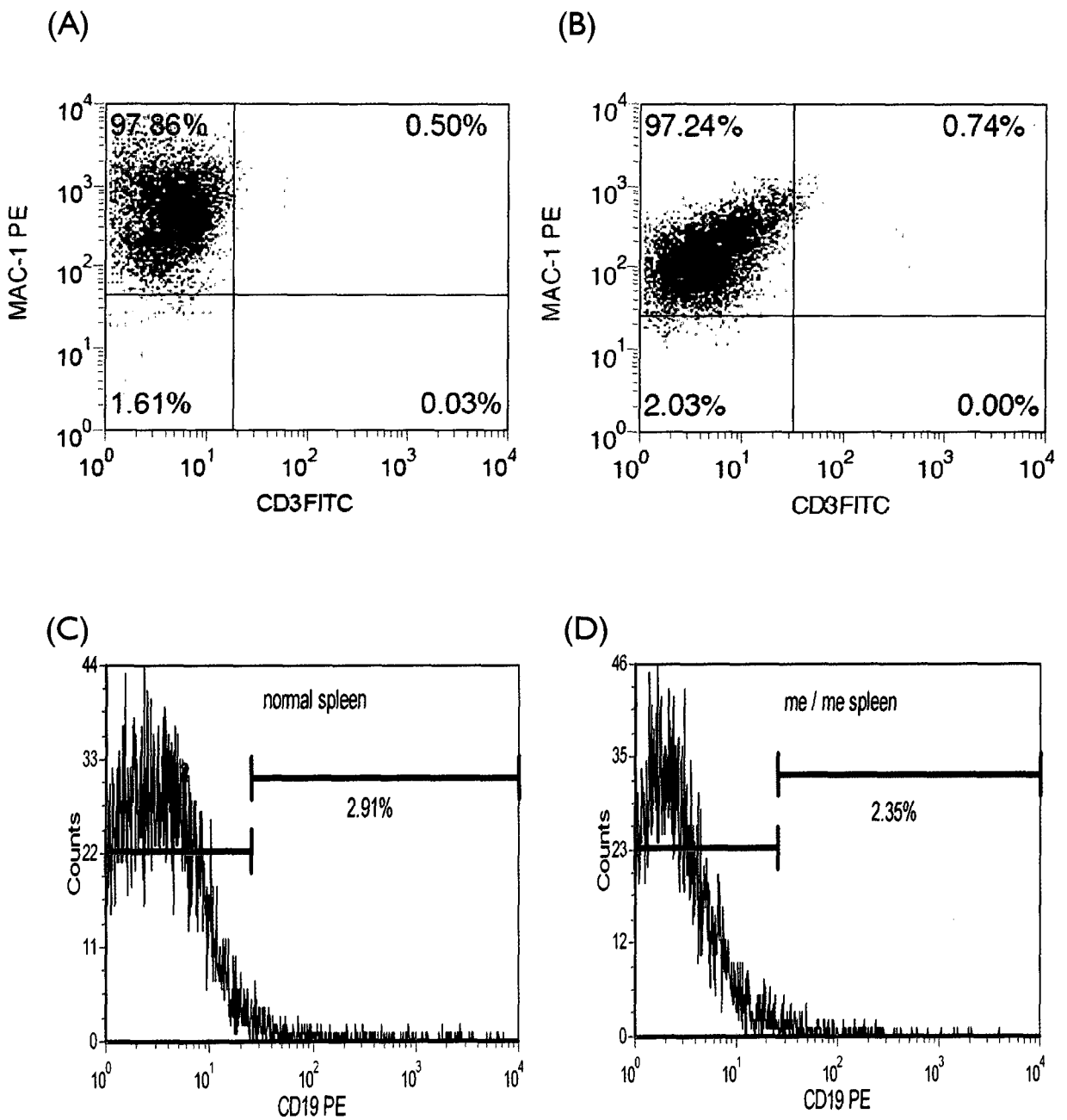

0.00%
0.18%
3.54%
96.29%
Gr-1 PE
F480FITC

Fig. III.4 Evaluation of B and T-cell population in six-day old normal and *me/me* splenocyte populations

Six-day old cultures of normal (A) and *me/me* (B) splenocytes were triple-stained using α-MAC-1-PE, α-CD-3-FITC and α-CD45-cychrome monoclonal Abs. Splenocytes were initially gated based on side-scatter and $CD45^+$ expression, followed by acquisition of MAC-1 $(CD\text{-}11b)^+$ and/or $CD\text{-}3^+$ populations (A and B) and for $CD19^+$ B cells in normal (C) and me/me (D) splenocytes. All experiments were repeated at least three times. Data from a representative experiment are depicted in the figure.


(A)
(B)
97.86%
0.50%
1.61%
0.03%
97.24%
0.74%
2.03%
0.00%
MAC-1 PE
CD3FITC
(C)
(D)
normal spleen
2.91%
me / me spleen
2.35%
Counts
CD19 PE

macrophages cultures are shown in Fig III.2C and III.2D. To further characterize the cultured population and to determine the level of contaminants by other cell types in culture, additional analyses were done by FACS for the presence of granulocytes, T cells and B cells. Using specific markers for these cells types namely, GR-1 PE, 3-FITC and CD-19 PE, respectively [4, 383, 384]. Results in Fig III.3A and III.3B show granulocyte ($Gr\text{-}1^+$) populations of 0.37% and 0.00% in normal and *me/me* cultures, respectively. Fig III.4A and III.4B show $CD\text{-}3^+$ T cell populations of 0.04% and 0.00% for normal and *me/me* cultures, respectively. Fig III.4C and III.4D show B cell ($CD19^+$) populations of 2.91% and 2.35% for normal and *me/me* cultures, respectively. Collectively, these low numbers of contaminating cells were consistently observed in all three independent experiments performed. The low populations of granulocytes, B cells and T cells observed in six-day old normal and *me/me* splenic macrophage cultures raised the possibility that Gr-1-PE, CD-19-PE and CD-3-FITC monoclonal Abs used might have been defective. To discount this possibility, whole blood samples from normal mice were examined. FACS analyses of whole blood samples from normal mice showed much higher levels of B cells, T cells and granulocytes (Table III.1) hence confirming the efficacy of respective antibodies used.

III.2 PRO-INFLAMMATORY CYTOKINE AND CHEMOKINE PROFILES IN LPS TREATED NORMAL AND *ME/ME* SPLENIC MACROPHAGES.

After establishing the procedure for generating splenic macrophages from spleens of normal and *me/me* mice, the next objective was to profile the levels of the

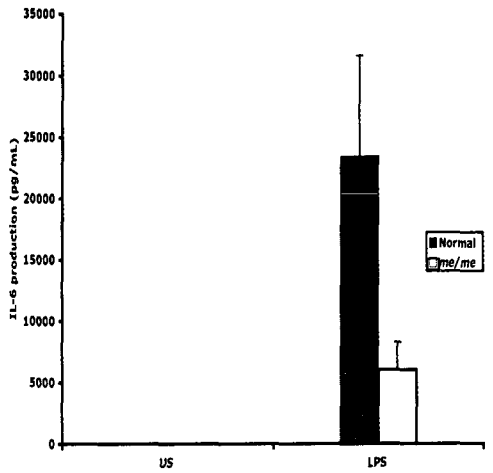
TABLE III.1.0 : PERIPHERAL BLOOD IMMUNOPHENOTYPING IN NORMAL MICE.

Monoclonal Antibody	**Positive Cells (%)**		
	Lymphocyte gate	Monocyte Gate	Granulocyte Gate
CD45 Cychrome	99.5	99.5	99.7
CD-3 FITC	57.8	25.7	9.8
MAC-1 PE	10	82.6	98.8
CD-19 PE	32.4	23.9	15.4
Gr-1 PE	6.9	71.9	95
F4/80 FITC	2.1	20.8	16.2

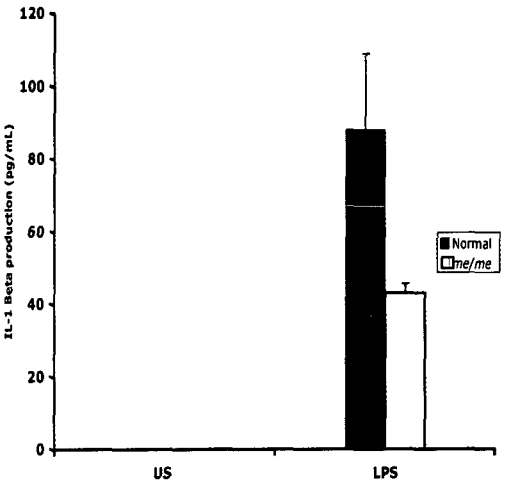
Fig III.5 Anomalous production of IL-6, IL-1β, TNF-α and MIP-2 in LPS treated *me/me* splenic macrophage cultures.

IL-6 (A), IL-1β (B), TNF-α(C) and MIP-2 (D) production levels in culture supernatants of normal (■) and *me/me* (□) splenic macrophages, was measured by ELISA. Splenic macrophages cultured at a density of approximately 5 X 10^6 cells/mL were either untreated or treated with LPS (50μg/mL) for 72 h. Culture supernatants were harvested after 72 h and tested for IL-6, IL-1β, TNF-α and MIP-2. The inset in C panel shows the amount of TNF-α produced by untreated normal and *me/me* splenic macrophages. All experiments were repeated at least three times. Data from a representative experiment are depicted in the figures. For clarity and because the amount of measurable cytokines and chemokine induced by LPS varies, data are depicted on different graphs with different scale ranges. ELISA data are represented as arithmetic mean of duplicate wells ± variation from the mean within a single experiment.

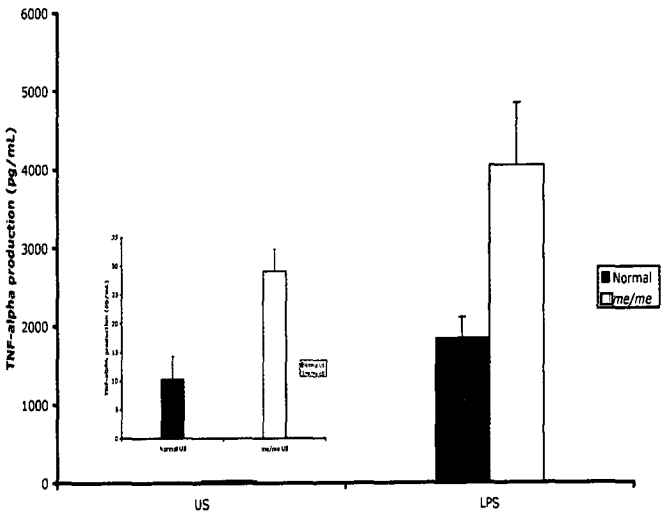
A


IL-6 production (pg/mL)
35000
30000
25000
20000
15000
10000
5000
0
US
LPS
Normal
me/me

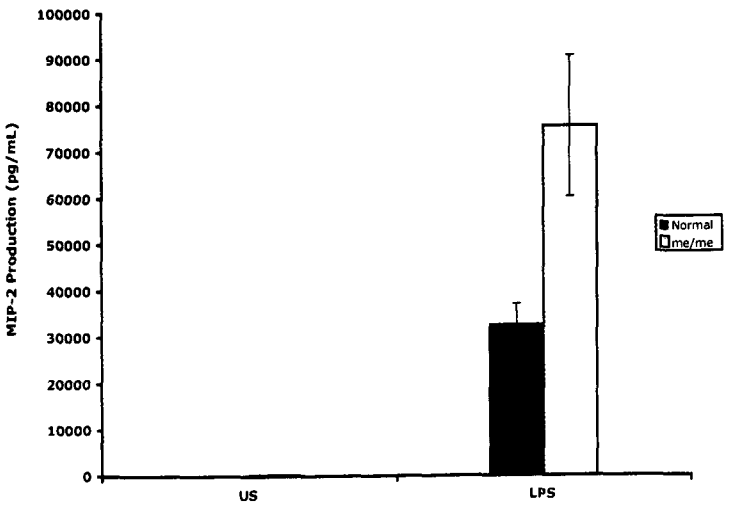
B


IL-1 Beta production (pg/mL)
120
100
80
60
40
20
0
US
LPS
Normal
me/me

C


TNF-alpha production (pg/mL)
6000
5000
4000
3000
2000
1000
0
US
LPS
Normal
me/me

D


MIP-2 Production (pg/mL)
100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
US
LPS
Normal
me/me

pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and chemokines MIP-2, in LPS treated normal and *me/me* splenic macrophages.

III.2.1 Analysis of pro-inflammatory cytokines in LPS treated normal and *me/me* mice

To determine the role of SHP-1 in inflammatory responses, the profiles of IL-6, IL-1β, TNF-α and MIP-2 in LPS treated normal and *me/me* splenic macrophages, were compared by ELISA. Supernatants from cells treated with 50 μg/mL LPS for 72 h were harvested and measured. Results comparing the IL-6 production levels in LPS treated normal and *me/me* splenic macrophages showed on average, a 3-fold decrease in IL-6 levels in cultures of *me/me* splenic macrophages compared to normal splenic macrophages (Fig III.5A). IL-1β levels in LPS treated *me/me* splenic macrophages were slightly lower compared to normal splenic macrophages (Fig III.5B). Next, TNF-α, a key pro-inflammatory cytokine induced by LPS and responsible for mediating the secondary effects of LPS [146, 147, 152, 157, 159, 173], was examined. ELISA analysis comparing the levels of TNF-α production in LPS treated normal and *me/me* splenic macrophages showed on average, a 2-fold increase in TNF-α production in LPS treated *me/me* splenic macrophages compared to normal splenic macrophages (Fig III.5C).

Me/me mice are characterized by infiltration and accumulation of neutrophils in the skin causing alopecia and necrotic patches on skin surfaces [365, 367]. MIP-2, an IL-8 equivalent in humans, is a chemokine responsible for neutrophil recruitment to sites of inflammation [52, 385]. This prompted the analysis of MIP-2 levels in *me/me* splenic macrophages. ELISA results obtained from supernatants of LPS treated normal

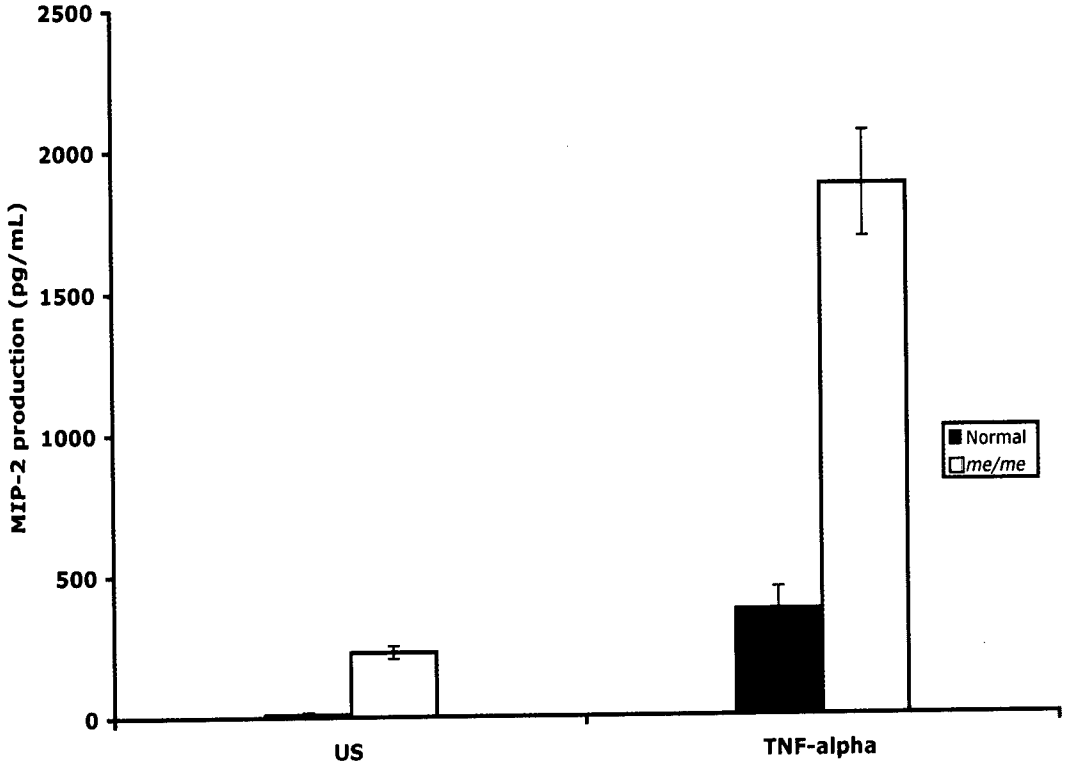
and *me/me* splenic macrophages showed, a 2-fold increase in MIP-2 production in *me/me* splenic macrophages compared to normal controls (Fig III.5D). The observation of high levels of MIP-2 in LPS treated *me/me* splenic macrophages raised the possibility that this was a direct consequence of elevated levels of TNF-α. It was therefore of interest to examine the effects of TNF-α on MIP-2 production in normal and *me/me* splenic macrophages. Normal and *me/me* splenic macrophages treated with 100 ng/mL of TNF-α for 24 h revealed an increase in MIP-2 production compared to untreated normal and *me/me* splenic macrophages. In addition, the levels of MIP-2 production in TNF-α treated *me/me* splenic macrophages were, 4-fold higher compared to normal splenic macrophages (Fig III.6A), suggesting that the elevated levels of TNF-α produced by *me/me* splenic macrophages induce high levels of MIP-2 and contribute to the accumulation of neutrophils and other innate immune cells at sites of inflammatory lesions. Based on this, I further explored LPS-mediated TNF-α production.

III.2.1.1 Kinetics of LPS-mediated TNF-α production

The observation that the levels of TNF-α were elevated in *me/me* splenic macrophages treated with LPS for 72 h prompted a kinetic analysis of TNF-α production in LPS treated normal and *me/me* splenic macrophages. For this, normal and *me/me* splenic macrophages were treated with LPS for 0, 6, 12, 24, 36, 48 and 72 h. Elevated levels of TNF-α were observed between 12 to 72 h in *me/me* splenic macrophages compared to normal controls (Fig III.7A). The observed increase in

Fig III.6 Elevated MIP-2 production in TNF-alpha treated *me/me* splenic macrophages.

Normal (■) and *me/me* (□) splenic macrophages plated at a density of approximately 5 X 10^6 cells/mL were either untreated or treated with TNF-α (100ng/mL) for 72 h. Culture supernatants were harvested after 72 h and assessed by ELISA for MIP-2 production. All experiments were repeated twice. Data from a representative experiment are depicted in the figure. ELISA data are represented as arithmetic mean of duplicate wells ± variation from the mean within a single experiment.


2500
2000
1500
1000
500
0
MIP-2 production (pg/mL)
US
TNF-alpha
Normal
me/me

TNF-α production in *me/me* splenic macrophages was therefore not linked to the duration of LPS treatment. Given that these elevated levels of TNF-α protein production were not time dependent, it was necessary to determine if this was a consequence of altered transcription. Therefore, total RNA was isolated from normal and *me/me* splenic macrophages treated with LPS for 7h. Total RNA was converted to cDNA by reverse transcription and TNF-α was amplified using specific primers. Amplified products were analyzed by gel electrophoresis. Results show elevated TNF-α mRNA in *me/me* splenic macrophages (Fig III.7C) compared to normal macrophages when normalized to actin mRNA. These results were further confirmed by time-dependent measurement of mRNA levels of TNF-α in LPS treated normal and *me/me* splenic macrophages by semi-quantitative real time RT-PCR (SQ-RTPCR). Normal and *me/me* splenic macrophages were treated with LPS for 4,5,6 and 7 h, total mRNA was extracted and TNF-α mRNA was quantified relative to the untreated control. SQ-RTPCR results (Fig III.7B) show at least a 3-fold increase in TNF-α mRNA levels in LPS-treated *me/me* splenic macrophage compared to normal controls, thus, indicating that increased TNF-α production was due to increased transcription. Comparison of LPS treated samples (at the 4,5 and 6 h time points) to untreated controls showed a 120-fold and a 40-fold increase in TNF-α mRNA induction in *me/me* and normal splenic macrophages, respectively.

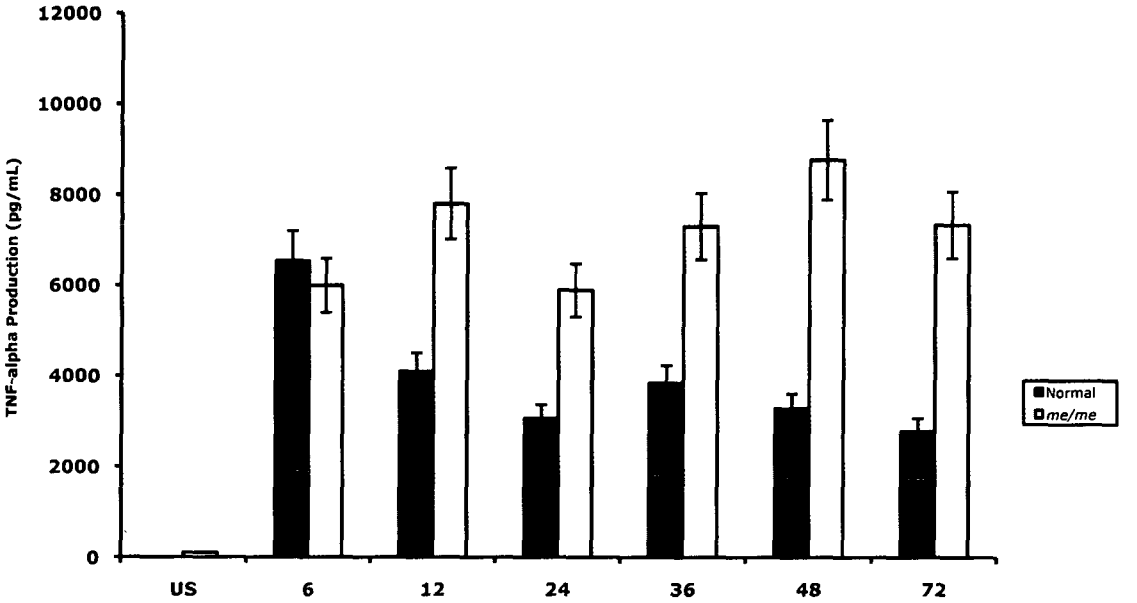
Fig III.7 Time dependent analysis of TNF-α protein and mRNA levels in normal and *me/me* splenic macrophages.

(A) Normal (■) and *me/me* (□) splenic macrophages plated at a density of approximately 5 X 10^6 cells/mL were either untreated or treated with LPS (10 μg/mL) for 0-72 h. Culture supernatants were harvested after 0,6,12, 24, 36, 48 and 72 h and assessed by ELISA for TNF-α production. All experiments were repeated at least three times. Data from a representative experiment are depicted in the figure. ELISA data are represented as arithmetic mean of duplicate wells ± variation from the mean within the representative experiment.

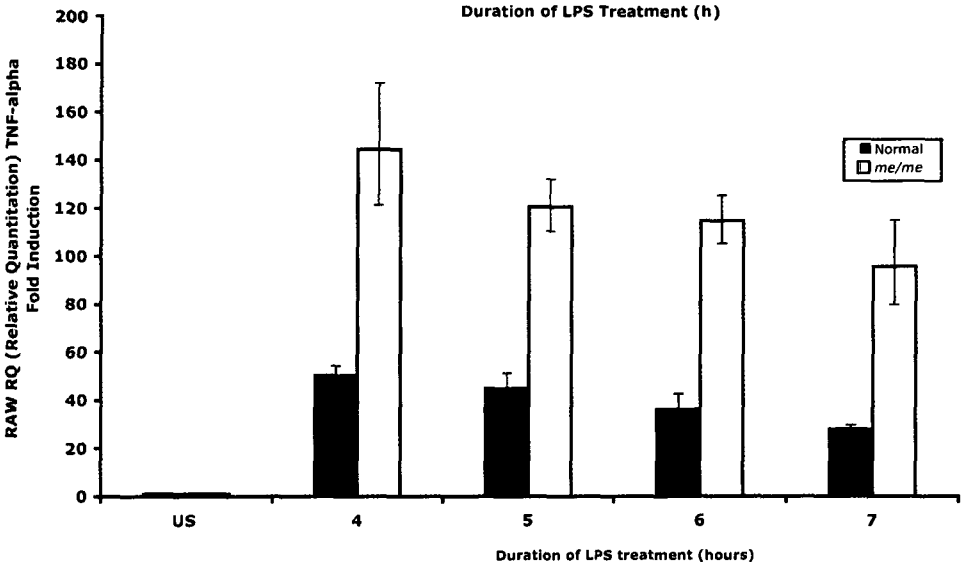
(B) Real time RT-PCR analysis of TNF-α transcripts in LPS treated normal (■) and *me/me* (□) splenic macrophages. Serum starved splenic macrophages (1 X 10^7 cells/ml) were either untreated or treated with LPS (10 μg/mL) for 0,4,5,6,7h. Total RNA was extracted followed by cDNA synthesis. TNF-α transcripts were amplified by Q-PCR using β-actin as a reference control. To measure the relative changes in TNF-α induction, results of LPS treated samples were normalized to the untreated control within the experiment. Data were represented as fold change versus calibrator sample, which is the untreated control. All experiments were performed at least three times. Data from a representative experiments are depicted in the figure. Error bars represent the upper and lower levels of variation from the mean within a single experiment.

(C) RT-PCR analysis of TNF-α transcripts. Serum starved normal and *me/me* splenic macrophages (5 X 10^6 cells/mL) were treated with LPS (10 μg/mL) for 7h. Total RNA was extracted and expression of TNF-α (top panel) was determined by semiquantitative RT-PCR. RT-PCR products were visualized on PAGE gels. β-actin transcripts were used as a standard control (lower panel). All experiments were performed at least three times however data from a single experiment is depicted in the figure.

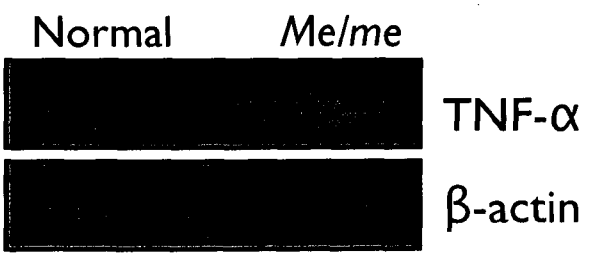
A


12000
10000
8000
6000
4000
2000
0
TNF-alpha Production (pg/mL)
US
6
12
24
36
48
72
Duration of LPS Treatment (h)
Normal
me/me

B


200
180
160
140
120
100
80
60
40
20
0
RAW RQ (Relative Quantitation) TNF-alpha Fold Induction
US
4
5
6
7
Duration of LPS treatment (hours)
Normal
me/me

C


Normal
Me/me
TNF-α
β-actin

III.2.1.2 Dose response analysis of LPS-mediated TNF-α production

Elevated levels of TNF-α production in *me/me* splenic macrophages may be a function of the dose of LPS used for treatment. To discount this possibility, I analyzed the levels of TNF-α production in normal and *me/me* splenic macrophages treated with varying and increasing doses of LPS (0, 0.1, 1, 5, 10, 25, 50 μg/mL). Results of ELISA analysis (Fig III.8) revealed increased levels of TNF-α production in *me/me* splenic macrophages compared to normal controls at all doses of LPS that were significant at 1, 5, 10 and 50 μg/mL and showed that high TNF-α production was not observed only at high doses of LPS.

III.2.2 Analysis of anti-inflammatory cytokines in LPS treated normal and *me/me* splenic macrophages

IL-10 is a well-recognized anti-inflammatory cytokine with known inhibitory activity on TNF-α biosynthesis. In view of my findings that TNF-α production was elevated in *me/me* splenic macrophages, it was of interest to evaluate IL-10 production in these cells. To examine this, I assessed the levels of LPS-induced IL-10 in *me/me* splenic macrophages and compared them to normal controls at 72 h post treatment with 50 μg/mL LPS. The amount of IL-10 in cultures of *me/me* splenic macrophages showed a 2-3-fold decrease in IL-10 levels compared to normal splenic macrophages (Fig III.9).

Fig III.8 LPS Dose dependent analysis of TNF-α production in normal and *me/me* splenic macrophages.

Normal (■) and *me/me* (□) splenic splenic macrophages plated at a density of approximately 5 X 10^6 cells/mL were either untreated (US) or treated with increasing doses of LPS (0, 0.1, 1, 5, 10, 25, 50 μg/mL) for 24 h. Culture supernatants were harvested and TNF-α production was evaluated by ELISA. All experiments were repeated at least three times. Data from a representative experiment are depicted in the figure. ELISA data are represented as arithmetic mean of duplicate wells ± variation from the mean within a single experiment.

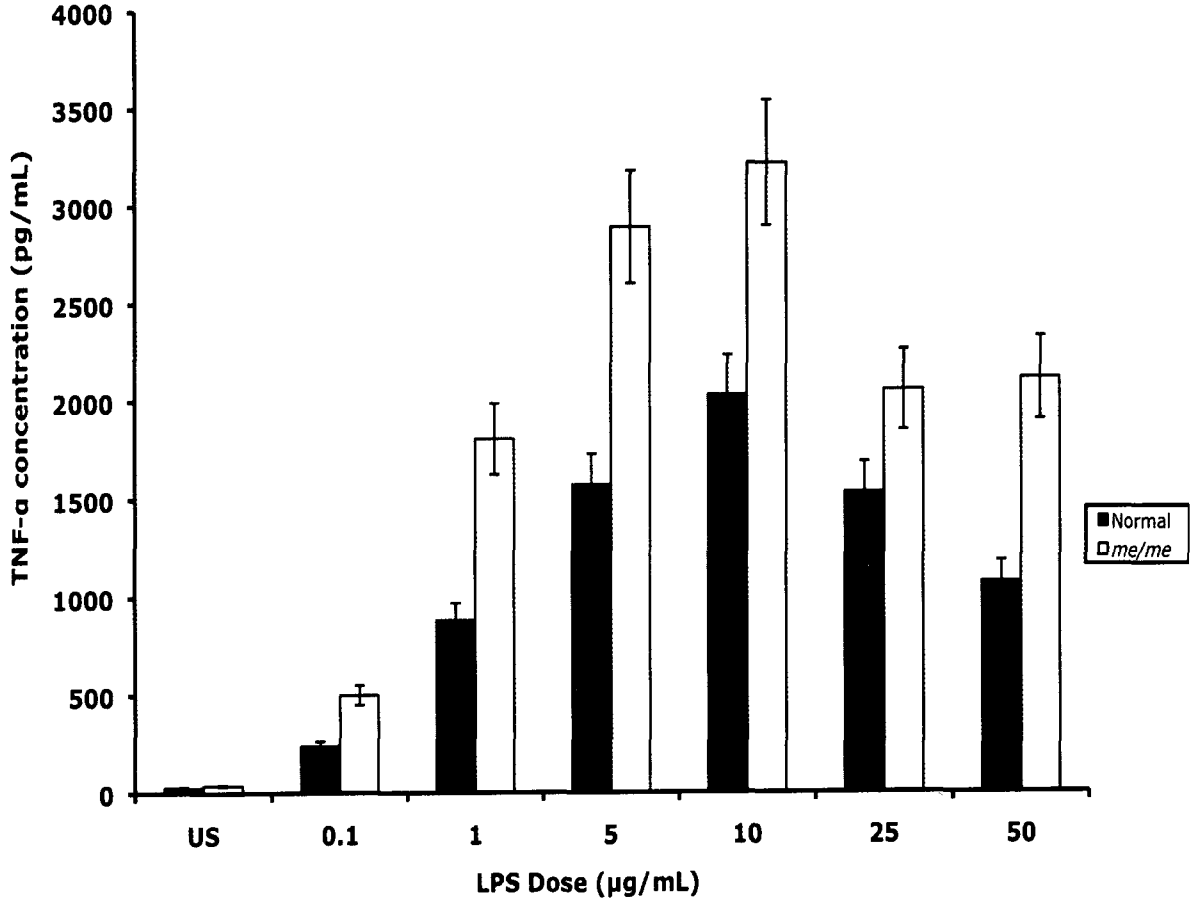
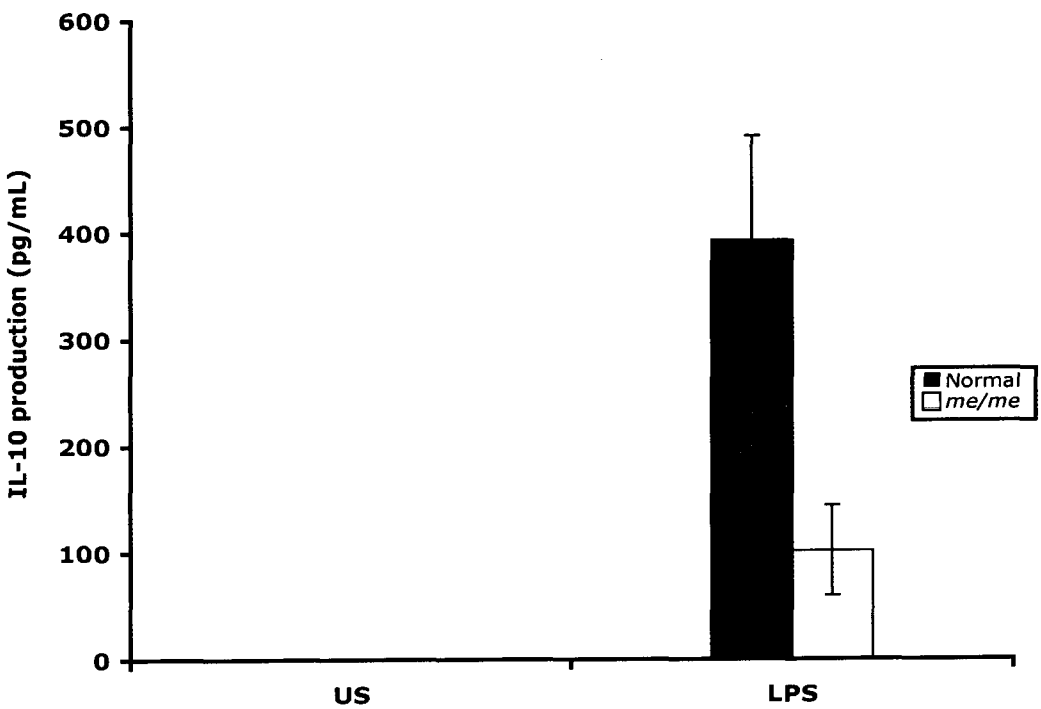

4000
3500
3000
2500
2000
1500
1000
500
0
TNF-α concentration (pg/mL)
US
0.1
1
5
10
25
50
LPS Dose (μg/mL)
Normal
me/me

Fig III.9 Assessment of IL-10 production in LPS treated normal and *me/me* splenic macrophages.

