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Regulation of IL-12 Family Cytokines in Normal and HIV-1-infected Human Monocytic Cells

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Regulation of IL-12 Family Cytokines in Normal and HIV-1-infected Human Monocytic Cells

By

Ali Akbar Rahim Rahimi

A thesis submitted to the Faculty of Graduate Studies
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

Department of Biochemistry, Microbiology, and Immunology
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Abstract

HIV infection results in progressive loss of general and HIV-specific cellular immunity. HIV employs a variety of mechanisms to undermine the effectiveness of the host immune system including dysregulation of IL-12 family of Th1 cytokines such as IL-12, IL-23 and IL-27. These cytokines are abundantly produced by monocytic cells following stimulation with various TLR ligands that are components of viruses, bacteria, or microbial products such as LPS and cytokines such as IFNγ. Monocytic cells in addition to being key sources of Th1 cytokines, act as a bridge between innate and adoptive immune responses and play a crucial role in HIV immunopathogenesis. It is well established that IL-12 production by monocytic cells is decreased in HIV-infected individuals and following *in vitro* infection of monocytic cells with HIV. However, the role played by IL-23 and IL-27 in HIV immunopathogenesis is not clear. Moreover, whether expression of IL-23 and IL-27 is altered in monocytic cells from HIV-infected individuals and following *in vitro* infection of monocytic cells remains unknown.

To understand the molecular mechanisms underlying the loss of protective Th1 responses in HIV infection, it is imperative to investigate the role and regulation of IL-23 and IL-27. Therefore, I have investigated the molecular mechanisms by which LPS alone or in combination with IFNγ regulate the expression of IL-23 and IL-27 in human monocytic cells and human monocytic cell lines as model systems. I show that LPS-induced IL-23 proteins production is regulated through the activation of JNK, p38 MAPKs and PI3K signalling pathways in THP-1 cells, but following stimulation with IFNγ and LPS, IL-23 is regulated primarily by the PI3K pathway. In normal monocytes, IFNγ- or IFNγ/LPS- induced IL-23 protein production is regulated through the activation of PI3K and calcium pathways. In terms of IL-27 regulation in normal monocytes, I have demonstrated that p38 MAPK and PI3K are the essential signalling pathways that regulate IFNγ/LPS-induced IL-27 production. Moreover, LPS- and IFNγ/LPS-induced IL-27 expression is regulated by JNK, p38 MAPKs and PI3K pathways in THP-1 cells.

Subsequently, I have elucidated the effects of HIV infection of human monocytes, and monocytic cell line (THP-1 cells) on spontaneous as well as LPS-induced production of IL-23 and IL-27. My results suggest that HIV-infection transiently increases IL-23p19, IL-12/23p40 mRNA and IL-23 proteins, but in terms of IL-27 it only increases IL-27EBI3 mRNA expression and does not affect on IL-27 proteins production. In addition, I show that HIV-infection does not affect LPS-induced IL-27 production, but it has a remarkable inhibitory effect on LPS-induced IL-23 production in THP-1 cells and PBMCs.

Taken together, this study provides basic understanding of the major signaling pathways involved in the regulation of IL-23 and IL-27 in activated monocytic cells as well as the effects of *in vitro* HIV infection on the expression of these cytokines. These results may suggest potential strategies aimed at enhancing immune responsiveness.

Dedications

To all those without whom this thesis might not have been written.

To my wife-Nahid- and to the lights of my eyes-Mohammad and Amir-, who offered me unconditional love and support throughout the course of this thesis.

To my mother (Zahra Safari, 1931-2010), who passed away after 7 years soffering from a strange brain stroke, and to my dearest sister -Malihe- who was taking care of our mom all the time after her disease.

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List of abbreviations

AIDS	acquired immunodeficiency syndrome
AIF	Apoptosis inducing factor
Akt	acutely transferring retrovirus Akt8 in rodent T-cell lymphoma
amp	ampicillin
AP-1	Activator Protein-1
APCs	Antigen Presenting Cells
ATCC	American type culture collection
bp	base pair
BSA	bovine serum albumin
cAMP	3, 5-cyclic adenosine monophosphate
Caspase	cysteinyl aspartate-specific protease
CD	Cluster of differentiation
CD127	IL-7R
CD14	specific receptor for LPS
CD45	leukocyte common antigen
CD54	intercellular adhesion molecule-1 (ICAM-1)
CD80	B7.1 (co-stimulatory factor)
CD86	B7.2 (co-stimulatory factor)
cDNA	complementary deoxyribonucleic acid
CMIR	cell mediate immune response
CpG	cytosine-phosphate-guanosine oligodinucleotides
c-Ras	non oncogenic forms of Ras (are present in all cells)
CREB	cAMP response element binding protein
CSIF	cytokine synthesis inhibiting factor
CTLs	cytotoxic T lymphocytes
CXCR4	chemokine receptor
DAG	diacyl-glycerol
DC	Dendritic Cell
DC-SIGN	Dendritic Cell-Specific ICAM-3 Grabbing Nonintegrin; CD209
DC-SIGNR	DC-SIGN-related (also called L-SIGN and DC-SIGN2)
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxy ribonucleic acid
DTT	dithiothreitol
EAE	experimental autoimmune encephalitis
E. coli	Escherichia coli
EB	ethidium bromide
ECL	Enhanced Chemo-Luminescence
EDTA	ethylene diamine tetra acetate
EGF	epidermal growth factor
ELISA	enzyme linked immuno sorbent assay
EMSA	electrophoretic mobility shift assay
Env	HIV envelope glycoprotein

ERK	External Signal-Regulated Kinase
FACS	Fluorescence Activated Cell Sorting
FACScan	fluorescence-activated cell scan
FAM	fluorophore 6-carboxyfluorescein
FcR	Fc receptor
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
G proteins	guanine nucleotide-binding proteins
gag	glycosylated antigen
GAM	Goat Anti Mouse Antibody
GAR	Goat Anti Rabbit Antibody
gp120 (SU)	surface glycoprotein
gp41 ™	transmembrane glycoprotein
GPCRs	G protein-coupled receptors
GPI	glycosylphosphatidyl-inositol
Grb2	growth factor receptor binding protein-2
HAART	highly active antiretroviral therapy
HEPES	N-2-Hydroxyethylpiperazine-N-2-ethanesulphoric acid
HIV-1	human immunodeficiency virus type-1
HRP	horseradish peroxidase
IκB	inhibitors of κB
IBD	inflammatory bowel disease
IFIG	interferon-inducible genes
IC_{50}	Inhibitory Concentration 50%
IFN	interferon
IFN-γ	Interferon-γ
IL	interleukin
IL-1Ra	IL-1 receptor agonist
IMDM	Iscove's modified Dulbecco's modified Eagle's medium
IRAK	interleukin-1 receptor-associated kinase
IRF	Interferon regulatory factors
ISGF-3	interferon stimulated gene factor-3
ISRE	interferon-stimulated regulatory element
Jak	Janus family tyrosine kinase
JNK	Jun N-terminal protein kinase
JNK/SAPK	c-Jun kinase/stress activated protein kinase
kb	kilo base
kDa	kilo Dalton
LBP	LPS-binding protein
LPS	lipopolysaccharide
LTNP	Long Term Non Progression
LTR	long terminal repeat
mAb	monoclonal antibody
Mac1	CD11b
MAP	mitogen-activated protein

MAPKs	mitogen-activated protein kinases
M-CSF	monocyte-colony stimulating factor
MDDCs	Monocyte derived dendritic cells
MDMs	monocyte-derived macrophages
MEK	MAPK/ERK kinase
MEKK	MEK kinase
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MIP-1	macrophage inflammatory protein-1
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
MS	multiple sclerosis
mTOR	mammalian target of Rapamycin
mTOR	mammalian target of rapamycin (signalling pathway)
MyD88	a myeloid differentiation marker that functions as an adapter molecule
Nef	negative factor
NF-κB	nuclear factor κB
NFAT	nuclear factor activated T cell
NK cells	natural killer cells
OD	optical density
ORF	Open reading frame
PAMP	pathogen associated molecular patterns
PBMCs	Peripheral Blood Mononuclear Cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pDC	Plasmacytoid dendritic cell
PDK1	PtdIns (3,4,5) P3-dependent protein kinase-1
PHA	phytohemagglutinin
PI3K	phosphatidyl inositol (PI) 3-kinase
PIAS family	Protein inhibitors of activated STAT family
PKC	protein kinase C
PLA2	phospholipase A2
PLC	a plasma membrane-associated enzyme of the phospholipase C family
PMA	phorbol myristate acetate
PMA	12-O-tetradecanoylphorbol-13-acetate (TPA)
PP2B	Calcineurin
PTB	phosphotyrosine binding domain
PTGS	post transcriptional gene silencing
PTKs	protein tyrosine kinases
PTPs	phosphotyrosine phosphatases
RA	rheumatoid arthritis
RANTES	Regulated upon Activation Normal T cell-Expressed and secreted
rev	regulator of virus expression
RNA	ribonucleic acid
RNAse	ribonuclease

RT	room temperature
RTKs	receptor tyrosine kinases
RT-PCR	reverse transcriptase-polymerase chain reaction
sCD14	soluble CD14
SDS	sodium dodecyl sulphate
SEK1	stress-activated protein/ERK kinase 1
shRNA	short hairpin RNA
SIV	simian immunodeficiency virus
SLE	Systematic lupus erythematosus
smac	second mitochondrial activator of caspases
SOCS	suppressor of cytokine synthesis
STAT	signal transducer and activator of transcription
Tat	trans-activator of virus transcription
Th1	T helper 1 cells
Th2	T helper 2 cells
TLRs	Toll-like receptors
TNF-α	Tumor Necrosis Factor-α
TRAF	TNF-α receptor associated factor
Tris	tris[hydroxymethyl]aminomethane
Tyk2	tyrosine kinase 2
u	unit
UNAIDS	joint united nations programme on HIV/AIDS
UV	ultra violet
vif	virion infectivity factor
Vpr	virus protein regulatory
vpu	virus protein unknown

1. CHAPTER I

INTRODUCTION

Human Immunodeficiency Virus type I (HIV-1) infection represents one of the most serious challenges to global public health, since more than 33 million people are living with HIV/AIDS worldwide and more than 20 million have died because of this infection [1]. Several viruses that induce chronic infections can evade immune responses to establish infection leading to disease progression. HIV-1 is a prototype of this class of pathogens. Not only does it mutate rapidly and make its surface components difficult to access by neutralizing antibodies, it also creates cellular hideouts, establishes proviral latency, modulates cell-surface receptors and destroys immune effectors to escape eradication [2]. HIV-1 employs a variety of mechanisms to undermine the effectiveness of the host immune system including dysregulation of Th1 cytokines, such as interleukin (IL)-12. IL-12 is an immunoregulatory cytokine that plays an important role in the development of protective cell-mediated immune responses (CMIR) [3]. The loss of CMIR function in diseases such as HIV/AIDS leads to increased susceptibility to opportunistic infections and their consequences. Recently, two heterodimeric cytokines, IL-23 and IL-27 that are structurally related to IL-12 and exhibit biological functions similar to IL-12 with respect to the Th1 differentiation pathway, have been described [4-6]. IL-23 and IL-27 are abundantly produced by monocytes/macrophages and dendritic cells (DCs) [4, 5, 7]. Monocytes/macrophages and DCs which, are not only efficient antigen presenting cells but also are important sources of Th1 cytokines, act as a bridge between innate and adaptive immune responses and play a key role in HIV-1 immunopathogenesis [8-13].

Macrophages contribute to innate and adaptive immune responses against viruses by secreting interferon (IFN)-αβ, IL-12, and IL-18, which synergistically induce IFNγ

production in natural killer (NK) cells and cytotoxic T cells [14]. It is well established that IL-12 production is decreased in HIV-1-infected individuals and following *in vitro* HIV-1 infection of monocytic cells [15-22]. By site directed mutagenesis it has been shown that HIV-1 infection of THP-1 cells suppresses IL-12p40 expression by inhibiting LPS-induced nuclear factor binding to the NF-κB, AP-1, and Sp1 sites of the IL-12p40 promoter [20, 21]. Moreover, monocytes infected *in vitro* with HIV-1 displayed decreased IL-12p35 expression and p70 production, suggesting that such decreased IL-12 expression may contribute to reduced IL-12 production in HIV-1 infected individuals [23]. IL-12 expression has been shown to be regulated by CD40-CD40 ligand interaction and LPS-induced JNK-MAPK dependent AP-1 and NF-κB activation [24, 25]. In the recent past, signalling pathways involved in IL-12 in murine and human monocytic cells and dendritic cells have been investigated [3, 25-33]. However, how the production of IL-23 and IL-27, the members of IL-12 family cytokines, is regulated in normal healthy monocytes/macrophages and following *in vitro* infection with HIV remains unknown. The regulation of IL-23 and IL-27 in monocytes/macrophages and the other cells types has not been properly addressed because of the unavailability of reliable ELISA kits in the last couple of years. There are reports about the regulation of IL-23 and IL-27 subunits at the mRNA level [34-38]. However, these reports did not provide biologically significant observations in the absence of their analysis at the protein level.

Therefore, this thesis will deal with the following questions: a) How IL-23 and IL-27 production is regulated by human monocytic cells; b) what are the possible mechanisms and the signalling pathways involved in this regulation; and finally c) how *in*

vitro HIV-1 infection regulates IL-23 and IL-27 production in normal human monocytic cells.

BACKGROUND

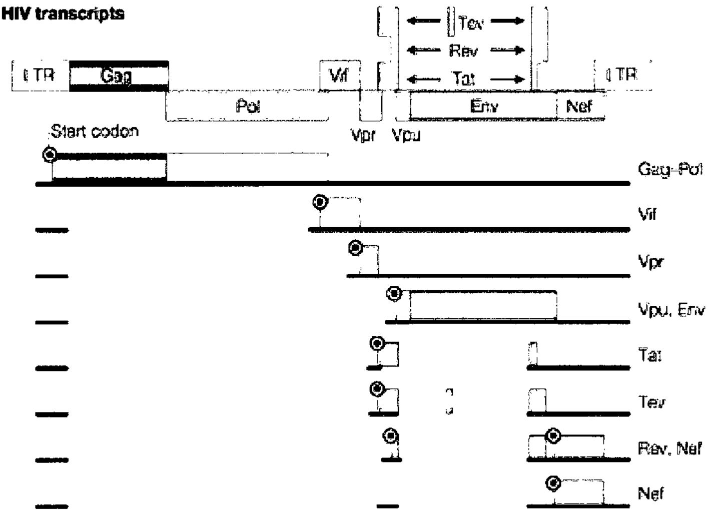
HIV-1:

HIV-1 belongs to a Lentivirus genus in the subfamily of Orthoretrovirinae in the family of Retroviridae. Virions are spherical, enveloped and 80-100 nm in diameter. Virion buoyant density is 1.16-1.18 g/cm3 in sucrose. Virions are sensitive to heat, detergents and formaldehyde, but are relatively resistance to UV light. The virus genome characteristic of members of the subfamily Orthoretrovirinae consists of a dimer of linear, positive sense, single strand RNA, each monomer 7 to 11 kb in size. The RNA constitues about 2% of the virion dry weight. The monomers are held together by hydrogen bonds. Whereas HIV-1 proteins constitute about 60% of the virion dry weight [The eighth report of the International Committee on Taxonomy of Viruses (ICTV)-2005]. Prior to integration, the viral DNA can be detected in the nucleus in three forms, Linear, 1-long terminal repeat (LTR) or 2-LTR circles. HIV regulatory proteins Tat and Rev are produced in basal amounts from these circle DNA in the nucleous of the host cells. The integrated form of HIV-1, also known as the provirus, is approximately 9.8 kilo bases in length. Both ends of the provirus are flanked by a repeated sequence known as the LTRs. The genes of HIV are located in the central region of the proviral DNA and encode at least nine proteins (Fig. 1.1). These proteins are divided into three classes: the structural proteins (Gag, Pol, and Env), the regulatory proteins (Tat and Rev), and the accessory proteins (Vpu, Vpr, Vif, and Nef). The principal activity of the accessory proteins appears to be evasion from various forms of cell-mediated (or intrinsic) immune responses, antiviral resistance and to be important for efficient infection *in vivo*.

Figure 1.1. The HIV-1 genome, transcripts and proteins

A) HIV transcripts: The provirus gets activated when the environmental or internal signal cause the transcription factors stimulate transcription of proviral DNA. The 10-kb HIV genome contains open reading frames for 16 proteins that are synthesized from at least 10 transcripts. The final outcome of transcription process is mass production of viral proteins and viral RNA.

B) HIV proteins: the structural proteins (Gag, Pol, and Env), the regulatory proteins (Tat and Rev), and the accessory proteins (Vpu, Vpr, Vif, and Nef)



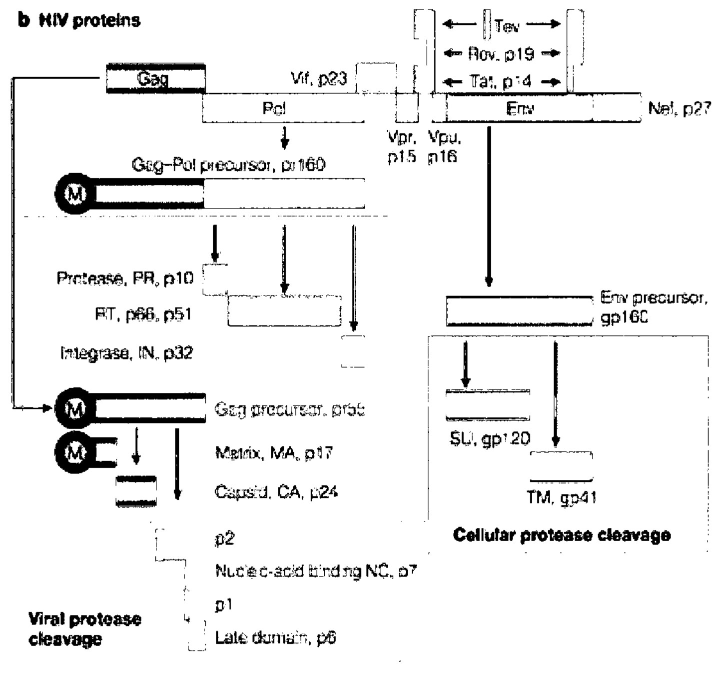


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Figure 1.1

Broadly speaking, the HIV-1 accessory proteins modify the local environment within infected cells to ensure viral persistence, replication, dissemination, and transmission [39].

Monocytes / macrophages and HIV-1 infection:

HIV mainly infects the cells by attaching to CD4 receptors and the CCR5 or CXCR4 co-receptors, expressed on the CD4 subset of T lymphocytes and cells of myeloid lineage including monocytes, macrophages and dendritic cells. The co-receptors for HIV-1 entry, CCR5 and CXCR4 are G protein-coupled receptors (GPCRs), and are also important for the cellular tropism of HIV-1. The β-chemokine receptor, CCR5, is the major co-receptor for macrophage tropic (M tropic) strains of HIV-1, whereas the α-chemokine receptor, CXCR4, facilitates entry of T-tropic HIV-1 strains. Some primary isolates of HIV-1 may be dual-tropic, exhibiting features of both M-tropic and T-tropic isolates and use either CXCR4 or CCR5 for infection of T cells or macrophages. Only a very small proportion of blood monocytes (0.001 to 0.01) are infected with HIV-1 at any time throughout the course of infection *in vivo* [40]. Both adult and neonatal monocytes express CD4. The mRNA transcripts for CCR5 and CXCR4 are present in freshly isolated blood monocytes. Although CCR5 surface expression is not detected on these cells [41], it can be detected on monocytes from adult donors after 24 hours of adherence to plastic [42]. CXCR4 is expressed at high levels as mRNA and membrane protein on freshly isolated monocytes [41, 43]. Monocyte-derived macrophages (MDMs) are susceptible to infection with both clinical and laboratory strains of HIV-1 *in vitro* and are

commonly used as a model to assess HIV-1 infection of tissue macrophages [41, 44]. Tissue macrophages are major targets for HIV-1 infection. Resident tissue macrophages including alveolar macrophages, peritoneal macrophages, perivascular macrophages and microglia in brain are readily susceptible to HIV-1 infection *in vitro*. The proportion of macrophages infected by HIV within tissues is relatively high, ranging from 1- 50% depending on the site and stage of infection [45-49]. HIV can be detected in 10-20% CD16+ monocytic cells in patients on highly active antiretroviral therapy (HAART).

Monocytes/macrophages are professional phagocytes capable of inducing adaptive immunity by presenting antigens to T cells. Mononuclear phagocytes are widely recognized as cells that play a central role in the regulation of immune and inflammatory activities as well as in tissue remodelling. The execution of these activities is mediated by complex and multifactorial processes, which involve products secreted by activated macrophages. These low molecular-weight proteins known as cytokines are produced by virtually all cells of the innate and adaptive immune systems and, in particular, by T helper (Th) cells and monocytes/macrophages. Cytokines act non-enzymatically in picomolar to nanomolar concentrations to regulate host cell function. Their major functional activities are concerned with the regulation of the development and behaviour of immune effector cells such as activation, proliferation, differentiation and migration [50]. Cytokines serve as chemical messengers within the immune system, although they also communicate with certain cells in other systems, including those of the nervous system. Thus, they can function in an integrated fashion to facilitate homeostasis. In addition, they also play a significant role in driving hypersensitivity and inflammatory responses and in some cases they can promote acute or chronic distress in tissues and

organ systems [50]. In general, monocyte activation in response to bacterial endotoxin or lipopolysaccharide (LPS) interaction with its receptor, CD14, induces pro-inflammatory (IL-1, TNF-α, etc.) and anti-inflammatory (IL-10, soluble TNF-R, IL-1Rα) cytokines [51-53].

HIV-1 and modulation of cell surface markers:

HIV infection leads to characteristic alterations of lymphocytes subsets, which could be reflected by the altered expression levels of cell surface molecules with various implications of immune functions. A number of cell surface antigens on lymphocytes have been investigated for their association with HIV infection and disease progression, including activation markers such as CD38 and HLA-DR. During HIV infection, both CD38 and HLA-DR are significantly up-regulated on CD4+ and CD8+ T cells [54, 55]. Decreased expression of CD38 on CD8+ T cells is highly correlated with therapy response [56], and lack of expression of CD38 and HLA-DR on CD4+ T cells relates to long-term non-progression in HIV/AIDS [57]. In addition, a shift from a naïve to a memory phenotype (CD45RA+CD45RO- to CD45RA-CD45RO+) has also been observed in the CD8+ subsets when comparing HIV+ individuals to healthy controls [58]. Other frequently used activation markers include CD95 (Fas-receptor) which plays a role in cell apoptosis, CD57 associated with cell replicate senescence [59, 60], and CD71 related to T cell proliferation [61].

In addition to activation markers, cytokine receptors and cell adhesion molecules also have implications in HIV infection and disease progression. It has been shown that HIV infection leads to progressive decrease in the CD127+CD132- (IL-7R alpha and

gamma chain, respectively) subset and increases in the CD127-CD132+ subset on CD4+ and CD8+ T cells [62]. Recently, HIV-Tat protein and IL-7 in a synergistic function were shown to decrease the expression of IL-7R on CD8 T cells [63].

The loss of antigen-specific CD4+ T cells during primary HIV infection is linked to infection of CD127+CD4+ T cells [62]. Recently, the increased concentration of CD127 in the plasma of HIV positive individuals has been reported [64]. With respect to adhesion molecules on T cell surface, the increased number of CD8+CD11b+ T cells has been correlated with HIV disease progression in asymptomatic patients [65]. Closely related to CD11b, the up-regulation of CD11c has been correlated with HIV infection and disease progression [66].

HIV-1 infection and cytokine networks:

Dysregulation of cytokine profiles is a well known consequence of HIV infection. Cytokines play an important role in controlling the homoeostasis of the immune system. The switching of cytokine production profiles from Th1 to Th2 in CD4+ T cells contribute to HIV/AIDS disease progression by impairing cell-mediated immune responses leading to opportunistic infections, malignancies, and autoimmune diseases. Effects of HIV infection on cytokine production *in vivo* and *in vitro* have been examined by several laboratories and are listed in Table 1.1. In addition, cytokines and chemokines have been shown to exhibit multiple effects on HIV-1 infection and replication, which can either be inhibitory, stimulatory or bifunctional [67]. Effects of cytokines on HIV-1 replication in T cells, monocytes/macrophages as well as other cell types have been investigated in details and are summarized in Table 1.2. During the course of HIV-1

Table 1.1. Effect of HIV infection on cytokine production

Cytokine	Secretion	Source of cells	HIV infection
IL-1	↑	MDM	*in vitro*
	↑	monocytes	*in vivo*
IL-2	↓	CD4+ T cells	*in vitro*
	↓	PBMC, CD4+ T cells	*in vitro*, gp160 stimulation
	↓	PBMC	*in vivo*
IL-4	↑	PBMC, CD4+ T cells	*in vivo*
	no change	CD4+ T cells	*in vivo*
	↓	CD4+ T cells	*in vivo*
IL-6	↑	MDM	*in vitro*
	↑	THP-1 cell line, PBMC	*in vitro*, gp41, gp120 stimulation
	↑	serum	*in vivo*
IL-8	↑	MDM	*in vitro*
	↑	serum, bronchoalveolar fluid	*in vitro*
IL-10	↑	monocytes, MDM	*in vitro*
	↑	PBMC, monocytes, MDM	*in vitro*, gp41, gp120 stimulation
	↑	PBMC, bronchoalveolar fluid	*in vivo*
IL-12	↑	monocytes, MDM	*in vitro*, gp120 stimulation
	↓	PBMC	*in vivo*
IL-13	↓	PBMC, CD4+, CD8+ T cells	*in vivo*
IFN-α	↓	monocytes, MDM	*in vitro*
	↑	CD4+ T cells, monocytes	*in vitro*, gp120 stimulation
	↑	serum	*in vivo*
IFN-β	↑	monocytes, MDM	*in vitro*, gp120 stimulation
IFN-γ	↓	CD4+ T cells	*in vitro*
	↑	CD4+ T cells	*in vivo*
	↓	PBMC	*in vitro*
TNF-α	↑	PBMC, CD4+ T cells, MDM	*in vitro*
	↑	monocytes, PBMC, serum	*in vivo*
TNF-β	↑	PBMC, CD4+ T cells	*in vitro*
	↑	cerebrospinal fluid	*in vivo*
MIP-1α, β	↑	CD4+ T cells, MDM	*in vitro*
RANTES	↑	PBMC, plasma	*in vitro*

Taken from Kedzierska and Crowe, 2001. ↑, increase; ↓, decrease.

Table 1.2. Effects of cytokines on HIV replication

Cytokine	Secretion	Source of cells	HIV infection
IL-1	↑	PBMC, MDM, U1	*in vitro*
IL-2	↑	PBMC, T cell line	*in vitro*
IL-4	↑	PBMC	*in vivo*
	↑	monocytes, MDM, PBMC, U937	*in vitro*
	↓	monocytes, MDM	*in vitro*
IL-6	↑	PBMC, MDM, U1	*in vitro*
	no change	plasma	*in vivo*
IL-7	↑	PBMC	*in vitro*
	↑	PBMC	*in vivo*
IL-10	↓	MDM, U1	*in vitro*
IL-12	↑	PBMC, T cell lines	*in vitro*
	↓	MDM	*in vitro*
IL-13	↓	MDM	*in vitro*
IL-15	↑	PBMC, T cell lines	*in vitro*
	↑	PBMC	*in vivo*
IL-16	↓	CD4+ T cells, PBMC, MDM, DCs	*in vitro*
IL-18	↑	U1	*in vitro*
IFN-α, IFN-β	↓	monocytes, MDM, U937, T cells	*in vitro*
	↓	plasma	*in vivo*
IFN-γ	↓	monocytes, MDM	*in vitro*
	↑	U1, U937, PBMC, CD4+ T cells	*in vitro*
TNF-α, TNF-β	↑	T cells, MDM, U1	*in vitro*
MIP-1α, β, RANTES	↓	monocyte, MDM, PBMC, migroglial cells	*in vitro*

Taken from Kedzierska and Crowe, 2001. ↑, increase; ↓, decrease.

infection, secretion of Th1 cytokines, such as IL-2, antiviral IFNγ and IL-12 is generally decreased, whereas that of Th2 cytokines such as IL-10, IL-8, and IL-4 is increased.

Among all abovementioned immune regulatory factors in HIV infection, herein I will briefly discussing the general roles of IFNγ and IL-12 family of cytokines, which regulate cell-mediate immune responses against intracellular infections and are the major focus of my study.

IFNγ

IFNγ belongs to the type II interferons and is a Th1 cytokine. IFNγ is produced by T cells and NK cells from a single gene found on the long arm of chromosome 12 [68]. It has been reported that IFNγ secretion positively correlates with CD4+ T cell counts and negatively with HIV-1 viral load in plasma [69]. Down regulation of IFNγ production is detected in both CD4+ and CD8+ T cells and associated with reduced number of cells secreting IFNγ [70]. IFNγ has inhibitory effects on the HIV-1 entry phase by down-regulation of CD4 receptors on the cell surface of human primary monocytes [71]. Furthermore, IFNγ also inhibits viral replication by inhibiting the reverse transcriptase activity in this lineage of cells and results in a strong protection against HIV-1 infection in human primary monocytes [68, 72].

IL-12

IL-12 is an immunoregulatory cytokine that plays an important role in the development of protective CMIR against intracellular infections caused by bacteria,

parasites, and viruses including HIV [3]. It has been demonstrated that exogenous IL-12 enhances IL-2 production leading to cell proliferation and development of cell mediated immunity and enhanced CTL responses in HIV-infected patients [73]. The loss of CMI function in diseases such as HIV/AIDS leads to increased susceptibility to opportunistic infections and consequent progressive immunodeficiency. A remarkable reduction in IL-12p40 production has been found in PBMCs from HIV positive patients. In addition, an *in vitro* study on HIV-infected human monocytes has shown a remarkable reduction in IL-12p40 production [15, 74]. This reduction in IL-12p40 level has been considered as a major malfunction in HIV-associated immunodeficiency [22, 75, 76]. Although the mechanisms responsible for IL-12p40 reduction is not clear, in a study on macaques it has been shown that a wild-type SIV infection in monocytic cells significantly suppressed the IL-12p40 levels compared to the cells infected with Nef-deficient strains [77]. A recent study in our laboratory has shown that Nef plays an important role in down-regulation of IL-12p40 production via JNK MAPKs and NFκB inhibition [78]. Chambers et al. have shown that HIV infection of THP-1 cells resulted in decreased LPS-induced nuclear factor binding to the NFκB, AP-1, and Sp1 sites of the IL-12p40 promoter [20]. Monocytes infected with HIV-1 *in vitro* also displayed decreased IL-12p35 expression and p70 production [23].

Role of HIV-1 proteins in cytokine regulation:

In addition to containing large amount of Tat and Nef in the cytoplasm and membrane, HIV-1-infected cells release Tat, an 80-104 amino acid protein essential for

HIV-1 replication, and Nef into the extracellular environment [79-81]. Tat and Nef are not only crucial for high levels of HIV-1 replication, but also have important roles in promoting viral immune evasion. The HIV-1 proteins Tat and Nef, which are expressed early after infection, trigger key steps in the viral life cycle and optimize the intracellular milieu of the host cell for viral replication [82]. HIV Tat binds to its RNA response element, called TAR, the host cellular co-factor, and positive transcription elongation factor B (P-TEFb), which induces the expression of two proteins, cyclin T1 and CDK9. Activation of P-TEFb stimulates phosphorylation of the C-terminal domain of RNA polymerase II, converts this LTR-bound enzyme into an effectively elongating polymerase. Production of full-length viral RNA promotes increased expression of Tat that leads to even higher viral gene expression [2, 82]. In the absence of Tat, the host cell RNA polymerase II engages the LTR but cannot elongate effectively down the full length of the viral DNA template resulting in the production of short ineffective viral transcripts [83]. Tat is found in the plasma of infected individuals, where it can also modulate functions of uninfected cells. For example, Tat induces apoptosis in T cells and cell death in neurons. Tat can drive transcription of a number of cytokines, such as IL-6, IL-2, TNF-α, and ICAM-1 [84-91]. Tat inhibits the phagocytosis of apoptotic tumour cells by dendritic cells, possibly leading to defective processing and presentation of tumour-associated antigens, and prevents secretion of IL-12 [92]. Tat blocks granzyme a secretion by NK cells and inhibits killing of tumour targets by NK cells [93]. Extracellular Tat stimulates the growth of Kaposi's sarcoma cells, and promotes chemotaxis and invasive behaviour by monocytes [79, 84, 86, 90, 94-98]. In our lab, Gee et al. have shown that Tat induces IL-10 transcription through an ERK MAPK-dependent

CREB-1 transcription factor pathway [99]. Tat has also been suggested to cause immune disorders by inducing T cell apoptosis, and by interfering with immune responses through modulation of major histocompatibility complex (MHC) proteins expression, synthesis of IL-12 and IL-10, and NK cell activity [90, 94-98, 100]. *The role of Tat on IL-12 family cytokines remains unknown.*

The *nef* gene is highly conserved in all primate lentiviruses and the encoded Nef protein appears to be a virulence factor critical for the development of HIV/AIDS [101]. Nef down-regulates surface expression of CD4, a key receptor for HIV-1, and decreases surface expression of MHC class I proteins. In addition to surface down-modulation of CD4, Nef also accelerates internalization of the CD28 co-stimulatory molecule, which is necessary for maximal T-cell activation. Nef increases the state of T cell activation, rendering the environment in T cells more favourable for viral replication, and prevents apoptosis in the virus-producing T cells [82, 101-108]. Moreover, when CD4+ T cells are relatively normal, exogenous Nef suppresses CD40-dependent IgG, IgA and IgE class switching in bystander B cells, thereby enabling HIV-1 to escape potentially protective T cell-dependent antibody responses [109]. It has been also shown that Nef down-regulates IL-12/23p40 production by NFκB and JNK inhibition [78]. Inhibition of CD4, CD28, MHC-I, and IL-12p40 proteins by Nef may mediate immune evasion. This immune evasion strategy could be particularly important at an early stage of HIV-1 infection. HIV-1 Nef and Vpu regulate the surface expression and localization of host cell membrane proteins [39]. Furthermore, transgenic animals expressing HIV-1 *nef*/3'-LTR gene in their T cells, exhibit profound immunodeficiency with striking similarities to AIDS [102, 103, 110].

Virus protein R (Vpr) is one of the HIV-1 proteins packaged in great quantities into the nucleocapsid. Vpr, a 14 kDa 96 amino acid protein, is the most conserved and multifunctional regulatory protein, but whose function has been difficult to elucidate [111]. Vpr has been detected in the extracellular fluids such as cerebrospinal fluid and plasma of HIV-infected patients [112]. It plays a key role in virus replication by causing nuclear translocation of the HIV-1 provirus to efficiently infect nondividing cells such as monocytes/macrophages and resting lymphocytes [112, 113]. Vpr induces apoptosis in a variety of cell types including T cells, neutrophils, macrophages, and neuronal cells following HIV-1 infection [114, 115]. The bulk of data indicates that the cell death phenotype induced by Vpr is linked to the pathway leading to G2 arrest. In addition, HIV-1 Vpr also facilitates infection of macrophages [39]. In our lab Mishra S. et al. have shown that JNK MAPK-dependent signalling pathway activation is required for Vpr-induced apoptosis in human monocytic cells [116]. Vpr also remarkably enhances glucocorticoid receptor (GR) activity in monocytes/macrophages and lymphocytes, functioning as a potent coactivator of the receptor. It is well known that glucocorticoids are potent inhibitor of IL-12 production in peripheral monocytes/macrophages. Mirani et al. have demonstrated that extracellular Vpr induces GR activity leading to reduction of IL-12p70, but not IL-12p40 production in monocytes/macrophages [112]. A recent study by Majumder et al. also has shown the inhibitory effects of HIV Vpr on IL-12p70 production by applying different approaches. They have demonstrated the inhibitory effects of Vpr on IL-12p70 production in monocytes/macrophages, which were treated with HIV Vpr^{+} strain comparing to HIV Vpr^{-} virus [117]. HIV Vpr also has an effect on the maturation of dendritic cells and modulates cell surface markers on this initial target

of the virus. Vpr impairs surface markers such as CD80, CD83, and CD86. It also has been reported that Vpr inhibits IL-12 production and upregulates IL-10 in DCs [118].

Expression of IL-12 family of cytokines and their receptors:

For over a decade, IL-12 has been recognized as the canonical cytokine that links innate and adaptive immunity, and with the discovery of IL-23 and IL-27 as cytokines related to IL-12, there has been a concerted effort to understand the relationship between these cytokines. Recent studies have provided new insights into the developmental pathways that promote the differentiation and function of $CD4^+$ T helper cells and offer a dramatically altered perspective on the cause and prevention of autoimmune disease. Besides cell-cell contacts that provide activation signals via peptide-MHC/TCR and classical costimulatory interactions (B7/CD28), antigen-presenting cells communicate with T cells via cytokine production. As a consequence of interacting with various microbial products, antigen-presenting DCs and monocytes/macrophages, as well as other cell types, produce a variety of these soluble factors that are responsible for the expansion and differentiation of naïve T cells to generate mature phenotypes such as Th1 and Th2 cells [119]. IL-12, discovered in 1989, is well known as a central player in promoting the differentiation of naïve $CD4^+$ T cells into mature Th1 effector cells and is a potent stimulus for NK cells and $CD8^+$ T cells to produce IFNγ [119].

All cytokines of the IL-12 family are produced by activated DCs and macrophages, which function as antigen-presenting cells. Expression of IL-12p35, IL-23p19, and IL-27p28 has been shown in many different cell types. However, IL-12p40 transcription appears to be restricted to antigen-presenting cells [120]. Stimuli for the expression of IL-

12 family cytokines include pathogen-associated molecular patterns (PAMPs), which are ligands for toll-like receptors (TLRs) on monocytes/macrophages and DCs. The production of cytokines of the IL-12 family by monocytes/macrophages and DCs is triggered when TLR are activated by pathogen-associated molecular patterns present on bacterial, fungal, and viral microbes [121]. In addition, optimal production of IL-12 (and probably also of IL-23 and IL-27) requires cytokines such as IFNγ, IL-4, and IL-13 depending on the cell types [120, 122]. Target cells for all IL-12 family cytokines are NK cells and T cells. In this study I have used IFNγ (10 ng/ml) to prime the monocytes to induce IL-23 and IL-27 expression following LPS stimulation. To date, the exact role of IFNγ-induced IL-23 and IL-27 expression in monocytes/macrophages is not known. The known roles for IFNγ-mediated IL-12 family cytokines expression are summarized on page 37. Monocytes/macrophages and DCs appear to express functional receptors for IL-12 and IL-23 [123-125]. For IL-27, a receptor has also been described on monocytes/macrophages, Langerhans' cells, activated DCs, and endothelial cells [120, 126, 127]. The individual members of the IL-12 family cytokines have overlapping, but also distinct, activities. This may partially be based on different receptor components expressed on different target cells or due to different developmental stages of the target cells (naïve vs. memory T helper cells) [120]. Naïve T helper (Th) cells express receptors for IL-27 and IL-12, but not for IL-23 [128]. On the other hand, memory Th cells express receptors for IL-23, but have only low or no expression of receptors for IL-27 or IL-12 [120, 129, 130].

Many assays previously performed to measure IL-12 levels were based on measuring the amount of the IL-12p40 subunit, but now it appears that many of those assays may have also been measuring some IL-23 as well.

IL-23 and its biological properties

For several years, IL-12-dependent Th1 cells were thought to be essential for the induction of autoimmunity, based on the use of neutralizing IL-12p40 antibodies or IL-12p40-deficient mice. Recently, IL-23 has been demonstrated to play a key role in the development of several autoimmune diseases such as multiple sclerosis (MS) and experimental autoimmune encephalitis (EAE). In November 2000, Oppmann et al. demonstrated that the IL-23p19 subunit, a four-α helix cytokine with an overall sequence identity of approximately 40% to IL-12p35, has no biological activity by itself, but binds to IL-12p40 to form IL-23, with biological activities similar as well as distinct from IL-12. IL-23 induces proliferation of mouse memory T cells, but IL-12 does not. IL-23 has biological functions that are similar to IL-12, such as IFNγ production from CD4 T cells, antitumor and antimetastatic activity. Similar to IL-12, IL-23 induces IFNγ production in phytohemagglutinin (PHA)-stimulated T cells [119, 129]. Some earlier reports also suggest that IL-23 induces a more robust and sustained cytotoxic T lymphocyte and Th1 immune response than IL-12 [123, 131]. Furthermore, IL-23N220L, an N-glycosylation mutant of this protein that shows reduced expression of excess IL-12p40 but has no inhibitory effects upon IL-23 levels, has been proposed as an effective adjuvant for DNA vaccine to induce antigen-specific T cell immunity [132, 133]. Daily injections of IL-23

in wild-type mice led to a psoriasis-like phenotype with visually apparent erythema and induration and is associated with prominent dermal papillary blood vessel formation and possibly vasodilatation as soon as 2 days after starting treatment [134]. Histological and immunohistochemical examination of IL-23-treated skin show epidermal hyperplasia and a mixed dermal infiltrate consisting of neutrophils, macrophages, CD11c$^+$ DCs, and CD4$^+$ T cells as early as 1 day after IL-23 treatment. Importantly, these activities are unique to IL-23, as IL-12 does not induce changes in epidermal thickness when injected into skin [119]. The impact of IL-23 on autoimmunity and chronic inflammation is well established. Increased level of IL-23p19 subunit expression in microglia cells from multiple sclerosis lesions have been reported [135]. However, its role in protection against infectious diseases is poorly understood and our current understanding of the biology of IL-23 is still considerably less than IL-12, which to-date has been studied more extensively. IL-12 is critical for the generation of CMIR to infectious agents including the HIV-1 virus, as well as having potent anti tumour activity, and playing a significant role in autoimmunity [3, 120].

The biological functions of IL-12 and IL-23 may be attributed to the sharing of IL-12 and IL-23 receptor subunits. The IL-23 receptor (R) is also composed of two subunits, the IL-12Rβ1 chain shared by IL-12 and an IL-23-specific chain IL-23R [131, 136, 137]. Like IL-12, IL-23 is primarily secreted by DCs and macrophages and has been implicated in autoimmune disease such as EAE and MS [123, 138, 139]. IL-23, *in vitro,* has been shown to induce IFNγ production by DCs [6, 123], suggesting that it may regulate T cell-mediated immunity. However, IL-23 induces longer-lasting CD4$^+$ and CD8$^+$ T cell immunity than IL-12 [5, 131]. Sentinel dendritic cells and macrophages

produce IL-23 within a few hours after exposure to LPS and other microbial products [140]. This, in turn, triggers rapid IL-17 responses from tissue-resident T cells including α-β, γ-δ and NK T lymphocytes [140]. IL-17 promotes the production of IL-1, IL-6, IL-8, and TNF-α in stromal, epithelial and endothelial cells, and also in a subset of monocytes [140]. The IL-23/IL17 axis is an important mediator of inflammation. In transgenic mouse models, over-expression of IL-23 leads to a systemic inflammatory response [141]. IL-23 effects on IL-17-producing T cells may also enhance the development of several models of autoimmune disease including EAE, collagen-induced arthritis (CIA), colitis, and diabetes [138, 139, 142-146]. IL-23 may also play a role in increased tumour growth associated with chronic inflammation [147].

The impact of IL-23 on autoimmunity and chronic inflammation is well established. In humans IL-23 has been found to be upregulated in several diseases with dysregulated immune function including psoriasis, Crohn's disease, and multiple sclerosis [134, 148, 149]. Together, these proinflammatory cytokines, IL-23 and IL-17, rapidly recruit neutrophils to the site of infection. This rapidly acting IL-23/IL-17 response provides time for adaptive responses to eliminate the infection. However, over activation of IL-23/IL-17 pathway can lead to the development of a chronic inflammatory response and persistent immunopathology [134, 140, 147-149].

Very little information exists on the roles of IL-23 in HIV-1 pathogenesis. One recent study showed that IL-23p19 is reduced in HIV-1 patients on effective antiretroviral therapy (ART) with a corresponding reduction in IFNγ production [150]. Of note, this decreased IL-23p19 expression was observed in a cohort of patients with severe immunodeficiency (CD4 counts <50/μl) prior to initiating ART.

IL-27 and its biological properties

It is well known that IL-12 is a dominant factor in driving the development of Th1 cells leading to secretion of IFNγ, which stimulates the immune response to eradicate intracellular pathogens. Efficient Th1-driven adaptive immune responses require activation of the T cell stimulatory cytokine IL-12 by activated antigen-presenting cells. In 2002, a new heterodimeric cytokine termed IL-27 that consists of Epstein-Barr virus-induced gene 3 (EBI3) and p28 was discovered [151]. IL-27EBI3, a 33-34 kDa glycosylated protein is related to the IL-12p40 subunit of IL-12 [152] and IL-27p28 is related to the IL-12p35 polypeptide. IL-27 is involved in early Th1 initiation and possesses anti-inflammatory properties [153]. IL-27 may also play a role in the induction of T-bet expression, a master transcriptional regulator for Th1 differentiation, and regulation of immunoglobulin class switching in B cells. Its action is mediated through IL-27 receptor consisting of the orphan cytokine receptor WSX-1/TCCR and gp130. The IL-27/WSX1 signalling system induces STAT1-mediated T-bet expression to initiate Th1 differentiation [151, 154].

By comparison to IL-12 and IL-23, which share a similar structural makeup, IL-27 differs in that its subunits are not linked by a disulfide bond. The absence of the disulfide bond theoretically allows for production of the two subunits by distinct cells followed by extracellular association [155]. IL-27 has attracted considerable interest as an anticancer agent due to its similarities with IL-12. IL-12 is thought to be effective against tumours because it promotes Th1 polarization and, thereby, promotes cellular immune responses and proliferation of cytotoxic T lymphocytes (CTL) [155]. IL-27 enhancement of CTL activity strengthens the case that IL-27 could be a potent antitumor agent. Over

expression of IL-27 in mouse colon carcinoma C26 cells, TBJ neoblastoma cells, or B16F10 melanoma cells led to protection against tumour growth [155]. It has been shown that IL-27 has anti-angiogenic properties, suppressing neovascularization of tumours as well as chick embryonic angiogenesis [155]. On the other hand, it has also been shown that IL-27 suppresses the secretion of proinflammatory cytokines, including IL-23-induced IL-17 from activated T cells [156]. Specifically, suppression of IL-2 and IL-17 expression and inhibition of IL-6 function are likely to contribute significantly to the effects of IL-27 [155].

It has been shown that there is an over expression of IL-27EBI3 by EBV-transformed B cell lines and human T-cell leukemia virus type 1 (HTLV-1)-infected cell lines, but there was no significant IL-27p28 or IL-27 expression detected in these cells. This selective over expression of IL-27EBI3 by transformed cells suggests that IL-27EBI3 may play a role, independently from its association to IL-27p28, in regulating anti-viral or anti-tumoral immune responses [157]. The antitumor effects and protective immunity of IL-27 has been shown in mice [158]. IL-27p28 and IL-27EBI3 transcripts are strongly up-regulated in Crohn's disease. The stimulatory effects of these cytokines on naïve T cells in addition to a strongly synergistic action with IL-12 to trigger IFNγ production may contribute to the perpetuation of the inflammatory process in patients with Crohn's disease [159].

Given the similarities in structure between IL-12 family of cytokines and their receptors, it is possible that additional heterodimeric complexes can form. For example, an association between IL-27EBI3 and IL-12p35 has been reported in 1997 [119, 160] and recently two different groups provided evidence that $CD4^+$ $CD25^+$ $FoxP3^+$ regulatory

T cells produce IL-27EBI3 and IL-12p35 in combination leading to a biologically active cytokine that inhibits T regulatory cells function [161, 162]. This heterodimeric cytokine (IL-27EBI3 and IL-12p35) has been designated as IL-35.

Initial attempts to purify IL-23p19 and IL-27p28 from the supernatant of transiently transfected cells were unsuccessful. This observation, combined with the knowledge that IL-12p35 requires IL-12p40 for secretion, led to studies that revealed that secretion of IL-23p19 and IL-27p28 depends on their ability to partner with the small family of secreted type I cytokine receptors, which include IL-12p40, CLF-1, and IL-27EBI3 [119, 129]. Table 1.3 shows the major producers and target cells for all three IL-12 family cytokines. Since the IL-12 family of cytokines share component parts and receptor usage, some of the functions of IL-12, IL-23, and IL-27 are overlapping [4, 7, 131, 163]. However, these cytokines also exhibit unique behaviours, thus eliminating redundancy in their effects on the intracellular milieu [4, 7, 163]. A major role for IL-27 has been defined as a key regulator in the early stages of Th1 development while a predominant role for IL-23 has been defined in the activation, proliferation and IFNγ production in CD4+ memory T cells [4, 7, 163]. In support of this, it has been shown that naïve T cells do not respond to IL-23 whereas activated/memory T cells do respond [5, 7, 123, 130]. IL-27 acts through the IL-27 receptor which is expressed widely within the immune system, and functional effects of IL-27R signalling have been observed in various cell types including mast cells, B cells, NK cells, DCs, macrophages, and neutrophils [126, 127, 151, 164]. IL-27R is composed of a type 1 orphan cytokine receptor, T cell cytokine receptor (TCCR/WSX-1), a subunit predominantly expressed on

	T cells	B cells	NK cells	Macrophages	Dendritic cells	Mast cells	Endothelial cells
IL-12 producers	□	□	□	■	■	□	□
IL-12 responders	■	□	■	□	□	□	□
IL-23 producers	□	□	□	■	■	□	□
IL-23 responders	■	□	■	■	□	□	□
IL-27 producers	□	□	□	■	■	□	□
IL-27 responders	■	□	■	□	□	□	□

Table 1.3. Common functional features of IL-12 family of cytokines. All three cytokines are able to regulate Th1 cell response and to induce IFNγ production. They play crucial roles in CMIR and against intracellular pathogens.

naïve CD4$^+$ T cells and NK cells, and gp130, the second receptor chain [126, 130, 150, 151, 154]. IL-27 induces T-bet expression responsible for maintaining IL-12Rβ2 expression after CD4$^+$ T cell activation and suppresses expression of GATA-3, a key transcription factor in the Th2 differentiation pathway [5, 131, 154, 165]. This is important in HIV-1 infection, as Th2 T cells support greater HIV-1 replication and inhibit cellular immune responses to HIV-1. Moreover, recently IL-27 has been introduced as a novel anti-HIV cytokine similar to IFNα. Imamichi et al. have shown that IL-27 induces 28 IFN-inducible genes (IFIG) in human monocytes derived macrophages and 5 IFIG in CD4 T cells [166]. Also very recent studies have shown an inhibitory effect of IL-27 on HIV [167] and HBV [168] replication. Still there is much more research to be done to define the role of IL-23 and IL-27 in HIV pathogenesis.

Signal transduction pathways:

Since one of the main aims of my research project is to understand the signalling pathways involved in the stimulatory effects of LPS on IL-12 family cytokines induction in human monocytic cells, I will describe briefly the major signalling pathways activated following interaction of LPS with its receptor. Activation of kinases in the cytosolic domain of the receptor and G protein activation are two major types of signal transduction pathways. In general, cytokine receptors have protein kinase activity in its cytosolic domain. The receptor kinases are activated when ligand binds to its extra-cellular domain. The kinase phosphorylates its own cytoplasmic domain; this auto-phosphorylation enables the receptor to associate with and activate a target protein, which

in turn acts upon new substrates within the cell. The most common kinase receptors are tyrosine kinases, but there are also some serine/threonine kinase receptors. An alternate type of signal transduction through G protein activation occurs when the inactive form of the G protein, the trimmer bound to GDP, gets converted to the active form by replacement of GDP with GTP and dissociation of the G protein into a single subunit carrying GTP and a dimer of the two other subunits [169].

