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GRADE / DEGREE

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Involvement of tyrosine phosphatase SHP-1 in the regulation of IL-12p40 production in murine splenic macrophages

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A Thesis Submitted to the

School of Graduate Studies

University of Ottawa

In Partial Fulfillment of the Requirements for the Degree of

Master of Science

Department of Biochemistry, Microbiology and Immunology

Faculty of Medicine

By

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Your file Votre référence
ISBN: 978-0-494-52357-5
Our file Notre référence
ISBN: 978-0-494-52357-5

ABSTRACT

Defects in IL-12p40 production characterize pathological conditions including autoimmunity. SHP-1 null Motheaten (*me/me*) mice, used as a model, develop autoimmunity due to deregulated production of pro-inflammatory and regulatory cytokines. LPS stimulated *me/me* splenic macrophages showed significant suppression of IL-12p40 production. Interfering with SHP-1 expression/function in normal splenic macrophages or reconstitution of SHP-1 expression in *me/me* macrophages affected LPS induced IL-12 production. To understand the involvement of SHP-1 in LPS induced IL-12p40, we investigated the role of signaling pathways. Data revealed roles for calmodulin/calcinuerin pathways as critical components of IL-12p40 production in LPS stimulated splenic macrophages. JNK and P38 MAPK were not involved in IL-12p40 production in murine splenic macrophages, while activation of PI3K or ERK pathways inhibited LPS induced IL-12p40 production, suggesting a negative role of these pathways on calcium induced IL-12p40 gene expression. In addition, IL-12p40 promoter analyses showed alternative binding of calcium activated NFAT, NFκB and AP1.

ACKNOWLEDGEMENT

I would like to thank to all people who supported me and were involved in one way or another in the preparation of this thesis. With the biggest contribution to this thesis, I would like to thank my thesis supervisor, Dr. Maya Kozlowski. I have been in her project since 2003 when I started my M.Sc. assignment. During these years I have known Maya as a sympathetic and principle-centered person. I owe her lots of gratitude for having me shown this way of research. She could not even realize how much I have learned from her. Besides of being an excellent supervisor, Maya was as close as a relative and a good friend to me. I am really glad that I have come to get know Dr. Maya Kozlowski in my life. Her extraordinary culture and knowledge of the physical and virtual worlds made possible the Masters of Microbiology and Immunology Program and inspired me to do this thesis. Dr. Kozlowski managed to re-direct my thinking towards the world of the virtual reality and brought new dimensions to my professional culture. She supported me with her encouragement and many fruitful discussions.

I would also like to thank my committee members Dr. A. Kumar, Dr. K. Wright, Dr. P. Liston, and Dr. D. Franks for their helpful and critical suggestions over the course of my project.

I feel a deep sense of gratitude for my parents who formed part of my vision and taught me the good things that really matter in life. I owe my loving thanks to my mother who sacrificed her valuable time to take care of my daughter so I can finish this thesis on time. Also, I would like to thank my father who has advised me in every aspects of my life. I wish to express my warm and sincere thanks to my parents in-laws for their faith

and encouragement. I am grateful for my brother Alireza for rendering me the sense and the value of brotherhood. Without their encouragement and understanding, it would have been impossible for me to finish this work.

Finally, I would also like to express my deepest gratitude for the constant support, understanding and love that I received from my husband Amir Akhavan. I am very grateful for my husband Amir, for his love and patience during the M.Sc. period. One of the best experiences that we lived through in this period was the birth of our beautiful daughter Sharmin, who provided an additional and joyful dimension to our life mission.

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LIST OF ABBREVIATIONS

AP-1 – Activating protein-1

APC – Antigen presenting cell

ATCC – American Type Culture Collection

ATP – Adenosine triphosphate

BCR – B cell receptor

β-gal – Beta galactosiadase

bp – base pair

BSA – Bovine serum albumin

CaM - Calmodulin

cAMP – Cyclic Adenosine monophosphate

cDNA – Complementary DNA

C/EBP - CCAAT enhancer binding protein

Cn – Calcineurin

CsA – Cyclosporine A

CSF – Colony stimulating factor

CTL – Cytotoxic T lymphocytes

DCs – Dendritic cells

DDT – Dichloro-dipenyl-trichloro-ethane

Del – Deletion mutant

DEPC – Diethyl pyrocarbonate

DMSO – Dimethyl sulfoxide

DNA – Deoxy ribonucleic acid

dNTP – 2'-deoxyribonucleotide triphosphate

DTT – Dithiotreitol

EAT - Experimental autoimmune thyroiditis

EBV - Epstein-Barr virus

ECL – enhanced chemiluminescence

EDTA – Ethylene diamine tetra acetic acid

ELISA – Enzyme linked immuno-sorbant assay

EMSA – Electrophoretic mobility shift assay

ERK – Extracellular-signal regulated kinase

FL - Full length

FBS – Fetal bovine serum

GFP - Green florescent protein

GM-CSF – granulocyte-macrophage CSF

Hcph6 - hematopoietic cell phosphatase

Hepes – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

hr – Hour

HRP – Horseradish peroxide

IC50 – 50% inhibition dose

ICSBP - interferon consensus sequence binding protein

IFN-αβ - Interferon αβ receptor

IFN-γ - Interferon γ receptor

Ig – Immunoglobulin

IL – Interleukin (e.g, IL-12)

IPTG – Isopropyl-beta-D-thiogalactopyraoside

ITIMs - Immunoreceptor tyrosine-based inhibitory motifs

JNK – c-Jun N-terminal kinase

kDa - Kilodalton

LPS – Lipopolysaccharide

LBP – LPS binding protein

mAb – monoclonal antibody

MAPK – Mitogen activated protein kinase

M-CSF – macrophage CSF

me/me mice - motheaten mice

2-ME – 2-mercaptoethanol

MEK1- MAPK activating kinase

min – minute

μg – Micro gram

μL – Microlitre

MKK3 – MAP kinase kinase kinase

μM – Micro Mole

mL – Millilitre

mM – Milli Mole

mRNA – messenger RNA

MOI – Mutiplicity of infection

mRNA – messenger ribonucleic acid

NFAT – Nuclear factor of activated T cells

NFκB – Nuclear factor κB

NK cell – Natural killer cell

NOD mice - None obese diabetic mice

PAGE – Poly acrylamide gel electrophoresis

PBMC - Peripheral blood mononuclear cell

PBS – Phosphate-buffered saline

PCR – Polymerase chain reaction

pg – Pico gram

PI3K – Phosphatidyl inositol-3-kinase

PLC – Phospholipase C

PMSF – Phenyl methyl sulfonyl fluride

PTC - Peltier Thermal Cycler

PTK – Protein trosine kinase

PTP – Protein tyrosine phosphatase

RNA – Ribonucleic acid

RT-PCR – Reverse transcriptase polymerase chain reaction

SCID – Severe combined immunodeficiency

SD – Standard deviation

SDS – Sodium dodecyl sulfate

sec - second

SHP-1 – SH2 domain containing phosphatase-1

SHP-2 - SH2 domain containing phosphatase-2

Src – Sarcoma

TAE – Tris acetate EDTA buffer

TBE – Tris boric acid-EDTA

TBS – Tris buffered saline

TBST – TBS with tween20

TCR – T cell receptor

TGF-β - Transforming growth factor-β

Th cell – T helper cell

TLR – Toll like receptor

TNF-α - Tumor necrosis factor-α

UV - Ultraviolet

V – Volt

INTRODUCTON

Cytokines are small proteins (~25kDa) that are the core of communication between immune system cells. They are actively produced by immune cells as well as other cell types, usually in response to a stimulus. Cytokines can act by binding to their specific receptors on the cell membrane, and each receptor allows a distinct signal pathway to start in the cell that eventually results in biochemical changes in the target cells. Interleukins (abbreviated IL) are a group of cytokines that are produced by and acting on *leuko*cytes (hence the leukin) as a means of *inter*cellular communication (inter-). Different interleukins are identified by assigned numbers. [1] For example, the cytokines produced by macrophages and induced by pathogens include IL-1, IL-6, IL-12, Tumor Necrosis Factor (TNF-α), and IL-8. [1] There are three structural groups of cytokines: the hematopoietic cytokine receptor family like interleukins and growth hormones, the TNF family, and the chemokine family. [1]

1. Protein Characteristics of Interleukin 12 (IL-12)

IL-12 is a heterodimeric 70kDa glycoprotein composed of two disulfide-linked subunits, 35kDa light chain (known as p35 or IL-12α) and 40 kDa heavy chain (known as p40 or IL-12β). The expression of the two genes encoding either the p40 protein chain or the p35 protein is regulated independently of each other. Expression of the gene encoding p35 protein is constitutive in contrast the gene encoding p40 is inducible. While the p35 protein is expressed in many tissue types, p40 mRNA is found in macrophages and any cell type that secretes IL-12p70 [23]. The p35 protein resembles other single chain

cytokines, whereas p40 chain belongs to the hematopoietic cytokine-receptor family and shows some homology with the extracellular domain of the receptor for IL-6 receptor α-subunit. [2, 3] Recently, p40 has also been shown to be a subunit of another composite cytokine, which associates with another molecule p19 to form a new cytokine known as, IL-23. [4, 5] To produce a bioactive IL-12p70, both p35 and p40 genes have to be expressed coordinately in the same cells. It is shown that p35 can be functional only when associated with p40; however, p40 can be secreted as a monomer or a homodimer even when there is no IL-12p35 or IL-23p19. Interestingly, studies demonstrated that there is an overproduction of p40 chain compared to p35 chain. p40 chain forms homodimers $(p40)_2$ and competes with the bioactive p70 heterodimer for binding to the IL-12 receptor. Thus, p40 homodimer may act as an IL-12 antagonist, but this possibility still remains to be further determined experimentally. [2, 3, 4]

2. Biological Activities of IL-12 (Fig 1.1)

A. Cellular Sources of IL-12: Originally IL-12 was discovered as a protein, secreted by Epstein-Barr virus (EBV) transformed human B cell lines. [10] The main producer cells of IL-12 are cells of the innate immune system and phagocytic cells: antigen-presenting cells (APC) such as monocyte/macrophages, professional APCs (dendritic cells), and neutrophils, which are involved in the early innate immunity. Other cell types that secrete IL-12 include mast cells from the connective tissue type (not the mucosal type), skin Langerhans cells, microglia and probably keratinocytes. Cellular sources of IL-12 are indicators of major physiological roles for IL-12 as an early pro-

inflammatory cytokine, and an immuno-regulatory cytokine to maintain cell-mediated immune responses. [2, 4]

B. Regulation of Th1/Th2 Responses by IL-12: IL-12 can be regarded as a cytokine that connects the innate immune system and acquired immunity. The physiologically most important target cells of IL-12 action are $CD4^+$ T cells. IL-12 is recognized for its ability to regulate the balance between T Helper1 (Th1) and T Helper2 (Th2) cells (functional subsets of $CD4^+$ T cells). Th1 cells produce type-1 cytokines, IL-2 and IFN-γ, leading to cell mediated immunity; however, Th2 cells produce IL-4, IL-5, IL-6, IL-10, and IL-13, thus promoting humoral immunity.[3, 4] Th1 subtypes are associated with eradication of intracellular pathogens like some bacteria, viruses and also with eradication of tumor cells; however, Th2 cells mediate elimination of certain parasites, such as intestinal worms.[18] Researchers using both human and mice models have demonstrated that IL-12 promotes proliferation and differentiation of T cells toward Th1 subtypes while suppressing Th2 responses (Fig1.1).[7, 8, 10] In contrast, under some experimental condition, IL-12 was shown to induce Th2 responses. [3, 12, 13] This can be justified by cytokine microenvironment or maturational state of the T cells. [3]

IL-12 promotes Th1 responses in three ways: a) IL-12 facilitates Th1 differentiation from naïve $CD4^+$ T cells and IFN-γ secretion following stimulation with antigen and costimlatory molecules, b) it acts as a costimulus needed for maximum production of IFN-γ by Th1 cells, c) it promotes the differentiation of resting memory T cells, encountering with an antigen to which they have been already exposed, toward IFN-γ producing Th1 cells.[3, 9] Therefore, IL-12 is an excellent trigger for IFN-γ production and it has been shown that it synergizes with other cytokines, such as IL-2, IL-18, and IL-27

to further augment IFN-γ production.[24] Although IL-2 is considered a type1 cytokine, IL-12 does not regulate IL-2 production.[3]

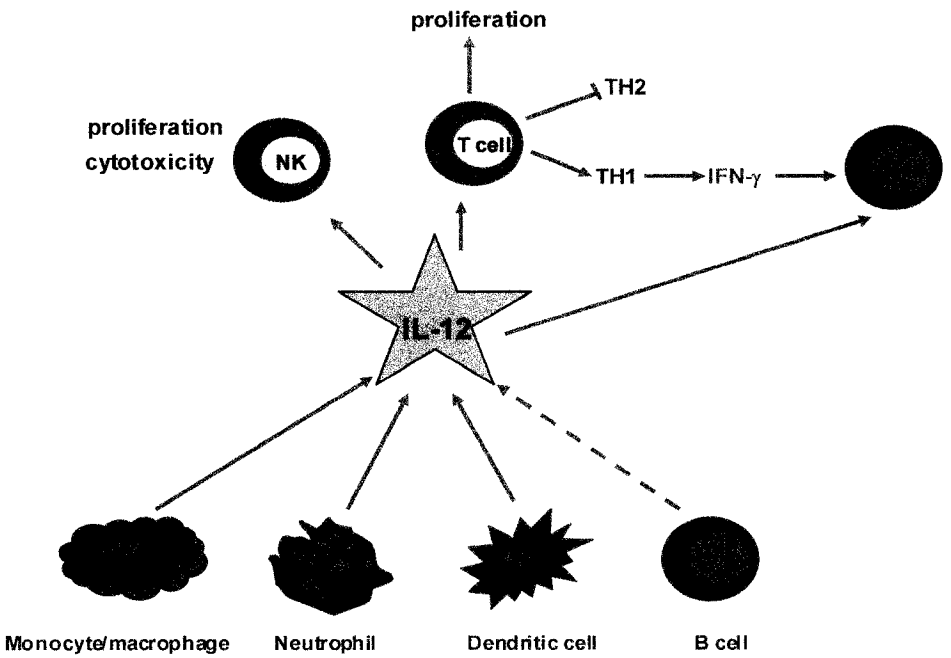
C. Regulation of Humoral Immunity: IL-12 can induce or suppress antibody responses. Studies in mice have indicated that IL-12 can enhance humoral immunity by affecting IgG2a, IgG2b, and IgG3 antibody responses to antigens. These Ig isotypes are related to Th1 responses. In contrast, IL-12 can also enhance Th2 responses by down-regulating IgG1. This was observed when antibody levels were assayed after primary immunization. Overall, most studies have demonstrated that the effects of IL-12 on humoral responses are mediated through Th1 cells and Th1 cell produced IFN-γ; however, IL-12 might affect B cells directly (Fig1.1).[3]

D. Other Functions of IL-12: IL-12 can increase the proliferation of pre-activated (but not resting) cytotoxic T lymphocytes (CTLs), lymphokine activated killer cells (LAK cells) and natural killer cells (NK cells). (Fig1.1) It promotes the lytic activity of LAK and NK cells, and augments the cytotoxic activity of CTLs by up-regulating the expression of major cytotoxins, such as granzymes (A and B) and perforin, and also adhesion molecules (CD11a but not CD11b).[4, 6, 10, 21] IL-12 also induces NK cell production of IFN-γ, leading to antimicrobial responses. This is shown to be critical in elimination of many bacterial and parasitic pathogens, such as *Listeria monocytogens, Toxoplasma gondii, and Toxoplasma Cruzi.*[22] Moreover, IL-12 enhances survival, proliferation and differentiation of hematopoietic stem cells through synergizing with stem cell factor, IL-3 and other hematopoietic growth factors.[3, 10]

Figure1.1: Summary of the biology of IL-12.

This figure represents the cellular sources of IL-12, regulation of Th1/Th2 responses by IL-12, regulation of humoral immunity, and the other functions of IL-12 including its effect on proliferation and enhancement of cytotoxicity of NK and NKT cells.

Figure1.1


proliferation
TH2
proliferation
cytotoxicity
NK
T cell
TH1
IFN-γ
IL-12
Monocyte/macrophage
Neutrophil
Dendritic cell
B cell

3. Positive Regulation of IL-12 Production

The triggers of IL-12 secretion by cells of the innate immune system are live intracellular bacteria, (such as *L. monocytogenes* and various *Mycobacteria*), bacterial products, intracellular parasites, (such as *Leishmania major and Toxoplasma gondii*), and some viruses.[2, 10, 14, 18] Also, it has been shown that the complex glycolipid lipopolysaccharide (LPS), a cell membrane component of gram-negative bacteria, is a potent stimulus for the production of both IL-12p70 and p40 from human myeloid cell lines as well as human neutrophils. [10] There are two pathways for IL-12 secretion: a) T cell independent pathway which is initiated by a potent IL-12 stimulus like *Toxoplasma gondii* or LPS, and b) T cell dependent pathway that is induced by engagement of CD40 on APCs with CD40 ligand (CD40L) on activated T cells. Additional costimlatory molecules, which contribute in IL-12 secretion, are CD58 /CD2 and B7/CD28, expressed on APCs and T cells respectively.[2, 10]

Studies have demonstrated that IFN-γ and bacteria and/or microbial products can augment IL-12 secretion from human monocytes and macrophage cells synergistically.[3, 11] In parallel, another study on peripheral blood mononuclear cells (PBMCs) shows that IFN-γ alone does not promote p40 gene expression, while LPS/IFN-γ treatment induces monocyte cells to produce IL-12 p40.[14] The ability of IFN-γ to augment IL-12 production makes a positive feedback loop during Th1 responses resulting in a strong immune/inflammatory response against antigen. Moreover, other cytokines such as TNF-α, granulocytes-macrophage colony-stimulating factor (GM-CSF) can also enhance IL-12 production. [2, 18] Surprisingly, although IL-4 and IL-13 are considered as Th2 cytokines, they also increase IL-12 secretion from DCs. Following the first 24 hour of

stimulation with either IL-4 or IL-13, there is a decreased production of IL-12p40 while at later times binding of these cytokines enhance expression of the genes encoding both p40 and p35, and eventually IL-12p70. [4]

4. Negative Regulation of IL-12 Production

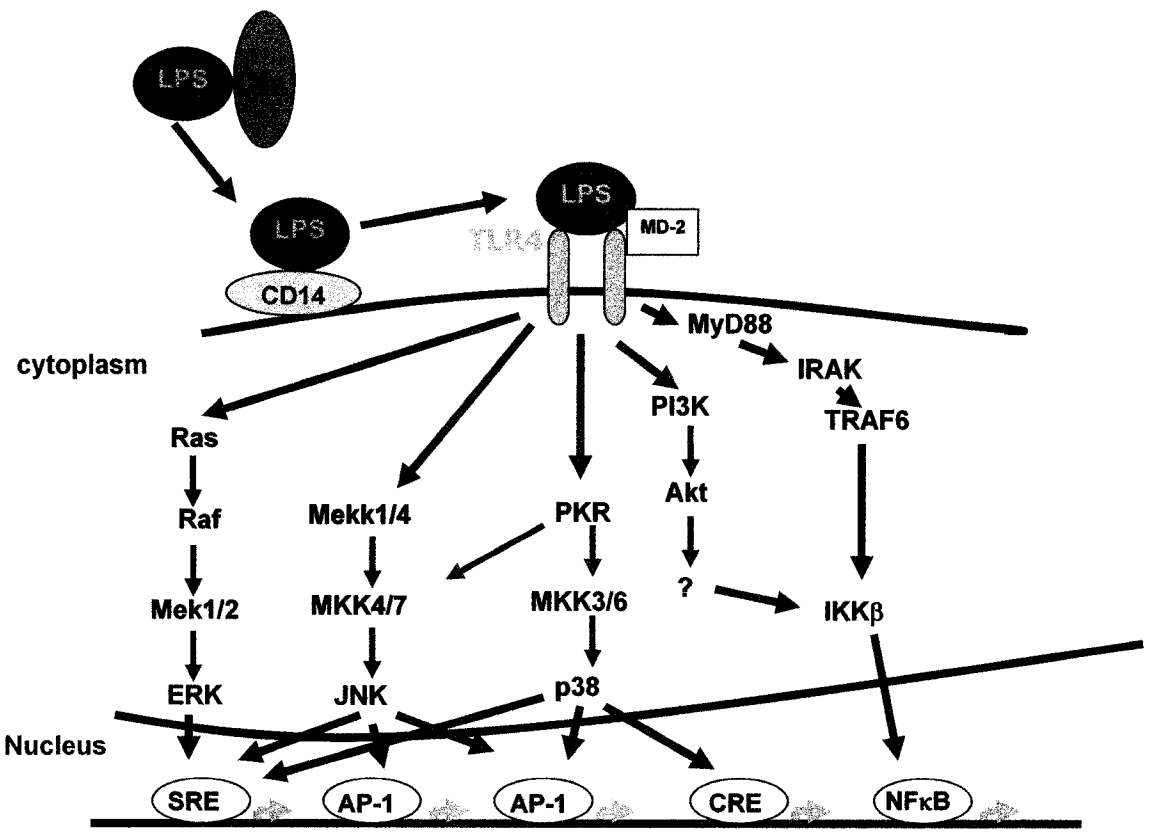
As mentioned before, there is a positive feed back loop between IL-12 and IFN-γ production: IL-12 augments IFN-γ production, and IFN-γ enhances IL-12 secretion. To prevent this system from going out of control and developing cell mediated autoimmune disease; there are certain cytokines, such as IL-10, IL-4, IL-13, and Transforming Growth Factor-β (TGF-β) whose secretion blocks this feedback loop. [11, 15, 16, 18] Studies in mice and human have shown that IL-10 (anti-inflammatory cytokine) is the most important blocker of IL-12 secretion by inhibiting the transcription of p40 and p35 subunits of IL-12. For example, it has been shown that destructive colitis in IL-10 deficient mice can be prevented by treatment with antibodies to IL-12 [20]. Interestingly, the production of IL-10 is induced by IL-12, obeyed at the later time during the immune response, and in this way IL-12 limits its own pro-inflammatory effects. [116] Therefore, the positive regulatory effect of IL-12 on secretion of both IFN-γ (Th1 type cytokine) and IL-10 (a Th2 type cytokine) at the same time creates a complex regulatory network composing of various cell types that is still unclear. [2, 17] Also IFN-α and IFN-β are shown to compete with IL-12 in induction of IFN-γ and Th1 responses. In addition, studies in LPS stimulated human monocyte-derived macrophages have shown that after 16 hours pre-incubation period with TNF-α, there was an inhibitory effect on IL-12p40 expression but not on IL-

12 p35. This suggested that TNFα also plays a role in the production of IL-12p70 by influencing the expression of IL-12p40. [4, 19]

5. Involvement of Signaling Pathways Activated by TLR4: IL-12 secretion is an important indicator of macrophage's ability to modulate immune responses. IL-12 is produced by antigen presenting cells such as macrophages and DCs in response to microbial pathogens. [51] LPS, a major component of the outer membrane of gram-negative bacteria, is a potent immune stimulant that is responsible for many of the cellular responses to gram negative microorganisms. Macrophage stimulation by LPS results in some changes in the cytoskeleton (spreading); cytokine and chemokine secretion, and antimicrobial activities. LPS stimulation also regulates cell surface receptors, antigen presentation molecules, and co-stimulatory molecules. [52] TLRs (toll-like receptor) have been implicated as the major receptors for bacterial pathogen binding on the cell membrane. LPS binds to TLR4 resulting in activation of macrophages through stimulation of signal transduction pathways. Signal transduction induced by LPS is enhanced after the association of LPS with LPS-binding protein (LBP) and the binding of this complex with the CD14/TLR4 complex (Fig 1.2). CD14 (a myeloid marker antigen) is a primary LPS receptor which is expressed predominantly on monocytes and macrophages as glycophosphoinositol-linked protein (cell surface antigen). CD14 also exists in a soluble form that has been shed in response to monocyte activation or differentiation and can substitute for membrane bound CD14. [53, 62, 98]

Fig 1.2. LPS stimulation of monocytes and activation of signaling pathways: LPS interacts with the serum protein LBP. Then the LPS/LBP complex is transferred to the CD14/TLR4 and as a result LPS interacts with TLR4 receptor and the accessory protein MD-2. Multiple signal transduction pathways are activated upon LPS stimulation of monocytes/macrophages. These pathways include MAP kinases (ERK, JNK, and p38), Ikk pathway, and PI3K/Akt pathway. These pathways directly or indirectly activate different transcription factors as it is shown.

Figure. 1.2


LPS
LPS
LPS
TLR4
MD-2
CD14
cytoplasm
MyD88
IRAK
TRAF6
PI3K
Akt
?
Ras
Raf
Mek1/2
ERK
Mekk1/4
MKK4/7
JNK
PKR
MKK3/6
p38
IKKβ
Nucleus
SRE
AP-1
AP-1
CRE
NFκB

It has been estimated that the eukaryotic cells contains more than 1000 signalling molecules and only 10 second messengers. Second messengers are molecules that mediate cell activity by relaying signals from extracellular molecules bound to the receptors on the cell surface. In addition, they increase the strength of the signals. These signals on the cell surface are converted into specific cellular responses through activation of distinct signal transduction pathways. There are three important classes of second messenger: a) Cyclic nucleotides (e.g., cAMP), b) inositol triphosphate (IP3) and diacylglycerol (DAG), and c) calcium ions (Ca^{2+}). [56, 57]

A. Calcium signalling and cell activation: There is some evidence suggesting the involvement of calcium signaling pathways in the regulation of IL-12p40 production in DCs and monocytes. [62, 63, 64, 67, 110] Calcium pumps in the endoplasmic reticulum membrane (SERCA pumps) pump calcium into the endoplasmic reticulum (ER) at the expense of ATP hydrolysis. In response to signalling, ER spreads calcium into the cytosol. The concentration of cytosolic calcium is modulated by transport systems such as Ca^{2+} channels and Na^{+} $(H^{+})/Ca^{2+}$ exchangers in the plasma membrane, the endo(sarco)plasmic reticulum, and/or mitochondria. The function of these transporters regulates calcium concentration not only in the cytosol but in the nucleus and intracellular organelles. Free calcium plays a key role in modulation of multiple cellular functions, and the concentration of released calcium into the cytosol is modulated by a variety of calcium binding proteins, such as calmodulin (CaM).[58, 59, 60] CaM, as a calcium receptor, has been found to be involved in many cellular functions, such as synthesis and degradation of cyclic nucleotides, the phosphorylation/dephosphorylation

cycle of proteins, gene transcription and the modulation of different transport systems. Therefore, CaM is capable of regulation of signaling pathways contributing to the control of cellular metabolism, cytoskeletal organizations, cytokine production, muscle contraction, intercellular communication, cell proliferation, differentiation and apoptosis.[60]

One of the major CaM binding protein is a serine/threonine phosphatase known as calcineurin (Cn). Calcineurin is expressed in different kinds of tissues and plays a critical role in the immune system, particularly lymphocyte function. This enzyme directly modulates the activity of NFAT (Nuclear Factor of Activated T cells) protein. Activation of calcineurin is mediated through induction of different classes of cell surface receptors, including immuno-receptors and receptor tyrosine kinases which in turn activate phospholipase C (PLC)-γ. [61, 93] PLC-γ hydrolyses phosphatidylinositol (PI)-4, 5-biphosphate (PIP2) at the plasma membrane, thereby releasing the two main second messengers diacylglycerol (DAG) and inositol-1, 4, 5-triphosphate (IP3). The former (DAG) activates protein kinase C (PKC), whereas IP3 interacts with IP3 receptor (IP3R) in ER and results in increased Ca^{2+} levels in the cytoplasm by depletion of ER Ca^{2+} stores and Ca^{2+} influx. Increased level of calcium activates many CaM-dependent enzymes including calcineurin. [93] For example, when TCR interacts with an antigen, the intracellular concentration of calcium in the cell rises. The major source for calcium increase following TCR engagement is Ca^{2+} influx through calcium release-activated calcium (CRAC) channels. This cytoplasmic calcium increase activates Cn. Cn induces different transcription factors including NFAT in such that Cn interacts with regulatory subunits of NFAT and dephosphorylates these proteins. This results in translocation of

NFAT to the nucleus, binding to the promoter or enhancer sequences of different genes, resulting in gene transcription. [66] For instance, NFAT has been originally identified as an important factor for of IL-2 gene induction. IL-2 activates T-helper lymphocytes and induces the production of other cytokines. In this way, it governs the action of cytotoxic lymphocytes and NK cells. The amount of IL-2, produced by the T-helper cells is believed to significantly affect the extent of the immune response. [61, 66]

B. MAP kinase pathway: There are many studies indicating the involvement of MAP kinase pathways in the regulation of IL-12p40 production in human and murine macrophages. [82, 84, 85, 88, 91 95] The MAP kinases are a critical group of serine/threonine signaling kinases that regulate phosphorylation and activation of transcription factors and hence link transmembrane signaling with cytokine gene induction in the nucleus. MAP kinases constitute three major subfamilies which include the extracellular signal-regulated kinases (ERKs), the c-jun amino-terminal kinases (JNKs), and the p38 MAP kinases. MAP kinases contribute to functional responses of cells including proliferation, differentiation and apoptosis, inflammation and the responses to environmental stress. Many studies have demonstrated that all three subfamilies of MAPKs are activated by LPS in a variety of macrophage cell types [103] Upon LPS stimulation, TLR4 (a signaling receptor for LPS) interacts with another molecule MD-2 which is necessary for LPS recognition. This interaction with MD-2 has also been shown to be required for activation of MAP kinases. [103] In addition, upon LPS/TLR4 activation, TLR4 binds to adaptor protein MyD88, which in turn recruits IL-1 receptor-associated kinase (IRAK) to the receptor. Once activated, IRAK interacts with TNF associated factor 6 (TRAF6)

resulting in activation of various signaling pathways including MAP kinases and NFκB gene expression. [104] (Fig 1.2) Also, PKC activation upon LPS stimulation has been reported to activate MAP kinase pathway in a manner dependent on Raf and independent on Ras. There is a study in alveolar macrophages showing that activation of PLC-γ upon LPS stimulation is related to activation of ERK pathway. However the exact pathway for activation of MAP kinases upon LPS stimulation in macrophages remains to be understood. [103] The MAP kinases are all activated by phosphorylation of both threonine and tyrosine residues. This phosphorylation is performed by distinct upstream dual-specifity MAP kinase kinases (MAPKK). The MAPKK are in turn activated by MAP kinase kinase kinases (MAPKKK) through phosphorylation of serine or threonine residues. [81, 82, 83]

The ERK pathway is activated by mitogens, growth factors, and hormones whereas JNK and p38 pathways are reported to be activated by both environmental stress (such as osmotic changes and heat shock) and inflammatory cytokines (such as TNF-α and IL-1). The JNK and p38 pathways are also called stress-activated MAP kinase (SAPK) pathways. Thus, the physiological functions of these two pathways may be overlapping.[84] Biochemical and genetic studies have indicated the roles for regulation of cytokine production, B and T cells proliferation and differentiation, the innate immune responses, and apoptosis. JNK pathway activation is maintained by the phosphorylation of tyrosine and threonine residues. The full activation of JNKs requires activation of both MKK4 and MKK7. Activation of MEKK1-MKK4/JNK pathway leads to up-regulation of some pro-inflammatory cytokines, such as IFN-γ, IL-2 and TNF-α, through activation of the transcription factor activator AP-1. [84, 86, 87]

C. PI3K pathway: PI3Ks are heterodimeric enzymes that contain regulatory and catalytic domains. Binding of regulatory domain of heterodimeric protein to phosphotyrosine residues of cellular receptors, results in PI3K activation. [80] PI3Ks are lipid kinases that phosphorylate membrane associated lipids of the phosphoinositide family in response to various stimuli. Once these kinases become activated, they phosphorylate the plasma membrane phospholipid phosphatidylinositol 4, 5-bisphosphate to generate the second messenger phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)*P3*]. PtdIns(3,4,5) P_3 is metabolized by enzymes called phosphatases to phosphatidylinositol 3,4-bisphosphate, which itself is a second messenger, and then to phosphatidylinositol 3-phosphate. One of the most important targets of PI3K is the serine/threonine protein kinase Akt. [76]

PI3Ks are implicated in controlling many cell responses including mitogenesis, cell survival, differentiation and activation, cytoskeletal remodeling and vesicular trafficking. In particular, they play a critical role in the immune system.[76] For example, it is found that PI3K/Akt pathway plays role in the regulation of inflammatory cytokine production in both human and mouse monocytes/macrophages. [77, 78] Upon LPS stimulation of macrophages, protein kinase C (PKC) is activated through a PI3K dependent pathway.[103]

Involvement of PI3K pathway was considered based on data showing that PI3K knockout mice produce a high level of Th1-asociated cytokines including IL-12. Thus, PI3K activity has been shown to have a negative regulatory effect on IL-12 synthesis. Similarly, another study has demonstrated that inhibition of PI3K-Akt pathway, activated by *P. gingivalis* LPS, results in an increased level of IL-12 production. [80]

Since IL-12 is a central cytokine involved in the modulation of normal immune responses, deregulation of signal transduction pathways, leading to the induction of IL-12, has profound effects on the function of the immune system. Consequently, deregulation of IL-12 production has been implicated in numerous autoimmune diseases.

