The effects of HIV on the regulation of IL-12 family cytokines, IL-12, IL-23, and IL-27 production in human monocyte-derived macrophages

By

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ABSTRACT

IL-12 family cytokines IL-23 and IL-27 play an important role linking innate and adaptive immunity, and regulating T-cell responses. The production of IL-12, a structurally similar cytokine, is decreased in chronic HIV infection; therefore IL-23 and IL-27 may also be influenced by HIV infection. I hypothesized that HIV inhibits LPS-induced IL-23 and IL-27 production in human MDMs by suppressing the activation of signalling pathways regulating their expression. *In vitro* HIV-infection of MDMs did not have any effect on basal secretion of IL-23 or IL-27; however, HIV inhibited LPS-induced production of IL-12/23 p40 and IL-23 p19, and IL-27 EBI3 and IL-27 p28 mRNA expression, and IL-23, IL-12/23 p40 and IL-27 secretion. In order to evaluate the molecular mechanisms by which HIV inhibits IL-23 and IL-27 in LPS-stimulated MDMs, the signalling pathways regulating their expression were evaluated. The PI3K, p38 MAPK, and JNK MAPK pathways were found to positively regulate LPS-induced IL-27 secretion. Interestingly, *in vitro* HIV infection inhibited LPS-induced p38 and JNK MAPK activation in MDMs. In summary, I have shown that HIV inhibits IL-23 and IL-27 production in LPS-stimulated MDMs and that HIV may inhibit LPS-induced IL-27 production through the inhibition of p38 and JNK MAPK activation.

It is currently unknown whether PKCs regulate LPS-induced IL-23 or IL-27 in human monocytes/macrophages. I demonstrated that classical PKCs differentially regulate LPS-induced IL-23 and IL-27 secretion within THP-1 cells, primary monocytes, and MDMs. Classical PKCs were found to positively regulate LPS-induced IL-12/23 p40 and IL-27 p28 mRNA expression and IL-12/23 p40, IL-23, and IL-27 secretion in primary human monocytes. Similarly, the classical PKCs were found to positively regulate IL-27 p28 mRNA

expression and IL-27 secretion in THP-1 cells. However, classical PKCs did not regulate LPS-induced IL-27 production in MDMs, or LPS-induced IL-23 production in THP-1 cells. Overall, this demonstrates that classical PKCs differentially regulate LPS-induced IL-23 and IL-27 production in different myeloid cells.

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HIV:	Human Immunodeficiency Virus Type 1
AIDS:	Acquired Immunodeficiency Syndrome
HAART:	Highly Active Antiretroviral Therapy
cART:	Combination Antiretroviral Therapy
CD:	Cluster of Differentiation
T-cells:	T-lymphocyte Cells
DCs:	Dendritic Cells
Mos:	Monocytes
MΦs:	Macrophages
M-tropic:	Macrophage-Tropic
CCR5:	CC-Chemokine Receptor Type-5
T-tropic:	T-cell Tropic
CXCR4:	CXC-Chemokine Receptor Type-4
B-cells:	B-Lymphocyte Cells
TLR:	Toll-Like Receptor
TNF:	Tumor Necrosis Factor
NF-κB:	Nuclear Factor-Kappa B
DC-SIGN:	Dendritic Cell-Specific Intracellular Adhesion Molecule-3-Grabbing Non-Integrin
IFN:	Interferon
IRF:	Interferon Regulator Factor
IL:	Interleukin
NK cells:	Natural Killer cells
Th1:	T-helper 1
MDMs:	Monocyte-Derived Macrophages
gp120:	Glycoprotein-120
MIP:	Macrophage Inflammatory Protein
RANTES:	Regulated on Activation, Normal T-Expressed and Secreted
Env:	Envelop
SIV:	Simian Immunodeficiency Virus
LN:	Lymph Node
GALT:	Gut-Associated Lymphoid Tissue
TGF:	Transforming Growth Factor
MCP-1:	Monocyte Chemotactic Protein
IP-10:	IFNγ-Induced Protein-10
PBMCs:	Peripheral Blood Mononuclear Cells
Vpr:	Viral Protein R
Tat:	Trans-Activator of Transcription
Th2:	T-helper 2
DNA:	Deoxyribonucleic Acid
CTL:	Cytotoxic T-Lymphocyte
IL-12R:	IL-12 Receptor
IL-23R:	IL-23 Receptor