LPS signalling pathways:

LPS is the principal component of the outer membrane of gram negative bacteria. It binds to the cell surface receptor CD14 present mostly on myeloid cells including monocytes [170]. The interaction of LPS with CD14 is mediated by LPS-binding protein (LBP) [171] which is present in plasma at 3-10 ng/ml, but whose levels rise dramatically after an acute phase response. CD14 is also present in plasma in a soluble form (sCD14) that helps the preparation of LPS, LBP, and CD14 complex. The complex of LPS, LBP, and CD14 is a transducing signal mediated by LPS to the CD14 negative cells to TLR4 on the cell surface of monocytes/macrophages [172, 173]. CD14 is expressed on the surface of myeloid cells via a glycosyl phosphatidylinositol (GPI) tail, which anchors the protein to the membrane without a transmembrane segment responsible for transduction of signals into the cell [174, 175]. It suggests that CD14 is not the LPS signalling receptor. The signal is mediated by toll like receptor 4 (TLR4) with a leucine rich motif to the extracellular domain for interaction with the ligand such as LPS [176] (Fig. 1.2). In addition to TLR4, MD2 is associated with LPS signalling in monocytes. MD2 is also required for LPS activation of MAPK pathways [177, 178]. LPS-induced NFκB

signalling is mediated by MyD88, IL-1 receptor associated kinase (IRAK), and TNF-α receptor associated factor (TRAF) 6 [179, 180]. TRAF6 activates either MEKK1 or TAK1 that leading to phosphorylation of IκB kinase (IKKs). Normally, NFκB remains in the cytoplasm in association with IκB in a complex. The phosphorylated IκB kinase activates IκB and NFκB is released and translocated into the nucleus for further gene expression. Binding of LPS to TLR4 also induces PI3K activation without involvement of MyD88, IRAK, and TRAF6 [181]. PI3K activates the NFκB pathway [181], however the intermediate molecules have not been identified yet. In addition to PI3K and NFκB pathways, LPS also induces PKC and MAPKs signalling pathways in human monocytic cells [182-184]. In response to LPS, tyrosine kinase in the cytoplasmic domain of the receptor is phosphorylated which leads to activation of Ras-Raf-MEK1/2 followed by phosphorylation of ERK1/2 [185] (Fig. 1.2). However, there is also evidence for LPS activation of ERK1/2 pathway in a c-Raf independent manner [186]. The substrates for ERK1/2 include ElK1, and SRF [187]. LPS also activates the JNK pathway via activation of MEKK1/4 (MAPKKK) followed by MKK4/7 (MAPKK) [188]. The downstream signalling molecules in JNK pathway include c-Jun, ATF2 and Elk-1 transcription factors [189]. The upstream signalling molecules in LPS induced p38 signalling pathway include CdC42, PAK, and Rac1 [179]. The MAPKKKs involved in that cascade are protein kinase RNA regulated (PKR), ASK1, and TAK1 and the MAPKKs include dual specificity kinase MKK3/6 [190]. The p38 MAPK phosphorylates and activates transcription factors that include ATF2, Elk1, CHOP, and MEF2C [181].

Figure 1.2. LPS is one of the best stimulator of monocytes/macrophages and activates various signalling pathways and transcription factors [185].

LPS binds to the serum protein LBP and is transferred to the CD14 at the cell surface. LPS, LPS-binding protein and CD14 complex interacts with the signalling receptor TLR4 and its accessory protein MD-2. LPS stimulates the activation of various MAPK pathways. These pathways directly or indirectly phosphorylate and activate various transcription factors, including Elk-1, c-Jun, c-Fos, ATF-1, ATF-2, SRF, and CREB. In addition, LPS activates the IKK pathway via MyD88, IRAK, and TRAF6. TAK1–TAB2 and MEKK1–ECSIT complexes phosphorylate IKKβ, which in turn phosphorylates IκBs. Subsequent degradation of IκBs permits nuclear translocation of NF-κB/Rel complexes, such as p50/p65. The PI3K–Akt pathway phosphorylates and activates p65 via an unknown kinase.

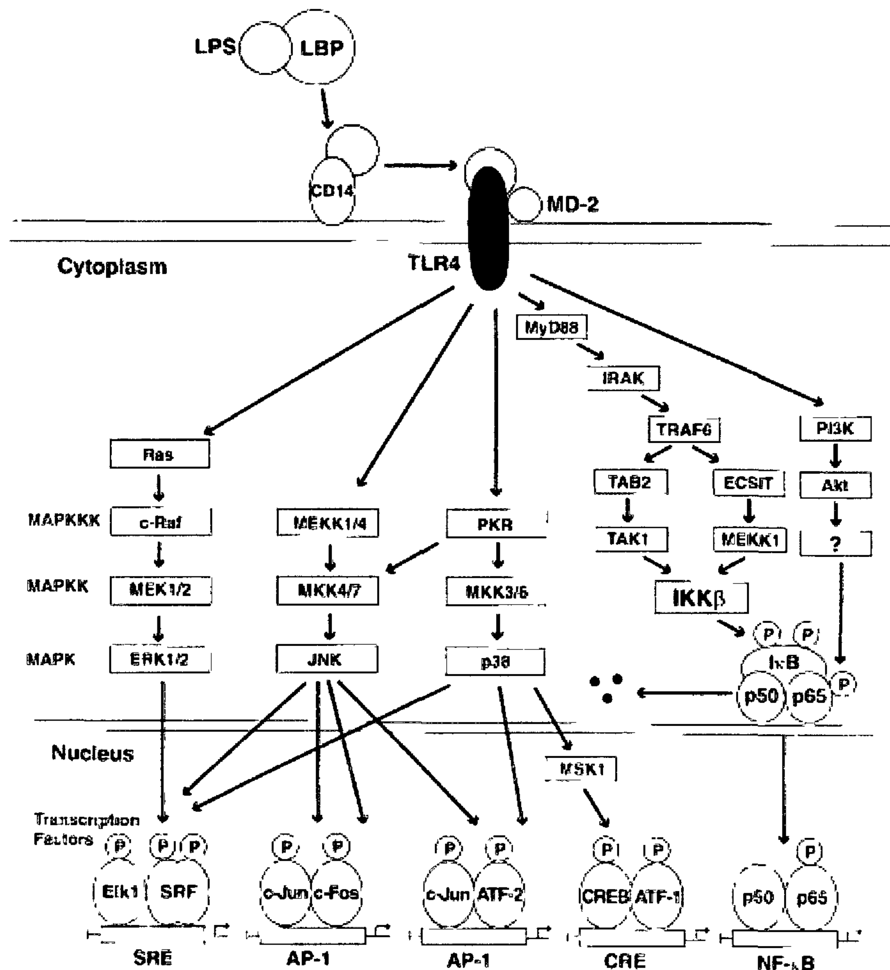


Figure 1.2

The Mitogen Activated Protein Kinases (MAPKs) Signalling Pathway

The best characterized pathway that is initiated by receptor tyrosine kinases involves the activation of a monomeric GTP-binding protein to activate a cascade of cytosolic kinases. In mammalian cells, the cascade is often initiated by activation of a tyrosine kinase receptor, such as EGF or PDGF receptors. The receptor activates the Ras pathway by means of an "adaptor" protein. The activation of Ras leads to the activation of the Raf Ser/Thr kinase, which in turn activates the kinase MEK. In general, MEK phosphorylation induces MAPK activation and it leads to the phosphorylation of transcription factors that trigger changes in cell phenotype varying from growth to differentiation, depending on the cell type (Fig. 1.2). Other targets for the kinases include cytoskeletal proteins that may directly influence cell structure [169]. MAPKs are key players in cellular responses such as proliferation, differentiation, and apoptosis [191]. The three main families of MAPKs are the extracellular signal-regulated protein kinase (ERK1 and ERK2), the C-jun N-terminal kinases (JNKs), and p38 MAPK/stress-activated protein kinases. ERKs respond to mitogens and growth factors that regulate cell proliferation and differentiation, whereas JNK and p38 MAPKs are predominantly activated by stress and inflammatory cytokines (eg. IL-1β and TNF-α) [191].

The Phosphatidylinositol 3-kinases (PI3K) Signalling Pathway

Over the past decade, research on PI3K has demonstrated that this family of enzymes contains important regulators of cellular signalling. They are activated by G-protein-coupled receptors or receptors with an intrinsic or associated protein tyrosine

kinase activity and/or proteins that are tyrosine phosphorylated in response to extra cellular stimuli [192]. Another way in which phosphatidylinositol 3-kinases are activated is by a direct interaction with the small GTPase Ras [193, 194]. In response to extracellular stimuli, phosphoinositides are phosphorylated on the 3-position of the inositol ring by PI3K [195, 196]. The products of PI3K, namely phosphatidylinositol(3)monophosphate [PtdIns(3)P] or PIP, phosphatidylinositol(3,4)biphosphate [PtdIns(3,4)P] or PIP2, and phosphatidylinositol-(3,4,5) triphosphate [PtdIns(3,4,5)P] or PIP3, govern many cellular events, such as cell growth and survival, cytoskeletal remodeling and the trafficking of intracellular organelles (Fig. 1.2) [195-197]. The PI3Ks are divided into four classes, referred to I_A, I_B, II and III, on the basis of their structural characteristics and substrate specificity [197].

Class I phosphatidylinositol 3-kinases generate PtdIns(3)P, PtdIns(3,4)P2, and PtdIns(3,4,5)P3 and consist of four mammalian p110 catalytic isoforms (p110α, β, γ, and δ) that associate with the p85-family of regulatory subunits, except for p110γ which binds to a p101 adaptor [198, 199]. The majority of tyrosine-kinase coupled transmembrane receptors can activate class Ia PI3K. The well-known characterized down stream target for class I PI3K is protein kinase B (PKB). PKB is also known as Akt, the mammalian homologue of the viral oncogene (v-Akt). Akt is activated by two phosphorylation events, both of which are most likely induced by the phosphoinositide-dependent kinase, PDK1, following PI3-kinase activation. Downstream of Akt lie a number of enzymes implicated as effectors of the actions of insulin on glucose metabolism and protein synthesis, and of PI3-kinase signalling on cell survival and cellular proliferation [200]. The phosphatidylinositol 3-kinases of class II generate PtdIns(3)P and PtdIns(3,4)P2.

Enzymatically, the class II kinases are distinguished by their virtual inability to phosphorylate PtdIns(4,5)P2 *in vitro*. A further, distinguishing feature of these enzymes is that they are predominantly membrane-associated, in contrast to class I kinases, which, in the resting state, are cytoplasmic [200]. The class III enzymes only produce PtdIns(3)P [192, 201].

The JAK/STAT signalling pathways

Although cytokines activate multiple signalling pathways, the activation of Jak-STAT pathway plays an important role in mediating their biological effects. The Jak-STAT pathway triggered by a number of cytokines including IL-10 and IFNγ, allows rapidly the transduction of an extracellular signal into the nucleus. In general, the interaction of a cytokine with its ligand-binding receptor α subunit is the first step in the formation of a signalling-competent receptor complex. This process involves the oligomerization of the ligand-bound subunit with either another subunit or a separate, signal-transducing β subunit [202, 203]. This oligomerization initiates the process of signal transduction by activation of the receptor-associated Janus family tyrosine kinases (JAKs) through cross-phosphorylation (Fig. 1.3). Immediate targets of the activated JAKs are the cytoplasmic portions of the receptors and receptor associated proteins. The tyrosine phosphorylated sites become docking elements for Src homology 2 (SH2) - and phosphotyrosyl-binding domain-containing proteins present in the membrane or the cytoplasmic compartment. Prominent among these are the signal transducer and activator of transcriptions (STATs). Receptor-recruited STATs are phosphorylated on a single

tyrosine residue in the carboxyl terminal portion. The modified STATs are released from the cytoplasmic region of the receptor subunits to form homodimers or heterodimers through reciprocal interaction between the phosphotyrosine of one STAT and the SH2 domain of another. Following dimerization, STATs rapidly translocated to the nucleus and interact with specific regulatory elements to induce target gene transcription [204]. Seven members of the STAT family of transcription factors have been identified in mammalian cells: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6. STAT isoforms lacking regions of the c-terminal domain have a competitive dominant-negative effect on gene induction mediated by the STAT pathway, counteracting the effects of the full-length isoform STATα [205-208]. Constitutive activation of STAT1, STAT3, and STAT5 has been demonstrated to be associated with malignant transformation induced by various oncoproteins [208-210]. This phenomenon has been reported in a number of malignant cell lines and human cancers as well [211]. Dimerization of STAT proteins in the cytoplasm by phosphotyrosine-SH2 interaction is a critical step in STAT activation and subsequent gene transcription. Ideal candidates to interfere with dimerization would be SH2-like peptides recognizing phosphotyrosine residues of the STATs or small molecule peptide mimetic with phosphotyrosine residues that specifically bind to SH2 sequence of STATs. Disruption of STAT3 dimerization by the SH2 domain-binding phosphotyrosyl peptide, PYpLKTK, was demonstrated to block STAT3-mediated DNA binding activity, gene regulation, and cell transformation *in vitro* and *in vivo* [212]. Since applying PYpLKTK directly and selectively inhibits STAT proteins' function; nonspecific side effects are theoretically expected to be much less than with other strategies that block STAT upstream signalling [204].

Figure 1.3. JAK/STAT signal transduction pathway [187]

Ligand-induced receptor oligomerization leading to JAKs tyrosine residues phosphorylation. STATs are phosphorylated by the JAKs on a conserved tyrosine residue to form STATs homodimers or heterodimers. STATs dissociate from the receptor after the dimerization and translocate into the nucleus. In the nucleus, homodimer or hetero-dimer of STATs bind to specific response elements and induce gene transcription.

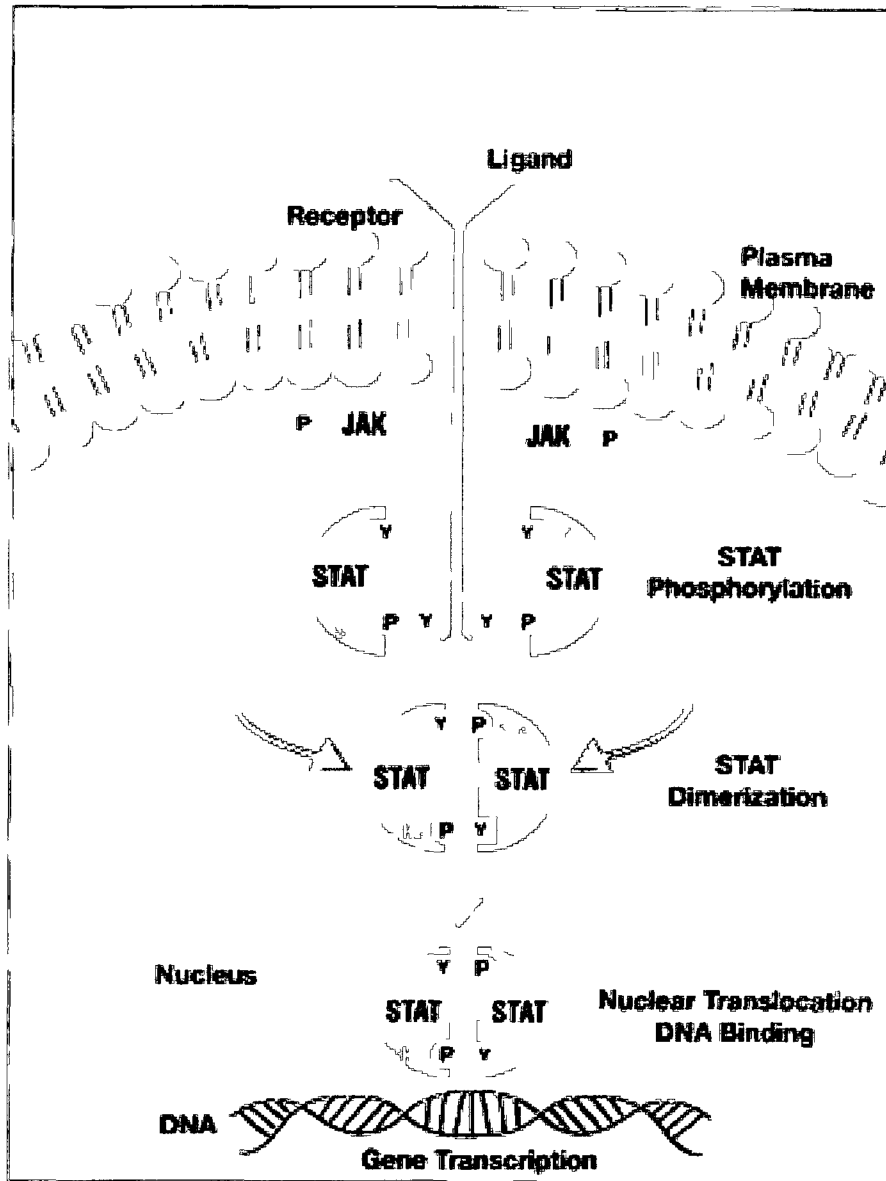


Figure 1.3

Ca2+ signalling pathway:

Ca2+ is a highly versatile intracellular signalling molecule that can regulate various cellular functions [213]. It may also synergize with other signalling pathways [196, 197]. One study suggests the involvement of intracellular sensors of the cytosolic Ca^{2+} levels such as calmodulin (CaM), protein kinase C (PKC) and the $p21^{ras}$ / PI3 kinase / Akt pathway simultaneously link the Ca^{2+} second messenger to NFκB activity [195]. PKC may integrate two types of signals, one from membrane receptors acting through phospholipase C (PLC) [214] and one elicited by Ca^{2+} for Ca^{2+} sensitive PKCs [215]. The ras pathway acts similarly, integrating membrane receptor signals through G proteins and Ca^{2+} signals through ras-GRF and it may activate PI3K [216, 217]. CaM, a Ca^{2+} activated protein regulates phosphatases and kinases, among which calcineurin and calmodulin kinase II (CAMKII) have been reported to be involved in activation of NFκB in neuronal cells [195]. Induction of NFκB by various stimuli has already been shown to require Ca^{2+} for proper signal transduction [195, 218, 219]. Since Ca^{2+} is upstream of all the major kinase signalling pathways, I investigated the involvement of calcium signalling pathway in LPS/IFNγ-induced IL-23 and IL-27 expression in human monocytic cells.

Triggers for expression of IL-12 family cytokines:

Activated monocytes/macrophages and DCs are the major source for IL-12 family cytokines expression. Many pathogens are able to induce the production of IL-12 family cytokines in a variety of normal and/or transformed human monocytic cells. Examples of this include: *Francisella tularensis* [220], *Salmonella enteritidis* [221], *Bordetella*

pertussis [222], *Mycobacterium tuberculosis* [223], Theiler's murine encephalomyelitis virus (TMEV) [224], Cytomegalovirus (CMV) [225], Sendai virus [226], Hepatitis B virus [227], Aspergillus famigatus [228], and Lactobacillus plantarum [133]. Toll-like receptor (TLR) agonists (including LPS, CpG, and PloyI:C) also enhance expression of the IL-12 family cytokine subunits, resulting in the release of IL-12, IL-23, or IL-27 cytokines at the mRNA and/or protein level in monocytes/macrophages and DCs [119, 121, 222]. In addition to human monocytes/macrophages, it has been shown that murine bone marrow derived myeloid DCs and plasmacytoid DCs upon treatment with specific ligands for TLR 3, 4, 7, and 9 are able to produce IL-23p19, but in plasmacytoid DCs IL-23p19 transcripts were not translated into protein and there was no detectable IL-23 protein produced by pDCs [229]. Moreover, some host signals such as CD40, Prostaglandin E2 (PGE2), and adenosine tri-phosphate (ATP) can regulate the balance between IL-12 and IL-23 production [119].

IFNγ mediated IL-12 family cytokines expression:

The enhancement of IL-12 production in IFNγ-primed monocytes has been documented. Although IFNγ alone does not induce the production of IL-12p40, it primes the IL-12p40 promoter so that transcription can occur in response to a second stimulus such as LPS [74, 230-232]. IFNγ promotes IL-12p40 transcription through the induction of transcription factors, interferon regulatory factors (IRFs). IRF-1, 2, 5, 7 and 8 are involved in the regulation of IL12p35 and IL-12p40 transcription [233, 234]. In several studies it has been shown that IRF1&2 knock out mice are defective in IL-12p35 and IL-

12p40 expression, while mice lacking IFR8 fail to express IL-12p40 expression [235-237]. On the other hand, it is well known that over expression of IL-12 may cause inflammatory response leading to autoimmune diseases such as multiple sclerosis. There is a natural negative feedback to control this potential disorder. Among the known negative factors, IL-10 is a potent inhibitor of IL-12 production [28, 238]. IL-10 exerts its inhibitory effects by decreasing NFκB and AP-1 activation [239, 240]. In addition, IFNγ enhances IL-23 production during *Francisella tularensis* infection of human monocytes [220].

Signalling pathways involved in IL-23 regulation:

Infection of monocytes/macrophages by human pathogenic micro-organisms initiates the innate immunity response through the production of immune system modulating cytokines. Examples of this include: IFNβ, IFNγ, nitric oxide, and IL-12, all of which play critical role as anti-viral cytokines [241]. As I explained earlier in this chapter, many different micro-organisms such as bacteria and viruses are able to induce IL-12p40, IL-12p35, IL-23p19, IL-27p28, and IL-27EBI3 expression at mRNA levels and IL-12, IL-23, IL-27 at protein levels [119, 121, 133, 220-229]. However, the potential role of the major intracellular signalling pathways in LPS- and IFNγ-induced IL-23 and IL-27 expression in human monocytic cells is not clear.

To date, there are just a few published papers that reported the role of LPS and micro-organisms in intracellular signalling pathways activation leading to IL-23 and IL-27 cytokines expression in human monocytic cells. Mitsuyoshi et al. demonstrated in

2006 that Rac1 negatively regulates LPS-induced IL-23p19 mRNA expression in human macrophages and DCs [242]. In addition, our previous studies using an extensive deletion analysis of the IL-12p40 promoter, showed that LPS-induced IL-12p40 production in THP-1_{CD14} cells was regulated through AP-1 and NF-κB via the activation of JNK MAPKs [25]. Another study on LPS-induced IL-23 expression has been done in human microglia cell lines. Yonghai et al. have shown that p38 MAPK and NFκB transcription factor positively regulate inducible IL-23p19 expression as the p38 inhibitor (SB203580) significantly decreased the IL-23p19 mRNA induction in microglia cells [243]. Another study in human monocytes demonstrated that *Francisella tularensis*-induced IL-23p19 and IL-12p40 expression were dependent on the PI3K/Akt pathway activation, which leads to NFκB activation followed by IL-23p19 and/or IL-12p40 expression [244]. Yang et al. have shown that *M. tuberculosis*-induced IL-23 production in human MDMs was negatively regulated by Ribosomal S6 kinase 1 (S6K1) activation; and blockade of the mammalian targets of rapamycin (mTOR)/S6K1, downstream mediators of PI3K, remarkably increased IL-12/23p40 and IL-23p19 mRNA as well as IL-23 protein expression [223, 245]. Furthermore, p38 MAPKs inhibition significantly reduced IL-12p40 and IL-23p19 mRNA and IL-23 protein expression [223]. In addition, in a mice splenic DC model system, PI3K pathway was shown to negatively regulate IL-12p40 proteins production [246].

Taken together, it has been suggested that MAPKs and PI3K signalling pathways are involved in the regulation of IL-23 expression in human monocytic cells, which were stimulated with bacteria (such as *F. tularensis*, *M. tuberculosis*, or *S. aureus*) or bacterial products (LPS).

***Signalling pathways involved in IL-27 regulation*:**

The biologically active heterodimer IL-27 as a member of IL-12 family of cytokines has been recently characterized. However, the signalling pathways involved in the expression of this cytokine have not been described. Activated human monocytes/macrophages and dendritic cells are a remarkable source of IL-27p28 and IL-27EBI3 expression [151, 247, 248]. Increased levels of IL-27 transcripts have been shown in monocytes/macrophages and DCs in LPS/TLR4-activated cells [151, 221]. It has also been demonstrated that in the Lewis rat, splenocytes treated with heat-killed *M. tuberculosis* expressed an increased level of IL-27p28 and IL-27EBI3 genes [247]. Furthermore, different stimulators such as LPS and E. *coli* were able to induce IL-27p28 gene expression in bone marrows-derived DCs from Lewis rats [248]. In terms of the role of signalling pathways involved in IL-27 expression regulation, there are a few reported investigations in mice and two reports in human macrophages and DCs. Hause et al. have shown that Theiler's murine encephalomyelitis virus (TMEV)-induced expression of IL-27p28 in RAW264.7, a mouse macrophage cell line, depends on TLR3 and TLR7 activation of JNK-MAPK-kinases [224]. In addition, in two other mouse models, bone marrow derived macrophages (BMDMs) stimulated with *S. enteritidis* or LPS expressed IL-27 subunits via the activation of TLR4/MyD88 signalling pathway [153, 249]. Moreover, Wirtz et. al. demonstrated that TLR ligands significantly upregulate IL-27EBI3 expression and this upregulation occurs via TLR-2, -4, -9, MyD88, and NFκB activation in mouse splenic DCs [250]. Another group has shown that TLR ligands such as LPS, R848, and CPG induce IL-27p28 upregulation through the activation of IRF3 [251]. There are only two reports to date regarding the IL-27 subunit gene expression in

human monocytes. TLR agonists upregulated IL-27p28 and IL-27EBI3 gene expression through IRFI activation in human MDMs; and this IL-27p28 upregulation was inhibited with neutralizing antibodies against IFNα [252]. In the second study, DCs stimulated with TLR ligands or following infection with CMV expressed IL-27p28 through the activation of TLR4 and IRF3 signalling pathways [251]. Overall, these reports suggest that JNK MAPKs activation through MyD88, IRFI, and IRF3 signalling are involved in IL-27 regulation induced by TLR-3, -4, and -7 ligands. However, there is no information available regarding the signalling pathways involved in the regulation of IL-27 in human monocytic cells stimulated with either IFNγ or IFNγ and LPS.

Hypothesis:

I hypothesize that HIV-1 infection will result in changes/modulations in the intracellular signal transduction cascades in monocytic cells, which will result in altered IL-23 and IL-27 expression.

Rationale:

HIV-1 can use several strategies to subvert the host immune response by modulating host cellular gene expression. Our increasing understanding of the functions and mechanisms of action of HIV and its immunopathogenecity, and the availability of several *in vitro* assays to study its activity, should facilitate the discovery of suitable therapeutic anti-HIV-1 inhibitors. Such inhibitors, combined with agents targeting reverse transcriptase, proteases or other factors that are crucial for viral replication, could constitute a basis for the 'pharmaco-vaccination' of HIV-1-infected individuals [2]. In the last few years, the role and regulation of IL-12 in monocytic cells by HIV-1 was the major research area in our laboratory [19-21, 253]. Our results suggest that three distinct signalling proteins regulate LPS-induced IL-12p40 production in human monocytic cells: JNK, p100α subunit of PI3K, and calmodulin-dependent protein kinase-II (CaMK-II) through the activation of NFκB and AP-1 transcription factors [33]. Our preliminary results also suggest that the HIV-1 regulatory protein Nef down regulates LPS-induced IL-12p40 production in monocytic cells by regulating the JNK pathway and the downstream effector NFκB [25]. However, the molecular mechanisms underlying the regulation of IL-23 and IL-27 in particular in human monocytic cells remain unknown.

Moreover, how HIV-1 or its regulatory proteins, affect the molecular mechanisms underlying regulation of IL-23 and IL-27 remain unknown. *Therefore, in this study, I first identified the LPS-induced signalling pathways responsible for the production of IL-23 and IL-27 from human monocytes and a monocytic cell line (THP-1). Subsequently, I investigated how in vitro infection with HIV-1 modulates spontaneous or LPS-induced expression of the IL-23 and IL-27 in human monocytic cells with THP-1 as a model system.*

Monocytes/macrophages are known to harbour HIV-1 virus and serve as viral reservoirs. Through the use of monocytes as a model system, the results of this study will be useful towards devising novel strategies to eliminate virus from infected individuals.

Main Aim and Specific Objectives:

The overall aim of this study is to elucidate the molecular mechanisms by which HIV-1 itself or HIV-1 regulatory protein, Tat, alters the expression of the IL-12 family of Th1 cytokines IL-23 and IL-27 in monocytes and THP-1 (a promonocytic cell line), as model systems. The specific aims are as follows:

Specific Aim 1: To determine the signalling pathways involved in the regulation of IL-23 in LPS-stimulated human monocytic cell lines and primary monocytes.

Specific Aim 2: To elucidate the signalling pathways involved in the regulation of IL-27 in LPS-stimulated human monocytic cell lines and primary monocytes.

Specific Aim 3: To elucidate the molecular mechanisms by which HIV-1 or HIV-1 Tat protein modulate the activity of signalling proteins involved in LPS-induced expression of IL-23 and IL-27 in monocytic cells.

Specifically, I have investigated the role of MAPKs, PI3K, and calcium-signalling pathway involved in the regulation of LPS-induced expression of IL-12 family cytokines.

Significance and Long-term Goals:

The results from these investigations will provide a broad basic understanding of HIV-1-mediated modulation of Th1 cytokines. These data also could lead us to address approaches for controlling HIV-1-associated immune deficiency. It is currently not possible to eliminate HIV-1 infection from the tissue reservoirs in patients receiving anti-retroviral therapy. The approaches directed at understanding the regulation of Th1 cytokines produced by HIV-1-infected monocytic cells may be helpful in devising novel strategies to enhance CMIR and facilitate immune reconstitution and potentially eliminate virus from the body. *Therefore, the long-term goal is to specifically identify the signalling molecules that interact with or are modulated by HIV-1in monocytic cells with the ultimate aim of enhancing Th1 cytokine production and restoring CMIR to facilitate the elimination of virus from the body.*

2. CHAPTER II

MATERIALS & METHODS

Cell lines, cell culture, and reagents

THP-1, a promonocytic cell line derived from an acute lymphocytic leukaemia patient, was obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in ISCOVE'S Modified Dulbecco's Medium (IMDM) (WISENT INC.) supplemented with 10% fetal bovine serum (FBS) (Invitrogen/Life Technologies, Grand Island, NY), penicillin (100 U/mL), and gentamicin (100 μg/mL).

PD98059, an inhibitor of mitogen-activated protein ERK kinase-1 (MEK1) that selectively blocks the activity of ERK MAPKs, the pyridinyl imidazole, SB202190, a potent and specific inhibitor of p38 MAPK, and LY294002, a cell permeable, potent and specific phosphatidylinositol 3-kinases (PI3Ks) inhibitor were obtained from Calbiochem (San Diego, CA). JAK inhibitor I, a broad range inhibitor for JAK kinases was purchased from Calbiochem. Lipopolysaccharide (LPS) derived from *E. coli 0111:B4* and all other chemicals used for Western blotting were obtained from Sigma-Aldrich (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) (Tables 2.1 & 2.2).

Monocytes isolation

Peripheral blood mononuclear cells (PBMCs) were prepared from blood obtained from healthy adult volunteers following approval of the protocol by the Ethics Review Committee of the Children's Hospital of Eastern Ontario. The PBMCs were isolated by density gradient centrifugation over Ficoll Hypaque (Amersham Biosciences, Piscataway, NJ), the cell layer containing mononuclear cells was collected and washed three times in PBS. Purified, nonactivated monocytes were isolated by negative selection by depletion

of T cells and B cells using the AutoMACS separator. Briefly, using the Monocyte Isolation Kit, human monocytes were isolated by depletion of non-monocytes. Non-monocytes were indirectly magnetically labeled with a cocktail of biotin-conjugated monoclonal antibodies, as primary labeling reagent, and anti-biotin monoclonal antibodies conjugated to MicroBeads, as the secondary labeling reagent. In between the two labeling steps no washing steps were required. The magnetically labeled non-monocytes were depleted by retaining them on a MACS® Column in the magnetic field of a MACS Separator, while the unlabeled monocytes passed through the column.

The mononuclear cells obtained contained <5% $CD2^+$ T cells and $CD19^+$ B cells as determined by flow-cytometric analysis.

Table 2.1. Pharmacological inhibitors, used doses, targets, and their sources

	Inhibitor	**Concentrations Used**	**Target**	**Source**
1	PD98059	5-50 μM	ERK MAPK	Calbiochem
2	SP600125	5-50 μM	JNK MAPK	Calbiochem
3	SB202190	5-50 μM	p38 MAPK	Calbiochem
4	LY294002	5-50 μM	PI3K	Calbiochem
5	EGTA	2-10 mM	Calcium chelator	Calbiochem
6	SKF96365	20-100 μM	Receptor mediated calcium entry	Calbiochem
7	KN93	5-50 μM	CAMKII	Calbiochem
8	FK506	0.5-5 μM	Calcineurin	AG Scientific
10	JAK inhibitor-I	5-100 nM	JAK kinases	Calbiochem

Table 2.2. Pharmacological inhibitors, generic names, and molecular formula

	Inhibitor	Generic Name	Molecular Formula
1	PD98059	2′-Amino-3′-methoxyflavone	$C_{16}H_{13}NO_3$
2	SP600125	Anthra[1,9-*cd*]pyrazol-6(2*H*)-one	$C_{14}H_8N_2O$
3	SB202190	4-(4-Fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole	$C_{20}H_{14}FN_3O$
4	LY294002	2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one	$C_{19}H_{17}NO_3$
5	EGTA	Ethyleneglycol-*bis*(β-aminoethyl)-N,N,N′,N′-tetraacetic Acid	$C_{14}H_{24}N_2O_{10}$
6	SKF-96365	1-[□-[3-(4-Methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H-imidazole, HCl	$C_{22}H_{26}N_2O_3$. HCl
7	KN-93	2-[N-(2-hydroxyethyl)]-N-(4-methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine)	$C_{26}H_{29}ClN_2O_4S$
8	FK506	Tacrolimus, Fujimycin	C44H69NO12
10	JAK inhibitor-I	2-(1,1-Dimethylethyl)-9-fluoro-3,6-dihydro-7H-benz[h]-imidaz[4,5-f]isoquinolin-7-one	$C_{18}H_{16}FN_3O$

HIV infection of THP-1 cells

In order to generate stock of the virus, a dual tropic strain of HIV-1 (HIV-1_{CS204}) was propagated based on the propagation of primary HIV isolates (NIH AIDS Research & Reference Reagent Program). Expansion cultures were established by infection of THP-1_{CD14} cells with infectious culture supernatant (kindly obtained from Dr. Jonathan Angel, OHRI, University of Ottawa). At day 10 post infection, supernatants were collected followed by measurement of HIV-1p24 using an antigen capture assay kit (NCI_ Frederick, Frederick, MD). The stock with known concentration of the virus (40 ng/ml p24 Ag) was kept at - 174^{oC}. All experiments were performed with the same batch of the virus.

To determine the effect of HIV-1 infection on LPS-induced IL-12 family cytokine expression, THP-1 cells were maintained in IMDM supplemented with 10% FBS, penicillin (100 U/ml) and gentamicin (100μg/ml). For HIV-1 infections of THP-1 cells, cells were cultured in suspension in 12-well plates at a concentration of $5x10^5$cells/ml in the presence of Polybrene (2 μg/ml). THP-1 cells were mock infected (with media from the cell line used to grow HIV), or infected with cell-free HIV isolates containing 5 to 20 ng/ml of p24 Ag. After 2 hrs of exposure to virus at 37^{oC}, cells were gently washed two times with PBS to remove the unadsorbed inoculum following which cells were resuspended with fresh culture medium. THP-1 cells infected with HIV were maintained for 7 days in culture. HIV infected THP-1 cells cultured for 0, 2, 4, and 6 days were stimulated with LPS for 4 hrs for RNA detection or 24 hrs for measurement of cytokines by ELISA. Supernatants collected after 24 hrs were analysed for production of p24 Ag

and IL-12 family cytokine proteins by ELISA (eBioscience). Cells harvested after 4 hrs of stimulation were analysed for the expression of IL-12 family cytokine mRNAs by Real-Time RT-PCR. HIV-1 replication in THP-1 cells was confirmed by measuring the elevated amounts of HIV-1p24 antigen in supernatants.

Cell stimulation

To determine the effect of ERK, JNK, p38 MAPKs, PI3K, and the Calcium signalling pathways on LPS-induced IL-12 family cytokines expression, monocytes (1 x 10^6 cells/ml) and THP-1 cells (1 x 10^6 cells/ml) were incubated with various concentrations of the MEK-1 inhibitor PD98059 (5 to 50 μM), the JNK MAPKs inhibitor, SP600125 (5 to 50 μM), the p38 MAPK inhibitor, SB202190 (5 to 50 μM), the PI3K inhibitor, LY294002 (2 to 40 μM) and the Calcium pathway inhibitors, EGTA (2-10 mM), SKF-96365 (2-50 μM), or KN93 (2-50 μM) for 2 hours in 12 well culture plates (Falcon, Becton-Dickinson, Frankland Lakes, NJ). Cells were left untreated or stimulated with LPS (1 μg/ml) for 4 or 24 hours in the presence or the absence of the above mentioned inhibitors. The cells and supernatants were then analyzed for IL-12 family cytokines expression by Real-Time RT-PCR or ELISA. All experiments were performed at least three times. Human monocytes and THP-1 cells continuously exposed for 24 hours to the signalling pathway inhibitors in the presence of 10% FBS, and without a change of medium, were >95% viable by microscopic analysis based on Trypan blue staining.

Western blot analysis

Phosphorylation of MAPKs (ERK, p38, and JNK) and the PI3K downstream substrate, Akt, was determined by Western blot analysis using phospho-MAPKs, and phospho-Akt specific antibodies, respectively, according to the standard procedures as previously described[53]. Normal human monocytes and THP-1 cells were pretreated with indicated concentrations of inhibitors followed by stimulation at 37°C / 5% CO_2 for 0-120 min with 1 μg/ml of LPS. Subsequently, cells were placed on ice followed by washing with cold PBS. Cell lysates were prepared by lysing the cell pellets at 4^0C for 30-45 minutes with lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 100 mM NaF, 100 mM sodium orthovanadate, and 1 mM EGTA pH 7.7), followed by centrifugation for 20 min at 11500 x g at 4°C. The protein concentration of the supernatants was determined using the Bio-Rad protein determination assay (Bio-Rad laboratories, Hercules, CA). Total cell proteins were subjected to electrophoresis on 12% polyacrylamide SDS-PAGE gels and the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (BioRad). The membranes were probed with either rabbit anti-human-phospho-p38 (Thr180/Tyr182) mAb (New England Biolabs, Mississauga, Ontario, Canada), mouse anti-human-phosho-ERK MAPKs (Thr202/Tyr204) mAb (Santa Cruz, Biotechnologies, Inc., Santa Cruz, CA), mouse anti-human phospho-JNK (Thr183/Tyr185) antibodies (Santa Cruz, Biotechnologies, Inc., Santa Cruz, CA) or rabbit anti-human-phospho-Akt (Ser473) (Cell Signalling Technology), followed by horseradish peroxidase conjugated goat anti-rabbit or goat anti-mouse polyclonal antibodies (Bio-Rad). The membranes were stripped of the

primary antibodies by incubating the blots in stripping buffer (62.5 mM Tris-HCl, pH 6.7, 100mM 2ME, 2% SDS, and 710 μl DTT) for 30 min and reprobed with rabbit polyclonal antibodies specific for the unphosphorylated forms of either ERK, p38, JNK MAPKs (Santa Cruz), or Akt, (Cell Signalling Technology). All immunoblots were visualized by enhanced chemiluminescence (ECL, Amersham, Baie d'Urfe, PQ, Canada).

Transfection of small interfering RNA (siRNA)

Transfection of ERK siRNA, P38 si RNA, and p85 PI3K siRNA— THP-1 Cells (10^6) were transfected with siRNA SMARTpool ERK, p38, or PI3K p85α or nonspecific control pool (siRNA control) using DharmaFECT™ 2 transfection reagent as per the manufacturer's instructions (Dharmacon). Following transfection, cells were stimulated with LPS (1 μg/ml) for 8 hours for Real-Time PCR or for 30 min for Western blotting for ERK, p38, or p85 PI3K. The supernatants were collected after 24 hours of stimulation for ELISA.

THP-1 cells were seeded in 12 well plates (Falcon) at a concentration of 25 x 10^4 cells/ml in serum-free IMDM media. Cells were transfected with either 1 μg of small interfering RNAs specific for ERK, p38, PI3Kp85 or control vector (Dharmacon) using FuGene 6 (Roche Applied Biosciences, Laval, Quebec, Canada) as described by the manufacturer. The siRNA for PI3Kp85 was a mix of up to three different plasmids. Briefly, the cells were washed once in sterile PBS and then resuspended in serum-free IMDM media. Fugene 6 was allowed to complex with 1 μg of the siRNA plasmids in a total volume of 100 μl of serum free IMDM media for 15 min prior to drop-wise addition

to the cell culture. Following transfection, cells were incubated in serum-free media for 6 hours and were cultured overnight for an additional 16 hours with 2% fetal calf serum. 1 μg/ml of LPS was added 24 hours after transfection and following an additional 24 hr period, the cells were harvested and analyzed for IL-12 family cytokines expression by Real-Time PCR.

Reverse-transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from either THP-1 cells or human primary monocytes using RNeasy kit (QIA GENE) according to the manufacturer's instructions. First-strand cDNA was constructed from 1 μg total RNA (Applied Biosystems).

Total RNAs were analyzed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) using specific primers (Applied Biosystems) and the one-step quantitative RT-PCR system (Applied Biosystems). All the primers were labeled at their 3' terminus with a reporter fluorophore 6-carboxyfluorescein (FAM). Real-Time PCR was performed using specific primer sets for human IL-23p19, IL-27p28, IL-27EBI3, IL-12p35, IL-12p40, and β Actin (see the primer sequences in table 2.3).

Relative Quantitative Real-Time PCR

Expression level of each mRNA was quantitatively analyzed by a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) with the cDNA prepared as described above under "RT-PCR analysis" and TaqMan Universal Master Mix (Applied Biosystems). The expression level of IL-12 family cytokines mRNA detected by TaqMan

Gene Expression Assay (Hs00372324_ml; Hs00377366_ml; Hs00194957_ml; Hs00233688_ml; Hs00168405_ml) is shown as the ratio to that of β Actin, which was determined with human β Actin (Hs99999903_ml) control reagents (Applied Biosystems), by calculation of cycle threshold (Ct) values in amplification plots with a 7500 SDS software (Applied Biosystems).

Table 2.3. Primer sequences used for Real-Time RT-PCR experiments

Target gene primer	**Product ID***	**Sequence**
IL-23p19	Hs00372324_ml	GTGGGACACATGGATCTAAGAGAAG
IL-27p28	Hs00377366_ml	GCCGCCTCTCTGACCCGGAGCGTCT
IL-27EBI3	Hs00194957_ml	GCGCGCTTCCACCGGGTGGGGCCCA
IL-12p35	Hs00168405_ml	TGCTCCAGAAGGCCAGACAAACTCT
IL-12p40	Hs00233688_ml	CAAAAGCAGCAGAGGCTCTTCTGAC
β-actin	Hs99999903_ml	GCCTCGCCTTTGCCGATCCGCCGCC

The concentration of IL-23 (p19/p40) in the supernatants of stimulated Monocytic cells was measured with ELISA Ready-Set-Go kits with pre-coated plates (eBioscience) in which the capture Ab specifically recognizes IL-23p19 subunit and the detection Ab is anti-IL-12p40 mAb.

To study signal pathways involved in IL-23p19 regulation, activated human monocytes were cultured in the presence of signal-specific inhibitors, including

PD98059, SP600125, SB202190, LY294002, EGTA, SKF-96365, and FK506. These inhibitors were dissolved in DMSO. Less than 0.1% DMSO was added to the cells. Real-time PCR was performed to quantify mRNA levels in human monocytic cells. The TaqMan probe for IL-12p35, IL-12/23p40, IL-23p19, IL-27p28, IL-27EBI3 and one endogenous control, β-actin, were purchased from Applied Biosystems. Total RNA extraction and reverse transcript into cDNA were carried out following the instructions of the cDNA synthesis kit (Applied Biosystems). PCR was performed in 96-well microtiter plates under the following conditions: 2 min at 50 °C, 10 min at 95 °C and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Beta-actin was used as endogenous control. To control for cross-contamination, a sample consisting of distilled water was also submitted.

Statistical analysis

Data were graphed and analyzed using GraphPad Prism 5.0 or Microsoft Excel software 2003. Statistical significance was tested using the Student's *t*-test or analysis of variance (ANOVA) with Dunnett post-test pairwise comparison as appropriate. Quantitative data are presented as mean±SE of three or more independent experiments using THP-1 cells or primary monocytes from different blood donors. For all tests, $p < 0.05$ was considered statistically significant. The immunoblot images shown are representative of experiments done using cells from at least three different donors.

3. CHAPTER III

Results

Part I

LPS- and IFNγ/LPS-induced IL-23 Regulation in Human Monocytic Cells

INTRODUCTION:

IL-23, a member of the IL-12 family of Th1 cytokines [129], is produced primarily by monocytic and dendritic cells (DCs) and plays a key role in host defences against invading pathogens by coordinating the innate and adaptive immune responses [4]. It is a heterodimeric cytokine composed of the p40 subunit, which is shared with IL-12, and the p19 subunit unique to IL-23 [4, 128, 129, 254]. Expression of the two subunits is tightly regulated and IL-23p19 is poorly secreted in the absence of IL-12/23p40 [129, 255, 256]. Similar to IL-12, IL-23 induces differentiation and proliferation of Th1 cells, T cell-mediated immunity and IFNγ production [4, 257, 258]. Specifically, IL-23 induces the proliferation of $CD4^+$ memory T cells and IFNγ production; however, naïve T cells are not responsive to this cytokine [4]. The main distinguishing feature of IL-23 is its ability to induce generation of Th17 cells and production of IL-17 [259]. The IL-23-Th17 pathway plays a key role in the induction of inflammatory cytokines contributing to several autoimmune diseases including experimental autoimmune encephalitis, multiple sclerosis, psoriasis, colitis and Crohn's disease and infection with extracellular pathogens [138, 139, 149, 260-262]. Recently, IL-23 was implicated as a crucial cytokine in the pathogenesis of experimental autoimmune encephalomyelitis and in animal models of rheumatoid arthritis [263]. Moreover, anti-IL-23 antibodies have been used effectively in various animal models of inflammatory and autoimmune diseases [263].

The regulation of IL-23 in human monocytic cells in response to mitogens, microbial products, bacteria, and viruses is poorly understood. IL-23p19 and IL-12/23p40

subunits are encoded by two different genes. Recently, LPS-induced IL-23p19 was found to be negatively regulated by Rac1 through the NFκBp65 transactivation-dependent and IκB-independent pathway in PMA-treated THP-1 macrophages and human DCs [242] without affecting IL-12/23 p40 induction [242]. IL-23p19 expression was also found to be regulated by c-Rel NFkB in murine macrophages stimulated with TLR-2, 3, 4, 6 and 9 agonists [264]. There is some evidence to suggest that JNK MAPKs negatively regulates whereas p38 MAPKs positively regulates LPS- and C3bgp-induced production of IL-12p40 and IL-23 in human PBMCs [265]. Herein, I investigated the regulation of IL-12p40 and IL-23 expression in primary human monocytic cells in response to stimulation with either IFNγ alone or IFNγ and LPS. I show for the first time that IFNγ/LPS-induced IL-23 and IL-12p40 expression in primary human monocytic cells is regulated by the p38 MAPKs as well as via the calcium pathway and is independent of JAK/STAT signalling.

IFNγ- and IFNγ/LPS-induced IL-23 regulation in human primary monocytes

To study the regulation of IL-23 production in primary human monocytes, I first investigated the ability of LPS to induce IL-23 expression in primary monocytes. LPS alone enhanced IL-12/23p40 expression. However, it induced relatively low levels of IL-23p19 and IL-12p35 mRNA and failed to induce IL-23 expression (Fig 3.1.A, 3.1.B). These observations were similar to the results shown by other researchers who had described the necessity of priming with IFNγ followed by LPS stimulation to induce IL-12 family cytokines gene expression in human monocytic cells. Optimal expression of IL-23p19 and IL-12/23p40 mRNA *in vitro* requires specific priming of monocytes by IFNγ for 8-16 hrs before LPS stimulation [74, 230-232]. Therefore, the human primary monocytes were treated with IFNγ (10 ng/ml) for 16 hrs either alone or followed by LPS stimulation for 4 and 24 hrs to measure IL-23 and IL-12 mRNA transcripts in addition to IL-23 proteins, respectively. IFNγ stimulation of monocytes resulted in an increase in IL-23p19, IL-12/23p40, and IL-12p35 mRNA levels (Fig 3.1A) as well as an increase in IL-23 proteins production (Fig 3.1.B) compared to the unstimulated cells. Stimulation of cells with both IFNγ and LPS synergistically induced the expression of IL-23p19 ($p=0.0001$) and p40 mRNA ($p=0.01$). However, IFNγ and LPS stimulation induced IL-23 protein expression to the same extent as IFNγ alone (Fig. 3.1.A&B).

Figure 3.1. Effect of IFNγ and LPS on IL-23p19 and IL-12/23p40 mRNA expression and IL-23 protein production in human primary monocytes.

A) Isolated human primary monocytes ($1x10^6$/ml) were treated with either IFNγ (10 ng/ml) for 16 hrs, LPS (1μg/ml) for 4 hrs, or with both IFNγ and LPS for 16 hrs and 4 hrs, respectively. Cell pellets were collected and IL-23p19, IL-12/23p40, and IL-12p35 mRNA expression were quantified by relative quantitative Real-Time PCR (R.Q. Real-Time PCR). Error bars represent the SEM of 3 to 5 independent experiments. IL-12p35 graph (lower graph) is representing a mean of 2 independent experiments.

B) Human primary monocytes ($1x10^6$/ml) were treated with either IFNγ (10 ng/ml) for 16 hrs, or LPS (1μg/ml) for 24 hrs, or with both IFNγ and LPS for 16 hrs followed by stimulation with LPS for another 24 hrs. IL-23 protein production was then determined from the cell culture supernatants by commercially available eBioscience ELISA kits. Error bars represent the SEM of 4 independent experiments.

T-test was used to determine significant differences.

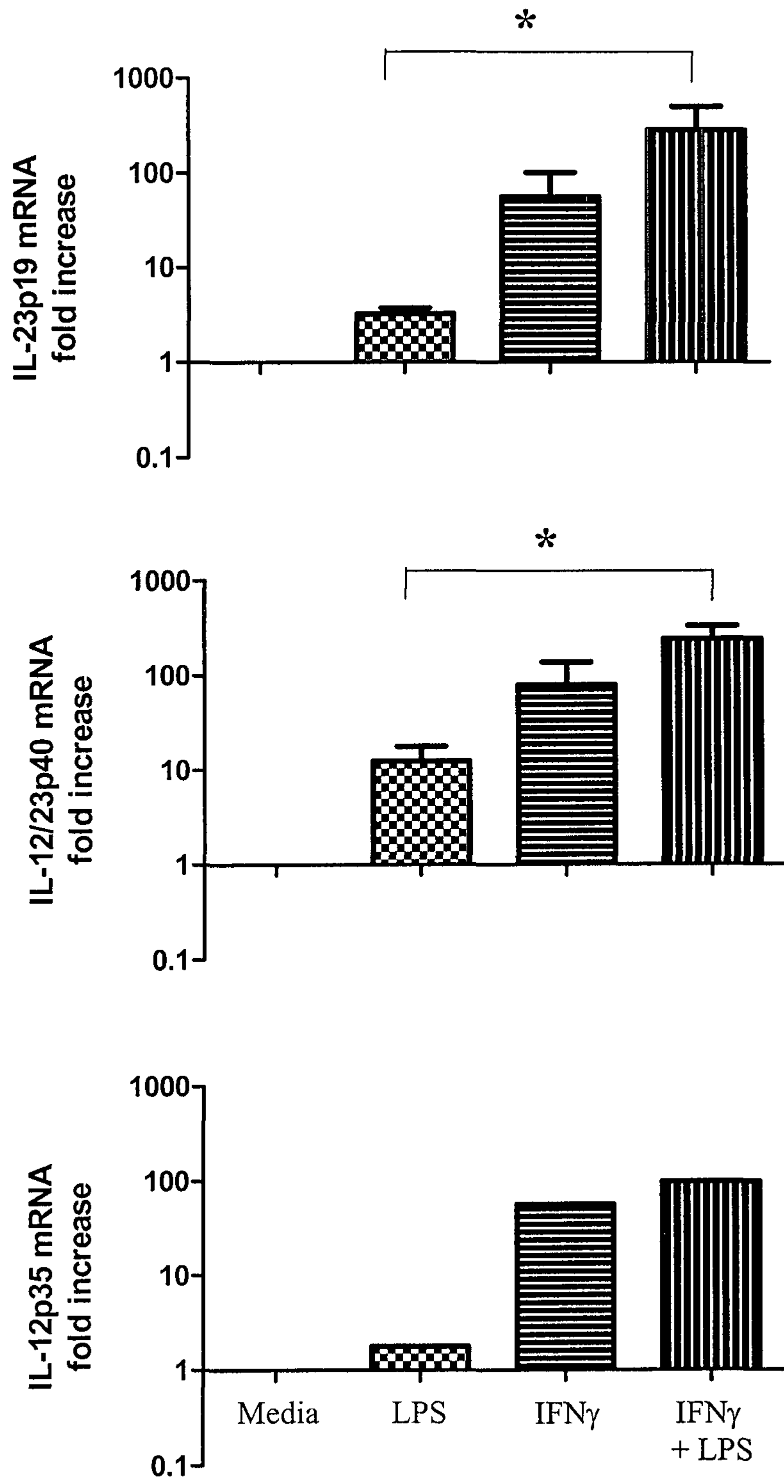


Figure 3.1.A

B.

