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FACULTY OF GRADUATE AND
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Ph.D. (Microbiology and Immunology)

GRADE / DEGREE

Department of Microbiology and Immunology

FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

Regulation of IL-12p40 Expression by HIV-Nef in Human Monocytic Cells

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**Regulation of IL-12p40 Expression by HIV-Nef
in Human Monocytic Cells**

By

Wei Ma

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
Faculty of Medicine
University of Ottawa

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ISBN: 978-0-494-49378-6

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Abstract

Impaired cellular immunity because of dysregulation of Th1 cytokines is a major feature of AIDS. Interleukin-12 (IL-12) is an important Th1 cytokine, which plays a critical role in cellular immunity by producing IFN γ from T and NK cells. Biologically active IL-12 is a 70 kD glycoprotein composed of two disulfide-linked subunits, p35 and p40. The p40 subunit is an appropriate indicator for IL-12 due to its expression in IL-12p70 producing cells including monocytes/macrophages in response to various stimuli, such as bacterial endotoxin, LPS. However, how LPS induces IL-12p40 in monocytes is not clear. A severely decreased IL-12p40 production has been found in PBMCs and macrophages from HIV positive individuals and is believed to be correlated to HIV-associated immunodeficiency. The mechanism underlying the impaired IL-12 production in HIV infected monocytes/macrophages remains unknown. HIV-1 Nef protein, an AIDS pathogenetic factor, has been shown to downregulate Th1 cytokines. In this study, I investigated the potential signaling pathways involved in the downregulation of IL-12p40 production by intracellular Nef in LPS-activated human monocytic cells. LPS activates monocytes by coupling to CD14/TLR4 receptor on the cell surface and activating MAPK pathways, calcium/PI3K pathways and several transcription factors including Ets2, NF κ B, and AP-1. These signaling components have been shown to be implicated in downregulation of IL-12p40 production by several immunosuppressive drugs such as dexamethasone (Dxm), FK506 and cyclosporine A (CyA). By utilizing these immunosuppressive drugs and the specific inhibitors that are involved in MAPKs and calcium/PI3K signaling pathways. I demonstrated that Dxm and FK506/CyA mediated downregulation of IL-12p40 production is regulated by two distinct and independent signaling pathways, JNK and CaMKII activated PI3K pathways in LPS-stimulated human monocytic cells, in which, Dxm regulating via the JNK whereas FK506/CyA regulating via the CaMKII. By extensive mutative analyses of the IL-12p40 promoter, I further demonstrated that the *cis*-binding motifs of transcription factor AP-1 and NF κ B were required for IL-12p40 transcription in both signaling pathways. I then focused on intracellular Nef as a potential repressive molecule in regulation of MAPKs, CaMKII activated PI3K and transcriptional factors involved in LPS-induced IL-12p40 expression. I demonstrated that intracellular Nef downregulates IL-12p40 in human monocytes by specifically inhibition the JNK MAPK and transcription factor NF κ B. These studies may provide new therapeutic tools for the treatment of inflammation and autoimmune diseases and a further understanding for significant functions of intracellular Nef in AIDS pathogenesis.

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Dedicated to

*My Son who provided all the motivation
for completing this thesis.*

Acknowledgments

I would like to sincerely thank my supervisor, Dr. Ashok Kumar for all his guidance, critical insights, constant inspiration and encouragement. This thesis would not have been possible without his great support that I have received throughout all these years.

I would like to sincerely thank my thesis advisory committee: Dr. Kathryn E. Wright, Dr. Francisco Diaz-Mitoma, and Dr. Maya Kozlowski for all the helpful advice. In particular I would like to express my sincere thanks to Dr. Wright for her patient guidance and rewarding discussions.

I would like to sincerely thank Dr. Marko Kryworuchko for spending time reading and correcting my proposals for my course work. I would like to sincerely thank my friends: Sasmita Mishra for taking part in my project and for excellent and skillful laboratory work; Katrina Gee, Wilfrid Lim and Jyoti Mishra for co-authorship and for valuable helps with Western Blot and EMSA; Ali Akbar Rahim Rahami for interesting discussions about all, and Rita Frost for great help with all kinds of administrative issues. I would also like to thank all former and present colleagues at division of virology for numerous help and for making the division a nice place to work in.

I thank my family for their constant encouragement and never ending support through all these years.

These studies were supported by the grants from University of Ottawa and NSERC (Natural Sciences and Engineering Research Council of Canada).

List of Abbreviations

Ab	Antibody
AIDS	Acquired immunodeficiency syndrome
AP1	Activator protein 1
ASK	Apoptosis signal regulating kinase-1
ATP	Adenine triphosphate
ATF	Activating transcription factor 1
bp	Base pair
Bcl2	B cell follicular lymphoma
BclXL	Bcl2-X long form
CAF	CD8+ cell-antiviral factor
CaM	Calmodulin
CAMKII	Calmodulin kinase II
cAMP	Cyclic adenosine monophosphate
CCL4	Macrophage inflammatory protein-1 β
CCL5	Chemokine (C-C motif) ligand 5
CCR5	Chemokine (C-C motif) receptor 5
cGMP	Cyclic guanosine monophosphate
CD	Cluster of differentiation
cDNA	Complementary DNA
C/EBP	CCAAT/enhancer binding protein
CMV	Cytomegalovirus
cpm	Counts per minute
CREB	cAMP-response element-binding protein
CTL	Cytotoxic T lymphocyte
CXCR4	Chemokine (C-X-C motif) receptor 4
CyA	Cyclosporin A
DC	Dendritic cell
Dxm	Dexamethasone
EAE	Experimental autoimmune encephalomyelitis
EBI3	Epstein-Barr virus induced protein 3
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERK	Extracellular-related kinase
Fas	Fibroblast associated
GM-CSF	Granulocyte macrophage – colony stimulating factor
GTP	Guanosine triphosphate
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HRPO	Horseradish peroxidase
IFN	Interferon
IL	Interleukin
IRF	IFN γ regulatory factors
JAK	Janus associated kinase
JNK	c-jun N-terminal kinase

Kb	Kilobase
LPS	Lipopolysaccharide
LTR	Long terminal repeat
mAb	Monoclonal antibody
MAPK	mitogen-associated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPKK kinase
MEK	MAP kinase/ERK kinase
MIP1 α	Murine macrophage inflammatory protein 1 alpha
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
mRNA	Messenger RNA
MyD	Myeloid differentiation factor
MS	Multiple sclerosis
NEF	Negative factor
NF κ B	Nuclear factor κ B
NK	Natural killer
nt	Nucleotides
PARP	Poly-(ADP-ribose)-polymerase
PBMC	Peripheral blood mononuclear cells
PGE2	Prostaglandin E2
PI3K	Phosphatidylinositol 3 kinase
PIC	Preintegration complex
PKA	Protein kinase A
PMA	Phorbol 12-myristate 13-acetate
PTK	Protein tyrosine kinase
RT-PCR	Reverse transcription-polymerase chain reaction
SAPK	Stress-activated protein kinase
SEK	Stress activated protein/ERK kinase
SH2	Src-homology 2 domain
SRE	Serum response element
Stat	Signal transducer and activator of transcription
TGF β	Transforming growth factor- β
Th1	Type1 T helper cells
TNF	Tumor necrosis factor
TRAF6	TNF receptor-associated factor 6
Tyk2	Tyrosion kinse 2
WHO	World health organization

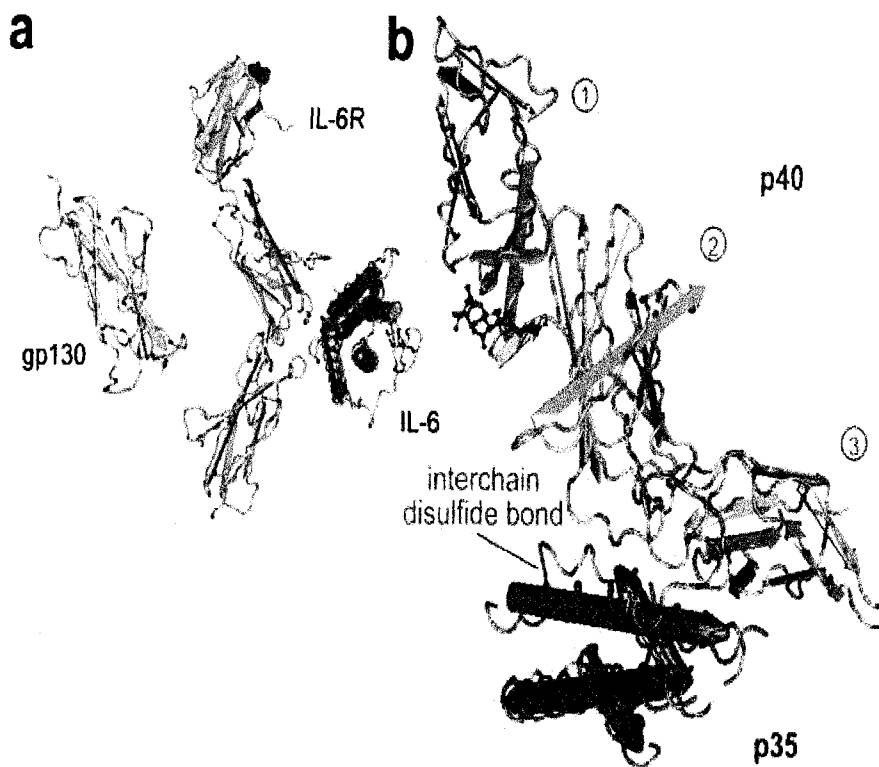
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Chapter I

Introduction

Acquired Immune Deficiency Syndrome (AIDS) caused by Human Immunodeficiency Virus (HIV), was first reported in the United States in 1981 and has since become a major worldwide epidemic [1]. Estimated by the end of the year of 2006, nearly 50 million people are living with the HIV virus and 28 million people have died from AIDS [2]. HIV is a subgroup of retroviruses known as lentivirus [3]. This family of viruses is known for their latency, persistent viremia and attacks on the host immune system [4]. HIV has high affinity for CD4+ T lymphocytes and monocytes/macrophages. The virus replicates itself in these cells by generating a DNA copy by reverse transcriptase. Viral DNA becomes incorporated into the host DNA, enabling further replication [5].

Depletion of CD4+ T cells has been considered as a hallmark of disease early on and in the late stages of AIDS [6]. The median time from infection to AIDS diagnosis now exceeds 10 years [7]. Like CD4+ T cells, monocytes/macrophages also play a crucial role in the generation and regulation of immunity. Moreover, in HIV-1 infection and AIDS development, monocytes/macrophages play dual roles. They represent a viral reservoir acting as primary target and viral carriers, and trigger innate and adaptive immunities as primary effectors. Unlike T cells, infection of macrophages with HIV-1 is not cytopathic; therefore they may serve as long-lived reservoirs during all stages of HIV-1 infection. Monocytes/macrophages perform their effects by secreting cytokines, the potent modulators of immune system that play a critical role in HIV immunopathogenesis [8;9]. In HIV infection, cytokines have been demonstrated to induce differentiation and cellular responses of specific target cells and contribute to the pathogenesis of AIDS [10]. HIV-1 positive patients have a progressive loss of type 1

cellular immune responses because of decreased secretion of Th1 cytokines including heterodimeric IL-12 family, and increased Th2 cytokines, IL-4 and IL-10. This leads to impaired antiviral immune responses and development of AIDS [11;12].

IL-12 plays a critical role in the development of cell mediated immunity against intracellular infections caused by bacteria, parasites and viruses including HIV [13]. IL-12 is a 70 kD regulatory cytokine composed of two disulfide-linked subunits p35 and p40 which are encoded by two distinct differently regulated genes. IL-12p40 is also shared by another Th1 cytokine, IL-23. The p35 is consistently expressed in various cell types, whereas p40 is inducible in bioactive IL-12p70 producing antigen presenting cells and is transcriptionally regulated. The p40 subunit, hence, is considered as an indicator of biologically active IL-12 [14;15], however, at present, is not well understood the regulation of IL-12p40 in human monocytes/macrophages.

In HIV infection, a dramatic decrease in IL-12p40 production has been found in PBMCs from HIV positive individuals and viral infected monocytes in vitro [16;17]. This impaired IL-12p40 production has been considered to be correlated with HIV-associated immunodeficiency [18;19]. We and others have also shown that exogenous IL-12 enhances IL-2 production, cell proliferation and development of cell mediated cytotoxicity of HIV antigen stimulated PBMC from HIV positive individuals [20-22]. Although the mechanisms responsible for IL-12p40 inhibition in HIV infection is not clear, it has been recently shown that IL-12p40 production is suppressed following infection of monocytic cells with wild type SIV compared to the cells infected with Nef-deficient strains [23]. Nef is a 27 kD myristoylated protein. Besides its well known functions of downregulating the cell surface molecules CD4 and MHCI, Nef can

uniquely interact with numbers of intracellular signaling molecules through its myristoyl moiety, leading to the dysregulation of host immune responses [24-26]. Therefore, I hypothesized that by interfering with signaling molecules, such as MAPKs and transcription factors, NF κ B and AP-1, Nef may negatively regulate IL-12 production in human monocytic cells, leading to the dysfunction of Th1 immune responses. Thus, my thesis project deals with the signaling pathways involved in induction of IL-12p40 and how HIV-Nef regulates IL-12p40 in human monocytic cells.

1. Interleukin-12 (IL-12)

IL-12 is an important immunoregulatory cytokine produced primarily by monocytes/macrophages, dendritic cells and B cells. The major functions of IL-12 include induction of IFN γ production from natural killer (NK) cells and T cells, enhancement of cytotoxicity of NK cells and cytotoxic CD8⁺ T cells (CTLs) and differentiation of naïve T cell into Th1 effectors. Therefore, IL-12 plays a critical role in the development of cell-mediated immunity against intracellular infections caused by mycobacterium, parasites and viruses, including HIV [13;27]

1.1. Structure of IL-12

IL-12 is a 70 kD heterodimeric protein folding into a bundle of four alpha-helices and composed of two disulfide-linked subunits, designated p35 and p40. Both subunits are required for the biological activity of IL-12. The genes encoding the human p40 and p35 subunits are unrelated, map to chromosomes 5q31-33 and 3q12-3q13.2, respectively, and bear no apparent homology [28]. However, p35 shares homology with other single-chain cytokines such as IL-6 [29], whereas p40 is homologous to the hematopoietin receptor family, particularly the extracellular domain of the IL-6 receptor α chain [30]

(Fig. 1.1-1). This sequence similarity suggested that IL-12 is evolutionally a common primordial cytokine and structurally linked to a soluble cytokine-cytokine receptor complex [29]. The genes encoding the murine p40 and p35 exhibit 70 and 60% sequence homology to the human p40 and p35 genes, respectively [31]. IL-12p40 is expressed and secreted as monomer and homodimer [32;33]. The p40 monomer seems to have no biological function, while the homodimer serves as a natural antagonist of IL-12p70. There is evidence to suggest that p40 homodimers block the activities of IL-12p70 via competitively binding to the IL-12 receptor and reducing IL-12p70-mediated Th1 responses in vivo [34]. The p40 homodimers have also been shown to enhance alloantigen-specific Th1 development [35], suggesting that p40 homodimers may function similarly to the biologically active IL-12p70 under certain conditions.

Recently, two other heterodimeric cytokines, IL-23 and IL-27 have been grouped together as the IL-12 family and contribute to the development of Th1 responses [36]. IL-23 is a heterodimer of IL-12 p40 and p19 that is homologous to p35 [37]. Compared to IL-12, IL-23 is a late-phase factor in Th1 responses by inducing the proliferation of memory CD4⁺ T cells [38], and maintenance of CTL responses [39]. In contrast to IL-12, IL-23 promotes presentation of tolerogenic peptides by APCs [40] implicating a causal relationship in autoimmune disease states. IL-27 is a heterodimer of EBI3 and p28. EBI3 is homologous to IL-12p40 and p28 is structurally related to IL-12p35. IL-27 has been shown to stimulate naïve T cell differentiation in the early phase in the presence or absence of IL-12. IL-27 also induces the expression of functional IL-12 receptors in naive CD4⁺ T cells, making these cells sensitive to IL-12-mediated Th cell development [41].

Fig. 1.1-1 IL-12p70 complex and their structural evolution:

Ribbon diagram of the IL-12p70 complex and their structural evolution. IL-12 resembles a soluble class 1 cytokine-receptor complex. IL-12p35 subunit with 4 helices shares homology with four helix bundle cytokines represented by IL-6 (green). IL-12p40 subunit with three domains labeled 1–3 is most resembles the extracellular domain of the IL-6R-subunit. Both subunits of the IL-12R belong to the gp130 subgroup of the cytokine receptor superfamily. The interchain disulfide bonds are shown in red [42].

1.2. IL-12 Receptor and Signal Transduction

The biological activities of IL-12 are mediated by binding to its specific cell surface receptors through tyrosine kinase 2 (Tyk2)/ Janus associated kinase 2 (Jak2)/ signal transducer activator transcription 4 (Stat4) signaling pathway. The functional IL-12 receptor is a heterodimer composed of IL-12R β 1 and IL-12R β 2 chains which are structurally related to the type I cytokine receptor superfamily and are homologous to gp130 [43;44]. The IL-12R β 2 chain is a signaling component of the receptor expressed on NK cells and activated T cells and functions as a molecular switch in determining T cell phenotype. It is upregulated by cytokines that promote Th1 cell development and inhibited by those that promote Th2 cell development. The IL-12R β 1 is a type I transmembrane protein with a disulfide-linked oligomer, which is required for binding of IL-12 [45;46]. The intracellular signaling pathway of IL-12 involves 2 members of the Jak family, Jak2 and Tyk2 and several members of the Stat transcription activator family, including Stat1, Stat4, and Stat5 [47;48]. Among the Stats that are involved in IL-12 signaling, Stat4 plays a critical role. The binding of IL-12 to its receptors induces the phosphorylation of Tyk2/Jak2, which further phosphorylates Stat4 and results in expression of IL-12 responsive genes encoding IFN γ (Fig. 1.1-2). Human and mice lacking either IL-12R β 1/ β 2, Tyk2/Jak2 or Stat4 showed an impaired IL-12 signaling in T cells and NK cells [49;50].

1.3. Biological Functions of IL-12

The Role in Cellular Immune Responses

The most important function of IL-12 is its ability to produce IFN γ , through which, IL-12 regulates Th1 and Th2 responses. IL-12 produces IFN γ in several ways:

promotes the differentiation of naïve T cells directly towards Th1 effectors that enable them to produce IFN γ [51;52]; acts as a costimulator to enhance the differentiated Th1 cells to produce IFN γ ; induces the switch of IFN γ -producing memory Th1 cells to their effective stages and enables them to secrete IFN γ [53] and activates NK cells to produce IFN γ . IL-12 induces IFN γ in NK cells and T cells through Jak2/STAT4 signaling pathway. The binding of IL-12 to its receptor results in the phosphorylation of Jak2 followed by STAT complexes, which translocate to the nucleus and bind to the transcription coactivator elements to stimulate transcription of the IFN γ gene. [54;55].

IFN γ is a critical mediator in the development of Th1 cellular immunity. It regulates Th1/Th2 responses through upregulation of Th1 cytokines IL-2 and IL-12 and downregulation of Th2 cytokines, IL-4 and IL-10 [56]. The roles of IFN γ in Th1 cellular immunity against various invading pathogens, in particular viruses, are shown to be decisive for the outcome of disease and virus clearance. Investigators have demonstrated that virus-specific CTL and NK cells against various viral infection are depending on their ability to produce IFN γ [57;58]. Antiviral activity of IFN γ is mediated by its receptors and the Jak2/Stat1 signaling pathway, through which, IFN γ induces a number of antiviral protein expression including nitric oxide (NO), RNA-dependent protein kinase (PKR) and IFN γ regulatory factors (IRFs) that, in turn mediate antiviral effects by interfering with regulation of synthesis and degradation of both viral and host immune molecules [59;60]. Mice deficient in IFN γ and its receptors, or the signaling molecules Jak2/Stat1 have severely impaired viral specific Th1 immune responses. Besides its Th1 activity, IFN γ is also a macrophage-activating cytokine [61]. Thus, IFN γ represents the first line of host defense and serves in both innate and adaptive immunity.

Enhancement of differentiation and cytotoxicity of NK cells and CTLs

Similar to induction of IFN γ , IL-12 also promotes differentiation of NK cells and CTLs and enhances their cytotoxicity by the Jak2/Stat4 signaling pathway [62]. IL-12 acts as an autocrine growth factor with its receptors on NK cells and T cells, leading to IL-12 responsive gene products, such as IFN γ . In addition, IL-12 has been shown to serve as a direct chemotactic factor for NK cell infiltration, further increasing their cytotoxic lysis [63].

NK cells are important effectors of immune system, particularly in innate host defense against viruses and tumors. Without prior priming, NK cells can directly lyse virus infected cells, tumor cells and other target cells by releasing the perforin and granzyme-containing granules. NK cells also produce a variety of regulatory cytokines including IFN γ , TGF β 1, TNF α , IL-1 β , IL-10, G-CSF and GM-CSF, and CC-chemokines, such as RANTES, MIP-1 α and MIP-1 β , which are involved in antigen-specific immune responses in the elimination of intracellular pathogens. Hence, NK cells are also critical regulators of both innate and adaptive immune responses [64]. Of note, the direct killing without prior priming is the main advantage that NK cells have over antigen-specific lymphocytes in antiviral immunity. This enables NK cells to exert their effect early in the course of viral infection. NK cells thus are particularly crucial in the clearance of viral infections [65;66].

The critical role of CTLs in controlling viremia has been well demonstrated in CMV or EBV infection. CTLs lyse antigen-bearing target cells through granule- and Fas-mediated apoptosis: granule exocytosis targets any antigen-bearing cell [67] whereas Fas-mediated cytotoxicity kills only Fas-expressing cells and does not require antigen

expression [68]. Although the cytolytic role of CTL in HIV infection remains elusive, its product IFN- γ has also a negative correlation with the plasma viral loads. HIV specific CTLs are able to suppress viral replication in CD4⁺ infected T cells by a non-cytolytic mechanism, involving secreted CD8⁺ cell-antiviral factor (CAF) [69]. In addition, HIV specific CTLs produce TNF α and chemokines such as CCL4 and RANTES (CCL5), which are critical in suppression of viral entry and replication [70].

The Role in Humoral Immunity

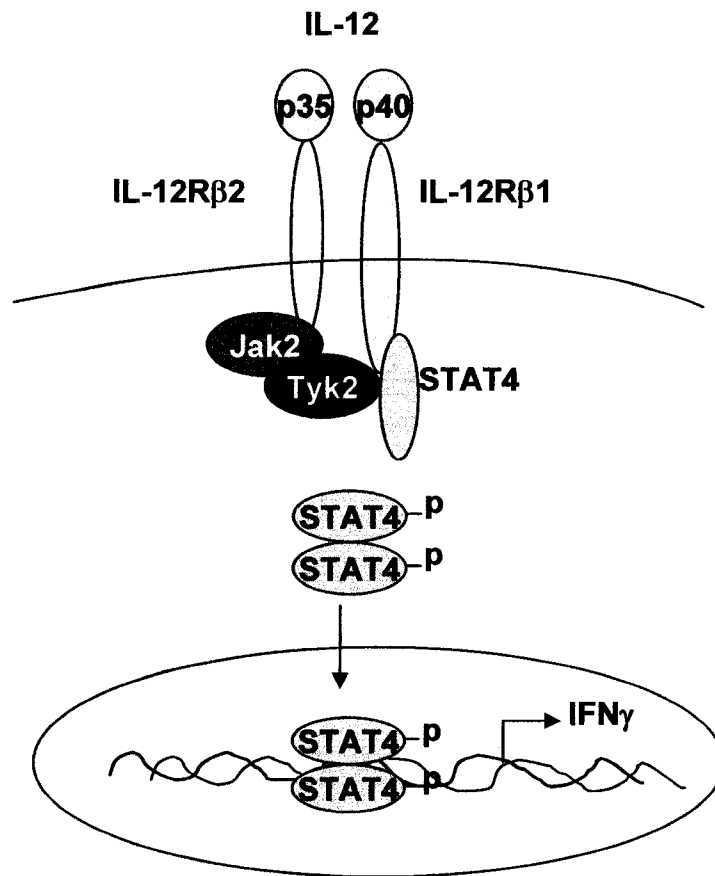
Studies have shown that although IL-12 might directly affect B-cell proliferation, differentiation and IFN γ production [71], the effect of IL-12 on B-cell activation and production of particular immunoglobulin isotypes (enhanced IgG1, IgG2a and decreased IgE, IgA) are mainly mediated indirectly by its product, IFN γ . In addition, IL-12 synergizing with IL-18 induces IFN γ in Th1 cells and B cells, leading to Th1 type of IgG2a switching in B cells [72;73].

In Infectious Diseases and Autoimmune Disorders

Because of its ability of inducing cell-mediated Th1 immune response, IL-12 is central in the control of various intracellular infections caused by intracellular pathogens including *Leishmania major*, *Toxoplasma gondii*, *Trypanosoma cruzi*, *Cryptococcus neoformans* and *mycobacteria* [74-78]. During these infections, early produced IL-12 by monocytes/macrophage and dendritic cells is prerequisite for developing protective cellular Th1 immune response [79]. The importance of IL-12 in cell-mediated immune responses to these pathogens is highlighted in humans and mice with deficient IL-12 or IL-12R genes. This leads to dangerous defects in cellular immunity and increased susceptibility to those intracellular pathogens [80;81]. IL-12, therefore, has been considered as a Th1

Fig. 1.1-2 The IL-12/IL-12R and signal transduction

The IL-12R comprises of two components, the IL-12R β 1 and the IL-12R β 2. Both receptor subunits belong to the gp130 subgroup of cytokine receptors. The biological activity of IL-12 at its receptor complex requires the initial interaction of IL-12p40 with IL-12R β 1 and the interaction of IL-12p35 with IL-12R β 2. The IL-12R β 2 subunit appears to function as the signal transducing component of the high-affinity receptor complex. The interaction of IL-12 with its receptor induces the phosphorylation of Jak2 and Tyk2, which in turn phosphorylate and activate Stat1, Stat3, Stat4 and Stat5, but the cellular effects of IL-12 are mainly mediated by Stat4 phosphorylation. Adopted from [82].



adjuvant in the treatment of these intracellular infections.

In contrast, IL-12-induced Th1 cellular immune responses could be harmful to certain autoimmune disorders, such as endotoxin induced shock, multiple sclerosis (MS), rheumatoid arthritis, inflammatory bowel and graft versus-host disease. In these cases, an increased IL-12-dependent Th1 immune response appears to be pivotal to the disease progression [83;84]. Recent studies indicated that IL-12-induced Th1 responses initiate allograft rejection by promoting cytotoxic T-cell activities and IFN γ -mediated delayed-type hypersensitivity reactions [85], while IL-12 knockout mice are resistant to experimental autoimmune encephalomyelitis (EAE) [86]. In this regard, a variety of immunosuppressive drugs including dexamethasone (Dxm), chemically unrelated immunosuppressant FK506 (tacrolimus) [87] and cyclosporine A (CyA) have been utilized to prevent Th1-dependent immune disorders. Dxm is a glucocorticoid which regulates IL-12R expression and IL-12 responsiveness, and is important in the balance of Th1- and Th2-type responses [88]. CyA is a cyclic polypeptide produced as a fungal metabolite and consists of 11 amino acids whereas FK506 is a macrolide lactone [89]. By interacting with their own binding proteins, cyclophilin A and the 12 kDa FK506-binding protein, FKBP12, CyA and FK506 bind to the same target, calcineurin. CyA and FK506 have been shown to suppress activation of NFAT in T cells through calcium signals[90;91], and to suppress production of Th1 cytokines including IL-12 in LPS-stimulated myeloid DCs [92] and in heart allografts [93]. However, how Dxm, FK506 and CsA suppress the Th1 responses through the regulation of IL-12 remain to be elucidated.

The role of IL-12 in viral infections is also controversial. IL-12-mediated effects can be either protective or detrimental, depending upon the type of infection [94]. For example, endogenous IL-12 was induced during influenza pneumonia [95], while decreased IL-12p40 production in macrophages from measles virus infection directly attributed to the cell mediated immunosuppression [96]. In HBV transgenic mice, IL-12 treatment induced IFN α/β and IFN γ , inhibiting hepatitis B virus replication in liver and kidney [97]. IL-12 also exhibits potent antiviral activities for herpes simplex virus by inducing IFN γ [98]. In HIV infection, PBMC and macrophages from HIV-1-infected patients have significantly impaired IL-12 and IL-12p40 production [16]. However, how IL-12 is inhibited in HIV infection remains unclear.

1.4. Regulation of IL-12

Positive Regulation

In a T cell-independent manner, killed and live bacteria, and some of their specific products including bacterial cell wall components (such as LPS, Peptidoglycan), CpG containing bacterial DNA, bacterial flagellin, viral RNA, intracellular parasites and fungi that signal Toll like receptors (TLRs) strongly induce IL-12 in monocytes/macrophages and dendritic cells [99]. However, the mechanism of IL-12 induction in response to these stimuli is not well understood. I have investigated the signaling pathways involved in IL-12 production in response to LPS in human monocytes [100]. IL-12p40 is also produced during the antigen-specific phase of the immune response in a T cell dependent manner. It occurs when T cells meet their cognate antigen presented by activated macrophages or dendritic cells followed by signaling via the TCR, which results in the up-regulation of

CD40 ligand. The interaction of CD40 ligand with CD40 on the macrophage or dendritic cell activates IL-12 production [101].

In addition, IL-12 production is upregulated by IFN- γ in a positive feedback mechanism, in which, IL-12 promotes NK cells and T cells to secrete IFN- γ , which in turn activates monocytes/macrophages for further IL-12 production [102]. This positive feedback loop is required for optimal production of IL-12 for certain pathogens who induce low levels of IL-12, such as *Leishmania major* or Bacille Calmette-Guerin [103]. IFN γ performs this effect by activation of transcription factors, IRFs. Among the identified eight members of IRFs (1 to 8), IRF1, 2 and 8 are believed to be involved in the regulation of p35 and p40 gene transcriptions and this is proved in animal studies [104-107].

Negative Regulation

Although IL-12 is important for host defense; overexpression of IL-12 could cause persistent inflammation leading to autoimmune disorders such as multiple sclerosis. Therefore appropriate downregulation of IL-12 is equally important in protecting the host from harmful immune responses. Among the known negative factors, the Th2 cytokine IL-10 is the most potent inhibitor of IL-12 production from phagocytic cells both at the mRNA and protein levels [15;108]. IL-10 inhibits IL-12 by decreasing the activation of NF κ B and AP-1 [109;110] and the association of IL-12p40 promoter to RNA polymerase [111]. In IL-10 knockout mice, the serum IL-12 levels are positively correlated with prolonged survival in viral infection by increasing Th1 response and antiviral infection activity. IL-10 knockout mice infected with *Toxoplasma gondii*

showed an uncontrolled production of IL-12 and sensitivity to LPS [112]. IL-12 production is also inhibited by transforming growth factor (TGF β) [113], IL-11, IL13, INF- α/β [114;115], prostaglandin E2 (PGE2), histamine, FcR, measles receptor CD46 and cholera toxin [116] (Fig. 1.1-3 A).

Regulation of IL-12 Gene Transcription

IL-12p35

Although p35 is widely expressed in cells, analysis of p35 regulation has been difficult, due to its expression at low levels and only secreted in order to dimerize with the p40 subunit to form the bioactive IL-12p70. Evidence suggests that the expression of p35 is regulated at both levels of transcription and post-transcription [117]. Human and murine p35 promoters contain binding sites for several transcription factors that have been shown to be involved in IL-12p35 transcription. These transcription factors include IRF-1, IRF-3, SP1, IRF8 and NF κ B family members c-Rel and p65 [107;118].

IL-12p40

IL-12p40 gene is highly inducible and expressed in IL-12-producing cells; therefore, most regulatory factors which influence IL-12p70 expression have similar effects on p40 gene expression. Analysis of transcription and translation levels of IL-12 has demonstrated that the human IL-12p40 gene is primarily regulated at the transcriptional level. The transcription of endogenous p40 is cell type specific. IL-12p40 promoters linked to a luciferase reporter gene has been transfected into various IL-12-producing and non-producing cell lines. The results suggest that it is constitutively active in EBV-transformed B cell lines (RPMI-8866, CESS), and inducible in myeloid cell lines (THP-1 and RAW 264.7), but inactive in T cell lines (Molt-13 and Jurkat) [17].

Fig. 1.1-3 Regulation of IL-12 production

A, Regulation of IL-12 production: Various pathogenic organisms induced high levels of IL-12 production, including bacterial cell wall components, LPS and PGN, CpG containing bacterial DNA, bacterial flagellin, viral RNA, intracellular parasites and fungi. IL-12 is also produced in a T cell dependent manner through the engagement of CD40 on antigen presenting cells with its ligand CD40 on T cells. IL-12 production is also unregulated by IFN γ in a positive feedback loop. IL-12 is also negatively regulated by endogenous molecules, IL-10, TGF β , IFN $\alpha\beta$, 1.25-dihydroxyvitamin D3 and PGE2; the pathogens HIV, Measles, Leishmania and receptor ligations, CD46 and Fc γ R. Adopted from [31]

B, Regulation of *IL-12p40* gene transcription: *IL-12p40* gene is regulated at the level of transcription. The human *p40* promoters have been studied and several putative binding motifs have been demonstrated for the corresponding transcription factors including C/EBP, NF κ B, PU.1, Ets2, SP-1, NF-IL6 and IRF. Adopted from [31].

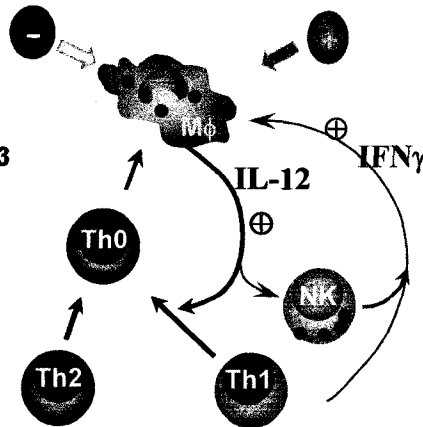
A, Regulation of IL-12 production

Negative feed back

Endogenous molecules:
IL-10, TGF- β , IFN- $\alpha\beta$
1.25-dihydroxyvitamin D3
PGE2

Pathogens:
HIV, measles, virus,
Leishmania,

Receptor ligation:
CD46, Fc γ R,



Positive feed back

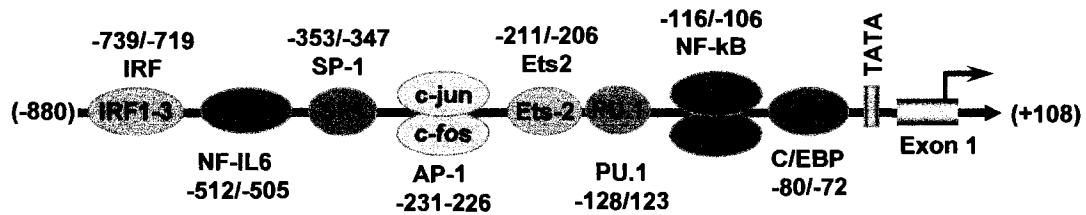
Microbial products:
LPS/CD14

Lipotoichoic acid/CD14

Inflammation:
Hyaluronan/CD44

Immune response:
CD40/CD40L

B, Regulation of IL-12 Gene Transcription



The regulation of the p40 promoter is very complex and involves multiple transcription factor binding elements (Fig. 1.1-3 B) [31]. Characterization of the IL-12p40 promoter reveals an NF κ B element which is found to be necessary for induction of promoter activity by LPS both in humans and mice [119]. Subsequently, other transcription factors such as Ets-2 (a member of the Ets family of transcription factors), PU-1, IRF1, IRF2, IRF8 and c-Rel are demonstrated to be involved in regulation of IL-12p40 transcription by LPS [105;120;121]. Studies with the murine IL-12p40 promoter indicate that both C/EBP and AP-1 elements have a crucial role in IL-12p40 promoter activation via a c-Fos- and c-Jun-based mechanism [122]. Furthermore, the murine IL-12 p40 promoter harbors a positioned nucleosome called nucleosome 1 in the proximal promoter region encompassing the NF κ B, C/EBP, AP-1 and IRF8 sites. Upon activation with LPS and IFN- γ , nucleosome 1 was selectively remodelled [123]. In addition, a GATA sequence in the IL-12 p40 promoter (GA-12) is the only known repressor element in the IL-12p40 promoter that has been reported. The GA-12 element is located between the NF κ B and the Ets sites and occupied by a GA-12 binding protein (GAP12) in unstimulated cells. GAP12 suppresses IL-12p40 transcription through binding to two known inhibitors of IL-12, IL-4 and PGE2 [124].

1.5. Therapeutic Potential of IL-12 in HIV-1 Infection

Studies indicated that natural IL-12 could provide a regulatory link between innate resistance and the development of the antigen-specific adaptive immune response. Moreover, 1) The recombinant IL-12 also has therapeutic potential because of its activity against tumors and infections, and its effectiveness as an adjuvant enhancing cell-mediated immunity in vaccination; 2) IL-12 is deficient in activated

monocytes/macrophage and T cells from HIV-1 positive individuals; 3) It restores IFN γ secretion by macrophages of HIV-1 infected individuals; 4) It can act in combination with other cytokines to inhibit the expression and infectivity of HIV-1 in macrophages; 5) It induces the expansion of T cells that secrete putative immunoregulatory factors able to protect against HIV-1 infection and finally, and 6) It has been used as a potential therapeutic factor in infectious diseases and in cancer patients. Thus, IL-12 could be a potent candidate as adjunct immunotherapy to increase vaccine efficacy in both healthy and immunocompromised populations, and in its potential future clinical use to inhibit HIV-1 replication *in vivo*.

2. HIV-Nef Protein

HIV-1 is a compact lentivirus consisting of nine open reading frames encoding three structural proteins, Gag, Pol, and Env that are incorporated into virus particles, and six regulatory proteins (Tat, Vif, Rev, Nef, Vpr, and Vpu) that are required for a growth process such as gene expression (Fig. 1.2-1A). Nef is a key factor for viral infectivity and pathogenicity [125]. It is abundantly produced in the early stage of infection by all lentiviruses and predominantly resides in the cytoplasm, plasma membranes, nucleus and nuclear membrane. Nef can also exist in the soluble form and can be detected in serum from HIV patients [126]. The well known functions of Nef are downregulation of the surface expression of CD4 and MHC I molecules, enhancement of viral replication and infectivity, and modulation of cellular signal transduction pathways [127;128].

2.1. Structure of Nef

General structure

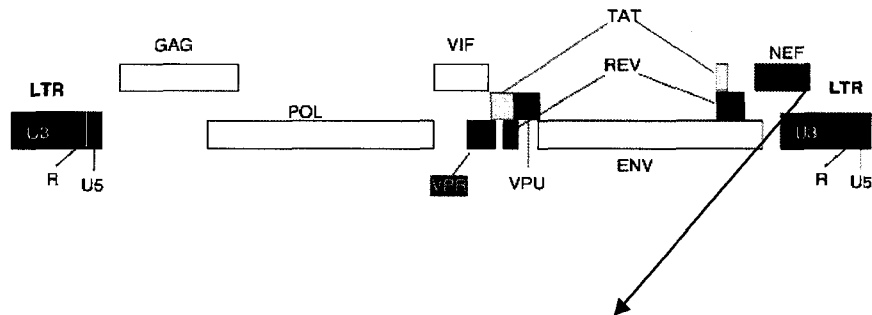
Fig. 1.2-1 HIV genome and the structure model of the full length, myristoylated HIV-1 Nef protein, strain NL4-3:

A, HIV-1 genome: The integrated form of HIV-1 is approximately 9.8 kilobases in length.(1) Both ends of the provirus are flanked by a repeated sequence known as the long terminal repeats (LTRs). The genes of HIV are located in the central region of the proviral DNA and encode at least nine proteins including structure proteins Gag, Pol, and Env and regulatory proteins which are Tat, Rev, Vpu, Vpr, Vif, and Nef [129].

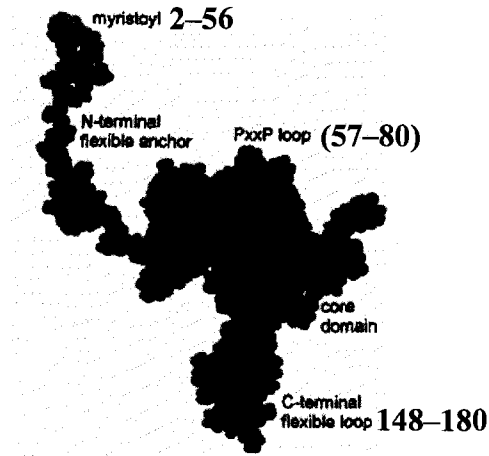
B, Molecular surface presentation. Three flexible regions within the Nef structure are highlighted: the myristoylated anchor domain (myr2–56, light blue), the PxxP loop (57–80, middle blue) and the C-terminal flexible loop (148–180, dark blue). The N-terminal myristoyl moiety is labeled [130].

C, Functional motifs of Nef: Basrd on tne full-length structure assembly of HIV Nef, motifs involved in Nef-mediated CD4, MHC I and CD1d downregulation have been demonstrated. Adopted from [131;132].

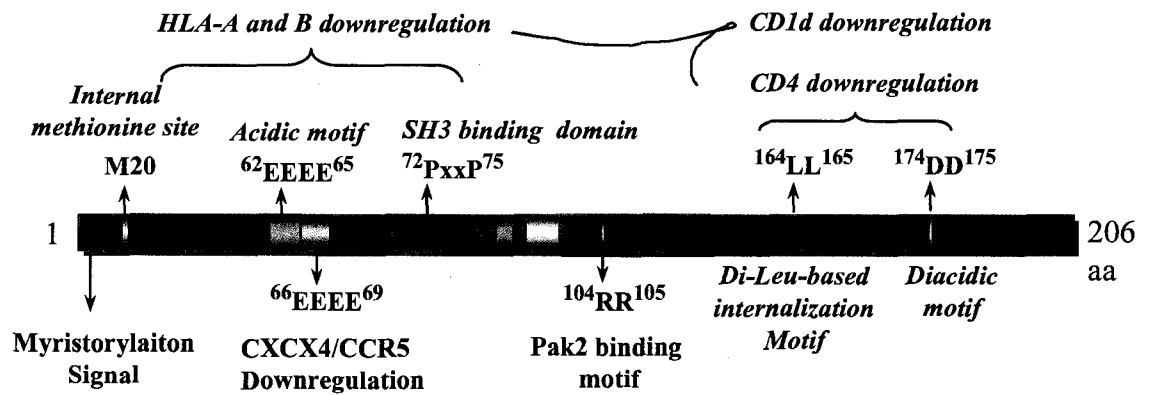
A



B



C



The Nef open reading frame is located at the N-terminal end of the viral genome, partially overlapping the N-terminal long terminal repeat (LTR). It exhibits sequence polymorphism, and is actively transcribed early after infection and translated from multiple spliced viral mRNA [133]. HIV-1 Nef is a 27 kD, myristoylated protein of 206 amino acids, whereas Nef protein of HIV-2 and SIV has an additional C-terminal sequence of 10-30 amino acids long. HIV-1 Nef can also be translated from an internal AUG 57 base pairs downstream from initiating AUG, resulting in the production of a truncated, non-myristoylated 25 kD protein [134]. Nef is also phosphorylated on both tyrosine and serine residues and the role of this modification is ambiguous [135]. The Nef protein contains two major domains: the N-terminal myristoylated anchor domain (residues 1-57) and the core domain (residues 56-206) [130;136]. By using X-ray crystallography and nuclear magnetic resonance (NMR)-spectroscopy, the structure of a C-terminal core domain and partial N-terminal anchor domain of Nef have been elucidated, but as yet there is no information about the structure of the full-length myristoylated form. The core domain contains a type II polyproline helix located at residues 70-77 which is followed by a central anti-parallel β -sheet of four strands flanked at the N-terminal end by two long anti-parallel α -helices (residue 80-120) and at the C-terminal end by two short α -helices between residues 187-203. The first three helices of Nef form an opening cavity for easy interaction with various components. In addition, Nef has two large flexible regions: the first one is 56 amino acids at the N-terminus and a second one is a 30 amino acid extended loop (residues 148-178) projected out of the core domain. The N-terminal membrane anchor domain is diverse and structurally flexible containing two α -helices which are stabilized by myristoylation and are involved in

membrane binding. In fact, virtually all biological activities of Nef require its association with cellular membranes which is achieved by N-terminal myristoylation of the protein [137-149] (Fig. 1.2-1.B). A series of sequential motifs have been identified in Nef that are highly conserved among Nef alleles of all HIV-1 subtypes and are at different stages of disease. These motifs also mediate a multitude of interactions in protein modification, trafficking and signaling events (Fig. 1.2-1 C).

Functional Motifs

In Intracellular Signals

An N-terminal proline rich motif known as ⁷²PxxP⁷⁵ represents the main binding site for the Src family kinases and mediates the interaction between Nef and the SH3 domain of the Src family kinases including Hck, Lck, Lyn, Fyn and other tyrosine kinases [140-143]. This motif also interacts with several serine/threonine kinases, including p21-activated protein kinase (PAK), θ isoform of protein kinase C, Vav and MAPKs [11;144-147]. This proline rich motif thus is central to many Nef-induced biological effects. Recently, two 11-mer peptides spanning motifs, ⁶⁶EEEE⁶⁹ have been shown to be required for downregulation of chemokine receptors CCR5 and CXCR4 [148].

In Downregulation of CD4

CD4 downregulation is important to viral replication in activated T cells [149]. Five sequence motifs within N-terminal and core domains are identified to be involved in CD4 downregulation. The first motif is the myristoylation signaling sequence that anchors Nef to the membrane where Nef directly binds to cytoplasmic tail of CD4 through the second motif, centered on residues 57-59, targeting CD4 into clathrin coated

pits (CCPs). Thereafter, three other binding motifs in the loop region of the core domain continually direct the Nef/CD4 complex into the endocytotic pathways. The first one, a di-Leu-based internalization motif (¹⁶⁴LL¹⁶⁵) in the middle of the loop connects Nef with clathrin-associated adaptor protein (AP) complexes and recruits CD4 molecules into clathrin-coated pits (COP). Simultaneously, Nef also interacts with a subunit of the v-ATPase via a second diacidic motif, (¹⁷⁴DD¹⁷⁵) located at the C-terminal end of the loop that may facilitate AP2 recruitment. This is followed by the association of Nef with β -COP coatomers in the endosome through a third diacidic motif, ¹⁵⁴EE¹⁵⁵, which is located at the N-terminal end of the loop. Together, this directs CD4 to lysosome, where CD4 is degraded [138;139].