6. IL-12 and Autoimmune Disease

Autoimmunity is a pathological condition that arises from overactive adaptive immune responses against self antigen. In autoimmune disease, the immune effector pathways result in inflammatory damages to tissues; however, what causes the immune response to self is still unknown. In the past it was believed that autoimmunity is dependent on antibodies and immune complexes while recently it has been found that many autoimmune diseases, especially tissue specific are T cell mediated.[2, 25] IL-12 's specific role in autoimmunity appears to be variable.

Deregulated positive feed back loop between IL-12 and IFN-γ may lead to development of cell mediated autoimmunity such as autoimmune encephalitis, uveitis, diabetes, Crohn's disease and arthritis. Therefore, IL-12 is an important cytokine that contributes to the development of cell mediated autoimmune diseases.[2] For instance, studies using mouse model for diabetes (NOD, None obese diabetic mice) indicated that administration of recombinant IL-12 to these mice greatly enhanced the severity of disease; however, administration of $(p40)_2$, an antagonist for IL-12, resulted in a reduced Th1 responses and inactivation of Th2 responses. These observations indicate a role for IL-12 in pathogenesis of diabetes mellitus in mice.[3, 25] Similarly, immunohistochemical analysis of tissues from the colons of human patients with Crohn's disease showed an increased number of macrophages producing IL-12 and T cells producing IFN-γ. [3]

In contrast to a pathological effect of IL-12 over-secretion in some autoimmune diseases, downregulation of IL-12 can also contribute to pathogenesis of certain inflammatory diseases in such that IL-12 deficiency leads to hyperactivation of Th2 immune response. [18] For example, studies on asthmatic patients have revealed a hyperactivation of Th2 subtypes compared to Th1 cell types. Additionally, expression of IL-12 message in bronchial biopsies from asthmatic patients was significantly decreased, compared to normal controls. [26] Also, it was found that IL-12 treatment is useful in reduction of IL-4 and IL-5 expression in the airways of the murine model of asthma [18]; moreover, it inhibits eosinophil accumulation in the lungs [3]. Similarly, in experimental autoimmune thyroiditis (EAT) induced by thioglycolate + LPS, IL-12p40 knock out mice were more susceptible to EAT disease. Therefore, IL-12 can play a key role in decreasing the severity of the disease and reduces autoantibody levels. [27, 28]

IL-12 is a central cytokine regulating Th1 responses and its deregulation results in pathological conditions including autoimmunity. To understand the regulation of this cytokine, I employed the motheaten mouse model (*me/me*) with a defined genetic lesion affecting the expression and function of the signal transduction protein tyrosine phosphatase SHP-1 whose function regulates a number of signal transduction pathways in immune cells.

7. Characteristics of Motheaten Mice (*me/me*)

Motheaten *(me)* mutation and its less severe allelic form, viable motheaten *(me^v)* in the C57BL/6J mice were first observed at the Jackson Laboratory. [29] Homozygosity for the *me* and *me^v* mutations results in the most severe abnormalities in hematopoietic cell development and deregulation of immune cell functions, and as a consequence these mice

develop both immunodeficiency and systemic autoimmunity.[30, 31] Moreover, these mice also have some non-immunological defects such as infertility and decreased body weight and as a result reduced size. [29] The motheaten locus occupies a region on mouse chromosome 6 which has a homology with human chromosome 12p12-p13. [31] This region is known to include the gene for a human protein tyrosine phosphatase. Defects in motheaten mice are due to the mutations within the structural gene (*Hcph6*, hematopoietic cell phosphatase,) encoding a specific nontransmembrane protein-tyrosine phosphatase (PTP) SHP-1 expressed predominantly in cells of hematopoietic lineage. The gene encoding SHP-1 had been cloned by several groups of researchers and given different names- PTP-1C, HCP, SHP, SHPTP1, and PTPN6. [29] Later on this protein tyrosine phosphatase was renamed as SHP-1(Src homology 2 domain-containing phophatase1), and I will be using this term in this text.

A. Hematologic and Immunologic Characteristics: *me/me* mice can usually be recognized by three days of age, when one or several small abscesses appear on the surface of their skin. Myeloid/monocytic cells and granulocytes, particularly neutrophils, accumulate in the epidermis, and displace hair follicles, which leads to patchy inflammation and alopecia. As a consequence they will have a motheaten appearance. [32, 33, 34] Similarly, marked increase and infiltration of myeloid/monocytes and granulocytes to the tissues such as spleen and lungs lead to splenomegaly and development of hemorrhagic pneumonitis that kills the mice in 2 weeks. [33, 34] This immediate death is due to the accumulation of neutrophils and macrophages in pulmonary alveoli with a consequent of focal intra-alveolar hemorrhages. [32] In addition, the *me/me* mice develop progressive arthritis, and limb necrosis. [33, 34] The important role for monocytic cells in

developing inflammation in *me/me* mice is supported by data showing that anti-Mac-1antibody, Ab for a macrophage marker, can be used as a treatment for reducing inflammatory phenotype in these mice. [39, 112]

In general, *me/me* mice exhibit abnormalities in immunological functions, such as defects in several hemopoietic cell lineages and in particular, impaired lymphocyte growth, differentiation and function. The abnormalities in lymphocyte development is evidenced by premature thymic involution as a result of impaired migration of pro-thymocytes to the thymus, diminished proliferative T cell responses to antigens and mitogens, defects in cytotoxic T cell responses, and depletion of bone marrow B cell precursors(surface $B220^{+}$) or conventional B cells. In contrast, studies in *me/me* mice have shown that this downregulation of conventional B cells is compensated by a high percentage of normally minor B cell subpopulation (B1 cells), which express the pan-T cell surface glycoprotein CD5 (Ly-1). There is evidence that in *me/me* mice in addition to all the B-lymphocytes, the splenic macrophages also express CD5.[38] CD5 interacts with B cell receptor (BCR) on B1 cells; however, its function is not well understood. B1 cells are the majority of the peripheral B cell population and are associated with the IgM autoantibody production and consequently autoimmunity development. [30, 31, 32, 33, 34, 35, 36] The level of IgM in the sera of *me/me* mice is 25-50 times higher than in normal mice.[32] Furthermore, motheaten mice also exhibit abnormalities of macrophages, dendritic cells, natural killer cells, and erythroid cell precursor populations. For instance, SHP-1 deficiency results in hyper activation of macrophages expressing CD5. CD5 is usually expressed by T cells and B1B cells and maybe expressed by activated macrophages. Also, studies on *me/me* mice demonstrated that intrathymic macrophages are increased

while intrathymic dendritic cells are decreased. So, these abnormalities in numbers of macrophages and DCs may be associated with the early thymic involution. [36, 37] In addition to thymic atrophy, *me/me* mice suffer from splenomegaly as a result of huge proliferation of monocytic/macrophage and erythrocytes cell lineages. The size of the lymph nodes of these mice increases due to the expansion of neutrophils, plasma cells and multi nucleated giant cells. This is while the number of lymphocytes is down in the lymph nodes. In *me/me* bone-marrow, there is a decreased level of erythrocytes and increased level of myeloid cells. [32]

B. What is Tyrosine Phosphatase SHP-1? Control of many cellular processes including cell survival, proliferation, and differentiation is regulated by the phosphorylation of cellular proteins, often on tyrosine residues. Tyrosyl phosphorylation is a reversible process that can change enzymatic activity, protein localization, protein assembly, which in turn, regulates cellular signaling pathways. Regulation of tyrosine phosphorylation is mediated by two types of enzymes: protein-tyrosine kinases (PTKs), which phosphorylate tyrosine residues, and protein-tyrosine phosphatases (PTPs), which de-phosphorylate tyrosine residues and thus antagonize the action of PTKs. [30, 40, 41] Since these two groups of enzymes regulate intracellular signal transduction pathways activated by extracellular stimuli, it is important to understand their functions as well as to reveal their intracellular targets.

Most of the PTPs seem to be expressed in all kinds of tissues, but some of them such as CD45, SHP-1 and HePTP are mostly expressed in hematopoietic cells.[30] PTPs contain a superfamily (~90 human members) which is structurally characterized by a conserved active–site signature motif. Classic PTPs (~40) comprise a 240-250 amino

acid PTP domain, surrounded by different regulatory sequences, and they are very specific for hydrolyzing phosphotyrosine residues. They are two groups of classic PTPs: transmembrane-receptor like (RPTP), and non-transmembrane class which is recognized by distinct N- and/or C-terminal regulatory sequences. [40] Tyrosine phosphatase SHP-1 is nontransmembrane PTP that contains two N-terminal SH2 domains (N-SH2 and C-SH2), encoded by nucleotides 10-300 and 327-615, a catalytic PTP domain (nucleotides 795-1542) and an inhibitory C-terminal tail including two sites for tyrosine phosphorylation (Fig1.2).[33, 40, 44] There are two isoforms (68 and 71kDa) of SHP-1 that have been recognized in wild type hematopoietic tissues.[30, 44]

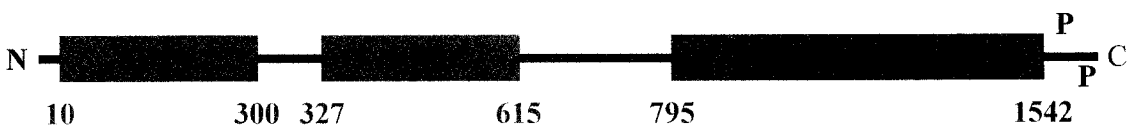
B.1. Role of the SH2 domain: Src homology (SH2) domains act as protein phospho-tyrosine binding domains, and mediate the interaction of PTP with its substrates. In hematopoietic cells many proteins, which interact with a SHP-1-SH2 domains, have one or more immunoreceptor tyrosine-based inhibitory motifs (ITIMs). SHP-1 interacts with ITIM motifs of these proteins through binding of both the N- and C-SH2 domains. Also, it is known that catalytic activity of SHP-1 is regulated by structural rearrangement of the SH2 domains. For instance, removal of the N-terminal SH2 domain of SHP-1, but not the C terminal SH2 domain, activates the enzyme, indicating that the catalytic activity is inhibited by an intramolecular binding involving the N-terminal SH2 domain. It has been shown that the inhibitory activity of the SH2 domain of SHP-1 is mediated by interaction of this domain with a tyrosine phosphorylated residue present in the C-terminal tail of the protein. Binding of this phosphotyrosine residue to N-SH2 results in the folding the enzyme and obstruction of the phosphatase domain. Furthermore, addition of a phosphotyrosyl peptide that interacts with the N-SH2 markedly enhances catalysis of

the enzyme. This was supported by the data showing that ligands containing two phosphotyrosine residues, interacting with both SH2 domains, are able to stimulate catalysis probably by releasing the inhibitory N-terminal SH2 domain by displacing the phosphotyrosine residue in the C-terminal tail. [33, 40] Sequencing analysis of the regions of normal and *me/me* SHP-1 gene indicated the absence of a cytidine residue at position 228 in the N terminal SH2 domain, and as a consequence, no functional SHP-1 protein is produced in *me/me* mice. [30]

B.2. Role of C-tail: In addition to the role of C-tail in the regulation of SHP-1 activity the C-tail plays also a role in the nuclear localization of the protein, although this is still controversial. The C-tail has a functional nuclear localization sequence (NLS). SHP-1 can be located in the nucleus in epithelial cells; however, in hematopoietic cells, SHP-1 remains in the cytoplasm (under basal conditions). However, upon cytokine stimulation, nuclear translocation of SHP-1 occurs with delayed kinetics (more than 1 hour post stimulation) in hematopoietic cells. This observation suggests that a new protein is synthesized to either assist nuclear import of SHP-1 or to facilitate activation of SHP-1. [40, 42, 43]

Fig 1.3: **Structure of SHP-1 protein:** The SHP-1 (68kDa protein) contains two N-terminal SH2 domains and one C-terminal PTP domain. Two phosphorylated sites in the C-terminal are shown. Numbers are indicators of nucleotide position. [30]

Figure. 1.3



C. Targets Of SHP-1: SHP-1 has been implicated in the regulation of signaling pathways through interacting with a number of receptors and protein tyrosine kinases, including ZAP70, CD3, CD5, and interleukin-2R in T cells; IL-3R, IL-4R, IL-13, IFN-αβ R, colony stimulating factor 1 receptors, erythropoietin receptor, stem cell factor receptor c-kit in hematopoietic cells; antigen receptor co-modulators such as CD22 and FcγRIIB, BCR, TCR, SLP76, and CD72 in B cells, the killer cell inhibitory receptor in NKcells, and cytosolic signaling molecules such as Vav and Grb2/Sos1 that contribute to Ras activation. [44, 48, 49, 113, 114] SHP-1 has been shown to negatively regulate the signaling cascades in hematopoietic cells activated by cytokines and antigens. For instance, there is hyperactivation of Src family kinases and hyper-responsiveness of thymocytes upon TCR stimulation in *me/me* mice. [45, 46] Also, in Jurkat T cells, TCR cross-linking promotes the SHP-1/ZAP70 kinase interaction, resulting in dephosphorylation of ZAP70 tyrosine kinase and activation of downstream signaling proteins including Fyn and Lck.[44]

Furthermore, SHP-1 is implicated in negative regulation of signaling pathways that promote myeloid cell growth, survival and activation. SHP-1 is also involved in suppression of β2-integrin mediated adhesion activity. This observation is based on the fact that SHP-1 deficient macrophages have been found to be hyper adherent to β2-integrin ligands. However, SHP-1 targets in integrin signaling remain to be understood.[33, 40, 47]

SHP-1 regulates many facets of myeloid cell behavior through interactions with the diverse range of molecules. For example, it is found that SHP-1 binds and negatively regulates growth factor receptor, such as the c-kit receptor (a protein tyrosine kinase

receptor), a conclusion consistent with an enhanced mitogenic signal in SHP-1 null bone marrow macrophages. [48] Similarly, studies have shown a marked increase of *me/me* macrophages in response to growth factors such as granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage stimulating factor (M-CSF), and IL-3. This was a consequence of hyper-phosphorylation of both M-CSF and IL-3 receptors in response to the stimulus. [33] These data showed that SHP-1 interacts with these signaling effectors to predominantly downregulate activation signals transduced via these receptors.

D. SHP-1 and Autoimmunity: As mentioned before, lack of SHP-1 function in *me/me* mice leads to a variety of hematopoietic cell abnormalities including a vast expansion of monocytes, erythrocytes and a paucity of B cells. However, there is an enrichment of $CD5^+$ B cells (B1B cells) in these mice, which is associated with autoantibody production and consequently autoimmunity development.

Studies in asthma disease have shown that there is a hyperactivation of $CD4^+$ Th2 cells which leads to allergic airway inflammation. [26, 50] Also, studies in heterozygous motheaten (*me/+*) mice, whose SHP-1 expression is lower than in wild type (about one third), have demonstrated that Th2 cell differentiation and Th2 cytokine production were enhanced compared to normal controls. [50, 115] Therefore, SHP-1 seems to act as a negative regulator in the generation of asthma phenotype. This is consistent with the context that in *me/me* mice, SHP-1 deficiency leads to severe inflammation and premature death consequent to hemorrhagic pneumonitis.

8. Overview

Cytokines are important mediators of immune responses and defects in cytokine production characterize many pathological conditions including development of autoimmunity. Motheaten (*me/me*) SHP-1 null mice, develop severe immune disorders and autoimmunity consequent, at least in part, to deregulated production of pro-inflammatory IL-6 and TNF-α cytokines and a regulatory cytokine IL-10. (Kozlowski's laboratory observations, unpublished, 110, 111)

Aberrant levels of IL-12 have been implicated in autoimmune diseases. To understand the involvement of SHP-1 in the regulation of IL-12 production, *me/me* mouse model was employed. *me/me* macrophages derived from bone marrow and spleen exhibit morphological and cellular changes suggesting deregulation of signal transduction cascades leading to the production of cytokines. SHP-1 is known to negatively regulate a number of signaling pathways activated by cytokine and antigen receptors. However, the signal transduction pathway(s) and signaling proteins responsible for the altered behavior of *me/me* macrophages remain largely unknown. To better define the role of this phosphatase in autoimmunity and in controlling the functions of monocytic/macrophage cell lineages we were interested in understanding how SHP-1 regulates LPS mediated induction of IL-12 in splenic macrophages. These are the underlying thoughts leading the research described in this thesis.

9. Rational: Lack of SHP-1 leads to autoimmunity in *me/me* mice. [30, 31] Lack of IL-12 has been implicated in the progression of autoimmunity. [27, 28] Therefore, autoimmunity in *me/me* mice may be due at least in part to deregulation of the immuno-regulatory cytokine IL-12.

10. Hypothesis

SHP-1 is an important positive regulator of IL-12p40 production in LPS stimulated macrophages.

11. Objectives

I. Loss of SHP-1 and IL-12 production

A. To compare the levels of IL-12p40 production in normal and *me/me* splenic macrophages stimulated with LPS.

B. To determine the effect of IFN-γ on LPS induced IL-12p40 production in normal and *me/me* splenic macrophages.

C. To interfere with SHP-1 expression in normal macrophages using anti-sense oligonucleotides and conversely to reconstitute SHP-1 expression in *me/me* macrophages using SHP-1 adenoviral vectors.

II. SHP-1 signaling in IL-12 production

To explore the signal transduction pathways regulated by SHP-1 and required for IL-12p40 production in LPS stimulated splenic macrophages.

III. SHP-1 in IL-12 gene regulation

To identify transcription factors regulating IL-12p40 gene transcription by employing deletion analyses of the IL-12p40 promoter.

MATERIALS AND METHODS

1. Animals

Wild type (WT) and *me/me* mice were bred and raised under specific pathogen-free conditions in the animal facilities of Health Canada, Ottawa, ON. Mice were bred and used according to the protocol approved by animal care committee Health Canada. Mice used for experiments were all from the C3H/HE-me strain (Jackson Laboratory, USA) that had been propagated by mating heterozygotes (*me/+*) for SHP-1 mutation. Mice generated from intercrosses of *me/+* were genotyped in our laboratory using genomic DNA obtained from the tail tissue of WT mice and mice bearing mutation at the SHP-1 locus using DNeasy Tissue kit (QIAGEN, Mississauga, ON). The ages of the *me/me* and WT mice at the time of sacrifice were between 9-12 days old. *me/me* mice can usually be recognized by 3 days of age, when one or several small abscesses appear on the surface of the skin.

2. Reagents:

LPS (Lipopolysaccharide) from E.coli was purchased from Sigma, St, Louise, MO, USA. IFN-γ was obtained from R&D systems (Minneapolis, MN, USA). Anti-sense SHP-1, anti-sense SHP-2 and anti-sense CD4 were made by the University Core DNA services at University of Calgary. Mouse monoclonal anti SHP-1 antibodies was purchased from BD Biosciences, Canada. Inhibitors (PTP-1, LY294002, SB203580, PD98059, SP600125) were all purchased from Calbiochem, Lajolla, CA. Inhibitors (SKF-96365, KN-93, cyclosporine A, W7, 2APB) were all obtained from Cedarlane Laboratories,

Hornby, ON, Canada. All the inhibitors upon arrival were reconstituted in DMSO or in water and kept in -20 ° C in dark.

3. Isolation and culture of splenic macrophages

Spleens were dissected from normal and *me/me* mice and crushed between glass slides. For each experiment at least 2-4 spleens were pooled. Single cell suspensions were placed in $75cm^2$ medium polystyrene flasks (NUNC Brand products). Splenic Mϕs were isolated and cultured in Optimem medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) (Cansera, Burlington, Ontario,), 1% penicillin-streptomycin (Gibco), 3 μL of 2-mercapto-ethanol (Sigma, St Louise, MO), supplemented with 20% L929CLL1 stimulation medium (mouse cell line strain L) at 37°C in a 5% CO2/ air mixture. Medium was changed every 3-4 days until the cells become confluent.

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

$1x10^6$ splenocytes derived from either normal or *me/me* spleens were cultured in 200μL of Optimem medium containing 10% FBS, 1% penicillin- streptomycin, 3 μL of 2-mercapto-ethanol (0.5g/L) + 20% L929 in 96 well flat bottom plates and incubated at 37°C in a 5% CO2/air mixture in for 3 days until they were differentiated toward macrophages. After 3 day incubation, cells were washed thoroughly with 200 μL of PBS (phosphate buffered saline) (Gibco), resuspended in Optimem without L929. Then, the cells were stimulated with 1μg/mL LPS for 24 hours. If there was any treatment with inhibitors, they were added at specific concentrations 2 hours prior to LPS stimulation. Each condition was conducted in four replicas. Cell supernatants were collected and

assayed for IL-12p40 production, using mouse IL-12-p40 DuoSet (R&D Systems) according to the manufacture's protocol. The ELISA consisted of rat anti-murine IL-12p40 capture antibody (Ab), biotinylated goat anti-mouse IL-12p40 Detection Ab, and Horse radish peroxidase (HRP)-conjugated streptavidin detection reagent. The plates were coated overnight with the capture antibody at 4°C at a final concentration of 4 μg/mL. The plates were washed three times with 300 μL of wash buffer [0.05% Tween 20 (Sigma) in PBS, PH=7.2-7.4] Then they were blocked by adding 300 μL of Reagent Diluent (1%BSA in PBS, PH=7.2-7.4), washed three times as described earlier, and dried under vaccum for 10 min. 100 μL of IL-12p40 Standard in reagent diluent or samples without any dilutions were added and incubated for 2 hours; the aspiration/wash was repeated for 3 times; 100 μL of Detection Ab at a final concentration of 400 ng/mL was added and incubated for 2 hours; the aspiration/ wash was repeated again for 3 times; 100 μL of the working dilution of streptavidin-HRP was added and incubated for 20 min. ELISAs were developed with R&D substrate reagents. The reaction was stopped by stop solution (2N H_2SO_4) and absorbance was determined, using a microplate reader (MRX revelation, Dynex Technologies) set to 450 nm. The mean of duplicate wells were calculated based on a standard curve that was constructed for each assay, using recombinant murine IL-12p40 standard included in the kit. (R&D Systems)

5. RT-PCR (reverse transcription-polymerase chain reaction)

A. RNA extraction:

RNA was extracted from cell cultures after different time courses of LPS stimulation, using RNeasy Mini Kit (QIAGEN) as per the manufacture's protocol. The quality and quantity of complementary DNA is critically dependent on the integrity of mRNA

(messenger RNA) used as the template. Therefore, there are certain precautions that should be taken to prevent the risk of RNase contamination. Disposable gloves should be used at all times and changed often. All glassware, nondisposable plasticware and electrophoresis apparatus should be kept separately from other labware. Glassware should be soaked and rinsed in a solution of 0.1% diethyl pyrocarbonate (DEPC) and autoclaved. To remove DNA contamination from RNA, RNA samples were treated with 1 μL of (10x) DNA reaction buffer and 1 μL DNAaseI Amplification grade, using DNAaseI Amplification Grade kit (Invitrogen, Carisbad, CA) at room temperature (RT) for 15 minutes (min). Then 1 μL of EDTA (2 mM) was added to the mixture and incubated for 10 min at 65°C. The RNA samples were stored at -80°C freezer.

B. complementary DNA (cDNA) synthesis [Reverse transcriptase(RT) reaction, using cDNA Cycle^{TM} kit], (Invitrogen) used a recommended by supplier.

B1. Before performing the RT reaction, 5 μg of total sample RNA + 1 μL oligo dt primer in a 11.5 μL volume and 2 μL of control RNA + 9.5 μL of water + 1 μL of random primer were heated at 65°C for 10 min and quenched on ice for 2min.

B2. The following components were set up in a sterile RNAase-free microcentrifuge tubes and added to both mRNA sample and control reaction (Test RNA).

RNAase inhibitor (1 μL), (5x) RT buffer (4 μL), (100 mM) dNTP (deoxy nucleotide tri phosphate) (1 μL), Sodium pyrophosphate (1 μL), and AMVRT (Avian myeloblastosis virus reverse transcriptase) enzyme (1 μL).

The samples were kept at 42°C for 60 min, and cDNAs were denatured at 95°C for 2 min, using PTC-200 Peltier Thermal Cycler, MJ Research.

C. PCR Reaction:

The mRNA sample and control reactions were prepared as described below:

mRNA sample: RT sample (3 μL), PCR buffer (5 μL), dNTPs (1 μL), mouse IL-12p40 Primer Pair™(4 μL) [provided from R&D Systems) , Taq DNA polymerase(1 μL), and Nuclease-free water to final volume of 50 μL.

Control Reaction: RT sample (2 μL), PCR buffer (5 μL), dNTP (1 μL), forward Primer (4 μL), reverse primer (4 μL), Taq DNA polymerase (1 μL), Nuclease-free water to a final volume of 50 μL.

In addition, as a control the transcripts for constitutively expressed β-actin gene were examined in each sample to evaluate the integrity of the cDNA as well as to determine the comparability of RNA input for each cDNA synthesis.

The reactions were all carried out in the PTC-200 Peltier Thermal Cycler, MJ Research.

The PCR program used in the experiment was as follows: denaturation at 95°C for 2 min, annealing at 58°C for 1 min, extension at 72°C for 1 min, 27 cycles of denaturation at 95°C, and final elongation at 72°C for 7 min.

The PCR products were electrophoresed on 1.2% agarose gel and visualized by ethidium bromide staining under ultraviolet light. The gel was scanned using Typhoon phosphoro imager, (Amersham, Montreal, Quebec). The digital image was further analyzed and the density of each band was determined. The peak height and the area measurements were determined for each band on the gel track using Gelbase software and the ratios of IL-12 message to β-actin were calculated.

6. Adenoviral (Ad) transduction of splenic macrophages

*A. **ELISA:*** $5x10^5$ splenocytes from *me/me* mice were placed in 24-well plates in triplicate, and grown in 500 μL conditioned medium as described earlier. After 3 day culture, the medium was aspirated, and splenic Mϕs were washed with 500 μL PBS and resuspended in 200 μL Optimem medium, containing 10% FBS, without L929. Then the macrophages were infected with increasing MOIs, Multiplicity of Infection, (0, 5, 10, 20, 50, and 100) of [Ad-GFP (Green florescent protein), Ad-SHP1, Ad-Dominant negative SHP-1 (substrate trapping SHP-1 mutant), and Ad-Dominant negative SHP-1 (phosphatase inactive mutant)]. Adenoviral constructs were all provided by Dr Peter Liston (University Of Ottawa, CHEO, Ottawa, ON). Then the plates were incubated at 37°C in a 5% CO2/ air mixture. After adding viruses to splenic macrophages, plates were rocked every 15 min and put back to the incubator. This was done for a total time period of 90 min. Following 90 min incubation, the volume was increased to 500 μL and the cells were further incubated for 24 hours. The next day, splenic macrophages were stimulated with 1 μg/mL LPS for 24 hours. Following the stimulation the cell supernatants were collected and examined for IL-12p40 production, using IL-12p40 specific ELISA kit, as described earlier.

In addition, for Ad-GFP transduction, cells were viewed under a florescent microscope (kindly provided by Dr. Peter Liston). The procedure was as follows: After transduction of Mϕs with Ad-GFP, cells were washed with PBS twice and fixed by 250 μL of 1% glutaraldehyde in PBS at 4°C for 10 min. Then the cells were stained in X-gal buffer (1 mg/mL X-gal, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2mM $MgCl_2$, 0.05% TritonX-100 in PBS, PH=7.2-7.5) at 37°C incubator for 4 hours, and photographed under

microscope. All the reagents, used in this protocol, were provided from Dr. Liston's laboratory.

B. Western Immunoblot analysis:

B.1. Preparation of cell lysates: After transduction of splenic Mϕs with Ad-SHP1, Ad-Dominant negative SHP-1 (substrate trapping SHP-1), and Ad-Dominant negative SHP-1 (phosphatase inactive mutant), cells were harvested from the wells 48 hours post-transduction, using scraper. Splenic Mϕs were lysed with Laemmli sample buffer (Bio Rad), boiled for 5 min, and sonicated in water bath for 10-15sec. The lysates were stored at -80° C.