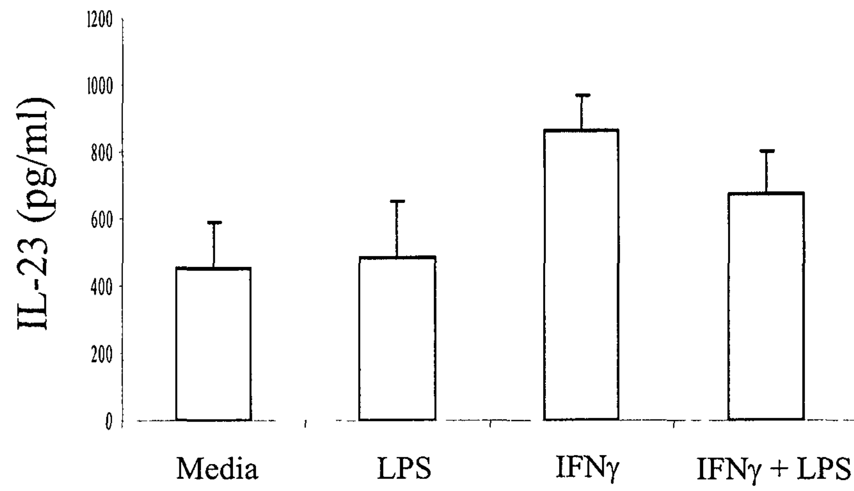


Figure 3.1.B

IFNγ/LPS-induced IL-23 production in human monocytes is not regulated by the JAK/STAT signalling pathway

Since monocytes were primed with IFNγ followed by LPS stimulation, and because IFNγ mediates its biological effects through the activation of JAK/STAT pathway, it was at interest to determine the involvement of IFNγ-activated JAK/STAT pathway in the expression of IL-23 mRNA subunits and IL-23 protein production. The role of the JAK/STAT pathway in regulating IFNγ and IFNγ/LPS-induced IL-23 expression was therefore investigated by employing the general JAK inhibitor, JAK inhibitor-1, to inhibit the JAK/STAT pathway. For this, highly purified primary monocytes from healthy donors were pretreated with a general JAK kinase inhibitor, JAK inhibitor-1, for 2 hrs followed by stimulation with IFNγ for 16 hrs alone or IFNγ for 16 hrs followed by LPS stimulation for 24 hrs and measurement of protein production by ELISA.

Interestingly, pretreatment with JAK inhibitor-1 caused an increase in IL-23p19 mRNA (data not shown) as wells as an increase in IL-12/23p40 protein production in cells stimulated with either IFNγ alone ($p=0.0001$) (Fig. 3.2.A) or with IFNγ and LPS together (Fig. 3.2.B). The JAK inhibitor-1 in the absence of IFNγ/LPS stimulation did not effect IL-23p19 and IL-12/23p40 expression (data not shown). As a control, similar concentrations of JAK inhibitor-1 inhibited IFNγ-induced IL-27 expression ($p<0.05$) (Fig 3.3.B). These results suggest that the JAK/STAT pathway negatively regulates the IFNγ/LPS-induced IL-23 expression.

Figure 3.2. Effect of JAK Inhibitor I –JAK kinases inhibitor- on IFNγ and IFNγ/LPS-induced IL-23 production in human primary monocytes.

A) Monocytes ($1x10^6$/ml) were treated with JAK inhibitor I at concentrations ranging from 0 to 100 nM for 2 hrs followed by IFNγ (10 ng/ml) for 16 hrs.

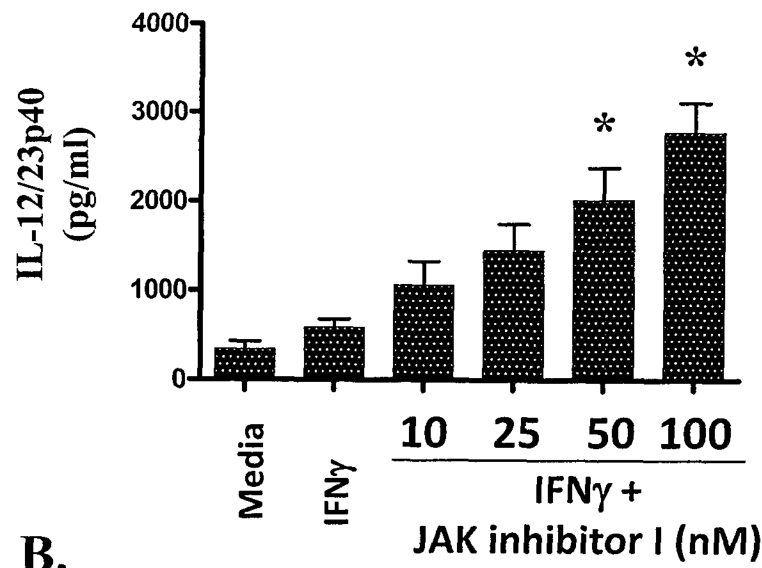
B) Monocytes ($1x10^6$/ml) were treated with JAK inhibitor I at concentrations ranging from 0 to 100 nM for 2 hrs followed by IFNγ (10 ng/ml) for 16 hrs and then LPS (1μg/ml) stimulation for 4 **(upper panel)** or 24 hrs **(lower panel)**.

Cell pellets and culture supernatants were collected for IL-23p19 mRNA assay by Real-Time PCR, and IL-23 or IL-12/23p40 proteins measurement by ELISA.

Error bars represent the SEM of 3 independent experiments.

ANOVA was used to determine significant differences. The asterisk sign on top of each column shows significant difference domparing to the positive control.

A.



B.

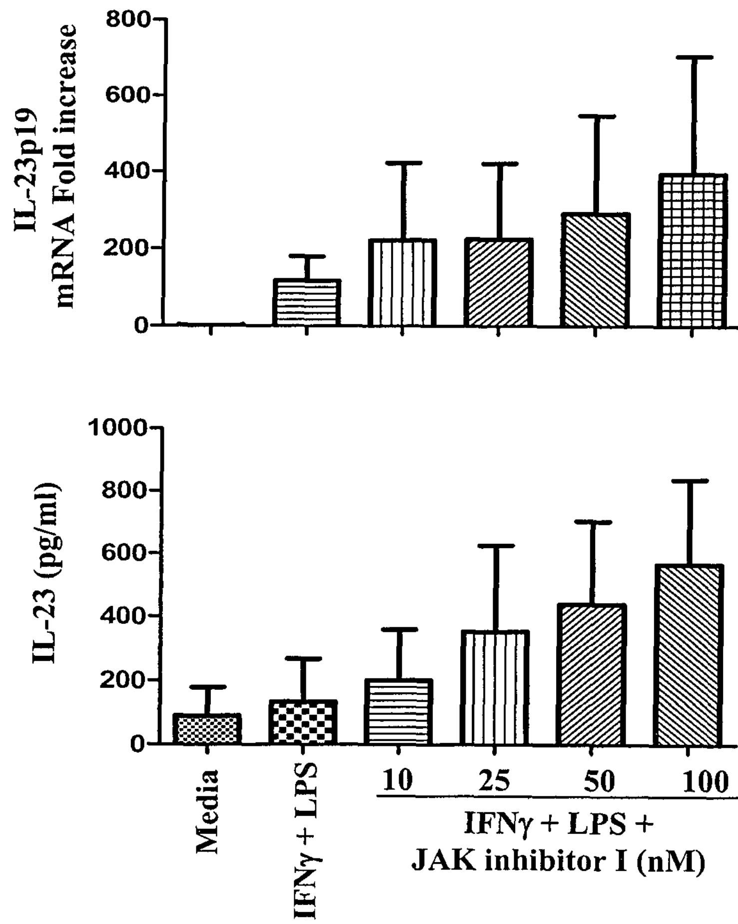


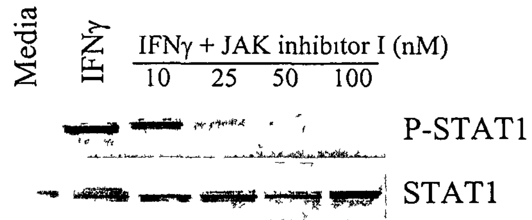
Figure 3.2

Figure 3.3. JAK kinase inhibitor, JAK inhibitor-1, inhibits IFNγ-induced IL-27 protein production in THP-1 cells

A) Monocytes ($1x10^6$/ml) were treated with increasing concentrations of Jak inhibitor-1 (0 to 100 nM) for 2 hours followed by IFNγ (10 ng/ml) stimulation for 30 min. Cell pellets were collected and STAT-1 phosphorylation was determined by Western blotting.

B) THP-1 cells ($1x10^6$/ml) were treated with increasing concentrations of Jak inhibitor-1 (0 to 100 nM) for 2 hours followed by IFNγ (10 ng/ml) stimulation for 24 hrs. Culture supernatants were collected and IL-27 proteins production was determined by ELISA. Error bars represent the SEM of 3 independent experiments. An ANOVA was used to analyse data for significant differences.

A.



B.

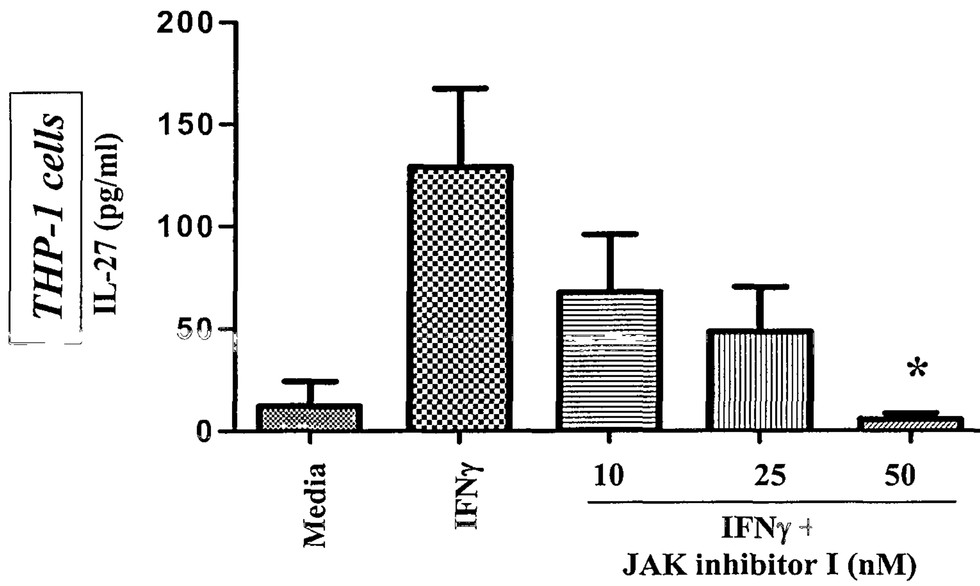


Figure 3.3

The biological activity of Jak-inhibitor-1 was examined by determining its effect on STAT-1 phosphorylation. Cells pre-treated with the Jak inhibitor-1 showed a significant reduction in IFNγ-induced STAT-1 phosphorylation (Fig. 3.3.A). These results suggest that the well known classical IFNγ-JAK/STAT signalling pathway is not involved in IFNγ or IFNγ/LPS-induced IL-23 regulation in human monocytic cells. Therefore, I hypothesized that IFNγ may induce IL-23 regulation via an alternate pathway like calcium signalling or MAPKs pathways in normal human monocytic cells.

PI3K does not regulate IFNγ/LPS-induced IL-23 expression in normal human monocytes

It has been previously demonstrated that LPS-induced IL-12p40 expression is regulated by the PI3K pathway in human monocytic cells [33]. Moreover, IL-12p40 has also been shown to be negatively regulated by the PI3K pathway in murine and human monocyte-derived macrophages [246]. Additionally, IL-23 expression was shown to be regulated by the PI3K pathway [223, 244]. Since IL-12p40 is a subunit of IL-23, we determined the role of the PI3K signalling in IFNγ and IFNγ/LPS-induced IL-23 expression by employing the pharmacological PI3K inhibitor, LY294002.

First, I demonstrated that IFNγ induced the activation of PI3K/Akt pathway and pre-treatment with LY294002 inhibited IFNγ-induced Akt activation in primary human monocytes (Fig. 3.4.A).To determine the role of PI3K signaling, monocytes pretreated with LY294002 were stimulated with IFNγ alone or with IFNγ and LPS and IL-23 expression was determined by Real-Time PCR and ELISA. Interestingly, results show

that IL-23p19 transcripts were significantly enhanced (p=0.006) in cells treated with PI3K specific inhibitor in a dose-dependent manner (Fig. 3.4.B). Moreover, IL-23 (Fig. 3.4.C left panel) and IL-12/23p40 proteins (Fig. 3.4.C right panel) levels also were increased in the presence of LY294002. Similar results were obtained following stimulation of cells with IFNγ alone in LY294002 pretreated cells (data not shown). These results suggest that PI3K signalling pathway negatively regulates IFNγ/LPS-induced IL-23 expression in normal human monocytes.

LPS and/or IFNγ/LPS-induced IL-23 expression is regulated by the p38 MAPK pathway

We and others have previously demonstrated that LPS-induced IL-12p40 expression is regulated by the JNK pathway [25, 78]. In addition, p38 MAPK has been shown to regulate IL-12p40 expression in murine and human macrophages [25, 78, 243, 266-268]. The MAPK pathway has also been reported to be an important regulator of IL-23 expression [245, 256]. Therefore, it was of interest to determine the role of MAPKs in the regulation of IFNγ/LPS-induced IL-23p19, IL-12p40 gene expression and IL-23 proteins production. To determine the role of MAPK signalling on IL-23 production, the pharmacological inhibitors specific for p38 (SB202190), ERK (PD98059) and JNK (SP600125) MAPKs [269-271] were employed. IFNγ alone activated ERK, JNK, and p38 MAPKs in primary monocytes and PD98059, SP600125, and SB202190 inhibited phosphorylation of their respective MAPKs in response to IFNγ (Fig. 3.5).

Figure 3.4. Effect of LY294002 –PI3K signalling pathway inhibitor- on LPS-induced IL-23 production in primary monocytes.

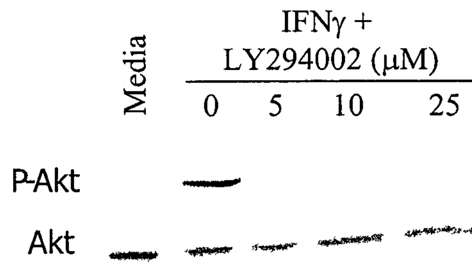
A) Monocytes ($1x10^6$/ml) were treated with increasing concentrations of PI3K inhibitor (0 to 50 μM) for 2 hours followed by IFNγ (10 ng/ml) stimulation for 30 min. Cell pellets were collected and Akt phosphorylation was determined by Western blotting.

B) Monocytes ($1x10^6$/ml) from three different individuals were treated with LY294002 at concentrations ranging from 0 to 50 μM for 2 hrs followed by IFNγ (10 ng/ml) stimulation for 16 hrs and then LPS (1μg/ml) stimulation for 4 hrs. Cells were harvested for mRNA isolation. IL-23p19 gene expression was determined by R.Q. Real-Time PCR analysis.

C) Monocytes ($1x10^6$/ml) were treated with LY294002 at concentrations ranging from 0 to 50 μM for 2 hrs followed by IFNγ (10 ng/ml) for 16 hrs and then LPS (1μg/ml) stimulation for 24 hrs. Cell culture supernatants were collected for total IL-23 **(left panel)** and IL-12/23p40 **(right panel)** protein measurement by ELISA.

Error bars represent the SEM of 3 independent experiments. An ANOVA was used to analyse data for significant differences.

A.



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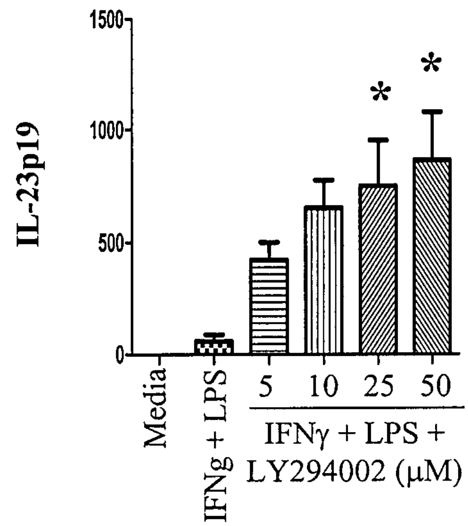

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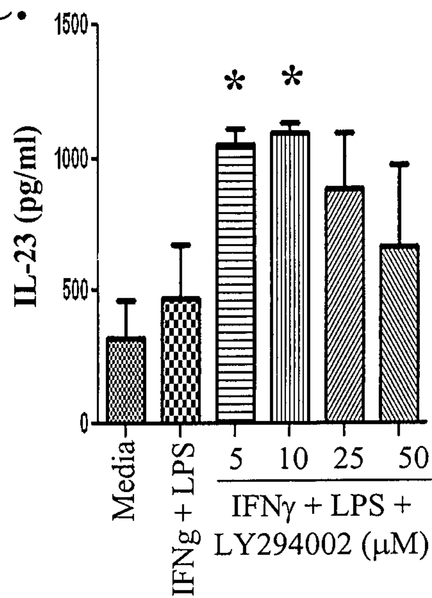


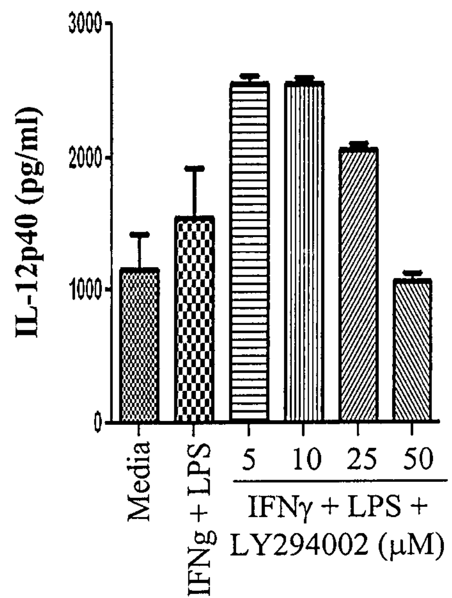


Figure 3.4

To determine the involvement of MAPKs in IL-23 regulation, monocytes were cultured with increasing concentrations of each MAPKs inhibitor and stimulated with either IFNγ alone or IFNγ and LPS followed by analysis of IL-23p19, IL-12p40 and IL-23 expression. Inhibition of the ERK MAPKs pathway in IFNγ-primed and LPS-stimulated monocytes resulted in a significant decrease (p=0.01) in the levels of IL-23p19 mRNA expression (Fig. 3.6.A), however, PD98059 did not affect IL-23 protein production (Fig. 3.6.B left panel). Similarly, IFNγ/LPS-induced IL-12/23p40 protein production was unaffected by PD98059 (Fig. 3.6.B right panel). Similar results were obtained when cells were stimulated only with IFNγ (data not shown). These results suggest a lack of correlation between IL-23 p19 and p40 genes expression and IL-23 protein production.

In terms of the role of JNK MAPKs in IL-23 regulation, interestingly, my results show that JNK MAPK plays a negative regulatory role in IFNγ/LPS-induced IL-23 expression. IL-23p19 transcripts were significantly enhanced (p=0.04) in primary monocytes, which were treated with JNK MAPKs specific inhibitor (SP600125 ranging from 5 to 50 μM) in a dose-dependent manner (Fig. 3.7.A). Moreover, total IL-23 and IL-12/23p40 protein levels also were increased in the presence of SP600125, but were not statistically significant (Fig. 3.7.B). Similar results were obtained following stimulation of cells with IFNγ alone (data not shown). These results suggest that the JNK pathway negatively regulates IFNγ/LPS-induced IL-23p19 expression at mRNA level in normal human monocytes. In contrast, inhibition of the p38 MAPK enhanced IFNγ and LPS-induced IL-23p19 mRNA expression, although ANOVA analysis doesn't show a significant increase (Fig. 3.8.A), Interestingly, low concentrations (5 to 20 μM) of p38

inhibitor significantly increased IL-23 and IL-12p40 protein expression and high doses (25 to 50 μM of SB202190) significantly decreased both IL-12/23p40 and IL-23 proteins production (Fig. 3.8.B). Similar results were obtained when the role of p38 MAPK in the regulation of IL-23p19 mRNA expression was analyzed in monocytic cells stimulated with IFNγ alone (Fig. 3.8.C), but p38 inhibitor significantly decreases the level of IL-12/23p40 and IL-23 proteins at all concentrations of the inhibitor. Taken together, the results suggest that inhibition of JNK MAPKs in monocytes stimulated with IFNγ and LPS showed increased IL-23p19 mRNA as wells as IL-12/23p40 and IL-23 protein levels (Fig. 3.7). In contrast, inhibition of p38 MAPK significantly decreased IL-12/23p40 ($p=0.001$) and IL-23 protein ($p=0.03$) production (Fig. 3.8.D). These results suggest that IL-23 and IL-12p40 expression induced by either IFNγ or IFNγ and LPS is negatively regulated by JNK MAPKs and positively regulated by the p38 MAPK pathway.

Figure 3.5. The effect of MAPKs pharmacological inhibitors on IFNγ-induced IL-23 expression.

Monocytes (1×10^6/ml) were treated with PD98059, SP600125, or SB202190 at concentrations ranging from 0 to 50 μM for 2 hrs before IFNγ (10 ng/ml) stimulation. Total proteins (50 μg) were analyzed by Western blot analysis using anti-phospho-ERK (P-ERK), anti-phospho-JNK (P-JNK), or anti-phospho-p38 (P-p38) antibodies. To control for equal protein loading, the membranes were stripped and reprobed with anti-ERK, anti-JNK, or anti-p38 antibodies, respectively.

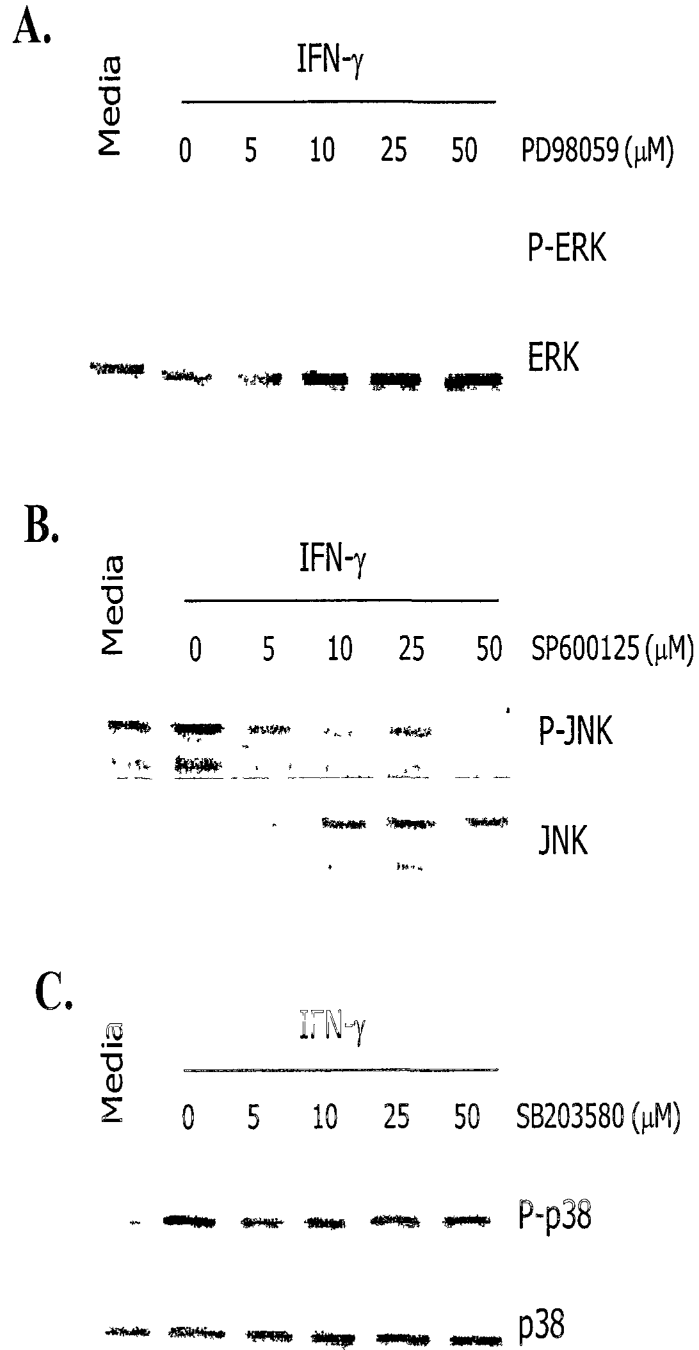
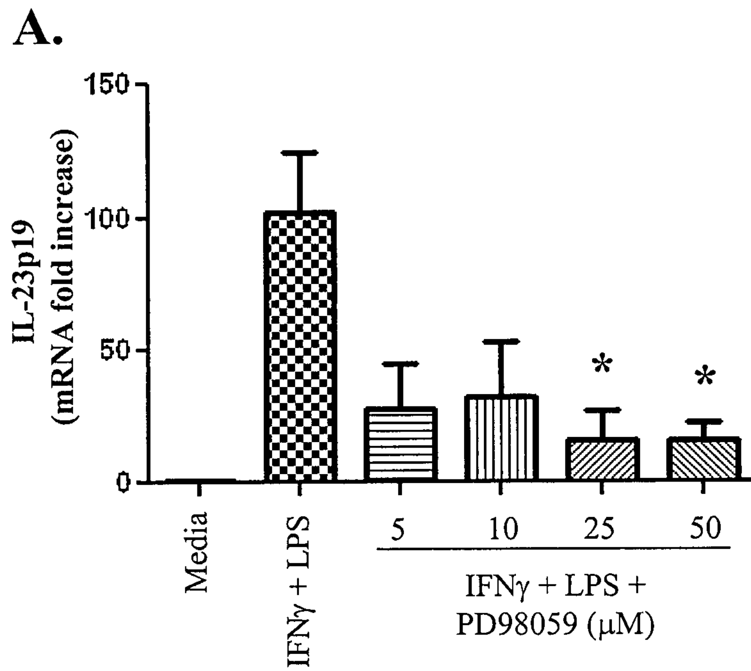


Figure 3.5

Figure 3.6. Effect of PD98059 -ERK MAPKs inhibitor- on LPS-induced IL-23 production in primary monocytes.

A) Monocytes ($1x10^6$/ml) were treated with PD98059 at concentrations ranging from 0 to 50 μM for 2 hrs followed by IFNγ (10 ng/ml) stimulation for 16 hrs and then LPS (1μg/ml) stimulation for 4 hrs. Cells were harvested for mRNA isolation. IL-23p19 gene expression was determined by R.Q. Real-Time PCR analysis. Error bars represent the SEM of 3 independent experiments. An ANOVA was used to analyse data for significant differences.

B) Monocytes ($1x10^6$/ml) were treated with PD98059 at concentrations ranging from 0 to 50 μM for 2 hrs followed by IFNγ (10 ng/ml) for 16 hrs and then LPS (1μg/ml) stimulation for 24 hrs. Cell culture supernatants were collected for total IL-23 and IL-12/23p40 protein measurement by ELISA. Error bars represent the SEM of 3 to 4 independent experiments.



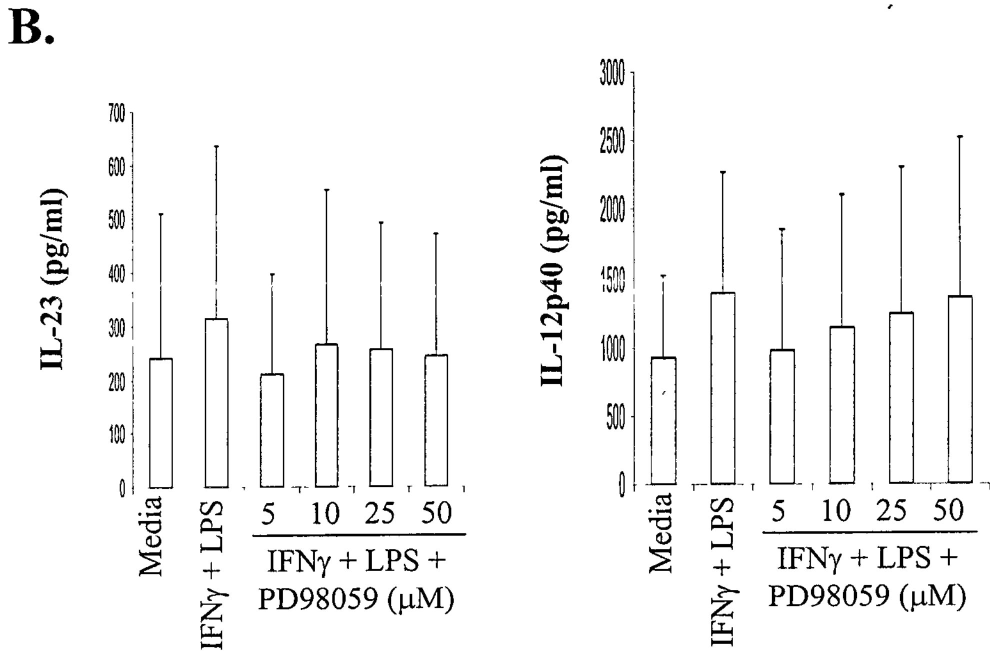


Figure 3.6

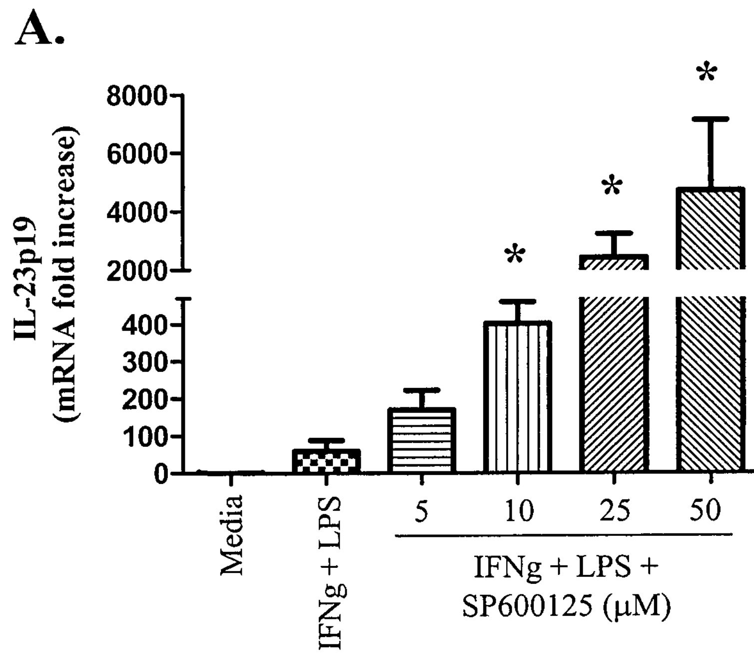
Figure 3.7. Effect of SP600125 -JNK MAPKs pharmacological inhibitor- on LPS-induced IL-23 production in primary monocytes.

A) Monocytes ($1x10^6$/ml) were treated with SP600125 at concentrations ranging from 0 to 50 μM for 2 hrs followed by IFNγ (10 ng/ml) stimulation for 16 hrs and then LPS (1μg/ml) stimulation for 4 hrs. Cells were harvested for mRNA isolation. IL-23p19 gene expression was determined by R.Q. Real-Time PCR analysis. Error bars represent the SEM of 3 independent experiments.

B) Monocytes ($1x10^6$/ml) were treated with SP600125 at concentrations ranging from 0 to 50 μM for 2 hrs followed by IFNγ (10 ng/ml) for 16 hrs and then LPS (1μg/ml) stimulation for 24 hrs. Cell culture supernatants were collected for total IL-23 and IL-12/23p40 protein measurement by ELISA. Error bars represent the SEM of 3 to 4 independent experiments.

ANOVA was used to analyse data for significant differences.

A.



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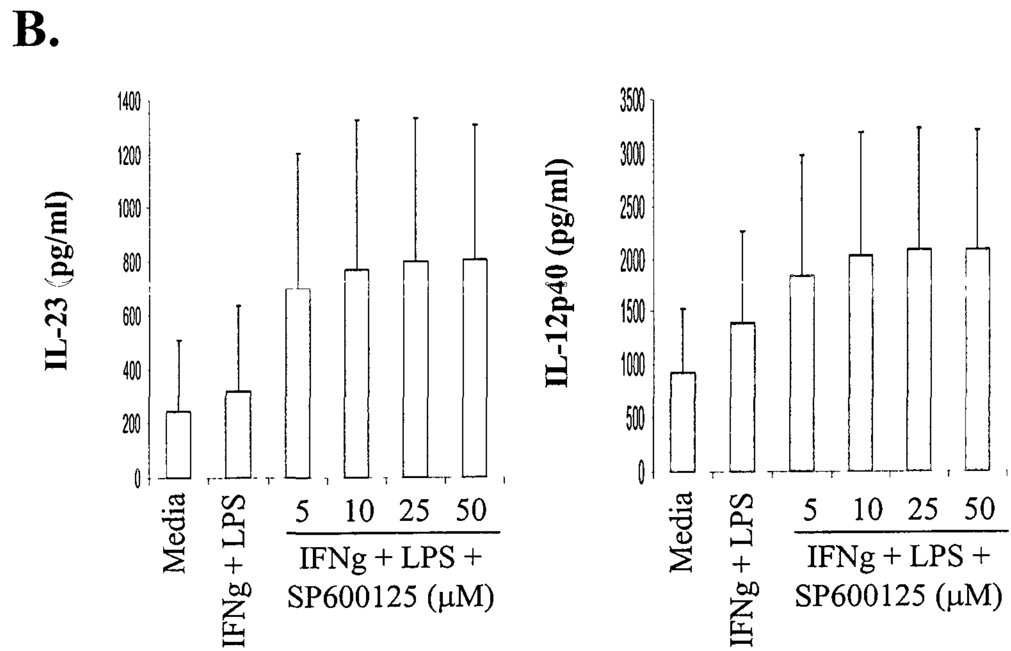


Figure 3.7

EGTA inhibits IL-23 and IL-12/23p40 proteins production in IFNγ/LPS-treated normal human monocyes

It has been previously demonstrated that LPS-induced IL-12p40 expression is regulated by the calcium signalling pathway [33]. However, IL-12p40 regulation has also been shown to be negatively regulated by calcium signalling in murine and human macrophages [246]. Therefore, I hypothesized that IFNγ- and IFNγ/LPS-induced IL-23 expression may be regulated by the calcium signalling pathway. The role of calcium signalling pathway was analyzed by employing calcium-specific inhibitor, EGTA. The results show that EGTA enhanced IL-23p19 gene expression (Fig. 3.9.A), but significantly decreased IL-23 ($p=0.007$) and IL-12/23p40 protein ($p=0.001$) production in a dose-dependent manner in primary monocytes (Fig. 3.9.B).

Figure 3.8. Effect of SB202190 –p38 MAPK inhibitor- on IFNγ and IFNγ/LPS-induced IL-23 production in primary monocytes.

A) Monocytes ($1x10^6$/ml) were treated with SB202190 at concentrations ranging from 0 to 50 μM for 2 hrs followed by stimulation with IFNγ (10 ng/ml) for 16 hrs and then stimulation with LPS (1μg/ml) for another 4 hrs. Cells were harvested for mRNA isolation. IL-23p19 gene expression was determined by R.Q. Real-Time PCR analysis.

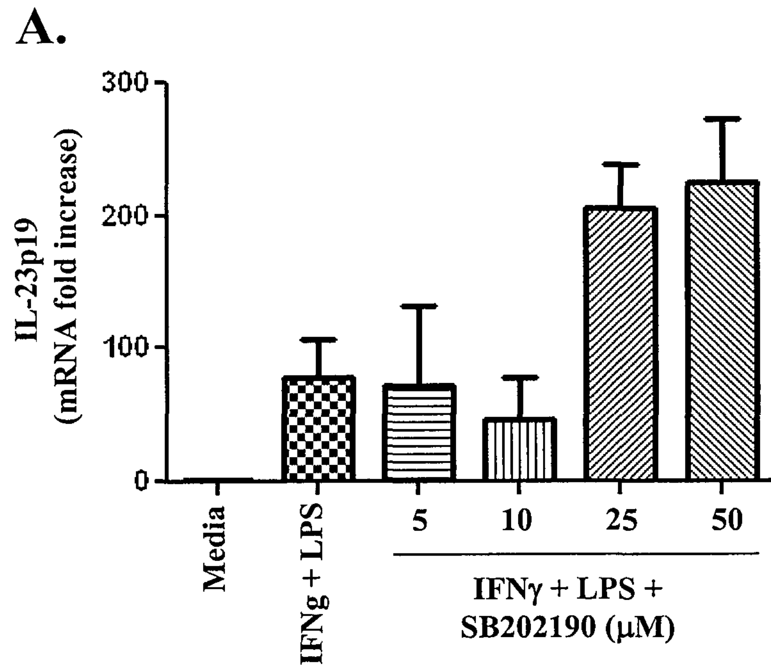
B) Monocytes ($1x10^6$/ml) were treated with SB202190 at concentrations ranging from 0 to 50 μM for 2 hrs followed by stimulation with IFNγ (10 ng/ml) for 16 hrs and then stimulation with LPS (1μg/ml) for another 24 hrs. Cell culture supernatants were collected to measure IL-23 and IL-12/23p40 proteins by ELISA. An ANOVA test was used to analyse data for significant differences.
(**) Two asterisks on top of each column show **significant increase**.

(*) One asterisk on top of each column shows **significant decrease**.

C) Monocytes ($1x10^6$/ml) were treated with SB202190 at concentrations ranging from 0 to 50 μM for 2 hrs followed by stimulation with IFNγ (10 ng/ml) for 4 hrs. Cells were harvested for mRNA isolation. IL-23p19 gene expression was determined by R.Q. Real-Time PCR analysis. The histogram is a representative of 2 independent experiments.

D) Monocytes ($1x10^6$/ml) were treated with SB202190 at concentrations ranging from 0 to 50 μM for 2 hrs followed by stimulation with IFNγ (10 ng/ml) for 16 hrs. Cell culture supernatants were collected to measure IL-23 and IL-12/23p40 proteins by ELISA.

Error bars represent the SEM of 3 to 4 independent experiments. ANOVA used to analyse significant differences.



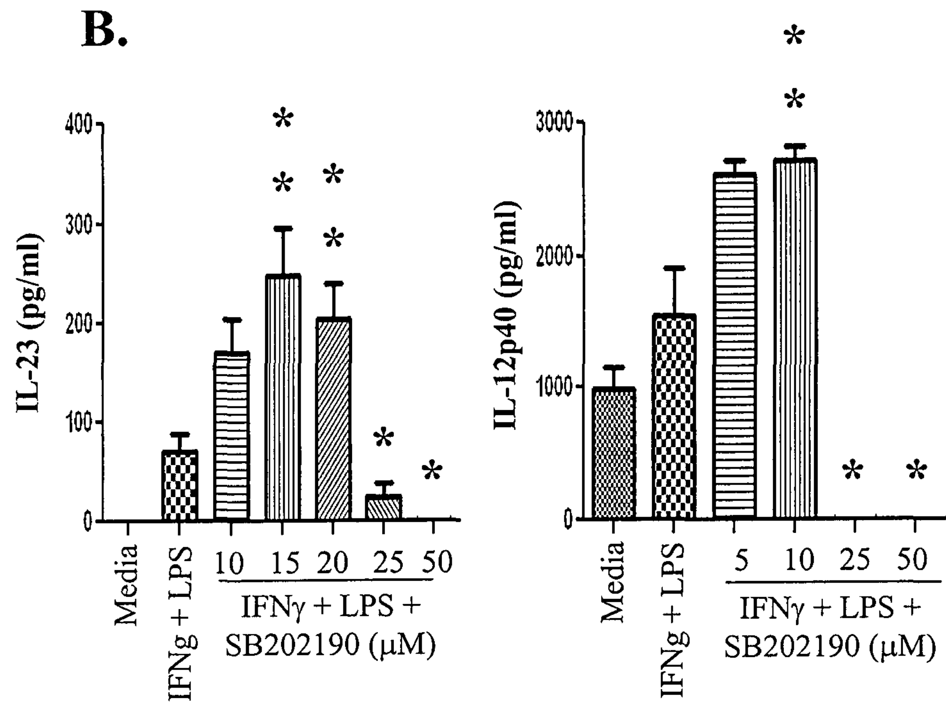
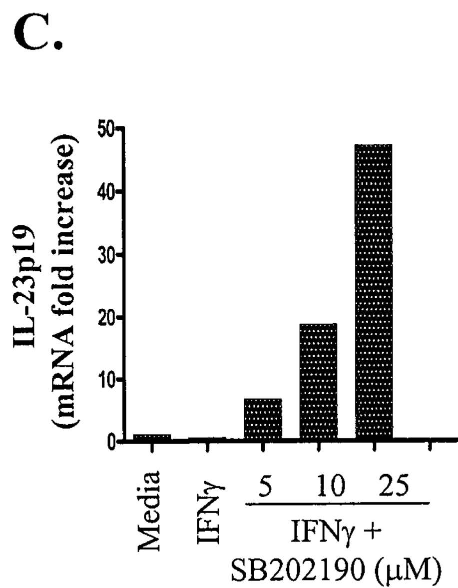


Figure 3.8 A&B

C.



D.

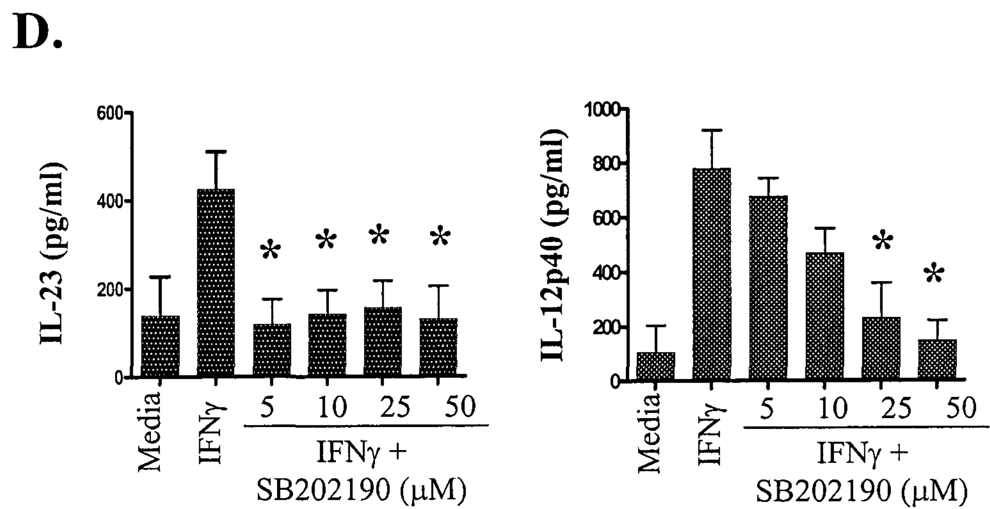


Figure 3.8 C&D

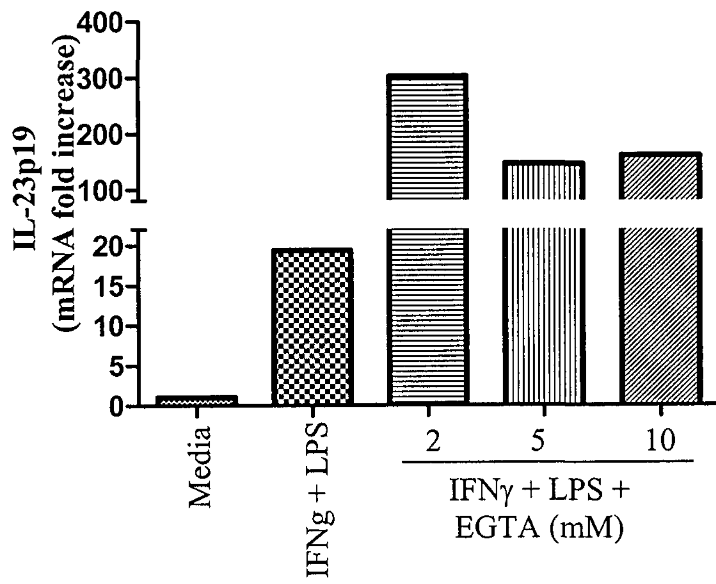
Figure 3.9. Role of EGTA on IFNγ/LPS-induced IL-23 production in human monocytes.

A) Monocytes ($1x10^6$/ml) were treated with EGTA at concentrations ranging from 0 to 10 mM for 2 hrs followed by IFNγ (10 ng/ml) stimulation for 16 hrs and then LPS (1μg/ml) stimulation for 4 hrs. Cells were harvested for mRNA isolation. IL-23p19 gene expression was determined by R.Q. Real-Time PCR analysis. The histogram is representing a mean of 2 independent experiments.

B) Monocytes ($1x10^6$/ml) were treated with EGTA at concentrations ranging from 0 to 10 mM for 2 hrs followed by IFNγ (10 ng/ml) for 16 hrs and then LPS (1μg/ml) stimulation for 24 hrs. Cell culture supernatants were collected for total IL-23 and IL-12/23p40 protein measurement by ELISA. Error bars represent the SEM of 3 to 5 independent experiments.

ANOVA test was used to analyse data for significant differences.

A.



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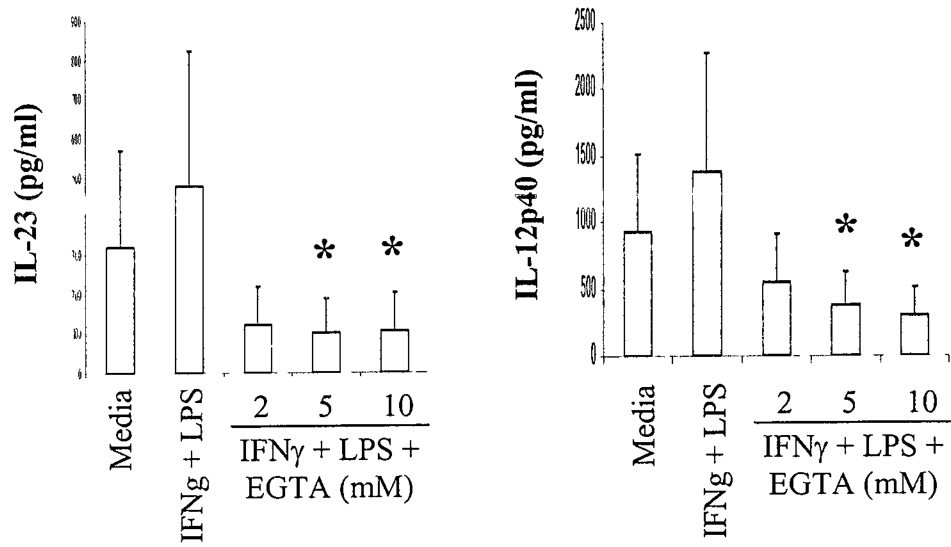


Figure 3.9

IFNγ- and IFNγ/LPS-induced IL-23 regulation in THP-1 cells

LPS significantly induces IL-23p19 and IL-12/23p40 mRNA expression and IL-23 protein production in THP-1 cells.

THP-1 cells (human acute promonocytic cell line) serve as a suitable alternative to peripheral blood monocyte models as these cells can develop macrophage functions following the addition of stimuli such as LPS and the other natural TLRs ligands [272]. Since LPS acts following binding to the LPS-binding protein/CD14 complex on the cells, THP-1 cells stably transfected with CD14 receptor (THP-1_{CD14}) were used in the present study and hereafter are interchangeably referred as THP-1 cells. In this study, *I characterized the regulation of IL-23 in THP-1 cells stimulated either with LPS (1 μg/ml) alone or LPS (1 μg/ml) and IFNγ (10 ng/ml) followed by analysis of IL-23 gene expression and protein production.*

LPS stimulation of THP-1 cells induced maximum expression of IL-23p19 ($p=0.01$) and IL-12/23p40 ($p=0.006$) genes at 4 hrs post-stimulation, as determined by R.Q. Real-Time PCR. However, the expression of IL-12/23p40 mRNA in these cells was found to be significantly greater than that of the IL-23p19 gene (Fig. 3.10.A). IL-23 production in the culture supernatants of LPS-stimulated THP-1 cells harvested at 24 hrs post-stimulation was measured by ELISA (eBioscience). LPS stimulation significantly ($p=0.001$) induced IL-23 protein production in the range of approximately 10000 pg/ml (Fig. 3.10.B) suggesting that THP-1 cells following LPS stimulation can produce IL-23 at both protein and mRNA levels. Since IFNγ priming was necessary to induce IL-23 production in normal monocytes, I determined if IFNγ and LPS can synergize to induce IL-23 in THP-1 cells using the same protocol employed for primary monocytes. Interestingly, stimulation

with IFNγ and LPS significantly enhanced the expression of IL-23 p19 and p40 transcripts compared to the cells stimulated with LPS alone (Fig. 3.11.A). However, at protein levels, LPS alone induced the production of IL-23 which was approximately 5 times more than the cells stimulated with IFNγ and LPS together (Fig. 3.11.B upper panel). However, stimulation of cells with either LPS or LPS and IFNγ together produced similar levels of IL-12/23p40 proteins (Fig. 3.11.B lower panel). In contrast to the primary monocytes, IFNγ did not significantly induce either IL-23 or IL-12p40 proteins in THP-1 cells.

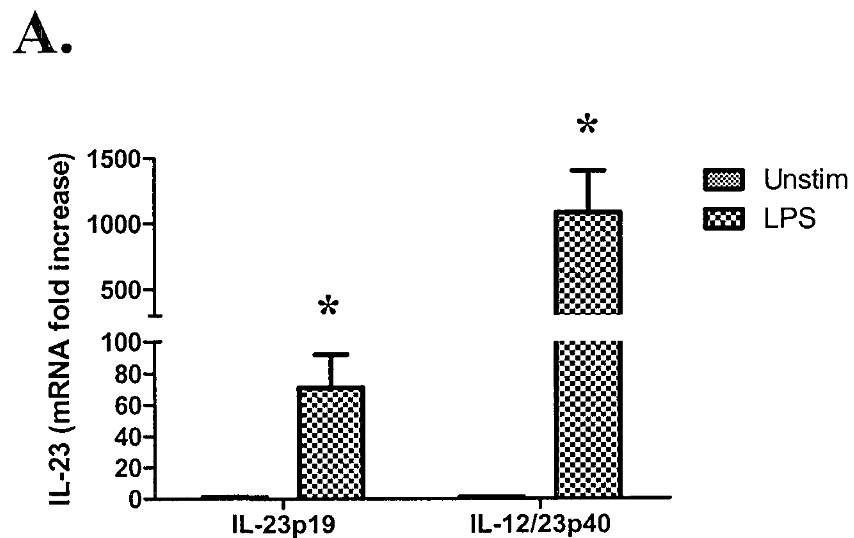
Figure 3.10. Effect of LPS stimulation on IL-23p19 and IL-12/23p40 mRNA expression and IL-23 protein production in THP-1 cells.

A) THP-1 cells ($1x10^6$/ml) were treated with LPS (1μg/ml) for 4 hrs followed by analysis for IL-23p19 and IL-23p40 mRNA expression by relative quantitative Real-Time PCR (R.Q. Real-Time PCR).

B) THP-1 cells ($1x10^6$/ml) were treated with LPS (1μg/ml) for 24 hrs and the supernatants were collected for IL-23 protein measurement. IL-23 protein production was determined by commercially available ELISA kits.

