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Regulation of Interleukin-12 p40 Synthesis in HIV-Infected Myeloid Cells

By

Kelley A. Chambers

THESIS
Submitted to the School of Graduate Studies in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

Department of Biochemistry, Microbiology & Immunology
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ABSTRACT

Progressive immunodeficiency in HIV infection is paralleled by a decrease in IL-12 production, a cytokine crucial for cellular immune function. This thesis reports an examination of the requirement for productive viral infection, the role of other cytokines, and the intracellular molecular targets of HIV-mediated suppression of IL-12 p40 expression, with particular emphasis on regulation of the IL-12 p40 promoter, and LPS-induced activation of MAP kinases.

The reduction in LPS-induced IL-12 p40 protein and mRNA following acute in vitro HIV infection of THP-1 cells and monocyte-derived macrophages (MDM) was not attributed to IL-10 or TGF-β activity, and was not restored by priming with IL-4, IL-13, or IFN-γ. Suppression of IL-12 was dependent upon productive viral infection, and was due, at least in part, to impaired transcription of IL-12 p40. HIV infection of THP-1 cells reduced nuclear factor binding to the NF-κB, AP-1, and Sp1 sites of the IL-12 p40 promoter. Nuclear factor binding to these sites, in addition to the Ets-2 element, was also altered by HIV infection of MDM. By site-directed mutagenesis, each of these transcription factor binding sites were determined to be necessary for IL-12 p40 promoter activation. Binding of NF-κB p50, p65, c-Rel, c-Fos, c-Jun, Sp1 and Sp3 proteins to the IL-12 p40 promoter was reduced by HIV infection of THP-1 cells, while p50, c-Rel, c-Fos, c-Jun, IRF-1, ICSBP, and Ets-2 binding was altered by HIV in MDM. HIV infection did not affect cellular levels of the nuclear factors, with exception of NF-κB p65 in THP-1 cells, and c-Fos in MDM. HIV infection of myeloid cells decreased levels of

phosphorylated JNK MAPK, impaired phosphorylation of p38 MAPK, and suppressed phosphorylation and degradation of IκBα, while ERK 1/2 MAPK was unaffected.

Taken together, these results suggest that impaired IL-12 production in HIV-infected myeloid cells occurs, in part, via disruption of IL-12 p40 transcription, and is dependent on productive infection, and independent of the activity of other cytokines. Inhibition of IL-12 p40 transcription is associated with disruption of nuclear factor binding to the NF-κB, AP-1, Sp1, and Ets-2 elements of the human IL-12 p40 promoter, which may be a consequence of altered MAPK activation.

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DEDICATION

This thesis is dedicated to the memory of Giuseppe Parato, Lionel Vincent Chambers, and Annie Elizabeth Hindley.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES AND ILLUSTRATIONS

LIST OF ABBREVIATIONS

AP-1	Activator protein-1
APC	Antigen presenting cell
ASA	Acetyl salicylic acid
BSA	Bovine serum albumin
C/EBP	CCAAT enhancer binding protein
CHX	Cycloheximide
CMI	Cell-mediated immunity
CNS	Central nervous system
CTL	Cytotoxic T lymphocyte
DAPI	4,6-Diamidino-2-phenylindole
DC	Dendritic cell
DNA	Deoxyribonucleic acid
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-barr virus
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal regulated kinase
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GA-12	GATA sequence in the IL-12 promoter
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

GM-CSF	Granulocyte-macrophage colony stimulating factor
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
ICSBP	Interferon consensus sequence binding protein
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRAK	IL-1 receptor associated kinase
IRF	Interferon response factor
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
LC	Langerhans' cell
LCMV	Lymphocytic choriomeningitis virus
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MAPK	Mitogen activated protein kinase
MCMV	Murine cytomegalovirus
M-CSF	Macrophage colony stimulating factor
MDM	Monocyte-derived macrophage
MNC	Mononuclear cell

MEK	MAP kinase kinase
MIP	Macrophage inflammatory protein
MMLV	Moloney murine leukemia virus
m.o.i.	multiplicity of infection
MTB	*Mycobacterium tuberculosis*
MyD88	Myeloid differentiation factor 88
NF	Nuclear factor
NK	Natural killer
NOD	Nonobese diabetic
OPD	Orthophenylenediamine-HCl
PACAP	Pituitary adenylate cyclase activating polypeptide
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PE	Phycoerythrin
PGE2	Prostaglandin E2
PHA	Phytohemagglutinin
PI3K	Phosphatidyl inositol 3 kinase
PKC	Protein kinase C

PKR	Protein kinase R
RLU	Relative light units
RNA	Ribonucleic acid
RPA	Ribonuclease protection assay
RT	Reverse transcription
SAC	*Staphylococcus aureus*
SAPK	Stress-activated protein kinase
SH	Src homology
SMNC	Splenic mononuclear cell
SOCS-1	Suppressor of cytokine signaling 1
STAg	Soluble tachyzoite antigen
STAT	Signal transducer and activator of transcription
$TCID_{50}$	Tissue culture infectious dose 50
TGF	Transforming growth factor
Th	T helper
TIR	Toll/IL-1 receptor
TLR	Toll like receptor
TMB	Tetramethylbenzidine
TNF	Tumour necrosis factor
Tollip	Toll/IL-1 interacting protein
TRAF	TNF receptor associated factor
VIP	Vasoactive intestinal peptide

I. INTRODUCTION

Interleukin (IL)-12 was first isolated from a human EBV-transformed B cell line in 1989 by Kobayashi et al., and originally named natural killer cell stimulatory factor (NKSF). The first description of NKSF was as a novel heterodimeric cytokine, which when added to human peripheral blood leukocytes induced IFN-γ production, enhanced natural killer cell (NK) cytotoxicity, and enhanced mitogenic responses of T lymphocytes treated with mitogenic lectins and phorbol diesters (1). Since its discovery, IL-12 has been demonstrated to be a key immunoregulatory cytokine, produced by a variety of antigen presenting cells (APC), and functioning to link innate and adaptive immunity (reviewed in (2)). IL-12 can be elicited by early innate pathogen sensing mechanisms of APC, or via interactions between APC and activated T lymphocytes via the CD40/CD40 ligand interaction, and therefore, IL-12 is crucial for both initiation and propagation of an appropriate adaptive immune response according to the nature of the immunological challenge (3). As such, a broad array of roles for IL-12 has been described in host defence against intracellular bacterial or parasitic challenge, viral infection, tumour cells, and in regulation of autoimmune disease and allergic responses, with implications for immunotherapy and vaccine design. Therefore, understanding IL-12 regulation is essential for comprehension of numerous infectious and autoimmune disorders, and may provide insight into novel approaches to regulate or circumvent these disease states.

The immune dysfunction associated with HIV infection and disease is multi-faceted. Among the defects in immune competence is a dramatic decline in cell-mediated immunity, vital for control of many opportunistic infections and neoplasms in

parallel with a decline in stimulus-induced IL-12 production. This thesis provides an evaluation of the inhibition of IL-12 in HIV-infected monocytes and the myeloid THP-1 cell line, the role of other cytokines in this inhibition, the requirement for productive cellular infection in suppression of IL-12, and examines in detail the underlying molecular mechanisms of regulation of IL-12, with emphasis on transcriptional control and signalling in response to pathogen sensing.

A. Interleukin-12:

1. Biologically active IL-12 is a 70kDa heterodimer: Interleukin (IL)-12 is a 70 kDa heterodimeric cytokine, and is the founding member of a novel class of heterodimeric cytokines, which has recently expanded to include IL-23 and IL-27 (4, 5). The biologically active form of IL-12 consists of p40 and p35 monomeric subunits covalently linked by a disulfide bond (1, 6). Coordinated expression of both IL-12 p35 and p40 subunits is required for synthesis and release of the bioactive p70 molecule, yet the two monomers are located on separate chromosomes (IL-12 p40 on chromosome 5, IL-12 p35 on chromosome 3) and are differentially regulated (7). Expression of the p35 subunit is considered to be constitutive or only moderately regulated in many cell types, some of which do not express IL-12 p70 (8), yet p35 production is rate limiting in the formation of p70 (9). Conversely, expression of the p40 subunit is highly inducible in a manner similar to that of p70, and is restricted to cell types capable of producing bioactive IL-12 p70 (8, 10, 11).

The disparity in regulation between the two subunits is highlighted by the observation that in response to numerous stimulatory and inhibitory factors, expression of IL-12 p40 mRNA correlates closely with induction of p70 production, while p35

mRNA levels remain unchanged (12-16). In response to stimulation of PBMC with *Staphylococcus aureus* (SAC), expression of p40 mRNA correlates closely with induction of p70 production to a greater extent than p35 (12). TNF-α suppresses IL-12 p70 production in LPS- or *S. aureus*- stimulated human macrophages (15) in a manner that decreases p40 without affecting p35 mRNA expression. CD40 ligand induction of IL-12 in macrophages occurs in a manner that augments IL-12 p40 expression while p35 mRNA accumulation is CD40L-independent (13). Infection of macrophages with *M. leprae* reduces p70 and p40 without a detectable effect on p35 mRNA expression. Enhanced IL-12 p70 production in response to GM-CSF corresponds with increased p40 expression but not that of p35 (14). As IL-12 p40 protein and mRNA closely parallel production of the p70 molecule, understanding regulation of the IL-12 p40 gene is of paramount importance in understanding production of bioactive IL-12 p70.

2. Production of IL-12 p40 monomers, homodimers, and IL-12 p70 heterodimers: Following stimulation of IL-12 producing cells (see below), the IL-12 p40 subunit is produced in dramatic excess over the bioactive p70 heterodimer (8). This p40 is secreted in monomeric form, or as disulfide-bonded homodimers. Both of these forms of the p40 subunit are devoid of IL-12-specific biological activity. The p40 homodimer can bind to the IL-12 receptor, but is incapable of eliciting the signal transduction events and downstream biological responses that occur following receptor ligation with p70 (17). In the murine system, in vivo production of IL-12 p40 homodimers may function as an IL-12 antagonist, via competition with p70 for binding to the IL-12 receptor (18, 19). No biological function has been ascribed to free monomeric p40. While in vivo production of p40 homodimers with an antagonistic function has not been described in humans,

both p40 monomers and p40 homodimers have in vitro inhibitory action for IL-12 p70 binding to the KIT225/K6 human T cell line expressing IL-12R, with the monomer being a 20-fold less potent inhibitor of p70 binding than the p40 homodimer (20). Secretion of the free p35 monomer has not been described to date.

Production and secretion of biologically active IL-12 p70 is tightly regulated, likely a reflection of the need to control the magnitude and duration of production of this potent proinflammatory and immunomodulatory cytokine. The p35 subunit is extensively modified with sialic acid adducts to N-linked oligosaccharides, which is essential for both correct assembly and secretion of IL-12 p70 heterodimers (21). On the contrary, the p40 subunit is not posttranslationally modified by glycosylation, and its secretion in monomeric or homodimeric form is not dependent upon such modifications (21). Additional studies from this group illustrated that human IL-12 p70 exists intracellularly in an immature form, which is glycosylated on the p35 chain upon stimulation, allowing rapid secretion of mature p70 (21).

3. IL-12 binding to the IL-12 Receptor: The dimeric IL-12 receptor consists of IL-12Rβ1 and β2 subunits, each a type I transmembrane glycoprotein and binds IL-12 p70 with high affinity in mice and humans (22, 23). While the majority of studies evaluating expression and regulation of the IL-12 receptor have focused on T and NK cells, the dimeric IL-12 receptor has also been reported to be expressed on dendritic cells (24) and microglia (25). Recognition of p70 via the IL-12 receptor occurs via binding of β1 to IL-12 p35 subunit, while the β2 receptor binds IL-12 p40. In both murine and human cells, the β2 subunit appears to be responsible for initiating intracellular signalling events following receptor ligation (26), and as such, modulation of expression of the β2 subunit

plays a significant role in induction of IL-12 responses, and in physiological regulation of IL-12 responsiveness. This is highlighted by the observation that IL-12Rβ2 expression is upregulated in the Th1 phenotype of CD4+ T cells (27, 28), and may play a role in perpetuation or exacerbation of Th1-driven autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE), the murine model of Multiple Sclerosis (29, 30). Deficient expression of IL-12Rβ2 has been linked to impaired IFN-γ production in HIV infected patients (12) and impaired T lymphocyte responses to *C. neoformans* in vitro (31).

Binding of IL-12 p70 to the IL-12 receptor induces tyrosine phosphorylation of Janus-family kinases (JAKs) and subsequent activation of STATs (signal transducer and activator of transcription). Specifically, IL-12 receptor ligation results in activation of TYK2 and JAK2, which interact with the cytoplasmic domains of IL-12Rβ1 and β2, respectively (23). Recruitment of JAKs to the IL-12R cytoplasmic domain and subsequent phosphorylation of Rβ2 residues leads to phosphorylation and binding of numerous STATS (STAT 1, 3, 5, 4) to Rβ2 via their SH2 domains (22). Following activation, STATs dimerize and translocate to the nucleus to activate transcription of IL-12 responsive genes. Although STATs 1, 3, 4, and 5 are activated by IL-12 receptor ligation, the majority of biological effects of IL-12 on NK cells and T lymphocytes are mediated by STAT4 activation as demonstrated in STAT4-knockout mice which are impaired in IFN-γ production, and Th1 responses (32).

4. Cells that produce IL-12 p70: The main cell populations which produce the IL-12 p70 heterodimer in response to CD40 ligation or a broad range of pathogen-derived stimuli (discussed below) are professional antigen presenting cells (APC), particularly

monocytes/macrophages and dendritic cells (33, 34). IL-12 production by APC at sites of infection in the periphery, or in T cell areas of lymphoid tissues is vital for early induction of innate immune responses to pathogenic challenge via induction of interferon-γ (35, 36), and for coordination of T-cell mediated adaptive immune responses (discussed in detail in section A.9 of the Introduction).

In addition to professional APC, numerous reports have illustrated that neutrophils represent an additional source of IL-12 production, which may be particularly important in early pathogen sensing and innate immune responses. Neutrophils in mice contain preformed stores of IL-12 (37) that are rapidly released upon in vivo infection with *T. gondii* (38). The presence of preformed intracellular stores of IL-12 protein ready for rapid release following pathogen challenge contributes to the role of IL-12 in early innate pathogen sensing in peripheral tissues, to initiate a rapid immune response. Keratinocytes have also been demonstrated to produce IL-12, as reported by Yawalkar et al., in an investigation of in situ IL-12 production in skin biopsies from human patients during a contact hypersensitivity reaction (39). Microglia are another source of IL-12 (25), and as such, likely play a role in cell-mediated immune responses in the CNS (40). Although IL-12 was initially isolated from an EBV-transformed B cell line, IL-12 production from primary B cells has not been confirmed.

5. Induction of IL-12 production: Interleukin-12 production can be induced through one of two major routes. The first is through recognition of bacteria or bacterial products or PAMPs (pathogen-associated molecular patterns) via TLR (Toll-like receptor) family members with subsequent activation of a myriad of intracellular signalling events, culminating in MAP kinase activation and activation of numerous

cellular transcription factors (see below). The second route of IL-12 induction is via ligation of CD40 on the surface of APCs such as monocytes/macrophages and dendritic cells following interaction with activated T lymphocytes expressing CD40 ligand (CD40L) (13). IL-12 induction through either of these mechanisms is subject to regulation by numerous factors, which will be discussed in detail in section A.6.

i. Induction of IL-12 Biosynthesis: Lipopolysaccharide (LPS) is a major constituent of the outer membrane of gram-negative bacteria such as *E. coli*, which can cause systemic inflammatory reactions and septic shock following infection (41). IL-12 p70 protein production is induced by, and is required for immunological defence against a broad array of pathogens. Intracellular bacteria including *Mycobacterium tuberculosis* and *Listeria monocytogenes*, and intracellular parasites such as *Leishmania major* and *Toxoplasma gondii* require IL-12 and/or IL-12-induced IFN-γ production for eradication of these infections. Although many of these organisms infect and reproduce in cells of the monocyte/macrophage lineage, inducing IL-12 production in the process, pathogen sensing and IL-12 synthesis by dendritic cells in the periphery, in the absence of intracellular infection, play an important role in early immune detection of infectious challenge, and aid in setting the stage for an adaptive immune response. Recognition of pathogen-associated molecules or structures including lipoteichoic acid (42), lipoarabinomannan (43), and peptidoglycan (44), in addition to LPS, via CD14, contributes to induction of IL-12 from myeloid cells, yet until the discovery of mammalian Toll-like receptors (TLR), the molecular mechanism of signalling production of IL-12 or other proinflammatory cytokines was poorly understood.

ii. CD14 is an LPS Receptor: CD14, present in soluble form in serum or membrane-bound on monocytes, macrophages and dendritic cells has long been recognized as an LPS receptor (45). However, CD14 is a glycosyl-phosphatidylinositol (GPI)-anchored glycoprotein, and as such has no cytoplasmic domain and therefore cannot transduce intracellular signals upon LPS recognition on its own (45). Earlier studies indicated that the Src tyrosine kinase Lyn associated with CD14 following LPS stimulation of monocytes, and along with other Src tyrosine kinases Fgr and Hck, might be responsible for activation of myeloid cells in response to endotoxin (46). However, the observation that peritoneal and bone-marrow derived macrophages from $fgr^{-/-}/hck^{-/-}/lyn^{-/-}$ mice exhibited normal nitrite production and IL-1, IL-6, and TNF-α expression following LPS stimulation, indicated that LPS-induced activation of macrophages could occur independently of Src family tyrosine kinases, eluding to the existence of other LPS-signalling receptors (47).

iii. Toll-like Receptors: The Toll-like receptor (TLR) family of receptors represents an evolutionarily conserved superfamily of pattern-recognition receptors (PRRs) that contribute to innate immune defence against pathogen challenge. Members of this family are represented in organisms as diverse as *Drosophila* (48), and tobacco plants (49). The first mammalian homologue of Toll was first characterized in 1997 (50), and was quickly followed by the identification of TLR4 as an endotoxin receptor, with the observation that defective LPS signalling in C3H/HeJ mice was associated with a mutation in the tlr4 gene locus (51). Since this discovery, the TLR family has expanded to include at least 10 members (TLR1 through 10). The description of multiple TLR family members with discreet, albeit possibly overlapping ligands, with differential TLR

expression on different leukocyte classes (52, 53), suggests that the innate immune system is in fact selective, and capable of discriminating among specific categories of pathogens (3, 54). This property of the innate immune system suggests that the innate immune system is capable of discriminating among pathogens, allowing tailoring of adaptive immune responses according to the nature of the pathogenic challenge.

iv. TLRs and TLR ligands: Of the 10 mammalian TLRs identified to date, pathogen-associated ligands have only been reported for five. TLR2 recognizes and elicits a biological response following exposure to lipoproteins of gram positive and gram negative bacteria (55, 56), glycolipids from *M. tuberculosis* (57), lipoteichoic acid from *S. pneumoniae* and *S. aureus* (42), and bacterial peptidoglycan from gram-positive bacteria (56, 58). Bacterial flagellin from flagellated bacteria such as *S. typhimurium* or *L. monocytogenes* is recognized by TLR5 (59), while CpG DNA is recognized by TLR9 in lysosomal compartments (60). Double-stranded RNA, a common molecular pattern observed in viral infections, may be the ligand for TLR3 (61). In the mammalian system, TLR4 has been demonstrated to be an LPS receptor, and unlike other TLRs, TLR4 must be co-expressed with the MD-2 adaptor protein in order to elicit LPS-induced biological responses (62-64). Recently, it has been described that interactions between different TLRs upon substrate recognition may confer additional specificity of cellular responses upon pathogen challenge. When transfected into HEK293 cells, TLR2 and TLR6 cooperate to enhance responsiveness to stimulation with modulin from *Staphylococcus epidermidis*, and this stimulation was inhibited in the presence of TLR1 (65). Although ligands for all TLRs have not yet been assigned, recent evidence suggests that heat-shock proteins and fibronectin proinflammatory extradomain A may elicit inflammatory

responses through TLR4-mediated recognition (66, 67), providing an additional danger recognition mechanism for the innate immune system.

v. Signal transduction via TLR4 in response to LPS: The TLR family shares homology in the cytoplasmic domain with the IL-1 receptor, and as such, shares much of its intracellular signalling machinery, and imparts overlapping effector outcomes following receptor ligation, including proinflammatory cytokine synthesis, maturation of dendritic cells and upregulation of costimulatory molecules on APCs. The signal transduction events propagated by recognition of LPS by TLR4 are depicted in figure 1.

LPS bound by the serum protein LBP (LPS-binding protein) interacts with soluble or membrane-associated CD14, which acts as a shuttle for the physical transfer of LPS to TLR4 in complex with MD-2 on the APC surface (62). Following TLR4/MD-2 ligation by LPS, the adaptor molecule MyD88 (myeloid differentiation factor 88) is recruited to the cytoplasmic domain of TLR4 via homologous TIR (Toll/IL-1 receptor) domains, facilitated by Tollip (toll interacting protein) (68). Subsequently the serine/threonine kinase IRAK (IL-1 receptor associated kinase) is recruited to MyD88 via interactions between death domains (69), IRAK is autophosphorylated and dissociates from MyD88 (70). Phosphorylated IRAK associates with TRAF6 (TNF receptor associated factor), which promotes activation of all three classes of mitogen activated protein kinase (MAPK) and activates the IκB kinase (IKK) complex and downstream NF-κB activation (reviewed in (71)). Intracellular signalling events initiated by MyD88 recruitment to TLR are largely responsible for proinflammatory cytokine production, including production of IL-12 (72). However, LPS stimulation of myeloid cells also activates protein kinase C (PKC), Src tyrosine kinases, small G proteins, and

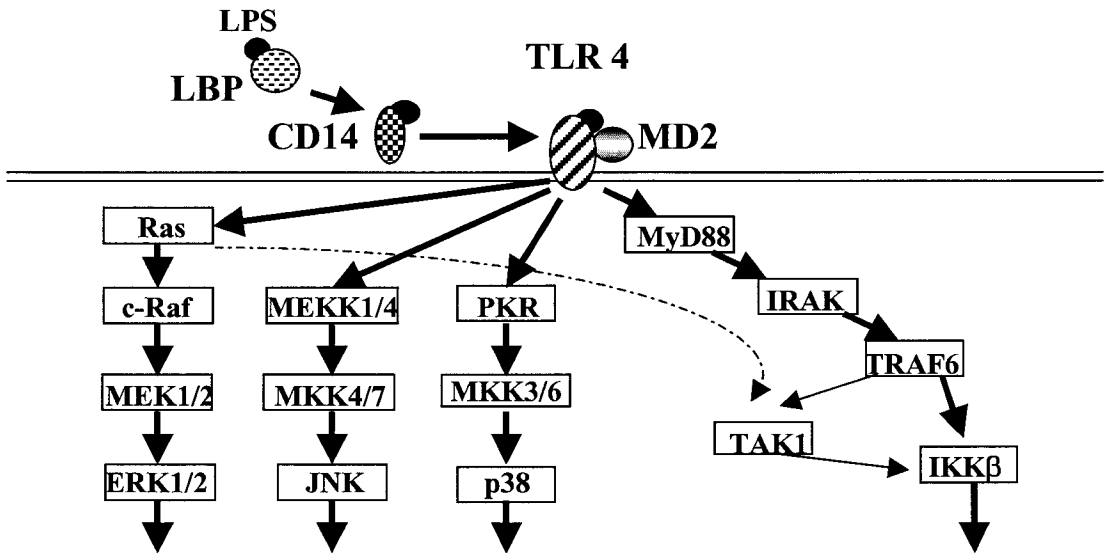

LPS
LBP
CD14
TLR 4
MD2
Ras
c-Raf
MEK1/2
ERK1/2
MEKK1/4
MKK4/7
JNK
PKR
MKK3/6
p38
MyD88
IRAK
TRAF6
TAK1
IKKβ
Activation of transcription factors
NF-κB activation

FIGURE 1: *LPS-Mediated Signalling via Toll-Like Receptor (TLR) 4:*

Lipopolysaccharide (LPS) bound by the serum LPS-binding protein (LBP) is recognized by soluble or membrane-associated CD14, and is shuttled to TLR4. LPS in complex with TLR4 and MD-2 on the myeloid cell surface initiates a series of intracellular events leading to MAP kinase and NF-κB activation, by both MyD88-dependent and –independent mechanisms. Adapted from M. Guha *et. al.* (71).

PI3 kinase (73-75), which may contribute to MyD88-independent TLR signalling and downstream biological outcomes, including IFN-inducible gene expression, caspase activation, and induction of costimulatory molecules (72, 76, 77).

Due to the severity of consequences of excessive inflammatory responses and proinflammatory cytokine synthesis, signalling via TLR and TLR expression itself are likely subject to tight regulation. The monocyte/macrophage-specific IRAK homologue IRAK-M is a negative regulator or LPS-induced activation. IRAK-M is recruited to MyD88 upon LPS activation and prevents dissociation from MyD88 and subsequent interaction with TRAF6 (78). The physiological relevance of IRAK-M is illustrated by reduced endotoxin tolerance observed in IRAK-M deficient mice (78). Additional evidence suggests that SOCS-1 (suppressor of cytokine signalling-1) that is induced in murine macrophages by LPS, functions as a negative regulator of TLR4 signalling, conferring protection from endotoxic shock (79). Endotoxin tolerized mice exhibit impaired MAP kinase, NF-κB, and AP-1 activation and deficient upregulation of TLR4 mRNA during subsequent LPS exposure, indicating that TLR expression itself may be a potential target of regulation of inflammatory responses (80).

Finally, while different TLR family members share their cytoplasmic domains and to some extent, intracellular signalling machinery, there appears to be some degree of unique signalling components distinguishing intracellular responses elicited by TLRs. THP-1 cells tolerized with lipoteichoic acid (LTA) from *S. aureus* are hyporesponsive to a secondary challenge with LTA but not to LPS. On the contrary, THP-1 cells tolerized with LPS are hyporesponsive to subsequent challenge with either LPS or LTA, indicating some disparity between TLR2- versus TLR4-mediated intracellular signalling

events (81). This may reflect a greater breadth in recognition of pathogens, and a greater degree of adaptability of this innate immune response mechanism.

vi. CD40/CD40L interaction: In addition to stimulation of APC (monocytes, macrophages, dendritic cells) via recognition of PAMPs by TLR family members, IL-12 is also induced by the ligation of CD40 by CD40L on activated T lymphocytes during antigen presentation (13). In this regard, IL-12 production by CD40-engaged APCs aids in enhancement of IFN-γ production and proliferation of T lymphocytes, and subsequently promotes Th1-biased adaptive immune responses (82, 83), and provides positive feedback for IL-12 production and additional APC activation.

Ligation of CD40 on dendritic cells promotes receptor trimerization, and association of CD40 with the Src tyrosine kinase Lyn in membrane rafts (84). Recruitment of Src tyrosine kinases leads to downstream activation of MEK 1/2 and ERK 1/2 activation, and activation of transcription of proinflammatory cytokines (84). In addition, receptor oligomerization promotes recruitment of TRAF to CD40 and with subsequent activation of p38 MAPK and IL-12 p40 synthesis (84).

While IL-12 induction in APCs following detection of pathogens via TLRs is vital for initiation of adaptive immune responses and enhancement of innate immune responses, CD40/CD40L-dependent IL-12 production lends further protection for hosts from infection by intracellular pathogens including *M. tuberculosis* (85, 86) or *T. gondii* (87). CD40L engagement of CD40 on APC synergizes with bacterial stimuli to enhance protective immune responses to intracellular pathogens (88, 89), and exogenous addition of CD40L to PBMC in vitro can augment or restore defective IL-12 production in HIV seropositive individuals (83, 90). Due to the potency of CD40L in eliciting IL-12

production and supporting cell-mediated immunity, CD40L along with microbial products or IL-12 itself, may be exploited as an adjuvant to enhance vaccine efficacy and host protection against tumours (91) or infectious diseases (92).

6. Regulation of Interleukin-12 Expression: While interleukin-12 is induced by microbial stimulation or CD40 ligation by CD40 ligand on activated T cells, production of IL-12 is regulated or influenced by numerous factors, most importantly, other cytokines. Although cytokines elicit a potent positive or negative regulatory influence over IL-12 production, cytokines alone are insufficient for induction of IL-12.

i. Positive IL-12 regulation by cytokines: One of the most potent cytokines eliciting a positive regulatory role over IL-12 production and which plays a vital role in the downstream biological effects of IL-12 (discussed in section A.9) is IFN-γ. While bacterial stimuli such as LPS induce IL-12 p40/p70 synthesis in PBMC or monocytes, IFN-γ dramatically enhances this production (8, 10). IFN-γ functions in a positive feedback loop with IL-12: IL-12 induced from an APC following pathogen challenge or CD40 ligation elicits IFN-γ production from NK cells or T lymphocytes, which enhances APC activation and IL-12 production. Numerous studies evaluating the priming effect of IFN-γ production have demonstrated that IFN-γ enhances IL-12p70 expression via enhancing transcription of both p35 and p40 subunit mRNAs (10). In addition to this priming effect, IFN-γ also enhances IL-12 responsiveness via augmenting and maintaining expression of IL-12Rβ2 on Th1 cells (93).

Other cytokines that prime microbe-induced IL-12 production include IL-4 and IL-13. These are typically considered to be Th2-type cytokines, and if administered to PBMC in the short-term prior to stimulation with LPS or *S. aureus*, IL-4 and IL-13 are

inhibitory for IL-12 production (94). However, with prolonged exposure to either cytokine (greater that 20 hours) prior to addition of microbial stimulus, IL-12 production is enhanced (8, 95). This priming effect in PBMC corresponds to enhanced transcription of both p35 and p40 subunit mRNAs (95).

ii. Negative Regulation of IL-12 by Cytokines: Interleukin-10 is a potent and particularly physiologically relevant negative regulator of IL-12 expression, and of inflammatory responses in general. IL-10, like IL-12, is induced by microbial stimuli such as LPS, and feeds back to downregulate IL-12 production, thus limiting the production of IFN-γ induced by IL-12. Inhibition of IL-12 by IL-10 occurs at the level of transcription of the IL-12 p40 and p35 subunits, and is cycloheximide-sensitive indicating that IL-12-mediated inhibition occurs indirectly via the synthesis of an additional protein intermediate (96). While IL-10 is a potent regulator of IL-12 and is induced by overlapping stimuli, induction of IL-10 does not always result in decreased IL-12 production. For example, butyrate impairs IL-12 p40 and p35 mRNA expression in parallel with augmented IL-10 production in *S. aureus*-stimulated monocytes, yet IL-12 inhibition by butyrate is IL-10-independent (97). However, in IL-10-deficient mice challenged with avirulent *T. gondii*, augmented IL-12 production and deleterious inflammatory responses occurred, highlighting the importance of IL-10 in physiological control of IL-12 synthesis (98).

TGF-β is another potent inhibitor of IL-12 synthesis, and does so by reducing transcription and stability of the p40 subunit mRNA (99). TNF-α, which is also induced by diverse microbial stimuli, is also inhibitory for IL-12 production. TNF-α-mediated suppression of IL-12 production in LPS- or *S. aureus*-treated human monocyte-derived

macrophages is IL-10-independent (15). IFN-α/β are other cytokines involved in the innate immune response to pathogen challenge and facilitate cell-mediated immune responses, which are also inhibitors of IL-12 production by monocytes (100) and murine splenocytes treated with *S. aureus* (101) via inhibition of transcription of the IL-12 p40 subunit.

iii. Negative regulation of IL-12 Induction by Receptor Ligation: Aside from regulation of IL-12 production by cytokines, ligation of Fc receptors and G-protein coupled receptors are an additional mechanism of IL-12 downregulation. FcγR ligation on bone marrow derived monocytes with IgG-opsonized erythrocytes impairs transcription of IL-12 p35 and p40 subunits (102). Similarly, treatment of murine splenocytes with *S. aureus* and IFN-γ or CpG DNA in the presence of the G-protein inhibitor pertussis toxin augments IL-12 production (103). Accordingly, Balb/c mice infected with *L. major* which normally results in a non-healing phenotype marked by insufficient IL-12 production, revert to a healing phenotype with inclusion of pertussis toxin (103). Other examples of ligands of G-protein coupled receptors that impair IL-12 production include vasoactive intestinal peptide (VIP) or pituitary adenylate cyclase activating peptide (PACAP) binding to the VPAC-1 receptor (104). However, ligation of G-protein coupled receptors does not exclusively result in inhibition of IL-12. For example, during *T. gondii* infection, binding of STAg-induced MIP-1β to CCR5 is required for IL-12 induction and IL-12-dependent pathogen control (105).

iv. Inhibition of IL-12 production by prostaglandin E2: Another important soluble mediator of inflammation is prostaglandin E2 (PGE2), a lipid by-product of arachidonic acid. PGE2 is a potent inhibitor of IL-12, acting in part via the induction of

inhibitory signals following PGE2 binding to its cognate G-protein coupled receptors EP1, 2, 3, or 4 (106). PGE2 production from LPS-stimulated bone marrow dendritic cells inhibits IL-12 indirectly by induction of IL-10 synthesis (107). Another study suggests that addition of PGE2 to TNF-α-treated immature human dendritic cells enhances the ratio of p40/p70, thereby increasing the presence of antagonistic free p40 monomer or homodimer to compete with p70 for IL-12 receptor occupancy (108), although in LPS- or CD40L-treated dendritic cells both p40 and p70 protein production was inhibited.

The control mechanisms in place for regulation of IL-12 expression are vital for ensuring limitation of proinflammatory responses to microbial challenge or during a downstream antigen-specific immune response. Dysregulation of any of these control elements directly at the level of intracellular regulation of IL-12 synthesis or indirectly via alterations in IL-12 receptor expression or production of other cytokines, may lead to deleterious Th1- or IL-12-mediated autoimmunity, or may allow immunological escape of microbes that require IL-12 for pathogen control. Elevated levels of IL-12 production in macrophages from nonobese diabetic (NOD) mice are thought to predispose this mouse strain to the development of diabetes (109), and mice susceptible to autoimmune myocarditis deficient in IL-12 p40 resist development of disease (110). In addition, IL-12 deficient mice exhibit impaired IFN-γ production in response to LPS, and reduced antigen-specific Th1 responses (111).

*7. **Transcriptional Control of IL-12 p40:*** The human IL-12 p40 promoter contains at least eight putative transcription factor binding sites, including C/EBP, NF-κB, PU.1, AP-1, Ets-2, Sp1, IRF-1, and NF-IL-6, depicted in figure 2 (2). An additional control

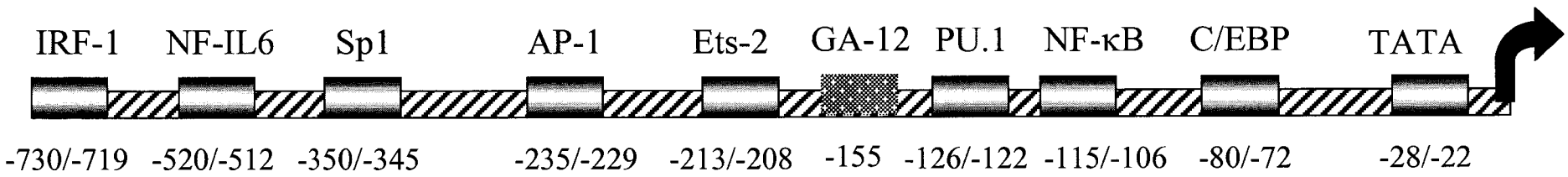

IRF-1
NF-IL6
Sp1
AP-1
Ets-2
GA-12
PU.1
NF-κB
C/EBP
TATA
-730/-719
-520/-512
-350/-345
-235/-229
-213/-208
-155
-126/-122
-115/-106
-80/-72
-28/-22

FIGURE 2: *Proximal Human Interleukin-12 p40 Promoter:*

Schematic representation of the proximal human IL-12 p40 promoter, indicating the positions of eight putative transcription factor binding sites (C/EBP, NF-κB, PU.1, AP-1, Ets-2, Sp1, NF-IL-6, IRF-1) and a recently identified repressor element (GA-12). Adapted from G. Trinchieri (2).

element has been recently identified, termed GA-12 (GATA sequence in the IL-12 promoter) (112). This element is located at –155 in the human IL-12 p40 promoter between the Ets-2 and NF-κB binding sites, and negatively regulates promoter activation by IL-4 and PGE2 (112). The protein binding to the GA-12 element following IL-4 or PGE2 treatment has not been identified. The relative contribution of each of the transcription factor binding sites in promoter activation remains poorly understood and most studies are limited to the murine model.

In murine macrophages, the C/EBP and NF-κB elements exhibit a functional synergy, without cooperative binding, in response to heat-killed *Listeria monocytogenes* (113). In addition, stimulation of RAW264.7 murine macrophages with CpG DNA regulates IL-12 p40 transcription in a manner requiring C/EBP and NF-κB (114). Presence of the NF-κB element in murine macrophages is essential for pathogen-induced promoter activation (115), and both p50/p65 and p50/c-Rel NF-κB heterodimers bind to the NF-κB site following LPS stimulation (116). While both NF-κB heterodimers bind to the NF-κB site with similar affinity, c-rel$^{-/-}$ murine macrophages are deficient in IL-12 p40 expression, while p65$^{-/-}$ macrophages are not (116). This selective requirement for c-Rel in IL-12 p40 transcription is independent of the TLR-dependent nucleosome remodeling events necessary for transcriptional activation of IL-12 p40 (117). A large multiprotein complex forms at the Ets-2 site in response to LPS stimulation in RAW264.7 cells (118, 119), comprising p50, c-Rel, ICSBP (interferon consensus sequence binding protein), IRF-1 (interferon regulatory factor-1), PU.1 and Ets-2.

Numerous pharmacological inhibitors of IL-12 production do so by impairing nuclear factor binding to the NF-κB element (eg. VIP/PACAP, retinoids, ASA, and 1,25-dihydroxyvitamin D_3)(104, 120-122), while FcRγ-mediated suppression of IL-12 p40 expression is due, in part to diminished binding of nuclear protein to the Ets-2 element (102). IRF-1-/- and ICSBP-/- mice have impaired IL-12 production, however the role of the IRF-1 site in IL-12 p40 promoter activation has not been determined (123-125). In addition, the contributions of the NF-IL-6, Sp1, and AP-1 sites in IL-12 p40 transcription have not been evaluated.

8. MAP kinases and IL-12: Signalling through TLRs in response to pathogen-associated molecular patterns, such as LPS, initiates numerous intracellular signalling events culminating in activation of MAP kinases and phosphorylation and degradation of IκBα, with subsequent activation of NF-κB (reviewed in (71)). Several recent reports have implicated MAPK activation in regulation of production of cytokines, including IL-12 (126). Activation of p38 MAPK following treatment of murine macrophages with berberine (127) or LPS (128) is required for IL-12 p40 transcription (127). Similarly, treatment of RAW264.7 murine macrophages with CpG DNA activates IL-12 p40 expression via p38 MAPK (129). A strict requirement for p38 in LPS-induced IL-12 p40 expression is evident from studies of mice deficient in the p38 activator Mkk3, which are deficient in p40 expression (130). Activation of p38 by LPS may be dependent on the dsRNA-dependent kinase PKR, as PKR-/- mice have impaired IL-12 production in response to in vitro or in vivo LPS challenge (131).

On the contrary, activation of ERK MAPK is inhibitory for IL-12 production (132). Treatment of murine spleen-derived dendritic cells with TNF-α to induce

maturation inhibits IL-12 via ERK activation (133). Activation of ERK via pathogen challenge and subsequent impairment in IL-12 production may reflect an additional level of regulation to maintain tight control to limit deleterious endotoxic responses. Unfortunately, regulation of IL-12 via MAPKs, particularly ERK, provides an opportunity for invading pathogens to evade an immune response. *Leishmania* phosphoglycans, which promote parasite survival, do so via activation of ERK MAPK, thereby limiting IL-12 production (128). Although the roles of p38 and ERK MAPK in pathogen-mediated regulation of IL-12 production have been described in murine APCs, studies of MAPK involvement in IL-12 regulation in the human system, and studies of the role of JNK in IL-12 induction specifically, are lacking.

9. Biological Functions of Interleukin-12: Interleukin-12 is a potent inducer of cell-mediated immune responses, important in immunological control of infections with intracellular pathogens, and in anti-tumour immunity. The majority of the biological function of IL-12 is mediated via its ability to elicit IFN-γ production from T and NK cells, and to induce polarization of CD4+ T cells into the Th1 subset of IFN-γ producing T lymphocytes, either directly or in synergy with other cytokines or APC costimulatory molecules.

Early pathogen sensing by APCs elicits IL-12 production, which in turn evokes IFN-γ production from NK cells and T lymphocytes (134, 135). IFN-γ produced in this manner is responsible for several of the biological effects of IL-12. IFN-γ feeds back to the APC and further augments IL-12 production, and activates the APC. IFN-γ enhances costimulatory molecule expression and MHC expression (136), increasing the efficacy of APC to stimulate naïve T lymphocytes. IFN-γ also enhances IL-12-mediated effects

on IL-12 receptor-expressing cells by augmenting and maintaining expression of the IL-12 receptor (123). While IL-12 is integral for induction of IFN-γ, IL-18 synergizes with IL-12 to maximize IFN-γ production (137). Interleukin-12 is also a potent and critical inducer of the differentiation of the Th1 phenotype of CD4+ T cells (138-142), which produces IL-2 and IFN-γ and supports cell-mediated immune responses. In this manner, IL-12 and IL-12-induced IFN-γ aid in promoting proliferation of activated T cells (143) and NK cells (144). The cell-mediated/Th1 response also promotes cytotoxicity of CD8+ cytotoxic T lymphocytes and NK cells (145-147), thereby aiding in antiviral and anti-tumour antigen-specific immune responses (148-150). One of the key functions of IL-12-elicited IFN-γ from NK cells and Th1 cells is activation of microbicidal functions necessary for eradication of intracellular infections within phagocytes, such as *L. major, T. gondii,* and *M. tuberculosis* (139, 151-153). These antimicrobial activities include production of nitric oxide via iNOS induction, production of superoxide and other oxygen-derived products that are directly toxic for bacteria, enhancing lysosomal fusion with phagosomes, and increase in costimulatory molecule expression to render cells better APCs for directing adaptive immune responses (reviewed in (154)).

A role for IL-12 in viral infections is less clear. Inclusion of IL-12 in vaccine regimens or during antigenic priming improves anti-viral and anti-tumour immunity and antigen-specific CTL generation (155-159). IL-12 also enhances expression of granzyme B and perforin in CTL (160). However, in many instances viral infection does not induce or require IL-12 for viral clearance, including infection with influenza or lymphocytic choriomenigitis virus (LCMV) (148, 161). On the contrary, infection of mice with murine cytomegalovirus (MCMV), or patients in the acute phase of varicella

zoster infection show elevated levels of IL-12 production (148, 149, 162), which may play a role in eliciting early IFN-γ production from NK cells, aiding in control of viral replication (148, 149, 163). It appears that generation of CTL effector cells is not dependent on IL-12, but is augmented both at the level of precursor frequency and cytotoxic activity, and therefore the role of IL-12 in viral infection may primarily be to induce early IFN-γ production and elicit Th1 development to supply help during secondary immune responses.

Due to the potent capacity of interleukin-12 to elicit Th1 development and IFN-γ production, IL-12 is vital for host resistance to a variety of infectious challenges. Intracellular bacteria and intracellular parasites are strong inducers of IL-12 production, and IL-12 and IL-12-induced IFN-γ production/Th1 cell development is critical for eradication of infection with these pathogens. The potent proinflammatory and Th1-inducing capacity of IL-12 has recently been exploited via application of IL-12 as a vaccine adjuvant to improve cell-mediated immunity, for use against a wide variety of pathogens and anti-tumour immune reactions. The deficiency in stimulus-induced IL-12 production which occurs in HIV infection may contribute to reduced cellular immune responses and enhanced susceptibility to opportunistic infections that accompany HIV disease.

B. HIV Immunopathogenesis:

HIV disease is characterized by severe immune suppression, which advances with disease progression. A hallmark of HIV infection is a gradual loss of CD4+ T cell number (164), and eventual susceptibility to opportunistic infections and neoplasms. As

CD4+ T cells are vital components of the adaptive immune response, and coordinate humoral and cellular immune responses in an antigen-specific manner, loss of the CD4+ T cell population and the influence of this loss on the immune response are central to the immune defects observed with HIV infection. Although the hallmark of HIV infection is a loss of CD4+ T cell numbers, impaired CD4+ T cell function can be observed prior to a decline in number (165), highlighting the importance of understanding early aspects of immune dysfunction that may contribute to a loss of virologic control and impaired cell-mediated immunity and immune competence despite an intact CD4+ T cell count.

1. T cell defects in HIV infection: CD4+ T cells provide help for the differentiation of antigen-specific effector CD8+ cytotoxic T lymphocytes (CTL), probably via activation of APC to upregulate costimulatory molecules (166). The loss of the CD4+ T cell compartment may therefore contribute to the decline in CTL function against a variety of pathogens, including a decline in HIV-specific CTL-mediated immunity. In addition, during late-stage disease with low CD4+ T cell counts, IFN-γ levels are reduced, which may contribute to the reduced cell-mediated immunity observed in HIV disease (167).

During progressive HIV infection and disease CD4+ T cells may be lost via numerous mechanisms. Direct infection of CD4+ T cells resulting in apoptosis, CD8+ T cytotoxic T cell or NK-mediated killing, and syncytia formation are well-documented modes of loss of infected T cells (168). However, the high level of plasma viremia with a low level of prevalence of CD4+ T cells infected with HIV suggests that CD4+ T cell killing by direct HIV infection is insufficient to explain the dramatic immunodeficiency associated with HIV infection (169), and that other indirect mechanisms of CD4+ T cell

loss are likely at play including activation-induced cell death, apoptosis induced by engagement of CD4 by HIV-1 gp120, infection of thymocytes and reduced thymopoeisis (168, 170). Loss of CD4+ T cell function or anergy in response to HIV antigens may additionally contribute to the deficiency in the CD4+ T cell compartment (169, 171). Infection of APCs with HIV may provide antigenic stimulation with insufficient costimulation resulting in HIV-specific CD4+T cell anergy (169).

Although CD4+ T cells are not always required for CD8+ T cell activation (166), CD4+ T cells do appear to be necessary for continued CTL activity in persistent viral infection. Whatever the nature of events culminating in loss of CD4+ T cells number and function, the lack of HIV-specific CD4 help likely contributes significantly to impaired CTL responses. This is the case with other models of chronic viral infection such as LCMV, wherein CTL activity is impaired in the absence of adequate T cell help (172). In fact, a strong protective HIV-specific CTL response is observed when a robust CD4+ T cell response occurs (173), and CTL activity appears to be essential for control of viremia and limiting disease progression (174, 175). Despite reduced plasma viremia and augmented CD4+ T cell numbers following highly active antiretroviral therapy (HAART), HIV-specific CD4 and CD8 functions remain impaired, unless HAART is administered during primary infection (176), emphasizing the need to understand key features in early immune defects that exacerbate disease.

*2. **Antigen presenting cells and HIV infection:*** As innate immune-sensing of pathogens and subsequent activation, maturation and/or differentiation of APCs is vital for shaping the character of the adaptive immune response, numerous studies have also addressed HIV-mediated effects on APCs and other key players of the innate immune response. Cells of the myeloid lineage are key players in the immunopathogenesis of

HIV infection. Tissue-resident macrophages and dendritic cells are among the first susceptible host cells exposed to HIV infection in the periphery (177) and while circulating monocytes in blood are infrequently found to be infected (178), HIV-infected macrophages can be readily identified in the central nervous system, lungs, tonsils, and adenoids (179-182). As myeloid cells are generally not susceptible to the cytopathic effects of HIV infection (183) and macrophages persist in peripheral tissues, allowing consistent low-level virus reproduction despite antiretroviral therapy (184), cells of the myeloid lineage represent key players in promoting HIV infection and spread via establishment of reservoirs of viral infection and replication.

Dendritic cells play an additional vital role in viral infection. As dendritic cells also reside in the periphery, they are among the first cells to become exposed to HIV at mucosal sites of infection (reviewed in (185, 186)). Due to the capacity of dendritic cells to migrate from the periphery into T cell areas of lymphoid tissues and become potent activators of naïve T cells, dendritic cells represent a mode of viral transmission from mucosal/peripheral tissues to target CD4+ T cells, ideally situated to become activated and permissive for HIV infection. While the existence of virus-producing dendritic cells in vivo is controversial, several in vitro and ex vivo studies have illustrated that dendritic cells harbor infectious HIV virus particles (181, 182), and migrate to T cell areas of draining lymph nodes of mucosal tissues, facilitating HIV spread by disseminating HIV to CD4+ T lymphocytes (187-190). At least two types of immature DC can be identified and purified from whole blood: myeloid DC (mDC) of myeloid origin (CD11c+, CD1c+), and plasmacytoid DCs (pDC), of proposed lymphocytic origin (191-193). Both types are readily infected with HIV in vitro and are reduced in numbers in HIV-infected individuals (194-197).

*3. **APC defects in HIV infection:*** Examination of mononuclear phagocytes from HIV-seropositive individuals ex vivo illustrated diminished respiratory burst and release of β-glucuronidase in response to *C. neoformans* (198). In vitro treatment of macrophages with HIV-1 gp120 impaired phagocytosis of yeast (199), and macrophages infected in vitro are less effective at intracellular killing of *Candida pseudotropiate* (200). Monocyte-derived macrophages obtained from PBMC from HIV seropositive donors exhibit a reduced capacity for killing of *T. gondii*, in an environment deficient in IFN-γ production (201). Taken together, these studies indicate that macrophages in the setting of HIV infection are defective in several aspects of antimicrobial activity.

With respect to the role of APCs in directing adaptive immune responses, Langerhans' cells (LC) from HIV seropositive donors exhibit normal levels of HLA-DR, but LCs and macrophages were less potent stimulators for allogeneic T lymphocytes than LCs and macrophages from healthy donors, and also exhibited decreased recall antigen and mitogen-induced T cell responsiveness (202). However, acute in vitro infection of a macrophage cell line suppressed HLA-DR protein and mRNA expression, and impaired proper localization of HLA-DR to late endosomes or lysosomes (203). Monocytes isolated from PBMC from HIV-infected individuals have reduced levels of surface expression of the B7 costimulatory molecule (204). Consistent with ex vivo observations of monocytes from HIV infected donors, APC treatment with gp120 interferes with costimulation of T cells via interruption of CD40/CD40L and CD28/B7.1 interactions (205). As APCs from HIV seropositive individuals clearly are impaired in innate and adaptive arms of the immune response, and are important targets of infection and sources of replicating virus and viral antigen, the APC compartment is likely an

important contributor to HIV immunopathogenesis, and may be partly accountable for the decline in HIV-specific and antimicrobial responses in general.

4. Altered Cytokine Production in HIV Infection: Early studies regarding cytokine imbalances occurring during HIV infection characterized an overproduction of Th2 cytokines, especially IL-10, but also TGF-β and IL-4, and an underproduction of Th1 cytokines such as IL-2 and IFN-γ ((206, 207) and reviewed in (208)). In addition, stimulus-induced IL-12 production from PBMC from HIV seropositive donors, and from monocytes infected with HIV-1 in vitro is dramatically impaired (209). As the cytokine milieu in the microenvironment present during antigen presentation is vital for determining the makeup of the adaptive immune response, cytokine imbalances in HIV-infected persons, particularly the underproduction of IL-12 has been suggested as a causative or vital contributing factor to the immune defects observed with HIV disease. The shift in Th-derived cytokines to a Th2 phenotype rather than a Th1 phenotype may prevent establishment of cell-mediated immune responses needed for eradication of intracellular pathogens, many of which are important sources of opportunistic infections that establish themselves in persons infected with HIV.

C. Interleukin-12 and HIV:

HIV disease is characterized by dysfunction in cell-mediated immunity, which is illustrated by numerous ex vivo measures of immune function including diminished proliferative responses to antigen, as well as a reduction in HIV-specific cytotoxic T lymphocyte activity (208, 210, 211). The impairment of T lymphocyte function occurs in asymptomatic individuals, prior to the reduction in CD4+ T cell numbers and

progresses over time (165, 206, 207). In parallel with this impaired cell-mediated immunity, peripheral blood mononuclear cells (PBMC) of HIV seropositive individuals produce less IL-12 in response to bacterial stimuli than PBMC from seronegative individuals (12, 209). This defect in IL-12 induction by HIV infection is somewhat specific, as production of other proinflammatory cytokines such as TNF-α and IL-1β is not reduced (209). As well, IL-12 may have a direct role in promoting HIV-specific immunity. Addition of IL-12 in vitro enhances the immune effector functions of PBMC from HIV seropositive individuals, as indicated by augmented IL-2 secretion and proliferation of PBMC in response to HIV envelope peptides (211, 212). IL-12 also enhances PBMC and CD4+ T cell proliferation and IFN-γ secretion in response to HIV-1 p24 antigen (213), and augments HIV-specific cytotoxic T lymphocyte (CTL) activity (171). In addition, prolonged suppression of viral load in HIV patients receiving antiretroviral therapy results in improved parameters of immune function, including enhanced IL-12 production (25, 214, 215). These observations suggest a possible reversibility of the impaired cell-mediated immunity during HIV infection, and indicate a potential role for IL-12 in promoting HIV-specific adaptive immune responses.

Numerous reports in the literature have demonstrated that IL-12 production in response to bacterial stimulation is impaired in HIV seropositive individuals, and that this defect may contribute to impaired cell-mediated immune responses, including HIV-specific adaptive immunity (83, 171, 209). To date, the mechanism by which HIV infection suppresses IL-12 expression remains unclear. Treatment of monocytes with soluble HIV-1 gp120 suppresses LPS-induced IL-12 production via induction of IL-10, a potent antagonist of IL-12 expression (8, 99, 216, 217). While enhanced production of

IL-10 has been documented in some HIV-infected individuals (11, 206, 218), other studies have shown no increase in IL-10 production in HIV-infected PBMC or monocytes cultured with bacterial stimuli (219, 220), and that inhibition of IL-12 induction from HIV+ PBMC is not reversed by neutralizing antibodies against IL-10 (209). In addition, exogenous HIV-1 Tat or Vpr protein impairs stimulus-induced IL-12 production in human monocytes (221, 222). Several reports have shown that IL-12 production defects in PBMC from HIV-positive patients may be overcome by addition of IFN-γ, IL-4, or IL-13 prior to bacterial stimulation (95, 223). Although IFN-γ, IL-4, and IL-13 can promote optimal IL-12 production under some circumstances, and IL-10 and TGF-β inhibit IL-12, it remains unclear whether the activity of these cytokines, or lack thereof, is responsible for impaired IL-12 induction by HIV infection of myeloid cells. Others have proposed that increased prostaglandin E2 production (224) or defective CD40 ligand induction (83) may contribute to reduced IL-12 production in HIV-infected individuals. While acute infection of monocytes in vitro results in suppression of IL-12 protein production following bacterial stimulation (209), an absolute requirement for productive cellular infection and a direct impact on IL-12 production in infected myeloid cells have not been clearly established.