B.2. Polyacrylamide gel electrophoresis (PAGE): Protein analysis was performed in 10% SDS-PAGE resolving gel. The resolving gel was composed of deionized distilled water, 4X lower gel buffer [91g ultra pure Tris Base (Gibco), 2 g electrophoresis purity reagent SDS (Bio-Rad) bringing the PH to 8.81 in a final volume to 500 mL with deionized distilled water], 30% Acrylamide/Bis solution (Bio-Rad), TEMED (GibcoBRL), 10% APS (Ammonium Persulfate, Bio-Rad). The 5% stacking gel was composed of deionized distilled waster, 30% Acrylamide/Bis solution, stacking gel buffer (60.6 g ultra pure Tris Base, bringing the PH to 6.8, in a final volume of 500 mL with deionized distilled water), 10% electrophoresis purity reagent SDS, TEMED, and 10% APS. Prestained broad range protein markers (Bio-Rad) were used to estimate molecular mass. Electrophoresis was carried out in 1X Running buffer (1/10 dilution of 10X Tris/Glycin/SDS buffer solution, Bio-Rad) at 107 volt for approximately 2 hours until the bromophenol blue dye could be seen dripping off the bottom edge of the gel.

B.3. Electrophoretic Transfer: The samples were analyzed on sodium dodecyl sulfate (SDS)–10% polyacrylamide gels electrophoresis (SDS-PAGE) following which resolved proteins were transferred to an Immobilon transfer membrane (Millipore, Bedford, MA, USA) at 100 volts for 1 hour, using 1X Transfer buffer (1/10 dilution of 10X Tris Glycin buffer; Bio-Rad).

B.4. Immunoblotting: The membrane was blocked with 15 mL of Blocking buffer [5% bovine serum albumin in 1X TBST (containing1M Tris-HCl, 5 M NaCl, 0.05% Tween 20)] for 1 hour at RT on a shaker. The membrane was probed by using mouse monoclonal anti-SHP-1 antibody at RT for 1 hour. Three 10 minute washes with 1X TBST were performed at RT on a shaker. The membrane was incubated with HRP conjugated goat anti-mouse polyclonal antibody (Bio-Rad, Richmond , CA) for 1 hour at RT on a shaker. Three 10 minute washes with 1X TBST were repeated. The specific proteins were visualized by incubating the membrane in the ECL solution (enhanced chemiluminescence detection) (Santa Cruz Biotechnology, CA) at RT for 2 min and exposed for various amounts of time to BioMax X-ray film (Kodak). The film was developed using the Kodak M35A X-omat Processor.

7. Preparation of constructs for IL-12p40 promoter

A. PCR: Full length (FL) and one 5' deletion mutant (Del1) of murine IL-12p40 promoter region; fragment -983 to +355 base pairs (bp) (see Fig.2.1); Gene Bank^{TM}accession number (S79628); was amplified by PCR method from mouse tail genomic DNA.

The following conditions were used for the 50 μL PCR reaction: 5 μL of 10x High fidelity (HF) PCR reaction buffer, 0.5-1 μL of DNA template, 0.5 μL of each 5' and 3' primers (10 μM each), 0.25 μL of 100 mM dNTPs, 1 μL of 50X Advantage-HF Polymerase Mix, and 42.25-42.75 μL of Ultra-purified water. The reagents used for PCR were all obtained from Advantage-HF PCR kit, BD Biosciences Clontech. The dNTPs used, was from Invitrogen Inc. The primers with restriction sites (XhoI and HindIII) used to amplify the murine IL-12 promoter fragments were designed using Sci Ed Central software and shown in Table1. All the primers were purchased from the University Core DNA services in University of Calgary.

Table1-The sequence of each murine IL-12 promoter 5' primer

Primer Name	Position	Sequence
Full length	-983(bp)	5'AAAAAACTCGAGCAGGCCAGGACAGGAATGGA3'
Deletion1	-378(bp)	5'AAAAAACTCGAGGTGGCATGAACCATGAACTC3'

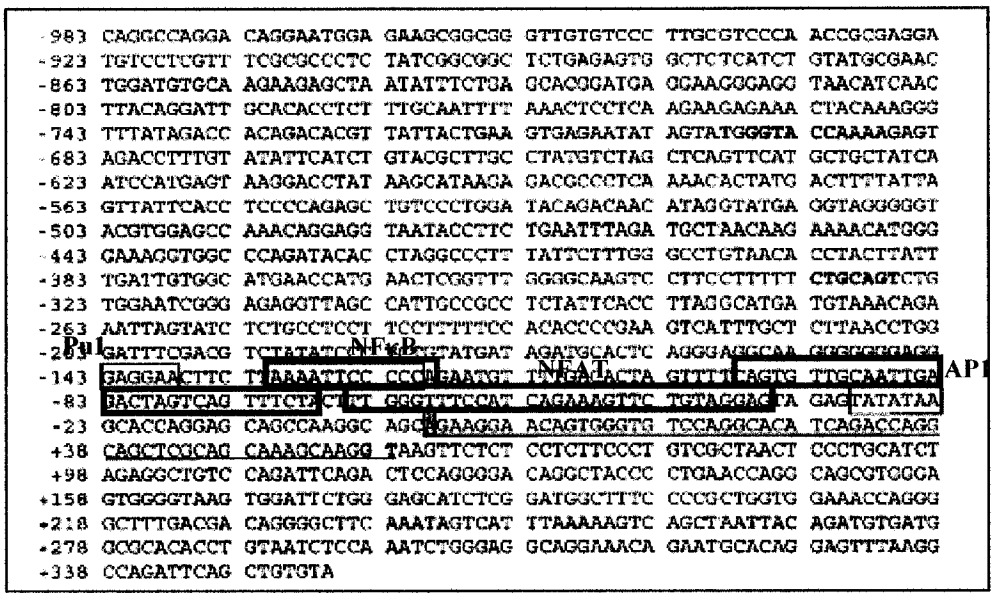
FL and Del1 were generated using the same 3' primer positioned at +355bp (5'AAAAAAAAGCTTTGTTCCTTCTGCTGCCTTGG-3').

The PCR reactions were carried out in the PTC-200 Peltier Thermal Cycler, MJ Research with the following program: denaturation at 94°C for 2 min, 30 cycles of denaturation at 94°C for 20 sec, annealing at 60°C for 30 sec, and extension at 72°C for 3 min, and final elongation at 72°C for 7 min.

Figure2.1: murine IL-12p40 promoter region (fragment -983 to +355)

A schematic representation of the murine IL-1240 promoter. Gene Bank™accession number (S79628). Murine IL-12p40 promoter-luciferase plasmids were generated by PCR using murine genomic DNA. Genomic sequence of the murine p40 promoter region. Numbers are with reference to the start site of transcription. Boxes indicate, respectively, a Pu.1consensus site, an NF-kB consensus site, AP1 consensus site, NFAT consensus site, and a TATA box, and the start site of transcription (shaded).

Figure.2.1


-983 CAGGCCAGGA CAGGAATGGA GAAGCGGCGG GTTGTGTCCC TTGCGTCCCA ACCGCGAGGA
-923 TGTCCTCGTT TCGCGCCCTC TATCGGCGGC TCTGAGAGTG GCTCTCATCT GTATGCGAAC
-863 TGGATGTGCA AGAAGAGCTA ATATTTCTGA GCACGGATGA GGAAGGGAGG TAACATCAAC
-803 TTACAGGATT GCACACCTCT TTGCAATTTT AAACTCCTCA AGAAGAGAAA CTACAAAGGG
-743 TTTATAGACC ACAGACACGT TATTACTGAA GTGAGAATAT AGTATGGGTA CCAAAAGAGT
-683 AGACCTTTGT ATATTCATCT GTACGCTTGC CTATGTCTAG CTCAGTTCAT GCTGCTATCA
-623 ATCCATGAGT AAGGACCTAT AAGCATAAGA GACGCCCTCA AAACACTATG ACTTTTATTA
-563 GTTATTCACC TCCCCAGAGC TGTCCCTGGA TACAGACAAC ATAGGTATGA GGTAGGGGGT
-503 ACGTGGAGCC AAACAGGAGG TAATACCTTC TGAATTTAGA TGCTAACAAG AAAACATGGG
-443 GAAAGGTGGC CCAGATACAC CTAGGCCCTT TATTCTTTGG GCCTGTAACA CCTACTTATT
-383 TGATTGTGGC ATGAACCATG AACTCGGTTT GGGGCAAGTC CTTCCTTTTT CTGCAGTCTG
-323 TGGAATCGGG AGAGGTTAGC CATTGCCGCC TCTATTCACC TTAGGCATGA TGTAAACAGA
-263 AATTAGTATC TCTGCCTCCT TCCTTTTTCC ACACCCCGAA GTCATTTGCT CTTAACCTGG
-203 GATTTCGACG TCTATATCCT TGGCGTATGAT AGATGCACTC AGGGAGGCAA GGGGGGAGG
-143 GAGGAACTTC TTAAAATTCC CCCTGAATGT TNFGACTA GTTTTCAGTG TTGCAATTGA
-83 GACTAGTCAG TTTCTCCTCT GGGTTTCCAT CAGAAAGTTC TGTAGGAGTA GAGTATATAA
-23 GCACCAGGAG CAGCCAAGGC AGCTGAAGGA ACAGTGGGTG TCCAGGCACA TCAGACCAGG
+38 CAGCTCGCAG CAAAGCAAGG TAAGTTCTCT CCTCTTCCCT GTCGCTAACT CCCTGCATCT
+98 AGAGGCTGTC CAGATTCAGA CTCCAGGGGA CAGGCTACCC CTGAACCAGG CAGCGTGGGA
+158 GTGGGGTAAG TGGATTCTGG GAGCATCTCG GATGGCTTTC CCCGCTGGTG GAAACCAGGG
+218 GCTTTGACGA CAGGGGCTTC AAATAGTCAT TTAAAAAGTC AGCTAATTAC AGATGTGATG
+278 GCGCACACCT GTAATCTCCA AATCTGGGAG GCAGGAAACA GAATGCACAG GAGTTTAAGG
+338 CCAGATTCAG CTGTGTA
PuI
NFκB
NFAT
AP1

B. Agarose gel electrophoresis: The result of the PCR was checked by running 1.2% agarose gel electrophoresis in 1X Tris-Acetate buffer containing ethidium bromide (TAE; 40 mM Tris-acetate, 1 mM EDTA, PH=8). Samples were prepared by combining 3-5 μL of the PCR product with 2-3 μL of gel loading buffer. Then gel was photographed by using an AEphaEase™ imager with a U.V.light box (Alpha Innotech Corporation).

C. Purification of DNA: PCR purification was performed, using QIAquick PCR purification kit (Qiagen), prior to DNA cloning into pCR® 2.1 vector. PCR purification protocol was provided along with each kit and no changes were made away from the steps recommended. The purity of DNA fragment was verified by running the samples on 1.2% agarose gel electrophoresis as described in section B.

D. cloning of the IL-12p40 promoter fragments into pCR® 2.1 vectors: The following conditions were used for the ligation of IL-12p40 (FL) promoter or Del1 in a total volume of 10 μL: 1 μL of 10X Ligation buffer, 0.5 μL of pCR® 2.1 vector (25ng/μL), 1-2 μL of purified DNA product, 0.5 μL T4 DNA ligase, and 7.5 μL of Ultra-purified water. All the reagents were obtained from TA cloning® kit (Invitrogen Inc) and were used according to manufacture recommendation. Ligation was performed in the PTC-200 Peltier Thermal Cycler, MJ Research at 15°C overnight. Following ligation, XL1-Blue E coli cells were transformed with either FL or Del1 ligation mixtures.

E. Transformation of XL1-Blue Chemically Competent E.coli Cells:

The competent cells are extremely fragile, so it is very important that a great care is taken while working with these cells. Therefore, no deviation was made away from the protocol, provided in the kit. The plasmids were introduced into cells in a heat shock step.

The volume of plasmid DNA added to the reaction was limited to a maximum of 10% of the total cell volume. Then the bacterial cells were cultured in a small volume to allow for the expression of the antibiotic resistance gene encoded by the plasmid. 200 μL of the transformed cell mixture was spread on 2YT-Broth (Yeast Trypton) medium (Gibco) agar plates supplemented with 50 μg/mL ampicillin (Sigma, dissolved in autoclaved water and filtered with 0.2 μm filter paper), using a sterile spreader. Transformants containing IL-12p40 inserts were recognized by white color of colonies arising on 2YT-Broth ampicillin plates supplemented with IPTG (20 mM) and X-gal (80 μg/mL) , both from Invitrogen Inc To ensure even spreading of IPTG and X-gal on agar plates, 100 μL of S.O.C medium (0.5% Yeast extract, 2.0% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM $MgSO_4$, 20 mM glucose), a complex cell growth medium that ensures maximum transformation efficiency, (Invitrogen Inc) was loaded onto the plate prior to addition of 7.77 μL of 432 mM IPTG and 40 μL of 5% X-gal. This step was necessary properly dissolve IPTG and X-gal. Then the plates were incubated at 37°C overnight. The white colonies are indicator the pCR® 2.1 vector containing insert while the blue colonies indicate the presence of pCR® 2.1 vector without any insert.

F. Restriction enzyme analysis of the insert in pCR® 2.1 vector: 6-7 white colonies were picked for plasmid DNA isolation. For this, white colonies were inoculated into 2 mL of 2YT-Broth containing ampicillin (50 μg/mL) and cultured on a shaker at 37°C overnight. Overnight cultures were then used for mini-preparation of plasmids (using GenElute™ Plasmid Mini-prep Kit, obtained from Sigma). The procedure was followed exactly as on the protocol provided in the kit. To confirm the insertion of murine IL-12p40 promoter (FL or Del1) into the pCR® 2.1 vector, restriction digestion of

the plasmids with XhoI and HindIII restriction enzymes was carried out at 37°C overnight. The restriction enzyme reaction was as follows: 11.5 μL of DNA plasmid, 1.5 μL of 10X buffer B, and 1 μL of each XhoI and HindIII and the total volume was adjusted with water to 15 μL. The completion of plasmid DNA digestion was confirmed by DNA gel electrophoresis. For subcloning, DNA fragments corresponding to IL-12p40 promoter were isolated using Qiaex® II Gel Extraction Kit (Qiagen). Protocol of gel extraction was provided along with each kit and no changes were made away from the steps recommended. DNA was loaded onto 1.2% agarose gel for extraction of desired promoter fragments. The bands corresponding to either FL or Del1 were then cut from the agarose gels in agar and purified according to the instructions provided by Qiaex® II Gel Extraction Kit. After DNA gel extraction, purified DNA was quality checked by 1.2% agarose gel electrophoresis by loadind 3-4 μL of the final product onto the gel. Purified fragments were then ligated with PGL3-Basic vector (Promega, Maidson, WI). The ligation reaction was as follows: 1 μL of 10X ligation buffer, 1μL of PGL3-Basic vector, 5-7.5 μL of purified DNA fragment, 1 μL of T4 DNA ligase, and the total volume was adjusted to 10 μL with ultra-purified water. Ligation was performed at 15°C overnight. Then ligation mixture was used for transformation XL1-Blue cells.

G. Transformation of XL1-Blue chemically competent E.coli cells: The protocol for transformation was exactly the same as mentioned earlier except that X-gal and IPTG were not included in the 2YT ampicillin plates.

After overnight incubation of plates at 37°C, the colonies cells were analyzed for the presence of the desired plasmids.

H. Verification of DNA inserts in the PGL3-Basic vector containing luciferase reporter gene: 6-7 colonies were picked and separately cultured in 2 mL of 2YT-Broth containing ampicilin (50 μg/mL) on a shaker at 37°C overnight, followed by mini-preparation of DNA. The protocol for mini-preparation was the same as mentioned before. To confirm the insertion of murine IL-12p40 promoter (FL or Del1) into the PGL3 basic vector, restriction digestion of the plasmids with XhoI and HindIII restriction enzymes was carried out at 37°C overnight. The restriction enzyme reaction was as follows: 5 μL of DNA plasmid, 1 μL of 10X buffer B, 1 μL of HindIII, 1 μL of XhoI, and 2 μL of ultra-purified water to reach the total volume of 10 μL. The completion of plasmid DNA digestion was confirmed by DNA gel electrophoresis.

I. Maxi-preparation (maxi-prep) of plasmids: After confirming the presence and correct size of DNA insert in PGL-Basic vector maxi-preparation of plasmid DNA was performed, to obtain sufficient amount of DNA to do the transfection, using EndoFree® Plasmid Maxi Kit (Qiagen). The procedure of maxi-prep was identical to the protocol provided in the kit. The final DNA pellet was vacuum-dried at medium heat for 5 min. DNA was dissolved in 300-500μL of EndoFree®TE buffer, and the concentration was measured using spectrophotometer. If DNA plasmids were used frequently, they were stored in 4°C to prevent from freeze and thaw cycle.

8. Transfection of RAW264.7 cells:

A. cell culture: RAW264.7 cells, a mouse macrophage cell line established from ascites of a tumor induced in a male BALB/c mouse by the intraperitoneal injection of Abelson murine leukemia virus, were purchased from the American Type Culture Collection (ATCC) (Rockville MD). The cells were grown in Optimem medium

containing 10% FBS, 1% penicillin-streptomycin, and 3 μL of 2-mercapto-ethanol. RAW264.7 cells were grown in $75cm^2$ medium sized flasks in a 5% CO2/air mixture.

B. Transfection of plasmids and expression of luciferase reporter gene: $2x10^6$ of RAW264.7 cells were placed in 6 well plates and grown in 2 mL of Optimem medium, containing 10% FBS and 3 μL of 2-mercapto-ethanol. The plates were incubated in a 5% CO2/air mixture for 24 hours. After 1 day incubation, cells were washed with 2mL PBS and resuspended in Optimem media +10% FBS. No antibiotics were added to media during transfection as this would cause cell death. Transfection of RAW264.7 cells was carried out, using Lipofectamine 2000 reagent (Life technologies) according to its 6 well plate protocol. To measure luciferase activity 2 μg of FL/PGL3 or its deletion constructs, were co-transfected with 2 μg of pSV-β-galactosidase control vector in RAW264.7 cells. Cells were stimulated with 1 μg/mL LPS post- transfection and harvested using scraper 24 hours following stimulation. When inhibitors were used, they were added at specified concentrations 2 hours prior to stimulation with LPS. The samples were stored at -80°C until ready to prepare lysates.

C. Luciferase assay: The reagents were provided by luciferase assay system (Promega, Maidson, WI). Luciferase assay procedure was carried out as follows: 50 μL of 1X Reporter Lysis buffer was added to frozen cell pellets; pellets were thawed by incubating at 37°C water bath. Cell pellets were then put at -80°C for 10-15 min and allowed to thaw in a 37°C water bath for 5 min. All the lysates were then vortexed for 10-15 sec; and followed by spin at 12000 rpm in the eppendorf bench top microfuge for 15 sec. Finally, the supernatants were transferred to fresh 0.5 mL eppendorf tubes. 20 μL of each supernatant was placed in a Corning/Costar 96 well assay plate (Dynex plate) and

used for luciferase and β-gal assay, respectively. The luciferase activity was measured using Lumistar instrument (BMG, LAB Tech) and Lumistar Galaxy software.

D. β-galactosidase assay: The reagents were provided from β-galactosidase Enzyme Assay System (Promega). The protocol used was as follow: 20 μL of 2X assay buffer was added to lysates and to the β-galactosidase reagent that comes with kit as a positive control. The plate was then covered by adherent plastic seal and incubated at 37°C incubator (no CO2) for 30 min. The reaction was stopped by adding 150 μL of 1M Na_2CO_3 solution. Activity of β-galactosidase activity is measured by the conversion of the X-gal substrates which is manifested by the development of the yellow color in the wells. The absorbance reading was done using Dynex MRX Revelation Plate Reader (405 nm).

To calculate the RLU (relative luciferase units) for each condition, the mean luciferase activity was divided by the mean β-galactosidase activity.

9. Electrophoretic Mobility Shift Assay:

A. Preparation of Nuclear extracts: Normal and *me/me* Splenic macrophages were grown in 75cm^2 medium polystyrene flasks until cells became confluent (cells were cultured in 3 medium flasks for each experimental condition). Macrophages were scraped, suspended in PBS buffer and spinned at 2000 rpm for 5 min. Following centrifugation the cell pellets were resuspended in 1 mL of Optimem medium (without FBS) and stimulated with 1 μg/mL LPS for different times at 37°C incubator. After LPS stimulation, the cells were spinned at 4 °C for 5 min at 200x g. The cell pellets were washed in 1 mL of cold working buffer A [10 mM Hepes (Gibco), 10 mM KCL, 1.5 mM $MgCl_2$, 0.5 mM DTT (Gibco), 0.5 mM PMSF (Sigma), PH=7.9], spinned at 4 °C for 5

min at 200X g, resuspended in 25 μL of working buffer A + 0.1% NP40, and lysed on ice for 10 min. Then, the cells were spinned at 4 °C at 20000Xg for 5 min, and the supernatants, containing cytoplasmic proteins, were removed. The cell pellets containing nuclei were resuspended in 25 μL of cold working buffer B[20mM Hepes, 420 mM NaCl (EMD, Gibbstown), 1.5 mM $MgCl_2$, 0.2 mM EDTA (Gibco), 25% glycerol, 0.5 mM DTT, 0.5mM PMSF, 0.5 mM Spermidine (Sigma) (, 0.15 mM Spermine (Sigma), 5 μg/mL Aprotinin (Sigma), 5μg/mL Leupeptin, 5 μg/mL PepstatinA (Sigma)] (PH=7.9-8.2); kept on ice for 15 min, mixed gently and spinned at 4 °C at 20000xg for 10 min. Finally, the supernatants consisting of nuclear proteins were collected and stored at -80°C.

Procedure and all the reagents for Electrophoretic Mobility Shift Assay (EMSA) was provided by the LightShift Chemiluminescent EMSA Kit (Pierce Biotechnology, Rockford, IL, USA).

B. Binding Reactions: 5 μg of nuclear proteins were mixed with 20 femtomoles (fmol) of each biotin labeled NFAT, NFκB and AP1 oligunucleotide probes at room temperature for 20 min. The oligonucleotide sequences corresponding to NFAT, NFκB and AP1 binding sites within the murine IL-12 p40 promoter were as follows: **NFAT**, 5'-TTG GGT TTC CAT CAG AAA GTT CTG TAG GAG-3'and 3'-ACC CCA AAG GTA GTC TTT CAA GAC ATC CTC -5'; **NFκB**, 5'-CTT CTT AAA ATT CCC CCA GAA TGT TTT GAC-3' and 3'-GAA GAA TTT TAA GGG GGT CTT ACA AAA CTG-5'; **AP1**, 5'-CAG TGT TGC AAT TGA GAC TAG TCA GTT TCT-3' and 3'-GTC ACA ACG TTA ACT CTG ATC AGT CAA AGA-5'. [89, 90] All the oligonucleotides were made by the university core DNA services in University of Calgary.

The reaction mixture (20μL) consisted of Ultrapure Water, 2 μL of 10X Binding buffer, 1 μL of Poly (dI.dC), 5 μg of protein extract, and 20 fmol of Biotin End-Labeled Target DNA. After 20 min binding reaction, 5 μL of 5X Loading Buffer (obtained from the kit) was added to the mixture.

C. Electrophorase Binding Reactions: The DNA-protein complexes were subjected to a 6% polyacrylamide gel [3mL of 30% Acrylamide(Bio Rad, Hercules, CA), 1.5 mL of 5X TBE (Tris Borate electrophoresis buffer), 10.250 mL of autoclaved water, 200 μL of 10% APS, and 50 μL of Temed (Gibco)] electrophoresis in 0.5X TBE. 5X TBE was prepared as follow: Tris base (450 mM) (Sigma), Boric Acid (450 mM) (AnalaR® EM Sience, Darmstadt, Germany), PH=8.3. Before running the samples, the gel was pre-electrophorased for 60 min.

D. Electrophoretic Transfer of Binding Reactions to Nylon Membrane: The nylon membrane (Biotrans™ Nylon membranes, ICN, Irvine, CA) was soaked in 0.5X TBE for 10 min. Electrophoretic transfer was performed at 100 V for 45 min.

E. Cross-link Transferred DNA to Membrane: The membrane containing transfer DNA was placed on a dry paper towel with the bromophenol blue side up and the DNA was cross-linked at120mJ/cm^2 using a commercial UV-light cross linker instrument for 45-60 sec.

F. Detection of Biotin-labeled DNA by Chemiluminescent: The steps of this stage were identical to the protocol provided in the LightShift Chemiluminescent EMSA Kit.

10. Statistical analysis: Means were compared by Student's T test. The results are expressed as means ± Standard deviation (SD).

Results

I. Loss of SHP-1 and IL-12 production:

1.1. Decreased level of IL-12p40 production in SHP-1 null *me/me* macrophages

To evaluate whether SHP-1 participates in the regulation of IL-12p40 production in splenic macrophages, I investigated the levels of IL-12p40 production in normal and SHP-1 null splenic macrophages following LPS stimulation. LPS binds to TLR4/CD14 receptor complex resulting in activation of macrophages through stimulation of signal transduction pathways resulting in induction of cytokine genes and secretion of cytokines. (Fig 3.1) Macrophages were obtained from spleens, dissected from young (12-14 days old) normal and *me/me* mice. Macrophage cells were stimulated with 10 μg/mL of LPS for 24 hours and culture conditioned medium was assessed for IL-12p40 secretion by ELISA. The data demonstrated that *me/me* splenic macrophages upon LPS stimulation exhibit 3 fold (75%) suppression of IL-12p40 production compared to normal controls. (Fig3.2)

Figure 3.1. LPS signaling and cytokine secretion: The LPS binding protein conveys LPS to CD14. LPS then comes into contact with TLR4 and MD2. This LPS/TLR4 interaction leads to activation of various signaling proteins and as a result activation of different transcription factors inducing multiple cytokine gene expression.

Figure. 3.1

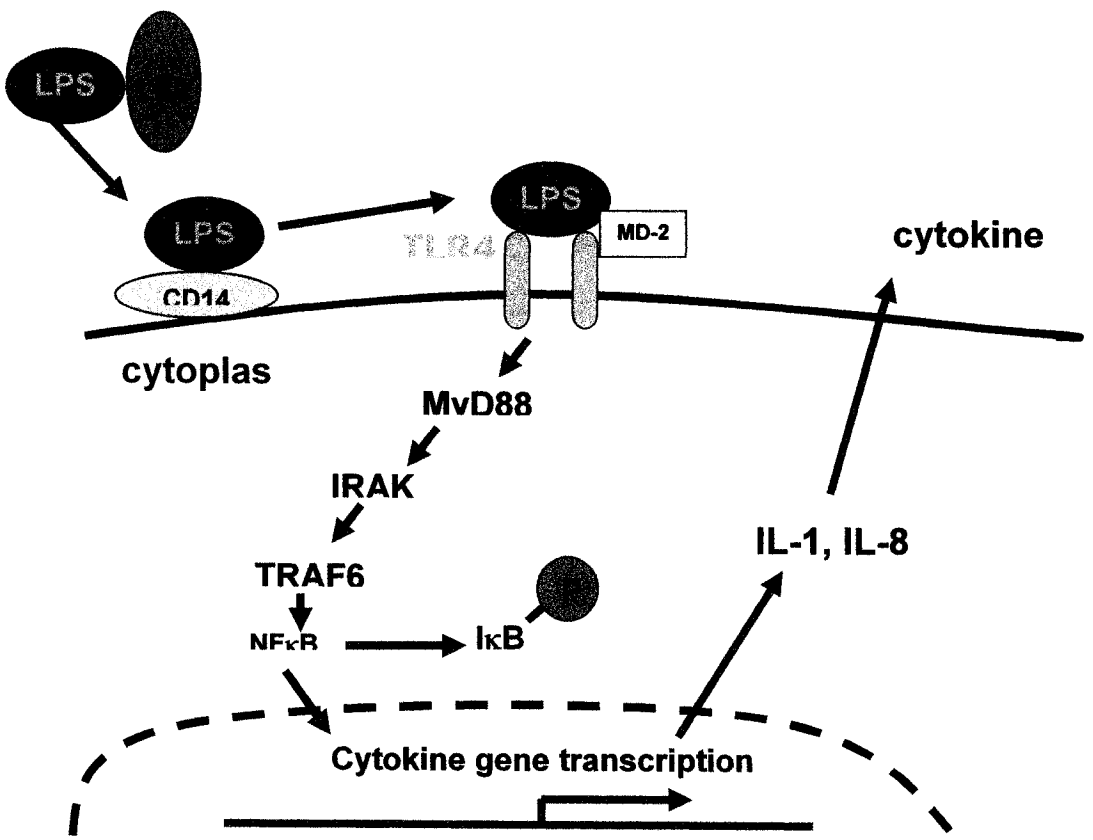
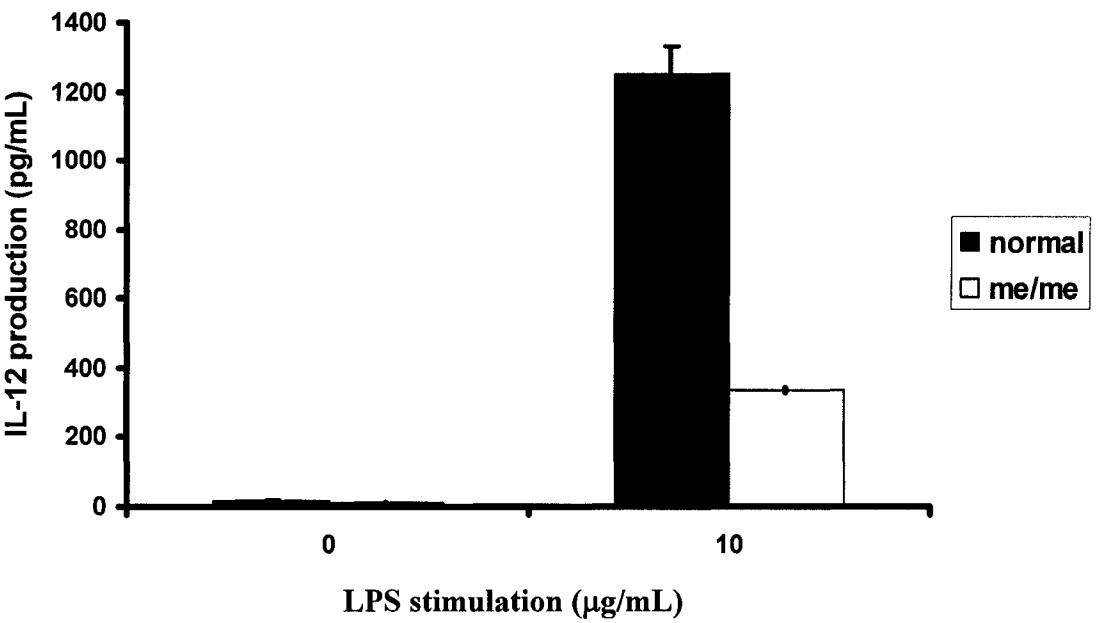

LPS
LPS
CD14
LPS
TLR4
MD-2
cytokine
cytoplas
MyD88
IRAK
TRAF6
NFκB
IκB
IL-1, IL-8
Cytokine gene transcription

Fig 3.2. IL-1240 production in normal and *me/me* splenic macrophages stimulated with LPS for 24 hours:

Spleens of normal and *me/me* mice were removed and crushed between glass slides. 10^6 cells were suspended in 200 μL of Opti-Mem media +10% FBS +20% CLL-1, and incubated in 96 well plates at 37°C in a 5% CO2 air mixture for 3 days. Macrophages were left unstimulated or stimulated with 10 μg/mL LPS for 24 hours. The cells for every condition were seeded in four wells. Cell supernatants were collected and IL-12p40 production was measured by ELISA. The absorbance was determined, using a microplate reader set to 450 nm. The mean of duplicate wells were calculated based on a standard curve that was constructed for each assay. Error bars represent standard deviation. This experiment has been done three times. Similar results were obtained in a total of three independent experiments. One of three independent experiments with similar results is shown.