Error bars represent the SEM of 3 independent experiments.
T-test was used to analyse significant differences.

A.



B.

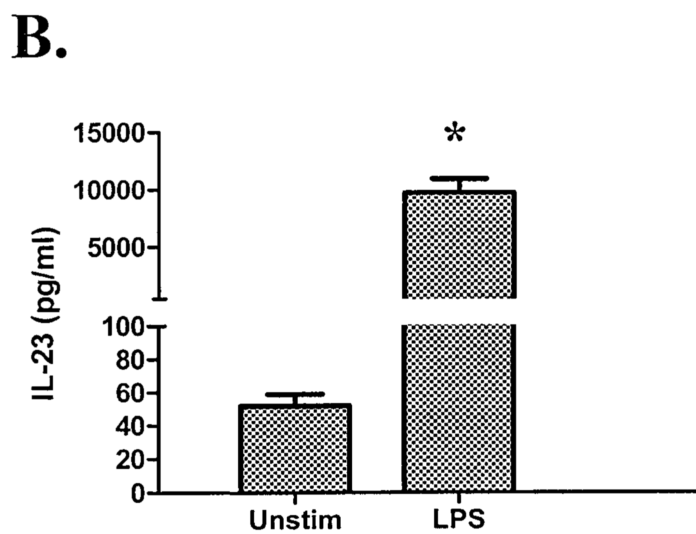


Figure 3.10

LPS- and IFNγ/LPS-induced IL-23 expression is regulated by PI3K in THP-1 cells

Since PI3K has been shown to play a significant role in the regulation of LPS-induced IL-12p40 production [33], I determined whether PI3K activation regulates IL-23 production in LPS- and IFNγ/LPS-stimulated THP-1 cells by employing LY249002, a pharmacological inhibitor specific for PI3K. LY249002 was found to be biologically active in THP-1 cells as LPS induced the activation of Akt which was inhibited by LY249002 in a dose-dependent manner (Fig. 3.12.A). LY294002 did not affect the expression of IL-23p19 subunit in THP-1 cells stimulated with either LPS alone (Fig. 3.12.B) or IFNγ and LPS together (Fig. 3.13. A). These results suggests that PI3K signalling pathway has no positive regulatory effect on IL-23p19 mRNA expression. However, LPS-induced IL-23 ($p=0.02$) and IL-12/23p40 ($p=0.007$) protein production was significantly down regulated by LY294002 (Fig. 3.12.C). In terms of the IFNγ and LPS-induced IL-23 production, the results show that LY294002 significantly inhibited IL-12p40 mRNA ($p=0.04$) expression (Fig. 3.13.A) and IL-23 ($p=0.0001$) and IL-12/23p40 ($p=0.004$) protein production (Fig. 3.13.B) in THP-1 cells. The role of PI3K pathway in IL-23p19 expression was confirmed by transfecting THP-1 cells with PI3Kp85α subunit siRNA. Transfection of THP-1 cells with p85 siRNA significantly inhibited the phosphorylation of p85 following LPS stimulation (Fig 3.14 upper panel). Similar to the results obtained with LY249002, p85 transfected cells exhibited an increase in IL-23p19 expression suggesting that there was no positive regulatory role for PI3K signalling pathway in LPS-induced IL-23p19 mRNA expression in THP-1 cells

(Fig. 3.14 lower panel). These results suggest that PI3K signalling pathway is a potent signalling pathway that regulates LPS- and IFNγ/LPS-induced IL-23 proteins production in THP-1 cells, but not the expression of IL-23p19 subunit.

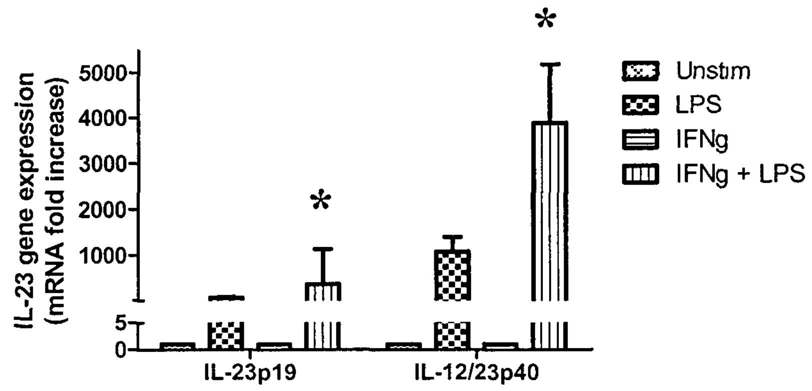
Figure 3.11. LPS as well as the combinations of IFNγ and LPS are able to induce IL-23 production in THP-1 cells.

A) THP-1 cells ($1x10^6$/ml) were treated with IFNγ (10 ng/ml) for 16 hrs followed by LPS (1μg/ml) for 4 hrs. Cells were subjected to analyze for IL-23p19 and IL-23p40 mRNA expression by relative quantitative Real-Time PCR (R.Q. Real-Time PCR).

B) THP-1 cells ($1x10^6$/ml) were treated with IFNγ (10 ng/ml) for 16 hrs followed by LPS (1μg/ml) for 24 hrs. Cell culture supernatants were collected for IL-23 and IL-12/23p40 protein measurement. IL-23 protein production was determined by the commercially available ELISA kits.

Error bars represent the SEM of 3 independent experiments.

A.



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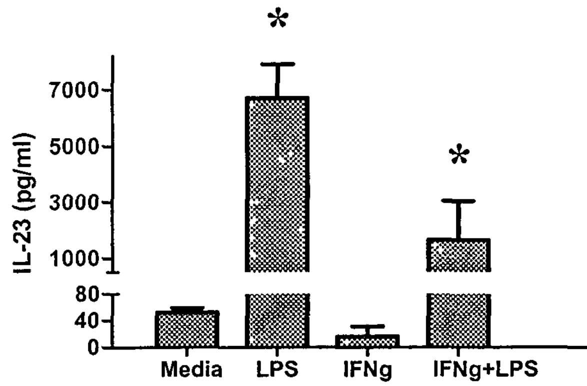


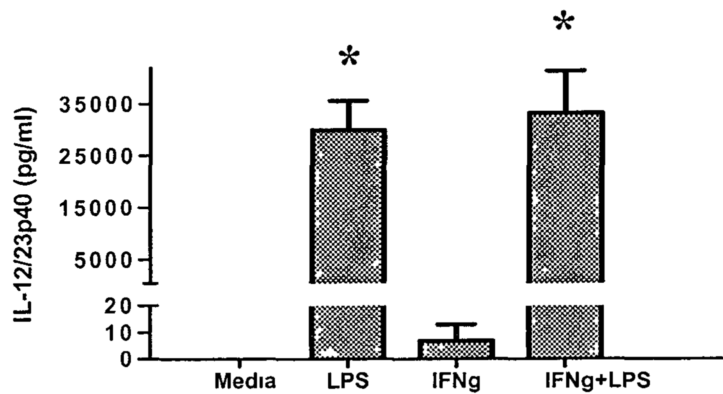


Figure 3.11

Figure 3.12. Role of PI3K pathway in LPS-induced IL-23 expression in THP-1 cells.

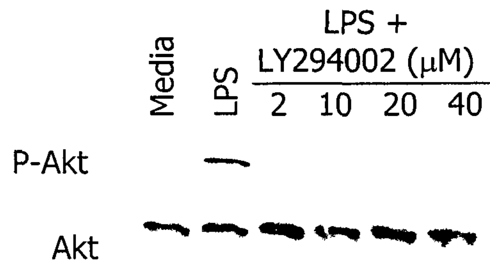
A) THP-1 cells ($1x10^6$/ml) were treated with LY294002 at concentrations ranging from 0 to 40 μM for 2 hrs before LPS (1μg/ml) stimulation. Total proteins (50 μg) were analyzed by Western blot analysis using anti-phospho-Akt (p-Akt) antibodies. To control for equal protein loading, the membrane was stripped and reprobed with anti-Akt antibodies.

B) THP-1 cells ($1x10^6$/ml) were treated with LY294002 at concentrations ranging from 0 to 40 μM for 2 hrs followed by LPS (1μg/ml) stimulation for 4 hrs. Cells were harvested for mRNA isolation. IL-23p19 gene expression was determined by R.Q. Real-Time PCR analysis.

C) THP-1 cells ($1x10^6$/ml) were treated with LY294002 at concentrations ranging from 0 to 40 μM for 2 hrs followed by LPS (1μg/ml) stimulation for 24 hrs. Supernatants were collected for total IL-23 and IL-12/23p40 proteins measurement by ELISA.
Error bars represent the SEM of 3 independent experiments.

ANOVA test was used to analyse data for significant differences.

A.



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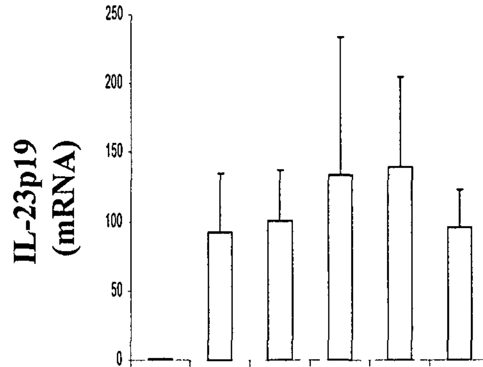


C.

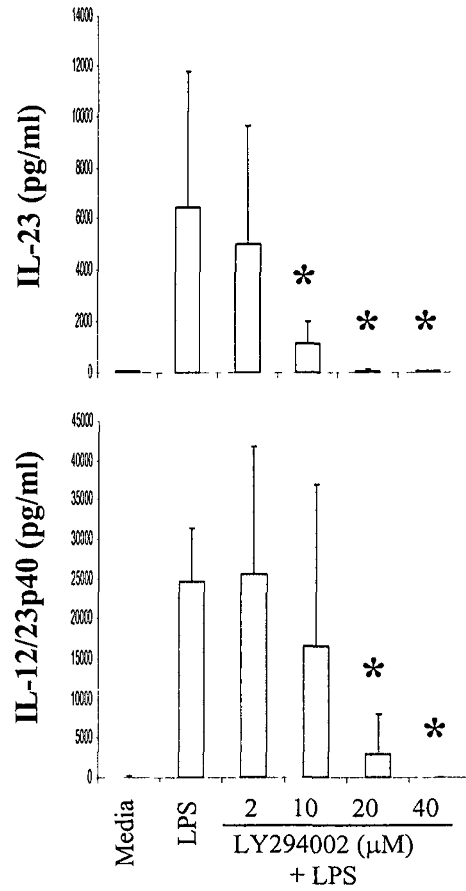


Figure 3.12

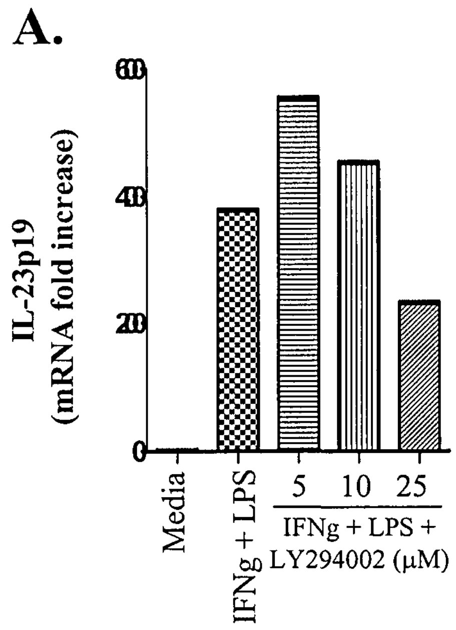
Figure 3.13. PI3K pathway regulates IFNγ/LPS-induced IL-23 protein production in THP-1 cells.

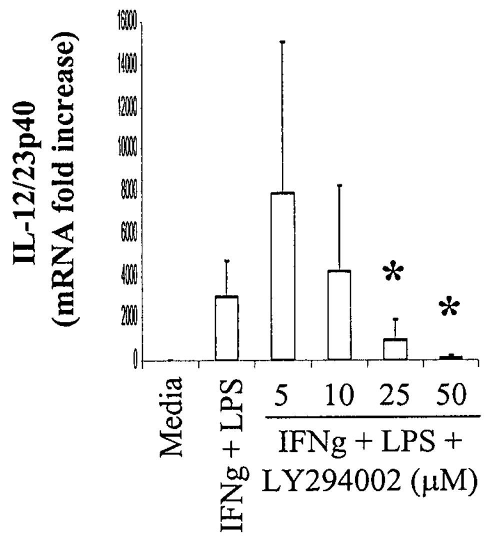
A) THP-1 cells ($1x10^6$/ml) were treated with LY294002 at concentrations ranging from 0 to 50 μM for 2 hrs followed by IFNγ (10 ng/ml) stimulation for 16 hrs and then LPS (1μg/ml) stimulation for 4 hrs. Cells were harvested for mRNA isolation. IL-23p19 and IL-12/23p40 gene expression were determined by R.Q. Real-Time PCR analysis. The left panel is a representative of 2 experiments.

B) THP-1 cells ($1x10^6$/ml) were treated with LY294002 at concentrations ranging from 0 to 50 μM for 2 hrs followed by IFNγ (10 ng/ml) stimulation for 16 hrs and then LPS (1μg/ml) stimulation for 24 hrs. Cell culture supernatants were collected for total IL-23 and IL-12/23p40 protein measurement by ELISA.

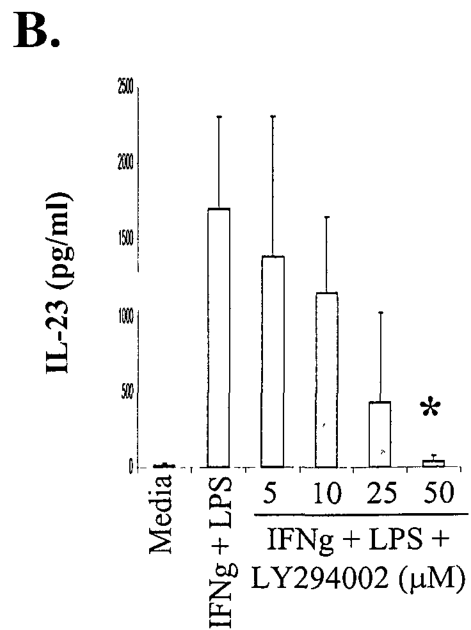
Error bars represent the SEM of 3 independent experiments.
ANOVA test used to show the significant changes.

A.





B.



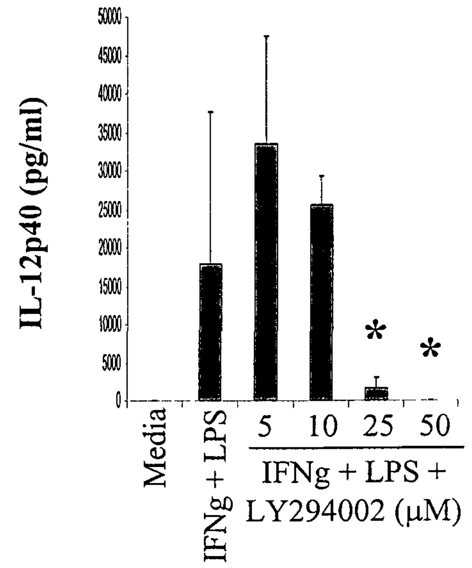


Figure 3.13

JNK and p38 MAPKs regulate LPS-induced IL-23 protein production in THP-1 cells

JNK and p38 MAPKs have been shown to regulate IL-12p40 expression in murine and human macrophages [25]. The ERK MAPKs have also been reported to be important regulators of IL-23 expression [256]. However, the role of MAPKs in the regulation of IFNγ/LPS-induced IL-23p19, IL-12p40 and IL-23 expression in monocytic cells is not known. To investigate the role of MAPKs, the biological activity of all three pharmacological inhibitors of MAPKs in THP-1 cells was determined. THP-1 cells were treated with MAPKs specific inhibitors for 2 hrs prior to stimulation with LPS for another 30 minutes followed by analysis of ERK, JNK and p38 MAPKs phosphorylation by Western blotting. LPS stimulation induced the phosphorylation of all three members of MAPKs that was inhibited by prior treatment of cells with PD98059, SP600125, or SB202190 in a dose-dependent manner (Fig. 3.15.A). To determine the effect of MAPKs inhibitors on IL-23 expression at protein and mRNA levels, cells were treated with various MAPKs inhibitors for 2 hrs, prior to 4 or 24 hrs stimulation with LPS. Treatment of cells with ERK, JNK, and p38 specific inhibitors increased the expression of IL-23p19 at mRNA level (Fig. 3.15.B). At the protein level, production of IL-23 proteins in LPS-stimulated cells was significantly down regulated in the presence of JNK- ($p=0.001$) and p38-MAPK ($p=0.009$) inhibitors (Fig. 3.15.C middle and lower panels). In contrast, PD98059 did not significantly decreased IL-23 protein production (Fig. 3.15.C upper panel). However, MAPKs did not significantly downregulate IL-23 expression following stimulation of cells with IFNγ and LPS. PD98059 (Fig. 3.16.A&B), SP600125

(Fig. 3.17.A&B), and SB202190 (Fig. 3.18. A&B) did not affect the expression of IL-23p19 and p40 mRNA and IL-12p40 and IL-23 proteins induced following stimulation with IFNγ and LPS. The above results were confirmed by using siRNAs against ERK and p38 MAPKs. Cells were transfected with ERK, P38 siRNA or control siRNA using DharmaFECT™ 2 transfection reagent as per the manufacturer's instructions (Dharmacon). Following transfection, cells were stimulated with LPS (1 μg/ml) for 8 hrs for R.Q. Real-Time PCR and for 30 min for Western blotting for ERK and p38 MAPKs. Transfection with ERK and p38 specific siRNA significantly reduced LPS-induced ERK and p38 phosphorylation, respectively (Fig. 3.19 A&B upper panels). Furthermore, there was no positive regulatory role either for ERK or p38 MAPK in LPS-induced IL-23p19 expression (Fig. 3.19 A&B lower panels).

Figure 3.14. Using deficient p85 PI3K THP-1 cells to confirm the role of PI3K signalling pathway in LPS-induced IL-23p19 expression.

Upper panel: THP-1 cells were transfected with the either control siRNA or p85 PI3K siRNA for 24 hrs followed by stimulation with LPS (1μg/ml) for 30 min for the determination of phosphorylated p85-PI3K by Western blot analysis.

Lower panel: THP-1 cells were transfected with the either control siRNA or p85 PI3K siRNA for 24 h followed by stimulation with LPS (1μg/ml) for 8 hrs for IL-23p19 gene expression assay by R.Q. Real-Time PCR. The histogram is representing a mean of 2 independent experiments.

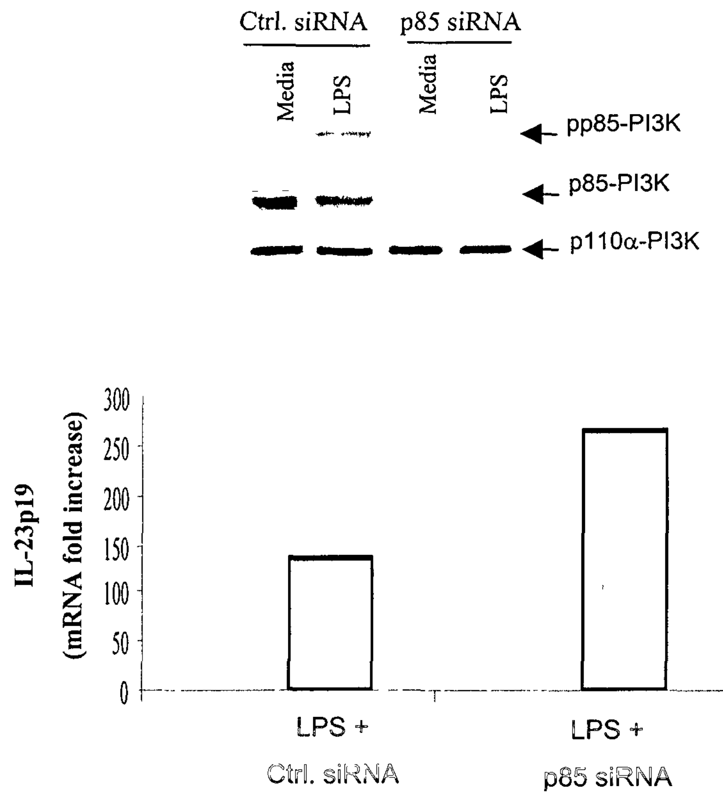


Figure 3.14

Calcium signalling pathway does not regulate LPS- and IFNγ/LPS-induced IL-23 expression in THP-1 cells

The calcium signalling pathway has been shown to regulate IL-12p40 production [33, 246]. Since Ca^{2+} is upstream of all the major kinase signalling pathways including MAPKs, PI3K, and protein kinase C [195, 218, 273], and because PI3K regulated IFNγ/LPS-induced IL-23 expression, I investigated the involvement of calcium signalling pathway in LPS- and LPS/IFNγ-induced IL-23 expression. To elucidate the role of Ca^{2+} and calcium signalling pathways in LPS- or IFNγ/LPS-induced IL-23 regulation, THP-1 cells were treated with two well known calcium inhibitors, EGTA (0-10 mM) and SKF96365 (0-50 μM), for 2 hrs followed by stimulation with LPS alone or the combination of IFNγ (for 16 hrs) and LPS (for 4 or 24 hrs). I first determined whether EGTA is able to inhibit the LPS- and IFNγ/LPS-induced IL-23p19 expression as well as IL-23 production. The results show that IL-23 p19 mRNA expression and IL-23 proteins production were not inhibited in the cells treated with either EGTA (Fig. 3.20.A&B and 3.21.A&B) or SKF96365 (Fig. 3.22 & 3.23). These results suggest that the calcium signalling pathway may not play a role in LPS- or IFNγ and LPS-induced IL-23 regulation in THP-1 cells.

Figure 3.15. The effect of MAPKs pharmacological inhibitors on LPS-induced IL-23 expression.

A) THP-1 cells ($1x10^6$/ml) were treated with PD98059, SP600125, or SB202190 at concentrations ranging from 0 to 50 μM for 2 hrs before LPS (1μg/ml) stimulation. Total proteins (50 μg) were analyzed by Western blot analysis using anti-phospho-ERK (P-ERK), anti-phospho-JNK (P-JNK), or anti-phospho-p38 (P-p38) antibodies. To control for equal protein loading, the membranes were stripped and reprobed with anti-ERK, anti-JNK, or anti-p38 antibodies, respectively.

B) THP-1 cells ($1x10^6$/ml) were treated with PD98059, SP600125, or SB202190 at concentrations ranging from 0 to 50 μM for 2 hrs followed by LPS (1μg/ml) stimulation for 4 hrs. Cells were harvested for mRNA isolation. IL-23p19 gene expression was determined by R.Q. Real-Time PCR analysis.

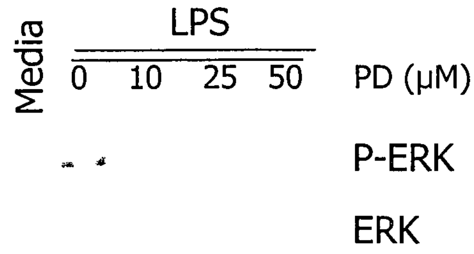
C) THP-1 cells ($1x10^6$/ml) were treated with PD98059, SP600125, or SB202190 at concentrations ranging from 0 to 50 μM for 2 hrs followed by LPS (1μg/ml) stimulation for 24 hrs. Supernatants were collected for total IL-23 and IL-23p40 protein measurement by ELISA.

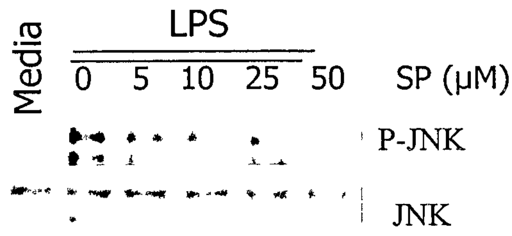
ANOVA test was used to analyse data for significant differences.

In B&C, error bars represent the SEM of 3 independent experiments.

A.

THP-1





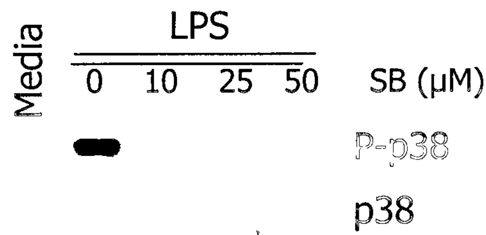
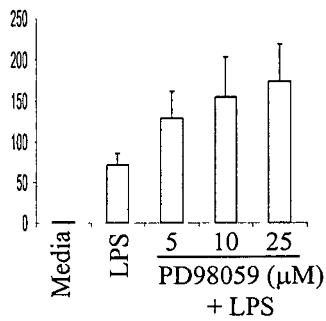
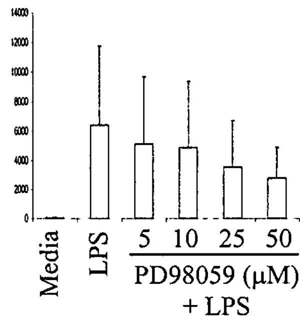
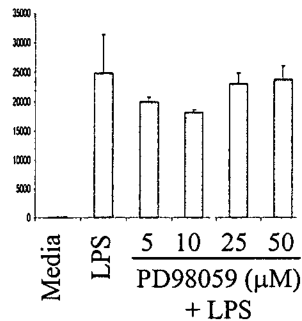


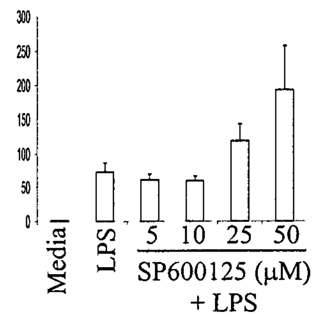
Figure 3.15.A

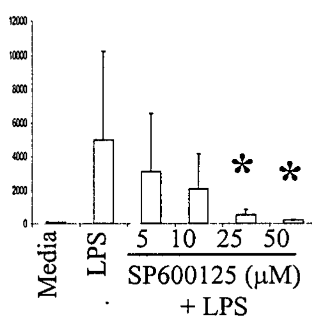
B. **C.**

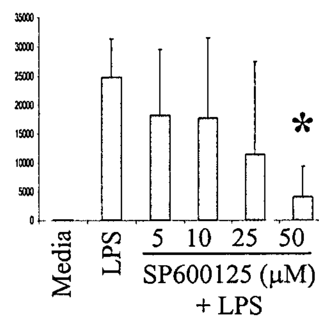


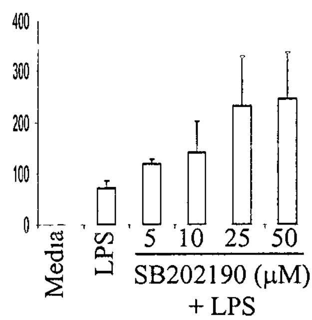


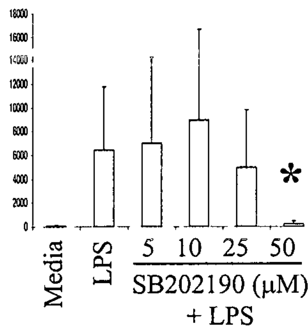












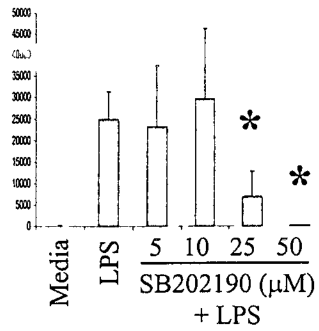


Figure 3.15.B&C

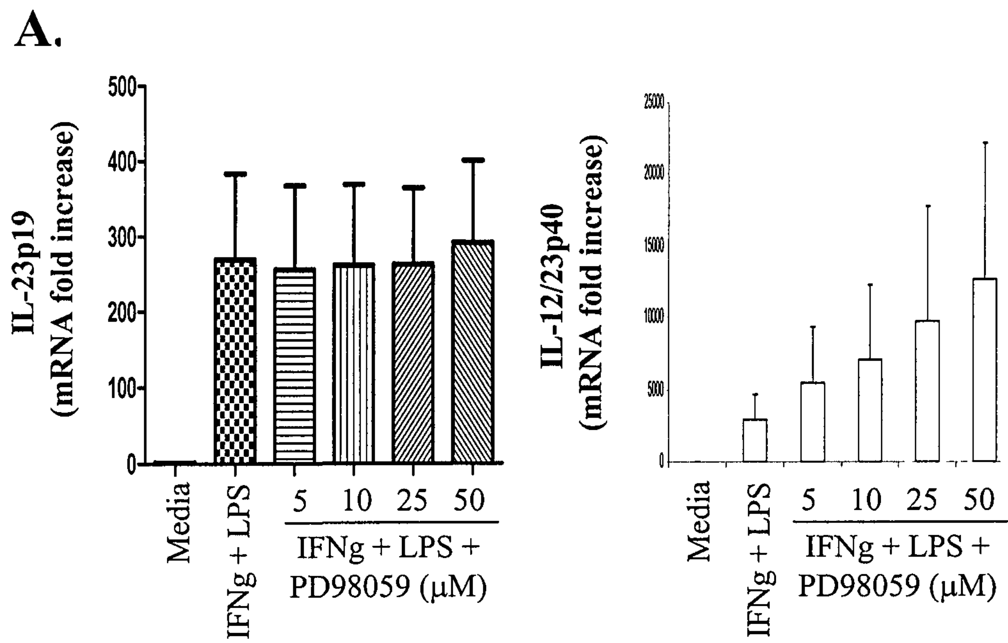
Figure 3.16. ERK MAPKs do not affect in IFNγ/LPS-induced IL-23 expression in THP-1 cells.

A) THP-1 cells ($1x10^6$/ml) were treated with PD98059 at concentrations ranging from 0 to 50 μM for 2 hrs followed by IFNγ (10 ng/ml) stimulation for 16 hrs and then LPS (1μg/ml) stimulation for 4 hrs. Cells were harvested for mRNA isolation. IL-23p19 and IL-12/23p40 gene expression were determined by R.Q. Real-Time PCR analysis. For IL-23p19 expression, each line represents results from monocytes from one individual.

B) THP-1 cells ($1x10^6$/ml) were treated with PD98059 at concentrations ranging from 0 to 50 μM for 2 hrs followed by IFNγ (10 ng/ml) stimulation for 16 hrs and then LPS (1μg/ml) stimulation for 24 hrs. Cell culture supernatants were collected for total IL-23 and IL-12/23p40 protein measurement by ELISA.

ANOVA test was used to analyse data for significant differences.
Error bars represent the SEM of 3 independent experiments.

A.



B.

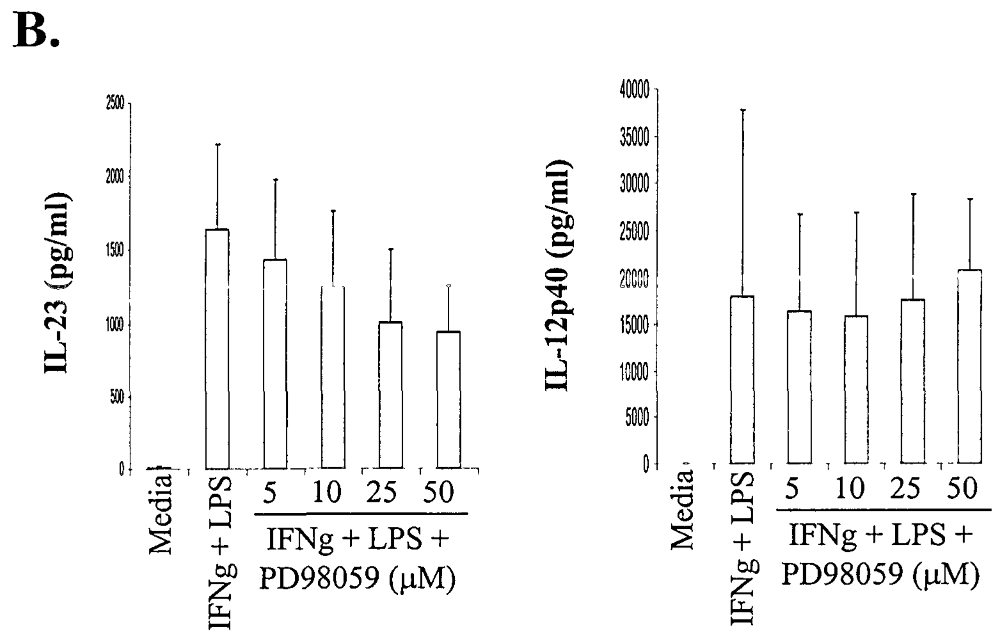


Figure 3.16

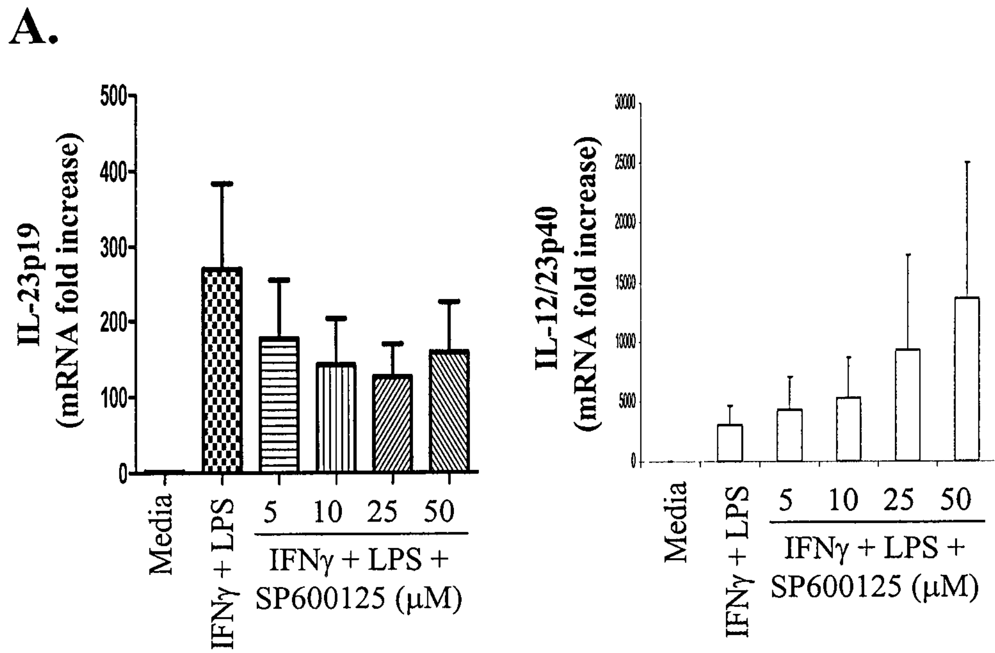
Figure 3.17. JNK MAPKs do not mediate IFNγ/LPS-induced IL-23 expression in THP-1 cells

A) THP-1 cells ($1x10^6$/ml) were treated with SP600125 at concentrations ranging from 0 to 50 μM for 2 hrs followed by IFNγ (10 ng/ml) stimulation for 16 hrs and then LPS (1μg/ml) stimulation for 4 hrs. Cells were harvested for mRNA isolation. IL-23p19 and IL-12/23p40 gene expression were determined by R.Q. Real-Time PCR analysis. Each line represents results from monocytes from one individual.

B) THP-1 cells ($1x10^6$/ml) were treated with SP600125 at concentrations ranging from 0 to 50 μM for 2 hrs followed by IFNγ (10 ng/ml) stimulation for 16 hrs and then LPS (1μg/ml) stimulation for 24 hrs. Cell culture supernatants were collected for total IL-23 and IL-12/23p40 protein measurement by ELISA.

Error bars represent the SEM of 3 independent experiments.

A.



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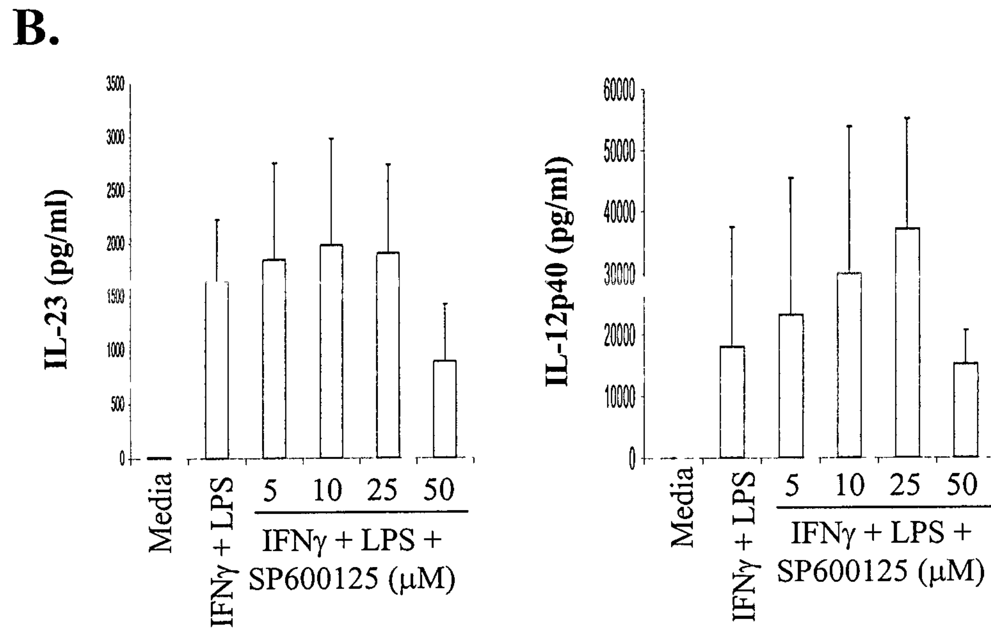


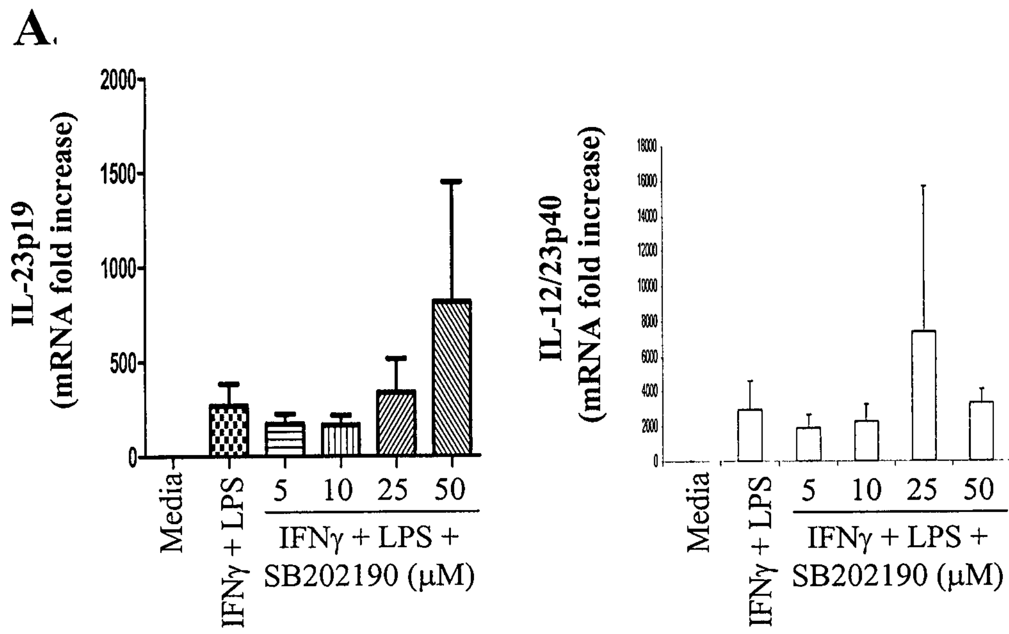
Figure 3.17

Figure 3.18. p38 MAPK inhibitor does not inhibit IFNγ/LPS-induced IL-23 protein production in THP-1 cells

A) THP-1 cells ($1x10^6$/ml) were treated with SB202190 at concentrations ranging from 0 to 50 μM for 2 hrs followed by IFNγ (10 ng/ml) stimulation for 16 hrs and then LPS (1μg/ml) stimulation for 4 hrs. Cells were harvested for mRNA isolation. IL-23p19 and IL-12/23p40 gene expression were determined by R.Q. Real-Time PCR analysis. Each line represents results from monocytes from one individual.

B) THP-1 cells ($1x10^6$/ml) were treated with SB202190 at concentrations ranging from 0 to 50 μM for 2 hrs followed by IFNγ (10 ng/ml) stimulation for 16 hrs and then LPS (1μg/ml) stimulation for 24 hrs. Cell culture supernatants were collected for total IL-23 and IL-12/23p40 protein measurement by ELISA.

Error bars represent the SEM of 3 to 5 independent experiments.



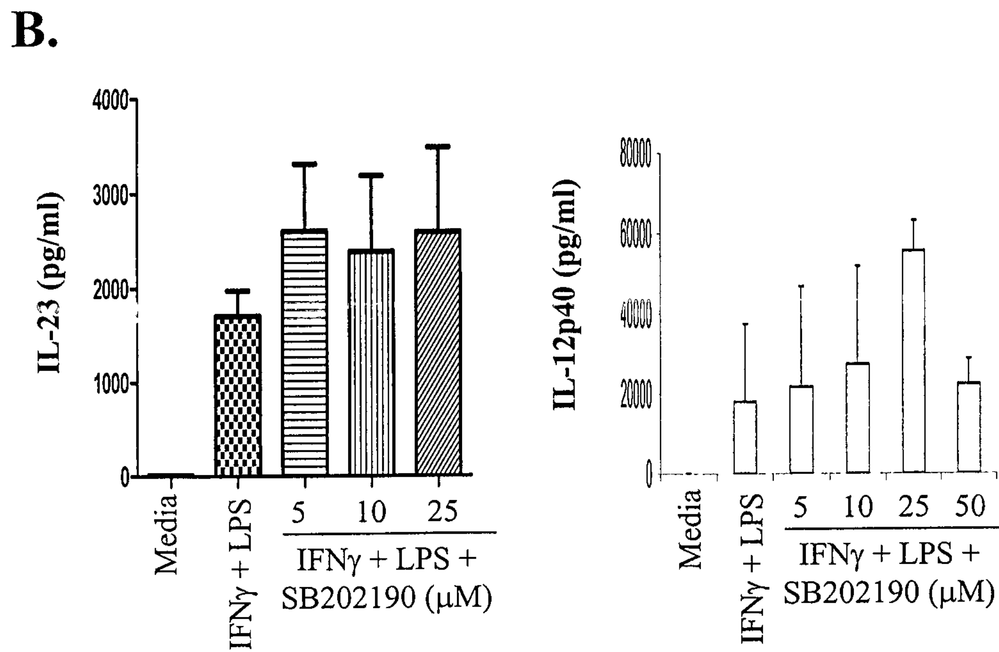


Figure 3.18

Figure 3.19. The effect of ERK and p38 MAPKs siRNA on LPS-induced IL-23 expression.

A)

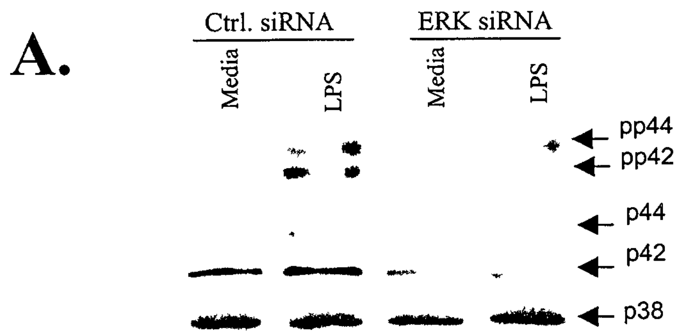
Upper panel: THP-1 cells were transfected with the either control siRNA or ERK MAPK siRNA for 24 hrs followed by stimulation with LPS (1μg/ml) for 30 min for the determination of phosphorylated ERK MAPK by Western blot analysis.
Lower panel: THP-1 cells were transfected with the either control siRNA or ERK MAPK siRNA for 24 h followed by stimulation with LPS (1μg/ml) for 8 hrs for IL-23p19 gene expression assay by R.Q. Real-Time PCR.

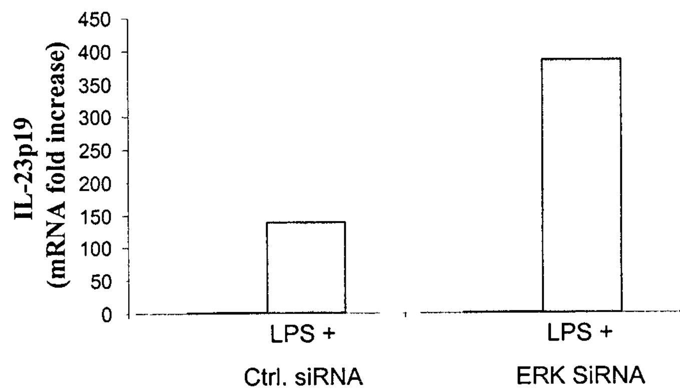
B)

Upper panel: THP-1 cells were transfected with the either control siRNA or p38 MAPK siRNA for 24 hrs followed by stimulation with LPS (1μg/ml) for 30 min for the determination of phosphorylated p38 MAPK by Western blot analysis.
Lower panel: THP-1 cells were transfected with the either control siRNA or p38 MAPK siRNA for 24 h followed by stimulation with LPS (1μg/ml) for 8 hrs for IL-23p19 gene expression assay by R.Q. Real-Time PCR.

Each histogram is a representative from 2 indipendent experiments.

A.





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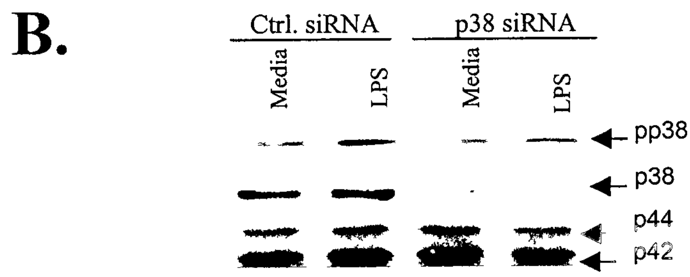


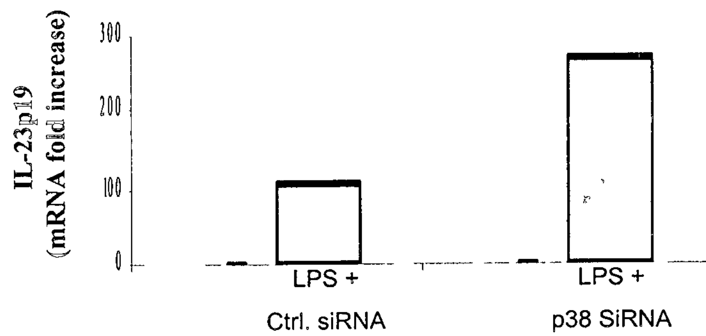


Figure 3.19

IFNγ/LPS-induced IL-23 production in THP-1 cells is not regulated by JAK/STAT pathway

Since THP-1 cells were treated with IFNγ followed by LPS stimulation, it was of interest to determine if the JAK/STAT pathway activated by IFNγ impacted on IL-23 production in cells stimulated with IFNγ and LPS together. To determine the role of JAK/STAT pathway in IFNγ/LPS-induced IL-23 expression, cells were treated with a general JAK kinase inhibitor – Jak inhibitor1- for 2 hrs followed by IFNγ stimulation for 16 hrs followed by LPS stimulation. Results show that inhibition of JAK/STAT pathway has no positive regulatory effect on IFNγ/LPS-induced IL-23 gene expressions or IL-23 protein production in THP-1 cells (Fig. 3.24 A and 3.24.B).

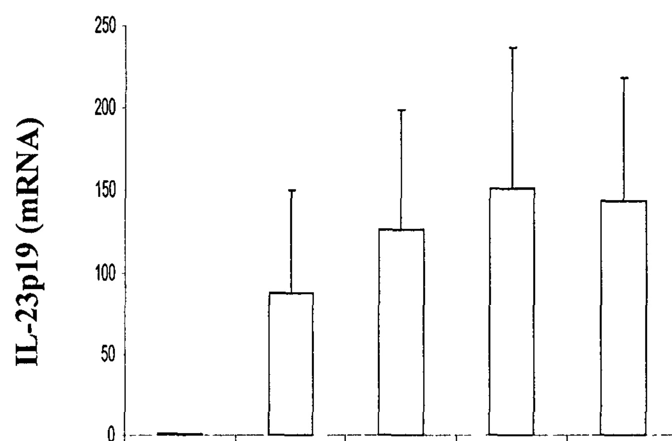
Figure 3.20. Calcium signalling does not regulate IL-23 expression in LPS-stimulated THP-1 cells.

A) THP-1 cells ($1x10^6$/ml) were treated with EGTA at the concentrations ranging from 0 to 10 mM for 2 hrs followed by LPS (1μg/ml) stimulation for 4 hrs. Cells were harvested for mRNA isolation. IL-23p19 gene expression was determined by R.Q. Real-Time PCR analysis.

B) THP-1 cells ($1x10^6$/ml) were treated with EGTA at the concentrations ranging from 0 to 10 mM for 2 hrs followed by LPS (1μg/ml) stimulation for 24 hrs. Supernatants were collected for total IL-23 and IL-23p40 proteins measured by ELISA.

Error bars represent the SEM of 3 independent experiments.

A.



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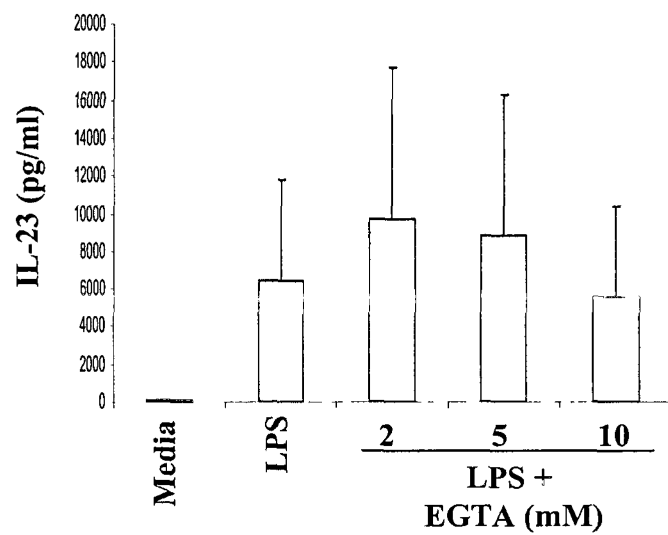


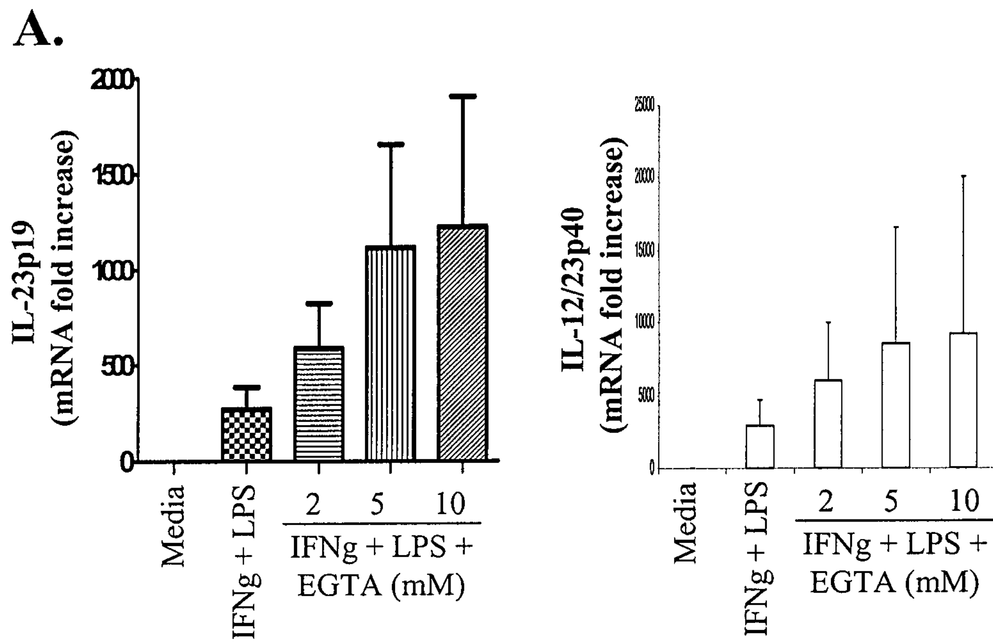
Figure 3.20

Figure 3.21. Calcium signalling does not regulate IFNγ/LPS-induced IL-23 expression in THP-1 cells.