Normal (■) and *me/me* (□) splenic macrophages cultured at a density of approximately 5 X 10^6 cells/mL were either untreated or treated with LPS (50μg/mL) for 72 h. Culture supernatants were harvested after 72 h and evaluated by ELISA for IL-10 production. All experiments were repeated at least three times. However, data from a representative experiment showing similar trends are depicted in the figure. ELISA data are represented as arithmetic mean of duplicate wells ± variation from the mean within a single experiment.


600
500
400
300
200
100
0
IL-10 production (pg/mL)
US
LPS
Normal
me/me

III.2.2.1 Kinetics of LPS-mediated IL-10 production

The diminished levels of LPS-induced IL-10 production observed in *me/me* splenic macrophages might be dependent on the duration of LPS treatment. To test this, normal and *me/me* splenic macrophages were treated with LPS (10 μg/mL) for 0, 6, 12, 24, 36, 48 and 72 h. ELISA analysis (Fig III.10A) of supernatants revealed that IL-10 production in *me/me* splenic macrophages was consistently diminished as early as 6 h after stimulation with LPS up to 72 h. At all time points and in all three experiments that were performed, at least a 3-fold reduction in IL-10 production was observed in LPS treated *me/me* splenic macrophages when compared with normal controls. These results suggested that the observed low levels of IL-10 production in LPS-treated *me/me* splenic macrophages were not time dependent. The observation that IL-10 levels were significantly reduced in *me/me* splenic macrophages was extended by examining IL-10 mRNA induction in LPS treated normal and *me/me* splenic macrophages. Therefore, total RNA was isolated from normal and *me/me* splenic macrophages treated with LPS for 7h. Total RNA was converted to cDNA by reverse transcription and IL-10 was amplified using specific primers. Amplified products were analyzed by gel electrophoresis. Results show diminished levels of IL-10 mRNA in *me/me* splenic macrophages (Fig III.10C). These results were further confirmed by time-dependent measurement of mRNA levels over time using real time RT-PCR (SQ-RTPCR). Results from SQ-RTPCR analysis of IL-10 mRNA levels (Fig III.10B) demonstrated a significant decrease in LPS-mediated induction of IL-10 mRNA in *me/me* splenic macrophages compared to normal controls. In normal splenic macrophages treated with LPS, a 20 to 25-fold induction in IL-10 mRNA

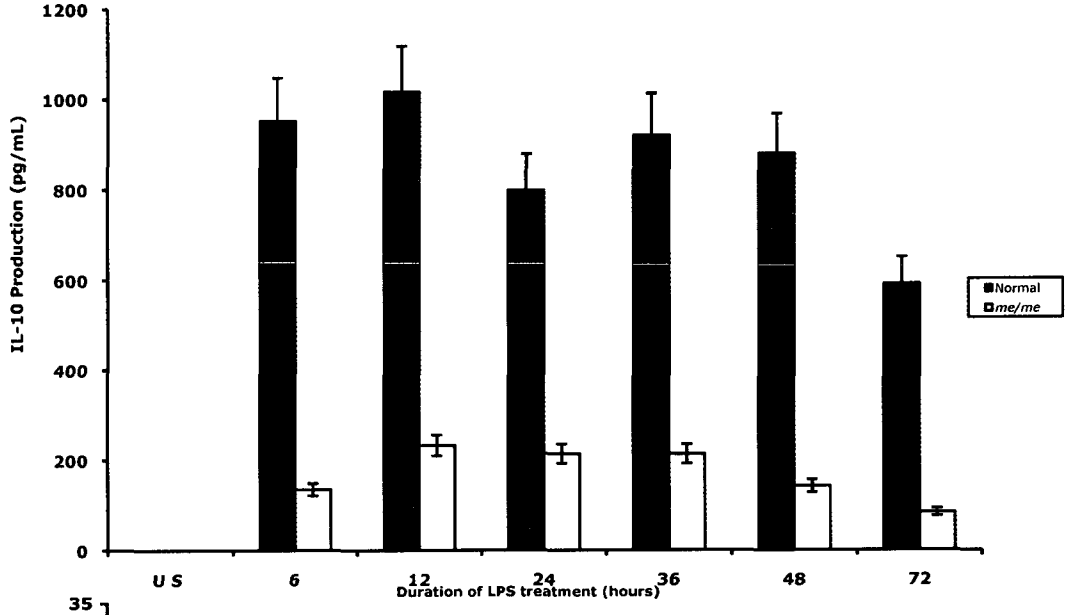
Fig III.10 Time dependent analysis of IL-10 protein and mRNA levels in normal and *me/me* splenic macrophages.

(A) Normal (■) and *me/me* (□) splenic macrophages plated at a density of approximately 5 X 10^6 cells/mL were either untreated (US) or treated with LPS (10 μg/mL) for 0-72 h. Culture supernatants were harvested after 0,6,12, 24, 36, 48 and 72 h and assessed for IL-10 production by ELISA. All experiments were repeated at least three times. Data from a representative experiment are depicted in the figure. ELISA data are represented as arithmetic mean of duplicate wells ± variation from the mean within a single experiment.

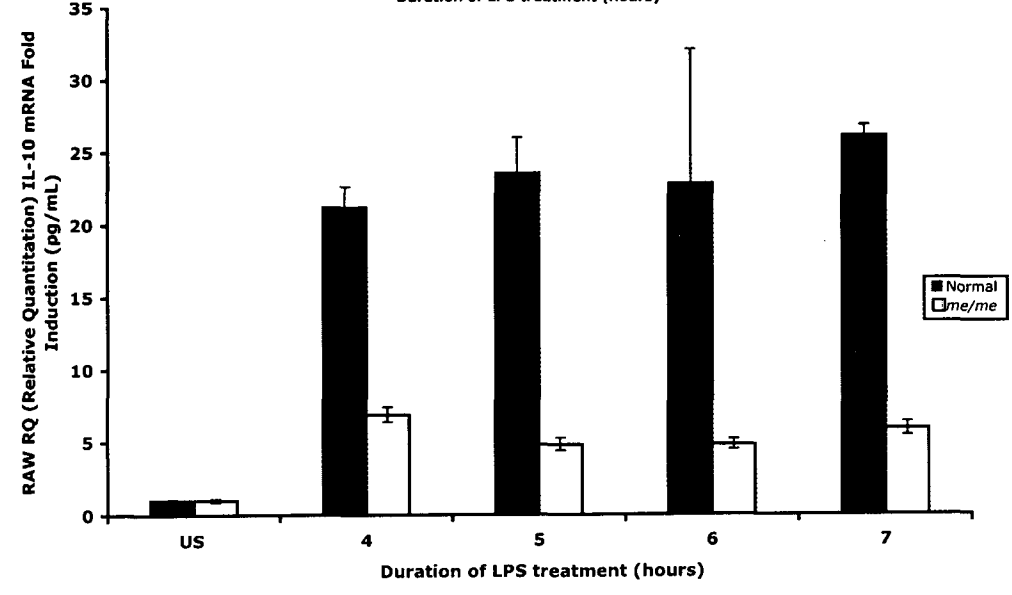
(B) Real time RT-PCR analysis of IL-10 transcripts in LPS treated normal (■) and *me/me* (□) splenic macrophages. Serum starved splenic macrophages (1 X 10^7 cells/ml) were either untreated or treated with LPS (10 μg/mL) for 0,4,5,6,7h. Total RNA was extracted followed by cDNA synthesis. IL-10 transcripts were amplified by Q-PCR using β-actin as a reference control. To measure the relative changes in IL-10 induction, results of LPS treated samples were normalized to the untreated control within the experiment. Data were represented as fold change versus calibrator sample, which is the untreated control. All experiments were performed at least three times. Data from a representative experiments are depicted in the figure. Error bars represent the upper and lower levels of variation from the mean within a single experiment.

(C) RT-PCR analysis of IL-10 transcripts. Serum starved normal and *me/me* splenic macrophages (5 X 10^6 cells/mL) were treated with LPS (10 μg/mL) for 7h. Total RNA was extracted and expression of IL-10 (top panel) was determined by semi-quantitative RT-PCR using β-actin as a standard control (lower panel). All experiments were performed at least three times however data from a single experiment is depicted in the figure.

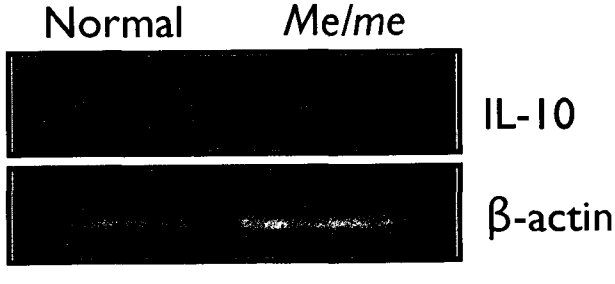
A


1200
1000
800
600
400
200
0
IL-10 Production (pg/mL)
Normal
me/me
U S
6
12
24
36
48
72
Duration of LPS treatment (hours)

B


35
30
25
20
15
10
5
0
RAW RQ (Relative Quantitation) IL-10 mRNA Fold Induction (pg/mL)
Normal
me/me
US
4
5
6
7
Duration of LPS treatment (hours)

C


Normal
Me/me
IL-10
β-actin

was observed compared to untreated normal controls (Fig. III.10B). In contrast to normal splenic macrophages, only a 3 to 5-fold induction in IL-10 mRNA was observed in LPS treated *me/me* splenic macrophages when compared to untreated *me/me* controls.

III.2.2.2 Dose response analysis of LPS-mediated IL-10 production

The low levels of IL-10 in LPS treated *me/me* splenic macrophages may be dependent on the dose of LPS used. Therefore, normal and *me/me* splenic macrophages were treated with different doses of LPS (0, 0.1, 1, 5, 10, 25, 50 μg/mL) for 24 h followed by ELISA analysis of IL-10 production. ELISA analysis (Fig III.11A) show >3-fold decrease in IL-10 production in LPS treated *me/me* splenic macrophages compared to normal controls. The observed relative decrease in IL-10 production in *me/me* splenic macrophages was independent of LPS dose suggesting that the diminished levels of IL-10 production in *me/me* splenic macrophages were not a function of LPS treatment dose.

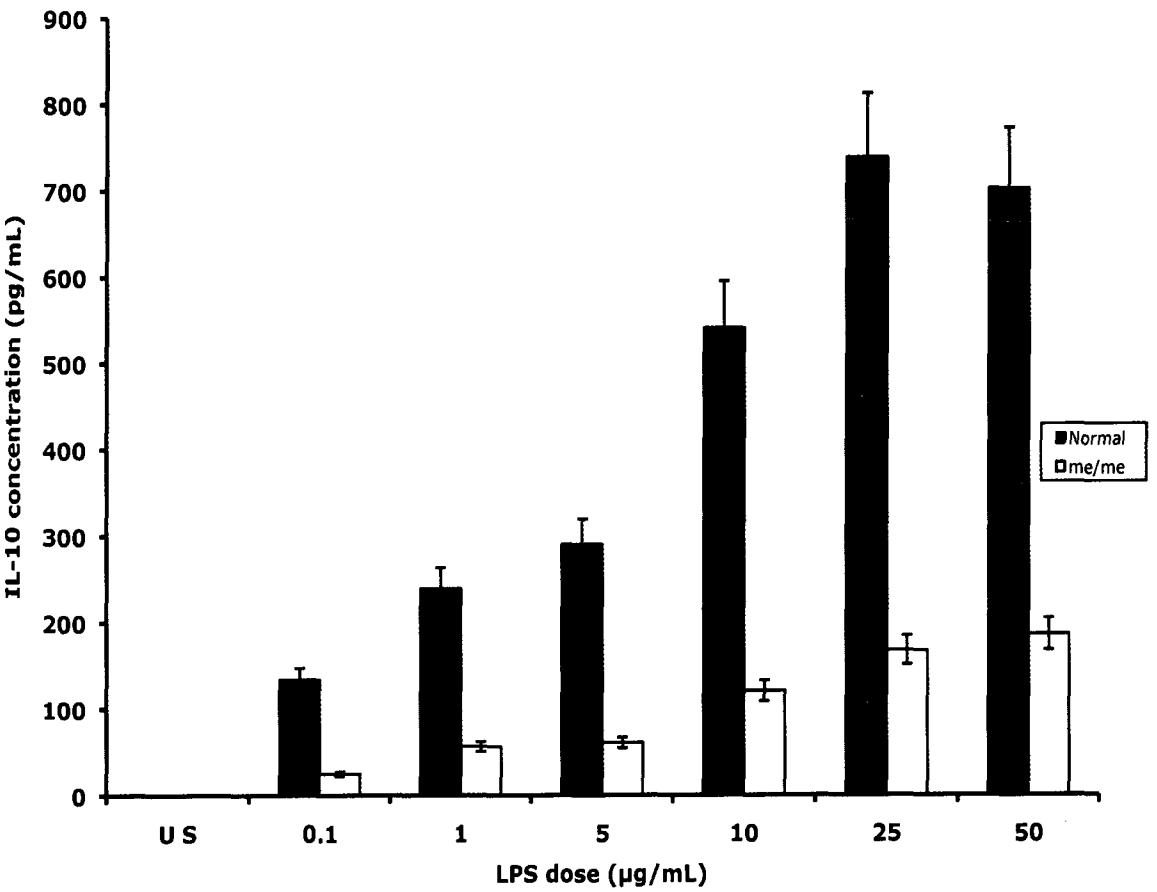
III.2.3 Elevated TNF-α levels maybe a result of poor IL-10 production in *me/me* splenic macrophages.

The precise mechanism(s) of IL-10 inhibition of TNF-α action remains poorly understood. To understand the role IL-10 plays in TNF-α expression in *me/me* splenic macrophages, normal and *me/me* splenic macrophages were treated with varying doses of exogenous recombinant murine IL-10 (rmIL-10) (0 to 50 ng/mL) for 2 h

Fig III.11 LPS Dose dependent analysis of IL-10 production in normal and *me/me* splenic macrophages.

(A) Normal (■) and *me/me* (□) splenic macrophages plated at a density of approximately 5 X 10^6 cells/mL were either untreated (US) or treated with increasing doses of LPS (0, 0.1, 1, 5, 10, 25, 50 μg/mL) for 24 h. Culture supernatants were harvested and evaluated for IL-10 production by ELISA. All experiments were repeated at least three times. Data from a representative experiment are depicted in the figure. ELISA data are represented as arithmetic mean of duplicate wells ± variation from the mean within a single experiment.

A


900
800
700
600
500
400
300
200
100
0
IL-10 concentration (pg/mL)
U S
0.1
1
5
10
25
50
LPS dose (µg/mL)
Normal
me/me

prior to LPS treatment for 24 h, after which the levels of TNF-α were analysed by ELISA (Fig III.12A, B and C). Addition of exogenous rmIL-10 in cultures of *me/me* splenic macrophages resulted in a 3 to 6-fold decline (in all experiments performed) in TNF-α production compared to *me/me* splenic macrophages treated with LPS only. TNF-α production in normal splenic macrophages was also further decreased in normal splenic macrophages pretreated with rmIL-10. These decreases were also observed when very small doses of rmIL-10 ranging from 0 to 2000 pg/mL were used (Fig III.12C). As a negative control, I also analyzed supernatants from normal and *me/me* splenic macrophages that were untreated or treated with rmIL-10 only (Fig III.12A). TNF-α production levels in splenic macrophages treated with rmIL-10 only, was identical to that produced in untreated macrophages and suggests that rmIL-10 only treatment did not induce TNF-α production in these cells.

Since pretreatment with exogenous rmIL-10 diminished TNF-α production in both normal and *me/me* splenic macrophages, and TNF-α mediates the production of secondary messengers such as MIP-2 (Fig III.6), it was of interest to evaluate if MIP-2 production would also decline in splenic macrophages pretreated with rmIL-10. For this, normal and *me/me* splenic macrophages were pretreated with rmIL-10 at 50 ng/mL for 2 h prior to treatment with either LPS or rmTNF-α for 24h. ELISA analysis showed that MIP-2 production was diminished in *me/me* splenic macrophages pretreated with rmIL-10 prior to LPS (Fig III.13A) or rmTNF-α (Fig III.13B).

Fig III.12 Pretreatment with exogenous rmIL-10 prior to LPS treatment diminishes TNF-α production in normal and *me/me* splenic macrophages.

Normal (■) and *me/me* (□) splenic macrophages plated at a density of approximately 5 X 10^6 cells/mL were either untreated (US) or pretreated with rmIL-10 at (A) 50 ng/mL, (B) 3.125 – 50 ng/mL and (C) 0.1 to 2 ng/mL for 2 h prior to treatment with LPS for 24 h. Culture supernatants were harvested and analyzed by ELISA for TNF-α production. (A) and (B) were repeated three times and (C) is a single experiment. Data depicted in the figures are from single representative experiments showing similar trends. ELISA data are represented as arithmetic mean of duplicate wells ± variation from the mean within a single experiment.

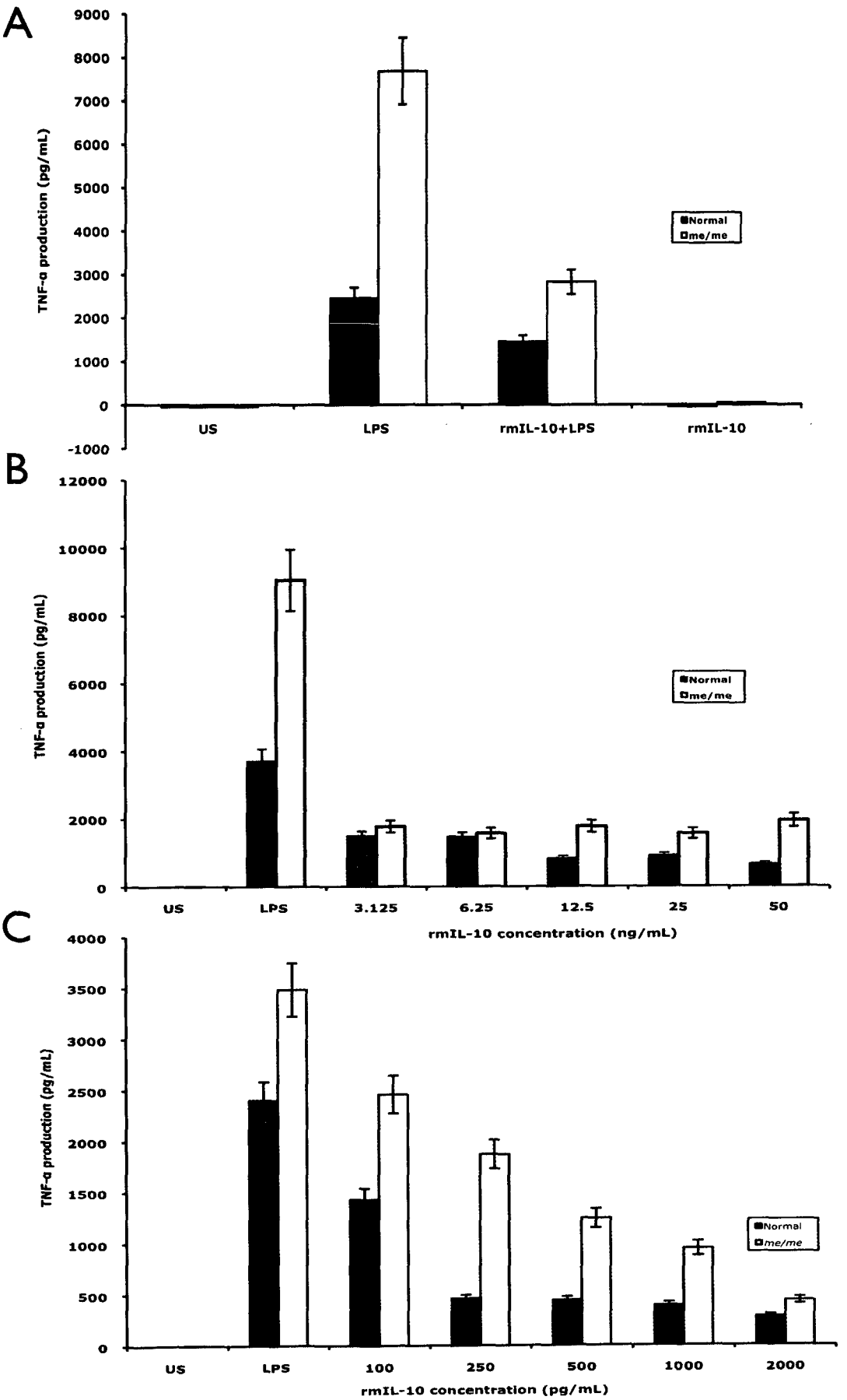
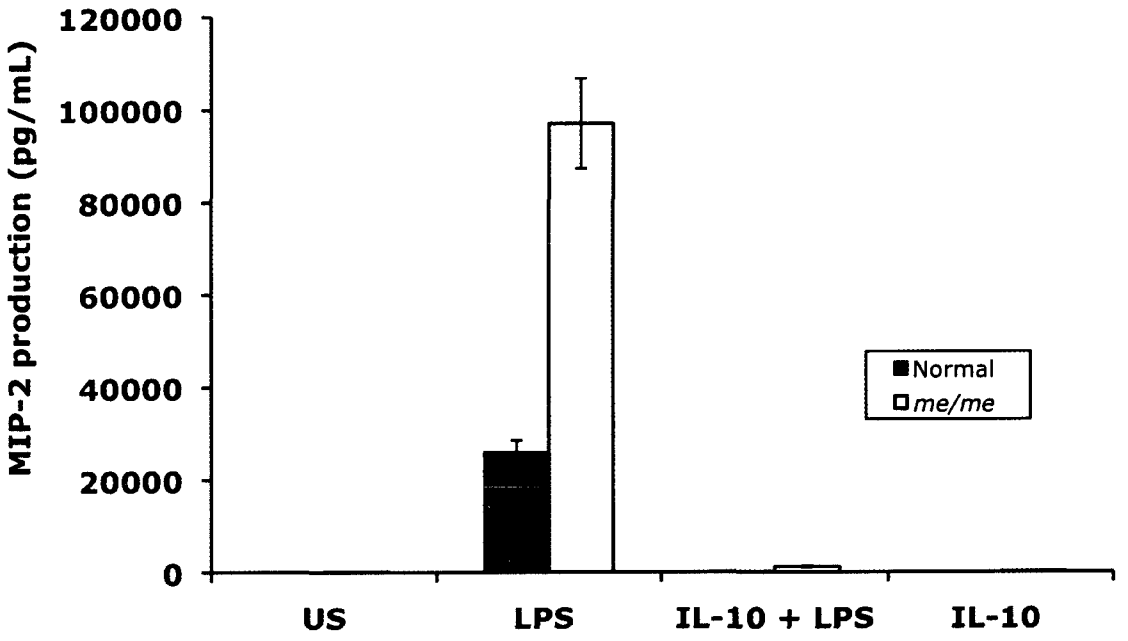

A
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
-1000
TNF-α production (pg/mL)
Normal
me/me
US
LPS
rmIL-10+LPS
rmIL-10
B
12000
10000
8000
6000
4000
2000
0
TNF-α production (pg/mL)
Normal
me/me
US
LPS
3.125
6.25
12.5
25
50
rmIL-10 concentration (ng/mL)
C
4000
3500
3000
2500
2000
1500
1000
500
0
TNF-α production (pg/mL)
Normal
me/me
US
LPS
100
250
500
1000
2000
rmIL-10 concentration (pg/mL)

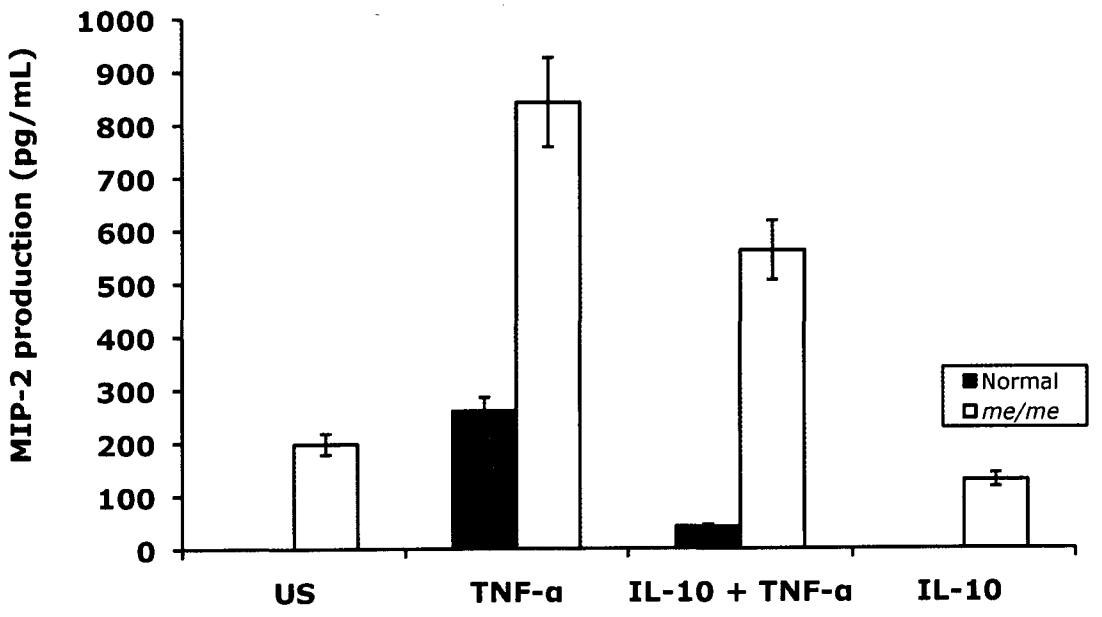
Fig III.13 Pretreatment with exogenous rmIL-10 prior to LPS or TNF-α treatment diminishes MIP-2 production in normal and *me/me* splenic macrophages.

Cytokine ELISA measuring MIP-2 production in culture supernatants of normal (■) and *me/me* (□) splenic macrophages. Normal and *me/me* splenic macrophages plated at a density of approximately 5 X 10^6 cells/mL were either untreated (US) or pretreated with rmIL-10 at 50 ng/mL, for 2 h prior to treatment with (A) LPS (10 μg/mL) or (B) TNF-α (100 ng/mL) for 24 h. Culture supernatants were harvested and analyzed by ELISA for MIP-2 production. All experiments were repeated at least twice. Data from representative experiments are depicted in the figure. ELISA data are represented as arithmetic mean of duplicate wells ± variation from the mean within a single experiment.

A


MIP-2 production (pg/mL)
120000
100000
80000
60000
40000
20000
0
US
LPS
IL-10 + LPS
IL-10
Normal
me/me

B


MIP-2 production (pg/mL)
1000
900
800
700
600
500
400
300
200
100
0
US
TNF-a
IL-10 + TNF-a
IL-10
Normal
me/me

III.2.4 Excess TNF-α production and poor IL-10 production in splenic macrophages are consequences of SHP-1 loss.

(i) Ablation of SHP-1 protein expression using anti-sense SHP-1 oligonucleotides increases TNF-α and inhibits IL-10 production in normal splenic macrophages.

The finding that TNF-α production is elevated and IL-10 production is diminished in LPS-treated SHP-1 deficient splenic macrophages suggests a central role for SHP-1 in regulating TNF-α and IL-10 production in splenic macrophages. To explore the involvement of SHP-1 in these events, I mimicked the *me/me* phenotype in normal splenic macrophages by interfering with SHP-1 expression using SHP-1-specific antisense oligonucleotides. This was achieved by culturing splenic macrophages with phosphorothioate-modified (to enhance stability by preventing nuclease activity) antisense oligonucleotide specific for the translational start site of SHP-1. As controls for specificity, a related anti-SHP-2 oligonucleotide and an unrelated anti-CD4 oligonucleotide with modified phosphorothioate backbones were used. Normal splenic macrophages were treated with respective antisense oligonucleotides for 2 h prior to stimulation with LPS for 24 h. ELISA analysis of culture supernatants for TNF-α and IL-10 production revealed a 3-fold increase in TNF-α production (Fig III.14B) and a 2 to 3-fold decrease in IL-10 production (Fig III.14A) in normal splenic macrophages treated with SHP-1 antisense oligonucleotides when compared to controls treated with LPS only. Controls from culture supernatants from anti-SHP-2 or anti-CD-4 oligonucleotide-treated normal splenic macrophages did not show a significant difference in IL-10 or TNF-α production when compared to normal splenic macrophages treated with LPS only (Fig III.14 A and B). These results

indicate that the expression of SHP-1 is required for IL-10 production in splenic macrophages. To confirm these results, a different antisense approach was also used. For this normal splenic macrophages were permeated using *Streptolysin O* (SLO), treated with antisense oligonucleotides, resealed with 100 mM $CaCl_2$ and incubated for 18 to 24 h. Cells were then treated with LPS for 24 h. Results from ELISA analysis (Fig III.14C) of culture supernatants showed a 40% decline in IL-10 production in normal splenic macrophages treated with 2.5 μM anti-SHP-1 antisense oligonucleotide when compared to those treated with LPS (+SLO) only. No significant difference in IL-10 production was observed in normal splenic macrophages treated with control scrambled antisense oligonucleotides (Fig III.14C). To confirm that SHP-1 antisense oligonucleotide treatment blocked SHP-1 expression, the levels of SHP-1 protein expression in lysates of normal splenic macrophages treated with SHP-1 antisense oligonucleotides were determined by western immunoblotting. Results of western immunoblotting confirmed that SHP-1 protein expression levels were drastically declined in normal splenic macrophages treated with anti-SHP-1 antisense at 2.5 μM concentration (Fig III.14D).

(ii) Restoration of the moth-eaten phenotype by expressing SHP-1 gene in me/me splenic macrophages.