At least eight putative transcription factor binding sites have been identified in the human IL-12 p40 promoter, yet the relative contributions of each of these sites in stimulus-induced transcription are poorly understood, and modulation of IL-12 p40 expression by HIV acting at one or several of these sites has not been addressed. Similarly, the effect of HIV infection on LPS-induced intracellular signalling for activation of IL-12 p40, particularly through MAP kinases, and any downstream

consequences on host cell transcriptional machinery acting at the IL-12 p40 promoter has not been examined.

D. Rationale:

Given the crucial role of interleukin-12 in modulating immune responses, and considering that IL-12 may enhance HIV-specific immunity, elucidating factors that influence IL-12 production by myeloid cells will advance the understanding of the immunopathogenesis of HIV infection and potentially lead to novel approaches to therapy. As IL-12 p40 protein and mRNA expression closely parallels production of the p70 heterodimer in response to numerous stimulatory and inhibitory agents, evaluation of HIV-mediated suppression of IL-12 production has been directed towards regulation of the highly inducible p40 subunit. Therefore, the purpose of the work described herein was to delineate the mechanisms by which HIV inhibits LPS-induced IL-12 p40 transcription in isolated myeloid cells.

E. Hypotheses:

1. HIV infection of myeloid cells in isolation (moncyte-derived macrophages (MDM) and THP-1 cells) suppresses induction of IL-12 p40 expression in response to bacterial stimulation.
2. Suppression of IL-12 p40 expression occurs directly, independent of the activity of other host- or virus-derived soluble factors, and requires productive infection by HIV.
3. HIV infection of myeloid cells affects transcription factor binding to the IL-12 p40 promoter, and this aberrant binding may be a result of impaired upstream LPS-induced intracellular signal transduction.

F. Specific Objectives:

1. To compare IL-12 p40 protein and mRNA expression in mock- and HIV-infected myeloid cells following bacterial stimulation;
2. To determine whether HIV-mediated inhibition of IL-12 p40 expression occurs directly, or indirectly requiring de novo protein synthesis;
3. To examine whether neutralization of antagonistic cytokines, specifically IL-10 and TGF-β, reverses the suppression of IL-12 p40 production by HIV infection;
4. To evaluate whether addition of IL-12-enhancing cytokines to HIV-infected myeloid cells restores deficient IL-12 p40 expression;
5. To ascertain whether suppression of IL-12 p40 induction in HIV-infected myeloid cell cultures is soluble factor-mediated, or is dependent upon productive infection by HIV;

6. To determine whether HIV impairs IL-12 p40 gene expression at the level of transcription;
7. To determine the effects of HIV infection on transcription factor binding to the IL-12 p40 promoter in myeloid cells;
8. To confirm that any transcription factor binding sites in the IL-12 p40 promoter which are affected by HIV infection are indeed implicated in LPS-induced promoter activation;
9. To examine whether HIV infection causes aberrations in LPS-induced signalling, particularly via MAP kinases.

II. MATERIALS & METHODS

A. Cell Lines and Virus Stocks:

1. Cell lines: The monocytic THP-1 cell line and the 8E5 CEM-derivative T cell line were obtained from the American Type Culture Collection (Manassas, VA). The THP-1 cell line was selected as a representative myeloid model allowing for productive HIV infection and IL-12 expression superior to HL-60 and U937 cells. Each cell line was maintained in suspension in T-75 tissue culture flasks in RPMI-1640 medium (Gibco/Invitrogen, Burlington, ON) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco), and 2.5 mg/L Fungizone (Bristol Myers Squibb, Montreal). Cells were replated at a density of 0.5 x 10^6 cells/ml every 3-4 days, and incubated at 37°C, 5% CO_2.

2. Virus Stocks: The dual-tropic HIV-1 clinical isolate, cs204, was a gift from Dr. F. Diaz-Mitoma (Children's Hospital of Eastern Ontario, Ottawa, Canada). HIV-1_{IIIB} was obtained from the National Institutes of Health AIDS Research and Reference Reagent Program (Bethesda, MD).

B. Monocyte Isolation and Culture:

1. PBMC Isolation: Peripheral blood mononuclear cells (PBMC) were isolated from HIV-seronegative healthy donors by diluting whole blood in a 1:1 ratio with 1X phosphate buffered saline (PBS; Gibco), and overlaying on Ficoll-Paque Plus (Amersham-Pharmacia Biotech, Baie d'Urfe, PQ) in 50 ml conical centrifuge tubes at a ratio of 2:1 diluted blood to Ficoll. Tubes were centrifuged at 400 x g for 30 minutes

with no brake, after which time the buffy coat layer was collected and washed twice in 4-fold excess PBS with centrifugation at 400 x g.

2. Isolation of Monocytes from PBMC: After washing of PBMC, cells were counted and resuspended in RPMI-1640 supplemented with 5% human type AB serum (Sigma, Oakville, ON), 2.5 mg/L Fungizone, 100 U/ml penicillin, and 100 μg/ml streptomycin, and plated at a concentration of 10^6 cells/ml in T-75 tissue culture flasks. After 90 minutes in culture at 37°C and 5% CO_2, non-adherent mononuclear cells were removed by gently washing twice with 1X PBS, and adherent monocytes were collected by scraping. Monocytes were pelleted at 400 x g and subsequently maintained in culture in RPMI-1640 medium supplemented with 5% AB serum and antibiotics. As monocytes isolated in this manner were subsequently cultured for a total of 12 days, including 7 days of mock or HIV infection and allowing for significant differentiation, this myeloid cell type will henceforth be referred to as monocyte-derived macrophages (MDM).

C. In Vitro HIV Infection:

1. Preparation and Expansion of Virus Stocks: The laboratory strain HIV-1_{IIIB} and the HIV-1 clinical isolate, cs204, were each expanded in THP-1 cells, and titrated for use in subsequent experiments in either THP-1 cells or MDM. Briefly, 5 x 10^6 THP-1 cells were incubated with 500 μl of each virus stock in a 1 ml total volume, and after 8 hours of incubation at 37°C, 5% CO_2, cells were washed once with 1X PBS, and plated in T-25 tissue culture flasks at 0.5 x 10^6 cells/ml. After 3 days, 10 ml of medium (RPMI + 10% FBS) were added, and cells were transferred into T-75 flasks, with addition of fresh medium every 3-4 days. On days 0, 7, and 14, culture supernatant aliquots of 1 ml

were harvested and stored at –80°C, and on day 14 of culture, HIV replication was verified by assaying supernatants for HIV-1 p24 antigen production by specific ELISA (NEN Life Science Products, Boston, MA). Once HIV-1 p24 antigen levels in culture exceeded 50 ng/ml, the total culture volume was harvested, centrifuged at 400 x g, and cell-free virus-containing supernatants were aliquoted and stored at –80°C for future use.

2. $TCID_{50}$ Determination of HIV-1 Virus Stocks: The 50% tissue-culture infectious dose ($TCID_{50}$) of each virus stock was determined against both THP-1 cells and MDM by titration of serial dilutions of each virus stock against a constant number of target cells. Virus stocks were typically generated to a titer of 10^5/$TCID_{50}$/ml. Briefly, in a 96-well flat-bottomed tissue culture cluster plate, 200 µl of virus stock diluted 1:12 with medium were plated in triplicate in one row. To the next 6 rows, 150 µl of medium (RPMI + 10% FCS in the case of THP-1 cells, or RPMI + 5% AB serum for MDM) are added in triplicate. Using a multichannel pipettor, 50 µl of virus-containing medium from the first row were transferred to the adjacent row, mixed, and virus was transferred in this manner across each of the next 5 rows, producing a series of 4-fold dilutions of virus. To every well in the plate, 50 µl of cells (4 x 10^6/ml) were added, and the plate was incubated at 37°C, 5% CO_2. On day 4 of culture, 150 µl of medium were discarded and replaced with fresh medium, followed by an additional 3 days of culture. On day 7, supernatants were harvested from each well, centrifuged to remove cells, and assayed for HIV-1 p24 antigen production by commercial ELISA (NEN Life Science Products, Boston, MA; see below). By this method a well was scored as positive if the p24 content exceeded 50 pg/ml (225). After all wells are scored, $TCID_{50}$ for the stock is calculated by the Spearman-Karber Method as follows (reviewed in (225)):

$M = xk + d[0.5 - (1/n)(\Sigma r)]$

Where xk = the dose of highest dilution

r = the number of negative wells

d = the spacing between dilutions

n = the number of wells of each dilution (i.e. 3)

$\therefore$ The 50% endpoint = 4^{-M}

And to convert to the 50% titer (10^x):

$x = M \bullet \log 4$

Finally, $TCID_{50}$/ml is determined by multiplying by a factor of 5 to correct for the original dilution (i.e. 1000 µl ÷ 200 µl).

3. HIV-1 p24 Antigen Quantitation: Monitoring productive infection of myeloid cells by HIV-1 and titration of resulting virus stocks was achieved, in part, by quantitation of HIV-1 core p24 antigen production by specific ELISA (NEN) according to manufacturers' instructions. Undiluted or 10-fold serially diluted virus-containing supernatants, or a serially diluted HIV-1 p24 protein standard of known concentration (100-12.5 pg/ml), were added to individual wells in a 96-well antibody-coated microtitre assay plate, along with 20 µl of 5% Triton X-100. Samples and standards were incubated for 2 hours at 37°C prior to washing and aspirating 4 times with an Elx50 Auto Strip Washer (Bio-Tek Instruments, Winooski, VT), followed by addition of a biotinylated α-p24 detector antibody. After 1 hour of incubation at 37°C and subsequent washing, streptavidin-conjugated horseradish peroxidase (HRP) was added to bind p24 antigen-detector antibody complexes, and incubated for 30 minutes at room temperature. HIV-1 p24 antigen in each sample or standard preparation was detected by the activity of HRP

against its substrate orthophenylenediamine-HCl (OPD), which produces a yellow colour with an intensity proportional to the concentration of HIV-1 p24 antigen protein present. The absorbance of each sample at 492 nm was quantitated with a Spectramax 190 microplate reader (Molecular Devices, Sunnyvale, CA). The lower limit of detection of this assay was 12.5 pg/ml.

4. In vitro infection of THP-1 and MDM for Examination of Impact of HIV Infection on IL-12 Synthesis: THP-1 cells (6 x 10^6) were incubated with 10^4 $TCID_{50}$ of HIV-1 virus isolate (multiplicity of infection 0.002) for 6 hours at 37°C, 5% CO_2, followed by washing with 1X PBS and subsequent culture at 0.5 x 10^6 cells/ml. Conversely, mock-infected cells were incubated with 500 µl of culture medium in lieu of virus, and thereafter treated in a manner identical to HIV-infected cultures. In preliminary investigations, a dose of 10^4 $TCID_{50}$ against 6 x 10^6 THP-1 cells was sufficient to dramatically suppress LPS-induced IL-12 p40 protein production. Alternatively, monocytes (6 x 10^6) were cultured for 5 days prior to infection with HIV-1 (multiplicity of infection 0.002), to allow sufficient adherence-induced activation of monocytes to promote productive HIV infection (226, 227). Cell culture medium was replenished every 3-4 days.

D. Cell Culture Conditions for Cytokine Induction:

1. Induction of Interleukin-12 Synthesis: As bacteria and bacterial products are potent inducers of interleukin-12 synthesis in myeloid cells, mock- and HIV-infected THP-1 and MDM (10^6/ml) were stimulated with either 1 µg/ml lipopolysaccharide (LPS: Sigma, Oakville, ON), 7.5 µg/ml *S. aureus* Cowan strain-1 (SAC) Pansorbin cells

(Calbiochem, Hornby, ON), or 100 μg/ml heat-killed *M. tuberculosis* (MTB) (Difco Laboratories, Detroit, MI), following 7 days of culture post-infection. In all experiments evaluating IL-12 p40 expression, THP-1 cells and MDM were incubated with stimulants for a total of either 4 or 24 hours, determined in preliminary investigations to be optimal for detection of IL-12 p40 mRNA and protein production, respectively. As initial experiments demonstrated that LPS was the most effective agent for the induction of IL-12 synthesis (figure 7A), subsequent investigations into the mechanisms of suppression of IL-12 synthesis by HIV infection were limited to the use of LPS as the IL-12-inducing stimulant.

2. Inhibition of de novo protein synthesis: To begin to examine the molecular mechanism(s) by which HIV infection of myeloid cells inhibits LPS-induced IL-12 expression, investigations were conducted to address whether HIV impaired IL-12 induction directly, or whether these suppressive effects were dependent upon de novo protein synthesis. After 7 days in culture, mock- and HIV-infected THP-1 cells and MDM were incubated with 10 μg/ml of the protein synthesis inhibitor cycloheximide (CHX) (Sigma) for 1 hour prior to stimulation with 1 μg/ml LPS (14). Cells were stimulated with LPS for a total of 24 hours prior to supernatant harvest for quantitation of IL-12 p40 protein production, and in parallel cultures for 4 hours prior to RNA extraction and evaluation of IL-12 p40 mRNA production.

3. Evaluation of the Role of Cytokines in HIV-Induced Suppression of IL-12:

i. Neutralization of IL-10 and TGF-β: To delineate any potential role for the activity of IL-12-inhibiting cytokines in HIV-mediated suppression of IL-12, myeloid cells were treated with neutralizing antibodies against IL-10 and TGF-β. Specifically, on

day 7 of culture, mock- and HIV-infected THP-1 cells and MDM were stimulated with 1 μg/ml LPS in the presence of saturating quantities of neutralizing antibodies to IL-10 (0.5 μg/ml; monoclonal mouse $IgG2_b$; R&D Systems, Minneapolis, MN) and/or TGF-β (1 μg/ml; total rabbit IgG antibody; R&D Systems).

ii. Priming with IFN-γ or IL-4/IL-13: In some experiments, cells were treated with priming cytokines prior to addition of LPS. Specifically, day 7 mock- and HIV-infected THP-1 cells and MDM (10^6/ml) were preincubated with IFN-γ (100 ng/ml; R&D Systems) for 14 hours (223), or with IL-4 and IL-13 (10 ng/ml each, R&D Systems) for 24 hours prior to stimulation with LPS (95).

iii. Determination of Role of Productive Viral Infection and Replication in HIV-Mediated Suppression of Interleukin-12 Synthesis:

a. Treatment of myeloid cells with replication-incompetent virus: THP-1 cells and MDM (6×10^6) were incubated for 6 hours with supernatants from 8E5 cells containing syncytium-inducing HIV-1 virions defective in reverse-transcriptase activity (10 ng HIV-1 p24/ml), in lieu of infection with replication-competent virus. After 7 days in culture, cells were stimulated with medium or LPS (1 μg/ml) in parallel cultures for 4 or 24 hours, prior to harvesting RNA or IL-12 p40-containing supernatants as described.

b. Incubation of myeloid cells with soluble HIV-1 gp120: THP-1 and MDM (10^6/ml) were incubated with 3 μg/ml soluble $HIV\text{-}1_{IIIB}$ gp120 (NIH AIDS Research and Reference Reagent Program) (216), for 24 hours prior to treatment with medium or LPS (1 μg/ml).

iv. Examination of role of soluble factors in HIV-mediated inhibition of IL-12 p40 induction by LPS: THP-1 cells were incubated with ultraviolet (UV)-irradiated

supernatants from mock- and HIV-infected THP-1 for 24 hours prior to LPS stimulation. Briefly, cryopreserved supernatants from cultures of THP-1 cells mock- or HIV-infected for 7 days were exposed to ultraviolet light for 1 hour inside a biological safety cabinet (228), prior to addition to uninfected THP-1 cells. After 24 hours of additional stimulation with medium or LPS (1 μg/ml), supernatants were harvested and frozen at –80°C, until IL-12 p40 protein was quantitated by specific ELISA (R&D Systems, see below).

E. IL-12 p40 Protein Quantitation:

1. Supernatant harvest: Following 24 hours of stimulation with bacterial stimuli as specified (1 μg/ml LPS, 7.5 μg/ml SAC, 100 μg/ml heat-killed MTB), supernatants were harvested into 1.5 ml eppendorf tubes and centrifuged for 5 minutes at 1500 x g. Cell pellets were discarded and cell-free supernatants were transferred into fresh 1.5 ml tubes and frozen at –80°C until future use.

2. IL-12 p40 ELISA: To quantitate IL-12 p40 protein production by THP-1 cells or MDM, stored supernatants were thawed, and assayed for IL-12 p40 protein content by commercial sandwich ELISA according to manufacturers' instructions (R&D Systems). Briefly, neat or appropriately diluted experimental supernatants, or a serially diluted IL-12 p40 protein standard of known concentration (2000-31.25 pg/ml), were added to individual wells in a 96-well microtitre assay plate. Samples and standards were incubated for 2 hours at room temperature, prior to washing and aspirating 4 times with an ELx50 Auto Strip Washer, followed by addition of a horseradish peroxidase (HRP)-conjugated anti-human IL-12 p40 antibody. After 1 hour of incubation at room

temperature and subsequent washing, IL-12 p40 protein in each sample or standard preparation was detected by the activity of HRP against its substrate tetramethylbenzidine (TMB). The reaction of HRP with TMB produces a yellow colour with an intensity proportional to the concentration of IL-12 p40 protein present, which was quantitated with a Spectramax 190 microplate reader with an absorbance filter for 450 nm), using SoftMax Pro software. The limit of detection of this assay was 15 pg/ml.

F. IL-12 p40 mRNA Quantitation:

1. Total RNA Extraction from THP-1 Cells: Following 4 hours of treatment with specified stimuli, total cellular RNA was extracted from mock- and HIV-infected THP-1 cells using TriPure Isolation Reagent according to manufacturers' instructions (Roche, Laval, QC). THP-1 cells (10^7) were collected, pelleted at 400 x g in a polypropylene centrifuge tube, and lysed in 1 ml of TriPure Isolation Reagent. 200 µl chloroform (Sigma) were added to each sample, and following 10 minutes at room temperature, the samples were centrifuged at 12,000 x g for 15 minutes in a 4°C chilled Eppendorf 5417R microcentrifuge (Brinkmann Instruments, Ltd., Mississauga, ON). The upper clear aqueous phase containing total cellular RNA was removed, placed into a fresh polypropylene microcentrifuge tube, and precipitated with 0.5 ml isopropanol (Sigma) for 10 minutes at room temperature. Samples were centrifuged at 12,000 x g for 10 minutes at 4°C, and pelleted RNA was washed with 1 ml of 75% ethanol. Following centrifugation at 7500 x g for 5 minutes at 4°C, RNA pellets were allowed to dry, resuspended in diethylpyrocarbonate (DEPC, Sigma)-treated water and heated to 60°C for 10 minutes. RNA extracts were frozen at –80°C until further use, at which time

samples were thawed, and RNA quantity and purity was determined by measuring absorbance at 260 nm and 280 nm wavelengths, using a Spectramax 190 spectrophotometer. Nucleic acid purity was calculated as the ratio of A_{260}/A_{280}, and was considered acceptable between 1.75 and 2.0. RNA concentration was calculated as A_{260} x 40 μg/ml.

2. Total RNA Extraction from MDM: Due to limitations in the number of MDM which could be obtained from healthy blood donors, total RNA was harvested from cultured MDM using the RNeasy RNA extraction system (Qiagen; Mississauga, ON), which reliably allowed extraction of sufficient quantity and purity of RNA from only 2 x 10^6 MDM grown in a 6-well tissue culture plate, compared with the TriPure method. After 4 hours in culture with LPS, MDM were rinsed twice with 1X PBS and lysed directly in the tissue culture plate using 350 μl of RNeasy Lysis Buffer RLT. After 5 minutes at room temperature, lysates were collected and homogenized by centrifugation at 12,000 x g in a Qiashredder column (Qiagen). To the homogenized lysate, 350 μl of 70% ethanol were added, and the resulting mixture was transferred to an RNeasy mini column, and centrifuged at 12,000 x g for 15 seconds. The flow-through was discarded, and the column was washed twice with 700 μl of wash buffer, with centrifugation at 12,000 x g for 15 seconds. RNA was eluted from the column by the addition of 50 μl of RNase-free water and centrifugation at 12,000 x g for 1 minute. RNA extracts were frozen at –80°C until further use, at which time samples were thawed, and RNA quantity and purity was determined as described above.

3. Quantitation of IL-12 p40 mRNA Expression in THP-1 Cells by Ribonuclease Protection Assay (RPA): IL-12 p40 mRNA expression in THP-1 cells was quantitated

using the RiboQuant ribonuclease protection assay system (Pharmingen, Mississauga, ON) according to manufacturers' instructions.

i. Probe Synthesis: Radiolabelled antisense mRNA riboprobes were transcribed from template cDNA for IL-12 p40 and GAPDH (50 ng) in a reaction mixture including the RNase inhibitor RNasin (40 U) a nucleotide pool containing dGTP, dATP, dCTP, dUTP (2.75 mM each), dithiothreitol (DTT, 5 mM), transcription buffer, 100 μCi of α-^{32}P-UTP (Amersham), and 20 U T7 RNA polymerase. The probe synthesis reaction proceeded at 37°C for 1 hour, and was terminated by the addition of 2 U RNase-free DNase, and further incubation at 37°C for 30 minutes. Radiolabelled riboprobes were isolated by phenol:chloroform extraction by addition of 26 μl of 20 mM EDTA, 25 μl of Tris-buffered phenol, 25 μl of chloroform/isoamyl alcohol (50:1), and 2 μg yeast tRNA. This mixture was vortexed and microcentrifuged at maximum speed for 5 minutes, and the upper aqueous phase was precipitated with 50 μl of 4M ammonium acetate and 250 μl 100% ethanol, with incubation at –80°C for 30 minutes. Riboprobes were pelleted by centrifugation for 15 minutes at 12,000 x g at 4°C, washed in 90% ethanol, dried, and resuspended in 50 μl hybridization buffer. To quantitate ^{32}P-incorporation into the riboprobe cocktail, duplicate 1 μl aliquots of the probe were counted in a Beckman LS6500 scintillation counter (Beckman-Coulter, Mississauga, ON) in the absence of scintillation fluid. The probe cocktail was diluted with hybridization buffer to a concentration of 3 x 10^5 cpm/μl for use in the hybridization reaction.

ii. Preparation and Hybridization of RNA Samples: Twenty μg of total cellular RNA, or 2 μg of yeast tRNA (negative control) or 2 μg of human positive control RNA were dried in an Eppendorf Concentrator 5301 vacuum evaporator centrifuge

(Brinkmann), and resuspended in 8 µl of hybridization buffer per sample. To each sample, 2 µl of diluted riboprobe cocktail (3 x 10^5 cpm/µl) were added, and following heating to 90°C, the reaction mixture was slowly cooled to 56°C, and hybridization proceeded overnight (12-16 hours). Prior to RNase treatment of the hybridization reaction, samples were equilibrated to 37°C for 15 minutes.

iii. RNase Digestion Reaction: To each hybridization mixture, 100 µl of RNase cocktail were added (25 ng RNase A and 150 U RNase T1), and incubated for 45 minutes at 30°C. This digestion reaction was terminated by the addition of 18 µl of proteinase K cocktail (including 15 µg proteinase K) and incubation at 37°C for 15 minutes. Digested, protected double-stranded mRNA transcripts for IL-12 p40 and GAPDH were isolated by phenol:chloroform extraction by the addition of 65 µl of Tris-buffered phenol and 65 µl chloroform/isoamyl alcohol (50:1). Following centrifugation at 12,000 x g for 5 minutes, the upper aqueous phase was extracted, and precipitated with 4M ammonium acetate and 650 µl 100% ethanol at –80°C for 30 minutes. Hybridized, digested RNA was pelleted by centrifugation at 12,000 x g for 15 minutes at 4°C, washed with 90% ethanol, and completely air-dried. To each sample, 5 µl of loading buffer were added, and each sample was denatured at 95°C for 3 minutes prior to gel loading.

iv. Resolution of Protected IL-12 p40 and GAPDH mRNA Fragments: Hybridized, RNase-digested RNA samples were resolved on a 6% denaturing polyacrylamide gel (19:1 acrylamide:bis-acrylamide, 7M urea). Following 30-60 minutes for polymerization, the gel was pre-run in a Kodak BioMax STS 45I sequencing gel apparatus (Interscience; Markham, ON) for 45 minutes at 40 watts with a Power Pak

1000 power source (Bio-Rad Laboratories, Mississauga, ON) in 0.5X TBE (45 mM Tris pH 8.3, 45 mM boric acid, 1 mM EDTA). Protected mRNA fragments were resolved by electrophoresis for 2 hours at 50 watts. Gels were dried onto Whatmann 3mm chromatography paper for 1.5 hours at 80°C using a Model 583 gel drier and HydroTech vacuum pump (Bio-Rad), exposed to a Phosphor-Screen (Molecular Dynamics/ Amersham), and visualized using a Storm Phosphorimager and ImageQuant software (Molecular Dynamics/Amersham).

4. Determination of IL-12 p40 mRNA Expression in MDM by Semi-Quantitative RT-PCR: As the number of MDM, and hence, the amount of RNA that could be obtained from one individual was insufficient to enable quantitation of IL-12 p40 mRNA by Ribonuclease Protection Assay, IL-12 p40 mRNA levels in MDM were assessed by semi-quantitative RT-PCR.

i. Reverse Transcription: One μg of total monocyte RNA was reverse transcribed using the 1st-STRAND cDNA Synthesis Kit (Clontech, Palo Alto, CA) according to manufacturers' instructions. The 20 μl reverse-transcription reaction mixture included 1 μg total cellular RNA, 20 pmol oligo $(dT)_{18}$ primer, 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 0.5 mM each of dATP, dTTP, dCTP, dGTP, 40 U RNase inhibitor, and 200 U MMLV reverse transcriptase. The reverse transcription reaction was carried out in a Perkin-Elmer 9600 Thermocycler (Perkin-Elmer, Mississauga, ON) for 1 hour at 42°C, and was terminated by heating to 94°C for 5 minutes.

ii. Determination of Input cDNA: Using an cDNA sample derived from reverse-transcription of an RNA sample from LPS stimulated MDM, 5 μl of neat or

serial 2-fold dilutions of cDNA were PCR amplified with gene-specific primers (see below for oligonucleotide sequences and reaction conditions) for IL-12 p40 or GAPDH housekeeping gene control, in order to determine the appropriate dilution of cDNA that would be used to best compare alterations in mRNA expression between experimental samples. The quantity of starting cDNA to be used in experimental PCR reactions was selected based on the dilution of cDNA which appeared to be sub-saturating for the PCR reaction, based on visualization of PCR products resulting from serial dilutions of input cDNA.

iii. PCR Amplification of IL-12 p40 and GAPDH cDNA: Total cellular RNA was extracted from MDM, and reverse-transcribed as described. Five µl of appropriately diluted cDNA from each experimental sample were PCR amplified with gene-specific primers for either human IL-12 p40 or for GAPDH, using the Advantage cDNA PCR Kit (Clontech). Each 50 µl PCR reaction mixture included 1 µl 50X Advantage cDNA Polymerase Mix (1% glycerol, 0.8 mM Tris-HCl pH 7.5, 1 mM KCl, 0.5 mM $(NH_4)_2SO_4$, 2 µM EDTA, 0.1 mM β-mercaptoethanol, 0.005% Thesit, 1.1 µg/µl TaqStart Antibody, 1.1 µg/µl KlenTaq-1 DNA polymerase), 5 µl 10X cDNA PCR reaction buffer (40 mM Tricine-KOH pH 9.2, 15 mM KOAc, 3.5 mM $Mg(OAc)_2$, 3.75 µg/ml Bovine serum albumin), 1 µl 50X dNTP mix (0.2 mM each dATP, dTTP, dCTP, dGTP), and 50 pmol each of sense and antisense primer. Gene-specific primers for PCR were as follows: IL-12 p40 sense 5'-GGACCAGAGCAGTGAGGTCTT-3'; IL-12 p40 antisense: 5'-CTCCTTGTTGTCCCCTCTGA-3'; GAPDH sense 5'-CCACCCATGG CAAATTCCATGGCA-3'; GAPDH antisense: 5'-TCTAGACGGCAGGTCAGGTCC ACC-3') (Stratagene, La Jolla, CA). PCR cycling was carried out in a Perkin-Elmer

9600 Thermocycler (Perkin-Elmer) for 25 and 30 cycles for GAPDH and IL-12 p40, respectively, as follows: 95°C for 30 seconds, 63°C for 30 seconds, and 72°C for 40 seconds. PCR reactions were preceded by 5 minutes of denaturation at 94°C, and were followed by a final 10-minute extension step at 72°C. PCR products were electrophoretically separated on a 1% agarose gel in 0.5X TBE at 90 V for approximately 90 minutes. Following electrophoresis, agarose gels were stained in ethidium bromide (0.5 μg/ml; Sigma) for 30 minutes, followed by brief destaining in water, prior to visualization and recording using a UVP White/UV transilluminator (Diamed, Mississauga, ON).

5. Interleukin-12 Bioassay: As production of the human bioactive IL-12 p70 heterodimer was often found to be below the limit of detection of a commercial ELISA (human IL-12 p70 ELISA, R&D Systems), production of IL-12 p70 in myeloid cell cultures was confirmed using a bioassay based on the ability of IL-12 p70 to augment PHA-induced IFN-γ production from human T lymphocytes (143).

i. Isolation of human T lymphocytes by magnetic bead selection: Whole blood was obtained from HIV-seronegative volunteers, and PBMC were isolated by density gradient centrifugation on Ficoll as described. Primary T lymphocytes were obtained by positive selection using magnetic beads conjugated to anti-human CD4 and anti-human CD8 antibodies (Miltenyi Biotech, Auburn, CA) according to manufacturers' instructions. Briefly, PBMC were resuspended in PBS to a concentration of 10^7 cells in 80 μl, and incubated with 20 μl anti-CD8 microbeads per 10^7 cells, for 20 minutes at 4°C. Cells were washed once in PBS, and following centrifugation at 400 x g for 5 minutes, cells were resuspended in 500 μl PBS per 10^8 cells, and applied to an LS type

column suspended in a MiniMACS magnet (Miltenyi). Flow-through cells were kept, the column was removed from the magnet, and the CD8+ fraction of cells was eluted in PBS. The remaining fraction of PBMC were resuspended to 10^7 cells in 80 µl, and incubated with 20 µl of anti-CD4-conjugated microbeads for 20 minutes at 4°C. Cells were washed once with PBS, resuspended in 500 µl PBS per 10^8 cells, and applied a new LS column supported in the MiniMACS magnet. Flow-through cells were discarded, and the CD4+ fraction was eluted with PBS, and pooled with the previously isolated CD8+ cellular fraction.

ii. Induction of IFN-γ: Primary T lymphocytes (2 x 10^6/1 ml total volume) were stimulated with medium or PHA (15 µg/ml; Gibco) for 48 hours in the presence or absence of cryopreserved supernatants derived from medium or LPS-stimulated THP-1 cells. In parallel cultures, anti-human IL-12 p70 neutralizing antibody was included (5 µg/ml; R&D Systems) to confirm the specificity of any IFN-γ enhancing effect for bioactive IL-12 p70. Alternatively, T lymphocytes were stimulated with medium or PHA, in the presence or absence of recombinant human IL-12 p70 (5 ng/ml; R&D Systems) as a positive control. Supernatants were harvested and cryopreserved, until secreted IFN-γ in T cell cultures was quantitated by commercial ELISA (R&D Systems).

iii. Quantitation of IFN-γ production by ELISA: IFN-γ production in PHA-stimulated human T lymphocytes was measured by commercial sandwich ELISA according to manufacturers' instructions (R&D Systems). Briefly, neat or appropriately diluted experimental supernatants, or a serially diluted IFN-γ protein standard of known concentration (1000-15.6 pg/ml), were added to individual wells in a 96-well microtitre assay plate. Samples and standards were incubated for 2 hours at room temperature,

prior to washing and aspirating 4 times with an ELx50 automated plate washer, followed by addition of a horseradish peroxidase (HRP)-conjugated anti-human IFN-γ antibody. After 1 hour of incubation at room temperature and subsequent washing, IFN-γ protein in each sample or standard preparation was detected by the activity of HRP against its TMB substrate, producing a yellow colour with an intensity proportional to the concentration of IFN-γ present. Absorbance at 450 nm was measured using a Spectramax 190 microplate reader and SoftMax Pro software. The limit of detection of this assay was 8 pg/ml.

G. Flow Cytometric Analysis of Intracellular Interleukin-12 p40 and HIV-1 p24 Antigen Production:

As an indirect indication as to whether productive infection was required for HIV-mediated suppression of IL-12 p40 production, flow cytometry was employed to evaluate IL-12 p40 protein expression at the single cell level in mock- and HIV-infected THP-1 cells. After 7 days in culture, mock- and HIV-infected THP-1 cells (10^6/ml) were treated with medium or LPS (1 μg/ml) for 24 hours, with addition of 8 μl of GolgiStop (BD Pharmingen, Mississauga, ON) for the last 12 hours of culture to prevent protein secretion. Cells were washed with PBS + 0.5% bovine serum albumin (BSA, Sigma) + 2 mM EDTA, centrifuged at 400 x g, and resuspended in 100 μl of fixation reagent (Caltag Fix-and-Perm; Cedarlane, Hornby, ON) for 15 minutes at 4°C, and washed again in PBS + 0.5% BSA + 2 mM EDTA. Cell pellets were resuspended in 100 μl permeabilization reagent (Caltag Fix-and-Perm Kit) for 10 minutes at 4°C. Prior to incubation with antibodies, permeabilized cells were incubated with PBS + 0.5% BSA +

5% human AB serum for 20 minutes at 4°C, to minimize nonspecific antibody binding. Cells were aliquoted into 12 x 75 mm polystyrene test tubes at 2 x 10^5 cells per sample, and incubated with 5 µl RD1-labelled anti-HIV-1 p24 antibody (mouse IgG_1, Beckman-Coulter, Mississauga, ON), or 2.5 µg PE-labelled anti-human IL-12 p40/p70 antibody (mouse IgG_1, BD Pharmingen), or with 2.5 µg of PE-labelled mouse IgG_1 isotype control (BD Pharmingen) for 30 minutes at 4°C. Intracellular expression of IL-12 p40/p70 protein and HIV-1 p24 antigen was measured by flow cytometric analysis using an ALTRA flow cytometer Expo32 analysis software (Beckman-Coulter).

H. Immunofluorescence Microscopy:

To further evaluate IL-12 p40 protein production in HIV-infected THP-1 cells, day 7 mock- and HIV-infected THP-1 were stimulated with medium or LPS in the presence of GolgiStop (Pharmingen) as described above. Cells were washed with PBS and immobilized on poly-L-lysine (0.1 mg/ml, Sigma) coated cover slips at 10^6 cells/ml for 15 minutes at room temperature. Cover slips were fixed in 4% paraformaldehyde for 20 minutes at room temperature, and permeabilized in 0.5% Triton-X-100 in PBS with 10% AB serum for an additional 10 minutes at room temperature. As a control for intracellular expression of IL-12 p40/p70 and HIV-1 p24 antigen, additional samples were fixed in 4% paraformaldehyde without permeabilization. Specimens were incubated in blocking buffer (3% BSA, 10% human AB serum) for 30 minutes prior to staining with 10 µg monoclonal anti-human IL-12 p40/p70 primary antibody (Pharmingen) for 1 hour at room temperature. After washing in PBS, 10 µg FITC-labelled anti-mouse-IgG_1 (Pharmingen) secondary antibody was added for 1 hour. Cells

were washed and subsequently stained with 20 µl monoclonal anti-HIV-1-p24-RD1 (KC57: Beckman-Coulter), or with 10 µl of PE-mouse IgG_1 isotype control (BD Pharmingen) and with DAPI (4,6-Diamidino-2-phenylindole, Sigma) nuclear stain (0.1 µg/ml) for 20 minutes. Cover slips were dried, mounted in 50% glycerol, and visualized using an Axioplan2 fluorescence microscope (Zeiss, Germany).

I. Nuclear Run-On Transcription Assay:

The relative rate of transcription of THP-1 cells following HIV infection was evaluated using a nuclear run-on transcription assay.

1. Isolation of intact nuclei: Mock- and HIV-infected THP-1 ($5x10^7$ total cells at 10^6/ml) were stimulated for 4 hours with medium or LPS (1 µg/ml), after which time the cells were harvested and washed twice in ice-cold PBS with centrifugation at 400 x g. Cells were resuspended in 1 ml of solution A per 3 x 10^7 cells (3M sucrose, 60 mM KCl, 15 mM NaCl, 15 mM Hepes pH 7.5, 2 mM EDTA pH 8.0, 0.5 mM EGTA pH 8.0, 0.15 mM spermine, 0.5 mM spermidine, 0.5 mM DTT, 0.1 mM phenylmethanesulfonyl fluoride (PMSF), supplemented with 157 µg/ml benzamidine, 190 µg/ml iodoacetamide, 1 µg/ml trypsin inhibitor, and 1 µg/ml leupeptin). This mixture was incubated on ice for 5 minutes in the presence of 5 µl of 10% NP-40 (BDH Chemicals; Toronto, ON) and mechanically homogenized with 20 strokes with a chilled Dounce homogenizer. The homogenate was transferred to a 1.5 ml polypropylene microcentrifuge tube and centrifuged at 500 x g for 5 minutes at 4°C. Pelleted nuclei were resuspended with 50 µl of solution E (50% glycerol, 20 mM Tris pH 7.9, 75 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 100 µM PMSF, and 10 U/ml RNase inhibitor).

*2. **RNA Transcription Reaction:*** The transcription reaction proceeded at 28°C for 45 minutes, in a 200 µl reaction mixture including 50 µl of isolated nuclei, 250 µCi α-^{32}P-UTP, reaction buffer (0.3 M ammonium sulfate, 0.1 M Tris pH 7.9, 4 mM $MgCl_2$, 4 mM $MnCl_2$, 0.2 M NaCl, 0.4 mM EDTA pH 8.0, and 0.1 mM PMSF) and 1.5 mM DTT, 10 mM creatine phosphate, 10 mM each GTP, ATP, CTP, 0.25 U/µl RNase inhibitor, 1 mM PMSF, and 5% glycerol. The transcription reaction was terminated by the addition of RNase-free DNase (0.1 U/µl; Sigma) and yeast tRNA (0.55 µg/µl; Gibco), followed by incubation at 37°C for 30 minutes. Proteinase K was added (200 µg/ml; Gibco) in the presence of 10 mM Tris-HCl pH 7.9, 10 mM EDTA, and 0.5% SDS, and incubated with the reaction mixture at 42°C for 30 minutes.

Radiolabelled nascent transcripts were extracted from nuclei using TriPure Isolation Reagent (Roche) according to manufacturers' instructions, as described in section F.1 of the Methods. RNA was precipitated at –20°C overnight, prior to preparation for hybridization to target cDNA.

*3. **Preparation and Immobilization of Target cDNA:*** Target cDNA was prepared from PCR products for human IL-12 p40 and the GAPDH housekeeping gene control, inserted into the T/A cloning vector pT-Adv (Clontech) or the PCR cloning vector pPCR-Script Amp SK(+) (Stratagene), respectively, for use as target sequences to detect IL-12 p40 and GAPDH mRNA transcripts.

i. T/A cloning of human IL-12 p40 PCR products: Target cDNAs for IL-12 p40 transcripts were prepared by directly subcloning PCR products for human IL-12 p40 into the pT-Adv TA cloning vector, using the AdvanTAge PCR Cloning Kit (Clontech). IL-12 p40 cDNA was PCR-amplified from RNA isolated from LPS-stimulated THP-1 cells,

as described using previously optimized PCR reaction and cycling conditions (see above).

a. Ligation: PCR products for IL-12 p40 were directly ligated into the pT-Adv vector (Clontech) in a 1:1 insert to vector molar ratio, in a 10 µl reaction mixture containing 50 ng pT-Adv, and 4 U of T4 DNA ligase. The ligation reaction proceeded at 14°C overnight in a Perkin-Elmer 9600 thermocycler, prior to transformation into DH5α supercompetent cells.

b. Transformation: DH5α cells were thawed on ice, combined with 2 µl of each ligation reaction, and incubated on ice for 30 minutes. Transformation reactions were heat-pulsed at 42°C for 45 seconds, and incubated on ice for an additional 2 minutes. To each transformation, 250 µl SOC medium (Gibco/Invitrogen) warmed to 37°C were added, followed by shaking at 225 rpm at 37°C for 1 hour in an orbital shaker (Model 4535, Forma Scientific/Fisher, Ottawa, ON). Cells were plated onto 100 mm LB agar plates (10 g/L NaCl, 10 g/L tryptone, 5 g/L yeast extract, 20 g/L agar) supplemented with 50 µg/ml ampicillin, 100 µl of 2% 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal) and 100 µl of 10 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG), and incubated overnight at 37°C.

c. Verification of insertion of IL-12 p40 cDNA into pT-Adv: White colonies were selected from overnight cultures of transformed DH5α cells, and spiked into 5 ml LB broth cultures, supplemented with 50 µg/ml ampicillin, and incubated overnight with shaking at 225 rpm, 37°C in an orbital shaker. Plasmid DNA was extracted from these cultures using the QIAprep Spin Miniprep Kit (Qiagen) according to manufacturers' instructions. Briefly, bacterial cells were pelleted in 1.5 ml

microcentrifuge tubes, by centrifugation at 1000 x g for 5 minutes. Cells were resuspended in 250 µl buffer P1 (50 mM Tris-Cl pH 8.0, 10 mM EDTA, 100 µg/ml RNase A), and lysed for 5 minutes on ice with 250 µl buffer P2 (200 mM NaOH, 1% SDS). Lysates were neutralized by addition of 350 µl buffer N3, followed by centrifugation at 12,000 x g for 10 minutes. Supernatants were added to the spin columns, centrifuged at 12,000 x g for 1 minute, washed with 750 µl buffer PE, and eluted with 75 µl 10 mM Tris-HCl, pH 8.5. Plasmid DNA extracted in this manner was used as a template for PCR amplification with IL-12 p40 gene-specific primers, followed by agarose gel electrophoresis, to confirm incorporation of IL-12 p40 cDNA into pT-Adv.

ii. PCR cloning of human GAPDH PCR products: Target cDNA for GAPDH transcripts were prepared by subcloning PCR products for GAPDH into the pPCR-Script Amp SK(+) cloning vector, using the PCR-SCRIPT Amp Cloning Kit (Stratagene). GAPDH cDNA was PCR-amplified from RNA isolated from LPS-stimulated THP-1 cells, as described, using standardized PCR reaction and cycling conditions (see above).

a. Purification and polishing of PCR insert: Prior to ligation of GAPDH cDNA into pPCR-Script Amp SK(+), PCR products were first purified from PCR reactions by incubation with Microspin 400 Columns (Amersham), and centrifugation at 735 x g for 2 minutes to remove primers, enzymes, and excess nucleotides. Ends of purified PCR products were polished in a 13.3 µl reaction including 2.5 mM each dNTP, and 0.5 U *Pfu* DNA polymerase. This reaction proceeded at 72°C for 30 minutes in a Perkin-Elmer 9600 thermocycler. In parallel, the pT-Adv vector from the Clontech TA cloning kit (above) and the pPCR-Script cloning vector were polished for subsequent

ligation in the absence of PCR product insert, to serve as empty vector controls in the nuclear run on assay.

b. Ligation and transformation: PCR products were ligated with 10 ng of pPCR-Script cloning vector in a 40:1 insert-to-vector molar ratio, in a 10 µl reaction containing 1 µl 10X ligation buffer, 0.5 µl 10 mM rATP, 5 U *SrfI*, and 4 U T4 DNA ligase. In parallel reactions, polished pT-Adv and pPCR-Script vectors were ligated in the absence of PCR inserts, in reactions omitting the *SrfI* restriction enzyme. The ligation reactions were incubated at room temperature for 1 hour, followed by heating to 65°C for 10 minutes prior to transformation into DH5α supercompetent *E. coli* cells, as described. Transformations were plated onto LB agar plates supplemented with 50 µg/ml ampicillin, 100 µl of 2% X-gal, and 100 µl of 10 mM IPTG as described, and white colonies were cultured and screened as described above, to verify incorporation of GAPDH cDNA. Blue colonies were selected from cultures of transformations resulting from ligations of control plasmids without cDNA inserts.

iii. Large-scale plasmid purification: Following verification of bacterial colonies containing IL-12 p40- or GAPDH-incorporating vectors, or empty control pT-Adv and pPCR-Script vectors, these cultures were upscaled for large-scale isolation of these plasmids using the Qiagen Plasmid Maxi Kit (Qiagen). Briefly, cells from 250 ml of each bacterial culture were pelleted by centrifugation at 6000 x g for 15 minutes at 4°C in a Beckman Avanti J-25I Centrifuge (Beckman-Coulter). Bacterial pellets were resuspended in 10 ml of buffer P1 (50 mM Tris-Cl pH 8.0, 10 mM EDTA, 100 µg/ml RNase A), and lysed in 10 ml buffer P2 (200 mM NaOH, 1% SDS) for 5 minutes. Lysates were neutralized by the addition of 10 ml buffer P3 (3M potassium acetate pH

5.5), and incubated on ice for 20 minutes prior to centrifugation at 20,000 x g for 30 minutes at 4°C. Supernatants were centrifuged again immediately at 20,000 x g for 15 minutes at 4°C and added to a QIAGEN-tip 500 column, equilibrated in buffer QBT (750 mM NaCl, 50 mM MOPS pH 7.0, 15% isopropanol, 0.15% Triton X-100). Flow-through was discarded and columns were washed twice with 30 ml of buffer QC (1M NaCl, 50 mM MOPS pH 7.0, 15% isopropanol), and plasmid DNA was eluted with 15 ml buffer QF (1.25M NaCl, 50 mM Tris-Cl pH 8.5, 15% isopropanol). Flow-through was precipitated with 0.7 volumes of isopropanol, centrifuged at 15,000 x g for 30 minutes at 4°C, washed with 70% ethanol, dried, and resuspended in 500 µl 10 mM Tris-HCl, pH 8.5.

iv. Immobilization of target cDNA: Plasmids containing target cDNA for IL-12 p40 and GAPDH, or empty control vectors, were linearized and denatured prior to blotting onto nitrocellulose membrane.

a. Linearization and denaturation of plasmid DNA: The IL-12 p40/pT-Adv vector or empty control plasmid were linearized by restriction digestion with 3 U/µg DNA of *SacI* (Amersham), while the GAPDH/pPCR-Script vector or empty control plasmid were linearized with 3 U/µg DNA of *HindIII* (Amersham). Digests were carried out overnight at 37°C, and heat inactivated by incubation for 15 minutes at 60°C for *SacI*, and at 90°C for *HindIII* digests. Linearized vectors were extracted with 25:24:1 phenol/chloroform/isoamyl alcohol, and precipitated with 40 µl 3M sodium acetate and 1 ml 100% ethanol. Precipitation proceeded at –20°C overnight, followed by centrifugation at 12,000 x g for 15 minutes at 4°C, and washing with 75% ethanol.

Linearized DNA was dried, resuspended in 10 mM Tris-HCl pH 8.5, and quantitated using the Spectramax 190 spectrophotometer.

b. Membrane Blotting: Linearized target plasmid was dotted (10 μg/dot) onto Hybond-ECL nitrocellulose membrane (Amersham) using a Bio-Dot Microfiltration Apparatus (Bio-Rad). Each preparation of linearized target or control vector DNA was denatured by the addition of an equal volume of 1N NaOH and subsequent heating at 65°C for 30 minutes. Samples were neutralized with 1.6 volumes of 2M ammonium acetate, and set on ice. Nitrocellulose was pre-soaked in 6X SSC (90 mM sodium citrate pH 7.0, 0.9 M NaCl) for 10 minutes prior to assembly of the dot-blot apparatus. Target or control DNA was dotted at 10 μg/dot under gentle vacuum, followed by rinsing of each well with 2X SSC (30 mM sodium citrate pH 7.0, 0.3 M NaCl). Nitrocellulose was allowed to dry overnight, prior to baking for 2 hours at 80°C in a vacuum oven. Prior to use for detection of mRNA transcripts, nitrocellulose membranes were prehybridized overnight at 42°C in hybridization buffer (see below).

v. Hybridization of nuclear mRNA transcripts to target cDNA: Following overnight isopropanol precipitation, labelled transcripts were pelleted by centrifugation at 12,000 x g for 15 minutes at 4°C, washed once with 70% ethanol, and resuspended in 400 μl of hybridization solution (50% deionized formamide, 5X SSPE [50 mM phosphate buffer pH 7.4, 745 mM NaCl, 5 mM EDTA], 1X Denhardt solution [0.2 mg/ml Ficoll, 0.2 mg/ml polyvinyl pyrrolidone, 0.2 mg/ml BSA], 0.1% SDS, and 0.4 mg/ml salmon sperm DNA [Invitrogen]). RNA in hybridization solution was heated at 65°C for 15 minutes, and 10 μl aliquots were counted using a Beckman LS6500 scintillation counter. Radiolabelled RNA samples were resuspended in hybridization

solution and denatured by heating at 85°C for 10 minutes, prior to hybridization with denatured target cDNA immobilized on nitrocellulose membrane.

Nascent radiolabelled mRNA transcripts were hybridized to target IL-12 p40 and GAPDH cDNA in a total volume of 500 µl in 1 ml polystyrene screw-cap tubes for 48 hours at 42°C, with constant rotation in a Shake 'n' Stack hybridization oven (Hybaid Ltd., Interscience). Membranes were washed once with 2X SSC/0.1% SDS at room temperature, twice with 1X SSC/0.1% SDS at room temperature, and once with 1X SSC/0.1% SDS at 42°C. Nitrocellulose membranes were air-dried, and hybridized mRNA transcripts were visualized following exposure to a Phosphor Screen and subsequent imaging and analysis with a Storm Phosphorimager and ImageQuant Software (Molecular Dynamics/ Amersham).

J. Electrophoretic Mobility Shift Assays (EMSA):

In order to assess alterations in binding of nuclear factor complexes to the human IL-12 p40 promoter following HIV infection, electrophoretic mobility shift assays (EMSA) were performed using nuclear extracts derived from THP-1cells and MDM.

1. Oligonucleotide Preparation and Labelling:

i. Annealing single-stranded oligonucleotides: Oligonucleotide sequences used for EMSA analysis of nuclear factor binding to the human IL-12 p40 promoter are listed in table 1, and were synthesized along with their complementary sequences by Gibco (NF-κB, C/EBP) or by The University of Ottawa Biotechnology Research Institute (AP-1, Sp1, Ets-2, IRF-1, NF-IL-6). Oligonucleotides were resuspended in TEN buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA, 100 mM NaCl) to a final concentration of 1 µg/µl, and complementary strands were mixed in an equimolar ratio in a 0.5 ml polypropylene

microcentrifuge tube, and denatured at 95°C. Oligonucleotides were annealed by gradually equilibrating to room temperature overnight.

TABLE 1: *Oligonucleotide Sequences for EMSA Analysis of Nuclear Factor Binding to the Human IL-12 p40 Promoter:*

Binding Site	Oligonucleotide Sequence
C/EBP	5'-TTG TTT TCA ATG TTG CAA CAA GT-3'
NF-κB	5'-AGG AAC TTC TTG AAA TTC CCC CAG AAG GTT TT-3'
Ets-2	5'-AAA GTC ATT TCC TCT TAG TTC ATT A-3'
AP-1	5'-TCC TTC CTT ATT CCC CAC CCA-3'
Sp1	5'-GTC TGA CCG CCC CTT GGC-3'
NF-IL-6	5'-GTA TGT TTC TGA AAT TAA AGG-3'
IRF-1	5'-TGA GGG TAT TTC ACT TTC TGC TCC-3'

ii. End-labelling dsDNA probes: Double-stranded oligonucleotide probes were end-labelled with γ-^{32}P-ATP prior to use in electrophoretic mobility shift assays. The 30 µl labelling reaction mixture was composed of 20 U T4 polynucleotide kinase (Amersham), 0.1 µg of double-stranded oligonucleotide, 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, and 50 µg/ml BSA, and proceeded at 37°C for 1 hour. The end-labelling reaction was terminated by the addition of 1 µl 0.5M EDTA, and unincorporated nucleotides were removed using MicroSpin G-50 Columns (Amersham) according to manufacturers' instructions. The resin in the column was resuspended by vortexing, and the column was pre-spun at 735 x g for 3 minutes to remove TE equilibration buffer. Each labelling reaction was added to a column, centrifuged at 735 x g for 3 minutes, and rinsed with 200 µl TE buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA). Following removal of excess unincorporated nucleotides, end-labelled probes in the column flow-through were precipitated in 1/10 volume of 3M sodium acetate and 2

volumes of 95% ethanol, at –20°C overnight. Probes were pelleted by centrifugation at 12,000 x g for 15 minutes at 4°C, washed once with 75% ethanol, dried, and resuspended in 50 µl ddH_2O for use in EMSA reactions.

2. Nuclear Extract Preparation from THP-1 Cells and MDM: Mock- and HIV-infected THP-1 and MDM (5 x 10^6 cells/ condition, cultured at 10^6 cells/ml) were cultured with medium or 1 µg/ml LPS for 2 hours prior to extraction of nuclear DNA binding proteins. THP-1 cells were washed with ice-cold PBS and centrifuged at 400 x g for 5 minutes. Alternatively, MDM were collected by incubation for 10 minutes in 1X trypsin-EDTA (Invitrogen), prior to centrifugation and washing with ice-cold PBS. THP-1 or monocyte cell pellets were resuspended in hypotonic lysis buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, pH 7.9) for 5 minutes on ice, and cytoplasmic membranes were disrupted by mechanical lysis with 20 strokes in a Dounce homogenizer. Nuclei were pelleted by centrifugation at 12,000 x g for 5 minutes at 4°C, and nuclear DNA-binding protein was stripped from nuclei by incubation on ice for 15 minutes in high-salt buffer (20 mM HEPES, 25% glycerol, 420 mM KCl, 1.5 mM $MgCl_2$, 2 mM EDTA, pH 7.9), followed by addition of a neutralization buffer (20 mM HEPES, 25% glycerol, 0.2 mM EDTA, pH 7.9). Each buffer was supplemented with 2 µg/ml pepstatin A, 10 µg/ml aprotonin, 2 µg/ml leupeptin, 0.5 mM DTT, and 0.575 mM PMSF prior to use. Cellular debris was pelleted by centrifugation at 12,000 x g for 10 minutes at 4°C, and supernatants containing nuclear DNA binding proteins were aliquoted and stored at -80°C until use in DNA binding assays.