Figure.3.2


1400
1200
1000
800
600
400
200
0
IL-12 production (pg/mL)
0
10
LPS stimulation (μg/mL)
normal
me/me

1.2. Suppressed IL-12p40 production in SHP-1 null macrophages does not depend on LPS dose or duration of stimulation

To assess whether different doses of LPS had differential effects on the IL-12p40 production from activated macrophages, splenic macrophages were cultured with various concentrations of LPS (0, 0.1, 1, 5, 10, 12.5, 25, and 50 μg/mL). Then, IL-12p40 production was examined in the supernatants by ELISA after 24 hours of LPS stimulation. As shown in Figure 3.3a, the data revealed that decreased level of IL-12p40 production in *me/me* splenic macrophages, compared to normal controls, was not dependent on LPS dose. This experiment also confirmed earlier observation and showed that more than 80% suppression of IL-12p40 production in *me/me* macrophages was observed for all LPS doses used. Furthermore, secretion of IL-12p40 from *me/me* splenic macrophages peaked when 1 μg/mL of LPS was used in both normal and *me/me* splenic macrophages.

To characterize the kinetics of IL-12p40 production in normal and *me/me* splenic macrophages, IL-12p40 levels were next measured in supernatants collected during 5 sequential intervals (0, 6, 12, 24, 36, and 48 hours) of LPS stimulation (1 μg/mL) , using ELISA. As shown in Figure 3.3b, IL-12p40 secretion was suppressed in *me/me* macrophages, an effect observed irrespective of duration of LPS stimulation. In addition, IL-12p40 production peaked after 24 hours of LPS stimulation in normal splenic macrophages, and was delayed in *me/me* macrophages where it peaked at 36 hours post-stimulation.

Fig 3.3a: IL-12p40 production in normal and *me/me* splenic macrophages stimulated with different doses of LPS.

Spleens of normal and *me/me* mice were removed and crushed between glass slides. 10^6 cells were suspended in 200 μL of Opti-Mem media +10% FBS +20% CLL-1, and incubated in 96 well plates at 37°C in a 5% CO2 air mixture for 3 days. Macrophages were either left unstimulated or stimulated with various doses of LPS (0.1, 1, 5, 10, 12.5, 25, and 50 μg/mL) for 24 hours. Every condition was repeated by seeding the cells in four wells. Cell supernatants were collected and IL-12p40 production was measured by ELISA. The absorbance was determined, using a microplate reader set to 450 nm. The mean of duplicate wells were calculated based on a standard curve that was constructed for each assay. This experiment has been done three times. Similar results were obtained in a total of three independent experiments. One of three independent experiments with similar results is shown.

Figure. 3.3a

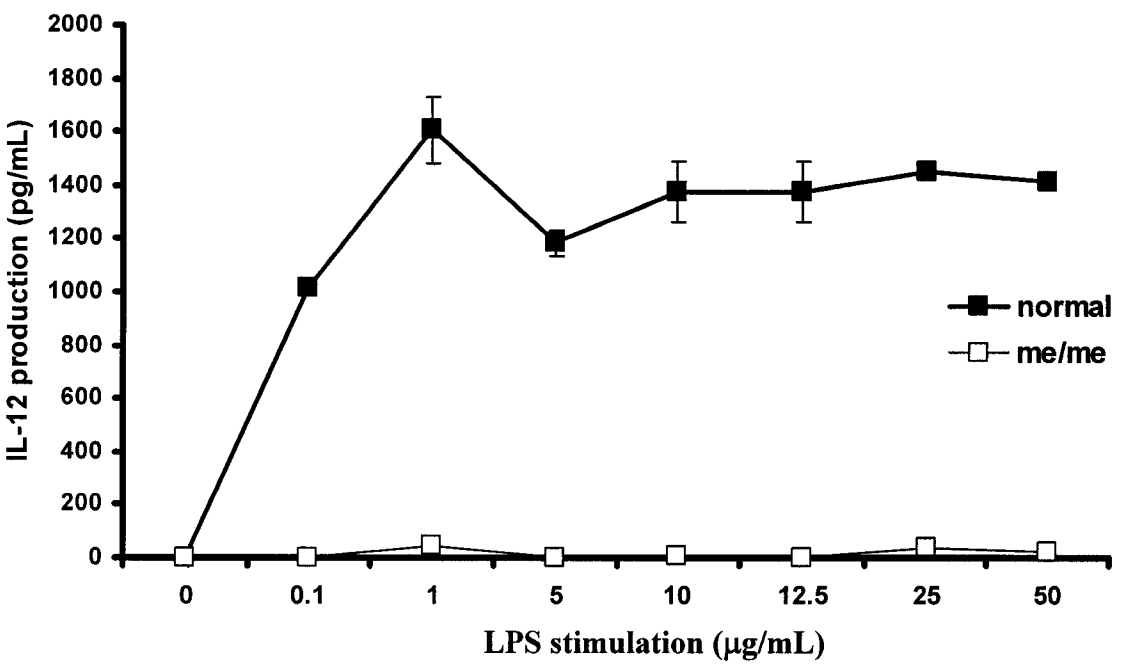
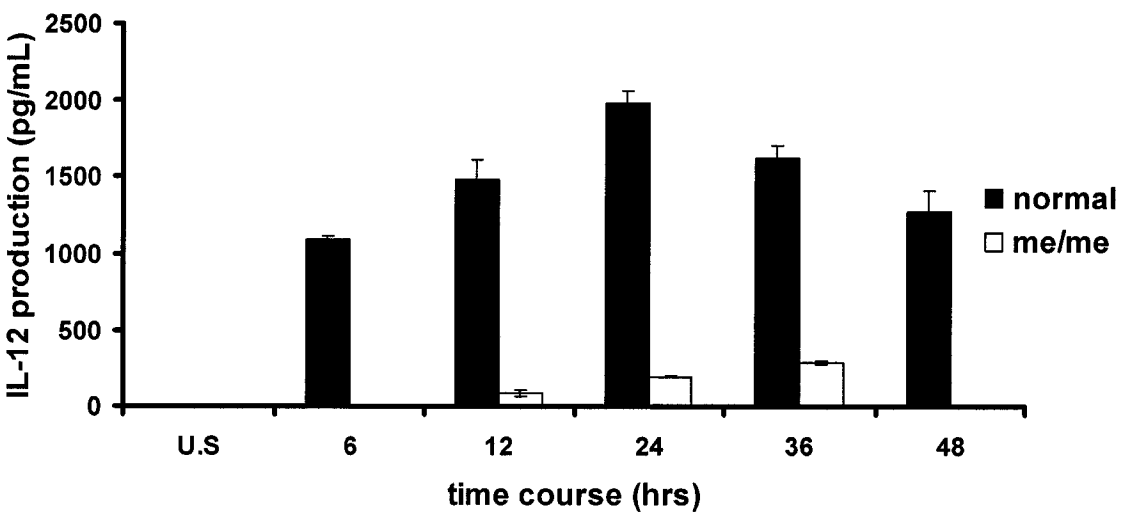

2000
1800
1600
1400
1200
1000
800
600
400
200
0
IL-12 production (pg/mL)
normal
me/me
0
0.1
1
5
10
12.5
25
50
LPS stimulation (μg/mL)

Fig 3.3b: Time course analysis of IL-12p40 production in normal and *me/me* splenic macrophages stimulated with LPS.

Spleens of normal and *me/me* mice were removed and crushed between glass slides. 10^6 cells were suspended in 200 μL of Opti-Mem media +10% FBS +20% CLL-1, and incubated in 96 well plates at 37°C in a 5% CO2 air mixture for 3 days. Macrophages were stimulated with or without 1 μg/mL LPS in a time course over 48 hours. Every condition was repeated by seeding the cells in four wells. Cell supernatants were collected and IL-12p40 production was measured by ELISA. The absorbance was determined, using microplate reader set to 450 nm. The mean of duplicate wells were calculated based on a standard curve that was constructed for each assay. This experiment has been done three times. Similar results were obtained in a total of three independent experiments. One of three independent experiments with similar results is shown.

Figure. 3.3b

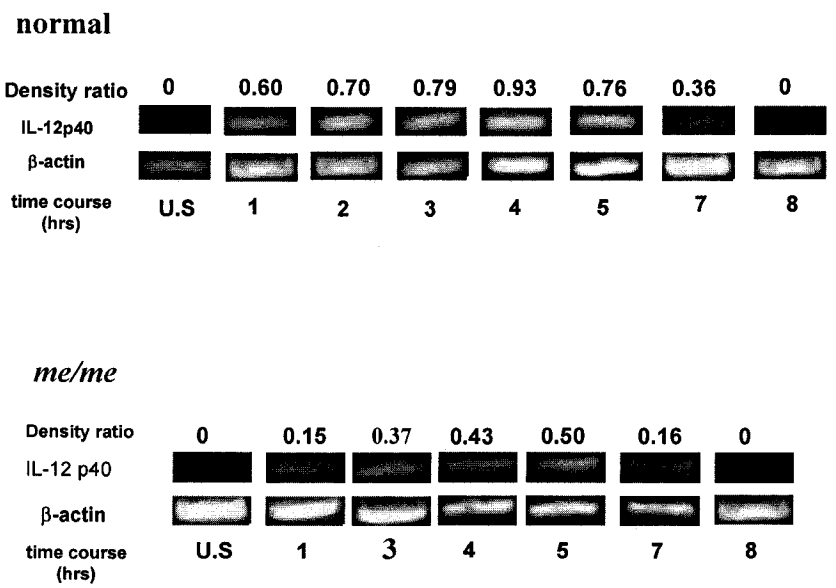

IL-12 production (pg/mL)
2500
2000
1500
1000
500
0
U.S
6
12
24
36
48
time course (hrs)
normal
me/me

To further determine whether the suppression of IL-12p40 production in LPS stimulated *me/me* splenic macrophages occurs at the level of gene transcription, I performed time course analysis of IL-12p40 messenger RNA (mRNA) from normal and *me/me* splenic macrophages after LPS stimulation (from 0-to 8 hours), using RT-PCR. As shown in Figure 3.4, following 0 to 8 hours of LPS stimulation, the level of IL-12p40 transcript in normal splenic macrophages was increased and peaked after 4 hours of LPS stimulation. In contrast, IL-12p40 message in SHP-1 null mice was induced at the much lower level and delayed (peaking at 5 hours of LPS treatment) compared to normal macrophages. RT-PCR results for each time point were normalized against β-actin mRNA as an internal standard. Therefore, comparison of kinetics of IL-12p40 message in normal and SHP-1 null *me/me* splenic macrophages by RT-PCR, further confirmed that lack of SHP-1 in *me/me* macrophages leads to decreased level of IL-12p40 production.

Taken together, SHP-1 null *me/me* splenic macrophages produce a decreased level of IL-12p40 upon LPS stimulation. This effect was observed irrespective of LPS dose and duration of stimulation.

Fig 3.4: Time course analysis of IL-12p40 message in normal and *me/me* splenic macrophages after LPS stimulation: Spleens were dissected from normal and *me/me* mice and crushed between glass slides. Single cell suspensions were placed in 75cm^2 medium polystyrene flasks. Splenic macrophages were isolated and cultured in Opti-mem medium containing 10% FBS, supplemented with 20% L929 medium at 37°C in a 5% CO2/ air mixture. After 6 days culture, the cells were treated with or without LPS for a time period of 1 to 8 hours and RNA was extracted. The level of IL-12p40 message was measured using RT-PCR. Results are represented as the relative expression of IL-12p40 mRNA normalized to β-actin mRNA and shown as density ratio. This experiment has been done once for each time point condition in both normal and *me/me* splenic macrophages.

Figure. 3.4


normal
Density ratio
0
0.60
0.70
0.79
0.93
0.76
0.36
0
IL-12p40
β-actin
time course (hrs)
U.S
1
2
3
4
5
7
8
me/me
Density ratio
0
0.15
0.37
0.43
0.50
0.16
0
IL-12 p40
β-actin
time course (hrs)
U.S
1
3
4
5
7
8

1.3. Impact of IFN-γ treatment on IL-12p40 production in *me/me* macrophages

It is known that IL-12p40 gene is induced at the level of transcription and protein production in response to IFN-γ/LPS stimulation in human PBMC (peripheral blood mononuclear cell). Similarly, analysis of human p40 promoter regulation in the transfected RAW264.7 cells showed that IL-12p40 gene is modulated primarily at the transcriptional level in response to IFN-γ and LPS stimulation. [14] The IFN-γ capacity to induce IL-12 production makes a positive feedback loop during Th1 responses resulting in a strong immune/inflammatory response directed against an antigen.

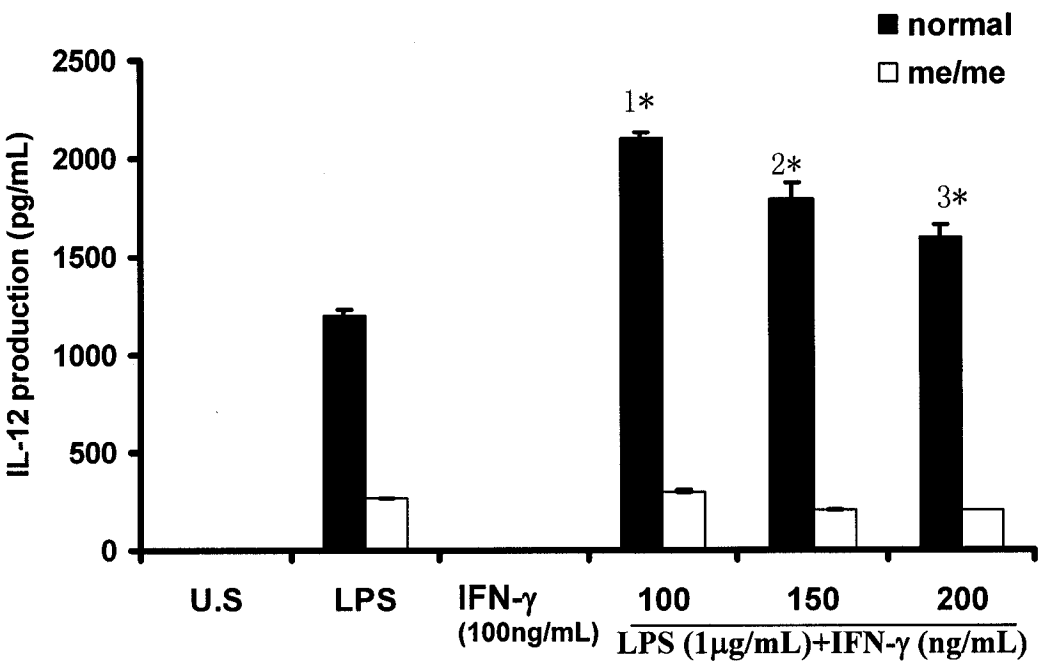
To determine whether IFN-γ can enhance suppressed level of IL-12p40 secretion in *me/me* macrophages, normal and *me/me* splenic macrophages were treated with different doses of recombinant mouse IFN-γ (100, 150, 200 ng/mL) simultaneous with LPS (1 μg/mL) for 24 hours. Supernatants were then collected and IL-12p40 secretion was measured by ELISA. As shown in Figure 3.5, IFN-γ alone did not induce any IL-12p40 production in either normal or *me/me* macrophages. However, normal splenic macrophages treated simultaneously with IFN-γ and LPS produced an increased level of IL-12p40. This synergistic effect led to an increase in IL-12p40 production at all three doses of IFN-γ used, and there was a 2 fold increase when 100 ng/mL concentration of IFN-γ was used. In contrast, IFN-γ/LPS stimulation did not augment IL-12p40 production in *me/me* splenic macrophages.

Fig. 3.5: IL-12p40 production in normal and *me/me* splenic macrophages stimulated with LPS and IFN-γ for 24 hrs:

Spleens of normal and *me/me* mice were removed and crushed between glass slides. 10^6 cells were suspended in 200 μL of Opti-Mem media +10% FBS +20% CLL-1, and incubated in 96 well plates at 37°C in a 5% CO2 air mixture for 3 days. The cells were left unstimulated or stimulated with LPS alone (1 μg/mL), IFN-γ alone (100 ng/mL) or with LPS in combination with different doses of IFN-γ (100,150, and 200 ng/mL) for 24 hours. Unstimulated cells were also cultured in parallel without LPS and IFN-γ. Every condition was repeated by seeding the cells in four wells. Cell supernatants were collected and IL-12 production was measured by ELISA. The absorbance was determined, using a microplate reader set to 450 nm. The mean of duplicate wells were calculated based on a standard curve that was constructed for each assay. This experiment has been done three times. Similar results were obtained in a total of three independent experiments. One of three independent experiments with similar results is shown.

	P (T<=t) one-tail
1*	0.022699
2*	0.023626
3*	0.04939

Figure. 3.5


normal
me/me
IL-12 production (pg/mL)
2500
2000
1500
1000
500
0
1*
2*
3*
U.S
LPS
IFN-γ
(100ng/mL)
100
150
200
LPS (1μg/mL)+IFN-γ (ng/mL)

1.4. Loss of IL-12 production as a consequence of SHP-1 loss

To further expand my observation that SHP-1 is involved in the regulation of IL-12p40 production following LPS stimulation, I interfered with SHP-1 function in normal macrophages by using a SHP-1 inhibitor (PTP-1). PTP-1 binds to catalytic domain of the SH2 domain-containing phosphatase SHP-1 and thus impairing SHP-1 catalytic function. (Calbiochem)

Normal splenic macrophages were treated with different concentrations of SHP-1 inhibitor (1, 2.5, 5, and 10 μM) for 2 hours before LPS (1 μg/mL) stimulation (for 24 hours). Then supernatants were harvested, and IL-12p40 levels were measured by ELISA. As shown in Figure 3.6a, there was a downregulation of IL-12p40 production in a dose dependent manner in cells treated with increasing concentration of PTP-1. Almost 50% suppression of IL-12 production was observed after treatment of cells with 2.5 μM of PTP-1 and little IL-12p40 produced when 5 μM of PTP-1 was used.

To further demonstrate the role for SHP-1 in LPS induced IL-12p40 production, I also interfered with SHP-1 expression in normal splenic macrophages using SHP-1 specific anti-sense oligonucleotides. The SHP-1 anti-sense oligonucleotides interfere with SHP-1 mRNA before it can be translated into amino-acids which makeup SHP-1 proteins.

As for PTP-1 experiments, normal splenic macrophages were treated with different doses of anti-sense oligonucleotides to SHP-1 (15 and 30 μM/mL) for 2 hours before LPS (1 μg/mL) stimulation (for 24 hours). To ensure the response is specific to the effect of SHP-1 anti-sense oligonucleotides on IL-12p40 production, the cells were also treated with unrelated oligonucleotides specific to either SHP-2 (30 μM/mL) or CD4 (30

μM/mL). Culture supernatants were collected and IL-12p40 measured by ELISA. Consistent with the previous experiment, there was a dose dependent decrease in the level of IL-12p40 production in cells expressing suppressed level of SHP-1, as demonstrated in (Figure3.6b).

Fig 3.6a: IL-12p40 production after treatment of normal macrophages with SHP-1 inhibitor (PTP-1):

Spleens of normal and *me/me* mice were removed and crushed between glass slides. 10^6 cells were suspended in 200 μL of Opti-Mem media +10% FBS +20% CLL-1, and incubated in 96 well plates at 37°C in a 5% CO2 air mixture for 3 days. Macrophages were incubated either with 1 μg/mL LPS alone for 24 hours, or with different concentrations of PTP-1 (1, 2.5, 5, and 10 μM) for 2 hours prior to stimulation with 1 μg/mL LPS for 24 hours. In parallel, unstimulated cells were also cultured without LPS and without PTP-1. Four wells were prepared for each condition. Cell supernatants were collected and IL-12 production was measured by ELISA. The absorbance was determined, using a microplate reader set to 450 nm. The mean of duplicate wells were calculated based on a standard curve that was constructed for each assay. This experiment has been done three times. Similar results were obtained in a total of three independent experiments. One of the three independent experiments with similar results is shown.

	P (T<=t) one-tail
1*	0.028684
2*	0.004517
3*	0.012737

Figure. 3.6a

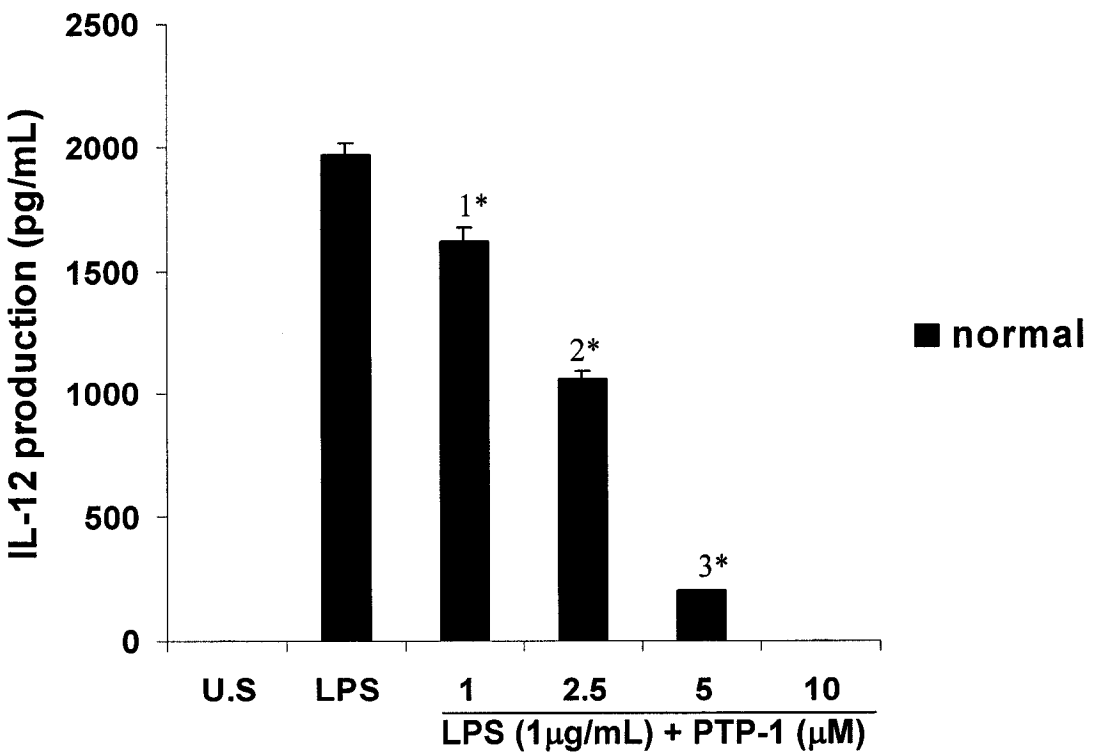
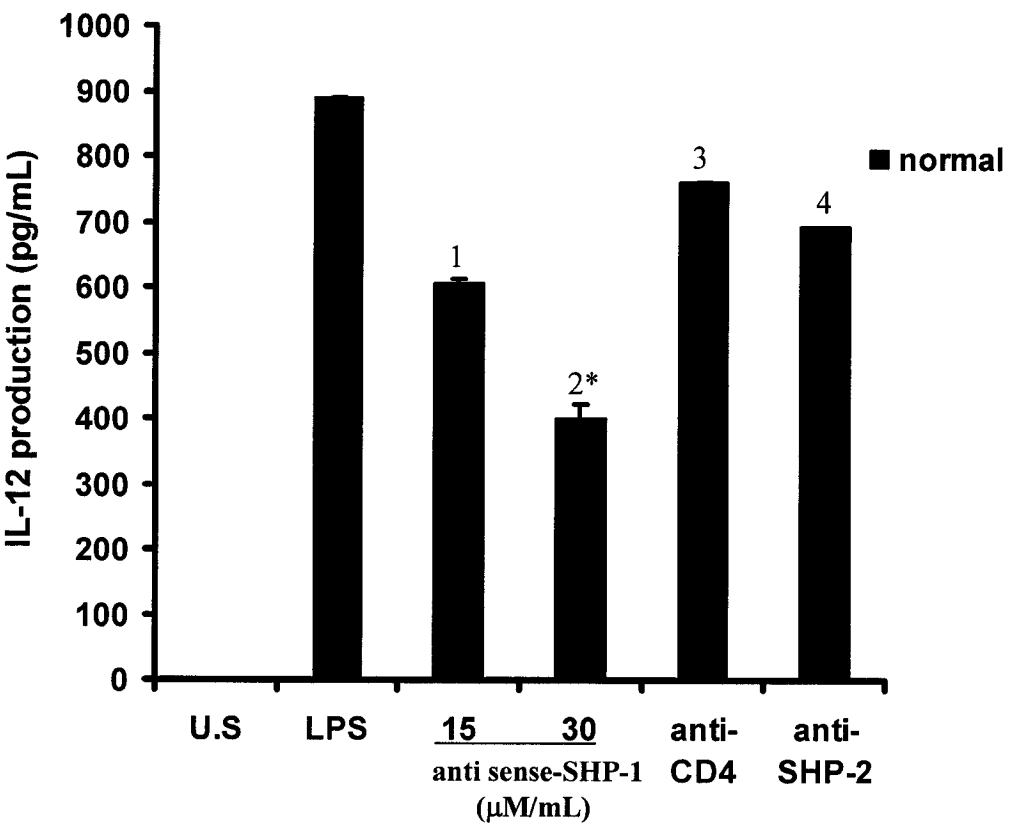

2500
2000
1500
1000
500
0
IL-12 production (pg/mL)
1*
2*
3*
normal
U.S
LPS
1
2.5
5
10
LPS (1μg/mL) + PTP-1 (μM)

Fig 3.6b: IL-12p40 production after using anti-sense oligonucleotides to SHP-1:

Spleens of normal and *me/me* mice were removed and crushed between glass slides. 10^6 cells were suspended in 200 μL of Opti-Mem media +10% FBS +20% CLL-1, and incubated in 96 well plates at 37°C in a 5% CO2 air mixture for 3 days. The cells were incubated either with 1 μg/mL LPS alone for 24 hours, or with different concentrations of specific anti-sense oligonucleotides to SHP-1 (15 and 30 μM/mL) for 2 hours prior to stimulation with 1 μg/mL LPS for 24 hours. In parallel unstimulated cells were also cultured without LPS or anti-sense oligonucleotides to SHP-1. Unrelated anti-sense oligonucleotides to SHP-2 and CD4 (30 μM/mL) were used as negative controls. Four wells were set up for each experimental condition. Cell supernatants were collected and IL-12p40 production was measured by ELISA. The absorbance was determined, using a microplate reader set to 450 nm. The mean of duplicate wells were calculated based on a standard curve that was constructed for each assay. This experiment has been done three times. Similar results were obtained in a total of three independent experiments. One of three independent experiments with similar results is shown.

	P (T<=t) one-tail
1	0.070185
2*	0.018801
3	0.212173
4	0.197474

Figure3.6b


1000
900
800
700
600
500
400
300
200
100
0
IL-12 production (pg/mL)
normal
1
2*
3
4
U.S
LPS
15
30
anti sense-SHP-1 (μM/mL)
anti-CD4
anti-SHP-2

Furthermore, I reconstituted SHP-1 in *me/me* splenic macrophages using SHP-1 adenoviral vectors. Splenic macrophages were infected with increasing MOIs (0, 5, 10, 20, and 100) of an adenoviral vector containing SHP-1 for 24 hours. Then the cells were stimulated with 1 μg/mL LPS for 24hours. The supernatants were collected and assayed for IL-12p40 production using ELISA. Expression of SHP-1 in *me/me* macrophages resulted in increased level of IL-12p40 secretion in reconstituted cells, compared to controls. (Figure 3.7a) Infection of macrophages with SHP-1 adenoviral vector at MOI 100 led to 50-60% increased production of IL-12p40 by transduced *me/me* splenic macrophages. In parallel, the *me/me* macrophages were also infected with increasing MOIs (0, 5, 10, 20, and 100) of negative controls, such as Adenoviral vector construct containing GFP or adenoviral vector expressing dominant negative SHP-1 (substrate trapping mutant of SHP-1), or an adenoviral vector expressing SHP-1 phosphatase inactive mutant. Then transduced macrophages were treated with 1 μg/mL LPS for 24 hours. The supernatants were harvested and examined for IL-12p40 secretion by ELISA. There was no increased level of IL-12p40 production after transduction of macrophages with negative control (Ad-GFP, Ad-substrate trapping SHP-1, Ad-phosphatase inactive SHP-1). In addition, the cells transduced by Ad-GFP with increasing MOIs (0, 5, 10, 20, and 100), were viewed under a confocal microscope and photographed. Fluorescence photographic images showed increased level of GFP expression in motheaten macrophages relative to the increasing MOIs. (Fig 3.7b)

Conversely, normal splenic macrophages were also infected with Ad-dominant negative mutant of SHP-1 containing only SH2 domains of SHP-1 protein. Then the cells were stimulated with 1 μg/mL LPS, and IL-12p40 were measured by ELISA. As shown in

Figure 3.7c, expression of SH2 domains of SHP-1 resulted in marked downregulation of IL-12p40 production (about 80%), compared to untransduced controls.