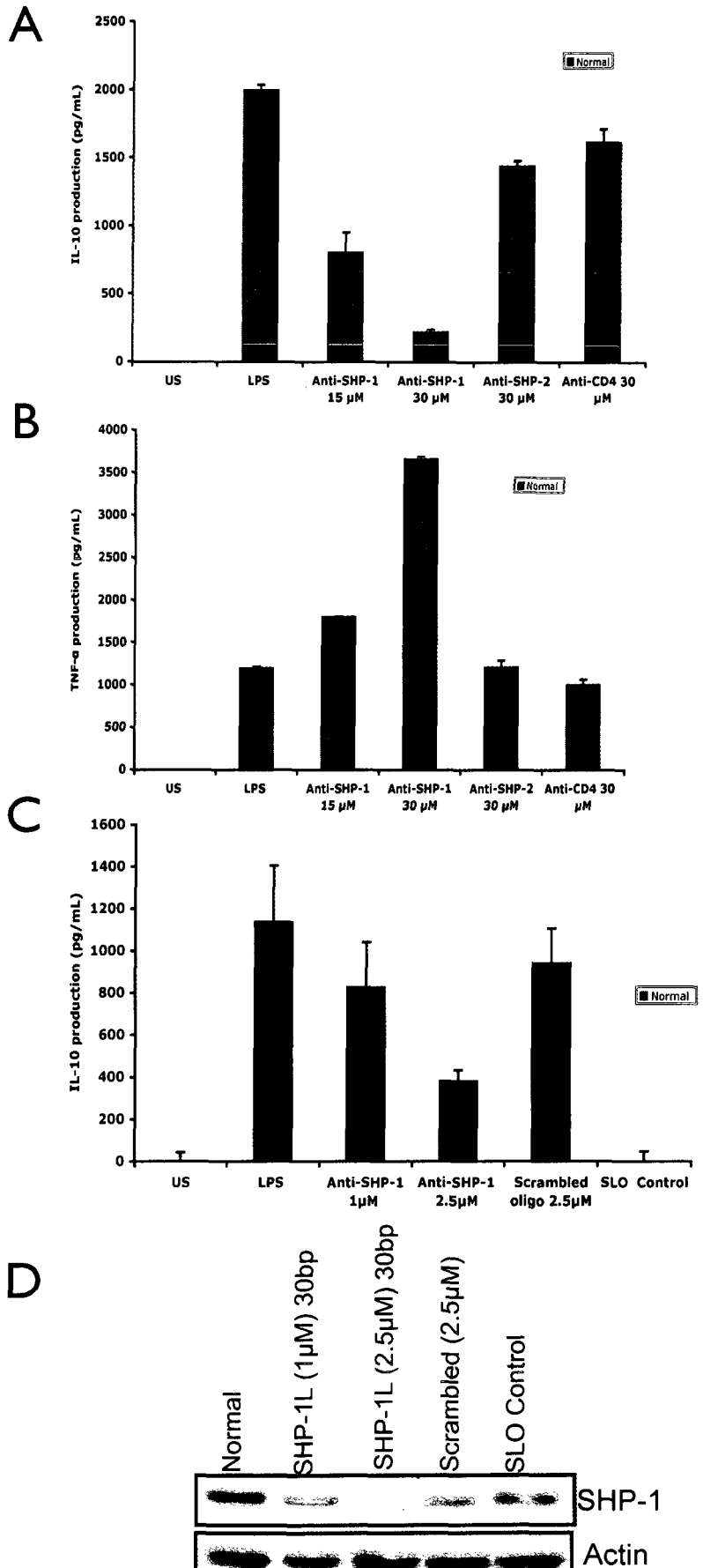
To confirm the central role of SHP-1 in LPS-mediated IL-10 and TNF-α production in splenic macrophages, *me/me* splenic macrophages were reconstituted with the SHP-1 gene. This was achieved by transducing *me/me* splenic macrophages with adenoviral vectors containing a full-length mouse SHP-1 gene (Ad-SHP-1). In the first experiment, four to six-day old *me/me* splenic macrophages were transduced

Fig III.14 Treatment with SHP-1 antisense oligonucleotides inhibits IL-10 but enhances TNF-α production in normal splenic macrophages.

Normal (■) splenic macrophages plated at a density of approximately 5 X 10^6 cells/mL were either untreated (US) or pretreated with SHP-1 antisense oligonucleotides or with control SHP-2 or CD-4 antisense oligonucleotides for 2 h prior to treatment with LPS (10 μg/mL) for 24 h. Culture supernatants were harvested and analyzed by ELISA for (A) IL-10 (B) TNF-α production. All experiments were repeated at least three times however data from a representative experiment are depicted in the figure. Due to a shortage in SHP-1 antisense oligonucleotides, individual points in experiments were done on different days utilizing splenic macrophages from different animal pools. ELISA data are represented as arithmetic mean of duplicate wells ± variation from the mean within a single experiment.

(C) SHP-1 antisense oligonucleotide treatment using reversible permeabilization delivery approach. Normal splenic macrophages plated at a density of approximately 5 X 10^6 cells/mL were reversibly permeated using SLO. Normal splenic macrophage cultures were then either untreated (US) or pretreated with SHP-1 antisense oligonucleotides (or scrambled) for 20 h followed by LPS treatment for 24 h. SLO only treatments (without LPS) were used as controls. Culture supernatants were harvested and analyzed by ELISA for IL-10 production. All experiments were repeated at least three times however data from a representative experiment showing similar trends are depicted in the figure. ELISA data are represented as arithmetic mean of duplicate wells ± variation from the mean within a single experiment.

(D) SHP-1 antisense oligonucleotide treatment suppresses SHP-1 protein expression in normal splenic macrophages. Serum starved normal splenic macrophages (1 X 10^7 cells/mL per condition) were permeated using SLO and were either left untreated or treated with SHP-1 antisense oligonucleotide (and other controls as labeled) for 24 h. Cultures were harvested and subjected to Western blot procedure using monoclonal α-SHP-1 Ab (upper panel) followed by reprobing the membranes using α-actin Ab to ensure equal loading (lower panel). Results shown in this figure are representative of two independent experiments showing similar trends.


A
IL-10 production (pg/mL)
2500
2000
1500
1000
500
0
Normal
US
LPS
Anti-SHP-1 15 μM
Anti-SHP-1 30 μM
Anti-SHP-2 30 μM
Anti-CD4 30 μM
B
TNF-α production (pg/mL)
4000
3500
3000
2500
2000
1500
1000
500
0
Normal
US
LPS
Anti-SHP-1 15 μM
Anti-SHP-1 30 μM
Anti-SHP-2 30 μM
Anti-CD4 30 μM
C
IL-10 production (pg/mL)
1600
1400
1200
1000
800
600
400
200
0
Normal
US
LPS
Anti-SHP-1 1μM
Anti-SHP-1 2.5μM
Scrambled oligo 2.5μM
SLO Control
D
Normal
SHP-1L (1μM) 30bp
SHP-1L (2.5μM) 30bp
Scrambled (2.5μM)
SLO Control
SHP-1
Actin

with Ad-SHP-1. The multiplicity of infection (MOI) was calculated by adjusting the adenoviral titre to the average cell number per well. *Me/me* splenic macrophages were infected with Ad-SHP-1 MOI 10 and MOI 20 for 24 h followed by LPS treatment for 24 h. The following controls were also performed; untreated *me/me* splenic macrophages, untransduced but LPS-treated *me/me* splenic macrophages (MOI 0) and me/me splenic macrophages transduced with Ad-GFP (adenovirus expressing green fluorescent protein) and treated with LPS. Further controls included *me/me* splenic macrophages transduced with either Ad-GFP or Ad-SHP-1 without LPS treatment. Restoration of SHP-1 expression in *me/me* splenic macrophages resulted in a 50% increase in LPS-mediated IL-10 production at MOI 20 compared to MOI 0 (Fig III.15A). Additionally, TNF-α ELISA analysis showed a 50% decrease in TNF-α production at MOI 20 compared to MOI 0 (Fig III.15B). Reconstitution of *me/me* splenic macrophages with the control virus, Ad-GFP, did not impact LPS-mediated IL-10 or TNF-α production. The levels of IL-10 or TNF-α produced in Ad-GFP transduced *me/me* splenic macrophages were similar to the levels observed in cells treated with LPS only (MOI 0 in Fig III.15A and B). The expression of SHP-1 in Ad-SHP-1 transduced *me/me* splenic macrophages was confirmed by western blot analysis. Result of western blot analysis revealed increasing SHP-1 expression in an MOI dependent manner (Fig III.15C). It is interesting to note that the levels of SHP-1 expressed in *me/me* splenic macrophages transduced with Ad-SHP-1 at MOI 20 (Lane 3) correspond to the levels of SHP-1 in normal splenic macrophages (Lane 7 in Fig III.15C).

Fig III.15 Reconstitution of SHP-1 in *me/me* splenic macrophages enhances IL-10 and moderates TNF-α production.

Me/me (□) splenic macrophages plated at a density of approximately 5 X 10^6 cells/mL were either uninfected (MOI 0) or infected with Adeno-SHP-1 (or control Adeno-GFP) for 24 h prior to treatment with LPS (10 μg/mL) for another 24 h. Other controls included treatment with adeno-SHP-1 and adeno-GFP only (no LPS). Culture supernatants were harvested and analyzed by ELISA for (A) IL-10 (B) TNF-α production. (C) Cultures infected with adenoviruses as described above were harvested and cell pellets lysed. Lysates were subjected to SDS-PAGE followed by Western blot analysis using α-SHP-1 monoclonal Ab (*upper panel*). Blots were stripped and reprobed with α-actin antibody as a control for loading (*lower panel*). All experiments were repeated at least three times. Data shown are from representative experiments showing similar trends. ELISA data are represented as arithmetic mean of duplicate wells ± variation from the mean within a single experiment.

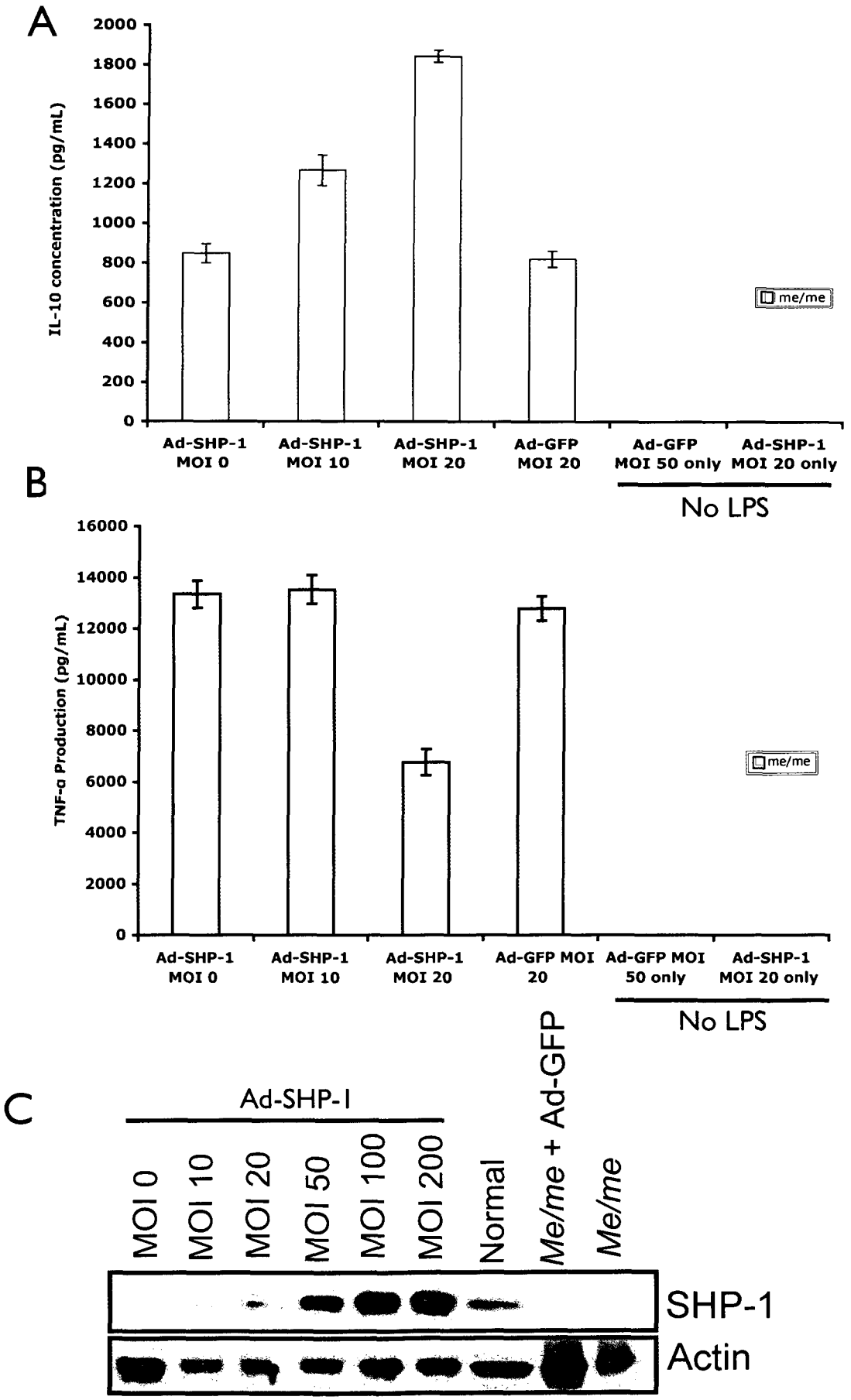
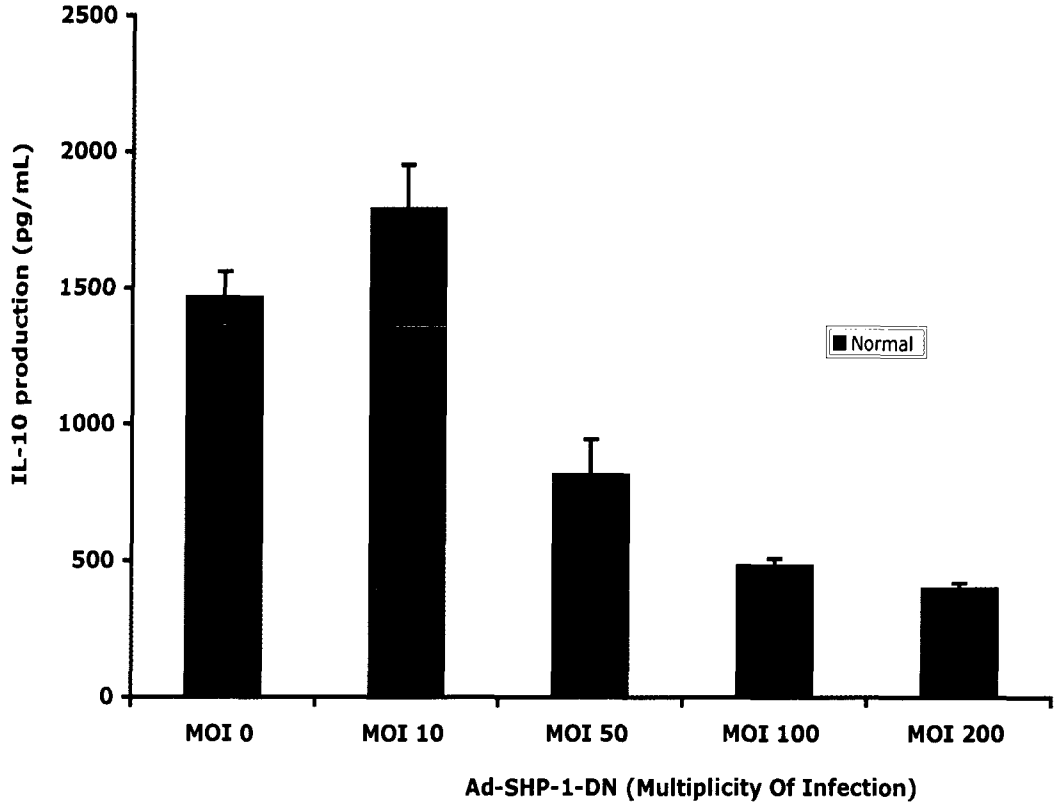

A
IL-10 concentration (pg/mL)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
me/me
Ad-SHP-1 MOI 0
Ad-SHP-1 MOI 10
Ad-SHP-1 MOI 20
Ad-GFP MOI 20
Ad-GFP MOI 50 only
Ad-SHP-1 MOI 20 only
No LPS
B
TNF-α Production (pg/mL)
16000
14000
12000
10000
8000
6000
4000
2000
0
me/me
Ad-SHP-1 MOI 0
Ad-SHP-1 MOI 10
Ad-SHP-1 MOI 20
Ad-GFP MOI 20
Ad-GFP MOI 50 only
Ad-SHP-1 MOI 20 only
No LPS
C
Ad-SHP-I
MOI 0
MOI 10
MOI 20
MOI 50
MOI 100
MOI 200
Normal
Me/me + Ad-GFP
Me/me
SHP-1
Actin

Fig III.16 The phosphatase domain of SHP-1 is required for IL-10 production in normal splenic macrophages.

Normal (■) splenic macrophages plated at a density of approximately 5 X 10^6 cells/mL were either uninfected (MOI 0) or infected with Ad-SHP-1-DN (adenoviral vector containing only the SH-2 domains of SHP-1) for 24 h prior to treatment with LPS (10 μg/mL) for another 24 h. Culture supernatants were harvested and analyzed by ELISA for (A) IL-10. All experiments were repeated at least three times. Data shown are from representative experiments showing similar trends. ELISA data are represented as arithmetic mean of duplicate wells ± variation from the mean within a single experiment.

Schematic illustrations showing (B) adenoviral vector with full length SHP-1 gene and (C) adenoviral vector containing dominant negative SHP-1 gene, expressing SH-2 domains (in green) of SHP-1 but lacking SHP-1 phosphatase (in red) domain. Y536 and Y564 depict two tyrosine phosphorylation sites that modulate SHP-1 activity in the c-terminus. Depicted amino acid numbers indicate positioning of amino acids and domain sizes.

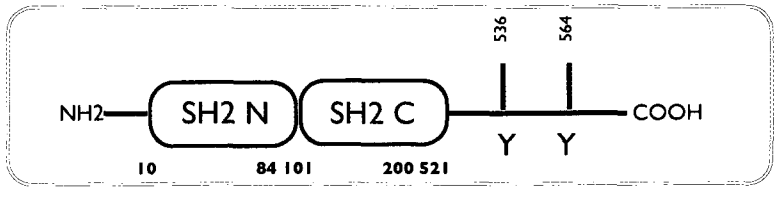
A



B

Ad-SHP-1

C



Ad-SHP-1-DN

(iii) Phosphatase domain of SHP-1 is required for LPS-mediated IL-10 production.

To examine the importance of SHP-1 phosphatase activity in LPS-mediated IL-10 production, an Adenoviral vector encoding a SHP-1 gene with mutations (dominant negative), (Ad-SHP-1-DN), lacking the catalytic activity of SHP-1 was prepared by Dr. Peter Liston (Children's Hospital of Eastern Ontario (CHEO), Ottawa, ON). This dominant negative SHP-1 mutant vector expresses a modified form of full length SHP-1. It contains two SH2 domains of SHP-1 but lacks the phosphatase domain of SHP-1 and consequently, has no phosphatase activity (Fig. III.16B and C). The protein expressed from this construct, retains its ability to bind SHP-1 target proteins. The competitive binding of SHP-1-DN results in the sequestration of SHP-1 target proteins, ultimately leading to the accumulation of tyrosine-phosphorylated proteins and the disruption of normal cellular functions [386-389]. To determine if SHP-1 phosphatase activity is required for IL-10 production, normal splenic macrophages were transduced with increasing MOI of Ad-SHP-1-DN for 24 h followed by LPS treatment for another 24 h. ELISA analyses revealed an MOI-dependent, 2 - 4 fold decline in LPS-mediated IL-10 production compared to untransduced but LPS treated normal splenic macrophages (Fig. III.16A).

III.3.0 SUMMARY OF KEY RESULTS EXAMINING THE PROFILES OF IL-10 AND TNF-α IN LPS TREATED NORMAL AND SHP-1–NULL, *ME/ME* SPLENIC MACROPHAGES.

Thus far, the role of the tyrosine phosphatase, SHP-1, in modulating LPS-mediated IL-10 and TNF-α production in splenic macrophages has been

demonstrated. A comparison of LPS-mediated IL-10 and TNF-α production in normal and *me/me* splenic macrophages revealed that IL-10 levels were diminished by an average of 50% in *me/me* cultures while TNF-α levels were elevated by an average of 50% in SHP-1 null splenic macrophages. Further analysis of IL-10 and TNF-α production in *me/me* and normal splenic macropahges, showed that observed differences were not a function of LPS dose or the duration of LPS treatment. Furthermore, analysis of IL-10 and TNF-α mRNA levels in LPS treated normal and *me/me* splenic macrophages also revealed that IL-10 mRNA induction was suppressed and TNF-α mRNA induction was elevated in LPS treated *me/me* splenic macrophages. Exogenous pretreatment of *me/me* splenic macropahges with rmIL-10 prior to LPS treatment decreased the levels of TNF-α production. These results suggest that optimal levels of IL-10 are required to effectively control TNF-α levels in splenic macrophages. Direct involvement of SHP-1 function in LPS-induced IL-10 production was examined by interfering with SHP-1 expression in normal splenic macrophages using SHP-1 antisense oligonucleotides. Intereference of SHP-1 expression resulted in a decline in IL-10 production thus mimicking the *me/me* phenotype. On the contrary, reconstitution of SHP-1 gene expression in *me/me* splenic macrophages using SHP-1 adenoviral vectors restored optimal IL-10 production. Treatment of normal splenic macrophages with adenoviral vectors expressing the SH2 domains of SHP-1 (SHP-1-DN) revealed a dependence of LPS-mediated IL-10 production on SHP-1 phosphatase activity.

Taken together, these results suggest that the elevated levels of TNF-α observed in *me/me* splenic macrophages are a consequence of low IL-10 production in SHP-1

null cells. This study therefore focuses on the role of SHP-1 in regulating the signaling pathways and mechanism(s) that govern IL-10 production in LPS treated splenic macrophages.

III.4.0 SIGNAL TRANSDUCTION PATHWAYS ACTIVATED BY LPS IN NORMAL AND *ME/ME* SPLENIC MACROPHAGES AND RESULTING IN IL-10 PRODUCTION.

LPS signaling through TLR-4 is known to result in the activation of 259 genes in macrophages [257, 275, 279]. A complex constituting CD-14, TLR-4 and MD2 is required to effectively transduce LPS signals [274-276] irrespective of the downstream pathways activated. In normal macrophages, stimulation with LPS induces the expression of many cytokines including IL-10 (Fig III.9, 10 and 11) as demonstrated in the earlier results of this study. The activation of MyD88/IRAK/TRAF and NF-κB pathways in LPS stimulated cells has been well documented and represents the classical pathways activated by LPS via TLR4 (Fig I.2). More recently, LPS–mediated activation of MAPK pathways has been shown to play a critical role in induction of specifc genes and in cytokine production including, IL-10, in human and murine monocytes [268, 286, 288, 289]. The precise mechanism, and in particular the role of protein tyrosine kinases and tyrosine phosphatases in activating cytokine genes, especially IL-10, remain unknown. Therefore the objective of this part of the study was to systematically examine the role of SHP-1 in the activation of MAPKs and PTKs required for LPS-induced IL-10 production in murine splenic macrophages.

III.4.1 Signal transduction pathways

III.4.2 MAPK pathway(s)

The MAP kinases, P38, ERK1/2 and JNK1/2/3 activated in response to different external stimuli such as UV stress, mitogens and antigens have been implicated in the regulation of cell activation, migration, proliferation and cytokine synthesis [285, 286, 288, 295]. Although the treatment of macrophages with LPS has been shown to upregulate the IL-10 gene through the involvement of MAPKs in human alveolar macrophages and human macrophage cell lines [22, 390], the function of SHP-1 in LPS-mediated activation of MAPK resulting in IL-10 production remain unknown. Thus, to examine the impact of SHP-1 on MAPK pathway(s) in LPS-mediated IL-10 production, I inhibited the individual members of the MAPK family in normal and *me/me* splenic macrophages using specific pharmacological inhibitors and evaluated IL-10 production. To highlight possible roles or differences associated with SHP-1 expression and activity, *me/me* splenic macrophages were included in all inhibition experiments despite the low levels of IL-10 observed in these cells.

III.4.2.1 Role of MAP kinases in LPS-mediated IL-10 production in normal and *me/me* splenic macrophages

(i) *ERK1/2 and JNK1/2/3 MAPK pathway*

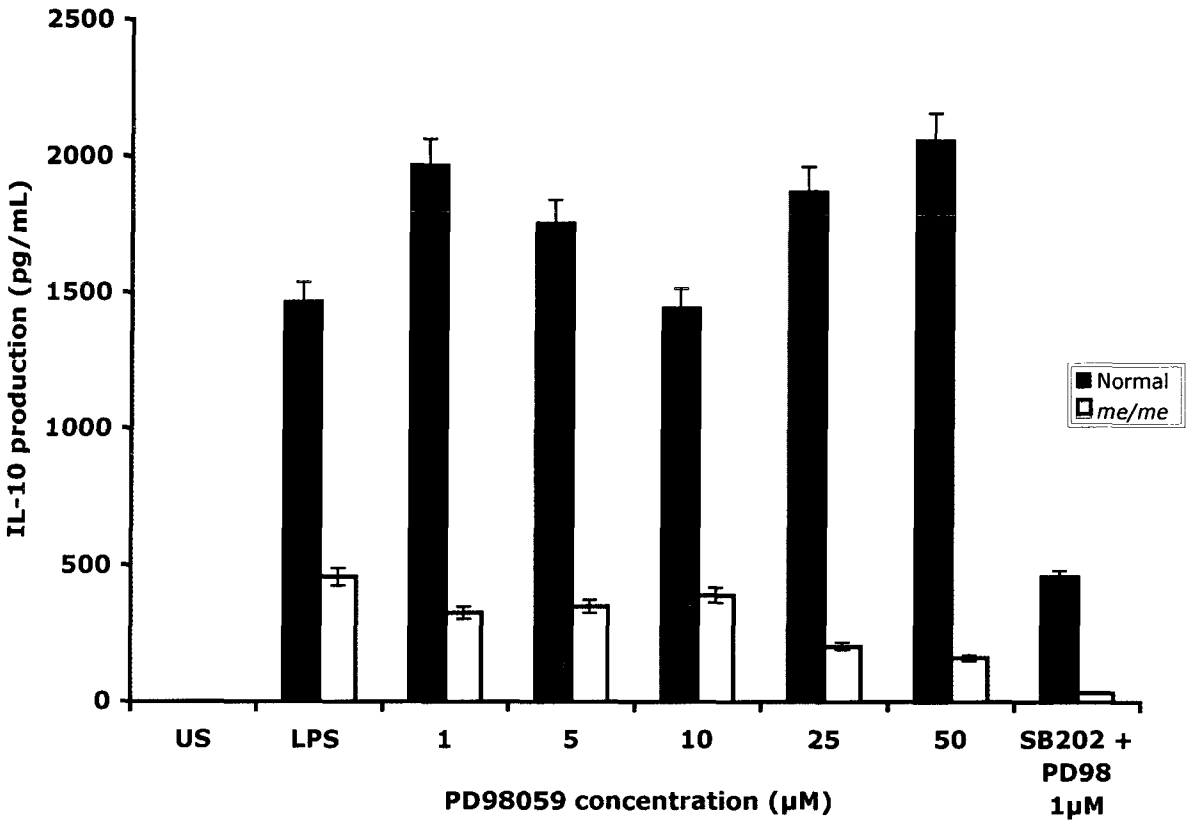
In order to determine whether the activation ERK1/2 MAPK pathway was essential for IL-10 production in response to LPS, the activation of the ERK1/2 MAPK was inhibited by treatment of normal and *me/me* splenic macrophages with a pharmacological inhibitor of ERK1/2 MAPK, PD98059, for 2 h followed by LPS

Fig III.17 LPS induced IL-10 production in ERK1/2 MAPK-inhibited normal or *me/me* splenic macrophages.

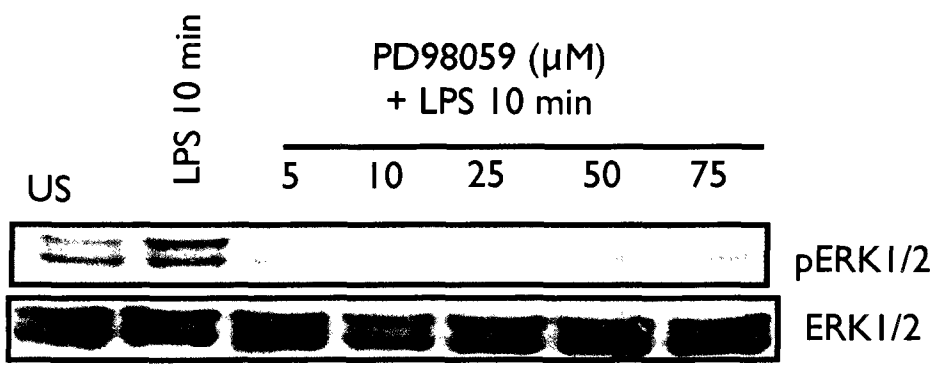
(A) Normal (■) and *me/me* (□) splenic macrophages plated at a density of approximately 5 X 10^6 cells/mL were either untreated (US) or treated with the ERK1/2 MAPK specific inhibitor PD98059 at indicated concentrations (0 to 50 μM) for 2 h prior to LPS (10 μg/mL) treatment for 24 h. Culture supernatants were harvested after 24 h and assessed by ELISA for IL-10 production. All experiments were repeated three times. Data from a representative experiment are depicted in the figure. ELISA data are represented as arithmetic mean of duplicate wells ± variation from the mean within a single experiment.

(B) Serum starved normal splenic macrophages (1 X 10^7cells/mL) were either untreated (US) or pretreated with PD98059 at indicated concentrations (0 - 75 μM) for 2 h followed by LPS treatment for 10 min. Cell pellets were lysed and total protein lysates (100 μg) were subjected SDS-PAGE followed by Western blot analysis using phospho-ERK1/2 monoclonal Ab. Membranes were stripped and reprobed using ERK1/2 Ab as a control for equal loading. Result shown is a representative of three independent experiments showing very similar trends.

A


2500
2000
1500
1000
500
0
IL-10 production (pg/mL)
Normal
me/me
US
LPS
1
5
10
25
50
SB202 + PD98 1μM
PD98059 concentration (μM)

B

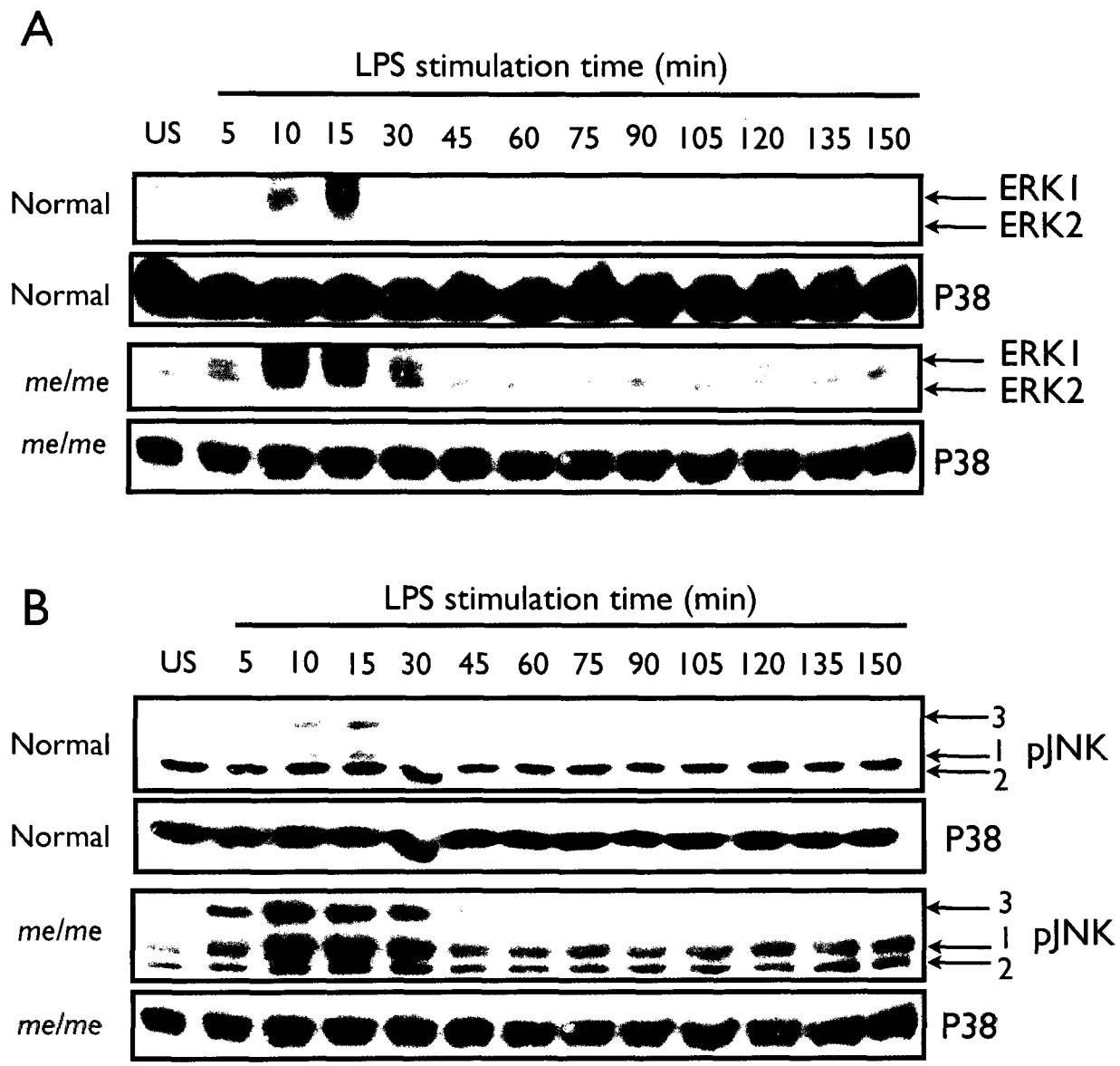

LPS 10 min
PD98059 (μM)
+ LPS 10 min
US
5
10
25
50
75
pERK1/2
ERK1/2

treatment for 24 h. Supernatants from normal and *me/me* splenic macrophage cultures were collected and examined for IL-10 production. Analysis showed that inhibition of ERK1/2, did not suppress IL-10 production in LPS treated normal splenic macrophages (Fig III.17A), even at 50 μM doses. A slight suppression in IL-10 production was observed in *me/me* splenic macrophages at higher doses of PD98059 and may be attributed at least in part to decreased specificity of the Erk inhibitor, PD98059 at higher concentrations. To confirm ERK1/2 inhibitor activity and its specificity, normal splenic macrophages were treated with increasing concentrations (0 to 50 μM) of PD98059 for 2 h prior to LPS treatment for 10 min and lysates were evaluated for ERK1/2 phosphorylation by western immunoblotting. Results in Fig III.17B demonstrate that doses as low as 5μM concentration of PD98059 effectively inhibited ERK1/2 phosphorylation. Similar experiments using the JNK1/2/3 inhibitor SP600125 yielded inconclusive results, data obtained from those experiments are not shown in this thesis.