3. Electrophoretic Mobility Shift Assay Reactions: To assess LPS-inducible IL-12 p40 promoter binding activity of nuclear factors from THP-1 cells and MDM, nuclear

extracts were incubated with ^{32}P-labelled double-stranded oligonucleotide probes, representing putative transcription factor binding sites represented in the IL-12 p40 promoter. Five μg of nuclear extract from each treatment condition were incubated in a reaction mixture (1 mM Tris-HCl pH 7.9, 0.1 mM EDTA, 4 mM NaCl, 0.1 mM β-mercaptoethanol, 0.4% glycerol, 1 mM DTT, 50 μg/ml poly dI·dC, 50 μg/ml BSA) containing a ^{32}P-labelled double-stranded oligonucleotide probe, representing one of the individual nuclear factor binding sites. As some of the samples were derived from HIV-infected cell cultures, nuclear extracts were first incubated in a 1:1 mixture of 5% Triton X-100, prior to inclusion in EMSA reactions. Preliminary investigations demonstrated that inclusion of Triton X-100 had no demonstrable effect on nuclear factor binding of nuclear extracts. As a negative binding control, EMSA binding reactions were conducted in parallel, which included labelled probe, but omitted nuclear extract. To illustrate specificity of nuclear factor binding for each specific probe, parallel EMSA reactions were incubated with a 50-fold molar excess of cold (unlabelled) probe. Independent EMSA analysis of the PU.1 site was not performed as the NF-κB oligonucleotide overlaps this element.

4. Supershift Analysis of Nuclear Factor Binding to the Human IL-12 p40 Promoter: Subsequent supershift analyses were conducted to identify specific proteins whose binding capability was altered by HIV infection of myeloid cells. EMSA reactions were set up as described above, but included specific antibodies directed against relevant nuclear factors for the sites affected by HIV infection of THP-1 cells or MDM. Specifically, nuclear extracts were combined with the NF-κB oligonucleotide in the presence of NF-κB p50, p65, or c-Rel antibodies; with the AP-1 oligonucleotide in

the presence of c-Fos or c-Jun antibodies; with the Sp1 probe and Sp1 or Sp3 antibodies; and with the Ets-2 probe and Ets-2, IRF-1, ICSBP, NF-κB p50, or c-Rel antibodies. All antibodies were included in the EMSA/supershift reaction mixtures at a final concentration of 20 μg/ml. In addition, a goat IgG control was included in control binding reactions to verify binding specificity of transcription factor-specific antibodies. The sources and isotypes of each antibody used in these supershift experiments are listed in table 2.

5. Gel Electrophoresis of EMSA and Supershift Binding Reactions: Bound and unbound ^{32}P-labelled oligonucleotides were resolved on a non-denaturing 6% polyacrylamide gel (19:1 acrylamide:bis-acrylamide). The gel was pre-run in a Hoefer Vertical Slab Gel Unit model SE 400 (Amersham) at 80V for 1 hour, prior to addition of 5 μl gel loading buffer (20% w/v glycerol, 1 mg/ml bromophenol blue) to each sample. Gels were run at 80V for 3.5 hours (Power Pak 1000, Bio-Rad) prior to drying between Whatmann paper and Gel Air Cellophane support film (Bio-Rad) for 1.5 hours. Dried gels were exposed to a Phosphor Screen, and documented using a Storm Phosphorimager and ImageQuant software (Molecular Dynamics).

K. Western Blotting of Nuclear Factors and Signalling Kinases:

1. Detection of Nuclear Factor Proteins in THP-1 and Monocyte Cellular Lysates:

i. Culture and lysis of THP-1 cells and MDM: To examine the impact of HIV infection on myeloid cell nuclear factor expression, mock- and HIV-infected THP-1 cells or

TABLE 2: *Antibodies Used for Supershift/EMSA Reactions:*

Antigen	Antibody Isotype	Form	Source
NF-κB p50	IgG1	Mouse monoclonal	Santa Cruz
NF-κB p65	IgG1	Mouse monoclonal	Santa Cruz
c-Rel	IgG1	Mouse monoclonal	Santa Cruz
Sp1	IgG	rabbit polyclonal	Upstate Biotechnology
Sp3	IgG	rabbit polyclonal	Santa Cruz
c-Fos	IgG	rabbit polyclonal	Upstate Biotechnology
c-Jun	IgG	rabbit polyclonal	Santa Cruz
Ets-2	IgG	rabbit polyclonal	Santa Cruz
IRF-1	IgG	rabbit polyclonal	Santa Cruz
ICSBP	IgG	rabbit polyclonal	Santa Cruz

MDM (5 x 10^6 cells per condition at 10^6/ml) were stimulated with medium or LPS (1 μg/ml) for 1 hour, and washed twice in ice-cold PBS prior to lysis. Pelleted THP-1 cells were lysed in 400 μl RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/ml each aprotonin, leupeptin, pepstatin A, 1 mM Na_3VO_4, 1 mM NaF) and incubated at 4°C for 30 minutes. Adherent monocyte cultures in 6-well plates were lysed with 250 μl RIPA buffer directly in the tissue culture plate. Lysates were collected and centrifuged at 12,000 x g for 10 minutes at 4°C to pellet cellular debris, and supernatants were aliquoted and stored at –80°C until further use.

ii. Quantitation of Protein Content of Cellular Extracts: Total protein present in whole-cell lysates, or nuclear extracts (above) was quantitated using the Pierce BCA assay kit according to manufacturers' instructions (Pierce, Rockford, IL). Briefly, 25 μl of each sample or appropriately diluted BSA standard (2000-25 μg/ml) were added to

one well of a 96-well microtitre plate. To each well, 200 µl of a 50:1 mixture of BCA reagent A and BCA reagent B were added and incubated for 30 minutes at 37°C. The resulting colour change was quantitated using a Spectramax 190 microplate reader, measuring absorbance at 562 nm, and calculation of protein content of each sample against the standard curve was performed with SoftMax Pro software.

iii. SDS-PAGE: Fifty µg of cell lysate from each treatment were combined with 1X loading buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 25% glycerol, 0.01 % bromophenol blue), and heated at 90°C for 5 minutes prior to loading onto a 4-15% Tris-HCl Ready-gel (BioRad). Electrophoresis was conducted at 150 V for 1.5 hours in Tris/glycine/SDS running buffer (25 mM Tris-HCl pH 7.5, 192 mM glycine, 0.1% SDS) in a Mini-Protean 3 Electrophoresis Cell (Bio-Rad). Following denaturing gel electrophoresis, proteins were transferred to PVDF membrane (Immobilon-P, Millipore, Bedford, MA), using a wet-transfer setup in a Mini Trans-Blot Cell (Bio-Rad). PVDF membrane was pre-soaked in methanol for 5 minutes, and the membrane and gel were equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3) for 10 minutes prior to assembly in the transfer apparatus. The transfer was conducted at 100 V for 1 hour, after which time membranes were blocked in 5% skim milk in PBS for 1 hour at room temperature, with agitation. After five washes in TBST buffer (20 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.05% Tween-20, pH 7.5) for 5 minutes each, with shaking, membranes were incubated with primary antibodies against NF-κB p50, p65, c-Rel, Sp1, Sp3, c-Fos, c-Jun, Ets-2, IRF-1, or ICSBP. These antibodies were diluted in 5% skim milk in PBS, at concentrations listed in table 3. Membranes were stained with primary antibodies overnight at 4°C with shaking, and washed five times in

TBST. Secondary HRP-conjugated antibodies, diluted in 5% skim milk and PBS, were incubated with membranes at room temperature, with shaking, for 1 hour, and were then washed five times in TBST buffer. After washing, bound antibodies were detected by chemiluminescence by incubation for five minutes with Supersignal West Pico Chemiluminescent Substrate (Pierce), and visualized by autoradiography. Following exposure, membranes were stripped in TBST + 0.7% β-mercaptoethanol at 50°C for 20 minutes, washed, and reprobed with an anti-PCNA antibody (Santa Cruz) to ensure uniform loading of samples.

iv. Detection of Phosphorylated and Unphosphorylated IκBα, and p38, JNK, ERK 1/2 MAP Kinases: Similarly, expression levels and phosphorylation status of IκBα and MAP kinases in THP-1 cells and MDM were monitored by Western Blotting of whole-cell lysates. Mock- and HIV-infected THP-1 cells or MDM (5 x 10^6 cells per condition at 10^6/ml) were stimulated with medium or LPS (1 μg/ml) for 1 hour, and washed twice in ice-cold PBS prior to lysis. Pelleted THP-1 cells were lysed in 400 μl RIPA buffer, and adherent monocyte cultures were lysed with 250 μl RIPA buffer directly in the tissue culture plate, as described above.

Fifty μg of total cell lysate from each experimental condition were resolved by SDS-PAGE and transferred to PVDF membrane as described above. After blocking with 5% skim milk and PBS, membranes were blotted with antibodies against IκBα, p38α, JNK 1/2, and ERK 1/2, in their native or phosphorylated states. Antibodies were diluted in 5% skim milk and PBS to concentrations stated in table 3.

TABLE 3: *Antibodies Used for Western Blotting of Nuclear Factors and Signalling Mediators:*

Antigen	Isotype	Form	Concentration	Source
NF-κB p50	IgG1	mouse monoclonal	1:500	Santa Cruz
NF-κB p65	IgG1	mouse monoclonal	1:500	Santa Cruz
c-Rel	IgG1	mouse monoclonal	1:500	Santa Cruz
Sp1	IgG	rabbit polyclonal	1:1000	Upstate Biotech.
Sp3	IgG	rabbit polyclonal	1:1000	Santa Cruz
c-Fos	IgG	rabbit polyclonal	1:250	Upstate Biotech.
c-Jun	IgG	rabbit polyclonal	1:500	Santa Cruz
Ets-2	IgG	rabbit polyclonal	1:500	Santa Cruz
IRF-1	IgG	rabbit polyclonal	1:500	Santa Cruz
ICSBP	IgG	rabbit polyclonal	1:250	Santa Cruz
PCNA	IgG2a	mouse monoclonal	1:1000	Santa Cruz
p38 MAPK	IgG1	mouse monoclonal	1:1000	Santa Cruz
phospho-p38	IgG	rabbit polyclonal	1:500	Santa Cruz
JNK ½	IgG1	mouse monoclonal	1:1000	Santa Cruz
phospho-JNK 1/2	IgG1	mouse monoclonal	1:500	Santa Cruz
ERK ½	IgG	rabbit polyclonal	1:1000	Santa Cruz
phospho-ERK 1/2	IgG2a	mouse monoclonal	1:500	Santa Cruz
IκBα	IgG	rabbit polyclonal	1:500	Santa Cruz
Phosphor-IκBα	IgG2b	mouse monoclonal	1:500	Santa Cruz

To compare alterations in protein levels among experimental samples, autoradiographs were scanned using an HP ScanJet 7400c scanner (Hewlett-Packard, Mississauga, ON), and images were analyzed using Scion Image software (Scion Corporation, Frederick, MD).

L. Mutation of the NF-κB, AP-1, Sp1, and Ets-2 Elements in the IL-12 p40 Promoter:

In order to evaluate the role of each transcription factor binding site affected by HIV infection, a 3.3 kb IL-12 p40 promoter-luciferase reporter construct was mutated and assessed for LPS-induced promoter activation in THP-1 cells and MDM.

1. IL-12 p40 promoter construct: A 3.3kb IL-12 p40 promoter-containing luciferase reporter vector, p3.3kb-luc (7), was a kind gift of Dr. Giorgio Trinchieri and Dr. Xiaojing Ma. As this vector was based on the low-copy pXP2 backbone, a *XhoI* restriction fragment encompassing the entire IL-12 p40 promoter region was subcloned into the smaller high-copy pGL3 basic luciferase vector (Promega; Maddison, WI) to facilitate propagation. Briefly, 10 μg of 3.3kb-luc and 5 μg of pGL3 vector were digested with 3 U/μg of *XhoI* (Amersham) in 100 μl reactions overnight at 37°C. The linearized pGL3 vector was purified by ethanol precipitation as described, while the 3.3 kb promoter-containing fragment was gel purified using the GFX Gel Purification Kit (Amersham/Pharmacia) according to manufacturers' instructions. The p3.3kb-luc *XhoI* digest reaction was resolved on a 1% agarose gel, and slices of agarose containing the 3.3 kb fragment were excised and melted in 10 μl capture buffer per 10 mg of agarose, at 60°C for 10 minutes. Each sample was added to a GFX column, microcentrifuged at 12,000 x g, washed with 500 μl of a Tris/EDTA/ethanol wash buffer, and eluted with ddH_2O. Purified digested pGL3 vector (400 ng) was treated with calf intestinal alkaline phosphatase (1 U; MBI Fermentas, Burlington, ON) for 30 minutes at 37°C, heat inactivated at 75°C for 15 minutes, and extracted with phenol/chloroform as described in section F.3.i. Following ethanol precipitation, the pGL3 vector was ligated with the 3.3 kb promoter insert in a 5:1 insert-to-vector ratio with 1 μl T4 DNA ligase (New England

Biomedical, Northborough, MA) overnight at room temperature. Ligations were transformed into supercompetent DH5α as described in section I.3.i.b, and plated onto LB agar plates supplemented with 50 μg/ml ampicillin.

Ten colonies were selected, grown in 5 ml LB broth (10 g/l NaCl, 10 g/l tryptone, 5 g/l yeast extract) supplemented with 50 μg/ml ampicillin overnight at 37°C in the orbital shaker, and plasmids were purified using the QIAprep Plasmid Miniprep Kit, as described in section I.3.i.c. Plasmid DNA was screened for incorporation of the 3.3kb promoter fragment in the correct orientation, by restriction digestion of 0.5 μg plasmid with 10 U *BglII* (Amersham). Digests were resolved by agarose gel electrophoresis, and digests producing 5414 and 2704 bp fragments were indicative of correct incorporation of the IL-12 p40 promoter *XhoI* fragment. Colonies transformed with the pGL3 vector containing the 3.3 kb IL-12 p40 promoter in the forward orientation were subsequently upscaled in 250 ml cultures in LB broth with 50 μg/ml ampicillin, and pGL3p40 plasmids were purified using the Qiagen Plasmid Maxi Kit (section I.3.iii).

2. Wild-Type IL-12 p40 Promoter/Luciferase Recombinant Adenovirus Construction: Preliminary experiments with the 3.3kb IL-12 p40 promoter-luciferase vector demonstrated that this vector was not readily transfected into THP-1 cells by numerous methods attempted. With this, and considering the intention to evaluate promoter activation in both THP-1 cells and primary MDM, the IL-12 p40 promoter-luciferase reporter cassette was assembled into a recombinant adenovirus vector by Dr. Robin Parks at the Ottawa Health Research Institute. The strategy for recombinant adenovirus construction is depicted in figure 4, and the plasmids used to conduct Cre-lox recombination to generate recombinant adenovirus are illustrated in figure 3A.

Preliminary investigations using a wild-type *lacZ*-containing adenovirus preparation confirmed that adenovirus could readily infect THP-1 cells (data not shown).

From this pGL3p40 vector, recombinant adenovirus containing the IL-12 p40-luciferase reporter cassette was constructed by Cre-lox recombination (figure 4). The entire IL-12 p40 promoter/luciferase cassette was subcloned as a 5.1 kb *SalI* fragment into the E1 region of the pRP2159 shuttle vector in front of a loxP site, as illustrated in figure 3A. The resulting promoter-containing shuttle vector (pKC2a) was cotransfected using Superfect according to manufacturers' instructions (Qiagen), into E1-complementing 293 cells, along with the pBHGbloxΔE1,3Cre (229) adenoviral backbone plasmid. Following Cre-mediated recombination between loxP sites in the shuttle and backbone plasmids, E1/E3-deleted, replication-defective IL-12 p40 promoter-luciferase containing recombinant adenovirus (AdKCp40) was purified by cesium chloride buoyant density centrifugation according to standard methods (230).

3. Promoter Mutagenesis and Recombinant Adenovirus Construction:

i. Site-Directed Mutagenesis: To evaluate the respective roles of the NF-κB, AP-1, Sp1, and Ets-2 binding sites in LPS-induced IL-12 p40 promoter activation, individual mutants of these sites within pKC2a were generated by site-directed mutagenesis, using the QuickChange Mutagenesis kit (Stratagene, La Jolla, CA). Mutants were synthesized by Dr. Michael Montpetit at Health Canada, and are shown in figure 5. The strategy used to generate recombinant adenovirus carrying mutant IL-12 p40 promoter-luciferase constructs is illustrated in figure 6, and the vectors used for this construction are depicted in figure 3B.

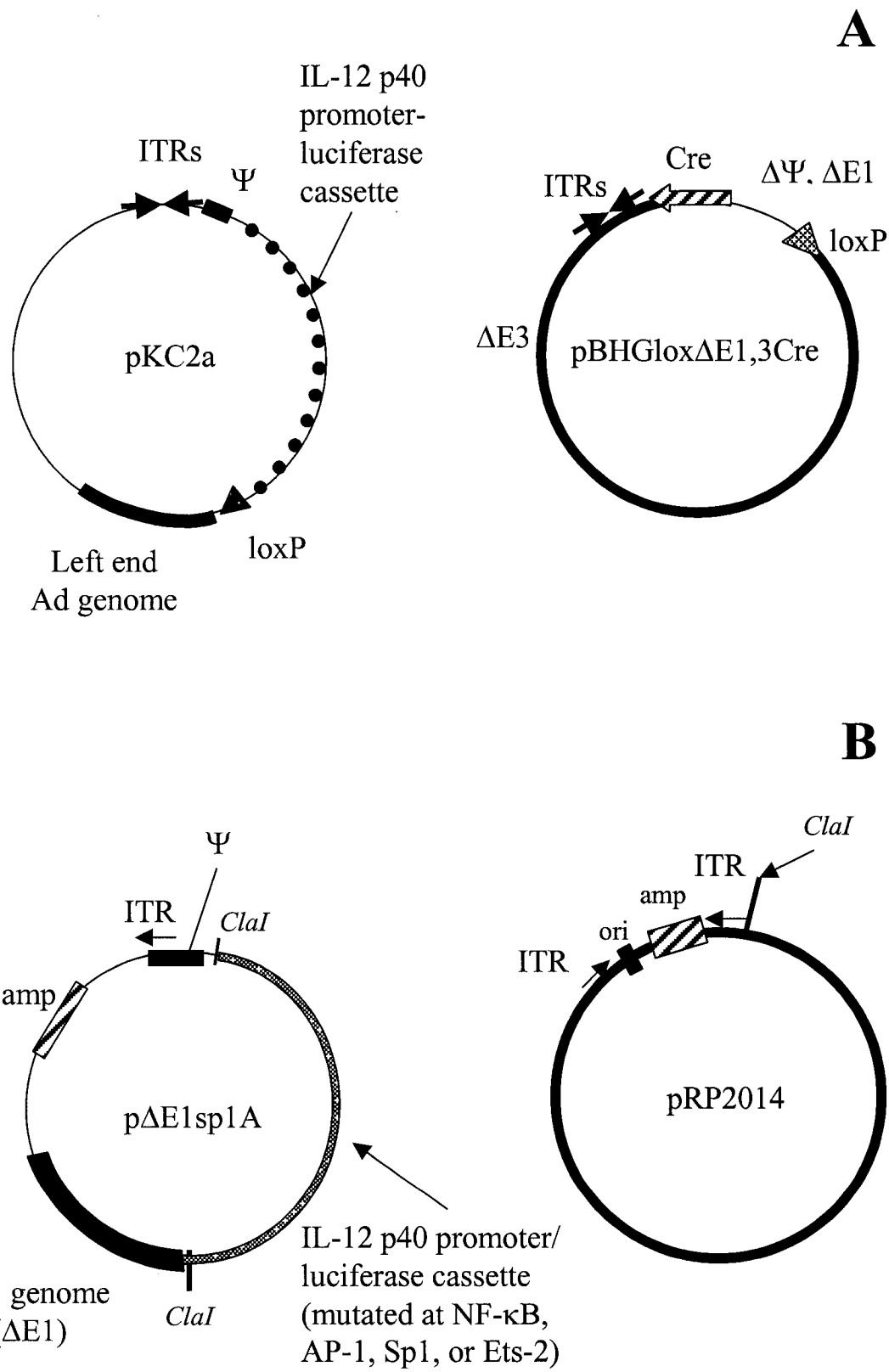

A
IL-12 p40
promoter-
luciferase
cassette
ITRs
Ψ
pKC2a
Left end
Ad genome
loxP
Cre
ΔΨ, ΔE1
ITRs
loxP
ΔE3
pBHGloxΔE1,3Cre
B
Ψ
ITR
ClaI
amp
pΔE1sp1A
Ad5 genome
(ΔE1)
ClaI
IL-12 p40 promoter/
luciferase cassette
(mutated at NF-κB,
AP-1, Sp1, or Ets-2)
ClaI
ITR
amp
ori
ITR
pRP2014

FIGURE 3: *Plasmid Maps for the Generation of Recombinant Adenovirus Vectors Containing the IL-12 p40 Promoter:*

Schematic representation of plasmids used to generate recombinant adenoviral vectors to facilitate delivery of the IL-12 p40 promoter-luciferase reporter constructs to THP-1 cells and MDM. **(A)** pKC2a shuttle vector and pBHGloxΔE1,3Cre genomic vector used to generate recombinant adenovirus by Cre-lox recombination. **(B)** pΔE1sp1A shuttle vector and pRP2014 genomic vector used to generate recombinant adenovirus containing mutated IL-12 p40 promoter-luciferase constructs, by bacterial recombination. Arrows indicate inverted terminal repeats (ITR); ori denotes origin of replication; Ψ indicates a packaging signal; amp indicates ampicillin resistance gene; ΔE1 and ΔE3 denote regions of the adenovirus genome deleted of E1 and E3, respectively; thick lines denote adenovirus-derived DNA; dotted lines indicate IL-12 p40 promoter-luciferase insert. All vectors were supplied by Dr. Robin Parks at the Ottawa Health Research Institute.

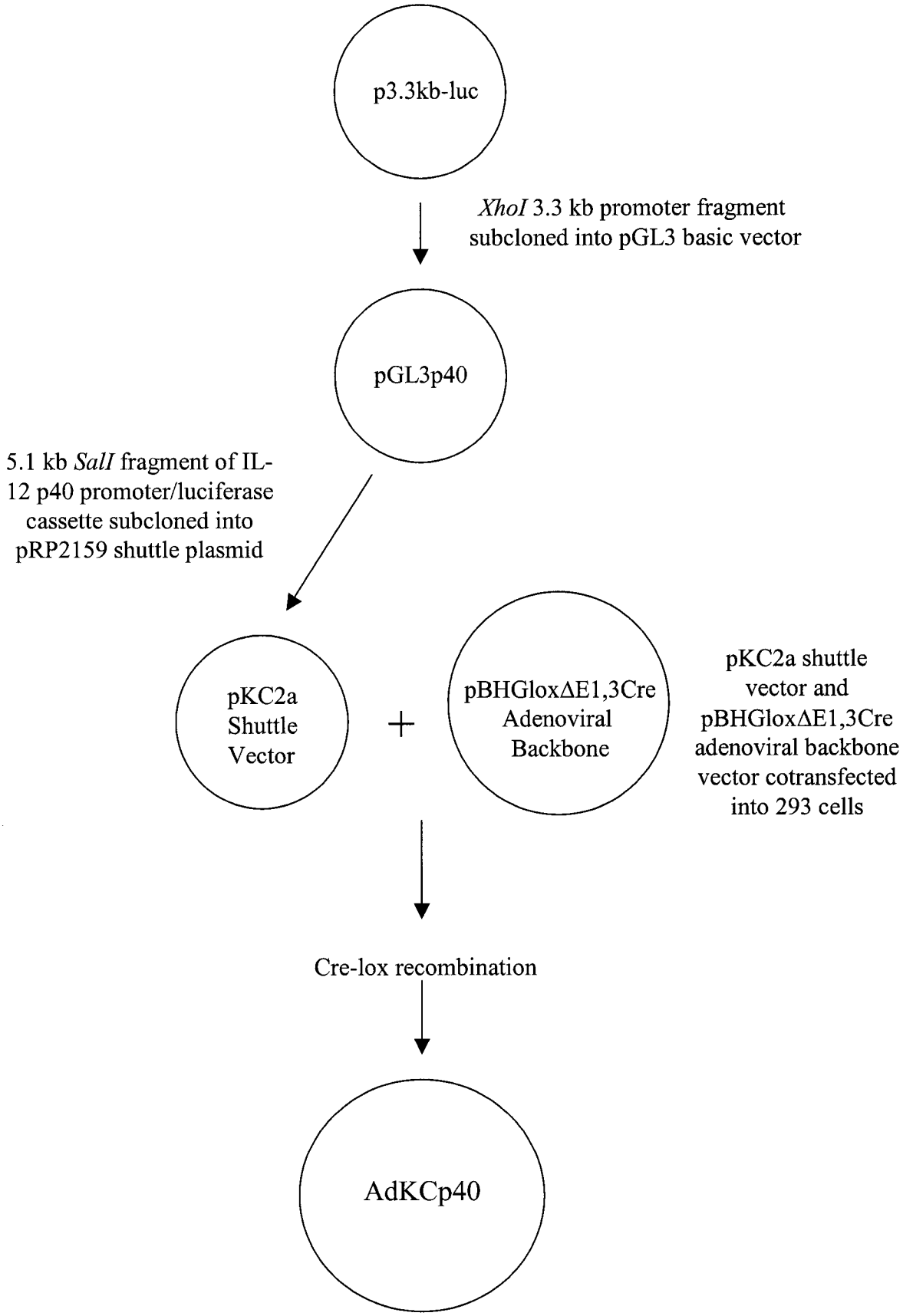

p3.3kb-luc
XhoI 3.3 kb promoter fragment subcloned into pGL3 basic vector
pGL3p40
5.1 kb *SalI* fragment of IL-12 p40 promoter/luciferase cassette subcloned into pRP2159 shuttle plasmid
pKC2a Shuttle Vector
+
pBHGloxΔE1,3Cre Adenoviral Backbone
pKC2a shuttle vector and pBHGloxΔE1,3Cre adenoviral backbone vector cotransfected into 293 cells
Cre-lox recombination
AdKCp40
Purification of Recombinant Adenovirus

FIGURE 4: *Generation of Wild-Type IL-12 p40 Promoter-Luciferase Reporter Containing Recombinant Adenoviral Vector by Cre-Mediated Recombination:*

Schematic representation of construction of a recombinant adenoviral vector carrying the wild-type human IL-12 p40 promoter upstream of a luciferase reporter construct. Assembly of the recombinant adenovirus vector was conducted by Dr. Robin Parks at the Ottawa Health Research Institute. Key features of plasmids used for construction of recombinant adenovirus vectors are illustrated in figure 3.

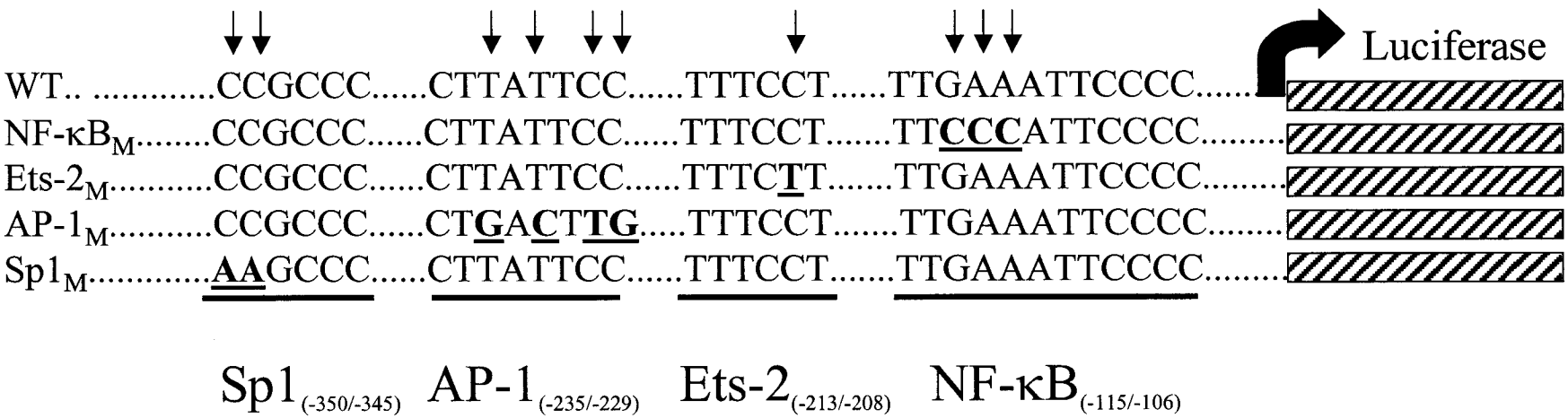

Luciferase
WT.. .....CCGCCC.....CTTATTCC.....TTTCCT.....TTGAAATTCCCC.....
NF-κB$_M$.....CCGCCC.....CTTATTCC.....TTTCCT.....TTCCCATTCCCC.....
Ets-2$_M$.....CCGCCC.....CTTATTCC.....TTTCTT.....TTGAAATTCCCC.....
AP-1$_M$.....CCGCCC.....CTGACTTG.....TTTCCT.....TTGAAATTCCCC.....
Sp1$_M$.....AAGCCC.....CTTATTCC.....TTTCCT.....TTGAAATTCCCC.....
Sp1$_{(-350/-345)}$ AP-1$_{(-235/-229)}$ Ets-2$_{(-213/-208)}$ NF-κB$_{(-115/-106)}$

FIGURE 5: *Wild-Type and Mutant IL-12 p40 Promoter Sequences for Luciferase Reporter Assays:*

Schematic representation of NF-κB(113), Ets-2, AP-1, and Sp1(231) mutations in the human IL-12 p40 promoter within a luciferase reporter construct, relative to the wild-type promoter. Mutants have been assembled into recombinant adenovirus vectors for delivery to myeloid cells as described in section L.3 of the Materials and Methods, and as illustrated in figure 6.

To facilitate mutagenesis, a 1.4 kb *SmaI* fragment encompassing the proximal IL-12 p40 promoter region was excised by restriction digestion, and substituted into a pUC19 vector, resulting in creation of pUC19p40. Sense and antisense (125 ng each) mutagenic oligonucleotides (Tm > 78°C, GC content approximately 40%) were annealed to 10 ng pUC19p40, and cycled 12 times in the presence of *Pfu* Turbo DNA polymerase as follows: 95°C for 30 seconds, 55°C for 1 minute, 68°C for 2 minutes/kb of DNA template. Following digestion with 10 U *DpnI* for 1 hour at 37°C, reaction mixtures were transformed into supercompetent bacteria as described in section I.3.i.b. Mutants were confirmed by sequencing and the 1.4 kb mutant IL-12 p40-containing *SmaI* fragments within the pUC19 vectors were substituted back into the parent pKC2a vector following *SmaI* restriction digestion and gel purification using the GFX DNA purification kit (Amersham), as described in section L.1. To confirm proper orientation of the *SmaI* fragments, pKC2a preparations containing mutated IL-12 p40 promoter sites were digested with *PstI* (Amersham), and proper orientation was verified with production of a 2749, 3359, and 3598 bp restriction fragments.

ii. Recombinant IL-12 p40 Promoter Mutant Adenovirus Construction:

Following *ClaI* digestion of mutant IL-12 p40/luciferase containing plasmids, fragments containing the entire IL-12 p40 mutant promoter/luciferase constructs were inserted into the pΔE1sp1a shuttle plasmid (229, 232), and contransformed with pRP2014 adenoviral vector backbone plasmid into the *E. coli* BJ5183 strain. Following homologous recombination between overlapping portions of the shuttle and backbone vectors, recombinant E1/E3-deleted IL-12 p40 mutant promoter/luciferase-containing adenoviral

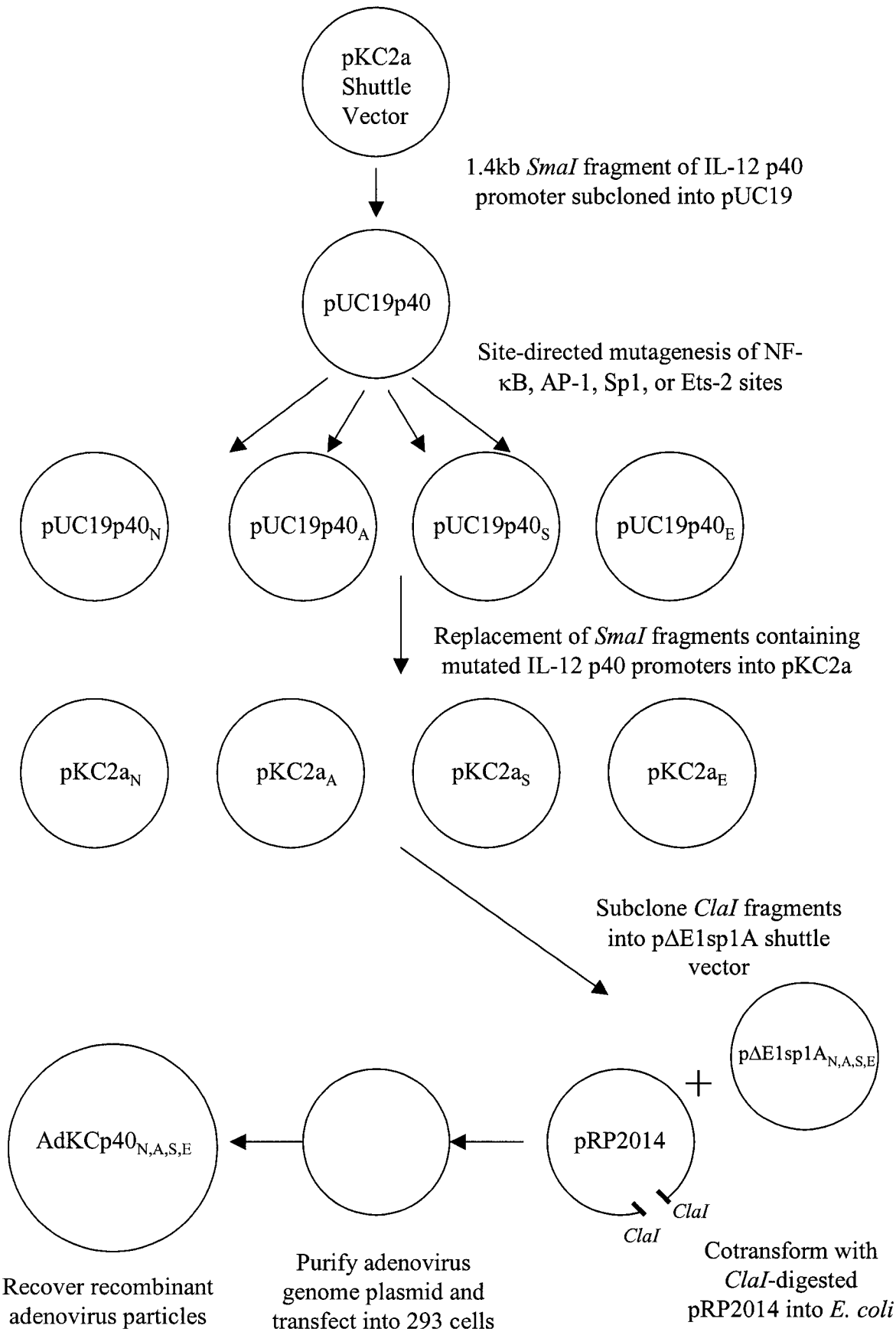

pKC2a Shuttle Vector
1.4kb *SmaI* fragment of IL-12 p40 promoter subcloned into pUC19
pUC19p40
Site-directed mutagenesis of NF-κB, AP-1, Sp1, or Ets-2 sites
pUC19p40N
pUC19p40A
pUC19p40S
pUC19p40E
Replacement of *SmaI* fragments containing mutated IL-12 p40 promoters into pKC2a
pKC2aN
pKC2aA
pKC2aS
pKC2aE
Subclone *ClaI* fragments into pΔE1sp1A shuttle vector
pΔE1sp1AN,A,S,E
pRP2014
ClaI
ClaI
Cotransform with *ClaI*-digested pRP2014 into *E. coli*
Purify adenovirus genome plasmid and transfect into 293 cells
AdKCp40N,A,S,E
Recover recombinant adenovirus particles

FIGURE 6: *Generation of NF-κB, AP-1, Sp1, and Ets-2 Mutant IL-12 p40 Promoter-Luciferase Reporter Containing Recombinant Adenoviral Vectors:*

Schematic representation of construction of a recombinant adenoviral vectors carrying the human IL-12 p40 promoter-luciferase reporter construct, containing mutations in either the NF-κB, AP-1, Sp1, or Ets-2 elements of the IL-12 p40 promoter. Mutagenesis was performed by Dr. Michael Montpetit. Construction of recombinant adenovirus particles containing the promoter-luciferase constructs was carried out in the lab of Dr. Robin Parks at the Ottawa Health Research Institute. Key features of plasmids used for construction of recombinant adenovirus vectors are illustrated in figure 3. Plasmids containing mutations in NF-κB, AP-1, Sp1, or Ets-2 are indicated with the subscripts N, A, S, or E, respectively.

genome vectors were purified by alkaline lysis, and transfected into 293 cells for packaging of replication-defective recombinant adenovirus particles ($AdKCp40_N$, $AdKCp40_A$, $AdKCp40_S$, $AdKCp40_E$), which were purified by cesium chloride buoyant density centrifugation.

4. Evaluation of Wild-Type and Mutant IL-12 p40 Promoter Activation in THP-1 Cells and MDM:

i. Recombinant Adenovirus Infection: THP-1 cells and MDM ($2x10^6$/ml) were infected with recombinant adenovirus vectors containing the wild-type (AdKCp40) or mutant IL-12 p40 promoter/luciferase vectors ($AdKCp40_N$, $AdKCp40_A$, $AdKCp40_S$, $AdKCp40_E$) at m.o.i. = 50. After 24 hours, cells were stimulated with medium or LPS (1 μg/ml) for an additional 48 hours, determined in preliminary experiments to be optimal for luciferase detection from the LPS-stimulated wild-type promoter.

ii. Luciferase Assay: To quantitate LPS-induced promoter activation of wild-type and mutant IL-12 p40 promoters, adenovirus-infected LPS-stimulated THP-1 cells and MDM were lysed and luciferase activity was detected using the Enhanced Firefly Luciferase Assay Kit (BD Biosciences/Pharmingen; Mississauga, ON), according to manufacturers' instructions. Cells were washed twice in PBS, and lysed in 1X lysis/assay buffer for 20 minutes at room temperature. After brief centrifugation at 12,000 x g to pellet cellular debris, 100 μl of sample were added to 12 x 75 mm assay cuvettes, and 100 μl of reagent A (enhancing solution including ATP and $MgCl_2$) were manually added to each sample. Using a Lumat LB9507 luminometer (Berthold Technologies/Fisher Scientific, Ottawa, ON), 100 μl of reagent B (luciferin substrate) were automatically dispensed, and emitted light was quantitated for 10 seconds per

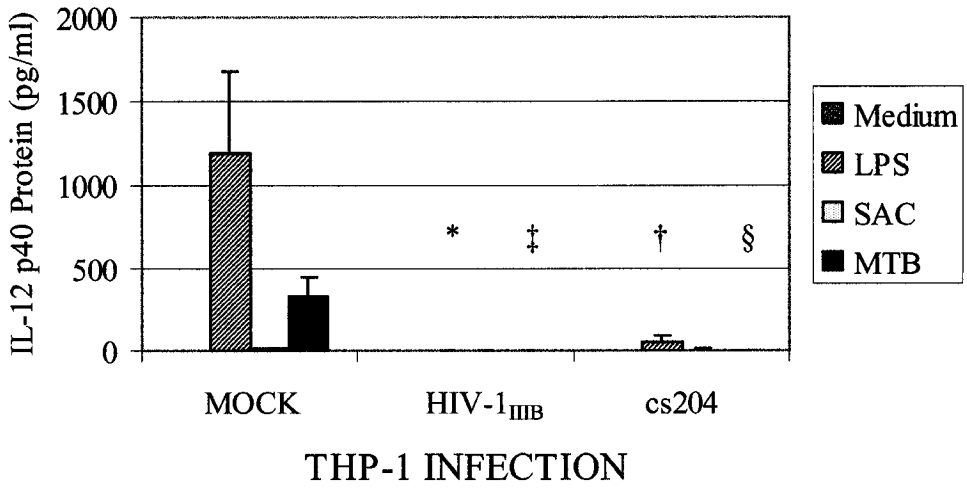
sample. Luciferase activity among the different experimental conditions was normalized according to the total protein content of cellular extracts, as determined by the Pierce BCA Protein Assay, described earlier.

III. RESULTS

A. HIV Infection of THP-1 Cells and MDM Impairs Stimulus-Induced IL-12 Expression:

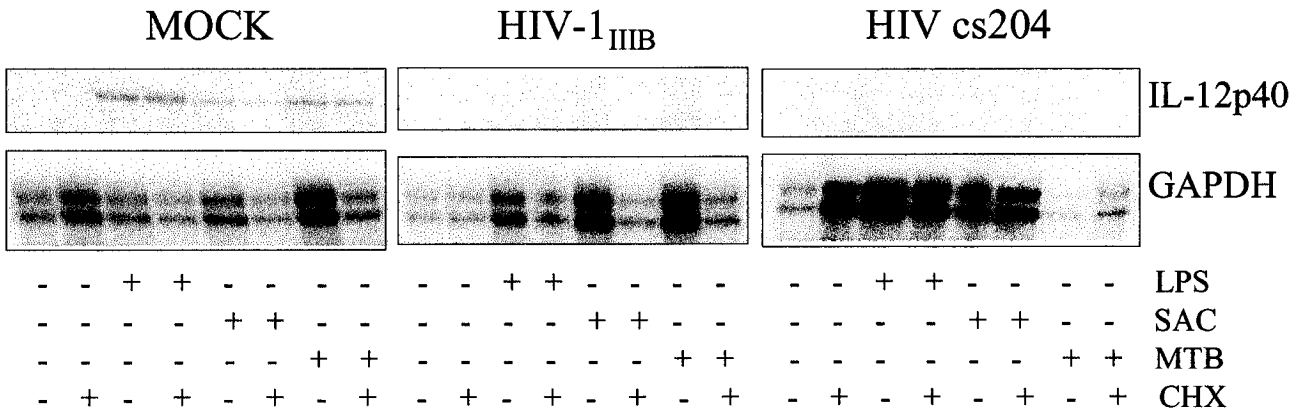
1. Effect of HIV infection on IL-12 p40 protein and mRNA expression in response to bacterial stimulation: Previous reports have demonstrated an impaired ability of peripheral blood mononuclear cells (PBMC) or MDM from HIV-seropositive individuals to produce IL-12 in response to bacterial stimuli (12, 209). To confirm this effect in an acute infection model of myeloid cells in isolation, MDM from HIV-seronegative volunteers, and cells of the THP-1 myeloid line were infected in vitro with the laboratory strain HIV-1_{IIIB}, or with a dual-tropic clinical isolate, cs204. Seven days post-infection, mock- or HIV-infected THP-1 and MDM were assayed for IL-12 p40 protein and mRNA expression in response to bacterial stimuli, including lipopolysaccharide (LPS), *Staphylococcus aureus* Pansorbin cells (SAC), or heat-killed *Mycobacterium tuberculosis* (MTB). IL-12 p40 production by uninfected THP-1 cells and MDM was undetectable following incubation in medium alone (figure 7A and 8A, respectively), and in THP-1 cells, IL-12 p40 secretion was maximal with LPS stimulation compared to stimulation with SAC or MTB preparations (figure 7A). Enhanced production of IL-12 p40 protein in THP-1 cells and MDM in response to bacterial stimulation was paralleled by increased detection of IL-12 p40 mRNA by ribonuclease protection assay and semi-quantitative RT-PCR (figure 7B and 8B, respectively). After stimulation with LPS, SAC, or heat-killed MTB (figure 7A), THP-1 cells infected with either HIV-1_{IIIB} or the clinical isolate, cs204, showed dramatic reduction in IL-12 p40 protein production relative to mock-infected controls.

A



THP-1 INFECTION

B



C

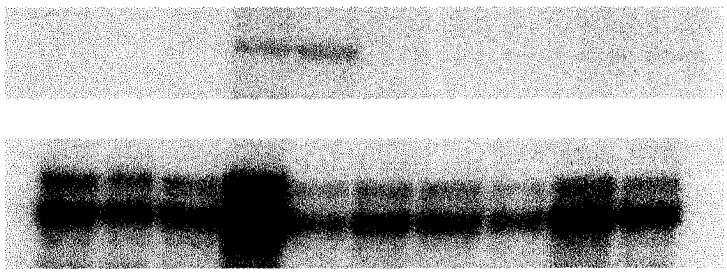
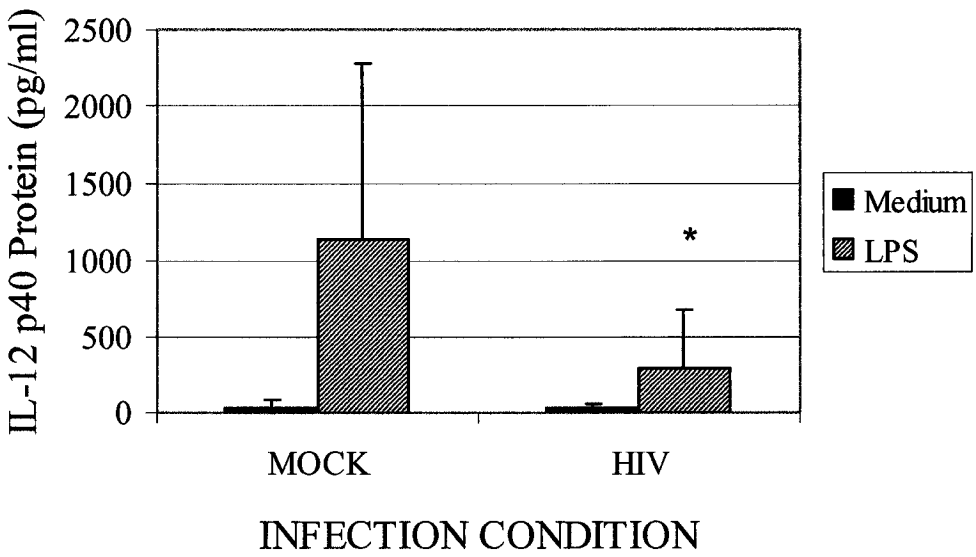


FIGURE 7: *Inhibition of stimulus-induced IL-12 expression by HIV-1 infection of THP-1 cells, in the presence or absence of cycloheximide.*

THP-1 cells were mock-infected or infected with HIV-1_{IIIB} or cs204, and 7 days later, treated with or without CHX, and stimulated with medium, LPS, SAC, or MTB. **(A)** 24 hours after addition of stimuli, supernatants from THP-1 cells were harvested, and assayed for total IL-12 p40 protein by ELISA. Mean ± SD, n=5. * p= 0.0499, ‡ p= 0.0392, † p= 0.048, § p= 0.039 by Student's t-test for HIV-infected versus mock-infected, similarly stimulated controls. **(B)** THP-1 were pretreated for 1 hour with or without CHX, and after 4 hours of stimulation, total RNA was harvested and assayed for IL-12 p40 mRNA expression by ribonuclease protection assay. The images shown are representative of 5 similar experiments. **(C)** THP-1 were stimulated with LPS (1 μg/ml) for 0, 1, 2, 4, or 12 hours prior to RNA extraction and IL-12 p40 mRNA quantitation by RPA as in **(B)**.

A


IL-12 p40 Protein (pg/ml)
2500
2000
1500
1000
500
0
*
Medium
LPS
MOCK
HIV
INFECTION CONDITION

B

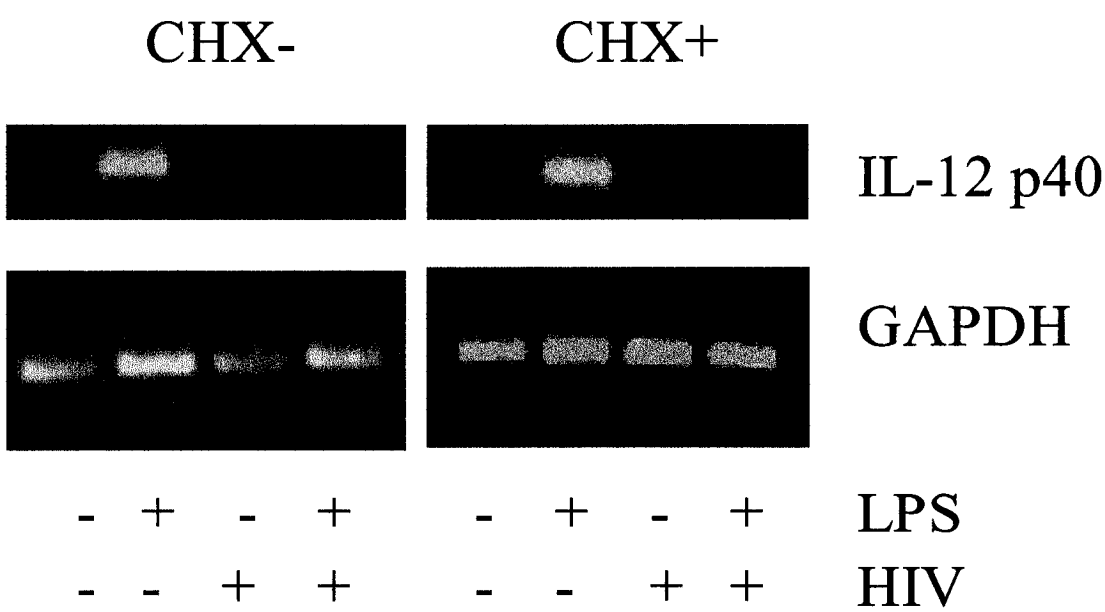
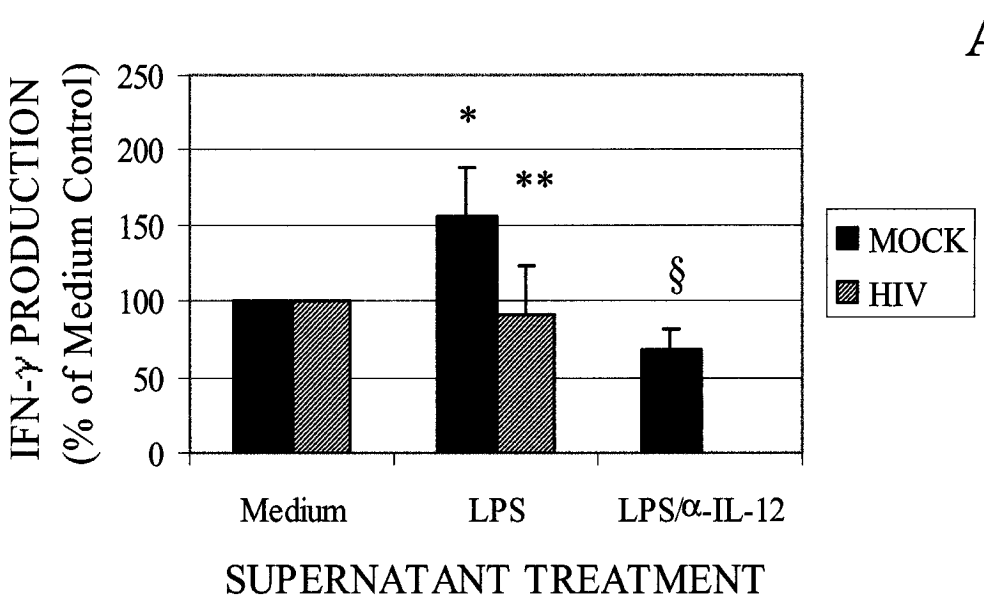

CHX-
CHX+
IL-12 p40
GAPDH
- + - + - + - +
LPS
- - + + - - + +
HIV

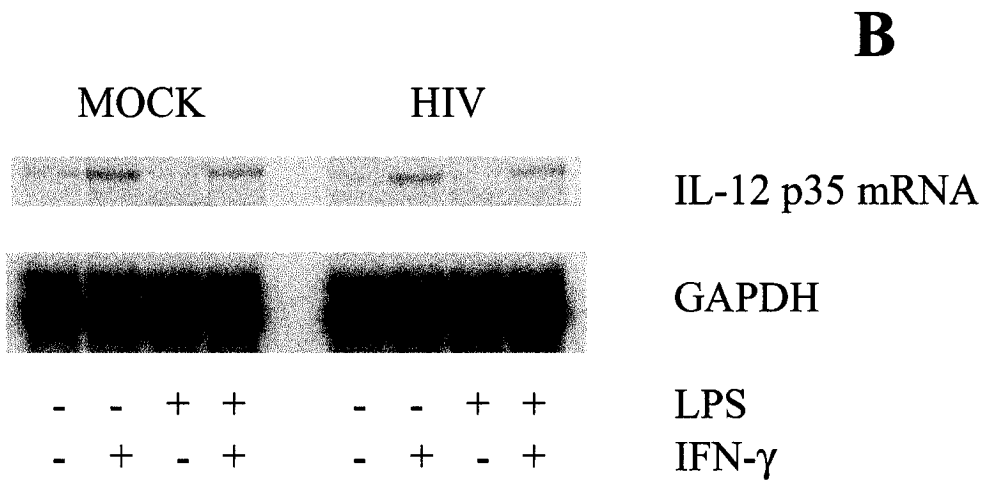
FIGURE 8: *Inhibition of stimulus-induced IL-12 expression by HIV-1 infection of MDM, in the presence or absence of cycloheximide.*

MDM were infected with HIV-1 cs204 and on day 7 were treated with or without CHX for 1 hour prior to stimulation with medium or LPS. **(A)** Following 24 hours of stimulation in the presence of medium or LPS, supernatants were harvested and assayed for total IL-12 p40 protein production by ELISA. Bars represent the mean IL-12 p40 protein production (pg/ml) ± standard deviation, n=3. * p= 0.0308 by Student's t-test for HIV-infected LPS-stimulated cells versus mock-infected controls. **(B)** Total RNA from MDM treated with or without CHX and LPS was reverse-transcribed, PCR-amplified with gene-specific primers for GAPDH and IL-12 p40, and visualized by electrophoresis in a 1.2% agarose gel and staining with ethidium bromide. The gel shown is representative of 3 independent experiments.