A) THP-1 cells ($1x10^6$/ml) were treated with EGTA at concentrations ranging from 0 to 10 mM for 2 hrs followed by IFNγ (10 ng/ml) stimulation for 16 hrs and then LPS (1μg/ml) stimulation for 4 hrs. Cells were harvested for mRNA isolation. IL-23p19 and IL-12/23p40 gene expression were determined by R.Q. Real-Time PCR analysis.

B) THP-1 cells ($1x10^6$/ml) were treated with EGTA at concentrations ranging from 0 to 10 mM for 2 hrs followed by IFNγ (10 ng/ml) stimulation for 16 hrs and then LPS (1μg/ml) stimulation for 24 hrs. Cell culture supernatants were collected for total IL-23 and IL-12/23p40 protein measurement by ELISA.

Error bars represent the SEM of 3 to 4 independent experiments. No significant differences were obtained.



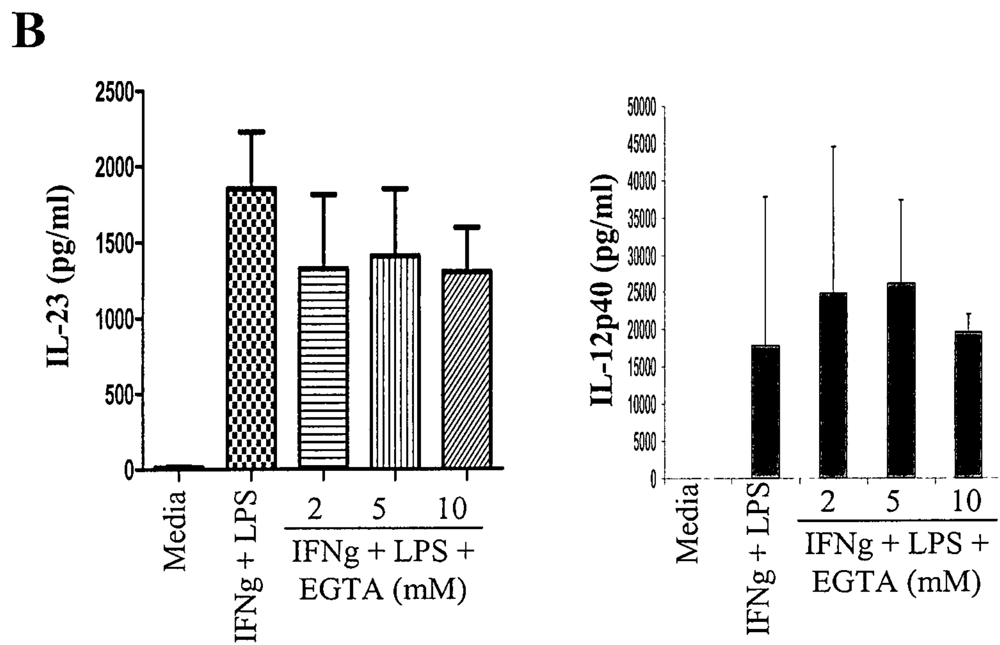


Figure 3.21

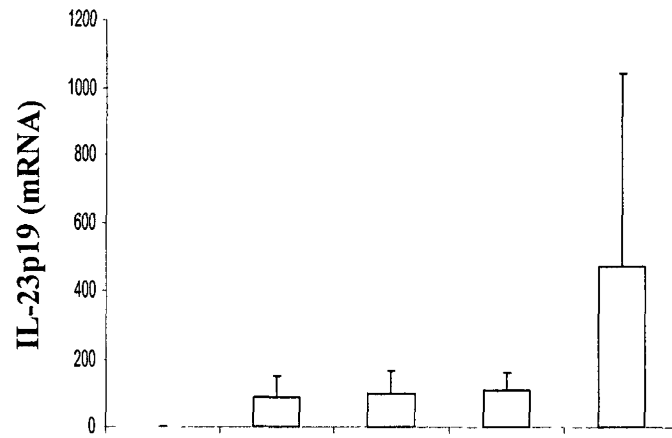
Figure 3.22. Calcium signalling inhibitor, SKF96365, does not inhibit IL-23 expression in LPS-stimulated THP-1 cells.

A) THP-1 cells ($1x10^6$/ml) were treated with SKF96365 at the concentrations ranging from 0 to 50 μM for 2 hrs followed by LPS (1μg/ml) stimulation for 4 hrs. Cells were harvested for mRNA isolation. IL-23p19 gene expression was determined by R.Q. Real-Time PCR analysis.

B) THP-1 cells ($1x10^6$/ml) were treated with SKF96365 at the concentrations ranging from 0 to 50 μM for 2 hrs followed by LPS (1μg/ml) stimulation for 24 hrs. Supernatants were collected for total IL-23 and IL-23p40 proteins measured by ELISA.

Error bars represent the SEM of 3 independent experiments.

A.



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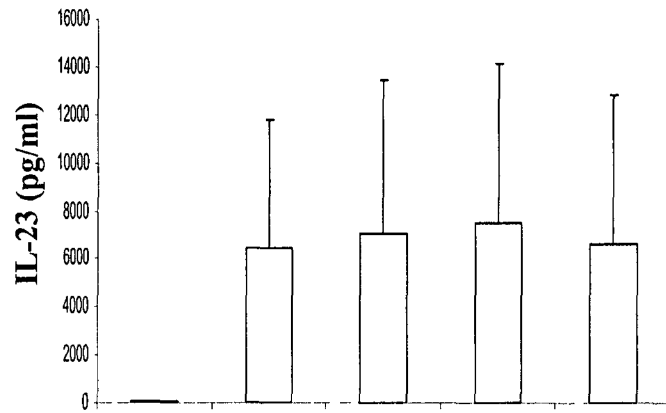


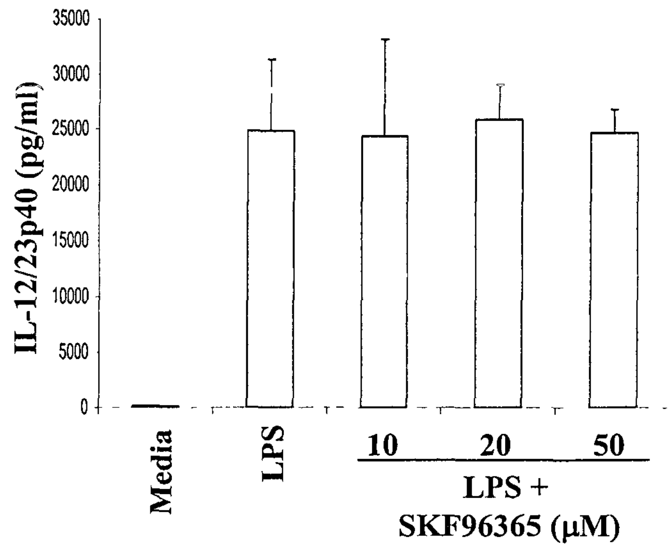


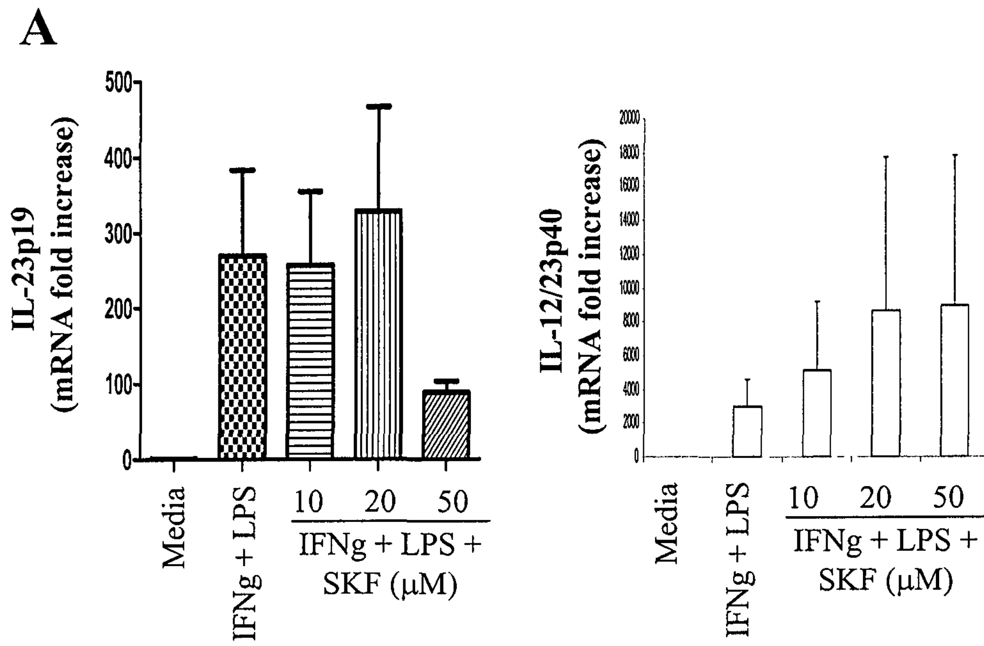
Figure 3.22

Figure 3.23. Calcium signalling inhibitor SKF96365 does not inhibit IFNγ/LPS-induced IL-23 expression in THP-1 cells.

A) THP-1 cells ($1x10^6$/ml) were treated with SKF96365 at concentrations ranging from 0 to 50 μM for 2 hrs followed by IFNγ (10 ng/ml) stimulation for 16 hrs and then LPS (1μg/ml) stimulation for 4 hrs. Cells were harvested for mRNA isolation. IL-23p19 and IL-12/23p40 gene expression were determined by R.Q. Real-Time PCR analysis.

B) THP-1 cells ($1x10^6$/ml) were treated with SKF96365 at concentrations ranging from 0 to 50 μM for 2 hrs followed by IFNγ (10 ng/ml) stimulation for 16 hrs and then LPS (1μg/ml) stimulation for 24 hrs. Cell culture supernatants were collected for total IL-23 and IL-12/23p40 protein measurement by ELISA.

Error bars represent the SEM of 3 to 4 independent experiments.



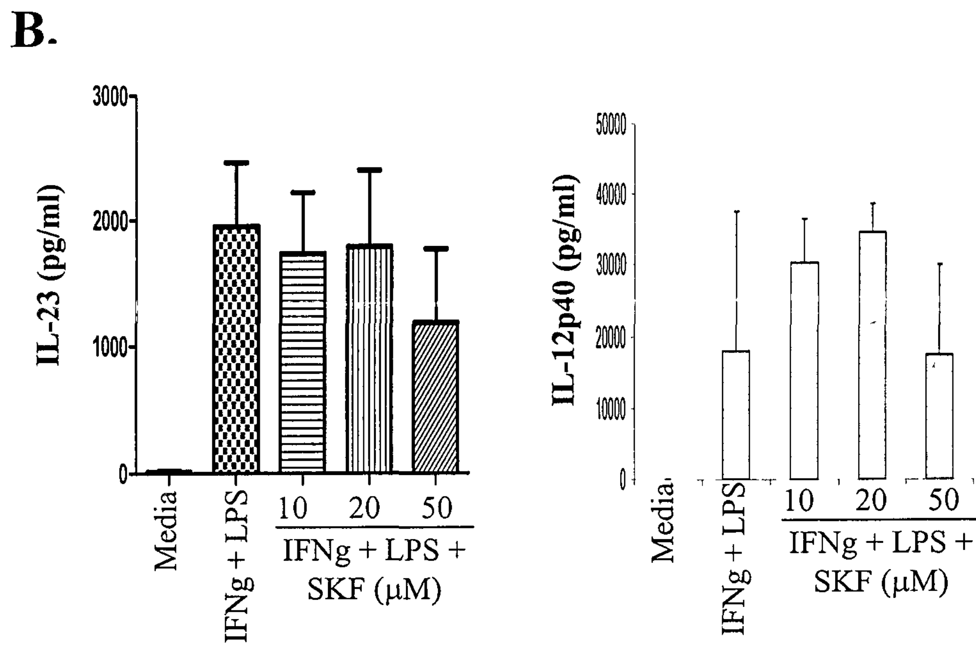


Figure 3.23

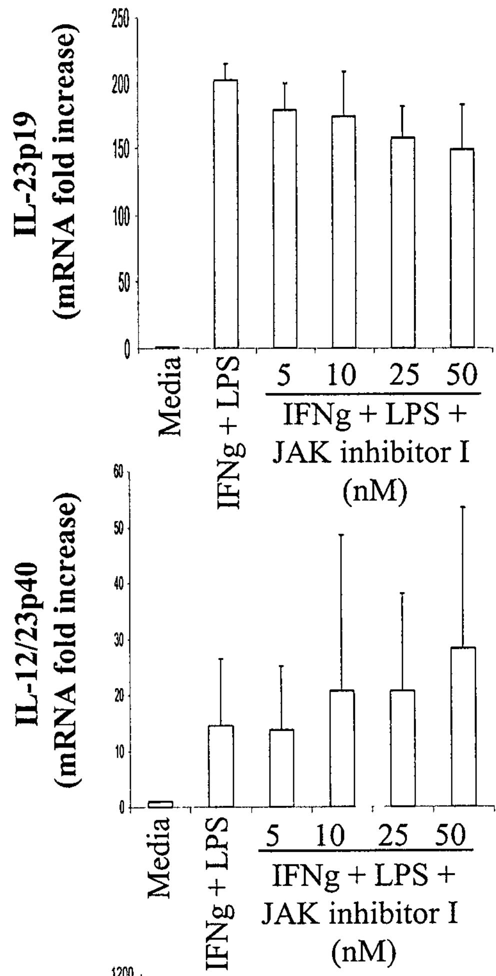
Figure 3.24. JAK/STAT signalling pathway does not regulate IFNγ/LPS-induced IL-23 expression in THP-1 cells.

A) THP-1 cells ($1x10^6$/ml) were treated with JAK inhibitor I at concentrations ranging from 0 to 50 μM for 2 hrs followed by IFNγ (10 ng/ml) stimulation for 16 hrs and then LPS (1μg/ml) stimulation for 4 hrs. Cells were harvested for mRNA isolation. IL-23p19 and IL-12/23p40 gene expression were determined by R.Q. Real-Time PCR analysis.

B) THP-1 cells ($1x10^6$/ml) were treated with JAK inhibitor I at concentrations ranging from 0 to 50 μM for 2 hrs followed by IFNγ (10 ng/ml) stimulation for 16 hrs and then LPS (1μg/ml) stimulation for 24 hrs. Cell culture supernatants were collected for total IL-23 protein measurement by ELISA.

Error bars represent the SEM of 3 to 4 independent experiments.

A.



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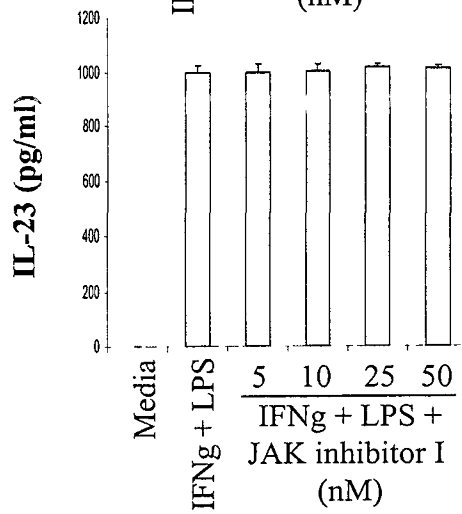


Figure 3.24

Part II

LPS- and IFNγ/LPS-induced IL-27 Regulation in Human Monocytic Cells

INTRODUCTION:

IL-27 is structurally and functionally related to IL-12, whose two subunits IL-12p35 and IL-12p40 structurally resemble IL-27p28 and EBI3, respectively. However, unlike the IL-12 subunits, IL-27p28 and EBI3 are not held together by disulphide bonds [119]. IL-27p28 and EBI3 are coexpressed primarily by macrophages and DCs. Monocytes and DCs are important sources of Th1 cytokines and act as a bridge between the innate and adaptive immune system [119, 274]. IL-27 is predominantly produced early on during microbial immune responses and plays a crucial role in activation and differentiation of naïve CD4 T cells to the Th1 lineage. IL-27 induces clonal proliferation of naïve, but not memory CD4 T cells. It synergizes with IL-12 to increase IFNγ production by naïve CD4 T cells [121, 156]. IL-27 causes upregulation of Th1-specific transcription factor T-bet and the transcriptional target of T-bet, IL-12Rα, on the surface of activated naïve CD4 T cells. It is also responsible for suppression of Th2-specific transcription factor GATA-3. Therefore, IL-27 blocks Th2 responses, as well as the production of Th2 cytokines [151, 154].

Most of the studies in this field have been conducted to elucidate the role of IL-27 in innate and cell-mediate immune responses [5, 119, 151, 155, 275]. Recently, a couple of reports have suggested a role for IL-27 in the inhibition of viral infections such as HIV and HCV [167, 168]. In terms of the signalling pathways involved in the regulation of IL-27 expression, very little information is available in the literature. For example, Theiler's murine encephalomyelitis virus-induced expression of IL-27p28 in RAW264.7, a mouse macrophage cell line, was shown to be dependent on TLR3- and TLR7-induced

activation of JNK-MAPK-kinases [224]. In addition, mice bone marrow-derived macrophages stimulated by *Salmonella enteritidis* or LPS expressed IL-27 subunits via the activation of TLR4/MyD88 signalling pathway [153, 249]. Moreover, TLR-2, TLR-4 and TLR-9 ligands were shown to upregulate IL-27EBI3 expression in mouse splenic DCs through MyD88, and NFκB activation [250]. Similarly, ligands for TLR-4 (LPS), TLR-7 (R848), and TLR-9 (CPG DNA) up regulated IL-27p28 through the activation of IRF3 [251]. To date there are only two reports showing the regulation of IL-27 subunit gene expression in human monocytes. TLR agonists were shown to upregulate IL-27p28 and IL-27EBI3 gene expression through IRFI activation in human MDMs. Furthermore, this IL-27p28 upregulation was inhibited by neutralizing antibodies against IFNγ [252]. In the second study, human DCs stimulated by TLR ligands or CMV infection induced IL-27p28 expression via the TLR4-activated IRF3 signalling pathway [251]. However, very little is known regarding the molecular mechanisms involved in the regulation of IL-27 production in human monocytic cells.

Given the importance of the role that IL-27 has in regulating host responses to invading pathogens, surprisingly, little it is known about its regulation. Here in this study *I investigated the molecular mechanisms involved in the regulation of IL-27 production in normal human primary monocytes and promonocytic cell line, THP-1$_{CD14}$ cells following activation by IFNγ or IFNγ and LPS.* My results suggest that LPS alone induces IL-27 expression in THP-1 cells, but not in normal human primary monocytes. In contrast, IFNγ alone has a limited effect on IL-27 expression in THP-1 cells. Moreover, priming with IFNγ was necessary to induce IL-27 expression by LPS-activated

monocytes. Overall results suggest that IFNγ-induced IL-27 expression is regulated by JNK MAPKs and PI3K in both primary monocytes and THP-1 cells. However, IL-27 expression was also regulated by p38 MAPK in IFNγ and LPS-stimulated human monocytes.

IFNγ- and IFNγ/LPS-induced IL-27 regulation in human primary monocytes

First, I determined if LPS was able to induce IL-27 expression in human primary monocytes. LPS alone failed to up-regulate IL-27p28 or IL-27EBI3 gene expression (Fig. 3.25). Optimal expression of IL-23p19 and IL-12/23p40 mRNA has been shown to require priming by IFNγ for 8-16 hrs prior to LPS stimulation [74, 230-232]. Therefore, I hypothesized that similar priming with IFNγ may also be necessary to induce IL-27 production. Human primary monocytes were stimulated with IFNγ (10 ng/ml) for 16 hrs either alone or followed by LPS stimulation for another 4 and 24 hrs to measure IL-27 transcripts and IL-27 proteins, respectively. Stimulation with IFNγ alone or IFNγ and LPS together significantly induced the expression of IL-27p28 gene transcripts whereas the induction of EBI-3 subunits following such stimulation was observed to be minimal (Fig. 3.25.A). Stimulation with IFNγ alone as well as IFNγ and LPS together induced significantly high levels of IL-27 proteins in these cells (Fig. 3.25.B).

IFNγ/LPS-induced IL-27 production in human monocytes is not regulated through the JAK/STAT signalling pathway

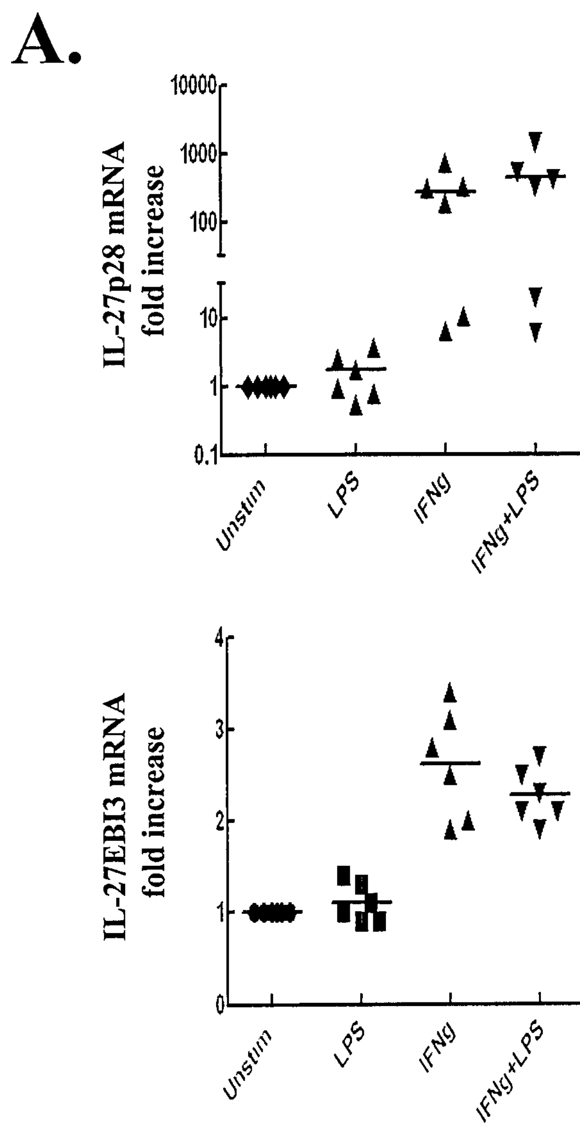
Since monocytes were primed with IFNγ followed by LPS stimulation, and because IFNγ mediates its biological effects through the activation of JAK-STAT pathway, I determined the involvement of IFNγ-activated JAK/STAT pathway in the expression of IL-27 mRNA subunits and IL-27 protein production by employing the JAK inhibitor-1 to inhibit the JAK/STAT pathway. To determine the role of JAK/STAT signalling in IFNγ/LPS- induced IL-27 expression, purified monocytes from healthy

Figure 3.25. Priming with IFNγ is required to induce IL-27 expression in human primary monocytes

A) Normal human monocytes ($1x10^6$/ml) were treated with IFNγ (10 ng/ml) for 16 hrs prior to stimulation with LPS (1μg/ml) for another 4 hrs followed by analysis for IL-27p28 and IL-27EBI3 mRNA expression by R.Q. Real-Time PCR. Each point represents IL-27 gene expression by monocytes from one individual.

B) Normal human monocytes ($1x10^6$/ml) were treated with IFNγ (10 ng/ml) for 16 hrs prior to stimulation with LPS (1μg/ml) for another 24 hrs followed by analysis for IL-27 production by ELISA.

Each line represents IL-27 protein expression by monocytes from one individual.



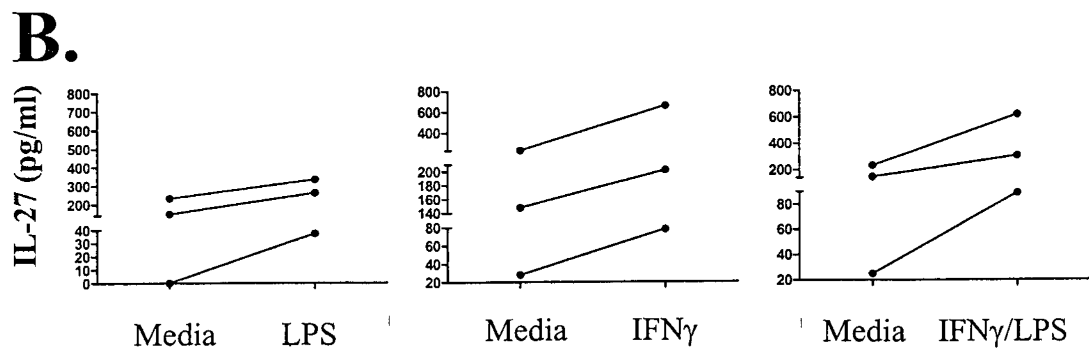


Figure 3.25

donors were treated with the Jak inhibitor-1 for 2 hrs followed by IFNγ treatment (16 hr) and LPS stimulation (4 hr for mRNA and 24 hr for protein production). The gene transcripts and protein production levels were measured by Real-Time PCR and ELISA, respectively. Results show that inhibition of Jak kinases did not affect IL-27p28 transcripts or IL-27 proteins expression in human monocytes stimulated by either IFNγ alone (Fig. 3.26 bottom) or IFNγ and LPS together (Fig. 3.26 top). As mentioned earlier in this chapter, I also investigated the biological activity of Jak-inhibitor-1 by determining its effect on STAT-1 phosphorylation. Cells pre-treated with the JAK inhibitor-1 showed significantly reduced STAT-1 phosphorylation, indicating the biological activity of the inhibitor (Fig. 3.3.A). As a positive control, the Jak-Inhibitor-1 significantly inhibited IL-27 proteins production in THP-1 cells stimulated with IFNγ alone (Fig. 3.3.B).

JNK and P38 MAPKs regulate IFNγ/LPS-induced IL-27 production in normal human monocytes

In our laboratory it has been shown previously that LPS-induced IL-12p40 production in human monocytic cells is regulated by the JNK MAPKs [25]. To determine the role of MAPKs, I first examined whether LPS induces phosphorylation of ERK, JNK, and p38 MAPKs, and whether this phosphorylation can be inhibited by their specific inhibitors. LPS induced the phosphorylation of ERK, JNK, and p38 MAPKs (Fig. 3.27.A) that was inhibited by their specific inhibitors in a dose-dependent manner (Fig. 3.27.A). IFNγ was also shown to induce the phosphorylation of all three members, ERK, JNK, and p38

MAPKs and this phosphorylation was inhibited by their respective MAPKs Inhibitors (Fig. 3.5). To determine the effect of MAPKs on IL-27 expression, purified human monocytes from healthy volunteers were treated with MAPKs specific inhibitors (PD98059, SP600125, or SB202190) for 2 hrs followed by priming for 16 hrs with IFNγ and subsequent stimulation for 4 hrs (gene expression) or 24 hrs (protein production) with LPS. The results show that PD98059, ERK inhibitor, did not modulate IFNγ and LPS-induced IL-27p28 gene expression or IL-27 protein production (Fig. 3.27.B&C), but the p38 inhibitor significantly inhibited ($p=0.0001$) IFNγ and LPS-induced IL-27 protein production (Fig. 3.27.C lower panel). Interestingly, none of the MAPKs inhibitors namely PD98059, SB202190 or SP600125 inhibited IL-27p28 gene expression in cells stimulated with either IFNγ alone (data not shown) or IFNγ and LPS together (Fig. 3.27.B). Significantly, JNK inhibitor, SP600125, inhibited IFNγ- ($p=0.02$) (Fig. 3.27.D lower panel) and IFNγ/LPS-induced ($p=0.03$) IL-27 protein productions (Fig. 3.27.C middle panel).

Figure 3.26. IFNγ/LPS-induced IL-27 production is not regulated by the JAK/STAT signalling pathway.

A) Normal human monocytes ($1x10^6$/ml) were treated with a broad range JAK kinase inhibitor (JAK inhibitor I) at concentrations ranging from 10 to 100 nM for 2 hrs followed by stimulation for 16 hrs with IFNγ (10 ng/ml) and for another 4 hrs with LPS (1μg/ml) **(upper panel)** or cells were stimulated with IFNγ for 4 hrs **(lower panel)**. Cells were harvested for mRNA isolation. IL-27p28 gene expression was determined by R.Q. Real-Time PCR analysis.

B) Normal human monocytes ($1x10^6$/ml) were treated with JAK inhibitor I at concentrations ranging from 10 to 100 nM for 2 hrs followed by stimulation for 16 hrs with IFNγ (10 ng/ml) and for another 24 hrs with LPS (1μg/ml) **(upper panel)** or cell were stimulated with IFNγ for 24 hrs **(lower panel)**. Supernatants were collected for IL-27 protein measurement by ELISA.

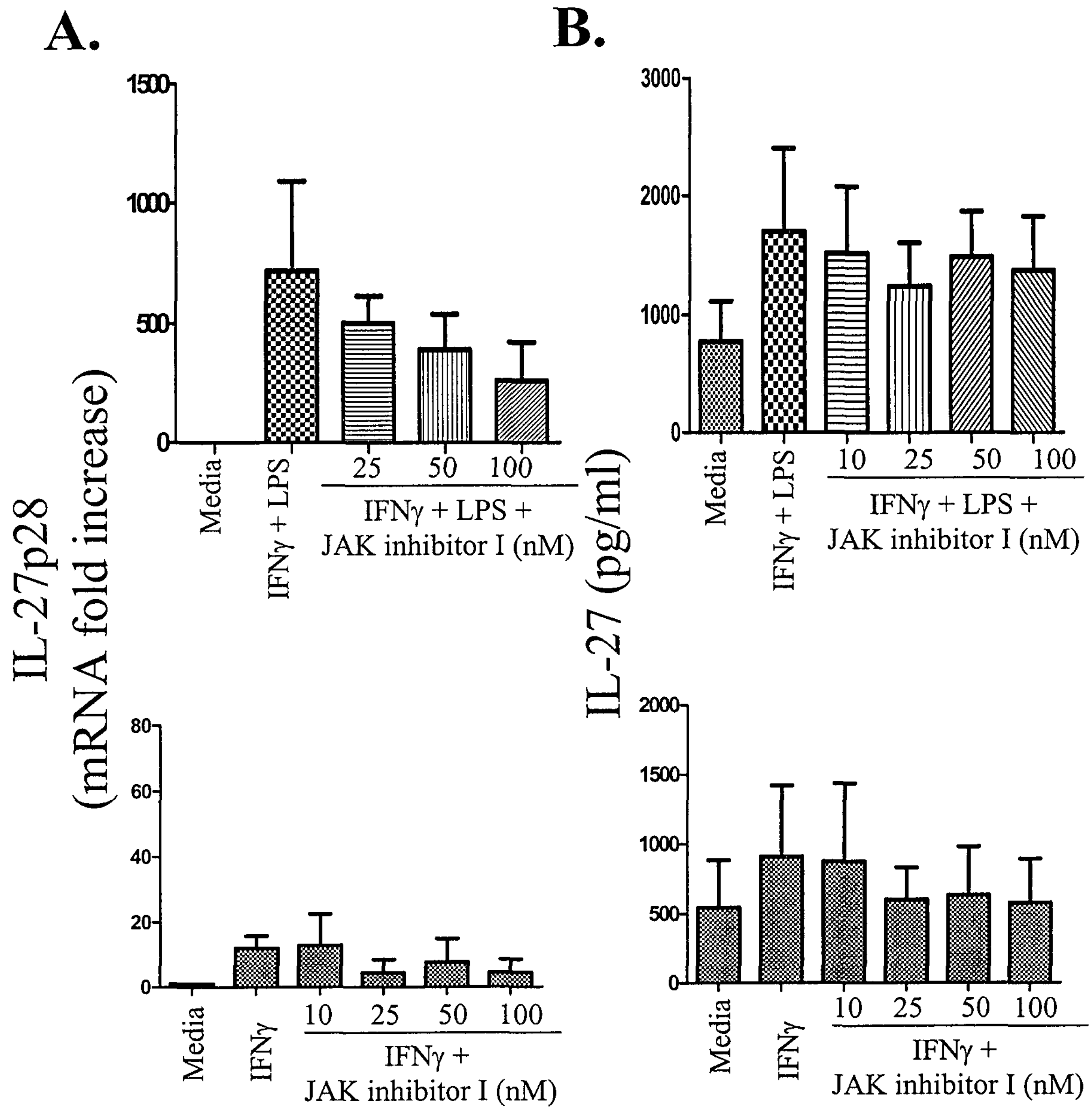


Figure 3.26

PI3K signalling pathway regulates IFNγ/LPS-induced IL-27 expression in normal human monocytes

It has been previously demonstrated in our laboratory that LPS-induced IL-12p40 production is regulated by the PI3K pathway [33]. Therefore, to determine the role of PI3K signalling in the regulation of IL-27, I examined if Akt, the well known downstream mediator of PI3K signalling, it phosphorylated with IFNγ or LPS stimulation and whether this phosphorylation can be inhibited by the PI3K specific inhibitor, LY249002 in purified human monocytes. Monocytes were pretreated with LY294002 for 2 hrs followed by stimulation with IFNγ or LPS for 15 min. IFNγ as well as LPS induced the phosphorylation of Akt that was inhibited by LY249002 in a dose-dependent manner (Fig. 3.28.A&C). Subsequently, to determine the role of PI3K in IFNγ and LPS-induced IL-27 expression, purified human monocytes were treated with LY294002 for 2 hr followed by stimulation with IFNγ for 16 hrs and LPS for another 4 or 24 hrs. IL-27p28 gene expression (Fig. 3.28.B upper panel) was not inhibited by LY294002 following stimulation with either IFNγ (data not shown) or IFNγ and LPS (Fig. 3.28.B upper panel). However, IL-27 protein production was significantly inhibited by LY294002 in cells stimulated with either IFNγ ($p=0.002$) (Fig. 3.28.D lower panel) or IFNγ and LPS ($p=0.02$) (Fig. 3.28.B lower panel). Moreover, LY249002 significantly inhibited IFNγ-induced IL-27 protein production without inhibiting the IL-27p28 gene expression (Fig. 3.28.D upper panel). These results suggest that PI3K activation plays an important role in IFNγ- as well as IFNγ/LPS-induced IL-27 expression in normal human monocytes.

Figure 3.27. Role of MAPKs in LPS-induced in IL-27 expression in primary monocytes

A) Normal human monocytes ($1x10^6$/ml) were treated with PD98059, SP600125, or SB203180 at concentrations ranging from 0 to 50 μM for 2 hrs before LPS (1μg/ml) stimulation. Total proteins (50 μg) were analyzed by Western blot analysis using anti-phospho-ERK (P-ERK), anti-phospho-JNK (P-JNK), or anti-phospho-p38 (P-p38) antibodies. To control for equal protein loading, the membranes were stripped and reprobed with anti-ERK, anti-JNK, or anti-p38 antibodies, respectively.

B) Normal human monocytes ($1x10^6$/ml) were treated with PD98059, SP600125, or SB202190 at concentrations ranging from 0 to 50 μM for 2 hrs followed by stimulation for 16 hrs with IFNγ (10 ng/ml) and for 4 hrs with LPS (1μg/ml). Cells were harvested for mRNA isolation. IL-27p28 and IL-27EBI3 gene expression were determined by R.Q. Real-Time PCR analysis.

C) Normal human monocytes ($1x10^6$/ml) were treated with PD98059, SP600125, or SB202190 at concentrations ranging from 0 to 50 μM for 2 hrs followed by stimulation for 16 hrs with IFNγ (10 ng/ml) and for 24 hrs with LPS (1μg/ml). Supernatants were collected for IL-27 protein measurement by ELISA.

D) Normal human monocytes ($1x10^6$/ml) were treated with SP600125 at concentrations ranging from 0 to 50 μM for 2 hrs followed by stimulation for 16 hrs with IFNγ (10 ng/ml). Cells and culture supernatants were collected for IL-27p28 mRNA and IL-27 protein production assay.

ANOVA test used to calculate the significant differences.

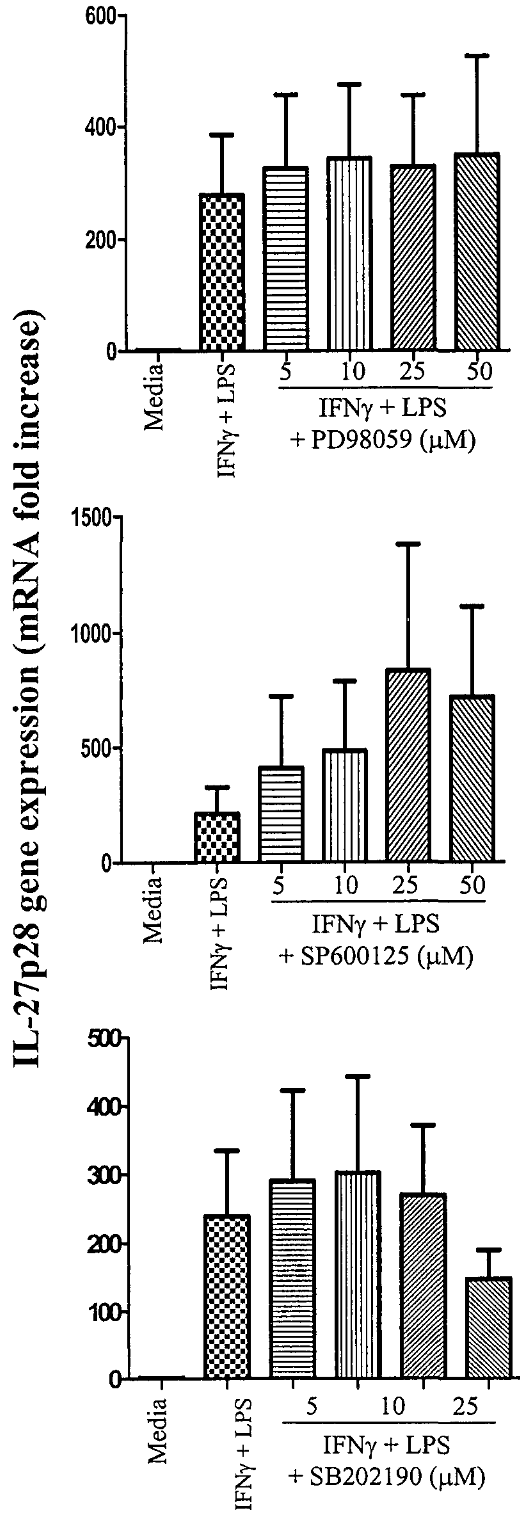
A.

Media
LPS
LPS +
PD98059 (μM)
5 10 25 50
p-ERK
ERK

Media
LPS
LPS +
SP600125 (μM)
5 10 25 50
P-JNK
JNK

Media
LPS
LPS +
SB203180 (μM)
10 25 50
P-p38
p38

Figure 3.27.A



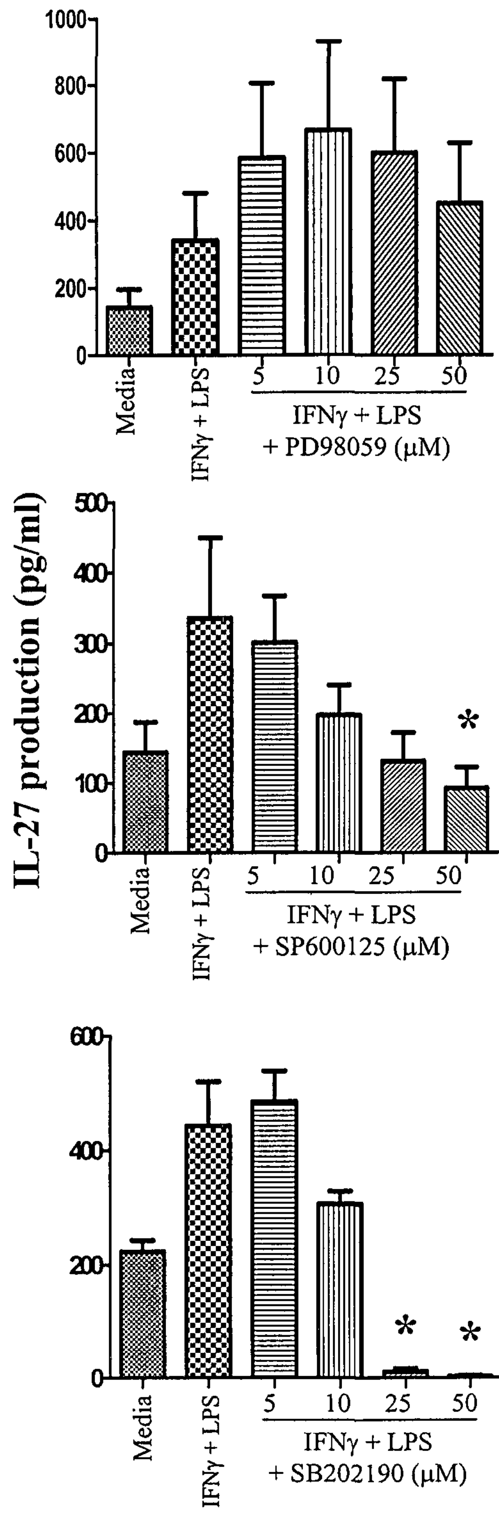


Figure 3.27.B&C

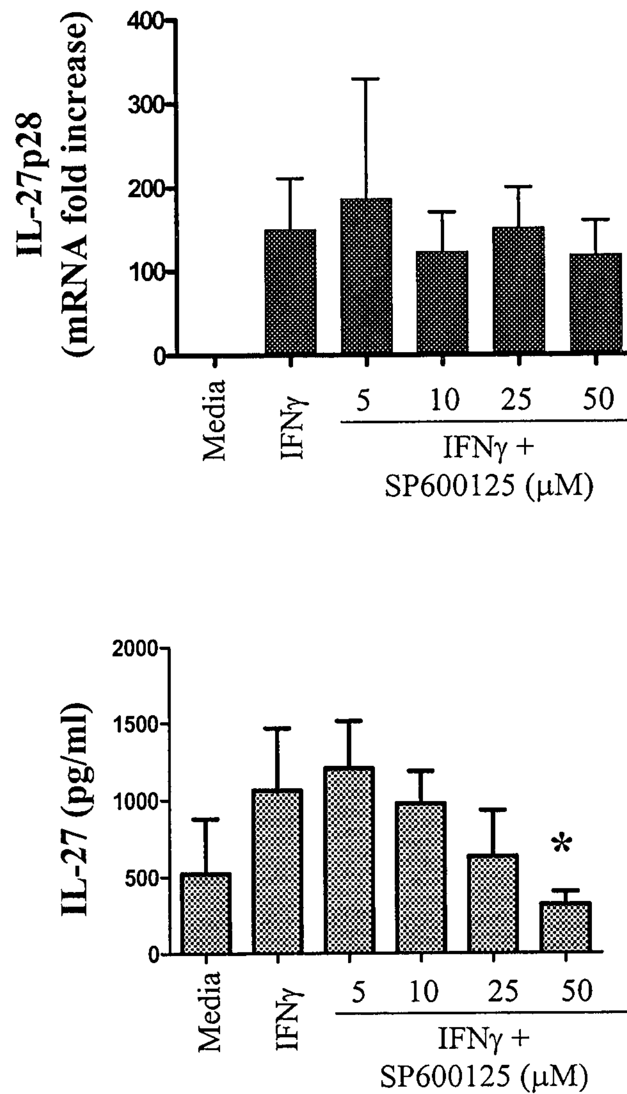


Figure 3.27.D

Calcium signalling pathway regulates IFNγ/LPS-induced IL-27 protein production in human monocytic cells

Our laboratory has previously demonstrated that LPS-induced IL-12p40 expression is regulated by the calcium signalling pathway [33]. However, IL-12p40 regulation has also been shown to be negatively regulated by calcium signalling in murine and human macrophages [246]. Therefore, I hypothesized that IFNγ- and IFNγ/LPS-induced IL-27 expression may also be regulated by the calcium signalling pathway in primary monocytes. To determine the regulatory role of the calcium signalling pathway, cells were treated with a well known calcium chelator, EGTA (2 to 10 nM) followed by IFNγ or IFNγ and LPS stimulation. The results show that EGTA significantly inhibited ($p=0.0004$) IL-27 protein production in human monocytic cells (Fig. 3.29.B), but failed to inhibit IL-27p28 gene expression (Fig. 3.29.A). These results suggest that the inhibitory effect of EGTA is mediated at the post-transcriptional level.

Figure 3.28. Role of PI3K pathway in IFNγ/LPS-induced IL-27 expression in normal human monocytes.

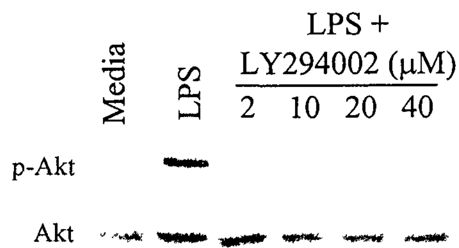
A) Normal human monocytes ($1x10^6$/ml) were treated with LY294002 at concentrations ranging from 0 to 40 μM for 2 hrs before LPS (1μg/ml) stimulation. Total proteins (50 μg) were analyzed by Western blot analysis using anti-phospho-Akt (p-Akt) antibodies. To control for equal protein loading, the membrane was stripped and reprobed with anti-Akt antibodies.

B) Normal human monocytes ($1x10^6$/ml) were treated with LY294002 at concentrations ranging from 0 to 25 μM for 2 hrs followed by stimulation for 16 hrs with IFNγ (10 ng/ml) and for another 4 **(upper panel)** or 24 hrs **(lower panel)** with LPS (1μg/ml). Cells pellets and cell culture supernatants were collected for mRNA isolation and protein measurement.

C) Normal human monocytes ($1x10^6$/ml) were treated with LY294002 at concentrations ranging from 0 to 40 μM for 2 hrs before IFNγ (10 ng/ml) stimulation. Total proteins (50 μg) were analyzed by Western blot analysis using anti-phospho-Akt (p-Akt) antibodies. To control for equal protein loading, the membrane was stripped and reprobed with anti-Akt antibodies.

D) Normal human monocytes ($1x10^6$/ml) were treated with LY294002 at concentrations ranging from 0 to 25 μM for 2 hrs followed by stimulation with IFNγ (10 ng/ml) for 4 hrs **(upper panel)** or 24 hrs **(lower panel)**. Cells pellets and cell culture supernatants were collected for mRNA isolation and protein measurement.

Error bars represent the SEM of 3 to 4 independent experiments.
ANOVA used to test significant differences.



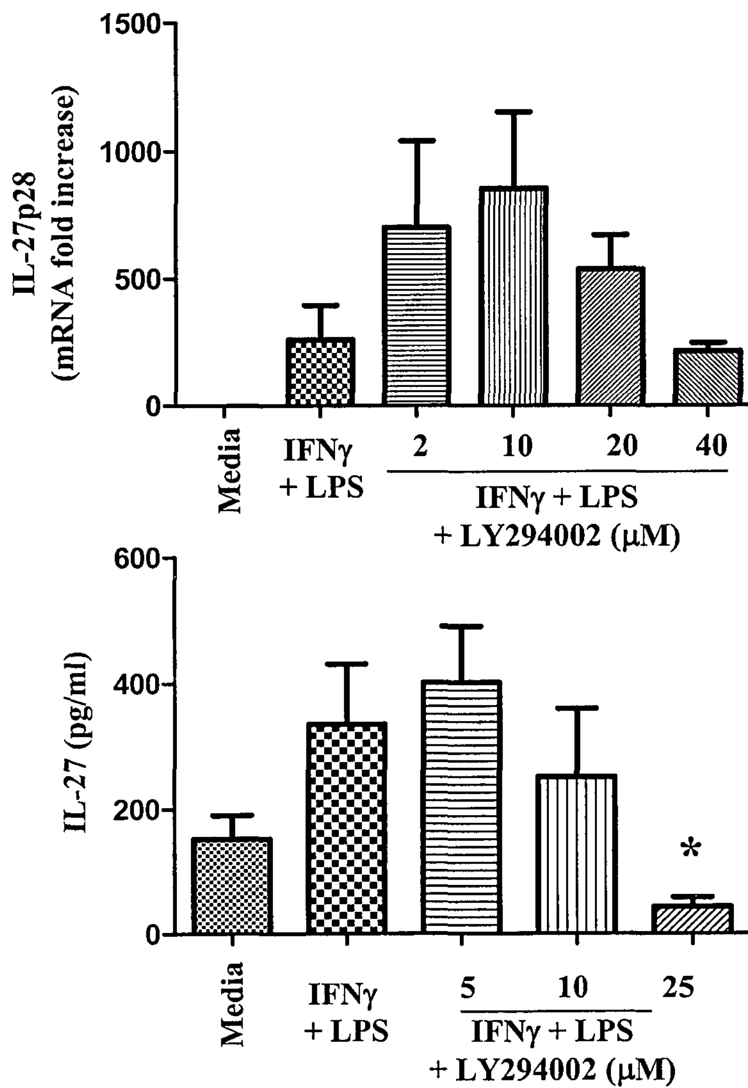
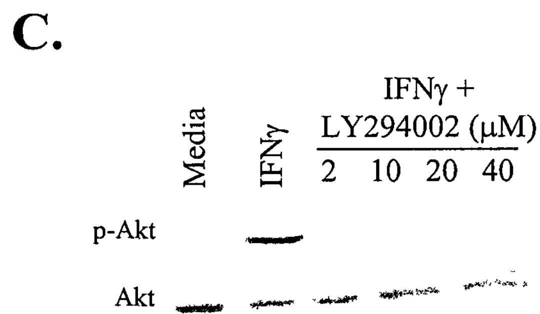
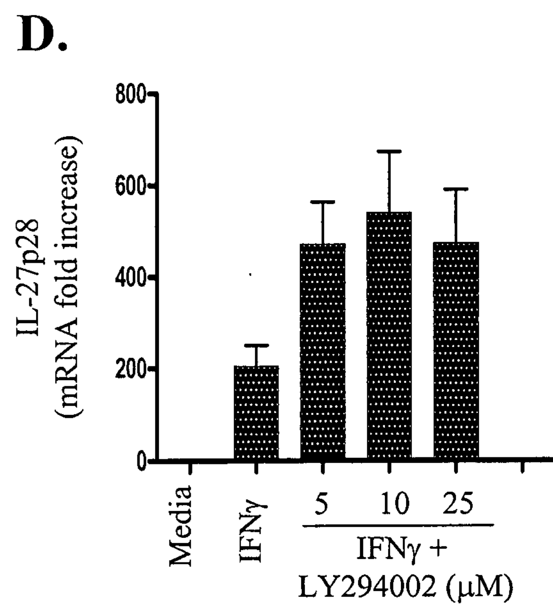


Figure 3.28.A&B

C.



D.



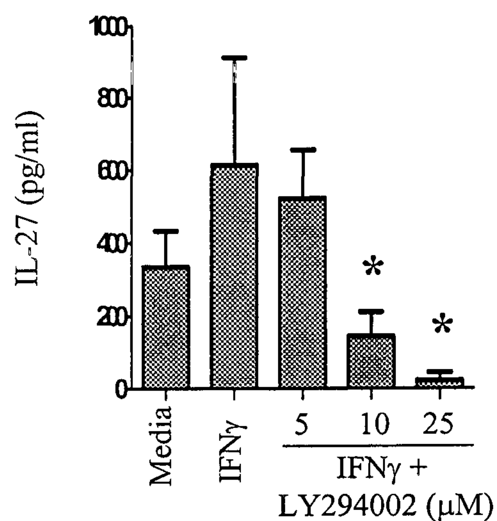


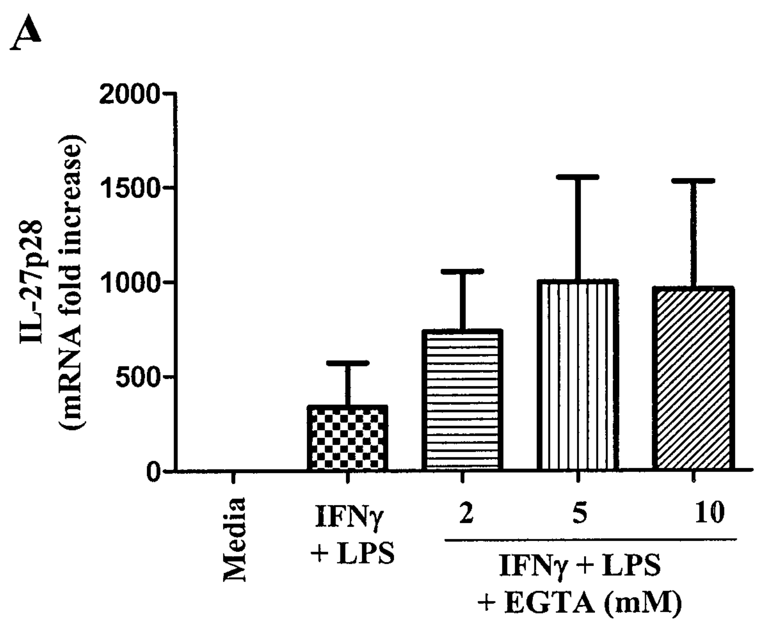
Figure 3.28.C&D

Figure 3.29. EGTA inhibits IL-27 protein production in normal human monocytes.