Although ERK1/2 did not appear to be important for IL-10 production in normal splenic macrophages, the lack of SHP-1 in me/me splenic macrophages may possibly alter the activation of MAPK. To assess if the lack of SHP-1 expression alters activation of MAP kinases, I compared the phosphorylation status of ERK1/2 and JNK 1/2/3 MAPKs in LPS treated normal and *me/me* splenic macrophages. For this, splenic macrophages were harvested; serum starved for 4 h then treated with LPS for 5 to 150 min. Lysates were probed by Western immunoblotting for phospho-ERK1/2 and phospho-JNK1/2/3 using phospho specific ERK1/2 and JNK1/2/3

Fig III.18 LPS-induced phosphorylation of ERK1/2 and JNK1/2/3 MAPK in normal and *me/me* splenic macrophages.

Serum starved normal and *me/me* splenic macrophages (approximately 1 X 10^7cells/mL per condition) were either untreated (US) or treated with LPS for indicated durations (5 - 150 min). Cell pellets were lysed and total protein lysates (100 μg) were subjected SDS-PAGE followed by Western blot analysis using (A) phospho ERK1/2 monoclonal Ab or (B) phospho JNK1/2/3 Ab. Arrows point to the different isoforms of ERK (A) and JNK (B). Membranes were stripped and reprobed using the anti-P38 Ab as a control for equal loading. Experiments assessing the activation of P38, ERK1/2 and JNK1/2/3 were done simultaneously, therefore equal loading controls were determined using P38 Ab. Results shown are representative of three independent experiments showing very similar trends.


A
LPS stimulation time (min)
US 5 10 15 30 45 60 75 90 105 120 135 150
Normal
ERK1
ERK2
Normal
P38
me/me
ERK1
ERK2
me/me
P38
B
LPS stimulation time (min)
US 5 10 15 30 45 60 75 90 105 120 135 150
Normal
3
1
2
pJNK
Normal
P38
me/me
3
1
2
pJNK
me/me
P38

antibodies respectively (Fig III.18A and B). Results revealed robust phosphorylation of ERK1/2 and JNK1/3 between 5 and 30 min in both *me/me* splenic macrophages and normal splenic macrophages (Fig III.18A and B). However, the phosphorylation of ERK1/2 and JNK1/3 MAP kinases diminished to levels comparable to those observed in untreated cells by 30 min post LPS stimulation. Overall, similar phosphorylation patterns for ERK1/2 and JNK1/2/3 MAP kinases were observed in both normal and *me/me* splenic macrophages. However, a closer comparison of individual experiments revealed that the phosphorylation levels for ERK1/2 and JNK1/3 within the 5 to 30 min time-span in *me/me* splenic macrophages appeared earlier and slightly elevated compared to normal controls (Fig III.18A and B).

(ii) *P38 MAPK pathway*

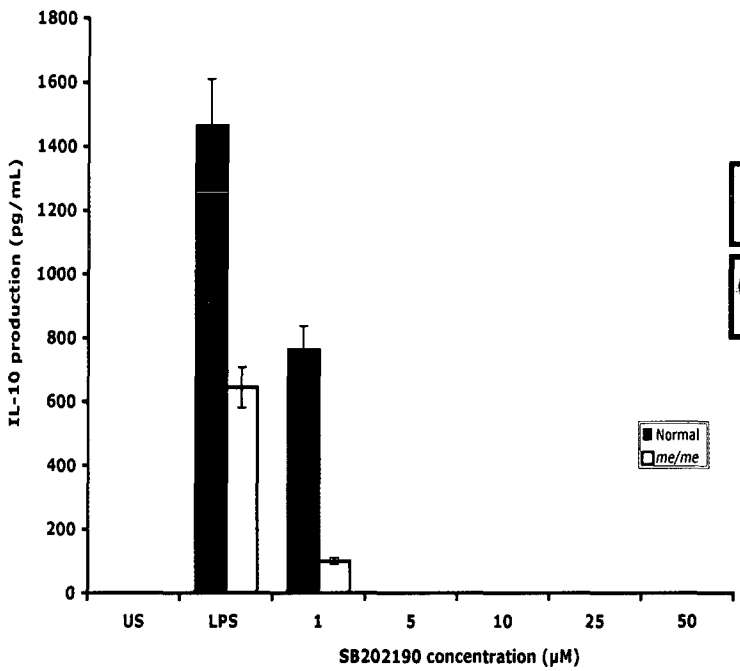
As noted earlier, LPS stimulation activates P38 MAPK, and this pathway is known to result in cytokine production in human macrophages. The role of P38 MAPK in IL-10 production was determined by specific inhibition of this kinase with its inhibitors, SB203580 or SB202190 for 2 h followed by LPS treatment for 24 h. Supernatants were collected and examined for IL-10 production. Two separate P38 MAPK inhibitors were used to ensure reliability of results obtained. SB 203580 acts by competing with ATP for binding on the ATP binding site of MEKK2, a kinase responsible for P38 activation while SB202190 acts by directly binding the ATP binding site of active P38 MAPK [391]. Both inhibitors induced a dose dependent abrogation of LPS-mediated IL-10 production in both normal and *me/me* splenic macrophages (Fig III.19A and B, respectively). Doses as low as 1 μM of SB202190 or 0.1 μM of SB203580 decreased IL-10 production in both normal and *me/me*

Fig III.19 LPS induced IL-10 production is regulated by P38 MAPK in normal or *me/me* splenic macrophages.

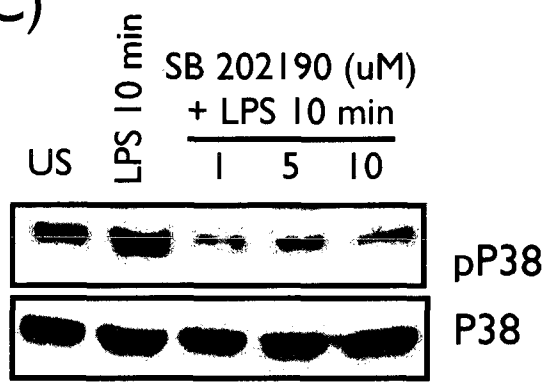
Normal (■) and *me/me* (□) splenic macrophages plated at a density of approximately 5 X 10^6 cells/mL were either untreated (US) or pretreated with (A) SB202190 (1-50 μM) or (B) SB203580 (0.1 - 5μM) for 2 h prior to LPS (10 μg/mL) treatment for 24 h. Culture supernatants were harvested after 24 h and assessed by ELISA for IL-10 production. All experiments were repeated three times. Data from a representative experiment are depicted in the figure. ELISA data are represented as arithmetic mean of duplicate wells ± variation from the mean within a single experiment.

Serum starved normal splenic macrophages (approximately 1 X 10^7cells/mL per condition) were either untreated (US) or pretreated with (C) SB202190 (1 – 10 μM) or (D) SB203580 (1 – 10 μM) for 2 h followed by LPS (10 μg/mL) treatment for 10 min. Cell pellets were lysed and total protein lysates (100 μg) were subjected SDS-PAGE followed by Western blot analysis using phospho-p38 MAPK Ab. To control for equal loading, membranes were stripped and reprobed using P38 MAPK Ab. Results shown are representative of two independent experiments showing similar trends.

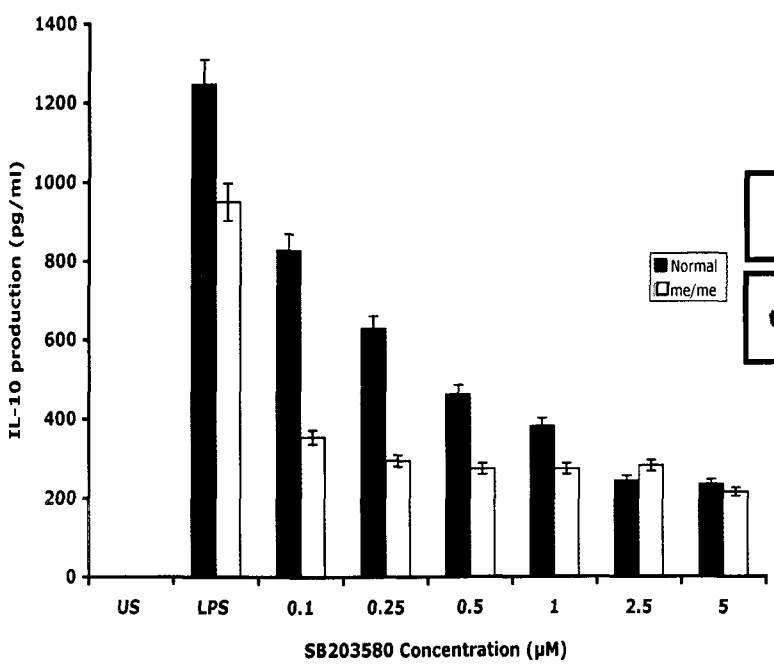
A


1800
1600
1400
1200
1000
800
600
400
200
0
IL-10 production (pg/mL)
Normal
me/me
US
LPS
1
5
10
25
50
SB202190 concentration (μM)

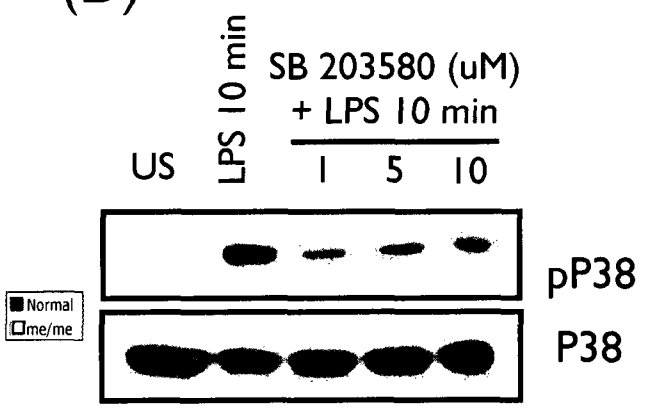
(C)


LPS 10 min
SB 202190 (uM)
+ LPS 10 min
US
1
5
10
pP38
P38

B


1400
1200
1000
800
600
400
200
0
IL-10 production (pg/ml)
US
LPS
0.1
0.25
0.5
1
2.5
5
SB203580 Concentration (μM)

(D)


LPS 10 min
SB 203580 (uM)
+ LPS 10 min
US
1
5
10
Normal
me/me
pP38
P38

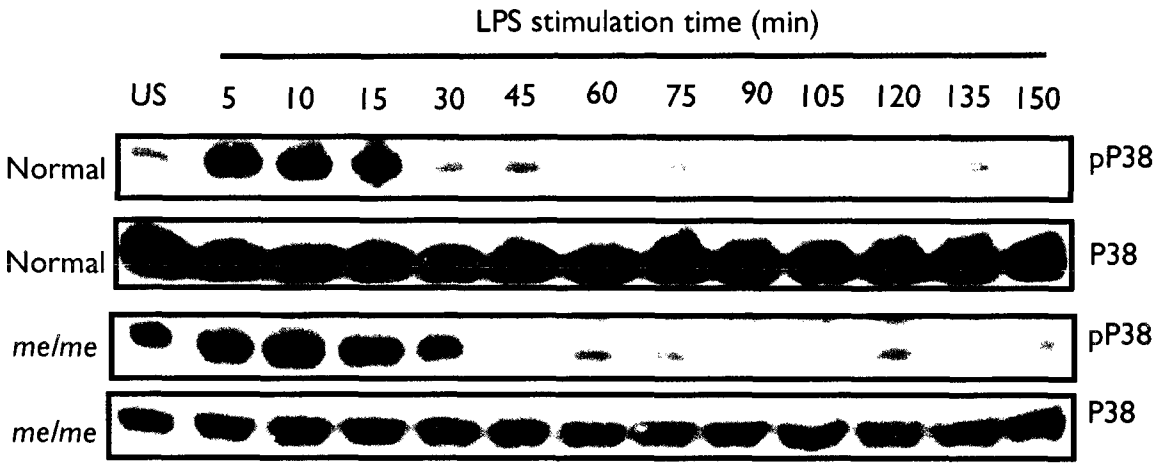
splenic macrophages. To control for P38 inhibitor activity, normal splenic macrophages were treated with increasing concentrations of either SB202190 or SB203580 for 2 h prior to LPS treatment for 10 min. Cell lysates were then evaluated for P38 phosphorylation by western immunoblotting using anti-phospho-P38 Ab. Results in Fig III.19C and D show that these inhibitors effectively diminished P38 phosphorylation. Since the results demonstrated that the activation of P38 is essential for LPS-induced IL-10 production in normal splenic macrophages, and that in the absence of SHP-1 IL-10 production in *me/me* splenic macrophages was reduced, it was of interest to examine if loss of SHP-1 impacts on the activation of P38 following LPS stimulation. For this, splenic macrophages were harvested, serum starved for 4h, then treated with LPS for 5 to 150 min (Fig III.20A) or 1 to 30 min (Fig III.20B). Lysates were subjected to western blot procedure and blots were probed for phospho-P38 using anti-phospho-P38 Ab to assess the levels of P38 phosphorylation in LPS treated normal and *me/me* splenic macrophages. Suprisingly, similar phosphorylation of P38 MAPK occurred in normal and *me/me* splenic macrophages between 5 and 30 min (Fig III.20A) or 1 and 30 min (Fig III.20B). In both sets of cells, the levels of P38 phosphorylation diminished to levels comparable to those observed in untreated cells by 30 min post LPS stimulation (Fig III.20A). Although similar P38 phosphorylation patterns were observed in both normal and *me/me* splenic macrophages, the phosphorylation levels for P38 MAPK at 30 min post LPS stimulation appeared slightly elevated and of longer duration in *me/me* splenic macrophages compared to normal controls (Fig III.20A) and was observed in all four experiments performed Another approach to determine whether P38 phosphorylation was altered in the

Fig III.20 LPS-induced phosphorylation of P38 MAPK in normal and *me/me* splenic macrophages.

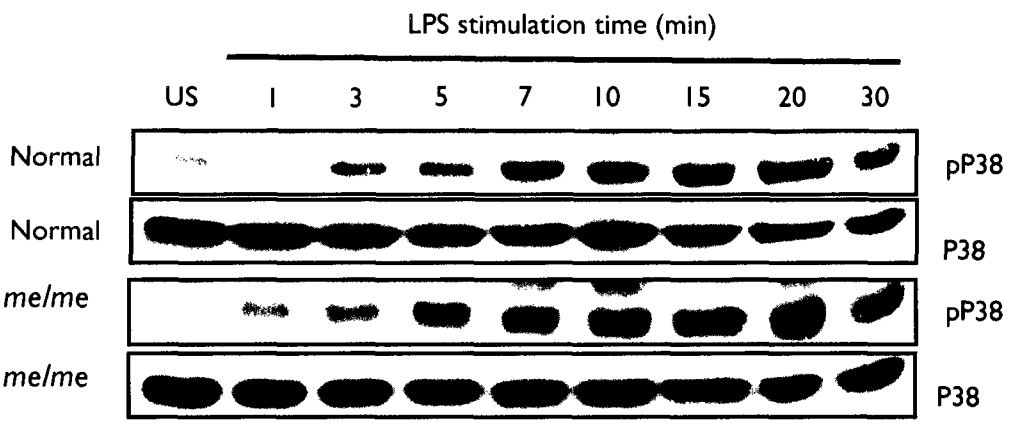
Serum starved normal and *me/me* splenic macrophages (approximately 1 X 10^7cells/mL per condition) were either untreated (US) or treated with LPS for (A) Long term duration (5 – 150 min) or (B) earlier and short term durations (1 -30 min). Cell pellets were lysed and total protein lysates (100 μg) were subjected to SDS-PAGE followed by Western blot analysis using anti-phospho p38 MAPK monoclonal Ab. To control for equal loading, membranes were stripped and reprobed using anti-P38 MAPK Ab. Results shown are representative of four independent experiments showing similar trends.

(C) Serum starved normal splenic macrophages (approximately 1 X 10^7cells/mL per condition) were either untreated (US) or pretreated with PTP-1 (SHP-1 inhibitor) at indicated concentrations (25 – 75 μM) for 2 h followed by LPS treatment for 10 min. Cell pellets were lysed and total protein lysates (100 μg) were subjected SDS-PAGE followed by Western blot analysis using anti-phospho-p38 MAPK Ab. To control for equal loading, membranes were stripped and reprobed using P38 MAPK Ab. Results shown are representative of four independent experiments showing similar trends.

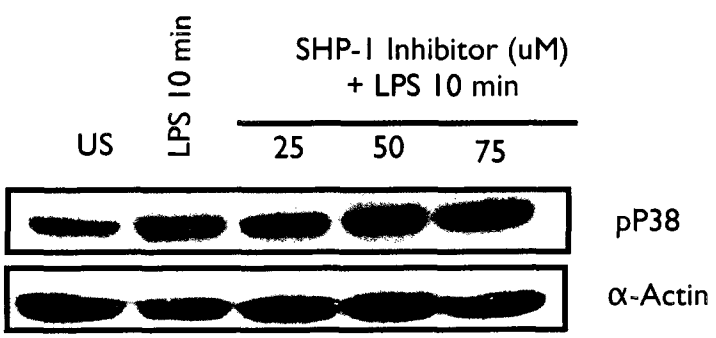
A


LPS stimulation time (min)
US
5
10
15
30
45
60
75
90
105
120
135
150
Normal
pP38
Normal
P38
me/me
pP38
me/me
P38

B


LPS stimulation time (min)
US
1
3
5
7
10
15
20
30
Normal
pP38
Normal
P38
me/me
pP38
me/me
P38

C


LPS 10 min
SHP-1 Inhibitor (uM)
+ LPS 10 min
US
25
50
75
pP38
α-Actin

absence of SHP-1 was to inhibit SHP-1 activity using the SHP-1 specific inhibitor, PTP-I and examined P38 phosphorylation. To accomplish this, normal splenic macrophages were treated with 25 to 75 μM of PTP-I inhibitor for 2 h followed by LPS treatment for 10 min and the extent of P38 phosphorylation was detected by Western immunoblotting using anti-phospho-P38 Ab. Both untreated and LPS treated controls were included. Results in Fig III.20C show that increasing doses of PTP-I inhibitor had no effect on P38 phosphorylation suggesting that SHP-1 is not required for LPS-mediated activation of P38 MAPK.

III.4.2.2 Conclusion on MAPK experiments

The analysis of ERK1/2 and JNK1/2/3 MAPKs demonstrate similar phosphorylation patterns of these kinases in normal and *me/me* splenic macrophages treated with LPS. The result also suggests that ERK1/2 is not required for LPS-induced IL-10 production in both normal and *me/me* splenic macrophages. In contrast, the inhibition of P38 effectively diminished IL-10 production in both normal and *me/me* splenic macrophages. This effect however, was not reflected in the phosphorylation levels of P38, which remained similar in both normal and *me/me* splenic macrophages. Taken together, these results imply that SHP-1 is not required for LPS-mediated phosphorylation of MAPKs in splenic macrophages. Although P38 MAPK plays a critical role in LPS-mediated IL-10 production, SHP-1 regulation of LPS-mediated IL-10 production appears independent of P38 MAPK. Hence, the reduced IL-10 production observed in me/me splenic macrophages is unlikely to be due to the effects or an effect of the loss of SHP-1 on P38.

III.4.3 Protein Tyrosine Kinase Pathways

(i) Role of cytoplasmic Protein Tyrosine Kinases in LPS mediated IL-10 production

In an effort to understand the involvement of SHP-1 in LPS-induced IL-10 production, it was of interest to explore the role of signaling proteins representing SHP-1 targets and known to be activated by LPS. Protein tyrosine kinases represent a class of proteins known to interact with SHP-1. Three families of cytoplasmic protein tyrosine kinases have been described in LPS signaling [275, 281, 296, 312, 316, 392]. These kinases include; the Itk/Btk family, Fak/Pyk2 family and Src family of protein tyrosine kinases.

III.4.3.1 Are Itk/Btk family kinases involved in LPS-mediated IL-10 production?

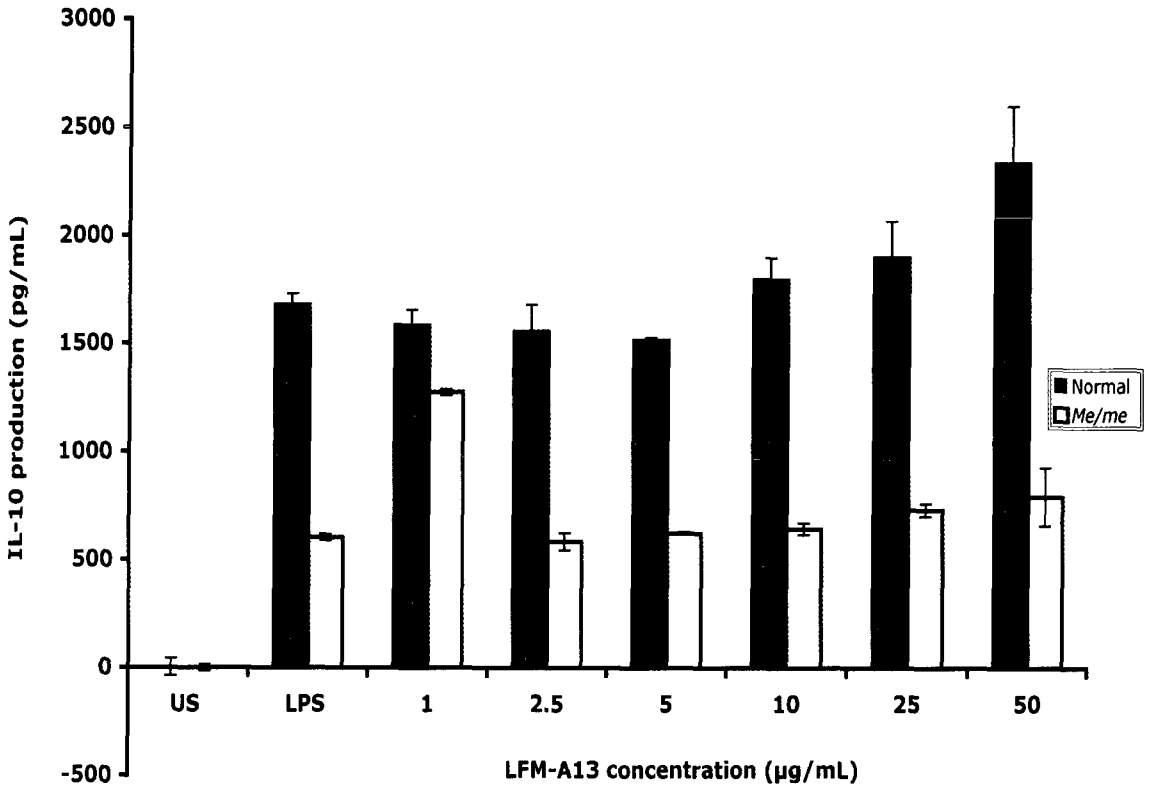
To assess the involvement of different PTK families in LPS-induced IL-10 production, I first examined the role of Bruton's tyrosine kinase (Btk). LFM-A13, an Itk/ Btk specific inhibitor was employed to disrupt the Itk/Btk activity in LPS treated normal and *me/me* splenic macrophages. Normal and *me/me* splenic macrophages were treated with increasing concentrations of LFM-A13 (0 to 50 μM) for 2 h prior to treatment with LPS for 24 h, after which IL-10 production in supernatants was measured. The results show that IL-10 production was not affected by Btk inhibition in either normal or *me/me* splenic macrophages even at 50 μM concentration (Fig III.21A). To confirm the activity of LFM-A13, normal splenic macrophages were treated with increasing concentrations of LFM-A13 for 2 h prior to LPS treatment for 10 min then the phosphorylation of Btk was assessed by western immunoblotting.

Fig III.21 The BTK Specific inhibitor LFM-A13 did not affect LPS-mediated IL-10 production in normal or *me/me* splenic macrophages.

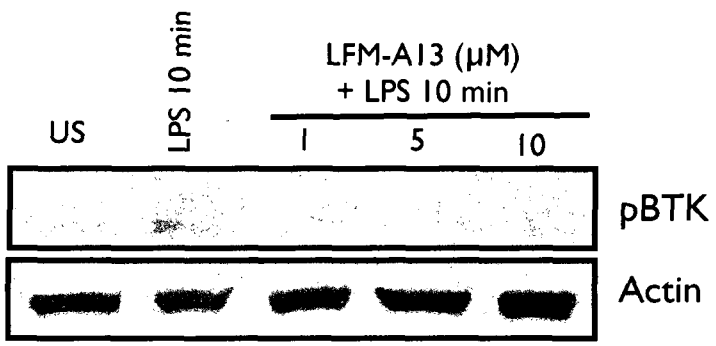
(A) Normal (■) and *me/me* (□) splenic macrophages plated at a density of approximately 5 X 10^6 cells/mL were either untreated (US) or pretreated with LFM-A13 (1 – 50 μM) for 2 h prior to LPS (10 μg/mL) treatment for 24 h. Culture supernatants were harvested after 24 h and assessed by ELISA for IL-10 production. All experiments were repeated three times. Data from a representative experiment are depicted in the figure. ELISA data are represented as arithmetic mean of duplicate wells ± variation from the mean within a single experiment.

(B) Serum starved normal splenic macrophages (approximately 1 X 10^7cells/mL per condition) were either untreated (US) or pretreated with LFM-A13 (1 – 10 μM) for 2 h followed by LPS (10 μg/mL) treatment for 10 min. Cell pellets were lysed and total protein lysates (100 μg) were subjected SDS-PAGE followed by Western blot analysis using anti-phospho-BTK monoclonal Ab. Membranes were stripped and reprobed using α-actin Ab as a control for equal loading. Results depicted are representative of two independent experiments showing similar trends.

A


3000
2500
2000
1500
1000
500
0
-500
IL-10 production (pg/mL)
US
LPS
1
2.5
5
10
25
50
LFM-A13 concentration (µg/mL)
Normal
Me/me

B


US
LPS 10 min
LFM-A13 (µM)
+ LPS 10 min
1
5
10
pBTK
Actin

The results confirm that LFM-A13, at low concentrations, was capable of inhibiting the phosphorylation of Btk (Fig III.21B).

III.4.3.2a Role of Src family kinase in LPS-mediated IL-10 production

To determine the role of Src family kinases in LPS-mediated IL-10 production in splenic macrophages, I used the specific Src inhibitors, PP2 and SU6656 [393] to block the activation of Src kinase family members. Again, normal and *me/me* splenic macrophages were treated with increasing concentrations (0 to 1 μM) of PP2 or SU6656 for 2h prior to LPS treatment for 24 h after which IL-10 production was measured. The results shown using PP2 reveal that IL-10 production was significantly inhibited in a dose dependent manner in normal splenic macrophages. In contrast, even high doses of 1 μM of PP2 did not affect IL-10 production in LPS treated *me/me* splenic macrophages (Fig III.22A) and suggest a requirement for Src family kinases in LPS-mediated IL-10 production in normal but not *me/me* splenic macrophages. Therefore, the reduced levels of IL-10 produced by *me/me* splenic macrophages may not be dependent on Src family kinases but on P38 MAPK. Control showing the inhibitory activity of PP2 on phosphorylation of Src-family kinases in normal splenic macrophages is shown in Fig III.22B. Similar results were obtained using the Src inhibitor, SU6656 (data not shown).

To further confirm the above observations, I examined IL-10 mRNA levels in PP2 treated cells by quantitative RT-PCR. To examine this, normal and *me/me* splenic macrophages were pretreated with PP2 (5,10 and 20μM) for 2 h prior to LPS treatment for 7 h after which IL-10 mRNA levels were measured by real time RT-

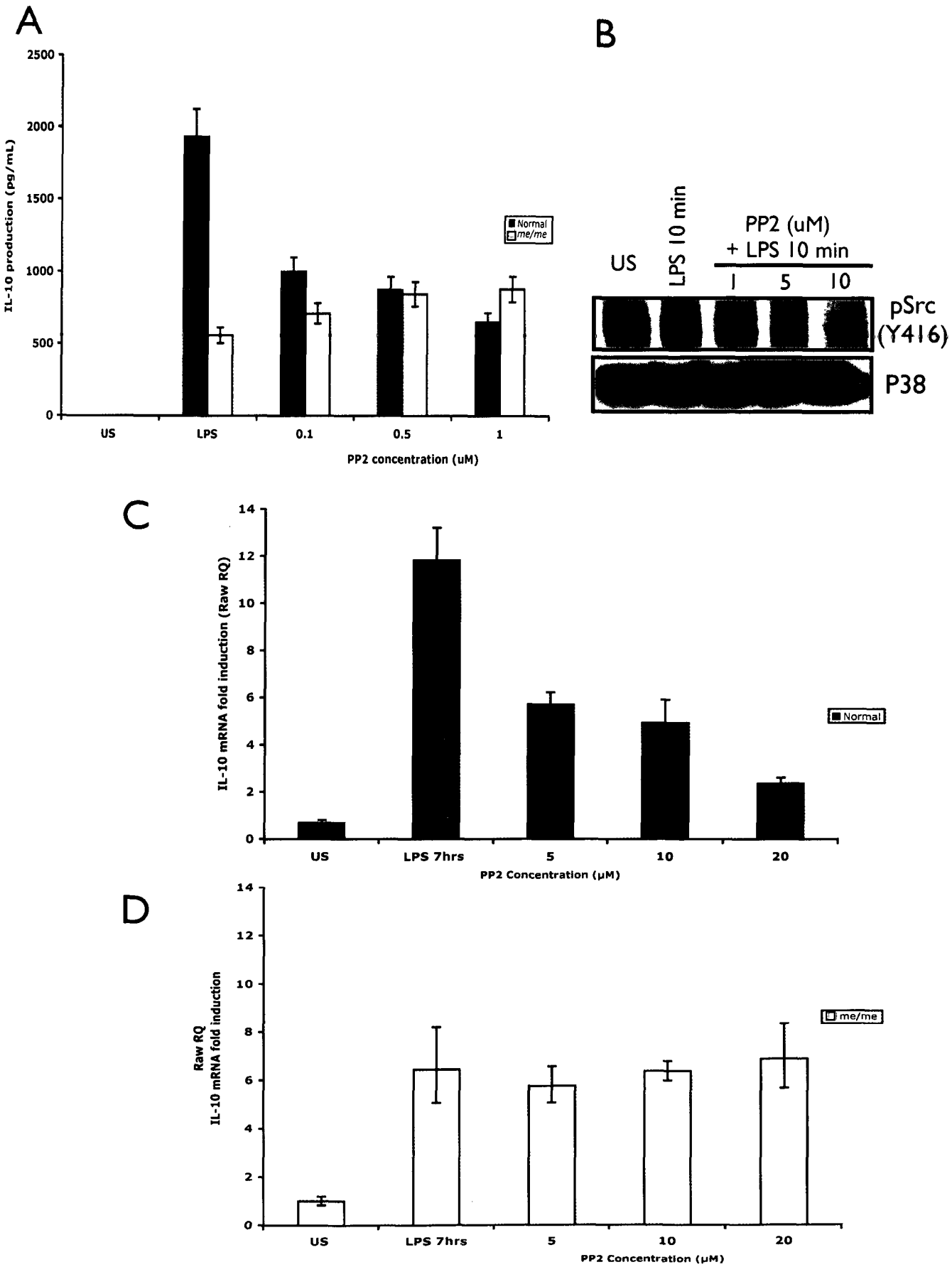
Fig III.22 LPS-induced IL-10 production is regulated by Src family kinases in normal but not in *me/me* splenic macrophages.

(A) Normal (■) and *me/me* (□) splenic macrophages plated at a density of approximately 5 X 10^6 cells/mL were either untreated (US) or pretreated with PP2 a specific inhibitor of Src family kinases at 0.1 – 1 μM concentrations for 2 h prior to LPS (10 μg/mL) treatment for 24 h. Culture supernatants were harvested after 24 h and assessed by ELISA for IL-10 production. All experiments were repeated three times. Data from a representative experiment are depicted in the figure. ELISA Data are represented as arithmetic mean of duplicate wells ± variation.

(B) Serum starved normal splenic macrophages (approximately 1 X 10^7cells/mL per condition) were either untreated (US) or pretreated with PP2 (1 – 10 μM) for 2 h followed by LPS (10 μg/mL) treatment for 10 min. Cell pellets were lysed and total protein lysates (100 μg) were subjected SDS-PAGE followed by Western blot analysis using anti-phospho-Src (Y416) Ab specific for all members of Src family kinases (SrcFK). Membranes were stripped and reprobed using p38 MAPK Ab as a control for equal loading. Results shown are representative of two independent experiments showing similar trends.