Accordingly, IL-12 p40 mRNA expression in HIV cs204-infected THP-1 cells stimulated with LPS, SAC, or MTB was undetectable by RPA (figure 7B). IL-12 p40 mRNA expression in THP-1 cells was similarly reduced following HIV-1_{IIIB} infection and stimulation by LPS, SAC, or MTB (figure 7B). Inhibition of IL-12 p40 expression following HIV infection was not likely due to delayed or altered IL-12 p40 mRNA production kinetics, as IL-12 p40 mRNA remained suppressed by RPA over 12 hours of stimulation of HIV-infected THP-1 cells with LPS (figure 7C). LPS-induced IL-12 p40 protein production was also dramatically suppressed following infection of MDM with HIV cs204 (figure 8A), in parallel with impaired production of IL-12 p40 mRNA (figure 8B), relative to mock-infected controls. As HIV-1_{IIIB} and HIV cs204 both impaired IL-12 expression dramatically in both THP-1 cells and MDM, and since laboratory strains of HIV are difficult to propagate in THP-1 cells (227), further investigations of HIV-mediated suppression of IL-12 were conducted with the clinical isolate, cs204.

*2. **Bioactive IL-12 p70 is reduced following HIV infection of myeloid cells, in the absence of alteration in IL-12 p35 mRNA:*** In preliminary investigations, production of the IL-12 p70 homodimer in THP-1 cells and MDM was undetectable by commercial ELISA with a lower limit of detection of 0.5 pg/ml. However, the presence of bioactive IL-12 p70 was confirmed in supernatants of LPS-stimulated THP-1 cells, using a bioassay to detect IL-12-specific augmentation of PHA-induced IFN-γ production from primary T lymphocytes. Supernatants from LPS-stimulated THP-1 cells incubated with PHA-stimulated primary T lymphocytes caused an enhancement in PHA-induced IFN-γ production (figure 9A). This increase in IFN-γ production was abrogated


A
IFN-γ PRODUCTION
(% of Medium Control)
250
200
150
100
50
0
*
**
§
MOCK
HIV
Medium
LPS
LPS/α-IL-12
SUPERNATANT TREATMENT


B
MOCK
HIV
IL-12 p35 mRNA
GAPDH
- - + + - - + +
- + - + - + - +
LPS
IFN-γ

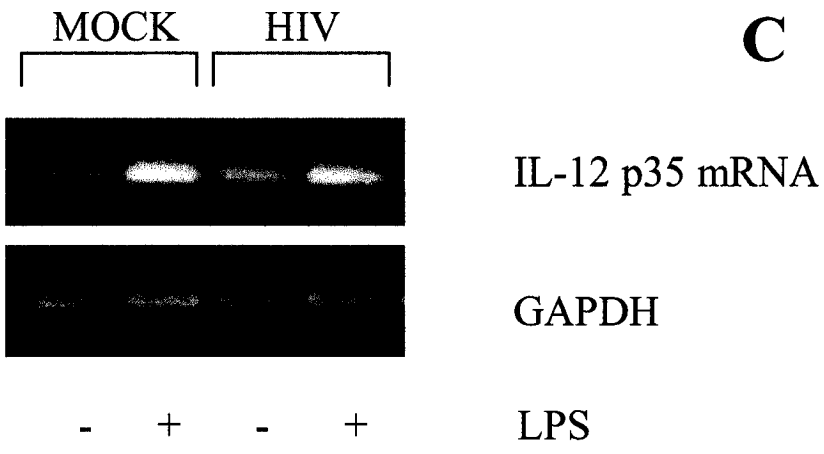

C
MOCK
HIV
IL-12 p35 mRNA
GAPDH
- + - +
LPS

FIGURE 9: *Detection of biologically active IL-12 p70, and its inhibition by HIV infection, in the absence of modulation of IL-12 p35 subunit mRNA.*

(**A**) Supernatants from mock- and HIV-infected, LPS stimulated THP-1 cells were added to PHA-stimulated primary T lymphocytes for 48 hours, prior to supernatant harvest and measurement of IFN-γ production by ELISA. Bars represent mean IFN-γ production expressed as a percentage of the medium control (± standard deviation, n=3). * $p = 0.05$ for LPS-stimulated, mock-infected THP-1 supernatants versus the medium control; ** $p = 0.04$ for LPS-stimulated mock- versus HIV-infected THP-1 supernatants; § $p = 0.008$ for LPS-stimulated THP-1 supernatants incubated with or without α-IL-12 antibody, all by Student's t-test. (**B**) THP-1 cells were mock- or HIV-infected for 7 days, and after 4 hours of stimulation with medium or LPS, IL-12 p35 mRNA was measured in mock- and HIV-infected THP-1 cells by ribonuclease protection assay. Image shown is representative of 3 independent experiments. (**C**) Similarly, total RNA was harvested from mock- and HIV-infected MDM, and IL-12 p35 mRNA was detected by RT-PCR. Gel shown is representative of 3 independent trials.

by addition of neutralizing anti-IL-12 antibody to T cell cultures (figure 9A), demonstrating that the IFN-γ-enhancing effect of supernatants from LPS-stimulated THP-1 cells could specifically be attributed to the presence of bioactive IL-12 p70.

In parallel with diminished LPS-induced IL-12 p40 protein and mRNA production (figure 7), HIV infection caused a reduction in the level of bioactive IL-12 p70 in supernatants of LPS-treated THP-1 cells (figure 9A), as determined by the described bioassay. In contrast, no alterations in IL-12 p35 mRNA expression were observed in LPS-stimulated HIV-infected THP-1 cells (Figure 9B) or MDM (figure 9C) following HIV infection with HIV cs204. As IL-12 p40 protein production parallels IL-12 p70 secretion in THP-1 cells in agreement with published reports (7, 13, 99), and HIV infection did not affect IL-12 p35 subunit expression, subsequent studies focused on examination of regulation of the IL-12 p40 subunit.

3. Inhibition of IL-12 p40 mRNA Induction by HIV Does Not Require de novo Protein Synthesis: To begin to delineate molecular mechanisms underlying HIV-mediated impairment of stimulus-induced IL-12 p40 expression, the protein synthesis inhibitor cycloheximide (CHX) was used to determine whether this suppression of IL-12 induction required synthesis of other host or viral factors. Mock- and HIV-infected THP-1 cells and MDM were incubated with CHX for 1 hour prior to, and for the duration of LPS stimulation. Protein synthesis inhibition by CHX failed to restore the ability of HIV-infected THP-1 to synthesize IL-12 p40 mRNA in response to stimulation with LPS, SAC, or MTB (figure 7B), as there was no restoration in detection of IL-12 p40 mRNA by RPA. Similarly, CHX did not enhance IL-12 p40 mRNA expression in LPS-stimulated MDM infected with HIV cs204, as IL-12 p40 mRNA expression in HIV-

infected MDM remained undetectable by RT-PCR, regardless of preincubation with CHX (figure 8B). Detection of LPS-induced IL-12 p40 mRNA in mock-infected THP-1 cells and MDM was unaffected by CHX treatment (figure 7B and figure 8B, respectively), consistent with a previous report by Marshall et al. (95). From these observations, HIV-mediated inhibition of IL-12 p40 expression in myeloid cells appears to occur directly, in the absence of any requirement for LPS-induced de novo host or viral protein synthesis.

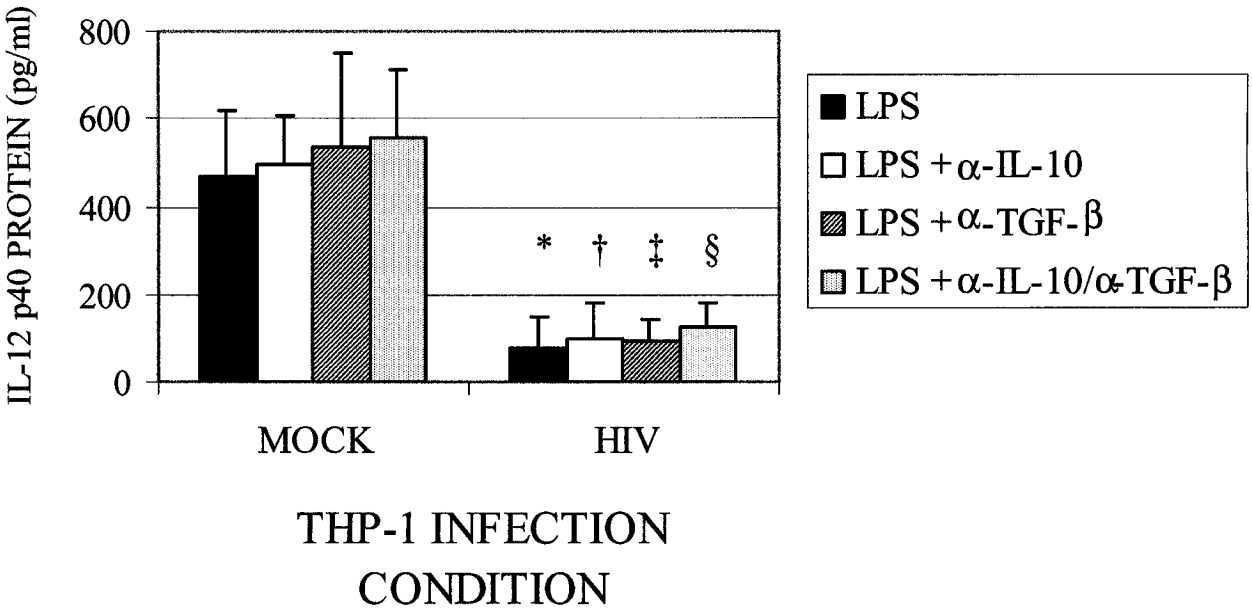
B. HIV-Mediated Inhibition of IL-12 p40 Expression is Independent of the Activity of Key IL-12 Modulating Cytokines:

1. Suppression of IL-12 p40 synthesis by HIV infection is independent of the inhibitory action of IL-10 and TGF-β: Although inhibition of LPS-induced IL-12 p40 expression by HIV infection of myeloid cells was independent of de novo protein synthesis, the possibility that an inhibitory factor may have been synthesized and may have accumulated during the 7 days in culture prior to treatment with CHX was addressed. Two candidates for such an inhibitory factor include the cytokines IL-10 and TGF-β; both are inhibitory for IL-12 expression (99, 216, 217), and both may be upregulated by HIV infection (8, 224). To examine whether IL-10 and/or TGF-β activity contributes to HIV-mediated suppression of IL-12 p40 expression, mock- and HIV-infected THP-1 cells were stimulated with LPS in the presence or absence of saturating quantities of neutralizing antibodies against either IL-10, TGF-β, or both. Stimulation of HIV-infected THP-1 cells with LPS in the presence of neutralizing antibodies to IL-10 or TGF-β did not restore production of IL-12 p40 protein, and in fact, did not affect IL-

12 p40 protein levels at all, regardless of HIV infection (figure 10A). Similarly, LPS-induced IL-12 p40 mRNA detection in HIV-infected THP-1 cells was unaffected by incubation with anti-IL-10 and/or anti-TGF-β neutralizing antibodies (figure 10B). In accordance with the observed effects of neutralizing antibodies on IL-12 p40 expression in THP-1 cells, treatment of HIV-infected MDM with anti-IL-10 and anti-TGF-β antibodies did not restore the ability of these cells to synthesize IL-12 p40 protein following LPS stimulation, and in fact had no effect on IL-12 p40 protein secretion regardless of HIV infection (figure 11A). In addition, IL-12 p40 mRNA detection in HIV-infected MDM was not augmented by addition of neutralizing IL-10 and TGF-β antibodies to monocyte cultures (figure 11B). Taken together, these results suggest that inhibition of LPS-induced IL-12 p40 protein and mRNA expression by HIV infection was independent of the activity of the IL-12-inhibiting cytokines IL-10 and TGF-β.

2. Priming HIV-infected myeloid cells with cytokines does not restore deficient IL-12 p40 production: While the inhibitory cytokines IL-10 and TGF-β did not appear to have a role in HIV-mediated suppression of IL-12 p40 expression, the reduced IL-12 p40 protein and mRNA levels in HIV-infected myeloid cell cultures may have been due, in part, to a lack of cytokines which augment IL-12 synthesis. Several reports in the literature have demonstrated that priming PBMC from HIV-infected donors with IFN-γ (219), IL-4, or IL-13 (220), prior to bacterial stimulation, enhances stimulus-induced IL-12 production. To determine whether suppression of IL-12 synthesis observed in an HIV infection model of isolated myeloid cells is due to a lack of one

A


IL-12 p40 PROTEIN (pg/ml)
800
600
400
200
0
*
†
‡
§
MOCK
HIV
THP-1 INFECTION CONDITION
LPS
LPS +α-IL-10
LPS +α-TGF-β
LPS +α-IL-10/α-TGF-β

B

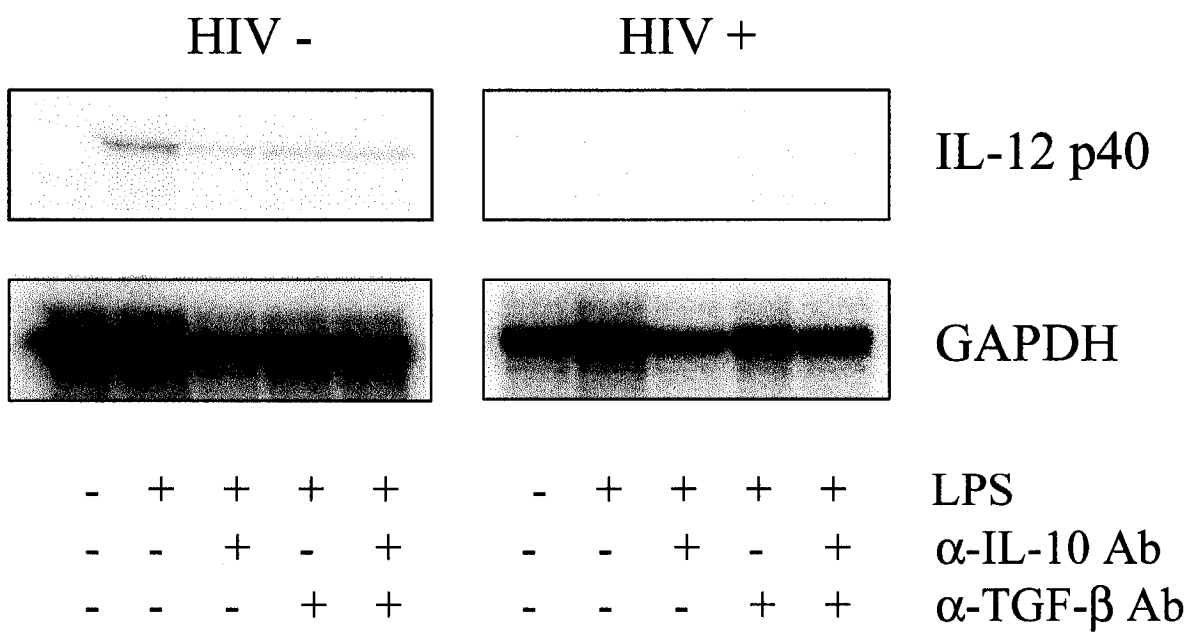
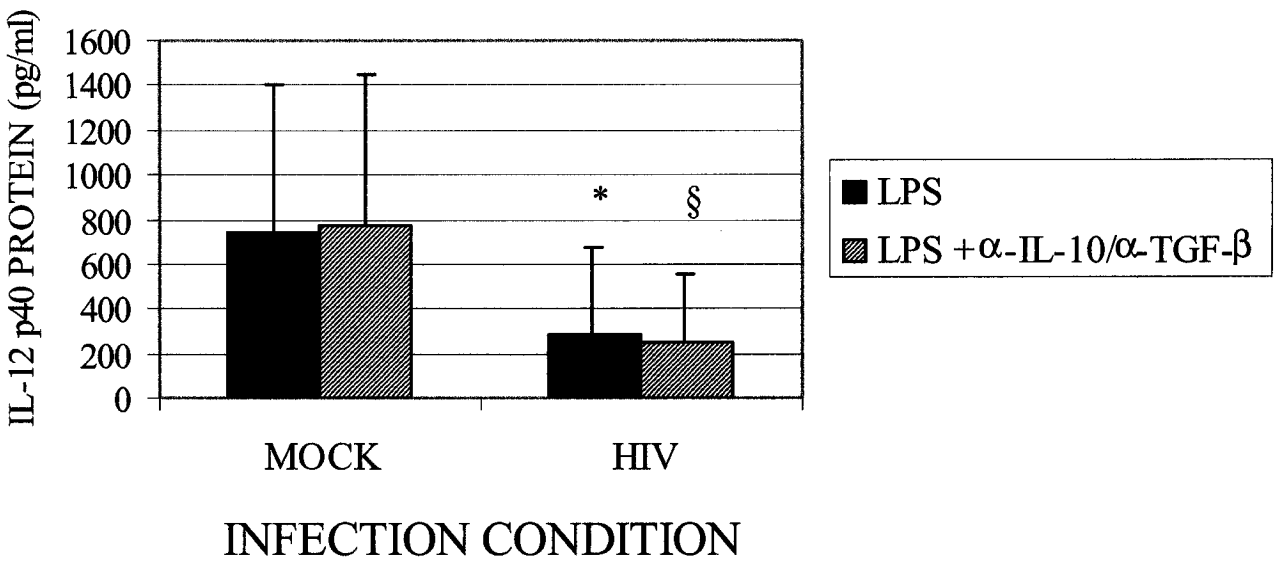

HIV -
HIV +
IL-12 p40
GAPDH
- + + + + - + + + + LPS
- - + - + - - + - + α-IL-10 Ab
- - - + + - - - + + α-TGF-β Ab

FIGURE 10: *Effect of IL-10 and TGF-β neutralization on IL-12 p40 expression in mock- and HIV-infected THP-1 cells.*

THP-1 cells were mock-infected or infected with HIV-1 cs204, and 7 days post-infection, stimulated with LPS in the presence or absence of neutralizing antibodies to IL-10 and TGF-β. **(A)** Supernatants were harvested from THP-1 cells after 24 hours of LPS stimulation in the presence or absence of antibodies to IL-10 or TGF-β, and assayed for total IL-12 p40 protein by ELISA. Bars indicate mean IL-12 p40 protein production ± standard deviation, n=3. * p= 0.0076, † p= 0.0072, ‡ p= 0.012, and § p= 0.013 by Student's t-test for HIV-infected THP-1 versus mock-infected LPS-stimulated controls. **(B)** In parallel cultures of THP-1 cells, total RNA was harvested after 4 hours of stimulation and IL-12 p40 mRNA was quantitated by ribonuclease protection assay. The image shown is representative of 3 experiments.

A


IL-12 p40 PROTEIN (pg/ml)
1600
1400
1200
1000
800
600
400
200
0
*
§
LPS
LPS +α-IL-10/α-TGF-β
MOCK
HIV
INFECTION CONDITION

B

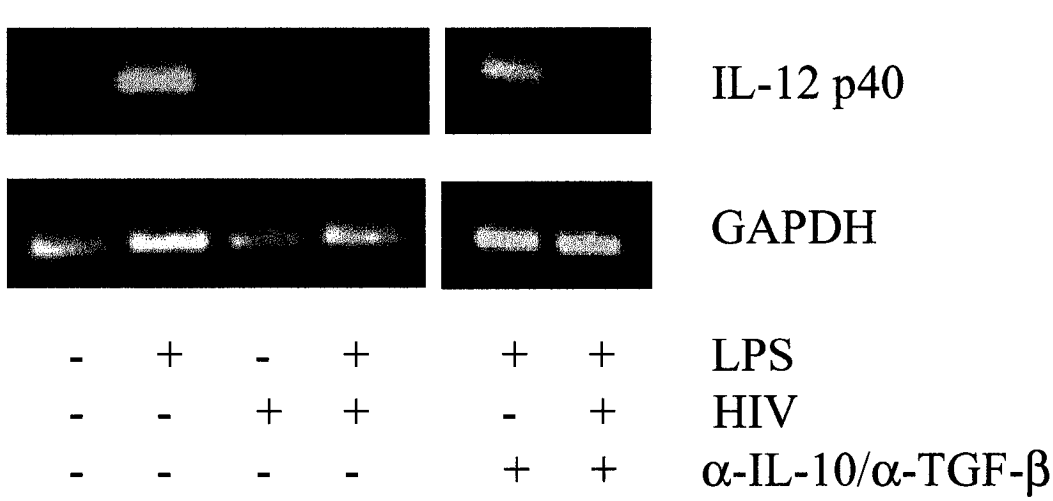

IL-12 p40
GAPDH
- + - + + +
- - + + - +
- - - - + +
LPS
HIV
α-IL-10/α-TGF-β

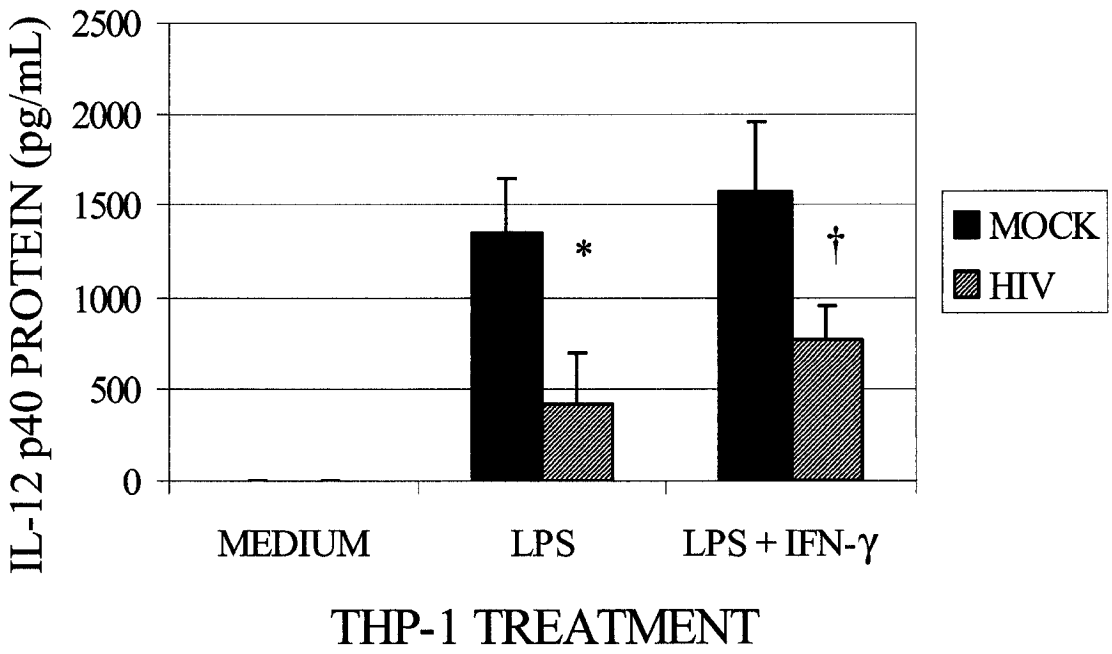
FIGURE 11: *Effect of neutralization of IL-10 and TGF-β on LPS-induced IL-12 p40 expression in MDM.*

MDM from HIV-seronegative volunteers were mock-infected or infected with HIV-1 cs204, and 7 days post-infection, stimulated with LPS in the presence or absence of neutralizing antibodies to IL-10 and TGF-β. **(A)** Supernatants were harvested from MDM after 24 hours of LPS stimulation in the presence or absence of antibodies to IL-10 or TGF-β, and assayed for total IL-12 p40 protein by ELISA. Bars represent mean IL-12 p40 protein production ± standard deviation, n=4. * p= 0.0485, and § p= 0.0453 by Student's t-test for HIV-infected MDM versus mock-infected controls. **(B)** In parallel cultures, total RNA was harvested from MDM following 4 hours in culture with medium or LPS, and 1 μg of total RNA was reverse-transcribed and PCR-amplified with gene-specific primers for IL-12 p40 or GAPDH. PCR products were visualized by agarose gel electrophoresis and ethidium bromide staining. The gel shown is representative of 3 experiments.

of these stimulatory cytokines, mock- and HIV-infected THP-1 and MDM were primed with IFN-γ, IL-4 or IL-13, prior to LPS stimulation. The decrease in LPS-induced IL-12 p40 protein synthesis and secretion observed following HIV infection of THP-1 cells was not restored by IFN-γ priming (figure 12A). In contrast to numerous literature reports demonstrating that IFN-γ enhances stimulus-induced IL-12 expression in PBMC and MDM (8, 10, 223), production of IL-12 p40 protein in LPS-stimulated THP-1 cells was unaffected by IFN-γ priming (figure 12A). In parallel with these observations at the level of IL-12 p40 protein production, detection of IL-12 p40 mRNA in HIV-infected THP-1 cells was also unaffected by IFN-γ priming prior to addition of LPS to mock- and HIV-infected cells (figure 12B). In similar experiments, priming THP-1 cells with IL-4, IL-13, or both, prior to LPS stimulation did not enhance production of IL-12 p40 protein in mock- or HIV-infected THP-1 cells (figure 14A), nor was IL-12 p40 mRNA affected by the addition of these IL-12-priming cytokines (figure 14B).

Contrary to the observed effects of IFN-γ treatment of THP-1 cells, IFN-γ priming of HIV-infected MDM significantly enhanced LPS-induced IL-12 p40 protein production to levels comparable to LPS-stimulated mock-infected MDM (figure 13A). However, this increased production of IL-12 p40 protein with IFN-γ priming occurred without enhancing accumulation of IL-12 p40 transcripts, as there was no IL-12 p40 mRNA detectable by RT-PCR in LPS-stimulated HIV-infected MDM that had been preincubated with IFN-γ (figure 13B). These results suggest that IL-12 p40 expression remains impaired by HIV infection in MDM, at least at the transcriptional level, regardless of IFN-γ priming.

A


IL-12 p40 PROTEIN (pg/mL)
2500
2000
1500
1000
500
0
*
†
MOCK
HIV
MEDIUM
LPS
LPS + IFN-γ
THP-1 TREATMENT

B

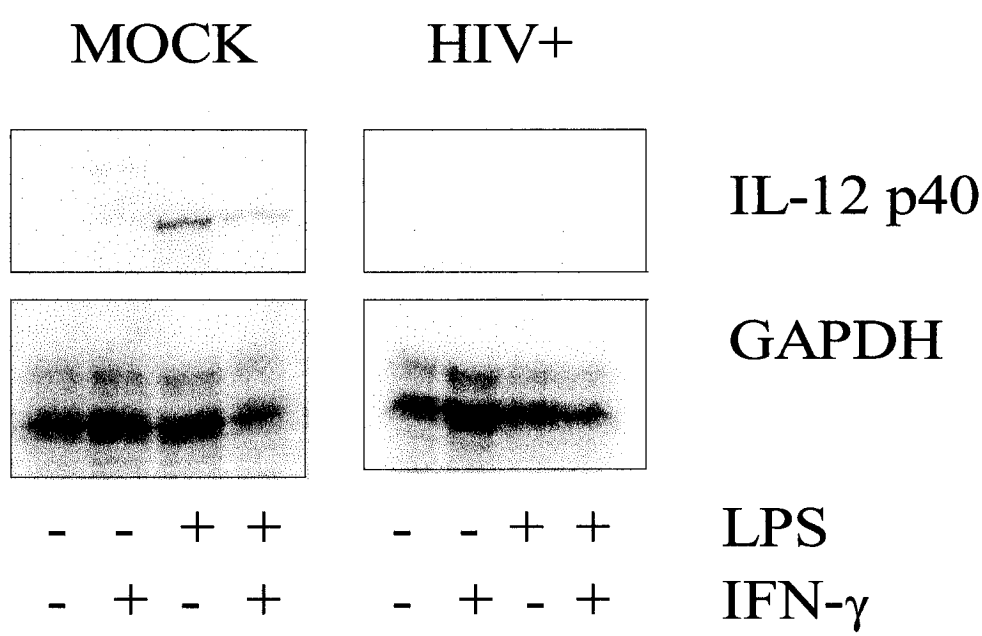
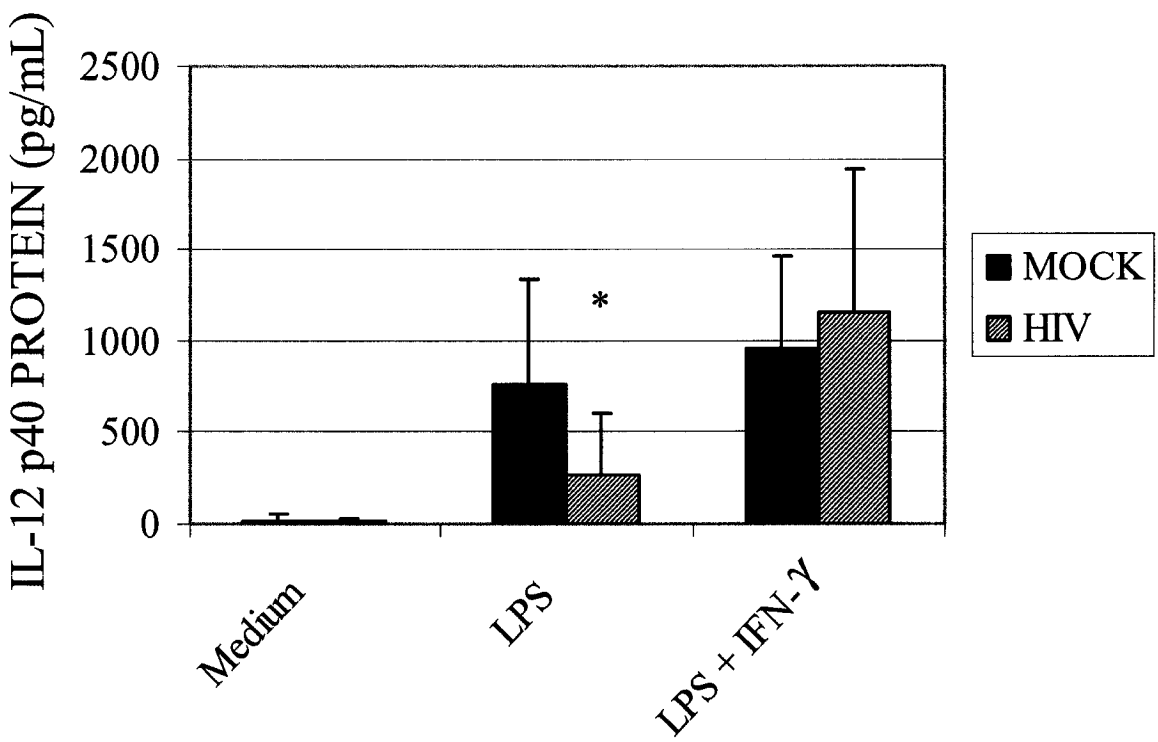

MOCK
HIV+
IL-12 p40
GAPDH
- - + + - - + + LPS
- + - + - + - + IFN-γ

FIGURE 12: *Priming HIV-infected THP-1 cells with IFN-γ prior to LPS stimulation.*

THP-1 cells were mock-infected or infected with HIV-1 cs204, and 7 days post-infection, incubated with or without IFN-γ for 14 hours prior to stimulation with medium or LPS. (**A**) Supernatants from THP-1 cells were harvested 24 hours after LPS stimulation and assayed for total IL-12 p40 protein secretion by ELISA. Bars indicate mean IL-12 p40 levels (pg/ml) ± standard deviation, n=3. * p= 0.04 for HIV-infected LPS stimulated THP-1 cells versus the mock-infected control, and † p=0.0408 for HIV-infected IFN-γ/LPS stimulated THP-1 cells versus similarly treated mock-infected controls, both by Student's t-test. (**B**) Total RNA was harvested from THP-1 primed with IFN-γ, after 4 hours of LPS stimulation, and changes in IL-12 p40 mRNA were detected by ribonuclease protection assay. The gel shown in B is representative of 3 experiments

A


IL-12 p40 PROTEIN (pg/mL)
2500
2000
1500
1000
500
0
*
MOCK
HIV
Medium
LPS
LPS + IFN-γ
MONOCYTE TREATMENT

B

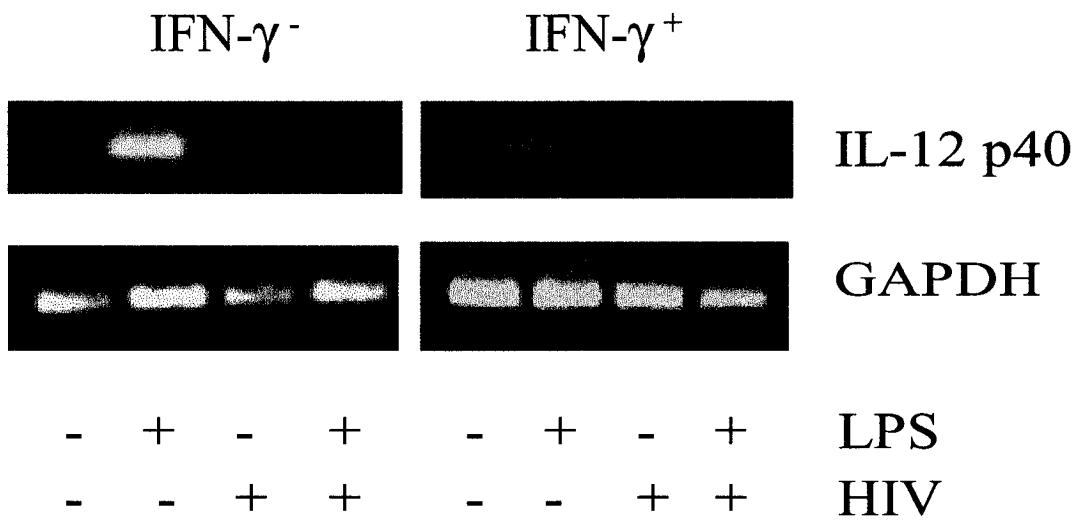
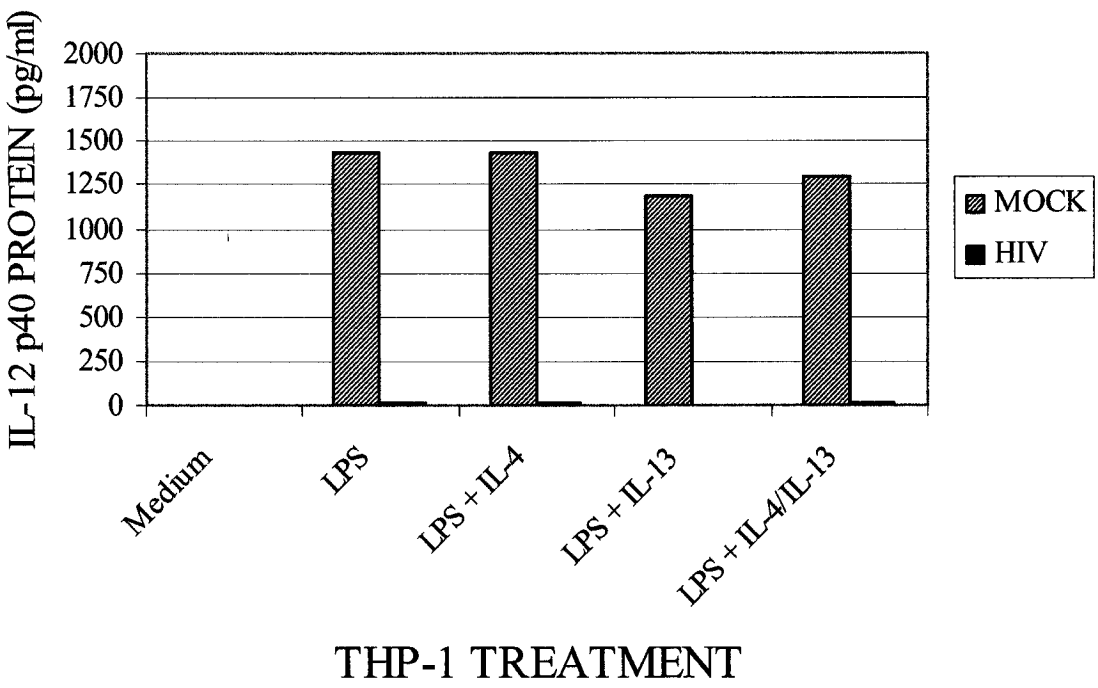

IFN-γ -
IFN-γ +
IL-12 p40
GAPDH
- + - + - + - + LPS
- - + + - - + + HIV

FIGURE 13: *Effect of IFN-γ priming on LPS-induced IL-12 p40 expression in HIV-infected MDM.*

MDM from HIV-seronegative donors were mock-infected or infected with HIV-1 cs204, and 7 days post-infection, incubated with or without IFN-γ for 14 hours prior to stimulation with medium or LPS. (**A**) Supernatants were harvested 24 hours after LPS stimulation and assayed for total IL-12 p40 protein secretion by ELISA. Bars represent mean IL-12 p40 protein production (pg/ml) ± standard deviation, n=5. *p=0.032 by Student's t-test versus mock-infected LPS-stimulated control. **(B)** In parallel cultures, total RNA was harvested from MDM primed with IFN-γ following 4 hours of stimulation with medium or LPS, reverse-transcribed and PCR-amplified with GAPDH and IL-12 p40 primers. The gel shown is representative of 3 independent trials.

A


IL-12 p40 PROTEIN (pg/ml)
2000
1750
1500
1250
1000
750
500
250
0
Medium
LPS
LPS + IL-4
LPS + IL-13
LPS + IL-4/IL-13
MOCK
HIV
THP-1 TREATMENT

B

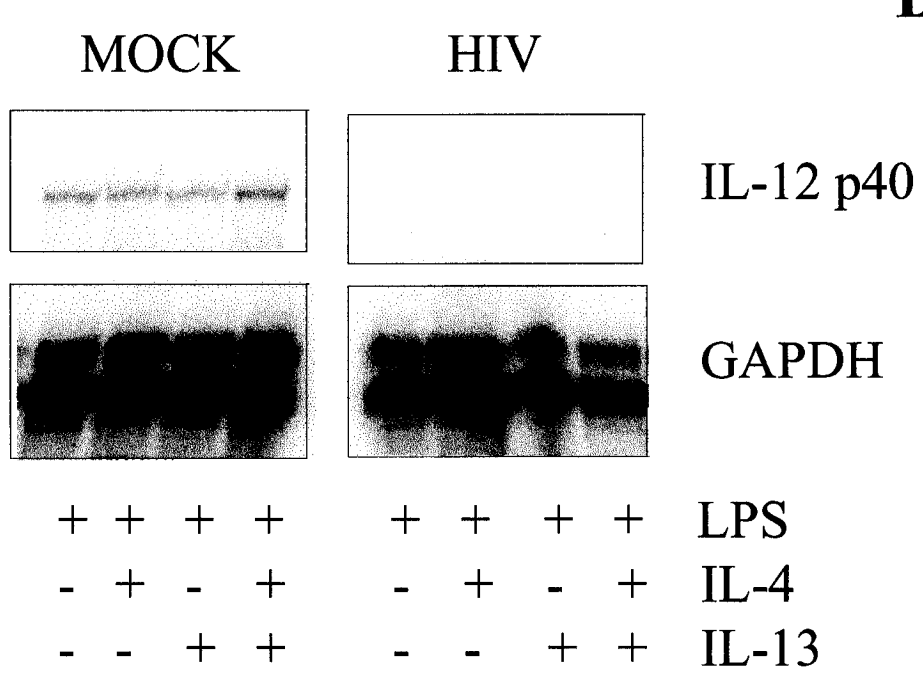

MOCK
HIV
IL-12 p40
GAPDH
+ + + + + + + + LPS
- + - + - + - + IL-4
- - + + - - + + IL-13

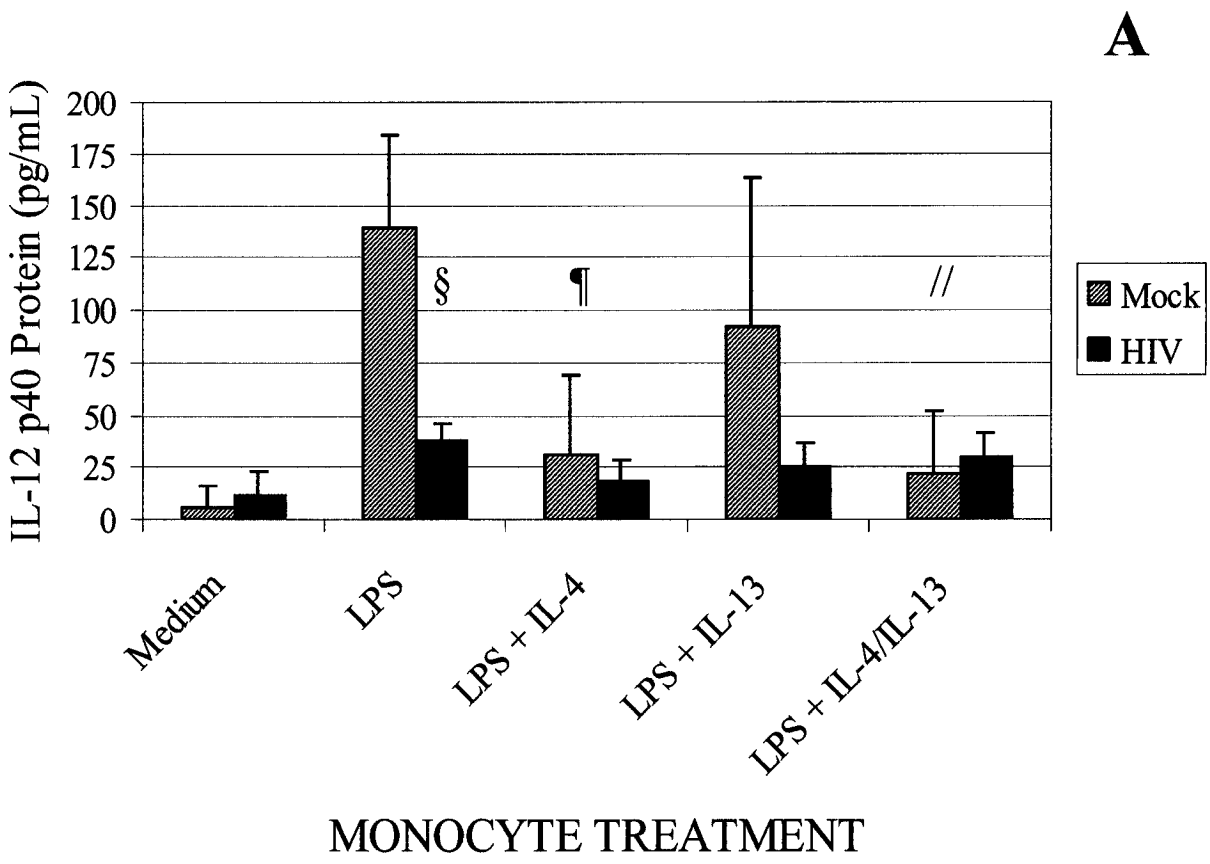
FIGURE 14: *The effects of priming HIV-infected THP-1 cells with IL-4, IL-13, or both, prior to LPS stimulation.*

THP-1 cells were mock-infected or infected with HIV-1 cs204, and 7 days post-infection, incubated with or without IL-4 or IL-13 for 24 hours prior to stimulation with medium or LPS. (**A**) Supernatants from THP-1 cells were harvested 24 hours after LPS stimulation and assayed for total IL-12 p40 protein secretion by ELISA. (**B**) Total RNA was harvested from THP-1 primed with IL-4/IL-13 after 4 hours of LPS stimulation, and changes in IL-12 p40 mRNA were detected by ribonuclease protection assay.

Priming HIV-infected MDM with IL-4, IL-13, or both, prior to LPS stimulation did not enhance IL-12 p40 protein (figure 15A) or IL-12 p40 mRNA (figure 15B), in accordance with the THP-1 data. In fact, incubation with IL-4 alone, or IL-4 and IL-13 in combination reduced LPS-induced IL-12 p40 protein production in uninfected MDM by 84.3% (figure 15A, $p= 0.004$), consistent with the primarily inhibitory role of IL-4 and IL-13 on monocyte/macrophage functions, including IL-12 production (233). The results of these investigations into roles for other key immunomodulatory cytokines in HIV-mediated suppression of IL-12 p40 expression indicate that inhibition of IL-12 p40 protein and mRNA is not a consequence of inhibitory actions of IL-10 or TGF-β, nor due to a lack of the agonistic cytokines IFN-γ, IL-4, or IL-13, although in MDM IFN-γ enhanced IL-12 p40 post-transcriptionally in HIV infected cells.

C. Evaluation of the Requirement for Productive Infection and HIV Replication in Impaired IL-12 p40 Expression in Myeloid Cells:

1. Treatment of myeloid cells with HIV-1 gp120 or replication-defective HIV virions: While HIV infection suppressed IL-12 p40 mRNA expression in myeloid cells in the absence of de novo protein synthesis, it remained to be determined whether this suppression is a consequence of productive infection. Other investigators have demonstrated that soluble HIV-1 gp120 alone suppresses IL-12 p40 protein production in MDM in an IL-10-dependent manner (216), and additionally affects production of numerous cytokines and chemokines including IL-4, IL-13, IL-6, TNF-α, MIP-1α, MIP-1β, and RANTES (234-236). To explore a specific requirement for productive


A
IL-12 p40 Protein (pg/mL)
200
175
150
125
100
75
50
25
0
§
¶
//
Mock
HIV
Medium
LPS
LPS + IL-4
LPS + IL-13
LPS + IL-4/IL-13
MONOCYTE TREATMENT

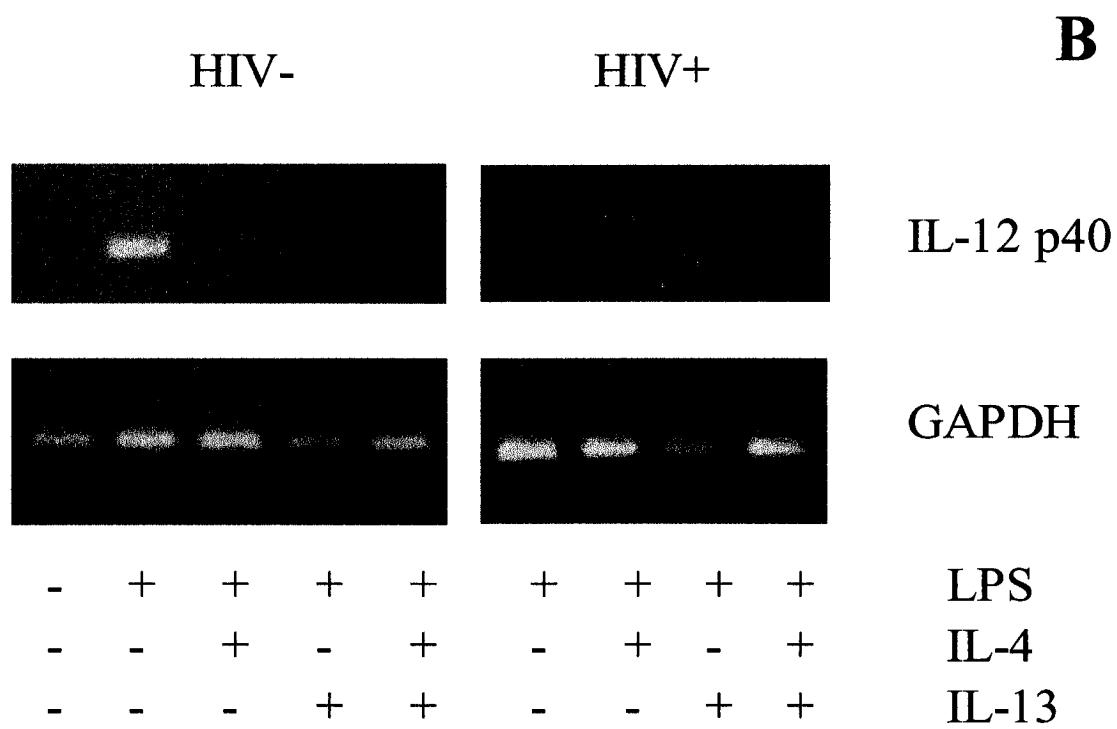

B
HIV-
HIV+
IL-12 p40
GAPDH
- + + + + + + + +
- - + - + - + - +
- - - + + - - + +
LPS
IL-4
IL-13

FIGURE 15: *Priming HIV-infected MDM with IL-4 and/or IL-13 prior to LPS stimulation.*

MDM were mock- or HIV-infected, and 7 days post-infection, incubated with or without IL-4 or IL-13 for 24 hours, prior to stimulation with medium or LPS. (**A**) Cell-free supernatants were harvested 24 hours after LPS stimulation and assayed for total IL-12 p40 protein secretion by ELISA. Bars represent mean IL-12 p40 protein levels (pg/ml) ± standard deviation, n=5. § p= 0.007 by Student's t-test for HIV-infected LPS-stimulated MDM versus the corresponding mock-infected control; ¶ p= 0.0098 for IL-4 primed and // p= 0.004 for IL-4/IL-13 primed THP-1 cells versus mock-infected, LPS-stimulated MDM, determined by Student's t-test. (**B**) Total RNA harvested from mock- or HIV-infected MDM, primed with IL-4/IL-13 prior to 4 hours of LPS stimulation was reverse-transcribed and PCR-amplified with GAPDH and IL-12 p40 primers. The gel shown is representative of 3 independent trials.

infection of myeloid cells in HIV-mediated suppression of IL-12 p40 induction, THP-1 cells and adherence derived MDM were mock- or HIV-infected, or treated with soluble HIV-1 gp120, or with supernatants from 8E5 cells, which shed HIV-1 virions defective in reverse-transcriptase. Incubation of THP-1 cells with RT-deficient virus (8E5 cell supernatant) or with soluble gp120 prior to LPS stimulation had no demonstrable effect on IL-12 p40 protein production (figure 16A). In addition, neither supernatant from 8E5 cells, nor soluble gp120 suppressed LPS-induced IL-12 p40 mRNA accumulation, while infection with HIV-1 completely inhibited production of IL-12 p40 mRNA (figure 16B). Inclusion of neutralizing anti-IL-10 antibody to THP-1 cells cultured with gp120 had no effect on LPS-induced IL-12 p40 mRNA expression (figure 16B), despite reports that gp120 treatment elicits IL-10 production from human MDM (216). The lack of influence of the RT-deficient virus on IL-12 induction compared to HIV infection was not due to disparity in the virion content of the 8E5 or HIV-containing supernatants, as the p24 antigen level of each treatment condition was comparable (10 and 8.3 ng/ml, respectively).