In Downregulation of HLA-A and B

Selective downregulation of HLA-A and B alleles is critical in viral immune evasion. Recently, the mechanism of downregulation of HLA-A and B alleles has been demonstrated in great detail. Three Nef motifs: acidic cluster ⁶²EEEE⁶⁵, ⁷²PxxP⁷⁵, and M²⁰ have been identified to be specifically required for this downregulation in an endocytosis/trans-Golgi network (TGN) pathway through a Nef/PACS-1/ARF6/PI3K axis [150-152]. This process is initiated by the binding of Nef to the cytoplasmic tail of HLA-A and B under the regulation of PI3 kinase whose activation requires the M²⁰ motif at the N-terminal of Nef. Subsequently, Nef links HLA-A and B to the trafficking proteins AP-1 and PACS-1 (adaptor protein-1/phosphofurin acidic cluster sorting protein-1) through its acidic cluster ⁶²EEEE⁶⁵. This is followed by ⁷²PxxP⁷⁵ mediated activation of ARF6 (ADP ribosylation factor 6), the activated ARF6 triggers transport of HLA-A and B from the cell surface to TGN through the ARF6 compartment in vesicles

[152;153]. In the TGN, HLA-A and B are degraded by endocytosis and Nef recycles back into the cytoplasm.

In Downregulation of CD1d

More recent studies showed that the four motifs of Nef are involved in downregulation of a non-classical MHC-I like molecule, CD1d by TGN internalization in a distinct but shared pathway with MHC-I and CD4 downregulation. This effect depends on a tyrosine-based motif present in the CD1d cytoplasmic tail as well as the actions of four Nef motifs including ⁶²EEEE⁶⁵, ⁷²PxxP⁷⁵, ¹⁶⁴LL¹⁶⁵ and ¹⁷⁴DD¹⁷⁵. These motifs are also known to be involved in the downregulation of MHC-I or CD4 as discussed above. Downregulation of CD1d molecules by Nef could affect NKT cell recognition, which may lead the virus to directly attack CD1-mediated immune responses, as well as avoid any virus-associated lipid antigen becoming a target for CD1-restricted NKT cells [154].

2.1. Biological Functions of Nef

The Nef protein has no known catalytic function. However, it is believed to promote viral pathogenicity by altering signaling pathways in infected cells through its interactions with cellular signaling proteins, such as Src tyrosine kinase family members including Hck, Lck, Lyn, Fyn and c-Src itself [141;142]. As mentioned earlier, Nef also interacts with several serine/threonine kinases, including p21-activated protein kinase (PAK), θ isoform of PKC, Vav, and MAPKs [144;147;155;156]. The interaction of Nef with these kinases can affect multiple cellular processes, leading to dysfunction of monocytes/macrophages, activation of T cells and dysregulation of cytokines, resulting in enhancement of viral replication, immune evasion, and enhanced survival in T-cells and macrophages [157].

2.2.1. Dysfunction of Monocytes/Macrophages

Monocytes/macrophages serve as antigen presenting cells and secrete inflammatory mediators to activate innate and adaptive immune cells. A growing number of studies implicate HIV-infected monocytes/macrophage as key factor in AIDS pathogenesis by representing a viral reservoir acting and by triggering innate and adaptive immunities [158]. HIV-1 Nef, as an essential factor for efficient viral replication and pathogenesis has been shown to alter monocytes/macrophages biology via mediating a variety of signaling molecules [159]. For instance, Nef can interact with Hck kinase that is strongly expressed in cells of the monocyte/macrophage lineage [160] and is found to be closely correlated with high titer replication of HIV-1 [161] and essential for AIDS [162]. In addition, Nef has been shown to impair abilities of phagocytosis and antigen presentation of monocytes/macrophages [163]. Nef expression in both monocytes and macrophages is implicated in regulation of $\text{TNF}\alpha$, $\text{IL-1}\beta$, IL-6 , and IL-10 , which are tightly connected to the vital specific immunodeficiency and viral replication [164;165]. Nef expressing macrophages also secrete a high level of $\text{MIP1}\alpha$ and $\text{MIP1}\beta$, leading uninfected cells to sites of infection, thus increasing viral infectivity [166].

2.2.2. In T cell Activation

In chronic HIV-1 infection, the virus exists persistently in T cells which causes hyperactivation and redundant cycles of T cell proliferation and death, eventually leading to T cells and immune system exhaustion [166]. Nef has been shown to disrupt several aspects of T cell receptor (TcR) signaling through its interactions with the Src family member Lck kinase [167]. Nef proteins from SIV can block TcR/CD3 signaling, preventing the activation and apoptosis of T cells. In contrast, Nef from human HIV-1 did

not have this blocking effect on T cells, thereby causing a massive cell death and progression to AIDS. Furthermore, as mentioned earlier, Nef is known to disrupt T cell activation through downregulation of CD4 and CD28 [168].

2.2.3. In Regulation of Cytokine Expression

As mentioned earlier, dysregulation of cytokine production is a main feature of AIDS. During the course of HIV infection, Th1 cytokines, such as IL-2, IFN- γ , and IL-12 are generally decreased, while Th2 cytokines, IL-4 and IL-10 are increased [24]. This leads to impaired antiviral immune responses and favors AIDS development. HIV-1 Nef is believed to be implicated in this impaired cellular immunity by regulation of host cytokine expression via interaction with intracellular signaling pathways. Nef gene products have been shown to decrease IL-2 and IFN γ expression in activated T cells through its effect on MAPKs signaling pathways and transcription factors, NF- κ B and AP-1 [24;169]. The reduction of IL-2 and IFN γ is also partially a result of Nef upregulating IL-10 expression, which has been observed in PBMC, T cell line (H9) and promonocytic cell line (U937), respectively [170]. Nef is also reported to increase expression of inflammatory cytokines and chemokines, IL-6, TNF α , IL-1b, MIP-1a and MIP-1b from macrophages, dendritic cells and PBMC through MAPK, PKC and calcium signals [165;171]. In human glial cells, Nef expression decreases TNF α -induced apoptosis via the activation of JNK MAPK [156]. More recent data indicates that exogenous Nef protein is able to inhibit the release of IL-18, a co-inducer of IFN γ with IL-12, from the human premonocytic cell line, THP-1 [172]. However, the molecular mechanisms involved in Nef mediated downregulation of cytokines, in particular IL-12p40, remain unknown.

2.2.4. In HIV-1 Pathogenicity

Nef protein has evolved in several ways to manipulate the biology of an infected cell to support viral replication, immune evasion, pathogenesis, and viral spread [173].

As an AIDS Factor

The *Nef* gene of HIV-1 is critical for AIDS pathogenesis. This was initially demonstrated by studies in rhesus macaques. Infection of the macaques with Nef-deleted or Nef-mutant variants of SIV exhibits extremely low viral burdens with either no AIDS-like symptoms or significantly delayed disease progression [174]. Similarly, human infected with a Nef-defective HIV-1 showed a long-term nonprogressive disease course with low viral copy numbers and normal CD4 T cell counts [175]. In the CD4/CCR5 transgenic mice with a wild type HIV-1 genome, a severe AIDS-like disease was observed, whereas these mice infected with Nef-defective HIV-1 prevented the development of AIDS-like pathology [176]. The mechanisms by which Nef speeds up progression of AIDS are not completely understood.

In HIV Replication and Infectivity

The well-conserved properties of HIV-1 Nef are enhancement of virion infectivity and stimulation of viral replication [177]. The mechanisms that mediate both of these Nef functions are poorly understood at present. A recent study showed that Nef protects macrophages from HIV-1-induced apoptosis and favors *in vivo* establishment of the virus-macrophage reservoirs [178]. Nef can also increase clustering of dendritic cells with T cells and disseminate virus to lymphocytes [179]. As a virion protein, Nef can increase viral infectivity by enhancing biosynthesis of lipid rafts and coupling of newly synthesized cholesterol to lipid rafts and virus particles, leading to increased release of

the core into the cytoplasm [180]. In addition, Nef has been found to augment efficiency of reverse transcription *in vitro* by increasing the affinity of reverse transcriptase (RT) for RNA [181]. Nef also increases proviral DNA synthesis via interaction with Tat (transactivator), a viral transcriptional activator and inhibitor of p53, which regulates HIV-1 gene expression by suppressing transcriptional activation of the LTR [182]. Furthermore Nef prevents immune killing of infected cells by downregulation of HLA-A and B surface expression [183], and by modulation of death signals driven by Fas and TNF α . Moreover, Nef downregulates cell surface expression of CD4 and CD28 molecules to limit superinfection [168]. CD4 downregulation also enhances virion release and envelop incorporation [184]. In addition, Nef stimulates secretion of inflammatory chemokines, such as MIP-1 α , MIP-1 β and IL-8 by viral infected macrophages, attracting the bystander uninfected CD4⁺ T cells to the site of infection and rendering these cells susceptible to virus infection [185].

In HIV Evasion

There are three major mechanisms by which Nef contributes to evasion of HIV infection [186]. The first one is that Nef down-regulates MHC I molecules, preventing the viral infected cell from CTL and NK cells mediated killing [187]. Nef selectively reduces the cell surface expression of HLA-A and B, resulting in impaired antigen uptake and CTL activity. Nef also comparatively increases the expression of HLA-C and E, known as major ligands for the NK cell inhibitory receptors (CD94/NKG2A), reducing the susceptibility of target cell to NK cell cytotoxicity [188]. The second one is that Nef inhibits Fas/TNF α -induced death in viral infected cells by binding and blocking the functions of apoptosis signal regulating kinase-1 (ASK1), a key signaling intermediate in

the Fas/TNF α -death signaling pathways [156;189;190]. Alternatively, Nef can block death signals in viral infected cells by inactivation of Bad through the phosphorylation of PAK and PI3 kinases. Bad is a proapoptotic member of the Bcl-2 family whose inactivation further inactivates the anti-apoptotic Bcl-2 family members (Bcl-2, Bcl-X_L and Mcl-1) [191;192]. In the third way, through the TcR/CD3 signaling pathway, Nef induces the surface expression of FasL by infected cells, which interacts with Fas on the surface of bystander cells including CTL, leading to cell death [193]. Nef also inhibits p53-dependent apoptosis within the infected cells. In addition, Nef is shown to reduce the expression of the non-classical MHC-I like molecule named CD1d, a lipid antigen-presenting molecule, which presents the lipid antigens to a subset of T cells known as CD1d-restricted NKT cells. NKT cells have the capacity to produce both cellular and humoral immune responses against various infections [131].

3. Intracellular Signaling Pathways

3.1. LPS Signaling Pathways in Monocytes/Macrophages

Monocytes/macrophages are involved in phagocytosis, production of cytokines, antigen-presentation to lymphocytes and killing bacteria, tumor cells and viral infected cells. In order to perform any of these functions, macrophages must be activated. LPS, an outer membrane component of Gram negative bacteria, is a potent activator of monocytes/ macrophages and triggers the secretion of many cytokines from these cells [203]. The LPS molecule consists of the glycolipid A, which anchors LPS to the outer membrane, the oligosaccharide core and the polysaccharide O-antigen. The activation of macrophages is initiated by binding of LPS to the extracellular signaling modulators including glycosylphosphatidylinositol (GPI)-anchored glycoprotein CD14 [195], LPS

binding protein (LBP) and adaptor protein, MD-2 [196] to form the LPS-signaling complex that subsequently binds to TLR4, a member of the toll-like receptor (TLR) family of pattern recognition receptors [197]. This leads to recruitment of the adaptor protein MyD88 and further activates a number of intracellular signaling pathways including MAPKs, PI3K, PKC, and others [198] to promote correlated genes transcription (Fig. 1.3-1).

3.2. MAPKs Signaling Pathways

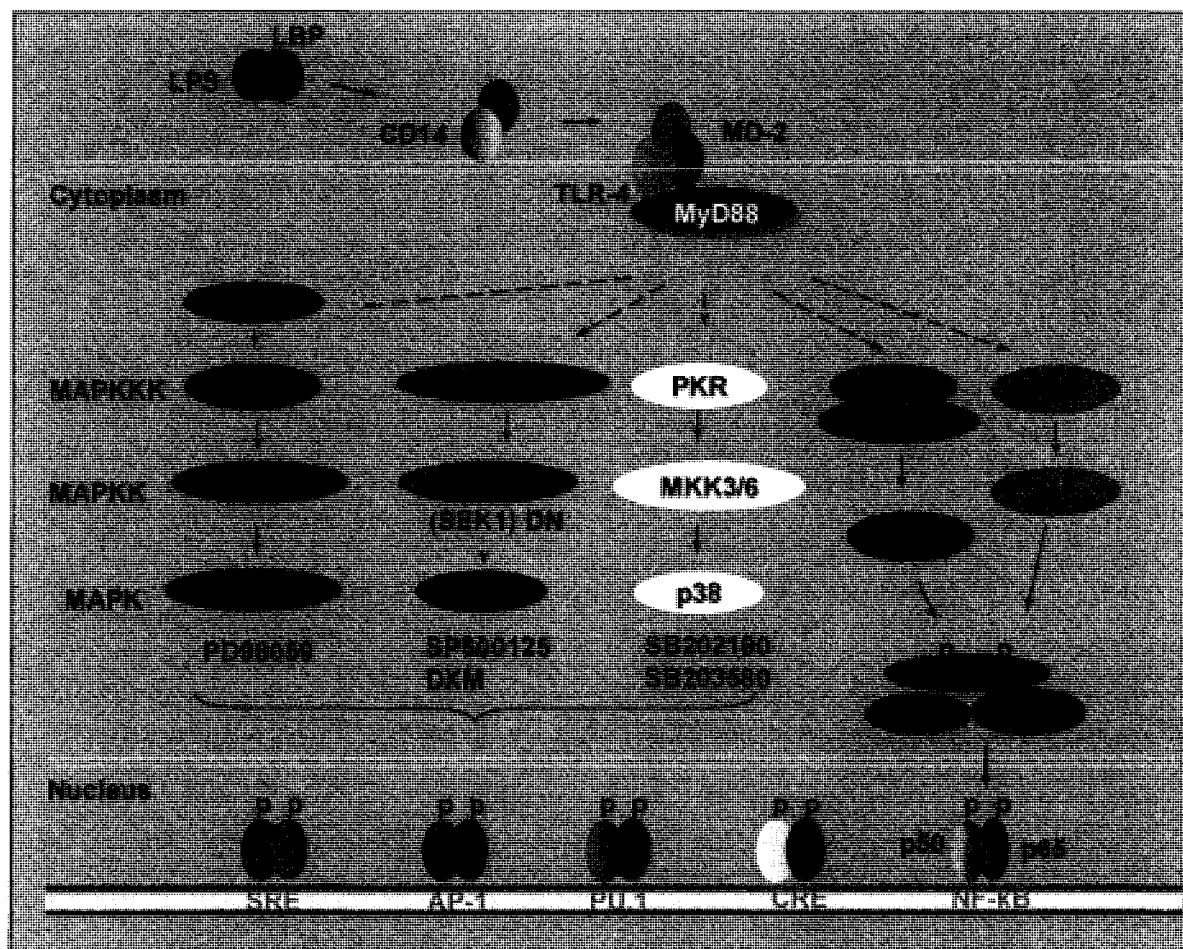
MAPKs (Mitogen-Activated Protein Kinases) are evolutionarily conserved enzymes connecting cell-surface receptors to their intracellular targets to regulate many cellular processes including cell growth, differentiation, and death. MAPKs are activated by a variety of extracellular stimuli via dual phosphorylation of threonine and tyrosine residues within a conserved T-X-Y motif [199] in the cytoplasm and are translocated into the nucleus, where they induce phosphorylation of transcription factors and co-activators. MAPK activity is regulated through three-tiered cascades composed of a MAPK, MAPK kinase (MAPKK, MKK or MEK) and a MAPKK kinase or MEK kinase (MAPKKK or MEKK)¹. The main members of MAPK family are extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs) and p38 [200-202]. They constitute the three key arms of MAPK signal transduction pathways (Fig. 1.3-1). In macrophages, the MAPK pathways have been shown to play important roles in the regulation of functions involved in inflammation and host defense [203]

ERK MAPKs

ERK, which consist of p44 ERK1 and p42 ERK2 enzymes (ERK1/2), was the first MAPK to be identified [200]. The ERK signaling pathway is preferentially activated

Fig. 1.3-1 LPS activated MAPK and PI3K signaling pathways and transcription factors in human monocytes:

The inhibitors used in this study are included. LPS binds to the serum protein LBP and cell surface molecule CD14 to form the LPS/LBP/CD14 complex which, in turn, interacts with the signaling receptor TLR4 and the accessory protein MD-2. This further activates MAPKs and PI3K pathways. The MAPK module contains a MAPK kinase kinase (MAPKKK), which phosphorylates a MAPK kinase (MAPKK). The activated MAPKKs can further activate the ERK, JNK, and p38 pathways. These pathways directly or indirectly phosphorylate and activate various transcription factors, including SRE (Serum response element), NFκB and CREB (cAMP-response element-binding protein). In addition, LPS activates the IKK pathway via MyD88/IRAK/TRAF6 (TNF receptor-associated factor 6) complex, which phosphorylate IKKb and IκBs. Subsequent degradation of IκBs permits nuclear translocation of NFκB. The PI3K/Akt pathway phosphorylates and activates NFκB via an unknown kinase [204].



in response to growth factors, cytokines, viral infections and LPS, ligands for G-protein coupled receptors and transforming agents. The ERK signaling pathway has been shown to be involved in regulation of cell proliferation and differentiation. The activation of ERK is initiated through activation of the small guanosine triphosphate (GTP) binding protein Ras. The GTP form of Ras binds to Raf (MAPKKK) at the plasma membrane. Activated Raf phosphorylates MEK1/MEK2 (MAPKKs) which in turn activate ERK1/ERK2 [205]. Activated ERK1/ERK2 subsequently phosphorylates a variety of proteins including the c-Jun and ELK-1 transcription factors. Recently, other forms of ERKs (ERK3, ERK4, and ERK5) have been characterized, but their regulation has remained unclear [200;206]. In mouse macrophages, the ERK pathway was shown to activate the transcription factor repressor element, GA-12 in response to LPS stimulation, leading to the repression of IL-12 p40 production [207].

P38 MAPKs

P38 has been shown to be involved in cellular stress response, cell proliferation, differentiation, and survival. There are five isoforms of p38 MAPK; p38 α (SAPK2), p38 β , p38 β 2, p38 γ (SAPK3), and p38 δ that are expressed in distinct tissues [210]. They are activated by a plethora of MAPKKKs which become activated in response to various physical and chemical stresses, such as oxidative stress, UV irradiation, hypoxia, ischemia, and various cytokines, including IL-1 and tumor necrosis factor alpha (reviewed in [211]). MKK6 activates all p38 isoforms by dual phosphorylation on Thr 180 and Tyr 182 [212], whereas MEK3 selectively phosphorylates the p38 α and p38 β isoforms at the same residues [213]. In addition, MKK4, which activates JNK, also stimulates p38 MAPK activity. p38 has been shown to be activated in macrophages and T

cells by numerous extracellular mediators of inflammation, such as chemoattractants, cytokines, chemokines, and LPS [209].

JNK MAPKs

JNK (c-Jun-N-terminal Kinase), also known as SAPK (Stress Activated Protein Kinase), is involved in cellular stress, defense responses, and cell survival. It is activated rapidly by a variety of environmental stresses including ultraviolet irradiation, proinflammatory stimuli: TNF- α , IL-1 β , LPS, double-stranded (ds) RNA, and protein synthesis inhibitors. The major isoforms of JNK are p46 JNK1, p54 JNK2 and p49 JNK3. JNK1 and JNK2 are ubiquitously expressed, whereas the expression of JNK3 is restricted to the brain, testis and heart [201;214;215]. Like all members of the MAP kinase family, JNKs are components of an evolutionarily conserved, three-tiered kinase cascade. An upstream MAP kinase kinase kinase (or JNK kinase kinase) phosphorylates and activates the relevant MAP kinase kinases: MKK4 (or SEK1) and MKK7, which in turn phosphorylate JNKs on Thr 183 and Tyr 185 within the tripeptide motif Thr-Pro-Tyr located in kinase sub-domain VIII causing increased transcriptional activity [270]. The MKK7 protein kinase is primarily activated by cytokines, such as TNF and IL-1, whereas MKK4 is mainly activated by environmental stress [217]. These two kinases are localized both in the cytosol and in the nucleus and may possibly activate JNK in the both places. Activated JNK phosphorylates a number of proteins including the transcription factors NF κ B and c-Jun which subsequently activates AP1 transcription factor complexes. Two JNK isoforms, JNK1 and JNK2, are found constitutively expressed in macrophages in response to LPS [218].

3.3. Calcium Signaling Pathways

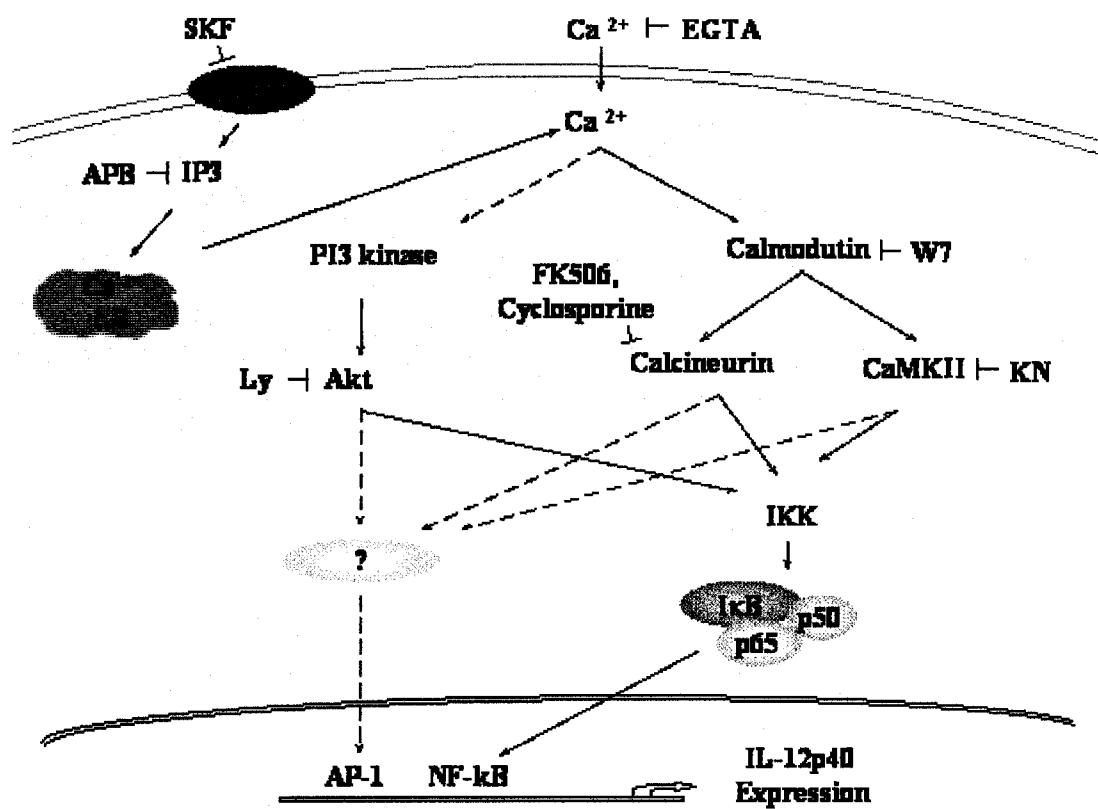
Ca^{2+} plays an essential role in a variety of intracellular signaling that is involved in many cellular activities [219]. Ca^{2+} acts by binding to calmodulin (CaM,) a small (148 amino acids), acidic protein capable of binding four calcium ions. The interaction of Ca^{2+} /CaM activates many different enzymes including CaM kinases II (CaMKII), protein phosphatases (calcineurin), nitric oxide synthase, inositol triphosphate kinase, nicotinamide adenine dinucleotide kinase, and cyclic nucleotide phosphodiesterase [220]. CaMKII is a multimeric Ca^{2+} /CaM-dependent protein kinase which contains 10-12 subunits. Once activated, CaMKII proceeds to phosphorylate itself and subsequently activates many other protein kinases, such as MAPKs, PKC and PKA [221;222]. Studies have suggested that Ca^{2+} signals, sometimes combining with other intracellular signals, are intensely involved in both innate and adaptive immunity. For example, Ca^{2+} induces the expression of $\text{IFN}\gamma$ and IL-2 through the calcineurin/NFAT signaling pathway, leading to the proliferation and activation of T cells [223]. Ca^{2+} is also an important factor for transcription of genes encoding the inflammatory cytokine $\text{IFN}\gamma$, IL-6, $\text{TNF}\alpha$ and IL-2 [224] (Fig. 1.3-2).

3.4. PI3K Signaling Pathways

PI3K (phosphoinositide 3-kinase) is a key component in growth factor pathways that regulate a multitude of cellular responses such as mitogenic responses, differentiation, apoptosis, cytoskeletal organization and various other aspects of monocyte function [225]. PI3 kinases can be grouped into four families (Ia, Ib, II, and III) with at least eight different lipid kinases according to their substrate preference and sequence homology, among which, class I is well characterized. Mammalian class-Ia PI3

Fig. 1.3-2 Calcium signaling pathway:

Binding of ligand to respective receptor induces calcium influx either through receptor mediated entry or through voltage dependent ion channel entry from external source. There is also release of calcium from endoplasmic reticulum through binding of IP3 to its receptor. As shown in the figure, influx of calcium activates calmodulin followed by CAMKII and calcineurin which leads to expression of various responsive genes. Adopted from [226].



kinases is a heterodimer composed of a regulatory subunit p85 (p85, p55 or other splice variants) and a catalytic subunit p110 (α , β or γ isoforms) [226]. The best characterized are the p85 and p110 α/β . Both are activated by small G-protein Ras-GTP or growth factor receptors through their phosphotyrosine TyrxxMet motifs by a SH2 domain. Activated class-I_A PI3 kinase induces phosphorylation of phosphatidylinositol (Ptdins) on the third carbon of the inositol ring to become Ptdins-(3)-P and Ptdins-(3,4,5)-P [227;228], which are able to recruit Akt and PDK1 (PI3K-phosphoinositide-dependent kinase 1) to the plasma membrane where Akt is phosphorylated and activated by PDK1. The activated PI3 kinase/Akt signal further regulates a number of transcription factors [226] (Fig. 1.3-2). Among them, NF κ B and AP-1 have been extensively studied [229].

3.5. Transcription Factors

NF- κ B

NF κ B is an inducible transcription factor of the Rel family and its activation is a hallmark of most infections including viral infections [230]. By regulating the expression of cytokines, growth factors, and effector enzymes in response to ligation of many receptors involved in immunity including TcR, BcR, the TLR family and others, NF κ B plays a critical role in many cellular processes, particularly in the immune system [231]. The mammalian NF κ B family is composed of five members including NF κ B1 (p50/p105), NF κ B1 (p52/p100), p65 (RelA), RelB, c-Rel, which exist as homo- or heterodimers[232]. NF κ B is normally found in the cytoplasm complexed with an inhibitory protein, I κ B, of which various isoforms exist including I κ B α , I κ B β , and I κ B ϵ [233]. Upon infection, signaling events are initiated leading to activation of MAP kinase kinase kinases (MAP3Ks), which promote the activation of a large kinase complex,

leading to phosphorylation of I κ B at two specific amino-terminal serine residues. Phosphorylated I κ B is subsequently targeted for degradation through the ubiquitin-dependent 26S proteasome pathway. Degradation of I κ B unmask the nuclear localization signal of NF κ B, which migrates to the nucleus and activates transcription.

Activation Protein 1 (AP-1)

AP-1 transcription factors play a central role in regulating the expression of key genes controlling cell proliferation, apoptosis, and cellular differentiation. AP-1 is a term that refers to a family of transcription factors, which form homo- or heterodimers. Members of this family are the Jun (c-Jun, JunB, JunD), Fos (c-Fos, FosB, Fra1, Fra2), or ATF (ATF2, ATFa, ATF3/LRF1, B-ATF) proteins [234;235]. The Jun proteins can either homo- or heterodimerize with members of the Fos or ATF while the Fos and ATF cannot associate with each other. All AP-1 factors share a common structural domain called bZIP (basic region leucine zipper) which enables these proteins to form dimers and bind to a common highly related DNA sequence, the AP-1 binding site. Studies with mice using knockout techniques showed that c-Jun is critical to embryonic development [236], whereas c-Fos is a key regulator in bone development [237]. Mice lacking JunD were also found to have retarded growth and defects in male reproductive function [238]

Rational

In HIV-1 infection and AIDS development, monocytes and macrophages play dual roles. They represent a viral reservoir acting as a primary target and viral carriers, and trigger innate and adaptive immunities as primary effectors. Monocytes are major inducer of the Th1 cytokine IL-12 with LPS stimulation. IL-12, which is represented by its p40 subunit, plays a critical role in the development of Th1 cellular immune responses

upon HIV infection. IL-12 production is severely decreased in HIV infected monocytes/macrophages. This is believed to be responsible for impaired HIV specific Th1 cellular immunity. However, the molecular mechanisms underlying the induction of IL-12p40 by LPS and this inducible IL-12p40 inhibition in HIV infection are poorly understood and remain to be investigated. Nef, one of the HIV regulatory proteins, is an early gene product and AIDS factor and has been shown to be implicated in dysfunctional Th1 immune responses and dysregulated cytokine expression. The intracellular Nef has been shown to inhibit Th1 cytokines IFN γ from T cells, and IL-18 from promonocytic cell line THP-1 cells. Nef is also reported to increase Th2 cytokines, IL-4 in T cells and IL-10 in monocytes, leading to further inhibition of Th1 cytokine production. Therefore, it is critical to determine how HIV-Nef causes downregulation of IL-12 and broadly Th1 cytokines and Th1 immune responses.

In this study, I investigated the molecular mechanism underlying the regulation of LPS-induced IL-12p40 production in human monocytic cells. Whether intracellularly expressed Nef causing downregulation of IL-12p40 production is subsequently examined. Furthermore, I determined the signaling pathways involved in downregulation of LPS-induced IL-12p40 production by HIV-Nef in human monocytic cells by employing retrovirus containing HIV-Nef gene. The retroviral vectors have been recently recommended as a powerful gene delivery tool and have been used in gene therapy in clinics. These viral vectors have been shown to maintain the transduction efficiency and to induce high levels of gene expression.

Hypothesis and Objectives

Hypothesis

LPS induces IL-12p40 production by activating intracellular signaling pathways such as MAPK and calcium signaling pathways through the activation of transcription factors in human monocytic cells. Further, impaired IL-12p40 production in HIV-1 infection is modulated by the HIV-1 regulatory protein Nef through its inhibitory effect on intracellular signaling pathways and the transcription factors involved in LPS-activated human monocytic cells.

Objectives

The main goal of my research project is to determine if the HIV-1 regulatory protein Nef suppresses LPS-induced IL-12p40 production in human monocytic cells and to investigate the intracellular signaling pathways involved in the Nef-induced downregulation of LPS-stimulated IL-12p40 production in human monocytic cells. The specific objectives of this thesis project are as follows:

1. To identify the intracellular signaling pathways involved in the regulation of LPS-induced IL-12p40 in human monocytic cells.
2. To elucidate the critical transcription factors involved in LPS-mediated IL-12p40 gene transcription in human monocytic cells.
3. To determine if intracellular Nef protein is involved in impaired IL-12p40 production in LPS-stimulated human monocytic cells.
4. To identify the signaling pathways and transcription factors involved in Nef-mediated IL-12p40 downregulation in LPS-stimulated human monocytic cells.

Chapter II

Materials and Methods

2.1. Cell lines and Cell Culture

THP-1, a promonocytic cell line derived from a human acute lymphocytic leukemia patient was obtained from the American Type Culture Collection (Manassas, VA). Five to 15% of THP-1 cells express CD14, and following LPS stimulation, the levels of CD14 expression is increased to ~50% (42). THP-1 cells transfected with a plasmid containing CD14 cDNA sequences (THP-1/CD14) were kindly provided by Dr. R. Ulevitch (The Scripps Research Institute, La Jolla, CA). THP-1/CD14 cells transfected with either pcDNA-3 plasmid expressing a dominant negative mutant of SEK1 (dsSEK1) or control pcDNA-3 plasmid were friendly provided by Dr. J. Woodget (Princess Margaret Hospital, Toronto, ON, Canada). THP-1 cells transduced with HR-p110 α 3 or HR-p110 α 1 were the gifts from Dr. Neil E. Reiner (University of British Columbia, Vancouver, BC, Canada). Retroviral packaging cell line, PT67 derived from HIN 3T3 cell line and package virus with a polytropic envelop was purchased from BD Biosciences Clontech (Mississauga, ON Canada). The cell lines used in this study are shown in Table 2.1. Cells were cultured in IMDM (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FBS (Invitrogen Life Technologies, Grand Island, NY), 100 U/ml penicillin, 100 μ g/ml gentamicin, 10 mM HEPES, and 2 mM glutamine.

2.2. Reagents

LPS derived from *Escherichia coli* 0111:B4 was obtained from Sigma-Aldrich Canada Ltd., Oakville, ON. The following MAPKs signaling inhibitors were employed (thire source and dose are showed in Table 2.2): JNK inhibitor SP600125, a reversible ATP-competitive inhibitor with >300-fold selectivity versus related MAPKs including ERK1 and p38-2 as well as PKA and IKK-2 was purchased from Biomol, Plymouth, PA.

Dexamethasone [(Dxm; (9-fluoro-16 α -methylprednisolone)] was also used as a JNK inhibitor. MAP/ERK kinase-1 inhibitor, PD98059, an inhibitor of MAP/ERK kinase-1, which selectively blocks the activity of ERK MAPK and has no effect on the activity of other serine threonine protein kinases including Raf1, p38 and JNK MAPK, was also purchased. The pyridinyl imidazole SB202190, a potent inhibitor of p38 MAPK used in this study, has no significant effect on the activity of ERK or JNK MAPK subgroups. The following calcium signaling inhibitors were also used: a calcium chelating agent EGTA was obtained from Sigma, SKF-96365 hydrochloride, a novel inhibitor of receptor-mediated calcium entry; 2-APB inhibits inositol (1,4,5) triphosphate (IP3) induced Ca²⁺ release from the ER; W7 hydrochloride, a calmodulin antagonist and KN-93, a specific cell permeable inhibitor of CaMKII; FK-506 interacts with FK506-binding protein (FKBP), forming a FK506–FKBP complex, which binds to and blocks calcineurin; and cyclosporin A binds to cyclophilin and inhibits the Ca²⁺ dependent phosphatases were all obtained from Calbiochem. The PI3K inhibitors Ly294002, a potent inhibitor that acts on the ATP binding site; and Wortmannin, a fungal metabolite that blocks the catalytic activity of PI3K without affecting the upstream signaling events were purchased from Calbiochem as well. Recombinant HIV-1 and SIV Nef proteins and anti-HIV-1 Nef antibodies were obtained from NIH AIDS Research and Reference Reagent Program. All antibodies and their source used in this study are shown in Table 2.3.

Table 2.1: Cell lines and their source used in this study

Name of the cell line	Source
THP-1 cell	American Type Culture Collection (Manassas, VA)
CD14/THP-1 cell	Dr. R. Ulevitch (The Scripps Research Institute, La Jolla, CA)
HR-p110a3/THP-1 cell	Dr. Neil E. Reiner (University of British Columbia, Vancouver, BC, Canada)
HR-p110a1/THP-1 cell	Dr. Neil E. Reiner (University of British Columbia, Vancouver, BC, Canada)
dsSEK1-CD14/THP-1 cell	Dr. J. Woodget (Princess Margaret Hospital, Toronto, ON, Canada)
PT67 derived from HIN 3T3 cell	BD Biosciences Clontech (Mississauga, ON Canada)

Table 2.2: Inhibitors and their source used in this study:

Inhibitors	Companies	Used Concentration	Cellular Targets
Dexamethasone (Dxm)	Sabex, Boucherville, Que	2-100 nM	JNK MAPK
SP600125	Calbiochem	5-50 μ M	JNK MAPK
SB202190	Calbiochem, San Diego, CA	5-50 μ M	P38 MAPK
SB203580	Calbiochem	5-50 μ M	P38 MAPK
PD98059	Calbiochem	5-50 μ M	ERK MAPK
FK 506	AG Scientific, San Diego, CA	0.5-5 μ M	Calcineurin
Cyclosporine A (CyA)	Sigma-Aldrich Oakvill ON Canada	0.5-5 μ M	Calcineurin
EGTA	Calbiochem	2-10 mM	Calcium chelator
SKF96365 HCl	Calbiochem	20-100 μ M	Receptor mediated Ca^{2+} entry
2-APB	Calbiochem	10-50 μ M	IP3 induced Ca^{2+} release
W7-HCl	Calbiochem	10-50 μ M	Calmodulin
KN-93	Calbiochem	5-50 μ M	CAMKII
Ly 294002	Calbiochem	5-50 μ M	PI3K
Wortmannin	Calbiochem	5-50 μ M	PI3K

Table 2.3: Antibodies and their source

Antibodies	Source
α -phospho-JNK MAPK	Cell Signaling
α -total-JNKMAPK	Santa Cruz Biotechnology
α -phospho-p38 MAPK	Cell Signaling, Danvers, MA
α -total-p38 MAPK	Santa Cruz Biotechnology, CA
α -phospho-ERK MAPK	Santa Cruz Biotechnology
α -total-ERK MAPK	Santa Cruz Biotechnology
α -phospho-CaMKII	Santa Cruz Biotechnology
α -total-CaMKII	Santa Cruz Biotechnology
α -phospho-Akt	Cell Signaling
α -total-Akt	Cell Signaling
α -rabbit-HRPO	Amersham Bioscience, Montreal, Canada
α -mouse-HRPO	Sigma-Aldrich Canada Ltd., Oakville, ON
c-fos (EMSA) (Rabbita)	Upstate Biotechnology, lake placid, NY
c-jun (EMSA) (Rabbit)	Santa Cruz Biotechnology
α -p50 (EMSA) (Mouse)	Santa Cruz Biotechnology
α -p65 (EMSA) (Mouse)	Santa Cruz Biotechnology

2.3. Isolation of Monocytes

Monocytes were purified from PBMC by a negative selection system. Briefly, PBMCs from healthy donors were isolated by density gradient centrifugation over Ficoll-Hypaque (Amersham Biosciences, Piscataway, NJ). Cells were incubated with magnetic polystyrene Dynabeads (DynaL Biotech, Oslo, Norway) coated with Abs specific for CD2 (T cells) and CD19 (B cells) for 30 min on ice for depletion of T cells and B cells. Cells were further incubated for 2 hr at 37⁰ C to eliminate nonadherent cells. The monocytes obtained contained <1% CD2+ T cells and CD19+ B cells as determined by flow-cytometric analysis. Cells were further cultured in IMDM in the presence or absence of specific stimuli.

2.4. Production of recombinant Nef- retroviruses in PT67 cells

Recombinant retroviral vector pSR α MSVtkneo containing Nef gene (pSR α -Nef) derived from HIV-1 SF2 strain (Gene Bank accession number K02007, nucleotide position 8504-9573) was a gift from Dr. T. Smithgall (University of Pittsburgh, PA). The control retrovirus was then prepared by using Klenow Fragment (NEB Biolabs). To produce high-titer pSR α -Nef stocks, PT67 cells were employed. These cells are designed not only for stably producing high-titer retrovirus, but also for efficiently infecting target cells by virus since virus produced by these cells can enter target cells via two surface molecules, the amphichroic retrovirus receptor RAM1 (Pit2) and the GALV (Pit1) receptors. Briefly, 1.5×10^6 cells were plated in 6 well dishes (Falcon) one day prior to transfection and transduced with 4 μ g of the Nef-retrovirus plus 12 μ g Fugen 6 (Roche, German) in 2ml of IMDM-FCS (10%)-polybrene (4mg/ml). After 24 hr, the culture medium was replaced with fresh IMDM-10% FCS and cells were selected by 300 μ g/ml

of G418. Seven days post-transfection, genomic DNA was purified for determining integration of the Nef gene by PCR (Primers - in Table 2.4.) and total proteins were extracted for measuring the expression of the Nef protein by western blotting with anti-Nef monoclonal antibody, EH-1 (NIH AIDS Research and Reference Reagent Program, Rockville, MD). To collect pSR α -Nef containing supernatants, 2×10^6 of pSR α -Nef producing PT67 cells were grown in 30 ml of IMDM plus 4 μ g/ml polybrene and 300 μ g/ml of G418 for 48 hrs. The supernatants were collected, filtered to remove contaminating cells and stored at -80°C. The viral load used for infection was optimized for viral titer by employing NIH 3T3 cells according to the manufacturer's protocol (BD Bioscience Clontech).

2.5. Generation of pcDNA-Nef stably expressing THP-1 cells

The full-length HIV-1-*Nef* gene obtained from pclonsnefSN (NIH AIDS Research and Reference Reagent Program) was subcloned into pcDNA3.1*zeo*⁺ expression vector (Invitrogen) at EcoRI restriction site. THP-1 cells were transfected with pcDNA3.1*zeo*⁺ containing Nef gene (pcDNA-Nef) by LipofectAMINE Reagent (Life Technologies, Inc.) according to the manufacturer's instructions. Briefly, cells (2×10^6) were plated in 6-well plates before transfection. 2 μ g of the pcDNA-Nef and 8 μ l of LipofectAMINE reagent were incubated with 200 μ l of Opti-MEM I Reduced Serum Medium (Life Technologies) for 30 min at room temperature to allow formation of DNA-liposome complexes. The resulting complexes were added to the cell culture. Forty eight hours post-transfection, the medium was replaced with fresh IMDM-10% FCS and cells were selected by 200 μ g/ml of zeocine. Seven days after transfection, cells were lysed and used for measuring Nef mRNA by PCR (primers showed in Table 2.4.) and the expression of Nef

protein by immunoblotting using a polyclonal anti-Nef antibody obtained from NIH AIDS Research and Reference Reagent Program.

2.6. Cell stimulation, infection and ELISA analysis

Purified monocytes (10^6) and THP-1 cells (0.5×10^6) were incubated in 24-well culture plates (Falcon; BD Biosciences, Franklin Lakes, NJ). Cells were left untreated or stimulated with LPS ($1 \mu\text{g/ml}$) for 48 h in the presence or the absence of various inhibitors. Cell supernatants were frozen at -70°C and thawed at the time of analysis for IL-12p40 production by ELISA. To ensure Nef biological activity in THP-1 cells infected with pSR α -Nef, I confirmed the expression of Nef mRNA and Nef protein by PCR and immunoblotting. Infection of monocytes and THP-1 cells with pSR α -Nef was performed at an MOI of 1 at 37°C . Cells were cultured in 3 ml of pSR α -Nef containing supernatant collected from packaging cells in the presence of 4 mg/ml polybrene for 24 hr and infected a second time under the identical condition for an additional 24 hr followed by stimulation with LPS for various times. Cells were harvested for protein and RNA extraction. The supernatants of 24 hr LPS stimulation were collected for measuring IL-12p40 production by ELISA with anti-IL-12p40 mAb (Systems of R & D).

2.7. Transfection of p85 PI3K siRNA

Cells (10^6) were transfected with siRNA SMARTpool PI3K p85 α and non-specific control pool (siRNA control) using DharmaFECTTM 2 transfection reagent as per the manufacturer's instructions (Dharmacon). Following transfection, cells were stimulated with LPS ($1 \mu\text{g/ml}$) for 8 h for real-time PCR and for 24 h for Western

blotting for p85 PI3K. The supernatants were collected after 24 h of stimulation for IL-12p40 ELISA.

2.8. RNA isolation and RT-PCR for IL-12p40 expression

Total RNA (1 µg) from LPS stimulated and/or virus infected monocytes and THP-1 cells were extracted using the RNeasy Plus Mini Kit ® (Qiagen) and reverse transcribed with Moloney murine leukemia virus reverse transcriptase (PerkinElmer, Foster City, CA). Equal aliquots (5 µl) of cDNA equivalent to 100 ng of RNA were subsequently amplified by PCR for IL-12p40 and β-actin. PCR was performed for 25 cycles at 95°C for 30 s, 58°C for 30s, and 72°C for 2 min, followed by 72°C for 10 min. The primers used for RT-PCR in this study for expression IL-12p40 were shown in Table 2.4.

2.9. Calcium influx Assay

THP-1 cells were infected with Nef-virus for 24 hr followed by second time infection for addition 24 hr. Cells were then stimulated with LPS for 0 to 120 minutes. The cells were washed twice with Ca²⁺ free PBS for 5 min at RT and resuspended in Buffer A (RPMI1640 containing 20 mM HEPES, pH 7.4 and 1mM Fluo-3/AM (Molecular Prodes, Inc. Eugene, OR) in 1mM Me2SO and 3.75% pluronic F-127 solution (Sigma) followed by incubation in dark for 45 min in a 37°C shaking water bath. The reaction was stopped by adding an equal volume of Buffer B (Buffer A containing 5% fetal bovine serum, pH 7.4), followed by incubation for 15 min in a 37° C water bath. The cells were washed and resuspended in Buffer B at a final concentration of 0.5 x 10⁶ cells/ml. The cells were washed again, aliquoted, and analyzed for Ca²⁺ levels by FACScan flow cytometer (BD Biosciences) equipped with CellQuest software, version

3.2.1 fl. Cell samples were maintained at 37° C during data acquisition. Intracellular Ca^{2+} levels at base line and followed stimulation with LPS (1 $\mu\text{g}/\text{ml}$) were measured. Ca^{2+} ionophore A23187 (20 mM) and 5 mM EGTA (Sigma) were used as positive and negative controls, respectively.

2.10. Western blotting analysis for kinases phosphorylation

Phosphorylation of MAPKs, CaMKII and Akt were determined by western blotting analysis using the corresponding MAPKs, CaMKII and Akt-specific Abs. Briefly, monocytes and THP-1 cells (2×10^6) were infected with Retro-Nef or control virus followed by LPS (1 $\mu\text{g}/\text{ml}$) stimulation for various times. Cells were harvested for protein extraction. The equal amounts of the proteins were subjected to 8% SDS-PAGE followed by transfer onto polyvinylidene difluoride membranes (Pall Gelman Laboratory, Ann Arbor, MI). The membranes were probed with the anti-human phospho-MAPKs followed by secondary polyclonal Abs conjugated to HRP (Bio-Rad). To determine CaMKII and Akt phosphorylation, immunoblotting analysis was performed by using specific anti-CaMKII and Akt Abs. For detection of even loading, the membranes were stripped and reprobed with antibodies specific for the total protein content of each kinase. The antibodies and their source used in this study are shown in Table 2.3. All immunoblots were visualized by ECL (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The subsequent densitometry analysis was preformed by CHEMIGENIUS2 XE Bio Imaging System (PerkinElmer LAS Canada, Inc, Woodbrige ON, Canada)

2.11. Plasmid Constructs of IL-12p40 promoter-luciferas reporter

Luciferase reporter gene vectors containing IL-12p40 promoter fragments were constructed as described earlier [100]. A series of hIL-12p40 promoter fragments (see

Fig. 5A; fragment -880 to -108; GenBank accession no. U89323) were amplified from genomic DNA by PCR. The primers with restriction sites used to amplify the hIL-12p40 promoter fragments from genomic DNA are shown in Fig. 3.1.4. The amplification consisted of denaturation at 95°C for 2 min followed by 30 cycles of the following: denaturation at 95°C for 30 s, annealing at 59°C for 2 min, and extension at 72°C for 2 min, and final elongation at 72°C for 10 min. The amplified promoter products were subcloned into the NheI/NcoI polylinker site of the basic luciferase reporter plasmid, pGL3B, and sequences were confirmed again. To introduce mutations in various transcription factor binding sites within hIL-12p40 promoter, site-directed mutagenesis was performed by PCR using mutagenic primers. The substitutive (site-directed) mutations (-232 to -108), including AP-1 binding sequence (wild type (wt), TTATTCC; mutant (m), ttttccc), Ets-2 binding sequence (wt, TTTCCT; m, ggacct), PU.1 binding sequence (wt, AAGGAA; m, ttcgaa), and NFκB binding sequence (wt, TTGAAATTCCC C; m, tgggttttggccc), were generated (Table 2.4. and Fig. 3.1.6). The lowercase letters indicate mutated oligonucleotides. The fragments containing these mutations then were inserted into the pGL3B reporter vector. The DNA sequencing was performed by the Biotechnology Research Institute, University of Ottawa.