Serum starved (C) normal (■) and (D) *me/me* (□) splenic macrophages (approximately 1 X 10^7cells/mL per condition) were either untreated (US) or pretreated with PP2 (5 – 20 μM) for 2 h prior to LPS (10 μg/mL) for 7 h. Total RNA was extracted followed by cDNA synthesis. IL-10 transcripts were amplified by Q-PCR using β-actin as a reference control. To measure the relative changes in IL-10 induction, results of LPS and PP2 + LPS treated samples were normalized to the untreated control within the experiment. Data were represented as fold change versus calibrator sample, which is the untreated control. To reflect the changes in IL-10 induction and to clearly demonstrate a differential effect of PP2 inhibitor on IL-10 production in *me/me* splenic macrophages compared to normal controls, the data are depicted on 2 separate graphs (C) Normal and (D) *me/me* All experiments were performed at least three times however data from a representative experiment showing similar trends are depicted in the figures. Error bars represent the variation within a single experiment.

*** pSrcFK (Y416) – pan anti-phospho-Src, specific to all members of the Src family.**


A
2500
2000
1500
1000
500
0
IL-10 production (pg/mL)
Normal
me/me
US
LPS
0.1
0.5
1
PP2 concentration (uM)
B
US
LPS 10 min
PP2 (uM)
+ LPS 10 min
1
5
10
pSrc
(Y416)
P38
C
14
12
10
8
6
4
2
0
IL-10 mRNA fold induction (Raw RQ)
Normal
US
LPS 7hrs
5
10
20
PP2 Concentration (μM)
D
14
12
10
8
6
4
2
0
Raw RQ
IL-10 mRNA fold induction
me/me
US
LPS 7hrs
5
10
20
PP2 Concentration (μM)

PCR. The results show a dose dependent decline in the IL-10 mRNA induction levels in normal splenic macrophages (Fig III.22C) but not in *me/me* splenic macrophages (Fig III.22D).

Results from both experiments revealed a differential involvement of Src family kinases in LPS-mediated IL-10 production between normal and *me/me* splenic macrophages. In normal splenic macrophages, the inhibition of Src family kinases resulted in diminished IL-10 production whereas in *me/me* splenic macrophages the inhibition of Src family kinases had no effect on IL-10 production. These results therefore suggest a role for Src family kinases in LPS-induced IL-10 production in normal splenic macrophages but not *me/me* splenic macrophages.

III.4.3.2b Role of SHP-1 in the activation of Src family kinase in LPS treated splenic macrophages.

(i) Src family kinases are poorly phosphorylated in LPS treated me/me splenic macrophages.

The differential involvement of Src family kinases in IL-10 production in normal and *me/me* splenic macrophages prompted me to examine the role of SHP-1 in the activation of Src family kinases upon LPS treatment. To determine if SHP-1 participates in the activation of Src family kinases, I compared phosphorylation of Src family kinases in LPS treated normal and *me/me* splenic macrophages. Splenic macrophages were harvested, serum starved for 4 h then treated with LPS for 5 to 150 min (Fig III.23A) or 1 to 30 min (Fig III.23B). Lysates were subjected to western blot procedure and blots were probed using anti-phospho-Src ($SrcFK_{Y416}$) specific for all members of src family kinases. Phosphorylation of Src family kinases on tyrosine 416

is indicative of maximal Src kinase activity [305, 394, 395]. Lysates from untreated cells were included as negative controls to define basal phosphorylation levels of Src family kinases. Results from western blot analyses revealed that Src phosphorylation was robust within 5 to 15 min (Fig III.23A and B) in normal splenic macrophages but was poor in *me/me* splenic macrophages, and remained at basal levels from 1 to 60 min in these cells (Fig III.23A and B). However, 60 min post LPS treatment, Src family kinases in *me/me* splenic macrophages were phosphorylated until 150 min (Fig III.23A).

(ii) Inhibition of SHP-1 in normal splenic macrophages attenuates Src family kinase phosphorylation.

To directly link SHP-1 to LPS-induced Src activation, the SHP-1 inhibitor, PTP-I was used to impede SHP-1 function in normal splenic macrophages. For this, normal splenic macrophages were harvested, serum starved for 4 h then treated with increasing concentrations of PTP-I (1 – 20 μM) for 2 h prior to LPS treatment for 10 min. Phosphorylation on tyrosine 416 (Y416) of Src kinase was determined by Western immunoblotting using anti-phospho-Src Ab, specific for Y416. Both untreated and LPS treated cell lysate controls were included. Results in Fig III.23C reveal a dose dependent decline in Src phosphorylation (Y416) in PTP-I treated cells, suggesting that LPS-induced Src family kinase phosphorylation is dependent on SHP-1.

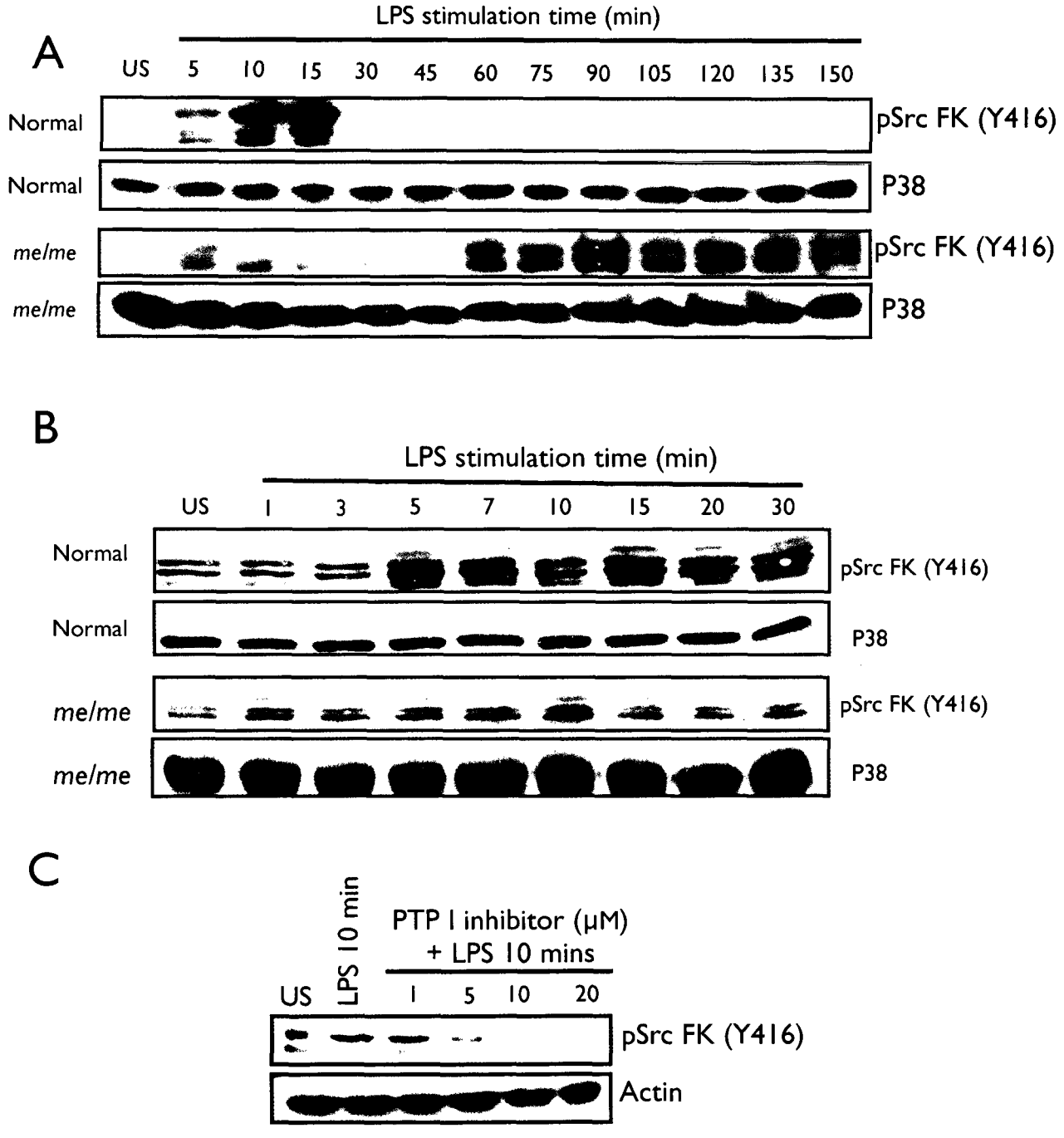
(iii) Src kinase activation is poor in LPS treated me/me splenic macrophages.

Tyrosine phosphorylation of signaling proteins is associated with conformational changes that can lead to the activation of signaling proteins [304, 306].

Fig III.23 Poor phosphorylation of Src family kinases in LPS treated *me/me* splenic macrophages.

Serum starved normal and *me/me* splenic macrophages (approximately 1 X 10^7cells/mL per condition) were either untreated (US) or treated with LPS for (A) Long term durations (5 – 150 min) or (B) short term durations (1 -30 min). Cell pellets were lysed and total protein lysates (100 μg) were subjected SDS-PAGE followed by Western blot analysis using pan anti-phospho-Src (Y416) Ab. Membranes were stripped and reprobed using the unrelated anti-p38 MAPK Ab as a control for equal loading. Results shown are representative of two independent experiments showing similar trends.

(C) Serum starved normal splenic macrophages (approximately 1 X 10^7cells/mL per condition) were either untreated (US) or pretreated with PTP-1 (SHP-1 inhibitor) at indicated concentrations (1 – 20 μM) for 2 h followed by LPS treatment for 10 min. Cell pellets were lysed and total protein lysates (100 μg) were subjected SDS-PAGE followed by Western blot analysis using pan anti-phospho-Src (Y416) Ab. Membranes were stripped and reprobed using α-Actin Ab as a control for equal loading. Results shown are representative of four independent experiments showing similar trends.


A
LPS stimulation time (min)
US 5 10 15 30 45 60 75 90 105 120 135 150
Normal
pSrc FK (Y416)
Normal
P38
me/me
pSrc FK (Y416)
me/me
P38
B
LPS stimulation time (min)
US 1 3 5 7 10 15 20 30
Normal
pSrc FK (Y416)
Normal
P38
me/me
pSrc FK (Y416)
me/me
P38
C
LPS 10 min
PTP I inhibitor (μM)
+ LPS 10 mins
US 1 5 10 20
pSrc FK (Y416)
Actin

It is well established that the binding of tyrosine 527 in the C-terminal tail of Src family kinases by *N*-terminal SH2 domains sequesters tyrosine 416 and it's associated activation site [394, 395]. To activate this enzyme, a dephosphorylation event acting on tyrosine 527 is required to expose tyrosine 416 [395]. Auto phosphorylation of tyrosine 416 within the catalytic kinase domain by intrinsic endogenous kinase activity leads to full activation of the enzyme [395, 396]. Protein tyrosine phosphatases, including SHP-1 have been implicated in the dephosphorylation of tyrosine 527 [304, 306, 394, 395, 397]. It is also noteworthy to mention that phosphorylation of Y416 or Y527 on Src exerts opposing effects on Src and therefore cannot occur simultaneously [396]. Thus, phosphorylation of Y416 of Src invariably implies dephosphorylation of Y527. Therefore, to examine if SHP-1 participates in LPS-induced phosphorylation of Src, (by dephosphorylating Y527 and enabling Y416 phosphorylation thus increasing Src activity) I compared Src kinase activity in LPS treated *me/me* splenic macrophages to normal controls. Normal and *me/me* splenic macrophages were harvested, treated with LPS (0 to 30 min) and Src was immunoprecipitated using a Src-specific antibody. Src activity was then measured in Src immunoprecipitates by a modified Src kinase assay employing fluorescence superquenching technique (Calbiochem). For this assay, a decrease in fluorescence is associated with an increase in Src kinase activity. An increase in Src kinase activity was observed in normal splenic macrophages treated with LPS for 5, 10, 15 and 30 min. In contrast, there was no increase in Src kinase activity in *me/me* splenic macrophages subjected to the same procedure (Fig III.24A). The function of SHP-1 in upregulating Src kinase activity was further verified by inhibiting SHP-1 function in

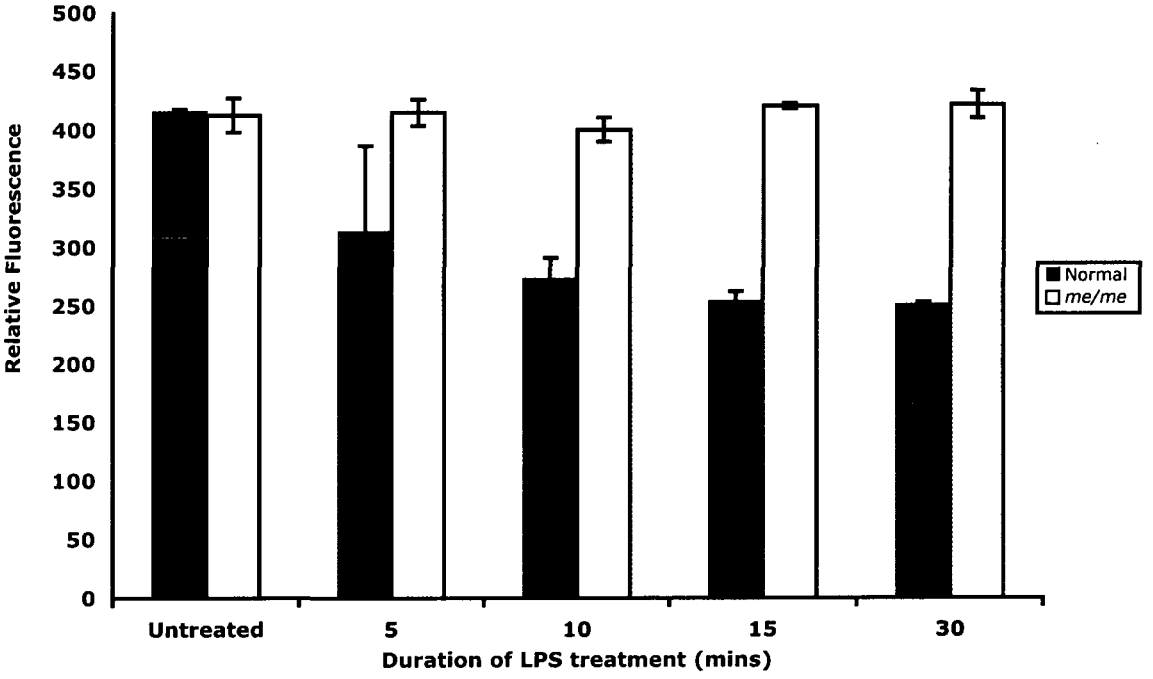
Fig III.24 Src kinase activity in LPS treated splenic macrophages is dependent on SHP-1 expression and activity

(A) Serum starved normal (■) and *me/me* (□) splenic macrophages (approximately 2 X 10^7cells/mL per condition) were either untreated (US) or treated with LPS (10 μg/mL) for 5 – 30 min. Cell pellets were lysed and total protein lysates (1000 μg) were subjected to Catch and Release immunoprecipitation procedure under non-denaturing conditions. To measure Src kinase activity, immunoprecipitates were subjected to a fluorescent superquencing-based kinase assay. Results shown are representative of three independent experiments showing similar trends.

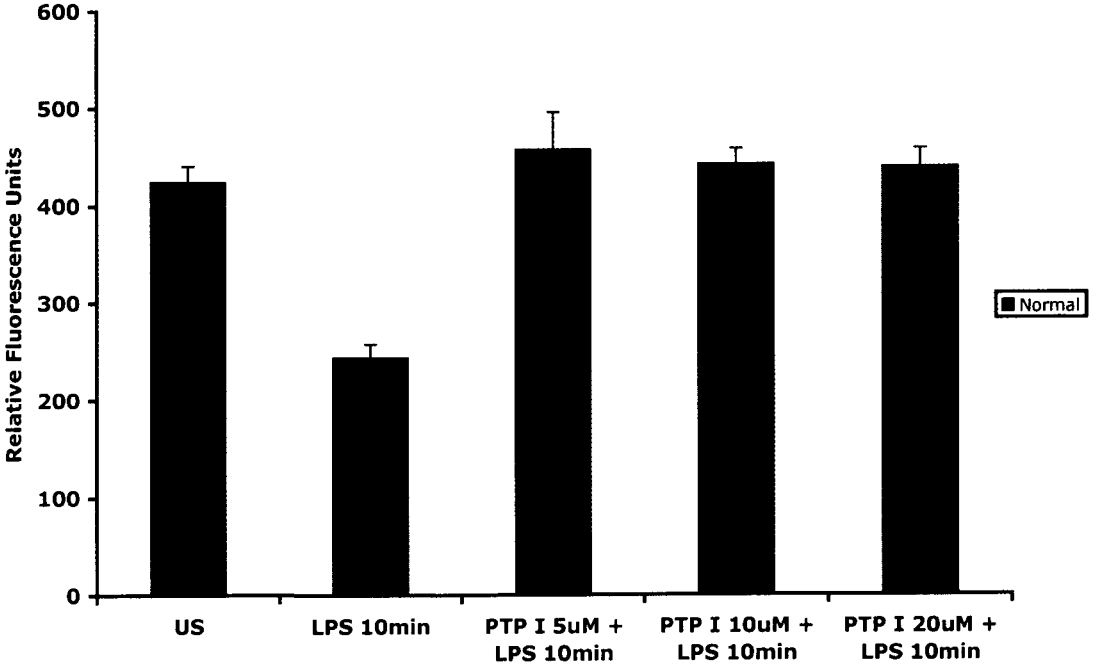
(B) Serum starved normal splenic macrophages (approximately 2 X 10^7cells/mL per condition) were either untreated (US) or pretreated with PTP-1 (SHP-1 inhibitor) at indicated concentrations (5– 20 μM) for 2 h followed by LPS treatment for 10 min. Cell pellets were lysed and total protein lysates (1000 μg) were subjected to Catch and Release immunoprecipitation procedure under non-denaturing conditions. To measure Src kinase activity, immunoprecipitates were subjected to fluorescent superquencing-based kinase assay. Results shown are representative of three independent experiments showing similar trends.

Please note that for fluorescent superquenching-based kinase assay, a decline in relative fluorescence corresponds to an increase in kinase activity.

(A)


500
450
400
350
300
250
200
150
100
50
0
Relative Fluorescence
Normal
me/me
Untreated
5
10
15
30
Duration of LPS treatment (mins)

(B)


600
500
400
300
200
100
0
Relative Fluorescence Units
Normal
US
LPS 10min
PTP I 5uM + LPS 10min
PTP I 10uM + LPS 10min
PTP I 20uM + LPS 10min

normal splenic macrophages using PTP-I inhibitor and examining Src kinase activity using fluorescent based-kinase assay. Briefly, normal splenic macrophages were treated with PTP-I (5-20 μM) for 2 h followed by LPS treatment (10 μg/mL) for 10 min, Src immunoprecipitation and Src kinase assay. Results in Fig III.24B show that treatment of normal splenic macrophages with PTP-I resulted in a decrease in Src kinase activity, comparable to levels observed in untreated controls differing markedly with Src kinase activity observed in cells treated with LPS only for 10 min. In three experiments completed, treatment with PTP-1 reduced Src kinase activity by 2-fold. Results of these experiments demonstrate that Src kinase is poorly activated in LPS treated SHP-1-deficient *me/me* splenic macrophages and that the inhibition of SHP-1 in normal splenic macrophages, reduces Src activity to levels observed in LPS treated *me/me* splenic macrophages.

(iv) SHP-1 does not co-immunoprecipitate with Src kinase in LPS stimulated normal splenic macrophages.

Since SHP-1 plays a role in the activation of Src kinase and both SHP-1 and Src are required for IL-10 production in LPS treated normal splenic macrophages, it was necessary to understand the nature of the interaction that occurs between SHP-1 and Src upon LPS stimulation. For this, normal splenic macrophages were harvested six days after culture, serum starved for 4 h and treated with LPS for 5 or 15 min. Lysates were subjected to either Src or SHP-1 immunoprecipitation followed by western immunoblotting using anti-SHP-1 or anti-Src antibodies. To accurately identify SHP-1 or Src band(s), the following immunoprecipitation/immunoblotting controls were also included; antibody and beads with no lysates, lysates only from normal splenic

macrophages and Ab control lysates from A431 cell lines provided by the manufacturer.

Results in Fig III.25A show that Src was not present in SHP-1 immunoprecipitates (Lane 4). Likewise, in Fig III.25B, results show that SHP-1 was not present in Src immunoprecipitates (Lanes 1,2 and 3). These results indicate that although SHP-1 influenced Src activation in LPS treated normal splenic, SHP-1 did not co-immunoprecipitate with Src.

SHP-1–Src interactions have been described in lympocytes and platelets in response to interleukin-2 and thrombin treatments respectively [397]. The present study utilizes splenic macrophages and LPS as a model. To ensure that the lack of SHP-1–Src interaction observed (Fig III.25A and B) was not due to technical problems but was rather intrinsic to the cell type and ligand used, I examined the relationship between SHP-1 and Src in PHA treated EL-4 cells, a mouse thymoma cell line. For this, EL-4 cells were grown to a density of 10^6 cells per mL and harvested. Cells were either left untreated or treated with PHA for 10 min. Cell lysates were subjected to Src or SHP-1 immunoprecipitation, followed by western immunoblotting using either anti-Src or anti-SHP-1 monoclonal antibody. Results in Fig III.26A (upper panel) show that SHP-1 co-immunoprecipitated with Src in PHA treated EL-4 cells but not in untreated cells. As a control, the blot was stripped and reprobed for Src. Fig III.26A (lower panel) demonstrates that Src kinase (red star) was present in SHP-1 immunoprecipitates from both untreated and PHA treated EL-4 cell lysates.

Fig III.25 SHP-1 does not co-immunoprecipitate with Src kinase in LPS stimulated normal splenic macrophages.

Serum starved normal splenic macrophages (approximately 2 X 10^7cells/mL per condition) were either untreated (US) or treated with LPS (10 μg/mL) for 5 and 15 min. Cell pellets were lysed and Src or SHP-1 (control lanes) was immunoprecipitated from total protein lysates (1000μg). Immunoprecipitation complexes were resolved on a 10% SDS-PAGE gel followed by Western blot analysis using (A) anti-Src monoclonal Ab. Membranes were stripped and reprobed using (B) anti-SHP-1 monoclonal Ab. To unambiguously identify bands of interest, the following controls were included; Ab and beads but no lysate (lane 5), total protein lysates from splenic macrophages (lane 6) and total protein lysates from A431 cell line (lane 7). Results shown are representative of three independent experiments showing similar trends.

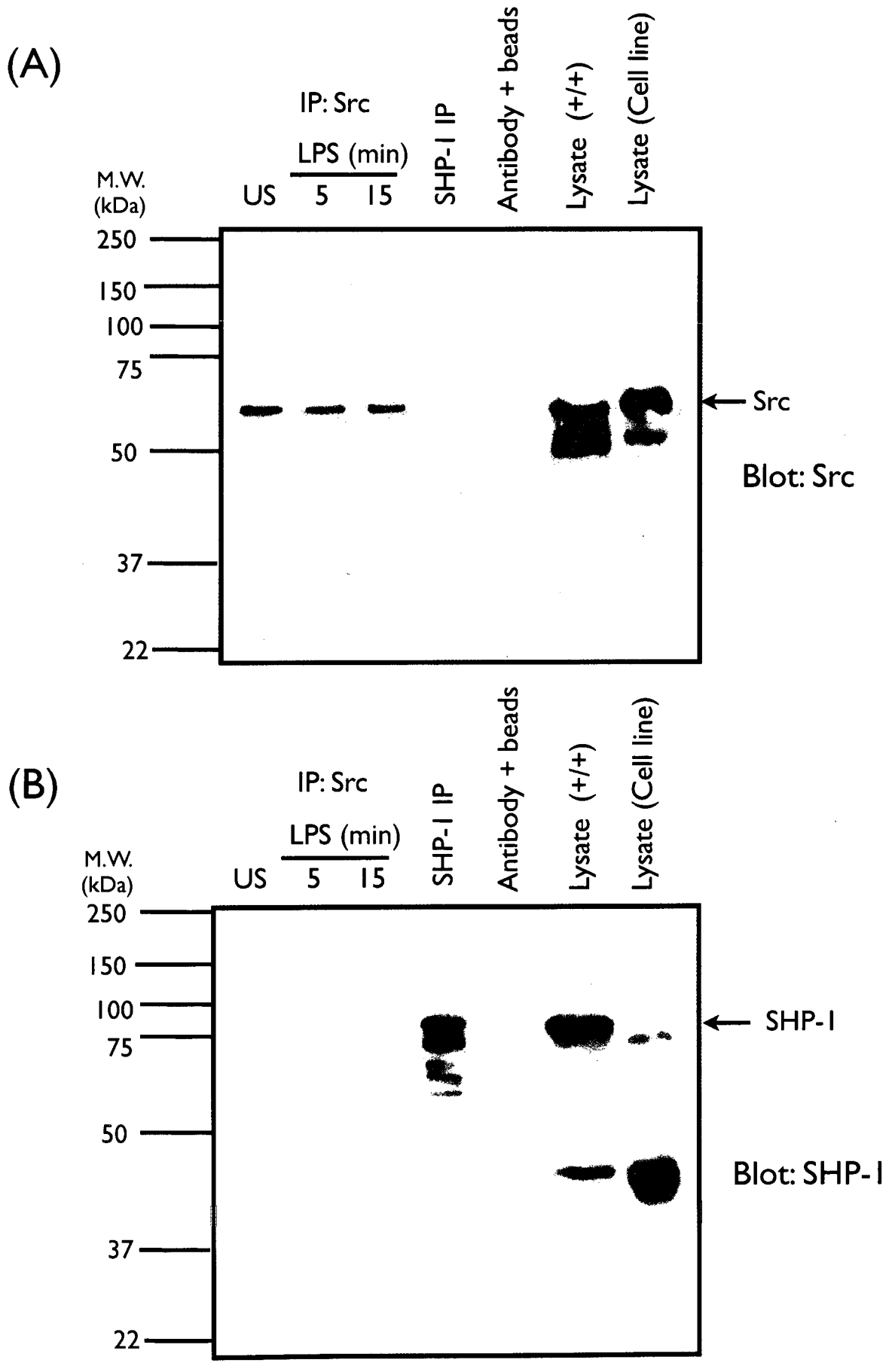
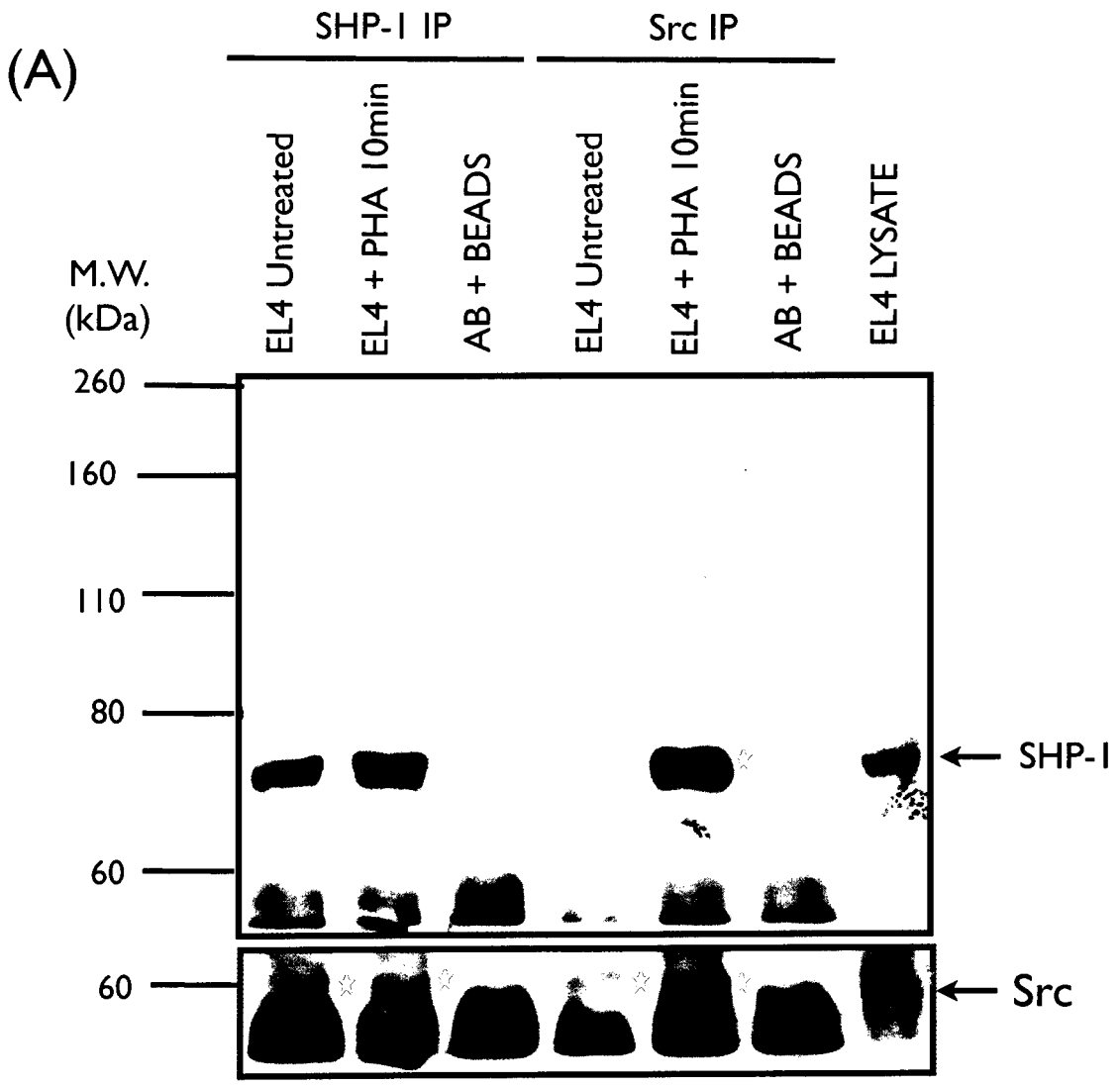

(A)
IP: Src
LPS (min)
US
5
15
SHP-1 IP
Antibody + beads
Lysate (+/+)
Lysate (Cell line)
M.W. (kDa)
250
150
100
75
50
37
22
Src
Blot: Src
(B)
IP: Src
LPS (min)
US
5
15
SHP-1 IP
Antibody + beads
Lysate (+/+)
Lysate (Cell line)
M.W. (kDa)
250
150
100
75
50
37
22
SHP-1
Blot: SHP-1

Fig III.26 Src co-immunoprecipitates with SHP-1 in PHA treated EL-4 mouse thymoma cells.

Serum starved EL-4 thymoma cells (approximately 2 X 10^7cells/mL per condition) were either untreated (US) or treated with PHA (20 ng/mL) for 10 min. Cell pellets were lysed and Src or SHP-1 was immunoprecipitated from total protein lysates (1000 μg). Immunoprecipitation complexes were resolved on 8% SDS-PAGE gels followed by Western blot analysis using anti-SHP-1 monoclonal Ab (top panel). Membranes were stripped and reprobed using anti-Src monoclonal Ab (bottom panel). To distinguish bands of interest, the following controls were also included; anti-SHP-1 Ab plus beads but no lysate and total protein lysates from EL-4 mouse thymoma cells. The red star identifies SHP-1 bands in Src immunoprecipitates. Results shown are representative of two independent experiments showing similar trends.

Please note that Src bands (56 to 60 KDa) have a close molecular weight to antibodies (55 kDa) used for immunoprecipitation. Hence, the Src bands closely migrate to antibody bands. To unambiguously identify Src bands a red star has been placed to the right of each Src band.