HIV-1 gp120 treatment of MDM prior to LPS stimulation also had no effect on IL-12 p40 protein (figure 17A) or mRNA expression (figure 17B), in agreement with the results in THP-1 cells. However, incubation of MDM with supernatant from 8E5 cells resulted in a 64.2% reduction in IL-12 p40 protein production relative to untreated, LPS stimulated control (figure 17A, $p=0.002$), though not to the level observed in HIV-infected MDM (figure 17A, $p=0.03$). This reduction in IL-12 p40 protein production was not accompanied by a decrease in IL-12 p40 mRNA production (figure 17B) as demonstrated by RT-PCR, indicating disparity in regulation of IL-12 p40 protein and

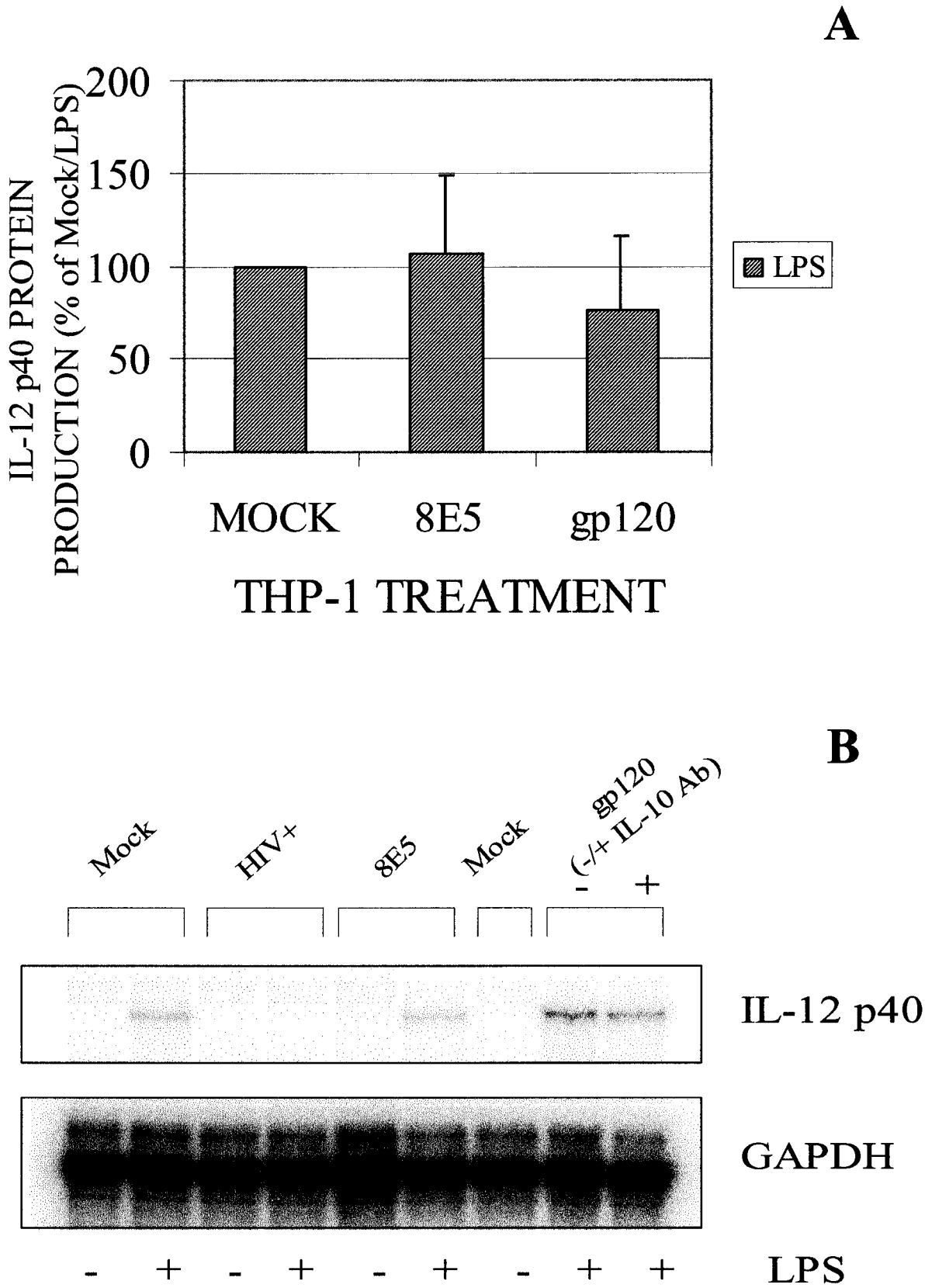
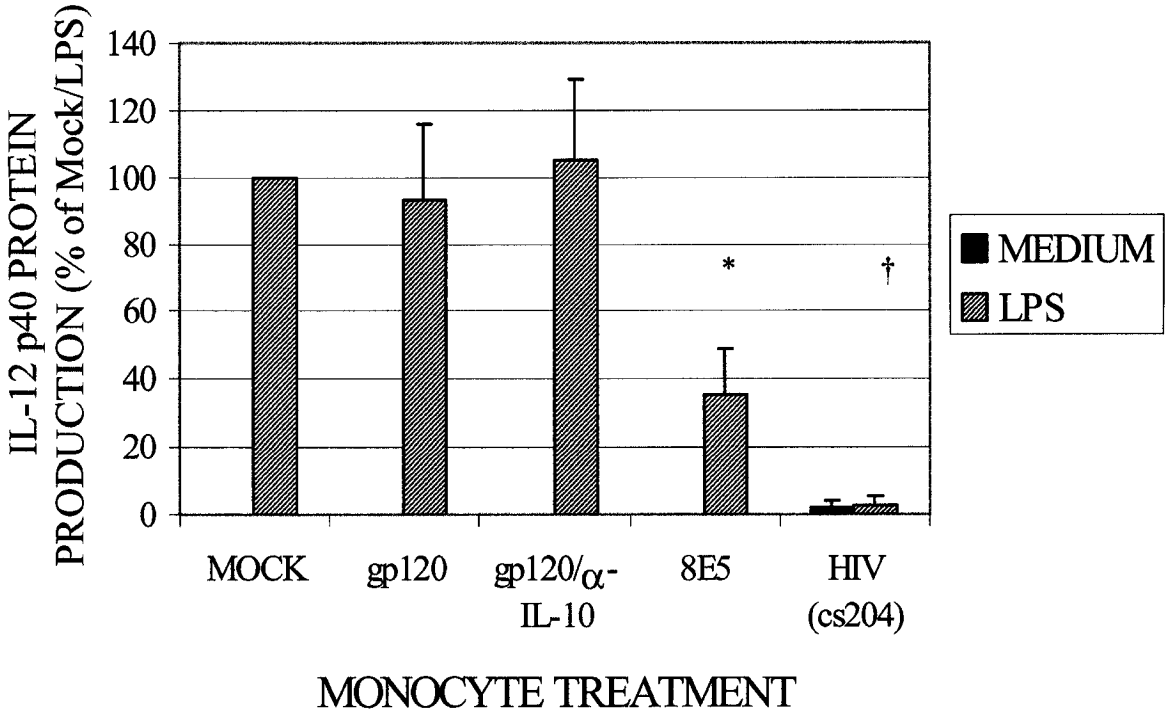

A
IL-12 p40 PROTEIN PRODUCTION (% of Mock/LPS)
200
150
100
50
0
LPS
MOCK
8E5
gp120
THP-1 TREATMENT
B
Mock
HIV+
8E5
Mock
gp120 (-/+ IL-10 Ab)
-
+
IL-12 p40
GAPDH
- + - + - + - + +
LPS

FIGURE 16: *Effect of soluble HIV-1 gp120 and RT-deficient HIV-1 on LPS-induced IL-12 expression in THP-1 cells.*

THP-1 cells were mock-infected, infected with HIV-1 cs204, incubated with 8E5 supernatants containing RT-deficient HIV-1 virions, or treated with soluble HIV-1 gp120, prior to stimulation with medium or LPS. (**A**) Total IL-12 p40 protein from culture supernatants of THP-1 cells stimulated with medium or LPS for 24 hours was quantitated by ELISA (n=3). Protein data for THP-1 cells are expressed as a percentage of IL-12 p40 protein production by mock-infected, LPS stimulated THP-1 cells ± standard deviation. **(B)** Total cellular RNA was harvested from THP-1 after 4 hours of LPS stimulation and changes in IL-12 p40 mRNA expression were detected by ribonuclease protection assay. Image shown is representative of 3 independent trials.

A


IL-12 p40 PROTEIN PRODUCTION (% of Mock/LPS)
140
120
100
80
60
40
20
0
*
†
MEDIUM
LPS
MOCK
gp120
gp120/α-IL-10
8E5
HIV (cs204)
MONOCYTE TREATMENT

B

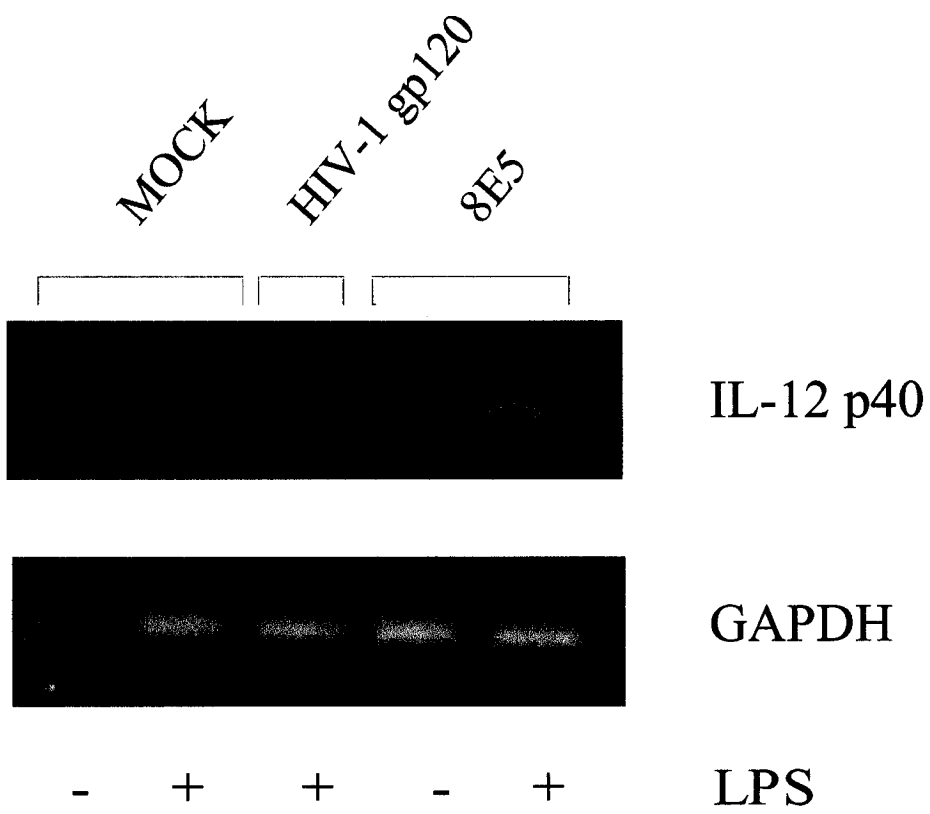

MOCK
HIV-1 gp120
8E5
IL-12 p40
GAPDH
- + + - +
LPS

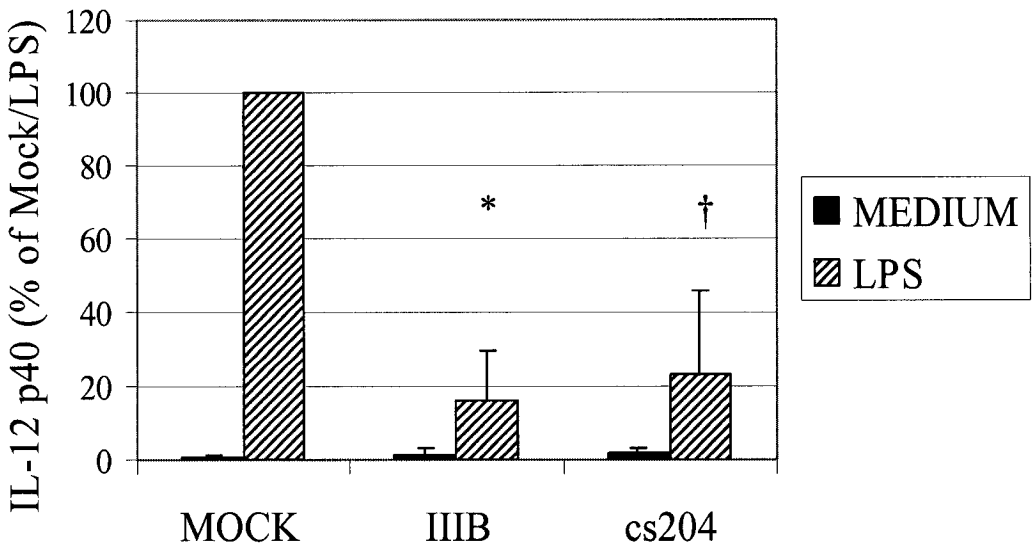
FIGURE 17: *Treatment of MDM with soluble HIV-1 gp120 and RT-deficient HIV-1, and the effect on LPS-induced IL-12 p40 expression.*

MDM from HIV-seronegative donors were mock-infected, infected with HIV-1 cs204, treated with 8E5 supernatants containing RT-deficient HIV-1 virions, or treated with soluble HIV-1 gp120, prior to stimulation with medium or LPS. (**A**) Total IL-12 p40 protein from culture supernatants stimulated with medium or LPS for 24 hours was quantitated by ELISA (n=3). Protein data are expressed as a percentage of IL-12 p40 protein production by mock-infected, LPS stimulated MDM, ± standard deviation. *p=0.002, † p=0.03 by Student's t-test versus mock-infected control. **(B)** Total RNA isolated from MDM after 4 hours of stimulation was reverse-transcribed and PCR-amplified with gene-specific primers for GAPDH or IL-12 p40. Gel shown is representative of 3 experiments.

mRNA synthesis in response to replication-competent and replication-defective HIV virions.

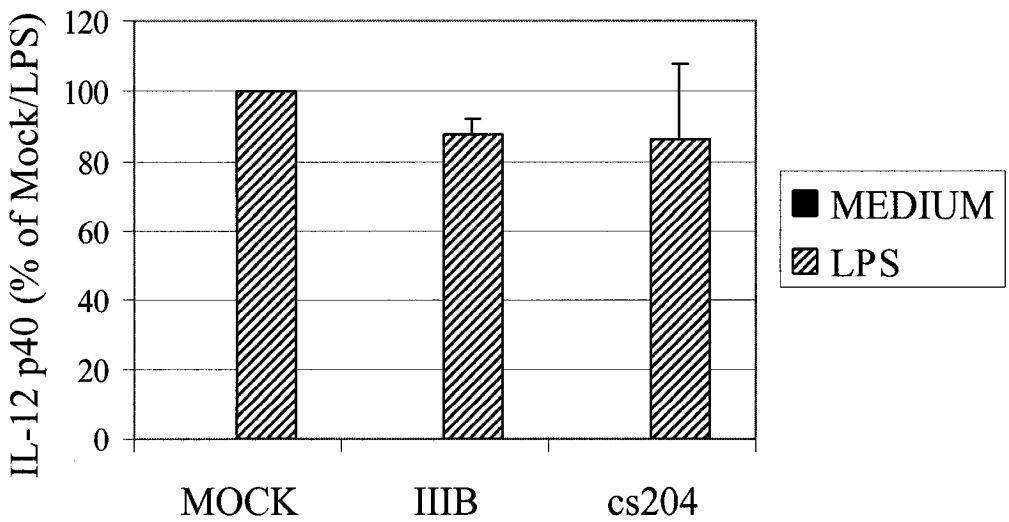
2. Treatment of THP-1 cells with UV-inactivated supernatants from HIV-infected THP-1 cell cultures: Having eliminated a role for de novo protein synthesis and select regulatory cytokines in HIV-mediated suppression of IL-12 p40 synthesis, investigations were conducted to confirm whether any host- or virus-derived soluble products present in infected cell cultures were responsible for suppression of IL-12 by HIV. Supernatants were collected from unstimulated THP-1 cells 7 days following infection with cs204 or HIV-1_{IIIB}, and subjected to UV radiation for 1 hour (228), prior to culture with fresh THP-1 cells. After 24 hours, THP-1 cells were stimulated with LPS, and supernatants were assayed for IL-12 p40 protein production. As illustrated in figure 18A and in agreement with earlier findings (figure 7A), infection of THP-1 cells with HIV-1_{IIIB} or the clinical isolate cs204 dramatically suppressed LPS-induced IL-12 p40 protein production. Supernatants from parallel mock- or HIV-infected THP-1 cells which did not previously receive LPS stimulation, and which were UV-irradiated prior to incubation with fresh THP-1 cells, failed to transfer suppression of LPS-induced IL-12 synthesis observed in the original infected THP-1 cell cultures (figure 18B). Neutralization of infectious virus by UV irradiation was confirmed by the failure of these inactivated supernatants to productively infect THP-1 cells during a 14 day culture period, as determined by HIV-1 p24 antigen quantitation described in the Methods. These findings suggested that suppression of IL-12 expression by HIV infection was not mediated by any soluble host or viral factors which may have accumulated in culture during the 7 days of culture prior to quantitation of IL-12 p40 protein and mRNA production.

A



HIV INFECTION & LPS
INDUCTION OF IL-12

B



LPS INDUCTION OF IL-12 IN THE
PRESENCE OF SUPERNATANT FROM
HIV-INFECTED THP-1

FIGURE 18: *Culture of THP-1 Cells with UV Irradiated Supernatants from HIV-1 Infected, Unstimulated THP-1 Cells.*

(A) THP-1 cells were mock-infected, or infected with HIV-1 cs204 or HIV-1_{IIIB}, and on day 7, cultured with medium or LPS for 24 hours. Supernatants were harvested and assayed for IL-12 p40 protein secretion by ELISA. IL-12 p40 protein production is expressed as a percentage of mock-infected, LPS-stimulated THP-1 cells, ± standard deviation, n=3; * p=0.009, † p=0.03 by Student's t-test versus mock-infected control. **(B)** Supernatants from HIV-infected cells cultured in medium alone (from experiment outlined in **A**) were subjected to UV irradiation for 1 hour, and were incubated with fresh THP-1 cells for 24 hours. THP-1 cells were subsequently stimulated with LPS for 24 hours, and IL-12 p40 protein production was quantitated by ELISA. IL-12 p40 protein production is expressed as a percentage of mock-infected, LPS-stimulated THP-1 cells, ± standard deviation, n=3.

3. Evaluation of intracellular IL-12 p40 protein and HIV-1 p24 antigen expression in THP-1 cells following HIV infection: In order to further characterize the requirement for productive HIV infection in suppression of IL-12 p40 production, intracellular IL-12 p40/p70 protein and HIV-1 p24 antigen expression in mock- and HIV-infected THP-1 cells were monitored by flow cytometry and immunofluorescence microscopy. As depicted in figure 19A, HIV-1 p24 antigen was undetectable in mock-infected THP-1 cells, but following HIV infection, 77.2% of cells stained positively for HIV p24. In addition, 72.8% of mock-infected LPS-stimulated THP-1 cells expressed intracellular IL-12 p40/p70 protein, while LPS-stimulated HIV-infected THP-1 cells expressed IL-12 protein at levels comparable to unstimulated controls (10.3% versus 8.0%) (figure 19B).

Similar results were obtained by immunofluorescence microscopy performed on mock- and HIV-infected THP-1 cells, as shown in figure 20. Intracellular IL-12 p40/p70 protein was readily detected in mock-infected, LPS-stimulated THP-1 cells (figure 20B), but only background levels of IL-12 protein were observed in HIV-infected cells (compare panel A and panel F). In comparison with total DAPI-stained cells, intracellular p24 protein was detected at varying intensities throughout HIV-infected (figure 20G) but not mock-infected THP-1 cells (figure 20C). The extensive HIV-1 p24 antigen detection throughout HIV-infected THP-1 cells was not due to surface expression of p24, as no staining was detected on HIV-infected THP-1 cell preparations that were not permeabilized (figure 20K). The dramatic reduction in intracellular IL-12 p40 protein detection following HIV infection of myeloid cells, in which the vast majority of cells are infected, lends further support to the suggestion that suppression of IL-12 expression by HIV is a result of direct cellular infection. These

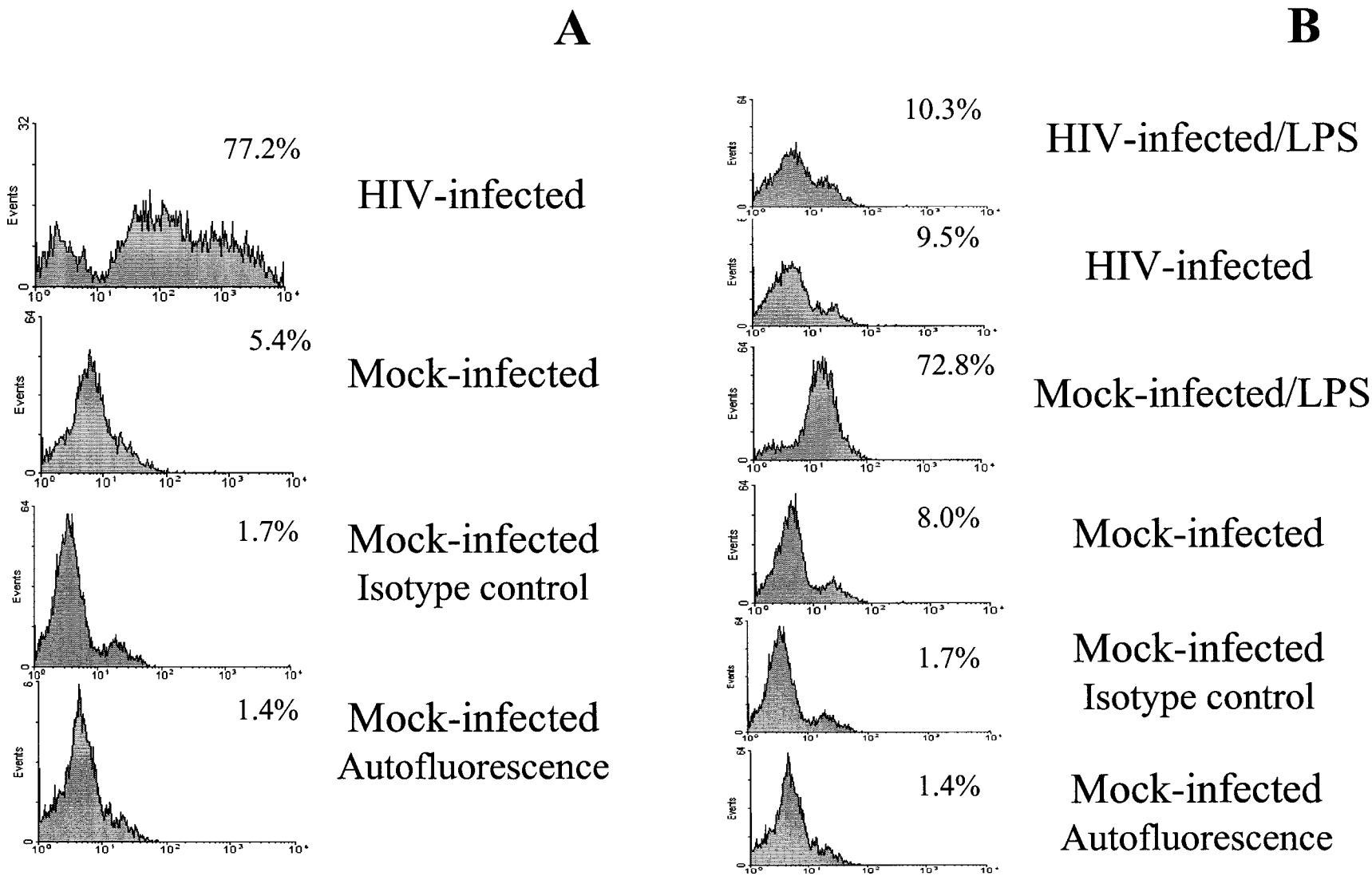

A
B
77.2%
HIV-infected
5.4%
Mock-infected
1.7%
Mock-infected
Isotype control
1.4%
Mock-infected
Autofluorescence
10.3%
HIV-infected/LPS
9.5%
HIV-infected
72.8%
Mock-infected/LPS
8.0%
Mock-infected
1.7%
Mock-infected
Isotype control
1.4%
Mock-infected
Autofluorescence
Events
α-HIV-1 P24 (PE)
α-IL-12 p40/p70 (PE)

FIGURE 19: *Quantitation of HIV-1 p24 and IL-12 p40 Expression in THP-1 Cells by Flow Cytometry.*

THP-1 cells were mock- or HIV-1 cs204-infected, and stimulated with medium or LPS in the presence of GolgiStop, and stained with PE-labeled anti-IL-12 p40/p70 or PE-labeled anti-HIV-1 p24 monoclonal antibodies, or PE-labeled mouse IgG_1 isotype control. (**A**) Flow cytometry histograms illustrating intracellular HIV-1 p24 antigen detection in mock- and HIV-infected, medium or LPS-stimulated THP-1 cells, compared with staining with a mouse IgG_1 isotype control. Labels indicate the percentage of THP-1 cells expressing HIV-1 p24 antigen under each treatment condition. (**B**) Intracellular human IL-12 p40/p70 protein detection in mock- and HIV-infected THP-1 cells, stimulated with medium or LPS as in **A**. Labels indicate the percentage of THP-1 cells infected with HIV (positively staining for IL-12 p40/p70 protein) under each treatment condition.

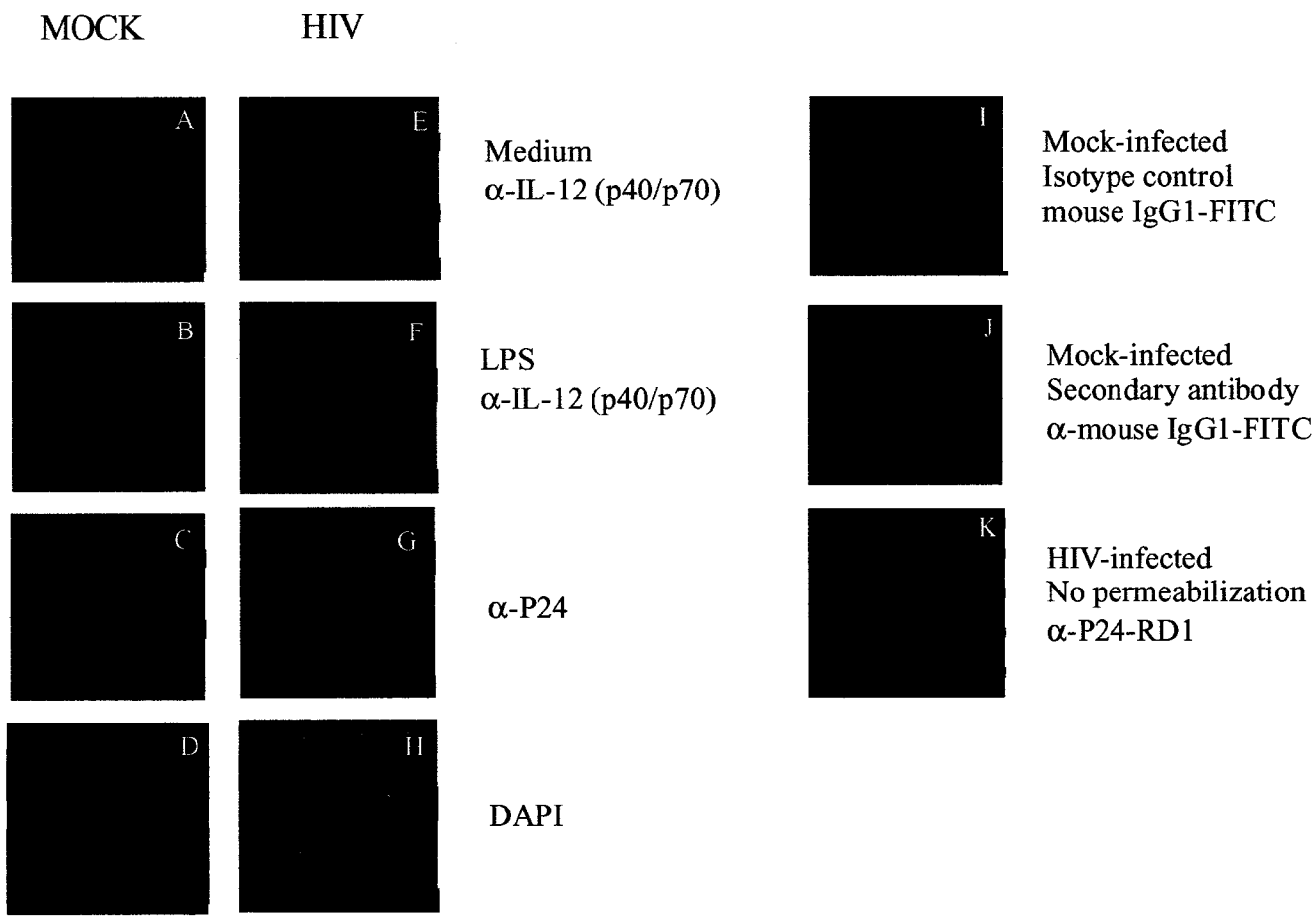

MOCK
HIV
A
E
Medium
α-IL-12 (p40/p70)
I
Mock-infected
Isotype control
mouse IgG1-FITC
B
F
LPS
α-IL-12 (p40/p70)
J
Mock-infected
Secondary antibody
α-mouse IgG1-FITC
C
G
α-P24
K
HIV-infected
No permeabilization
α-P24-RD1
D
H
DAPI

FIGURE 20: *Intracellular Detection of HIV-1 p24 and IL-12 p40 Expression in THP-1 Cells by Immunofluorescence Microscopy.*

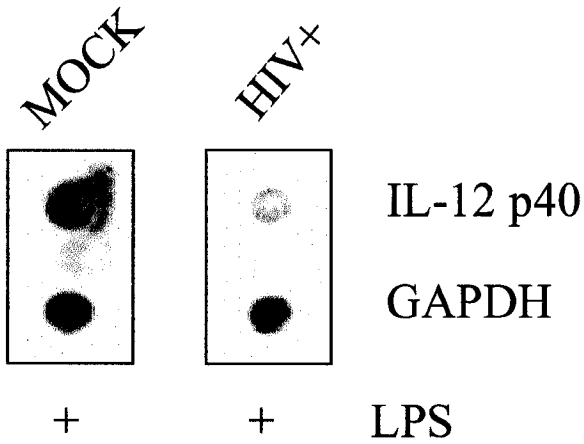
THP-1 cells were mock- or HIV-1 cs204-infected, and stimulated with medium or LPS in the presence of GolgiStop. (**A, E**) IL-12 p40/p70 protein detection in unstimulated THP-1; (**B, F**) IL-12 p40/p70 protein detection in mock- and HIV-infected LPS-stimulated THP-1 cells, respectively; (**C, G**) HIV-1 p24 antigen detection in mock- and HIV-infected THP-1 cells, respectively; (**D, H**) DAPI nuclear staining of mock- and HIV-infected THP-1 cells, respectively; (**I**) mouse IgG1-FITC isotype control staining of mock-infected THP-1 cells; (**J**) mouse IgG1-FITC staining of mock-infected THP-1 cells in the absence of anti-IL-12 p40/p70 primary antibody; (**K**) HIV-1 p24 staining of HIV-infected non-permeabilized THP-1 cells.

findings also validate the earlier observation of profound suppression of IL-12 p40 expression following HIV infection, and substantiate the notion that HIV-mediated suppression of IL-12 occurs, at least in part, at an intracellular level likely affecting the underlying molecular machinery involved in the initiation and synthesis of IL-12.

D. Impairment of Transcription of the IL-12 p40 Gene in .HIV-Infected Myeloid Cells:

1. Suppression of IL-12 p40 expression during HIV-1 infection of THP-1 cells occurs at the level of transcription: Previous reports in the literature have determined that LPS-induced transcription of the IL-12 p40 subunit gene is impaired in PBMC from HIV-infected patients (12). Having demonstrated that HIV infection impairs IL-12 synthesis in a manner that requires productive infection of myeloid cells, resulting in severe impairment of IL-12 p40 mRNA levels in myeloid cells, nuclear run-on transcription assays were conducted to determine whether HIV infection of isolated myeloid cells inhibits IL-12 expression at the level of transcription. Mock- and HIV-infected THP-1 were stimulated with LPS for 4 hours prior to isolation of intact nuclei and measurement of nascent nuclear transcripts for the IL-12 p40 gene and the GAPDH housekeeping gene control. Infection of THP-1 cells with HIV cs204 and subsequent LPS stimulation resulted in a dramatic reduction in IL-12 p40 mRNA hybridization to target IL-12 p40 cDNA, relative to mock-infected controls (figure 21A), indicative of significant reduction in the rate of transcription of the IL-12 p40 gene upon HIV infection. Transcription of the IL-12 p40 gene in HIV-infected LPS-stimulated THP-1 cells was in fact reduced to the level of transcription observed in unstimulated controls (data not shown). In a control experiment, incubation of ^{32}P-labelled total nuclear transcript with membranes containing empty vectors corresponding

A


MOCK
HIV+
IL-12 p40
GAPDH
+
+
LPS

B

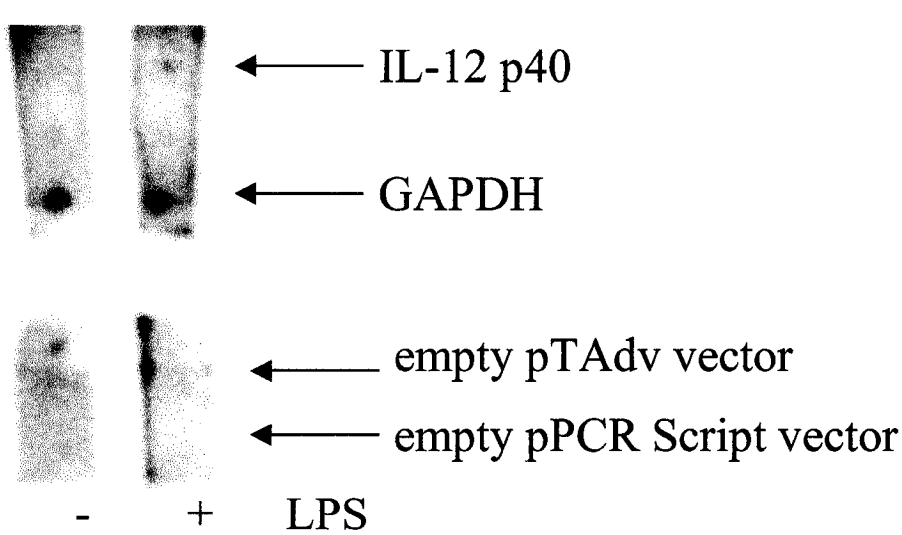

IL-12 p40
GAPDH
empty pTAdv vector
empty pPCR Script vector
-
+
LPS

FIGURE 21: *Effect of HIV Infection of Transcription of the IL-12 p40 Gene.*

THP-1 cells were mock-infected or infected with HIV-1 cs204, and stimulated with LPS for 4 hours prior to harvest and quantitation of nascent IL-12 p40 and GAPDH transcripts using a nuclear run-on assay. **(A)** Image depicts hybridization of ^{32}P-labelled IL-12 p40 and GAPDH mRNA to target cDNAs immobilized on nitrocellulose membranes. Image shown is representative of 3 experiments. **(B)** Control experiment illustrating the absence of hybridization of nuclear transcript to empty pT-Adv and pPCR-Script control vectors.

to the IL-12 p40 cDNA (pT-AdV) or GAPDH cDNA (pPCR-Script) targets demonstrated no hybridization to these empty control vectors, thereby illustrating specificity of the hybridization observed to IL-12 p40 and GAPDH target insert cDNAs (figure 21B).

2. Nuclear factor recruitment to the human IL-12 p40 promoter in HIV-infected myeloid cells: Having confirmed that HIV infection of myeloid cells suppresses IL-12 p40 gene expression, at least in part, at the level of transcription, studies were undertaken to evaluate the impact of HIV on nuclear factor binding to the IL-12 p40 promoter. Several putative transcription factor binding sites have been identified within the human and murine IL-12 p40 promoters, including C/EBP, NF-κB, PU.1, Ets-2, AP-1, Sp1, NF-IL-6, and IRF-1 (figure 1, Introduction), yet the significance of each site in stimulus-induced promoter activation is poorly understood. In addition, several reports have demonstrated that inhibitors of IL-12 synthesis can act via targeted disruption of nuclear factor binding to at least the NF-κB and Ets-2 promoter elements (102, 104, 120-122), yet any effects of HIV infection on nuclear factor binding to these or any other sites in the p40 promoter have not been evaluated. Therefore, each of the identified binding sites in the human IL-12 p40 promoter was surveyed for alterations in nuclear factor recruitment following HIV infection. To examine the effect of LPS on nuclear factor binding to the IL-12 p40 promoter, and the effect of HIV infection on this binding, EMSA analyses were performed on nuclear extracts from mock- and HIV-infected THP-1 cells and MDM, using ^{32}P-labelled oligonucleotide probes representing each of the individual putative transcription factor binding sites and corresponding flanking sequences derived from the human IL-12 p40 promoter.

i. Altered nuclear factor recruitment to the IL-12 p40 promoter following LPS treatment and/or HIV infection of THP-1 cells: In THP-1 cells, HIV exhibited a two-fold effect on nuclear factor binding to the NF-κB, AP-1 and Sp1 sites in the IL-12 p40 promoter. First, HIV infection reduced binding of nuclear protein to the NF-κB, AP-1 and Sp1 sites of the IL-12 p40 promoter (figures 22A, 23A, and 24A, respectively). LPS stimulation alone, in the absence of HIV infection also resulted in decreased binding to the promoter at each of these sites relative to unstimulated, mock-infected controls (figures 22A, 23A, 24A). Secondly, following HIV infection, this effect of LPS was reversed and binding was slightly increased at each of the NF-κB, AP-1 and Sp1 sites, though was still markedly reduced compared to LPS-stimulated uninfected THP-1 cells. At the Sp1 element in particular, EMSA analysis revealed formation of two specific complexes; in a number of replicate experiments, both complexes appear to be affected by HIV infection (figure 24A), however, in several other trials, only one of the two complexes was altered by HIV infection of THP-1 cells.

Subsequent investigations using supershift analysis with antibodies directed against relevant nuclear factors for these affected sites revealed that the decrease in binding of nuclear protein at the NF-κB binding site was due, at least in part, to reduced binding of NF-κB p50, p65, and c-Rel proteins to this element following HIV infection as indicated by the reduced intensity of the uppermost supershifted complex (figure 22B). Similarly, binding of c-Fos and c-Jun proteins to the AP-1 promoter element, as well as Sp1 and Sp3 protein binding to the Sp1 site were reduced by HIV infection

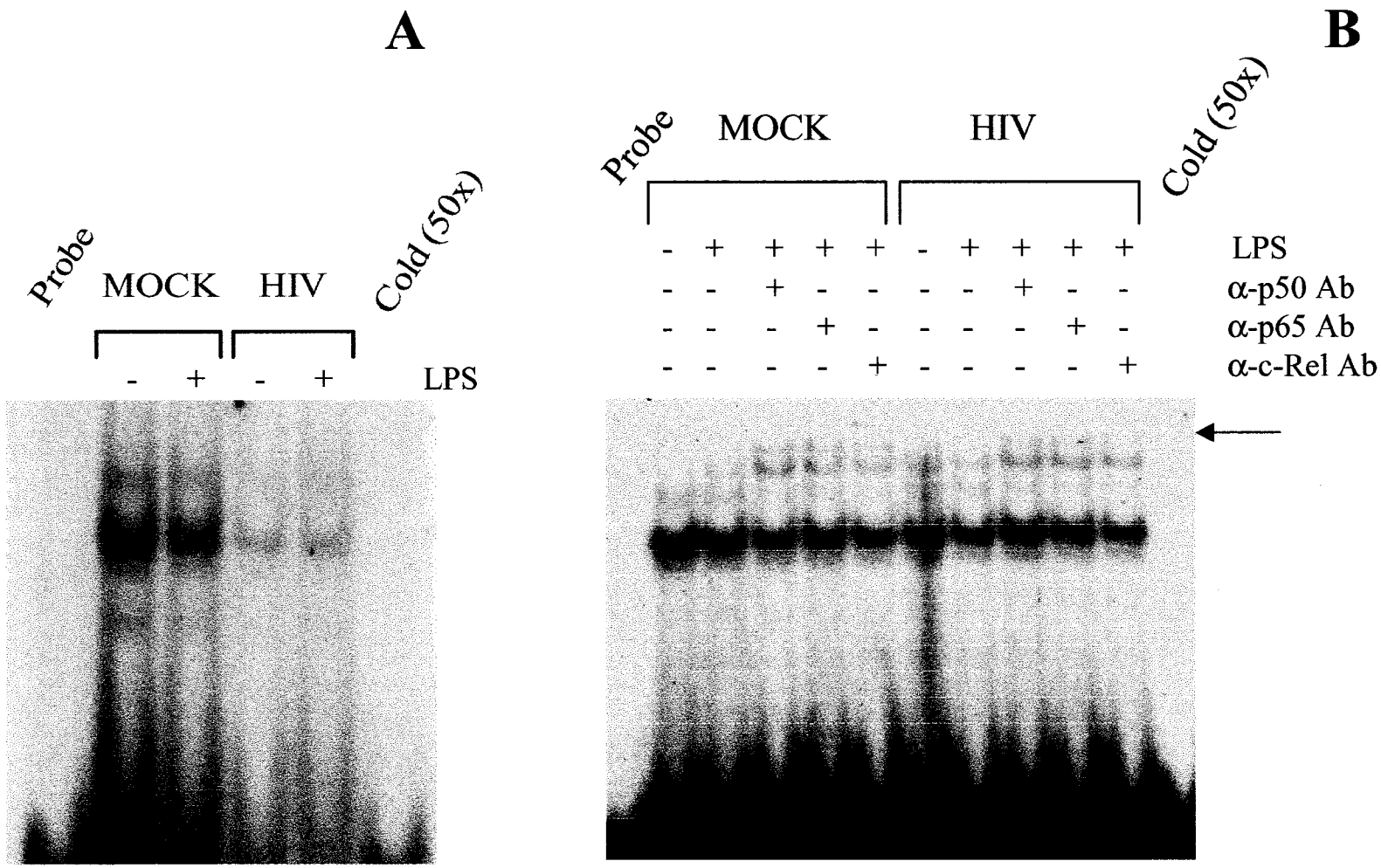

A
Probe
MOCK
HIV
Cold (50x)
- + - +
LPS
B
Probe
MOCK
HIV
Cold (50x)
- + + + + - + + + +
LPS
- - + - - - - + - -
α-p50 Ab
- - - + - - - - + -
α-p65 Ab
- - - - + - - - - +
α-c-Rel Ab

FIGURE #22: *EMSA and supershift analysis of nuclear factor binding to the NF-κB site in the human IL-12 p40 promoter in mock- and HIV-infected THP-1 cells.*

THP-1 cells were mock- or HIV-infected for 7 days prior to stimulation with medium or LPS (1 μg/ml). (**A**) Nuclear extracts were harvested after 2 hours and incubated with a ^{32}P-labelled oligonucleotide probe representing the putative NF-κB binding site derived from the human IL-12 p40 promoter. (**B**) Subsequent supershift analysis was conducted by incubation of EMSA binding reactions with antibodies (20 μg/ml) directed against NF-κB p50, p65, and c-Rel proteins. Images shown are representative of three independent experiments. Arrows indicate the positions of supershifted complexes in the presence of the indicated nuclear factor-specific antibodies.

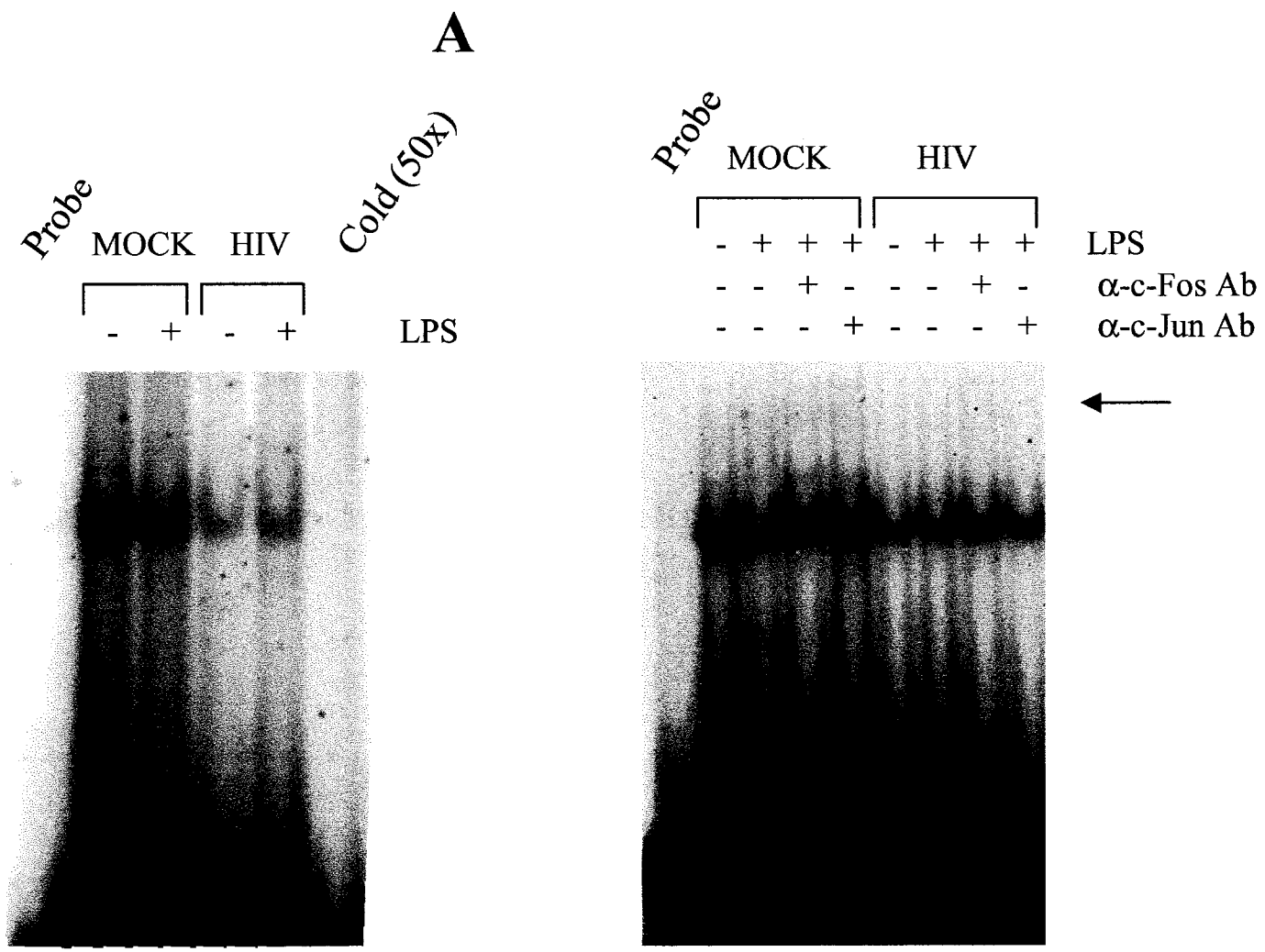

A
Probe
MOCK
HIV
Cold (50x)
- + - +
LPS
B
Probe
MOCK
HIV
- + + + - + + +
LPS
- - + - - - + -
α-c-Fos Ab
- - - + - - - +
α-c-Jun Ab

FIGURE #23: *EMSA and supershift analysis of nuclear factor binding to the AP-1 site in the human IL-12 p40 promoter in mock- and HIV-infected THP-1 cells.*

THP-1 cells were mock- or HIV-infected for 7 days prior to stimulation with medium or LPS (1 μg/ml). **(A)** Nuclear extracts were harvested after 2 hours and incubated with a ^{32}P-labelled oligonucleotide probe encompassing the AP-1 binding site and corresponding flanking DNA sequence derived from the human IL-12 p40 promoter. **(B)** Inclusion of antibodies (20 μg/ml) directed against the AP-1 binding proteins c-Fos and c-Jun in the DNA-protein binding reactions, produced supershifts indicative of binding of these factors to the IL-12 p40 promoter. Images shown are representative of three independent experiments. Arrows indicate the positions of supershifted complexes in the presence of the indicated nuclear factor-specific antibodies.

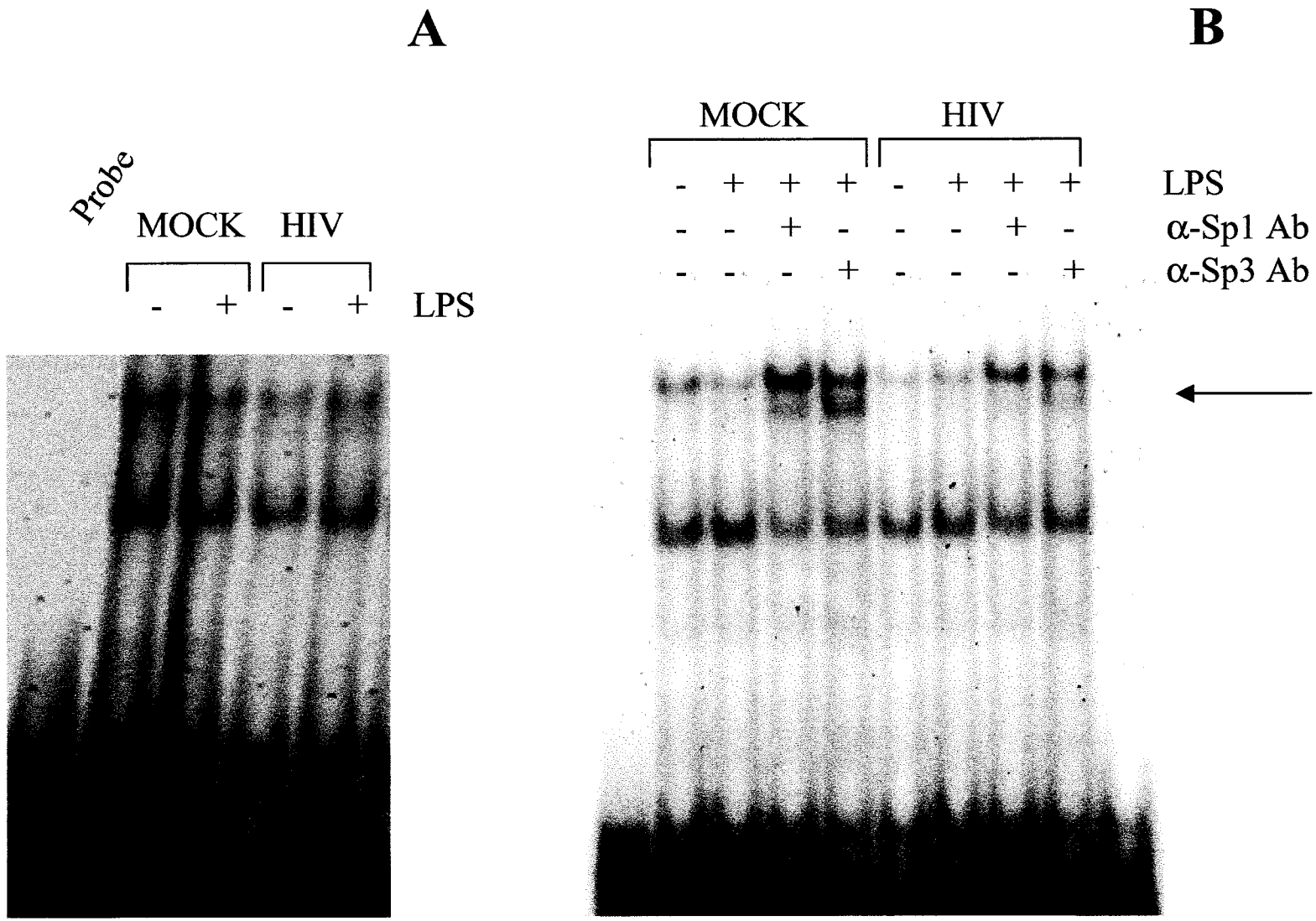
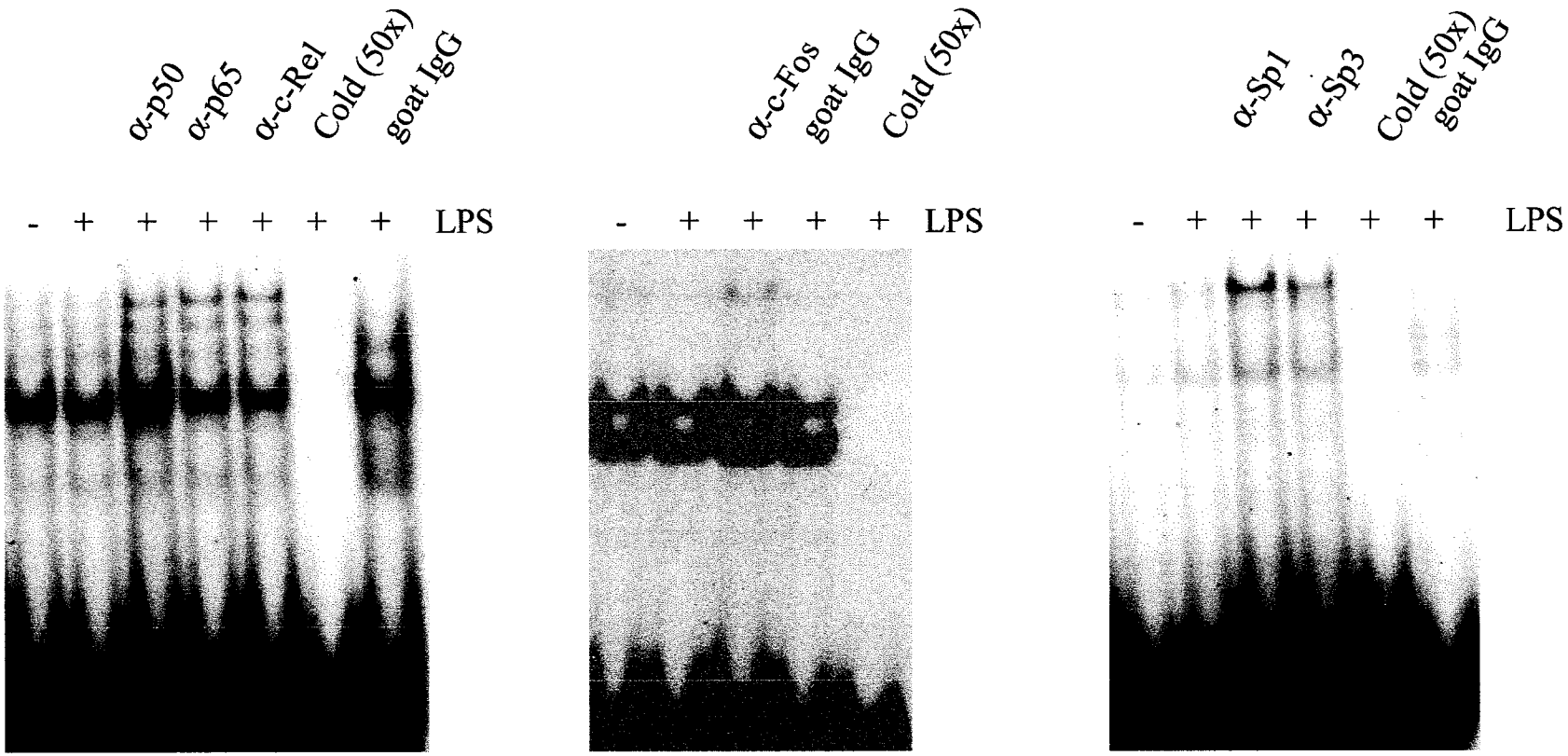

A
Probe
MOCK
HIV
- + - +
LPS
B
MOCK
HIV
- + + + - + + +
LPS
- - + - - - + -
α-Sp1 Ab
- - - + - - - +
α-Sp3 Ab

FIGURE #24: *EMSA and supershift analysis of nuclear factor binding to the Sp1 site in the human IL-12 p40 promoter in mock- and HIV-infected THP-1 cells.*

THP-1 cells were mock- or HIV-infected for 7 days prior to stimulation with medium or LPS (1 μg/ml). (**A**) Nuclear extracts were harvested after 2 hours and incubated with a ^{32}P-labelled oligonucleotide probe containing the putative Sp1 binding site represented in the human IL-12 p40 promoter. The gel shown is representative of 3 independent experiments. (**B**) Subsequent supershift analysis was performed by incubating DNA-protein binding reactions in the presence or absence of antibodies (20 μg/ml) directed against Sp1 and Sp3 proteins. Images shown are representative of three independent experiments. Arrows indicate the positions of supershifted complexes in the presence of the indicated nuclear factor-specific antibodies.

of THP-1 cells (figures 23B and 24B, respectively). Binding of nuclear factor-directed antibodies was specific, as supershifted complexes were not observed using a control goat-IgG antibody (figure 25). In contrast to the observed altered binding to the NF-κB, AP-1, and Sp1 sites in THP-1 nuclear extracts, nuclear factor recruitment to the C/EBP, Ets-2, NF-IL-6, and IRF-1 elements in THP-1 cells was apparently not LPS-inducible and was not affected by HIV infection of THP-1 cells (figures 26A through D, respectively).

ii. LPS-induced nuclear factor binding to the IL-12 p40 promoter in MDM and the effect of HIV infection: HIV-infection of MDM also exhibited a two-fold effect on IL-12 p40 promoter activation. However, unlike THP-1 cells, MDM infected with HIV exhibited an increase in the overall intensity of binding of nuclear factors to the NF-κB (figure 27A) and AP-1 (figure 28A) elements of the IL-12 p40 promoter, and suppressed transcription factor binding to the Ets-2 element relative to mock-infected controls (figure 30A). Similar to the observations in THP-1 cells, HIV infection of MDM additionally interfered with the effects of LPS on nuclear factor binding to these sites. Specifically, the LPS-induced increase in binding to the NF-κB and Ets-2 elements, as well as the LPS-induced decrease in binding to the AP-1 and Sp1 sites observed in mock-infected cells were reversed by HIV infection. While nuclear factor binding to the Sp1 element in unstimulated MDM was reduced by HIV infection, complex formation at Sp1 in HIV-infected LPS-stimulated MDM was enhanced, relative to mock-infected LPS-stimulated controls (figure 29A).