2.12. Transient transfection of THP-1 cells and luciferase reporter assay

DNA transfection was performed by Fugene 6 reagent as explained early (Roche, German). Briefly, 1μg of the test plasmid and 0.5μg of the pSV-β-galactosidase (transfection efficiency control) (Promega, Madison, WI) were incubated for 5 min at RT in 50 μl of IMDM with 10% FCS. The mixer was then incubated for 30 min at RT with 3μl of Fugene 6 reagent, which is in 50 μl of IMDM containing 10% FCS, to allow

formation of DNA-liposome complexes. These complexes were added to the cell suspension in each well. After 24hr of transfection, cells were stimulated with LPS for a further 24hr. Cells were harvested and then assayed for luciferase and β -galactosidase activity by using luciferase assay and β -galactosidase assay kits (Promega). Luciferase activities were normalized by measuring β -galactosidase value. To determine the involvement of calcium signaling in the activation of IL-12p40 promoter, THP-1 cells were transiently transfected with the test plasmid/control vector with pSV- β galactosidase for 48 hr and treated with the indicated concentration of inhibitors for 2 hr followed by stimulation with LPS (1 μ g/ml) for 24 hr. Luciferase activity and normalization were performed as above.

2.13. Electrophoretic mobility shift for NF κ B and AP-1 activation

Gel mobility assays were performed as per the standard technique and as described earlier [100]. Cells (10^7) were harvested in Tris-EDTA-saline buffer (pH 7.8) and centrifuged at 200 μ g for 5 min at 4°C. The cells were lysed for 10 min at 4°C with buffer A (10 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM DTT, and 0.5 mM PMSF (pH 7.9)) containing 0.1% Nonidet P-40. The lysates were centrifuged at 20,000 xg for 10 min at 4°C. The pellet containing the nuclei was suspended in buffer B (20 mM HEPES, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, and 25% glycerol) at 4°C for 15 min. Both buffers A and B contained the proteolytic inhibitors including DTT, PMSF, and spermidine at concentrations of 0.5 mM each, as well as 0.15 mM spermine, and 5 μ g/ml each of aprotinin, leupeptin, and pepstatin. The supernatant containing the nuclear proteins was collected and frozen at -80°C. Nuclear proteins (5 μ g) were mixed for 20 min at room temperature with either ³²P-labeled AP-1 or NF κ B oligonucleotide probes,

and the complexes were subjected to nondenaturing 5% PAGE for 90 min. The oligonucleotide probes contained sequences corresponding to the AP-1 or NFκB in the IL-12p40 promoter and have the following sequences (plus strand): NFκB, 5'-AGG AAC TTC TTG AAA TTC CCC CAG AAG GTT TT-3' and AP-1, 5'-TCC TTC CTT ATT CCC CAC CCA-3'. The mutant oligonucleotide sequences used as cold competitors corresponding to the NFκB and AP-1 binding sites were as follows (plus strand): mNFκB, 5'-AGG AAC TTC TTc ccA TTC CCC CAG AAG GTT TT-3'; AP-1, 5'-TCC TTC CTg AcT tgC CAC CCA-3'. The sequences of the probes used in this study are shown in Table 2.4. To illustrate specificity of nuclear factor binding for NFκB and AP-1 probes, parallel EMSA reactions were incubated with 50- to 200-fold excess of cold unlabelled probe. Subsequently, supershift analyses were also performed to identify the transcription factors NFκB and AP-1 by using specific mouse anti-NFκB p50 and p65 mAbs and rabbit anti-*c-fos* and anti-*c-jun* polyclonal Abs, respectively (Table 2.3.). Briefly, nuclear extracts were incubated with the NFκB oligonucleotides in the presence of anti-NFκB p50 or p65 Abs and anti-*c-fos* or -*c-jun* Abs, or control Abs at a final concentration of 20 µg/ml. The antibodies used in this study are shown in Table 2.3. The bound and unbound ³²P-labeled oligonucleotides were resolved by gel electrophoresis as described above. The gel was dried and exposed to x-ray film (Kodak, Rochester, NY).

2.14. Antisense oligonucleotides

c-jun and *c-fos* antisense oligonucleotides were purchased from Synthegen (Houston, TX). The oligonucleotide sequences are as follows: *c-jun*, 5'-TGC AGT CAT AGA AC-3'; *c-fos*, 5'-GAA GCC CGA GAA CAT CAT-3'; and control, 5'-ATG AGT GGC TTG-3'. The cells were incubated with the *c-jun* and *c-fos* antisense

Table 2.4: Sequence of primers used in PCR and oligonucleotides used in EM

Primer name	Primer sequence	Region Aplified (bp)	Product Length (bp)
For PCR			
pSR/-Nef, sense	5'-ATGGGTGGCAGTGGTCAAAACGTA-3'	.	237
pSR/-Nef, antisense	5'-GGAAAACCCACCTCTTCCTC-3'		
pcDNA-Nef, sense	5'-AGGCTAGCCTTCTTGAAATTCCTCCAGAGG-3'	.	639
pcDNA-Nef, anti sense	5'-ACCATGGGTGGCAAGTGGTCAAAACG-3'		
For RT-PCR			
IL-12p40, sense	5' GGACCAGAGCAGTGAGGTCTT-3'	.	373
IL-12p40, antisense	5'-CTCCTTGTGTCCCTCTGA-3'		
)-actin, sense	5-TGACGGGGTCACCCACACTGTGCCCATCTA-3'	.	663
)-actin, antisense	5'-CTAGAAGCATTTGCGGTGGACGATGGA-3'		
For IL-12p40 promoter			
Sense Primers:			
IL-12p40-C/EBP	5'-AGGCTAGCCAATGTTGCAACAAGTCAG-3'	-84 /+108	192
IL-12p40-wtNF/B	5'-AGGCTAGCCTTCTTGAAATTCCTCCAGAGG-3'	-120/+108	228
IL-12p40-mNFkB	5'-AGGCTAGCCTTCTgggtttgccc CAGAAGG-3'	-120/+108	228
IL-12p40-wtPU.1.NF/B	5'-CCTTCGAACTCTTGGTTTGGCCCCAG-3'	-131/+108	339
IL-12p40-mPU.1/wtNF/B	5'-CGTTCGAATTGTGTTGTCCTCTCTCTG-3'	-131/+108	339
IL-12p40-wtAP-1	5'-TGGCTAGCTTATTCCTCCACCCAAAAG-3'	-232/+108	340
IL-12p40-wtAP-1/mEst2,PU.1,NF/B	5'-AGGCTAGCTTATTCCTCCACCCAAAAGTCAGGACCTCTT-3'	-232/+108	340
IL-12p40-mAP-1,Est2,PU.1,NFkB	5'-ACGCGTGCTAGCTttttccc CCACCCAAAAG-3'	-232/+108	340
IL-12p40-Sp-1	5'-AAGCTAGCTGACCGCCCTTGGCCTTC-3'	-358/+108	466
IL-12p40-NF/IL-6	5'-TGGCTAGCTGTTTCTGAAATTAAGGAC-3'	-621/+108	724
IL-12p40-IRF	5'-AGGCTAGCGGGTATTACATTCTGTCTCC-3'	-733/+108	841
IL-12p40-"full length"	5'-TGGCTAGCCCACGAGATATC-3'	-880/+108	988
Antisense Primer:			
	5'-ATTCCATGGCTGCAGGCCACACGGGAG-3'		
For EMSA			
NF/B, sense	5'-AGG AAC TTC TTG AAA TTC CCC CAG AAG GTT TT-3'		
NF/B, sense	5'-AGG AAC TTC TTG AAA TTC CCC CAG AAG GTT TT-3'		
NF/B, antisense	3'-TCC TTG AAG AAC TTT AAG GGG GTC TTC CAA AA-5'		
mNF/B, sense	5'-AGG AAC TTC TTe ceA TTC CCC CAG AAG GTT TT-3'		
mNF/B, antisense	3'-TCC TTG AAG AAG ggT AAG GGG GTC TTC CAA AA-5'		
AP-1, sense	5'-TCC TTC CTT ATT CCC CAC CCA-3'		
AP-1, antisense	5'-AGG AAG GAA TAA GGG GTG GGT-3'		
mAP-1, sense	5'-TCC TTC CTg AcT tgC CAC CCA-3'		
mAP-1, antisense	3'-AGG AAG GAc TgA acG GTG GGT-5'		

oligonucleotides at different concentrations (1–10 μ M) for 4 h followed by stimulation with LPS (0.1–0.5 μ g/ml). The culture supernatants were harvested 48 h after stimulation for measurement of IL-10 and IL-12p40 production by ELISA.

2.15. Statistical analysis

Means were compared by two-tailed Student's paired *t* test. The results are expressed as mean $\pm$ SEM.

Chapter III

Signaling Pathways Involved in LPS-induced IL-12p40 in Human Monocytic Cells

3.1. Introduction

IL-12 plays a critical role in host defense, hence understanding the signaling mechanisms involved in the regulation of IL-12 expression, especially which of the inducible IL-12p40 component, may lead to the development of strategies for the treatment of autoimmune diseases, cancers and viral infections. LPS is the best-characterized monocytic mitogen and has been indicated as a potent inducer of IL-12p40 [239]. However, there is little information known on the role of intracellular signaling molecules that regulate IL-12p40 synthesis in human monocytic cells following LPS stimulation. To investigate this, I focused on the role of the MAPKs and calcium signaling pathways in LPS-induced IL-12 production, because these signaling pathways play a key role in lymphocytic proliferation, differentiation, and apoptosis [240;241]. IL-12 expression is known to be regulated at the level of transcription and multiple transcription factors and their complexes have been suggested to regulate IL-12p40 transcription in monocytic cells [121;124]. NF κ B has been shown to play a key role in regulating IL-12p40 transcription in IFN γ -stimulated murine and LPS-stimulated human monocytic cells [119]. The C/EBP transcription factor, in co-operation with the Rel/NF κ B complex, was also found to regulate the murine and human IL-12p40 gene [121]. Recently, several studies have also reported the key role for IRFs, NF κ B, Ets-2, AP-1 and PU.1 transcription factors in the regulation of murine and human IL-12p40 expression [120;121;242].

There are several immunosuppressive drugs including dexamethasone (Dxm), cyclosporine A (CyA), FK506 whose abilities are believed to inhibit cell-mediated Th1 immune responses. Dxm is a potent anti-inflammatory glucocorticoid and has been

widely used in the treatment of inflammations, allergic diseases, and a number of autoimmune disorders [243]. CyA and FK506 are also immunosuppressive drugs broadly used in anti-rejection and treatment of autoimmune diseases [244].

The immunosuppressive properties of glucocorticoids have been ascribed for their ability to suppress the synthesis of a number of cytokines including that of IL-12p40 [245]. The molecular mechanisms by which Dxm inhibits IL-12p40 production remain unknown. Glucocorticoids have been shown to mediate their biological effects on cytokine production primarily by down-regulating JNK MAPK activation [246;247], whereas Dxm has also been shown to exert its biological effects by down-regulating the NF κ B and AP-1 transcription factors [248;249]. Therefore, I investigated the intracellular signaling pathways and transcription factors involved in LPS-mediated IL-12p40 production in human monocytic cells and the possible mechanisms for Dxm to suppress Th1 immune responses. I first investigated the role of signaling molecules involved in MAPK pathways in LPS-stimulated normal human monocytic cells and in promonocytic THP-1 cells following treatment with special inhibitors of MAPKs. The results revealed that p38 and ERK MAPKs did not regulate IL-12p40 production, whereas Dxm treatment significantly inhibited LPS-induced IL-12p40 production by monocytes and THP-1 cells, suggesting a role for JNK MAPK in IL-12p40 regulation. This was confirmed by using JNK specific inhibitor, SP600125 [250] and a dominant-negative (dn) mutant of the SEK1 which is the upstream kinase of JNK. To understand the regulation of IL-12p40 transcription, I generated a series of IL-12p40 promoter deletion mutants linked to the luciferase reporter gene and examined the ability of these promoter fragments to drive the expression of the luciferase gene in LPS-stimulated THP-1 cells. The results of these

analyses suggest a previously unrecognized role of AP-1 in addition to that of NFκB in the regulation of the hIL-12p40 gene. The involvement of AP-1 in IL-12p40 transcription was further confirmed by interfering with the IL-12p40 production in cells using antisense c-fos and c-jun oligonucleotides. This data suggested the involvement of JNK in IL-12p40 production through the activation of AP-1 and NFκB transcription factors in LPS-stimulated human monocytic cells.

CyA and FK506 have been broadly used in a variety of disease conditions such as clinical post-organ transplantations and autoimmune diseases with a view to suppress T cell activation and cell-mediated Th1 immune responses [244;251]. Both CyA and FK506 are potent inhibitors of Ca^{2+} dependent T cell activation. CyA is a cyclic polypeptide produced as a fungal metabolite and consists of 11 amino acids whereas FK506 is a macrolide lactone [252]. CyA and FK506 bind to cyclophilin A and 12 kD FK506-binding protein, FKBP12, respectively. CyA-cyclophilin A and FK506-FKBP12 complexes further bind to the same target calcineurin [90;91], thereby inhibiting TcR/NFAT-signaling mediated T cell activation. CyA and FK506 not only affect T cells, but also modulate the biological functions of antigen presenting cells such as B cells, macrophages and dendritic cells. Recently, FK506 and CyA have been shown to inhibit Th1 responses by interfering with cytokine production including IL-12 by dendritic cells [253]. Thus, I investigated calcium signals and transcription factors involved in the regulation of LPS-induced IL-12p40 production in human monocytes, which may associate with CyA and FK506-mediated suppression of Th1 cellular immunity. In pretreated monocytes and THP-1 cells with CyA and FK506 followed by LPS stimulation, IL-12p40 production is significantly reduced. Of note, recent investigations

have indicated the negative regulatory role of calcium and PI3K pathways in LPS-induced IL-12p40 production in murine macrophages and dendritic cells [254;255]. However, in this study, I demonstrated that, in contrast to the regulation of murine IL-12p40 production, LPS-induced IL-12p40 production in human monocytic cells is positively regulated by the calcium-dependent PI3K pathways. Furthermore, CyA and FK506 inhibited IL-12p40 production by inhibiting the NF κ B and AP-1 transcription factors through interfering with calmodulin and calmodulin-dependent kinase-II (CaMKII) activated PI3K pathways.

Taking together, my results demonstrated for the first time that LPS-induced IL-12p40 production is regulated by two distinct and independent signaling pathways, JNK and CaM/CaMKII activated PI3K pathways in human monocytic cells [100;256].

3.2. Results

3.2.1. JNK, NF κ B and AP-1 in Dxm-mediated IL-12p40 production in LPS-stimulated human monocytes

3.2.1-1 LPS-induced IL-12p40 production by normal human monocytes does not involve the activation of either p38 or p42/44 ERK MAPKs

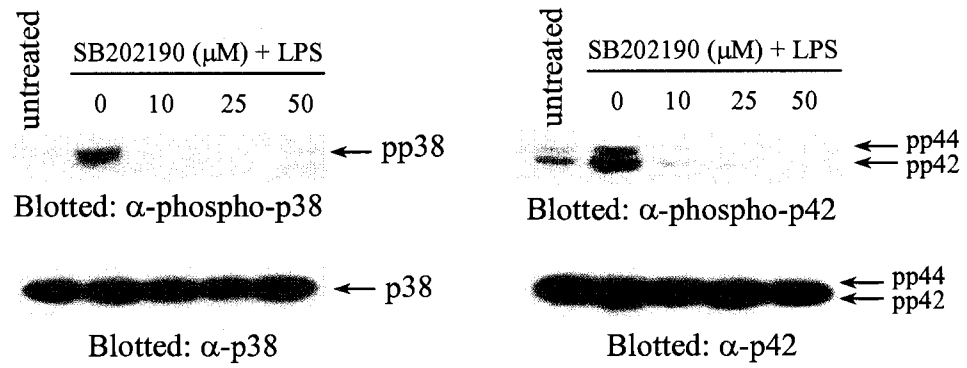
I first confirmed and show that purified normal human monocytes secrete IL-12p40 in response to LPS stimulation (Fig. 3.2.1-1.B). To investigate the role of MAPKs in LPS-induced IL-12p40 production, I examined the activation of ERK and p38 MAPKs in normal human monocytes. Purified monocytes isolated from healthy individuals were stimulated with LPS for 15 min and subjected to Western immunoblotting for p38 and ERK activation by using anti-phospho-p38 and anti-phospho-p42/44 ERK specific antibodies, respectively. The same blots were stripped and reprobed with anti-p38 and anti-42/44 Abs to ensure equal protein loading. The results showed that LPS stimulation

Fig. 3.2.1-1 p38 and p42/44 MAPK inhibitors do not affect LPS-induced IL-12p40 production in human monocytes:

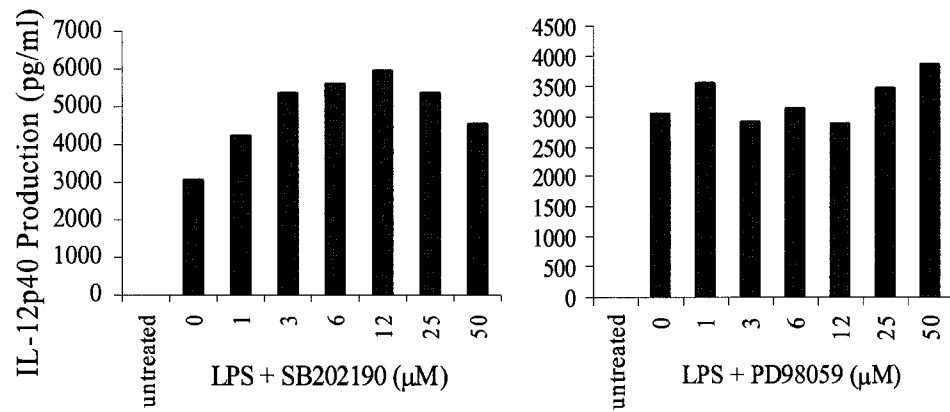
A, Purified normal human monocytes ($1 \times 10^6/\text{ml}$) were pretreated with either SB202190 or PD98059 at varying concentrations ranging from 0 to 50 μM for 2 h before LPS (1 $\mu\text{g}/\text{ml}$) stimulation for 10 min. Total proteins (50 μg) were subjected to SDS-PAGE followed by Western blot analysis using either antiphospho-p38 (pp38) or anti-phospho-p42/44 (indicated by arrows as pp42/44) Abs. To control for equal loading of proteins, the membranes were stripped and reprobed with either anti-p38 or anti-p42/44 Abs, respectively (indicated by arrows as p38 and p42/44).

B, Cells were treated with varying concentrations of inhibitors ranging from 0 to 50 μM for 2 h before stimulation with LPS (1 $\mu\text{g}/\text{ml}$). The supernatants were harvested after 48 h and analyzed by ELISA for IL-12p40 production. The results shown are representative of five independent experiments performed

A



B



induced the phosphorylation of both p38 and p42/44 ERKs (Fig. 3.2.1-1.A). To define the role of MAPKs involved in the regulation of LPS-induced IL-12p40 production, monocytes were treated with specific inhibitors of p38 (SB202190) and p42/44 ERKs (PD98059) at varying concentrations ranging from 0 to 50 μ M for 2 h followed by stimulation with LPS for 10 min. The results confirmed our earlier observations that both SB202190 and PD98059, at concentrations of 10 μ M, inhibited the phosphorylation of p38 and ERKs, respectively (Fig. 3.2.1-1.A).

To determine the role of p38 and p42/44 MAPKs, I analyzed IL-12p40 production in LPS-stimulated monocytes treated with the specific inhibitors of p38 and p42/44 ERKs. For this, purified monocytes were treated with SB202190 and PD98059 for 2 h before stimulation with LPS for 48 h. IL-12p40 production was not inhibited by either SB202190 or PD98059 at any concentration (Fig. 3.2.1-1.B). These results suggested that LPS-induced IL-12p40 production in normal monocytes does not involve the activation of either p38 or p42/44 ERKs. In fact, SB202190 enhanced IL-12p40 production (Fig. 3.2.1-1.B). I have previously shown that treatment of monocytes with SB202190 inhibits LPS-induced IL-10 production [100]. The modest but reproducible increase in IL-12p40 production following treatment of monocytes with SB202190 may be attributed to the decreased endogenous production of IL-10.

3.2.1-2 Dxm inhibits IL-12p40 production in LPS-stimulated monocytes and THP-1/CD14 cells

The lack of involvement of p38 and p42/44 ERKs in LPS-induced IL-12p40 production prompted me to examine the role of JNK, the third major member of the MAPK family. To determine whether the JNK signaling pathway was involved in IL-

IL-12p40 production, I took advantage of the fact that glucocorticoids inhibit the activation of JNK [100;247;252]. I examined whether LPS could induce JNK phosphorylation in normal human monocytes, and whether Dex could inhibit this phosphorylation. Monocytes were treated with Dex at varying concentrations for 2 h before stimulation with LPS. The results supported my earlier observations and showed that LPS-induced JNK phosphorylation in normal monocytes was inhibited by Dex in a dose-dependent manner (Fig. 3.2.1-2A). LPS-induced IL-12p40 production was inhibited by Dex at very low concentrations of 15 nM (Fig. 3.2.1-2A). To understand the molecular mechanism, and specifically the role of JNK in LPS-induced IL-12p40 production in human monocytic cells, I used the promonocytic THP-1/CD14 cell line which constitutively expressed the LPS receptor, CD14, on their surface membrane [100] (data not shown). Stimulation of THP-1/CD14 cells with LPS induced high levels of IL-12p40 production as determined by ELISA and RT-PCR (Fig.3.2.1-2B and C). To assess the involvement of JNK, I examined whether LPS could induce JNK phosphorylation in THP-1/CD14 cells in a manner similar to normal monocytes, and whether Dex could inhibit its phosphorylation. LPS induced phosphorylation of JNK, and this activation was inhibited in a dose-dependent manner by Dex (Fig.3.2.1-2C). LPS-induced IL-12p40 production in THP-1/CD14 cells was also highly sensitive to Dex in a manner similar to that of monocytes (Fig. 3.2.1-2C).

3.2.1-3 JNK MAPK plays a distinct role in LPS-induced IL-12p40 production in THP-1 cells

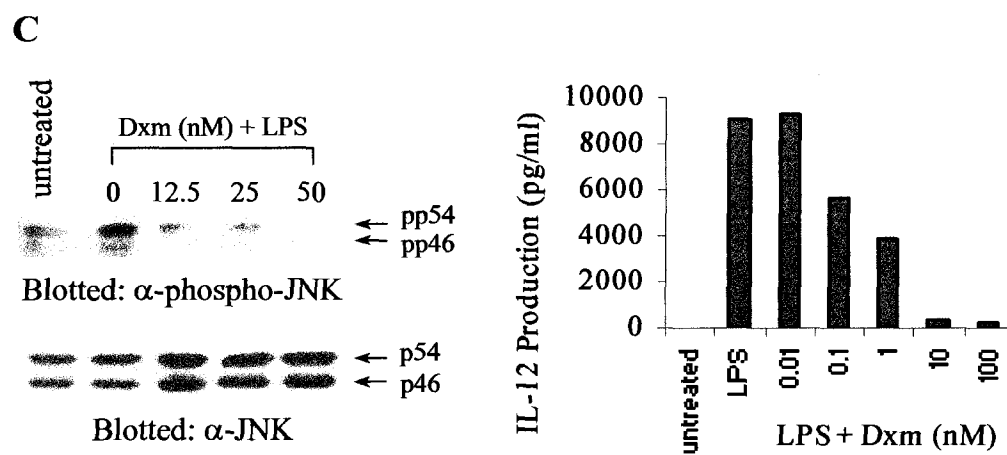
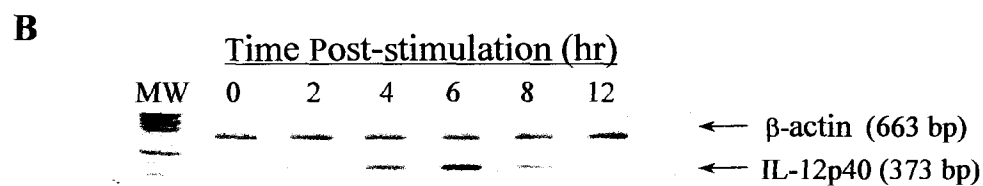
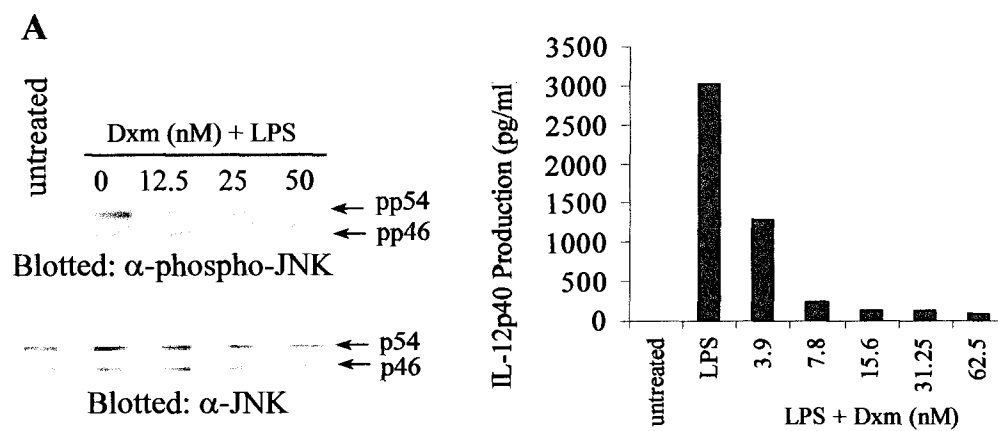
JNK is a cellular target of the SEK1 kinase and expression of the dnSEK1 interferes with JNK phosphorylation by competing out endogenous SEK1 [254;255]. To

Fig. 3.2.1-2 Dxm inhibits LPS-induced IL-12p40 production in normal monocytes and THP-1/CD14 cells:

A, Left panel, Normal monocytes ($1.0 \times 10^6/\text{ml}$) were pretreated with Dxm at concentrations ranging from 0 to 50 nM for 2 h before LPS ($1 \mu\text{g}/\text{ml}$) stimulation for 15 min. Total proteins ($50 \mu\text{g}$) were subjected to SDS-PAGE followed by Western blot analysis using anti-phospho-JNK1 Abs. Control protein levels were probed with anti-total JNK to verify equivalent protein loading. **Right panel,** Monocytes were pretreated with Dxm at concentrations ranging from 0 to 62.5 nM for 2 h before LPS stimulation for 48 h. Cell supernatants were harvested and analyzed for IL-12p40 production. The effects of Dxm on IL-12p40 production were qualitatively similar in seven different donors.

B, THP-1/CD14 cells were stimulated with LPS for various times ranging from 2 to 12 h. Cells were harvested for mRNA isolation, and IL-12p40 expression was determined by semiquantitative RT-PCR analysis using β -actin as a standard control. The results shown are representative of three independent experiments performed.

C, Left panel, THP-1/CD14 cells were pretreated with Dxm at concentrations ranging from 0 to 50 nM for 2 h before LPS stimulation for 15 min. Total proteins ($50 \mu\text{g}$) were subjected to SDS-PAGE followed by Western blot analysis. The membranes were blotted with anti-phospho-JNK1 Ab, and to control for protein loading, the membrane was stripped and reprobed with anti-JNK Ab. **Right panel,** THP-1/CD14 cells were pretreated with Dxm at concentrations ranging from 0 to 100 nM for 2 h before LPS stimulation for 48 h, following which cell supernatants were harvested and analyzed for IL-12p40 production. The results shown are representative of three independent experiments performed.



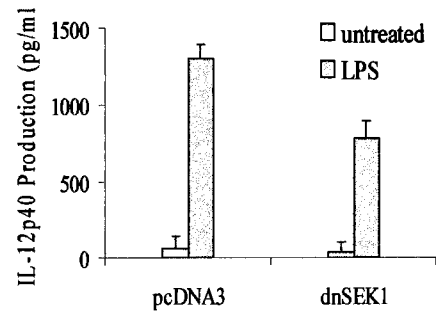
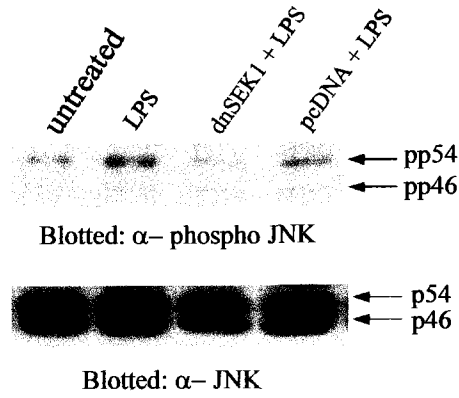
confirm the role of the JNK pathway in IL-12p40 production, THP-1/CD14 cells were transfected with either a plasmid expressing a dnSEK1 kinase mutant or with a control plasmid (pcDNA3). Cells transfected for 12 h with either the dnSEK1 or the control plasmids were stimulated with LPS and analyzed by ELISA for IL-12p40 production. The period of 12 h posttransfection before LPS stimulation was identified as the optimal time following cell stimulation with LPS for different time periods (data not shown). IL-12p40 production was significantly reduced in dnSEK1-transfected cells compared with the cells transfected with the control plasmid (Fig. 3.2.1-3A; $p < 0.05$). The effect of transfection of THP-1/CD14 cells with dnSEK1 on IL-12p40 production was selective, because the expression of CD14 and the IL-10 production following LPS stimulation remained unaffected [257] (data not shown). Recently, a specific JNK inhibitor, SP600125, has become commercially available. To confirm the involvement of JNK, THP-1/CD14 cells were pretreated with varying concentrations of SP600125 before stimulation with LPS. SP600125 inhibited JNK phosphorylation induced by LPS in a dose-dependent manner (Fig. 3.2.1-3B). To confirm the specificity of SP600125, the same blots were stripped and probed with either Anti-phospho p38 or anti-phospho p42/44 Abs. The results show that SP600125 did not inhibit the phosphorylation of either p38 or p42/44 MAPKs (Fig. 3.2.1-3B). Analysis of the IL-12p40 promoter region required for IL-12p40 transcription results obtained with Dxm, SP600125 inhibited LPS-induced IL-12p40 production in a dose-dependent manner (Fig. 3.2.1-3B). Furthermore, both Dxm (data not shown) and SP600125 did not affect LPS-induced IL-10 production (263 ± 11 vs. 235 ± 54 pg/ml). These results suggest that JNK MAPK activation may be involved in LPS-induced IL-12p40 production in normal human monocytic cells.

Fig. 3.2.1-3 JNK signal involved in LPS-induced IL-12p40 production in THP-1 cells:

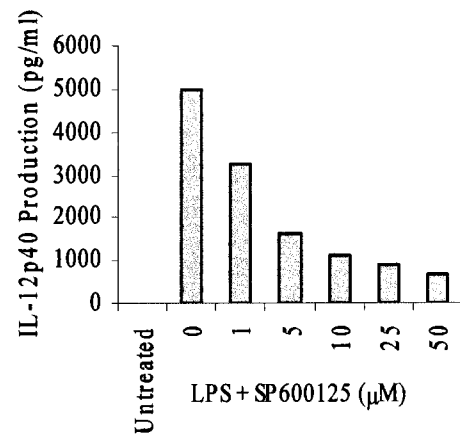
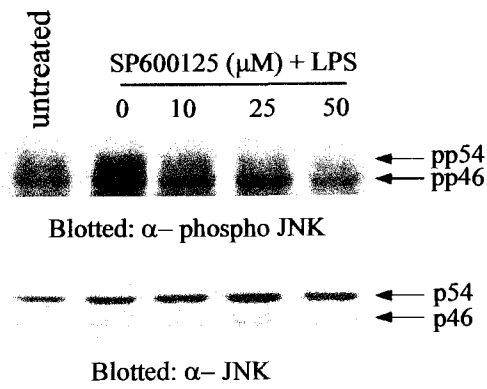
A, Left panel, JNK phosphorylation is inhibited by LPS stimulation in dnSEK1-transfected THP-1 cells. Cells were transfected with either a dnSEK1 kinase mutant or with control vector followed by stimulation with LPS for 10 min. Total proteins (50 µg) were subjected to SDS-PAGE analysis followed by Western blot analysis. The membranes were blotted with an anti-phospho JNK rabbit polyclonal Ab, and to control for equal loading of proteins, the membranes were stripped and reprobed with anti-JNK rabbit polyclonal Ab. **Right panel,** dnSEK1 kinase mutant inhibits LPS-induced IL-12p40 production. THP-1/CD14 cells were transfected with a dnSEK1 construct. After 12 h of transfection, cells were treated with LPS (1 µg/ml) for 48 h, following which IL-12p40 production was analyzed in the cell supernatants by ELISA. The results shown are a mean of three experiments performed.

B, SP600125 inhibits LPS-induced IL-12p40 production in THP-1/CD14 cells. Left panel, THP-1/CD14 cells (1×10^6 /ml) were treated with SP600125 at concentrations ranging from 0 to 50 µM for 2 h before LPS stimulation (1 µg/ml) for 15 min. Total proteins were analyzed for JNK phosphorylation using an anti-phospho-JNK rabbit polyclonal Ab. The same membranes were stripped and reprobed for the phosphorylation of p38 and p42/44 ERK MAPKs using the anti-phospho-p38 (pp38) or anti-phospho-p42/44 (pp42/44) Abs. To control for protein loading, the membranes were stripped and reprobed with anti-JNK rabbit polyclonal Abs. **Right panel,** Cells (0.5×10^6 /ml) were treated with SP600125 at concentrations ranging from 0 to 50 µM for 2 h before stimulation with LPS followed by analysis of IL-12p40 production by ELISA. The experiment shown is representative of two different experiments

A



B



3.2.1-4 Analysis of the IL-12p40 promoter region required for IL-12p40 transcription

The hIL-12p40 promoter has been characterized [120;121;124]. To understand the regulation of IL-12p40 gene transcription in LPS-stimulated THP-1/CD14 cells, I PCR cloned the IL-12p40 promoter fragment from 5'-880 to 3' +108 bp relative to the +1 transcription start site (Fig. 3.2.1-4.). The amplified promoter fragment was subcloned into the NheI/NcoI polylinker sites of the basic luciferase reporter plasmid, pGL3B. THP-1 and THP-1/CD14 cells were transiently transfected with the IL-12p40 promoter/luciferase reporter construct (pIL-12Pr-GL3B). Twenty-four hours posttransfection, cells were stimulated with LPS for periods of time ranging from 6 to 36 h, following which relative luciferase activity was assessed. The results showed that luciferase activity could be detected by 12 h and peaked at 24 h following LPS stimulation (Fig. 3.2.1-5A). The maximum increase in luciferase activity ranged from 6- to 8-fold relative to the unstimulated cells and the cells transfected with the control plasmid pGL3B. (Fig. 3.2.1-5B). Similar results were obtained for THP-1 cells, although the increase in luciferase activity was relatively lower than for the THP-1/CD14 cells transfected with pIL-12Pr-GL3B (Fig. 3.2.1-5B). To identify the DNA sequences required for IL-12p40 transcription, I cloned a series of hIL-12p40 promoter fragments (from 5'-880 to 3'+108 bp) by chopping up from the 5' end and linked with pGL3B. The exact size of the amplified product and the location of consensus sequences for different transcription factors identified within the IL-12p40 promoter are depicted in Figs. 3.1.4. and 3.1.6. Transfection of THP-1/CD14 cells with pGL3B containing various deletions of the IL-12p40 promoter revealed that deletion of sequences from -880 to -120 bp did not

Fig. 3.2.1-4 Nucleotide sequence of the first exon and 5'-flanking promoter region of the hIL-12p40 gene (GenBank accession no. U89323.):

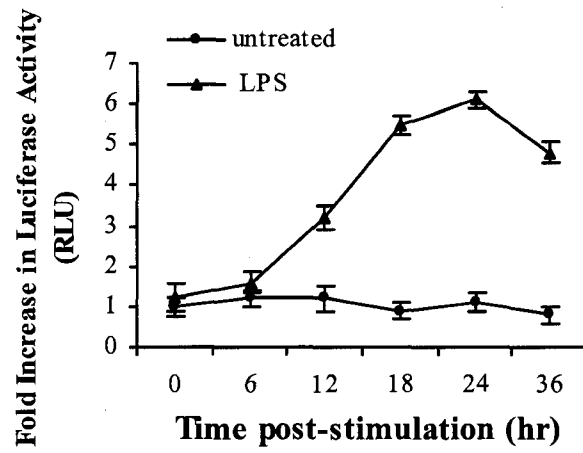
Putative cis-regulatory elements are boxed. The first exon is underlined and in bold type.

-880 CCACCACGAGAGATATC T TTATTCCGCT AT TCCT GT GCATCT GCACG GA GCCCCTAGG GCCATAGATTTG
 -810 TG TGCAAATGAAAT GAGGATGTAGTCTGGGTGCCCCAAGGGGGGGT GCCTT GAGT GT GGTT GTCTGTATGC
 IRF -730/-719
 -740 CT CCCT GAGGG **TATTTCACTTTC** T GCTCCCATCCG CCCCTAT GAGCGAGTACCTATGAGCACAGGATGTG
 -670 CACATATTT GAGTCTTATTAGT GGTACACGCAGTTTATCATCTCCCCAG GTCT GTGTCT GTAT GAAATGTG
 -600 CAT GGGT GTGT GT GT GCACGCGT GT GT TCCCACTCGGGG AAT GT GGGGAGAG GTGCATGGAGCCAAGA
 NF/IL-6 -512/-505
 -530 TGGGT GGTAATAGTATGT **TTCTGAAA** TTAAA GGAATAAT GTGGAGGAAGGCGCCCCAGATGTACTAAAC
 -460 CCTTT GCC TTCATCTCATCCTCTCTGACTT GGG AAG AACCAGGATTTT GTTTTAAAGCCCTTGGGCATACAG
 SP-1 -353/-347
 -390 TT GT TCCATCCC GACAT GA ACTCAGCCTCCC GTCT GA **CCGCCC** C T TG G CCT TCCT TCTTC CT CGATCT
 -320 GT GGAACCCAGGG AATCTGCCTAGT GCT GTCTCCAA GCACCTT GGCCATGATG TAAACCCAGAGAAATTA
 AP-1 -232/-226 Ets-2 -211/-206
 -250 GCATCTCCATCTCCT TCC **TTATTCC** CCACCC AAAAGTCA **TTTCC** CT TAG TCAT T ACCT GGG ATTTT GA
 PU.1 -128/-123 NF-kB
 -180 TGTCTAT GTT CCCT CCTCGTTATTGATACACACACAGAGAGAGACAAACAAA **AAGGAA** CTTCTT **GAAAT**
 -116/-106 C/EBP -80/-72
 -110 **TCCC**CC AGAAGGT TTT GAGAGTT GTTTTCA **ATGTTGCAA** CAAGTCAGTTTCTAGT T TAAGT T TCCATCAG
 TATA box
 -40 AAAGGAGTAGAG **TATATAA** GTTCCAGTACCAGCAACAGCAGCAGCAGAAGAAACAACATCTGTTTCAGGGGCCA
 ↑ exon 1
 +30 **TTGGACTCTCCGTCCTGCCCAGAGCAAGGTAAGCA**CTTCCCAAGCCCCTACC TCCCTCCCCTCCCT GTGGGC
 ↑ intron 1
 +100 CTGCAG

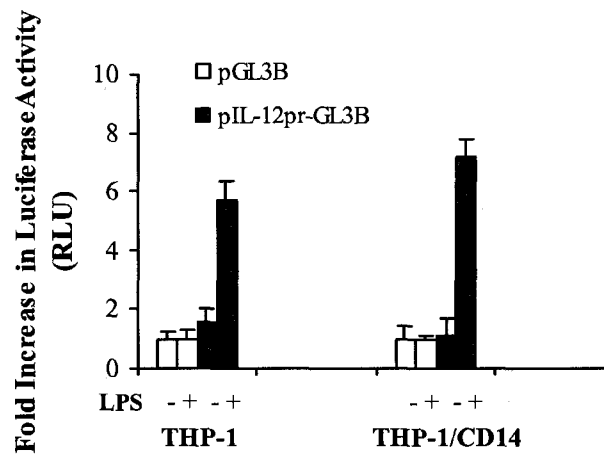
Fig. 3.2.1-5 Luciferase activity in LPS-stimulated THP-1 and THP-1/CD14 cells transfected with a hIL-12p40 promoter/luciferase construct:

THP-1 and THP-1/CD14 cells (1.5×10^6) were transiently cotransfected with 6 μg of either pIL-12pr-GL3B or vector control, and with 3 μg of β -galactosidase control plasmid, and allowed to grow for 24 h. **A**, THP-1 cells were treated with 1 $\mu\text{g}/\text{ml}$ LPS for 6, 12, 18, 24, and 36 h. **B**, The transfected THP-1 and THP-1/CD14 cells were treated with 1 $\mu\text{g}/\text{ml}$ LPS for 24 h. Following stimulation with LPS, luciferase and β -galactosidase activities were determined in the cell lysates. Cells transfected with vector pGL3B alone served as a negative control. Luciferase activity was normalized for β -galactosidase activity to give relative luciferase units (RLU). The results shown are the mean $\pm$ SD of four experiments performed in triplicate and normalized for β -galactosidase activity.

A



B



affect luciferase activity compared with the cells transcribed with the entire promoter sequence. However, deletion of sequences upstream of -84 bp abrogated luciferase activity (Fig. 3.2.1-6). Similar results were obtained for THP-1 cells (data not shown). These results suggest that DNA sequences located between -120 and -84 bp are necessary for IL-12p40 gene transcription in LPS-stimulated THP-1/CD14 cells.

3.2.1-5 The AP-1 and NFκB binding sites within the hIL-12p40 promoter are required for LPS-induced IL-12p40 production

IL-12p40 gene transcription has been shown to be regulated by multiple transcription factors including NFκB, PU.1, and Ets-2 [121;124]. A computer-aided analysis of the hIL-12p40 promoter sequence between -120 and -84 bp revealed the existence of a consensus sequence for NFκB (Fig. 3.2.1-4). To examine the role of NFκB in LPS-induced hIL-12p40 gene transcription, I generated site-directed mutagenesis in the NFκB sequence by PCR (Fig. 3.2.1-7A) and cloned into pGL3B. THP-1/CD14 cells transfected with this plasmid showed marked reduction in luciferase activity when compared with cells containing the plasmid encoding the wild-type NFκB sequence (Fig. 3.2.1-7B). To determine the role of PU.1, I cloned IL-12p40 fragment from -131 to -108 bp with the mutant PU.1 binding sequences and wtNFκB binding site, designated as IL-12p40PrNmGL3B. Transfection of THP-1/CD14 cells with IL-12p40PrNmGL3B significantly reduced the luciferase activity compared with the cells transfected with the plasmid containing wild-type NFκB and PU.1 sequences. Furthermore, transfection of cells with the IL-12p40 promoter (-131 bp) containing mutations only in the PU.1 binding site did not reduce luciferase activity as compared with the cells transfected with the promoter containing wtNFκB and PU.1 binding sites (data not shown). These results

Fig. 3.2.1-6 Transcriptional activities of deletion mutants of IL-12p40 promoter in LPS-stimulated THP-1/CD14 cells:

Top, The line diagram summarizes the position of potential regulatory elements relative to the structure of eight deletion constructs used in the experiment. The putative binding sites for the transcription factors IFN regulatory factor (IRF), NF/IL-6, Sp-1, AP-1, Ets-2, PU.1, NF- κ B, and C/EBP are shown.

Bottom, THP-1/CD14 cells were cotransfected with 10 μ g of either IL-12p40 promoter deletion mutant construct or vector control, and with 5 μ g of β -galactosidase control plasmid. After 24 h, cells were stimulated with LPS (1 μ g/ml) for another 24 h. Cell lysates from unstimulated and LPS-stimulated cells were assayed for luciferase and β -galactosidase activities. Luciferase activity was normalized for β -galactosidase activity to give relative luciferase units (RLU). The results shown are the mean $\pm$ SD of four experiments performed in triplicate.