(A)
SHP-1 IP
Src IP
EL4 Untreated
EL4 + PHA 10min
AB + BEADS
EL4 Untreated
EL4 + PHA 10min
AB + BEADS
EL4 LYSATE
M.W.
(kDa)
260
160
110
80
60
60
SHP-1
Src

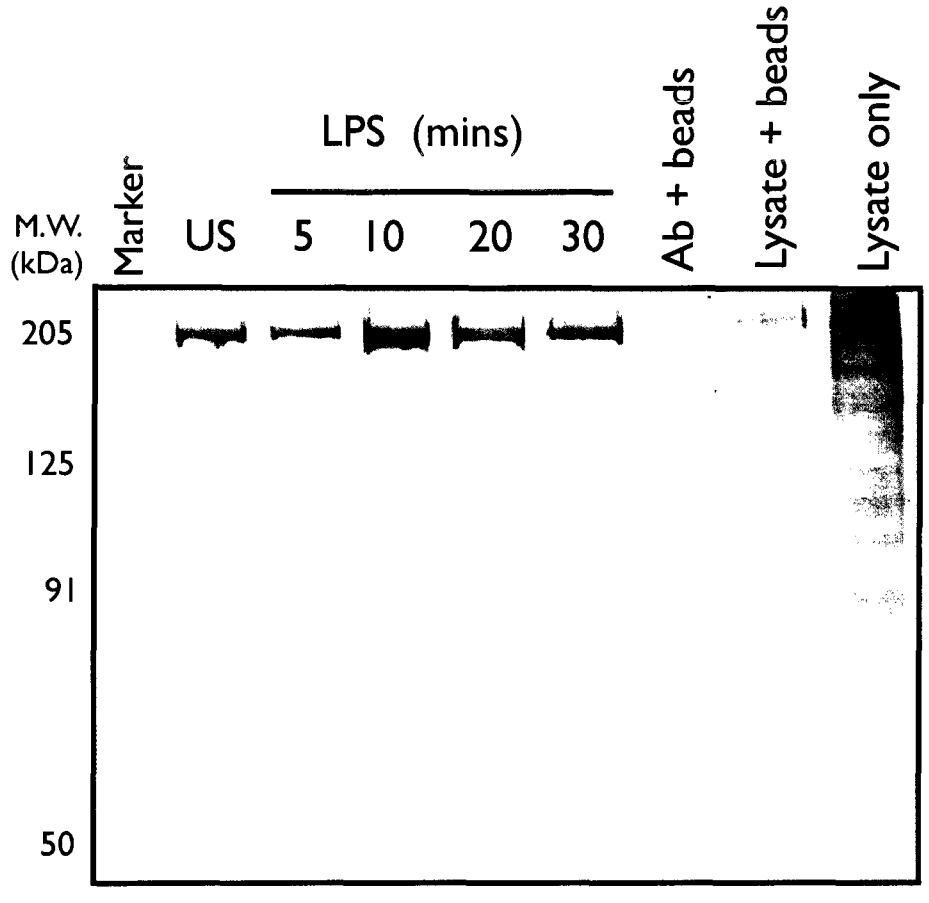
III.4.4 SHP-1 interacts with other proteins in splenic macrophages

Results from experiments examining the interaction of Src and SHP-1 in LPS treated normal splenic macrophages showed that Src did not co-immunoprecipitate with SHP-1 and vice versa and suggests lack of a direct physical interaction between Src and SHP-1. These findings prompted me to search for other possible proteins that may directly interact with SHP-1 in LPS signaling. To accomplish this, normal splenic macrophages were either not treated or treated with LPS for 5 to 30 min, and resulting lysates were subjected to SHP-1 immunoprecipitation. SHP-1 immunoprecipitates were resolved on SDS-PAGE gels, immunoprecipitating proteins were stained with sypro Tangerine and the gels were scanned using a Typhoon phospho-imager (GE Healthcare). Negative controls were included to unambiguously identify distinct bands representing proteins that interact with SHP-1. These controls included, antibody plus protein A Sepharose beads, protein lysate plus protein A Sepharose beads and protein lysates only. Results of SHP-1 immunoprecipitation shown in Fig III.27A revealed 5 distinct bands in both untreated and LPS treated lysates but not found in negative controls. No bands were observed at the 60 kDa level which is where the Src family kinases should be located. Hence Src family kinase members were likely not present in SHP-1 immunoprecipitates. Two bands were observed just below 90 kDa possibly representing SHP-1 isoforms (68 and 72 kDa). Two other bands were also observed at ~ 110 and 125 kDa respectively. These bands were repeatedly observed in at least four experiments.

Based on the above results, I further explored those PTK members which migrate in the 110 to 125 kDa range, that are known to be expressed in macrophages

Fig III.27 Preliminary identification of SHP-1 interacting partners.

Serum starved normal splenic macrophages (approximately 2 X 10^7cells/mL per condition) were either untreated (US) or treated with LPS (10 μg/mL) for 5 - 30 min. Cell pellets were lysed and SHP-1 was immunoprecipitated from total protein lysates (1000 μg) using anti-SHP-1 polyclonal antibody. Immunoprecipitation complexes were resolved on 8% SDS-PAGE gel followed by a Sypro tangerine gel staining for 90 min. To unambiguously identify bands of interest, the following controls were included; Ab and protein A Sepharose beads but no protein lysate, protein lysates and protein A Sepharose beads but no antibody and total protein lysates from normal splenic macrophages. Red stars higlight distinct protein bands of interest. Results shown are representative of at least four independent experiments showing similar trends.



SHP-1 IP

Stain: Sypro Tangerine

Detection limit ≥ 3ng

and activated by LPS stimulation [314-316, 398]. The Focal adhesion kinase (FAK) and Proline-rich tyrosine kinase (Pyk2) family of PTKs matched these criteria.

III.4.5 Interaction of SHP-1 with FAK/PYK2 family of PTKs

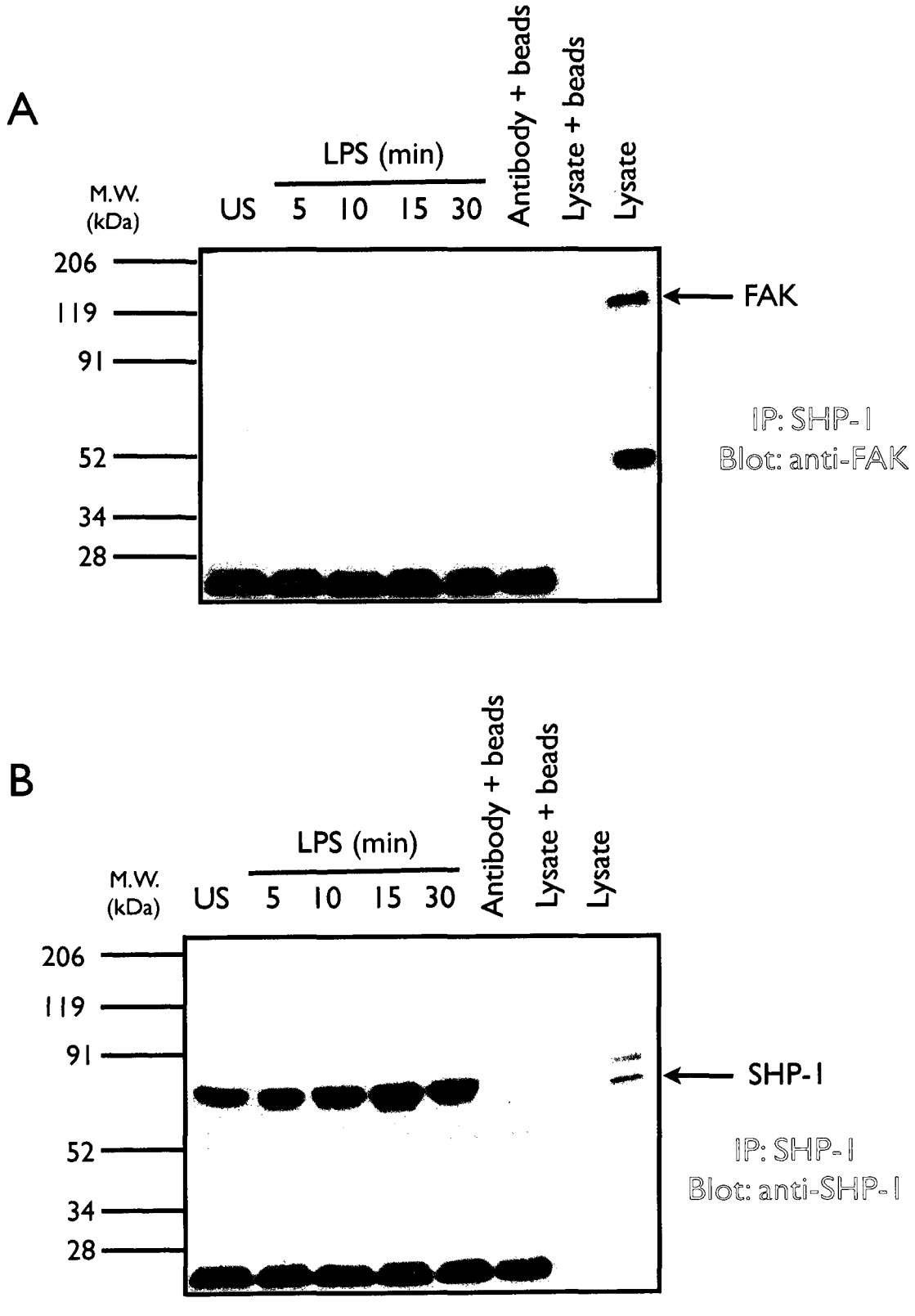
The FAK/Pyk2 signaling proteins are structurally related tyrosine kinases that play essential roles in cell migration and mitosis and specifically are associated with cytoskeletal rearrangement [398-402]. The phosphorylation and activation of FAK and Pyk2 upon LPS treatment in macrophages and leading to cytokine production has been extensively described [313-316]. However, the association of SHP-1 and FAK or Pyk2 in an LPS activated signaling pathway(s) has not been investigated.

(i) SHP-1 does not co-immunoprecipitate with FAK in LPS treated murine splenic macrophages.

To determine if FAK associates with SHP-1, SHP-1 was immunoprecipitated from lysates of untreated and LPS treated (5 to 30 min) normal splenic macrophages followed by Western immunoblotting using anti-FAK monoclonal antibody. Results in Fig III.28A show that FAK was not present in SHP-1 immunoprecipitates but was clearly identified in total protein lysates. As a control and to ensure that SHP-1 was present in immunoprecipitates, blots were stripped and reprobed with anti-SHP-1 monoclonal antibody. Results in Fig III.28B confirmed efficient immuprecipitation of SHP-1. These results suggest that FAK does not co-immunoprecipitate with SHP-1 following LPS treatment and therefore it appears unlikely that SHP-1 and FAK directly interact during LPS signaling in splenic macrophages.

Fig III.28 FAK does not co-immunoprecipitate with SHP-1 in normal splenic macrophages.

Serum starved normal splenic macrophages (approximately 2 X 10^7cells/mL per condition) were either untreated (US) or treated with LPS (10 μg/mL) for 5 - 30 min. Cell pellets were lysed and SHP-1 was immunoprecipitated from total protein lysates (1000 μg) using anti-SHP-1 polyclonal antibody. Immunoprecipitation complexes were resolved on a 10% SDS-PAGE gel followed by Western blot analysis using (A) anti-FAK monoclonal Ab. Membranes were stripped and reprobed using (B) anti-SHP-1 monoclonal Ab. To identify bands of interest, the following controls were included; Ab and protein A Sepharose beads but no protein lysate, protein lysates and protein A Sepharose beads but no Ab and total protein lysates from normal splenic macrophages. Arrows point to bands corresponding to either FAK or SHP-1. Figures shown are representative of at least three independent experiments showing similar results.


A
LPS (min)
M.W. (kDa)
US
5
10
15
30
Antibody + beads
Lysate + beads
Lysate
206
119
91
52
34
28
FAK
IP: SHP-1
Blot: anti-FAK
B
LPS (min)
M.W. (kDa)
US
5
10
15
30
Antibody + beads
Lysate + beads
Lysate
206
119
91
52
34
28
SHP-1
IP: SHP-1
Blot: anti-SHP-1

(ii) Pyk2 co-immunoprecipitates with SHP-1 in LPS treated normal splenic macrophages

The same experiment was performed to determine if Pyk2 co-immunoprecipitates with SHP-1 in LPS stimulated splenic macrophages using anti-Pyk2 monoclonal antibody. In all 3 experiments completed, Pyk2 did coprecipitate with SHP-1 (Fig III.29A). The amount of Pyk2 co-immunoprecipitating with SHP-1 increased with time after LPS treatment peaking at the 10 min time point and decreased after 30 min. As with previous experiments and to ensure that SHP-1 was present in immunoprecipitates, blots were stripped and reprobed with anti-SHP-1 monoclonal antibody (Fig III.29B), to confirm the presence of SHP-1 in the immunoprecipitates. These results suggest that SHP-1 and Pyk2 physically interact.

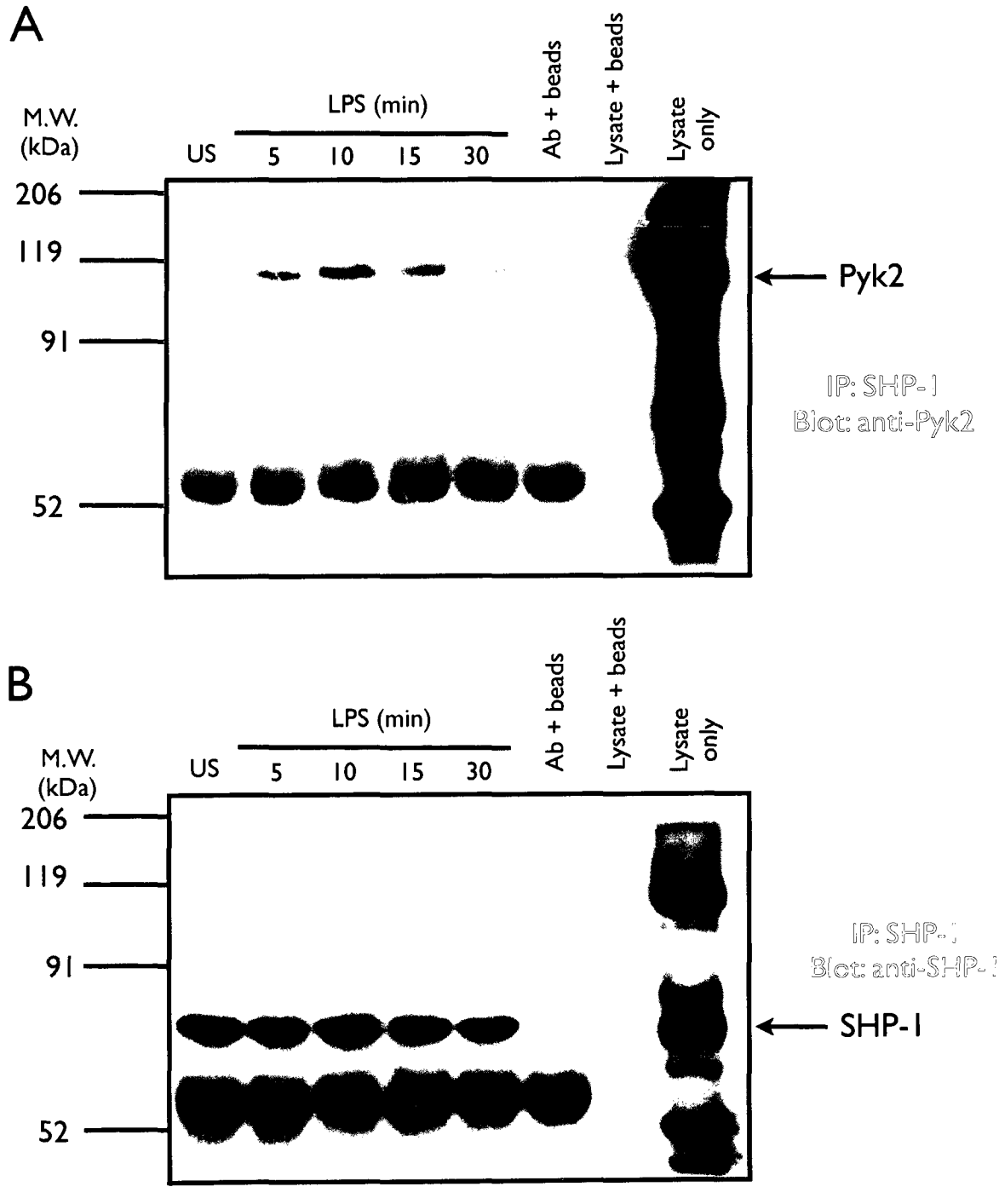
III.4.5.1 Role of Pyk2 in LPS-mediated IL-10 production

(i) Pyk2 expression is required for IL-10 production

Given that SHP-1 interacts with Pyk2 in splenic macrophages, it was crucial to examine the role of Pyk2 in LPS-mediated IL-10 production in normal splenic macrophages. To do this, Pyk2 expression was obstructed in normal splenic macrophages using Pyk2 antisense oligonucleotides for 24 h prior to stimulating with LPS for another 24 h. To control for oligonucleotide specificity, one sample was treated with an irrelevant oligonucleotide sequence (IRR SQ) and a second sample was treated with *Streptolysin O* (SLO) only. Culture supernatants were assayed for IL-10 production. In all 3 experiments completed, a 3-fold decline in IL-10 production was observed in normal splenic macrophages treated with Pyk2 antisense oligonucleotide and is

Fig III.29 Pyk2 co-immunoprecipitates with SHP-1 in LPS treated normal splenic macrophages.

Serum starved normal splenic macrophages (approximately 2 X 10^7cells/mL per condition) were either untreated (US) or treated with LPS (10 μg/mL) for 5 - 30 min. Cell pellets were lysed and SHP-1 was immunoprecipitated from total protein lysates (1000 μg). Immunoprecipitation complexes were resolved on a 10% SDS-PAGE gel followed by Western blot analysis using (A) α-Pyk2 monoclonal Ab. Membranes were reprobed using (B) α-SHP-1 monoclonal Ab. To detect bands of interest, the following controls were included; Ab and protein A Sepharose beads but no protein lysate, protein lysates and protein A Sepharose beads but no Ab and total protein lysates from normal splenic macrophages. Arrows point to bands corresponding to either Pyk2 or SHP-1. Figures shown are representative of two independent experiments showing similar results.


A
M.W. (kDa)
LPS (min)
US
5
10
15
30
Ab + beads
Lysate + beads
Lysate only
206
119
91
52
Pyk2
IP: SHP-1
Blot: anti-Pyk2
B
M.W. (kDa)
LPS (min)
US
5
10
15
30
Ab + beads
Lysate + beads
Lysate only
206
119
91
52
SHP-1
IP: SHP-1
Blot: anti-SHP-1

depicted in Fig III.30A. There was no significant decline in IL-10 production in cells treated with either IRR SQ or *Streptolysin O* (SLO) only.

To confirm Pyk2 antisense activity, normal splenic macrophages were treated with Pyk2 antisense oligonucleotides for 18 to 24 h and protein lysates were examined for Pyk2 expression by Western blot. Negative controls to determine the specificity of Pyk2 antisense oligonucleotide treatment include, untreated, Pyk2 sense oligonucleotide, IRR SQ and SLO. Results in Fig III.30B reveal that the treatment of normal splenic macrophages with Pyk2 antisense oligonucleotides efficiently diminished Pyk2 expression while negative controls did not have an effect on Pyk2 expression.

(ii) Pyk2 phosphorylation is essential for LPS-induced IL-10 production in normal splenic macrophages.

To determine if Pyk2 phosphorylation is required for LPS-induced IL-10 production in splenic macrophages, I inhibited Pyk2 activation using the Pyk2 specific inhibitor, AG-17. Normal splenic macrophages were treated with increasing concentrations (5, 10, 20 μM) of AG-17 for 2 h prior to treatment with LPS for 7 h. IL-10 induction was assessed by measuring IL-10 mRNA using real time RT-PCR. Results in Fig III.30C reveal that IL-10 levels were significantly diminished in splenic macrophages treated with AG-17 compared to those treated with LPS only. To confirm the activity of AG-17 on Pyk2 activation/phosphorylation, normal splenic macrophages were either untreated or treated with increasing concentrations of AG-17 for 2 h prior to treatment with LPS for 10 min. Western immunoblotting was performed using phosphoPyk2 antibody specific to Y402, to determine the levels of Pyk2 phoshorylation. Results show that Pyk2 phosphorylation was

Fig. III.30 Pyk2 expression and activation are required for LPS-induced IL-10 production in splenic macrophages.

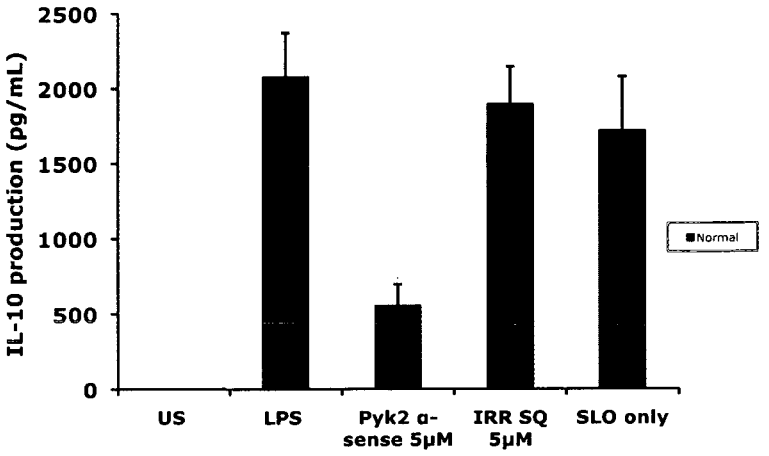
(A) Normal (■) splenic macrophages were subjected to Pyk2 antisense treatment using reversible permeabilization delivery approach. Normal splenic macrophages cultures were plated at a density of approximately 5 X 10^6 cells/mL were either untreated (US) or pretreated with α-Pyk-2 antisense, oligonucleotides for 20 - 24 h followed by LPS treatment for 24 h. To control for Pyk2 antisense specificity, IRR SQ and SLO-only treatments were included. Culture supernatants were harvested and analyzed by ELISA for IL-10 production. All experiments were repeated at least three times. Data shown are representative experiments showing similar trends. ELISA data are represented as arithmetic mean of duplicate wells ± variation.

(B) Pyk2 antisense oligonucleotide treatment suppresses SHP-1 protein expression in normal splenic macrophages. Serum starved normal splenic macrophages (1 X 10^7 cells/mL per condition) were permeated using SLO and were either left untreated (US) or treated with Pyk2 antisense oligonucleotide (or other controls as indicated) for 20 h. Cultures were harvested and subjected to Western blot procedure using monoclonal α-Pyk2 Ab (upper panel). Membranes were stripped and reprobed using α-actin Ab for equal loading control (lower panel). Results shown in this figure are representative of two independent experiments showing similar trends.

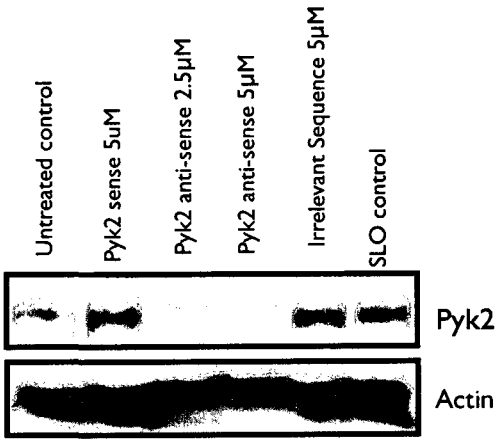
(C) Serum starved normal (■) splenic macrophages (approximately 1 X 10^7cells/mL per condition) were either untreated (US) or pretreated with AG-17 (5 – 20 μM) for 2 h prior to LPS (10 μg/mL) for 7 h. Total RNA was extracted followed by cDNA synthesis. IL-10 transcripts were amplified by Q-PCR using β-actin as a reference control. To measure the relative changes in IL-10 induction, results of LPS and AG-17 + LPS treated samples were normalized to the untreated control within the experiment. Data were represented as fold change versus calibrator sample, which is the untreated control. All experiments were performed at least three times Data from a representative experiment are depicted in the results. Error bars represent the variation within a single experiment.

(D) Serum starved normal splenic macrophages (approximately 1 X 10^7cells/mL per condition) were either untreated (US) or pretreated with AG-17 (Pyk2 inhibitor) at indicated concentrations (5 – 20 μM) for 2 h followed by LPS treatment for 10 min. Cell pellets were lysed and total protein lysates (100 μg) were subjected to SDS-PAGE followed by Western blot analysis using α-phospho-Pyk2 (Y402) monoclonal Ab (Top panel). As a control for equal loading membranes were stripped and reprobed using α-Actin Ab (bottom panel). Results shown are representative of two independent experiments showing similar trends.

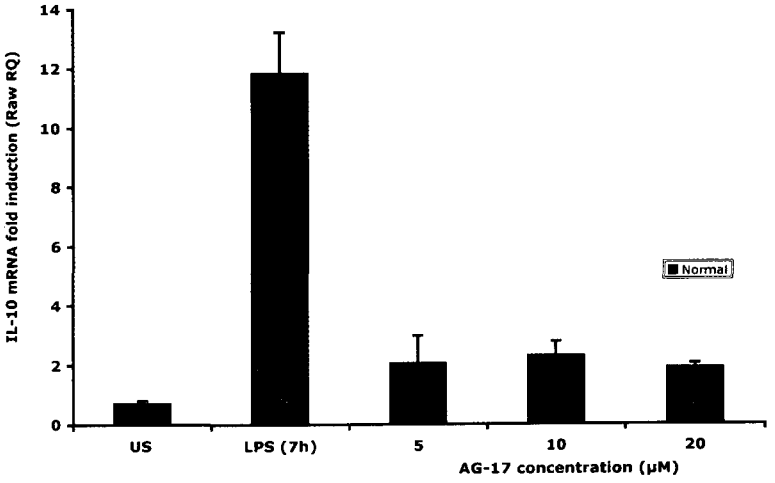
A


IL-10 production (pg/mL)
2500
2000
1500
1000
500
0
US
LPS
Pyk2 α-sense 5μM
IRR SQ 5μM
SLO only
Normal

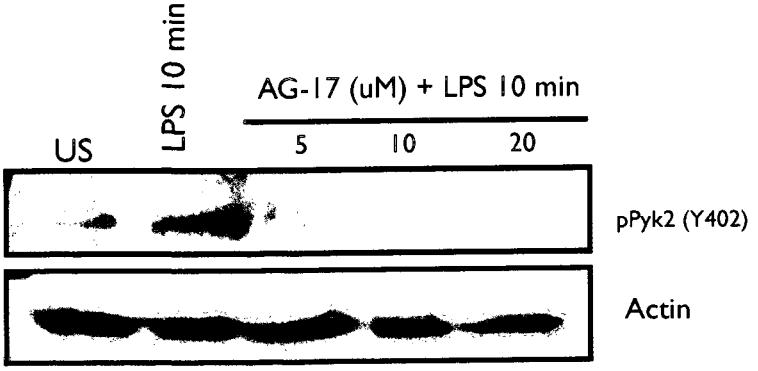
B


Untreated control
Pyk2 sense 5uM
Pyk2 anti-sense 2.5μM
Pyk2 anti-sense 5μM
Irrelevant Sequence 5μM
SLO control
Pyk2
Actin

C


IL-10 mRNA fold induction (Raw RQ)
14
12
10
8
6
4
2
0
US
LPS (7h)
5
10
20
AG-17 concentration (μM)
Normal

D


LPS 10 min
AG-17 (uM) + LPS 10 min
US
5
10
20
pPyk2 (Y402)
Actin

diminished in AG-17 treated cells compared to cells treated with LPS only (Fig III.30D), suggesting that AG-17 was functional as a Pyk2 inhibitor.

III.4.6 Role of Pyk2 and Src in LPS induced IL-10 production

In light of the findings that SHP-1, Src and Pyk2 all play a role in LPS-mediated IL-10 production and SHP-1 directly interacts with Pyk2 but not Src, it was of interest to understand the relationship between Pyk2 and Src in LPS signaling. There are few reports in literature describing Pyk2-Src interaction. For example, it was shown that Pyk2 and Src interaction is required for G-protein-coupled receptor(s) induced cytoskeletal rearrangements in monocytes [403, 404]. It was also shown that Pyk2 and Src interaction induced by G-protein coupled receptors does not involve activation of the MAPK pathway. However, there is no information on the interaction between Pyk2 and Src in LPS signaling or their interactive involvement in regulating IL-10 production.

(i) Src co-immunoprecipitates with Pyk2 in normal and me/me splenic macrophages

To understand the mechanism by which Src and Pyk2 might regulate SHP-1-dependent IL-10 production in LPS treated splenic macrophages, I examined Src and Pyk2 interaction by co-immunoprecipitation, using LPS treated normal and *me/me* splenic macrophages. For this, normal and *me/me* splenic macrophages were either not treated or treated with LPS for 5 to 30 mins and cell lysates were subjected to Src or Pyk2 immunoprecipitations followed by Western immunoblotting with the alternate antibody. Src immunoprecipitates in Fig III.31A and B, reveal that Pyk2 co-

precipitates with Src in both normal and *me/me* splenic macrophages. Similarly, results in Fig III.32A and B show that Src was present in Pyk2 immunoprecipitates in both normal and *me/me* splenic macrophages. Controls using antibody plus protein A Sepharose beads but no protein lysates were negative for the presence of either Src or Pyk2 (Lane 6 in Fig III.31 A, B, C and D and Fig III.32 A, B, C and D).

To control for loading and to ensure that Src or Pyk2 was successfully immunoprecipitated from total lysates, blots were stripped and re-probed with either anti-Src or anti-Pyk2 monoclonal antibodies, respectively. Results in Fig III.32C and D demonstrated that Src was present in Src immunoprecipitates in both normal and *me/me* splenic macrophages respectively. Equally, Fig III.31C and D show that Pyk2 was present in Pyk2 immunoprecipitates in both normal and *me/me* splenic macrophages.

III.4.7 The SHP-1-Pyk2-Src Tripartite

(i) Inhibition of SHP-1 or Pyk2 diminishes Src phosphorylation in LPS treated normal splenic macrophages.

Thus far, the results obtained provide evidence that LPS-induced IL-10 production in splenic macrophages is regulated by SHP-1. This regulation also involves two other signaling proteins, Src and Pyk2. The results also suggest that although SHP-1 directly interacts with Pyk2, its regulation of Src appears to be indirect. The evidence further suggests that Src and Pyk2 are intimate partners. To further delineate the relationships among the three proteins and to understand the mechanism by which they regulate LPS-induced IL-10 production in splenic macrophages, experiments were conducted to examine the role of Pyk2 and SHP-1 in

Fig III.31 Src co-immunoprecipitates with Pyk2 in LPS treated normal and *me/me* splenic macrophages

Serum starved normal splenic macrophages (approximately 2 X 10^7cells/mL per condition) were either untreated (US) or treated with LPS (10 μg/mL) for 5 - 30 min. Cell pellets were lysed and Pyk2 was immunoprecipitated from total protein lysates (1000 μg) using α-Pyk2 polyclonal Ab. Immunoprecipitation complexes were resolved on 8% SDS-PAGE gels followed by Western blot analysis using α-Src monoclonal Ab (A) normal splenic macrophages (B) *me/me* splenic macrophages. Membranes were stripped and reprobed using α-Pyk2 monoclonal Ab to control for loading (C) normal splenic macrophages (D) *me/me* splenic macrophages. To detect bands of interest, the following controls were included; Ab and beads but no lysate, lysates and beads but no Ab and total protein lysates from normal splenic macrophages. Red arrows indicate Src and Pyk2 bands. Figures shown are representative of two independent experiments showing similar results.