α-p50
α-p65
α-c-Rel
Cold (50x)
goat IgG
- + + + + + + LPS
α-c-Fos
goat IgG
Cold (50x)
- + + + + LPS
α-Sp1
α-Sp3
Cold (50x)
goat IgG
- + + + + + LPS

NF-κB AP-1 Sp1

FIGURE 25: *Control goat IgG does not result in supershifted nuclear factor complexes.*

THP-1 cells (10^6/ml) were stimulated with LPS (1 μg/ml) and after 2 hours, nuclear extracts were harvested as described. Five μg of nuclear extract were incubated in EMSA reactions including ^{32}P-labelled oligonucleotide probes containing transcription factor binding sites for **(A)** NF-κB, **(B)** AP-1, or **(C)** Sp1 derived from the IL-12 p40 promoter, in the presence or absence of transcription factor-specific antibodies or a goat IgG control (20 μg/ml) as indicated. EMSA reactions were electrophoretically separated on 6% non-denaturing polyacrylamide gels.

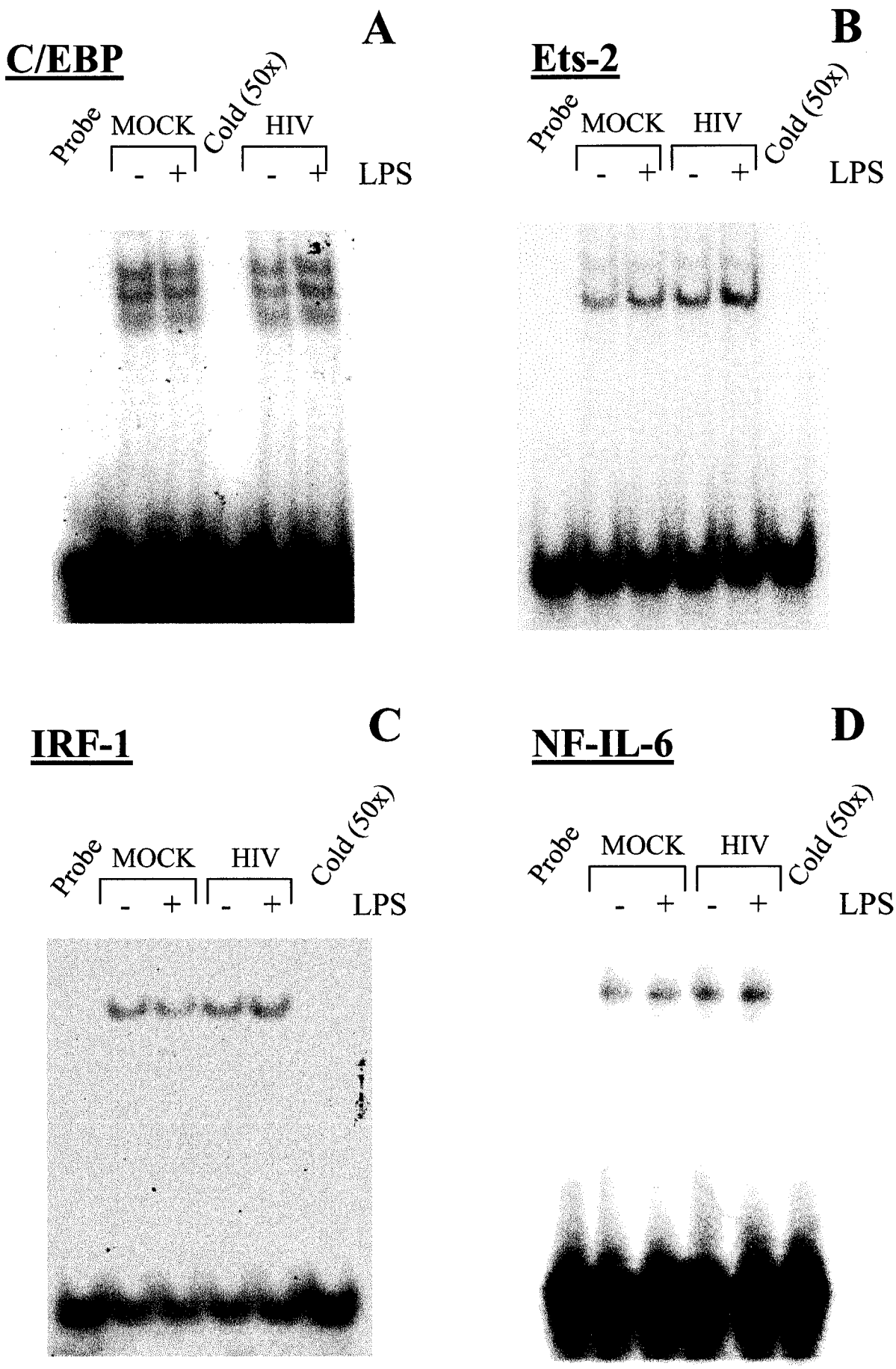

A
C/EBP
Probe
MOCK
Cold (50x)
HIV
- + - +
LPS
B
Ets-2
Probe
MOCK
HIV
Cold (50x)
- + - +
LPS
C
IRF-1
Probe
MOCK
HIV
Cold (50x)
- + - +
LPS
D
NF-IL-6
Probe
MOCK
HIV
Cold (50x)
- + - +
LPS

FIGURE 26: *HIV infection of THP-1 cells does not alter nuclear factor binding to the C/EBP, Ets-2, NF-IL-6, or IRF-1 sites of the human IL-12 p40 promoter.*

THP-1 cells were mock- or HIV-infected for 7 days prior to stimulation with medium or LPS (1 μg/ml) as in figures 22 through 24. Nuclear extracts were harvested and incubated with ^{32}P-labelled oligonucleotide probes containing putative binding sites for **(A)** C/EBP, **(B)** Ets-2, **(C)** NF-IL-6, or **(D)** IRF-1 and corresponding flanking DNA sequences derived from the human IL-12 p40 promoter. Gels shown are representative of three independent experiments.

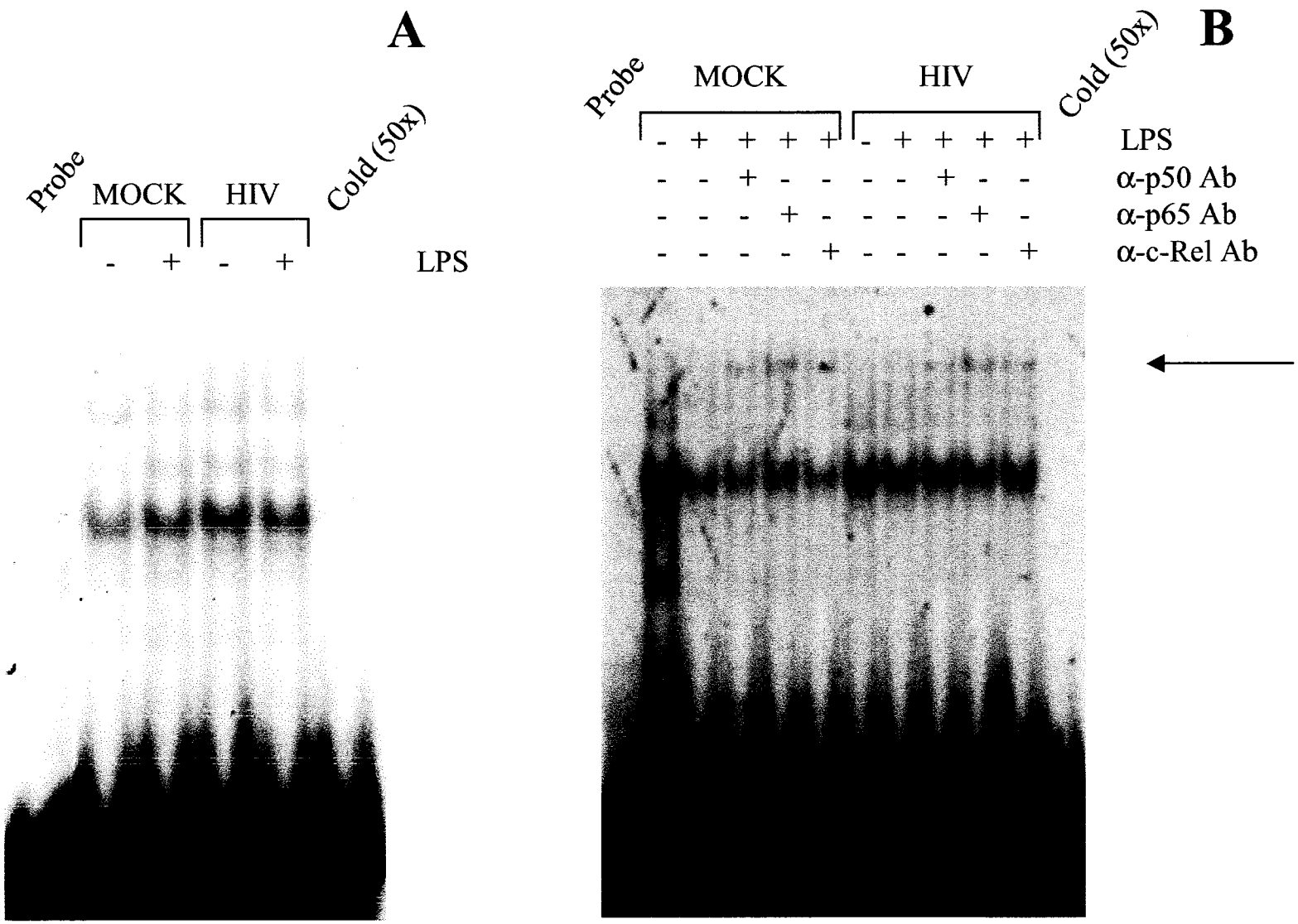

A
Probe
MOCK
HIV
Cold (50x)
LPS
- + - +
B
Probe
MOCK
HIV
Cold (50x)
LPS
α-p50 Ab
α-p65 Ab
α-c-Rel Ab
- + + + + - + + + +
- - + - - - - + - -
- - - + - - - - + -
- - - - + - - - - +

FIGURE 27: *EMSA and supershift analysis of nuclear factor binding to the NF-κB site of the human IL-12 p40 promoter in mock- and HIV-infected primary MDM.*

Primary MDM from HIV-seronegative volunteers were mock- or HIV-infected for 7 days prior to stimulation with medium or LPS (1 μg/ml). (**A**) Nuclear extracts were harvested and incubated with a ^{32}P-labelled oligonucleotide probe containing the NF-κB transcription factor consensus site derived from the human IL-12 p40 promoter. (**B**) Supershift analysis to identify DNA-binding proteins at this site were conducted by inclusion of antibodies directed against NF-κB p50, p65, and c-Rel, in EMSA binding reactions, as in figure 22. Images shown are representative of three independent experiments. Arrows indicate the presence and position of supershifted complexes.

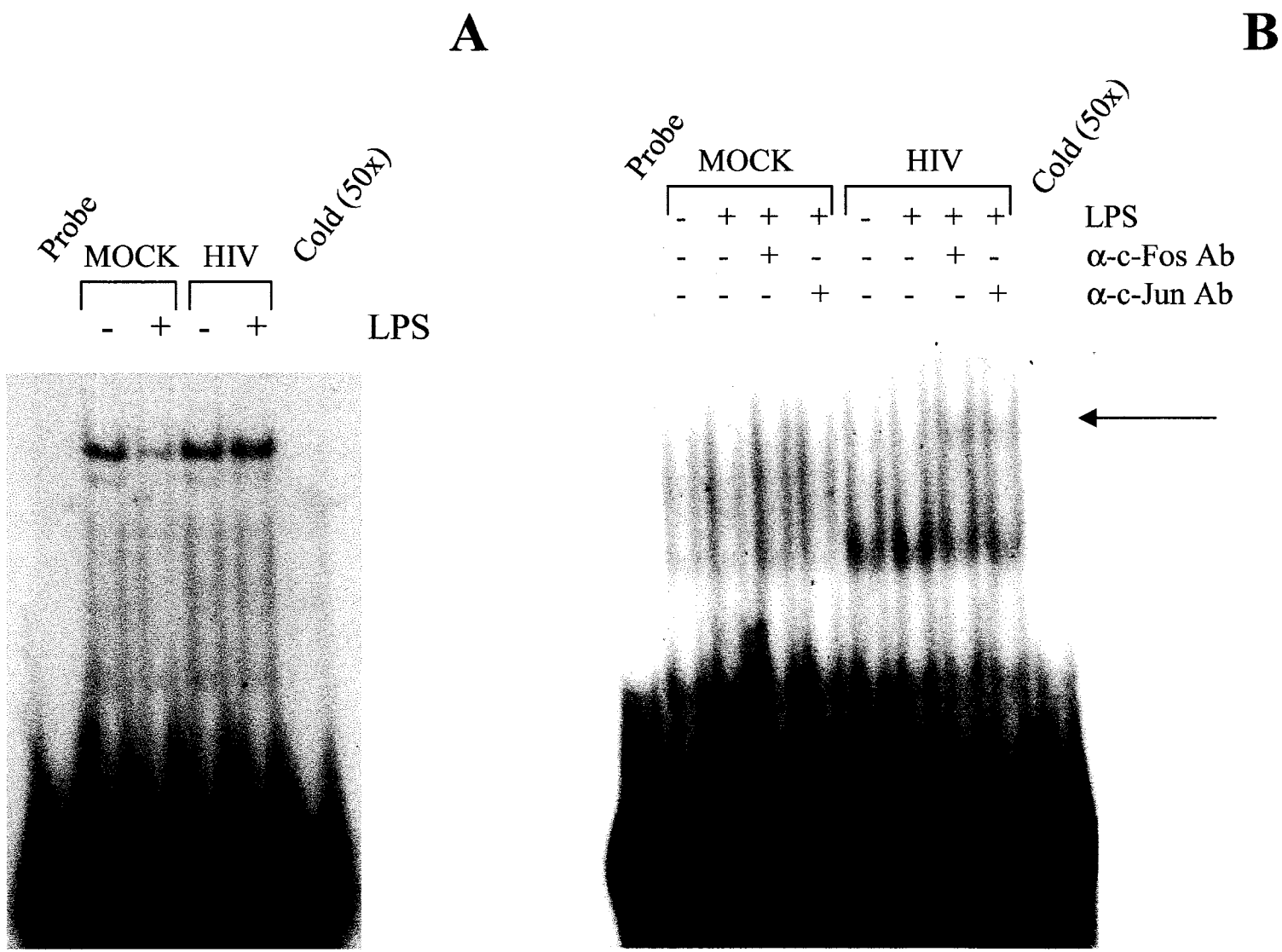

A
Probe
MOCK
HIV
Cold (50x)
- + - +
LPS
B
Probe
MOCK
HIV
Cold (50x)
- + + + - + + +
LPS
- - + - - - + -
α-c-Fos Ab
- - - + - - - +
α-c-Jun Ab

FIGURE 28: *EMSA and supershift analysis of nuclear factor binding to the AP-1 site of the human IL-12 p40 promoter in mock- and HIV-infected primary MDM.*

MDM from HIV-seronegative volunteers were mock- or HIV-infected for 7 days prior to stimulation with medium or LPS (1 μg/ml). **(A)** Nuclear extracts were harvested and incubated with a ^{32}P-labelled oligonucleotide probe containing the transcription factor consensus sequence for AP-1 binding. **(B)** Additional supershift analysis was performed using antibodies directed against the AP-1 binding proteins c-Fos and c-Jun, to evaluate alterations in binding of these proteins to the AP-1 element following HIV infection. Images shown are representative of three independent experiments. Arrows indicate the presence and position of supershifted complexes.

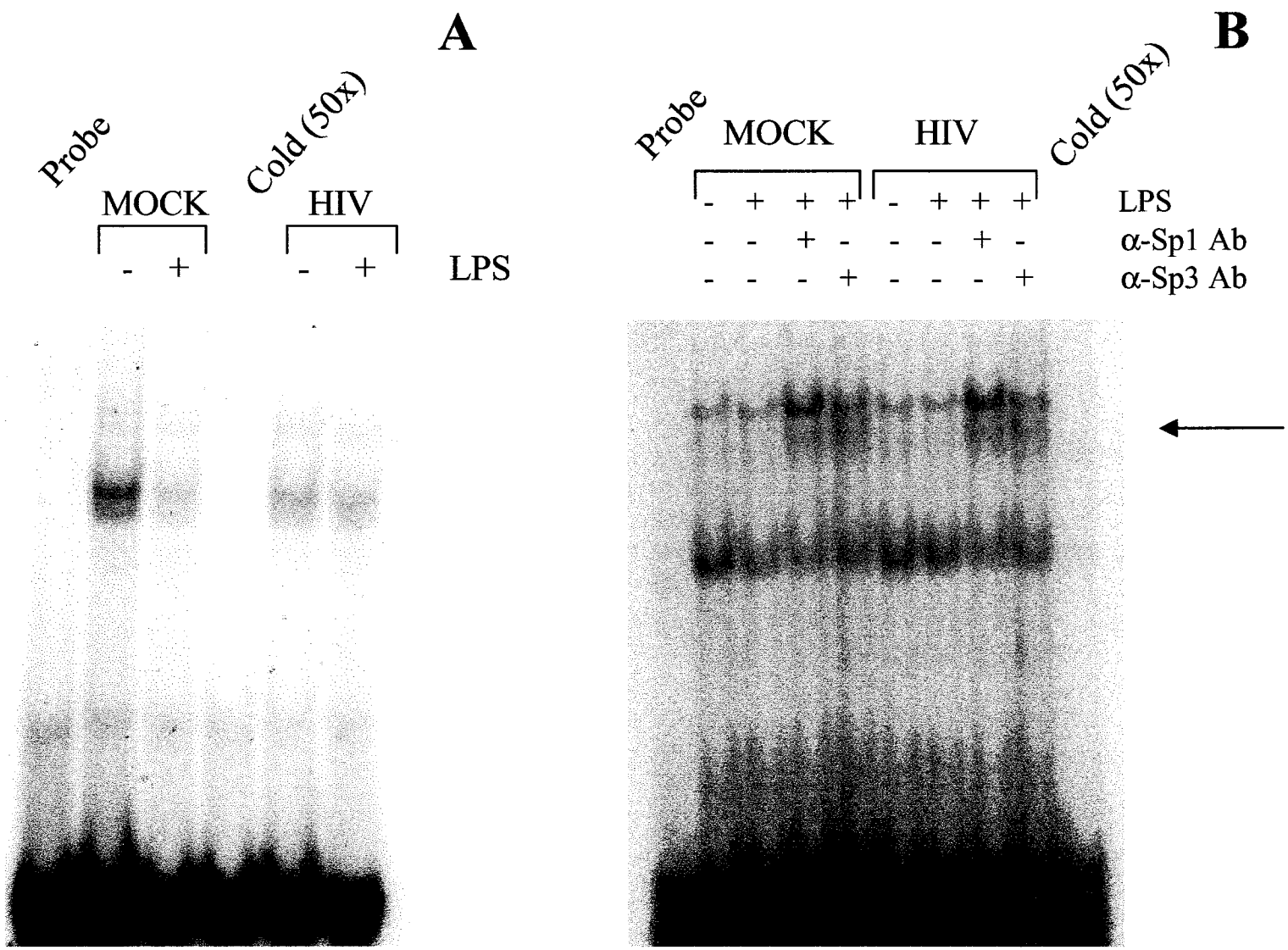

A
Probe
MOCK
Cold (50x)
HIV
- + - +
LPS
B
Probe
MOCK
HIV
Cold (50x)
- + + + - + + +
LPS
- - + - - - + -
α-Sp1 Ab
- - - + - - - +
α-Sp3 Ab

FIGURE 29: *EMSA and supershift analysis of nuclear factor binding to the Sp1 site of the human IL-12 p40 promoter in mock- and HIV-infected primary MDM.*

Primary MDM from HIV-seronegative volunteers were mock- or HIV-infected for 7 days prior to stimulation with medium or LPS (1 μg/ml). (**A**) Nuclear extracts were harvested and incubated with a ^{32}P-labelled double-stranded DNA probe consisting of the consensus sequence for Sp1 binding and flanking DNA sequences from the human IL-12 p40 promoter. (**B**) Additional supershift analysis was performed using antibodies against the Sp1 family members Sp1 and Sp3, to evaluate their promoter binding capability in mock- and HIV-infected MDM. Images shown are representative of three independent experiments. Arrows indicate the presence and position of supershifted complexes.

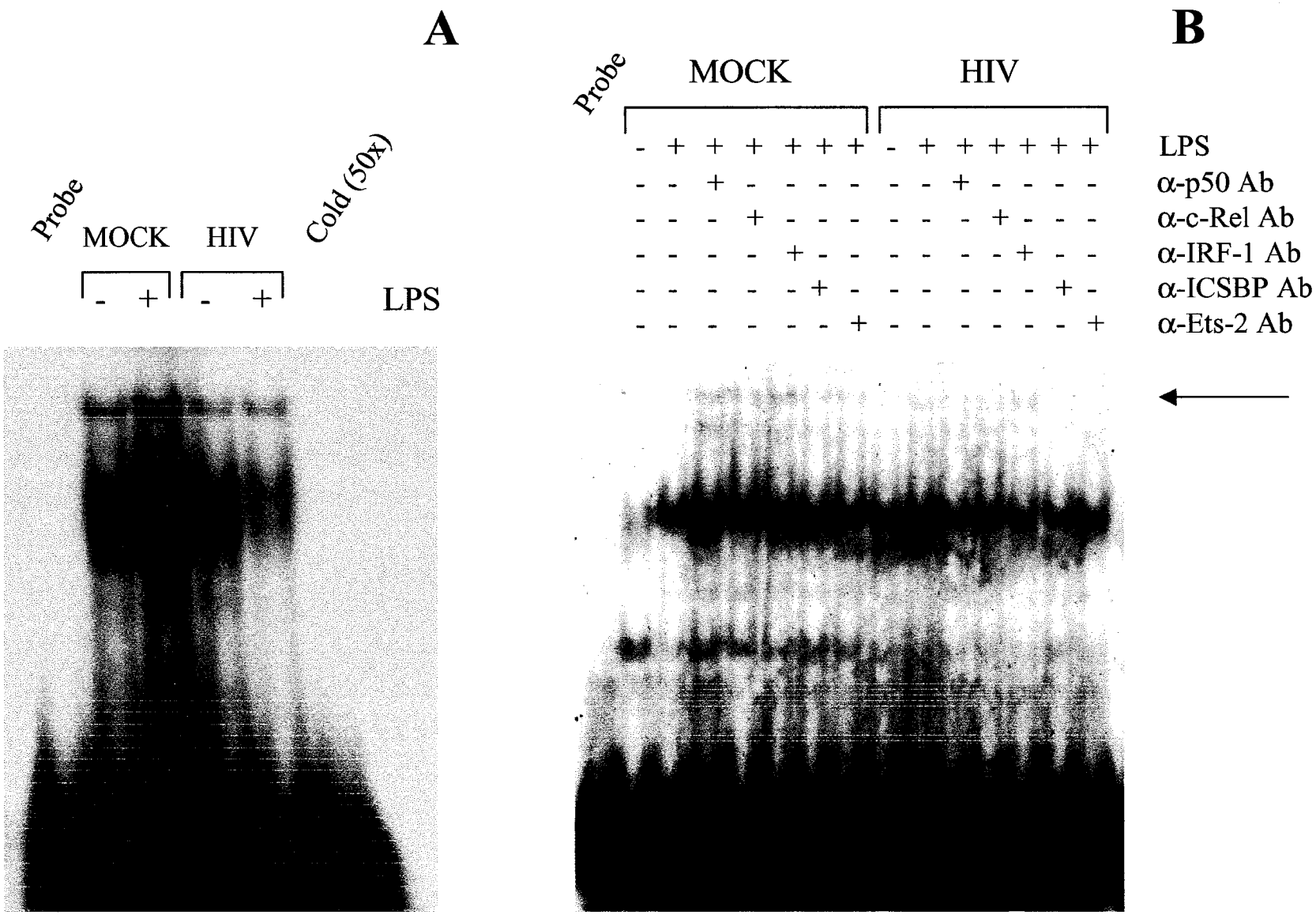

A
Probe
MOCK
HIV
Cold (50x)
LPS
- + - +
B
Probe
MOCK
HIV
- + + + + + + - + + + + + +
LPS
- - + - - - - - - + - - - -
α-p50 Ab
- - - + - - - - - - + - - -
α-c-Rel Ab
- - - - + - - - - - - + - -
α-IRF-1 Ab
- - - - - + - - - - - - + -
α-ICSBP Ab
- - - - - - + - - - - - - +
α-Ets-2 Ab

FIGURE 30: *Nuclear factor binding to the Ets-2 element of the human IL-12 p40 promoter in mock- and HIV-infected MDM.*

Primary MDM from HIV-seronegative volunteers were mock- or HIV-infected for 7 days prior to stimulation with medium or LPS (1 μg/ml). (**A**) After 4 hours of stimulation, nuclear extracts were harvested and incubated with a ^{32}P-labelled oligonucleotide probe containing the putative Ets-2 transcription factor consensus sequence. (**B**) Subsequent supershift analysis was performed by inclusion of antibodies directed against NF-κB p50, c-Rel, Ets-2, IRF-1, and ICSBP (20 μg/ml) in the DNA/protein binding reactions. Images shown are representative of three independent experiments. Arrows indicate the presence and position of supershifted complexes.

Subsequent supershift analysis of monocyte nuclear extracts revealed that the decreased binding of nuclear protein to the Ets-2 region of the IL-12 p40 promoter coincided with a decrease in binding of all of NF-κB p50, c-Rel, Ets-2, IRF-1, and ICSBP proteins following HIV infection (figure 30B) relative to mock-infected LPS-stimulated controls. In addition, enhanced binding of the AP-1 family proteins c-Fos and c-Jun to the AP-1 site was observed in HIV-infected MDM (figure 28B), however altered binding of nuclear factors to the NF-κB and Sp1 sites could not be attributed to altered binding of the cognate binding proteins for these promoter elements specifically (figure 27B and 29B, respectively). In agreement with the observations in THP-1 cells, HIV infection of MDM did not cause alteration in nuclear factor binding to the C/EBP, NF-IL-6, or IRF-1 sites in the human IL-12 p40 promoter (figure 31 panels A through C, respectively). However, unlike THP-1 cells, nuclear factor binding to these three elements exhibited LPS-inducible alterations, indicating that these sites likely participate in LPS stimulation of IL-12 p40 promoter activation. These observations, although somewhat contrasting between THP-1 and primary MDM, illustrate that the NF-κB, AP-1, Sp1, and Ets-2 region (-350/-105 relative to the transcription start site) of the IL-12 p40 promoter is indeed a target of transcriptional inhibition of the IL-12 p40 gene following HIV infection.

3. Functional Significance of IL-12 p40 Transcription Factor Binding Sites in LPS-Induced Promoter Activation: While the presence of the numerous transcription factor binding sites had been mapped in the human and murine IL-12 p40 promoters by virtue of their consensus sequences, it remains unclear what the relative contribution of each individual site is to the activation of the promoter in response to LPS stimulation in

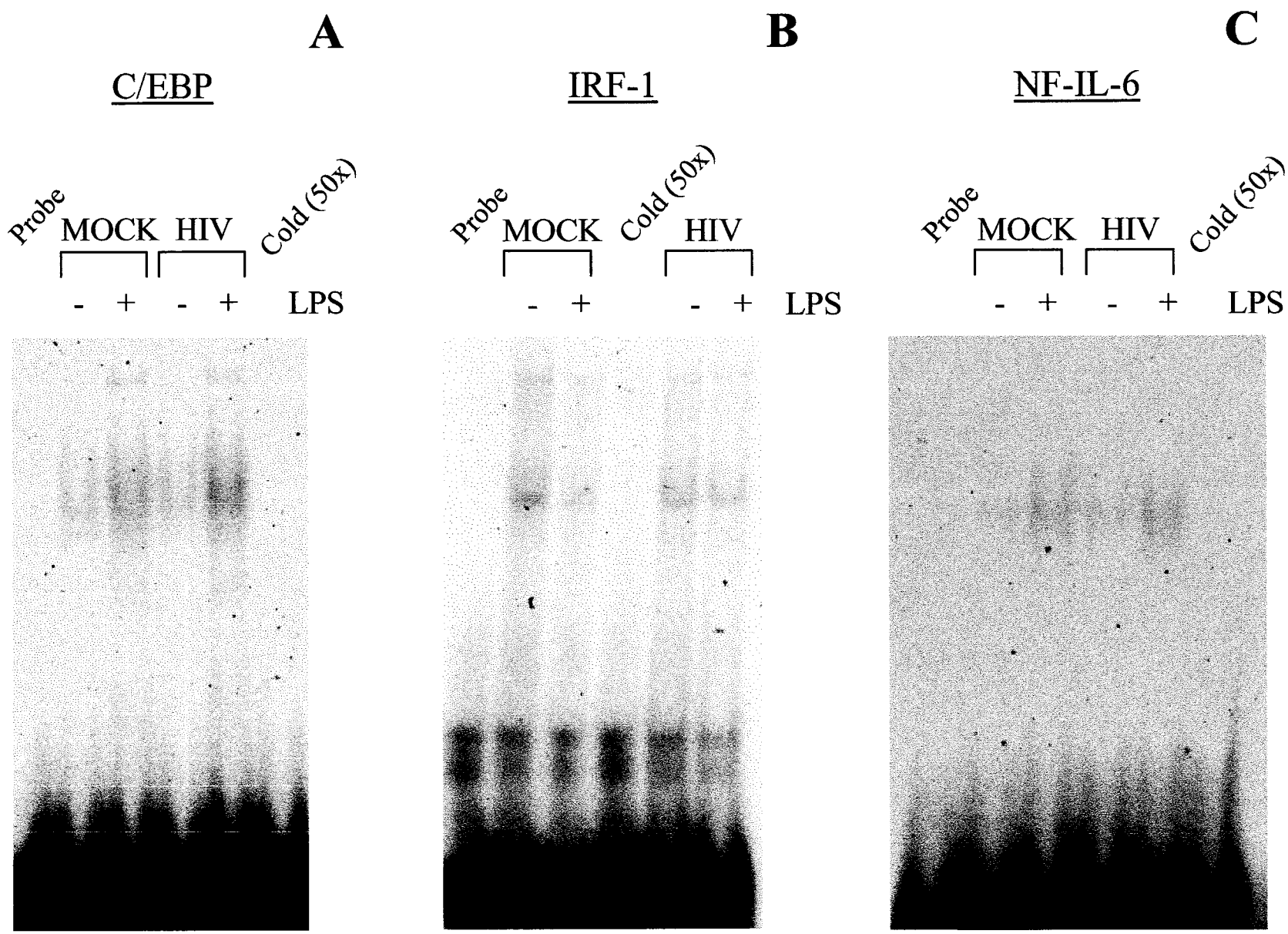

A
C/EBP
Probe
MOCK
HIV
Cold (50x)
- + - +
LPS
B
IRF-1
Probe
MOCK
Cold (50x)
HIV
- + - +
LPS
C
NF-IL-6
Probe
MOCK
HIV
Cold (50x)
- + - +
LPS

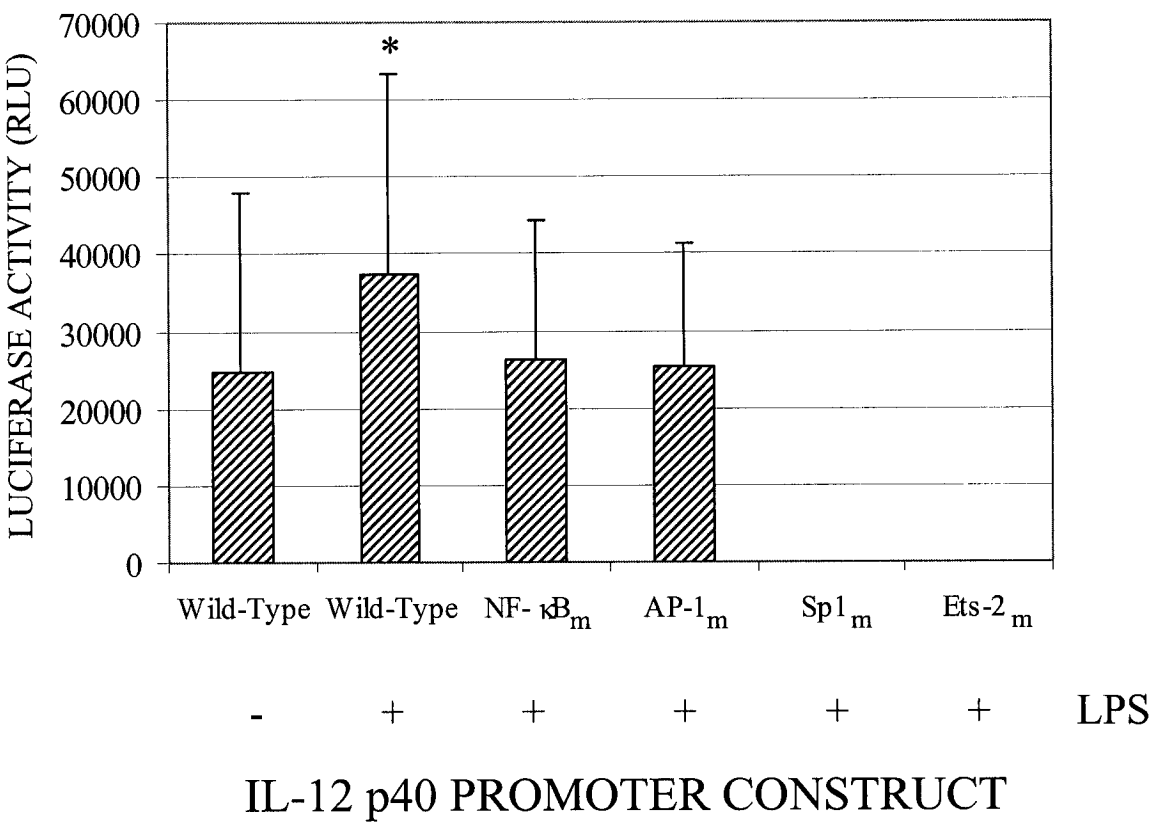
FIGURE 31: *HIV Infection of MDM Does Not Affect Recruitment of Nuclear Factors to the C/EBP, NF-IL-6, or IRF-1 Elements of the IL-12 p40 Promoter.*

MDM were mock- or HIV-infected, medium- or LPS-stimulated as in figures 26 through 30. Nuclear extracts were incubated with ^{32}P-labelled probes containing putative **(A)** C/EBP, **(B)** NF-IL-6, or **(C)** IRF-1 binding sites represented in the human IL-12 p40 promoter. Gels shown are representative of three independent trials.

human myeloid cells. To determine whether the sites which exhibited alterations in nuclear factor binding following HIV infection of myeloid cells are actually required for activation of the IL-12 p40 promoter by LPS, site-directed mutagenesis of the NF-κB, AP-1, Sp1, and Ets-2 sites was performed. Although Ets-2 nuclear factor binding was unaffected by HIV infection of THP-1 cells, mutational analysis of this site was included to clarify dependence of the IL-12 p40 promoter on the Ets-2 element in myeloid cells in general.

From a 3.3 kb IL-12 p40 promoter construct (a kind gift of Dr. Giorgio Trinchieri and Dr. Xiaojing Ma), site-directed mutants of the individual NF-κB (NF-κB_m), AP-1 (AP-1_m), Sp1 (Sp1_m), or Ets-2 (Ets-2_m) sites were generated, and are shown relative to the wild-type promoter sequence in figure 5 (Methods). These wild-type and mutant IL-12 p40 promoter constructs driving a luciferase reporter gene were assembled into recombinant adenoviral vectors for delivery to THP-1 cells and primary MDM, according to the schemes illustrated in figures 4 and 6 (Methods). THP-1 cells infected with recombinant adenovirus carrying the wild-type promoter illustrated LPS-induced enhancement in luciferase activity (figure 32; $p=0.033$). This LPS-inducible promoter activity was reduced to that of the wild-type promoter construct treated with medium alone, when either the NF-κB or AP-1 sites were mutated. Furthermore, mutation of either of the Sp1 or Ets-2 elements within the 3.3 kb promoter completely abrogated transcriptional activation of the IL-12 p40 promoter-luciferase construct in THP-1 cells (figure 32). EMSA analysis of nuclear factor binding to mutant or wild-type NF-κB, AP-1, Sp1 or Ets-2 oligonucleotides verified that each individual binding site mutation

THP-1 Cells

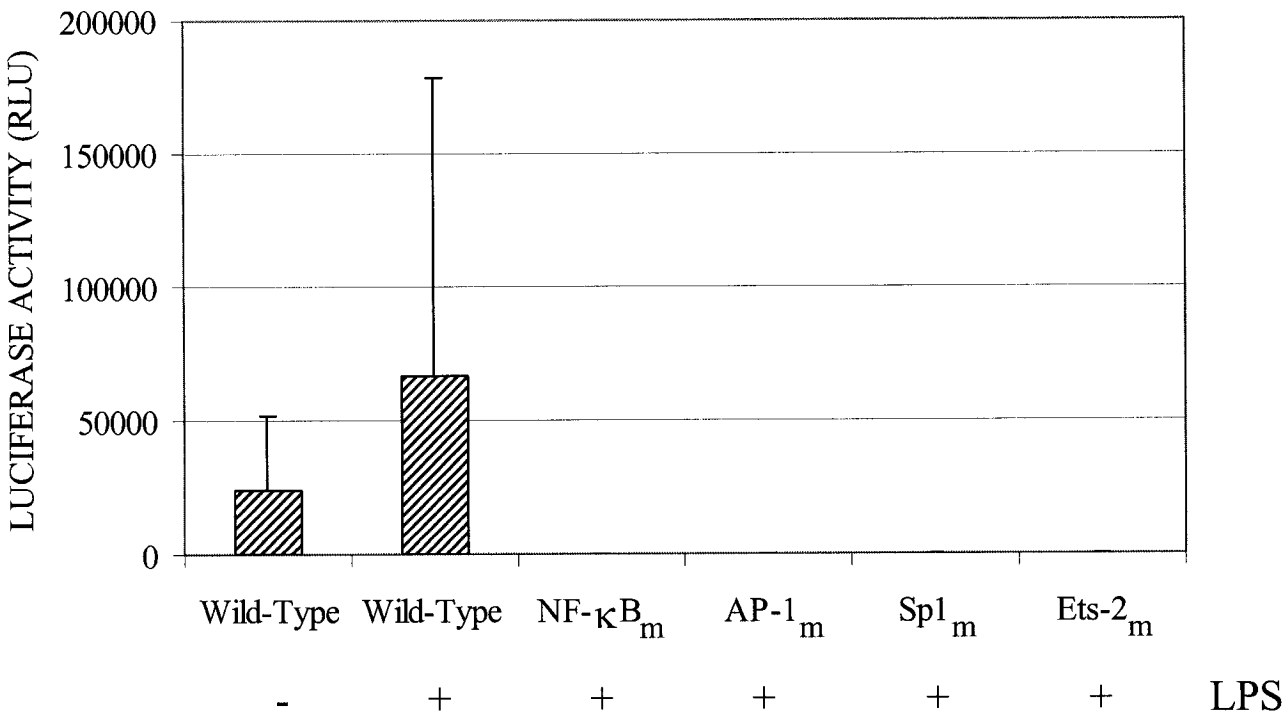


IL-12 p40 PROMOTER CONSTRUCT

FIGURE 32: *The NF-κB, AP-1, Sp1 and Ets-2 Elements of the Human IL-12 p40 Promoter are Each Required for LPS-Induced Promoter Activation in THP-1 Cells.*

Recombinant adenoviral vectors containing a wild-type IL-12 p40 promoter, or a promoter containing mutations of the NF-κB (NF-κB_m), AP-1 (AP-1_m), Sp1 (Sp1_m) or Ets-2 (Ets-2_m) sites individually, driving expression of a luciferase reporter cassette were used to infect THP-1 cells (m.o.i. = 50). Cells were stimulated with medium or LPS (1 μg/ml) for 48 hours prior to lysis and luciferase activity determination. Raw luciferase activity for THP-1 cells infected with recombinant adenovirus delivering wild-type, NF-κB_m, AP-1_m, Sp1_m, or Ets-2_m IL-12 p40 promoters is reported as RLU (relative light units) and was normalized between samples according to total protein content of cellular extracts. Bars represent mean RLU ± standard deviation; n=3. * p=0.033 by Student's T-test comparing medium- versus LPS-induced activation of the wild-type IL-12 p40 promoter.

Monocytes



IL-12 p40 PROMOTER CONSTRUCT

FIGURE 33: *The NF-κB, AP-1, Sp1 and Ets-2 Elements of the Human IL-12 p40 Promoter are Each Required for LPS-Induced Promoter Activation in Myeloid Cells.*

Recombinant adenoviral vectors containing a wild-type IL-12 p40 promoter, or a promoter containing mutations of the NF-κB (NF-κB_m), AP-1 (AP-1_m), Sp1 (Sp1_m) or Ets-2 (Ets-2_m) sites upstream of a luciferase reporter cassette were used to infect primary MDM (m.o.i. = 50). Cells were stimulated with medium or LPS (1 μg/ml) for 48 hours prior to lysis and luciferase activity determination. Luciferase activity for primary MDM infected with adenovirus carrying wild-type IL-12 p40 promoter compared with NF-κB_m, AP-1_m, Sp1_m, or Ets-2_m mutant promoters, is reported as RLU (relative light units) and was normalized between experimental samples according to the total protein content of cellular extracts. Bars represent mean RLU ± standard deviation; n=5.

dramatically inhibited LPS-induced nuclear factor binding (figure 34). Delivery of wild-type and mutant IL-12 p40 promoter/luciferase-containing recombinant adenovirus to MDM demonstrated that mutation of any of the NF-κB, AP-1, Sp1, or Ets-2 elements completely blocked LPS-induced transcriptional activation of the human IL-12 p40 promoter in primary MDM (figure 33). Together, these results indicate that each of the sites within the human IL-12 p40 promoter which had been demonstrated to be affected by HIV infection of myeloid cells (NF-κB, AP-1, Sp1, Ets-2) are vital for LPS-induced activation of the IL-12 p40 promoter.

4. Effect of HIV Infection on Nuclear Factor Expression in Myeloid Cells:

i. Expression of NF-κB, AP-1, and Sp1-binding proteins in mock- and HIV-infected THP-1 cells: Following the observation that HIV infection altered binding of numerous nuclear factors to the NF-κB, AP-1, and Sp1 sites in both THP-1 cells and MDM, and additionally affected the Ets-2 site in MDM alone, Western Blotting was used to examine the impact of HIV infection on overall expression of these defined nuclear factors, to lend further insight into the mechanism of their altered binding to the IL-12 p40 promoter. Whole-cell lysates were isolated from mock- or HIV-infected THP-1 cells and MDM after 7 days in culture, and levels of nuclear factor expression were determined by Western Blotting. As illustrated in figure 35, HIV infection of THP-1 cells did not alter expression of NF-κB p50 or c-Rel proteins (panels A, C), however the level of NF-κB p65 protein was reduced relative to the mock-infected control (panel B), potentially contributing to the observed reduction in p65 binding to the NF-κB site of the IL-12 p40 promoter (figure 22B). By densitometric analysis this corresponded to a

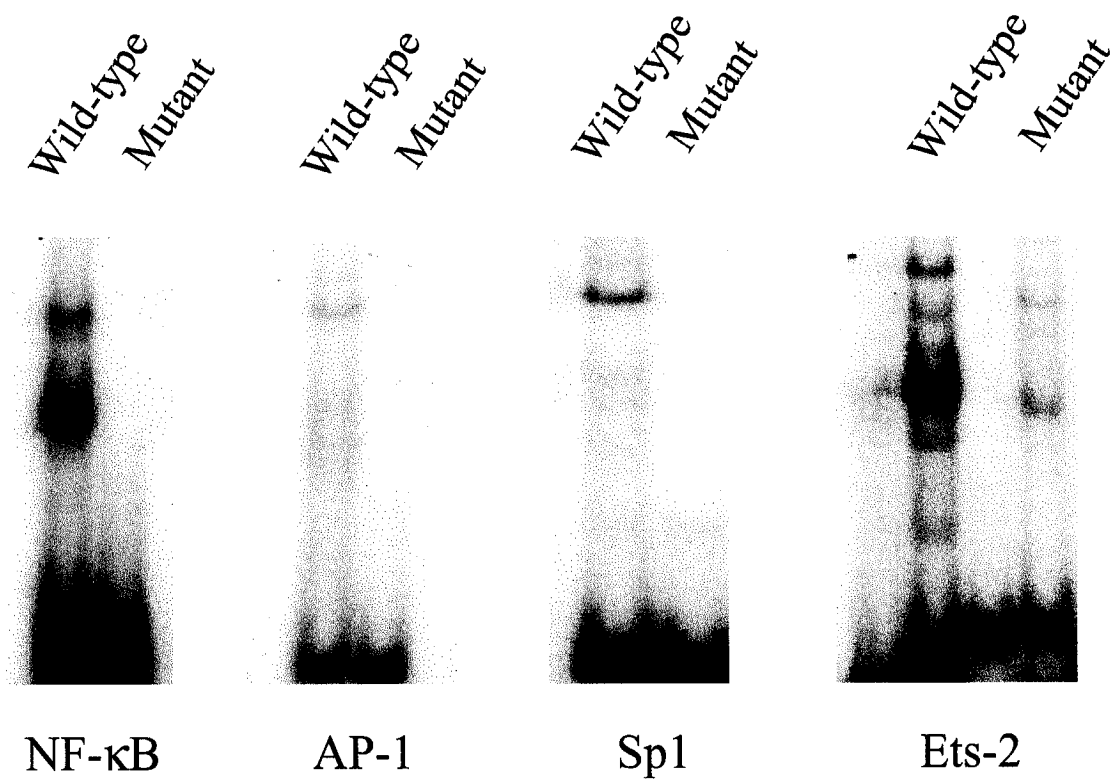

Wild-type
Mutant
Wild-type
Mutant
Wild-type
Mutant
Wild-type
Mutant
NF-κB
AP-1
Sp1
Ets-2

FIGURE 34: *Point mutations at the NF-κB, AP-1, Sp1, and Ets-2 elements of the human IL-12 p40 promoter abrogate nuclear factor binding.*

THP-1 cells (10^6/ml) were stimulated with LPS (1 μg/ml) and after 2 hours, nuclear extracts were harvested as described. Five μg of nuclear extract were incubated in EMSA reactions including ^{32}P-labelled wild-type oligonucleotides, or oligonucleotides containing mutations in the **(A)** NF-κB, **(B)** AP-1, **(C)** Sp1, or **(D)** Ets-2 sites, as listed in table 1 in the Materials & Methods. EMSA reactions were electrophoretically separated on 6% non-denaturing polyacrylamide gels.

THP-1 Cells

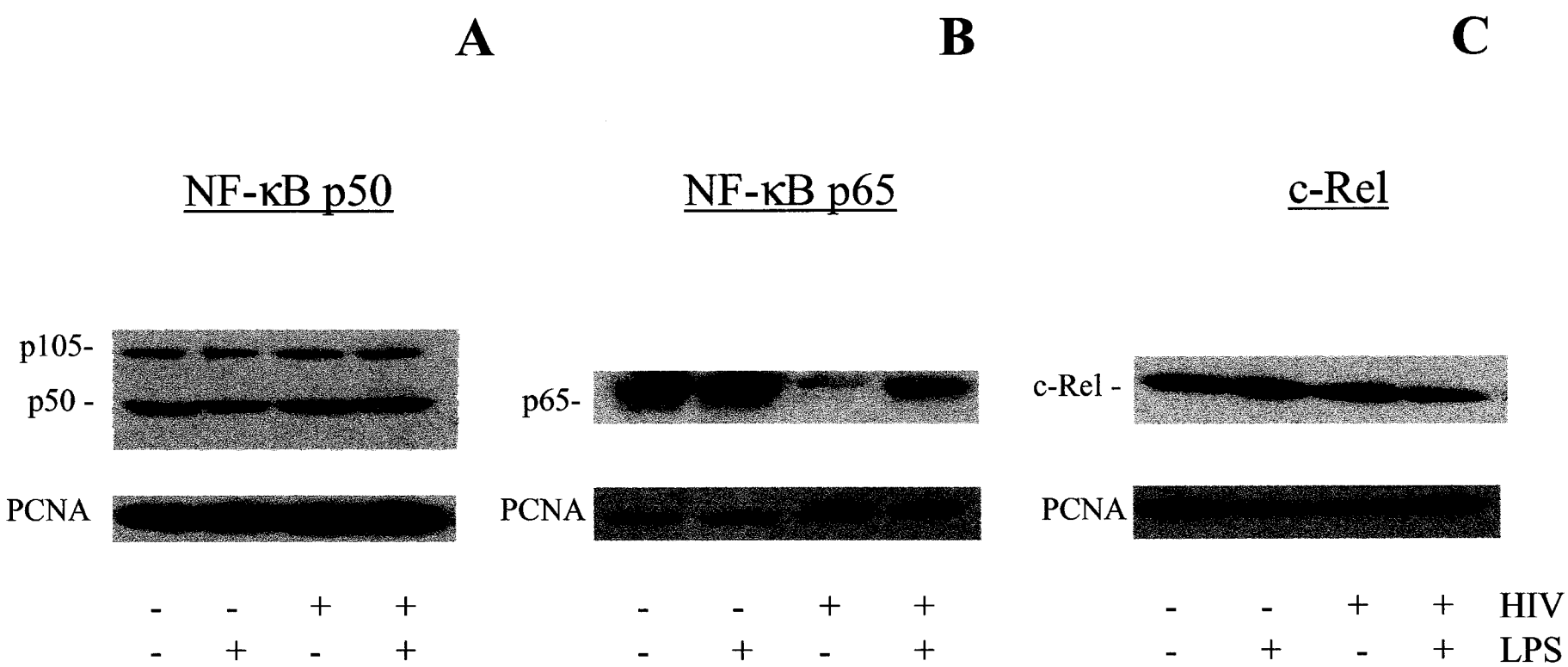

A
B
C
NF-κB p50
NF-κB p65
c-Rel
p105-
p50 -
p65-
c-Rel -
PCNA
PCNA
PCNA
- - + +
- + - +
- - + +
- + - +
- - + +
- + - +
HIV
LPS

FIGURE 35: *Effect of HIV infection on expression of NF-κB proteins in THP-1 cells.*

THP-1 cells were mock- or HIV-infected and after 7 days, stimulated with medium or LPS (1 μg/ml) as in figure 22. Fifty μg of whole-cell lysate were electrophoretically separated by SDS-PAGE, transferred to PVDF membrane and blotted with antibodies against **(A)** NF-κB p50, **(B)** p65, and **(C)** c-Rel proteins, or against PCNA as a loading control. HRP-conjugated secondary antibodies were detected by chemiluminescence and subsequent autoradiography. Gels shown are representative of three independent experiments.

76.9 % (± 3.8%) decrease in p65 expression in LPS-stimulated HIV-infected THP-1 cells relative to mock-infected LPS-stimulated controls. The overall cellular expression of the Sp1 family proteins Sp1 and Sp3, as well as AP-1 proteins c-Fos and c-Jun were also unaffected by HIV infection of THP-1 cells (figure 36 and 37, respectively).

ii. Effect of HIV infection on cellular expression of NF-κB, AP-1, Sp1 or Ets-2-binding nuclear factors: Similar analyses conducted in primary MDM showed no effect of HIV infection on NF-κB p50 or p65 expression (figure 38A and B). However, by densitometric analysis of Western Blots of cellular lysates from HIV-infected LPS-stimulated MDM, relative to mock-infected LPS-stimulated controls, c-Rel expression was augmented by 77.7 % (± 97%) (figure 38C). While Sp1 and Sp3 protein expression (figure 40) and c-Jun expression (figure 39A) was not modulated by HIV, c-Fos levels were elevated by greater than 4-fold in HIV-infected MDM (figure 39B). A survey of proteins known to bind to the Ets-2 site in addition to NF-κB p50/c-Rel illustrated that Ets-2, IRF-1, and ICSBP levels were unchanged by HIV infection in MDM (figure 41 A through C). These data suggest that with exception of altered NF-κB p65 expression by HIV infection of THP-1 cells, and augmented c-Rel and c-Fos expression in MDM, modulation of expression of nuclear factors does not appear to explain the altered binding of these factors to the IL-12 p40 promoter observed following HIV infection of myeloid cells.