A) Normal human monocytes ($1x10^6$/ml) were treated with EGTA at concentrations ranging from 2 to 10 mM for 2 hrs followed by stimulation for 16 hrs with IFNγ (10 ng/ml) and for another 4 hrs with LPS (1μg/ml). Cells were harvested for mRNA isolation. IL-27p28 gene expression was determined by R.Q. Real-Time PCR analysis.

B) Normal human monocytes ($1x10^6$/ml) were treated with EGTA at concentrations ranging from 2 to 10 mM for 2 hrs followed by stimulation for 16 hrs with IFNγ (10 ng/ml) and for another 24 hrs with LPS (1μg/ml). Supernatants were collected for IL-27 protein measurement by ELISA.

Error bars represent the SEM of 3 to 4 independent experiments.
ANOVA used to test significant differences..



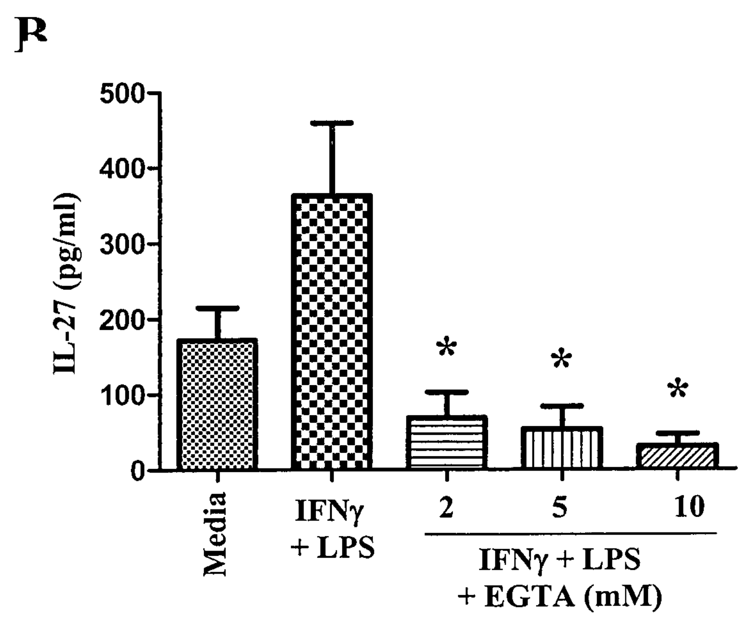


Figure 3.29

IFNγ- and IFNγ/LPS-induced IL-27 regulation in THP-1 cells

IL-27 protein is produced and secreted from LPS- and IFNγ/LPS stimulated THP-1 cells

The human promonocytic THP-1 cells serve as an excellent model to study the regulation of cytokines in human monocytic cells [272]. First, I investigated the expression of IL-27p28 and IL-27EBI3 genes following stimulation with LPS. Stimulation with LPS alone resulted in a maximum expression of IL-27p28 and IL-27EBI3 at 4 hr post-stimulation as determined by R.Q. Real-Time PCR. However, the expression of IL-27p28 mRNA in these cells was significantly higher than IL-27EBI3 genes (Fig. 3.30.A). Stimulation with LPS alone induced significantly high levels of IL-27 proteins ranging from 0 to about 600 pg/ml of IL-27 in a time-dependent manner (Fig. 3.30.B).

Since the stimuli and time courses applied in primary monocyes and THP-1 cells were not similar, it was interesting to determine whether signalling pathways involved in IL-27 regulation are similar in both experimental conditions. Therefore, THP-1 cells were treated with IFNγ for 16 hr followed by LPS for 4 or 24 hrs to measure the IL-27p28 gene transcripts and IL-27 protein production, respectively. LPS alone increased IL-27p28 gene expression at about 1100 folds higher than the unstimulated cells. However, the level of IL-27p28 gene induction following IFNγ stimulation alone was relatively lower (200 folds) than that observed with LPS alone (Fig. 3.31 upper panel). However, a significant synergistic upregulation of IL-27p28 expression was observed following stimulation with IFNγ and LPS together (25,300 fold). IFNγ induced IL-27p28, but it was lower as compared to human monocytes, and IL-27EBI3 expression is at the same level

as unstimulated THP-1 cells (Fig. 3.31 upper panel). Also IFNγ/LPS-induced IL-27EBI3 gene expression was lower than that with LPS alone (Fig. 3.31 middle panel). Surprisingly, at the protein levels, IFNγ/LPS-induced IL-27 production was similar to that observed following LPS stimulation alone (Fig. 3.31 lower panel). These results suggest differential regulation of IL-27 at transcriptional and translational level in THP-1 cells and normal human monocytes.

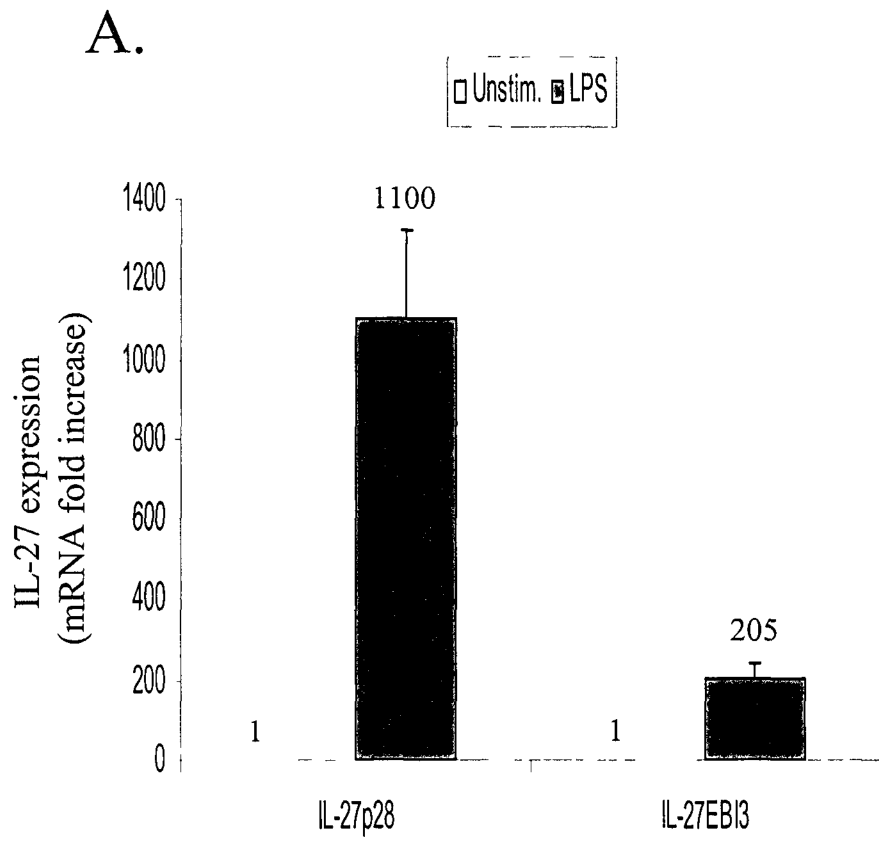
Figure 3.30. LPS induces IL-27 expression in THP-1 cells

A) THP-1 cells ($1x10^6$/ml) were stimulated with LPS (1μg/ml) for 4 hrs followed by analysis for IL-27p28 and IL-27EBI3 mRNA expression by relative quantitative Real-Time PCR (R.Q. Real-Time PCR).

B) THP-1 cells ($1x10^6$/ml) were stimulated with LPS (1μg/ml) for 0 to 30 hrs and the culture supernatants were collected for IL-27 protein measurement. IL-27 protein production was determined by ELISA.

Error bars represent the SEM of 3 independent experiments.

A.



B.

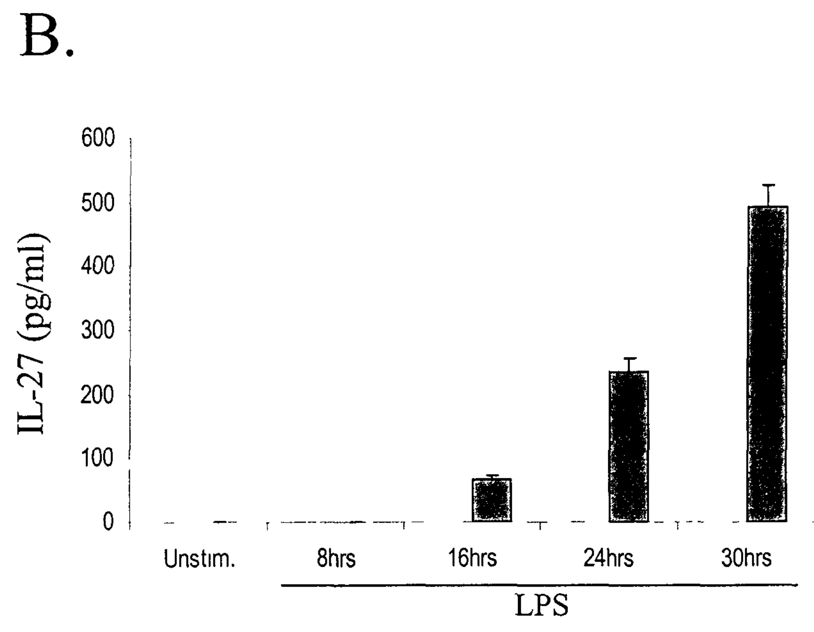


Figure 3.30

JNK and p38 MAPKs regulate LPS- and IFNγ/LPS-induced IL-27 production in THP-1 cells

The MAPKs have been shown to play a key role in the regulation of IL-12p40 and IL-12p70 proteins [25, 78, 245, 256, 268]. However, the involvement of MAPKs in LPS- and IFNγ/LPS-induced IL-27 regulation in human monocytic cells remains poorly understood. In order to investigate the role of MAPKs, I investigated the biological activity of all three pharmacological inhibitors specific for members of MAPKs in THP-1 cells. THP-1 cells were treated with inhibitors for 2 hrs prior to stimulation with LPS for another 30 min followed by analysis of ERK, JNK and p38 MAPKs phosphorylation by Western blotting. As shown earlier, LPS stimulation induced the phosphorylation of all three members of MAPKs which was inhibited by prior treatment of cells with PD98059, SP600125, or SB202190 (Fig. 3.15.A).

To determine the role of MAPKs in IL-27 regulation, cells were treated with various concentrations of MAPKs specific inhibitors for 2 hrs prior to stimulation with LPS for 4 hrs. Treatment of cells with ERK and JNK specific inhibitors either increased the level of IL-27 subunits p28 and EBI3 or had no significant effect at mRNA levels (Fig. 3.32.A upper and middle panels). In contrast, p38 MAPK inhibitor significantly down regulated the level of IL-27p28 transcripts ($p=0.008$) while it did not inhibit IL-27EBI3 gene expression (Fig. 3.32.A, lower panels). However, LPS-induced IL-27 production was significantly down regulated by JNK- ($p=0.001$) and p38-MAPKs ($p=0.005$) inhibitors (Fig. 3.32.B, middle and lower panels) whereas PD98059, the ERK inhibitor, did not affect IL-27 production (Fig. 3.32.B, upper panel). Similarly, IFNγ/LPS-induced

IL-27p28 and IL-27EBI3 gene expression and IL-27 protein production were not downregulated by ERK inhibitor. In contrast, the JNK inhibitor inhibited IL-27p28 expression under similar conditions. However, IL-27 proteins were positively regulated by the JNK MAPKs ($p=0.01$) whereas the ERK and p38 MAPKs inhibitors did not affect its expression (Fig. 3.33.B).

The involvement of p38 MAPK in the regulation of LPS-induced IL-27p28 gene expression was confirmed by employing siRNAs against ERK and p38 MAPKs. Cells were transfected with ERK, p38, or control siRNA using DharmaFECTTM 2 transfection reagents as per the manufacturer's instructions (Dharmacon). Following transfection, cells were stimulated with LPS (1 μg/ml) for 8 hrs for Real-Time PCR and for 30 min for for ERK and p38 MAPKs phosphorylation. The ERK-specific siRNA inhibited ERK phosphorylation following LPS stimulation (Fig. 3.34.A upper panel). There was no positive regulatory effect on IL-27p28 mRNA expression by the ERK specific siRNA (Fig. 3.34.A lower panel). However, p38 specific siRNA significantly reduced LPS-induced p38 phosphorylation (Fig. 3.34.B upper panel) as well as IL-27p28 mRNA expression (Fig. 3.34.B lower panel).

Figure 3.31. IFNγ/LPS combination significantly increases IL-27p28 mRNA and IL-27 proteins in THP-1 cells.

Upper panel: THP-1 cells were stimulated with LPS, IFNγ, or IFNγ + LPS. Cells were harvested for mRNA isolation. IL-27p28 gene expression was determined R.Q. Real-Time PCR analysis.

Middle panel: THP-1 cells were stimulated with LPS, IFNγ, or IFNγ + LPS. Cells were harvested for mRNA isolation. IL-27EBI3 gene expression was determined R.Q. Real-Time PCR analysis.

Lower panel: THP-1 cells were stimulated with LPS, IFNγ, or IFNγ + LPS. Supernatants were collected for IL-27 protein measurement by ELISA.
Error bars represent the SEM of 3 independent experiments.

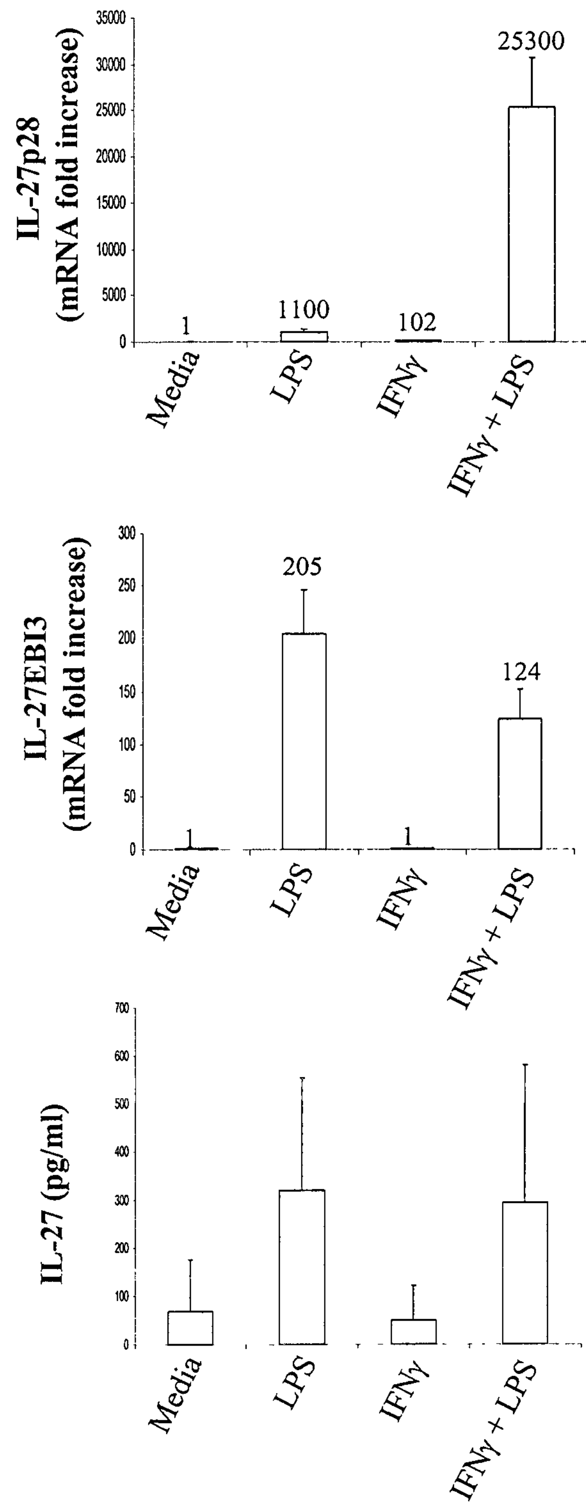


Figure 3.31

Figure 3.32. p38 and JNK MAPKs regulate LPS-induced IL-27 regulation in THP-1 cells

A) THP-1 cells ($1x10^6$/ml) were treated with PD98059, SP600125, or SB202190 at concentrations ranging from 0 to 50 μM for 2 hrs followed by LPS (1μg/ml) stimulation for 4 hrs. Cells were harvested for mRNA isolation. IL-27p28 and IL-27EBI3 gene expression were determined by R.Q. Real-Time PCR analysis.

B) THP-1 cells ($1x10^6$/ml) were treated with PD98059, SP600125, or SB202190 at concentrations ranging from 0 to 50 μM for 2 hrs followed by LPS (1μg/ml) stimulation for 24 hrs. Supernatants were collected for IL-27 protein measurement by ELISA.

Error bars represent the SEM of 3 to 5 independent experiments.
ANOVA used to test significant differences.

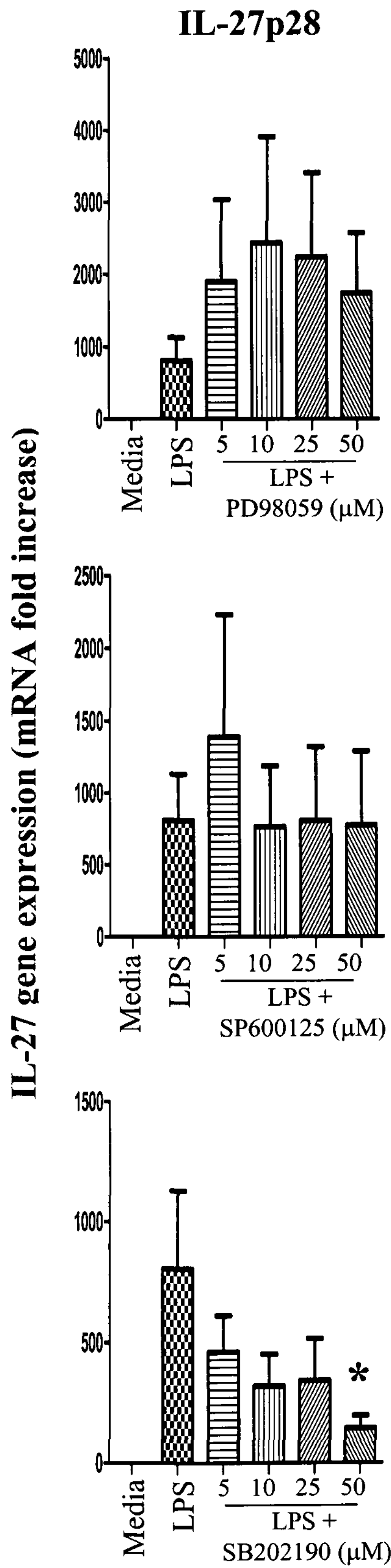


Figure 3.32

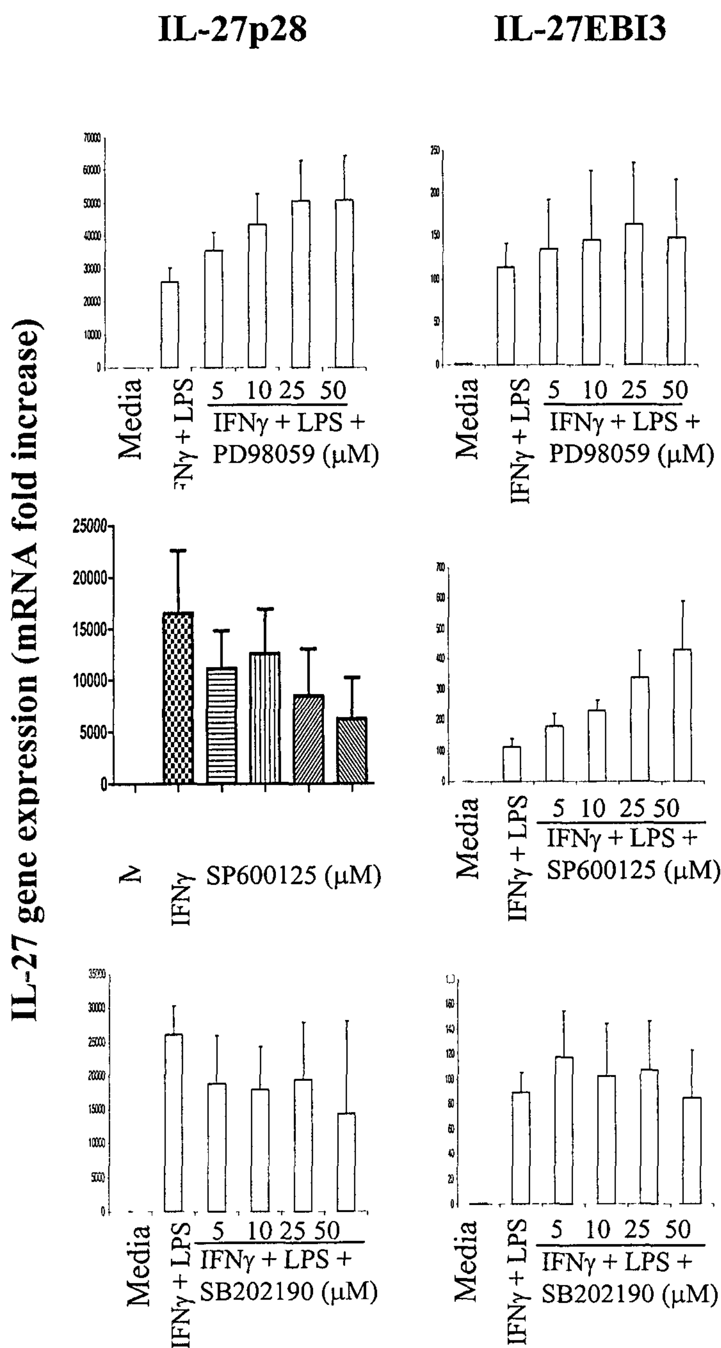
Figure 3.33. JNK MAPKs regulate IFNγ/LPS-induced IL-27 protein production in THP-1 cells.

A) THP-1 cells ($1x10^6$/ml) were treated with PD98059, SP600125, or SB202190 at concentrations ranging from 0 to 50 μM for 2 hrs followed by 16 hrs IFNγ (10 ng/ml) and 4 hrs LPS (1μg/ml) stimulation. Cells were harvested for mRNA isolation. IL-27p28 and IL-27EBI3 gene expression were determined by R.Q. Real-Time PCR analysis.

B) THP-1 cells ($1x10^6$/ml) were treated with PD98059, SP600125, or SB202190 at concentrations ranging from 0 to 50 μM for 2 hrs followed by 16 hrs IFNγ (10 ng/ml) and 24 hrs LPS (1μg/ml) stimulation. Supernatants were collected for IL-27 protein measurement by ELISA.

Error bars represent the SEM of 3 to 4 independent experiments.
ANOVA used to test significant differences.

A. **B.**



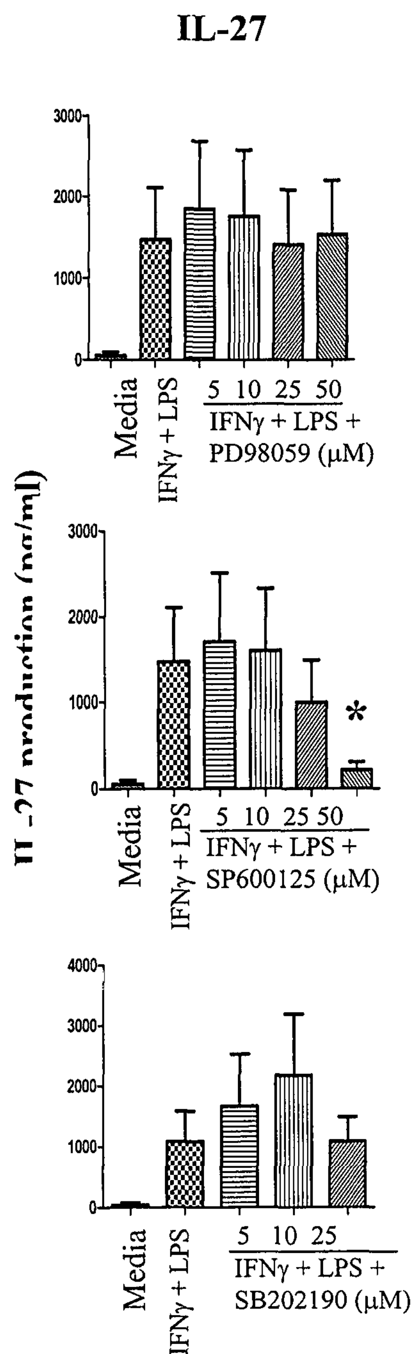


Figure 3.33

Figure 3.34. p38 MAPK siRNA inhibits the IL-27p28 mRNA expression in THP-1 cells.

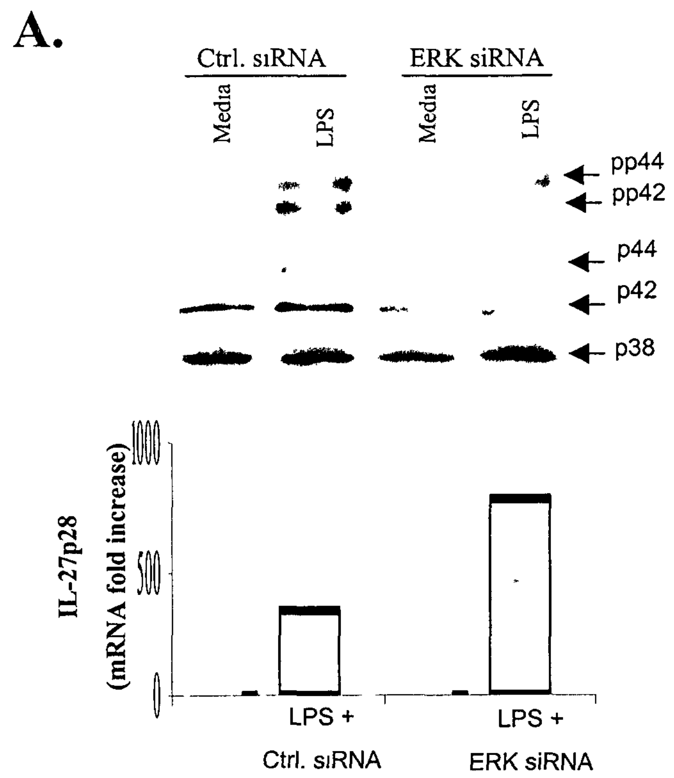
A)

Upper panel: THP-1 cells were transfected with the either control siRNA or ERK MAPK siRNA for 24 hrs followed by stimulation with LPS (1μg/ml) for 30 min for the determination of phosphorylated ERK MAPK by Western blot analysis.
Lower panel: THP-1 cells were transfected with the either control siRNA or ERK MAPK siRNA for 24 h followed by stimulation with LPS (1μg/ml) for 8 hrs for IL-27p28 gene expression assay by R.Q. Real-Time PCR.

B)

Upper panel: THP-1 cells were transfected with the either control siRNA or p38 MAPK siRNA for 24 hrs followed by stimulation with LPS (1μg/ml) for 30 min for the determination of phosphorylated p38 MAPK by Western blot analysis.
Lower panel: THP-1 cells were transfected with the either control siRNA or p38 MAPK siRNA for 24 h followed by stimulation with LPS (1μg/ml) for 8 hrs for IL-27p28 gene expression assay by R.Q. Real-Time PCR.

Each histogram is a representative of 2 independent experiments.



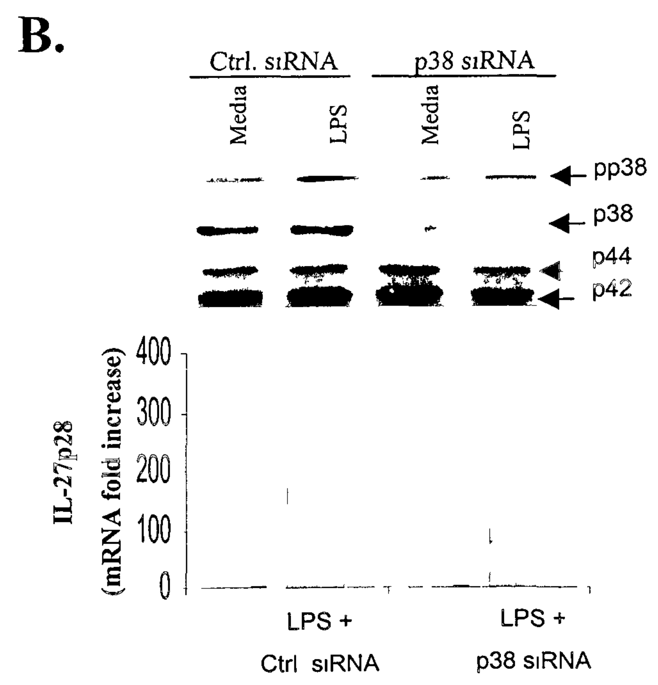


Figure 3.34

LPS- and IFNγ/LPS-induced IL-27 expression is regulated by the PI3K pathway

PI3K has been shown to regulate LPS-induced IL-12p40 expression in primary monocytes as well as human monocyte-derived macrophages [246, 276]. Additionally, IL-23 expression has previously been reported to be regulated via the PI3K pathway [223, 244]. Therefore, I examined whether the activation of PI3K regulates LPS- and IFNγ/LPS-induced IL-27 production in THP-1 cells by employing the PI3K-specific inhibitor, LY249002. LY249002 was found to be biologically active as it inhibited LPS-induced Akt phosphorylation in a dose-dependent manner in THP-1 cells (Fig. 3.35.A). Interestingly, LY294002 significantly inhibited LPS-induced IL-27p28 ($p=0.001$) and IL-27EBI3 ($p=0.005$) transcripts (Fig. 3.35.B) as well as LPS-induced IL-27 protein ($p=0.05$) production in a dose dependent manner (Fig. 3.35.C). Similarly, LY294002 significantly inhibited IFNγ/LPS-induced IL-27 p28 ($p=0.01$), and downregulated IL-27EBI3 transcripts (Fig. 3.36.A) as well as IL-27 ($p=0.03$) protein production in THP-1 cells (Fig. 3.36.B).

The role of the p85α regulatory subunit of PI3K in the regulation of IL-27p28 gene expression was confirmed by transfecting THP-1 cells with p85α siRNA. Results suggest that siRNA against p85α subunit significantly inhibited LPS-induced p85 phosphorylation as well as IL-27p28 gene expression (Fig. 3.35.D). Taken together, my results suggest that PI3K signalling pathway positively regulates LPS- and IFNγ/LPS-induced IL-27 gene expression and protein production in both THP-1 cells and primary human monocytes.

Figure 3.35. PI3K pathway regulates LPS-induced IL-27 expression in THP-1 cells.

A) THP-1 cells ($1x10^6$/ml) were treated with LY294002 at concentrations ranging from 0 to 40 μM for 2 hrs before LPS (1μg/ml) stimulation for 30 min. Total proteins (50 μg) were analyzed by Western blot analysis using anti-phospho-Akt (p-Akt) antibodies. To control for equal protein loading, the membrane was stripped and reprobed with anti-Akt antibodies.

B) THP-1 cells ($1x10^6$/ml) were treated with LY294002 at concentrations ranging from 0 to 40 μM for 2 hrs followed by LPS (1μg/ml) stimulation for 4 hrs. Cells were harvested for mRNA isolation. IL-27p28 and IL-27EBI3 gene expression were determined by R.Q. Real-Time PCR analysis.

C) THP-1 cells ($1x10^6$/ml) were treated with LY294002 at concentrations ranging from 0 to 40 μM for 2 hrs followed by LPS (1μg/ml) stimulation for 24 hrs. Supernatants were collected for IL-27 protein measurement by ELISA.

D)
Upper panel: THP-1 cells were transfected with the either control siRNA or p85 PI3K siRNA for 24 hrs followed by stimulation with LPS (1μg/ml) for 30 min for the determination of phosphorylated p85-PI3K by Western blot analysis.
Lower panel: THP-1 cells were transfected with the either control siRNA or p85 PI3K siRNA for 24 h followed by stimulation with LPS (1μg/ml) for 8 hrs for IL-27p28 gene expression assay by R.Q. Real-Time PCR. The histogram is a mean of 2 independent experiments.

Error bars represent the SEM of 3 independent experiments.
ANOVA used to test significant differences.

A.

Media

LPS + LY294002 (μM)

0 2 10 20 40

P-Akt

Akt

B.

IL-27p28 (mRNA)

100 80 60 40 20 0

Media LPS 2 10 20 40

LPS + LY294002 (μM)

IL-27EBI3 (mRNA)

Media LPS 2 10 20 40

LPS + LY294002 (μM)

C.

IL-27 (pg/ml)

1200 1000 800 600 400 200 0

Media LPS 2 10 20 40

LPS + LY294002 (μM)

D.

Ctrl. siRNA p85 siRNA

Media LPS Media LPS

pp85-PI3K

p85-PI3K

p110α-PI3K

IL-27p28 (mRNA fold increase)

350 300 250 200 150 100 50 0

LPS + Ctrl. siRNA

LPS + p85 SiRNA

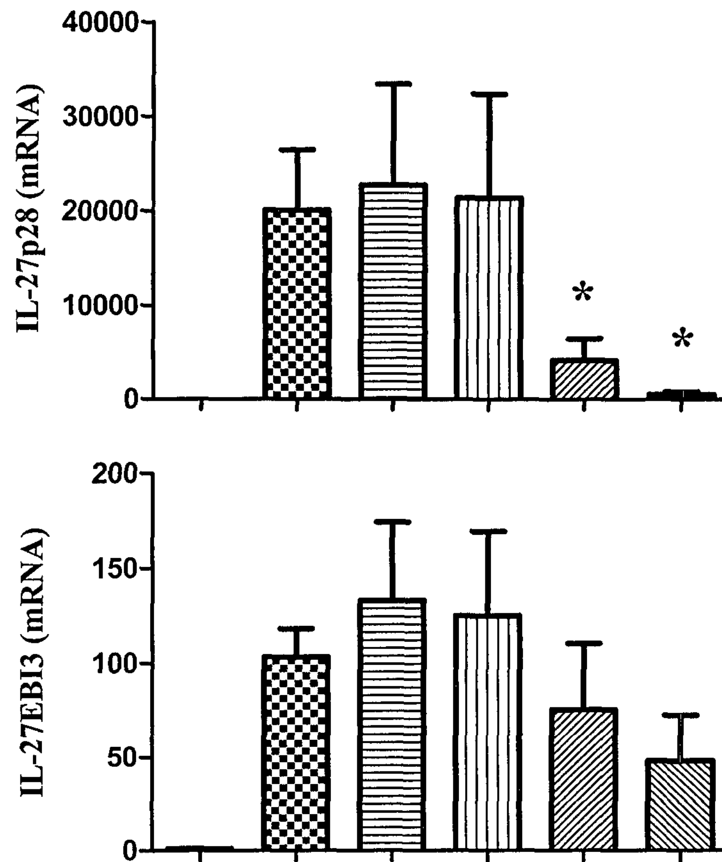
Figure 3.35

Figure 3.36. PI3K signalling pathway regulates IFNγ/LPS-induced IL-27 expression in THP-1 cells.

A) THP-1 cells ($1x10^6$/ml) were treated with LY294002 at concentrations ranging from 0 to 25 μM for 2 hrs followed by stimulation for 16 hrs with IFNγ (10 ng/ml) and another 4 hrs with LPS (1μg/ml). Cells were harvested for mRNA isolation. IL-27p28 and IL-27EBI3 gene expression were determined by R.Q. Real-Time PCR analysis.

B) THP-1 cells ($1x10^6$/ml) were treated with LY294002 at concentrations ranging from 0 to 25 μM for 2 hrs followed by stimulation for 16 hrs with IFNγ (10 ng/ml) and another 24 hrs with LPS (1μg/ml). Supernatants were collected for IL-27 protein measurement by ELISA.

Error bars represent the SEM of 3 independent experiments.
ANOVA used to test significant differences.



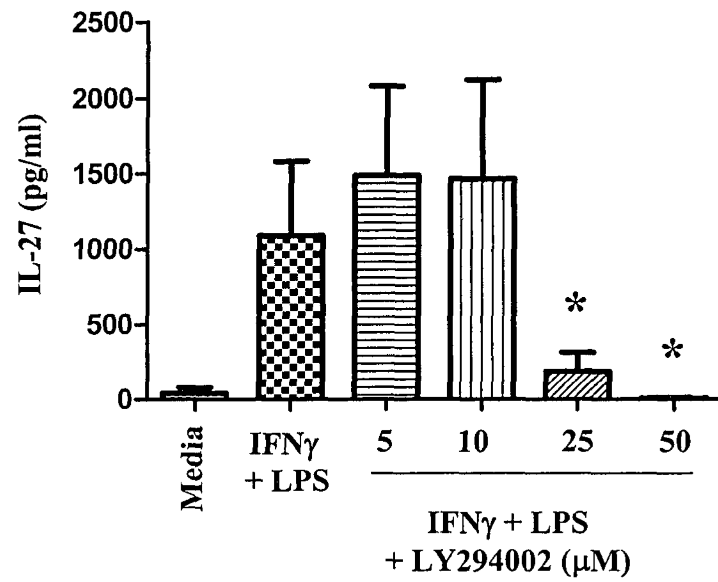


Figure 3.36

Cross-talk between PI3K and p38 MAPK pathways involved in LPS-induced IL-27 production in THP-1 cells

The proper functioning of the PI3K pathway like other signalling pathways depends on its constitutive and extensive communication with other signalling molecules, leading to synergistic or antagonistic effects and eventually biological outcomes. The nature of such signalling cross-talk is a complex situation and highly context-dependent. Since my results suggest that LPS- and IFNγ/LPS-induced IL-27 production in THP-1 cells is regulated by two major p38 and PI3K signalling pathways, I investigated whether IL-27 production is regulated by two distinct p38 and PI3K pathways or by a single pathway due to the cross-talk between these two pathways. This was investigated by determining if inhibition of p38 MAPK affects the activation of PI3K pathway and vice versa. The results show that inhibition of the p38 MAPK signalling pathway inhibited the phosphorylation of Akt following LPS stimulation (Fig. 3.37). However, in our lab the effect of inhibition of PI3K on p38 has been examined by Maria and she did not observe any effect on p38 phosphorylation. These results suggest that IL-27 production induced by LPS is governed by the p38 MAPK activated PI3K signalling pathway.

LPS- and IFNγ/LPS-induced IL-27 expression is not regulated by calcium signalling pathway

The changes in intracellular calcium ion concentration have a critical role in transcription, protein synthesis and survival of the cells. Calcium also acts as the upstream signalling mediator of the major signalling pathways such as MAPKs and PKC

Figure 3.37. Cross-talk between p38 MAPK and PI3K signalling pathways in LPS-induced Akt phosphorylation.

THP-1 cells ($1x10^6$/ml) were treated with either LY294002 or SB202190 at concentrations ranging from 0 to 50 μM for 2 hrs before LPS (1μg/ml) stimulation for 30 min. Total proteins (50 μg) were analyzed by Western blot analysis using anti-phospho-Akt (p-Akt) antibodies. To control for equal protein loading, the membranes were stripped and reprobed with anti-Akt antibodies.

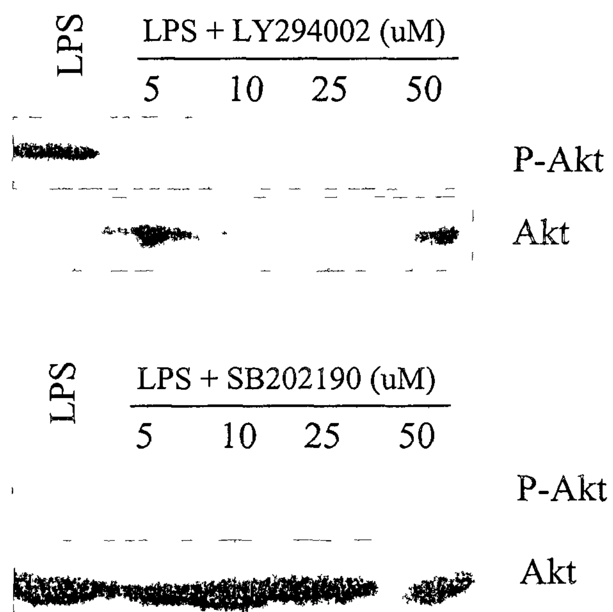


Figure 3.37

[273]. To study the role of calcium signalling, calcium signalling inhibitors, EGTA and SKF96365, were employed. THP-1 cells were pre-treated with EGTA (0-10 mM) or SKF96365 (0-50 μM) for 2 hrs followed by IFNγ for 16 hrs and LPS for 4 or 24 hrs followed by analysis of LPS- or IFNγ/LPS-induced IL-27 gene expression or protein production. The results suggest that calcium signalling inhibitors EGTA and SKF were not able to inhibit LPS- (Fig. 3.38.A&B) or IFNγ and LPS-induced IL-27p28 or IL-27EBI3 genes expression and protein production (Fig. 3.39.A&B). These results suggest that calcium signalling may not positively regulate IL-27 expression in LPS-stimulated THP-1 cells.

Figure 3.38. Calcium signalling pathway does not regulate LPS-induced IL-27 expression in THP-1 cells.

A) THP-1 cells ($1x10^6$/ml) were treated with EGTA, or SKF96365 at the optimized concentrations for 2 hrs followed by LPS (1μg/ml) stimulation for 4 hrs. Cells were harvested for mRNA isolation. IL-27p28 and IL-27EBI3 gene expression were determined by R.Q. Real-Time PCR analysis.

B) THP-1 cells ($1x10^6$/ml) were treated with EGTA, or SKF96365 at the optimized concentrations for 2 hrs followed by LPS (1μg/ml) stimulation for 24 hrs. Supernatants were collected for IL-27 protein measurement by ELISA.

Error bars represent the SEM of 3 independent experiments.
ANOVA used to test significant differences.

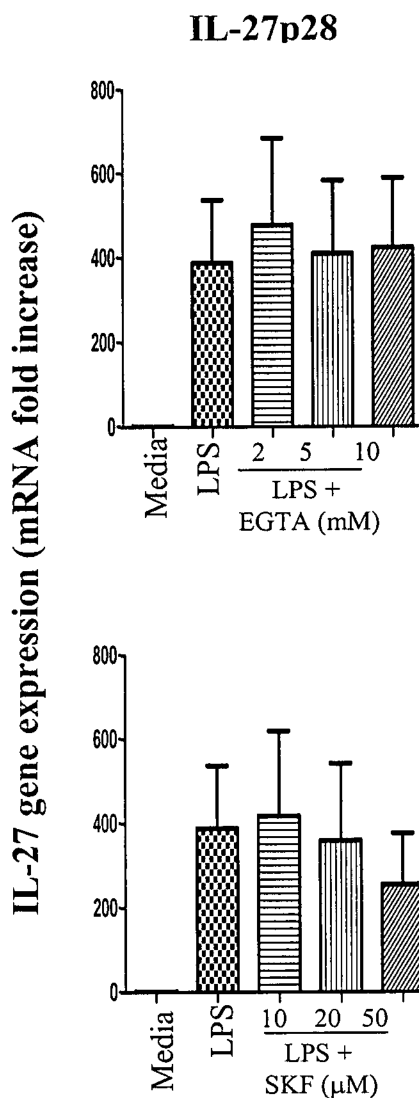
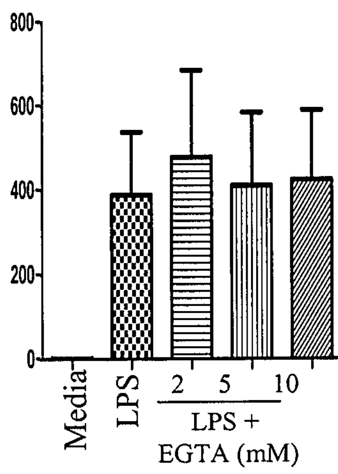
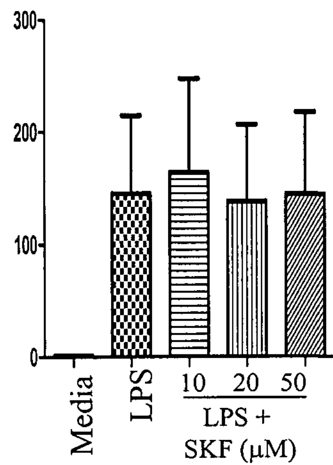
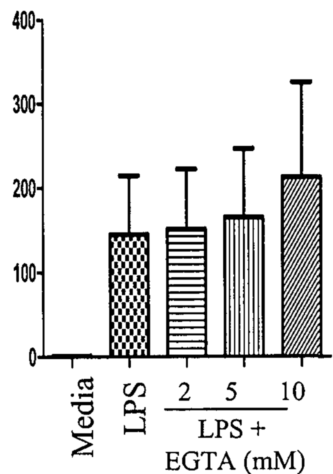
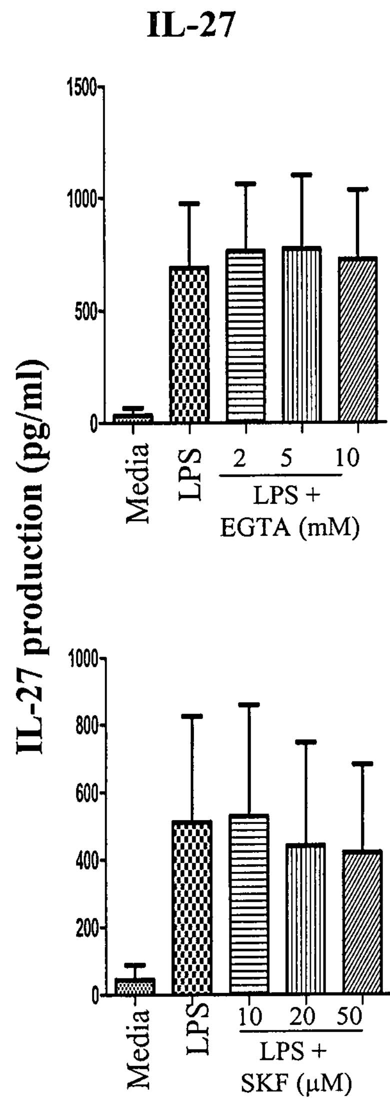
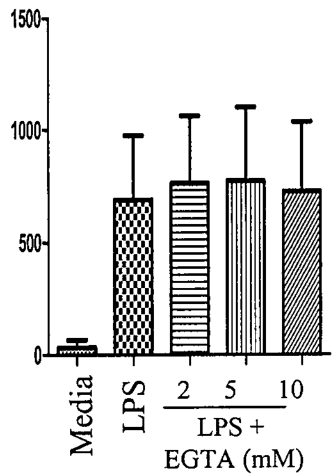







Figure 3.38

Figure 3.39. Role of calcium signalling pathway in the regulation of IFNγ/LPS-induced IL-27 in THP-1 cells.

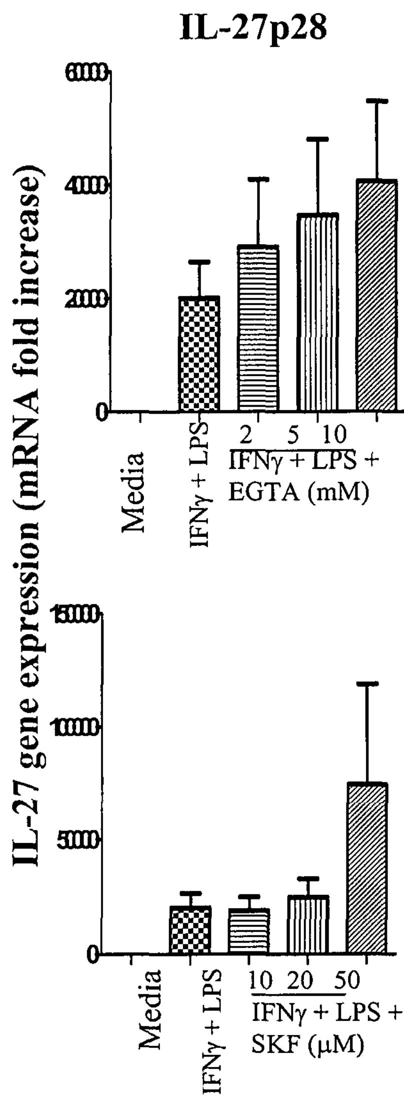
A) THP-1 cells ($1x10^6$/ml) were treated with EGTA **(upper panel)** at concentrations ranging from 2 to 10 mM or SKF96365 from 10-50 μM **(lower panel)** for 2 hrs followed by stimulation for 16 hrs with IFNγ (10 ng/ml) and for another 4 hrs with LPS (1μg/ml). Cells were harvested for mRNA isolation. IL-27p28 and IL-27EBI3 gene expression were determined by R.Q. Real-Time PCR analysis.

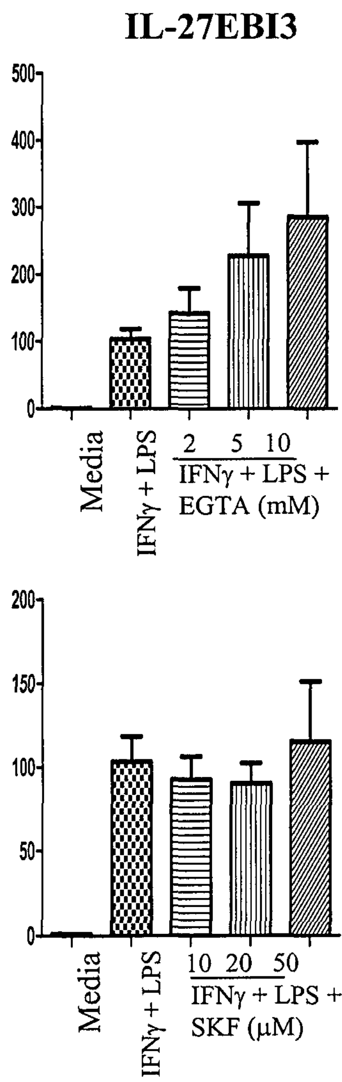
B) THP-1 cells ($1x10^6$/ml) were treated with EGTA at concentrations ranging from 2 to 10 mM for 2 hrs **(upper panel)** or SKF96365 from 10-50 μM **(lower panel)** followed by stimulation for 16 hrs with IFNγ (10 ng/ml) and for another 24 hrs with LPS (1μg/ml). Supernatants were collected for IL-27 protein measurement by ELISA

Error bars represent the SEM of 3 or 4 independent experiments. ANOVA used to test significant differences.

A.

B.