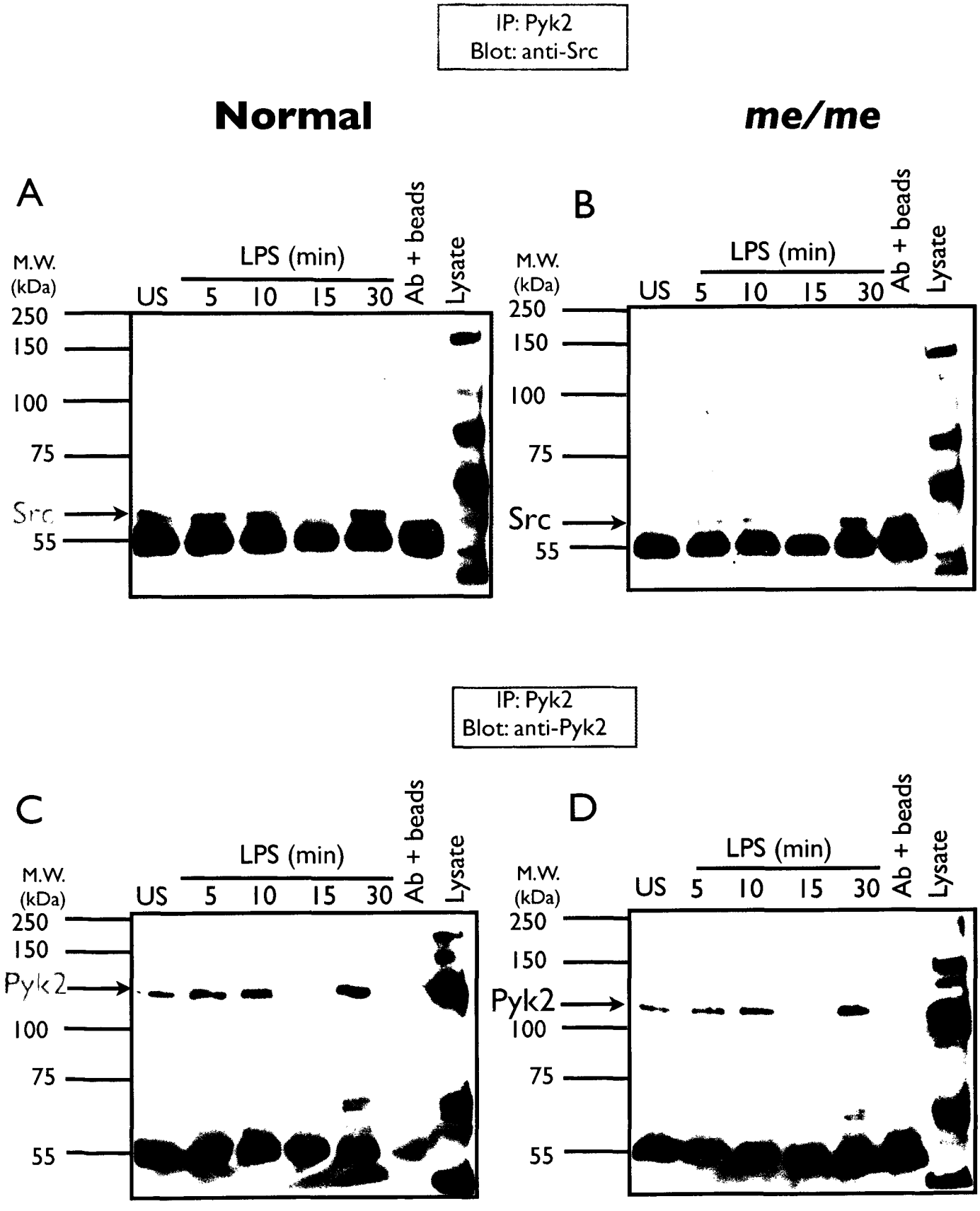
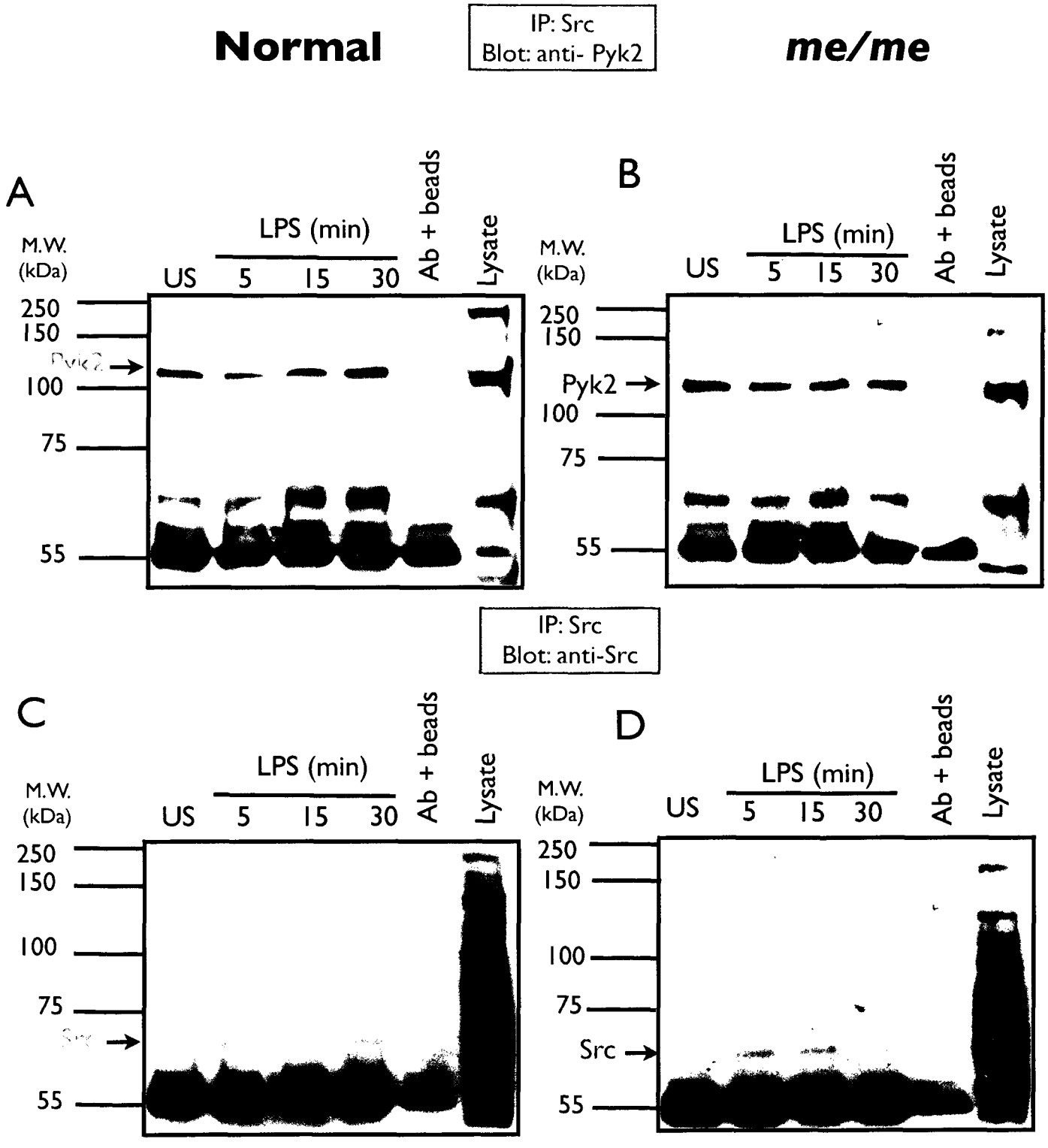

IP: Pyk2
Blot: anti-Src
Normal
me/me
A
B
M.W. (kDa)
LPS (min)
US
5
10
15
30
Ab + beads
Lysate
250
150
100
75
Src
55
IP: Pyk2
Blot: anti-Pyk2
C
D
Pyk2

Fig III.32 Pyk2 co-immunoprecipitates with Src in normal and *me/me* splenic macrophages

Serum starved normal splenic macrophages (approximately 2 X 10^7cells/mL per condition) were either untreated (US) or treated with LPS (10 μg/mL) for 5 - 30 min. Cell pellets were lysed and Src was immunoprecipitated from total protein lysates (1000 μg) using α-Src polyclonal Ab. Immunoprecipitation complexes were resolved on 8% SDS-PAGE gels followed by Western blot analysis using α-Pyk2 monoclonal Ab (A) normal splenic macrophages (B) *me/me* splenic macrophages. Membranes were stripped and reprobed using α-Src monoclonal Ab to control for loading (C) normal splenic macrophages (D) *me/me* splenic macrophages. To detect bands of interest, the following controls were included; Ab and beads but no lysate, lysates and beads but no Ab and total protein lysates from normal splenic macrophages. Figures shown are representative of two independent experiments showing similar results.


Normal
IP: Src
Blot: anti- Pyk2
me/me
A
B
M.W.
(kDa)
LPS (min)
US
5
15
30
Ab + beads
Lysate
250
150
100
75
55
Pyk2
IP: Src
Blot: anti-Src
C
D
Src

the activation of Src, a member of the Src family of kinases. To do this, SHP-1 or Pyk2 were inhibited in normal splenic macrophages using either PTP-I or AG-17, respectively. Splenic macrophages were treated with respective inhibitors for 2h prior to LPS treatment for 10 min. Lysates were subjected to Western immunoblotting and blots were probed with anti-phospho-Src monoclonal antibody. Results in Fig III.33A show that inhibition of SHP-1 with low concentrations (5 μM) of PTP-I diminished phosphorylation of Src. Likewise the inhibition of Pyk2 using low concentrations (5 μM) of AG-17 abrogated the phosphorylation of Src (Fig III.33B). The effect of AG-17 on Pyk2 phosphorylation was confirmed by, stripping respective blots and reprobing them with an anti-phospho-Pyk2 antibody (Fig III.33B middle panel). To control for protein loading, blots were stripped and re-probed with anti-actin antibody lower panels (Fig III.33A and B).

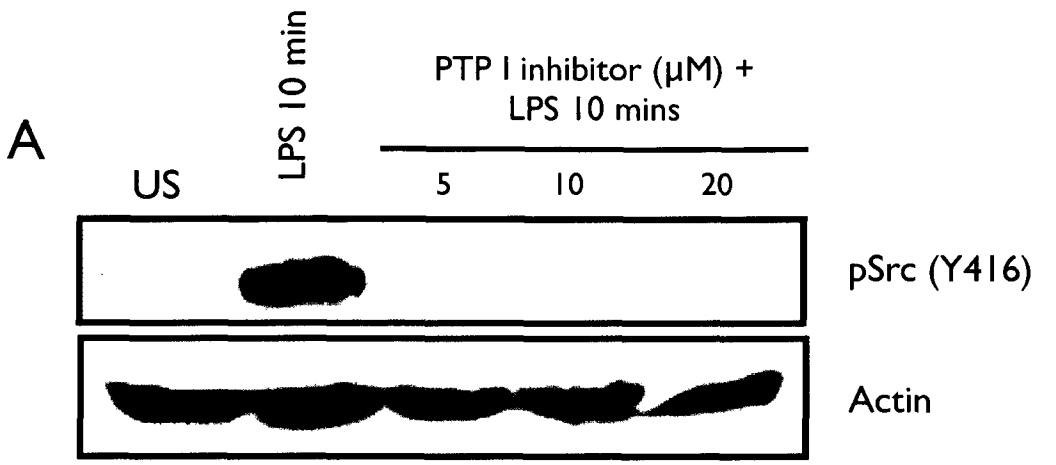
(ii) Src activity is diminished in Pyk2 immunoprecipitates from LPS-treated me/me splenic macrophages.

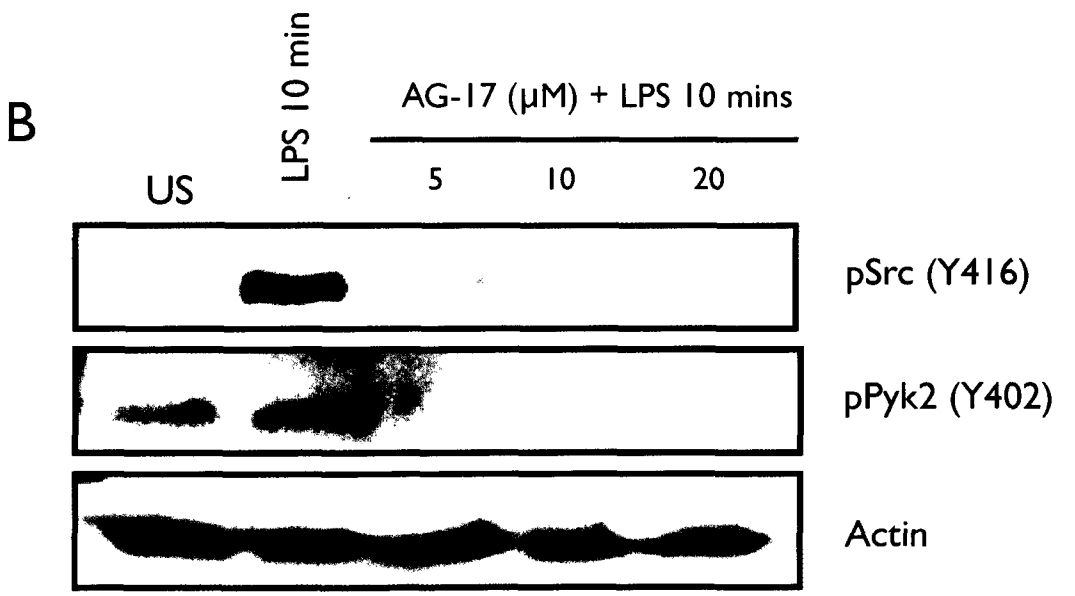
Pyk2 interacts with Src in both normal and *me/me* splenic macrophages and in normal splenic macrophages, Src phosphorylation appears to be dependent on Pyk2 phosphorylation (Fig III.33B). To understand this relationship, Src activity in Pyk2 immunoprecipitates derived from normal and *me/me* macrophages were compared. To achieve this, normal and *me/me* splenic macrophages were either untreated or treated with LPS for 5 to 30 min and resulting lysates were subjected to Pyk2 immunoprecipitation, then Src kinase activity was assayed in the immunoprecipitates. Results in Fig III.34A reveal an increase in Src kinase activity upon LPS treatment of normal splenic macrophages but not in LPS treated *me/me* splenic macrophages

Fig III.33 Inhibition of SHP-1 or Pyk-2 abrogates LPS-induced Src kinase phosphorylation in normal splenic macrophages.

(A) Serum starved normal splenic macrophages (approximately 1 X 10^7cells/mL per condition) were either untreated (US) or pretreated with PTP-1 (SHP-1 inhibitor) at indicated concentrations (5 – 20 μM) for 2 h followed by LPS treatment for 10 min. Cell pellets were lysed and total protein lysates (100 μg) were subjected to SDS-PAGE followed by Western blot analysis using α-phospho-Src (Y416) monoclonal Ab, specific to Src kinase (top panel). Membranes were stripped and reprobed using α-Actin Ab to control for equal loading (bottom panel). Results shown are representative of two independent experiments showing similar trends.

(B) Serum starved normal splenic macrophages (approximately 1 X 10^7cells/mL per condition) were either untreated (US) or pretreated with AG-17 (Pyk2 inhibitor) at indicated concentrations (5 – 20 μM) for 2 h followed by LPS treatment for 10 min. Cell pellets were lysed and total protein lysates (100 μg) were subjected to SDS-PAGE followed by Western blot analysis using α-phosphoSrc (Y416) monoclonal Ab, specific to Src member kinase (Top panel). Membranes were stripped and reprobed using α-phospho-Pyk2 (Y402) monoclonal Ab (middle panel) and α-Actin Ab to control for equal loading (bottom panel). Results shown are representative of two independent experiments showing similar trends.


A
US
LPS 10 min
PTP I inhibitor (μM) +
LPS 10 mins
5
10
20
pSrc (Y416)
Actin


B
US
LPS 10 min
AG-17 (μM) + LPS 10 mins
5
10
20
pSrc (Y416)
pPyk2 (Y402)
Actin

src kinase activity remains the same as in untreated controls. Therefore the result of this experiment suggests that Src activation is not dependent on the interaction between Pyk2 and Src as observed in *me/me* splenic macrophages (Fig III.31 and 32) but rather it may be dependent on the presence of SHP-1 in the complex.

III.4.8 LPS mediated IL-10 production is regulated by Src and P38 MAPK

(i) Src and P38 are both required for LPS-induced IL-10 production but are not interdependent.

Given that Src and P38 are both required for LPS-mediated IL-10 production in splenic macrophages, it was neccessary to determine if Src and P38 signaling pathways interact. To examine this, Src phosphorylation/activation was inhibited by treating normal splenic macrophages with increasing concentrations of the Src inhibitors PP2 or SU6656 for 2 h prior to LPS stimulation for 10 min. Lysates were subjected to Western immunoblotting and blots were probed using anti-phosphoP38 antibody. Results in Fig III.35A and B reveal unchanged phosphorylation of P38 in both PP2 and SU6656 treated normal splenic macrophages compared to LPS only controls. These findings indicate that optimal P38 phosphorylation is not Src dependent. This finding further supports earlier observations demonstrating that in LPS treated *me/me* splenic macrophages P38 phosphorylation remains the same as in normal controls but Src phosphorylation is diminished in LPS treated *me/me* splenic macrophages. These results therefore suggest that P38 and Src may be acting on divergent pathways and further suggest that Src but not the P38 pathway is SHP-1 dependent.

Fig III.34 Src activity is diminished in Pyk2 immunoprecipitates from LPS-treated *me/me* splenic macrophages.

(A) Serum starved normal (■) and *me/me* (□) splenic macrophages (approximately 2 X 10^7cells/mL per condition) were either untreated (US) or treated with LPS (10 μg/mL) for 5 - 30 min. Cell pellets were lysed and Pyk2 was immunoprecipitated from total protein lysates (1000 μg) using α-Pyk2 polyclonal Ab. To measure Src kinase activity, immunoprecipitates were subjected to fluorescent superquencing-based kinase assay. LPS treated samples were compared to untreated controls. A decline in relative fluorescence is indicative of an increase in Src kinase activity. Results shown are representative of three independent experiments showing similar results.

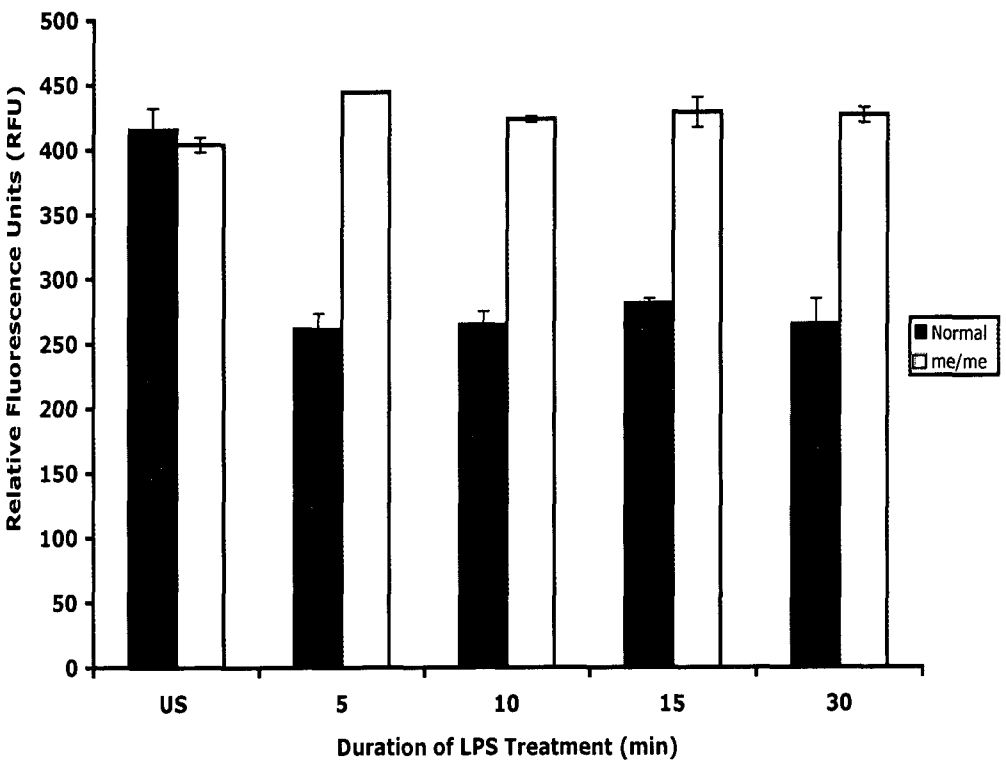
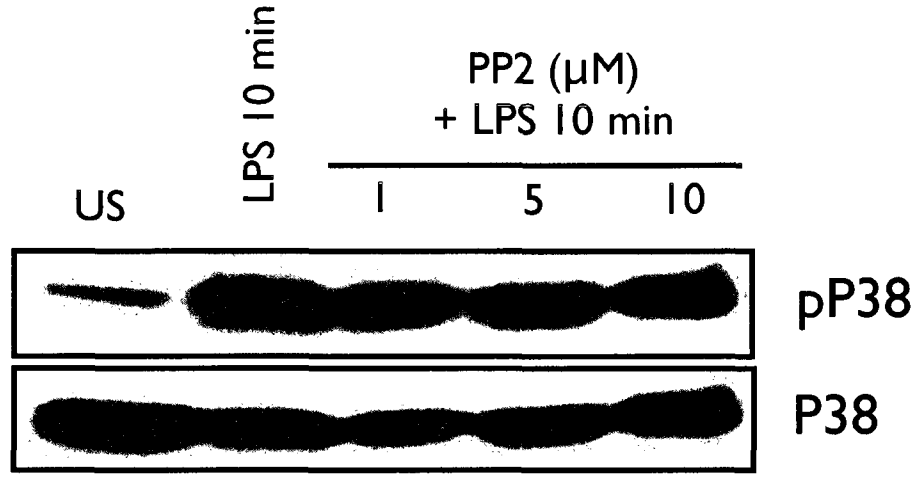

500
450
400
350
300
250
200
150
100
50
0
Relative Fluorescence Units (RFU)
US
5
10
15
30
Duration of LPS Treatment (min)
Normal
me/me

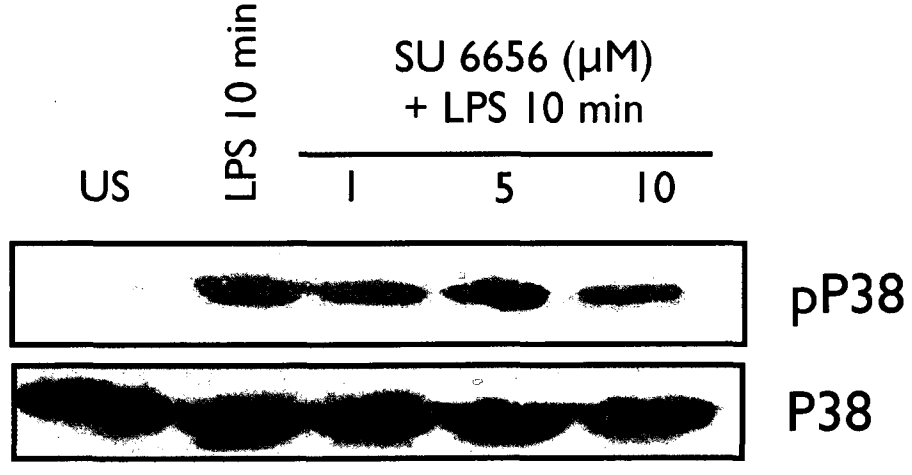
Fig III.35 P38 MAPK phosphorylation is not Src kinase dependent in normal splenic macrophages.

Serum starved normal splenic macrophages (approximately 1 X 10^7cells/mL per condition) were either untreated (US) or pretreated with Src inhibitors (A) PP2 (1 – 10 μM) or (B) SU6656 (1 – 10 μM) for 2 h followed by LPS treatment for 10 min. Cell pellets were lysed and total protein lysates (100 μg) were subjected SDS-PAGE followed by Western blot analysis using α-phosphoP38 polyclonal Ab. Membranes were stripped and reprobed using α-P38 polyclonal Ab as a control for equal loading. Results shown are representative of two independent experiments showing similar trends.

A


US
LPS 10 min
PP2 (μM)
+ LPS 10 min
1
5
10
pP38
P38

B


US
LPS 10 min
SU 6656 (μM)
+ LPS 10 min
1
5
10
pP38
P38

III.4.9 Summary of LPS-induced signal transduction pathway(s) required for IL-10 production in normal and *me/me* splenic macrophages.

Evaluation of the involvement of MAPK pathways in LPS stimulated splenic macrophages revealed that P38, ERK1/2 and JNK1/2/3 were similarly phosphorylated in both normal and SHP-1-deficient *me/me* splenic macrophages. Inhibition of ERK1/2 using PD98059 demonstrated that ERK1/2 is not involved in LPS-mediated IL-10 production in both normal and *me/me* splenic macrophages. In contrast, inhibition of P38 MAPK using SB203580 or SB202190 demonstrated a requirement for P38 in LPS-mediated IL-10 production in both normal and *me/me* splenic macrophages, therefore suggesting that P38 involvement in LPS-induced IL-10 production may not be dependent on SHP-1 function. This was further confirmed by showing that short and long-term phosphorylation of P38 in both normal and *me/me* splenic macrophages remained the same. Further investigation of PTK family members revealed that BTK was not involved in LPS-mediated IL-10 production. In contrast, inhibition of Src family kinases using PP2 showed a decline in IL-10 protein and mRNA induction in normal splenic macrophages but had no effect on IL-10 production in *me/me* splenic macrophages and thise correlated with lack of phosphorylation and activation of Src family kinases in LPS treated *me/me* splenic macrophages. Experiments where SHP-1 was inhibited by PTP-I showed the same reduced phosphophorylation of Src and confirmed that Src phosphorylation and activation is dependent on SHP-1. Evaluation of SHP-1 immunoprecipitates showed that Pyk2 co-immunoprecipitated with SHP-1 and this interaction appeared to be upregulated by LPS. Inhibition of Pyk2 using AG-17 diminished IL-10 mRNA

induction and Src phosphorylation, demonstrating a role for Pyk2 in Src dependent LPS-mediated IL-10 production in splenic macrophages. Inhibition of Src family kinases using SU6656 or PP2 had no effect on P38 phosphorylation revealing that divergent mechanisms are required for optimal LPS mediated IL-10 production.

IV. DISCUSSION

The LPS/TLR4 interaction is known to induce the tyrosine phosphorylation of numerous signaling proteins participating in various signaling cascades but thus far, the specific functions of these protein(s) have not been very well understood [15, 257, 268, 274-276, 279]. LPS has also been shown to elicit both pro-inflammatory and anti-inflammatory cytokine responses and although the functions of these cytokines have been the subject of intense studies, the mechanism(s) and the specific proteins involved in cascades leading to the production of anti-inflammatory cytokines are poorly understood [15, 273-275, 277, 278, 315]. Since the absence of SHP-1 in *me/me* mice has been associated with disruption in cytokine production [376, 405, 406], there is clearly a role for SHP-1 in LPS activated cytokine production.

The purpose of this study was to understand the role of SHP-1, in regulating both pro-inflammatory and anti-inflammatory cytokine production in a chronic inflammatory disease model. The results presented in this thesis support the hypothesis and show for the first time that in LPS-mediated signaling, SHP-1 acts both as a negative regulator of TNF-α and a positive regulator of IL-10 production. In addition, the studies in this thesis examine the relationship between SHP-1 and LPS-induced IL-10 production and reveal that LPS-induced IL-10 production in murine splenic macrophages is dependent on two distinct pathways: one which engages P38 MAPK and is SHP-1 independent and the other which is distinct from the P38 MAPK pathway and is dependent on SHP-1. Both pathways are required for efficient LPS-mediated IL-10 production.

SHP-1-deficient *me/me* mice are typified by poorly controlled activation and

expansion of hematopoietic cells [360, 366, 368, 369]. Chronic inflammatory pathologies observed in *me/me* mice are enhanced by the production of abnormally high and persistent levels of autoantibodies and pro-inflammatory cytokines that have been observed in sera of *me/me* mice [317, 364, 365, 376]. This study utilizes a relatively pure (>90%) splenic macrophage population to demonstrate dual and opposing roles for SHP-1 in regulating LPS-induced cytokine production in splenic macrophages. The findings from this study show that LPS mediated TNF-α production is persistently elevated in *me/me* splenic macrophages. It is noteworthy to point out that significant quantities of TNF-α, albeit at much lower levels compared to LPS treated macrophages, were also detected in untreated *me/me* splenic macrophages. These results parallel earlier findings reporting elevated levels of TNF-α in sera of *me/me* mice and in microglia cells, even under sterile conditions [376, 405]. The findings contradict reports suggesting that *me/me* splenocytes lack the ability to utilize LPS or stimuli-delivered signal [407]. Futhermore, these findings also significantly differ from other studies that have demonstrated elevated LPS-mediated TNF-α production in *me/me* mice sera [408] and microglia cells [405] but not in tissue macrophages such as alveoli or splenic macrophages that have been consistently implicated in *me/me* pathology [360, 369]. My results demonstrate that the impaired production of TNF-α in *me/me* splenic macrophages was not a function of LPS dose and further suggests that tissue macrophages may be an important source of TNF-α found in *me/me* mice sera. In addition, elevated levels of the murine neutrophil chemokine, MIP-2 were seen in both LPS and TNF-α treated *me/me* splenic macrophages. TNF-α induction of MIP-2 in *me/me* splenic macrophages

plausibly justifies enhanced and persistent migration of myeloid cells to sites of inflammation and suggests a likely link between the observed aberrant migratory patterns of neutrophils and persistent levels of TNF-α in *me/me* mice.

The time dependent quantification of TNF-α mRNA in *me/me* mice has not been previously examined in other studies. The examination of TNF-α mRNA did not categorically discount the possibility that increased levels of TNF-α protein in *me/me* splenic macrophages are a consequence of increased post-transcriptional processing of TNF-α message. However, studies showing that SHP-1 influences NF-κB [409, 410], a key transcription factor, required for TNF-α gene activation in macrophages suggest that the loss of SHP-1 unlikely results in increased TNF-α mRNA stability or its posttranscriptional modification but rather the results suggest that an increased production of TNF-α protein is a consequence of aberrant TNF-α gene expression. Since *me/me* splenic macrophages lack SHP-1 function, it is reasonable to postulate that SHP-1 regulates TNF-α gene expression by assuming a negative regulatory role in LPS signal transduction pathways leading not only to TNF-α but also to MIP-2 production.

The relationship between SHP-1 and the kinetics of TNF-α production has been investigated in microglia cells treated with LPS and implied that the loss of SHP-1 results in increased TNF-α production in LPS treated microglia cells [405]. Another similar study by Forget G. *et al.* also demonstrated a role for SHP-1 in regulating pro-inflammatory response including TNF-α production induced by Leishmania infection [411]. Recently, a study by Hardin A.O. *et al.* showed that SHP-1 inhibits LPS mediated TNF-α and iNOS (inducible Nitric Oxide Synthase) production in

RAW264.7 cells, a murine macrophage cell line, but not in primary cells [412]. The findings in this study examining the relationship between SHP-1 and TNF-α regulation support a growing consensus that SHP-1 is required to regulate TNF-α production in all cell types irrespective of the stimulus used.

A common and major limitation of some previous studies on this subject was the inability to delineate the precise mechanisms by which SHP-1 regulates TNF-α production. NF-κB is a well-studied transcription factor for TNF-α production and has been shown to be regulated by SHP-1 [409]. Increased NF-κB activation has also been demonstrated in the absence of SHP-1 [410]. It is therefore reasonable to speculate that SHP-1 regulation of TNF-α may be dependent on its negative regulation of NF-κB activation.

A previous study by Xiao Su et al showed that treatment of *me/me* mice with soluble TNFR or TNF binding protein (TBP) blocked the TNF/TNFR pathway and consequently ameliorated most inflammatory pathologies associated with *me/me* mice including pneumonitis, arthritis and alopecia [376]. Although the production of autoantibodies were not inhibited by sequestering TNF-α activity, the life span of *me/me* mice were prolonged from 2 to 6 weeks suggesting that TNF-α was central to the *me/me* pathology, thus highlighting the importance of an anti-TNF-α therapy.

The inability of *me/me* mice to self-resolve these inflammatory pathologies may conceivably be linked to their inability to produce anti-inflammatory cytokines in sufficient amounts. The observation of elevated, unregulated and persistent levels of TNF-α in *me/me* splenic macrophages prompted the evaluation of the natural inhibitor of TNF-α biosynthesis, IL-10, in *me/me* splenic macrophages. Profiling IL-

10 in LPS treated and untreated *me/me* splenic macrophages revealed that LPS induced IL-10 production was significantly reduced (>50%) in *me/me* splenic macrophages compared to normal controls. The possibility that the observed low levels of IL-10 in the absence of SHP-1 may be a function of LPS dose or duration of treatment was addressed by examining the dose response and kinetics of IL-10 production in *me/me* splenic macrophages. Results showed that the low levels of IL-10 production in *me/me* splenic macrophages were not dependent on LPS dose or duration of stimulation. In previous studies, Jie Zhao *et al.* investigated the production of IL-10 in *me/me* microglia cells following cochlear ablation in murine hindbrain and had noted marginally lower IL-10 production in these cells [406]. Although our study and that of Jie Zhao *et al.* [406] both show that IL-10 production is diminished in *me/me* mice, these studies markedly differ in the type of cells utilized, the ligand used to initiate activation, the timing of cytokine measurement and the magnitude of IL-10 decline. In this study, use of LPS, a potent ligand of TLR4, compared to ablation-induced activation [406], may partly explain the significant quantitative differences in IL-10 production in *me/me* macrophages observed in both systems. In contrast, a study by Khaled A.R. *et al.* [408] reported increased levels of IL-6, INF-γ, TNF-α and IL-10 in the sera of me^{v}/me^{v} mice and in activated T and B cells isolated from me^{v}/me^{v} mice. This prompted the examination of IL-10 mRNA levels in *me/me* splenic macrophages to ensure that message stability was not affected by the complete loss of SHP-1. SQ-RTPCR results evaluating IL-10 mRNA levels confirmed our earlier findings demonstrating diminished IL-10 message, thus contradicting the findings of Khaled A.R. *et al.* [408] and suggesting that SHP-1

impacts on LPS-induced IL-10 gene expression. Possible explanations for the discrepancy with the findings of Khaled *et al.* [408] include, but are not limited to, differences in cell types, purity of cell population, differences in signaling cascades induced by LPS treatment of B cells and CD3 stimulation of T cells compared to LPS treatment in macrophages and measurement of spontaneous cytokine production in mice sera. In addition, the residual 20% expression of SHP-1 in me^v/me^v mice [363] may account for some differences in cytokine production levels. Since macrophages are the dominant source of cytokines and since myeloid cells are the cells most affected by the loss of SHP-1, aberrant cytokine production, specifically in macrophages, may result in more severe outcomes in *me/me* pathology than aberrant cytokine production by other cell types and, therefore, represent the focus of this study.

A comparison of the kinetic profiles of IL-10 show that the amount of IL-10 produced in *me/me* splenic macrophages remained constitutively low contrasting with a robust production of IL-10 between 6 to 72 h post stimulation in normal splenic macrophages. Conversely, the kinetic profiles of TNF-α production in normal and *me/me* splenic macrophages after LPS exposure, revealed similar levels of TNF-α produced in both normal and *me/me* splenic macrophages at the 6 hr time point. In normal splenic macrophages, TNF-α production declined with an increase in time whereas in *me/me* splenic macrophages TNF-α levels increased and remained steady as time elapsed. Therefore, the kinetic profiles of TNF-α and IL-10 support the idea that the elevated and persistent levels of TNF-α may be a consequence of poor IL-10 production in *me/me* mice. Two possible explanations can be envisaged for the

incessant elevated levels of TNF-α in LPS stimulated *me/me* splenic macrophages. The levels of IL-10 produced in *me/me* splenic macrophages are either below the threshold required to suppress TNF-α overproduction in *me/me* splenic macrophages or *me/me* splenic macrophages are unresponsive to IL-10-induced signals, resulting in an inability to mediate the effects of IL-10, including suppressing TNF-α production. Exogenous treatment of *me/me* splenic macrophages with recombinant murine IL-10 showed suppression of both TNF-α and MIP-2 levels comparable to that of normal splenic macrophages, suggesting that IL-10 signal transduction mechanisms in *me/me* splenic macrophages are intact and may not be dependent on SHP-1 in mediating anti-TNF-α activities. Furthermore, the induction of increased levels of MIP-2 by TNF-α in *me/me* splenic macrophages indicates that these elevated levels of MIP-2 in LPS treated *me/me* splenic macrophages may directly stem from increased and sustained TNF-α production. Recent studies by Berlato *et al.* [413] and Qasimi *et al.* [414] imply that IL-10 mediated signaling specifically resulting in the inhibition of TNF-α requires Suppressor of Cytokine Signaling-3 (SOCS3). However, it remains unclear if SHP-1 regulates SOCS3 in an IL-10 mediated signaling context, making it difficult to predict the role SHP-1 assumes in IL-10 activated signaling pathways.