Fig 3.7a, b: IL-12p40 production in *me/me* splenic macrophages after transduction with SHP-1 adeno-virus:

Spleens of *me/me* mice were removed and crushed between glass slides. $5x10^5$cells were suspended in 500 μL of Opti-Mem media +10% FBS +20% CLL-1, and incubated in 24 well plates at 37°C in a 5% CO2 air mixture for 3 days. Then the macrophages were infected with increasing MOIs of (0, 5, 10, 20, 50, and 100) of (Ad-GFP, Ad-SHP1, Ad-Dominant negative SHP-1 (substrate trapping SHP-1), and Ad-Dominant negative SHP-1 (phosphatase inactive mutant) for 24 hours. The cells were then incubated with 1 μg/mL LPS for 24 hours. Also, in parallel, normal splenic macrophages were treated with 1 μg/mL LPS for 24 hours as a positive control. Three wells were set up for each experimental condition. Cell supernatants were collected and IL-12p40 production was measured by ELISA. The absorbance was determined, using a microplate reader set to 450 nm. The mean of duplicate wells were calculated based on a standard curve that was constructed for each assay. The black bar indicates the amount of IL-12p40 produced by normal macrophages in response to LPS stimulation. It is included as a positive control. The white and hatched bars depict the amount of IL-12p40 secreted by untransfected and transfected with different constructs, *me/me* macrophages following LPS stimulation. This experiment has been done two times. Similar results were obtained in a total of two independent experiments. One of two independent experiments with similar results is shown. (Fig3.7a) In addition, expression of green florescent protein by *me/me* macrophages transduced by Ad-GFP with increasing MOIs (0, 5, 10, 20, and 100) was monitored under a confocal microscope (Fig 3.7b)

	P (T<=t) one-tail
1*	0.031177
2*	0.024022
3*	0.00673
4*	0.001785
5*	0.002413

Figure. 3.7a

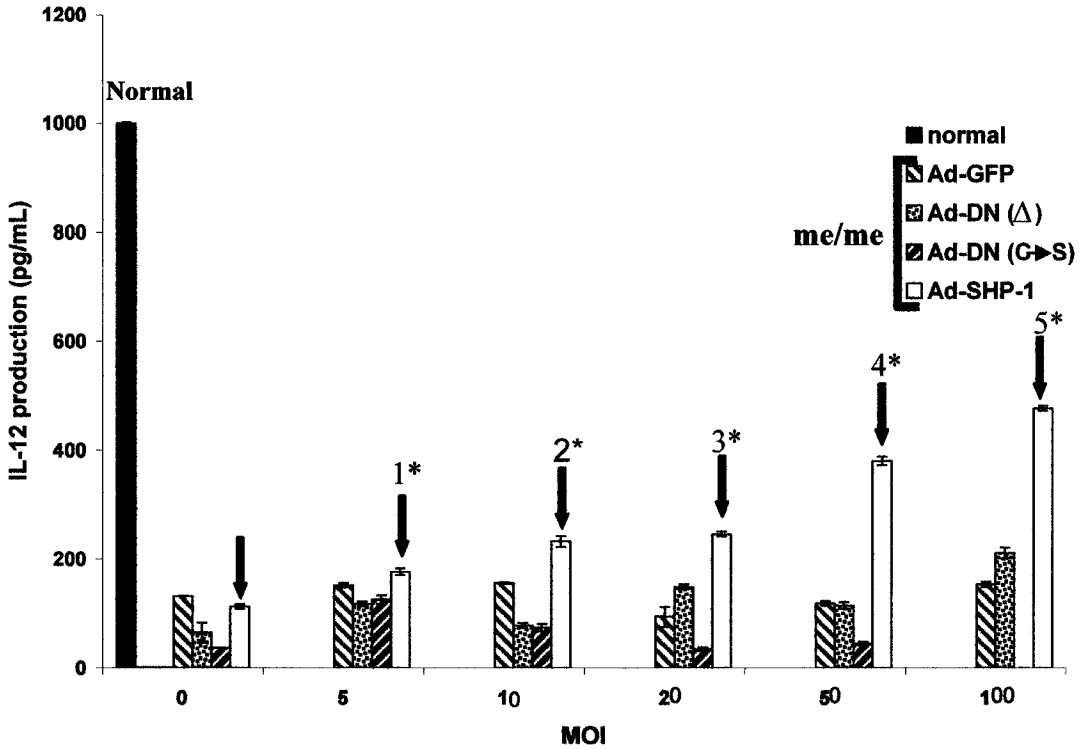


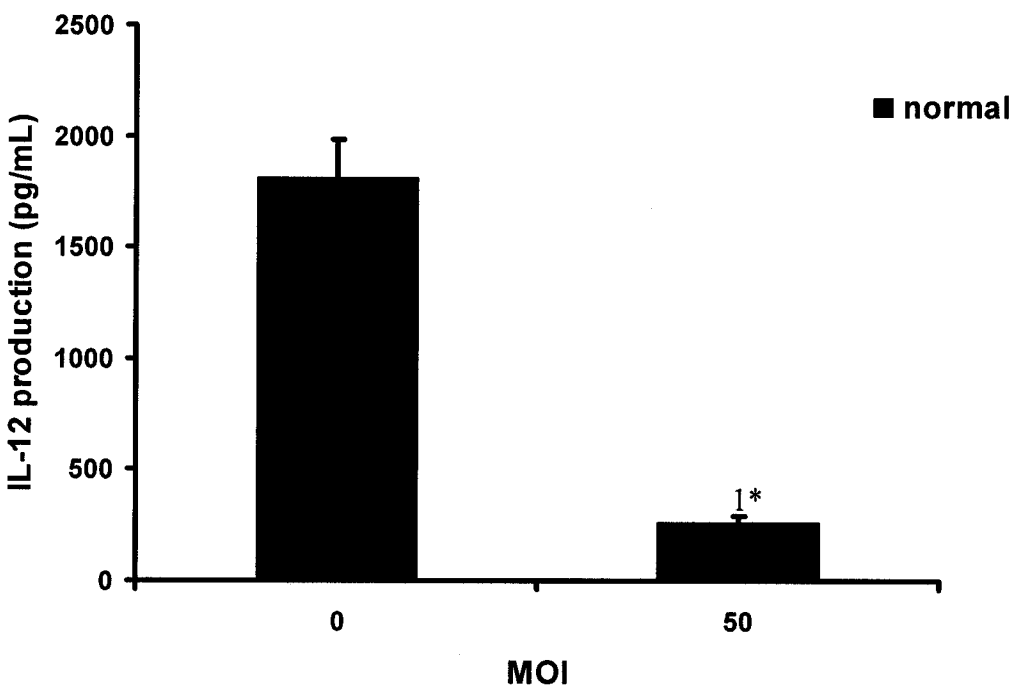
Figure. 3.7b

Fig 3.7c: IL-12p40 production in normal splenic macrophages after transduction with Ad-dominant negative SHP-1 (SH2 domain):

Spleens of normal mice were removed and crushed between glass slides. $5x10^5$cells were suspended in 500 μL of Opti-Mem media +10% FBS +20% CLL-1, and incubated in 24 well plates at 37°C in a 5% CO2 air mixture for 3 days. Then the macrophages were infected with MOI (0 and 50) Ad-Dominant negative SHP-1 (substrate trapping SHP-1) for 24 hours. The cells were then incubated with 1 μg/mL LPS for 24 hours. Three well were set up for each experimental condition. Cell supernatants were collected and IL-12p40 production was measured by ELISA. The absorbance was determined, using a microplate reader set to 450 nm. The mean of duplicate wells were calculated based on a standard curve that was constructed for each assay. This experiment has been done two times and similar results were obtained in a total of two independent experiments. The results of are shown here.

	P (T<=t) one-tail
1*	0.002589

Figure. 3.7c


2500
2000
1500
1000
500
0
IL-12 production (pg/mL)
normal
1*
0
50
MOI

To confirm the expression of SHP-1 in splenic macrophages transfected with adenoviral constructs, I performed Western Immunoblot analysis. Splenic macrophages were harvested from the wells 48 hours post-transduction. The cells were lysed and lysates were analyzed by western Immunoblot using anti-SHP-1 antibody. Western Immunoblot analysis revealed that the levels of SHP-1 protein increased accordingly to the increasing adenoviral MOI concentrations. (Fig3.8a) Western Immunoblot analysis showed increased levels of SHP-1 expression in cells transduced by either Ad-Dominant negative SHP-1 (substrate trapping SHP-1) or Ad-dominant negative SHP-1 (phosphatase inactive mutant) in a dose dependent manner. (Fig 3.8b, Fig 3.8c).

In Figure 3.8a due to technical difficulties SHP-1 expression in *me/me* macrophages infected by Ad- SHP-1 is shown only at MOI 5 and 10.

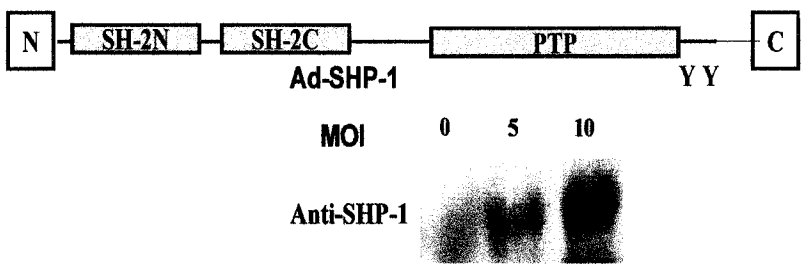
Overall, these results confirmed our hypothesis that differences in IL-12p40 production between normal and *me/me* splenic macrophages can be attributed to the lack of SHP-1.

Cellular changes in *me/me* macrophages derived from spleen suggest deregulation of signaling pathways leading to the production of cytokines. It was therefore important to compare in normal and *me/me* splenic macrophages signal transduction pathways which regulate IL-12p40 production and may depend on SHP-1.

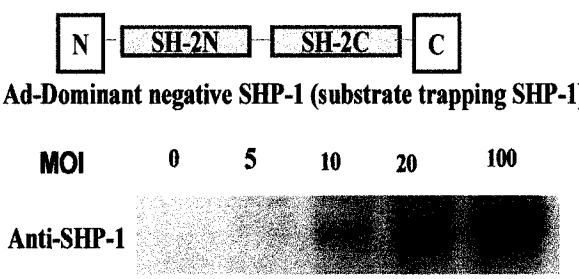
Fig 3.8: Expression of SHP-1 in cells transfected with adenoviral constructs:

Spleens of *me/me* mice were removed and crushed between glass slides. $5x10^5$cells were suspended in 500 μL of Opti-Mem media +10% FBS +20% CLL-1, and incubated in 24 well plates at 37°C in a 5% CO2 air mixture for 3 days. Then the macrophages were infected with increasing MOIs (0, 5, 10, 20, 50, and 100) of Ad-SHP1 **(Fig3.8a)**, Ad-Dominant negative SHP-1 (substrate trapping SHP-1) **(Fig3.8b),** and Ad-Dominant negative SHP-1 (phosphatase inactive mutant) **(Fig3.8c)** for 24 hours. The cells were then incubated with 1 μg/mL LPS for 24 hours. Then, cells were harvested from the wells 48 hours post-transduction. The lysates were subjected to SDS-PAGE followed by western blot analysis. The membranes were all blotted with anti-SHP-1 antibody.

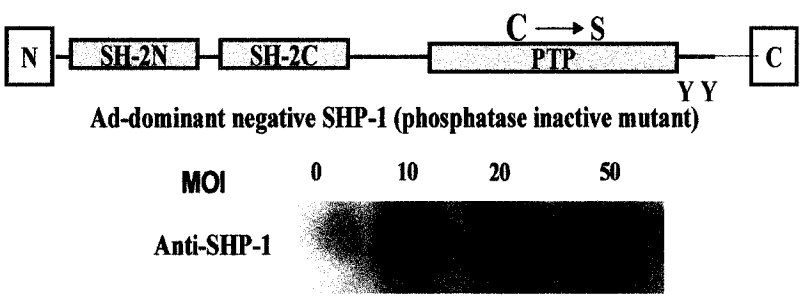
3.8a)


N
SH-2N
SH-2C
PTP
C
Ad-SHP-1
Y Y
MOI
0
5
10
Anti-SHP-1

3.8b)


N
SH-2N
SH-2C
C
Ad-Dominant negative SHP-1 (substrate trapping SHP-1)
MOI
0
5
10
20
100
Anti-SHP-1

3.8c)


C → S
N
SH-2N
SH-2C
PTP
C
Y Y
Ad-dominant negative SHP-1 (phosphatase inactive mutant)
MOI
0
10
20
50
Anti-SHP-1

II. SHP-1 signaling in IL-12p40 production

2.1. Involvement of SHP-1 in the regulation of IL-12p40 production through calcium signaling pathway

There is some evidence suggesting the involvement of calcium signaling pathways in the regulation of IL-12p40 production in DCs and monocytes. [62, 63, 64, 67, 110] However, the role of SHP-1 in these pathways has never been explored.

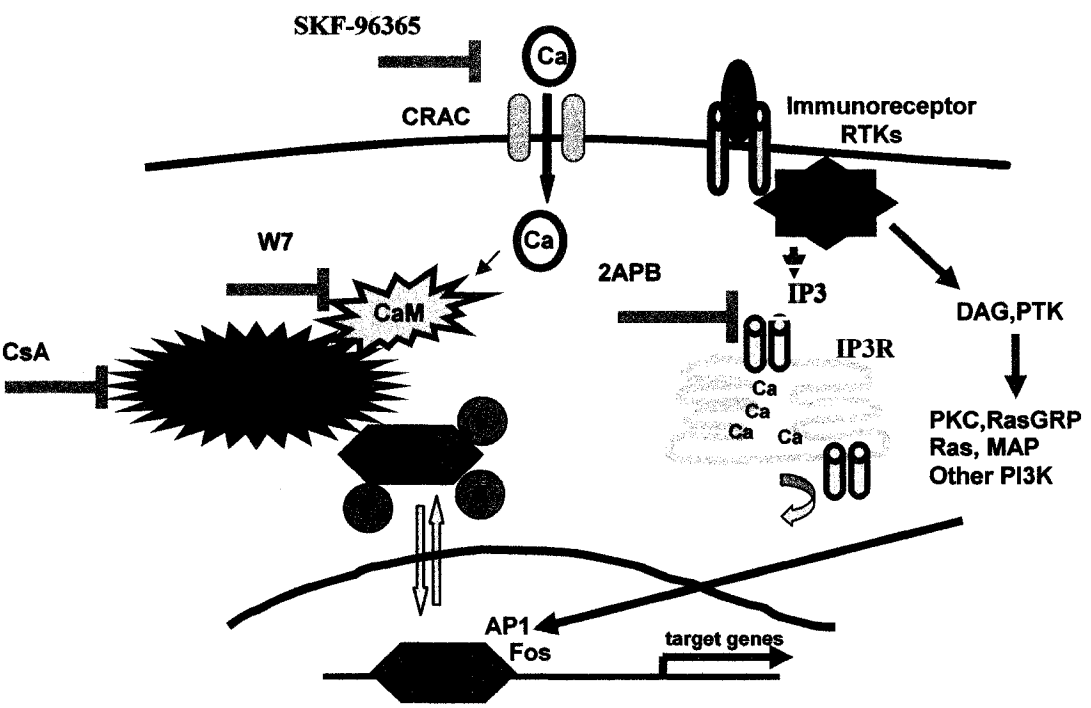
To investigate which calcium signaling pathways involving SHP-1 in the regulation of IL-12p40 production in macrophages derived from spleen, I studied the effect of different inhibitors of calcium signaling pathways including 2-aminoethoxydiphenylborate (2-APB), cyclosporine A (CsA), SKF-96365, W7, and KN-93. (Fig 3.9)

It has been reported that 2APB is a permeable regulator of IP3-induced calcium release. It suppresses thrombin-induced calcium release from platelets without affecting IP3 binding to its receptor. [68] It has been also shown that in liver cells, 2 APB inhibits store operated calcium channels (SOCs) via interacting with either the channel protein or a regulatory protein associated with the channel proteins.[69] Immunosuppressant cyclosporine A (CsA) binds to cyclophilin (a cytosolic receptor) and inhibits proline rotamase activity. The CsA-cyclophilin complex suppresses a type 2B phosphatase calcineurin.[70] SKF-96365, Hydrochloride (1-{β-[3-(4-methoxyphenyl) propyl]-4-methoxyphenethyl}-1H-imidazol, HCL) interferes with receptor mediated calcium entry in stimulated platelets, neutrophil, and endothelial cells, and thereby inhibits protein serine/threonine phosphatase and suppresses calcium entry.[71, 72, 73] W7 (N-(6-Aminohexyl)-5-chloro-1-naphthalene-sulfonamide.HCL) is a cell permeable inhibitor

Fig.3.9: The effect of different calcium inhibitors on calcium signaling pathways

2APB is a permeable regulator of IP3-induced calcium release. It suppresses thrombin-induced calcium release from platelets without affecting IP3 binding to its receptor. [68] It has been also shown that in liver cells, 2 APB inhibits store operated calcium channels (SOCs) via interacting with either the channel protein or a regulatory protein associated with the channel proteins.[69] Immunosuppressant **CsA** binds to cyclophilin (a cytosolic receptor) and inhibits proline rotamase activity. The CsA-cyclophilin complex suppresses a type 2B phosphatase calcineurin.[70] **SKF-96365**, interferes with receptor mediated calcium entry in stimulated platelets, neutrophil, and endothelial cells, and thereby inhibits protein serine/threonine phosphatase and suppresses calcium entry.[71, 72, 73] **W7** is a cell permeable inhibitor that binds to calmodulin and thereby suppressing Ca^{2+}-calmodulin regulated enzyme activity and it inhibits any enzyme that requires calmodulin binding. [74]

Figure. 3.9


SKF-96365
Ca
CRAC
Immunoreceptor
RTKs
Ca
W7
CaM
2APB
IP3
DAG,PTK
CsA
IP3R
Ca
Ca
Ca
Ca
PKC,RasGRP
Ras, MAP
Other PI3K
AP1
Fos
target genes

that binds to calmodulin and thereby suppressing Ca^{2+}-calmodulin regulated enzyme activity and it inhibits any enzyme that requires calmodulin binding. [74] KN-93, a cell permeable inhibitor that suppresses CaM kinaseII activity. [75]

To determine whether these inhibitors suppress IL-12p40 production from macrophages, normal and *me/me* splenic macrophages were incubated with different concentrations of each of the mentioned inhibitors for 2 hours prior to stimulation with 1 μg/mL of LPS for 24 hours. Culture supernatants were collected and IL-12p40 secretion was measured by ELISA. The results were as follows: addition of 2APB (Fig4.1), CsA (Fig4.2) and KN-93 (Fig4.3) had no effect on IL-12p40 production by the activated normal and *me/me* splenic macrophages compared with cells stimulated with LPS alone. In contrast, macrophages activated with LPS in the presence of W7 (Fig.4.4) and SKF-96365 (Fig4.5) demonstrated significantly decreased production of IL-12p40 in a dose dependent manner compared to negative controls. It was observed that IL-12p40 production was decreased about 3 fold upon treatment of cells with concentration as low as 5 μM of W7. Similarly, IL-12p40 production was decreased by 3 fold in cells treated with low concentration 25 μM of SKF-96365. Taken together, these data revealed that a) calcium signaling represent an important factor in the regulation of IL-12p40 in both normal and *me/me* macrophages following LPS stimulation. b) Among the calcium activated proteins, calcium channels and calmodulin/calcineurin pathway are required for IL-12p40 production following LPS stimulation of normal and *me/me* splenic macrophages. c) although both calmodulin and calcineurin enzymes play key roles in LPS stimulated IL-12p40 production in splenic macrophages, inhibition of these pathways affect IL-12p40 production in both normal and SHP-1 null *me/me* macrophages. Therefore, based on

these findings, it was reasonable to speculate that SHP-1 participates in IL-12p40 production by modulating signal transduction pathways other than those regulated by calcium in splenic macrophages. Hence, I examined the contribution of phosphoinositide 3-kinase (PI3K)/AKT pathway and Mitogen activated protein kinases (MAP kinases) in regulation of IL-12p40.

Fig4.1: The effect of 2APB on IL-12p40 production:

Spleens of normal and *me/me* mice were removed and crushed between glass slides. 10^6 cells were suspended in 200 μL of Opti-Mem media +10% FBS +20% CLL-1, and incubated in 96 well plates at 37°C in a 5% CO2 air mixture for 3 days. The cells were incubated either with 1 μg/mL LPS alone for 24 hours, or with different concentrations of 2APB (10, 12.5, 25, 50, 75 and 100 μM/mL), for 2 hours prior to stimulation with 1 μg/mL LPS for 24 hours. Unstimulated cells were also cultured in parallel without LPS and any inhibitor. Every condition was repeated by seeding the cells in four wells. Cell supernatants were collected and IL-12p40 production was measured by ELISA. The absorbance was determined, using a microplate reader set to 450 nm. The mean of duplicate wells were calculated based on a standard curve that was constructed for each assay. This experiment has been done three times. Similar results were obtained in a total of three independent experiments. One of three independent experiments with similar results is shown.

	P (T<=t) one-tail
1	0.202302
2	0.846516
3	0.956819
4	0.195305
5*	0.046134
6*	0.04661

Figure. 4.1

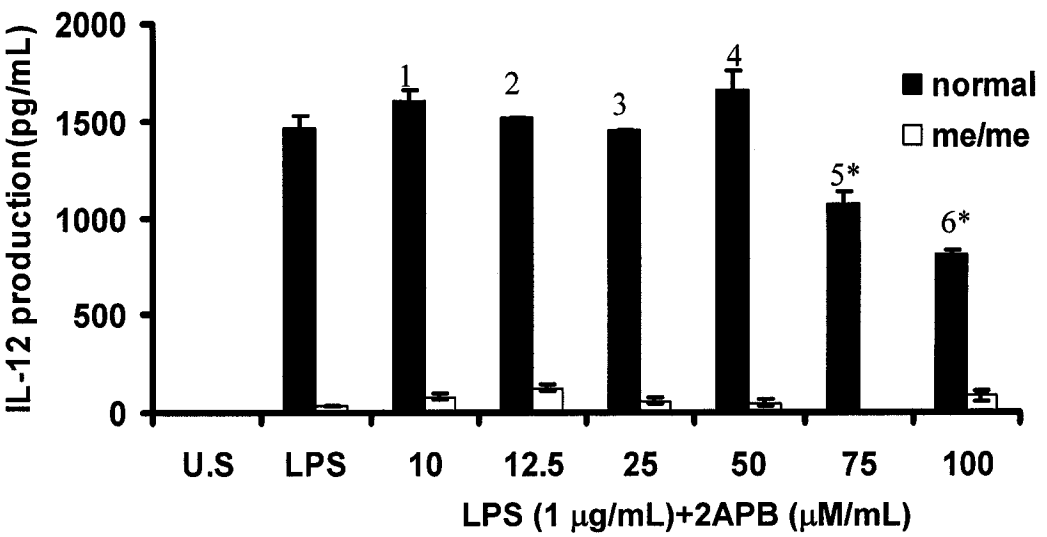

IL-12 production(pg/mL)
2000
1500
1000
500
0
1
2
3
4
5*
6*
normal
me/me
U.S
LPS
10
12.5
25
50
75
100
LPS (1 μg/mL)+2APB (μM/mL)

Fig 4.2: The effect of cyclosporine A on IL-12p40 production:

Spleens of normal and *me/me* mice were removed and crushed between glass slides. 10^6 cells were suspended in 200 μL of Opti-Mem media +10% FBS +20% CLL-1, and incubated in 96 well plates at 37°C in a 5% CO2 air mixture for 3 days. The cells were incubated either with 1 μg/mL LPS alone for 24 hours, or with different concentrations of CsA (100 nM, 250 nM, 500 nM, 1 μM, 2.5 μM and 5 μM), for 2 hours prior to stimulation with 1 μg/mL LPS for 24 hours. Unstimulated cells were also cultured in parallel without LPS and any inhibitor. Every condition was repeated by seeding the cells in four wells. Cell supernatants were collected and IL-12 production was measured by ELISA. The absorbance was determined, using a microplate reader set to 450 nm. The mean of duplicate wells were calculated based on a standard curve that was constructed for each assay. This experiment has been done three times. Similar results were obtained in a total of three independent experiments. One of three independent experiments with similar results is shown.

	P (T<=t) one-tail
1	0.148057
2	0.175809
3	0.726045
4	0.213698
5	0.202117
6	0.20236

Figure. 4.2

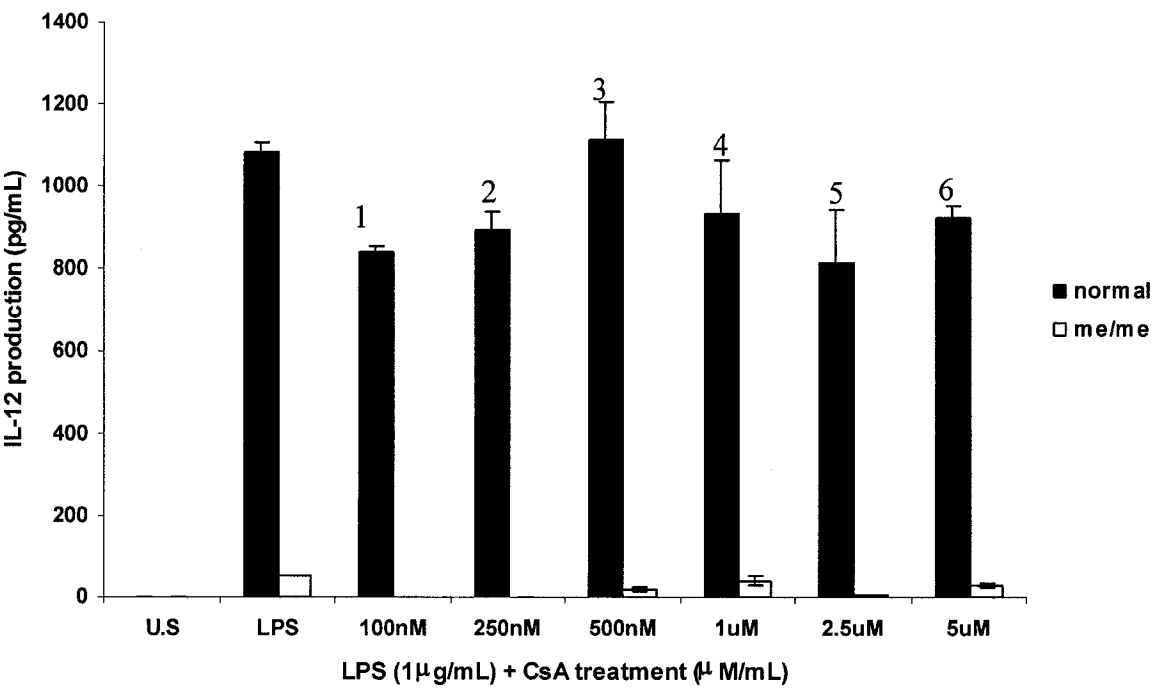

1400
1200
1000
800
600
400
200
0
IL-12 production (pg/mL)
1
2
3
4
5
6
U.S
LPS
100nM
250nM
500nM
1uM
2.5uM
5uM
LPS (1μg/mL) + CsA treatment (μ M/mL)
normal
me/me

Fig 4.3: The effect of KN-93 on IL-12p40 production:

Spleens of normal and *me/me* mice were removed and crushed between glass slides. 10^6 cells were suspended in 200 μL of Opti-Mem media +10% FBS +20% CLL-1, and incubated in 96 well plates at 37°C in a 5% CO2 air mixture for 3 days. The cells were incubated either with 1μg/mL LPS alone for 24 hours, or with different concentrations of KN-93(1, 2.5, 5, 10, 12.5 and 25 μM/mL), for 2 hours prior to stimulation with 1 μg/mL LPS for 24 hours. Unstimulated cells were also cultured in parallel without LPS and any inhibitor. Every condition was repeated by seeding the cells in four wells. Cell supernatants were collected and IL-12production was measured by ELISA. The absorbance was determined, using a microplate reader set to 450 nm. The mean of duplicate wells were calculated based on a standard curve that was constructed for each assay. This experiment has been done three times. Similar results were obtained in a total of three independent experiments. One of three independent experiments with similar results is shown.

	P (T<=t) one-tail
1	0.125066
2	0.187955
3	0.172974
4*	0.026921
5*	0.007293
6*	0.054138

Figure. 4.3

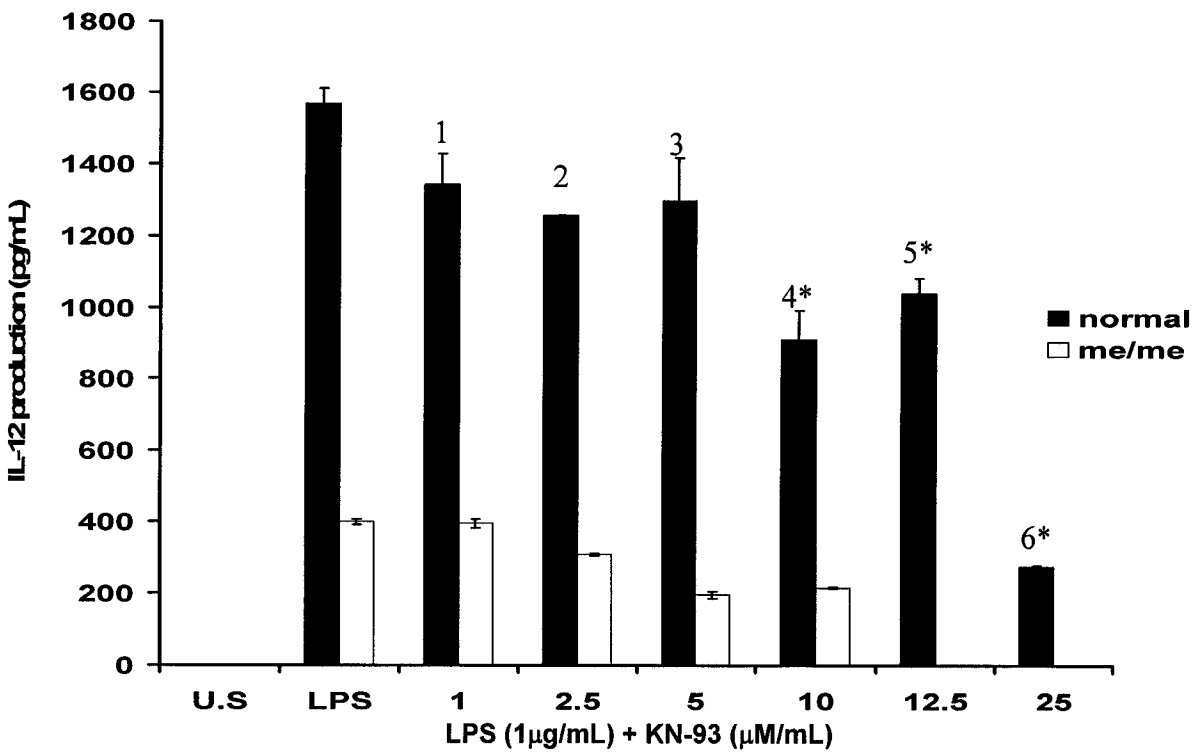

1800
1600
1400
1200
1000
800
600
400
200
0
IL-12 production (pg/mL)
1
2
3
4*
5*
6*
normal
me/me
U.S
LPS
1
2.5
5
10
12.5
25
LPS (1μg/mL) + KN-93 (μM/mL)

Fig 4.4: The effect of W7 on IL-12p40 production:

Spleens of normal and *me/me* mice were removed and crushed between glass slides. 10^6 cells were suspended in 200 µL of Opti-Mem media +10% FBS +20% CLL-1, and incubated in 96 well plates at 37°C in a 5% CO2 air mixture for 3 days. The cells were incubated either with 1µg/mL LPS alone for 24 hours, or with different concentrations of W7 (1, 2.5, 5, 10, 25, and 50 µM/mL), for 2 hours prior to stimulation with 1 µg/mL LPS for 24 hours. Unstimulated cells were also cultured in parallel without LPS and any inhibitor. Every condition was repeated by seeding the cells in four wells. Cell supernatants were collected and IL-12p40 production was measured by ELISA. The absorbance was determined, using a microplate reader set to 450 nm. The mean of duplicate wells were calculated based on a standard curve that was constructed for each assay. This experiment has been done three times. Similar results were obtained in a total of three independent experiments. One of three independent experiments with similar results is shown.