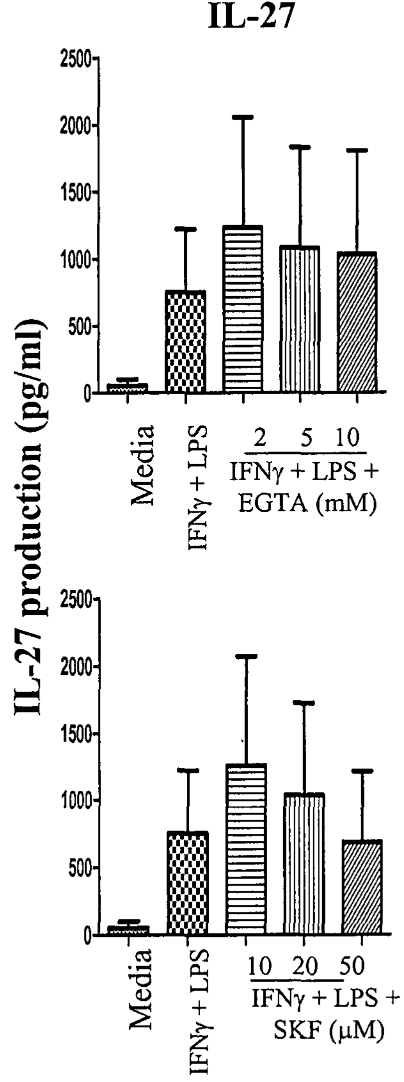


Figure 3.39

PART III

LPS-induced IL-12 family cytokines expression in HIV-infected THP-1 cells

INTRODUCTION:

Defects in cell-mediated immune responses (CMIR) play a key role in the pathogenesis of HIV infection. The failure of CMIR function facilitates the escape of virus from immune control and the collapse of the immune system [277]. Immune system dysfunction results in virus persistence, high viral load and disease progression. In the last few years, HIV-associated defects in CMIR including impaired $CD4^{+}$ T-cells and APCs functions have been investigated [278]. APCs such as monocyes/macrophages and dendritic cells play a crucial role in innate and adaptive immune responses and antigen presentation. APCs control the function of T and B cells by producing a variety of chemokines and cytokines such as IL-1, IL-8, IL-12, MCP-1, and TNF-α [279]. Monocytic cells, a key source of IL-12 family of Th1 cytokines, serve as a major reservoirs of HIV-1 infection and are functionally impaired in HIV-1 infected individuals [9-13, 118, 280-286].

Infection with HIV-1 results in the modulation of circulating levels of many host immune proteins including cytokines and chemokines that have the potential to influence virus replication [287]. HIV-1 employs a variety of mechanisms to undermine the effectiveness of the host immune system including dysregulation of Th1 cytokines, such as IL-12 [287, 288]. The loss of Th1 cytokines and eventual CMIR in diseases such as HIV/AIDS leads to increased susceptibility to opportunistic infections and their consequences. Recently, two heterodimeric cytokines, IL-23 and IL-27 that are structurally related to IL-12 and exhibit biological functions similar to IL-12 with respect to the Th1 differentiation have been described {Hunter, 2005 1578 /id;Langrish, 2004

1580 /id;Novelli, 2004 1581 /id}. It is well established that LPS-induced IL-12 production is down-regulated in HIV-infected individuals and following *in vitro* HIV infection of monocytic cells [15, 20, 21, 289]. HIV-1 infection of monocytic cells or their exposure to HIV-1 regulatory proteins may also alter the production of other members of IL-12 family of Th1 cytokines. The effect of HIV-1 infection on IL-23 and IL-27 production in human monocytic cells remains unknown. Therefore, ***I hypothesized*** that the expression of IL-23 and IL-27 is altered following *in vitro* HIV infection. This alteration in the expression of IL-23 and IL-27 may significantly contribute to the loss of CMIR, and subsequent HIV disease progression. Therefore, ***the main objective of this study was*** to evaluate the effects of HIV infection on spontaneous and LPS-induced IL-23 and IL-27 production in human monocytic cells with a long term objective to understand the molecular mechanisms responsible for modulation of these cytokines. Therefore, as a first step, in previous chapters, I characterized the molecular mechanisms involved in the regulation of LPS- and IFNγ/LPS-induced IL-23 and IL-27 expression in monocytic cells and THP-1 cells as model system. In this section, *I have investigated the impact of in vitro* $HIV\text{-}1_{CS204}$ *infection on spontaneous and LPS-induced IL-23 and IL-27 production in THP-1 cells.*

RESULTS:

HIV-1$_{CS204}$ properly replicates in THP-1 cells

First of all, I investigated the ability of the virus to infect and replicate in THP-1 cells. To determine whether the dual tropic clinical HIV-1 strain (HIV-1$_{CS204}$) replicates in THP-1 cells, the cells were infected with different doses of the virus for 2 hrs followed by washing off the infected cells with fresh media to remove unbound viruses. The cells were resuspended and cultured for different time periods. The cells and supernatants were collected for Real-Time PCR and protein production assay at day 2 and 4 post-infection. It is notable that all processes of cell infection and cell culture were done in presence of polybrene (4 μg/ml final concentrations) in order to have more efficient infectivity. The level of the virus replication was determined by measuring the level of HIV-1 p24gag proteins in the cell culture supernatants. The results show that HIV-1$_{CS204}$ can infect and replicate in THP-1 cells in a time- and dose-dependent manner (Fig. 3.40).

In vitro HIV-1$_{CS204}$ infection induces transient IL-23 expression in THP-1 cells

Next, I investigated whether *in vitro* HIV infection impacts the expression of IL-23 in unstimulated THP-1 cells. IL-23 genes expression was determined in mock uninfected and HIV-1$_{CS204}$-infected THP-1 cells by relative quantitative Real-Time PCR analysis. HIV infection of THP-1 cells significantly induced expression of IL-23p19 gene transcripts at day 2 post-infection. This increase in IL-23p19 expression directly correlated with the amount of virus used for infection of cells. IL-23p19 upregulation was

Figure 3.40. HIV-1CS204 properly replicates in THP-1 cells

THP-1 cells were infected with varying doses of HIV-1_{CS204}, a dual tropic HIV-1 strain; corresponding to 5-20 ng/ml of $p24_{gag}$ proteins.HIV-1_{CS204}-infected cells were cultured for four days. At day 2 post-infection supernatants were collected. The cells were washed off and cultured in the fresh media for 2 more days. HIV-1 replication was measured by a HIV-1p24 antigen capture assay kit.

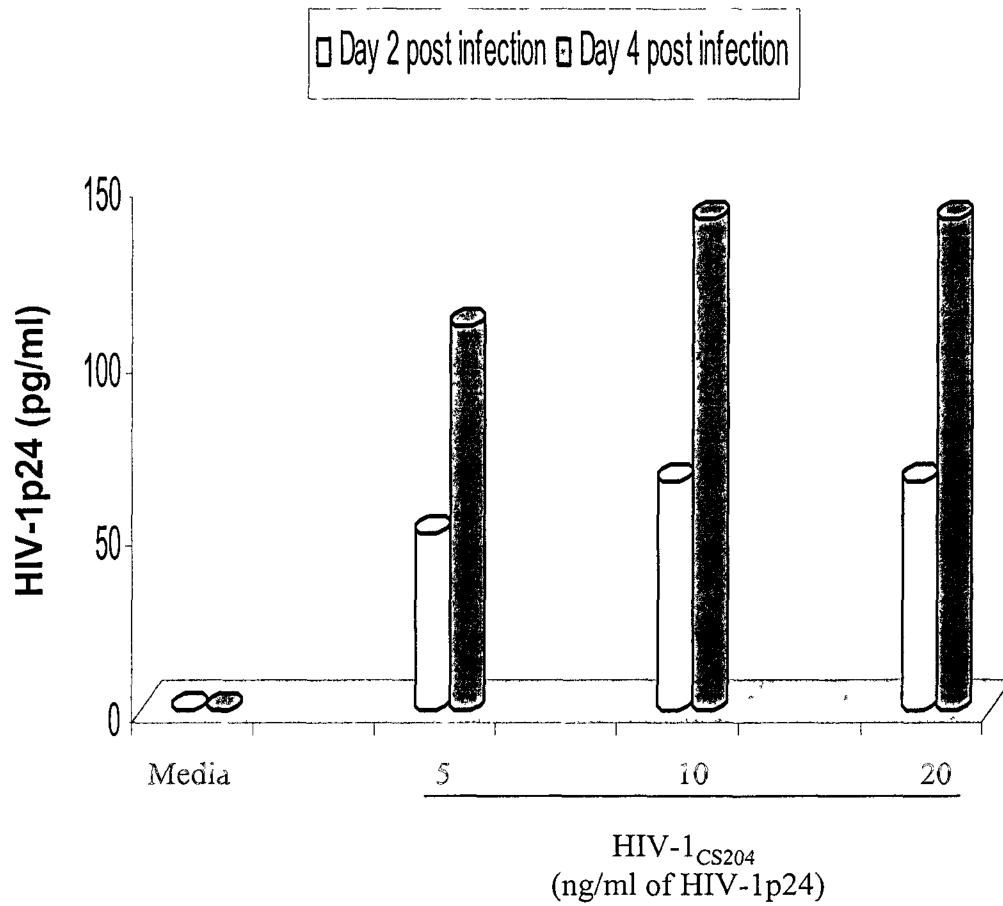


Figure 3.40

transient because the levels of IL-23p19 gene expression significantly decreased by day 4 post-infection (Fig. 3.41.A upper panel). Similarly, infection with HIV alone induced IL-12/23-p40 expression by day 2 post-infection that also correlated with the amount of virus used for infection of cells. However, by day 4, HIV infection caused a decrease in the expression of IL-12/23-p40 mRNA albeit not at levels observed with IL-23p19 subunit (Fig. 3.41.A lower panel).

Subsequently, I investigated whether IL-23 protein can be produced in HIV-infected cells. To this end, the culture supernatants of HIV-infected THP-1 cells were harvested at various time points (day 3 and day 5 post-infection) and IL-23 levels were measured by ELISA. Fig. 3.41.B upper panel shows that the level of IL-23 proteins at day 3 post-infection was significantly increased in a dose-dependent manner. This increase in IL-23 production could be observed even by day 5 post-infection particularly when cells were infected with 5 and 10 ng/ml of p24 (Fig. 3.41.B). However, in cells infected with high viral dose of 20 ng/ml of p24, IL-23 production was significantly decreased compared to cells infected with 5 and 10 ng/ml of p24 correlated with the amount of virus used for infection of cells. Overall, these results suggest that HIV-1_{CS204} infection in THP-1 cells significantly induces upregulation of IL-23 at both mRNA and protein levels.

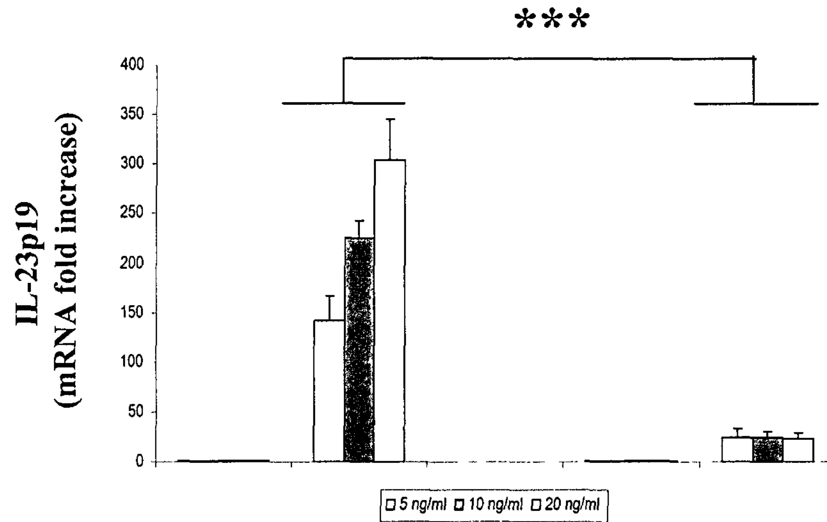
Figure 3.41. HIV-induces IL-23 gene expression and protein production in THP-1 cells

A) THP-1 cells ($1x10^6$/ml) were infected with various doses of the virus corresponding to 5-20 ng/ml of p24 antigen. Cell pellets were collected at day 2 and 4 post-infection followed by analysis for IL-23p19 and IL-12/23p40 mRNA expression by R.Q. Real-Time PCR. Error bars represent the SEM of 3 independent experiments.

B) THP-1 cells ($1x10^6$/ml) were infected with various doses of the virus corresponding to 5-20 ng/ml of p24 antigen. At days 3 and 5 post-infection, supernatants were collected for IL-23 protein measurement. IL-23 protein production was determined by a commercially available ELISA kit. Error bars represent the SEM of 4 independent experiments.

ANOVA test was used to show the significant differences.

A.



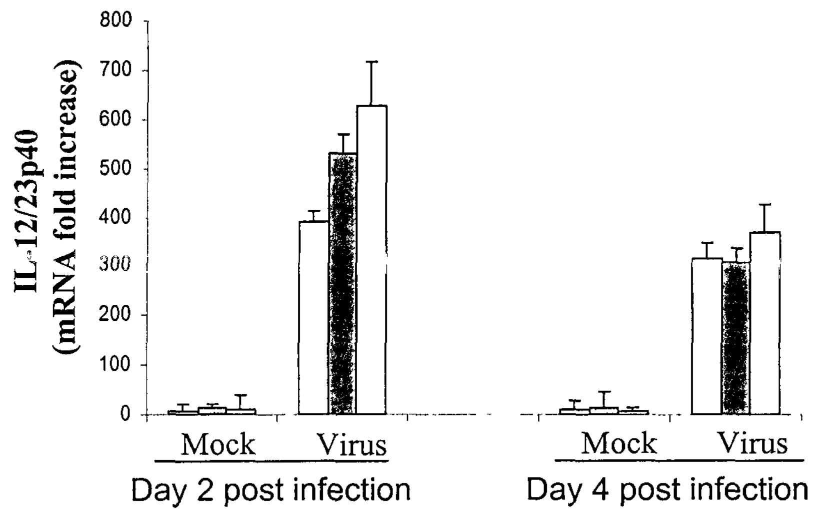


Figure 3.41.A

B.

Figure 3.41.B

In vitro HIV infection inhibits LPS-induced IL-23 production in THP-1 cells

In general, monocyte activation in response to bacterial endotoxin or LPS induces pro-inflammatory (IL-1, TNF-α, IL-12, etc.) and anti-inflammatory (IL-10, soluble TNF-R, IL-1Rα) cytokines [51-53]. As shown earlier in this chapter, LPS can induce the expression of IL-23. To determine if HIV-1_{CS204} infection affects LPS-induced IL-23 expression, THP-1 cells were infected with different doses of the virus ranging from 5-20 ng/ml of HIV-1p24 and for different time intervals ranging from 3-5 days followed by stimulation with LPS for another 24 and 48 hrs. IL-23 production in HIV infected cells was remarkably reduced by approximately 60% on both day 3 (Fig. 3.42.A) as well as day 5 (Fig. 3.42.B) post-infection and at 24 hrs (upper panel) and 48 hrs (lower panel) of stimulation with LPS as compared with LPS-stimulated and mock-infected THP-1 cells. Similar results were obtained on LPS-induced IL-23p19 gene expression in human peripheral blood mononuclear cells (PBMCs) following HIV infection. PBMCs were infected with 10 ng/ml of virus for 5 days. At day 2 and 5 post-infection, PBMCs were stimulated with 1 μg/ ml of LPS for 4 hrs followed by analysis of gene expression. HIV infection significantly reduced LPS-induced IL-23p19 gene expression on day 5 post-infection (Fig. 3.43). These results suggest that HIV infection reduces LPS-induced IL-23 production in PBMCs as well as THP-1 cells.

Figure 3.42. HIV-1CS204 infection inhibits LPS-induced IL-23 expression

THP-1 cells ($1x10^6$/ml) were infected with various doses of HIV-1_{CS204} corresponding to 5-20 ng/ml of p23 antigens. At day 3 **(A)** and day 5 **(B)** post-infection, cells were stimulated with LPS (1μg/ml) for 24 **(upper panel)** and 48 hrs **(lower panel)** followed by supernatants collection and IL-23 protein measurement by ELISA.

Error bars represent the SEM of 4 independent experiments.
The Student's t-test was used to analyse data for significant differences.

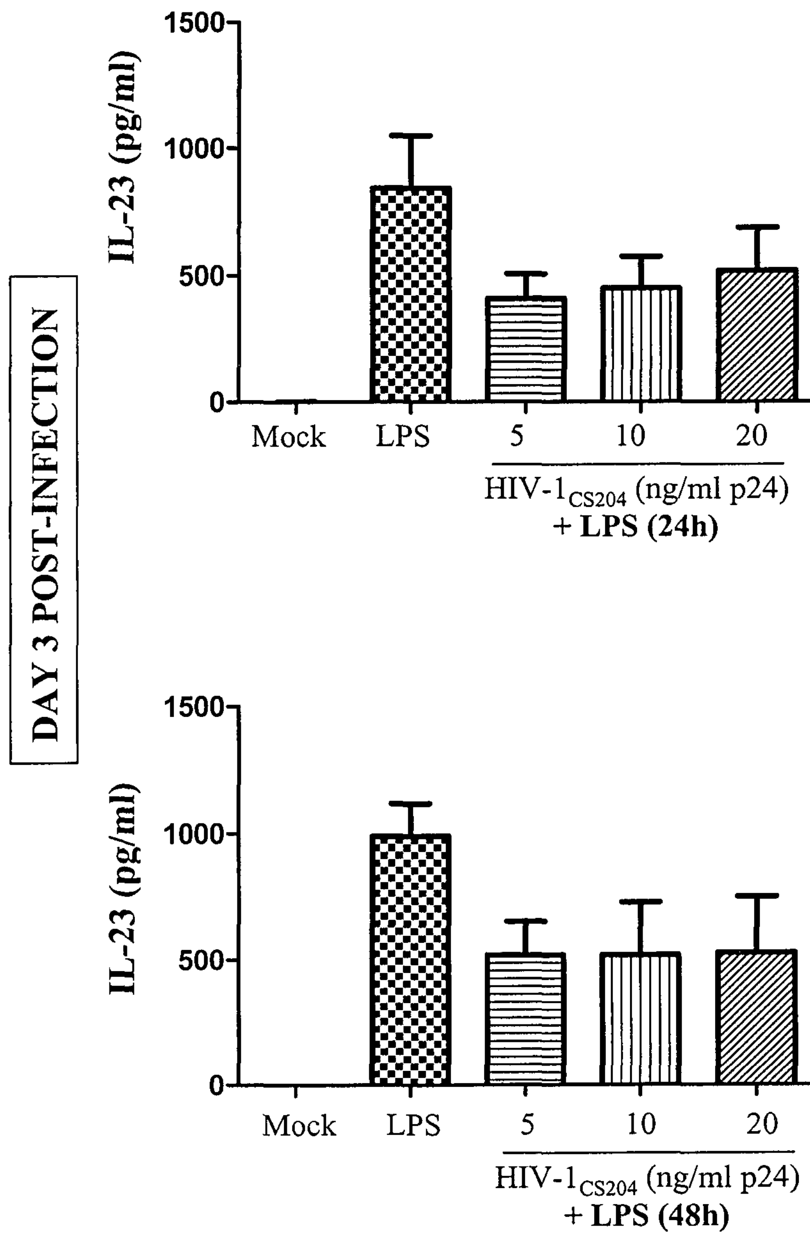


Figure 3.42.A

B.

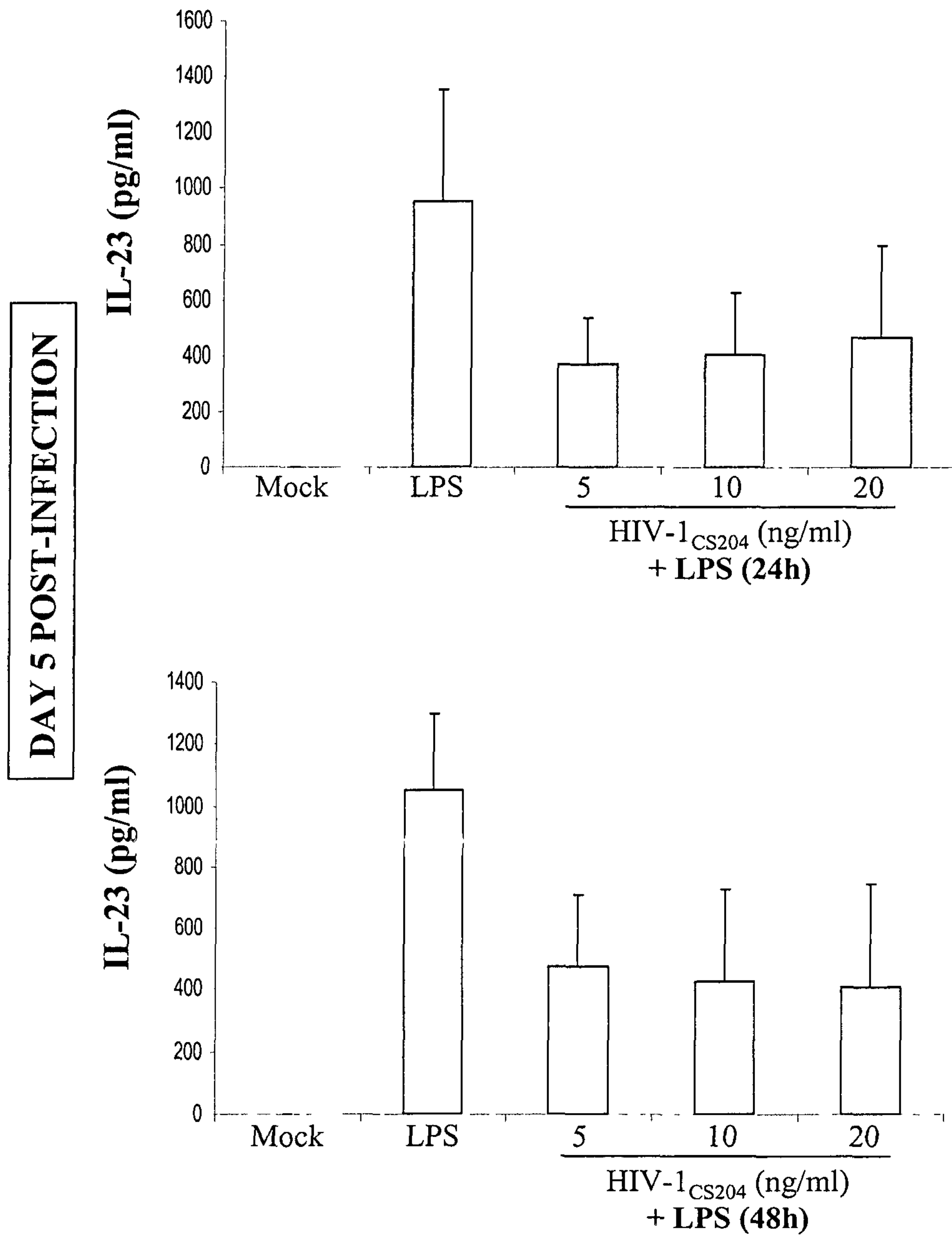


Figure 3.42.B

Figure 3.43. HIV-1CS204 infection inhibits LPS-induced IL-23p19 gene expression in PBMCs

PBMCs ($1x10^6$/ml) were infected with HIV-1_{CS204} virus (10 ng/ml). Cells were treated with LPS (1μg/ml) at day 0, 2, and 5 post-infection for 4 hrs followed by analysis for IL-23p19 mRNA expression by relative quantitative Real-Time PCR.
This experiment has been done just once.

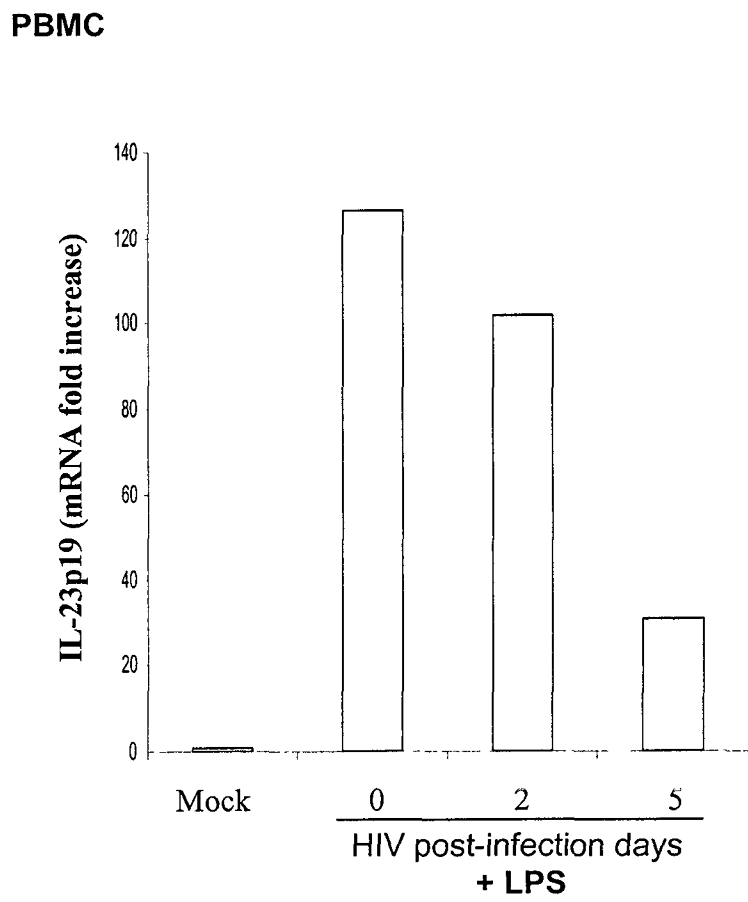


Figure 3.43

In vitro HIV-1$_{CS204}$ infection does not induce IL-27 proteins in THP-1 cells

A similar protocol as used for determining the effect of HIV-1 infection on IL-23 expression was used to investigate the effect of HIV-1 infection on IL-27 expression in THP-1 cells. THP-1 cells constitutively expressed low levels of the IL-27p28 subunit only. HIV infection at day 2 and 4 post-infection enhanced the levels of IL-27p28 expression but this increase was not significant (Fig 3.44.A upper panel). In contrast, HIV infection of THP-1 cells significantly upregulated the expression of IL-27EBI3 gene transcripts on days 2 and 4 post-infection, as determined by R.Q. Real-Time PCR (Fig. 3.44.A lower panel). Next, I investigated whether IL-27 protein can be produced in HIV-infected THP-1 cells. For this, IL-27 proteins were measured by ELISA in the culture supernatants of HIV-infected THP-1 cells harvested on days 3 and 5 post-infection. The level of IL-27 proteins in HIV-infected cells was not found to be different when compared with mock-infected cells even when cells were infected with high viral concentrations of 20 ng/ml of HIV-1p24 (Fig. 3.44.B). These results suggest that HIV-1$_{CS204}$ differentially regulates IL-23 and even IL-27 subunit expression in THP-1 cells.

HIV infection does not affect LPS-induced IL-27 expression

To investigate whether HIV-1$_{CS204}$ infection affects LPS-induced IL-27 expression, THP-1 cells were infected with different doses of the virus ranging from 5-20 ng/ml of HIV-1p24 for 3 and 5 days. The cells were subsequently stimulated with LPS for another 24 and 48 hrs. LPS induced the expression of IL-27 proteins on both days 3 and 5 in mock-infected as well as HIV-infected cells. In contrast to the effect of HIV on

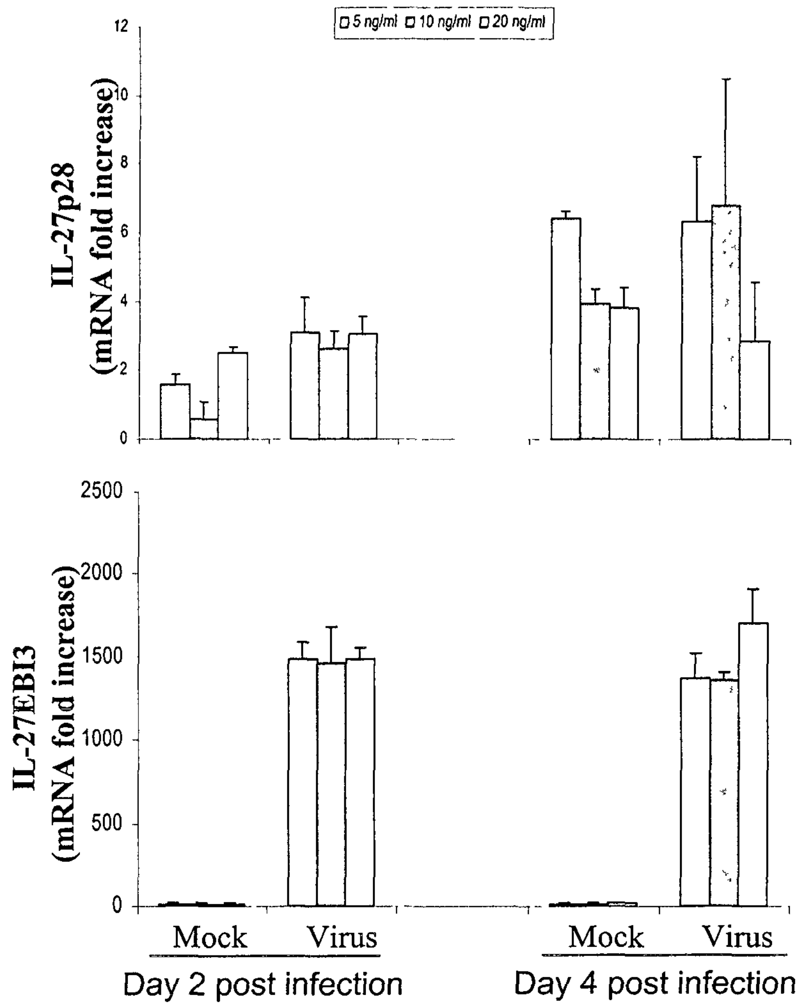
IL-23 expression, HIV infection in THP-1 cells did not affect LPS-induced IL-27 production either on day 3 (Fig 3.45.A) or on day 5 post-infection (Fig. 3.45.C). HIV infection did not affect IL-27 production even when cells were stimulated with LPS for 48 hr (Fig 3.45.B and 3.45.D). Taken together, these results suggest that HIV-infection does not affect LPS-induced IL-27 production, but it has a remarkable inhibitory effect on LPS-induced IL-23 production in THP-1 cells and PBMCs.

Figure 3.44. HIV infection differentially induce IL-27p28, IL-27EBI3, and IL-27 production in THP-1 cells

A) THP-1 cells ($1x10^6$/ml) were infected with various doses of the virus corresponding to 5-20 ng/ml of p24 antigens. Cell pellets were collected at day 2 and 4 post-infection followed by analysis for IL-27p28 and IL-27EBI3 mRNA expression by R.Q. Real-Time PCR. Error bars represent the SEM of 2 independent experiments.

B) THP-1 cells ($1x10^6$/ml) were infected with various doses of the virus corresponding to 5-20 ng/ml of p24 antigens. At 3 and 5 post-infection, supernatants were collected for IL-27 protein measurement. IL-27 protein production was determined by ELISA.
Error bars represent the SEM of 3 independent experiments.

A.



B.

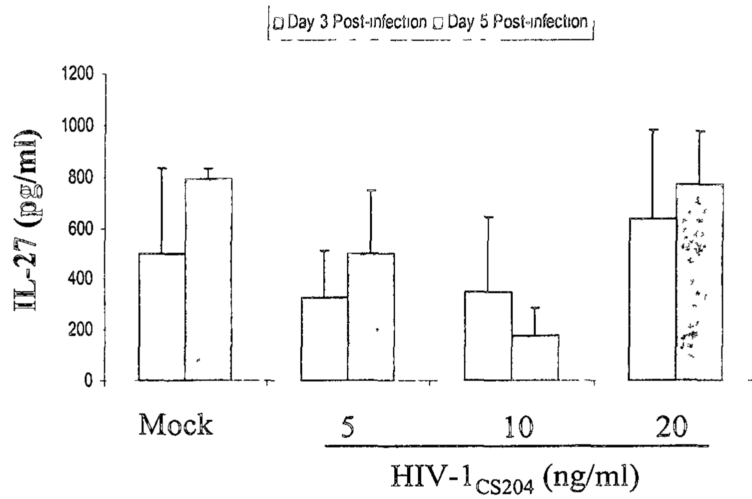


Figure 3.44

Figure 3.45. HIV-1CS204 infection does not inhibit LPS-induced IL-27 production in THP-1 cells

THP-1 cells ($1x10^6$/ml) were infected with various doses of HIV-1_{CS204} virus corresponding to 5-20 ng/ml of p24 antigens. At day 3 post-infection cells were stimulated with LPS (1μg/ml) for 24 hrs **(A)** and 48 hrs **(B)**. Similarly, on day 5 post-infection cells were stimulated with LPS for 24 **(C)** and 48 hrs **(D)**. Subsequently, supernatants were collect and IL-27 proteins were measured by ELISA.
Error bars represent the SEM of 3 independent experiments. .

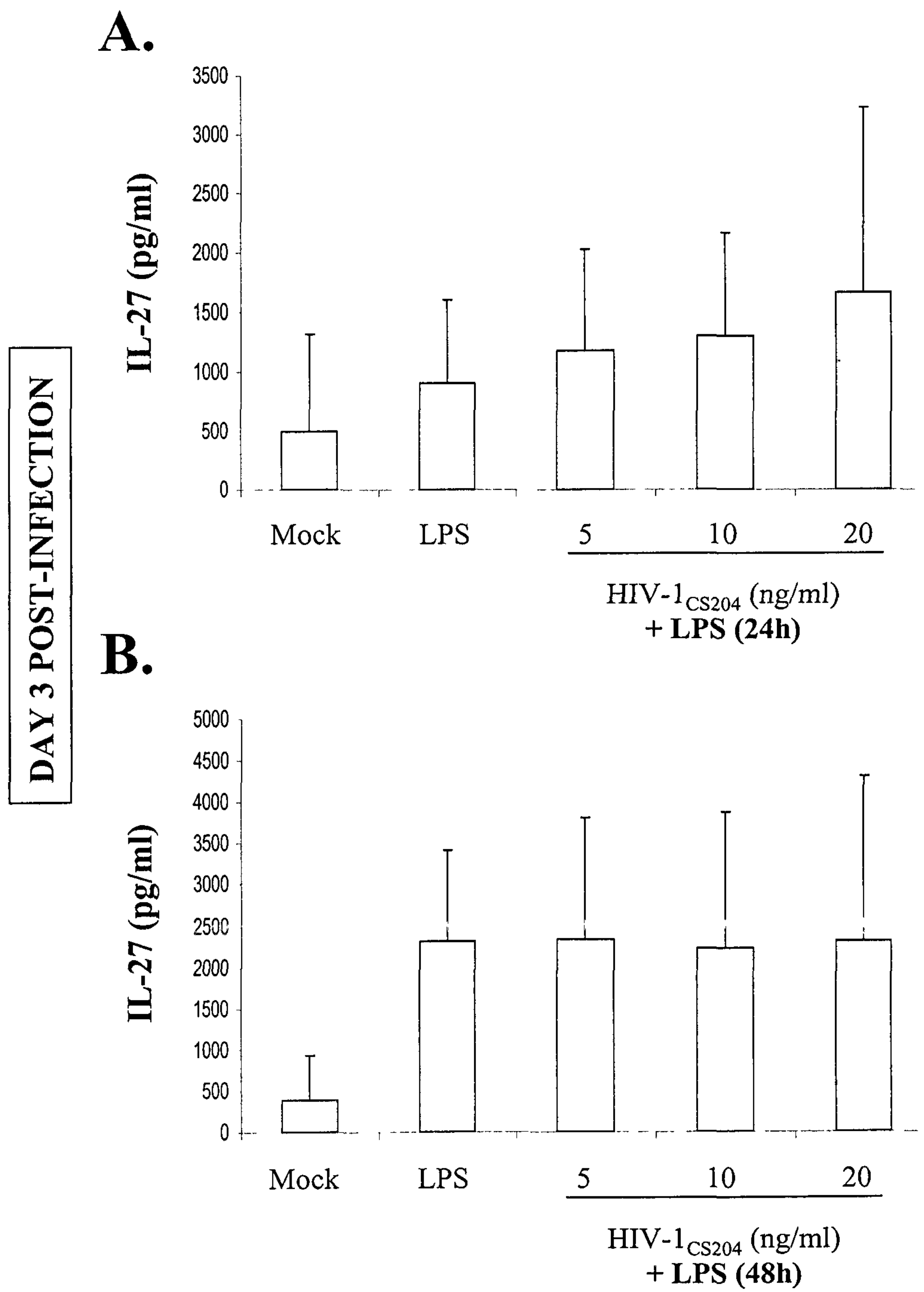
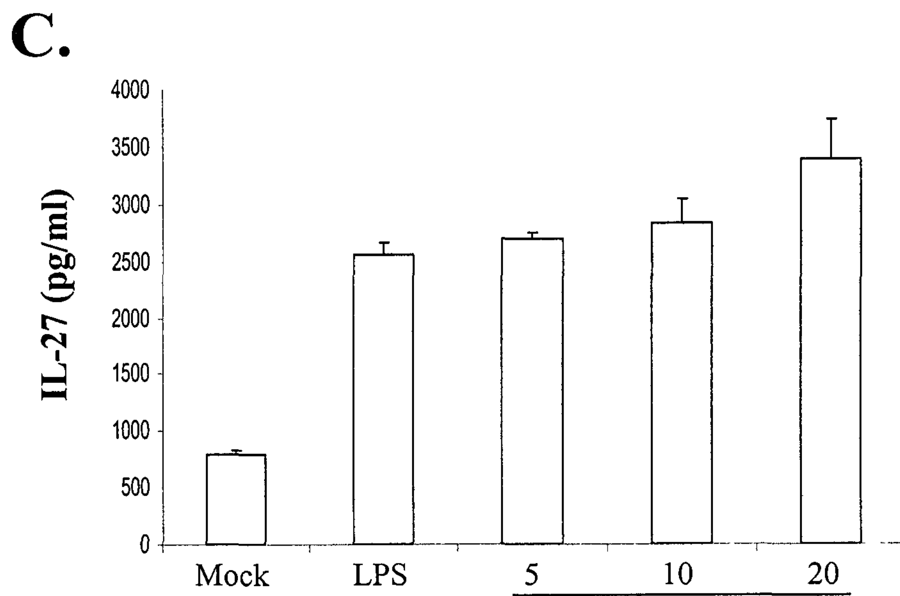


Figure 3.45.A&B

DAY 5 POST-INFECTION



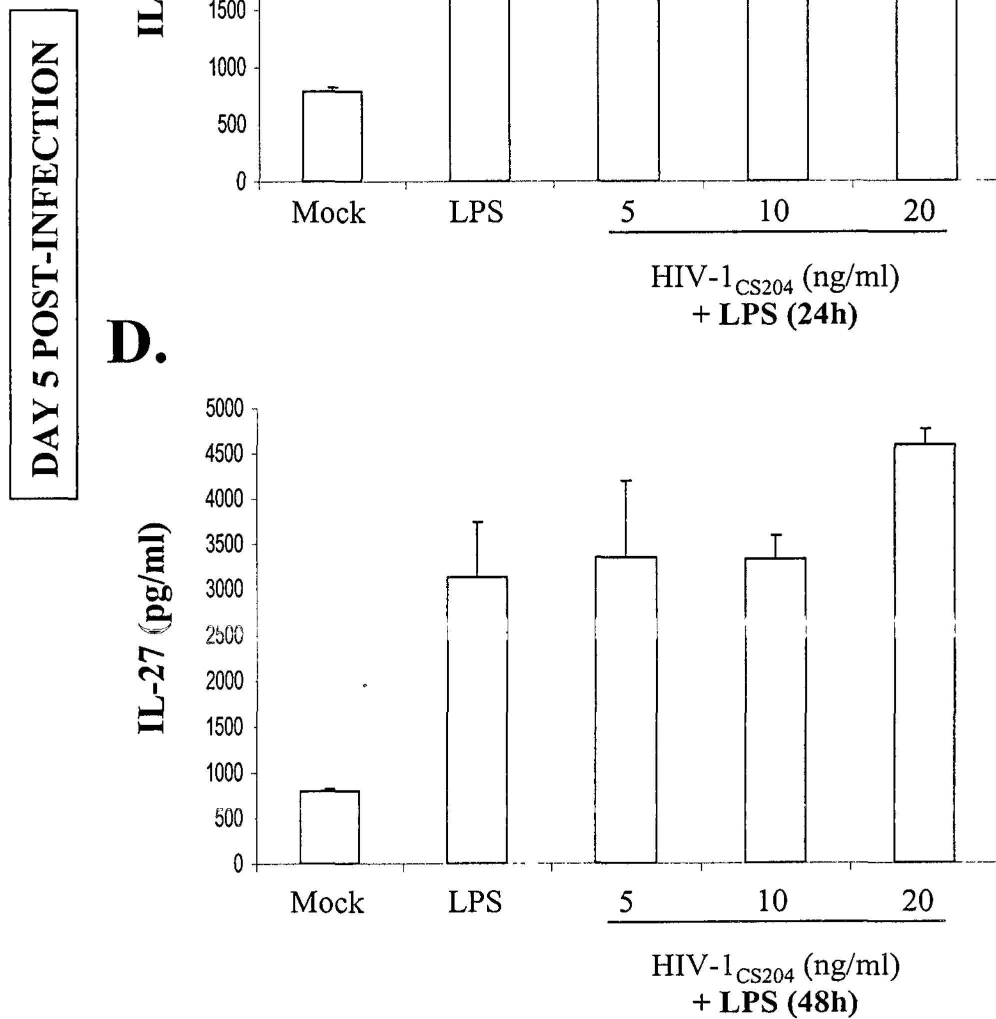


Figure 3.45.C&D

4. CHAPTER IV

DISCUSSION

Monocytes/macrophages are the major targets and main reservoirs of HIV infection. Recent studies have underlined the pivotal role of chemokines, cytokines, and their receptors in HIV pathogenesis [69, 290-294]. Numerous pathogens, including HIV, can stimulate the production of chemokines and cytokines, which in turn can modulate infected as well as bystander cell responses to the virus. This may profoundly influence the extent of viral replication, dissemination, and pathogenesis.

IL-12 family of Th1 cytokines are critical cytokines involved in the development of Th1 responses and are mainly produced by monocytes/macrophages and dendritic cells in response to various pathogenic stimuli including LPS. It is well established that IL-12 production is decreased in HIV-infected individuals and following *in vitro* HIV infection of monocytic cells [15, 20, 21, 289]. However, whether the expression of IL-23 and IL-27 following HIV infection is altered in monocytic cells remains unknown.

Therefore, to understand whether HIV infection alters the expression of IL-23 and IL-27 in monocytic cells, I first demonstrated that LPS is able to induce IL-23 and IL-27 production in THP-1 cells. I have also elucidated the necessity of priming the human primary monocytes with IFNγ in order to produce IL-23 and IL-27 following LPS stimulation.

The main *objective of this study was to* evaluate the effects of HIV infection on spontaneous and LPS-induced IL-23 and IL-27 production in human monocytic cells and to understand the molecular mechanisms responsible for modulation of these cytokines. Therefore, as a first step, I characterized the molecular mechanisms involved in the regulation of LPS- and IFNγ/LPS-induced IL-23 and IL-27 expression in monocytic cells and THP-1 cells as model system. The molecular mechanisms by which LPS induces

IL-12 production has been investigated. However, very little is known regarding the molecular mechanisms involved in the regulation of IL-23, IL-27 and their subunits by human Monocytic cells. We and others have demonstrated that IL-12 production in different model systems is regulated by MAPKs, PI3K and the calcium signalling [25, 33, 246, 268, 295, 296]. Most studies identify the MAPK signalling cascade as a positive regulator of IL-12 production (Kim L, et al. 2005; Ma W, et al. 2004; Ma W, et al. 2008) However, some reports suggest quite the opposite, and indicate a less important role for the MAPKs in controlling IL-12 expression (Dobreva ZG, et al. 2008; Utsugi M, et al. 2003). Therefore, based on these observations, I investigated the potential role of these signalling pathways that may regulate LPS- and/or IFNγ-induced IL-23 and/or IL-27 expression in human monocytic cells.

PART I

LPS- and IFNγ/LPS-induced IL-23 regulation in human primary monocytes:

To examine the signalling pathways involved in LPS- and IFNγ/LPS-induced IL-23 gene expression and protein production in human monocytic cells, I have shown that LPS significantly induces IL-23p19 and IL-12/23p40 gene expressions in addition to IL-23 protein production in THP-1 cells but not in human primary monocytes. LPS alone fails to induce the expression of IL-23p19 mRNA as well as IL-23 protein production in primary monocytes. The ability of LPS to stimulate IL-23p19 mRNA or IL-23 protein production in my experimental system contrasts with some previous reports, as LPS

alone has been reported to induce IL-23 expression in DCs [297], human monocytes [265, 298], and PBMCs [265, 299]. However, the level of stimulation of LPS-induced IL-23 protein production in human monocytes in these studies, when reported, has been generally very low relative to the amount of IL-23 protein reported in DCs stimulated with LPS or IFNγ/LPS (Barkman C, et al, 2008; Roses RE, et al, 2008). Moreover, primary monocytic cells are prone to activation and secrete varying basal levels of cytokines depending upon the method of cell isolation and purification. As such, it appears that primary monocytes may not be the significant producers of IL-23 protein in response to LPS.

In vivo, IFNγ stimulation of monocytes and antigen presenting cells is important because it enhances the ability of these cells to produce IL-12 and likely IL-23 [74, 230-232]. IFNγ-mediated increase in Th1 cytokine production, such as the IL-12 family of cytokines, provides a vital positive feedback mechanism for an enhanced immune response against invading pathogens [300]. My results show that IFNγ alone induces IL-23 production in primary human monocytes and enhances LPS-induced expression of IL-23 at both the mRNA (Fig. 3.1.A) and protein levels (Fig. 3.1.B). It is worth mentioning that there is a significant level of individual variation in the basal levels of IL-23 production which impacts the level of IL-23 induction following IFNγ and IFNγ/LPS stimulation. My results are consistent with reports from other laboratories that IL-23 expression is significantly enhanced following priming with IFNγ in monocytes, DCs, macrophages and microglia cells [36, 220, 236, 297, 299, 301-303].

Since IL-23p19 protein secretion is not detectable in cell culture supernatants [119, 129]. I measured the level of IL-12/23p40 and IL-23 proteins in the culture

supernatants by ELISA. Interestingly, IL-12/23p40 protein concentration levels in LPS-stimulated THP-1 cells are normally 10-20 times more than the IL-23 protein concentrations, but the trend of their histogram are very similar to each other (Fig. 3.15.C, right panels). In particular, similarities between the histogram patterns of IL-23 and IL-12/23p40 proteins in the presence of JNK MAPKs inhibitor further suggest the possibility of direct relation between the extracellular IL-23 protein production and the level of IL-12/23p40 protein concentration in the culture supernatants (Fig. 3.18.B). Furthermore, it is notable that IL-23p19 gene expression upregulation does not necessarily result in IL-23 protein production. It is possible that there are post transcriptional regulatory mechanisms that control the level of IL-23 protein production in stimulated monocytic cells.

A number of signalling pathways such as ERK, JNK, and p38 MAPKs are involved in LPS-mediated IL-12/23p40 expression in human monocytic cells and in different model systems have been identified by various investigators including our laboratory [20, 25, 78, 78, 266-268]. However, the signalling pathways involved in LPS- and IFNγ/LPS-induced IL-23 expressions in human monocytic cells are poorly understood. There is some evidence to suggest the involvement of MAPKs in LPS-induced IL-23 expression [241-243, 265, 268, 299]. For example, inhibition of p38 MAPK was shown to upregulate LPS-induced IL-12p40 production while at the same time down-regulating IL-23p19 expression in human monocytes and PBMCs [265]. My results suggest that IFNγ/LPS-induced IL-23 and IL-12/23p40 protein production is positively regulated by the p38 MAPK in human primary monocytes (Fig. 3.8.B) but does not affect IL-23p19 mRNA expression in the same cells. This positive regulatory

role of p38 MAPK is consistent with the results obtained in human MDDs stimulated with LPS and FcR agonists [267]. I have also shown that JNK does not positively regulate IFNγ/LPS-induced IL-23 production and rather serve as a negative regulator of IL-23 expression in primary monocytes and THP-1 cells (Fig. 3.7 & 3.17).

The results from this study suggest that post-transcriptional/post-translational mechanisms may regulate IL-23 expression and/or secretion in IFNγ/LPS-stimulated monocytic cells. IL-23p19 and IL-12/23p40 mRNA levels did not always correlate with IL-23 protein production specifically as seen in the p38 MAPK inhibition studies (Fig. 3.8; 3.15). In a mouse model study, Z. Waibler et al. have shown that bone marrow derived plasmacytoid DCs were unable to produce detectable amounts of IL-23p19 protein while they could express p19 mRNA constitutively [229]. Similarly, other studies have shown that IL-23p19 mRNA levels were not associated with an increase in IL-23 production in response to LPS stimulation in human monocytes/monocytes and dendritic cells [242, 265]. The lack of correlation between IL-23p19 and p40 mRNA and IL-23 protein production in our study may be attributed to a number of possible post-transcriptional/post-translational steps which may be regulated by a number of factors including IL-23 mRNA stability, protein translation, regulation of intracellular protein degradation, and secretion of newly synthesized proteins. The fact that inhibition of specific signalling pathways results in an increase in expression of IL-23p19 has been previously reported [241, 242, 265]. Dobreva *et al.* suggest that LPS-induced IL-23 production in human PBMCs and purified monocytes may be regulated by mechanisms other than at the transcriptional level [265]. Interestingly, the p38 MAPK pathway has been shown to be an important post-transcriptional regulator of mRNA stability [304]. It

is possible that the decrease in IL-23 protein production, despite an increase in IL-23p19 mRNA that I observed following inhibition of the p38 MAPK, may be due to altered IL-23p19 mRNA stability. Further studies are needed to determine the mechanism by which specific signalling pathways may be affecting cytokine synthesis at a post-transcriptional level. In fact, inhibition of the p38 MAPKs pathway in THP-1 cells inhibited LPS-induced TNF-α mRNA translation [305]. Similarly, another study has suggested that p38 MAPK regulates TNF-α production at the translational level in THP-1 cells and freshly purified primary monocytes [306]. Also in a recent study in our lab, the regulation of IL-12p40 expression in human primary monocytes and THP-1 cells was evaluated and the role of MAPKs signalling in transcription factor binding to the IL-12p40 promoter was determined. Studies related to the binding of transcription factors to the IL-12p40 promoter following LPS stimulation has shown several differences between monocytes and THP-1 cells (Boucher J, et al. 2010). In primary monocytes, ERK and p38 inhibition increased binding of AP-1 and SP1, respectively, to the IL-12p40 promoter, while JNK inhibition increased NF-κB, AP-1, and Sp1 binding. In THP-1 cells, p38, ERK, and JNK inhibition increased NF-κB and Sp1 binding to the IL-12p40 promoter, while inhibiting AP-1 binding (Boucher J, et al. 2010). These data clearly suggest that although LPS induces IL-12p40 expression in primary monocytes and THP-1 cells, distinct signalling pathways may be involved in different cell types.

Our laboratory has previously reported that LPS-induced IL-12/23p40 expression is regulated by the PI3K pathway in primary monocytes and THP-1 cells [33]. Recently, Butchar *et al.* have shown that IL-23 production is positively regulated by the PI3K pathway in human monocytes stimulated with *F. Novicida,* even though IL-23p19 mRNA

levels where unaffected by PI3K inhibition [220]. However, the role of this pathway in IL-23 expression mediated by IFNγ- or IFNγ/LPS stimulation in monocytic cells has not been examined. My results show that the PI3K pathway does not positively regulate IFNγ/LPS-induced IL-23 production in normal human primary monocytes (Fig. 3.4). However, PI3K plays a positive regulatory role in LPS- and IFNγ/LPS-induced IL-23 expression in THP-1 cells, as specific inhibition of the PI3K pathway significantly down-regulates IL-23 proteins production in this promonocytic cell line (Fig. 3.12 and 3.13). There is a significant difference between a promonocytic cell line and primary monocytes/macrophages. PI3K signalling pathway inhibitor had no inhibitory effect on IFNγ/LPS-induced IL-23 expression either at mRNA and protein levels in normal human monocytes. However, LPS-induced IL-23 regulation was affected only at the protein level in THP-1 cells.