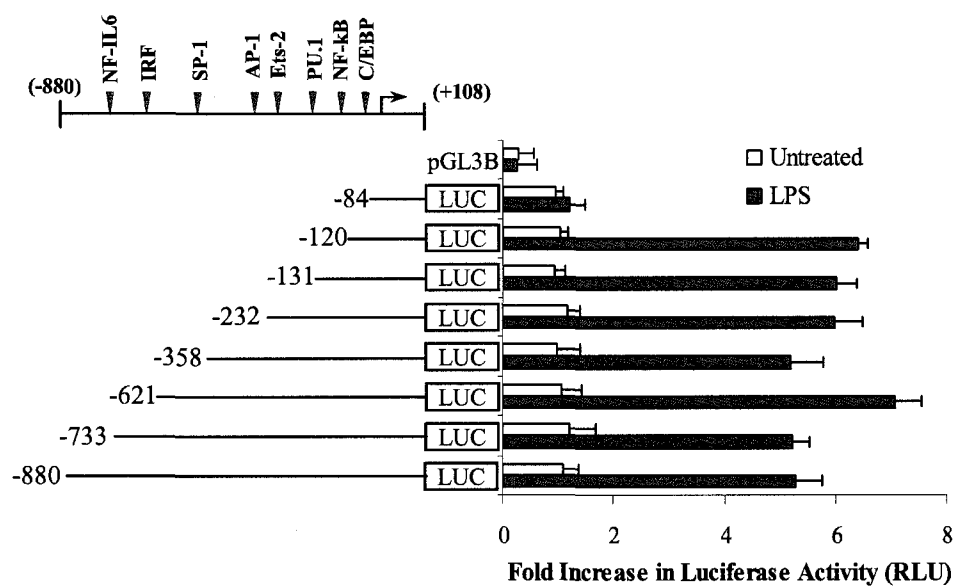
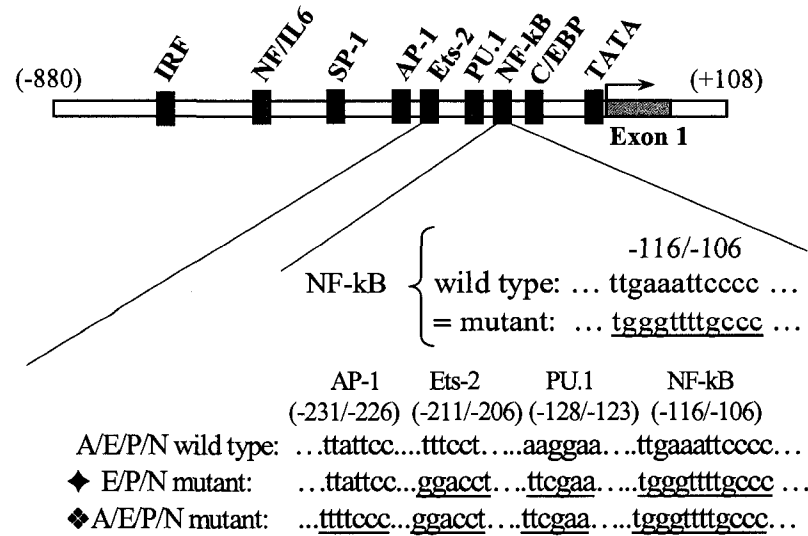


Fig. 3.2.1-7 The NF κ B and AP-1 binding site within the IL-12p40 promoter is required for LPS-induced IL-12p40 production:

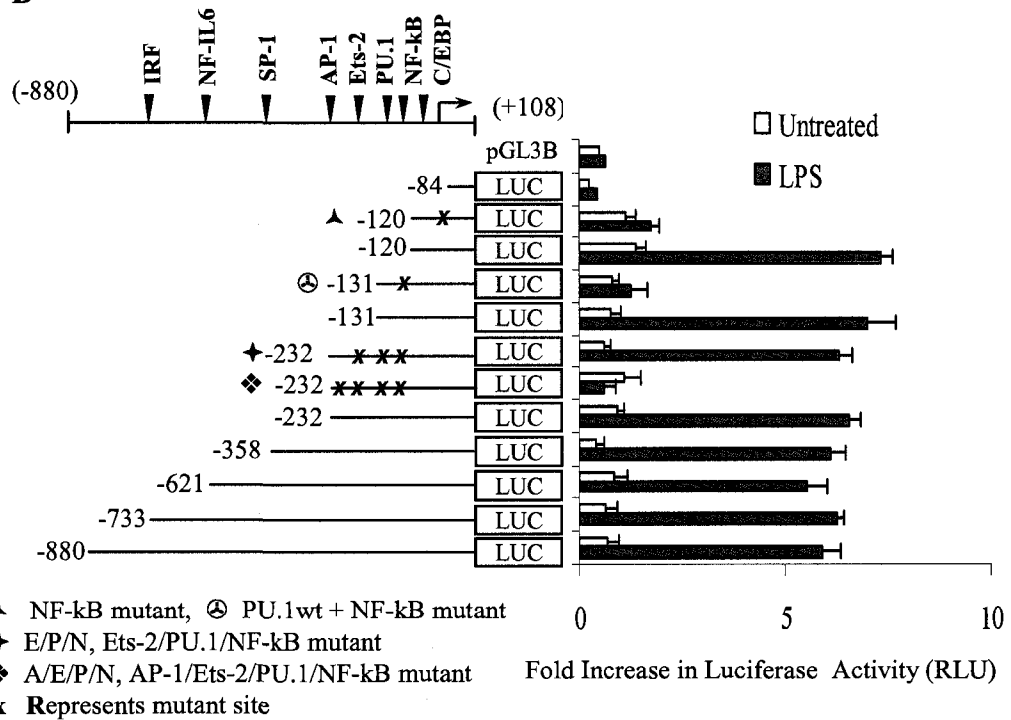
A, Line diagram depicts wild-type sequences and the mutations introduced in the binding sites of transcription factors NF κ B, PU.1, Ets-2, and AP-1 in the IL-12p40 promoter fragment. The IL-12p40 fragment (-120 to -108 bp) with a mutation in the NF κ B site is designated as NF κ B mutant; the IL-12p40 fragment (-131 to -108 bp) with a mutation in the NF κ B site is designated as PU.1wt - NF κ B mutant; the IL-12p40 fragment (-232 to -108 bp) with mutations in the AP-1, Ets-2, PU.1, and NF κ B sites is designated as A/E/P/N mutant; and the IL-12p40 fragment (-232 to -108 bp) with mutations in the Ets-2, PU.1, and NF κ B sites is designated as E/P/N mutant.

B, THP-1/CD14 cells were cotransfected with either 10 μ g of wild-type or mutant constructs A/E/P/N mutant, E/P/N mutant, PU.1wtNF κ B mutant, and NF κ B mutant, and with 5 μ g of β -galactosidase control vector. The transfected cells were stimulated with LPS (1 μ g/ml) for 24 h. Luciferase activity following normalization of β -galactosidase activity is shown as the mean $\pm$ SD of three experiments performed in triplicate.

A



B



suggested that, in the promoter fragment between -131 and -84 bp, NFκB plays a major role in LPS-induced IL-12p40 gene transcription. I have demonstrated that LPS-induced IL-12p40 production is highly sensitive to Dxm (Fig. 3.2.1-2). Because Dxm has been shown to exert its biological effects by down-regulating the AP-1 transcription factor [258] I hypothesized that, in addition to NFκB, AP-1 may also play a role in LPS-induced IL-12p40 gene transcription. To investigate the role of AP-1, I cloned a fragment ranged from -232 to -108 bp and linked to pGL3B. This fragment encodes, in addition to AP-1, the binding sites for NFκB, PU.1, and Ets-2 transcription factors that have been shown to be involved in IL-12p40 gene transcription [121;142]. To delineate the role of AP-1, I introduced mutations within the NFκB, PU.1, and Ets-2 sequences in the -232- to -108 bp promoter fragment and inserted into pGL3B (pIL-12Pr-E/P/Nm-GL3B). Surprisingly, transfection of THP-1/CD14 cells with pIL-12Pr-E/P/Nm-GL3B exhibited luciferase activity comparable with the cells transfected with the entire plasmid (Fig.3.2.1-7A and B). These results of the mutational analysis of the promoter region between -232 and -108 bp suggested the presence of DNA sequences other than those of NFκB, PU.1, and Ets-2 that are required for transcription of the IL-12p40 gene. Because this promoter fragment also encodes a binding site for AP-1, I investigated a role for AP-1 in LPS-induced IL-12p40 gene transcription. For this, I introduced a mutation in the consensus sequence of the AP-1 binding site, in addition to the mutations introduced earlier for the NFκB, PU.1, and Ets-2 binding sites. This fragment was cloned into pGL3B and designated as pIL-12Pr-A/E/P/Nm-GL3B. Transfection of THP-1/CD14 cells with pIL-12Pr-A/E/P/Nm-GL3B abrogated the luciferase activity compared with the plasmid containing the wtAP-1, PU.1, Ets-2, and NFκB sequences (Fig. 3.2.1-7B).

However, transfection of cells with IL-12p40 promoter (-232 bp) containing mutations only in the AP-1 binding site did not significantly reduce luciferase activity compared with the cells transfected with the promoter containing wtNFκB, PU.1, and Ets-2 binding sites (data not shown). These results suggested that AP-1, in addition to NFκB, may play a role in IL-12p40 gene transcription in LPS-stimulated human monocytic cells.

3.2.1-6 Antisense c-jun and c-fos (AP-1) oligonucleotides inhibit IL-12p40 production in LPS-stimulated THP-1/CD14 cells

To confirm the role of AP-1 in IL-12p40 expression, the antisense oligonucleotides for c-jun and c-fos, the AP-1 components, along with the control oligonucleotide containing equal number of base pairs were employed. Cells were treated with antisense

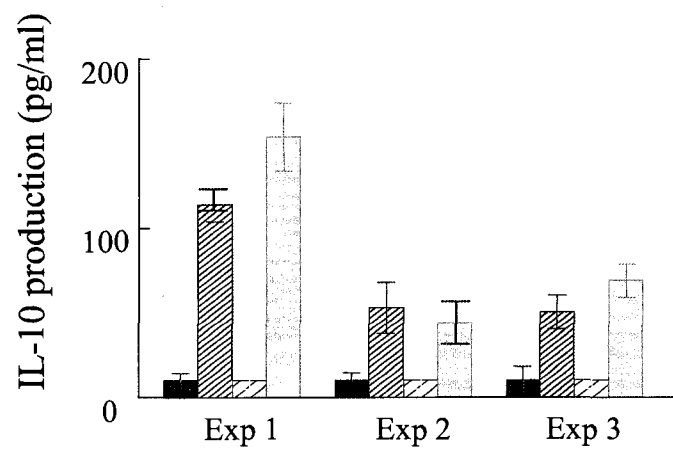
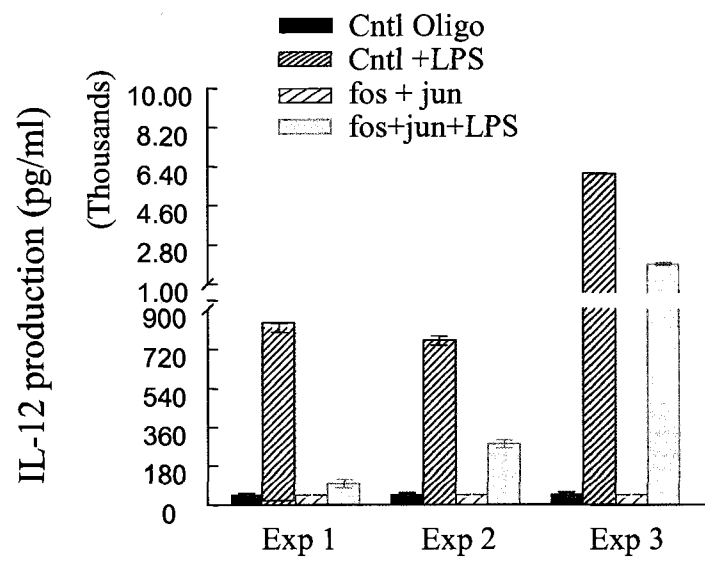
oligonucleotides for 4 h before stimulation with LPS (0.1 µg/ml in experiments 1 and 2, and 0.5 µg/ml in experiment 3) for 48 h. The results revealed that antisense c-jun and c-fos oligonucleotides significantly reduced IL-12p40 production. The c-fos and c-jun antisense oligonucleotides seemed to selectively inhibit IL-12p40 production, because IL-10 production (Fig. 3.2.1-8) and expression of CD44, CD80, or CD86 were not affected (data not shown).

3.2.1-7 Dxm and JNK inhibitor SP600125 down-regulate IL-12p40 expression by inhibiting AP-1 and NFκB activity

To investigate whether LPS-induced IL-12p40 expression is regulated by AP-1 through JNK activation, we used Dxm, and SP600125, a specific inhibitor of JNK MAPK. THP-1/CD14 cells were transfected with pGL3B containing a series of successive 5' deletions derived from -880 to -108 bp of the hIL-12p40 promoter as

Fig. 3.2.1-8 Antisense c-jun and c-fos oligonucleotides inhibit LPS-induced IL-12p40 production in THP-1/CD14 cells.

Cells were treated with the c-jun and c-fos antisense oligonucleotides (10 µg/ml) for 4 h followed by stimulation with LPS (0.1 µg/ml for experiments 1 and 2, and 0.5 µg/ml for experiment 3) for 48 h. The supernatants were analyzed for IL-12p40 and IL-10 production.

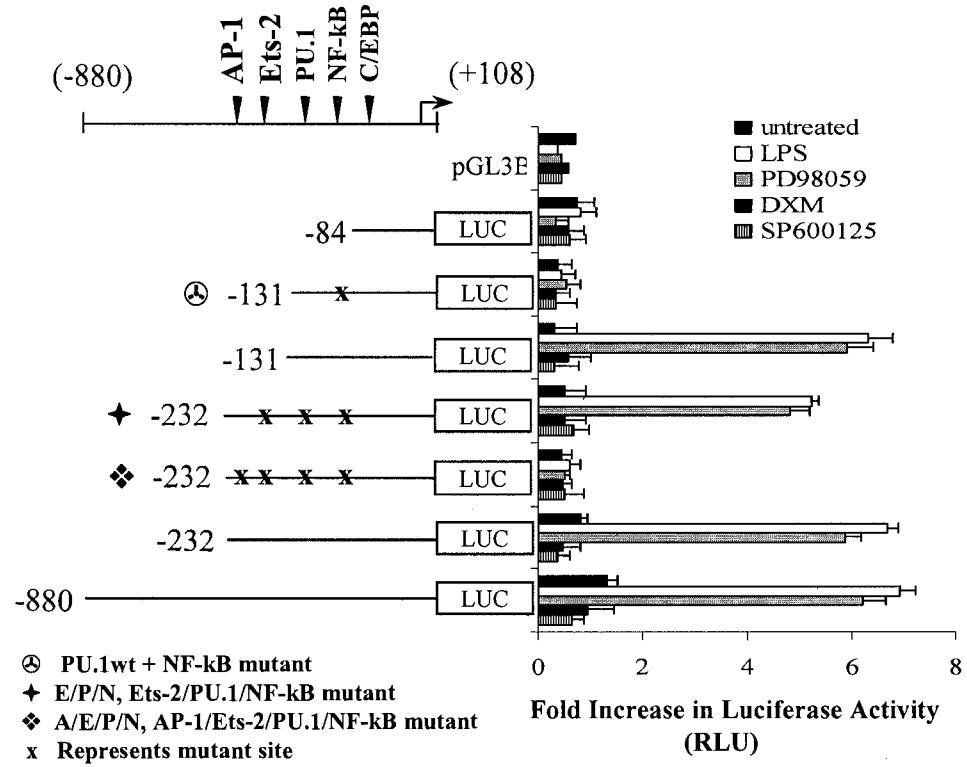
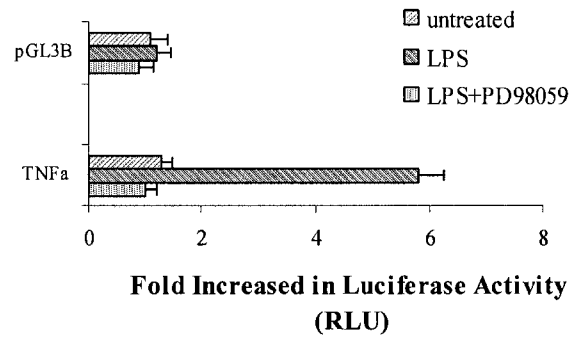


described above. The transfected cells were cultured for 2 h in the presence or the absence of either Dxm or SP600125. As a control, I treated transfected cells with PD98059, the ERK MAPK inhibitor, before stimulation with LPS. Luciferase activity was measured after 24 h. As observed above, transfection of THP-1/CD14 cells with plasmids containing deletion of sequences spanning -880 to -131 bp from the hIL-12 p40 promoter region revealed a 6- to 8-fold increase in the luciferase activity in LPS-stimulated cells, compared with the unstimulated cells or cells transfected with the control plasmid (Fig. 3.2.1-9A). Pretreatment of same cells with Dxm or SP600125 abrogated the luciferase activity (Fig. 3.2.1-9A). Similar results were observed in cells transfected with a plasmid (pIL-12Pr-E/P/Nm-GL3B) containing IL-12p40 promoter sequences (-232 to -108 bp) showing mutations in the binding sites for NFκB, PU.1, and Ets-2. Both Dxm and SP600125 inhibited the luciferase activity in THP-1/CD14 cells transfected with pIL-12Pr-E/P/Nm-GL3B following LPS stimulation (Fig.3.2.1-9A). As observed above, transfection of THP-1/CD14 cells with the plasmid, pIL-12-A/E/P/Nm-GL3B, containing mutations in the consensus sequences for AP-1, Ets-2, PU.1, and NFκB binding sites, did not increase the luciferase activity upon LPS stimulation. Our deletion analyses also demonstrated the sensitivity of NFκB activation to Dxm. Cells transfected with the constructs spanning from -131 to -108 bp that included a NFκB but not an AP-1 sequence, exhibited luciferase activity following LPS stimulation, and this activity was lost upon prior treatment with Dxm or SP600125 (Fig. 3.2.1-9A). Under the same experimental conditions, pretreatment of cells with PD98059 did not have any effect (Fig. 3.2.1-9A). To confirm that PD98059 can inhibit the luciferase activity of the reporter gene linked to the promoter of an Erk-responsive gene in our THP-1/CD14 cells

Fig. 3.2.1-9 JNK is involved in activation of AP-1 and NF κ B in THP-1/CD14 cells:

A, Dxm and SP600125 inhibit IL-12p40 expression by inhibiting AP-1 and NF κ B activity. THP-1/CD14 cells (1.5×10^6) were cotransfected with either 10 μ g of wild-type or mutant constructs A/E/P/N mutant, E/P/N mutant, and PU.1wt NF κ B mutant, and with 5 μ g of β -galactosidase control vector. The transfected cells were pretreated with either 25 nM Dxm, 25 μ M SP600125, or 15 μ M PD98059 as a control for 2 h followed by stimulation with 1 μ g/ml LPS for 24 h. Unstimulated, LPS-stimulated (LPS), LPS plus Dxm (Dxm)-, LPS plus SP600125 (SP600125)-, or LPS plus PD98059 (PD98059)-treated cells were harvested, and their lysates were assessed for luciferase and β -galactosidase activities. The results shown are the mean $\pm$ SD of three experiments performed in triplicate and normalized by β -galactosidase activity.

B, PD98059 can inhibit an ERK-responsive gene, TNF- α , in THP-1/CD14 cells. THP-1/CD14 cells transfected with the TNF- α promoter linked to the luciferase reporter gene were stimulated with LPS for 24 h followed by the assessment of luciferase activity as described above. The results shown are the mean $\pm$ SD of two experiments performed in triplicate following normalization for β -galactosidase activity.

A**B**

system, I transfected cells with TNF- α promoter linked to pGL3B (kindly provided by Dr. D. Wilkinson (Ottawa Health Research Institute, Ottawa, Ontario, Canada). I have previously shown that PD98059 inhibited LPS-induced TNF- α production in THP-1 cells in a dose-dependent manner [259]. In this study, I show that PD98059 inhibited LPS-induced luciferase activity in cells transfected with the plasmid containing TNF- α promoter compared with the cells transfected with the control plasmid (Fig. 3.2.1-9B). Taken together, these results suggest that both Dxm and SP600125 inhibited IL-12p40 transcription by inhibiting AP-1 activation in addition to that of NF κ B.

3.2.1-8 Dxm and SP600125 inhibit NF κ B and AP-1 binding to the IL-12p40 promoter in LPS-stimulated THP-1/CD14 cells and normal monocytes

To confirm the role of AP-1 and NF κ B in the regulation of hIL-12p40 gene transcription, I investigated whether LPS stimulation of THP-1/CD14 cells and normal monocytes induced the binding of NF κ B and AP-1 to their respective NF κ B and AP-1-binding sites present in the hIL-12p40 promoter. THP-1/CD14 cells and normal monocytes were stimulated with LPS over a period of time ranging from 0 to 60 min, and the nuclear extracts were analyzed in a gel shift assay for binding to AP-1 and NF κ B oligonucleotide probes corresponding to the AP-1 and NF κ B binding sites, respectively, in the IL-12p40 promoter. Because it was difficult to obtain sufficient numbers of monocytes from one donor, cells were stimulated for only 30 and 45 min. The results show that significant binding of AP-1 and NF κ B to the AP-1 and NF κ B oligonucleotides, respectively, occurred 30–45 min following stimulation of THP-1/CD14 cells (Fig. 3.2.1-10A) and normal monocytes (B) with LPS [257]. This binding was completely blocked

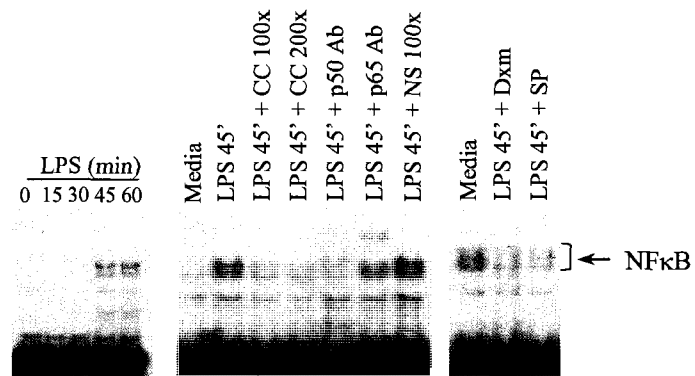
by competition with their respective cold AP-1 and NFκB oligonucleotides indicating their specificity (Fig. 3.2.1-10A). In contrast, mutant oligonucleotides (mNFκB and mAP-1), unlike their counterpart unmutated oligonucleotides, failed to compete for binding of NFκB and AP-1 transcription factors to their respective NFκB- and AP-1-labeled oligonucleotide probes (Fig. 3.2.1-10A). The mutations in the mNFκB and mAP-1 oligonucleotides corresponded to the mutations in the IL-12p40 promoter construct, pIL-12Pr-A/E/P/Nm-GL3B, used in the luciferase reporter assays as shown in Fig. 3.2.1-7. In addition, gel supershift analyses were performed to confirm the identity of the transcription factors NFκB and AP-1 by using specific mouse anti-NFκB p50 and p65 mAbs and rabbit anti-c-fos and rabbit anti-c-jun polyclonal Abs, respectively. Incubation of nuclear extracts obtained from both THP-1/CD14 cells and monocytes with oligonucleotide probes and either anti-p50 or p65 NFκB Abs, or anti-c-fos or anti-c-jun Abs revealed bands of higher molecular mass (Fig. 3.2.1-10). To determine whether Dxm and SP600125 inhibited binding of AP-1 and NFκB to their binding sites in the IL-12p40 promoter, THP-1/CD14 cells and normal monocytes were treated with Dxm or SP600125 for 2 h before LPS stimulation for 30 or 45 min followed by the analysis of AP-1 and NFκB binding to their corresponding oligonucleotide probes. The results show that both Dxm and SP600125 inhibited the binding of AP-1 and NFκB transcription factors to the AP-1 and NFκB probes, respectively, in both LPS-stimulated normal monocytes and THP-1/CD14 cells (Fig. 3.2.1-10). Taken together, the results suggest that IL-12p40 gene transcription may be regulated by AP-1 in addition to that of NFκB transcription factors through JNK activation.

Fig. 3.2.1-10 DXM and SP600125 inhibit NF κ B and AP-1 binding to the IL 12p40 promoter in LPS-stimulated THP-1/CD14 cells (**A**) and normal human monocytes (**B**).

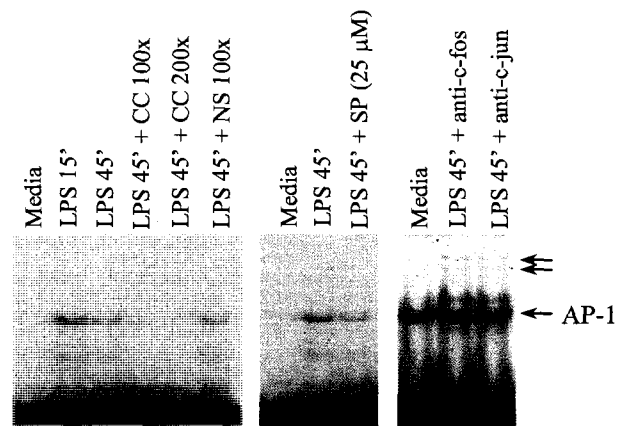
THP-1/CD14 cells and monocytes were stimulated with LPS (1 μ g/ml) for times ranging from 0 to 60 min. To determine the effects of Dxm and SP600125 on LPS-induced NF κ B and AP-1 activation, cells were treated with either Dxm (25 nM) or SP600125 (25 μ M) or 2 h before stimulation with LPS. Nuclear extracts containing 5 μ g of proteins were incubated for 1 h with 32 P-labeled oligonucleotides corresponding to the AP-1 or NF κ B sequences derived from the IL-12p40 promoter. To determine the specificity of AP-1 and NF κ B transcription factor binding, the nuclear extracts were incubated with unlabeled wild-type or mutant oligonucleotides (100–200 $\times$) corresponding to the AP-1 and NF κ B sequences, respectively. The supershift analysis for the NF κ B transcription factor was performed by treating the nuclear extracts obtained from both THP-1/CD14 cells (**A**) and monocytes (**B**) with oligonucleotide probes and either anti-p50 or -p65 NF κ B Abs, or the isotype control Abs. Similarly, for the AP-1 transcription factor, the nuclear extracts were incubated with oligonucleotide probes and anti-c-*fos*, anti-c-*jun* polyclonal Abs, or the control Abs. The supershift bands for NF κ B and AP-1 are indicated by arrows.

A. THP-1

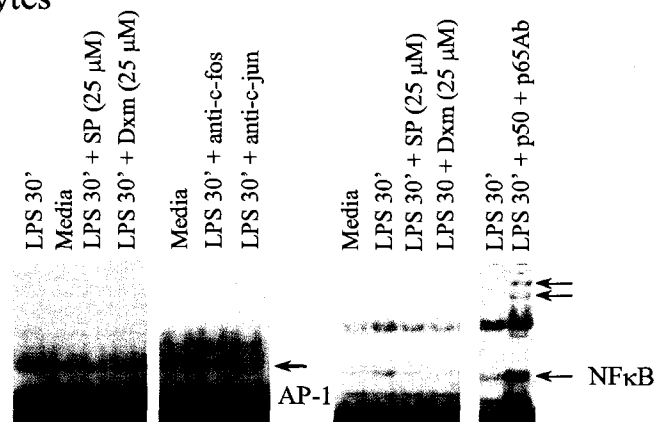
NF κ B



AP-1



B. Monocytes



3.2.2. CaMKII/Calcineurin/PI3K signals in CyA and FK506-regulated IL-12p40 Production in LPS-activated Human Monocytic Cells

3.2.2-1 K506 and CyA inhibit LPS-induced IL-12p40 production by inhibiting a calcium-regulated pathway in human monocytic cells

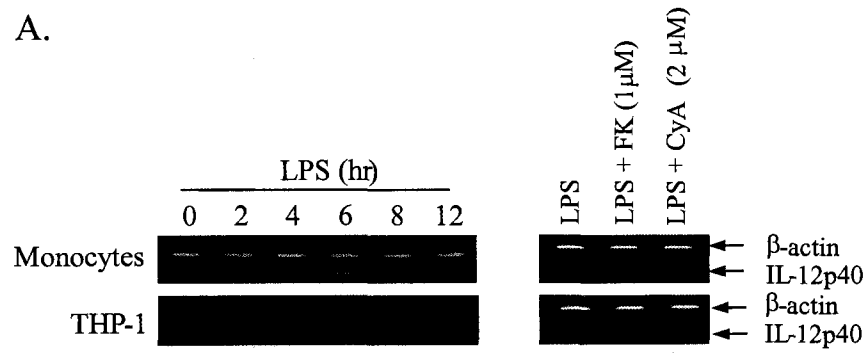
LPS stimulation of primary human monocytes and THP-1 cells induced maximum expression of IL-12p40 at 6 hr post stimulation. Treatment of cells with either CyA or FK506 for 2 hr prior to stimulation with LPS significantly inhibited IL-12p40 expression as determined by semi-quantitative RT-PCR (Fig. 3.2.2-1A) and ELISA (Fig 3.2.2-1B) in both primary monocytes and THP-1 cells. Since FK506 and CyA exert their biological effects by inhibiting the calcium-dependent signalling pathway [90;91], I determined the role of calcium signalling in LPS-induced IL-12p40 production. Stimulation of monocytes and THP-1 cells with LPS increased the levels of intracellular calcium after 4 min that was inhibited following treatment of cells with EGTA (Fig 3.2.2-2A). FK506 and CyA inhibited LPS-induced increases in intracellular calcium in both monocytes (from a MCF of 128 to the basal levels of 32) and THP-1 cells (from a MCF of 64 to the basal levels of 32) in a dose dependent manner (Fig. 3.2.2-2A). To confirm that FK506 and CyA are biologically active, Jurkat cells were treated with these agents for 2 hr prior to stimulation with PMA/ionomycin for another 5 min followed by analysis of NFAT-4 expression by Western blotting. NFAT-4 is constitutively expressed in resting Jurkat cells. PMA/ionomycin stimulation induces its translocation into the nuclei that is inhibited by prior treatment of cells with CyA/FK506. PMA and ionomycin treatment caused disappearance of the NFAT-4 band which was restored by pretreatment of cells with FK506 and CyA (Fig.3.2.2-2B). To determine a direct link for LPS-induced

Fig. 3.2.2-1 FK506 and CyA inhibit LPS-induced IL-12p40 expression in human monocytes and THP-1 cells:

A, monocytes and THP-1 cells (1×10^6) were treated with 1 μ M FK506 and 2 μ M CyA for 2 h and stimulated with 1 μ g/ml LPS for 0 –12 h for analysis of IL-12p40 mRNA expression by semiquantitative RT-PCR. The results shown are from one of three independent experiments with similar results.

B, monocytes and THP-1 cells pretreated with CyA and FK506 at various concentrations for 2 h were stimulated with LPS for 24 h followed by analysis of IL-12p40 production by ELISA. The results shown are a mean $\pm$ S.D. of three experiments.

A.



B.

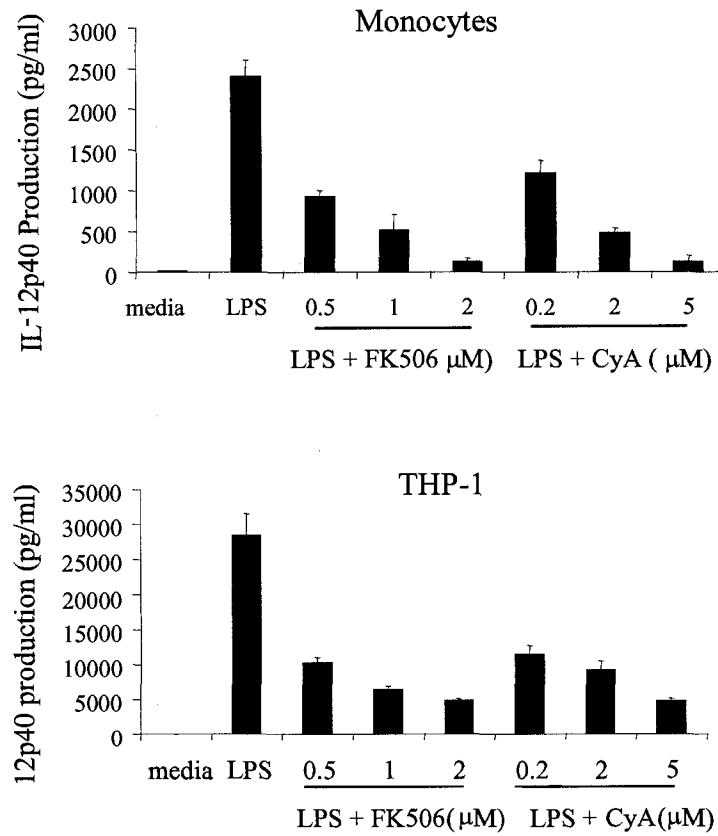


Fig. 3.2.2-2 Inhibition of IL-12p40 production by FK506 and CyA is calcium-dependent in LPS-activated monocytes and THP-1 cells:

A, FK506 and CyA inhibit LPS-induced increase in the levels of intracellular calcium in monocytes and THP-1 cells. Cells (0.5×10^6) were pretreated with varying doses of K506 (2–5 μ M) and CyA (2–5 μ M) for 2 h and loaded with Fluo-3/AM followed by LPS stimulation for 0–15 min. The levels of intracellular calcium were then measured by flow cytometric analysis.

B, the biological activity of FK506 and CyA was confirmed by their effect on NFAT-4 expression in Jurkat T cells. Jurkat T cells (2×10^6 /ml) were treated with FK506 and CyA for 2 h and stimulated with PMAplus ionomycin for 5 min. Cell lysates were analyzed for NFAT-4 expression by Western blotting using anti-NFAT-4 antibody (*top panel*). To ensure equal protein loading, membranes were stripped and reprobed with anti-CaMK-II Abs (*bottom panel*).

C, THP-1 cells and monocytes were pretreated with EGTA at concentrations ranging from 5 to 25mM for 2 h followed by LPS stimulation for another 24 h. IL-12p40 production in the supernatant was measured by ELISA. Results shown are a mean $\pm$ S.D. of three experiments.

D, IL-12p40 mRNA expression is regulated by CaM/CaMKII and PI3K pathways. Monocytes and THP-1 cells (5×10^6 /ml) were treated with various inhibitors at indicated concentrations for 2 h prior to stimulation with 1 μ g/ml LPS for 4 h followed by analysis for IL-12p40mRNA expression by semiquantitative RT-PCR analysis using β -actin as an internal control. The results shown are representative of three independent experiments.

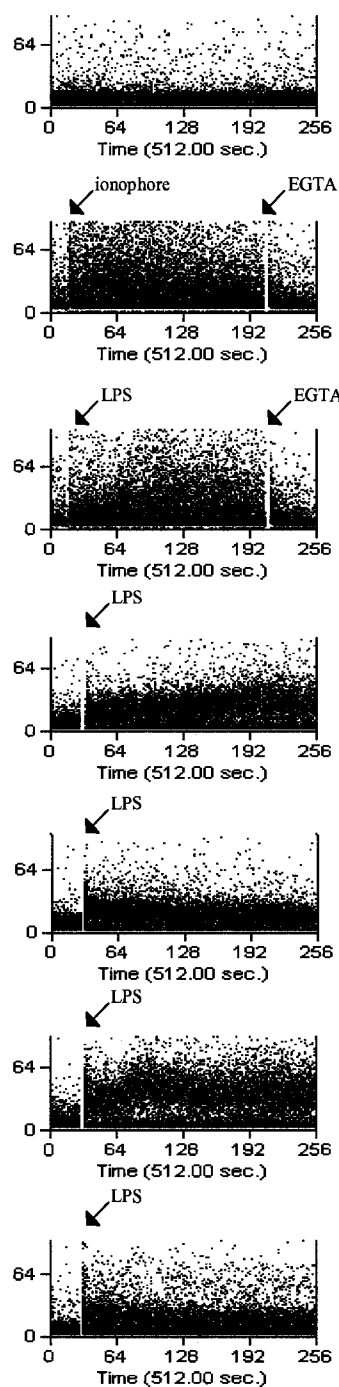
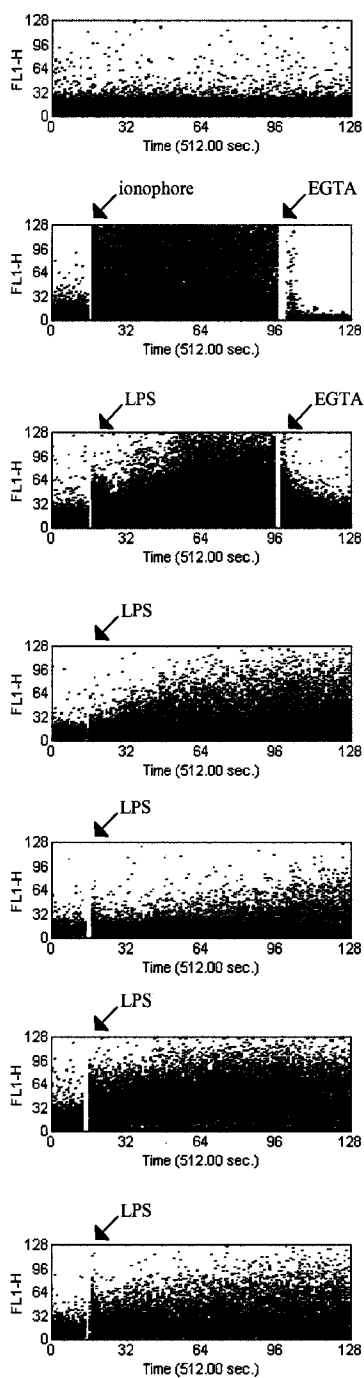
Fluorescence Intensity

Time stimulation

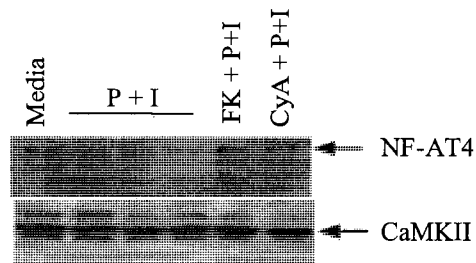
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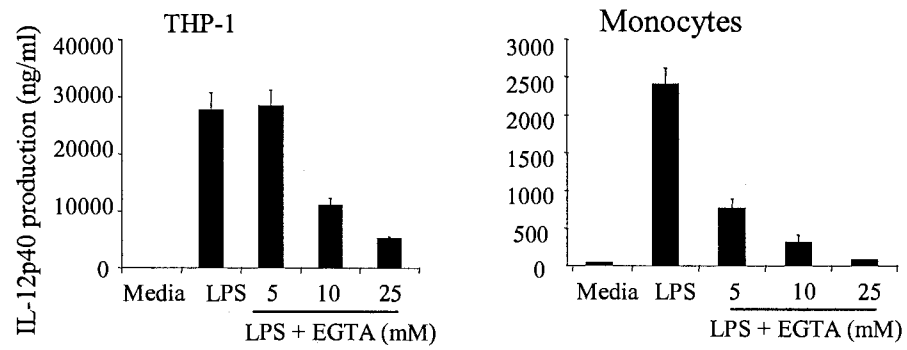
Media

FK (2 μ M)FK (5 μ M)CyA (2 μ M)CyA (5 μ M)

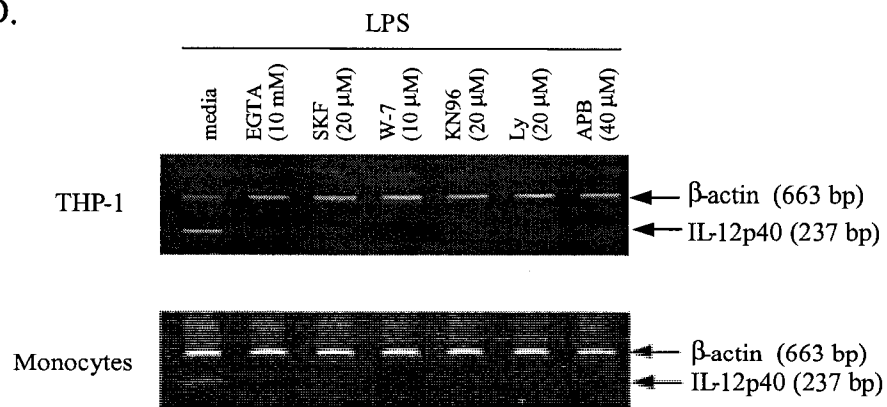
B.



C.



D.



increases in intracellular free calcium and IL-12p40 production, cells were incubated with EGTA prior to LPS stimulation for 24 hr. EGTA inhibited LPS-induced IL-12p40 production in both cell types as measured by ELISA (Fig. 3.2.2-2C) and semi-quantitative RT-PCR analysis (Fig 3.2.2-2D).

3.2.2-2 Calmodulin/CaMK-II regulates LPS-induced IL-12p40 production

To further delineate the role of calcium signalling, I used pharmacological inhibitors specific for members of the calcium signaling pathway. Increases in cytoplasmic calcium concentrations occurs by stimuli that activate voltage or ligand-gated calcium channels in the plasma membrane or following release of calcium present in intracellular stores, mainly in the endoplasmic reticulum (ER) [260;261]. The role of receptor-mediated entry of extracellular Ca^{2+} was studied by using SKF-96365 [262]. To determine if calcium release from the ER regulates IL-12p40 expression, we employed 2-APB, an inhibitor of the IP3 receptor which blocks calcium release from the ER by blocking IP3 receptor-gated channels [263]. Prior treatment of cells with either SKF-96365 or 2-APB significantly inhibited LPS-induced IL-12p40 production in both cell types as determined by ELISA (Fig. 3.2.2-3A) and semi-quantitative RT-PCR analysis (Fig 3.2.2-2D). It may be noted that monocytes were more sensitive to the effects of EGTA, SKF-96365 and 2-APB than were THP-1 cells. These results suggest that receptor-mediated Ca^{2+} entry as well as the Ca^{2+} release from the ER may be involved in LPS-induced IL-12p40 expression.

CaM, a major calcium receptor, is present in both cytoplasmic and nuclear compartments. The calcium/CaM complex regulates several downstream targets

including protein kinases and phosphatases [264;265]. To determine the role of CaM, I used a specific calmodulin inhibitor, W-7 [266;267]. W-7 inhibited LPS-induced IL-12p40 production in both cell types as determined by ELISA (Fig. 3.2.2-3B) and semi-quantitative RT-PCR analysis (Fig 3.2.2-2D). One major family of CaM effectors is the calmodulin-dependent protein kinase (CaMK), which includes a multifunctional kinase, CaMK-II. To gain further insight into the role of calcium/CaM, I examined the role of CaMK-II by employing the CaMK-II-specific inhibitor, KN93 [268;269] which significantly inhibited LPS-induced IL-12p40 production in both cell types as determined by ELISA (Fig. 3.2.2-3B) and semi-quantitative RT-PCR analysis (Fig. 3.2.2-2D). I also confirmed that LPS induced CaMK-II phosphorylation was inhibited by W-7 and KN93 in both cell types (Fig 3.2.2-3C).

3.2.2-3 The p110 α isoform of class I_A PI3K positively regulates LPS-induced IL-12p40 expression

The PI3K pathway negatively regulates IL-12p40 expression in murine DCs and monocytic cells [254;255]. Therefore, it was of interest to determine if PI3K regulates human IL-12p40 production in a manner similar to the calcium pathway. Therefore, I first demonstrated that LPS induced phosphorylation of Akt, a downstream substrate for PI3K, and LY294002 inhibited this phosphorylation in both monocytes and THP-1 cells (Fig. 3.2.2-4A). Interestingly, LY294002 significantly inhibited LPS-induced IL-12p40 expression in both cell types (Fig. 3.2.2-2D, and 3.2.2-4B).

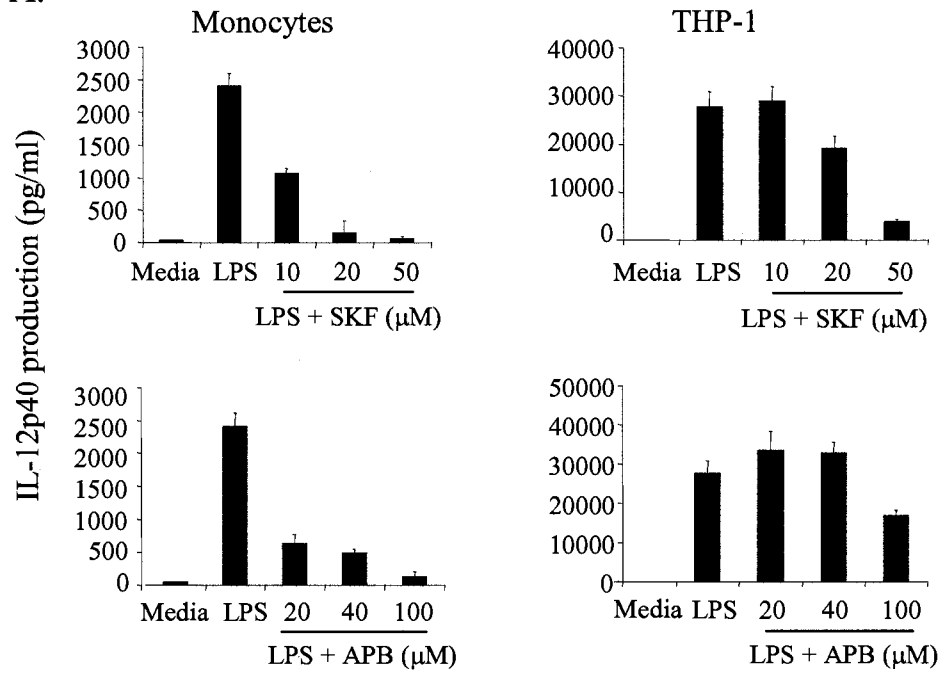
Mammalian class-I_A PI3K are heterodimers consisting of a regulatory subunit (p85 α , p85 β , p55 or other splice variants) and a p110 (α , β , or γ isoforms) catalytic unit [270-272]. Herein, we investigated the role of the p110 α isoform by employing THP-1

Fig. 3.2.2-3 LPS-induced IL-12p40 expression in monocytes and THP-1 cells is regulated by the CaM/CaMK-II pathway:

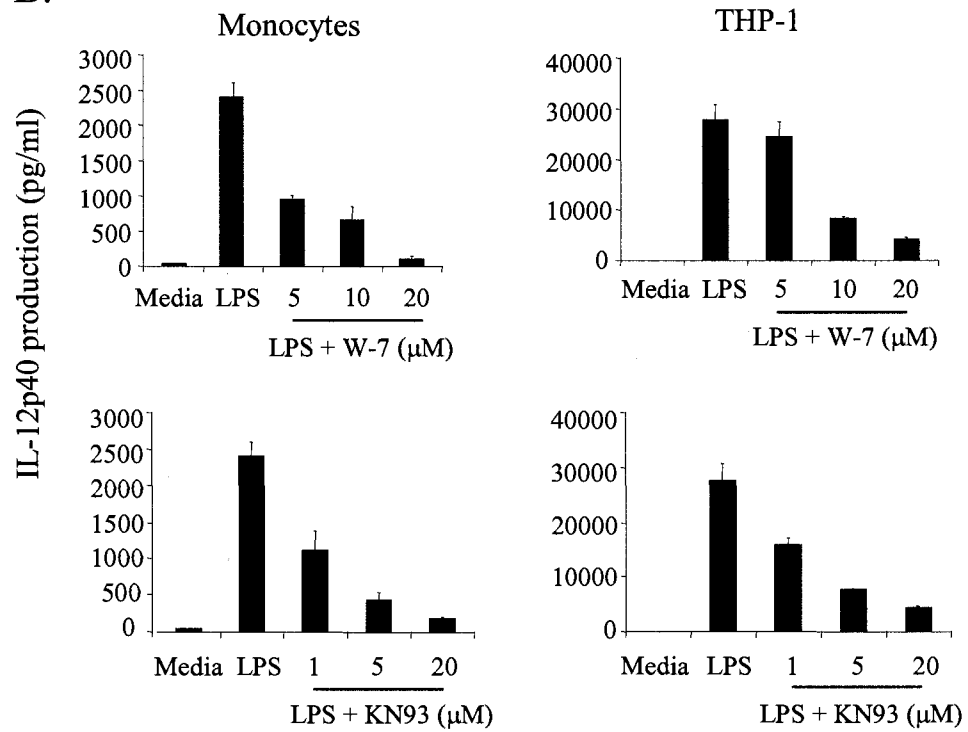
A & B, Monocytes (*left panel*) and THP-1 cells (*right panel*) at a concentration of 1×10^6 /ml were treated with varying concentrations of SKF, 2-APB (**A**), W7, or KN-93 (**B**) for 2 h prior to stimulation with LPS ($1 \mu\text{g}/\text{ml}$) for 24 h. The cell supernatants were analyzed for IL-12p40 production by ELISA. Results shown are a mean $\pm$ S.D. of three experiments.