Normal		***me/me***	
P (T<=t) one-tail		P (T<=t) one-tail	
1	0.152129	1'	0.527248
2*	0.032582	2'*	0.054604
3*	0.033471	3'*	0.058408
4*	0.023463	4'*	0.037541
5*	0.022453	5'*	0.014951
6*	0.021907	6'*	0.002056

Figure. 4.4

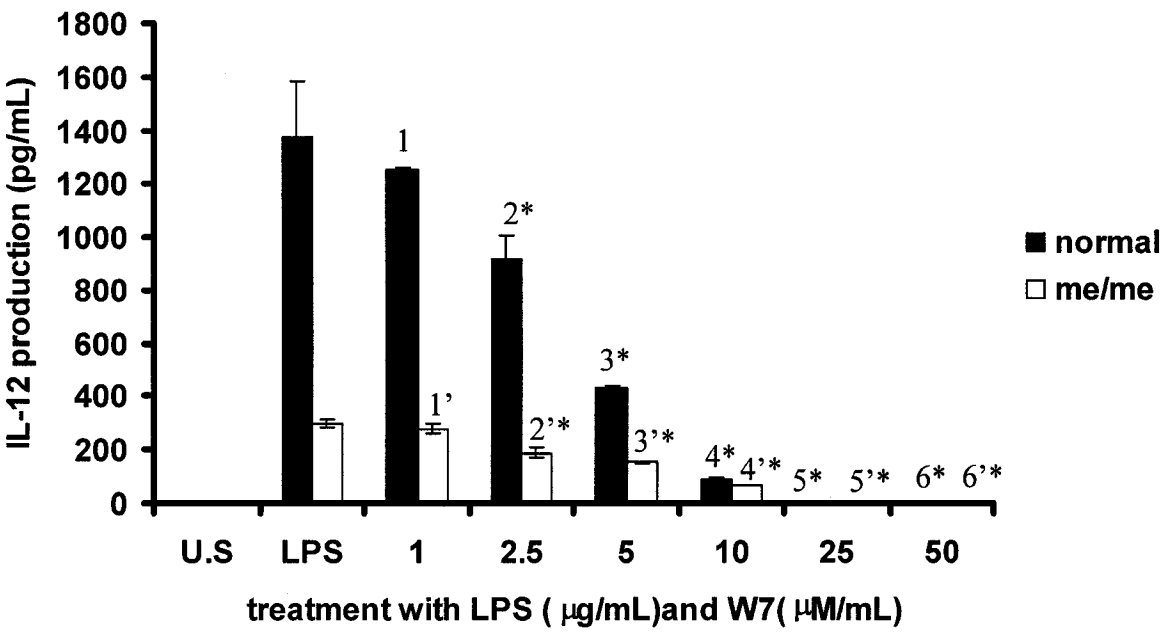
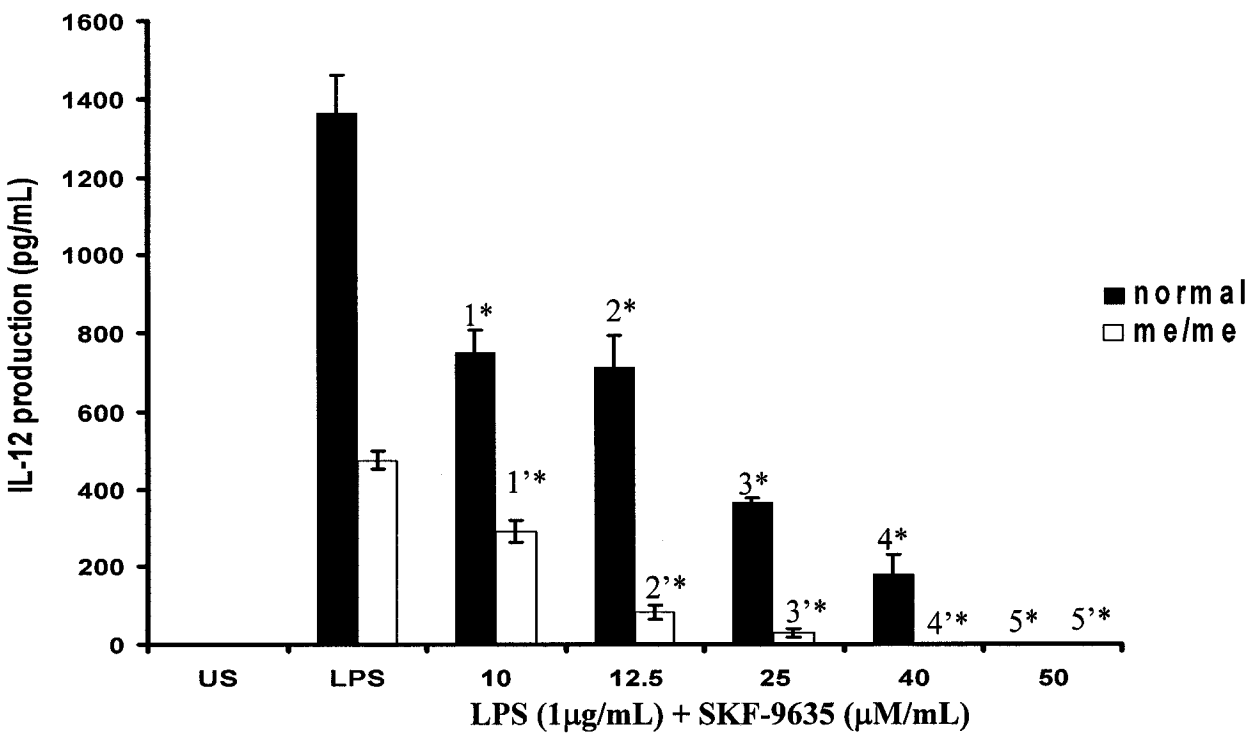

1800
1600
1400
1200
1000
800
600
400
200
0
IL-12 production (pg/mL)
1
2*
3*
1'
2'*
3'*
4*
4'*
5*
5'*
6*
6'*
normal
me/me
U.S
LPS
1
2.5
5
10
25
50
treatment with LPS (μg/mL)and W7(μM/mL)

Fig 4.5: The effect of SKF-96365on IL-12p40 production:

Spleens of normal and *me/me* mice were removed and crushed between glass slides. 10^6 cells were suspended in 200 μL of Opti-Mem media +10% FBS +20% CLL-1, and incubated in 96 well plates at 37°C in a 5% CO2 air mixture for 3 days. The cells were incubated either with 1μg/mL LPS alone for 24 hours, or with different concentrations of SKF-96365 (10, 12.5, 25, 40, and 50 μM/mL), for 2 hours prior to stimulation with 1 μg/mL LPS for 24 hours. Unstimulated cells were also cultured in parallel without LPS and any inhibitor. Every condition was repeated by seeding the cells in four wells. Cell supernatants were collected and IL-12p40 production was measured by ELISA. The absorbance was determined, using a microplate reader set to 450 nm. The mean of duplicate wells were calculated based on a standard curve that was constructed for each assay. This experiment has been done three times. Similar results were obtained in a total of three independent experiments. One of three independent experiments with similar results is shown.

Normal		***me/me***	
P (T<=t) one-tail		P (T<=t) one-tail	
1*	0.048143	1'*	0.052755
2*	0.032002	2'*	0.021739
3*	0.024176	3'	0.022137
4*	0.044029	4'*	0.053663
5*	0.007591	5'*	0.003735

Figure. 4.5


1600
1400
1200
1000
800
600
400
200
0
IL-12 production (pg/mL)
1*
1'*
2*
2'*
3*
3'*
4*
4'*
5*
5'*
normal
me/me
US
LPS
10
12.5
25
40
50
LPS (1μg/mL) + SKF-9635 (μM/mL)

2.2. Involvement of SHP-1 in the regulation of IL-12p40 production through MAP kinase pathway:

MAP kinase pathways have been implicated in the regulation of IL-12p40 production in human and murine macrophages. [82, 84, 85, 88, 91 95] In fact, the p38 MAP kinase was first found as a tyrosine phosphorylated 38 KDa protein upon LPS stimulation of murine macrophage cell lines.[84] To investigate whether SHP-1 is involved in the regulation of IL-12p40 production through p38 MAP kinase, first I studied the effects of inhibitor of p38 MAP Kinase on the production of IL-12p40 in normal and *me/me* splenic macrophages. To determine whether p38 was required for the production of IL-12 in response to LPS, normal and *me/me* splenic macrophages were treated with different doses of p38 MAPK inhibitor (SB203580) for 2 hours and then stimulated with 1 μg/mL LPS for 24 hours. SB203580 is a p38 inhibitor which is pyridinyl imidazol derivatives that interacts with ATP binding groove within the p38 MAP kinase kinase. It also can inhibit JNK2 activity obeyed at high doses. [84] IL-12p40 levels were measured in the culture supernatants by ELISA. (Fig4.6) The data revealed that treatment with the p38 inhibitor of LPS stimulated normal or *me/me* splenic macrophages did not have any effect on IL-12p40 production at any doses of SB203580 tested. This suggested that p38 MAPK is not required for the production of IL-12p40 in response to LPS.

It is well established that ERK MAP kinase is involved in LPS signal transduction in macrophages. [82] To find a role for SHP-1 in the regulation of IL-12p40, I tested the impact of ERK inhibitor (PD98059) on LPS-induced release of IL-12p40 from normal and *me/me* splenic macrophages. To examine the status of IL-12p40 production, macrophages were pretreated with PD98059 for 2 hours before LPS (1μg/mL) was added

for 24 hours. Supernatants were collected and IL-12p40 production was measured using ELISA technique. (Fig4.7a) Our results showed that treatment of splenic macrophages with PD98059 led to an increased level of IL-12p40 production, suggesting that ERK MAP kinases play a negative regulatory role in LPS mediated induction of IL-12p40 in both normal and SHP-1 null macrophages. However, this effect was much more pronounced in *me/me* macrophages where IL-12p40 production reached almost normal level when cells were treated with 10-25 μM/mL of PD98059. (Fig4.7b)

The JNK stress pathway is known to contribute to various intracellular signalling pathways that govern many cellular functions including cell growth, T cell activation and differentiation, transformation, apoptosis, and cytokine production. [84] To investigate the role of SHP-1 in the regulation of IL-12, I examined the effect of SP600125 (JNK inhibitor) on LPS-induced release of IL-12p40 from normal and *me/me* splenic macrophages. SP600125 is a specific ATP competitive inhibitor of JNK1, JNK2, and JNK3.[101, 102] Splenic macrophages were treated with increasing concentration of SP600125 for 2 hours. The cells, treated with inhibitor and untreated controls, were stimulated with LPS for 24 hours and IL-12p40 production was determined by ELISA. (Fig. 4.8) Treatment with JNK inhibitor did not show any effect on IL-12p40 production in both normal and *me/me* splenic macrophages in response to LPS. Therefore, JNK does not seem to play any role in IL-12p40 secretion in murine splenic macrophages.

Fig 4.6: The effect of SB203580 on IL-12p40 production:

Spleens of normal and *me/me* mice were removed and crushed between glass slides. 10^6 cells were suspended in 200 µL of Opti-Mem media +10% FBS +20% CLL-1, and incubated in 96 well plates at 37°C in a 5% CO2 air mixture for 3 days. The cells were incubated either with 1 µg/mL LPS alone for 24 hours, or with different concentrations of SB203580 (1, 2.5, 5, 10, 25, and 50 µM/mL), for 2 hours prior to stimulation with 1 µg/mL LPS for 24 hours. Unstimulated cells were also cultured in parallel without LPS and any inhibitor. Every condition was repeated by seeding the cells in four wells. Cell supernatants were collected and IL-12p40 production was measured by ELISA. The absorbance was determined, using a microplate reader set to 450 nm. The mean of duplicate wells were calculated based on a standard curve that was constructed for each assay. This experiment has been done three times. Similar results were obtained in a total of three independent experiments. One of three independent experiments with similar results is shown.

Normal		***me/me***	
P (T<=t) one-tail		P (T<=t) one-tail	
1	0.092072	1'	0.0718
2	0.08931	2'	0.216461
3	0.094965	3'	0.117225
4	0.196696	4'	0.191215
5	0.093841	5'	0.052807
6*	0.055184	6'*	0.220822

Figure. 4.6

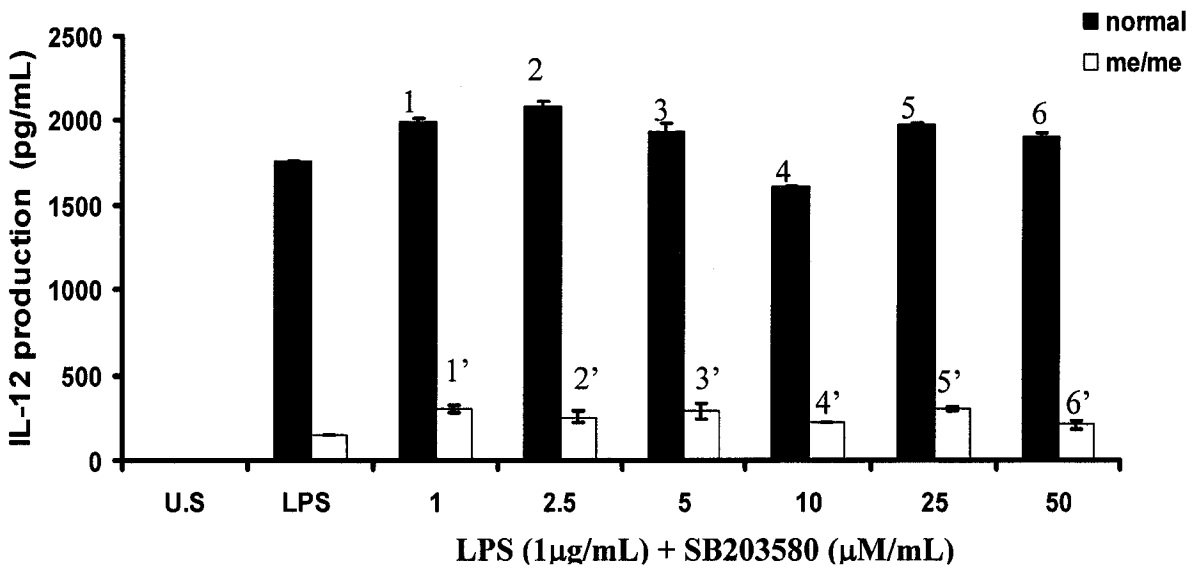

normal
me/me
IL-12 production (pg/mL)
2500
2000
1500
1000
500
0
1
1'
2
2'
3
3'
4
4'
5
5'
6
6'
U.S
LPS
1
2.5
5
10
25
50
LPS (1μg/mL) + SB203580 (μM/mL)

Fig 4.7: **The effect of PD98059 on IL-12p40 production:**

Figure4.7a: Spleens of normal and *me/me* mice were removed and crushed between glass slides. 10^6 cells were suspended in 200 µL of Opti-Mem media +10% FBS +20% CLL-1, and incubated in 96 well plates at 37°C in a 5% CO2 air mixture for 3 days. The cells were incubated either with 1 µg/mL LPS alone for 24 hours, or with different concentrations of PD98059 (1,5, 10, 25, and 50 µM/mL), for 2 hours prior to stimulation with 1 µg/mL LPS for 24 hours. Unstimulated cells were also cultured in parallel without LPS and any inhibitor. Every condition was repeated by seeding the cells in four wells. Cell supernatants were collected and IL-12p40 production was measured by ELISA. The absorbance was determined, using a microplate reader set to 450 nm. The mean of duplicate wells were calculated based on a standard curve that was constructed for each assay. This experiment has been done three times. Similar results were obtained in a total of three independent experiments. One of three independent experiments with similar results is shown.

Figure 4.7b: The data has also been shown as fold change in IL-12 level with /without inhibitor in order to better appreciate the influence of the inhibitor.

Normal		***me/me***	
P (T<=t) one-tail		P (T<=t) one-tail	
1*	0.043392	1'*	0.013693
2*	0.051706	2'*	0.001149
3*	0.01844	3'*	0.002189
4*	0.049404	4'*	0.05723
5*	0.049151	5'*	0.005766

Figure. 4.7a

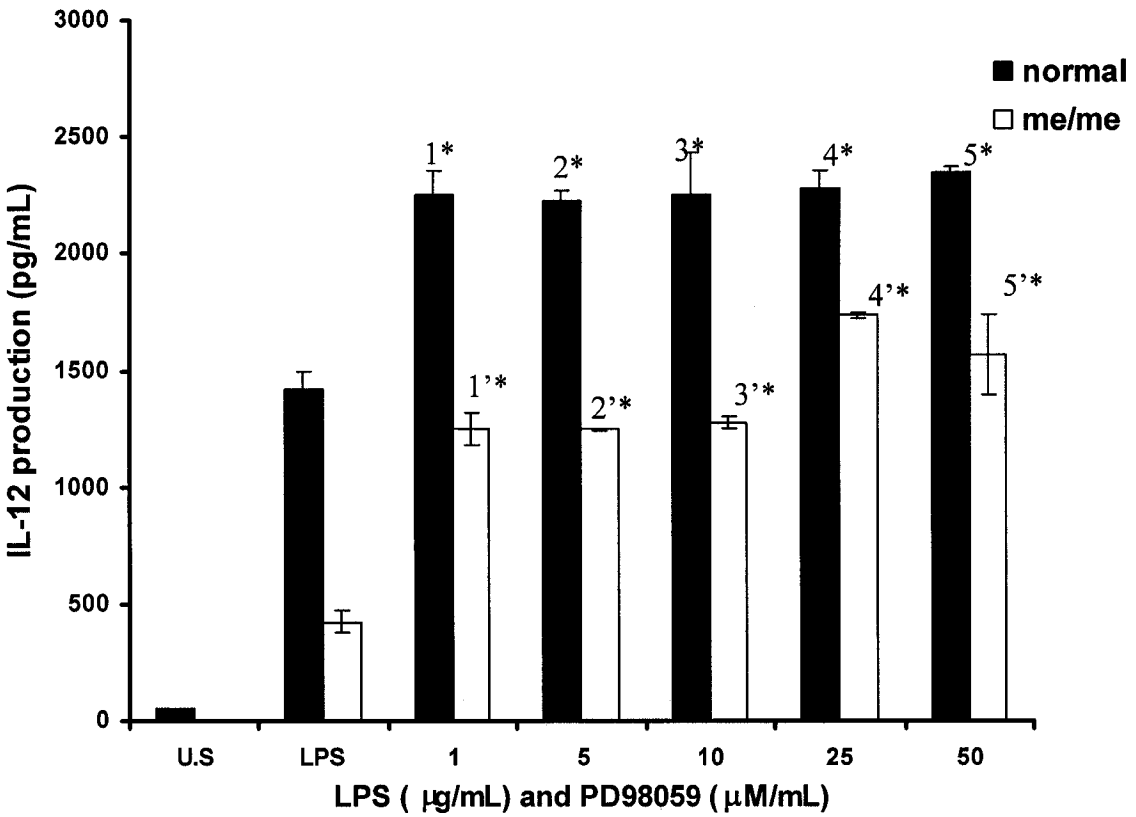


Figure. 4.7b

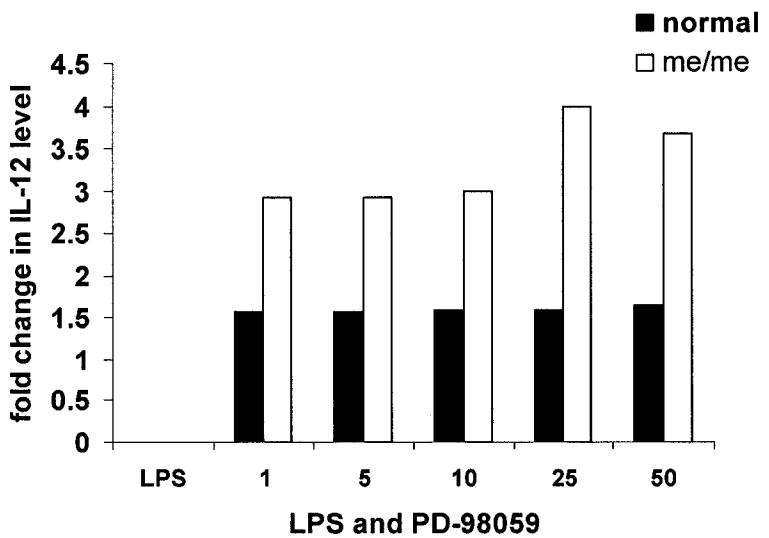
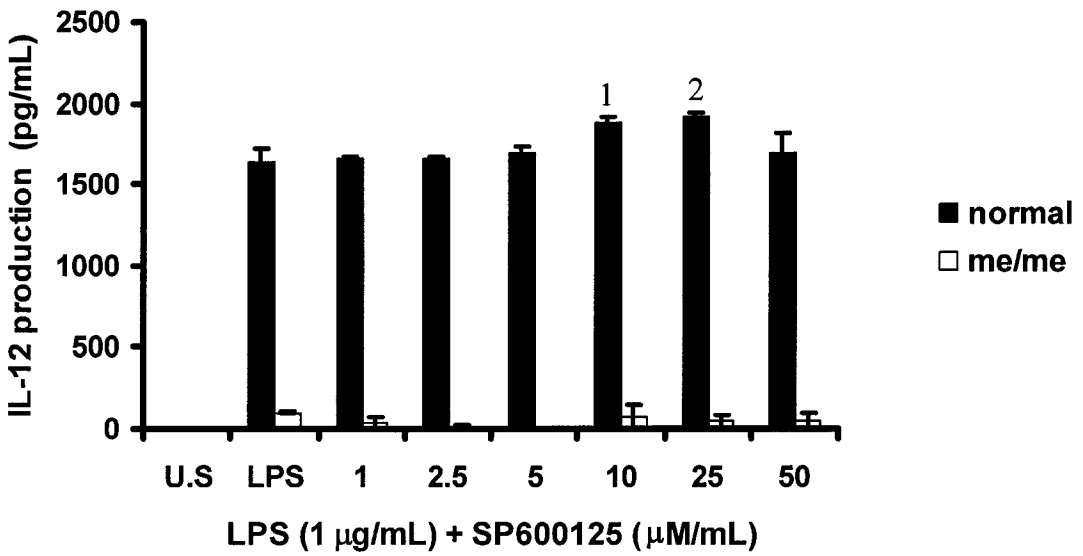


Fig 4.8: The effect of SP600125 on IL-12p40 production:

Spleens of normal and me/me mice were removed and crushed between glass slides. 10^6 cells were suspended in 200μL of Opti-Mem media +10% FBS +20% CLL-1, and incubated in 96 well plates at 37°C in a 5% CO2 air mixture for 3 days. The cells were incubated either with 1μg/mL LPS alone for 24 hours, or with different concentrations of SP600125 (1, 2.5, 5, 10, 25 and 50 μM/mL), for 2 hours prior to stimulation with 1μg/mL LPS for 24 hours. Unstimulated cells were also cultured in parallel without LPS and any inhibitor. Every condition was repeated by seeding the cells in four wells. Cell supernatants were collected and IL-12p40 production was measured by ELISA. The absorbance was determined, using a microplate reader set to 450 nm. The mean of duplicate wells were calculated based on a standard curve that was constructed for each assay. This experiment has been done three times. Similar results were obtained in a total of three independent experiments. One of three independent experiments with similar results is shown.

	P (T<=t) one-tail
1	0.189856
2	0.17087

Figure. 4.8


2500
2000
1500
1000
500
0
IL-12 production (pg/mL)
1
2
U.S
LPS
1
2.5
5
10
25
50
LPS (1 μg/mL) + SP600125 (μM/mL)
normal
me/me

2.3. Involvement of SHP-1 in the regulation of IL-12p40 production through PI3K pathway:

In addition to calcium signaling and MAP kinase signaling pathways the PI3K pathway has also been shown to be activated by LPS and implicated in IL-12 production.[80] However the role of SHP-1 in this process has never been addressed. To determine whether SHP-1 participates in IL-12p40 production by modulating PI3K activity downstream of LPS/TLR4 receptor signaling, I examined the IL-12p40 production in normal and *me/me* splenic macrophages. Normal and *me/me* splenic macrophages were incubated with different doses of PI3K inhibitor (LY294002 inhibits PI3K at IC50 of 1.4μM) for 2 hours (1, 5, 10, 25 μM/mL), followed by LPS stimulation (1 μg/mL) for 24 hours. Unstimulated controls were also included. Supernatants were taken and IL-12p40 concentration was measured using ELISA. (Fig 4.9a) Levels of IL-12p40 were increased (about 1.5 fold) following stimulation of normal splenic macrophages with LPS in the presence of LY294002 compared with cells stimulated with LPS alone. (Fig 4.9a) PI3K inhibited *me/me* macrophages showed 8-fold up-regulation of IL-12p40 production compared to cells stimulated with LPS alone. (Fig4.9b) This increase in IL-12p40 production in SHP-1 null macrophages was 4 times more of what was observed in normal LPS stimulated macrophages. Overall, inhibition of PI3K exhibits increased level of IL-12p40 production in both normal and *me/me* splenic macrophages. However, while in normal macrophages all doses of LY299002 led to an increase in IL-12p40 production, in *me/me* macrophages higher doses of LY29002 leveled of IL-12p40 production.

Fig 4.9: The effect of LY294002 on IL-12p40 production:

Figure4.9a: Spleens of normal and *me/me* mice were removed and crushed between glass slides. 10^6 cells were suspended in 200 µL of Opti-Mem media +10% FBS +20% CLL-1, and incubated in 96 well plates at 37°C in a 5% CO2 air mixture for 3 days. The cells were incubated either with 1µg/mL LPS alone for 24 hours, or with different concentrations of LY294002 (1, 5, 10, 25 µM/mL), for 2 hours prior to stimulation with 1 µg/mL LPS for 24 hours. Unstimulated cells were also cultured in parallel without LPS and any inhibitor. Every condition was repeated by seeding the cells in four wells. Cell supernatants were collected and IL-12 production was measured by ELISA. The absorbance was determined, using a microplate reader set to 450 nm. The mean of duplicate wells were calculated based on a standard curve that was constructed for each assay. This experiment has been done three times. Similar results were obtained in a total of three independent experiments. One of three independent experiments with similar results is shown.
Figure4.9b: The data has also been shown as fold change in IL-12 level with /without inhibitor in order to better appreciate the influence of the inhibitor.