In addition to MAPKs and PI3K signalling pathways I investigated the role of calcium signalling pathway in LPS- and IFNγ/LPS-induced IL-23 regulation. It is well documented that many processes such as cell differentiation, proliferation, transcriptional activation and apoptosis are depend on Ca^{2+} signals [214]. Binding of ligands to respective receptor induce calcium influx either through receptor mediated entry or through voltage dependent ion channel entry from external sources. Influx of calcium activates calmodulin followed by CAMKII and calcineurin, which leads to activation of NFκB resulting in expression of various genes. Calcium also activates PKC pathway in association with diacylglycerol leading to NFκB regulated gene expression. Recently, Ma et al. in our laboratory by using two specific pharmacological inhibitors, Cyclosporin A and FK506, for down stream mediators of calcium signalling pathway have shown a

calcium/calmudolin dependent IL-12/23p40 production regulation in human monocytic cells [33]. Another independent group has shown that treatment of cells with Azelnidipine (Aze), a potent calcium blocker, significantly upregulates LPS-induced IL-23 protein production in human MDDCs [267]. The role of the calcium signalling pathway in IL-23 regulation remains unknown. To investigate the role of calcium pathways in LPS- and IFNγ/LPS-induced IL-23 regulation I have used EGTA and SKF, which block calcium influx from exteracellular sources [217]. My results suggest that LPS-induced IL-23p19 gene expression and IL-23 protein production is not regulated by the calcium signalling pathway in THP-1 cells (Fig. 3.20, 3.21, and 3.22). However, IFNγ/LPS-induced IL-23 protein production is positively regulated by the calcium signalling pathway in human primary monocytes (Fig. 3.9). Further studies are needed to address the role of calcium signalling and particularly the CaMK-II in the regulation of IL-23 in THP-1 cells stimulated with LPS.

IFNγ is known to activate intracellular signalling cascades primarily via the JAK/STAT pathway [307, 308]. My data shows that JAK/STAT signalling pathway negatively regulates IL-23p19 mRNA subunit expression and IL-23 protein production in human primary monocytes (Fig 3.2). Furthermore, JAK/STAT signalling via STAT1 is not involved in the positive regulation of IFNγ/LPS-induced IL-23 production. The molecular mechanisms and the relationship between JAK/STAT and p38 MAPK pathways involved in the negative and positive regulation of IL-23 following IFNγ stimulation remains unknown. It has recently been reported that IFNγ can signal via an alternate STAT1-independent mechanisms [307, 309-311]. It has also been shown that IFNγ mediates signalling through the activation of a number of other intracellular

signalling pathways including the PI3K and MAPKs [312, 313]. The mechanism by which IFNγ activates MAPKs is not clear. It is possible that IFNγ signals via other STAT molecules such as STAT3 or STAT5, both of which have been shown to be activated by IFNγ [314, 315]. Interestingly, IFNγ has also been shown to recruit MyD88 [316], an important molecule in TLR ligand-induced gene expression, and may play a role in activating the downstream MAPKs and NF-κB [311] transcription factor. Sun et al. showed that IFNγ induced the association of MyD88, an important TLR-signalling molecule, with the IFNγ receptor and lead to the activation of p38 MAPK [316].

It may be noted that the inhibition of JAK/STAT and PI3K pathways strongly induced the expression of IL-23p19 subunit whereas inhibition of ERK MAPK inhibited the expression of p19 subunit in response to stimulation with either IFNγ or IFNγ and LPS together. We have not investigated the transcription factors involved in the regulation of IL-23p19 subunit in our model system. A number of transcription factors including NF-κB, SMAD3 and ATF-2 have been suggested to regulate LPS-induced transcription of IL-23p19 in macrophages [37]. However, the involvement of specific transcription factors in IFNγ or IFNγ/LPS-induced IL-23 expression remains unknown. IFNγ is a well known activator of a number of transcription factors including IRF-1, IRF-8, PU.1, C/EBP, CIITA, IRF-9 and c-Jun in various cell types but in a STAT1-dependent manner [311, 317-321]. Recently, STAT1-independent transcription factors including STAT3, STAT5, NF-κB and AP-1 have been shown to be activated by IFNγ [310, 315, 322-324]. Since IL-23 induction in response to IFNγ in our study is not dependent on the JAK/STAT pathway, it is likely that IFNγ- or IFNγ/LPS -induced IL-23 expression may be regulated by the activation of p38 MAPKs and independent of the STAT-1

transcription factors. A comprehensive examination of the downstream transcription factors involved in IFNγ/LPS-induced IL-23p19 or IL-23 expression will provide further insight into the regulation of this important Th1 cytokine. Studies are in progress in our laboratory to further understand the molecular mechanisms in the regulation of this subunit at the level of transcription factors and their activation by the upstream kinases particularly the p38, PI3K and the ERK and JNK MAPKs.

The regulation of cellular responses to IFNγ is very complex and is mediated by the equilibrium between the activities of the JAKs and STAT1, and a number of negative regulatory molecules, which include SOCS-1, protein inhibitor of activated STAT (PIAS), and protein tyrosine phosphatases (PTPs) [325]. The molecular mechanism by which JAK/STAT and PI3K negatively regulate IFNγ-induced IL-23p19 and p40 subunits or IL-23 expression is not known. There is evidence to suggest that the release of certain negative regulatory factors such as SOCS protein may cause the upregulation of certain cytokines. SOCS1 is a negative regulator of JAK/STAT signalling and is believed to be an inhibitor of IFNγ-induced IL-12 production in DCs [325]. It is likely that SOCS proteins may also play a role in the regulation of signalling pathways leading to differences in IL-23p19 and p40 mRNA levels and IL-23 protein production.

To dissect the signalling pathways involved in the regulation of IL-23 and IL-27 expression, most of the experiments were performed with small pharmacological inhibitors. Although these agents are specific for blocking the signalling kinases and the resulting pathways, these agents are also known to have some non-specific effects particularly when used at higher concentrations. We generally performed assays to determine their biological activities and used these agents at concentrations which were

not cytotoxic or apoptotic. For these reasons, it is always desirable to confirm the validity of such assays by using small interfering RNAs or knock out cell lines. The problem with identifying specific signalling pathways involved in primary human cells is that knock-out cell models do not exist and it is fairly difficult to transfect primary cells with siRNA resulting in changed monocyte phenotype and possibly regulation. However, we have used these siRNAs to reproduce the results in a monocytic cell line THP-1 at the mRNA level. Further studies are in progress in our laboratory to confirm results with additional siRNAs at the proteins levels.

Overall, my results suggest that IFNγ- and IFNγ/LPS-induced IL-23p19 gene expression is not positively regulated by JNK, p38 MAPKs, PI3K, or calcium signalling pathways, but ERK MAPKs play a positive regulatory role in IL-23p19 gene expression in human monocytes. Although ERK MAPKs do not regulate IFNγ- and IFNγ/LPS-induced IL-23 protein production, p38 MAPK and calcium signalling pathways play a positive regulatory role at protein level in these cells.

LPS- and IFNγ/LPS-induced IL-23 regulation in THP-1 cells

In THP-1 cells, LPS alone significantly induced the expression of IL-23p19 mRNA and IL-23 protein production (Fig. 3.10). This is consistent with other studies showing that LPS can induce IL-23 expression in human monocytes [265, 298], PBMCs [265, 299], and human monocyte-derived DCs [297]. Contrary to the higher levels of LPS-induced IL-12 expression obtained by priming primary monocytic and dendritic cells with IFNγ [36, 236, 297, 299, 301-303], IFNγ alone did not induce the production of IL-23 mRNA or protein in THP-1 cells, nor did it prime these cells for LPS-induced

IL-23 secretion. IFNγ priming of THP-1 cells may, in fact, make them less responsive to LPS-induced IL-23 protein production. However, to our knowledge, IFNγ/LPS-induced IL-23 expression has not yet been examined in THP-1 cells. It is possible that the regulation of IL-23 expression may differ depending on the specific cell type. This is evident from data obtained in our laboratory which indicate that LPS alone does not induce IL-23 protein production in primary human monocytes whereas IFNγ by itself can stimulate significant IL-23 secretion in these cells. My results suggest that substantial differences exist in the ability of LPS to induce IL-23 production in primary human monocytes and monocytic cell lines.

As mentioned earlier, the results from our laboratory and others have previously identified a number of signalling pathways involved in LPS-mediated IL-12/23p40 expression including the MAPK, PI3K and calcium signalling cascades [20, 78, 266, 326]. Herein, my results show that LPS-induced IL-23 production is positively regulated by the JNK and p38 MAPK pathways in THP-1 cells (Fig. 3.15.C). A number of studies have implicated the MAPKs in LPS-induced IL-23 expression in human monocytic cells [37, 241-243, 265, 299]. These data also appear consistent with a recent study showing that murine LPS-induced IL-23p19 mRNA and IL-23 protein expression are regulated by MAPKs pathways [327]. Interestingly, our data show that JNK and p38 inhibition negatively regulate IL-23p19 mRNA expression (Fig. 3.15.B) while at the same time decreasing IL-23 production (Fig. 3.15.C). A similar observation was shown in one study showing that inhibition of p38 MAPK up-regulated LPS-induced IL-12p40 while it reduced IL-23 production in human monocytes and PBMCs [265]. The observations that IL-23p19 mRNA is negatively regulated while IL-23 protein production is positively

regulated by the MAPK pathways are similar to those observed in primary monocytes suggest possible involvement of p38 MAPK in post-transcriptional mechanisms regulating IL-23 levels in response to LPS.

The role of MAPKs in regulating IFNγ-primed, LPS-induced IL-23 production was also determined. Our data show that priming of cells with IFNγ appears to affect the MAPK pathways regulating LPS-induced IL-23 mRNA and protein expression. As described above, cells pre-treated with any of the three MAPK inhibitors all displayed increased IL-23p19 mRNA expression in response to LPS alone. In contrast, when cells were primed with IFNγ before LPS stimulation, IL-23p19 mRNA expression did not significantly change (Fig 3.11.A). Similarly, LPS-induced IL-23 protein production in the presence of p38 and JNK MAPKs inhibitors was altered when cells were primed with IFNγ. It is likely that IFNγ may be affecting LPS-induced p38 or JNK signalling cascades with consequent effects on IL-23 mRNA as well as protein production.

It has been suggested that NF-κB, SMAD3, ATF-2 and AP-1 may regulate LPS-induced IL-23 expression [37, 327]. A comprehensive examination of the downstream transcription factors involved in IFNγ-induced IL-23p19 or IL-23 protein expression will provide further insight into the regulation of this important Th1 cytokine. The differences observed between IL-23 expression induced by LPS-alone or IFNγ/LPS co-stimulation may be due to differences in transcription factors activated by LPS and IFNγ. In particular, IFNγ is known to induce the expression of SOCS proteins which are important inhibitors of intracellular signalling cascades involved in cytokine expression [325]. It is possible that the reduced effect of LPS-induced IL-23 production is a result of the activation of SOCS expression by IFNγ. The SOCS proteins may impact MAPKs

signalling pathways regulating IL-23 production. This may explain differences in IL-23 mRNA levels and protein production seen between LPS and IFNγ/LPS stimulated THP-1 cells (Fig. 3.15, 3.17, and 3.18).

We have previously reported that IL-12/23p40 expression is regulated by PI3K in monocytic cells [33]. In this study, we also show that PI3K inhibition does not affect LPS- or IFNγ/LPS-induced IL-23p19 mRNA expression (Fig. 3.12.B and 3.13.A), but significantly regulates IL-23 as well as IL-12/23p40 protein production (Fig. 3.12.C and 3.13.B). My results are consistent with one report showing that IL-23 production is regulated by PI3K in human monocytes stimulated with *F. Novicida,* without affecting IL-23p19 mRNA levels [220].

Previous data from our laboratory show that IL-12p40 production is positively regulated by intracellular calcium signalling cascades [33]. To our knowledge, the role of calcium signalling in the regulation of IL-23 production has not been examined in THP-1 cells. My results suggest that calcium signalling does not affect IL-23 expression or its production in THP-1 cells. Using specific inhibitors of calcium influx (EGTA) and receptor-mediated calcium influx (SKF96365), we show that while both inhibitors increase LPS-induced IL-23p19 mRNA, they have no effect on IL-23 protein production (Fig. 3.20 & 3.22). Moreover, pre-treatment with EGTA or SKF96365 and priming with IFNγ had no effect on IL-23 production in LPS-stimulated cells (Fig. 3.21 and 3.23). Similar to LPS-alone, IFNγ/LPS treatment following EGTA inhibition resulted in an increase in IL-23p19 mRNA.

An increasing amount of data from our laboratory and others {1196} now suggests that there may be post-transcriptional mechanisms regulating IL-23 expression

and/or secretion. We show that the PI3K and MAPK pathways are positive regulators of IL-23 production while at the same time negatively regulate IL-23p19 mRNA expression. The fact that subunit mRNA levels go up while protein levels go down suggests that IL-23 secretion is regulated at a post-transcriptional level. A number of possible post-transcriptional steps may be regulated by the signalling pathways including regulation of IL-23 mRNA stability, protein translation, and regulation of intracellular protein degradation and/or secretion of newly synthesized protein. The fact that inhibition of specific signalling pathways results in an increase in expression of IL-23 mRNA and concomitant decrease in protein levels has been previously reported [241, 242, 265]. Other groups have also shown that IL-23p19 mRNA levels are not associated with an increase in IL-23 production in response to LPS [242, 265]. Interestingly, the p38 MAPK pathway has been shown to be an important post-transcriptional regulator of mRNA stability [304, 328]. It is possible that our observed decrease in IL-23 protein production, despite an increase in IL-23p19 mRNA following inhibition of the MAPK and PI3K signalling pathways, is a result of changes in IL-23p19 mRNA stability. This would then result in higher observed mRNA levels at early time-points post-stimulation but decreased levels of proteins after 24-48 hours of stimulation. Furthermore, inhibition of p38 MAPK in THP-1 cells was shown to cause a decrease in LPS-induced TNF-α mRNA translation [306]. This suggests that signalling pathways may be regulating the translation of newly synthesized IL-23. Further studies are required to elucidate the post-transcriptional mechanisms by which specific signalling pathways are regulating IL-23 synthesis.

In summary, I show that LPS-induced IL-23 production is regulated via the MAPKs and PI3K signalling pathways in THP-1 cells. I also show that IFNγ priming does not enhance the expression of IL-23 induced by LPS but it may play an inhibitory role in LPS-induced IL-23 production. The intracellular signalling mechanisms regulating IL-23 expression and the identification of the transcription factors involved in these pathways will be important steps in characterizing the function of this important pro-inflammatory cytokine (Figure 4.1).

Figure 4.1 Signalling pathways involved in IL-23 protein production in human monocytic cells

LPS- induced IL-23 proteins production is regulated through the activation of JNK, p38 MAPKs and PI3K signalling pathways in THP-1 cells, but following stimulation with IFNγ and LPS, IL-23 is regulated primarily by the PI3K pathway. In normal monocytes, IFNγ- or IFNγ/LPS- induced IL-23 protein production is regulated through the activation of PI3K and calcium pathways.

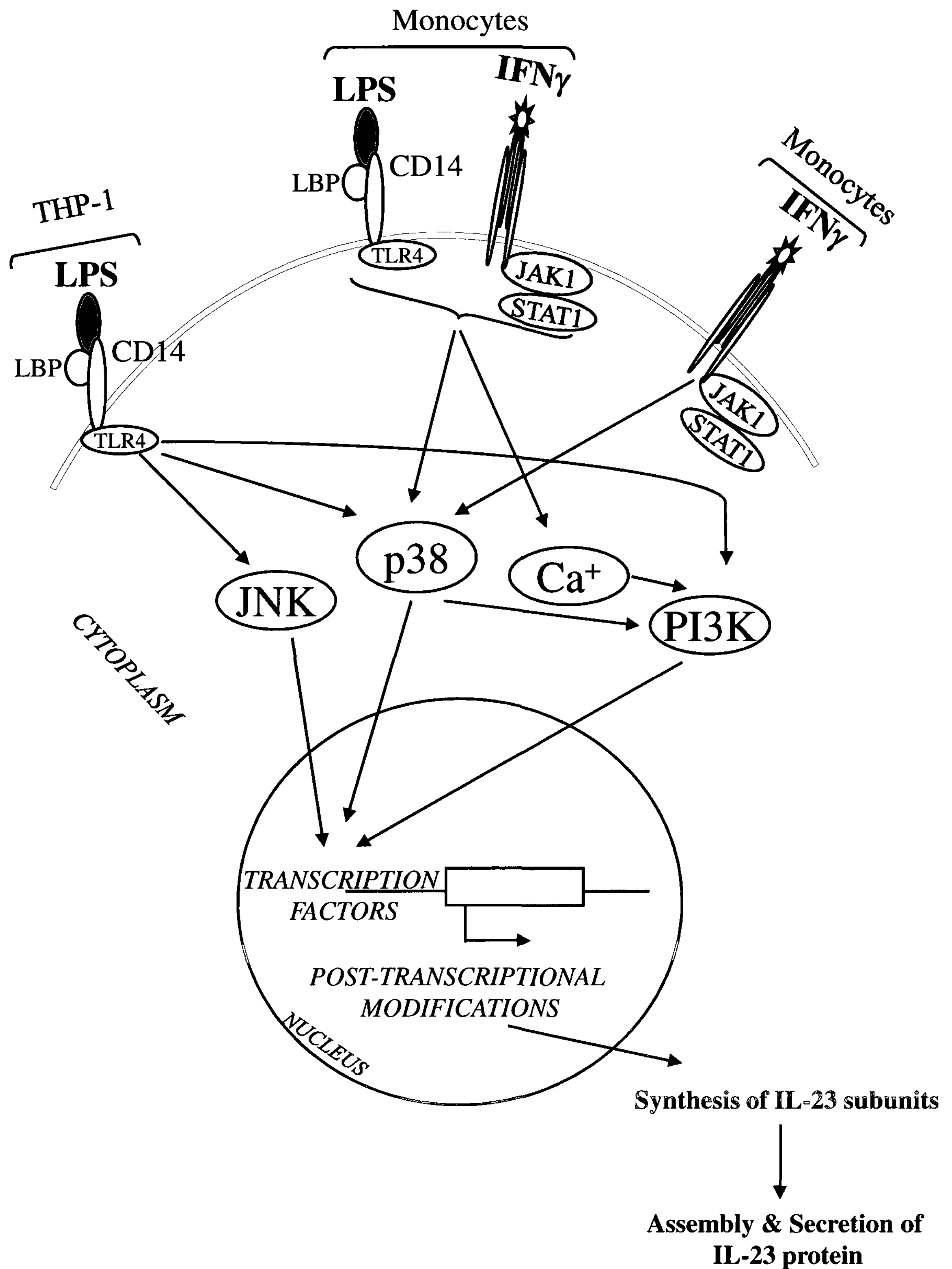


Figure 4.1

PART II

IFNγ- and IFNγ/LPS-induced IL-27 regulation in human primary monocytes and THP-1 cells

IL-27 is a key macrophage derived cytokine which controls Th cell-dependent, adaptive responses and inflammation following exposure to microbial pathogens [252]. In addition it has an important role in controlling the IL-23/IL-17 pathway [329-331]. Moreover, recent studies have shown significant roles for IL-27 in the host resistance against microbial infections [224]. Keeping in mind that monocytes/macrophages are the most important sources of these cytokines, very little is known regarding the intracellular signalling pathways and molecular mechanisms that govern the production of IL-27 in human monocytic cells. In particular, the regulation of IL-27 expression in LPS- and IFNγ/LPS-stimulated human monocytic cells has not been investigated. Herein, I demonstrated for the first time that IFNγ- and IFNγ/LPS-induced IL-27 expression in human primary monocytes is positively regulated by the JNK and p38 MAPKs and independent of the JAK/STAT signalling pathways. Similarly, LPS-induced IL-27 expression in THP-1 cells was regulated by the p38 and JNK MAPKs and the PI3K pathways.

LPS alone was not able to induce significant IL-27 expression in normal human monocytes. LPS-induced IL-27 expression required priming of cells with IFNγ. These observations were similar to those observed indicating the necessity of priming cells with IFNγ for LPS to induce IL-12 and L-23 subunit expression in human monocytic cells [74, 230-232]. The combination of LPS and IFNγ has also been shown to induce the expression of IL-27p28 and IL-27EBI3 mRNA in dendritic cells suggesting that priming

of cells with IFNγ is required for the production of high levels of IL-27 production in response to LPS stimulation [332]. However, in human monocytes derived macrophages it has been shown that both IL-27 subunits, p28 and EBI3, were expressed at RNA levels after LPS stimulation [252]. My results suggest that stimulation of highly purified monocytes with IFNγ alone or LPS and IFNγ resulted in an increase in IL-27p28 and IL-27EBI3 mRNA levels (Fig. 3.25.A) as well as increase in IL-27 protein production (Fig. 3.25.B) compared to the unstimulated cells. In contrast, LPS alone was sufficient to induce IL-27 expression at both mRNA and proteins levels in THP-1 cells. Surprisingly, IFNγ alone was not able to induce IL-27 expression at significant levels in these cells (Fig. 3.31).

LPS, a potent mitogen for activating cells of the monocytic lineage has been shown to induce IL-27p28 and EBI-3 subunits in murine dendritic cells. The molecular mechanism governing IL-27p28 and EBI-3 subunits following LPS stimulations are not well understood. LPS-induced IL-27p28 expression and IL-27 serum levels in LPS-injected mice were found to be critically dependent on Toll/IL-1R-containin adaptor-inducing IFNβ and its associated IFN regulatory factor (IRF) 3 transcription factor as demonstrated by promoter analysis [333]. Involvement of IRF-3 was also shown to regulate IL-27p28 expression in human dendritic cells as shown by chromatin immunoprecipitaion (CHIP) studies [251]. In addition, MyD88 was shown to regulate LPS-induced IL-27p28 expression through the activation of IRF-1 transcription factor [153, 334]. These studies suggest that multiple signalling pathways may be required for IL-27 regulation. In addition to TLR-4 ligand, LPS, TLR-2, TLR-3 and TLR7/8 have also been shown to regulate IL-27 gene expression in human macrophages, possibly through

the synthesis of IFNα [252]. IFNα-mediated IL-27 gene expression was also shown to be regulated through the activation of IRF-1 [252]. Similar results were obtained following stimulation of human DCs with IFNβ [334]. IFNβ -activated DCs exhibited IL-27p28 expression and this was regulated through the activation of IRF-1 [251, 334]. The signalling pathways and the molecular mechanisms that control IFNγ or IFNγ/LPS-induced IL-27 expression have not been addressed. Since IRFs control IFNγ-induced responses [335, 336], it is possible that IFNγ or IFNγ/LPS-induced IL-27 expression in human monocytic cells may be regulated by the IRF-1/3 transcription factors. Studies in our laboratory are in progress to address such issues.

The first question was whether IFNγ- or IFNγ/LPS-induced IL-27 expression is regulated via JAK/STAT signalling pathway. The results show that JAK/STAT signalling pathway negatively regulates IL-27p28 mRNA subunit expression and IL-27 protein production in human primary monocytes (Fig. 3.26) suggesting that JAK/STAT signalling via STAT1 is not involved in the positive regulation of IFNγ/LPS-induced IL-27 production. The relationship between the JAK/STAT, p38 and JNK MAPKs and the PI3K pathway with respect to negative and positive regulation of IL-27 expression remains unknown. As discussed earlier, with respect to the regulation of IL-23 in response to stimulation with IFNγ, IFNγ can signal via an alternate STAT1-independent mechanisms [307, 309-311] through the activation of a number of other intracellular signalling pathways including the PI3K and MAPKs [312, 313]. The mechanism by which IFNγ activates MAPKs is not clear. It is possible that IFNγ signals via other STAT molecules such as STAT3 or STAT5, [314, 315] and MyD88 [316], which may activate the downstream MAPKs and NF-κB transcription factor [311, 316].

Phosphoinositide kinases (PIK) are a family of similar enzymes capable of phosphorylation of the 3 position hydroxyl group of the inositol ring of phosphatidylinositol. PI3-kinases are linked to diverse group of cellular functions, including cell growth, proliferation, differentiation, motility, survival and intracellular trafficking [337]. Class Ia PI3K consist of a 85-kDa regulatory subunit (α, β, and γ isoforms) and a 110-kDa catalytic unit (α, β, and δ isoforms). Interaction of the p85 subunit with phosphorylated YxxM motifs in transmembrane receptors or adaptor molecules results in the recruitment of p85-p110 heterodimers to its substrate phosphatidylinositol 4,5-bisphosphate [338-342] that leads to the release of phosphatidylinositol 3,4,5-trisphosphates and eventual phosphorylation of a number of substrates including Akt, protein kinase A, and protein kinase C isoforms [195, 341]. There is considerable evidence to suggest that PI3K plays a key role in the regulation of IL-12p40 in murine and human monocytic cells and DCs [246, 343-345]. Our results show that PI3K plays a crucial role in the regulation of IL-27 expression in primary human monocytes as well as THP-1 cells following stimulation with LPS, IFNγ or IFNγ and LPS together.

I also determined the role of MAPKs in IFNγ/LPS-induced IL-27 expression in monocytic cells. The MAPKs signalling pathway is highly conserved and coordinates diverse cellular functions such as cell proliferation, cell survival/apoptosis and differentiation. MAPKs are serine/threonine-specific kinase proteins that respond to a multitude of extracellular stimuli. MAPKs include several subgroups, such as JNK, ERK and p38 MAPK, all being activated via a phosphorylation cascade which permit them to phosphorylate other protein kinases, phospholipases and transcription factors. The

MAPK pathway can be modulated by various mechanisms at various steps in the pathway. There is very little information available on the role of MAPKs in the regulation of IL-27 expression in human monocytes [224, 268]. The first is belongs to Lara Hause et al. that have shown Theiler's murine encephalomyelitis virus infection of RAW264.7 cells, a macrophage cell line, induces IL-27p28 mRNA expression through TLR3 and TLR7 and JNK-MAPK activation [224]. My results suggest that there are similarities in terms of signalling pathway activation leading to IL-27 expression in normal and cancerous human monocytic cells (THP-1 cells). It appears that JNK and p38 MAPKs are essential intracellular signalling mediators for IFNγ- and IFNγ/LPS-induced IL-27 production in human monocytic cells.

The JNK MAPK pathway includes JNK1, JNK2, and JNK3 [346]. JNK1 and JNK2 are widely expressed in several tissues, whereas JNK3 is more selectively expressed in brain, testis, and heart. The *JNK3* gene has been shown to be involved in neuronal cell death [347], whereas JNK1 and JNK2 have been implicated in Th1/Th2 cell differentiation [348, 349]. JNK1 has also been shown to regulate the development of T cell-mediated immunity against *Leishmania major* infections in an experimental mouse model [350]. Whether JNK1 or JNK2 regulates IL-27 expression in this system needs to be investigated.

Similar to the results obtained with IL-23 subunits, results with regulation of IL-27 expression suggest that the IL-27p28 mRNA levels did not always correlate with IL-27 protein production specifically as seen in the JNK and p38 MAPK inhibition studies (Fig. 3.27.B&C). These observations are indicative of the fact that post-transcriptional and/or post-translational mechanisms may regulate IL-27 expression and/or secretion in

IFNγ/LPS-stimulated monocytic cells. The lack of correlation between IL-2728 mRNA and IL-27 protein production in our study may be attributed to a number of possible post-transcriptional/post-translational steps which may be regulated by the signalling pathways including regulation of IL-27 mRNA stability, protein translation, regulation of intracellular protein degradation, and secretion of newly synthesized proteins. Similar observations have been made with respect to IL-23 regulation and have been discussed in detail in the discussion section of IL-23 regulation [229, 241, 242, 242, 265, 265, 304, 306]. Further studies are needed to determine the mechanism by which specific signalling pathways may be affecting IL-27 synthesis at a post-transcriptional level.

We obtained conflicting results on the role of calcium signalling in IL-27 expression in THP-1 cells and primary monocytes. While calcium signalling did not play a role in LPS-induced IL-27 production in THP-1 cells, calcium signalling was shown to regulate IL-27 expression in primary monocytes stimulated with LPS and IFNγ. The reasons for such conflicting results are not clear but may be attributed to differences in cell type and the mode of stimulation. Further studies are needed to address the precise calcium signalling pathways involved in IL-27 expression in primary monocytes stimulated with LPS and IFNγ.

Overall, my results show that p38 MAPK and PI3K are the essential signalling pathways that playing role in the regulation of IFNγ/LPS-induced IL-27 production (Figure 4.2).

Figure 4.2 Signalling pathways involved in IL-27 protein production in human monocytic cells

p38 MAPK and PI3K are the essential signalling pathways that regulate IFNγ/LPS-induced IL-27 production. Moreover, LPS- and IFNγ/LPS-induced IL-27 expression is regulated by JNK, p38 MAPKs and PI3K pathways in THP-1 cells.

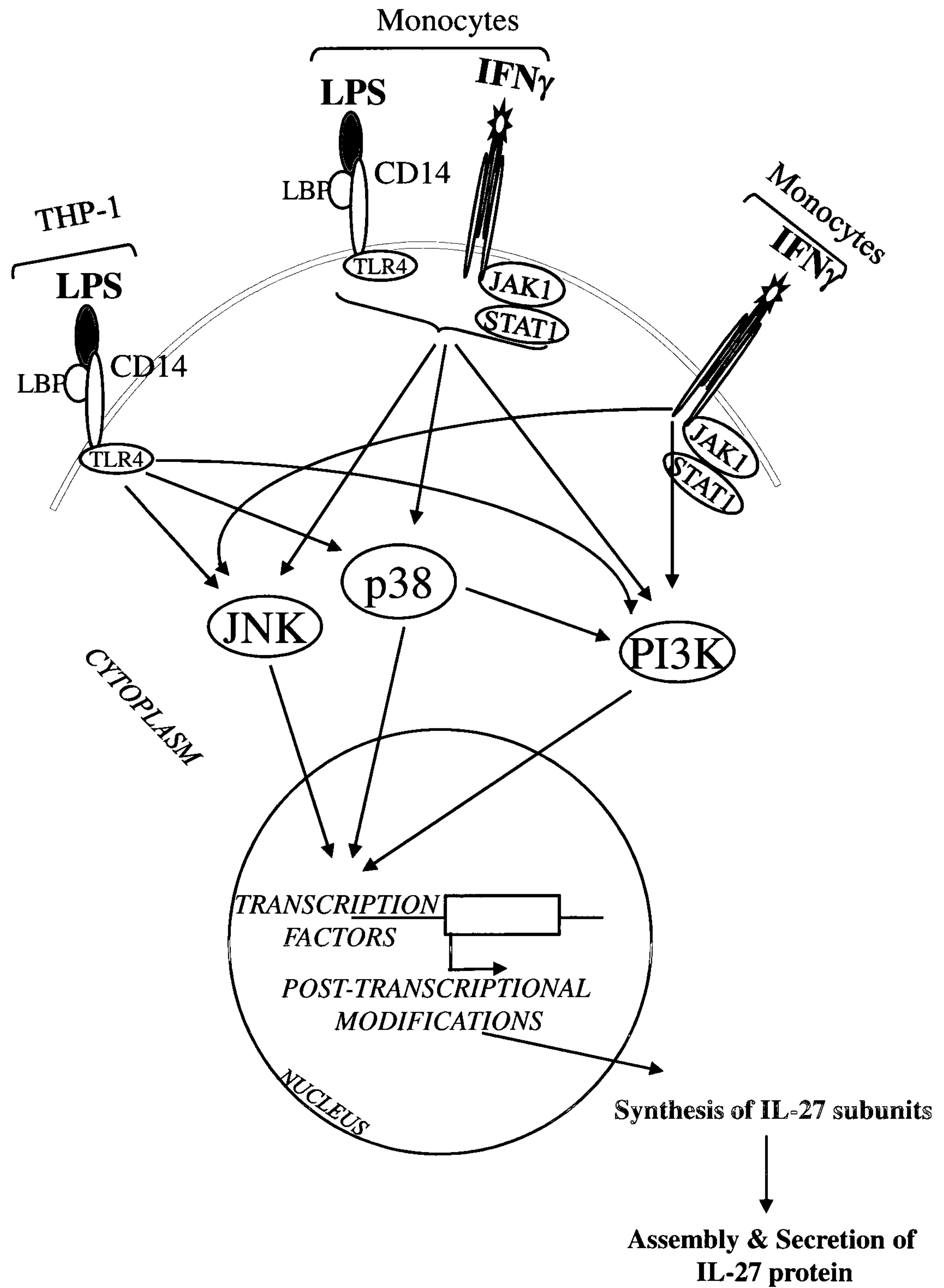


Figure 4.2

PART III

Impact of HIV infection on spontaneous and LPS-induced IL-23 and IL-27 expression in THP-1 cells

Expanding knowledge and better understanding of the immunopathogenesis of HIV-1 infection is a major prerequisite to improve and develop immunotherapeutics and prophylactic vaccines. HIV infection results in direct and indirect dysregulation of the immune systems. The direct effects of HIV infection on T cells and B cells and their functions are well documented [83, 101]. The indirect effects of HIV infection such as cytokine profile alteration *in vivo* or following infection *in vitro* contribute significantly towards immune dysregulation. In general, HIV infection results in the production of reduced levels of Th1 cytokines (IL-12, IFNγ) and enhanced levels of Th2 type cytokines (IL-4, IL-10) [294] and impaired cell mediated immunity as a consequence of abnormal cytokine regulation. Deficiency in IL-12 production by monocytes/ macrophages after HIV-1 infection has been identified as a potential factor responsible for impaired innate and cellular immune responses observed in AIDS patients [15, 22, 351]. However, the effect of HIV infection on the regulation of other members of IL-12 family cytokines such as IL-23 and IL-27 remains unknown. Because IL-23 and IL-27 have overlapping functions with IL-12 and all these cytokines are produced primarily by antigen presenting monocytic cell lineage, I hypothesized that HIV-1 infection will similarly affect the production of IL-23 and IL-27.

To study HIV immunopathogenesis and molecular mechanisms by which HIV exerts its role in the regulation of IL-23 and IL-27 in human monocytic cells, the impact of *in vitro* HIV infection on IL-23 and IL-27 production in THP-1 cells was investigated.

Relevant to HIV infection, THP-1 cells express low levels of CXCR4, CCR5, and CD4 receptors [352]. The susceptibility of THP-1 cells to macrophage tropic HIV-1 strains infection is controversial and different groups have reported different levels of HIV replication in this cell line [353, 354]. To increase the susceptibility of cells to HIV infection and replication, THP-1 and HL-60 cells were treated with polybrene based on the NIH standard protocols prior to infection with a dual tropic HIV-1_{CS204} strain. My results show that this dual tropic clinical strain of HIV-1 virus properly replicates in THP-1 cells (Fig. 3.40), but not in HL-60 (data not shown). Based on these results, subsequent HIV-1 experiments were performed in THP-1 cells infected with a dual tropic HIV-1_{CS204} strain.

My results suggest for the first time that HIV-1_{CS204} infection in unstimulated THP-1 cells significantly induces a transient upregulation of IL-23 at both mRNA and protein levels in a dose dependent manner (Fig. 3.41.A&B), but it fails to induce IL-27 gene or protein production under similar conditions (Fig. 3.44.A&B). Moreover, the levels of IL-23 production decreased with the increase in HIV replication over a period of time. Similar results have been obtained following infection of monocyte derived macrophages with HIV_{cs204} (data not shown). Interestingly, THP-1 cells produced constitutively detectable levels of IL-27 mRNA and IL-27 protein and HIV infection did not cause any change in the levels of this cytokine. However, HIV infection did induce significantly high levels of HIV EBI-3 mRNA in THP-1 cells. Whether *in vitro* infection of MDMs with HIV causes similar increase in IL-27 EBI-3 subunit and the physiological significance of EBI-3 upregulation remains to be investigated. It will also be interesting to determine if IL-23 and IL-27 subunits are produced constitutively throughout the

course of HIV infection and whether anti-retroviral treatment affects the production or expression of such cytokines or their subunits in HIV infected patients.

HIV/AIDS is associated with loss of Th1 cytokines, including IL-12 production. A dramatic reduction in IL-12p35 and IL-12p70 production in monocytes infected with HIV was reported in 1996 [23]. In addition, an investigation on PBMC of HIV-infected patients compared with healthy donors has revealed a dramatic reduction in both IL-12 subunits (p40 and p35 mRNA) resulting in lower IL-12 secretion from HIV-infected individuals [22]. Moreovder, Chambers K, et al. have shown that HIV infection directly impairs the rate of transcription of the IL-12p40 gene and this suppression of IL-12p40 transcripts expression was dependent upon active cellular infection followed by IL-12p40 gene expression disruption and not due to any soluble host or viral factors in HIV-infected cultures [21]. In addition, in another study by site-directed mutagenesis, it was shown that binding of NF-κB p50, c-Rel, p65, Sp1, Sp3, c-Fos, and c-Jun proteins to their cognate nuclear factor binding sites was somewhat impaired by HIV infection and this alteration in nuclear binding factor binding to numerous sites in the IL-12p40 promoter may contribute to the suppression in IL-12p40 transcripts [21]. HIV has been shown to enhance the production of several cytokines including IL-10 and TNF-α and their levels increase over a period of time and viral replication [99, 355]. The molecular mechanisms underlying enhanced production of IL-10 and TNF-α have been investigated. HIV accessory proteins Tat and Nef have been shown to enhance IL-10 production depending upon the cell type studies [84, 355]. Our laboratory has previously demonstrated that extracellular HIV-Tat-induced IL-10 transcription in primary human monocytes is

regulated by CREB-1 and Sp-1 transcription factors through the activation of calmodulin/ CaMK-II-dependent p38 MAPK [355]. On the other hand, intracellular HIV-Tat induced IL-10 transcription by ERK MAPK-dependent CREB-1 transcription factor activation through Ser_{-133} phosphorylation in THP-1 cells and primary monocytes [99]. Whether HIV accessory proteins can enhance transient production of IL-23 and IL-27-EBI-3 remains to be established. In the event HIV Tat or Nef causes upregulation of IL-23 and IL-27-EBI-3, it will be interesting to elucidate the underlying signalling pathways involved.

Very little is known about the role of HIV infection on monocytes/macrophages response to stimuli such as microbes and/or microbial products in terms of IL-23 and IL-27 expression. Therefore, we investigated the effect of LPS on THP-1 cells infected *in vitro* with HIV. Herein for the first time I have shown the differential impacts of HIV infection on LPS-induced IL-23 and IL-27 expression in THP-1 cells. LPS-induced expression of IL-23p19 (data not shown) as well as production of IL-23 proteins was significantly inhibited in HIV-infected THP-1 cells (Fig. 3.42). The level of inhibition increased over a period of 3-5 days post-infection. LPS-induced IL-23 proteins production in HIV-infected cells was significantly decreased at day 3 ($p = 0.01$) and day 5 ($p = 0.002$) post-infection, and this reduction was at maximum levels with the lowest titre of the virus corresponding to 5 ng/ml HIVp24 (Fig. 3.42.A&B). Similar results were shown in HIV-infected PBMCs (Fig. 3.43) and at day 5 post-infection the levels of LPS-induced IL-23 production was remarkably decreased. However, LPS-induced IL-27 expression or its subunits was not affected following *in vitro* infection with HIV under similar conditions at day 3 and 5 post-

infection (Fig. 3.45.A-D). Similar results have been obtained following LPS stimulation of monocyte-derived macrophages infected with HIV_{cs204} (data not shown).

In summary these studies provide basic information to further investigation on the molecular mechanism involved in the regulation of LPS-induced expression of IL-23 or its subunits. It will be interesting to determine whether HIV accessory or regulatory proteins alter the production of IL-23 or its subunits as HIV regulatory proteins Tat, Nef, and Vpr are known to modulate production of cytokines, including IL-12 [99, 294, 355-357]. Variable effects of Tat and Nef on IL-12 expression in B cells and monocytes have been observed possibly due to different cell types and the models used to deliver these genes/gene products into the cells. For example, Tat was shown to suppress IL-12 production in human PBMCs [90] and enhance its production in dendritic cells [358, 359]. We have previously shown that Tat did not affect IL-12p40 production in either unstimulated or LPS-stimulated monocytic cells [355] (data not shown). On the other hand, HIV Vpr protein was shown to inhibit IL-12 production by down-regulating IL-12p35 subunit without affecting the synthesis of IL-12p40 subunit [112]. Similarly, there are reports that Nef protein does not affect IL-12 expression in human PBMCs and U937 cells [90, 360]. However, introduction of Nef through adenoviruses and recombinant *nef* enhanced IL-12 production in immature dendritic cells [361]. Conversely, there is evidence for the inhibitory role for Nef in IL-12p40 production in monocytic cells. IL-12p40 production was suppressed in lymph nodes of macaques infected with simian immunodeficiency virus compared with those infected with the corresponding non-pathogenic *nef*-deleted strain [77].

5. CHAPTER V

Conclusion and Future Directions

Macrophages contribute to innate and adaptive immune responses against viruses by secreting IL-12, IL-18, and type one interferons which synergistically induce IFNγ production in CTL and NK cells. It is well established that IL-12 production is decreased in HIV-1-infected individuals and following *in vitro* HIV-1 infection of monocytic cells. However, the role of HIV infection on regulation of the recently described members of IL-12 family of cytokines is largely unknown. The main goal of my study was to investigate the intracellular signaling pathways involved in the regulation of IL-23 and IL-27 in normal human monocytic cells and following *in vitro* infection with HIV-1.

The first objective was to determine the signalling pathways involved in the regulation of IL-23 in LPS-stimulated normal human monocytic cells. I have demonstrated for the first time that IFNγ/LPS-induced IL-23 and IL-12p40 expression in primary monocytes is regulated by the p38 MAPK and the calcium pathways independent of the classical IFNγ intracellular JAK/STAT mediators. It is notable that in general, IFNγ- and IFNγ/LPS-induced IL-23p19 mRNA expression in monocytes and THP-1 cells were not inhibited by JNK, p38 MAPKs, PI3K, and calcium pathway specific inhibitors. In contrast, the signalling inhibitors for these pathways enhanced IL-23p19 transcript expression. It was necessary to reproduce these results with IL-23p19 proteins measurement in cell culture supernatants. Currently, IL-23p19 proteins are not detectable with the available protein assay kits and the measurement of this protein by western blotting was not successful (data not shown). Therefore, it will be of interest to determine the levels of intracellular or secreted LPS-induced IL-23p19 proteins in THP-1 cells and monocytes/macrophages by ELISA whenever available. I have shown that LPS induces

IL-23p19 expression through the activation of the calcium signalling pathway. However, further studies are needed to precisely elucidate the signalling mediators involved in IL-23p19 regulation by employing siRNA technology at least in THP-1 cells.

My second objective was to elucidate the signalling pathways involved in the regulation of IL-27 in human monocytic cells. I have shown that JNK MAPKs and PI3K are the essential signalling pathways that play a crucial role in the regulation of IFNγ/LPS-induced IL-27 production. In addition, p38 MAPK regulates LPS- and IFNγ/LPS-induced IL-27 regulation in THP-1 cells.

To confirm the role of MAPK and PI3K signalling pathways in the regulation of IL-23 and IL-27 gene expressions I have applied p38/ERK MAPKs and PI3K specific siRNAs. To reproduce these results it will be important to run similar experiments to measure IL-23 and IL-27 production at proteins level. Moreover, it will be of interest to elucidate any cross talk between signalling molecules involved in the regulation of IL-23p19 and/or IL-27p28 expression. As mentioned earlier, to identify the negative feedback regulatory role of PI3K or MAPKs in LPS-induced IL-23p19 and/or IL-27p28 expression, it will be important to employ dominant negative constructs or siRNA technology for PI3K or MAPKs.

It is important to illustrate the role of transcription factors involved in the regulation of IL-23p19, IL-12/23p40, IL-27p28, and IL-27EBI3 gene expression in monocytic cells stimulated with IFNγ or IFNγ and LPS. Characterization of transcription factors and the upstream signalling kinases regulating the expression of IL-12 family cytokines genes would involve promoter analysis, gel shift assay and/or CHIP analysis. The identification of promoter activity and transcription factors involved in these

pathways will be the next important steps in characterizing the function of these important pro-inflammatory cytokines.

My results clearly suggest that there is no correlation in the expression of IL-23 and IL-27 subunit genes and protein expression. The lack of correlation between IL-27p28 mRNA and IL-27 protein production in our study may be attributed to a number of possible post-transcriptional/post-translational steps which may be regulated by the signalling pathways including regulation of IL-23 and IL-27 mRNA stability, protein translation, regulation of intracellular protein degradation, and secretion of newly synthesized proteins. In addition it will be of interest to investigate the precise role of signalling molecules in assembly or synthesis of IL-23 and IL-27 subunits by applying specific pharmacological inhibitors, dominant negative constructs or specific siRNAs to knock down the target signalling molecules.

The results also suggest that JAK/STAT signalling via STAT1 is not involved in the positive regulation of IFNγ/LPS-induced expression of IL-23 and IL-27 and their subunits. The relationship between the JAK/STAT, MAPKs and the PI3K with respect to regulation of IL-23 and IL-27 expression remains unknown. As discussed earlier, IFNγ can signal through an alternate STAT1-independent mechanism following activation of a number of other intracellular signalling pathways including the PI3K and MAPKs. Understanding the role of JAK/STAT-independent pathway and its cross-talk with other major signalling molecules will be of the utmost significance in the IFNγ-mediated regulation of IL-12, IL-23 and IL-27.

Finally, the findings of my third objective suggest that HIV alone significantly induced IL-23 expression at mRNA and protein levels, but it did not induce IL-27

expression either at mRNA or at protein levels in THP-1 cells. It appears that HIV also induced IL-23 and IL-27 expression in MDMs (Kumar et al, unpublished observations). It will be of interest to investigate the molecular mechanisms by which HIV upregulates IL-23 expression in human monocytic cells.

The inhibitory role of HIV infection on LPS-induced IL-12p40 expression has been shown, but the role of HIV infection on LPS-induced IL-23 and IL-27 expression is poorly understood. The results suggest that HIV-infection significantly inhibited LPS-induced IL-23 expression at both mRNA and protein levels in THP-1 cells. However, HIV infection did not affect LPS-induced IL-27 expression. These observations suggest that HIV or its accessory proteins differentially modulate the activity of signalling proteins involved in LPS-induced expression of IL-23 and IL-27 in monocytic cells. It is not clear whether monocytic cells including primary monocytes / macrophages, monocytes derived macrophages (MDMs), and monocytes derived dendritic cells from HIV-infected individuals exhibit similar differential regulation of IL-12, IL-23 and IL-27. Differentially regulation of IL-23 and IL-27 in HIV infected cells leading to a differential cell response to micro-organisms or their structural particles may be a possible mechanism for HIV to escape the host immune response and disease progression.

Understanding the regulatory role of HIV or its accessory / regulatory proteins such as Tat, Nef or Vpr in the alteration of IL-23 subunits expression will be the next important step in this project. I have investigated the role of HIV-Tat protein on the regulation of IL-23 and IL-27. My results suggest that this protein does not affect IL-23 or IL-27 regulation in THP-1 cells (data not shown). In the event other HIV accessory proteins modify or down regulate these cytokines production, it will be interesting to determine the molecular

mechanisms and the signalling pathways involved in the modulation of IL-23 and IL-27. Our laboratory has previously shown that intracellular Nef expressed in primary monocytes and promonocytic THP-1 cells following transduction with retroviruses containing *nef* gene inhibited LPS-induced IL-12p40 transcription by inhibiting the JNK MAPKs without affecting the CaMK-II-activated pathway. In addition, Nef inhibited JNK-activated NFκB without affecting the AP-1 activity.

We are still far from being able to grasp the intricate network of interactions between the intracellular signalling pathways involved in IL-12 family of cytokines expression, but important pieces of this puzzle are now coming together. Overall my findings suggest that the major signalling pathways involved in IL-23 and IL-27 expression in human monocytic cells are p38 MAPK and PI3K. The implications of this knowledge are of course profound because a detailed understanding of the complex interactions that occur at the genetic and protein levels will provide attractive targets for the rational design of new drugs, for prevention and treatment approaches.

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STATEMENT

Maria is doing her PhD project to investigate the role of IFNγ on IL-12 family of cytokines regulation in human monocytic cells in our laboratory. I hereby would like to state that the Figures 3.2.A; 3.4.A; 3.5; 3.8C&D; 3.26 lower panels; 3.27.D; 3.28.C&D were generated by Ms Maria Blahoianu either alone or in collaboration with me.

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PUBLICATIONS:

- LPS-induced IL-23 expression in THP-1 cells is regulated by the MAPKs and PI3K signalling pathways
  Ali A. R. Rahimi*; Maria Blahoianu; Jonathan G. Boucher; Niranjala Gajanayaka; Ashok Kumar*
  (manuscript ready to submit)

- IFNγ Induces IL-23 Expression in Primary Human Monocytes via the p38 and ERK MAPKs and Calcium Pathways Independently of the JAK/STAT or PI3K Signaling
  Maria A. Blahoianu; ***Ali A. R. Rahimi****; Jonathan G. Boucher; Niranjala Gajanayaka; Jonathan B. Angel; Ashok Kumar*
  (manuscript ready to submit)

- STAT-1 Mediates the Stimulatory Effect of IL-10 on CD14 Expression in Human Monocytic Cells
  Ali A. R. Rahimi*; Katrina Gee; Sasmita Mishra; Wilfred Lim; Ashok Kumar*
  The Journal of Immunology, 2005, Vol. 174: 7823-7832

- *Quality Systems for Medical Laboratories*
  Translated by: **Ali A. R. Rahimi**; Safar Farajnia, 1999.
  WHO Regional Publications, Eastern Mediterranean Series 14

PAPER PRESENTATIONS:

- *Regulation of the IL-12 Family Cytokines IL-23 and IL-27 in Response to HIV of Human Monocytic Cells*

Ali A. R. Rahimi; Niranjala Gajanayaka; Katrina Gee; Ashok Kumar
XVII International AIDS Conference
Mexico City, Mexico, August 3-8, 2008

- *PI3K and P38 MAPK Differentially Regulate Expression of IL-12 Family Cytokines (IL-23 and IL-27) in LPS-induced Human Monocytic Cells*

Ali A. R. Rahimi; Maria Blahoianu; Niranjala Gajanayaka; Jyoti Mishra; Ashok Kumar
Research Conference 2007, Ontario HIV Treatment Network (OHTN)
Toronto, Ontario, Canada, November 19 and 20, 2007

- *PI3K and P38 MAPK Differentially Regulate Expression of IL-12 Family Cytokines (IL-23 and IL-27) in LPS-induced Human Monocytic Cells*

Ali A. R. Rahimi; Niranjala Gajanayaka; Jyoti Mishra; Ashok Kumar
The 50th Anniversary of the Discovery of Interferons (ISICR annual meeting)
Oxford, England, September 16-19, 2007

- *Regulation of the IL-12 Family Cytokines IL-23 and IL-27 in Response to HIV of Human Monocytic Cells*

Ali A. R. Rahimi; Katrina Gee; Niranjala Gajnayaka; Jonathan Angel; Ashok Kumar
Research Conference 2006, Ontario HIV Treatment Network (OHTN)
Toronto, Ontario, Canada, November 27 and 28, 2006

- *Dual Role of IL-10 in HIV Infection: Molecular Mechanisms Responsible for the Enhancement of HIV Replication in Dendritic Cells and its Inhibition in Macrophages*

Ali A. R. Rahimi; Charlene Young; Masoud Ghorbani; Marko Kryworuchko; Ashok Kumar
Research Conference 2005, Ontario HIV Treatment Network (OHTN)
Toronto, Ontario, Canada, November 24 and 25, 2005

- *STAT-1 Mediates the Stimulatory Effect of IL-10 on CD14 Expression in Human Monocytic Cells*

Ali A. R. Rahimi; Katrina Gee; Sasmita Mishra; Wilfred Lim; Ashok Kumar
25th Annual Conference of American Society for Reproductive Immunology
Providence, RI, USA, June 16-18, 2005

- *Prevalence of Hepatitis B Virus Among Laboratory Workers in N. W. of Iran.*

Ali A. R. Rahimi; R. Nategh; N. Rahsaz; N. Ghaem-maghami; A. Ghazanchaee.
9th International Congress on Infectious Diseases
Buenos Aires, Argentina, April 10-13, 2000.

- *Production & Evaluation of Coagglutination Test for Rapid Diagnosis of Vibrio Cholera.*

S. Farajnia; J. Poormajd; **Ali A. R. Rahimi**; M. Asgharzadeh; N. Rahsaz.
The Fourth International Congress of Immunology & Allergy
Isfahan, Iran, May 13-15, 1998

- *Evaluation of Urinary Tract Infections & Drug resistance in Tabriz (1996-1997).*

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10th International Congress of Geographic Medicine & 6th Iranian Congress of Infectious and Tropical Diseases
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- *Prospective study of serological HBV related markers in Donors with positive HBsAg.*

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- *Incidence Rate of Poliomyelitis in Tehran.*

Ali A. R. Rahimi; N. Saboori; H. Tabatabaei; A. Biniaz; R. Nategh
3rd Congress on Infectious & Tropical Diseases
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