C, Cells (1×10^6) were treated with W-7 and KN93 for 2 h prior to stimulation with LPS for 12 min. Cells were harvested for determining the phosphorylation of CaMKII by Western blot analysis using anti-phospho-CaMKII antibody (*top panel*). To ensure equal protein loading, the membrane were stripped and reprobed with anti-CaMKII Abs (*bottom panel*).

A.



B.



C.

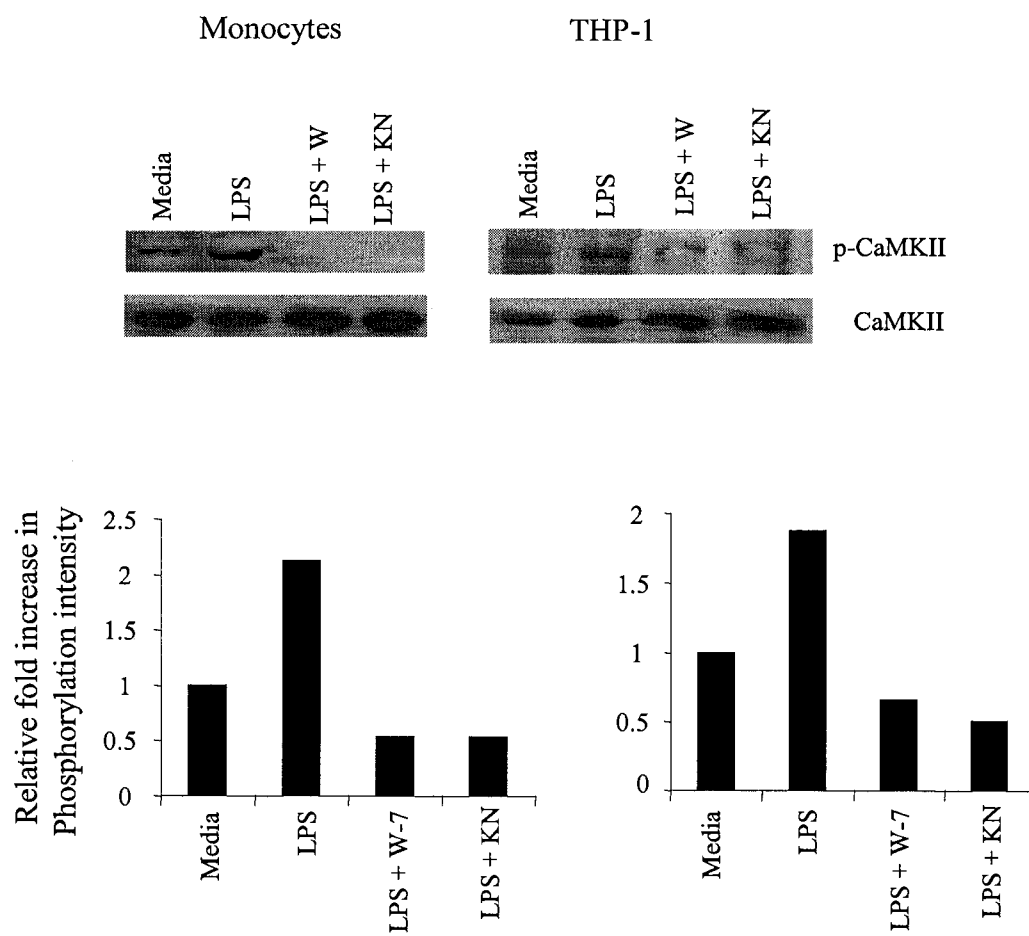


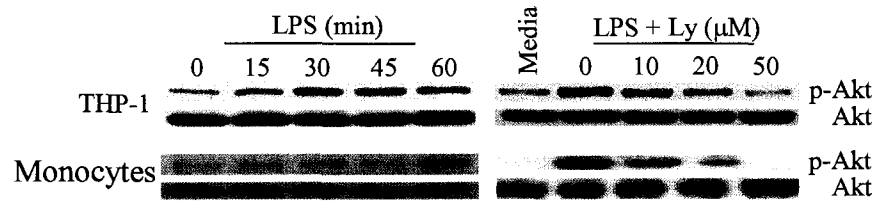
Fig. 3.2.2-4 LPS-induced IL-12p40 expression is positively regulated by the p110 α isoform of PI3K in human monocytic cells:

A, monocytes and THP-1 cells (1×10^6 /ml) were stimulated with LPS for 0–60 min (*left panel*). Monocytes and THP-1 cells (1×10^6 /ml) were cultured with various concentrations of Ly294002 ranging from 0 to 50 μ M for 2 h followed by stimulation with LPS for 60 min. Total proteins were subjected to Western blot analysis for Akt phosphorylation using anti-phospho-Akt Abs. The equal loading of protein was confirmed with anti-Akt Abs. The blot shown is a representative of three independent experiments.

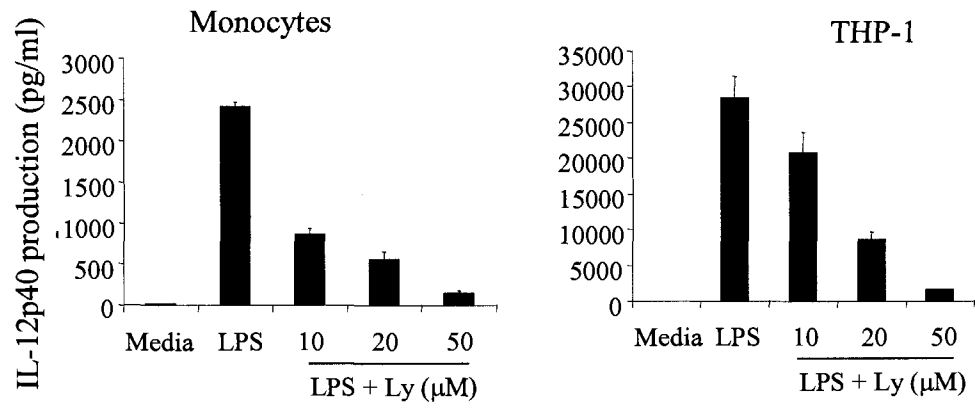
B, monocytes and THP-1 cells were pretreated with Ly294002 at indicated concentrations for 2 h followed by LPS (1 μ g/ml) stimulation for 24 h. IL-12p40 production in the supernatants was measured by ELISA. Results shown are a mean $\pm$ S.D. of three experiments.

C, normal THP-1 cells and THP-1 cells transduced with HR-p110 α 3 or HR-p110 α 1 (1×10^6 /ml) were stimulated with LPS (1 μ g/ml) for 24 h followed by analysis for IL-12p40 production by ELISA. The expression of PI3K p110 α and p85 isoforms in the above cells was determined by Western blotting by employing anti-p110 α and anti-p85 antibodies, respectively (*inset*). The results shown are representative of three independent experiments

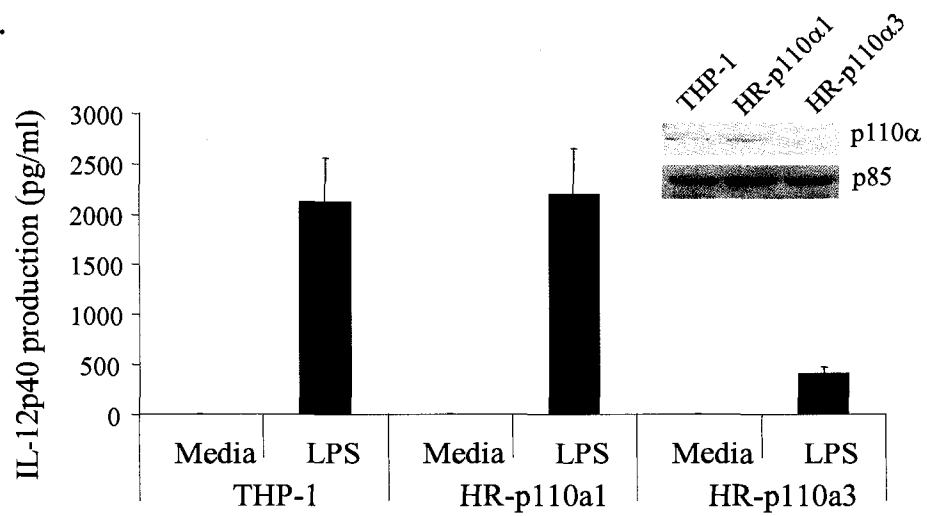
A.



B.



C.



cells stably deficient in the p110 α isoform [273]. Cells were stably transduced with a lentiviral vector expressing short hairpin RNA (shRNA) for the p110 α subunit (HR-p110 α 3). These cells exhibited complete inhibition of the PI3K p110 α isoform expression without affecting the expression of p85 (Fig 3.2.2-4C) or that of p110 β and p110 γ isoforms [273] and data not shown. Cells transduced with the control lentiviral vector HRp110 α 1 exhibited normal levels of the p110 α isoform (Fig. 3.2.2-4C) and [274]. To determine the involvement of the PI3K p110 α isoform, normal THP-1 cells, and THP-1 cells stably expressing HR-p110 α 3 and HR-p110 α 1 were stimulated with LPS for 24 hr and analyzed for IL-12p40 expression. The results show that IL-12p40 production was significantly reduced in THP-1 cells transfected with HR-p110 α 3 compared to the control (Fig. 3.2.2-4C) suggesting a key role for p110 α isoform of PI3K in LPS-induced IL-12p40 production. The role of p85 subunit of PI3K was confirmed by transfecting THP-1 cells with vectors containing p85-specific or a control siRNA. Transfection with p85-specific siRNA significantly reduced LPS-induced p85 phosphorylation (Fig 3.2.2-5A) and IL-12p40 expression as determined by RT-PCR (Fig. 3.2.2-5B) and ELISA (Fig. 3.2.2-5C).

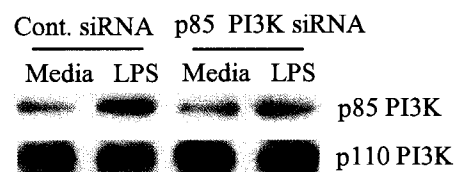
3.2.2-4 FK506 and CyA inhibit LPS-induced IL-12p40 production by inhibiting CaM/CaMKII-activated PI3K independent of the JNK pathway

I have previously demonstrated a key role for JNK in LPS-induced IL-12p40 production [113]. Since PI3K is involved in TLR-4-mediated signalling and Akt has been shown to regulate c-Raf (ERK), p38 and JNK MAPKs [275;276], I examined if activation of calmodulin/CaMKII, PI3K, and JNK by LPS constitute distinct pathways in IL-12p40 regulation. This was accomplished by pretreating cells for 2 hr with various

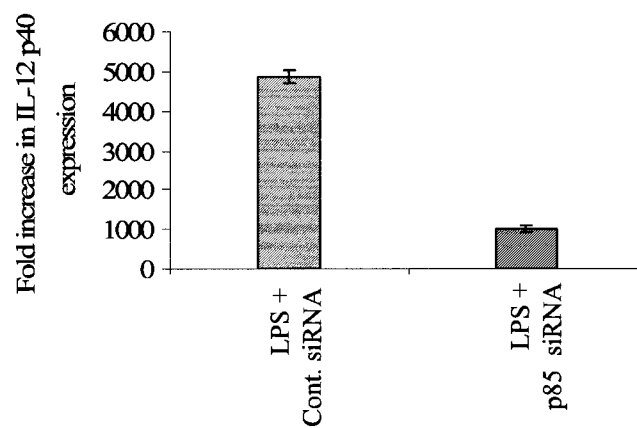
Fig. 3.2.2-5 LPS-induced IL-12p40 expression is positively regulated by p85 PI3K in human monocytic cells:

THP-1 cells were transfected with the vectors containing either the p85 or the control siRNA for 24 h followed by stimulation with LPS (1 µg/ml) either for 30 min for the determination of phosphorylated p85 by Western blot analysis (**A**), for 4 h for measurement of IL-12p40 RNA by real-time RT-PCR (**B**), or for 24 h for measurement of IL-12p40 by ELISA (**C**).

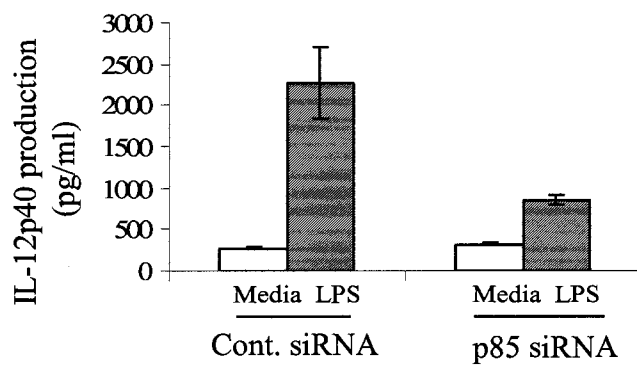
A.



B.



C.



concentrations of inhibitors specific for calcium-dependent signalling followed by LPS stimulation and analysis of Akt and JNK phosphorylation. EGTA, 2-APB, SKF, KN93, W7, FK506, and CyA suppressed phosphorylation of Akt without any effect on JNK phosphorylation in THP-1 cells and monocytes (Fig. 3.2.2-6). These results suggest that LPS-induced IL-12p40 production is regulated by two distinct pathways namely PI3K activated upstream by CaM/CaMKII, and the JNK pathway, independent of the latter. Moreover, CyA and FK506 inhibited LPS-induced IL-12p40 production through the selective inhibition of pathways involving calcium (Fig. 3.2.2-2A) and PI3K without affecting the JNK pathway (Fig 3.2.2-6A and 6B).

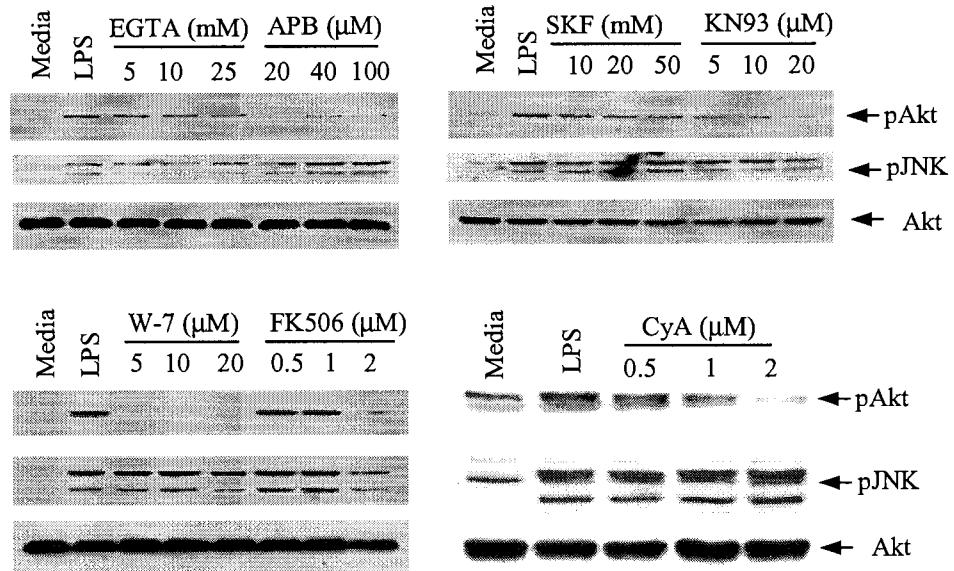
3.2.2-5 FK506 and CyA regulate LPS-induced IL-12p40 expression by NF κ B and AP-1 through the activation of CaM/CaMKII and PI3K pathways

I have previously shown that LPS-induced IL-12p40 production is regulated by JNK through the activation of NF κ B and AP-1 [100]. To determine if LPS-induced IL-12p40 production is regulated by CaM/CaMKII-activated NF κ B and/or AP-1, I confirmed my earlier results by transfecting cells with a series of 5'-deletion constructs containing IL-12p40 promoter (-880/+118 bp) sequences (Fig. 3.2.1-4) linked with the luciferase reporter plasmid, pGL3B. Subsequent LPS-induced luciferase activity analysis showed that transfection with the -880 to +118 construct induced significant luciferase activity compared to the cells transfected with the control vector whereas transfection with the -116 vector abrogated luciferase activity (Fig. 3.2.2-7). Transfection with vectors containing mutant NF κ B in the presence of PU.1 binding sequence (-128, pIL-12p40/wtPU.1, mNF κ B) abrogated the luciferase activity while transfection with vectors containing wild type AP-1 and mutant NF κ B sites (-232, pIL-12p40/wtAP-1.Ets-2.PU.1,

Fig. 3.2.2-6 LPS-induced Akt phosphorylation is regulated by the calcium signaling pathway:

THP-1 cells (**A**) and monocytes (**B**) ($2 \times 10^6/\text{ml}$) were treated with EGTA, 2-APB, SKF, W-7, CyA, or FK506 at indicated concentrations for 2 h prior to stimulation with LPS ($1 \mu\text{g}/\text{ml}$) for 30 min. Total proteins were subjected to SDS-PAGE analyzed for the phosphorylation of Akt and JNK using anti-phospho-Akt and anti-phospho-JNK Abs, respectively. For determining equal protein loading, membranes were reprobed with anti-Akt Abs. The results shown are representative of three independent experiments.

A. THP-1



B. Monocytes

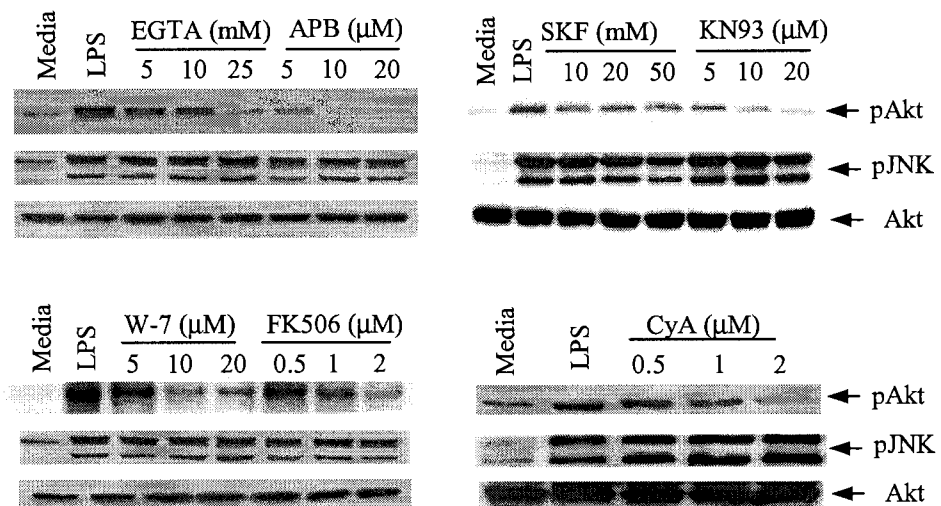


Fig. 3.2.2-7 LPS-induced expression of IL-12p40 is regulated by NF κ B and AP-1 transcription factors through the activation of calcium and PI3K pathways:

Pharmacological inhibitors specific for either calcium- or PI3K-regulated signaling inhibit LPS-induced activation of NF κ B and AP-1 in THP-1 cells. THP-1 cells (2×10^6) were pretreated with various inhibitors specific for either calcium (EGTA, SKF, 2-APB, W7, KN-93, CyA, and FK-506) or PI3K (Ly294002) at indicated concentrations for 2 h followed by transient transfection with 6 μ g of various hIL-12p40 promoter constructs containing either wild type or mutant AP-1 and NF κ B binding sequences, or control reporter vector, plus 3 μ g of β -galactosidase plasmid for 24 h. Cells were then stimulated with LPS for another 24 h and harvested for measuring luciferase and β -galactosidase activity. Luciferase activity was normalized with the β -galactosidase activity. The results shown are mean $\pm$ S.D. of four experiments each performed in triplicate.

mNFκB) still exhibited an induction of luciferase activity after LPS stimulation. However, when both AP-1 and NFκB sites were mutated (-232, pIL-12p40/mAP-1.Ets-2.PU.1.NFκB), LPS-induced luciferase activity was significantly decreased (Fig. 3.2.2-7, left panel). These results suggest that both AP-1 and NFκB regulate LPS-induced IL-12p40 production and that either one or the other are required inducing IL-12p40 transcription.

CaMKII has been shown to act as a mediator of IκB kinase activation in response to CD3/T cell receptor stimulation [255]. To determine if CaM/CaMKII regulated LPS-induced IL-12p40 expression through NFκB activation, cells transfected with the full length IL-12p40 promoter and the deletion constructs were treated with various pharmacological inhibitors of the calcium and PI3K pathways (EGTA, W7, 2-APB, SKF-96365, KN-93, CyA, FK506, and Ly294002) prior to LPS stimulation followed by measurement of luciferase activity. All these agents significantly decreased the LPS-induced luciferase activity in cells transfected with the full length (-880 to +112), the -232 (pIL-12p40/wtAP-1.Ets-2.PU.1, mNFκB), and -128 (pIL-12p40/wtPU.1.NFκB) promoter constructs (Fig. 3.2.2-7B). These results suggest that LPS-induced IL-12p40 transcription in human monocytic cells involves CaM/CaMKII- and PI3K-dependent activation of NFκB and/or AP-1.

3.2.2-6 FK506 and CyA inhibit binding of NFκB and AP-1 to their binding sites in the IL-12p40 promoter through the CaM/CaMKII and PI3K pathways

I confirmed my earlier results showing that NFκB and AP-1 regulate LPS-induced IL-12p40 transcription [100]. LPS stimulation induced significant binding of NFκB and AP-1. The specificity of NFκB and AP-1 binding was demonstrated by

competition with specific and non-specific oligonucleotides and by supershift analysis with anti-p50 and anti-p65 NFκB, and anti-c-jun and anti-c-fos antibodies for AP-1 (Fig. 3.2.2-8A, 8B). To determine whether binding of NFκB and AP-1 to their respective binding sites in the IL-12p40 promoter was regulated by CaM/CaMK-II and/or PI3-K, cells were treated with inhibitors of the calcium and PI3K signalling pathways before stimulation with LPS. As before, EGTA, W7, SKF-96365, KN-93, FK506, CyA, LY294002 inhibited the binding of NFκB and AP-1 to their respective probes in LPS-stimulated cells (Fig. 3.2.2-8A, 8B). These results suggest that LPS-induced IL-12p40 transcription is regulated by CaM/CaMKII as well as PI3K through NFκB and AP-1 activation. Moreover, CyA and FK506 inhibited IL-12p40 transcription by inhibiting AP-1 and NFκB activity.

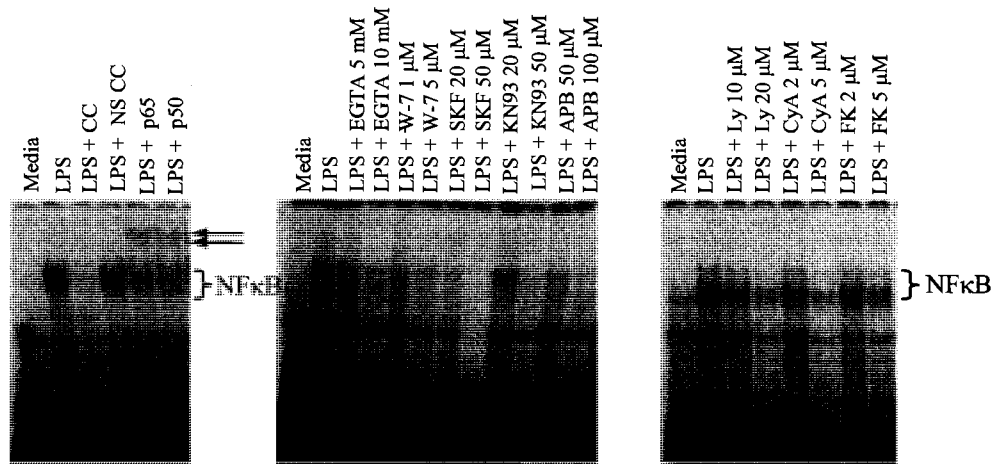
3.3. Discussion

IL-12 is a potent mediator of Th1 immune responses. This makes it an ideal target of immunosuppressive drugs in the treatment of Th1 cell mediated diseases. In this study, I utilized immunosuppressive agents, Dxm, FK506 and CyA, to investigate their role in signaling pathways that control IL-12p40 production in response to LPS in human monocytic cells. I demonstrated that all three drugs inhibit IL-12p40 production in monocytic cells responsive to LPS stimulation in distinct signaling pathways: Dxm was JNK MAPK pathway-dependent whereas FK506 and CyA were through their inhibitory effect on CaM/CaMKII and IP3K pathways. However, both pathways are through the same transcription factors, AP-1 and NFκB. The role of Dxm and FK506/CyA in the regulation of IL-12p40 will be discussed separately in the text.

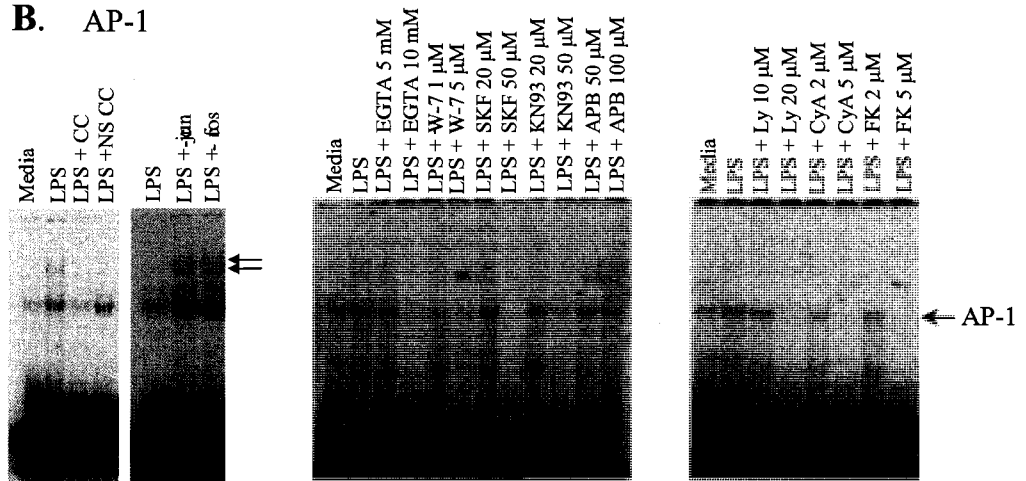
Fig. 3.2.2-8 Binding of AP-1 and NFκB to their binding sites in the human IL-12p40 promoter is inhibited by pharmacological inhibitors specific for calcium and PI3K-regulated pathways in LPS-stimulated THP-1 cells:

THP-1 cells were stimulated with LPS for 0 – 60 min. Nuclear proteins (5 μg) were incubated for 1 h with ³²P-labeled oligonucleotide corresponding to the NFκB or AP-1 sequences located in the IL-12p40 promoter. To determine the specificity of NFκB or AP-1 transcription factor binding, the nuclear extracts were incubated with 100 fold excess of unlabeled specific (CC) or nonspecific (NS CC) probes for 30 min before addition of labeled probes (**A** and **B**, left panel). The nuclear extracts were also incubated with anti-p50 and anti-p65 NFκB antibodies (**A**, left panel) or anti-c-Jun and anti-c-Fos antibodies along with their respective oligonucleotide probes for supershift analysis. THP-1 cells were incubated with various inhibitors specific for the calcium (EGTA, SKF, 2-APB, W7, KN-93, CyA, and FK-506) and PI3K (Ly294002) pathways at the indicated concentrations for 2 h followed by stimulation with LPS for 30 min (middle and right panels, **A** and **B**). Nuclear extracts were analyzed for binding to ³²P-labeled oligonucleotide probes corresponding to the NFκB (**A**) and AP-1 (**B**) binding sites in IL-12p40 promoter. The results shown are representative of three independent experiments.

A. NFκB



B. AP-1



JNK MAPK, NFκB and AP-1 transcription factors are involved in Dxm inhibiting IL-12p40 production in human monocytic cells and THP-1 cells

Dxm is an anti-inflammatory and immunosuppressive glucocorticoid that is widely used in the treatment of inflammation and a number of autoimmune disorders [274]. Dxm has been shown to inhibit the synthesis of LPS-induced IL-12 production by human monocytes [277;278]. However, the molecular mechanisms involved in this cellular event are not clear. In this study, I investigated the molecular mechanism by which Dxm inhibits IL-12p40 production and in particular the role of MAPK and the transcription factors involved in IL-12p40 regulation. I show that Dxm inhibited LPS-induced IL-12p40 production, suggesting an involvement of JNK. The role of JNK was confirmed by using specific inhibitors of JNK activation, SP600125 and a dnSEK-1 kinase mutant.

It has been shown that IL-10 produced endogenously by LPS-stimulated monocytes inhibits IL-12 production [279]. To avoid negative feedback regulation by the endogenous IL-10, I used THP-1/CD14 cells that exhibited enhanced LPS-mediated responses by constitutively expressed CD14 and are refractory to the IL-10-mediated biological effects [100]. Transfection of THP-1/CD14 cells with the IL-12p40 promoter linked to a luciferase gene revealed higher luciferase activity following LPS stimulation as compared with THP-1 cells transfected with the same constructs, and hence were used throughout this study.

Multiple transcription factors, including Ets-2, AP-1, NFκB, and PU.1 have been implicated in IL-12p40 gene transcription [121]. By cloning and mutagenesis of the IL-12p40 promoter, I provide evidence for the previously unrecognized role of AP-1 in

addition to that of NFκB in the regulation of LPS-induced IL-12p40 production. I show that mutation of the NFκB binding site in the IL-12p40 promoter constructs (-120bp and -131bp; pIL-12p40Pr-Nm-GL3B) reduced the luciferase activity, thereby confirming a role for NFκB. Because the IL-12p40 promoter construct (-131bp; pIL-12p40Pr-Nm-GL3B) also contains wtPU.1 in addition to mutant NFκB sequences, loss of luciferase activity following LPS stimulation suggest that PU.1 may not be involved in IL-12p40 gene activation in our model system. However, mutagenesis of NFκB, PU.1 and Ets-2 binding sites in the IL-12p40 promoter construct (-232bp; pIL-12Pr-E/P/Nm-GL3B) failed to reduce luciferase activity, suggesting the presence of binding sites for other transcription factors present in the IL-12p40 promoter that may cooperate with NFκB or may substitute for NFκB activity. Introduction of mutations in the AP-1 binding site, in addition to those of NFκB, PU.1 and Ets-2 (-232bp; pIL-12Pr-A/E/P/Nm-GL3B), abrogated the luciferase activity, thereby suggesting a role for AP-1 in IL-12p40 gene transcription. Conversely, mutagenesis of the AP-1 site alone in this IL-12p40 promoter construct failed to abrogate luciferase activity (data not shown). These results are contrary to the observations provided by Becker et al. [124] whereby mutations of NFκB, Ets and C/EBP sites reduced IL-12p40 promoter activity even though the AP-1 site was left intact. Although the reasons for this discrepancy are not clear, it appears that IL-12p40 gene transcription may be subject to differential regulation depending on the cell type and the nature of the external stimuli used. In contrast to our studies, Becker et al. have analyzed the IL-12p40 promoter by using murine RAW264 macrophage cells stimulated with LPS and NFκB. It is likely that stimulation of cells with IFNγ in addition

to LPS may activate a distinct set of transcription factors required for IL-12p40 gene activation, thereby revealing complex mechanisms involved in IL-12p40 gene regulation.

The role of AP-1 in IL-12p40 regulation was confirmed by an alternative approach using antisense oligonucleotides specific for c-jun and c-fos, which significantly inhibited IL-12p40 production in LPS-stimulated THP-1/CD14 cells. This inhibition was specific, because these oligonucleotides failed to inhibit IL-10 production under the same experimental conditions. While this work was in progress, Zhu et al. [280] demonstrated the involvement of an AP-1 sequence in murine IL-12p40 expression using RAW264.7. Although the human and murine IL-12p40 promoter sequences are distinct, results suggest that the AP-1 transcription factor may play a critical role in both human and murine IL-12p40 gene transcription.

AP-1 is a heterodimeric transcription factor comprised of members of the Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, Fra-1, Dra-2, FosB, and FosB2) proto-oncogene families. Members of the Fos and Jun families have been shown to dimerize via their leucine zipper domain with a variety of transcription factors including CREB/activating transcription factor, Maf, NFAT, and glucocorticoid receptor. Various Fos and Jun proteins interact with the promoters of cytokine genes either individually as AP-1 dimers, or in cooperation with other transcription factors such as NF κ B, NFAT, CREB/activating transcription factor, etc. [281;282]. For example, the regulation of IL-4, IL-5, and GM-CSF requires the formation of NFAT/AP-1 complexes in T cells [283;284]. Similarly, in LPS-stimulated THP-1 cells, c-Jun-containing complexes have been shown to interact with NF κ B proteins p50/p65, and synergistically enhance the TNF α promoter activity [285]. The results of this study reveal the involvement of NF κ B and AP-1 in IL-12p40

regulation. It is not clear whether both of these transcription factors can cooperate in IL-12p40 gene transcription in vivo. It is likely that the level of this putative cooperation is modulated by interaction with other cellular signaling molecules activated by different external stimuli depending on the cell type. Further studies are needed to understand the interaction of NF κ B and AP-1 and possibly other transcription factors necessary for IL-12p40 regulation.

The role of JNK in the regulation of IL-12p40 production was initially studied with Dxm, which mediates its effects by interfering with JNK phosphorylation and AP-1 activation [246;247]. I provide evidence for the role of JNK MAPK in the regulation of LPS-induced IL-12p40 production, by using the specific JNK inhibitor, SP600125, and by dnSEK1 kinase. SEK1 is immediately upstream of JNK and has been shown to phosphorylate JNK. Disruption of the SEK1 gene specifically blocks JNK activity [286;287]. The data presented here also suggest the involvement of JNK in IL-12p40 production through the activation of AP-1 and NF κ B transcription factors since both Dxm and SP600125 significantly decreased the activity of IL-12p40 promoter containing mutations in the binding sites for NF κ B and AP-1. In addition, by using gel shift assays, I show that both Dxm and SP600125 inhibited the binding of AP-1 and NF κ B to their respective oligonucleotides in LPS-stimulated THP-1/CD14 cells. Although it is not known whether JNK directly interacts with the members of the NF κ B transcription factors, JNK has been shown to influence the NF κ B pathway by regulating I κ B α activation [282]. Furthermore, c-Jun-containing complexes have been shown to interact with NF κ B proteins p50/p65 [288]. Keeping in view these observations, my results may

suggest a potential cooperation between AP-1 and NFκB pathways in the regulation of IL-12p40 production in human monocytic cells.

Taken together, my results suggest for the first time a role for JNK in hIL-12p40 production via the activation of AP-1. These results also suggest the involvement of JNK in the NFκB pathway leading to IL-12p40 production.

CaM/CaMKII /PI3K involved in CyA and FK506 downregulation of IL-12p40 production in human monocytes and THP-1 cells

CyA and FK506 are potent inhibitors of calcium-regulated signalling and exert their biological effects through calcineurin [90;91]. PI3K and calcium pathways were shown to negatively regulate LPS-induced IL-12p40 expression in murine macrophages and DCs [254;255]. In contrast, my results show that calcium signalling through CaM and CaMK-II, as well as through PI3K positively regulated LPS-induced IL-12p40 production in human monocytic cells. Moreover, both CyA and FK506 inhibited LPS-induced IL-12p40 production by inhibiting NFκB and AP-1 activity through the upstream CaM/CaMKII-activated PI3K and independent of the JNK pathway.

Inhibition of calcium signalling has been shown to enhance IL-12p40 production in LPS-stimulated murine monocytic cells. Ligation of Fc receptors on murine monocytic cells inhibited LPS-induced IL-12p40 production that was reversed by pretreatment with the calcium ion chelator, EGTA [289;290]. In contrast, my results show that inhibition of LPS-induced intracellular free calcium by EGTA significantly reduced IL-12p40 production in human monocytic cells. A positive role for calcium was further supported by employing specific inhibitors for CaM/CaMKII. CaM is a key signalling protein responsible for integrating Ca^{2+} signals to transcription factors. Two important

downstream targets of CaM are calcineurin and CaMK-II [291]. As with other kinases, CaMK-II undergoes autophosphorylation on a threonine residue contained in a phosphopeptide common to its α and β subunits thereby converting it into a Ca^{2+} /CaM independent enzyme [292]. The results obtained by employing specific inhibitors for CaM, calcineurin, and CaMKII, suggested that LPS-induced IL-12p40 production is regulated by the CaM/CaMKII pathway. The observation that both FK506 and CyA inhibited the LPS-induced increase in intracellular free calcium, CaMKII phosphorylation, and IL-12p40 production in monocytic cells suggest that this may be a mechanism by which these agents inhibit Th1 type responses by these cells. The observed enhanced sensitivity of primary monocytes to the calcium inhibitors compared to the THP-1 cells may be due to inherent differences between the primary versus the leukemic cells.

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 PI-4,5-P₂ that leads to the release of PI-3,4,5-P₃ triphosphates and eventual phosphorylation of a number of substrates including Akt, protein kinase A, and protein kinase C isoforms [270;293]. There is considerable evidence to suggest that PI3K plays a key role in the negative feedback regulation of IL-12p40 production by murine DCs and monocytic cells [255]. PI3K was shown to negatively regulate IL-12p40 production by human monocytic cells stimulated by the TLR-2 ligand, *Porphyromonas gingivalis* LPS (34) and following ligation of complement receptors C1q and C5a in LPS-stimulated human and murine

monocytic cells and DCs [294;295]. In contrast, we show that LPS-induced IL-12p40 production by primary human monocytes as well as THP-1 cells is regulated positively through PI3K activation.

Understanding the role and functions of PI3K family members has been difficult because of their resistance to genetic manipulation, broad biological activity of PI3K inhibitors to inhibit nearly all classes of PI3K isoforms [296], and embryonically lethal PI3K p110 α or p110 β gene knockouts [297]. There is evidence to suggest that the p85 subunit independent of the p110 α can regulate the expression of several genes including IL-2 [298]. Recently, using an RNAi-based approach, the p110 α subunit of PI3K was shown to regulate vitamin D3-induced adherence and upregulation of CD11b in monocytic cells [299]. Interestingly, herein, we have demonstrated that THP-1 cells deficient in the expression of the p110 α catalytic unit of PI3K produced significantly reduced levels of IL-12p40 in response to LPS without any effect on the expression of IL-10, CD44, B7.1, B7.2 and CD11b (data not shown). These results suggest that p85/p110 α PI3K selectively regulates LPS-induced IL-12p40 production.

The results of this study in concert with our earlier observations showing the regulation of LPS-induced IL-12p40 production through the JNK pathway suggest that LPS-induced IL-12p40 production is regulated by two distinct signalling pathways namely the CaM/CaMKII-activated PI3K pathway and the JNK pathway. Interestingly, both pathways regulated IL-12p40 production through the activation of NF κ B and AP-1 transcription factors. It is not clear why the JNK pathway failed to compensate for LPS-induced IL-12p40 production in the presence of inhibitors specific for the calcium pathway. In addition to signal transduction pathways, molecular mechanisms controlling

gene expression involve chromatin remodelling and DNA methylation [300]. Understanding the status of promoter methylation, and chromatin remodelling may elucidate the mechanisms underlying the involvement of distinct signalling pathways controlling IL-12p40 transcription.

Here, I have shown that IL-12p40 production is regulated by NF κ B and AP-1 through the activation of upstream calcium and PI3K pathways. NF κ B and AP-1 are known to be activated by the calcium/CaMKs [301;302] although it is not clear if Akt and CaM/CaMKII can directly interact with the members of the NF κ B and AP-1 family members. It is also not clear that in the presence of an intact promoter whether both of these transcription factors can synergize to induce IL-12p40 transcription. Although deletion mutation studies conducted to define the role of individual transcription factors did not reveal a synergistic effect at the level of luciferase activity, the potential cooperation between these two transcription factors cannot be ruled out. It is possible that the level of cooperation between these transcription factors is modulated following interaction with other signalling molecules activated by different external stimuli. Further studies are needed to elucidate the precise cooperation between NF κ B and AP-1 and possibly other factors involved in IL-12p40 transcription.

The unique role of IL-12p40 in the regulation of IL-12 and IL-23 suggest that it may be critically involved in the immunopathogenesis of Th1-mediated inflammatory and autoimmune disorders. Our investigations regarding the molecular mechanism underlying the inhibitory effects of CyA and FK506 on IL-12p40 production have revealed for the first time a positive role of calcium and PI3K and in particular a selective involvement of p110 α subunit of PI3K in regulating IL-12 production by human

monocytes. These findings raise the possibility of developing novel agents capable of inhibiting calcium and PI3K pathways as potential therapeutics for disorders related to excessive IL-12 production. The observations that calcium and PI3K signalling pathways positively regulate human IL-12p40 production in contrast to their negative regulatory role in murine IL-12p40 production highlight the importance of understanding in detail the mechanism involved in regulating the production of this critical cytokine.

In conclusion, the results of this study in concert with my earlier observations showing the regulation of LPS-induced IL-12p40 production through the JNK pathway suggest that LPS-induced IL-12p40 production is regulated by two distinct signaling pathways namely the CaM/CaMKII-activated PI3K pathway and the JNK pathway. Interestingly, both pathways regulated IL-12p40 production through the activation of NF κ B and AP-1 transcription factors. It is not clear why the JNK pathway failed to compensate for LPS-induced IL-12p40 production in the presence of inhibitors specific for the calcium pathway. In addition to signal transduction pathways, molecular mechanisms controlling gene expression involve chromatin remodelling and DNA methylation [300]. Understanding the status of promoter methylation, and chromatin remodelling may elucidate the mechanisms underlying the involvement of distinct signalling pathways controlling IL-12p40 transcription. These findings raise the possibility of the development of novel active analogs of Dxm, CyA and FK506 with more efficient inhibition of IL-12 production without side effects that may provide new therapeutic tools for the treatment of inflammation and autoimmune diseases.

Chapter IV

Conclusion Regulation of LPS-induced IL-12p40 Expression by Intracellular HIV-Nef

4.1. Introduction

HIV infection results in a 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 Th1 cytokines, such as IL-12. IL-12 plays a critical role in the development of cell mediated immunity and in the immunopathogenesis of various diseases including HIV infection, inflammation and autoimmune disorders [13;74] IL-12 promotes Th1 responses by inducing IFN- γ from NK cells and T cells and enhancing cytotoxicity of NK cells and CTL. IL-12 is mainly produced by monocytes/macrophages, dendritic cells and B cells. It is a 70 kD heterodimer composed of p35 and p40 subunits, which are disulfide linked together to form biological active IL-12. The p35 and p40 are encoded by two distinct genes and they are differentially regulated: the p40 gene is tightly regulated at the transcriptional level and detected only in biologically active IL-12 producing cells whereas the p35 gene is constitutively expressed in various cell types. IL-12 p40, therefore constitutes an indicator for IL-12 production [15;303].

Monocytes/macrophages play a key role in host defense against invading pathogens by coordinating and directing innate and adaptive immune responses. It is well established that monocytic cells serve as long term reservoirs in chronically infected HIV patients [390]. There is ample evidence to indicate that IL-12, and in particular, IL-12p40 production is impaired in HIV-infected individuals and in monocytic cells infected *in vitro* with HIV [398]. Results from our laboratory and others have shown that IL-12 production by peripheral blood mononuclear cells (PBMC) is decreased in HIV-infected patients. Furthermore, treatment of HIV-infected individuals with potent anti-retroviral

therapy enhanced IL-12 production [305]. We and others have also shown that exogenous addition of IL-12 enhances IL-2 production, cell proliferation and the development of cell mediated cytotoxicity of HIV-antigen stimulated PBMC from HIV positive individuals [306;307]

At present, there is a little information regarding the regulation and expression of IL-12 and in particular IL-12p40 in monocytic cells following HIV infection.

Recently, the role of intra-cellular signaling molecules that regulate IL-12p40 synthesis in human monocytic cells following LPS stimulation has been investigated. Multiple transcription factors including IFN- γ regulatory factors, NF κ B, Ets-2, AP-1, C/EBP, and PU.1 transcription factors and their complexes have been suggested to regulate IL-12p40 transcription in LPS-stimulated murine and human monocytic cells [100;308]. We and others have previously demonstrated the involvement of c-Jun-N-terminal kinase (JNK) in IL-12p40 production in LPS-stimulated human monocytic cells [100]. Recently, we have also demonstrated that LPS-induced IL-12p40 production is regulated by two distinct signaling pathways namely the CaM/CaMKII-activated PI3K pathway and the JNK pathways. Interestingly, both pathways regulated IL-12p40 production through the activation of NF κ B and AP-1 transcription factors.

In our study, IL-12p40 production was shown to be suppressed following infection of monocytic cells with wt-SIV compared to the cells infected with Nef-deficient strains [23]. Nef is a 27 kD myristoylated protein expressed early in HIV infection. In addition to the well known down-regulation of the cell surface molecules CD4 and MHCI molecules, Nef uniquely can interact with numbers of intracellular signaling molecules through its myristoyl moiety, leading to the dysregulation of host

immune responses. For example, by interfering with MAPK signaling molecules and transcription factors, NF κ B and AP-1, Nef gene products downregulate IL-2 and IFN γ in T cells, resulting in T cell dysfunction [156;309] In this study, I have demonstrated that intracellular expression of HIV-Nef in human monocytic cells resulted in inhibition of LPS-induced IL-12 p40 production. Furthermore, HIV-Nef inhibited IL-12p40 transcription by effectively inhibiting LPS-activated JNK without affecting the activation of p38 and ERK MAPK and calcium signalling including calcium influx and CaMKII. In addition, HIV-Nef inhibited JNK-activated NF κ B without affecting the AP-1 activity. Taken together, my results suggest for the first time that intracellular HIV-Nef down regulates LPS-induced IL-12p40 expression in human monocytic cells by selective inhibition of the JNK-activated NF κ B without affecting the CaMKII-activated PI3K pathway.

4.2. Results

4.2.1. Intracellular HIV-Nef inhibits LPS-induced IL-12p40 expression in primary monocytes and THP-1 cells

To determine the effect of intracellular HIV-Nef on the expression of IL-12p40 in human monocytic cells, HIV-Nef retroviruses were generated by transfecting amphotrophic packaging cell line PT-67 with either pSR α -Nef-retrovirus (pSR α -Nef) or the retrovirus containing the empty vector pSR α . The integration of pSR α -Nef in the transduced PT67 cells was verified by subjecting the genomic DNA purified from PT67 cells 7 days after transduction to PCR. The intracellular expression of Nef protein in PT67 cells was simultaneously determined by Western blot analysis. PT67 cells

transfected with pSR α -Nef exhibited Nef expression as determined by PCR (237 bp) and Western blot analysis (27 kD) (Fig. 4.2-1A). The PT67 cells stably transfected with pSR α -Nef were cultured for 3 days and the culture supernatants were used as a source of Nef containing retroviruses. The culture supernatants were assayed for determination of viral titers by infecting NIH 3T3 cells. Monocytes and THP-1 cells were infected with pSR α -Nef at a multiplicity of infection (MOI) of 1 for 24 hr. In order to obtain the maximal viral infectivity, cells were infected for a second time for another 24 hr. Monocytes and THP-1 cells infected with pSR α -Nef at MOI of 1 did not induce morphological changes and this level of infectivity was found to be optimal for influencing IL-12p40 expression (data not shown). Moreover, THP-1 cells and monocytes infected with pSR α -Nef at MOI of 1 expressed HIV-Nef as determined by PCR and Western blotting (Fig. 4.2-1B).