Normal		***me/me***	
P (T<=t) one-tail		P (T<=t) one-tail	
1*	0.040271	1'*	0.000152
2*	0.006978	2'*	0.029483
3*	0.016176	3'*	0.009776
4*	0.012721	4'	0.111682

Figure. 4.9a

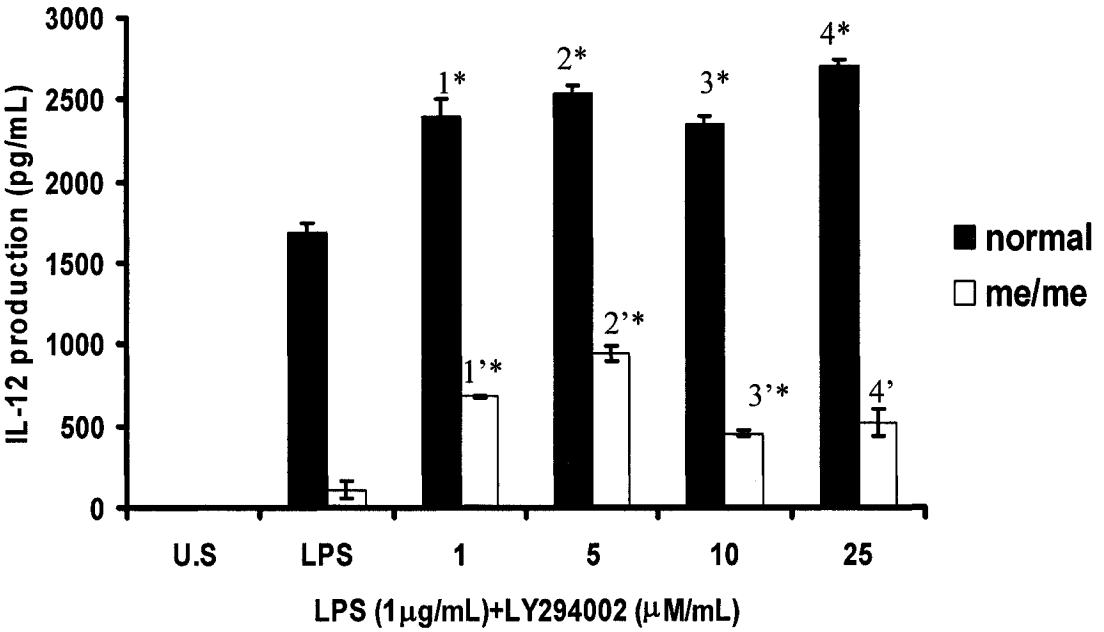
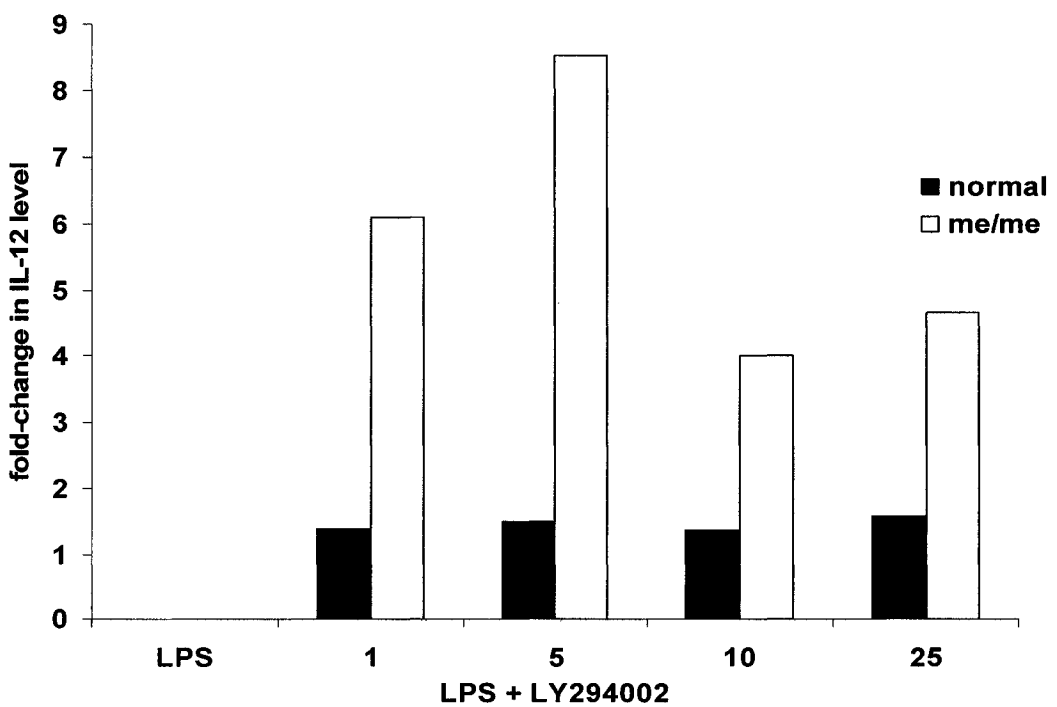


Figure. 4.9b



III. SHP-1 in IL-12 gene regulation

3.1. SHP-1 involvement in the regulation of IL-12p40 promoter in splenic macrophages

To analyse the mechanism involved in the regulation of IL-12p40 expression, I cloned and characterized murine IL-12p40 gene. I amplified and purified the full length (FL), IL-12p40 promoter fragment, -983 to +355 bp, and a 5' deletion mutant (Del1), fragment -378 to +355 bp, relative to +1 transcription start site, of IL-12p40 gene through PCR method. (Fig.2.1) Amplified fragments were introduced into PGL3 basic vector containing luciferase reporter gene. To study the regulation of IL-12p40 expression in macrophages through techniques such as promoter cloning, a transfectable system was required. Primary macrophages are not suitable for this purpose because they can not be readily transfected. Therefore, we turned to the mouse macrophage cell line RAW264.7 cells as a model system. The mouse macrophage cell line RAW264.7 cells were transfected with the IL-12p40 promoter luciferase reporter constructs (FL and Del1). 24 hours post-transfection, cells were stimulated with LPS for 24 hours and promoter activities were analyzed using the luciferase reporter assay system. I observed that in LPS stimulated cells, FL fragment led to a 4 fold increase in the luciferase activity compared to negative controls; however, the fusion construct containing the Del1 promoter fragment conferred only about a 2 fold increase in the luciferase activity suggesting the presence of sites for transcription factors in the deleted sequence of FL. (Fig5.1) Analysis of IL-12p40 promoter sequence from -983 to -378 by Genomatix Matinspector software revealed binding for OCT1 (octamer binding factor 1), ATBF1 (AT- binding transcription factor 1), AP4 (activator protein 4), NFY (Nuclear factor Y),

ETS2 (c-ETS2 binding site), AIRE (autoimmune regulator) transcription factors. Further studies are required to understand the involvement of these nuclear factors in LPS stimulated IL-12p0 gene transcription RAW264.7 cells.

To determine if SHP-1 plays a role in the regulation of IL-12p40 gene in macrophages, RAW264.7 cells, transfected with fusion constructs, were treated with different doses of SHP-1 inhibitor (PTP-1), followed by LPS stimulation. (Fig5.1) PTP-1 treatment of RAW264.7 cells containing FL fragment resulted in a significantly decreased level of luciferase activity in a dose dependent manner compared to negative controls. In contrast, the same treatment of RAW264.7 cells, containing Del1 construct, with low doses of 2.5 and 5 μM of PTP-1 did not produce a significant downregulation of luciferase activity compared to negative controls. However, treatment of RAW264.7 cells containing Del1 with 10 μM of PTP1 decreased luciferase activity. These results show a positive role for SHP-1 activity in regulation of FL of IL-12p40 promoter and IL-12p40 expression. This is consistent with our earlier observations using primary splenic macrophages.

Also, transfected RAW264.7 cells were treated by W7 and SKF-96365, followed by LPS stimulation. (Fig 5.2 and 5.3, respectively) Low concentrations of W7 (5 μM/mL) resulted in a 2 fold decrease in luciferase activity driven by a FL IL-12p40 promoter compared to the cells without inhibitor. Similarly, treatment of RAW264.7 cells expressing Del1 construct decreased luciferase activity by almost 50%. (Fig.5.2) Also, treatment with SKF-96365 (10 μM/mL), led to more than 2 fold decrease in luciferase activity of cells transfected with the FL construct compared to the cells which were not

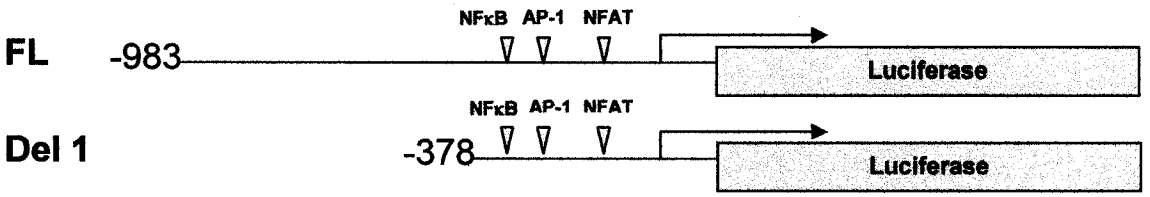
treated with SKF-96365. In contrast, SKF-96365 did not have a significant of luciferase activity in cells transfected with Del1 construct. (Fig.5.3) These results confirmed our previous data from ELISA experiment using supernatants from splenic macrophages showing the involvement of calmodulin/calcineurin pathways in regulation of IL-12p40 production. The results showed that while FL construct contained sites sensitive to both W7 and SKF-96365, Del1 construct lost the site sensitive to SKF-96365 treatment but retained the site sensitive to W7. Since CaM and calcineurin play roles in regulation of LPS stimulated IL-12p40 production, it was of interest to identify potential transcription factors, which are activated by calcium pathway, and regulate IL-12p40 gene transcription in normal and *me/me* splenic macrophages.

Fig 5.1: The expression of the luciferase activity of LPS stimulated RAW264.7 cells transfected with murine IL-12p40 (FL and Del1) fusion constructs and the effect of SHP-1 inhibitor (PTP-1) on the luciferase activity:

The IL-12p40 FL promoter and a 5' deletion mutant were synthesized by PCR using different 5' primers and a common 3' primer. PCR amplified fragments were cloned into the PGL3 basic luciferase reporter vector. $2x10^6$ of RAW264.7 cells were placed in 6 well plates and grown in 2 mL of Opti-Mem medium, containing 10% FBS and no antibiotics. The plates were incubated in a 5% CO2/air mixture for 24 hours to gain about 90% confluence. Transfection of RAW264.7 cells was carried out, using Lipofectamine 2000 reagent according to manufacture's protocol for 6 well plates. To measure luciferase activity 2 μg of FL/PGL3 or its deletion construct, were co-transfected with 2 μg of pSV-β-galactosidase control vector in RAW264.7 cells. Cells were treated with or without PTP-1 (SHP-1 inhibitor with different concentrations) for 2 hours and stimulated with 1 μg/mL LPS post- transfection and harvested 24 hours following stimulation, using scraper. Then the cells were lysed in 50 μL of lysis buffer. Luciferase and β-galactosidase activities were determined for the cell lysates following manufacture's manual. Lumistar instrument measured luciferase activity while the Dynex MRX Revelation Plate Reader (at OD 405 nm) measured β-galactosidase activities. Luciferase activity was normalized for β-galactosidase activity to give relative luciferase units (*RLU*).
This experiment has been done two times in duplicate. Similar results were obtained in a total of two independent experiments. One of the two independent experiments is shown, and normalized for β-galactosidase activity. Cells transfected with (-) LIPO, (-) DNA were taken as negative controls.
The upper panel shows IL-12p40 FL or Del1 luciferase fusion constructs. Binding sites for the major transcription factors were shown by arrow heads.

Full length		**Deletion 1**	
P (T<=t) one-tail		P (T<=t) one-tail	
		*	0.038131
1*	0.032487	1'	0.064615
2*	0.032368	2'	0.070686
3*	0.019721	3'*	0.003476

Figure. 5.1


FL
-983
NFκB AP-1 NFAT
Luciferase
Del 1
-378
NFκB AP-1 NFAT
Luciferase

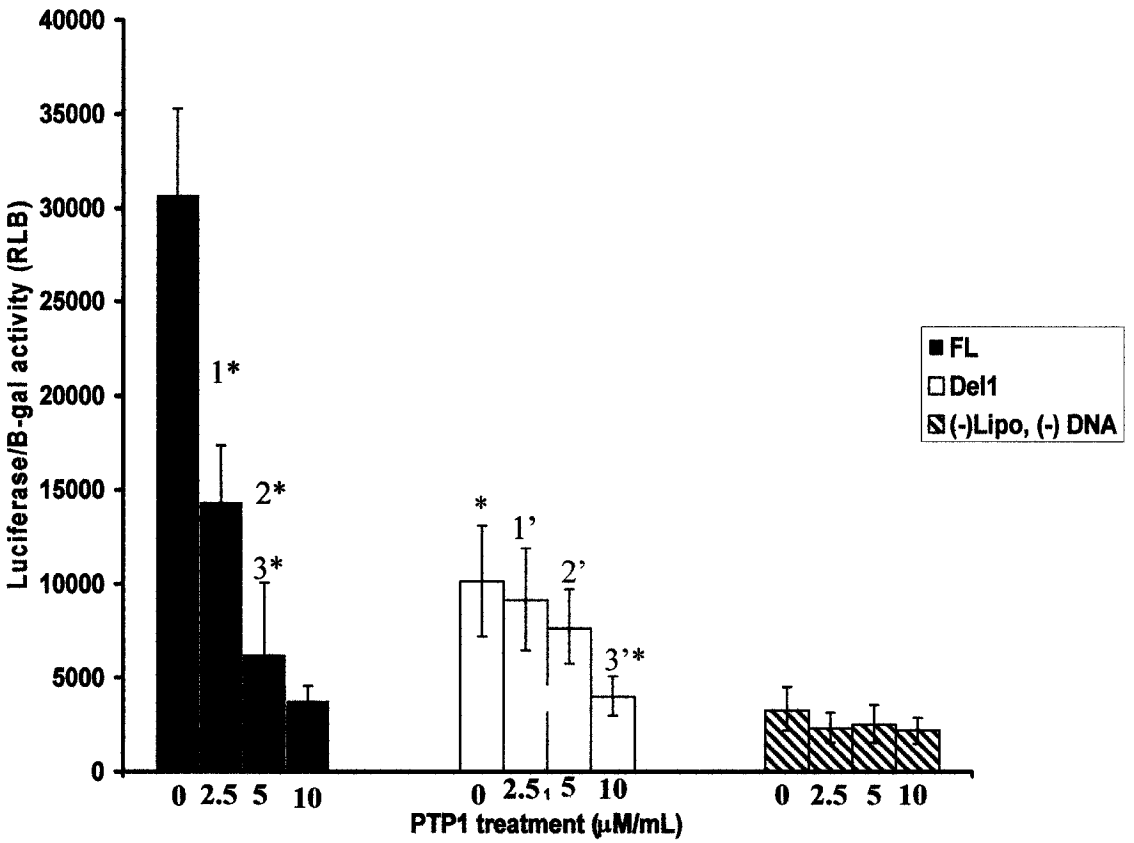

Luciferase/B-gal activity (RLB)
40000
35000
30000
25000
20000
15000
10000
5000
0
1*
2*
3*
*
1'
2'
3'*
0 2.5 5 10
0 2.5 5 10
0 2.5 5 10
PTP1 treatment (μM/mL)
FL
Del1
(-)Lipo, (-) DNA

Fig 5.2: The effect of W7 on the luciferase activity of LPS stimulated RAW264.7 cells transfected with murine IL-12p40 (FL and Del1):

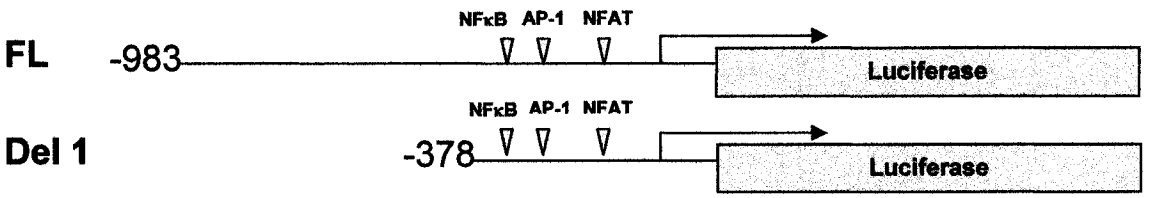
IL-12p40 promoter and 5' deletion mutant were synthesized by PCR, using different 5' primers and a common 3' primer, and cloned into the PGL3 basic luciferase reporter vector. $2x10^6$ of RAW264.7 cells were placed in 6 well plates and grown in 2 mL of Optimem medium, containing 10% FBS and no antibiotics. The plates were incubated in a 5% CO2/air mixture for 24 hours to gain about 90% confluence. Transfection of RAW264.7 cells was carried out, using Lipofectamine 2000 reagent according to the manufacture's protocol for 6 well plates. To measure luciferase activity 2 μg of FL/PGL3 or its deletion constructs, were co-transfected with 2 μg of pSV-β-galactosidase control vector in RAW264.7 cells. Cells were treated with or without W7 (5 μM/mL) for 2 hours and stimulated with 1 μg/mL LPS post- transfection and harvested 24 hours following stimulation, using scraper. Then the cells were lysed in 50 μL of lysis buffer. Luciferase and β-galactosidase activities were determined for the cell lysates following manufacture's manual. Lumistar instrument measured luciferase activity while the Dynex MRX Revelation Plate Reader (at OD 405 nm) measured β-galactosidase activities. Luciferase activity was normalized for β-galactosidase activity to give relative luciferase units (*RLU*). Conditions containing [(-) LIPO, (-) DNA], the PGL3 empty vector (basic PGL3), and [(+) Lipo, (-) DNA] were taken as negative controls. This experiment has been done three times in duplicate. Similar results were obtained in a total of three independent experiments. One of the three independent experiments with similar results is shown, and normalized for β-galactosidase activity.

P (T<=t) one-tail

1* 0.033218

2* 0.021364

Figure. 5.2


FL
-983
NFκB AP-1 NFAT
Luciferase
Del 1
-378
NFκB AP-1 NFAT
Luciferase

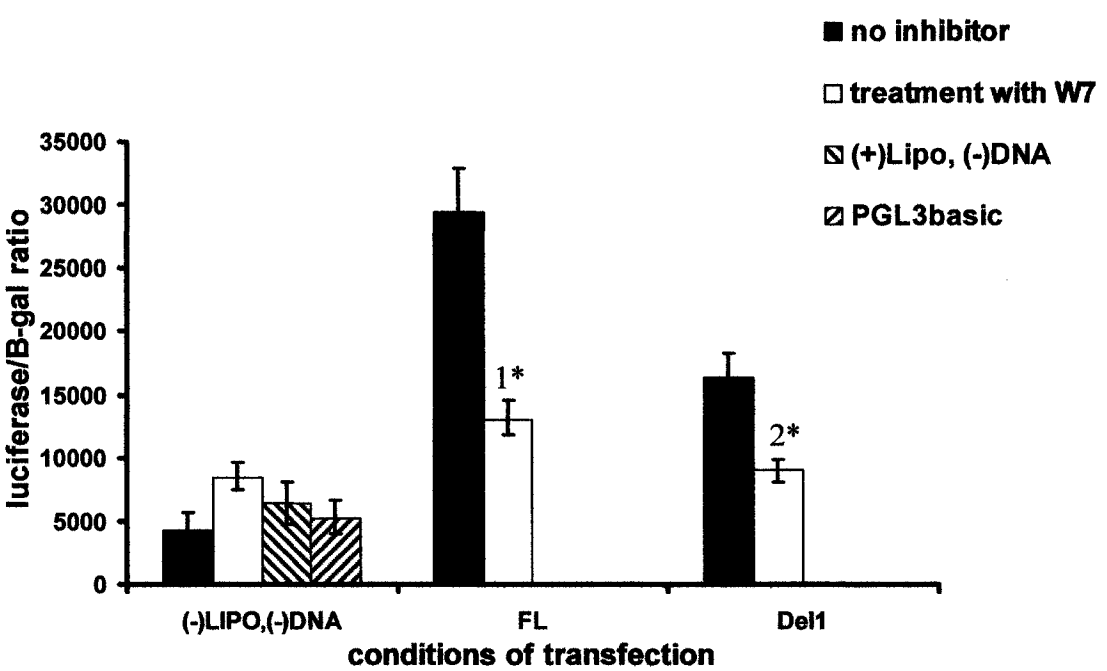

no inhibitor
treatment with W7
(+)Lipo, (-)DNA
PGL3basic
luciferase/B-gal ratio
35000
30000
25000
20000
15000
10000
5000
0
1*
2*
(-)LIPO,(-)DNA
FL
Del1
conditions of transfection

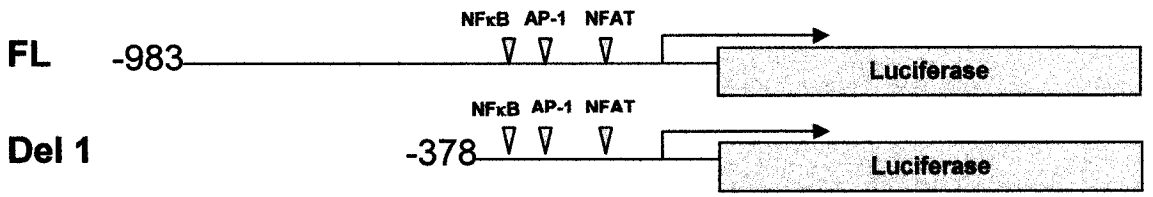
Fig 5.3: The effect of SKF-96365 on the luciferase activity of LPS stimulated RAW264.7 cells transfected with murine IL-12p40 (FL and Del1):

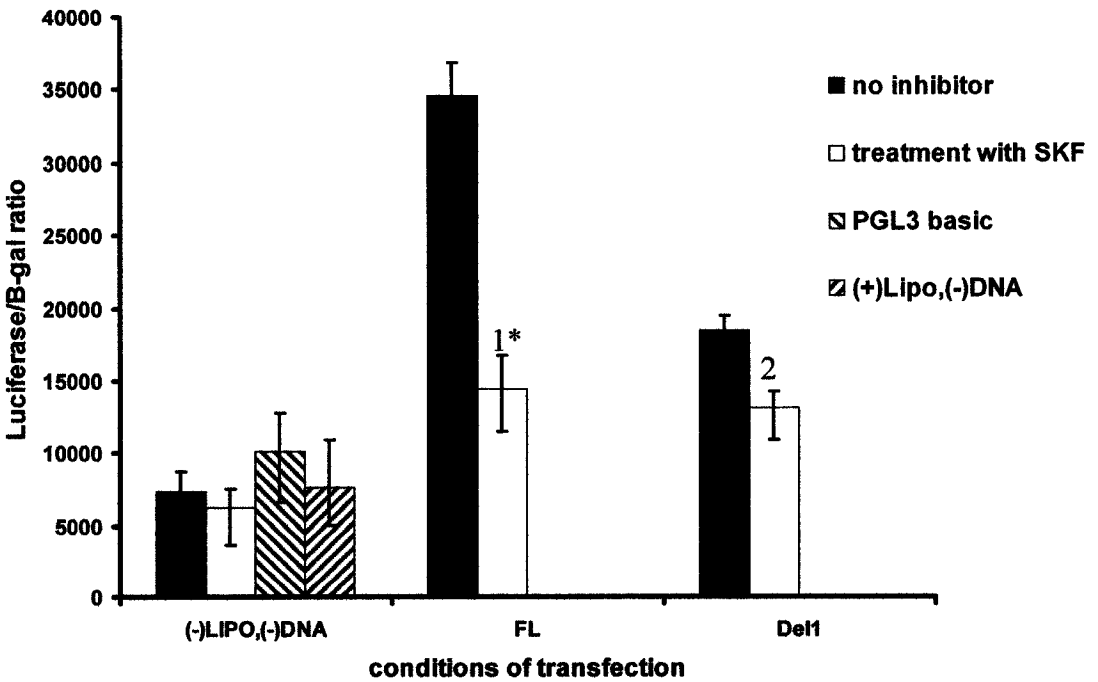
IL-12p40 promoter and 5' deletion mutant were synthesized by PCR, using different 5' primers and a common 3' primer and cloned into the PGL3 basic luciferase reporter vector. $2x10^6$ of RAW264.7 cells were placed in 6 well plates and grown in 2 mL of Optimem medium, containing 10% FBS and no antibiotics. The plates were incubated in a 5% CO2/air mixture for 24 hours to gain about 90% confluence. Transfection of RAW264.7 cells was carried out, using Lipofectamine 2000 reagent according to the manufacture's protocol for 6 well plates. To measure luciferase activity 2 μg of FL/PGL3 or its deletion construct, were co-transfected with 2 μg of pSV-β-galactosidase control vector in RAW264.7 cells. Cells were treated with or without SKF-96365 (10 μM/mL) for 2 hours and stimulated with 1 μg/mL LPS post- transfection and harvested 24 hours following stimulation, using scraper. Then the cells were lysed in 50 μL of lysis buffer. Luciferase and β-galactosidase activities were determined for the cell lysates following manufacture's manual. Lumistar instrument measured luciferase activity while the Dynex MRX Revelation Plate Reader (at OD 405 nm) measured β-galactosidase activities. Luciferase activity was normalized for β-galactosidase activity to give relative luciferase units (*RLU*). Conditions containing [(-) LIPO, (-) DNA], the PGL3 empty vector (basic PGL3), and [(+) Lipo, (-) DNA] were taken as negative controls. This experiment has been done three times in duplicate. Similar results were obtained in a total of three independent experiments. One of three independent experiments with similar results is shown, and normalized for β-galactosidase activity.

P (T<=t) one-tail

1*	0.014944
2	0.463711

Figure. 5.3


NFκB AP-1 NFAT
FL
-983
Luciferase
NFκB AP-1 NFAT
Del 1
-378
Luciferase


40000
35000
30000
25000
20000
15000
10000
5000
0
Luciferase/B-gal ratio
no inhibitor
treatment with SKF
PGL3 basic
(+)Lipo,(-)DNA
1*
2
(-)LIPO,(-)DNA
FL
Del1
conditions of transfection

3.2. Identification of transcription factors activated by calcium pathway which regulate IL-12p40 gene transcription in LPS stimulated splenic macrophages

Transcriptional regulation of IL-12p40 has been studied by several laboratories. It has been indicated that Rel/NFκB site between -122 and -132 bp, relative to the transcription start site is critical for murine IL-12p40 promoter activity stimulated by IFN-γ and also by LPS. There is also evidence demonstrating that the CCAAT enhancer binding protein (C/EBP) site, located downstream from the Rel/NFκB site, is important for mouse IL-12p40 promoter stimulation in cooperation with Rel/NFκB site. [90, 92] Analysis of the murine and human IL-12p40 promoters in RAW264.7 cells has indicated some control elements (such as a C/EBP binding site (between -96 and -88bp) and an AP1 site (from -81 to -75) cooperate to LPS activated expression of p40 gene. [89, 91] AP1 is a heterodimeric transcription factor composed of *jun* and *fos* families of proteins that plays a key role in expression of multiple cytokines. The members of these families have been shown to dimerize with different transcription factors including transcription factor nuclear factor of activated T cells (NFAT).[106, 107] Furthermore, the transcription factor interferon consensus sequence binding protein (ICSBP) has been shown to be necessary for the induction of murine IL-12p40 activity. Interestingly, studies in RAW264.7 cells have indicated that interaction between NFAT and ICSBP is required for IL-12p40 promoter stimulation. This is while IL-10 treatment, which is an anti-inflammatory cytokine that inhibits IL-12p40 expression, prevents from NFAT/ICSBP association in LPS/IFN-γ activated RAW264.7 cells. [89]

NFAT: There are five isoforms of NFAT family of proteins, which are designated as NFAT1 (known as NFATp or NFATc2), NFAT2 (known as NFATc or NFATc1),

NFAT3 (or NFATc4), NFAT4 (known as NFATx or NFATc3), and NFAT5 (non calcium regulated protein). [93] These five proteins are all related to the Rel/NFκB family; therefore, they all contain a Rel similarity domain for DNA binding and interaction with AP1 transcription factors. It has been reported that at least three of them (NFAT1, NFAT3, and NFAT4) are expressed in immune cells, such as lymphocytes: T cells, B cells and NK cells, and myeloid cells (macrophages and mast cells). [94] Activation of NFAT proteins is dependent on stimulation of different membrane proteins such as antigen receptors of T- and B-cells (TCR and BCR, respectively), Fc-receptors for IgG and IgE (FcRg and FcRe, respectively), and CD40 (a co-stimulator molecule of B-lymphocytes). [94] Upon activation, NFAT is translocated to the nucleus, where it interacts with the promoter, and leads to the induction of various cytokine genes. (Fig 5.4) There is a cooperative interaction between NFAT and another transcription factor AP1 which is required for integration of two major signaling pathways (calcium signaling and RAS-MAP kinase pathway) and finally induction of TNF-α, IFN-γ, Fas ligand and GM-CSF in macrophages.[93, 94] However, most of the studies addressing the mechanism of NFAT functions have been done using T cells. For example, it has been shown that formation of NFAT and AP1 in T cells is necessary for the regulation of IL-4, IL-5, and GM-CSF expression. [106, 108, 109]

In addition, NFAT expression is important for the proliferation and differentiation of effector T cells. For example, it has been shown that NFAT1 deficiency diminishes IFN-γ production by Th1 cells, induces differentiation of T cell toward Th2 cell types by increasing Th2 cytokines, and eventually causes severe interstitial pneumonitis. [93] Furthermore, studies on T cells from patients with a rare form of hereditary severe

combined immunodeficiency (SCID T cells) indicated an inactivation of NFAT, NFκB, and Ap1 transcription factors and as a result led to some cytokine deficiency (such as IFN-γ, TNF-α, and IL-2). The impairment of cytokine was due to lack of store-operated calcium entry which led to reduction in activation of all NFATs. [95, 96]

Considering the facts that a) NFAT has been shown to be expressed in macrophages, b) to be necessary for Th1 differentiation, and also due to my data showing that CaM and calcineurin play roles in regulation of LPS stimulated IL-12p40 production, it was a fair question to ask whether SHP-1 plays a role in the activation of NFAT in IL-12p40 gene regulation in splenic macrophages. To test this, I compared whether LPS stimulation of normal and *me/me* splenic macrophages induced the binding of NFAT, AP-1, and NFκB to their respective NFAT , AP-1 and NFκB-binding sites present in the murine IL-12p40 promoter. Therefore, I examined a time course (ranging from 0 to 60 minutes) of activation of NFAT, AP-1, and NFκB elements upon LPS stimulation in normal and *me/me* splenic macrophages using EMSA technique. (Fig 5.5, 5.6, 5.7 respectively) LPS activated nuclear extracts from normal and *me/me* splenic macrophages were examined in a gel shift assay for binding to oligonucleotide probes derived from murine IL-12p40 promoter containing NFAT, AP-1 and NFκB binding sites, respectively. The results show that significant binding of NFAT, AP-1 and NF-κB to the NFAT, AP-1 and NF-κB oligonucleotides, respectively, occurred within 15-20 min following LPS stimulation of normal splenic macrophages. Normal splenic macrophages showed that activation of NFAT and AP-1 elements peaked after 15 minutes post LPS stimulation, but induction of NFκB peaked after 20 minutes. In contrast, very weak binding of these transcription factors to the IL-12p40 promoter binding sites in *me/me* macrophages was observed.

Particularly, LPS activation on NFAT and NFκB in *me/me* splenic macrophages occurred at the later time (30-60minutes) and to a much lower degree compared to normal controls. AP-1 was also activated at the lower level but was induced at 5 min post-activation and diminished at 20 min post-stimulation. Although these experiments revealed diminished activation of transcription factors in LPS stimulated *me/me* nuclear extracts compared to normal controls, they need to be confirmed using cold competition controls. Therefore, suppression of IL-12p40 expression in SHP-1 null *me/me* splenic macrophages may occur due to the limited activation and subsequent binding of nuclear factors to the NFAT, NFκB and AP-1 sites of the murine IL-12p40 promoter. Interestingly, the impairment of NFκB in *me/me* macrophages was consistent with the data showing the aberrant expression of NFκB in *mev* mice lymphocytes which led to impairment of cytokine production and induction of autoimmunity. [97] My data support those bservation and further expand them to *me/me* splenic macrophages showing not only a weak binding of NFκB but also binding of different specious of NFκB element to the IL-12p40 promoter derived oligonucleotides.

Fig 5.4: Schematic view of NFAT activation in cells of lympho-myeloid complex:

Arrows point to NFAT-dependent activation and inhibition of Cytokine genes.