E. Inhibition of Activation of NF-κB and JNK and p38 MAP Kinases by HIV Infection:

LPS-stimulation of myeloid cells via toll-like receptor 4 (TLR4) initiates a broad array of intracellular signalling events, culminating in activation of NF-κB via IκBα

THP-1 Cells

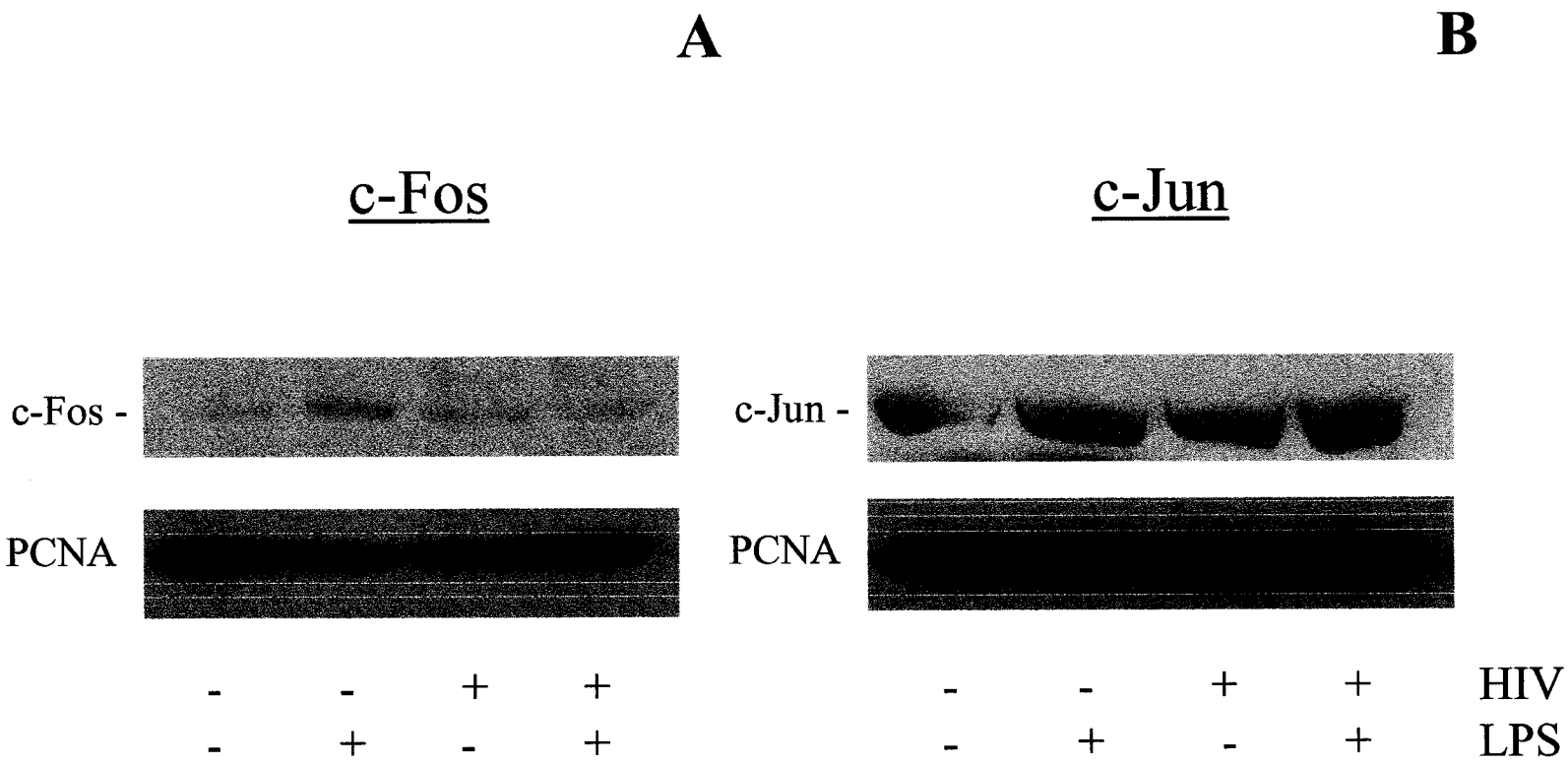


FIGURE 36: *Effect of HIV infection on cellular expression of AP-1 binding proteins in THP-1 cells.*

THP-1 cells were mock- or HIV-infected and after 7 days, stimulated with medium or LPS (1 μg/ml) as in figure 23. Fifty μg of whole-cell lysate were electrophoretically separated by SDS-PAGE, transferred to PVDF membrane and blotted with antibodies against **(A)** c-Fos or **(B)** c-Jun proteins, or against PCNA as a loading control. HRP-conjugated secondary antibodies were detected by chemiluminescence and subsequent autoradiography. Gels shown are representative of three independent experiments.

THP-1 Cells

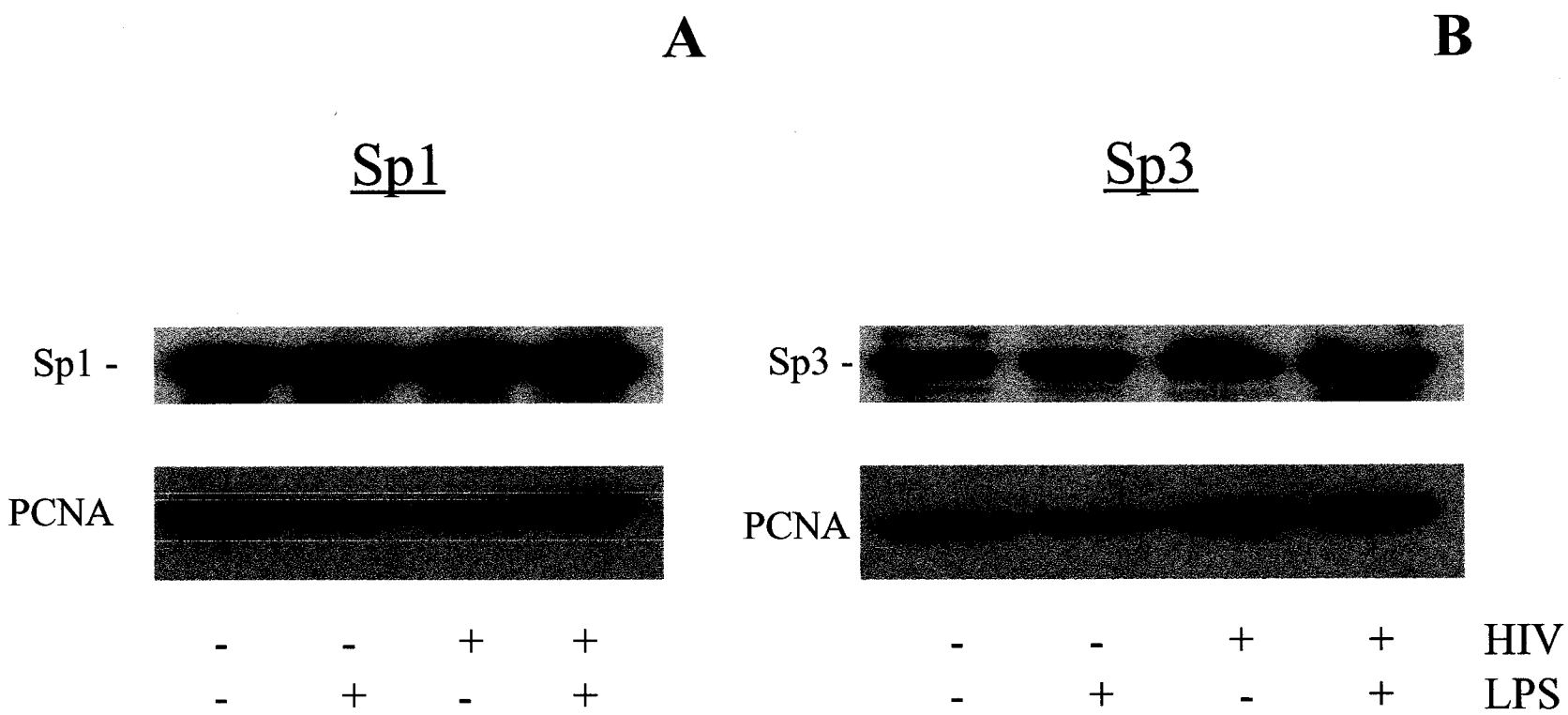


FIGURE 37: *Effect of HIV infection on expression of Sp1 family proteins in THP-1 cells.*

THP-1 cells were mock- or HIV-infected and after 7 days, stimulated with medium or LPS (1 μg/ml) as in figure 24. Fifty μg of whole-cell lysate were separated by SDS-PAGE, transferred to PVDF membrane and blotted with antibodies against **(A)** Sp1 or **(B)** Sp3 proteins, or against PCNA as a loading control. HRP-conjugated secondary antibodies were detected by chemiluminescence and subsequent autoradiography. Gels shown are representative of three independent experiments.

Monocytes

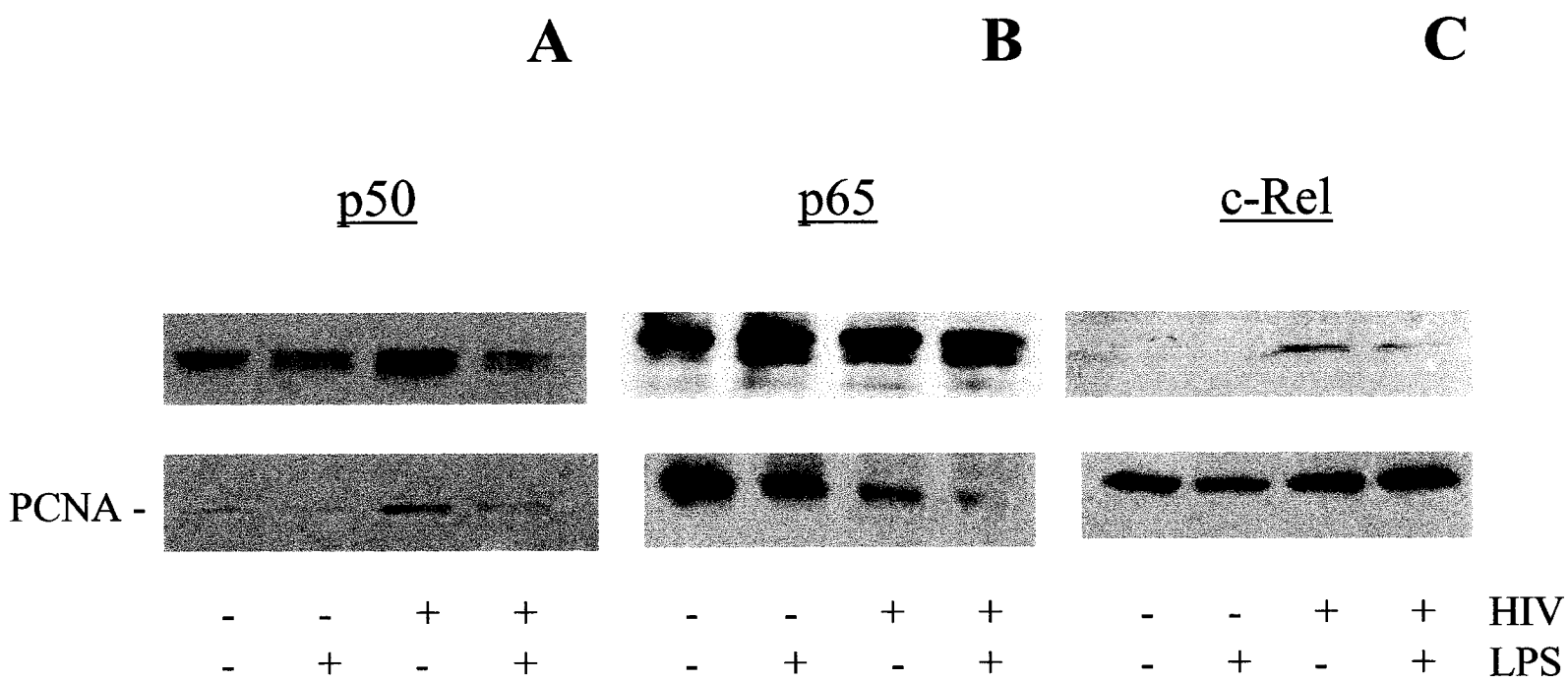


FIGURE 38: *Effect of HIV infection on NF-κB protein expression in primary MDM.*

Primary MDM from HIV-seronegative donors were mock- or HIV-infected and after 7 days, stimulated with medium or LPS (1 μg/ml) as in figure 26. Fifty μg of whole-cell lysate were electrophoretically separated by SDS-PAGE, transferred to PVDF membrane and blotted with antibodies against **(A)** NF-κB p50, **(B)** p65, and **(C)** c-Rel proteins, or against PCNA as a loading control. HRP-conjugated secondary antibodies were detected by chemiluminescence and subsequent autoradiography. Gels shown are representative of three independent experiments.

Monocytes

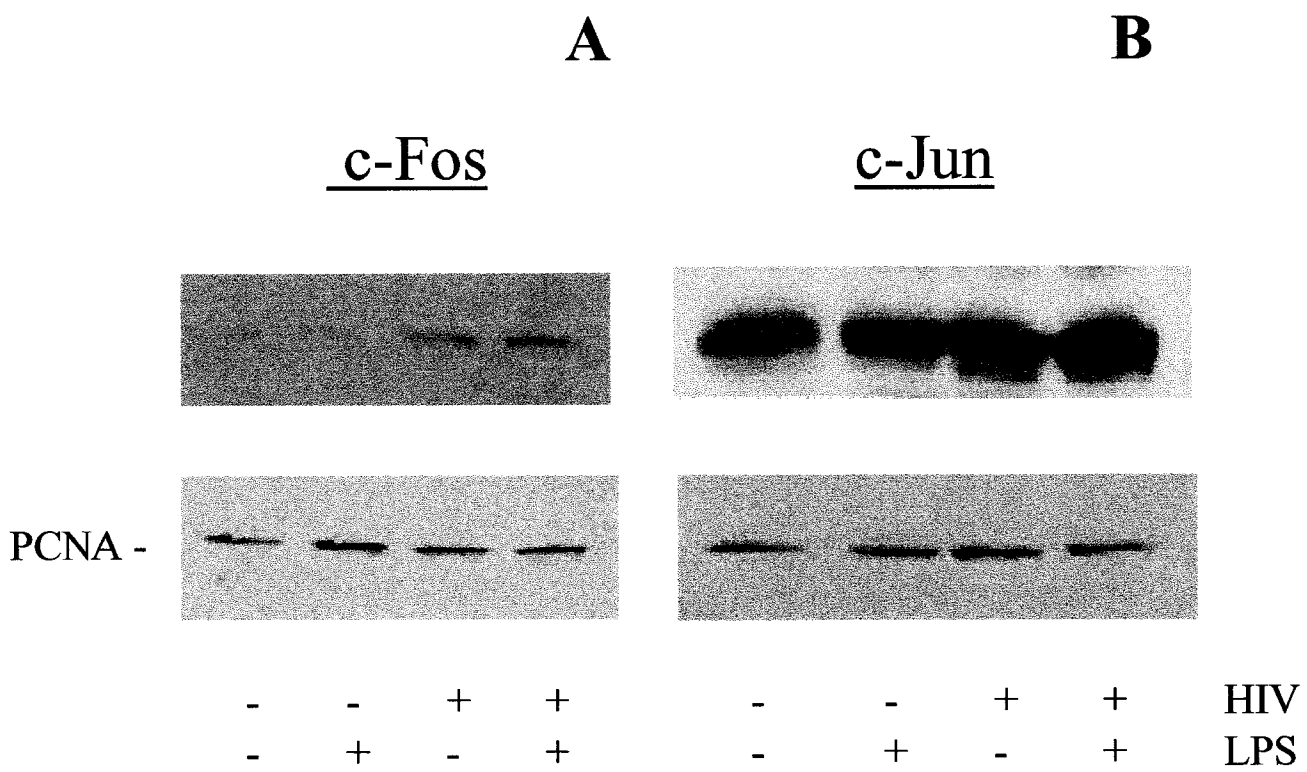


FIGURE 39: *Effect of HIV infection on expression of AP-1 binding proteins in primary MDM.*

MDM from HIV-seronegative donors were mock- or HIV-infected and after 7 days, stimulated with medium or LPS (1 μg/ml) as in figure 27. Fifty μg of whole-cell lysate were electrophoretically separated by SDS-PAGE, transferred to PVDF membrane and blotted with antibodies against the AP-1 binding proteins **(A)** c-Fos, or **(B)** c-Jun, or against PCNA as a loading control. HRP-conjugated secondary antibodies were detected by chemiluminescence and subsequent autoradiography. Gels shown are representative of three independent experiments.

Monocytes

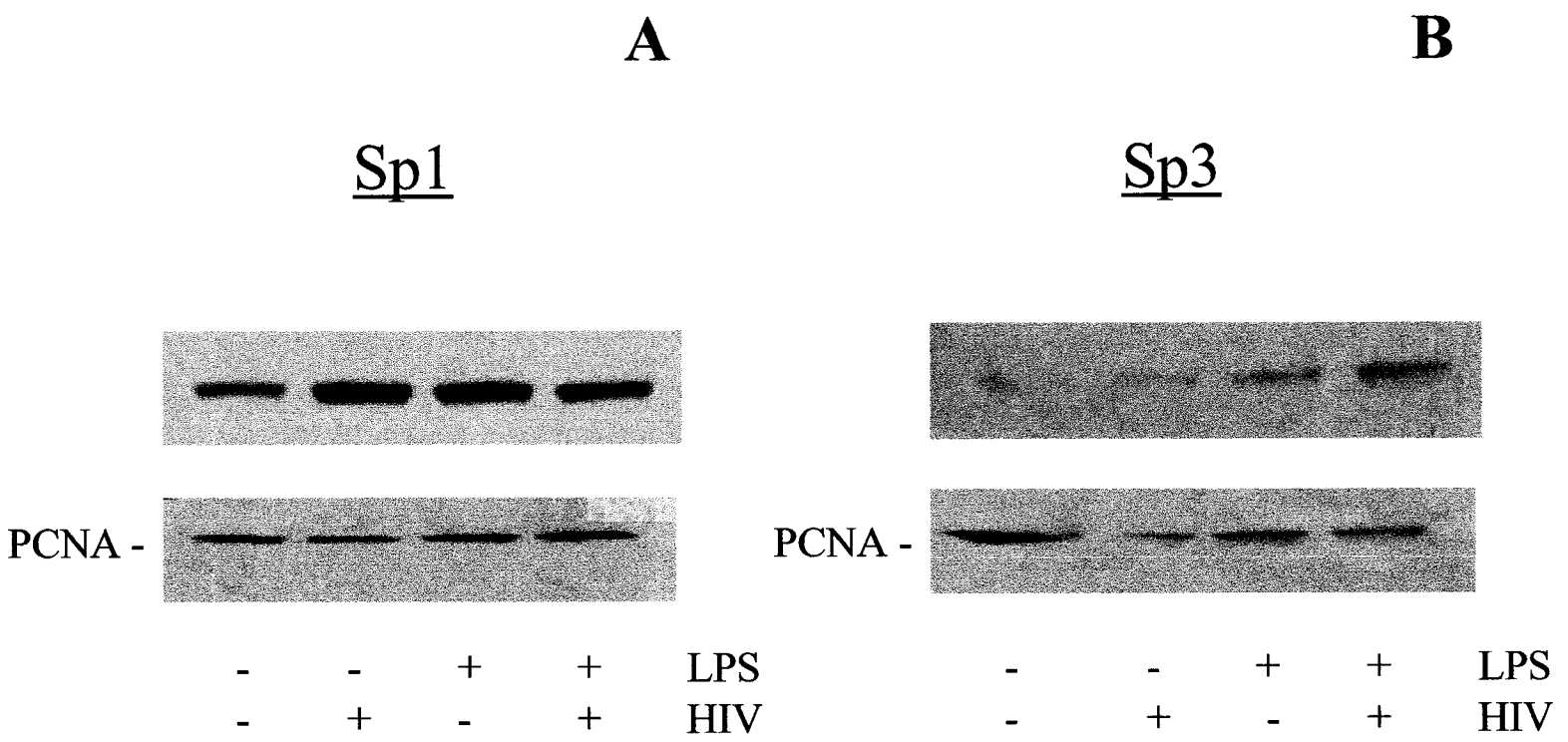


FIGURE 40: *Effect of HIV infection on Sp1 and Sp3 protein levels in primary MDM.*

Primary MDM from HIV-seronegative donors were mock- or HIV-infected and after 7 days, stimulated with medium or LPS (1 μg/ml) as in figure 28. Fifty μg of whole-cell lysate were electrophoretically separated by SDS-PAGE, transferred to PVDF membrane and blotted with antibodies against the Sp1 family members **(A)** Sp1, and **(B)** Sp3, or against PCNA as a loading control. HRP-conjugated secondary antibodies were detected by chemiluminescence and subsequent autoradiography. Gels shown are representative of three independent experiments.

Monocytes

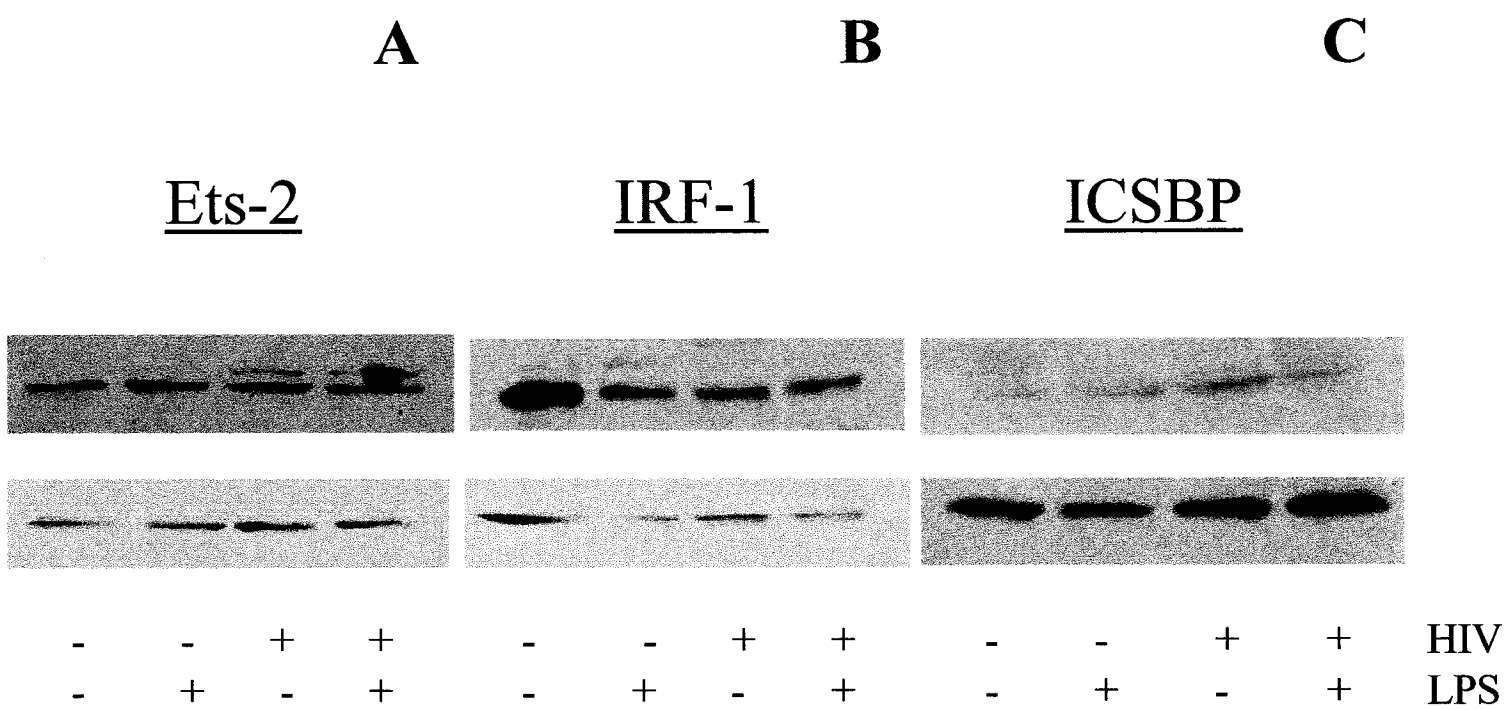


FIGURE 41: *Effect of HIV infection on expression of Ets-2 binding proteins in primary MDM.*

MDM from HIV-seronegative donors were mock- or HIV-infected and after 7 days, stimulated with medium or LPS (1 μg/ml) as in figure 29. Fifty μg of whole-cell lysate were electrophoretically separated by SDS-PAGE, transferred to PVDF membrane and blotted with antibodies against proteins identified to bind to the Ets-2 element of the human IL-12 p40 promoter, namely (**A**) Ets-2, (**B**) IRF-1, and (**C**) ICSBP, or against PCNA as a loading control. HRP-conjugated secondary antibodies were detected by chemiluminescence and subsequent autoradiography. Gels shown are representative of three independent experiments.

phosphorylation and degradation, and activation of p38, JNK and ERK MAP kinases and downstream transcription factor substrates (128). Since alterations in the level of expression of nuclear factors by HIV does not appear to be the principal mechanism of disruption of nuclear factor binding to the promoter, the impact of HIV infection on LPS-induced MAP kinase and NF-κB activation was subsequently addressed by Western Blotting with antibodies against MAP kinases and IκBα in their native and phosphorylated states.

1. Effect of HIV infection on MAP kinase and NF-κB activation in THP-1 cells: Mock- and HIV-infected THP-1 cells were stimulated with medium or LPS for 1 hour prior to whole-cell lysis and Western Blotting to detect MAP kinase expression and activation. As shown in figure 42, there was a decrease in phosphorylated JNK MAP kinase (48.9% ± 21.3% by densitometry) and in total JNK expression (51.5% ± 4.7% by densitometry) (panel 42A). In addition, phosphorylation of p38 MAP kinase was impaired by HIV infection of THP-1 cells, independent of any reduction in p38 MAPK expression (figure 42B). By densitometry, this represented a 73.5% ± 15.8% decrease in phospho-p38 MAPK. In contrast, no alterations in phosphorylation or expression of ERK 1/2 MAPK were observed between mock- and HIV-infected THP-1 cells (figure 43A). In addition to the effects of HIV on MAPK activation, a decrease in LPS-induced phosphorylation and degradation of IκBα was also observed in HIV-infected THP-1 cells (figure 43B), indicating a defect in overall NF-κB activation. By densitometric analysis, this was equivalent to a 59.1% ± 15.7% decrease in phosphorylation of IκBα in HIV-infected THP-1 cells relative to mock-infected controls. In addition, LPS-induced degradation of IκBα was impaired by HIV infection, producing a 19.7% ± 20.5%

THP-1 Cells

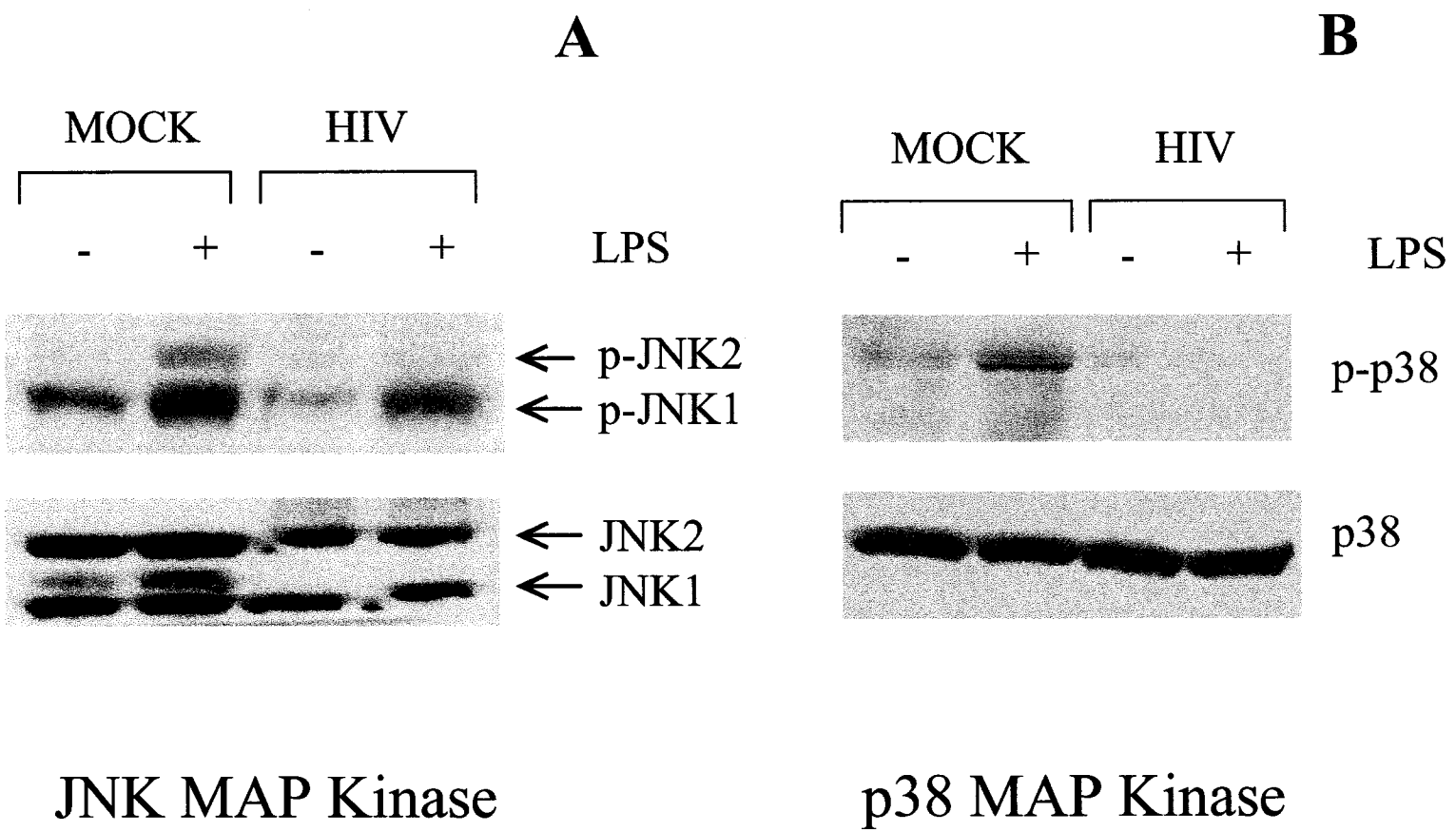
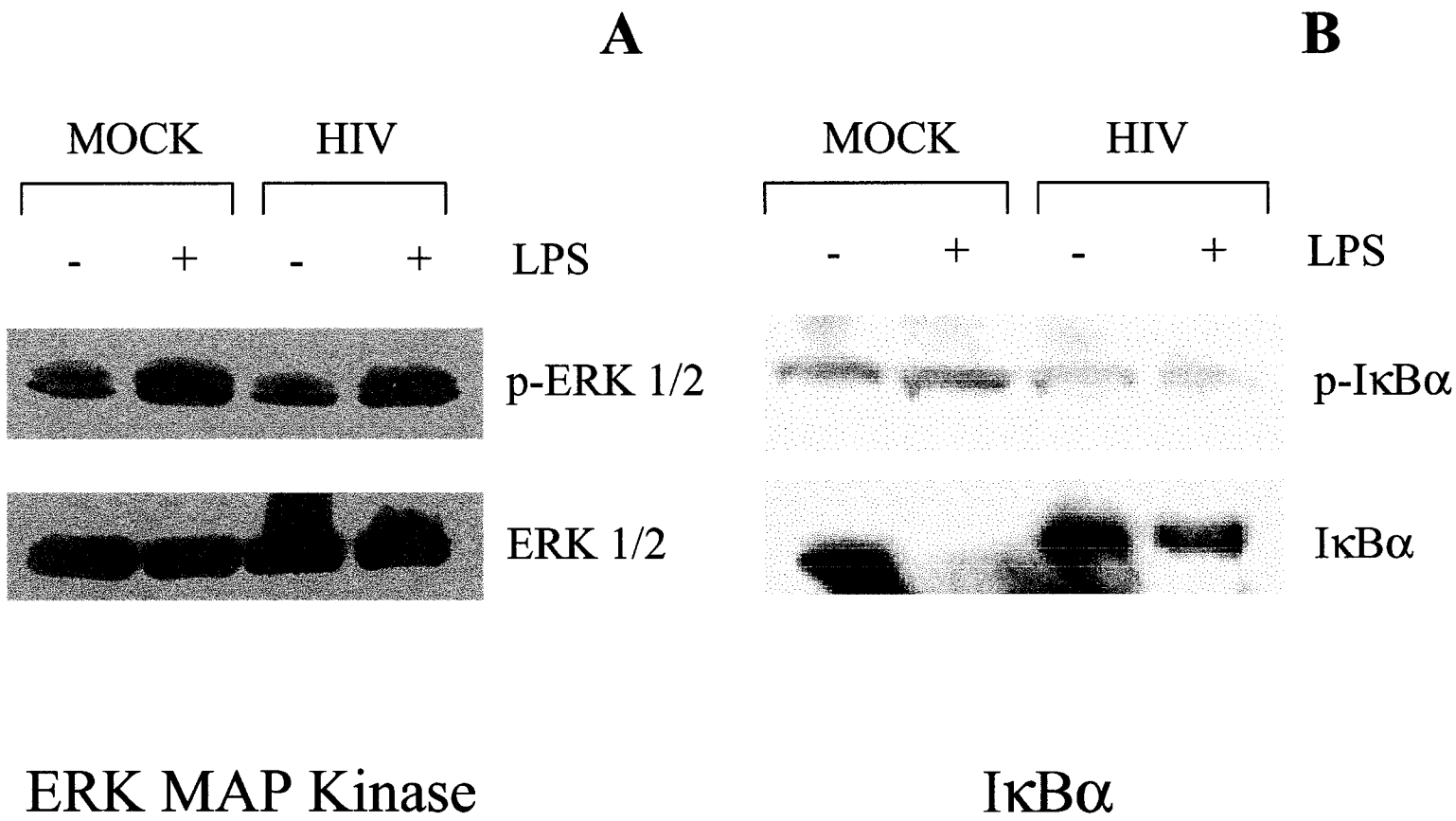

A
B
MOCK
HIV
MOCK
HIV
- + - + LPS
- + - + LPS
← p-JNK2
← p-JNK1
p-p38
← JNK2
← JNK1
p38
JNK MAP Kinase
p38 MAP Kinase

FIGURE 42: *HIV infection of THP-1 cells alters LPS-induced p38 and JNK MAP kinase activation.*

THP-1 cells were mock- or HIV-infected and after 7 days, stimulated with medium or LPS (1 μg/ml) for 1 hour prior to whole-cell lysis. Fifty μg of whole-cell lysate were electrophoretically separated, transferred to PVDF membrane and blotted with phospho-specific antibodies against **(A)** JNK MAP kinase and **(B)** p38 MAP kinase. After chemiluminescent detection and autoradiography, membranes were stripped and reprobed with antibodies directed against total JNK and p38 MAPK, respectively. Images shown are representative of 3 independent experiments.

THP-1 Cells



ERK MAP Kinase

IκBα

FIGURE 43: *Effect of HIV infection on LPS-induced ERK MAPK activation and IκBα phosphorylation and degradation in THP-1 cells.*

THP-1 cells were mock- or HIV-infected and after 7 days, stimulated with medium or LPS (1 μg/ml) for 1 hour prior to whole-cell lysis. Fifty μg of whole-cell lysate were electrophoretically separated, transferred to PVDF membrane and blotted with phospho-specific antibodies against **(A)** ERK 1/2 MAP kinase and **(B)** IκBα. After chemiluminescent detection and autoradiography, membranes were stripped and reprobed with antibodies directed against total cellular ERK 1/2 MAPK and IκBα, respectively. Images shown are representative of 3 independent experiments.

elevation in cellular IκBα levels.

2. Altered NF-κB and MAPK activation in HIV-infected MDM: Consistent with the observations in THP-1 cells, HIV infection of MDM resulted in diminished levels of phosphorylated JNK, although without a reduction in intracellular JNK levels relative to mock-infected controls (figure 44A). By densitometry, phospho-JNK was reduced by 62.7% ± 12.8%. LPS-induced phosphorylation of p38 MAPK was also suppressed (69.1% ± 15.03%), albeit without an apparent alteration in p38 MAPK expression (figure 44B). While phosphorylation and expression of ERK 1/2 MAPK was unaffected by HIV infection of MDM (figure 45A), phosphorylation and degradation of IκBα and therefore, NF-κB activation, was impaired (figure 45B). Specifically, LPS-induced phosphorylation of IκBα was reduced by 63.2% (± 16.99%), and while LPS induced 78.4% (± 20.8%) degradation in IκBα in mock-infected MDM, cellular IκBα was not degraded and in fact appeared to be enhanced (40.1% ± 38.1%) by HIV-infection of MDM. Taken together, these observations suggest that HIV infection of myeloid cells leads to selective impairment of LPS-induced JNK and p38 MAPK activation, and additionally suppresses MAPK dependent or independent NF-κB activation via inhibition of IκBα degradation. Overall, these LPS signalling defects likely contribute to the earlier observations of altered nuclear factor binding following HIV infection.

Monocytes

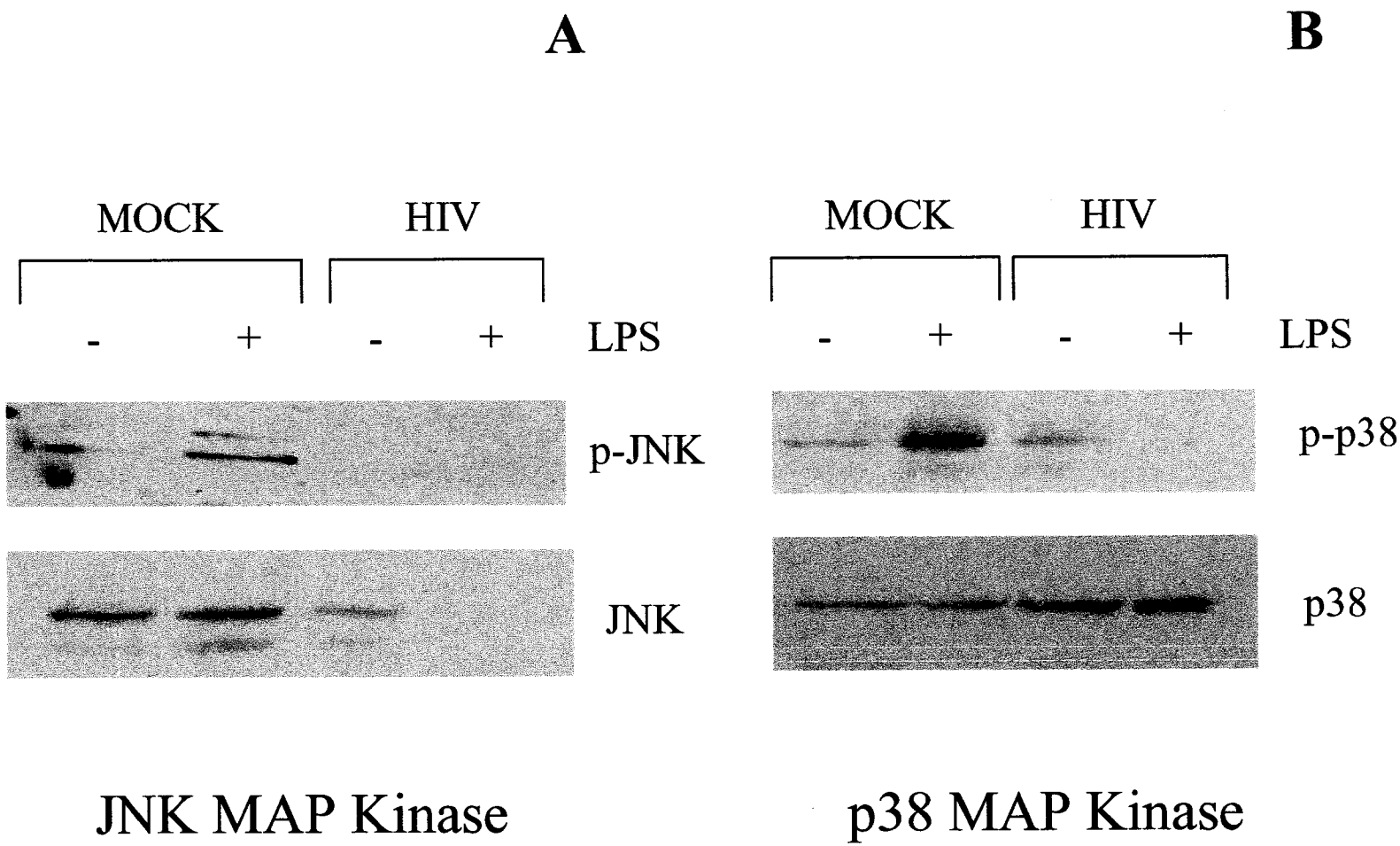
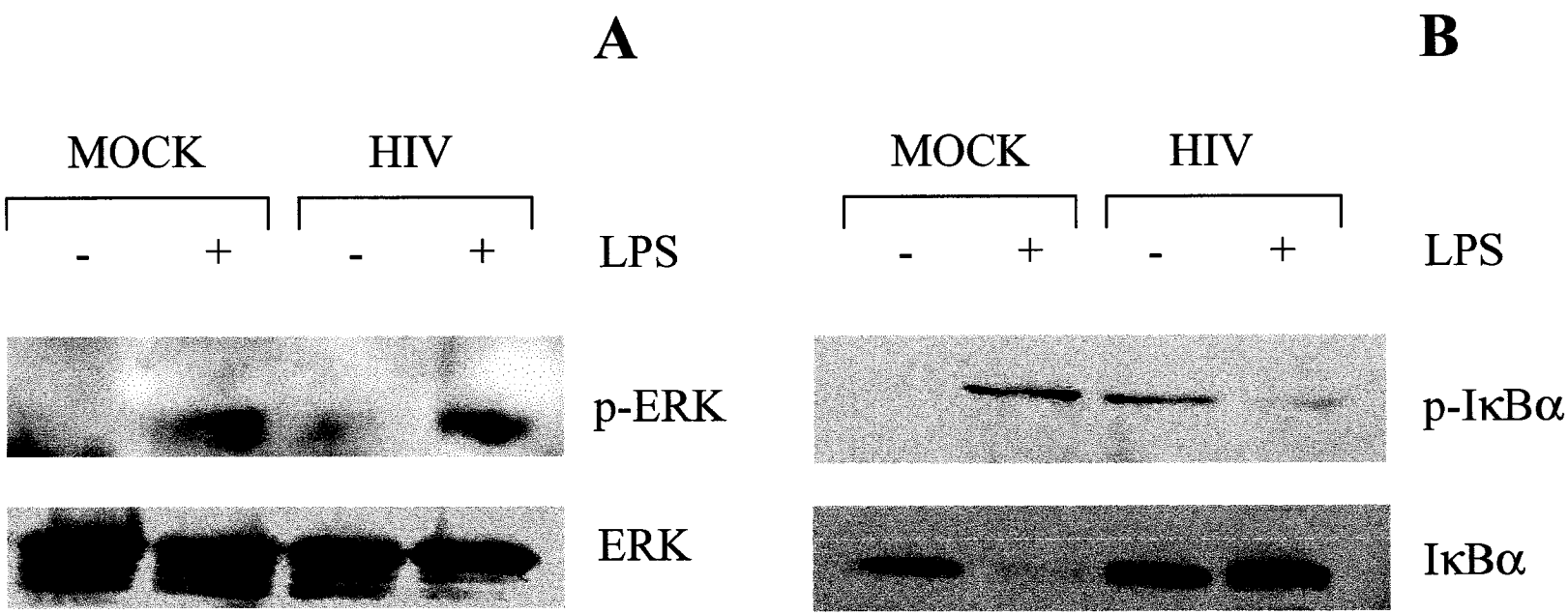

A
B
MOCK
HIV
MOCK
HIV
- + - + LPS
- + - + LPS
p-JNK
JNK
p-p38
p38
JNK MAP Kinase
p38 MAP Kinase

FIGURE 44: *HIV infection of primary MDM alters LPS-induced p38 and JNK MAP kinase activation.*

Primary MDM from HIV-seronegative volunteers were isolated by adherence, mock- or HIV-infected, and stimulated with medium or LPS (1 μg/ml) for 1 hour prior to whole-cell lysis. Fifty μg of whole-cell lysate were electrophoretically separated, transferred to PVDF membrane and blotted with phospho-specific antibodies against **(A)** JNK MAP kinase and **(B)** p38 MAP kinase. After chemiluminescent detection and autoradiography, membranes were stripped and reprobed with antibodies directed against total JNK and p38 MAPK, respectively. Images shown are representative of 3 independent experiments.

Monocytes



ERK 1/2 MAP Kinase

IκBα

FIGURE 45: *Effect of HIV infection on ERK MAP kinase and IκBα expression and activation in MDM.*

MDM from HIV-seronegative donors were mock- or HIV-infected and after 7 days, stimulated with medium or LPS (1 μg/ml) for 1 hour prior to whole-cell lysis. Fifty μg of whole-cell lysate were electrophoretically separated, transferred to PVDF membrane and blotted with phospho-specific antibodies against **(A)** ERK 1/2 MAP kinase and **(B)** IκBα. Membranes were additionally probed with antibodies against total ERK 1/2 MAPK and IκBα, as indicated. Images shown are representative of 3 independent experiments.

IV. DISCUSSION

Interleukin (IL)-12 is a proinflammatory and immunomodulatory cytokine, vital for coordination of adaptive cell-mediated immune responses and antimicrobial defences necessary for control of infection by a broad array of pathogens. The progressive decline in IL-12 production in parallel with impaired cell-mediated immunity that occurs during HIV disease may contribute to the immune dysfunction observed in HIV-infected individuals, rendering them susceptible to opportunistic infections and neoplasms. This impaired cytokine production is somewhat selective, as stimulus-induced production of other inflammatory cytokines including TNF-α, IL-1β, and IL-6 are unaffected by HIV infection (209). The mechanisms of this deregulation in stimulus-induced IL-12 production remain unclear. Considering the importance of IL-12 in directing immune responses, and that in vitro administration of IL-12 to PBMC from HIV seropositive donors augments HIV-specific adaptive immune responses (171, 209), examining the mechanisms underlying HIV-mediated IL-12 suppression is of paramount importance for understanding HIV immunopathogenesis. This may be particularly true due to the early immune defects independent of altered CD4+ T cell number (165, 237), which may be attributed in part to alterations in function of antigen-presenting cells, which have been shown to have numerous defects in individuals with HIV infection (reviewed in section B.3 of the Introduction).

The observation of cytokine imbalances occurring during HIV infection, including that of IL-12, suggests that IL-12 deregulation may be secondary to alterations in production of other cytokines such as IL-10, IFN-γ, and TGF-β. While hyperproduction of IL-10 has been documented in some HIV-infected individuals (11,

206, 218), other studies have shown no increase in IL-10 production in HIV-infected PBMC or monocytes cultured with bacterial stimuli (219, 220). Several reports have shown that IL-12 production defects in PBMC from HIV-positive patients may be overcome by addition of IFN-γ, IL-4, or IL-13 prior to bacterial stimulation (95, 223). Although IFN-γ, IL-4, and IL-13 can enhance IL-12 production under some circumstances, and IL-10 and TGF-β inhibit IL-12, it remains unclear whether the activity of these cytokines, or lack thereof, is responsible for impaired IL-12 induction by HIV infection of myeloid cells. While infection of monocytes in vitro results in suppression of IL-12 protein production following bacterial stimulation (209), an absolute requirement for productive cellular infection and a direct impact on IL-12 production in infected myeloid cells have not been clearly established.

Regarding intracellular molecular mechanisms of IL-12 p40 regulation, at least eight putative transcription factor binding sites have been identified in the human IL-12 p40 promoter, yet the relative contribution of each of these sites to LPS-induced promoter activation in human myeloid cells remains poorly defined. While several pharmacological inhibitors of IL-12 expression target the NF-κB or Ets-2 sites (104, 120-122), the impact of HIV infection on promoter activation via these or any of the described sites has not been addressed. Several studies have implicated p38 and ERK MAP kinases in the positive and negative regulation of IL-12, respectively (128-130, 132). In addition, the influence of HIV Tat and Nef proteins in MAP kinase activation have been intensely studied, albeit largely in T cell lines, yet the impact of HIV infection on MAP kinase activation in human myeloid cells has not been evaluated.

This thesis reports examinations into mechanisms underlying HIV-mediated suppression of IL-12 p40 gene expression. Specifically, this work highlights investigations into the requirement for viral replication and direct cellular infection in HIV-mediated inhibition of IL-12 p40, and the role of other cytokines. In addition, analysis of molecular aspects of regulation of IL-12 p40 by LPS and/or HIV infection with emphasis on transcription and promoter activation, and upstream signal transduction events is described.

A. HIV Impairs Stimulus-induced IL-12 p40 Protein and mRNA, in the Absence of De Novo Protein Synthesis, and Independent of Other Cytokines:

Infection of THP-1 cells and MDM with HIV-1 dramatically impaired IL-12 p40 protein and mRNA induction by several bacterial stimuli including LPS, SAC, and MTB. This inhibition was insensitive to cycloheximide treatment, indicating that suppression of IL-12 by HIV infection occurred directly, in the absence of de novo host or viral protein synthesis at the time of bacterial stimulation. This decrease in IL-12 p40 protein and mRNA occurred in parallel with a decrease in bioactive IL-12 p70 in HIV-infected THP-1 cells as detected in a bioassay based on alterations in PHA-induced IFN-γ production from primary T lymphocytes. This impaired IL-12 p40 and p70 production in HIV-infected THP-1 cells was independent of alterations in IL-12 p35 mRNA levels in THP-1 or MDM, in agreement with other reports illustrating discordant regulation of the p40 and p35 subunits in response to IL-12 stimulatory or inhibitory treatments (13-15). However, these data are in contrast to findings of Chougnet et al. (11), who have previously shown that IL-12 p35 mRNA is decreased to a greater extent than IL-12 p40

mRNA in monocytes/macrophages infected with HIV in vitro. In that report, the monocytes/macrophages had been cultured in GM-CSF, which may enhance IL-12 p40 expression (14) and suppress HIV replication (238). Both of these effects of GM-CSF may contribute to the different responses of the IL-12 p35 and p40 subunits to HIV infection observed by Chougnet's group. The decrease in bioactive IL-12 p70 in parallel with IL-12 p40 reported herein, in the absence of altered IL-12 p35, indicates that further examination of regulation of the IL-12 p40 subunit in the setting of HIV infection is warranted.

Since IL-10 and TGF-β strongly suppress IL-12 production (96, 99, 239) and are enhanced by HIV infection (218, 240), it has been speculated that elevation of these cytokines may contribute to the impaired IL-12 production and diminished cell-mediated immunity observed in HIV-infected individuals. Clearly, the results reported herein do not support this mechanism in infected myeloid cells in isolation, as neutralizing antibodies to either of these cytokines failed to enhance LPS-induced IL-12 p40 mRNA or protein production in THP-1 cells or MDM. However, these cytokines may be produced and play a role in impairing IL-12 production in unfractionated PBMC or in the in vivo setting. The observation that IL-10 activity did not contribute to suppressed IL-12 p40 expression may appear to conflict with one earlier report, which demonstrated increased IL-10 levels in response to SAC stimulation of PBMC from HIV seropositive donors, and improved IL-12 secretion in the presence of neutralizing anti-IL-10 antibodies (11). This differential requirement for IL-10 in HIV-mediated suppression of IL-12 may be attributed to the different stimuli and different cell populations examined in the two studies. Several subsequent publications indicate that suppression of IL-12

synthesis by HIV infection may be independent of IL-10 production or activity. Specifically, a report by Meyaard et al. found that PBMC from HIV seropositive and seronegative donors stimulated with SAC showed suppressed IL-12 p40 and p70 protein production, with no apparent change in IL-10 or PGE2 production (241). In addition, monocytes or M-CSF-treated macrophages infected in vitro with HIV, or isolated from HIV-seropositive donors, did not differ from uninfected controls in spontaneous or LPS-induced IL-10 production (219, 220). The finding that CHX did not restore IL-12 production in HIV-infected cells further corroborates the finding that IL-10 is not involved in HIV-mediated suppression of IL-12 in myeloid cells, since the mode of IL-10-mediated inhibition of IL-12 production has been shown to be CHX-sensitive (96). However it must be considered that immune complexes which may form upon neutralization of IL-10 or TGF-β with antibody, may bind FcRγ and actually impair IL-12 expression. The inability of UV-irradiated supernatants from HIV-infected THP-1 cells to transfer suppression of IL-12 production to uninfected cell cultures lends further support to the conclusion that activities of inhibitory cytokines such as TGF-β or IL-10 are not responsible for inhibition of IL-12 by HIV infection. The observed lack of enhancement of IL-12 production in mock-infected cells treated with anti-IL-10 antibody may reflect a level of IL-10 induction by LPS in HIV-infected cultures which is insufficient to influence IL-12 expression, in the absence of T cells which are additional important sources of IL-10 production.

Priming HIV-infected THP-1 cells with IFN-γ prior to LPS stimulation failed to restore IL-12 p40 protein or mRNA production. This observation may appear to be in contrast to another report demonstrating that priming PBMC with IFN-γ in vitro could

restore stimulus-induced IL-12 p70 protein production in parallel with enhanced IL-12 p40 mRNA detection in HIV-infected cells to the levels observed in healthy controls (223). IFN-γ priming did, however, enhance IL-12 p40 protein secretion in HIV-infected MDM, yet IL-12 p40 mRNA remained undetectable by RT-PCR. Recently, resting human macrophages were reported to contain bioactive preformed, membrane-associated IL-12 that is rapidly mobilized after contact with the intracellular pathogen *Leishmania donovani* (242). Similarly, eosinophils contain intracellular stores of RANTES that are mobilized following IFN-γ stimulation (243), and TNF-α, which is present in a preformed immature form and is cleaved upon activation, to release the active mature soluble form (244). Accumulation of stores of mature or rapidly activated immature cytokines likely represents important mediators of early immune responses upon contact or detection of pathogens, readily able to initiate cell-mediated responses, chemotaxis, and antimicrobial activity of phagocytes without the delay required for transcription and translation of these mediators.

The observation that IL-12 p40 protein is secreted from HIV-infected MDM following IFN-γ priming, in the absence of detectable mRNA, may be due to an as yet undefined IFN-γ-dependent release of such an IL-12 protein reservoir. Whether this IFN-γ-dependent IL-12 p40 release from HIV-infected MDM represents appropriately processed protein, and whether this secretion can be sustained or replenished in HIV-infected MDM in the apparent absence of new IL-12 p40 mRNA production, is unclear. In addition, it has not been determined whether this IL-12 p40 protein release represents release of bioactive p70, or just p40 monomers or homodimers. Therefore, it is premature to draw conclusions regarding the biological significance of this IL-12 p40

protein release. As well, it is clear that IL-12 p40 mRNA transcript accumulation is still impaired, indicating that IL-12 expression in response to LPS remains impaired to some degree in HIV-infected MDM.