To determine whether intracellularly expressed Nef affects IL-12p40 production in response to LPS-stimulation, I performed ELISA analysis. Monocytes and THP-1 cells were infected twice with pSR α -Nef or pSR α at a MOI of 1 followed by stimulation with LPS for another 24 hr. LPS-induced IL-12p40 production was significantly reduced by approximately 80% in pSR α -Nef infected monocytes compared to the control virus-infected monocytes as determined by ELISA (Fig. 4.2-1B). Similarly, intracellular Nef expression in THP-1 cells significantly inhibited LPS-induced IL-12p40 production compared to the cells infected with control virus (Fig. 4.2-1C). To determine if intracellularly expressed Nef inhibits LPS-induced IL-12p40 at the transcriptional level, total RNA was prepared from pSR α -Nef- or pSR α -infected monocytes and THP-1 cells stimulated with LPS for 4 hr followed by determination of IL-12p40 mRNA by semi-

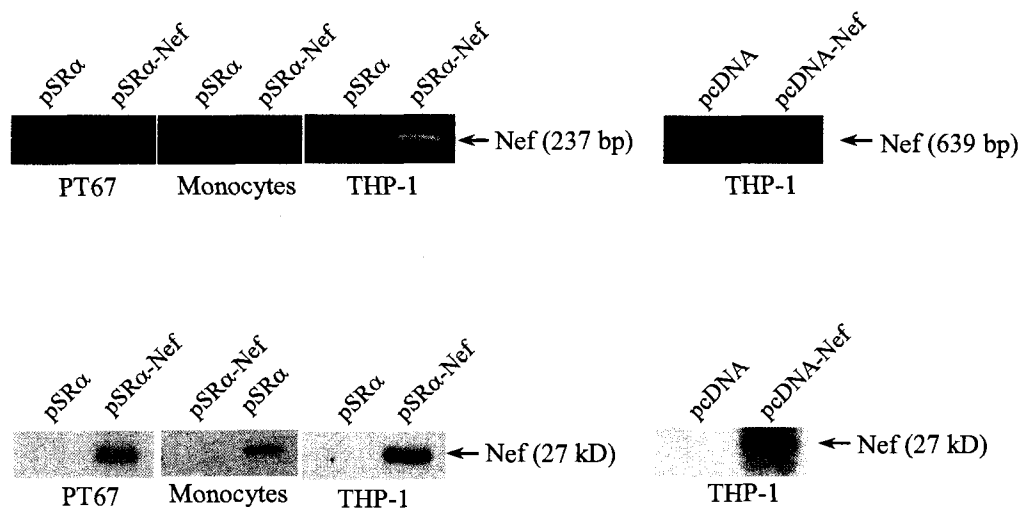
Fig. 4.2-1 IL-12p40 expression is reduced in Nef-infected monocytes and THP-1 cells:

A, Verification of Nef expression in pSR α -Nef transduced PT67 cells and THP-1 cells. Genomic DNA was purified from PT67 cells and THP-1 cells 7 days after transduction and subjected to PCR. The intracellular expression of Nef proteins in these cells were simultaneously determined by immunoblotting analysis using monoclonal anti-Nef antibody.

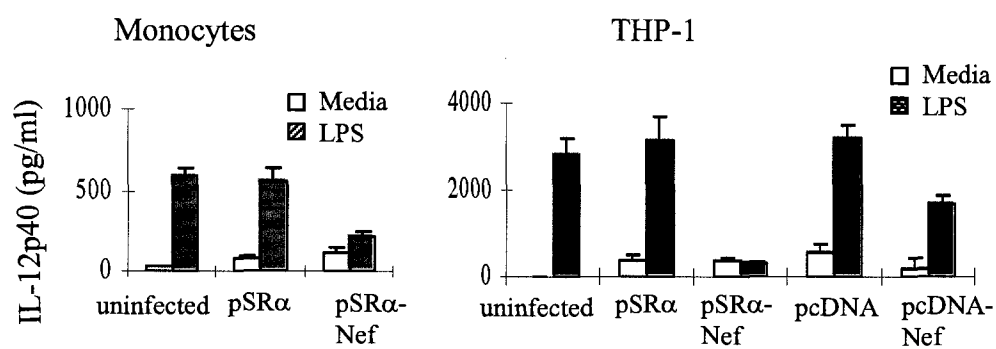
B, Nef inhibits LPS-induced IL-12p40 production in monocytes and THP-1 cells. 2×10^6 of monocytes (**left panel**) and THP-1 cells (**right panel**) were infected with either pSR α -Nef or control virus at a MOI of 1 at 37°C for 24 hr and infected second time. After 24 hr, cells were stimulated with 1 μ g/ml LPS for further 24 hr. The cell culture supernatants were collected and assayed for IL-12p40 production by ELISA.

C, The suppressed IL-12p40 mRNA expression in pSR α -Nef treated monocytes (**left panel**) and THP-1 cells (**middle panel**) is shown with their basal level in control virus infected cells. Cells (3×10^6) were infected with pSR α -Nef, twice, 24 hr for each infection followed by stimulation with 1 μ g/ml LPS for 4 hr. Total RNA was isolated and subject to RT-PCR. **In the right panel**, 3×10^6 pcDNA-Nef expressing THP-1 cells were treated with LPS (1 μ g/ml) for 4 hr. Total RNA was extracted and used for determine IL-12p40 mRNA by RT-PCR. β -actin is as a loading control.

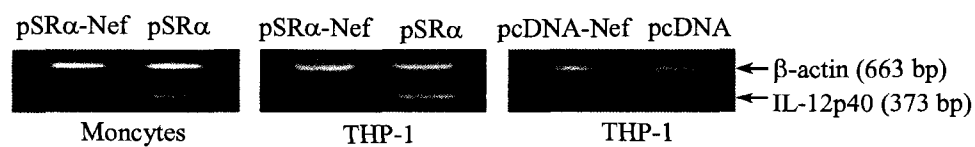
A



B



C



quantitative RT-PCR analysis. As shown in Fig. 4.2-1C, LPS-induced IL-12p40 mRNA expression was significantly reduced in pSR α -Nef-infected monocytes as well as THP-1 cells. I also confirmed that intracellular expression of HIV-Nef in human monocytic cells inhibits LPS-induced IL-12p40 production by stable transfection of THP-1 cells with Nef plasmid from another HIV strain (pcDNA-Nef). THP-1 cells transfected with pcDNA-Nef expressed Nef mRNA as well as Nef protein (Fig. 4.2-1A). Similar to the results obtained with pSR α -Nef-infected monocytic cells, LPS-induced IL-12p40 expression was significantly reduced in pcDNA-Nef transfected THP-1 cells compared to the cells transfected with the control pcDNA vector alone as determined by ELISA as well as semi-quantitative RT-PCR analysis (Fig. 4.2-1B and C).

4.2.2. Intracellular Nef downregulates IL-12p40 production through selective inhibition of JNK MAPK in human monocytic cells

I have previously demonstrated that LPS-induced IL-12p40 production in human monocytic cells is regulated by two distinct signaling pathways namely the c-Jun amino-terminal kinase (JNK) and the calmodulin-dependent protein kinase-II (CaMKII) pathways. Recently, Angle, et al [32] demonstrated the involvement of the p38 and JNK MAPKs in the impaired IL-12p40 production following infection of human monocytic cells with HIV (ref). It is also known that HIV-1 Nef interferes with the MAPKs in a variety of cell types [33,34]. Therefore, it is likely that HIV-Nef inhibits LPS-induced IL-12p40 expression through the inhibition of LPS-activated JNK or CaMKII alone or both JNK and CaMKII together. Therefore, we first confirmed our earlier findings that LPS-induced IL-12p40 expression is regulated by JNK as well as CaMKII in pSR infected THP-1 cells and primary monocytes by employing specific pharmacological inhibitors.

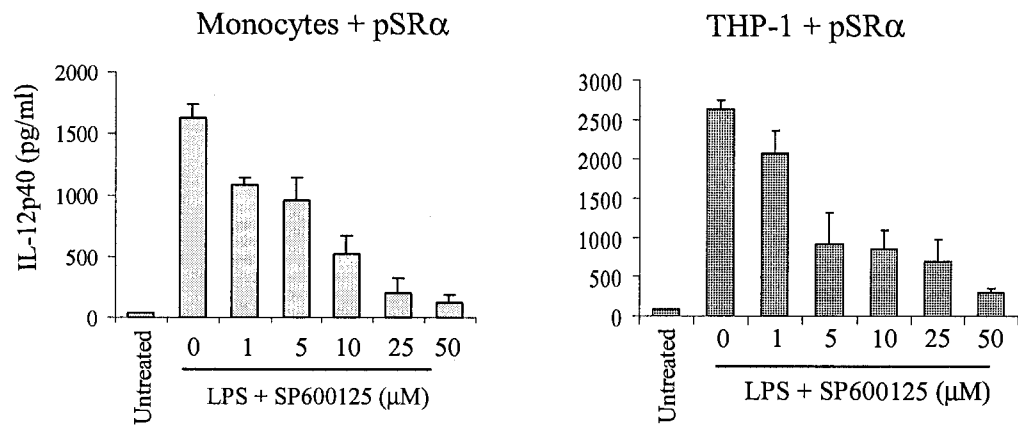
To ensure that the activity of JNK and $\text{Ca}^{2+}/\text{CaM}/\text{CaMKII}$ is not influenced by empty retroviral vectors, primary monocytes and THP-1 cells were infected twice with empty retrovirus vectors and two hr before LPS stimulation, cells were treated with various concentrations of either JNK inhibitor SP600125, calcium chelating agent EGTA, SKF96365, an inhibitor of receptor-mediated calcium entry, Calmodulin (CaM) antagonist W-7, and CaMKII inhibitor KN-93. After 24 hr, cell supernatants were analyzed for IL-12p40 production by ELISA. As expected, JNK and all calcium signaling inhibitors significantly reduced LPS-induced IL-12p40 production in a dose dependent manner (Fig. 4.2-2). As before, the biological activities of SP600125 were confirmed by demonstrating inhibition of LPS-activated JNK whereas W7 and KN-93 inhibited LPS-activated CaMKII phosphorylation (data not shown).

To determine if HIV-Nef affects activation of JNK and/or calcium signaling pathways following stimulation of monocytic cells with LPS, primary monocytes and THP-1 cells were infected twice with either pSR α -Nef or pSR α containing retroviruses followed by stimulation with LPS for 0 to 120 minutes. A five-fold reduction in activity of JNK phosphorylation was observed in pSR α -Nef-infected THP-1 cells compared to the cells infected with the control virus (Fig. 4.2-3A, left panel). Similarly, a seven-fold reduction was observed in pSR α -Nef-infected monocytes compared to the cells infected with the control virus (Fig. 4.2-3A, right panel). Because ERK MAPK was reported to be involved in reduced IL-12p40 production in HIV-infected monocytes, and because p38 phosphorylation has been implicated in IL-12p40 production in various cell types, I examined ERK and p38 phosphorylation following stripping of the same blots. The results show that the neither phosphorylation of ERK (Fig. 4.2-3B) or p38 (data not

Fig. 4.2-2 Inhibition of JNK and calcium signals is Nef-specific in monocytes and THP-1 cells:

SP600125 **(A)**, EGTA, SKF96365, W-7 and KN-93 **(B)** inhibit IL-12p40 production in control viral vector-infected monocytes and THP-1 cells in a dose dependent manner. Monocytes **(left panel)** and THP-1 cells **(right panel)** were infected with control virus vector for 24 hr. Two hr before second infection, cells were treated with various inhibitors at indicated concentration followed by simulation with 1 μ g/ml of LPS for 24 hr. The supernatant were then used for measuring IL-12p40 production by ELISA. The results are representative of four independent experiments, each performed in triplicate.

A



B

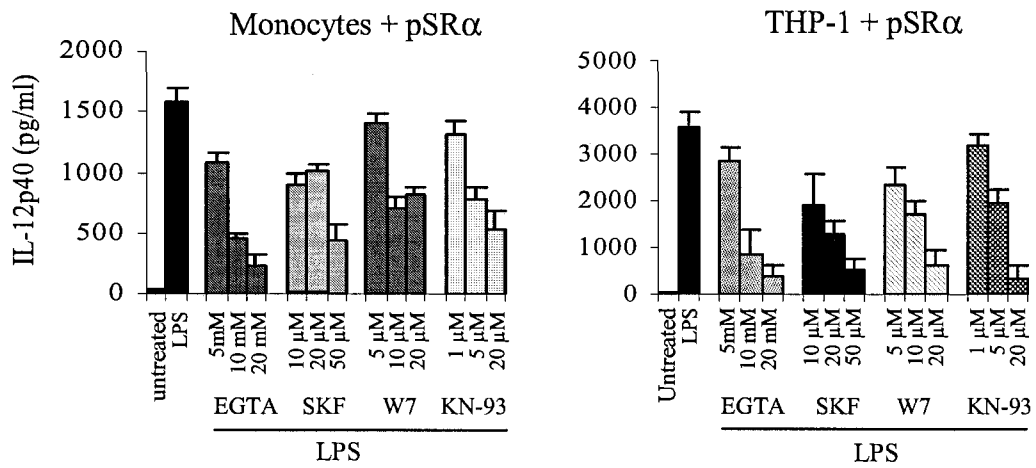
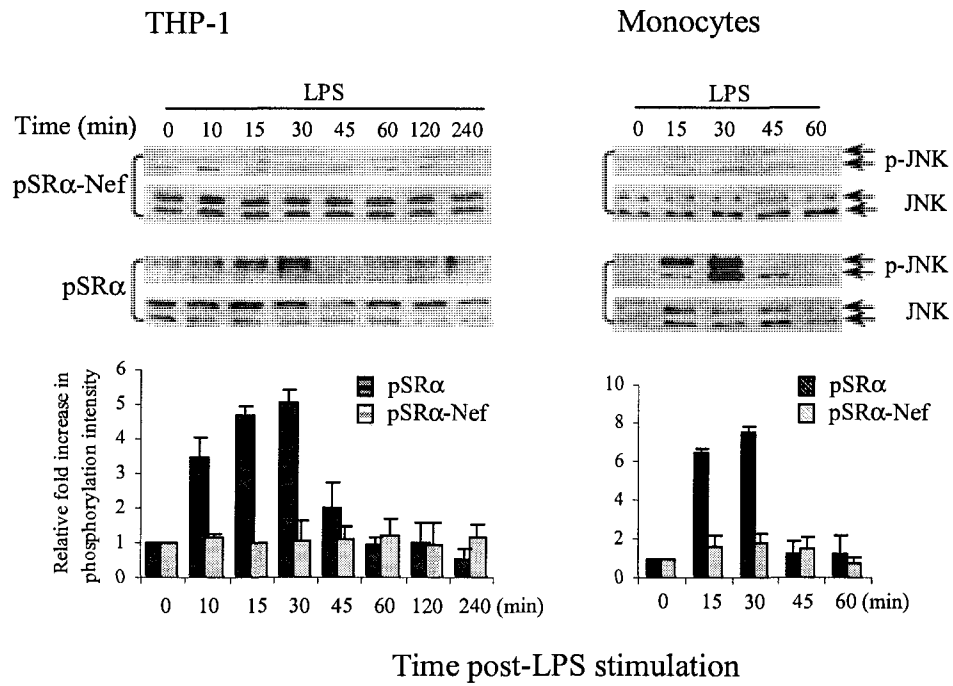


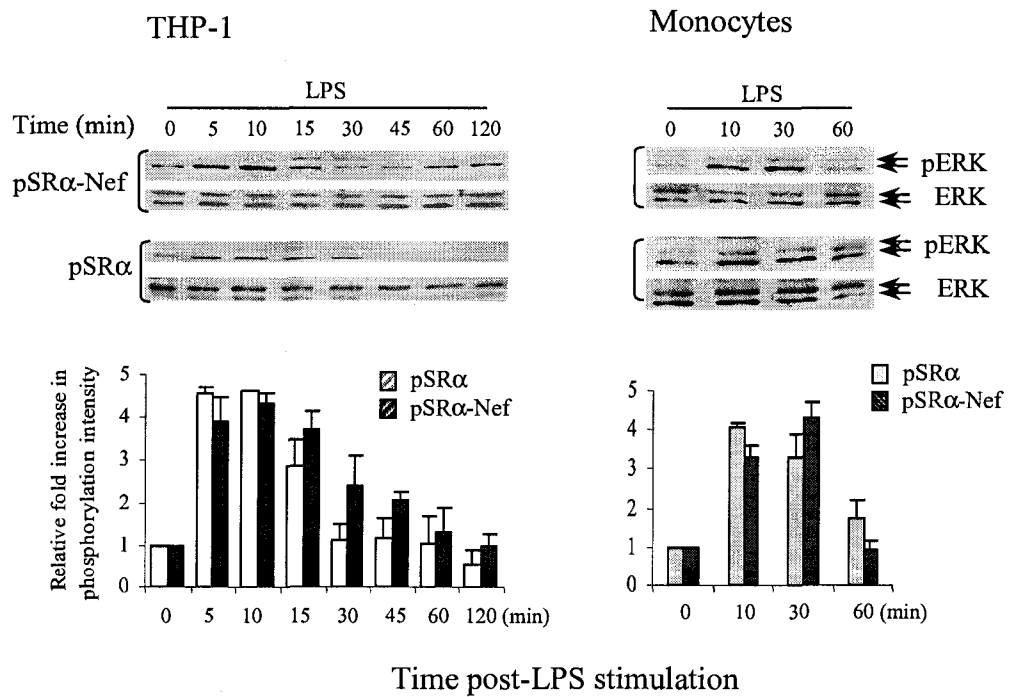
Fig. 4.2-3 Nef inhibits phosphorylation of JNK MAPK in LPS-activated monocytic cells:

Nef inhibits LPS-induced phosphorylation of JNK, but not ERK in monocytes and THP-1 cells. 2×10^6 of THP-1 cells (**left panels**) and monocytes (**right panels**) were infected twice with pSR α -Nef followed by stimulation with 1 μ g/ml LPS for the indicated time. Cells were lysed and analyzed for JNK (**A**) and ERK1/2 (**B**) phosphorylations by Western blot with anti-phospho-JNK and EEK1/2 Abs. The equal loading of protein were confirmed with anti-total JNK or ERK1/2 Abs. Quantitation and normalization as mean densitometry units to the kinase's content are shown at the bottom of each panel. Results are from three independent experiments.

A



B



shown) was significantly affected in pSR α -Nef-infected THP-1 cells or monocytes.

Stress-activated protein kinase/extracellular signal-regulated kinase 1 (SEK1) is a dual kinase immediately upstream of JNK, which phosphorylates JNK and p38 on threonine-183 and tyrosine-185 residues. I have previously demonstrated a critical role for SEK1 in the regulation of IL-12p40 by LPS-activated human monocytes (28). To further understand the role of intracellular Nef on the JNK signaling pathway, we determined if infection of cells with pSR α -Nef down regulated SEK-1 activity. LPS-induced activation of SEK-1 in pSR α -Nef infected monocytes and THP-1 cells was significantly reduced compared to the cells infected with the control pSR α -infected cells (Fig. 4.2-4). These results suggest that intracellular Nef downregulates LPS-induced IL-12p40 production by inhibiting LPS-activated SEK1 and JNK signaling pathway.

4.2.3. Intracellular Nef-mediated downregulation of LPS-induced IL-12p40 production does not involve calcium signaling and CaMK-II activity in human monocytic cells

As mentioned earlier, LPS-induced IL-12p40 production is also regulated by the calcium signaling pathway [256]. Based on the evidence that specific inhibitors for the calcium signaling including Ca²⁺ influx, CaM and CaMKII suppressed LPS-induced IL-12p40 production in pSR α control retrovirus-infected primary monocytes and THP-1 cells (Fig. 4.2-2 & 4.2-5), I determined if intracellular Nef inhibited LPS-induced calcium influx, and/or CaM/CaMKII activity. Since the function of CaMKII is calcium-dependent, we first investigated the effect of pSR α -Nef retrovirus infection on LPS-induced calcium influx in primary monocytes and THP-1 cells by flow cytometry using Fluo-3 as a calcium binding dye. LPS induced an increase in the levels of intracellular calcium at 12 min

Fig. 4.2-4 Nef inhibits SEK1 phosphorylation in LPS-activated monocytes and THP-1 cells:

Twice infection of THP-1 cells (**left panel**) and monocytes (**right panel**) with pSR α -Nef were stimulated with 1 μ g/ml LPS for 0 to 60 minutes. Total proteins were subjected to Western blot analysis for SEK1 phosphorylation using anti-phospho-SEK1 Abs. Total levels of SEK1 expression were determined with anti-total SEK. The entire experiments were repeated twice with comparable results.

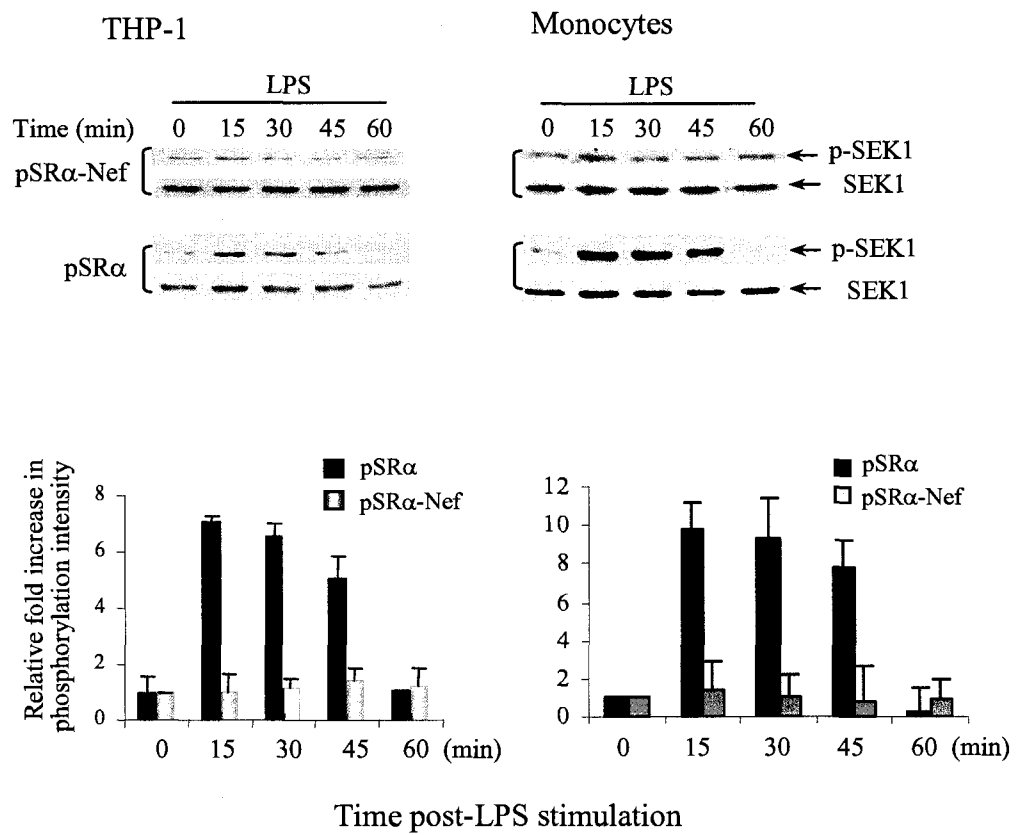
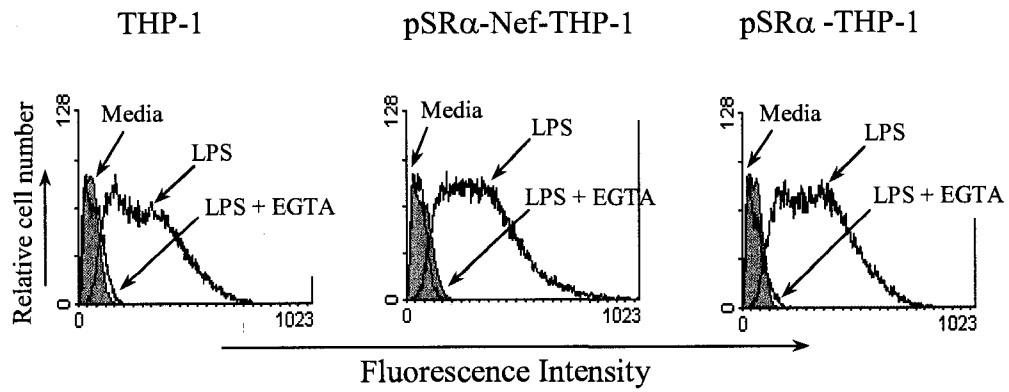


Fig. 4.2-5 Ca^{2+} /CaM/CaMKII signals are not involved in Nef-mediated IL-12p40 downregulation:

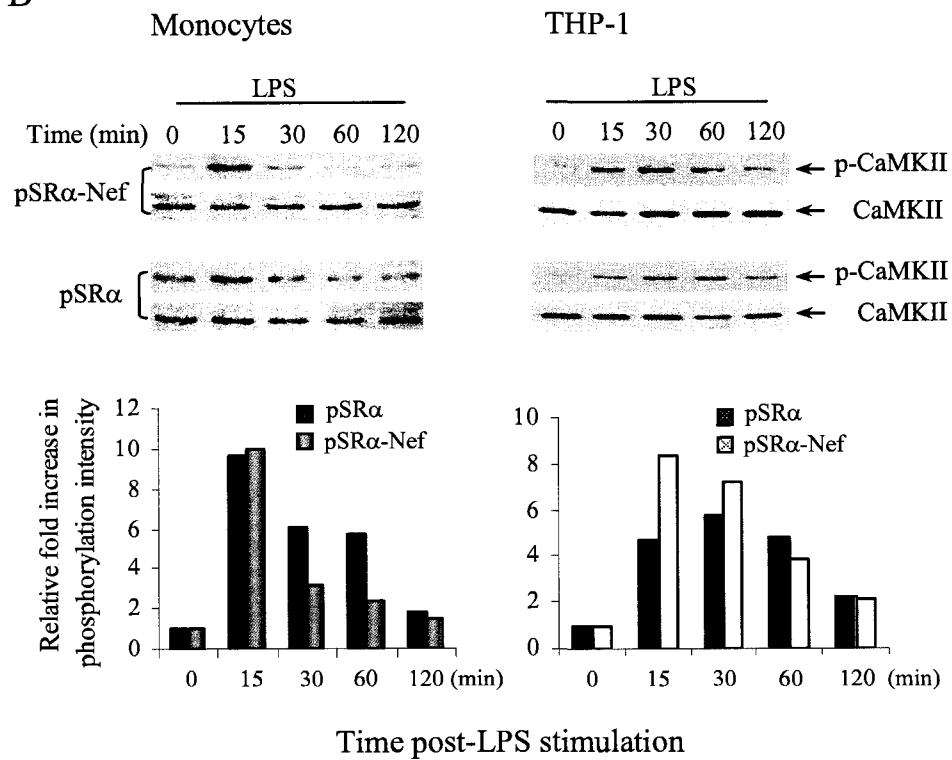
A, Calcium entry was not influenced by pSR α -Nef. THP-1 cells (5×10^6) were infected twice with pSR α -Nef or control vector, 24 hr for each infection and loaded with Fluo-3/AM followed by LPS stimulation for 0-15 min. The levels of intracellular calcium were then measured by flow cytometric analysis. These results represent three independent experiments.

B, The activity of CaMKII phosphorylation is not affected by pSR α -Nef. THP-1 cells (**left panel**) and monocytes (**right panel**) were infected with either pSR α -Nef or control virus followed by LPS stimulation for various times ranging from 0 to 120 minutes. Cells were then harvested for analysis of CaMKII phosphorylation by immunoblotting with anti-phospho-CaMKII antibody. The total CaMKII protein was detected with anti-total- t CaMKII antibody. Results are two separate experiments.

A



B



post-stimulation in primary monocytes and THP-1 cells infected with pSR α -Nef that was very similar to that observed in cells infected with the control retroviruses (Fig.4.2-5A). Similarly, similar levels of LPS-induced phosphorylation of CaM/CaMKII were observed in both monocytes and THP-1 cells infected with either pSR α -Nef or control retroviruses (Fig. 4.2-5B). These results suggested that the intracellular Nef may not affect LPS-induced calcium influx or CAMKII activity in primary monocytes and THP-1 cells. Taken together, our results further suggest that intracellular Nef down-regulates LPS-induced IL-12p40 production by selectively inhibiting the JNK pathway in human monocytic cells.

4.2.4. Involvement of IL-12p40 promoter region in Nef-mediated downregulation of IL-12p40 expression in LPS-stimulated THP-1 cells

I have previously demonstrated that LPS-induced IL-12p40 is regulated by NF κ B and AP-1 transcription factors in human monocytic cells [100]. To determine if intracellular expression of Nef inhibits LPS-induced IL-12p40 transcription by inhibiting the activity of NF κ B or AP-1, the transcriptional activity of the IL-12p40 promoter in response to intracellular Nef was measured by performing luciferase reporter assays. Therefore, we first confirmed our earlier observations by transfecting THP-1 cells infected with the control retroviruses with a series of 5'-deletion constructs containing the IL-12p40 promoter (full length, -880/+118 bp) sequences linked with the luciferase reporter plasmid, pGL3B. Cells infected with the control retrovirus were transfected with the promoter constructs for 24 hr prior to LPS stimulation. Subsequent luciferase activity analysis showed that transfection of cells with the -880 to +118 construct induced significant luciferase activity compared to the cells transfected with the control vector. In

contrast, transfection with the vector containing mutant NF κ B in the presence of wtPU.1 binding sequence (-128, pIL-12p40/wtPU.1,mNF κ B), abrogated luciferase activity [100] and data not shown. Transfection of cells with vectors containing wild type AP-1 and mutant NF κ B sites (-232, pIL-12p40/wtAP-1,m(Ets-2,PU.1,NF κ B) still exhibited an induction of luciferase activity after LPS stimulation (Fig4). However, when both AP-1 and NF κ B sites were mutated (-232, IL-12p40/wtAp-1, m(Ets-2,PU.1NF κ B), LPS-induced luciferase activity was significantly decreased (data not shown). These results suggest that both AP-1 and NF κ B regulate LPS-induced IL-12p40 production in THP-1 cells infected with the control retroviruses and that either one or the other are required to drive IL-12p40 transcription [100].

To investigate if intracellular Nef affected LPS-induced IL-12p40 transcription through the inhibition of NF κ B and/or AP-1 activity, THP-1 cells infected with pSR α -Nef retroviruses were transiently transfected with either the full length IL-12p40 promoter/luciferase reporter constructs (-880/+118 bp), wild types of AP-1(-232, pIL-12p40/wtAP-1,m(Ets-2,PU.1,NF κ B) and NF κ B containing constructs (-116). LPS-induced luciferase activity was significantly reduced in Nef-infected cells compared to the cells infected with control virus (Fig. 4.2-6, bottom panel). Similar results were obtained by cotransfecting THP-1 cells with the pcDNA-Nef and IL-12p40 promoter (-880/+118 bp). THP-1 cells cotransfected with the pcDNA-Nef and full length IL-12p40 promoter exhibited a significantly reduced LPS-induced luciferase activity compared to the cells transfected with the control pcDNA vector and the full length IL-12p40 promoter (Fig. 4.2- 6B).

Fig. 4.2-6 Nef inhibits IL-12p40 promoter activity through NFκB and AP-1 binding motifs in LPS-stimulated THP-1 cells:

A, Top panel shows the sequences of 5'-terminal truncated human IL-12p40 promoter. Numbers indicate nucleotide positions relative to the NFκB, AP-1 and other transcription factors, whose binding motifs are shown in capitals whereas the coordinated mutants are shown in lowercase. The sequences in the boxes are used as probes for EMSA. **Bottom panel** shows a decreased IL-12p40 promoter activity by pSRα-Nef through NFκB binding motif. 2×10^6 THP-1 cells were cultured with pSRα-Nef twice, 24 hr for each and transiently transfected with 4 μg of IL-12p40 promoter/luciferase constructs containing either AP-1 or NFκB binding motif plus 2 μg of β-galactosidase, an internal control for 24 hr. Cells were harvested following LPS (μg/ml) stimulation for addition 24 hr and lysed for analysis of luciferase activity. Luciferase activities were normalized with the base-line activity of control vector and β-galactosidase.

B, pcDNA-Nef inhibits IL-12p40 promoter transcription by suppression NFκB activity. Nef (driven by pcDNA plasmid) expressing THP-1 were transiently transfected with IL-12p40 promoter/luciferase constructs containing either AP-1 or NFκB binding motifs for 24 hr and treated with LPS for 24 hr. Cells were then harvested for luciferase and β-galactosidase activity assay in the same procedure explained above. Data shown in **A** and **B** are means ($\pm$ S.D.) of six separate experiments.

AP-1 -231/-225 Ets-2 -210/-205

-880CT TCC TTATTCC CCACCCAAAAGTCATTTCT CTTAG
 tttccc (m) *ggacct (m)*

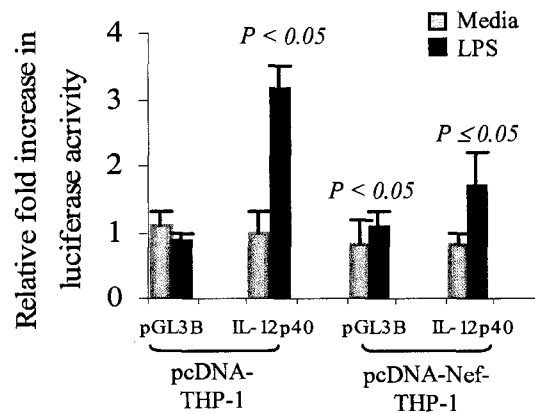
PU.1 -127/-122 NFκB -116/-106

-200 TCAT TA.....AAACAAAAAAGGAACCTTCTGAAATTCCCCCAGAA
 ttegaa (m) *tgggttttgccc (m)*

C/EBP -80/-72 TATA box

-100 GTTGTTTTCAATGTTGCAACAA.....AGTATATTAGTTCAGT
 +1 $\Rightarrow$ transcription Start

-10 AGCAACAGCA **GCAGAAGAAACAACATCTGTTTCAGGGCCATTGGA**..... +118



4.2.5. Nef inhibits IL-12p40 transcription through the selective suppression of NFκB in LPS-stimulated THP-1 cells

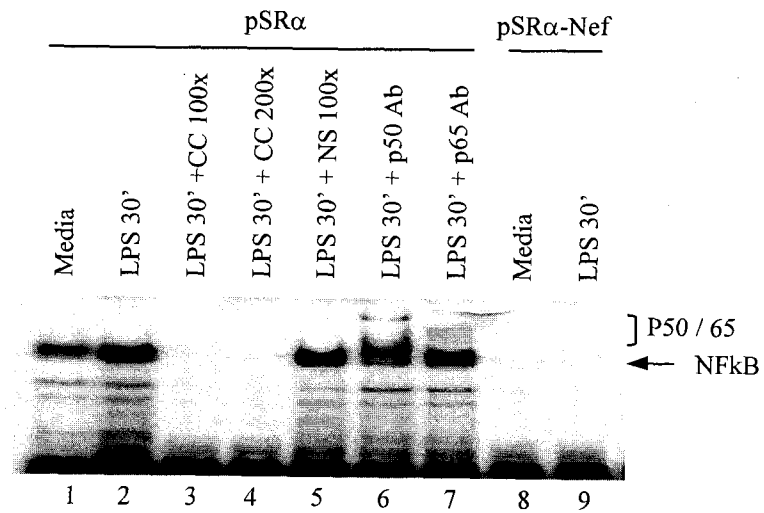
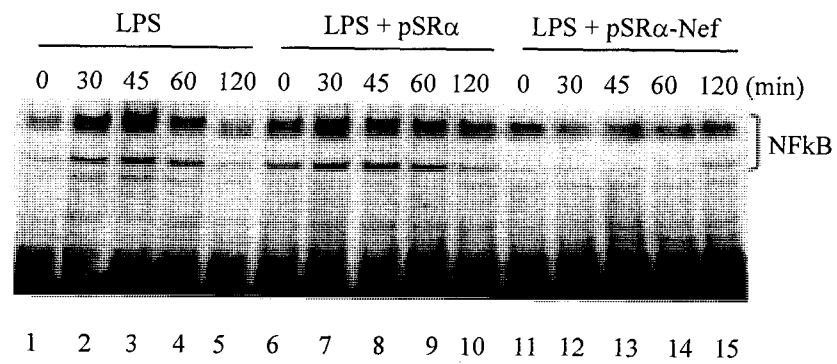
The above promoter analysis suggested that intracellular expression of Nef in human monocytic cells may be mediated through the down regulation of both NFκB and AP-1 activities. The involvement of NFκB and AP-1 activities in Nef-mediated down regulation of IL-12p40 transcription was further elucidated by determining the binding of NFκB and AP-1 transcription factors to their respective binding sites in the IL-12p40 promoter by gel shift assays. THP-1 cells were infected with either pSRα-Nef or control vector containing retroviruses followed by LPS stimulation for various periods of time and subsequent analysis of nuclear extracts for binding of NFκB and AP-1 to their binding sites on the γP^{32} labelled oligonucleotide probes containing NFκB or AP-1 sequences corresponding to the IL-12p40 promoter. Stimulation of pSRα-Nef retroviruses infected THP-1 cells with LPS failed to induce NFκB activity compared to the LPS-stimulated cells infected with the control retroviruses (Fig. 4.2-7A). The specificity of NFκB binding was demonstrated by competition with specific and nonspecific oligonucleotides and by supershift analysis with mouse anti-NFκB p50 and p65 antibodies (Fig. 4.2-7A). In contrast to NFκB, THP-1 cells infected with the pSRα-Nef did not show any decrease in the binding of AP-1 to its binding site on the IL-12p40 promoter compared to the cells infected with the control retroviruses following LPS stimulation (Fig. 4.2-7B). These results clearly suggest that intracellular expression of Nef selectively inhibits the binding of NFκB without affecting the binding of AP-1. Furthermore, Nef may induce inhibition of IL-12p40 transcription in human monocytic cells by selectively inhibiting the JNK-mediated activation of NFκB.

Fig. 4.2-7 Nef inhibits binding of NF κ B to its binding motif on IL-12p40 promoter in LPS-stimulated THP-1 cells:

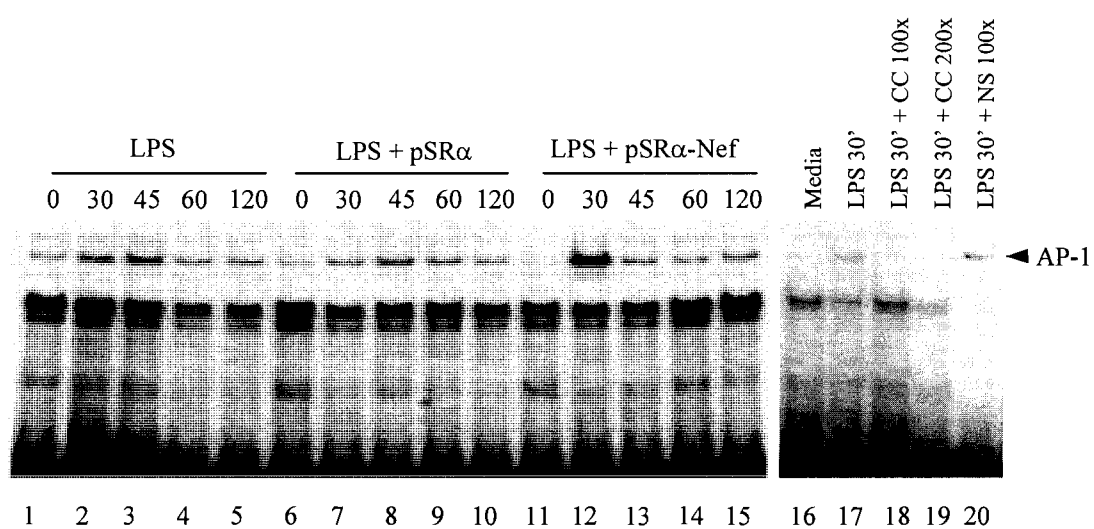
A, Top panel, the binding activity of NF κ B is inhibited by pSR α -Nef in a time course determined by gel-shift assay using the NF κ B probes. THP-1 cells were (5×10^6) were infected with either pSR α -Nef or control virus twice followed by stimulation with LPS for various times ranging from 30 to 120 minutes. Nuclear extracts (5 μ g) were incubated with 32 P-labeled oligonucleotides probes corresponding to NF κ B binding sequences derived from IL-12p40 promoter for the indicated time. The binding activity of NF- κ B was shown in parental THP-1 cells (lanes 1-5), control viral infected THP-1 cells (lanes 6-10) and pSR α -Nef infected THP-1 cells (lanes 11- 15). **Bottom panes,** the specificity of NF- κ B binding is interfered with pSR α -Nef by supershift assay. Cells were stimulated with 1 μ g/ml LPS for 30 min. 5 μ g of nuclear proteins were incubated with 32 P-labeled oligonucleotide probes corresponding to NF κ B. The specificity of NF κ B binding was determined by incubating nuclear proteins with unlabeled NF κ B (lanes 3-4) and non-specific oligonucleotides (lanes 5). The supershift analysis was performed by treating the nuclear proteins with oligonucleotide probes in the presence or the absence of anti-p50 or anti-p65 NF κ B antibodies and supershifted bands are indicated by arrows. The inhibitory effect of Nef on NF- κ B binding activity was shown in lanes 8-9. The experiments shown in A and B are representative of three different experiments.

B, AP-1 binding activity is not influenced by pSR α -Nef in a time course of gel shift assay. In the same experimental procedures, gel-shift assay was performed by incubating the nuclear extracts (5 μ g) with 32 P-labeled oligonucleotides probes corresponding to AP-1 binding sequences derived from IL-12p40 promoter for the indicated time. The binding activity for AP-1 was shown in parental THP-1 cells (lanes 1-5), control viral infected THP-1 cells (lanes 6-10), and pSR α -Nef infected THP-1 cells (lanes 11-15). Results indicated two independent experiments. The specificity of NF- κ B binding is interfered with pSR α -Nef by supershift assay (lanes 16-20).

A



B



4.3. Discussion

A severely decreased IL-12 by monocytes/macrophages after HIV-1 infection has been identified as a potential factor responsible for impaired innate and Th1 cell-mediated responses observed in AIDS patients. However, the mechanism by which HIV-1 infection downregulates IL-12 expression remains poorly understood. We have previously demonstrated that LPS-induced IL-12p40 production in human monocytic cells is regulated by NF κ B and AP-1 transcription factors through the activation of two distinct and independent upstream signaling pathways namely the JNK- and the CaMKII-activated pathways. In this study, I demonstrate for the first time that intracellularly expressed Nef inhibits IL-12p40 production by LPS-stimulated primary human monocytes and THP-1 cells through the selective inhibition of JNK-activated NF κ B pathway.

HIV regulatory proteins such as Tat, Nef and Vpr have been shown to modulate the production of cytokines including IL-12 [310]. 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 model systems used to deliver these genes/gene products into the cells and the presence of endotoxin in the preparations. For example, Tat has been shown to suppress as well as enhance IL-12 production in human PBMCs and dendritic cells [311]. I have previously demonstrated that intracellular as well as recombinant tat did not affect IL-12p40 production in either unstimulated or LPS-stimulated monocytic cells (ref and 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 [310]. Our unpublished results also suggest that treatment of THP-1 cells with Vpr

peptides did not affect LPS-induced IL-12p40 production in human monocytic cells. Similarly, there are reports that HIV Nef protein did not affect IL-12 expression in dendritic cells [312]. In contrast, IL-12p40 production was shown to be suppressed following infection of monocytic cells with HIV compared to infection with Nef-deficient HIV strains [313]. Our results suggest that intracellular expression utilizing infection with retroviral Nef constructs in THP-1 cells and primary monocytes inhibited LPS-induced IL-12p40 production.

My results suggest that intracellular Nef interferes with the LPS-activated signaling molecules to inhibit IL-12p40 production. LPS mediates its biological effects through the CD14/ TLR4 receptor complex [314] involving activation of tyrosine and serine/threonine protein kinases including PKC, calcium signaling and the MAPKs pathways. Nef has been shown to modulate cytokine expression in T cells and monocytes/macrophages by altering the activation of a variety of host cell signaling molecules. For example, IL-2 and IFN γ production was decreased in Nef-transduced Jurkat cells by inhibiting ERK and p38 MAPKs and NF κ B and NFAT transcription factors [24;315]. In this study, we show that HIV-Nef inhibits LPS-induced IL-12p40 expression by specifically inhibiting the LPS-activated JNK pathway.

JNK has been shown to play a critical role in Th1/Th2 cell differentiation, HIV Vpr-induced apoptosis of human monocytic cells, and LPS-induced production of proinflammatory cytokines [316;317]. JNK contains three members, JNK1, 2 and 3. JNK3 is selectively expressed in neuronal and cardiac tissues and associated with neuron cell apoptosis, whereas JNK1/2 are highly inducible in T cells and monocytic cells [318;319]. It is not clear whether JNK1 or JNK2 regulate IL-12p40 expression. JNK1 or

JNK2 specific siRNA and dnSEK1 employed previously in our laboratory inhibited both JNK1 and JNK2 isoforms making it difficult to pinpoint the role of JNK1 or JNK2 in IL-12p40 regulation [256]. My results in this study suggest that intracellular HIV Nef expression inhibited LPS-induced activation of both JNK1 and JNK2 isoforms while ERK and p38 MAPKs were not affected in either monocytes or THP-1 cells. JNK is activated by SEK1, a dual kinase upstream of JNK which phosphorylates both JNK and p38 MAPKs on threonine-183 and tyrosine-185 residues [255]. By employing a dominant negative SEK-1 construct, I previously demonstrated a critical role for SEK1 in IL-12p40 regulation by LPS-activated monocytes [100]. Here, my results also suggest that intracellular expression of Nef inhibited LPS-activated SEK-1 that may be responsible for inhibition of JNK activation and subsequent IL-12p40 transcription in both primary monocytes and THP-1 cells.

JNK is a serine/threonine kinase which activates various transcription factors including c-Jun, AP1, Sp-1, and NFκB in different cell types. Since LPS induced IL-12p40 transcription through JNK-activated NFκB and AP-1 [100], I investigated if intracellular Nef inhibited LPS-induced activation of NFκB and/or AP-1 transcription factors in the IL-12p40 production. My results suggest that in contrast to AP-1, intracellular nef selectively inhibited NFκB activation. Although it is not clear if Nef inhibited NFκB activity by directly inhibiting NFκB-JNK interactions, JNK has been shown to influence the NFκB pathway by regulating IκBα activation [320]. Since JNK can also activate c-Jun and c-Fos members of the AP-1 transcription factors and Nef did not affect AP-1 activation, it is likely that Nef may have inhibited NFκB activity independent of its interactions with the upstream JNK/SEK-1 kinases.