Multiple studies show that TNF-α is produced very early upon LPS stimulation and may trigger the late phase production of anti-inflammatory cytokines [45, 120, 146, 152, 157]. Interestingly, treatment of both normal and *me/me* splenic macrophages with TNF-α was unable to trigger the production of IL-10 in the absence of LPS (data not shown). This result is supported by a previous observation also demonstrating that TNF-α is unable to induce IL-10 production in macrophages

but does so in dendritic cells [131]. This highlights possible differences in the mechanism underlying IL-10 induction among myeloid cells and may partly explain why persistent levels of TNF-α in *me/me* mice sera are unable to trigger and mount an effective anti-inflammatory response aimed at resolving incessant TNF-α production.

Using a SHP-1 null murine model, this study suggests that SHP-1 differentially regulates LPS mediated production of MIP-2, TNF-α and IL-10 and further demonstrates that persistent and elevated levels of TNF-α are consequences of diminished levels of IL-10. The involvement and requirement for SHP-1 in LPS induced IL-10 production was demonstrated using two different approaches: 1) Intereference with SHP-1 expression using antisense oligonucleotides in normal splenic macrophages resulted in decreased IL-10 production and, 2) the reconstitution of functional SHP-1 activity in *me/me* splenic macrophages with SHP-1 gene using adenoviral vectors restored IL-10 production. Results showing a decline in IL-10 production in normal splenic macrophages transduced with adenoviral vectors expressing a dominant negative mutant of the SHP-1 gene, lacking a catalytically active phosphatase domain but expressing functional SH2 domains, demonstrated a requirement for SHP-1 binding and activity in LPS-mediated IL-10 production. This was in agreement with IL-10 gene expression studies demonstrating that the loss of SHP-1 impacts on IL-10 mRNA production. These results suggest that the LPS activated signal transduction pathway leading to IL-10 gene expression is controlled in a positive manner by SHP-1.

Since this study revealed a novel function of SHP-1 as a positive regulator of

IL-10 production, it was of interest to characterize the involvement of SHP-1 in LPS induced signaling pathways leading to IL-10 production. To date, the transcription factors Sp1, Sp3, AP-1 and STAT3 have been implicated in IL-10 production by monocytes and macrophages [390, 415-417]. Tone M. *et al.* [417] report a requirement for Sp-1 in LPS mediated IL-10 gene expression but failed to demonstrate an increase in Sp-1 or Sp-3 activation upon LPS treatment in human macrophages. The study further implies that the regulation of LPS-mediated IL-10 gene expression at the transcription factor level is not exclusively dependent on Sp-1 and Sp-3. The MAPK kinase, P38, has also been implicated in the regulation of IL-10 production via Sp-1 [390, 415]. It was therefore of interest to determine if P38 contributes to the observed suppressed levels of IL-10 in SHP-1 null *me/me* splenic macrophages. Splenic macrophages were treated with pharmacological inhibitors specific to distinct MAPK signaling pathways followed by LPS stimulation. The results using these approaches revealed that ERK1/2 MAPK inhibition did not alter IL-10 production even in the absence of SHP-1 but P38 inhibition significantly affected IL-10 production in both normal and *me/me* splenic macrophages thus supporting earlier reports showing that P38 MAPK is required for LPS induced IL-10 production in human monocytes [415]. The fact that inhibition of P38 MAPK exerted similar effects on IL-10 production in both normal and *me/me* splenic macrophages, suggest that the reduced (residual) levels of IL-10 produced in LPS treated *me/me* splenic macrophages are dependent on P38 MAPK. Therefore, P38 MAPK may not be exerting its effect on IL-10 production in a SHP-1-dependent manner, at least not in splenic macrophages.

Since both SHP-1 and P38 regulate LPS-mediated IL-10 production, it was of interest to determine if the loss or pharmacological inhibition of SHP-1 altered P38 activation. For this, the phosphorylation kinetics of specific MAPK family members in LPS treated normal and *me/me* splenic macrophages were examined. Interestingly, the loss of SHP-1 did not negatively affect the phosphorylation of ERK, JNK and P38 MAP kinases. Extension of LPS treatment time points reflecting long term treatment with LPS yielded similar outcomes. Collectively, these results suggest that SHP-1 likely regulates LPS-induced IL-10 production in a P38-independent manner. Both MyD88-dependent and MyD88-independent cascades are involved in LPS-mediated activation of P38 [274, 275, 277]. The exact mechanisms and signaling proteins mediating P38 activation in an LPS context are not clear, and are beyond the scope of this study.

Studies on LPS/TLR4 signaling have been devoted to understanding the MyD88-dependent and MyD88-independent pathway and little emphasis has been placed on understanding the role of PTKs in LPS signaling. Both SHP-1 and SHP-2 are unique among classical PTPs due to the expression of SH2 domains [323]. The modular nature of the SH2-domain enables the targeting of specific substrates, particularly tyrosine phosphorylated substrates [323-325]. The requirement for tyrosine phosphorylation and SHP-1-SH2 domain activity in LPS-mediated IL-10 production is supported by results from competitive binding experiments in normal splenic macrophages using adenoviral vectors containing SHP-1-DN mutant expressing only SH2 domains of SHP-1. A requirement for tyrosine phosphorylation invariably implicates a direct role for PTKs in LPS-mediated IL-10 production.

Interactions between SHP-1 and PTKs in diverse signaling cascades have been well studied [331, 356]. For example, SHP-1 has been shown to interact with Lck [418], and Lck is expressed in T cells, c-kit found in hematopoietic precursor cells [340], IL-3 receptor beta in myeloid cells [419] and EGF receptor in epithelial cells [338]. A limited number of studies have reported LPS activation of PTKs and their roles in cytokine production [281, 282, 309, 316]. An extensive literature review revealed three families of NRTKs that are activated by LPS. These include; Btk, a member of the tec family of NRTKs, Src family of NRTKs and FAK family of NRTKs. These NRTKs have been reported to interact with SHP-1 in various systems, to be expressed in macrophages [298, 304, 306] and to participate in cytokine production [296]. For example, Btk has been shown to play a role in LPS-mediated TNF-α production but not in IL-6 production [420]. So far, there has been only a limited speculation on possible involvement of PTKs in LPS mediated IL-10 production.

Although most studies demonstrate activation of Btk in LPS signaling [303, 312, 421] a single conflicting report by Perez de Diego R. *et al.* [422] suggest that Btk is not required in LPS-induced activation of human monocytes. Pharmacological inhibition of Btk in LPS treated macrophages in this study showed that Btk is not required for IL-10 production in murine splenic macrophages. This finding contradicts a single report by Schmidt N.W *et al.* [423], which suggested that Btk was required for IL-10 production in murine primary bone marrow derived macrophages (BMDM). These differences may be attributed to differences in cell types being examined (splenic macrophages versus BMDM) and the level of cell purity (< 80% were true macrophages according to flow data). In support of this study and further

contradicting the findings of Schmidt N.W. *et al.* [423], X-linked agammaglobulinemia (XLA) patients with significant mutations in the Btk gene produce sufficient levels of IL-10 [422, 424, 425].

There are multiple lines of evidence that demonstrate the involvement of Src family kinases in LPS signaling [282, 309, 426-429] where they have been implicated in the regulation of TNF-α and nitric oxide production in murine macrophages [392, 428]. Therefore, Src may play a crucial role in LPS signaling cascades that lead to cytokine production. Expression of SH2 domain modules is a distinguishing feature of the Src family kinases [297, 306, 307, 325], a characteristic shared also with SHP-1 and SHP-2 [323]. Tyrosine phosphorylation of Src family kinases enhances SH2 domain binding specificity and Src kinase activity [396]. Although both Src family kinases and SHP-1 are capable of binding phosphotyrosine proteins through their SH2 domains, they manifest opposing outcomes [325].

In the present study, pharmacological inhibition of Src family kinases in LPS treated splenic macrophages showed a requirement for Src family kinases in LPS-mediated IL-10 production in normal splenic macrophages. Examination of IL-10 mRNA in normal splenic macrophages pretreated with the Src inhibitor, PP2, also revealed similar results, supporting the notion that Src plays a role in LPS-mediated signaling cascade leading to IL-10 production. This observation is in agreement with a single study by Smolinska M.J. *et al.* [426] suggesting that inhibition of Src family kinase with PP2, inhibits IL-10 production but contradicts the study of Meng F. *et al.* [311] reporting that Src family kinases do not play significant roles in LPS-induced signaling in Hck/Fgr/Lyn triple knockout murine macrophages. A possible

explanation for this discrepancy may be the use of triple knockouts of the src family kinases; Hck, Fgr, and Lyn but not Src itself [311]. I speculate that specifically Src, but not Hck, Fgr or Lyn, may be responsible for mediating the effects of LPS that result in IL-10 production in splenic macrophages. Pharmacological inhibition of Src family kinases did not impact on either IL-10 protein or mRNA in LPS treated *me/me* splenic macrophages. *Me/me* splenic macrophages showed poor Src family kinase phosphorylation in the first 60 min of treatment with LPS. In addition, pharmacological inhibition of SHP-1 in LPS treated normal splenic macrophages also showed poor phosphorylation of Src. Results of this study clearly depict a requirement for SHP-1 in Src phosphorylation in LPS treated splenic macrophages and differ significantly with the findings of Smolinska M.J. *et al.* [426]. The observation that Src activation may be dependent on SHP-1 is not novel and has been described by Somani A.K. *et al.* [397] in human platelets and Jurkat T cells. Poor activation of Src in SHP-1 deficient thymocytes has been demonstrated by Somani A. K. *et al.* [397] but the outcome of SHP-1-dependent activation and the mechanism underlying SHP-1-dependent activation of Src remain unknown. The quest to understand the mechanism(s) governing SHP-1-dependent Src activation inspired studies to demonstrate direct interaction between SHP-1 and Src. Although, Somani A.K. *et al.* showed physical interaction of SHP-1 and Src in platelets [397], attempts, using immunoprecipitation experiments, to demonstrate a direct interaction between SHP-1 and Src in LPS treated normal splenic macrophages were unsuccessful. This lack of physical interaction of SHP-1 and Src in LPS activated splenic macrophages could not be attributed to technical difficulties since a direct interaction between

SHP-1 and Src was demonstrated in EL4 mouse thymoma cells. Although SHP-1 and Src can directly interact in human platelets and murine thymocytes [397, 430], LPS activated murine splenic macrophages appear unable to support such an intimate interaction. These differences may be ascribed to the use of different ligands, LPS compared to IL-2/thrombin, which engage distinct signaling pathways and signaling components in splenic macrophages compared to thymocytes/platelets and Chinese hamster ovary cells [397, 430]. However, Yamamoto Y. *et al.* [431] and Somani A.K. *et al.* [397] have demonstrated a positive regulation of Src kinase by SHP-1 in two different systems and these findings are consistent with our results. Both, Somani *et al.* [397] and Yamamoto *et al.* [431] suggest that SHP-1 facilitates the activation of Src kinase by dephosphorylating tyrosine residue 529 (Y529) (in mice, corresponding to Y530 in humans). Y529 is located in the C-terminal tail of Src and its phosphorylation by Csk (c-terminal src kinase) generates a binding site for *N*-terminus located SH2 domain leading to intramolecular rearrangement and sequestration of the Src kinase activation site consequently limiting kinase enzymatic activity [395]. The dephosphorylation of Src on Y529 is therefore essential to expose the kinase domain and auto-phosphorylation on tyrosine 416 for maximum enzyme activity [394].

The inability to demonstrate an interaction between SHP-1 and Src in normal splenic macrophages may be due to the nature of the interaction between SHP-1 and Src in our model system. I speculated that the interaction between SHP-1 and Src may either be indirect or of a transient nature.

Anand A.R. *et al.* [316] reported a role for the proline-rich tyrosine kinase

(Pyk2) in mediating LPS-induced IL-8 production in human endothelial cells. In addition, reports by William L.M. *et al.* [314] and Hazeki K. *et al.* [315] both described activation of Pyk2 in LPS treated murine monocytes and macrophages and further demonstrated a role for Src in tyrosine phosphorylation of Pyk2. Pharmacological inhibition of Pyk2 in LPS treated splenic macrophages resulted in suppression of IL-10 mRNA, suggesting a requirement for Pyk2 in LPS-mediated IL-10 production. In addition, inhibition of Pyk2 expression by antisense oligonucleotides also revealed a requirement for Pyk2 in IL-10 production. This appears to be a novel observation that has not been demonstrated previously. In support of this, my results show that Pyk2 coimmunoprecipitates with SHP-1 in normal splenic macrophages, and that LPS stimulation enhances this interaction. These results suggest that SHP-1 and Pyk2 form an LPS-induced complex in murine splenic macrophages. Adhesion-regulated multiprotein complexes involving SHP-1 and Pyk2 in macrophages have also been reported by Timms J.F. *et al.* [432] and my findings are in agreement with this report. Analyses of Pyk2 immunoprecipitates from untreated and LPS treated normal splenic macrophages also revealed the presence of Src kinase in Pyk2 immunopreciptates. Interestingly, the interaction between Src and Pyk2 appeared to be constitutive and was similar in both normal and *me/me* splenic macrophages. I envisage that this interaction may occur by binding of the SH3 domain of Src to the proline rich domains of Pyk2. The discovery of distinct interactions between Pyk2 and Src and also between SHP-1 and Pyk2 suggest that the mechanism governing SHP-1-mediated Src activation may be transient or indirect requiring the presence of Pyk2, possibly as a linker or adapter molecule.

Furthermore, pharmacological inhibition of either Pyk2 or SHP-1 in LPS treated normal splenic macrophages resulted in poor tyrosine phosphorylation of Src kinase, strongly suggesting a requirement for both SHP-1 and Pyk2 in Src activation. In line with these observations, Schauweinold D. *et al.* [433] described a requirement for Pyk2 in Src activation in thrombin treated vascular smooth muscle cells. This study further examined Src activity in Pyk2 immunoprecipitates, of both normal and *me/me* splenic macrophages and observed poor Src kinase activity in *me/me* but not normal Pyk2 immunoprecipitates indicating a requirement for SHP-1 in this complex for efficient LPS-mediated Src activation. Based on these results, it is logical to speculate that Pyk2 likely acts as a platform that independently recruits Src kinase and SHP-1. The proximity of both Src and SHP-1 may lead to the swift dephosphorylation of the inhibitory Y527 on Src, facilitating intramolecular rearrangement leading to complete activation of Src and mediating IL-10 production.

Using specific pharmacological agents aimed at inhibiting activation of either Src or p38 MAPK delineated two signal transduction pathways that regulate LPS-induced IL-10 production in splenic macrophages. The activation of Src and its requirement in LPS-induced IL-10 production was distinct but additive to the already described LPS-induced p38 MAPK pathway leading to IL-10 production. These approaches clearly demonstrated that inhibition of Src did not have any effect on LPS induced p38 MAPK phosphorylation and further strengthen the earlier findings of this study demonstrating that SHP-1 loss or SHP-1 inhibition had no effect on LPS-mediated p38 phosphorylation. This observation is in agreement with the findings of Smolinska M.J. *et al.* [426] that report no effect on p38 MAPK phosphorylation in

Src-inhibited, LPS-treated PBMC-derived primary human macrophages. It is therefore conceivable that, although P38 is required for LPS-mediated IL-10 production, a second pathway involving Src, Pyk2 and SHP-1 is responsible for approximately 50% of IL-10 production in murine splenic macrophages. Since the inhibition of p38 MAPK completely abolished LPS-mediated IL-10 production, I speculate that the pathway involving P38 engagement of the transcription factor, Specificity protein 1 (Sp1), may represent a first signal engaged by LPS to mediate IL-10 production in splenic macrophages. To prevent false induction of genes and to tightly control physiological processes, biological systems may have an inbuilt safety mechanism that is sometimes referred to as "two-hit signal" mechanism [434-438]. The second pathway engaging the Src-Pyk2-SHP-1 tripartite may therefore represent a second pathway or a "second hit" that confirms the first signal and initiates efficient and optimal IL-10 production. Although this study did not examine possible transcription factors involved in the second pathway, it is illogical to suggest that Sp1 would mediate the effects resulting from the Pyk2-Src-SHP-1 tripartite, since P38 directly controls Sp1 transcription leading to IL-10 [415]. We envisage that Signal Transducer and activator of Transcription 3 (STAT3), a transcription factor that has been implicated in both LPS signaling and IL-10 production may play a significant role in this pathway [416, 439-441] and would be interesting to explore further.

Mice deficient in IL-10 are characterized by gastro intestinal inflammatory disorders [442] such as inflammatory bowel disease/chronic colitis [235, 443]. Chronic inflammatory diseases resembling arthritis and pneumonitis have been reported in *me/me* mice [360, 365, 368, 369, 372, 374, 376]. Although these

disorders arise as a result of excessive and unregulated TNF-α and other pro-inflammatory cytokine production, this study strongly suggest that excessive TNF-α pro-inflammatory activity may be a direct consequence of inadequate IL-10 production. Numerous studies have repeatedly demonstrated major roles for IL-10 in protecting hosts from harmful effects that may be triggered by intense immune-inflammatory responses [235, 429, 442-445].

IV.1 CONCLUDING REMARKS

Using a unique moth-eaten mouse model system, this study describes, for the first time, a complex mechanism involving two independent signal transduction pathways essential for LPS-mediated IL-10 production in splenic macrophages. Data presented in this study demonstrate that in pathway 1, LPS induced IL-10 production is mediated through p38 MAPK activation and this pathway is SHP-1 independent (Fig IV.1B). This study further reveals, for the first time, a second critical pathway involving a Src-Pyk2-SHP-1 tripartite (Fig IV.1A). In this tripartite, Pyk2 acts as an adaptor molecule that links SHP-1 and Src, where SHP-1 is required for swift activation of Src. In *me/me* splenic macrophages, loss of SHP-1 from the tripartite complex results in poor activation of Src and ultimately in severe suppression of IL-10 production (Fig IV.2). Hence, the timely and efficient activation of Src by SHP-1

Fig IV.1 Models showing the two pathways that regulate LPS-induced IL-10 production in splenic macrophages.

The models shown here illustrate the two pathways that regulate LPS-mediated IL-10 production as demonstrated in the results section. Model (A) shows a SHP-1 dependent pathway requiring the SHP-1-dependent activation of Src kinase for IL-10 production as observed in normal splenic macrophages. Model (B) describes a SHP-1 independent pathway that does not require the activation of Src or expression of SHP-1. Instead, it relies on P38 MAPK expression, phosphorylation and activation for IL-10 production. This SHP-1 independent pathway is active in *me/me* splenic macrophages and is likely responsible for residual IL-10 production observed in LPS treated *me/me* splenic macrophages.

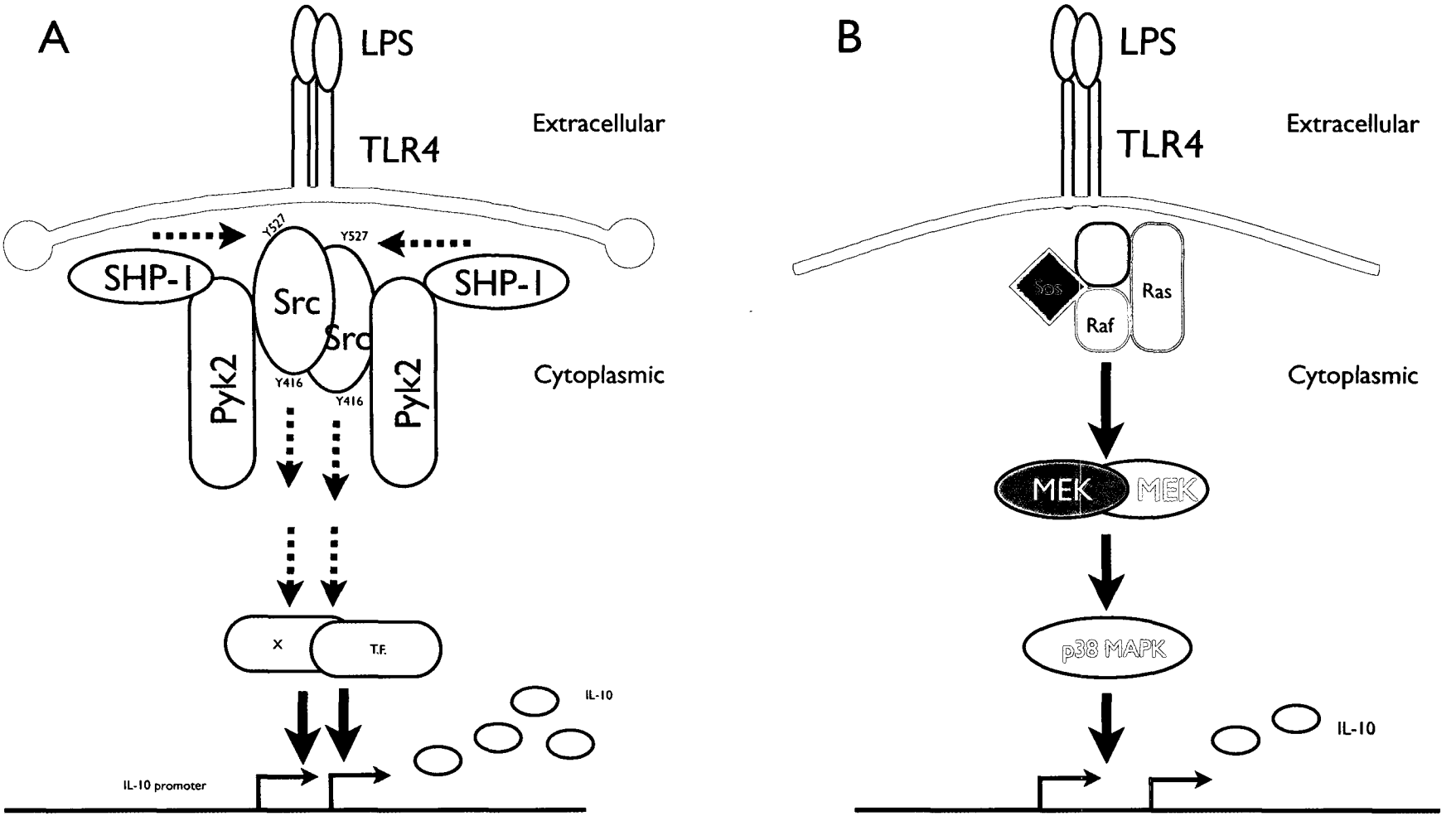
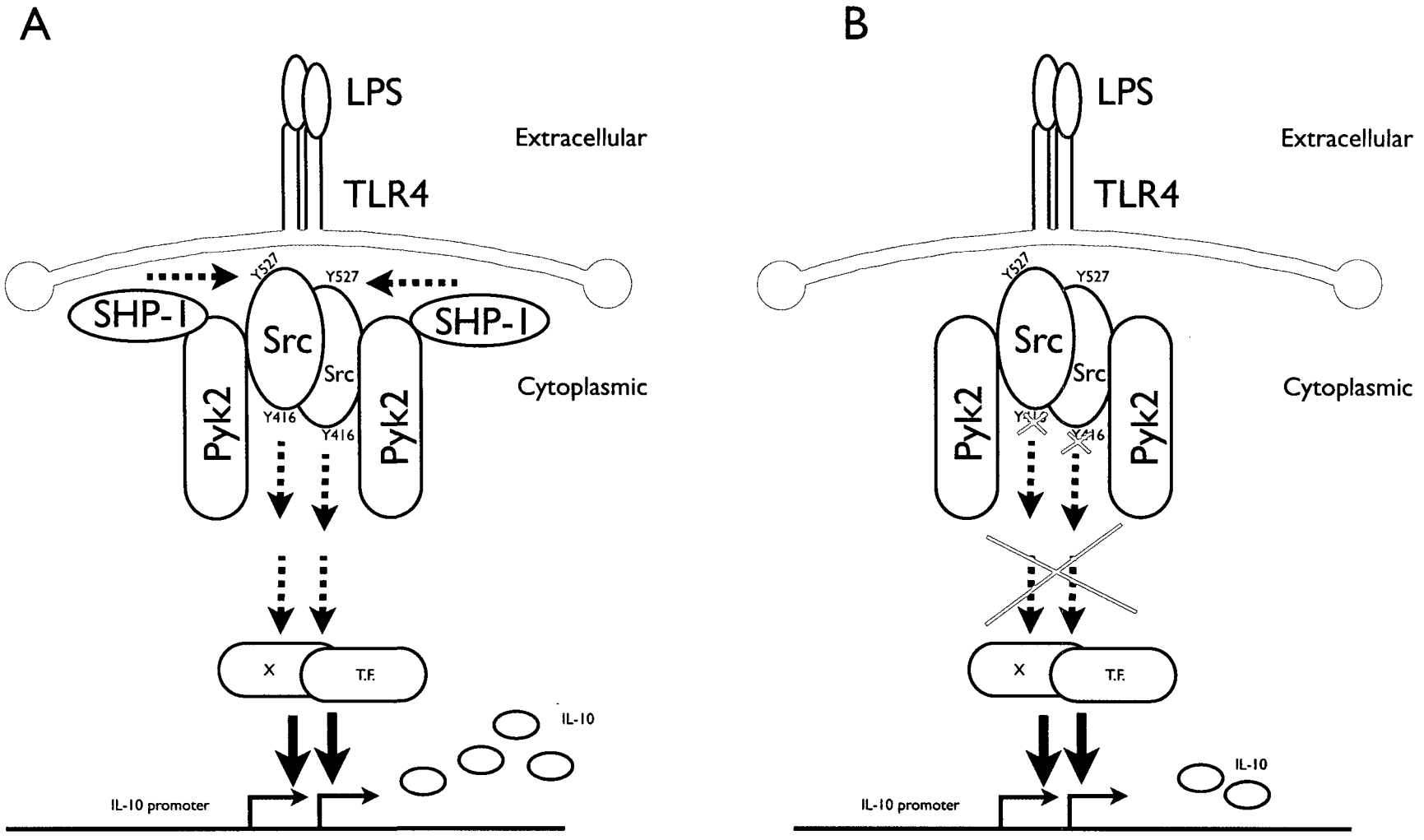

A
LPS
TLR4
Extracellular
Cytoplasmic
SHP-1
SHP-1
Src
Src
Y527
Y527
Y416
Y416
Pyk2
Pyk2
X
T.F.
IL-10
IL-10 promoter
B
LPS
TLR4
Extracellular
Cytoplasmic
SOS
Ras
Raf
MEK
MEK
p38 MAPK
IL-10

Fig IV.2 Models showing upstream activity of SHP-1-Pyk2-Src in LPS-mediated IL-10 production.

The models depicted in these figures are representative of the upstream activities of SHP-1 in LPS treated (A) Normal and (B) *me/me* splenic macrophages and are based our findings that the SHP-1-Pyk2-Src tripartite are required for optimal LPS-mediated IL-10 production. In model A, I demonstrate that in LPS treated normal splenic macrophages, SHP-1 complexes with Pyk2 and Pyk2 complexes with Src, but Src does not complex, at least not directly with SHP-1. However, SHP-1 and Pyk2 expression and activity are both required for Src activation. SHP-1 is required to deactivate Y527 on Src and Pyk2 likely is required to phosphorylate Src on Y416, consequently leading to optimal IL-10 production. In model (B) I show that in LPS treated *me/me* splenic macrophages, the lack of SHP-1 results in only a Pyk2-Src interaction. Pyk2 remains unable to phosphorylate the active site on Y416 on Src. This poor Src activation therefore leads to inadequate IL-10 production as observed in *me/me* splenic macrophages.


A
LPS
TLR4
Extracellular
Cytoplasmic
SHP-1
SHP-1
Src
Src
Y527
Y527
Y416
Y416
Pyk2
Pyk2
X
T.F.
IL-10
IL-10 promoter
B
LPS
TLR4
Extracellular
Cytoplasmic
Src
Src
Y527
Y527
Pyk2
Pyk2
X
T.F.
IL-10
IL-10 promoter

is critical for optimal IL-10 production in LPS treated normal splenic macrophages. This study further demonstrates that SHP-1 can assume a dual role in hematopoietic cells, as a negative regulator of TNF-α and as a positive regulator of IL-10. This conclusion is contrary to well established findings that predominantly implicate SHP-1 solely as a negative regulator of signal transduction pathways in hematopoietic cell function and development [324, 340, 342, 387, 412].

IV.2 SIGNIFICANCE OF THIS STUDY

This study expands our understanding of the mechanisms underlying the production of IL-10, as well as its regulatory and anti-inflammatory aspects. The study describes the mechanisms underlying IL-10 production and delineates the role of SHP-1 as a critical, positive regulator of LPS-mediated IL-10 production. Understanding the mechanisms that govern IL-10 production is essential, not only in understanding the basic biology of IL-10, but also in advancing our knowledge of chronic inflammatory disorders. It may also highlight potential therapeutic targets either in disorders where chronic production of IL-10 presents a problem, or where lack of IL-10 production exacerbates chronic inflammatory disorders.

The pleiotropic and anti-inflammatory properties exhibited by IL-10 designate it as a potential therapeutic agent in the treatment of anti-inflammatory diseases [124, 184]. IL-10 is currently undergoing clinical trials including safety and efficacy studies for the treatment of psoriasis, pancreatitis and other chronic inflammatory disorders (NCT 00040131, sponsored by Schering-Plough).

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VI. APPENDIX I

A. Buffers and Solutions.

1X TAE
40mM Tris-acetate (Sigma)
1 mM EDTA (Sigma)

5X TBE
450 mM Tris base (Sigma)
450 mM Boric acid (AnalaR R EM Science)
10mM EDTA pH 8.3 (Gibco-BRL)

10X PBS (for ELISA)
137mM NaCl (BDH)
2.7mM KCl (Gibco-BRL)
8.1mM Na2HPO4 (AnalaR R EM Science)
1.5mM KH2PO4 PH 7.2-7.4(Gibco-BRL)

Lysis buffer
20 mM Tris-HCl, pH 8 (Sigma)
150 mM NaCl (BDH)
1% (v/v) Nonidet P40 (NP-40) (Sigma)
10% (v/v) glycerol (BDH)
10 mM NaF (Sigma)
25 µg/mL aprotinin (Sigma)
25 µg/mL leupeptin (Sigma)
1 mM sodium orthovanadate (Sigma)

Stripping buffer
0.7 mM DTT (Sigma)
62.5 mM Tris-HCl, pH 6.7 (Sigma)
100 mM 2ME (Sigma)
2% SDS (Bio-Rad)

1X TBST
10 mM Tris-HCl, pH 8 (Sigma)
150 mM Nacl (BDH)
0.05% Tween-20 (Sigma)

Tris-EDTA-saline buffer
40 mM Tris-Hcl (Sigma)
1 mM EDTA (Gibco-BRL)
150 mM NaCl PH 7.8 (BDH)

Appendix II

Permission(s)

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Subject: **RE: Permission to use your illustration**
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Thank you for your email. Its fine with me if you use this figure. Usually you need to contact the journal directly because they usually have the copyright. Good luck with your thesis and defense
Best wishes
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Hello Dr. Ohashi,

I am presently a PhD student at the University of Ottawa, working on my doctoral dissertation which is related to LPS-mediated IL-10 production. I will like to include in my thesis an adopted and modified version of your LPS signal transduction figures from your review article ("LPS/TLR4 signal transduction pathway" cytokine 42(2008) 145-151). This will be strictly for use in describing the MyD88 dependent and independent pathway(s) in my thesis. I am therefore asking for your permission to use these figures in my thesis due at the end of August 2008.
Thanks for your kind consideration.
Sincerely,
Chinonso Okenwa

Chinonso Okenwa

Appendix II
Permission(s)

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Thank you for your email request. Permission is granted for you to use the material below for your [illegible] reapply for permission if you wish to distribute or publish your thesis/dissertation commercially.

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Hello Editor/publisher,

I am presently a PhD student at the University of Ottawa, working on my doctoral dissertation which is related to LPS-mediated TNF-alpha and IL-10 production. I will like to include in my thesis an adopted and modified version of your TNF-R structure figure (Fig.1) from the article by Camussi Giovanni et al. Eur. J.Biochem., 202,3-14(1991). This will be strictly for use in describing the differences in TNF receptors and pathway(s) in my thesis. I am therefore asking for your permission to use these figures in my thesis due in Sept. 2008.
Included below are the pictures from the journal and that I am planning to include in my thesis.

Thanks for your kind consideration.
Sincerely,
Chinonso Okenwa

Chinonso Okenwa