Figure. 5.4

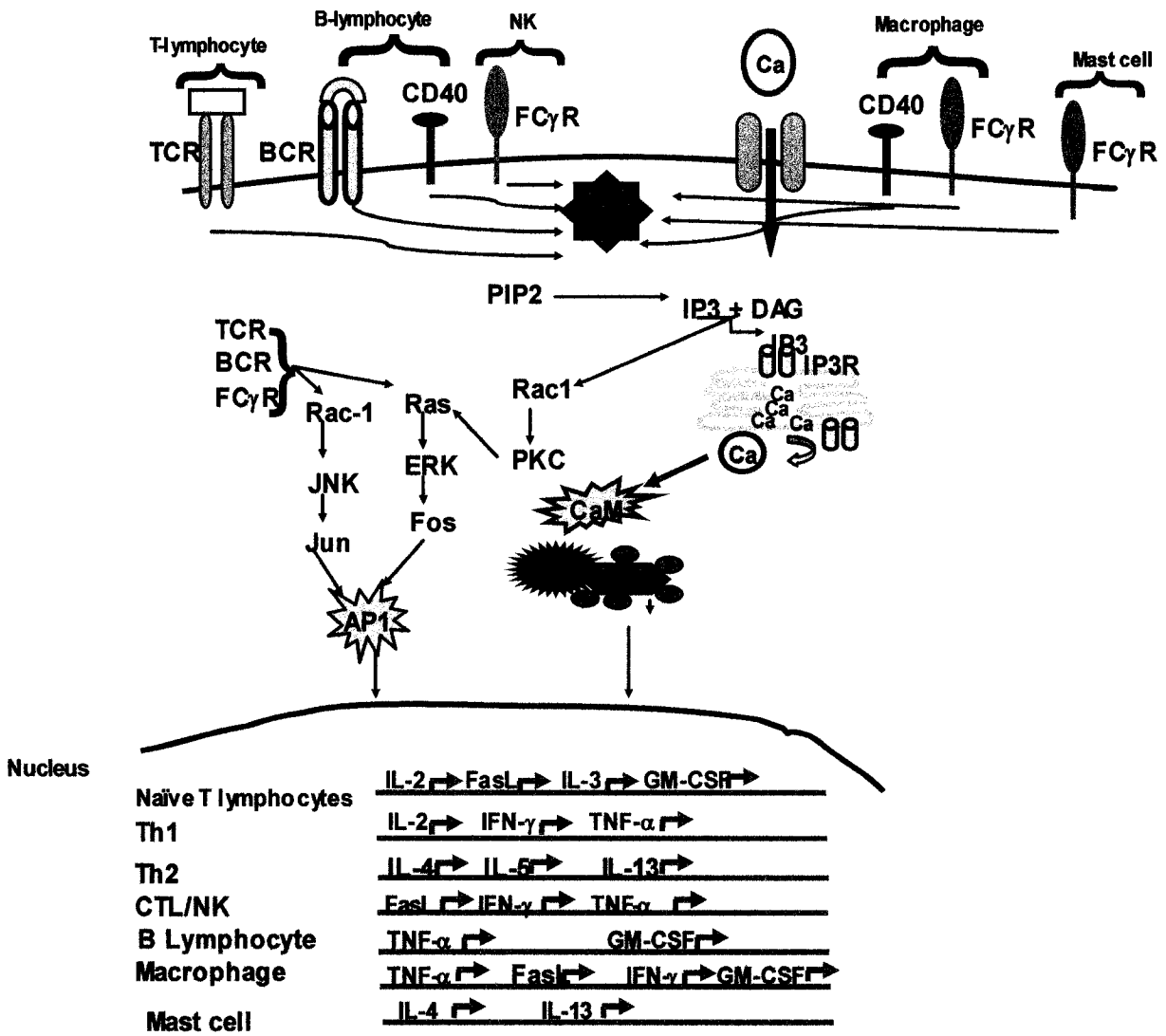
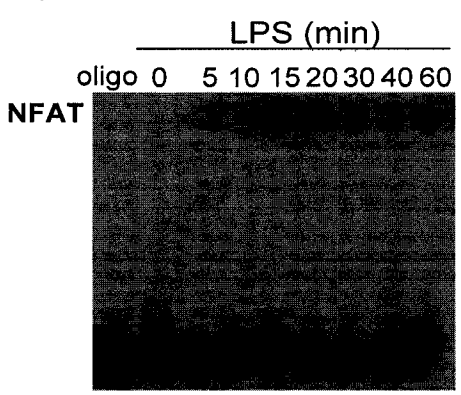

T-lymphocyte
B-lymphocyte
NK
Macrophage
Mast cell
TCR
BCR
CD40
FCγR
Ca
PIP2
IP3 + DAG
IP3
IP3R
Rac1
Rac-1
Ras
ERK
JNK
Fos
Jun
PKC
CaM
AP1
Nucleus
Naïve T lymphocytes
Th1
Th2
CTL/NK
B Lymphocyte
Macrophage
Mast cell
IL-2
FasL
IL-3
GM-CSF
IFN-γ
TNF-α
IL-4
IL-5
IL-13

Fig: 5.5: NFAT activation:

Normal and *me/me* splenic macrophages were grown in $75cm^2$ medium polystyrene flasks until cells became confluent (for each experimental condition the cells were cultured in 3 medium flasks). Normal **(Fig5.5a)** and *me/me* **(Fig5.5b)** splenic macrophages were stimulated with 1 μg/mL LPS for a period of time ranging from 0 to 60 minutes. Nuclear extracts were prepared from activated and inactivated normal and *me/me* splenic macrophages and analyzed for NFAT DNA binding by EMSA, as described under methods. The results are representative of four independent experiments for each of the normal and *me/me* macrophages.

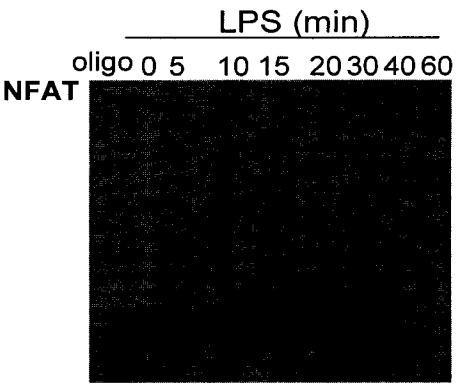
Figure. 5.5

5.5a)



Normal

5.5b)



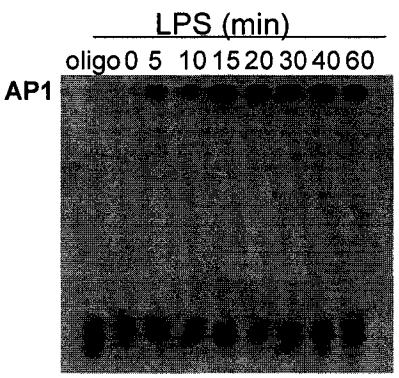
me/me

Fig: 5.6: AP1 activation:

Normal and *me/me* splenic macrophages were grown in $75cm^2$ medium polystyrene flasks until cells became confluent. (for each experimental condition the cells were cultured in 3 medium flasks). Normal **(Fig5.6a)** and *me/me* **(Fig5.6b)** splenic macrophages were stimulated with 1 μg/mL LPS for a period of time ranging from 0 to 60 minutes. Nuclear extracts were prepared from activated and inactivated normal and *me/me* splenic macrophages and analyzed for AP1 DNA binding by EMSA, as described under methods. The results are representative for four independent experiments for each of the normal and *me/me* macrophages.

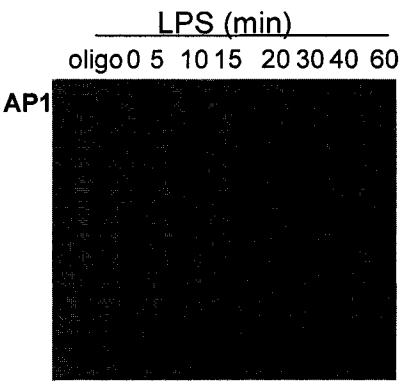
Figure. 5.6

5.6a)



Normal

5.6b)



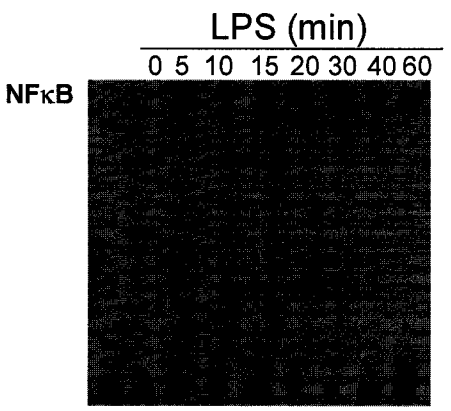
me/me

Fig: 5.7: NFκB activation:

Normal and *me/me* splenic macrophages were grown in 75cm^2 medium polystyrene flasks until cells became confluent (for each experimental condition the cells were cultured in 3 medium flasks) Normal **(Fig5.7a)** and *me/me* **(Fig5.7b)** splenic macrophages were stimulated with 1 μg/mL LPS for a period of time ranging from 0 to 60 minutes. Nuclear extracts were prepared from activated and inactivated normal and *me/me* splenic macrophages and analyzed for NFκB DNA binding by EMSA, as described under methods. The results are representative for three independent experiments for each of the normal and *me/me* macrophages.

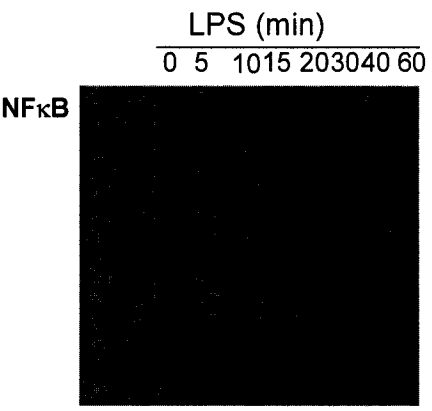
Figure. 5.7

5.7a)



Normal

5.7b)



me/me

Discussion:

The characterization of immunological defects in *me/me* mice has suggested that SHP-1 null mice develop severe inflammatory immune disorders combined with systemic autoimmunity. This might be a consequence of IL-12 dysregulation, a cytokine implicated in autoimmunity. Understanding how the immune system integrates the pleiotropic properties of IL-12p40 is a challenge, particularly so in motheaten mice since IL-12 is both a pro-inflammatory cytokine and an immuno-regulatory cytokine that plays a central role in both innate and adaptive immune response. The primary purpose of the current study was to assess the contribution of SHP-1 to the regulation of LPS activated signal transduction pathways leading to IL-12p40 production with a view to understand inflammatory and autoimmune disorders.

Initial evaluation of the cytokines secreted by LPS stimulated splenic and bone marrow macrophages revealed elevated levels of TNF-α, and decreased amount of IL-6 and IL-10 production in moth-eaten mice compared to normal controls. (Kozlowski's laboratory observations, unpublished, 110, 111)

The data reported herein reveal for the first time roles of SHP-1 in modulating the production of IL-12p40. The data suggest that the production of IL-12p40 (at the protein level) in LPS stimulated splenic *me/me* murine macrophages is significantly down-regulated (about 75%) compared to wild type splenic macrophages. This effect was observed irrespective of LPS dose and the length of stimulation with LPS. In parallel, time course analysis of IL-12p40 message showed a suppressed level of IL-12p40 gene transcription in LPS stimulated *me/me* macrophages compared to normal controls. This

suggests that SHP-1 is an important positive regulator of IL-12p40 production in LPS stimulated macrophages, and thereby it may be a key regulator of Th1 responses. Therefore, it may be hypothesized that there is a hyper-activation of Th2 responses in SHP-1 null *me/me* mice. Moreover, it might be concluded that elevated levels of SHP-1 protein might lead to the up-regulation of IL-12 and as a result elevated level of pro-inflammatory cytokines and consequent development of inflammatory responses.

Considering the fact that IL-12 knock out mice have been shown to be viable, fully fertile, and without any developmental abnormalities [100], IL-12 downregulation alone can not be responsible for all the characteristics of *me/me* mice. Therefore, other factors may be involved in progression of the disease of this phenotype.

One of the reasons, by which lack of IL-12 stands for development of autoimmunity in *me/me* mice, is IL-6 downregulation leading to depletion of bone marrow conventional B cells. In contrast, studies in *me/me* mice have shown that this downregulation of conventional B cells is compensated by a high percentage of normally minor B cell subpopulation (B1 cells), which express CD5 co-receptor. CD5+ B cells have been associated with autoantibody production, and thus cause autoimmunity. [30, 31,32]

Moreover, the low production of IL-12p40, as an immunoregulatory or anti-inflammatory cytokine, may explain why motheaten mice have a lower capacity to limit the protective inflammatory responses when compared to normal controls. This is further supported by the low levels of IL-10 and consequent high levels of TNF-α produced by *me/me* splenic macrophages. This cytokine imbalance makes these mice highly sensitive to development of inflammation. Therefore, other cytokines could compensate for the

lack of IL-12. For example, IL-18 has been reported to be capable of inducing a strong Th1 response and IFN-γ production following endotoxic shock. This effect has been shown to be independent of IL-12. [99] It is possible that *me/me* macrophages might compensate for IL-12 defect by producing more IL-18 with elevated levels of TNF-α and diminished IL-10 production. Despite the importance of IL-12 to both innate and adaptive immune response, the aspects governing the IL-12 production remain unknown.

Furthermore, addition of exogenous recombinant IFN-γ alone to normal and *me/me* splenic macrophages could not induce any IL-12p40 production, which confirms the data from Trinchieri et al. [14, 54] However, it contrasts with the data of Yoshida who found that upon IFN-γ stimulation alone, there is a marked increase of IL-12 p40 mRNA expression in murine macrophage cell line J774.[55] I also showed that in contrast to normal macrophages where stimulation with IFN-γ/LPS enhanced IL-12p40 production, IFN-γ/LPS stimulation did not augment IL-12p40 production in *me/me* macrophages. These results suggested a critical role for SHP-1 in regulating a potentially common signaling molecule or a protein complex, activated by both IFN-γ and LPS, and required for IL-12p40 production.

I further expanded my studies into the role of SHP-1 in the regulation IL-12p40 by interfering with SHP-1 function/expression in normal splenic macrophages using either SHP-1inhibitor (PTP-1) or antisense oligonucleotides specific to SHP-1. As a result, the normal macrophages expressing suppressed level of SHP-1 produced a decreased level of IL-12p40. Conversely, reconstitution of SHP-1 expression in *me/me* splenic macrophages using adenoviral vectors carrying SHP-1 gene demonstrated an increased level of IL-12p40 production in reconstituted cells compared to *me/me* controls.

Therefore, these experiments confirmed that loss of SHP-1 alone can account for the loss of IL-12p40 production in *me/me* mice, and thus it could be concluded for the first time that SHP-1 plays a dominant and a positive role in the regulation of IL-12p40 production. This is in contrast with the data showing that SHP-1 is predominantly involved in the negative regulation of signal transduction pathways in hematopoietic cells. For example, SHP-1 has been shown to downregulate signal transduction pathway activated by IL-2, IL-3, IL-4, EPO, and IFN-α/IFN-β signaling, and BCR, TCR, FCγ receptors, and co-receptors such as CD5, CD19 and CD22. [44, 48, 49, 113, 114] This study shows for the first time a positive involvement of SHP- 1 in the regulation of cytokine production. This notion was further supported by experiment in which the expression of a dominant negative mutant of SHP-1 containing only SH2 domains of SHP-1 led to a significant downregulation of IL-12p40 production compared to untransduced controls. This result further suggests that SHP-1 interacts through its SH2 domains with tyrosine phosphorylated proteins that are involved in the regulation of IL-12p40 production.

Since this study showed a novel function of SHP-1 as a positive regulator of IL-12p40 production, it was important to understand the role of this tyrosine phosphatase in the regulation of IL-12p40 and specifically to characterize its involvement in the positive regulation of signal transduction pathways leading to IL-12p40 production. To determine the mechanism of the inhibition of IL-12p40 secretion from *me/me* splenic macrophages, normal and *me/me* macrophages were treated with pharmacological agents specific to distinct signaling pathways following stimulation with LPS. Treatment of splenic macrophages with inhibitors such as 2APB, CsA, and KN-93, targeting calcium activated signal transduction proteins prior to their LPS stimulation did not affect the IL-12p40

production either in normal or *me/me* macrophages. Thus, the IL-12p40 production was not dependent on store operated calcium channels (SOCs), proline rotamase activity, or CaM kinaseII activity. However, it was dependent on calcium channel activated calmodulin/protein serine/threonine phosphatase (calcineurin) and Ca^{2+}-calmodulin regulated enzyme activity. The inhibition of IL-12p40 production in normal and *me/me* splenic macrophages only occurred when the assays were performed in the presence of SKF-96365 and W7 prior to LPS stimulation. These results are in contrast with the data showing that calcium influx following ligation of Fc-γ receptor results in the inhibition of IL-12 production in response to LPS. Conversely, calcineurin inhibitor has been shown to enhance IFN-γ-induced IL-12 production from murine macrophages suggesting that inhibition of Cn synergizes with calcium flux and IFN-γ activated signaling molecules (but not LPS) to increase IL-12 production. [63, 65] Furthermore, it has been demonstrated that calcium influx, which is induced following stimulation of murine macrophages with LPS, enhances the activation of ERK MAP kinase and thereby targeting IL-12p40 gene transcription by inhibiting the synthesis of transcription factor IRF-1. Therefore, these data suggested that calcium mediated suppression of IL-12p40 may be ERK and IRF-1 dependent. [62] It should be considered that there are some controversial reports stating that LPS induces calcium flux in macrophages. [63] Moreover, there is also some evidence implicating the contribution of calcium signaling pathways in the regulation of IL-12 in DCs. For example, studies have shown that mature DCs activated with either IFN-γ or TNF-α, or CD40L produce high quantities of IL-12p70 and calcium ionophore antagonized IL-12 production by DCs treated with these ligands. Interestingly, addition of calcineurin inhibitor, cyclosporine A (CsA), did not prevent the inhibition of IL-12

production by human DCs activated with IFN-γ or TNF-α, or CD40L. This suggested that inhibition of IL-12 secretion by calcium signaling is not associated with activation of calcineurin phosphatase. [64, 105] Further studies in immature human DCs, cultured with PB-TT, demonstrated that inhibition of calmodulin kinase II (a serine/threonine kinase), which is a critical signaling protein in mammalian cells, resulted in significant decrease in IL-12p70, IFN-γ, and IL-2 secretion [67] This was in contrast with our data showing that inhibition of CaM kinaseII activity did not have any effect on IL-12p40 production in splenic macrophages stimulated with LPS.

Since IL-12p40 production was affected in both normal and *me/me* macrophages upon inhibition of Ca^{2+}-calmodulin regulated enzyme activity and protein serine/threonine phosphatase (calcineurin), other pathways signaling for IL-12p40 production were hypothesized to be dependent on SHP-1.

To explore this, I further demonstrated a critical role for PI3K in negative regulation of the LPS induced IL-12p40 production by both normal and *me/me* murine splenic macrophages. Involvement of PI3K pathway was considered based on data showing that PI3K knockout mice produce a high level of Th1-asociated cytokines including IL-12. Thus, PI3K activity is implicated to have a negative regulatory effect on IL-12 synthesis. Similarly, another study has demonstrated that inhibition of PI3K-Akt pathway, activated by *P. gingivalis* LPS, results in an increased level of IL-12 production. [80] Also, it has been reported that SHP-1 deficient murine macrophages show a 10-15-fold increase in the 3-phosphorylated inositol products of PI3K, and hence the mice suffer from lethal infiltration of the lungs by myeloid cells, particularly macrophages [79]. This observation was consistent with our data showing that 5μM of

LY294002, the PI3K inhibitor, had a pronounced increase in IL-12p40 production by *me/me* macrophages (4 fold) compared to PI3K inhibitor treated normal macrophages (about1.5 fold). However, the PI3K inhibitor induced increase in IL-12p40 production by *me/me* macrophages was still not reaching the level of LPS induced IL-12p40 production in normal macrophages. Therefore, we may conclude that hyperactivation of PI3K in SHP-1 deficient macrophages is one of the reasons for impaired IL-12p40 production in moth-eaten mice.

To further define SHP-1 role in the regulation of IL-12p40, I also examined the contribution of MAP kinase pathway by employing the inhibitors of p38, JNK, and ERK MAP kinases. Normal and *me/me* splenic macrophages were treated with inhibitors and stimulated with LPS, and IL-12p40 production was subsequently measured. Our results demonstrated that p38 MAP kinases do not seem to play any role in IL-12p40 production in murine splenic macrophages. This result was consistent with a study showing no activation of P38 MAP kinases in IL-12p40 regulation in LPS stimulated human monocytes [(91)]. However, studies using $MKK3^{-/-}$ peritoneal macrophages demonstrated that IL-12 production was highly suppressed in the $MKK3^{-/-}$ macrophages upon LPS stimulation. This could be explained by the fact that in $MKK3^{-/-}$ peritoneal macrophages the p38 MAPK activity was reduced, compared to the wild type macrophage control. This is because MKK3 and MKK6 are known to be the specific upstream activators for p38 MAP kinase. This inhibition of IL-12 production was further confirmed by using SB203580 and SB202474, two inhibitor of p38 MAP kinase. [(84)] There is also evidence that IL-12 production by CD40/CD40 ligand engagement is decreased in the $MKK3^{-/-}$ DCs. [(84)] These results were consistent with another study showing that LPS mediated IL-

12 production was suppressed after using SB203580 (p38 inhibitor) in the murine macrophage cell line J774.[82] In contrast, Marriott et al. demonstrated that p38 has a suppressive effect on LPS-induced IL-12p40 production by human monocytes/macrophages.[88] Therefore, the contribution of p38 pathway in IL-12p40 regulation remains controversial.

Similar to p38, when LPS stimulated normal and *me/me* splenic macrophages were treated with JNK inhibitor (SP600125), there was no effect on IL-12p40 production. This is in contrast to study reporting that JNK has a negative role on LPS induced IL-12 production in human monocyte cell line THP-1. This was demonstrated by the experiment showing that using anti-sense oligonucleotides to JNK1 and JNK2 resulted in enhancement of IL-12 production in THP-1 cells. [85] JNK pathway has also been shown to regulate LPS induced IL-12p40 production in a positive manner. For example, treatment of LPS-stimulated normal human monocytic cells with JNK inhibitor (SP600125) led to decreased level of IL-12p40 production. [91]

Finally, when we made use of ERK inhibitor prior to LPS stimulation in normal and *me/me* splenic macrophages, there was an increased level of IL-12p40 compared to the untreated cells. This result was consistent with the data showing that ERK MAP kinases have a negative regulatory role in IL-12 secretion in both J774 macrophages and in murine peritoneal macrophages. [82] In contrast, studies of LPS stimulated THP-1 cells treated with ERK inhibitor (PD98059) revealed that ERK MAP kinases are not involved in IL-12 regulation. [91]

To further investigate the molecular mechanism by which the lack of SHP-1 inhibits IL-12p40 promoter activity, I cloned and purified IL-12p40 promoter containing the full length (FL), fragment -983 to +338 bp, and 5' deletion mutant (Del1), fragment -378 to +338 bp. In this study, I explored the regulation of the murine IL-12p40 promoter in murine RAW264.7 macrophage cell line following activation with LPS. Transfection of RAW264.7 cells with a plasmid containing Del1 promoter fragment did not result in high expression of the luciferase reporter, following LPS stimulation, compared to the cells transfected with FL promoter. Therefore, downregulation of luciferase activity in RAW264.7 cells harbouring Del1 construct suggests that the DNA sequence between -983 to -378 of the IL-12p40 promoter may contain information necessary for driving IL-12p40 gene transcription to high levels.

To further confirm the role of SHP-1 in the regulation of IL-12p40 gene transcription, I analysed the effect of SHP-1 inhibitor (PTP-1) on luciferase activity in RAW264.7 cells transfected with FL and Del1 IL-12p40 promoter constructs. Luciferase activity was significantly down-regulated in RAW264.7 cells containing the FL promoter upon treatment with PTP1. This result confirmed our previous data showing for the first time that SHP-1 has a positive effect on IL-12p40 regulation.

Moreover, to examine the effect of W7 and SKF-96365 on the regulation of IL-12p40 promoter in murine macrophages, RAW264.7 cells, transfected with either FL or Del1 constructs, were independently treated with either W7 or SKF-96365 followed by LPS stimulation. W7 significantly impaired luciferase activity in RAW264.7 cells transfected with either FL or Del1 promoter construct. This result confirmed our

previous data showing the involvement of calmodulin in the regulation of IL-12p40 promoter. SKF-96365 had also a significant effect on the transcription of FL promoter while its effect on luciferase activity of RAW264.7 cells transfected with Del1 was not significant, suggesting that the Del1 fragment, -378 to +355 bp is not an important target for SKF-96365.

The results of these studies support the conclusion that calcium channels and in particular calmodulin/calcineurin pathway are required for IL-12p40 production following LPS stimulation of normal and *me/me* splenic macrophages. Thus, calcium channels and calmodulin act physiologically as positive regulators of IL-12p40 gene expression. Another interesting feature of these studies was that JNK and P38 were not involved in the regulation of IL-12p40 production in LPS stimulated normal and *me/me* splenic macrophages while ERKMAP kinase and PI3K negatively regulate IL-12p40 production in both normal and *me/me* splenic macrophages. Moreover, LPS activated IL-12p40 gene transcription has been shown to be regulated by the activation of multiple transcription factors including NF-κB, AP1 and NFAT. [89, 90, 91, 92] This was consistent with our data showing a significant binding, within 15-20 min following LPS stimulation of normal splenic macrophages, of the transcription factors NFAT, AP-1 and NF-κB to the respective oligonucleotides derived from the murine IL-12p40 promoter. However, the binding of these transcription factors was very weak in *me/me* splenic macrophages. Thus, it maybe that NFAT, AP1, and NFκB activity toward IL-12p40 gene transcription could be increased through the opening the calcium channels and activation of calmodulin/calcineurin pathway but was not dependent on JNK and p38 MAP kinases. The inhibition of calcium pathways significantly downregulated IL-12p40 production,

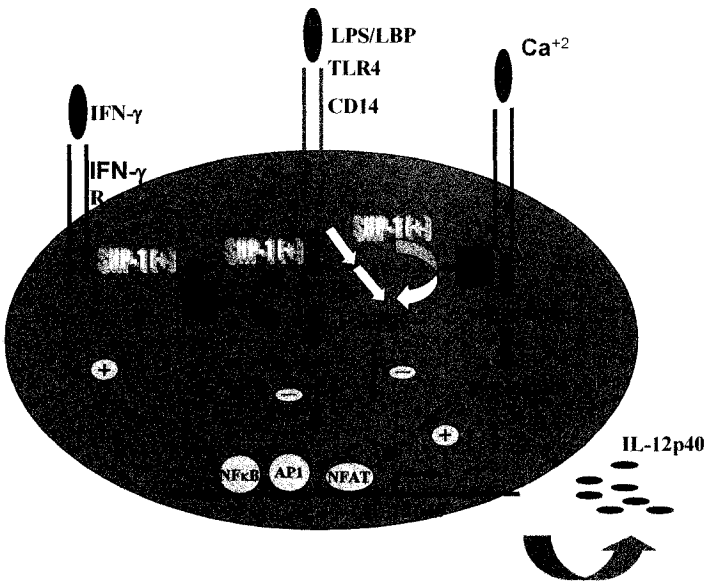
suggesting that calcium pathway has a predominantly positive effect on IL-12p40 production in both normal and *me/me* splenic macrophages. The high levels of IL-12p40 gene expression induced by calcium is modulated in a negative manner by PI3K and ERK pathways. Inhibition of either PI3K or ERK pathway using respective inhibitors led to increased IL-12p40 production in both normal and *me/me* macrophages. However, this increase in IL-12p40 was much greater in *me/me* macrophages. Consistent with this observation and with the notion that SHP-1 is a positive regulator of IL-12p40 production in LPS stimulated splenic macrophages,. SHP-1 may be involved in dephosphorylation of the members of PI3K and ERK signalling pathway leading to the activation of transcription factors such as NFAT , AP1, and NFκB and a consequently efficient IL-12p40 production. The lack of SHP-1 in *me/me* macrophages results in hyper-phosphorylation of PI3K and ERK signalling pathways. This may cause impairment in binding of transcription factors to their sites in the IL-12p40 promoter and downregulation of IL-12p40 gene transcription. Consistent with this model SHP-1 would act as a positive regulator of IL-12p40 production through dephosphorylation and downregulation of PI3K and ERK pathways. (Figure 6.1) Consequently, the take home message of this study is that SHP-1 functions a positive regulator of IL-12 p40 production in murine splenic macrophages by maintaining the balance between the positive and negative signalling induced by LPS.

Figure 6.1: A model showing a positive role of SHP-1 in the regulation of IL-12p40 production in normal and me/me splenic macrophages stimulated with LPS:

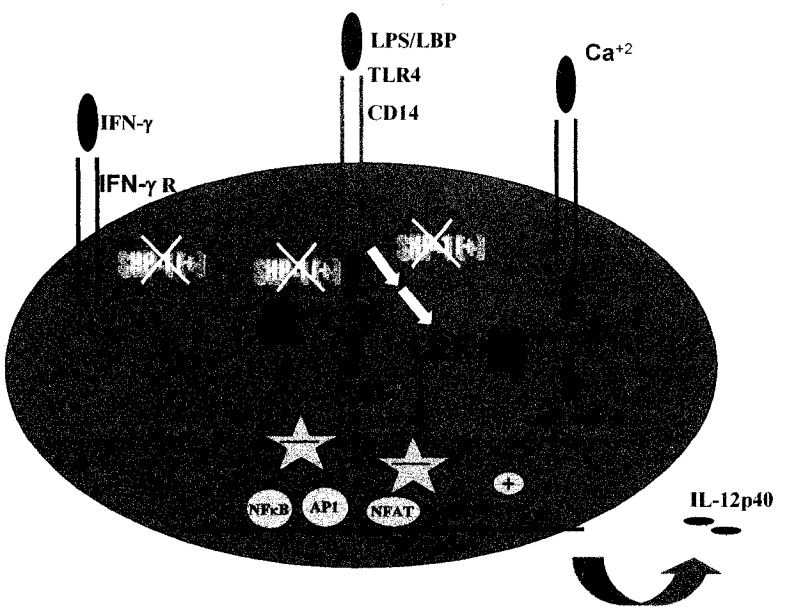
Calmodulin/calcineurin, PI3K and ERKMAP kinase signal transduction pathways synergise toward activation of IL-12p40 gene transcription. Calmodulin/calcineurin pathway predominantly has a positive effect on IL-12p40 production and this is depicted by a (+) sign. PI3K and ERK MAP kinase pathways are implicated as negative regulators and this function is mediated by SHP-1 in such that SHP-1 dephosphorylates members of these pathways allowing secretion of IL-12p40 in normal splenic macrophages. In contrast, in *me/me* macrophages, lack of SHP-1 leads to hyper-phosphorylation of PI3K and ERK MAP kinase pathways resulting in greater activation of these negative regulatory pathways and suppression of IL-12p40 production. Contribution of IFN-γ pathway to IL-12p40 production in normal but not in *me/me* macrophages is also shown.

Figure. 6.1

Normal



me/me



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