My previous results suggest that LPS-induced IL-12p40 production is regulated by two distinct signalling pathways namely the CaM/CaMKII and the JNK pathways. Interestingly, both pathways regulated IL-12p40 production through the activation of NFκB and AP-1 transcription factors. Since c-Jun-containing complexes have been shown to interact with NFκB proteins p50/p65 [321] it is likely that a potential co-operation between AP-1 and NFκB pathways regulates LPS-induced IL-12p40 transcription in human monocytic cells. In this study, I showed that although intracellularly expressed Nef did not inhibit AP-1 activity, Nef-mediated inhibition of NFκB abrogated LPS-induced IL-12p40 transcription. These results suggest that AP-1 in the presence of dysfunctional NFκB may not be able to drive LPS-induced IL-12p40 transcription.

How Nef inhibits JNK or NFκB to reduce LPS-induced IL-12p40 transcription is not clear at present. It is possible that Nef interferes with vacuolar ATPase which is important in phosphorylation of a number of protein kinases including JNK [165]. Nef could also interact with the regulatory domain of JNK leading to JNK phosphorylation. Glucocorticoids are highly associated with activation of JNK and IκB/NFκB signals. Glucocorticoids are also known to target HIV Vpr to suppress IL-12 in human monocytic cells [310]. Considering my earlier observation of a critical role for glucocorticoids in IL-12p40 expression through JNK activating NFκB, Nef could interact with glucocorticoid receptors that in turn increase the synthesis of IκB and block the nuclear translocation of NFκB. Interaction of Nef with glucocorticoid receptors could also inhibit activation of NFκB through a direct protein-protein interaction at the N-terminal Rel homology domain of NFκB, which mediates DNA binding, dimerization and interaction with IκB .

In addition, Nef could inhibit the formation of the p50-p50 homodimer or p50-p65 heterodimer that has been associated with transcription of IL-12 [322]. Besides signal transduction pathways, molecular mechanisms controlling gene expression involve chromatin remodelling and DNA methylation [300]. In addition to Nef-mediated inhibition of JNK and NF κ B activity, intracellular Nef may inhibit IL-12p40 transcription by altering the status of promoter methylation and chromatin remodeling. Further studies are necessary to elucidate the mechanisms underlying Nef-mediated down regulation of IL-12p40 transcription.

NF κ B plays a key role in the development of innate and adaptive immunity and is involved in the pathogenesis of a number of diseases including cancer, AIDS, and inflammatory disorders [323]. The biological significance of JNK-mediated activation of NF κ B signaling has been documented in cytokine production (IL-6, TNF- α , MIP-1 α) and susceptibility to bacterial and viral infection in animal models. In addition, NF κ B is a molecular basis for the aberrant growth and cytokine gene expression observed in AIDS [324-326]. Therefore, intracellular expression of Nef may not only down regulate IL-12 but may also be implicated in the down regulation of Th1 cytokines, Th1 responses and development of immunodeficiency.

Chapter V

Conclusion and Future Perspectives

IL-12 is a critical Th1 mediator mainly produced by monocytic cells in response to various pathogenic stimuli including LPS. IL-12 has been associated with the induction of strong cellular immune responses early in bacterial and viral infections and is critical for their clearance. Thus, its therapeutic potential has caused great attention. IL-12 production is found severely reduced in monocytic cells from HIV-1 infected individuals. The mechanisms by which IL-12p40 induced by LPS and its downregulation in HIV infection are not clear. Recent studies using infection with Nef-deficient HIV strains have shown that the HIV-1 regulatory protein Nef is involved in downregulation of Th1 cytokines in monocytes. Whether the impaired IL-12 production in HIV infected monocytes/macrophages is regulated by intracellular Nef and the molecular mechanisms involved in this regulation are both investigated in my thesis research.

LPS is a well known potent inducer of IL-12p40 from monocytes/macrophages. The molecular mechanism by which LPS induced IL-12p40 production is unknown. LPS is known to activate monocytic cells by binding to CD14/TLR4 receptor on the cell surface and by activating the entire three MAPKs and other protein kinase signaling pathways. In order to understand the signaling molecules involved in IL-12p40 production, I investigated the possible role of MAPKs in LPS-activated monocytes. Interestingly, only the JNK signaling pathway was required for IL-12p40 production. Since the JNK signaling pathway has been shown to be implicated in the regulation of Th1 responses and numbers of cytokine expression in the pathogenesis of infection diseases, autoimmune disorders and neurological disorders it is interesting to observe that JNK signaling is also involved in IL-12p40 production. In addition, I demonstrated for

the first time transcription factor AP-1 is a downstream target of JNK in addition to those previously established for the transcription factor NFkB.

The role of MAPKs in T cell differentiation and cytokine expression has been investigated. This has been associated with the immunosuppressive activity of antiinflammatory drugs, glucocorticoids such as Dxm. Indeed, the glucocorticoids have been shown to mediate their biological effects on cytokine production primarily by down-regulating activation of JNK MAPK as well as AP-1 and NFkB. In this study, I investigated the role of MAPKs and the transcription factors controlling IL-12p40 production in Dxm's biological activity. I showed for the first time that Dxm exerts its immunosuppressive ability by inhibition of IL-12p40 production via blocking the activation of JNK, AP-1 and NFkB. However, the mechanism by which Dxm inhibits JNK signaling is not well understood. The glucocorticoids regulate many biological processes through their intracellular glucocorticoid receptors which interact with specific DNA sequences termed glucocorticoid response elements (GREs) for transcription of glucocorticoid-responsive genes. Recently, the glucocorticoids have been shown to repress transcription by inhibiting the activity of a number of transcription factors including AP-1 and NFkB, independent of binding through the GREs. Whether Dxm inhibits IL-12p40 production through AP-1 and NFkB independent of GREs needs further investigation.

Two other immunosuppressive agents CyA and FK506 widely used in post-organ transplantation and autoimmune diseases are potent inhibitors of Ca^{2+} dependent T cell activation and have been shown to inhibit Th1 responses by interfering with cytokine production including IL-12. Since both FK506 and CyA are also inhibitors of calcineurin,

I investigated Ca^{2+} signaling in regulation of IL-12p40 production by CyA and FK506 and I demonstrated that calcineurin is a key factor in the downregulation of IL-12p40 by CyA and FK506. Calcineurin is tightly controlled by the Ca^{2+} /CaM complex. CaM is a major Ca^{2+} receptor existing in both the cytoplasm and nucleus. Ca^{2+} /CaM also activate CaMKII that I further illustrated as an important kinase in IL-12p40 regulation by using its pharmacological inhibitor. CaMKII has been shown to act as a mediator of I κ B kinase activation in response to CD3/TcR stimulation. In concert with my earlier observation showing the role of AP-1 in IL-12p40 transcription, I showed that CyA and FK506 inhibit binding of NF κ B and AP-1 to the IL-12p40 promoter through the CaM/CaMKII signaling. Since the Ca^{2+} /CaM complex highly controls the PI3K/Akt signaling pathway, I investigated the role of PI3K/Akt pathway to further understand Ca^{2+} /CaM signaling pathways involved in IL-12p40 induction. In contrast to the observation by others that PI3K signaling pathways negatively regulate LPS-induced murine IL-12p40 production, my results suggested that PI3K is a positive regulator in human IL-12p40 production. Furthermore, PI3K is CaM/CaMKII-dependent and JNK-independent in CyA and FK506-mediated IL-12p40 expression. Yet again, both AP-1 and NF κ B are involved in this event. These results suggested that LPS-induced IL-12p40 production is regulated by two independent pathways of CaM/CaMKII activated PI3K and JNK MAPK (Fig. 5.1). However, it is not clear why JNK pathway failed to compensate for LPS-induced IL-12p40 production in the presence of inhibitors specific for the calcium pathway. Moreover, since both pathways regulating IL-12p40 production are mediated through the same transcription factors, the molecular mechanisms controlling the activation of both AP-1 and NF κ B by these distinct pathways need further examination both *vitro* and *vivo*.

TLRs are key mediator of the immune response to mycobacterial and viral infections. In addition to TLR4, other TLRs are also implicated in IL-12 regulation in different cell types in response to different stimuli. For instance, TLR2 is required for IL-12 production in human macrophages and DCs in response to *M. tuberculosis*, whereas TLR11 is required for parasite-induced IL-12 production in DCs. It would be of interest to further study the signaling mechanism involved in the regulatory effects of these TLRs on IL-12 production by human macrophages and dendritic cells. Furthermore, CpG/TLR9 signaling triggers both B cell and DC producing IL-12. TLR9 as well as TLR7 are also known as viral ligands involved in regulation of various immune mediators by human macrophages and DCs. Future studies should identify which viral ligands and their TLRs are involved in HIV-mediated inhibition of IL-12 production by monocytic cells, DCs and B cells. In addition to signal transduction pathways, the mechanisms by which regulating gene expression involved in chromatin remodeling and DNA methylation remain to be clarified.

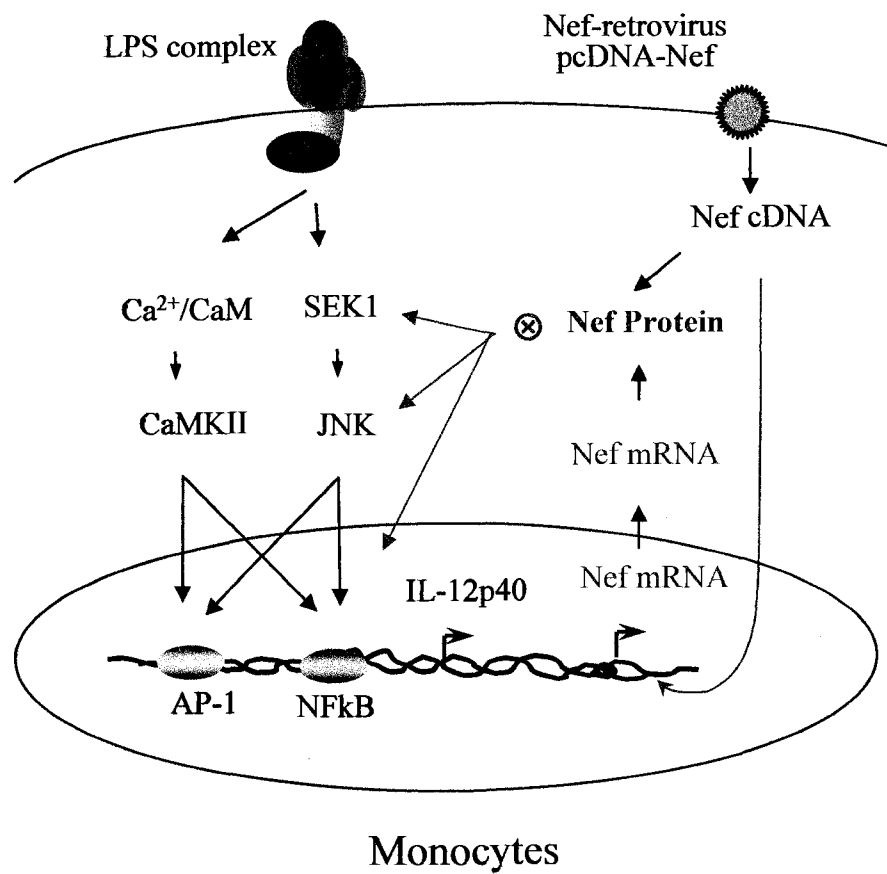
Nef is an early gene product and an AIDS factor that has been shown to be implicated in impaired Th1 immune responses and dysregulated cytokine expression. As I demonstrated earlier the critical role for JNK MAPK and CaM/CaMLII activated PI3K in IL-12p40 regulation, I then investigated the MAPKs and Ca²⁺ signals to understand the possible role of Nef in impaired IL-12p40 production from HIV-infected human monocytic cells. I demonstrated that intracellular Nef inhibits IL-12p40 production through JNK signaling by human monocytic cells and this is solely dependent on NFkB without affecting AP-1 activation (Fig. 5.1.). These results support the possibility of the role of Nef in damaged Th1 cellular immunity in the early stage of HIV infection.

However, several questions remain: 1) whether Nef directly interact with the signaling component mentioned above? What are other immune regulators that are possibly involved in Nef-mediated downregulation of IL-12p40? Since HIV-Vpr has also shown its inhibitory effect on IL-12p40 production, what is the possible relationship between these two proteins? Accordingly, future studies should focus on investigating how intracellular Nef and Vpr manipulate the activation of these signaling components in monocytes responsive to HIV infection. In addition, whether the IL-12p40 subunit is a key regulatory molecule in the immune remodeling observed in HIV infection needs to be further studied.

Overall, my results from these studies will provide a broad basic understanding of HIV-Nef mediated IL-12 expression and may suggest potential approaches for controlling immune deficiency in AIDS. Since it is difficult to eliminate HIV infection from the tissue reservoirs in patients when they receive anti-retroviral therapy, molecular approaches to understand the regulation of IL-12 by HIV may be helpful in designing novel strategies to eliminate virus from the body and enhance cellular immune responses to facilitate immune reconstitution.

Fig. 5-1 Signaling pathways involved in regulation of LPS-induced IL-12p40 production by HIV-1Nef in human monocytes:

LPS-induced IL-12p40 production in human monocytic cells is regulated by NF κ B and AP-1 transcription factors through the activation of two distinct upstream signaling pathways namely JNK and CaMKII-activated PI3K pathway. Furthermore, retroviral-mediated Nef gene inhibited LPS-induced IL-12-p40 expression through the inhibition of JNK-activated NF κ B without affecting the AP-1 activity.



Reference List

- [1] Majumdar D. An overview of the recent trends in HIV/AIDS in the United States. *Indian J Public Health* 2006 Jan;50(1):28-30.
- [2] Beyrer C. HIV epidemiology update and transmission factors: risks and risk contexts--16th International AIDS Conference epidemiology plenary. *Clin Infect Dis* 2007 Apr 1;44(7):981-7.
- [3] Emerman M, Malim MH. HIV-1 regulatory/accessory genes: keys to unraveling viral and host cell biology. *Science* 1998 Jun 19;280(5371):1880-4.
- [4] Fox R, Eldred LJ, Fuchs EJ, et al. Clinical manifestations of acute infection with human immunodeficiency virus in a cohort of gay men. *AIDS* 1987 May;1(1):35-8.
- [5] Hewson T, Lone N, Moore M, Howie S. Interactions of HIV-1 with antigen-presenting cells. *Immunol Cell Biol* 1999 Aug;77(4):289-303.
- [6] Fauci AS. HIV and AIDS: 20 years of science. *Nat Med* 2003 Jul;9(7):839-43.
- [7] Levy JA. Pathogenesis of human immunodeficiency virus infection. *Microbiol Rev* 1993 Mar;57(1):183-289.
- [8] Kedzierska K, Crowe SM. The role of monocytes and macrophages in the pathogenesis of HIV-1 infection. *Curr Med Chem* 2002 Nov;9(21):1893-903.
- [9] Imperiali FG, Zaninoni A, La ML, Tarsia P, Blasi F, Barcellini W. Increased *Mycobacterium tuberculosis* growth in HIV-1-infected human macrophages: role of tumour necrosis factor-alpha. *Clin Exp Immunol* 2001 Mar;123(3):435-42.
- [10] Baum MK, Miguez-Burbano MJ, Campa A, Shor-Posner G. Selenium and interleukins in persons infected with human immunodeficiency virus type 1. *J Infect Dis* 2000 Sep;182 Suppl 1:S69-S73.
- [11] Douglas SD, Durako S, Sullivan KE, Camarca M, Moscicki AB, Wilson CM. TH1 and TH2 cytokine mRNA and protein levels in human immunodeficiency virus (HIV)-seropositive and HIV-seronegative youths. *Clin Diagn Lab Immunol* 2003 May;10(3):399-404.
- [12] Punzon C, Resino S, Bellon JM, Munoz-Fernandez MA, Fresno M. Analysis of the systemic immune response in HIV-1-infected patients suffering from opportunistic *Candida* infection. *Eur Cytokine Netw* 2002 Apr;13(2):215-23.
- [13] Gately MK, Renzetti LM, Magram J, et al. The interleukin-12/interleukin-12-receptor system: role in normal and pathologic immune responses. *Annu Rev Immunol* 1998;16:495-521.
- [14] Gubler U, Chua AO, Schoenhaut DS, et al. Coexpression of two distinct genes is required to generate secreted bioactive cytotoxic lymphocyte maturation factor. *Proc Natl Acad Sci U S A* 1991 May 15;88(10):4143-7.

- [15] ste-Amezaga M, Ma X, Sartori A, Trinchieri G. Molecular mechanisms of the induction of IL-12 and its inhibition by IL-10. *J Immunol* 1998 Jun 15;160(12):5936-44.
- [16] Chehimi J, Starr SE, Frank I, et al. Impaired interleukin 12 production in human immunodeficiency virus-infected patients. *J Exp Med* 1994 Apr 1;179(4):1361-6.
- [17] Ma X, Montaner LJ. Proinflammatory response and IL-12 expression in HIV-1 infection. *J Leukoc Biol* 2000 Sep;68(3):383-90.
- [18] Marshall JD, Chehimi J, Gri G, Kostman JR, Montaner LJ, Trinchieri G. The interleukin-12-mediated pathway of immune events is dysfunctional in human immunodeficiency virus-infected individuals. *Blood* 1999 Aug 1;94(3):1003-11.
- [19] Magram J, Connaughton SE, Warriar RR, et al. IL-12-deficient mice are defective in IFN gamma production and type 1 cytokine responses. *Immunity* 1996 May;4(5):471-81.
- [20] Chehimi J, Marshall JD, Salvucci O, et al. IL-15 enhances immune functions during HIV infection. *J Immunol* 1997 Jun 15;158(12):5978-87.
- [21] Matsubara S, Takeda K, Kodama T, et al. IL-2 and IL-18 attenuation of airway hyperresponsiveness requires STAT4, IFN-gamma, and natural killer cells. *Am J Respir Cell Mol Biol* 2007 Mar;36(3):324-32.
- [22] Huang XL, Fan Z, Gupta P, Rinaldo CR, Jr. Activation of HIV type 1 specific cytotoxic T lymphocytes from semen by HIV type 1 antigen-presenting dendritic cells and IL-12. *AIDS Res Hum Retroviruses* 2006 Jan;22(1):93-8.
- [23] Zou W, Lackner AA, Simon M, et al. Early cytokine and chemokine gene expression in lymph nodes of macaques infected with simian immunodeficiency virus is predictive of disease outcome and vaccine efficacy. *J Virol* 1997 Feb;71(2):1227-36.
- [24] Collette Y, Chang HL, Cerdan C, et al. Specific Th1 cytokine down-regulation associated with primary clinically derived human immunodeficiency virus type 1 Nef gene-induced expression. *J Immunol* 1996 Jan 1;156(1):360-70.
- [25] Niederman TM, Hastings WR, Luria S, Bandres JC, Ratner L. HIV-1 Nef protein inhibits the recruitment of AP-1 DNA-binding activity in human T-cells. *Virology* 1993 May;194(1):338-44.
- [26] Pugliese A, Vidotto V, Beltramo T, Torre D. Regulation of interleukin-18 by THP-1 monocytoic cells stimulated with HIV-1 and Nef viral protein. *Eur Cytokine Netw* 2005 Sep;16(3):186-90.
- [27] Ma X, Trinchieri G. Regulation of interleukin-12 production in antigen-presenting cells. *Adv Immunol* 2001;79:55-92.
- [28] Sieburth D, Jabs EW, Warrington JA, et al. Assignment of genes encoding a unique cytokine (IL12) composed of two unrelated subunits to chromosomes 3 and 5. *Genomics* 1992 Sep;14(1):59-62.

- [29] Merberg DM, Wolf SF, Clark SC. Sequence similarity between NKSF and the IL-6/G-CSF family. *Immunol Today* 1992 Feb;13(2):77-8.
- [30] Gearing DP, Cosman D. Homology of the p40 subunit of natural killer cell stimulatory factor (NKSF) with the extracellular domain of the interleukin-6 receptor. *Cell* 1991 Jul 12;66(1):9-10.
- [31] Ma X, ste-Amezaga M, Trinchieri G. Regulation of interleukin-12 production. *Ann N Y Acad Sci* 1996 Oct 31;795:13-25.
- [32] Gillessen S, Carvajal D, Ling P, et al. Mouse interleukin-12 (IL-12) p40 homodimer: a potent IL-12 antagonist. *Eur J Immunol* 1995 Jan;25(1):200-6.
- [33] Podlaski FJ, Nanduri VB, Hulmes JD, et al. Molecular characterization of interleukin 12. *Arch Biochem Biophys* 1992 Apr;294(1):230-7.
- [34] Ling P, Gately MK, Gubler U, et al. Human IL-12 p40 homodimer binds to the IL-12 receptor but does not mediate biologic activity. *J Immunol* 1995 Jan 1;154(1):116-27.
- [35] Piccotti JR, Chan SY, Goodman RE, Magram J, Eichwald EJ, Bishop DK. IL-12 antagonism induces T helper 2 responses, yet exacerbates cardiac allograft rejection. Evidence against a dominant protective role for T helper 2 cytokines in alloimmunity. *J Immunol* 1996 Sep 1;157(5):1951-7.
- [36] Brombacher F, Kastelein RA, Alber G. Novel IL-12 family members shed light on the orchestration of Th1 responses. *Trends Immunol* 2003 Apr;24(4):207-12.
- [37] Oppmann B, Lesley R, Blom B, et al. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity* 2000 Nov;13(5):715-25.
- [38] Lankford CS, Frucht DM. A unique role for IL-23 in promoting cellular immunity. *J Leukoc Biol* 2003 Jan;73(1):49-56.
- [39] Ha SJ, Kim DJ, Baek KH, Yun YD, Sung YC. IL-23 induces stronger sustained CTL and Th1 immune responses than IL-12 in hepatitis C virus envelope protein 2 DNA immunization. *J Immunol* 2004 Jan 1;172(1):525-31.
- [40] Belladonna ML, Renauld JC, Bianchi R, et al. IL-23 and IL-12 have overlapping, but distinct, effects on murine dendritic cells. *J Immunol* 2002 Jun 1;168(11):5448-54.
- [41] Matsui M, Moriya O, Belladonna ML, et al. Adjuvant activities of novel cytokines, interleukin-23 (IL-23) and IL-27, for induction of hepatitis C virus-specific cytotoxic T lymphocytes in HLA-A*0201 transgenic mice. *J Virol* 2004 Sep;78(17):9093-104.
- [42] Holscher C. The power of combinatorial immunology: IL-12 and IL-12-related dimeric cytokines in infectious diseases. *Med Microbiol Immunol* 2004 Feb;193(1):1-17.
- [43] Naeger LK, McKinney J, Salvekar A, Hoey T. Identification of a STAT4 binding site in the interleukin-12 receptor required for signaling. *J Biol Chem* 1999 Jan 22;274(4):1875-8.

- [44] Jones D, Elloso MM, Showe L, Williams D, Trinchieri G, Scott P. Differential regulation of the interleukin-12 receptor during the innate immune response to *Leishmania major*. *Infect Immun* 1998 Aug;66(8):3818-24.
- [45] Wu CY, Gadina M, Wang K, O'Shea J, Seder RA. Cytokine regulation of IL-12 receptor beta2 expression: differential effects on human T and NK cells. *Eur J Immunol* 2000 May;30(5):1364-74.
- [46] Zhang GX, Yu S, Gran B, et al. Role of IL-12 receptor beta 1 in regulation of T cell response by APC in experimental autoimmune encephalomyelitis. *J Immunol* 2003 Nov 1;171(9):4485-92.
- [47] Bacon CM, McVicar DW, Ortaldo JR, Rees RC, O'Shea JJ, Johnston JA. Interleukin 12 (IL-12) induces tyrosine phosphorylation of JAK2 and TYK2: differential use of Janus family tyrosine kinases by IL-2 and IL-12. *J Exp Med* 1995 Jan 1;181(1):399-404.
- [48] Gollob JA, Murphy EA, Mahajan S, Schnipper CP, Ritz J, Frank DA. Altered interleukin-12 responsiveness in Th1 and Th2 cells is associated with the differential activation of STAT5 and STAT1. *Blood* 1998 Feb 15;91(4):1341-54.
- [49] Ozbek N, Fieschi C, Yilmaz BT, et al. Interleukin-12 receptor beta 1 chain deficiency in a child with disseminated tuberculosis. *Clin Infect Dis* 2005 Mar 15;40(6):e55-e58.
- [50] Mikus LD, Rosenthal LA, Sorkness RL, Lemanske RF, Jr. Reduced interferon-gamma secretion by natural killer cells from rats susceptible to postviral chronic airway dysfunction. *Am J Respir Cell Mol Biol* 2001 Jan;24(1):74-82.
- [51] Hsieh CS, Macatonia SE, Tripp CS, Wolf SF, O'Garra A, Murphy KM. Development of TH1 CD4+ T cells through IL-12 produced by *Listeria*-induced macrophages. *Science* 1993 Apr 23;260(5107):547-9.
- [52] Seder RA, Gazzinelli R, Sher A, Paul WE. Interleukin 12 acts directly on CD4+ T cells to enhance priming for interferon gamma production and diminishes interleukin 4 inhibition of such priming. *Proc Natl Acad Sci U S A* 1993 Nov 1;90(21):10188-92.
- [53] Tominaga K, Yoshimoto T, Torigoe K, et al. IL-12 synergizes with IL-18 or IL-1beta for IFN-gamma production from human T cells. *Int Immunol* 2000 Feb;12(2):151-60.
- [54] Nakahira M, Ahn HJ, Park WR, et al. Synergy of IL-12 and IL-18 for IFN-gamma gene expression: IL-12-induced STAT4 contributes to IFN-gamma promoter activation by up-regulating the binding activity of IL-18-induced activator protein 1. *J Immunol* 2002 Feb 1;168(3):1146-53.
- [55] O'Shea JJ. Jaks, STATs, cytokine signal transduction, and immunoregulation: are we there yet? *Immunity* 1997 Jul;7(1):1-11.
- [56] Marshall JD, Secrist H, DeKruyff RH, Wolf SF, Umetsu DT. IL-12 inhibits the production of IL-4 and IL-10 in allergen-specific human CD4+ T lymphocytes. *J Immunol* 1995 Jul 1;155(1):111-7.

- [57] Trinchieri G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat Rev Immunol* 2003 Feb;3(2):133-46.
- [58] Newport MJ, Huxley CM, Huston S, et al. A mutation in the interferon-gamma-receptor gene and susceptibility to mycobacterial infection. *N Engl J Med* 1996 Dec 26;335(26):1941-9.
- [59] Samuel CE, Ozato K. Induction of interferons-induced genes. *Biotherapy* 1996;8(3-4):183-7.
- [60] Bach EA, Aguet M, Schreiber RD. The IFN gamma receptor: a paradigm for cytokine receptor signaling. *Annu Rev Immunol* 1997;15:563-91.
- [61] Dalton DK, Pitts-Meek S, Keshav S, Figari IS, Bradley A, Stewart TA. Multiple defects of immune cell function in mice with disrupted interferon-gamma genes. *Science* 1993 Mar 19;259(5102):1739-42.
- [62] Yu CR, Lin JX, Fink DW, Akira S, Bloom ET, Yamauchi A. Differential utilization of Janus kinase-signal transducer activator of transcription signaling pathways in the stimulation of human natural killer cells by IL-2, IL-12, and IFN-alpha. *J Immunol* 1996 Jul 1;157(1):126-37.
- [63] Allavena P, Paganin C, Zhou D, Bianchi G, Sozzani S, Mantovani A. Interleukin-12 is chemotactic for natural killer cells and stimulates their interaction with vascular endothelium. *Blood* 1994 Oct 1;84(7):2261-8.
- [64] Biron CA, Nguyen KB, Pien GC, Cousens LP, Salazar-Mather TP. Natural killer cells in antiviral defense: function and regulation by innate cytokines. *Annu Rev Immunol* 1999;17:189-220.
- [65] Trinchieri G. Biology of natural killer cells. *Adv Immunol* 1989;47:187-376.
- [66] Lanier LL. On guard--activating NK cell receptors. *Nat Immunol* 2001 Jan;2(1):23-7.
- [67] Mueller YM, De Rosa SC, Hutton JA, et al. Increased CD95/Fas-induced apoptosis of HIV-specific CD8(+) T cells. *Immunity* 2001 Dec;15(6):871-82.
- [68] Badley AD, McElhinny JA, Leibson PJ, Lynch DH, Alderson MR, Paya CV. Upregulation of Fas ligand expression by human immunodeficiency virus in human macrophages mediates apoptosis of uninfected T lymphocytes. *J Virol* 1996 Jan;70(1):199-206.
- [69] Stranford SA, Skurnick J, Louria D, et al. Lack of infection in HIV-exposed individuals is associated with a strong CD8(+) cell noncytotoxic anti-HIV response. *Proc Natl Acad Sci U S A* 1999 Feb 2;96(3):1030-5.
- [70] Scott-Algara D, Buseyne F, Porrot F, et al. Not all tetramer binding CD8+ T cells can produce cytokines and chemokines involved in the effector functions of virus-specific CD8+ T lymphocytes in HIV-1 infected children. *J Clin Immunol* 2005 Jan;25(1):57-67.

- [71] Airolidi I, Gri G, Marshall JD, et al. Expression and function of IL-12 and IL-18 receptors on human tonsillar B cells. *J Immunol* 2000 Dec 15;165(12):6880-8.
- [72] Otani T, Nakamura S, Toki M, Motoda R, Kurimoto M, Orita K. Identification of IFN-gamma-producing cells in IL-12/IL-18-treated mice. *Cell Immunol* 1999 Dec 15;198(2):111-9.
- [73] Yoshimoto T, Okamura H, Tagawa YI, Iwakura Y, Nakanishi K. Interleukin 18 together with interleukin 12 inhibits IgE production by induction of interferon-gamma production from activated B cells. *Proc Natl Acad Sci U S A* 1997 Apr 15;94(8):3948-53.
- [74] Cooper AM, Magram J, Ferrante J, Orme IM. Interleukin 12 (IL-12) is crucial to the development of protective immunity in mice intravenously infected with mycobacterium tuberculosis. *J Exp Med* 1997 Jul 7;186(1):39-45.
- [75] Muller U, Kohler G, Mossmann H, et al. IL-12-independent IFN-gamma production by T cells in experimental Chagas' disease is mediated by IL-18. *J Immunol* 2001 Sep 15;167(6):3346-53.
- [76] Mattner F, Magram J, Ferrante J, et al. Genetically resistant mice lacking interleukin-12 are susceptible to infection with *Leishmania major* and mount a polarized Th2 cell response. *Eur J Immunol* 1996 Jul;26(7):1553-9.
- [77] Ely KH, Kasper LH, Khan IA. Augmentation of the CD8+ T cell response by IFN-gamma in IL-12-deficient mice during *Toxoplasma gondii* infection. *J Immunol* 1999 May 1;162(9):5449-54.
- [78] Kawakami K, Koguchi Y, Qureshi MH, et al. IL-18 contributes to host resistance against infection with *Cryptococcus neoformans* in mice with defective IL-12 synthesis through induction of IFN-gamma production by NK cells. *J Immunol* 2000 Jul 15;165(2):941-7.
- [79] Trinchieri G. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Annu Rev Immunol* 1995;13:251-76.
- [80] Scharton-Kersten TM, Yap G, Magram J, Sher A. Inducible nitric oxide is essential for host control of persistent but not acute infection with the intracellular pathogen *Toxoplasma gondii*. *J Exp Med* 1997 Apr 7;185(7):1261-73.
- [81] de JR, Altare F, Haagen IA, et al. Severe mycobacterial and *Salmonella* infections in interleukin-12 receptor-deficient patients. *Science* 1998 May 29;280(5368):1435-8.
- [82] Watford WT, Moriguchi M, Morinobu A, O'Shea JJ. The biology of IL-12: coordinating innate and adaptive immune responses. *Cytokine Growth Factor Rev* 2003 Oct;14(5):361-8.
- [83] Issazadeh S, Ljungdahl A, Hojeberg B, Mustafa M, Olsson T. Cytokine production in the central nervous system of Lewis rats with experimental autoimmune encephalomyelitis: dynamics of mRNA expression for interleukin-10, interleukin-12,

- cytolysin, tumor necrosis factor alpha and tumor necrosis factor beta. *J Neuroimmunol* 1995 Sep;61(2):205-12.
- [84] Williamson E, Garside P, Bradley JA, Mowat AM. IL-12 is a central mediator of acute graft-versus-host disease in mice. *J Immunol* 1996 Jul 15;157(2):689-99.
 - [85] Goriely S, Goldman M. The interleukin-12 family: new players in transplantation immunity? *Am J Transplant* 2007 Feb;7(2):278-84.
 - [86] Becher B, Durell BG, Noelle RJ. Experimental autoimmune encephalitis and inflammation in the absence of interleukin-12. *J Clin Invest* 2002 Aug;110(4):493-7.
 - [87] Gonschior AK, Christians U, Braun F, et al. Measurement of blood concentrations of FK506 (tacrolimus) and its metabolites in seven liver graft patients after the first dose by h.p.l.c.-MS and microparticle enzyme immunoassay (MEIA). *Br J Clin Pharmacol* 1994 Dec;38(6):567-71.
 - [88] Wu CY, Wang K, McDyer JF, Seder RA. Prostaglandin E2 and dexamethasone inhibit IL-12 receptor expression and IL-12 responsiveness. *J Immunol* 1998 Sep 15;161(6):2723-30.
 - [89] Hariharan S, Johnson CP, Bresnahan BA, Taranto SE, McIntosh MJ, Stablein D. Improved graft survival after renal transplantation in the United States, 1988 to 1996. *N Engl J Med* 2000 Mar 2;342(9):605-12.
 - [90] Kiani A, Rao A, Aramburu J. Manipulating immune responses with immunosuppressive agents that target NFAT. *Immunity* 2000 Apr;12(4):359-72.
 - [91] Crabtree GR. Generic signals and specific outcomes: signaling through Ca²⁺, calcineurin, and NF-AT. *Cell* 1999 Mar 5;96(5):611-4.
 - [92] Szabo G, Gavala C, Mandrekar P. Tacrolimus and cyclosporine A inhibit allostimulatory capacity and cytokine production of human myeloid dendritic cells. *J Investig Med* 2001 Sep;49(5):442-9.
 - [93] Egi H, Hayamizu K, Kitayama T, Ohmori I, Okajima M, Asahara T. Downregulation of both interleukin-12 and interleukin-2 in heart allografts by pretransplant host treatment with granulocyte colony-stimulating factor and tacrolimus. *Cytokine* 2002 May 7;18(3):164-7.
 - [94] Orange JS, Wolf SF, Biron CA. Effects of IL-12 on the response and susceptibility to experimental viral infections. *J Immunol* 1994 Feb 1;152(3):1253-64.
 - [95] Komatsu T, Bi Z, Reiss CS. Interferon-gamma induced type I nitric oxide synthase activity inhibits viral replication in neurons. *J Neuroimmunol* 1996 Aug;68(1-2):101-8.
 - [96] Karp CL, Wysocka M, Wahl LM, et al. Mechanism of suppression of cell-mediated immunity by measles virus. *Science* 1996 Jul 12;273(5272):228-31.
 - [97] Cavanaugh VJ, Guidotti LG, Chisari FV. Interleukin-12 inhibits hepatitis B virus replication in transgenic mice. *J Virol* 1997 Apr;71(4):3236-43.

- [98] Carr JA, Rogerson J, Mulqueen MJ, Roberts NA, Booth RF. Interleukin-12 exhibits potent antiviral activity in experimental herpesvirus infections. *J Virol* 1997 Oct;71(10):7799-803.
- [99] Moser M, Murphy KM. Dendritic cell regulation of TH1-TH2 development. *Nat Immunol* 2000 Sep;1(3):199-205.
- [100] Ma W, Gee K, Lim W, et al. Dexamethasone inhibits IL-12p40 production in lipopolysaccharide-stimulated human monocytic cells by down-regulating the activity of c-Jun N-terminal kinase, the activation protein-1, and NF-kappa B transcription factors. *J Immunol* 2004 Jan 1;172(1):318-30.
- [101] Kennedy MK, Picha KS, Fanslow WC, et al. CD40/CD40 ligand interactions are required for T cell-dependent production of interleukin-12 by mouse macrophages. *Eur J Immunol* 1996 Feb;26(2):370-8.
- [102] Lederer JA, Perez VL, DesRoches L, Kim SM, Abbas AK, Lichtman AH. Cytokine transcriptional events during helper T cell subset differentiation. *J Exp Med* 1996 Aug 1;184(2):397-406.
- [103] Reiner SL, Zheng S, Wang ZE, Stowring L, Locksley RM. Leishmania promastigotes evade interleukin 12 (IL-12) induction by macrophages and stimulate a broad range of cytokines from CD4+ T cells during initiation of infection. *J Exp Med* 1994 Feb 1;179(2):447-56.
- [104] Liu J, Cao S, Herman LM, Ma X. Differential regulation of interleukin (IL)-12 p35 and p40 gene expression and interferon (IFN)-gamma-primed IL-12 production by IFN regulatory factor 1. *J Exp Med* 2003 Oct 20;198(8):1265-76.
- [105] Ma X, Gri G, Trinchieri G. A novel ets-2-related nuclear factor is involved in transcriptional activation of the human interleukin-12 p40 gene promoter in response to interferon-gamma and LPS stimulation of monocytic cells. *Ann N Y Acad Sci* 1996 Oct 31;795:357-60.
- [106] Masumi A, Tamaoki S, Wang IM, Ozato K, Komuro K. IRF-8/ICSBP and IRF-1 cooperatively stimulate mouse IL-12 promoter activity in macrophages. *FEBS Lett* 2002 Nov 6;531(2):348-53.
- [107] Goriely S, Molle C, Nguyen M, et al. Interferon regulatory factor 3 is involved in Toll-like receptor 4 (TLR4)- and TLR3-induced IL-12p35 gene activation. *Blood* 2006 Feb 1;107(3):1078-84.
- [108] D'Andrea A, ste-Amezaga M, Valiante NM, Ma X, Kubin M, Trinchieri G. Interleukin 10 (IL-10) inhibits human lymphocyte interferon gamma-production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. *J Exp Med* 1993 Sep 1;178(3):1041-8.
- [109] Driessler F, Venstrom K, Sabat R, Asadullah K, Schottelius AJ. Molecular mechanisms of interleukin-10-mediated inhibition of NF-kappaB activity: a role for p50. *Clin Exp Immunol* 2004 Jan;135(1):64-73.

- [110] Rahim SS, Khan N, Boddupalli CS, Hasnain SE, Mukhopadhyay S. Interleukin-10 (IL-10) mediated suppression of IL-12 production in RAW 264.7 cells also involves c-rel transcription factor. *Immunology* 2005 Mar;114(3):313-21.
- [111] Zhou L, Nazarian AA, Smale ST. Interleukin-10 inhibits interleukin-12 p40 gene transcription by targeting a late event in the activation pathway. *Mol Cell Biol* 2004 Mar;24(6):2385-96.
- [112] Gazzinelli RT, Wysocka M, Hieny S, et al. In the absence of endogenous IL-10, mice acutely infected with *Toxoplasma gondii* succumb to a lethal immune response dependent on CD4⁺ T cells and accompanied by overproduction of IL-12, IFN- γ and TNF- α . *J Immunol* 1996 Jul 15;157(2):798-805.
- [113] Branton MH, Kopp JB. TGF- β and fibrosis. *Microbes Infect* 1999 Dec;1(15):1349-65.
- [114] McRae BL, Beilfuss BA, van Seventer GA. IFN- β differentially regulates CD40-induced cytokine secretion by human dendritic cells. *J Immunol* 2000 Jan 1;164(1):23-8.
- [115] Cousens LP, Orange JS, Su HC, Biron CA. Interferon- α/β inhibition of interleukin 12 and interferon- γ production in vitro and endogenously during viral infection. *Proc Natl Acad Sci U S A* 1997 Jan 21;94(2):634-9.
- [116] Karp CL, Wysocka M, Wahl LM, et al. Mechanism of suppression of cell-mediated immunity by measles virus. *Science* 1996 Jul 12;273(5272):228-31.
- [117] Murphy FJ, Hayes MP, Burd PR. Disparate intracellular processing of human IL-12 preprotein subunits: atypical processing of the P35 signal peptide. *J Immunol* 2000 Jan 15;164(2):839-47.
- [118] Liu J, Guan X, Tamura T, Ozato K, Ma X. Synergistic activation of interleukin-12 p35 gene transcription by interferon regulatory factor-1 and interferon consensus sequence-binding protein. *J Biol Chem* 2004 Dec 31;279(53):55609-17.
- [119] Murphy TL, Cleveland MG, Kulesza P, Magram J, Murphy KM. Regulation of interleukin 12 p40 expression through an NF- κ B half-site. *Mol Cell Biol* 1995 Oct;15(10):5258-67.
- [120] Ma X, Neurath M, Gri G, Trinchieri G. Identification and characterization of a novel Ets-2-related nuclear complex implicated in the activation of the human interleukin-12 p40 gene promoter. *J Biol Chem* 1997 Apr 18;272(16):10389-95.
- [121] Plevy SE, Gemberling JH, Hsu S, Dorner AJ, Smale ST. Multiple control elements mediate activation of the murine and human interleukin 12 p40 promoters: evidence of functional synergy between C/EBP and Rel proteins. *Mol Cell Biol* 1997 Aug;17(8):4572-88.
- [122] Matsumoto M, Einhaus D, Gold ES, Aderem A. Simvastatin augments lipopolysaccharide-induced proinflammatory responses in macrophages by differential

- regulation of the c-Fos and c-Jun transcription factors. *J Immunol* 2004 Jun 15;172(12):7377-84.
- [123] Zhu C, Gagnidze K, Gemberling JH, Plevy SE. Characterization of an activation protein-1-binding site in the murine interleukin-12 p40 promoter. Demonstration of novel functional elements by a reductionist approach. *J Biol Chem* 2001 May 25;276(21):18519-28.
 - [124] Becker C, Wirtz S, Ma X, Blessing M, Galle PR, Neurath MF. Regulation of IL-12 p40 promoter activity in primary human monocytes: roles of NF-kappaB, CCAAT/enhancer-binding protein beta, and PU.1 and identification of a novel repressor element (GA-12) that responds to IL-4 and prostaglandin E(2). *J Immunol* 2001 Sep 1;167(5):2608-18.
 - [125] Cullen BR. The role of Nef in the replication cycle of the human and simian immunodeficiency viruses. *Virology* 1994 Nov 15;205(1):1-6.
 - [126] Fujii Y, Otake K, Tashiro M, Adachi A. Soluble Nef antigen of HIV-1 is cytotoxic for human CD4+ T cells. *FEBS Lett* 1996 Sep 9;393(1):93-6.
 - [127] Sanchez-Velar N, Udofia EB, Yu Z, Zapp ML. hRIP, a cellular cofactor for Rev function, promotes release of HIV RNAs from the perinuclear region. *Genes Dev* 2004 Jan 1;18(1):23-34.
 - [128] Zhai S, Kang WZ, Yu X, et al. [Specific immune responses to human immunodeficiency virus type 1 Gag, Tat, Rev and Nef proteins]. *Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi* 2005 Mar;21(2):175-9.
 - [129] Gallo R, Wong-Staal F, Montagnier L, Haseltine WA, Yoshida M. HIV/HTLV gene nomenclature. *Nature* 1988 Jun 9;333(6173):504.
 - [130] Geyer M, Fackler OT, Peterlin BM. Structure--function relationships in HIV-1 Nef. *EMBO Rep* 2001 Jul;2(7):580-5.
 - [131] Cho S, Knox KS, Kohli LM, et al. Impaired cell surface expression of human CD1d by the formation of an HIV-1 Nef/CD1d complex. *Virology* 2005 Jul 5;337(2):242-52.
 - [132] Agopian K, Wei BL, Garcia JV, Gabuzda D. CD4 and MHC-I downregulation are conserved in primary HIV-1 Nef alleles from brain and lymphoid tissues, but Pak2 activation is highly variable. *Virology* 2007 Feb 5;358(1):119-35.
 - [133] Wu Y, Marsh JW. Early transcription from nonintegrated DNA in human immunodeficiency virus infection. *J Virol* 2003 Oct;77(19):10376-82.
 - [134] Welker R, Kottler H, Kalbitzer HR, Krausslich HG. Human immunodeficiency virus type 1 Nef protein is incorporated into virus particles and specifically cleaved by the viral proteinase. *Virology* 1996 May 1;219(1):228-36.
 - [135] Yang X, Gabuzda D. Regulation of human immunodeficiency virus type 1 infectivity by the ERK mitogen-activated protein kinase signaling pathway. *J Virol* 1999 Apr;73(4):3460-6.

- [136] Geyer M, Munte CE, Schorr J, Kellner R, Kalbitzer HR. Structure of the anchor-domain of myristoylated and non-myristoylated HIV-1 Nef protein. *J Mol Biol* 1999 May 28;289(1):123-38.
- [137] Schiavoni I, Muratori C, Piacentini V, Giammarioli AM, Federico M. The HIV-1 Nef protein: how an AIDS pathogenetic factor turns to a tool for combating AIDS. *Curr Drug Targets Immune Endocr Metabol Disord* 2004 Mar;4(1):19-27.
- [138] Arold ST, Baur AS. Dynamic Nef and Nef dynamics: how structure could explain the complex activities of this small HIV protein. *Trends Biochem Sci* 2001 Jun;26(6):356-63.
- [139] Piguet V, Schwartz O, Le GS, Trono D. The downregulation of CD4 and MHC-I by primate lentiviruses: a paradigm for the modulation of cell surface receptors. *Immunol Rev* 1999 Apr;168:51-63.
- [140] Saksela K, Cheng G, Baltimore D. Proline-rich (PxxP) motifs in HIV-1 Nef bind to SH3 domains of a subset of Src kinases and are required for the enhanced growth of Nef⁺ viruses but not for down-regulation of CD4. *EMBO J* 1995 Feb 1;14(3):484-91.
- [141] Arold S, Franken P, Strub MP, et al. The crystal structure of HIV-1 Nef protein bound to the Fyn kinase SH3 domain suggests a role for this complex in altered T cell receptor signaling. *Structure* 1997 Oct 15;5(10):1361-72.
- [142] Choi HJ, Smithgall TE. Conserved residues in the HIV-1 Nef hydrophobic pocket are essential for recruitment and activation of the Hck tyrosine kinase. *J Mol Biol* 2004 Nov 5;343(5):1255-68.
- [143] Briese L, Preusser A, Willbold D. Mapping the binding site of full length HIV-1 Nef on human Lck SH3 by NMR spectroscopy. *J Biomed Sci* 2005;12(3):451-6.
- [144] Sawai ET, Baur A, Struble H, Peterlin BM, Levy JA, Cheng-Mayer C. Human immunodeficiency virus type 1 Nef associates with a cellular serine kinase in T lymphocytes. *Proc Natl Acad Sci U S A* 1994 Feb 15;91(4):1539-43.
- [145] Nunn MF, Marsh JW. Human immunodeficiency virus type 1 Nef associates with a member of the p21-activated kinase family. *J Virol* 1996 Sep;70(9):6157-61.
- [146] Smith BL, Krushelnycky BW, Mochly-Rosen D, Berg P. The HIV nef protein associates with protein kinase C theta. *J Biol Chem* 1996 Jul 12;271(28):16753-7.
- [147] Fackler OT, Luo W, Geyer M, Alberts AS, Peterlin BM. Activation of Vav by Nef induces cytoskeletal rearrangements and downstream effector functions. *Mol Cell* 1999 Jun;3(6):729-39.
- [148] Michel N, Ganter K, Venzke S, Bitzegeio J, Fackler OT, Keppler OT. The Nef protein of human immunodeficiency virus is a broad-spectrum modulator of chemokine receptor cell surface levels that acts independently of classical motifs for receptor endocytosis and Galpha_i signaling. *Mol Biol Cell* 2006 Aug;17(8):3578-90.