In IL-4/IL-13 priming experiments, IL-4 alone, or IL-4 in combination with IL-13 decreased IL-12 p40 protein and mRNA expression in uninfected MDM, but had no demonstrable effect on IL-12 expression in HIV-infected MDM. The discrepancy between these findings and a previous report showing an enhancing effect of prolonged exposure to IL-4/IL-13 may be explained by the fact that the investigation here examines THP-1 cells and MDM cultured for 5 days prior to infection, followed by an additional 7 days in culture, whereas the report by Marshall et al examined PBMC and freshly isolated monocytes (95). Perhaps MDM following extensive culture, or in the absence of cellular interactions or soluble factors contributed by PMBC, are at a stage of maturation or differentiation that renders them refractory to the priming effects of IL-4 and IL-13. While monocytes/macrophages activated by microbial products or IFN-γ ("classically activated") have long been recognized to have augmented antimicrobial activity and intracellular pathogen killing by phagocytes, monocytes/macrophages activated by IL-4 ("alternatively activated") tend to suppress T lymphocyte proliferation and enhance polarization of Th2 cells (reviewed in (245)). Classically activated macrophages and alternatively activated macrophages do not vary in expression of HLA-DR, CD40, CD80, or CD86 (246), but IL-4-elicited alternatively activated macrophages are deficient in IL-12 production (247). In this regard, the failure of IL-4/IL-13 treatment of HIV-infected THP-1 and MDM to enhance IL-12 production is consistent with

polarization of myeloid cells towards the "M2" or alternatively activated, IL-12 deficient phenotype.

B. HIV-Mediated Inhibition of IL-12 p40 Expression in Myeloid Cells is Dependent Upon Productive Infection:

Further examination revealed that HIV-mediated inhibition of IL-12 synthesis was dependent upon direct cellular infection with replication-competent virus. Incubation of THP-1 cells with RT-deficient virus shed from the 8E5 cell line, or with soluble HIV-1 gp120 prior to LPS stimulation, failed to suppress IL-12 p40 protein or mRNA expression. MDM treated similarly showed no suppression of IL-12 p40 mRNA or protein with gp120, while RT-deficient virus caused partial, but not complete reduction of IL-12 p40 protein secretion, in the absence of a parallel decline in IL-12 p40 mRNA detection. This may represent an HIV-mediated post-transcriptional or translational mode of modulation of IL-12 p40 protein production and/or secretion, as with the effect of IFN-γ priming of infected MDM. This lack of IL-12 p40 mRNA and protein suppression by HIV-1 gp120 contradicts a published report wherein HIV-1 gp120 suppressed IL-12 expression in an IL-10-dependent manner (216), although the cells being studied by Taoufik et al., were fresh monocytes and 7-day M-CSF-stimulated macrophages. That study may therefore be discordant with the findings reported here based on the effects of different culture conditions and/or maturation of monocytes/macrophages, and the macrophage differentiation effects versus IL-12 inhibiting effects of M-CSF (248).

By immunofluorescence microscopy, only background levels of intracellular IL-12 p40/p70 production were observed in HIV-infected, LPS-stimulated THP-1. Most, if not all of these cells were infected, as determined by intracellular HIV-1 p24 antigen expression in the majority of DAPI-positive cells. Similar results were observed by intracellular flow cytometry. These results, along with the failure to detect any soluble suppressive host or viral products in UV-irradiated supernatants from HIV-infected THP-1 cells, underscore a requirement for direct cellular infection by replication-competent HIV to elicit full suppression of IL-12 production. However, cell viability following HIV infection cannot definitively be ruled out as a contributing factor to the profound suppression in IL-12 p40 expression following HIV infection, although expression of the GAPDH housekeeping gene and IL-12 p35 appear to remain intact. Additonally, modulation of CD14 and/or TLR4 LPS receptor expression was not addressed and may contribute to impaired IL-12 expression following HIV infection.

C. HIV Infection Impairs Transcription of the Human IL-12 p40 Gene:

Based on the results from nuclear run-on transcription assays, HIV infection of THP-1 cells suppressed IL-12 p40 expression at the level of transcription of the IL-12 p40 gene. This suppression was dramatic, and resulted in a rate of IL-12 p40 transcription similar to that of mock-infected unstimulated THP-1 cells. These results corroborate the observation of absent IL-12 p40 mRNA by RT-PCR and ribonuclease protection assay in HIV-infected MDM and THP-1 cells throughout this work.

Alteration of cytokine mRNA stability is a common mode of altering cytokine expression (249, 250), and therefore, enhanced IL-12 p40 transcript degradation by HIV as an additional mechanism of reducing IL-12 p40 expression cannot be excluded as a

contributing factor. In fact, TGF-β-mediated inhibition of IL-12 expression in the murine macrophage cell line ANA-1 impairs both transcription of IL-12 p40 and p35 subunits, and reduces the stability of the IL-12 p40 transcript (99). The dramatic reduction in IL-12 p40 mRNA detection observed in myeloid cells following HIV infection is not due to a shift in the kinetics of peak expression of this transcript, as IL-12 p40 mRNA was not detected in HIV-infected THP-1 over a 24 hour period, or as early as 2 hours after LPS stimulation.

The results presented thus far demonstrate that impaired IL-12 production in HIV-infected myeloid cells was due, at least in part, to the direct inhibition of IL-12 p40 gene expression. While there was obvious suppression of the rate of IL-12 p40 gene transcription, the molecular mechanisms underlying IL-12 suppression by HIV infection may encompass both transcriptional and translational control points. This was particularly evident in the IFN-γ and RT-deficient virus studies of MDM where there was disparity in the effects of these treatments on IL-12 p40 protein and mRNA production. Full inhibition of IL-12 expression by HIV-1 required productive infection by replication-competent virus, and was independent of the activity of cytokines known to influence IL-12 expression, that may themselves be affected by HIV infection. The direct impact of HIV on IL-12 synthesis illustrates the importance of examining IL-12 regulation in isolated myeloid cells, without the added complexity of secondary interactions occurring among different PBMC subpopulations. Although only a portion of monocytes isolated from peripheral blood of HIV-positive individuals is actually infected with HIV (178), ex vivo stimulus-induced IL-12 production among these cells is dramatically suppressed. These results suggest that direct cellular infection is required

for this suppression that cannot be transferred by soluble host or viral factors, although the impact of HIV infection on IL-12 p40 expression was not successfully addressed at the single-cell level. Therefore, further examination of the underling mechanisms of this suppression in vitro at a cellular level is crucial for understanding the modulation of this cytokine by HIV infection. Evaluating signalling cascades and transcription factor activity on the IL-12 p40 promoter may reveal points of regulation of IL-12 by HIV infection, and may illustrate how this cytokine is specifically suppressed while other inflammatory cytokines induced by LPS stimulation of myeloid cells remain unaffected or are augmented.

D. Alterations in Nuclear Factor Binding to the IL-12 p40 Promoter in HIV-Infected Myeloid Cells:

1. THP-1 Cells: Infection of THP-1 myeloid cells with HIV-1 reduced the level of nuclear factor binding to the NF-κB, AP-1, and Sp1 sites of the IL-12 p40 promoter, and altered the effects of LPS on this binding, but did not influence nuclear factor recruitment to the C/EBP, Ets-2, NF-IL-6, or IRF-1 sites. Site-directed mutagenesis studies demonstrated that each of these affected sites was vital for transcriptional activation of the IL-12 p40 promoter. The LPS-induced decrease in nuclear factor binding to the NF-κB, AP-1 and Sp1 sites observed in mock-infected THP-1 cells suggests that these sites may aid in recruitment of inhibitory factors to the promoter in resting cells, which are released upon activation. The nature of such factors, or whether this represents participation between these elements and the recently identified GA-12 repressor element at position -155 in the IL-12 p40 promoter remains to be determined

(112). The reversal of the LPS effect on the promoter following HIV infection, and the overall reduction in complex formation at NF-κB, AP-1 and Sp1 implies that inhibitory factors may remain bound to these elements in HIV-infected cells following LPS stimulation, and may also suggest an overall reduction in binding of stimulatory transcription factors to these sites. On one hand, the decrease in nuclear factor binding consistently observed with LPS stimulation at the NF-κB, AP-1 and Sp1 sites suggests the removal of an inhibitory factor present in resting cells. Yet these sites do not likely represent strictly repressive elements, as abrogation of binding to these sites by mutagenesis severely impairs transcriptional activation of the promoter. These findings suggest that these sites function to promote transcription in response to LPS, possibly via recruitment of basal transcription machinery, and may also be significant sites of regulation of promoter activation. The overall reduction in binding of nuclear factors to NF-κB, AP-1, and Sp1 sites in HIV-infected THP-1 cells may be indicative of decreased recruitment of stimulatory factors. Specifically, by supershift analysis, HIV infection of THP-1 cells produced a minor decrease in binding of NF-κB p50, p65, and c-Rel, and also impaired c-Fos and c-Jun protein binding at the AP-1 site, and suppressed Sp1 and Sp3 recruitment to the Sp1 element. This reduced binding is not a consequence of altered nuclear factor expression in HIV-infected THP-1 cells, with the exception of NF-κB p65, the expression of which was suppressed as determined by Western Blot analysis. If the NF-κB, AP-1, and Sp1 sites are subject to both positive and negative regulatory influences, then modulation of promoter activation by LPS stimulation and/or HIV infection may represent a balance between positive and negative regulatory factors.

Numerous reports have demonstrated that pharmacological inhibitors of IL-12 synthesis including ASA, 1,25-dihydroxyvitamin D_3, VIP and PACAP act via targeted disruption of nuclear complex formation at the NF-κB site (104, 121, 122). This is the first demonstration of HIV-mediated inhibition of nuclear factor binding to the IL-12 p40 promoter, and is also the first report implicating the AP-1 and Sp1 sites in regulation of IL-12. While there is no current literature evaluating the role of the AP-1 binding site in IL-12 p40 expression or describing specific proteins which may bind this element, c-fos-/- mice have an enhanced capacity for IL-12 expression (251), indicating that the AP-1 site or AP-1 family members may negatively regulate IL-12 p40 transcription. Recent characterization of an additional AP-1 site in the murine IL-12 p40 promoter revealed that removal of this site impairs LPS-induced IL-12 p40 promoter activation (252), consistent with the mutagenesis data presented here. As Sp1 family proteins are ubiquitously expressed and active in numerous promoters (reviewed in (253)), and considering that the Sp1 site is vital for IL-12 p40 promoter activation, decreased binding of Sp1 family proteins to this element likely contributes significantly to HIV-mediated suppression of IL-12 p40 transcription in THP-1 cells. Several published reports have implicated Ets-2 as a critical control element in LPS-induced IL-12 p40 promoter activation (118, 119), including a report that FcRγ ligation which impairs IL-12 synthesis acts in part by inhibition of binding of transcription factors to the Ets-2 site (102), and this is supported by the mutagenesis data in both THP-1 cells and MDM.

*2. **MDM:*** In contrast with the observations in THP-1 cells, EMSA analysis of primary MDM revealed increased nuclear factor binding to the NF-κB, AP-1, and Sp1 elements of the IL-12 p40 promoter following LPS stimulation of HIV-infected cells,

with a decrease in protein binding to the Ets-2 element. Recruitment of specific proteins known to bind the Ets-2 site, including NF-κB p50, c-Rel, IRF-1, ICSBP, and Ets-2 were all shown to be impaired by HIV infection of MDM using supershift analysis, in addition to slightly enhanced binding of c-Fos and c-Jun to the AP-1 element. Altered binding of these specific factors could not be explained by altered expression, with the exception of an increase in intracellular c-Fos protein levels in HIV-infected MDM. Although c-Rel expression was augmented, this finding is not consistent with a decrease in its binding at the Ets-2 site, nor with the observation that the increase in NF-κB binding following HIV infection was not accompanied with an increase in binding of any of the NF-κB proteins known to bind this site.

The NF-κB, AP-1, and Sp1 elements seem to be indispensable for promoter activation as illustrated by the mutagenesis data, yet EMSA studies revealed LPS-inducible enhancement in binding of nuclear protein to these sites following HIV infection. The functional significance of augmented binding to these sites remains to be determined, particularly since these findings differ from our observations in THP-1 cells. The increased binding of c-Fos and c-Jun proteins specifically to the AP-1 site of the IL-12 p40 promoter in HIV-infected MDM may indicate that in this system c-Fos and/or c-Jun may function in an inhibitory manner directly or indirectly via recruitment of inhibitory factors. The increase in nuclear factor binding to the NF-κB and Sp1 elements in HIV-infected MDM may reflect enhanced recruitment of inhibitory factors, or may reflect promoter occupancy by inactive NF-κB and Sp1 proteins, as we were unable to demonstrate that enhanced protein binding to these sites was associated with increased recruitment of NF-κB or Sp1 family proteins specifically. These observations may be

analogous to glucocorticoid receptor-mediated inhibition of NF-κB-mediated activation of proinflammatory genes where disruption of NF-κB DNA binding does not occur, but may instead be due to tethering inhibitory factors to the promoter (254). Several studies have illustrated that the transcriptional transactivation activity of NF-κB proteins can be impaired without effects on nuclear translocation or DNA binding (255, 256). Alternatively, while the NF-κB, AP-1, and Sp1 elements seem to be indispensable for promoter activation in MDM, nuclear factor recruitment to these sites may not be sufficient to drive IL-12 p40 transcription in a situation where nuclear factor binding to the Ets-2 element is decreased.

The Sp1 family member Sp3 has been shown to repress promoter activation by Sp1 and numerous other transcriptional activators (257). In HIV infected myeloid cells, it remains to be determined whether the mere presence of Sp3 on the promoter represses transcriptional activation mediated by Sp1 or other adjacent nuclear factors. An additional consideration is that Sp3 can also function as an activator of transcription (258), and therefore, whether Sp3 function is modulated by HIV infection of myeloid cells as a potential regulatory mechanism for IL-12 p40 expression remains to be determined. As demonstrated in this work, THP-1 cells show decreased Sp1 and Sp3 binding to the Sp1 element of the IL-12 p40 promoter following HIV infection, and in MDM the increase in nuclear factor binding to the Sp1 site is not apparently due to an increase in binding of Sp1 or Sp3. It therefore does not seem that enhanced recruitment of inhibitory Sp1 family proteins to the Sp1 site is a plausible mechanism for IL-12 p40 suppression by HIV, although the transcriptional activation or repression capacity of these promoter-bound proteins has not been specifically assessed. Therefore, differential

regulation of activation of Sp1 proteins bound to the IL-12 p40 promoter following HIV infection should be explored.

In addition, ICSBP (interferon consensus sequence binding protein) has both positive and negative influences over IFN-responsive promoters (reviewed in (259)). As ICSBP may repress IRF-1-mediated transcription, and IRF-1 and ICSBP participate in binding to the Ets-2 site, altered activity of ICSBP independent of its binding capability in HIV-infected MDM should be explored.

While there is disparity in the observations of HIV-mediated IL-12 p40 regulation in response to HIV infection among THP-1 cells and primary MDM, the fact that the same segment of the IL-12 p40 promoter is affected by HIV infection in both cell types indicates that this region is clearly an important target of IL-12 p40 transcriptional control and its impairment by HIV. Recent reports have demonstrated that a nucleosome, positioned over the NF-κB element in the murine IL-12 p40 promoter, spanning the Sp1, AP-1 and Ets-2 sites in the human promoter (-350/-150) is selectively remodelled following LPS/IFN-γ stimulation, allowing access of nuclear factors to chromatin and subsequent promoter activation (117). Regulation of chromatin structure in this central portion of the IL-12 p40 promoter may explain, in part, the targeting of this region in the human IL-12p40 promoter by HIV infection, and may add another level of regulation of host cell genes in general by HIV. Although the extent of binding alteration at each site individually may not be dramatic, particularly in the supershift data, the sum total of these changes, in the complete endogenous promoter with the potential additional effects on cooperation between neighbouring sites, may have a significant impact on transcriptional activation of the IL-12 p40 promoter. The

sum total of the observed alterations may dramatically destabilize the promoter, and cause a reduction in recruitment of basal transcription machinery (260).

The observed differences in promoter regulation between THP-1 and MDM are likely a consequence of the disparate physiology of the two cell populations. THP-1 cells are immortalized while MDM are primary adherent cell cultures, and the two populations represent different stages of differentiation within the myeloid cell lineage. These differences are additionally reflected in the observations of nuclear factor binding to the C/EBP, IRF-1, and NF-IL-6 sites of the IL-12 p40 promoter. Binding of nuclear factors to each of these sites was LPS-responsive in MDM, but LPS-unresponsive in THP-1 cells. The different responses of the IL-12 p40 promoter to LPS stimulation and HIV infection in THP-1 cells and MDM highlights that there are likely distinct IL-12 p40 transcription activation cascades in each cell population, that converge on a central important regulatory region. While there was variability in the magnitude of binding alterations among monocyte donors, future analyses correlating the level of in vitro infection with degree of IL-12 suppression and magnitude of binding alterations may highlight the importance of regulation of specific nuclear factor binding elements in HIV-mediated suppression of IL-12 p40.

E. LPS Signalling and MAP Kinase Activation in Mock- and HIV-Infected Myeloid Cells:

HIV infection of both THP-1 cells and MDM suppressed LPS-induced phosphorylation of p38 MAPK, and reduced levels of phosphorylated JNK MAPK. In addition, phosphorylation and degradation of IκBα in response to LPS stimulation was

impaired in HIV-infected THP-1 and MDM. As these kinases or signalling mediators regulate activation and function of Ets, NF-κB, AP-1, IRF, and Sp1 family members (261-263), and NF-κB and p38 MAP kinase have been strongly linked to IL-12 induction (127, 128), it is likely that transcriptional inhibition of IL-12 p40 by HIV infection occurs, at least in part, via repression of activation of these LPS-induced signalling mediators. The exact molecular targets of HIV in the LPS-mediated NF-κB and MAPK activation cascade remain to be identified. In HIV-infected THP-1 cells, the decreased phosphorylation and degradation of IκBα and subsequent defect in NF-κB activation is consistent with the observed decrease in binding of NF-κB proteins to the IL-12 p40 promoter, and with a role for the NF-κB site in promoter activation as supported in the literature and by the mutagenesis data presented. The association between impaired IκBα phosphorylation and degradation, and NF-κB activation is less obvious in MDM, given the enhanced binding to the NF-κB element in HIV-infected MDM. However, a decrease in binding of NF-κB p50 and c-Rel was observed at the Ets-2 element in HIV-infected MDM. The enhanced nuclear factor binding observed at the NF-κB site in MDM was not attributed to altered binding of NF-κB proteins themselves, therefore it is possible that the IκBα/NF-κB activation defect does not specifically involve the NF-κB element directly in MDM. Although the NF-κB site is vital for promoter activation, perhaps the change with HIV infection reflects recruitment of other factors, active repressors, or inactive cooperative transactivators to this site, acting alone or in cooperation with other positive or negative cis- or trans-activating factors.

As HIV Tat and Nef proteins reportedly alter MAPK activation in several systems (264-266), modulation of host-cell transcriptional machinery via aberrant

MAPK activation is a highly plausible mode of suppression of IL-12 p40 expression in HIV-infected myeloid cells. In the U937 myeloid cell line, exogenous Nef (264) or exogenous Tat treatment (267) enhanced activation of JNK MAP kinase. Similar JNK activation was observed in Jurkat T cells treated with soluble Tat (265) and H9 cells transfected with Tat (267). In addition, ERK activation is enhanced by expression of a GST-Tat fusion protein in Jurkat cells (266), and by incubation of T lymphocytes with Tat protein (268). SIV binding to Jurkat cells expressing CCR5 activates all three MAP kinases (269) and in vitro HIV infection of T lymphocytes enhances p38 MAPK activity (270). Although these studies imply that Tat or Nef likely enhances MAPK activation, these studies were mostly performed by transient transfection or exogenous addition of HIV proteins in lymphocytes as opposed to myeloid cells. As reported in this thesis, during HIV infection, activation of JNK and p38 in response to LPS stimulation is impaired. These findings likely differ from published studies based on the cells studied, and the culture conditions assessed.

The observations that activation of JNK and p38 MAP kinases is impaired in HIV-infected THP-1 cells and MDM are consistent with observations in our laboratory regarding the role of MAPKs in IL-12 p40 expression. Briefly, inhibition of p38 MAPK with SB203580 in LPS-treated THP-1 and MDM resulted in decreased IL-12 p40 protein expression, while inhibition of JNK MAP kinase with dexamethasone or SP600125 suppressed IL-12 p40 protein and mRNA. Conversely, inhibition of ERK MAP kinase with PD98059 had no demonstrable effect on IL-12 p40 protein or mRNA expression in MDM or THP-1 cells (personal communication with Marybeth Cameron and Fiona Frappier). These observations are consistent with literature reports in which p38 MAP kinase is linked to IL-12 p40 expression, and together with data presented in

this thesis, validate the proposition that HIV-mediated inhibition of IL-12 p40 expression in myeloid cells is attributed, in part, to decreased activation of p38 and JNK MAP kinases. ERK MAPK on the other hand, seems to be dispensable for IL-12 p40 expression, and in fact may be inhibitory (128, 132, 133). The observed decrease in phosphorylation and degradation of IκBα in HIV-infected LPS-stimulated THP-1 cells and MDM, indicative of a defect in NF-κB activation, is consistent with reports in the literature indicating that NF-κB is critical for p40 promoter activation (113, 115), and in agreement with the mutagenesis data reported herein.

Additional preliminary work ongoing in the laboratory supports the suggestion that inhibition of p38 and JNK MAP kinases produces alterations in LPS-inducible binding of nuclear factors to the NF-κB, AP-1, and Sp1 sites of the IL-12 p40 promoter in THP-1 cells. Specifically, inhibition of p38 MAPK impaired LPS-inducible nuclear factor binding to the AP-1 site, while inhibition of JNK MAPK suppressed transcription factor binding to the NF-κB and AP-1 IL-12 p40 promoter elements. Inhibition of ERK MAPK had no effect on nuclear factor binding to the NF-κB or AP-1 sites, and LPS-induced binding to the Sp1 element was insensitive to inhibition of any of the MAP kinases. Taken together with the results reported herein, these observations strengthen the link between impaired JNK and p38 MAPK phosphorylation and altered nuclear factor binding to at least the NF-κB and AP-1 sites of the IL-12 p40 promoter, and the contribution of these changes to impaired expression of IL-12 p40 observed in HIV-infected myeloid cells.

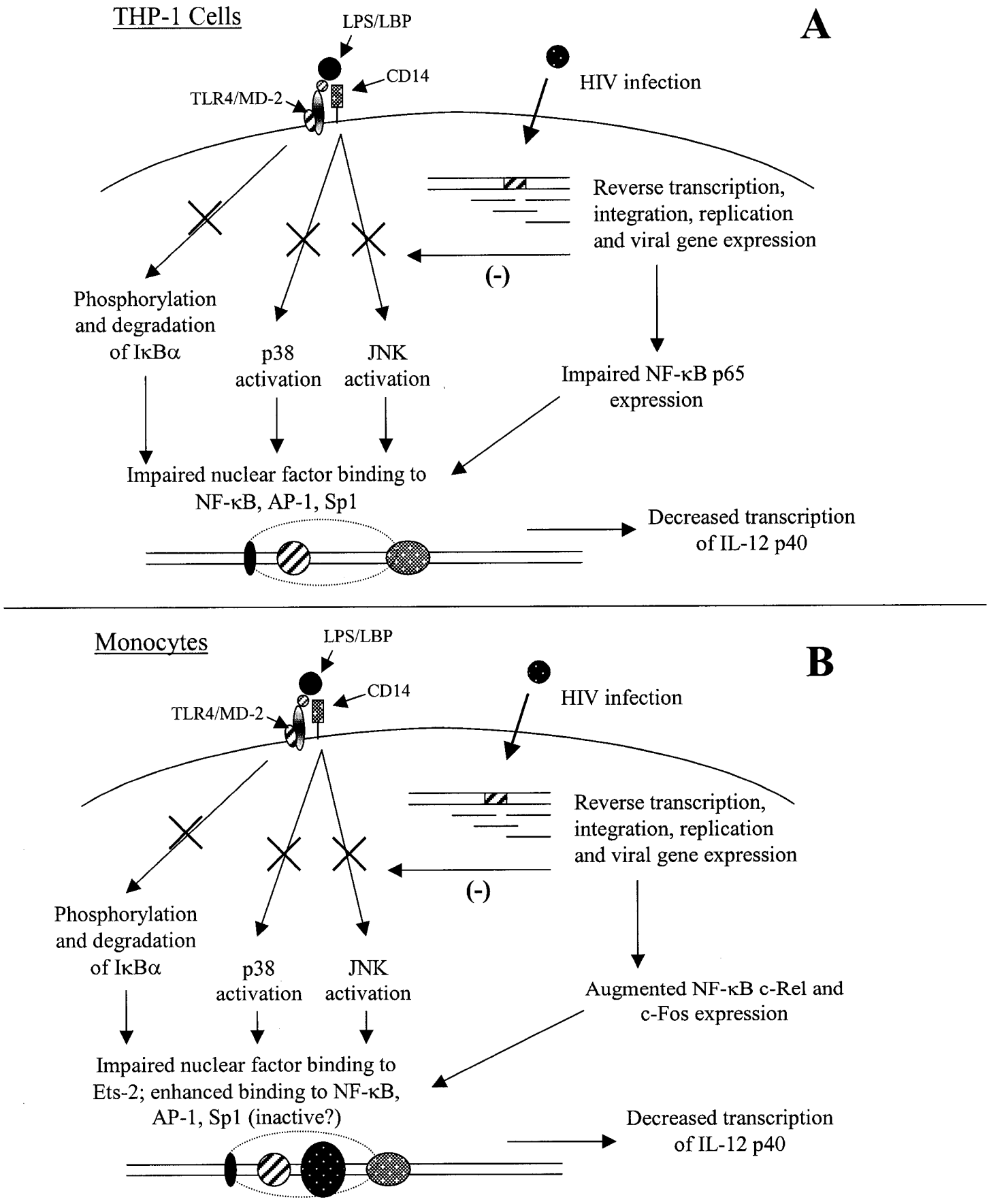

THP-1 Cells
A
LPS/LBP
CD14
TLR4/MD-2
HIV infection
Reverse transcription, integration, replication and viral gene expression
(-)
Phosphorylation and degradation of IκBα
p38 activation
JNK activation
Impaired NF-κB p65 expression
Impaired nuclear factor binding to NF-κB, AP-1, Sp1
Decreased transcription of IL-12 p40
Monocytes
B
LPS/LBP
CD14
TLR4/MD-2
HIV infection
Reverse transcription, integration, replication and viral gene expression
(-)
Phosphorylation and degradation of IκBα
p38 activation
JNK activation
Augmented NF-κB c-Rel and c-Fos expression
Impaired nuclear factor binding to Ets-2; enhanced binding to NF-κB, AP-1, Sp1 (inactive?)
Decreased transcription of IL-12 p40

FIGURE 46: *Proposed Mechanisms of HIV-Mediated Suppression of Interleukin-12 Expression in Myeloid Cells.*

(A) Schematic representation of proposed mechanism(s) of inhibition of IL-12 p40 expression following HIV infection of THP-1 cells. Following reverse-transcription, integration, and HIV viral gene expression, LPS-induced activation of p38 and JNK MAP kinases is impaired, in addition to inhibition of degradation of IκBα and subsequent NF-κB activation. These alterations in MAPK activity likely contribute to reduction in nuclear factor binding to the NF-κB, AP-1, and potentially the Sp1 site of the IL-12 p40 promoter, leading to impaired transcription and subsequent translation of the IL-12 p40 subunit. HIV infection also appears to reduce cellular levels of NF-κB p65 protein which may contribute to the observed decrease in its binding to the IL-12 p40 promoter. These effects of HIV are independent of the activity of IL-10, TGF-β, IL-4, IL-13, and IFN-γ, or of any host or viral soluble factors in infected THP-1 cell cultures.

(B) In HIV-infected MDM, similar defects in IκBα degradation and activation of p38 and JNK MAPK occur. These defects, along with decreased expression of c-Rel, and augmented expression of c-Fos may contribute to the observed alterations in nuclear factor binding to the IL-12 p40 promoter. Specifically, binding to the Ets-2 element is reduced, while nuclear factor binding to NF-κB, AP-1, and Sp1 is increased. Whether these increases are secondary to the decrease in binding of the large multiprotein complex at Ets-2, or represent binding of transcriptionally inactive factors remains to be determined. Aside from the conserved defects in MAPK activation in both cell types following HIV infection, the central Sp1/Ets-2/AP-1/NF-κB region of the IL-12 p40 promoter seems to be a common molecular target in the regulation of IL-12 p40 expression by HIV infection. The dotted oval indicates the position of a nucleosome over the IL-12 p40 promoter, which may be an additional target of IL-12 p40 gene regulation by HIV in this region of the promoter.

F. Model of IL-12 p40 Regulation in HIV-Infected Myeloid Cells:

The proposed mechanism of HIV-mediated inhibition of LPS-induced IL-12 p40 expression, based on the results reported in this work, is summarized in figure 46. In THP-1 cells (panel A), infection with HIV exerts several effects, all of which likely contribute to impaired LPS-inducible transcription of the IL-12 p40 gene. Activation of p38 and JNK MAP kinases in response to LPS is impaired in HIV-infected THP-1 cells, as is phosphorylation and turnover of IκBα, indicating a defect in NF-κB activation. As inhibition of these MAP kinases by pharmacological inhibitors (M. Cameron and F. Frappier, personal communication) blocks IL-12 p40 expression, HIV-mediated impairment of activation of these kinases likely contributes to the suppression of IL-12 p40 following HIV infection. This inhibition is likely related to the observed overall decrease in binding of nuclear factors to the NF-κB, AP-1, and Sp1 promoter elements (including impaired binding of NF-κB p50, p65, c-Rel, c-Fos, c-Jun, Sp1, and Sp3) and with the reversal of LPS-induced effects on nuclear complex formation at these sites, following HIV infection. Additionally, HIV infection seems to reduce NF-κB p65 protein expression in THP-1 cells, which may contribute to the observed reduction in binding of this protein to the NF-κB site.

MDM infected with HIV (panel B) also exhibit defects in p38 and JNK phosphorylation, and in IκBα phosphorylation and degradation. Again, as in THP-1 cells, these effects are likely significant contributors to diminished IL-12 p40 expression, in accordance with data from the literature and inhibitor studies (M. Cameron and F. Frappier), and likely reflect aberration in activation of myeloid cell nuclear factors. The overall enhancement in nuclear factor binding to the NF-κB, AP-1 and Sp1 sites, and the

reversal of the effects of LPS observed in mock-infected MDM may reflect increased attachment of inhibitory factors or inclusion of nuclear factors which bind but may be defective in transcription potential. The decrease in Ets-2 binding (including NF-κB p50, c-Rel, Ets-2, IRF-1, and ICSBP) may also reflect decreased recruitment of vital stimulatory transcription factors to the promoter following HIV infection. At the Ets-2 site, the decrease in c-Rel binding observed may be a significant defect, as c-Rel has been demonstrated to be particularly vital for p40 expression, along with IRF-1 and ICSBP as determined using murine knockout models (123, 124).

Although the exact alterations in nuclear factor binding occurring in THP-1 cells and MDM infected with HIV are empirically different, likely attributed to the different stages of maturation, and alternate physiology of the two cell types, several effects of HIV are conserved. In both cell types, p38, JNK, and NF-κB activation are affected, without an effect on ERK activation, indicating that p38, JNK, and IκBα are likely important targets of HIV-mediated inhibition of IL-12 expression. In addition, the conserved central region of the promoter being affected by HIV implies importance of this region in IL-12 regulation and modulation by HIV. The conserved location of the effect of HIV on the IL-12 p40 promoter may be consistent with impaired TLR-dependent nucleosome remodelling required for IL-12 p40 promoter activation (117). Although alterations in binding of specific nuclear factors to the IL-12 p40 promoter have begun to be identified, the ultimate functional consequences of these alterations cannot be appreciated until the complete makeup of factors binding these sites in mock- and HIV-infected cells is elucidated. The functional status of proteins bound to the promoter elements in terms of transactivation potential has not yet been determined.

In addition to these similarities, both THP-1 cells and MDM require infection with replication-competent virus to achieve full suppression of IL-12 p40 expression. This suppression was not mediated by soluble host or viral products, including cytokines, in either cell type.

G. Concluding Remarks:

Overall, this body of work makes a significant contribution to our understanding of the mechanism of HIV-mediated immunosuppression in infected myeloid cells. While in this system, decreased IL-12 p40/p70 occurred in the absence of altered expression of IL-12 p35 and was strictly dependent upon viral replication, and independent of other soluble host or viral factors or de novo protein synthesis, there are almost certainly additional mechanisms at work in unfractionated PBMC, and in the in vivo setting. The lack of a role for other cytokines in HIV-induced suppression of IL-12 production in isolated, infected MDM does not account for the contribution of cytokines produced by, and cell-cell contact with, other PBMC populations. In particular, T lymphocytes producing aberrant patterns of cytokines, and exhibiting altered expression of costimulatory molecules, may influence IL-12 production and HIV replication within monocytes/macrophages in vivo. While IFN-γ did not have a significant effect on IL-12 p40 expression in this system, IFN-γ is pivotal to IL-12 regulation and Th1-responses as a whole. This, in addition to the observation that IFN-γ levels may be compromised in HIV-infected individuals (223), likely adds to the defect in IL-12 production in response to stimulation.

The CD40/CD40 ligand axis of induction of IL-12 production was not examined in this work, but warrants future investigation. While the literature suggests that CD40 expression on antigen-presenting cells in HIV seropositive donors is not compromised and in fact may be augmented relative to healthy control subjects (271), CD40 ligand upregulation on activated T cells from HIV infected donors is impaired (90), and may add to the mechanisms contributing to impairment of IL-12 expression. Early findings in this series of investigations showed no modulation of IL-12 p35 mRNA in infected isolated myeloid cells, but this conflicts with observations in PBMC (11). In addition, a recent report demonstrated the presence of multiple IL-12 p35 mRNA species originating from different transcription start sites in the IL-12 p35 promoter depending on the presence or absence of stimulation with LPS (272). In the absence of stimulation, IL-12 p35 transcripts contained an additional ATG site in the 5' untranslated region which inhibited translation of this mRNA species, and following LPS treatment, transcription initiated from an alternate location, producing IL-12 p35 mRNA devoid of this upstream ATG, thereby allowing translation. This, and regulation of dimerization of the p35 and p40 subunits, and glycosylation of p35 allowing secretion of p70 may be additional levels of regulation of IL-12 production, and may be subject to modulation by HIV infection. While experiments reported herein focus on p40 subunit regulation, due to the close correlation between p40 expression and bioactive p70 production in our system and as reported by others, p35 is rate limiting and absolutely required for p70 formation, and therefore warrants consideration. However, given the close association between p40 and p70 expression, understanding discrete mechanisms of IL-12 p40 regulation in particular, at the cellular level is of paramount importance in understanding immunopathogenesis of HIV infection.

H. Relevance/Impact of the Current Work:

This body of work describes for the first time a mechanism of inhibition of IL-12 p40 gene expression by HIV infection that is independent of the activity of other cytokines, and strictly requires HIV replication. This suppression occurs at least in part, at the level of transcription of the IL-12 p40 gene, independent of de novo protein synthesis, and is not due to the presence of soluble host or viral inhibitory proteins in culture. This work is also the first demonstration of the effects of HIV infection on molecular regulation of LPS-induced activation of the human IL-12 p40 promoter, which may occur via inhibition of IκBα degradation and p38 and JNK MAP kinase activation in human myeloid cells. This is also the first demonstration that in addition to the NF-κB and Ets-2 IL-12 p40 promoter elements, the AP-1 and Sp1 sites are integral for LPS-induced transcription and are a targets of regulation of IL-12 p40 expression. Having identified the -350/-105 promoter region as a key control point in HIV-mediated suppression of IL-12 p40 transcription, future examination of interactions of HIV proteins with host cell signalling machinery will pinpoint the intracellular molecular mechanism of suppression of IL-12 by HIV.

Interleukin-12 is a crucial cytokine in the generation of cell-mediated immune responses necessary for defence against a variety of pathogens. As cell-mediated immunity and IL-12 production are compromised in HIV-infected individuals, gaining an understanding of how HIV modulates IL-12 production in myeloid cells may lead to novel approaches to therapy, improving cell-mediated immunity responses to opportunistic pathogens and enhancing HIV-specific adaptive immunity. Although

initial clinical trials of the subcutaneous administration of recombinant human IL-12 failed to demonstrate any evidence for improvement in host immune function (273), modulating the endogenous production of IL-12, or enhancing the expression of IL-12 in combination with other immune-based approaches may still prove to be beneficial in individuals with HIV infection. In addition, understanding intracellular molecular regulation of IL-12, and the influence of other PBMC populations and soluble mediators on this regulation, will eventually extend our knowledge to other disorders arising from or exacerbated by alterations in IL-12 expression or Th1 responses. In addition, a comprehensive understanding of control of IL-12 expression will facilitate the exploitation of IL-12 as an immunotherapeutic agent for tumours or infectious diseases directly or indirectly via its inclusion in vaccination regimens as a potent adjuvant.

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VII. CURRICULUM VITAE

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ACADEMIC BACKGROUND

September 1997 – present:

- Currently enrolled in the Ph.D. program in the Department of Biochemistry, Microbiology & Immunology at the University of Ottawa, under the supervision of Dr. Jonathan Angel at the Ottawa Health Research Institute (Molecular Medicine Program), 501 Smyth Road, Ottawa, Ontario, K1H 8L6.
- Successfully completed all coursework, seminar, and research requirements for the Ph.D. program, and permission to commence writing of the Ph.D. thesis was granted in December 2002.
- Courses completed: Advanced Topics in Immunology (MIC 8122, grade A), and Advanced Topics in Virology (MIC 8236, grade A).
- Ph.D. comprehensive exam successfully completed in June 1999 (grade A+).

September 1995 – April 1997:

- Successfully completed M.Sc. degree in the Department of Microbiology & Immunology at the University of Ottawa, under the thesis supervision of Dr. Lionel Filion and Dr. William Ross, 451 Smyth Road, Ottawa, Ontario, K1H 8M5
- Courses completed: Basic Immunology (MIC 5124, grade A), and Advanced Immunochemistry (MIC 8126, grade A)
- Thesis entitled "Immune Damage in Irradiated Mice: Contributions of Differential Radiosensitivity and Apoptosis in Mononuclear Cells, and Alterations in Natural Killer Cell Cytolytic Potential" successfully defended in July 1997

September 1991 – April 1995:

- Successfully completed B.Sc. Honours degree in the Department of Biology at the University of Ottawa, 550 Cumberland, Ottawa, Ontario K1N 6N5
- Conducted fourth year Honours research project under the supervision of Dr. William Ross and Dr. Joanne Dillon
- Graduated with Magna cum laude distinction

Scholarships and Awards:

- New Investigator's Award (Basic Science): Canadian Association of HIV Research, April 2002.
- OHTN Studentship Award: September 2001-August 2003
- University of Ottawa Excellence Scholarship: September 2001-August 2003
- Student and Fellow Travel Award from the 9th Conference on Retroviruses and Opportunistic Infections, February 2002
- MRC Doctoral Scholarship: May 1998 – April 2001
- Also awarded NSERC studentship and OGS studentship in 1998, which were declined upon acceptance of MRC Doctoral Scholarship (above)
- University of Ottawa Excellence Scholarship: May 1998 – April 2001
- Student and Fellow Travel Award from the 8th Conference on Retroviruses and Opportunistic Infections, February 2001
- Student and Fellow Travel Award from the 7th Conference on Retroviruses and Opportunistic Infections, February 2000
- University of Ottawa Admission Scholarship: September 1997 – May 1998
- Ontario Graduate Scholarship September 1996 – August 1997
- University of Ottawa Admission Scholarship: September 1995 – August 1996
- Canada Scholarship from the Department of Industry, Science, and Technology for high standing in science and mathematics (September 1991- April 1993)
- University of Ottawa Undergraduate Admission Scholarship for obtaining an overall OAC average above 90% (September 1991- April 1992)

Professional Development:

- Have given several poster and oral presentations at numerous national and international scientific conferences;
- Assisted in the supervision/mentoring of several graduate and Honour's students;
- Played an integral role in writing and submission of numerous operating grants, several of which were successfully awarded to Dr. Jonathan Angel;
- Taught an advanced level graduate lecture at the University of Ottawa (HIV Pathogenesis, course organizer: Dr. Karen Copeland).

EMPLOYMENT HISTORY

- Defense Research Assistant: May 1995 – August 1995
  - Defense Research Establishment, Ottawa
    Completed a study of biological dosimetry and immune suppression following exposure to ionizing radiation.

- Defense Research Assistant: May 1994 – August 1994
  - Defense Research Establishment, Ottawa
    Initiated a study of biological dosimetry to detect radiation injury, and standardized methods for future research in this area.

- Traffic Surveyor/ Statistical Data Collector: May 1993- August 1993
  - Regional Municipality of Ottawa-Carleton
    Performed traffic surveys under minimal supervision, contributing to regional traffic planning. Received training to supervise implementation of the program.

- Traffic Surveyor/ Statistical Data Collector: May 1992- August 1992
  - Regional Municipality of Ottawa-Carleton
    Performed traffic surveys under minimal supervision, contributing to regional traffic planning.

PUBLICATIONS, PRESENTATIONS, ABSTRACTS

Publications:

- ***Chambers KA***, Parks RJ, Angel JB (2003) Disruption of MAP Kinase Activation and Nuclear Factor Binding to the IL-12 p40 Promoter in HIV-Infected Myeloid Cells. (Submitted to Blood).

- ***Chambers KA***, Parato KG, Angel JB (2002) Active Cellular Infection of Myeloid Cells is Required for HIV-1-Mediated Suppression of Interleukin-12 p40 Expression. Cellular Immunology 215: 120-132.

- Parato KG, Kumar A, Badley AD, Sanchez-Dardon JL, ***Chambers KA***, Young CD, Lim WT, Kravcik S, Cameron DW, Angel JB (2002) Normalization of Natural Killer Cell Function and Phenotype with Effective Anti-HIV Therapy and the Role of Interleukin-10. AIDS 16(9): 1251-1256.

- Phenix BN, Angel JB, Mandy F, Kravcik S, Parato K, ***Chambers KA***, Gallicano K, Hawley-Foss N, Cassol S, Cameron DW, Badley AD (2000) Decreased HIV-associated T Cell Apoptosis by HIV Protease. AIDS Research and Human Retroviruses 16(6): 559-567

- ***Chambers KA***, Harrington NP, Ross WM, Filion LG (1998) Relative Alterations in Blood Mononuclear Cell Populations Reflect Radiation Injury in Mice. Cytometry 31(1): 45-52

- Harrington NP, ***Chambers KA***, Ross WM, Filion LG (1997) Radiation Damage and Immune Suppression in Splenic Mononuclear Cell Populations. Clinical and Experimental Immunology 107(2): 417-424

Abstracts and Conference Presentations:

- Frappier FM, ***Chambers KA***, Angel JB (2003) HIV Inhibition of IL-12 Synthesis is Mediated by Altering MAPK Activity and Nuclear Factor Binding to the IL-12 p40

Promoter. 10th Conference on Retroviruses and Opportunistic Infections, February 11-15 (Boston, MA).

- ***Chambers KA***, Parato KG, Angel JB (2003) Disruption of MAP Kinase Activation and Nuclear Factor Binding to the IL-12 p40 Promoter in HIV-Infected Myeloid Cells. Poster presented by ***KA Chambers*** at the Keystone Symposium on Regulatory and Effector Functions of Macrophages, January 30-February (Taos, NM).

- ***Chambers KA,*** Angel JB (2002) Suppression of IL-12 p40 Transcription by Acute HIV Infection: Disruption of Nuclear Factor Binding and Inhibition of MAP Kinase Activation. Oral presentation by ***KA Chambers*** at the 11th Annual Canadian Conference on HIV/AIDS Research, April 25-28 (Winnipeg, Manitoba).

- ***Chambers KA***, Angel JB (2002) Suppression of Interleukin (IL)-12 p40 Transcription by Acute HIV Infection: Disruption of Nuclear Factor Binding and Inhibition of MAP Kinase Activation. Poster presented by ***KA Chambers*** at the 9th Conference on Retroviruses and Opportunistic Infections, February 24-28 (Seattle, WA)

- ***Chambers KA***, Angel JB (2001) Acute Infection of Myeloid Cells with HIV-1 Alters Transcription Factor Binding to the IL-12 Promoter. Poster presented by ***KA Chambers*** at the 10th Annual Conference on HIV/AIDS Research, Toronto, Canada, May 2001.

- ***Chambers KA***, Angel JB (2001) Acute Infection of Myeloid Cells with HIV-1 Alters Transcription Factor Binding to the IL-12 Promoter. Poster presented by ***KA Chambers*** at the 8th Conference on Retroviruses and Opportunistic Infections, Chicago, USA, February 2001.

- ***Chambers KA***, Parato KG, Angel JB (2000) Suppression of Interleukin (IL)-12 Production by Acute HIV Infection & the Role of Other Cytokines. Oral presentation by ***KA Chambers*** at the 9th Annual Conference on HIV/AIDS Research, Montreal, Canada, April 2000.

- ***Chambers KA,*** Parato KG, Ahmed I, Angel JB (2000) Suppression of Interleukin (IL)-12 Production by Acute HIV Infection of Myeloid Cells Requires Productive Infection and May Occur Independently of the Activity of Key Modulatory Cytokines. Poster presented by ***KA Chambers*** at the Keystone Symposium on Cytokines and Disease, Snowbird Utah, USA, April 2000.

- ***Chambers KA***, Parato KG, Angel JB (2000) Suppression of Interleukin (IL)-12 Production by Acute HIV Infection & the Role of Other Cytokines. Poster presentation by ***KA Chambers*** at the 7th Conference on Retroviruses and Opportunistic Infections, San Francisco, USA, February 2000.

- Ross WM, ***Chambers KA***, Filion LG (1996) New R&D Thrusts in Biological Dosimetry in Canadian Defense Research. Oral presentation by WM Ross at 1996 RSG-23 (NATO Panel 8) Workshop on Assessment, Prophylaxis and Treatment of Ionizing Radiation Injury in Nuclear Environments, Ottawa, Canada, May 1996.

- ***Chambers KA***, Filion LG, Harrington NP, Ross WM (1996) Flow Cytometric Analysis of WBC from Irradiated Mice: Biological Indicators of Dose and Damage. Poster presentation by LG Filion at the International Society for Analytical Cytology XVIII Congress, Rimini, Italy, April 1996.

- Ross WM, ***Chambers KA***, Harrington NP, Filion LG (1995) Dose-dependent Changes in Numbers of Blood MNC in Irradiated Mice. Poster presentation by WM Ross at the 10th International Congress of Radiation Research, Wurzburg, Germany, August 1995.

- ***Chambers KA***, Harrington NP, Filion LG, Ross WM (1995) Flow Cytometric Analysis of Immune-Responsive Blood Mononuclear Cells in Irradiated Mice. Poster presentation by ***KA Chambers*** at the 9th International Congress of Immunology, San Francisco, USA, July 1995.

VIII. CONTRIBUTIONS OF COLLABORATORS

The research reported in this thesis was conducted exclusively by Kelley A. Chambers, with the following exceptions. The site-directed mutagenesis of the IL-12 p40 promoter, for use in recombinant adenovirus construction was performed by the late Dr. Michael Montpetit (Health Canada), although the mutagenic oligonucleotides used in site-directed mutagenesis were designed by Kelley A. Chambers. Sequencing was performed by the University of Ottawa Biotechnology Research Institute. The construction of recombinant adenovirus vectors was performed by Dr. Robin Parks and his research technicians Robert Meulenbroek and Kathy Sargent at the Ottawa Health Research Institute. The preliminary data mentioned in the Discussion regarding the use of MAP kinase inhibitors to assess the role of MAPK in IL-12 p40 expression was generated by Maribeth Cameron and Fiona Frappier in the laboratory of Dr. Jonathan Angel.

APPENDIX I: LIST OF CHEMICALS

RPMI-1640 medium (Gibco/Invitrogen, Burlington, ON)

Fetal bovine serum (FBS; Gibco)

Penicillin (Gibco)

Streptomycin (Gibco)

Fungizone (Bristol Myers Squibb, Montreal, QC)

Phosphate-buffered saline (PBS; Gibco)

Ficoll-Paque Plus (Amersham-Pharmacia Biotech, Baie d'Urfe, QC)

Human type AB serum (Sigma, Oakville, ON)

HIV-1 p24 antigen ELISA (NEN Life Science Products, Boston, MA)

Lipopolysaccharide (LPS; Sigma)

S. aureus Cowan strain-1 (SAC; Calbiochem, Hornby, ON)

Heat-killed *M. tuberculosis* (MTB; Difco Laboratories, Detroit, MI)

Cycloheximide (CHX; Sigma)

Anti-IL-10 antibody (R&D Systems, Minneapolis, MN)

Anti-TGF-β antibody (R&D Systems)

Interferon-γ (R&D Systems)

Interleukin-4 (R&D Systems)

Interleukin-13 (R&D Systems)

Interleukin-12 (R&D Systems)

Anti-human Interleukin-12 antibody (R&D Systems)

HIV-1_{IIIB} gp120 (NIH AIDS Research and Reference Reagent Program, Rockville, MD)

Human IL-12 p40 ELISA (R&D Systems)

TriPure Isolation Reagent (Roche, Laval, QC)

Chloroform (Sigma)

Isopropanol (Sigma)

Ethanol (Commercial Alcohols, Brampton, ON)

Diethylpyrocarbonate (DEPC; Sigma)

RNeasy RNA extraction kit (Qiagen, Mississauga, ON)

RiboQuant Ribonuclease protection assay kit (BD Pharmingen, Mississauga, ON)

Tris-buffered phenol (Gibco)

40% Acrylamide/bis-acrylamide (19:1) (Sigma)

30% Acrylamide/bis-acrylamide (19:1) (BioRad Laboratories, Mississauga, ON)

1^{st}-Strand cDNA synthesis kit (Clontech, Palo Alto, CA)

Advantage cDNA PCR kit (Clontech)

Ethidium bromide (Sigma)

CD4-microbeads (Miltenyi Biotech, Auburn, CA)

CD8-microbeads (Miltenyi Biotech)

Phytohemagglutinin (PHA; Gibco)

Human IFN-γ ELISA (R&D Systems)

GolgiStop (BD Pharmingen)

Bovine serum albumin (BSA; Sigma)

Caltag Fix-and-Perm Kit (Cedarlane, Hornby, ON)

RD1-conjugated anti-HIV-1 p24 antibody (Beckman-Coulter, Mississauga, ON)

PE-conjugated anti-human IL-12 p40/p70 antibody (BD Pharmingen)

PE-labeled mouse IgG_1 isotype control (BD Pharmingen)

Poly-L-lysine (Sigma)

Triton X-100 (Sigma)

Paraformaldehyde (Sigma)

4,6-Diamidino-2-phenylindole (DAPI; Sigma)

Glycerol (Sigma)

FITC-conjugated anti-mouse IgG_1 (BD Pharmingen)

Sucrose (Sigma)

KCl (Sigma)

NaCl (Sigma)

Hepes (Sigma)

EDTA (Sigma)

EGTA (Sigma)

Spermine (Gibco)

Spermidine (Gibco)

Dithiothreitol (DTT; Sigma)

Phenylmethanesulfonyl fluoride (PMSF; Sigma)

Benzamidine (Sigma)

Iodoacetamide (Sigma)

Trypsin inhibitor (Sigma)

Leupeptin (Sigma)

NP-40 (BDH Chemicals, Toronto, ON)

Tris (Sigma)

RNase inhibitor (Sigma)

α-^{32}P-UTP (Amersham)

γ-^{32}P-ATP (Amersham)

Ammonium sulfate (Sigma)

$MgCl_2$ (Sigma)

$MnCl_2$ (Sigma)

Creatine phosphate (Sigma)

GTP, ATP, CTP (Amersham)

RNase-free DNase (Sigma)

Yeast tRNA (Gibco)

Proteinase K (Gibco)

SDS (Sigma)

AdvanTAge PCR cloning kit (Clontech)

DH5α supercompetent cells (Gibco)

SOC medium (Gibco)

LB broth (Sigma)

Bacteriological agar (Sigma)

5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal; Gibco)

Isopropyl-1-thio-β-D-galactopyranoside (IPTG; Sigma)

Qiaprep Spin Miniprep kit (Qiagen)

PCR-Script Amp Cloning Kit (Stratagene, La Jolla, CA)

Microspin 400 columns (Amersham)

SacI restriction enzyme (Amersham)

HindIII restriction enzyme (Amersham)

Isoamyl alcohol (Sigma)

Sodium acetate (Sigma)

Hybond-ECL nitrocellulose membrane (Amersham)

NaOH (Sigma)

Ammonium acetate (Sigma)

20X SSC (Sigma)

20X SSPE (Sigma)

Deionized formamide (Sigma)

Ficoll (Sigma)

Polyvinyl pyrrolidone (Sigma)

Salmon sperm DNA (Gibco)

T4 polynucleotide kinase (Amersham)

Microspin G-50 columns (Amersham)

Trypsin-EDTA (Gibco)

β-mercaptoethanol (Sigma)

Sodium deoxycholate (Sigma)

Na_3VO_4 (Sigma)

NaF (Sigma)

BCA protein determination kit (Pierce, Rockford, IL)

4-15% Tris-HCl Ready-gel (BioRad)

Glycine (Sigma)

Immobilon-P PVDF membrane (Millipore, Bedford, MA)

Methanol (Sigma)

Tween-20 (Sigma)

SuperSignal west pico chemiluminescent substrate (Pierce)

XhoI restriction enzyme (Amersham)

pGL3 Basic vector (Promega, Maddison, WI)

GFX gel purification kit (Amersham)

Calf intestinal alkaline phosphatase (MBI Fermentas, Burlington, ON)

T4 DNA ligase (New England Biomedical, Northborough, MA)

BglII restriction enzyme (Amersham)

QuickChange Site-directed mutagenesis kit (Stratagene)

Enhanced firefly luciferase assay kit (BD Pharmingen)