- [149] Lundquist CA, Tobiume M, Zhou J, Unutmaz D, Aiken C. Nef-mediated downregulation of CD4 enhances human immunodeficiency virus type 1 replication in primary T lymphocytes. *J Virol* 2002 May;76(9):4625-33.
- [150] Lu X, Yu H, Liu SH, Brodsky FM, Peterlin BM. Interactions between HIV1 Nef and vacuolar ATPase facilitate the internalization of CD4. *Immunity* 1998 May;8(5):647-56.
- [151] Greenberg ME, Iafrate AJ, Skowronski J. The SH3 domain-binding surface and an acidic motif in HIV-1 Nef regulate trafficking of class I MHC complexes. *EMBO J* 1998 May 15;17(10):2777-89.
- [152] Le GS, Buseyne F, Trocha A, Walker BD, Heard JM, Schwartz O. Distinct trafficking pathways mediate Nef-induced and clathrin-dependent major histocompatibility complex class I down-regulation. *J Virol* 2000 Oct;74(19):9256-66.
- [153] Blagoveshchenskaya AD, Thomas L, Feliciangeli SF, Hung CH, Thomas G. HIV-1 Nef downregulates MHC-I by a PACS-1- and PI3K-regulated ARF6 endocytic pathway. *Cell* 2002 Dec 13;111(6):853-66.
- [154] Chen N, McCarthy C, Drakesmith H, et al. HIV-1 down-regulates the expression of CD1d via Nef. *Eur J Immunol* 2006 Feb;36(2):278-86.
- [155] Smith BL, Krushelnycky BW, Mochly-Rosen D, Berg P. The HIV nef protein associates with protein kinase C theta. *J Biol Chem* 1996 Jul 12;271(28):16753-7.
- [156] Robichaud GA, Poulin L. HIV type 1 nef gene inhibits tumor necrosis factor alpha-induced apoptosis and promotes cell proliferation through the action of MAPK and JNK in human glial cells. *AIDS Res Hum Retroviruses* 2000 Dec 10;16(18):1959-65.
- [157] Piguet V, Trono D. The Nef protein of primate lentiviruses. *Rev Med Virol* 1999 Apr;9(2):111-20.
- [158] Balestra E, Perno CF, Aquaro S, et al. Macrophages: a crucial reservoir for human immunodeficiency virus in the body. *J Biol Regul Homeost Agents* 2001 Jul;15(3):272-6.
- [159] Swingle S, Mann A, Jacque J, et al. HIV-1 Nef mediates lymphocyte chemotaxis and activation by infected macrophages. *Nat Med* 1999 Sep;5(9):997-103.
- [160] Martin JC, Bandres JC. Cells of the monocyte-macrophage lineage and pathogenesis of HIV-1 infection. *J Acquir Immune Defic Syndr* 1999 Dec 15;22(5):413-29.
- [161] Komuro I, Yokota Y, Yasuda S, Iwamoto A, Kagawa KS. CSF-induced and HIV-1-mediated distinct regulation of Hck and C/EBPbeta represent a heterogeneous susceptibility of monocyte-derived macrophages to M-tropic HIV-1 infection. *J Exp Med* 2003 Aug 4;198(3):443-53.
- [162] Hanna Z, Weng X, Kay DG, Poudrier J, Lowell C, Jolicoeur P. The pathogenicity of human immunodeficiency virus (HIV) type 1 Nef in CD4C/HIV transgenic mice is

abolished by mutation of its SH3-binding domain, and disease development is delayed in the absence of Hck. *J Virol* 2001 Oct;75(19):9378-92.

- [163] Biggs BA, Hewish M, Kent S, Hayes K, Crowe SM. HIV-1 infection of human macrophages impairs phagocytosis and killing of *Toxoplasma gondii*. *J Immunol* 1995 Jun 1;154(11):6132-9.
- [164] De SK, Venkateshan CN, Seth P, Gajdusek DC, Gibbs CJ. Adenovirus-mediated human immunodeficiency virus-1 Nef expression in human monocytes/macrophages and effect of Nef on downmodulation of Fcγ receptors and expression of monokines. *Blood* 1998 Mar 15;91(6):2108-17.
- [165] Olivetta E, Percario Z, Fiorucci G, et al. HIV-1 Nef induces the release of inflammatory factors from human monocyte/macrophages: involvement of Nef endocytotic signals and NF-κB activation. *J Immunol* 2003 Feb 15;170(4):1716-27.
- [166] Schindler M, Munch J, Kutsch O, et al. Nef-mediated suppression of T cell activation was lost in a lentiviral lineage that gave rise to HIV-1. *Cell* 2006 Jun 16;125(6):1055-67.
- [167] Bell I, Ashman C, Maughan J, Hooker E, Cook F, Reinhart TA. Association of simian immunodeficiency virus Nef with the T-cell receptor (TCR) zeta chain leads to TCR down-modulation. *J Gen Virol* 1998 Nov;79 (Pt 11):2717-27.
- [168] Swigut T, Shohdy N, Skowronski J. Mechanism for down-regulation of CD28 by Nef. *EMBO J* 2001 Apr 2;20(7):1593-604.
- [169] Niederman TM, Hastings WR, Luria S, Bandres JC, Ratner L. HIV-1 Nef protein inhibits the recruitment of AP-1 DNA-binding activity in human T-cells. *Virology* 1993 May;194(1):338-44.
- [170] Masood R, Lunardi-Iskandar Y, Moudgil T, et al. IL-10 inhibits HIV-1 replication and is induced by tat. *Biochem Biophys Res Commun* 1994 Jul 15;202(1):374-83.
- [171] Chirmule N, Oyaizu N, Saxinger C, Pahwa S. Nef protein of HIV-1 has B-cell stimulatory activity. *AIDS* 1994 Jun;8(6):733-4.
- [172] Pugliese A, Vidotto V, Beltramo T, Torre D. Regulation of interleukin-18 by THP-1 monocytoïd cells stimulated with HIV-1 and Nef viral protein. *Eur Cytokine Netw* 2005 Sep;16(3):186-90.
- [173] Huang MB, Jin LL, James CO, Khan M, Powell MD, Bond VC. Characterization of Nef-CXCR4 interactions important for apoptosis induction. *J Virol* 2004 Oct;78(20):11084-96.
- [174] Kestler HW, III, Ringler DJ, Mori K, et al. Importance of the nef gene for maintenance of high virus loads and for development of AIDS. *Cell* 1991 May 17;65(4):651-62.

- [175] Deacon NJ, Tsykin A, Solomon A, et al. Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients. *Science* 1995 Nov 10;270(5238):988-91.
- [176] Casartelli N, Di MG, Argentini C, et al. Structural defects and variations in the HIV-1 nef gene from rapid, slow and non-progressor children. *AIDS* 2003 Jun 13;17(9):1291-301.
- [177] Carl S, Greenough TC, Krumbiegel M, et al. Modulation of different human immunodeficiency virus type 1 Nef functions during progression to AIDS. *J Virol* 2001 Apr;75(8):3657-65.
- [178] Olivetta E, Federico M. HIV-1 Nef protects human-monocyte-derived macrophages from HIV-1-induced apoptosis. *Exp Cell Res* 2006 Apr 1;312(6):890-900.
- [179] Sol-Foulon N, Moris A, Nobile C, et al. HIV-1 Nef-induced upregulation of DC-SIGN in dendritic cells promotes lymphocyte clustering and viral spread. *Immunity* 2002 Jan;16(1):145-55.
- [180] Zheng YH, Plemenitas A, Fielding CJ, Peterlin BM. Nef increases the synthesis of and transports cholesterol to lipid rafts and HIV-1 progeny virions. *Proc Natl Acad Sci U S A* 2003 Jul 8;100(14):8460-5.
- [181] Khan M, Garcia-Barrio M, Powell MD. Restoration of wild-type infectivity to human immunodeficiency virus type 1 strains lacking nef by intravirion reverse transcription. *J Virol* 2001 Dec;75(24):12081-7.
- [182] Joseph AM, Ladha JS, Mojamdar M, Mitra D. Human immunodeficiency virus-1 Nef protein interacts with Tat and enhances HIV-1 gene expression. *FEBS Lett* 2003 Jul 31;548(1-3):37-42.
- [183] Le GS, Erdtmann L, Benichou S, et al. Nef interacts with the mu subunit of clathrin adaptor complexes and reveals a cryptic sorting signal in MHC I molecules. *Immunity* 1998 Apr;8(4):483-95.
- [184] Ross TM, Oran AE, Cullen BR. Inhibition of HIV-1 progeny virion release by cell-surface CD4 is relieved by expression of the viral Nef protein. *Curr Biol* 1999 Jun 17;9(12):613-21.
- [185] Quaranta MG, Tritarelli E, Giordani L, Viora M. HIV-1 Nef induces dendritic cell differentiation: a possible mechanism of uninfected CD4(+) T cell activation. *Exp Cell Res* 2002 May 1;275(2):243-54.
- [186] Xu XN, Screaton GR, McMichael AJ. Virus infections: escape, resistance, and counterattack. *Immunity* 2001 Dec;15(6):867-70.
- [187] Collins KL, Chen BK, Kalams SA, Walker BD, Baltimore D. HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocytes. *Nature* 1998 Jan 22;391(6665):397-401.

- [188] Cohen GB, Gandhi RT, Davis DM, et al. The selective downregulation of class I major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells. *Immunity* 1999 Jun;10(6):661-71.
- [189] Muthumani K, Choo AY, Hwang DS, et al. HIV-1 Nef-induced FasL induction and bystander killing requires p38 MAPK activation. *Blood* 2005 Sep 15;106(6):2059-68.
- [190] Geleziunas R, Xu W, Takeda K, Ichijo H, Greene WC. HIV-1 Nef inhibits ASK1-dependent death signalling providing a potential mechanism for protecting the infected host cell. *Nature* 2001 Apr 12;410(6830):834-8.
- [191] Wolf D, Witte V, Laffert B, et al. HIV-1 Nef associated PAK and PI3-kinases stimulate Akt-independent Bad-phosphorylation to induce anti-apoptotic signals. *Nat Med* 2001 Nov;7(11):1217-24.
- [192] Fackler OT, Baur AS. Live and let die: Nef functions beyond HIV replication. *Immunity* 2002 Apr;16(4):493-7.
- [193] Van PL, Biuckians A, Abbas AK. Functional roles of Fas and Bcl-2-regulated apoptosis of T lymphocytes. *J Immunol* 1998 Mar 1;160(5):2065-71.
- [194] Bastian NR, Hibbs JB, Jr. Assembly and regulation of NADPH oxidase and nitric oxide synthase. *Curr Opin Immunol* 1994 Feb;6(1):131-9.
- [195] Stefanova I, Horejsi V, Ansotegui IJ, Knapp W, Stockinger H. GPI-anchored cell-surface molecules complexed to protein tyrosine kinases. *Science* 1991 Nov 15;254(5034):1016-9.
- [196] Shimazu R, Akashi S, Ogata H, et al. MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *J Exp Med* 1999 Jun 7;189(11):1777-82.
- [197] Poltorak A, He X, Smirnova I, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 1998 Dec 11;282(5396):2085-8.
- [198] Sweet MJ, Hume DA. Endotoxin signal transduction in macrophages. *J Leukoc Biol* 1996 Jul;60(1):8-26.
- [199] Ray LB, Sturgill TW. Insulin-stimulated microtubule-associated protein kinase is phosphorylated on tyrosine and threonine in vivo. *Proc Natl Acad Sci U S A* 1988 Jun;85(11):3753-7.
- [200] Boulton TG, Cobb MH. Identification of multiple extracellular signal-regulated kinases (ERKs) with antipeptide antibodies. *Cell Regul* 1991 May;2(5):357-71.
- [201] Kyriakis JM, Banerjee P, Nikolakaki E, et al. The stress-activated protein kinase subfamily of c-Jun kinases. *Nature* 1994 May 12;369(6476):156-60.
- [202] Han J, Richter B, Li Z, Kravchenko V, Ulevitch RJ. Molecular cloning of human p38 MAP kinase. *Biochim Biophys Acta* 1995 Mar 16;1265(2-3):224-7.

- [203] Lu HT, Yang DD, Wysk M, et al. Defective IL-12 production in mitogen-activated protein (MAP) kinase kinase 3 (Mkk3)-deficient mice. *EMBO J* 1999 Apr 1;18(7):1845-57.
- [204] Guha M, Mackman N. LPS induction of gene expression in human monocytes. *Cell Signal* 2001 Feb;13(2):85-94.
- [205] Burgering BM, Bos JL. Regulation of Ras-mediated signalling: more than one way to skin a cat. *Trends Biochem Sci* 1995 Jan;20(1):18-22.
- [206] Zhou G, Bao ZQ, Dixon JE. Components of a new human protein kinase signal transduction pathway. *J Biol Chem* 1995 May 26;270(21):12665-9.
- [207] Saito S, Matsuura M, Hirai Y. Regulation of lipopolysaccharide-induced interleukin-12 production by activation of repressor element GA-12 through hyperactivation of the ERK pathway. *Clin Vaccine Immunol* 2006 Aug;13(8):876-83.
- [208] Hale KK, Trollinger D, Rihaneck M, Manthey CL. Differential expression and activation of p38 mitogen-activated protein kinase alpha, beta, gamma, and delta in inflammatory cell lineages. *J Immunol* 1999 Apr 1;162(7):4246-52.
- [209] Ono K, Han J. The p38 signal transduction pathway: activation and function. *Cell Signal* 2000 Jan;12(1):1-13.
- [210] Derijard B, Raingeaud J, Barrett T, et al. Independent human MAP-kinase signal transduction pathways defined by MEK and MKK isoforms. *Science* 1995 Feb 3;267(5198):682-5.
- [211] Chen Z, Gibson TB, Robinson F, et al. MAP kinases. *Chem Rev* 2001 Aug;101(8):2449-76.
- [212] Ashwell JD. The many paths to p38 mitogen-activated protein kinase activation in the immune system. *Nat Rev Immunol* 2006 Jul;6(7):532-40.
- [213] Enslen H, Raingeaud J, Davis RJ. Selective activation of p38 mitogen-activated protein (MAP) kinase isoforms by the MAP kinase kinases MKK3 and MKK6. *J Biol Chem* 1998 Jan 16;273(3):1741-8.
- [214] Liu ZG, Hsu H, Goeddel DV, Karin M. Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-kappaB activation prevents cell death. *Cell* 1996 Nov 1;87(3):565-76.
- [215] Derijard B, Hibi M, Wu IH, et al. JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell* 1994 Mar 25;76(6):1025-37.
- [216] Gupta S, Barrett T, Whitmarsh AJ, et al. Selective interaction of JNK protein kinase isoforms with transcription factors. *EMBO J* 1996 Jun 3;15(11):2760-70.
- [217] Tournier C, Whitmarsh AJ, Cavanagh J, Barrett T, Davis RJ. The MKK7 gene encodes a group of c-Jun NH2-terminal kinase kinases. *Mol Cell Biol* 1999 Feb;19(2):1569-81.

- [218] Whitmarsh AJ, Yang SH, Su MS, Sharrocks AD, Davis RJ. Role of p38 and JNK mitogen-activated protein kinases in the activation of ternary complex factors. *Mol Cell Biol* 1997 May;17(5):2360-71.
- [219] Clapham DE. Calcium signaling. *Cell* 1995 Jan 27;80(2):259-68.
- [220] Cuzalegui FH, Bading H. Calcium-regulated protein kinase cascades and their transcription factor targets. *Cell Mol Life Sci* 2000 Mar;57(3):402-10.
- [221] Nguyen A, Chen P, Cai H. Role of CaMKII in hydrogen peroxide activation of ERK1/2, p38 MAPK, HSP27 and actin reorganization in endothelial cells. *FEBS Lett* 2004 Aug 13;572(1-3):307-13.
- [222] Yao WD, Wu CF. Distinct roles of CaMKII and PKA in regulation of firing patterns and K(+) currents in *Drosophila* neurons. *J Neurophysiol* 2001 Apr;85(4):1384-94.
- [223] Kim J, Sharma RP. Calcium-mediated activation of c-Jun NH2-terminal kinase (JNK) and apoptosis in response to cadmium in murine macrophages. *Toxicol Sci* 2004 Oct;81(2):518-27.
- [224] Xie QW, Kashiwabara Y, Nathan C. Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase. *J Biol Chem* 1994 Feb 18;269(7):4705-8.
- [225] Wymann MP, Pirola L. Structure and function of phosphoinositide 3-kinases. *Biochim Biophys Acta* 1998 Dec 8;1436(1-2):127-50.
- [226] Vanhaesebroeck B, Higashi K, Raven C, et al. Autophosphorylation of p110delta phosphoinositide 3-kinase: a new paradigm for the regulation of lipid kinases in vitro and in vivo. *EMBO J* 1999 Mar 1;18(5):1292-302.
- [227] Fruman DA, Meyers RE, Cantley LC. Phosphoinositide kinases. *Annu Rev Biochem* 1998;67:481-507.
- [228] Rodriguez-Viciana P, Warne PH, Dhand R, et al. Phosphatidylinositol-3-OH kinase as a direct target of Ras. *Nature* 1994 Aug 18;370(6490):527-32.
- [229] Copeland KF. Modulation of HIV-1 transcription by cytokines and chemokines. *Mini Rev Med Chem* 2005 Dec;5(12):1093-101.
- [230] Hatada EN, Krappmann D, Scheidereit C. NF-kappaB and the innate immune response. *Curr Opin Immunol* 2000 Feb;12(1):52-8.
- [231] Bonizzi G, Karin M. The two NF-kappaB activation pathways and their role in innate and adaptive immunity. *Trends Immunol* 2004 Jun;25(6):280-8.
- [232] Spiecker M, Darius H, Liao JK. A functional role of I kappa B-epsilon in endothelial cell activation. *J Immunol* 2000 Mar 15;164(6):3316-22.
- [233] Zandi E, Chen Y, Karin M. Direct phosphorylation of IkappaB by IKKalpha and IKKbeta: discrimination between free and NF-kappaB-bound substrate. *Science* 1998 Aug 28;281(5381):1360-3.

- [234] Mechta-Grigoriou F, Gerald D, Yaniv M. The mammalian Jun proteins: redundancy and specificity. *Oncogene* 2001 Apr 30;20(19):2378-89.
- [235] Chang YH, Hsieh SL, Chen MC, Lin WW. Lymphotoxin beta receptor induces interleukin 8 gene expression via NF-kappaB and AP-1 activation. *Exp Cell Res* 2002 Aug 15;278(2):166-74.
- [236] Hilberg F, Aguzzi A, Howells N, Wagner EF. c-jun is essential for normal mouse development and hepatogenesis. *Nature* 1993 Sep 9;365(6442):179-81.
- [237] Matsuo K, Owens JM, Tonko M, Elliott C, Chambers TJ, Wagner EF. Fosl1 is a transcriptional target of c-Fos during osteoclast differentiation. *Nat Genet* 2000 Feb;24(2):184-7.
- [238] Thepot D, Weitzman JB, Barra J, et al. Targeted disruption of the murine junD gene results in multiple defects in male reproductive function. *Development* 2000 Jan;127(1):143-53.
- [239] Fernandez-Lago L, Rodriguez-Tarazona E, Vizcaino N. Differential secretion of interleukin-12 (IL-12) subunits and heterodimeric IL-12p70 protein by CD-1 mice and murine macrophages in response to intracellular infection by *Brucella abortus*. *J Med Microbiol* 1999 Dec;48(12):1065-73.
- [240] Lewis TS, Shapiro PS, Ahn NG. Signal transduction through MAP kinase cascades. *Adv Cancer Res* 1998;74:49-139.
- [241] Bilancio A, Okkenhaug K, Camps M, et al. Key role of the p110delta isoform of PI3K in B-cell antigen and IL-4 receptor signaling: comparative analysis of genetic and pharmacologic interference with p110delta function in B cells. *Blood* 2006 Jan 15;107(2):642-50.
- [242] Ma X, Chow JM, Gri G, et al. The interleukin 12 p40 gene promoter is primed by interferon gamma in monocytic cells. *J Exp Med* 1996 Jan 1;183(1):147-57.
- [243] Wilckens T, De RR. Glucocorticoids and immune function: unknown dimensions and new frontiers. *Immunol Today* 1997 Sep;18(9):418-24.
- [244] Jiang H, Wynn C, Pan F, Ebbs A, Erickson LM, Kobayashi M. Tacrolimus and cyclosporine differ in their capacity to overcome ongoing allograft rejection as a result of their differential abilities to inhibit interleukin-10 production. *Transplantation* 2002 Jun 15;73(11):1808-17.
- [245] Franchimont D, Galon J, Gadina M, et al. Inhibition of Th1 immune response by glucocorticoids: dexamethasone selectively inhibits IL-12-induced Stat4 phosphorylation in T lymphocytes. *J Immunol* 2000 Feb 15;164(4):1768-74.
- [246] Hirasawa N, Sato Y, Fujita Y, Mue S, Ohuchi K. Inhibition by dexamethasone of antigen-induced c-Jun N-terminal kinase activation in rat basophilic leukemia cells. *J Immunol* 1998 Nov 1;161(9):4939-43.

- [247] Shalom-Barak T, Quach J, Lotz M. Interleukin-17-induced gene expression in articular chondrocytes is associated with activation of mitogen-activated protein kinases and NF-kappaB. *J Biol Chem* 1998 Oct 16;273(42):27467-73.
- [248] Jonat C, Rahmsdorf HJ, Park KK, et al. Antitumor promotion and antiinflammation: down-modulation of AP-1 (Fos/Jun) activity by glucocorticoid hormone. *Cell* 1990 Sep 21;62(6):1189-204.
- [249] Auphan N, DiDonato JA, Rosette C, Helmberg A, Karin M. Immunosuppression by glucocorticoids: inhibition of NF-kappa B activity through induction of I kappa B synthesis. *Science* 1995 Oct 13;270(5234):286-90.
- [250] Bennett BL, Sasaki DT, Murray BW, et al. SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. *Proc Natl Acad Sci U S A* 2001 Nov 20;98(24):13681-6.
- [251] Sauma D, Fierro A, Mora JR, et al. Cyclosporine preconditions dendritic cells during differentiation and reduces IL-2 and IL-12 production following activation: a potential tolerogenic effect. *Transplant Proc* 2003 Nov;35(7):2515-7.
- [252] Hariharan S, Johnson CP, Bresnahan BA, Taranto SE, McIntosh MJ, Stablein D. Improved graft survival after renal transplantation in the United States, 1988 to 1996. *N Engl J Med* 2000 Mar 2;342(9):605-12.
- [253] Schreiber SL, Crabtree GR. The mechanism of action of cyclosporin A and FK506. *Immunol Today* 1992 Apr;13(4):136-42.
- [254] Fukao T, Koyasu S. PI3K and negative regulation of TLR signaling. *Trends Immunol* 2003 Jul;24(7):358-63.
- [255] la SA, Gadina M, Kelsall BL. G(i)-protein-dependent inhibition of IL-12 production is mediated by activation of the phosphatidylinositol 3-kinase-protein 3 kinase B/Akt pathway and JNK. *J Immunol* 2005 Sep 1;175(5):2994-9.
- [256] Ma W, Mishra S, Gee K, et al. Cyclosporin A and FK506 Inhibit IL-12p40 Production through the Calmodulin/Calmodulin-dependent Protein Kinase-activated Phosphoinositide 3-Kinase in Lipopolysaccharide-stimulated Human Monocytic Cells. *J Biol Chem* 2007 May 4;282(18):13351-62.
- [257] Lim W, Ma W, Gee K, et al. Distinct role of p38 and c-Jun N-terminal kinases in IL-10-dependent and IL-10-independent regulation of the costimulatory molecule B7.2 in lipopolysaccharide-stimulated human monocytic cells. *J Immunol* 2002 Feb 15;168(4):1759-69.
- [258] Jonat C, Rahmsdorf HJ, Park KK, et al. Antitumor promotion and antiinflammation: down-modulation of AP-1 (Fos/Jun) activity by glucocorticoid hormone. *Cell* 1990 Sep 21;62(6):1189-204.
- [259] Gee K, Kozlowski M, Kumar A. Tumor necrosis factor-alpha induces functionally active hyaluronan-adhesive CD44 by activating sialidase through p38 mitogen-activated protein kinase in lipopolysaccharide-stimulated human monocytic cells. *J Biol Chem* 2003 Sep 26;278(39):37275-87.

- [260] Berridge MJ, Bootman MD, Roderick HL. Calcium signalling: dynamics, homeostasis and remodelling. *Nat Rev Mol Cell Biol* 2003 Jul;4(7):517-29.
- [261] Kasri NN, Holmes AM, Bultynck G, et al. Regulation of InsP3 receptor activity by neuronal Ca²⁺-binding proteins. *EMBO J* 2004 Jan 28;23(2):312-21.
- [262] Merritt JE, Armstrong WP, Benham CD, et al. SK&F 96365, a novel inhibitor of receptor-mediated calcium entry. *Biochem J* 1990 Oct 15;271(2):515-22.
- [263] Bootman MD, Collins TJ, Mackenzie L, Roderick HL, Berridge MJ, Peppiatt CM. 2-aminoethoxydiphenyl borate (2-APB) is a reliable blocker of store-operated Ca²⁺ entry but an inconsistent inhibitor of InsP3-induced Ca²⁺ release. *FASEB J* 2002 Aug;16(10):1145-50.
- [264] Kasri NN, Parys JB, Callewaert G, Missiaen L, De SH. Calmodulin and calcium-release channels. *Biol Res* 2004;37(4):577-82.
- [265] Stull JT. Ca²⁺-dependent cell signaling through calmodulin-activated protein phosphatase and protein kinases minireview series. *J Biol Chem* 2001 Jan 26;276(4):2311-2.
- [266] Lilienbaum A, Israel A. From calcium to NF-kappa B signaling pathways in neurons. *Mol Cell Biol* 2003 Apr;23(8):2680-98.
- [267] Mishra S, Mishra JP, Gee K, McManus DC, Lacasse EC, Kumar A. Distinct role of calmodulin and calmodulin-dependent protein kinase-II in lipopolysaccharide and tumor necrosis factor-alpha -mediated suppression of apoptosis and antiapoptotic c-IAP2 gene expression in human monocytic cells. *J Biol Chem* 2005 Sep 9;
- [268] Braun AP, Schulman H. The multifunctional calcium/calmodulin-dependent protein kinase: from form to function. *Annu Rev Physiol* 1995;57:417-45.:417-45.
- [269] Tombes RM, Faison MO, Turbeville JM. Organization and evolution of multifunctional Ca(2+)/CaM-dependent protein kinase genes. *Gene* 2003 Dec 11;322:17-31.:17-31.
- [270] Vanhaesebroeck B, Leevers SJ, Ahmadi K, et al. Synthesis and function of 3-phosphorylated inositol lipids. *Annu Rev Biochem* 2001;70:535-602.:535-602.
- [271] Vanhaesebroeck B, Jones GE, Allen WE, et al. Distinct PI(3)Ks mediate mitogenic signalling and cell migration in macrophages. *Nat Cell Biol* 1999 May;1(1):69-71.
- [272] Siddhanta U, McIlroy J, Shah A, Zhang Y, Backer JM. Distinct roles for the p110alpha and hVPS34 phosphatidylinositol 3'-kinases in vesicular trafficking, regulation of the actin cytoskeleton, and mitogenesis. *J Cell Biol* 1998 Dec 14;143(6):1647-59.
- [273] Lee JS, Scott CB, Komaki R, Ettinger DS, Sause WT. Impact of institutional experience on survival outcome of patients undergoing combined chemoradiation therapy for inoperable non-small-cell lung cancer. *Int J Radiat Oncol Biol Phys* 2002 Feb 1;52(2):362-70.

- [274] Wilckens T, De RR. Glucocorticoids and immune function: unknown dimensions and new frontiers. *Immunol Today* 1997 Sep;18(9):418-24.
- [275] Rommel C, Clarke BA, Zimmermann S, et al. Differentiation stage-specific inhibition of the Raf-MEK-ERK pathway by Akt. *Science* 1999 Nov 26;286(5445):1738-41.
- [276] Park HS, Kim MS, Huh SH, et al. Akt (protein kinase B) negatively regulates SEK1 by means of protein phosphorylation. *J Biol Chem* 2002 Jan 25;277(4):2573-8.
- [277] Blotta MH, DeKruyff RH, Umetsu DT. Corticosteroids inhibit IL-12 production in human monocytes and enhance their capacity to induce IL-4 synthesis in CD4+ lymphocytes. *J Immunol* 1997 Jun 15;158(12):5589-95.
- [278] DeKruyff RH, Fang Y, Umetsu DT. Corticosteroids enhance the capacity of macrophages to induce Th2 cytokine synthesis in CD4+ lymphocytes by inhibiting IL-12 production. *J Immunol* 1998 Mar 1;160(5):2231-7.
- [279] de Waal MR, Abrams J, Bennett B, Figdor CG, de Vries JE. Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J Exp Med* 1991 Nov 1;174(5):1209-20.
- [280] Zhu C, Gagnidze K, Gemberling JH, Plevy SE. Characterization of an activation protein-1-binding site in the murine interleukin-12 p40 promoter. Demonstration of novel functional elements by a reductionist approach 1. *J Biol Chem* 2001 May 25;276(21):18519-28.
- [281] Mechta-Grigoriou F, Gerald D, Yaniv M. The mammalian Jun proteins: redundancy and specificity. *Oncogene* 2001 Apr 30;20(19):2378-89.
- [282] Chang YH, Hsieh SL, Chen MC, Lin WW. Lymphotoxin beta receptor induces interleukin 8 gene expression via NF-kappaB and AP-1 activation. *Exp Cell Res* 2002 Aug 15;278(2):166-74.
- [283] Rooney JW, Hoey T, Glimcher LH. Coordinate and cooperative roles for NF-AT and AP-1 in the regulation of the murine IL-4 gene. *Immunity* 1995 May;2(5):473-83.
- [284] Wang CY, Bassuk AG, Boise LH, Thompson CB, Bravo R, Leiden JM. Activation of the granulocyte-macrophage colony-stimulating factor promoter in T cells requires cooperative binding of Elf-1 and AP-1 transcription factors. *Mol Cell Biol* 1994 Feb;14(2):1153-9.
- [285] Yao J, Mackman N, Edgington TS, Fan ST. Lipopolysaccharide induction of the tumor necrosis factor-alpha promoter in human monocytic cells. Regulation by Egr-1, c-Jun, and NF-kappaB transcription factors. *J Biol Chem* 1997 Jul 11;272(28):17795-801.
- [286] Zanke BW, Boudreau K, Rubie E, et al. The stress-activated protein kinase pathway mediates cell death following injury induced by cis-platinum, UV irradiation or heat. *Curr Biol* 1996 May 1;6(5):606-13.

- [287] Whitmarsh AJ, Shore P, Sharrocks AD, Davis RJ. Integration of MAP kinase signal transduction pathways at the serum response element. *Science* 1995 Jul 21;269(5222):403-7.
- [288] Chang NS, Schultz L, Heath J. Suppression of IkappaBalpha expression is necessary for c-Jun N-terminal kinase-mediated enhancement of Fas cytotoxicity. *Biochem Biophys Res Commun* 2000 Jul 21;274(1):4-10.
- [289] Grazia CM, Sutterwala FS, Trinchieri G, Mosser DM, Ma X. Suppression of Il-12 transcription in macrophages following Fc gamma receptor ligation. *J Immunol* 2001 Apr 1;166(7):4498-506.
- [290] Sutterwala FS, Noel GJ, Clynes R, Mosser DM. Selective suppression of interleukin-12 induction after macrophage receptor ligation. *J Exp Med* 1997 Jun 2;185(11):1977-85.
- [291] Kim Y, Moon JS, Lee KS, et al. Ca²⁺/calmodulin-dependent protein phosphatase calcineurin mediates the expression of iNOS through IKK and NF-kappaB activity in LPS-stimulated mouse peritoneal macrophages and RAW 264.7 cells. *Biochem Biophys Res Commun* 2004 Feb 13;314(3):695-703.
- [292] Hong SY, Yoon WH, Park JH, Kang SG, Ahn JH, Lee TH. Involvement of two NF-kappa B binding elements in tumor necrosis factor alpha -, CD40-, and epstein-barr virus latent membrane protein 1-mediated induction of the cellular inhibitor of apoptosis protein 2 gene. *J Biol Chem* 2000 Jun 16;275(24):18022-8.
- [293] Engelman JA, Luo J, Cantley LC. The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. *Nat Rev Genet* 2006 Aug;7(8):606-19.
- [294] Waggoner SN, Cruise MW, Kassel R, Hahn YS. gC1q receptor ligation selectively down-regulates human IL-12 production through activation of the phosphoinositide 3-kinase pathway. *J Immunol* 2005 Oct 1;175(7):4706-14.
- [295] Hawlisch H, Belkaid Y, Baelder R, Hildeman D, Gerard C, Kohl J. C5a negatively regulates toll-like receptor 4-induced immune responses. *Immunity* 2005 Apr;22(4):415-26.
- [296] Domin J, Pages F, Volinia S, et al. Cloning of a human phosphoinositide 3-kinase with a C2 domain that displays reduced sensitivity to the inhibitor wortmannin. *Biochem J* 1997 Aug 15;326 (Pt 1):139-47.
- [297] Bi L, Okabe I, Bernard DJ, Nussbaum RL. Early embryonic lethality in mice deficient in the p110beta catalytic subunit of PI 3-kinase. *Mamm Genome* 2002 Mar;13(3):169-72.
- [298] Luo J, Cantley LC. The negative regulation of phosphoinositide 3-kinase signaling by p85 and its implication in cancer. *Cell Cycle* 2005 Oct;4(10):1309-12.
- [299] Lee JS, Hmama Z, Mui A, Reiner NE. Stable gene silencing in human monocytic cell lines using lentiviral-delivered small interference RNA. Silencing of the p110alpha isoform of phosphoinositide 3-kinase reveals differential regulation of adherence

induced by 1 α ,25-dihydroxycholecalciferol and bacterial lipopolysaccharide. *J Biol Chem* 2004 Mar 5;279(10):9379-88.

- [300] Saha A, Wittmeyer J, Cairns BR. Chromatin remodelling: the industrial revolution of DNA around histones. *Nat Rev Mol Cell Biol* 2006 Jun;7(6):437-47.
- [301] Hughes K, Edin S, Antonsson A, Grundstrom T. Calmodulin-dependent kinase II mediates T cell receptor/CD3- and phorbol ester-induced activation of I κ B kinase. *J Biol Chem* 2001 Sep 21;276(38):36008-13.
- [302] Ho N, Gullberg M, Chatila T. Activation protein 1-dependent transcriptional activation of interleukin 2 gene by Ca²⁺/calmodulin kinase type IV/Gr. *J Exp Med* 1996 Jul 1;184(1):101-12.
- [303] Gubler U, Chua AO, Schoenhaut DS, et al. Coexpression of two distinct genes is required to generate secreted bioactive cytotoxic lymphocyte maturation factor. *Proc Natl Acad Sci U S A* 1991 May 15;88(10):4143-7.
- [304] Chambers KA, Parks RJ, Angel JB. Disruption of MAP kinase activation and nuclear factor binding to the IL-12 p40 promoter in HIV-infected myeloid cells. *Clin Exp Immunol* 2004 Aug;137(2):329-40.
- [305] Angel JB, Kumar A, Parato K, et al. Improvement in cell-mediated immune function during potent anti-human immunodeficiency virus therapy with ritonavir plus saquinavir. *J Infect Dis* 1998 Apr;177(4):898-904.
- [306] Clerici M, Lucey DR, Berzofsky JA, et al. Restoration of HIV-specific cell-mediated immune responses by interleukin-12 in vitro. *Science* 1993 Dec 10;262(5140):1721-4.
- [307] Dybul M, Mercier G, Belson M, et al. CD40 ligand trimer and IL-12 enhance peripheral blood mononuclear cells and CD4⁺ T cell proliferation and production of IFN- γ in response to p24 antigen in HIV-infected individuals: potential contribution of anergy to HIV-specific unresponsiveness. *J Immunol* 2000 Aug 1;165(3):1685-91.
- [308] Utsugi M, Dobashi K, Ishizuka T, et al. c-Jun N-terminal kinase negatively regulates lipopolysaccharide-induced IL-12 production in human macrophages: role of mitogen-activated protein kinase in glutathione redox regulation of IL-12 production. *J Immunol* 2003 Jul 15;171(2):628-35.
- [309] Niederman TM, Hastings WR, Luria S, Bandres JC, Ratner L. HIV-1 Nef protein inhibits the recruitment of AP-1 DNA-binding activity in human T-cells. *Virology* 1993 May;194(1):338-44.
- [310] Mirani M, Elenkov I, Volpi S, Hiroi N, Chrousos GP, Kino T. HIV-1 protein Vpr suppresses IL-12 production from human monocytes by enhancing glucocorticoid action: potential implications of Vpr coactivator activity for the innate and cellular immunity deficits observed in HIV-1 infection. *J Immunol* 2002 Dec 1;169(11):6361-8.

- [311] Ito M, Ishida T, He L, et al. HIV type 1 Tat protein inhibits interleukin 12 production by human peripheral blood mononuclear cells. *AIDS Res Hum Retroviruses* 1998 Jul 1;14(10):845-9.
- [312] Quaranta MG, Mattioli B, Spadaro F, et al. HIV-1 Nef triggers Vav-mediated signaling pathway leading to functional and morphological differentiation of dendritic cells. *FASEB J* 2003 Nov;17(14):2025-36.
- [313] Kuwata T, Miura T, Haga T, Kozyrev I, Hayami M. Construction of chimeric simian and human immunodeficiency viruses that produce interleukin 12. *AIDS Res Hum Retroviruses* 2000 Mar 20;16(5):465-70.
- [314] Guha M, Mackman N. LPS induction of gene expression in human monocytes. *Cell Signal* 2001 Feb;13(2):85-94.
- [315] Pugliese A, Vidotto V, Beltramo T, Torre D. Regulation of interleukin-18 by THP-1 monocytoïd cells stimulated with HIV-1 and Nef viral protein. *Eur Cytokine Netw* 2005 Sep;16(3):186-90.
- [316] Swantek JL, Cobb MH, Geppert TD. Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) is required for lipopolysaccharide stimulation of tumor necrosis factor alpha (TNF-alpha) translation: glucocorticoids inhibit TNF-alpha translation by blocking JNK/SAPK. *Mol Cell Biol* 1997 Nov;17(11):6274-82.
- [317] Pruett SB, Zheng Q, Schwab C, Fan R. Sodium methylthiocarbamate inhibits MAP kinase activation through toll-like receptor 4, alters cytokine production by mouse peritoneal macrophages, and suppresses innate immunity. *Toxicol Sci* 2005 Sep;87(1):75-85.
- [318] Yang DD, Conze D, Whitmarsh AJ, et al. Differentiation of CD4+ T cells to Th1 cells requires MAP kinase JNK2. *Immunity* 1998 Oct;9(4):575-85.
- [319] Constant SL, Dong C, Yang DD, Wysk M, Davis RJ, Flavell RA. JNK1 is required for T cell-mediated immunity against *Leishmania major* infection. *J Immunol* 2000 Sep 1;165(5):2671-6.
- [320] Santos-Sierra S, Golenbock DT, Henneke P. Toll-like receptor-dependent discrimination of streptococci. *J Endotoxin Res* 2006;12(5):307-12.
- [321] Ju KD, Yu JH, Kim H, Kim KH. Role of mitogen-activated protein kinases, NF-kappaB, and AP-1 on cerulein-induced IL-8 expression in pancreatic acinar cells. *Ann N Y Acad Sci* 2006 Dec;1090:368-74.
- [322] Sanjabi S, Hoffmann A, Liou HC, Baltimore D, Smale ST. Selective requirement for c-Rel during IL-12 P40 gene induction in macrophages. *Proc Natl Acad Sci U S A* 2000 Nov 7;97(23):12705-10.
- [323] Luo JL, Kamata H, Karin M. IKK/NF-kappaB signaling: balancing life and death--a new approach to cancer therapy. *J Clin Invest* 2005 Oct;115(10):2625-32.

- [324] Maeda S, Chang L, Li ZW, Luo JL, Leffert H, Karin M. IKKbeta is required for prevention of apoptosis mediated by cell-bound but not by circulating TNFalpha. *Immunity* 2003 Nov;19(5):725-37.
- [325] Kamata H, Honda S, Maeda S, Chang L, Hirata H, Karin M. Reactive oxygen species promote TNFalpha-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. *Cell* 2005 Mar 11;120(5):649-61.
- [326] Maeda S, Kamata H, Luo JL, Leffert H, Karin M. IKKbeta couples hepatocyte death to cytokine-driven compensatory proliferation that promotes chemical hepatocarcinogenesis. *Cell* 2005 Jul 1;121(7):977-90.

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PUBLICATIONS:

1. **Ma, W.**, Mishra S., Mishra J., Angel, J and Kumar A. Intracellular HIV-1 Nef protein downregulates IL-12p40 production by modulation of JNK MAPK activated NF- κ B pathway in LPS-stimulated human monocytic cells. Submitted.
2. **Ma, W.**, Mishra S, Gee, K., Mishra J., Angel, J and Kumar A. Calaium signaling and PI3 kinase pathways are positive regulators of IL-12p40 production in LPS-stimulated human monocytic cells. *J. Biol. Chem.* Vol.282:13351-13362, May. 2007.
3. Gee, K., Angel, J, **Ma, W.**, Sasmita Mishra and Kumar, A. Intracellular HIV-tat expression induced IL-10 synthesis by the CREB-1 transcrioiton factor through SER133 phosphorylaiton and its regylation by the ERK1/2 MAPK in human monocytic cells. In press *J. Biol. Chem.* Vol. 281:31647-31658, Oct.2006.
4. **Ma, W.**, Gee, K., Lim, W., Kozlowski, and Kumar. A. Dexaamethasone inhibits IL12p40 production in lipopolysaccharide-stimulated human monocytic cells by down-regulation the activity of c-Jun N-terminal kinase, the AP-1 and NF- κ B transcription factors *J. Immunol.* Vol.17:318-330, Jan. 2004.
5. Gee, K., Lim, W., **Ma, W.**, Diaz, M., Kozlowski, M., and Kumar. A. Differential Regulation of CD44 Expression by LPS and Tumor Necrosis Factor-alpha in Human Monocytic Cells. *J. Immunol* Vol. 16:5660-5672, Nov. 2002.
6. Lim, W., **Ma, W.**, Gee, K., Aucoin, S., Diza, M., and Kumar, A. Distinct Roles of p38 and c-Jun N-Terminal Kinase in IL-10-dependent and IL-10-independent Regulation of Co-stimulatory Molecule B7.2 in LPS-Stimulated Human Mononuclear Cells. *J. Immunol.* Vol.168 (4):1759-69, Feb. 2002
7. **Ma, W.**, Lim, W., Gee, K., Aucoin, S., Nandan, D., Kozlowski, M., Diaz, M., and Kumar,A. The p38 Mitogen Activated Kinase Pathway Regulates the Human IL-10 Promoter via the Activation of Sp1 Transcription Factor in Lipopolysaccharide-Stimulated Human Macrophages. *J. Biol. Chem.* Vol. 276(17):13664-13674, April, 2001
8. Jiang, Y., **Ma, W.**, Kozasa T., Hattori, S., and Huang, X. The G protein G α 12 stimulates Bruton's Tyrosine Kinase and a Ras-GAP through a Conserved PH/BM Domain. *Nature* Vol. 395(22:808-13, Oct. 1998
9. Zhou, B., **Ma, W.**, and Huang, X. Specific Antibodies to the External Vestibule of Voltage-gated Potassium Channels Block Current. *J. General Physiology* Vol.111 (4):555-563, April 1998

10. Bence, K., **Ma, W.**, (*Co-first author*), Kozasa, T., and Huang, X. Directly Stimulation of Bruton's Tyrosine Kinase by Gαq. *Nature* Vol.389 (18):196-199, Sept. 1997
11. **Ma, W.** and Zheng, Z. Nutritional evaluation of food soybean proteins. *J. Shenyang Medical School*, Vol. 7:15-18, July 1994
12. **Ma, W.** "Biochemistry" (Text Book for undergraduate students, wrote two chapters: Blood and Biological Oxidation), Edited by Gu T., Published by *Publishing House of University of Sciences and Technology of China* 1992
13. **Ma, W.**, Du, C. X., and Guang, X.Y. A Negative Correlation between Activity of Superoxide Dismutase and Fluoride Content in Fluorosis Patients of the Hui Nationality in High Fluorine Areas, *Endemi Disease Bulletins* Vol. 3:64-66, April 1991
14. **Ma, W.** and Du, C. X. An Improving Method of Measuring Activity of Superoxide Dismutase. *J. Ningxia Medical School*, Vol. 1:29-31, Jan. 1989.

ABSTRACTS PRESENTED:

1. **Ma, W.**, Mishra, S and Kumar, A. Intracellular HIV-1 Nef protein inhibits IL-12p40 production by modulation of JNK MAPK, NF-κB pathway in LPS-stimulated human monocytic cells. **16th Annual Canadian Conference on HIV/AIDS Research. April, 20-30, 2007.**
2. **Ma, W.** and Kumar, A. Involvement of MAPKs and other protein kinases signaling transduction pathways in regulation of IL-12p40 by intracellular HIV-1 Nef in response to bacterial endotoxin, lipopolysaccharide (LPS), in human monocytic cells. **12th International Conference of Immunology. Montreal, Canada July 18-23, 2004.**
3. **Ma, W.**, Kumar A. Molecular mechanisms in signaling pathways involved in regulation of IL-12p40 by HIV-1 Nef in LPS-activated human monocytic cells. **Ontario HIV Treatment Network Research Conference. Toronto, Canada, Nov. 3-5, 2003.**
4. **Ma W.**, Gee, K., Lim, W., and Kumar, A. 2004. Dexamethasone inhibits IL-12p40 production in LPS stimulated human monocytes by down-regulating the activity of JNK, AP-1 and NF-κB. **11th International Congress of Immunology and 4th Annual Conference of FOCIS. 239-42. Stockholm, Sweden, July 22-28, 2001.**
5. **Ma, W.**, Lim, W., Gee, K., Diaz, M., and Kumar, A. The p38 Mitogen Activated Kinase Pathway Regulates the Human IL-10 Promoter via the Activation of Sp1 Transcription Factor in LPS-Stimulated Human Macrophages. **Ontario HIV Treatment Network Research Conference. Toronto, Canada, Nov. 1-5, 2001**
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