JAK:	Janus Kinase
STAT:	Signal Transducer and Activator of Transcription
TKY:	Tyrosine Kinase
Th17:	T-helper 17
mRNA:	Messenger Ribonucleic Acid
EBI3:	Epstein Barr Virus-Induced Gene-3
IL-27R:	IL-27 Receptor
gp130:	Glycoprotein-130
TCCR:	T-cell Cytokine Receptor
GATA-3:	GATA Binding Protein-3
Tr1:	Type-1 T-Regulatory
Treg:	T-Regulatory
APOBEC:	Apolipoprotein B mRNA Editing, Enzyme-Catalytic, Polypeptide-like
IL-1R:	IL-1 Receptor
TIR:	TLR/IL-1R Homology
MyD88:	Myeloid Differentiation Primary Response Gene 88
TIRAP:	TIR Domain Containing Adapter Protein
TRIF:	TIR-Domain-Containing Adapter-Inducing IFNβ
TRAM:	TRIF-Related Adapter Molecule
PI3K:	Phosphoinositide-3-Kinase
BCAP:	B-cell Adapter for PI3K
PIP2:	Phosphatidylinositol 4,5-Bisphosphate
AKT:	Protein Kinase B
IRAK:	IL-1R-Associated Kinases
TRAFs:	TNF-Associated Factors
TAK-1:	TGFβ-Activated Kinase 1
PKC:	Protein Kinase C
MAPK:	Mitogen Activated Protein Kinase
cPKCs:	Classical Protein Kinase Cs
nPKCs:	Novel Protein Kinase Cs
aPKCs:	Atypical PKCs
ATP:	Adenosine Triphosphate
JNK:	c-Jun N-Terminal Kinase
ERK:	Extracellular Signal-Regulated Kinase
TAB1:	TAK-Binding Protein 1
MKK:	Mitogen Activated Protein Kinase Kinase
MEK:	Extracellular Signal-Regulated Kinase Kinase
LPS:	Lipopolysaccharide
AP-1:	Activator Protein-1
FCS:	Fetal Calf Serum
CD14-THP-1 cells:	THP-1 cells Stably Transfected with CD14 cDNA Sequences
M-CSF:	Macrophage Colony-Stimulating Factor
PE:	Phycoerythrin
qRT- PCR:	Quantitative Real Time Polymerase Chain Reaction
ELISA:	Enzyme Linked Immunosorbant Assay
PBS:	Phosphate Buffered Saline

TMB:	3,3’,5,5’-Tetramethylbenzibidine
HRP:	Horse Radish Peroxidase
BSA:	Bovine Serum Albumin
siRNA:	Small Interfering RNA
ssRNA:	Single-Stranded RNA
M1:	Type-1 (MΦs)
M2:	Type-2 (MΦs)
GM-CSF:	Granulocyte Macrophage Colony-Stimulating Factor
shRNA:	Small-Hairpin RNA

CHAPTER I: GENERAL INTRODUCTION

Human immunodeficiency virus infection:

Human immunodeficiency virus type 1 (HIV) infection causes severe immune dysregulation which, if left untreated, leads to the development of acquired immunodeficiency syndrome (AIDS). Chronic immune activation and viral persistence are two of the main driving forces causing immune dysregulation in HIV. These two forces are capable of amplifying each other, creating a vicious cycle promoting immune dysfunction. Prior to the introduction of highly active antiretroviral therapy (HAART)/combination antiretroviral therapy (cART) in 1996, being diagnosed with HIV was considered a death sentence. The introduction of therapy has significantly decreased the number of AIDS-related deaths; however, the life expectancy of HIV-infected individuals still remains lower than healthy people (1). Two of the most characteristic changes observed in HIV infection are the depletion of cluster of differentiation (CD)-4 T-lymphocyte cells (T-cells), and increased viral loads. HIV-infected individuals who respond successfully to HAART have increased CD4 T-cell counts and decreased viremia. Although chronic immune activation is reduced in HIV-infected individuals on HAART, these patients still have higher levels of immune activation markers compared to uninfected individuals (2, 3). There is increasing evidence to suggest that chronic immune activation in HIV infection is a leading cause to premature aging and increased susceptibility to age-related diseases including cancer, dementia, and cardiovascular disease (4).

One mechanism by which HIV contributes to chronic immune activation in individuals on therapy is through viral persistence and the development of viral reservoirs.

HIV is capable of infecting CD4 T-cells, dendritic cells (DCs), monocytes (Mos), and macrophages (MΦs), however, both Mos and DCs are less susceptible to HIV infection (5, 6). MΦs are one of the first cells to become infected with HIV (7), and they are resistant to the cytopathic effects of HIV (8). Replication competent HIV has been isolated from Mos of HIV-infected patients on HAART who have achieved increased CD4 T-cells numbers, and decreased viral loads less than 50 copies/mL (9). This suggests that HAART is unsuccessful at eliminating Mo/MΦ HIV reservoirs. Increased activation of MΦs in HIV-infected individuals on HAART has also been linked to increased microbial translocation (10). Mos and MΦs and the IL-12 family of cytokines are important mediators of innate and adaptive immune responses. Thus, infection of Mos and MΦs with HIV and the subsequent dysregulation of the IL-12 family of cytokines likely play a significant role in the sustained immune activation, and immune dysfunction observed in HIV-infected individuals on and off HAART. Further understanding of how HIV affects MΦ function and cytokine production will be pivotal in developing better therapeutics to reduce chronic immune activation and improve disease outcome.

Monocytes/macrophages in HIV infection:

MΦs are among the first cells to become infected with HIV (7). Mos and MΦs are more susceptible to macrophage-tropic (M-tropic)/ CC-chemokine receptor type-5 (CCR5) tropic HIV strains (11, 12), however, T-cell tropic (T-tropic)/CXC-chemokine receptor type-4 (CXCR4) and dual tropic infection of Mos and MΦs have been observed (13-16). Mos are less susceptible to HIV infection compared to MΦs, with only 0.001-1% of peripheral blood Mos becoming infected over the course of infection (6, 17). During the differentiation process of Mos into MΦs, CCR5 is up-regulated, enhancing the susceptibility of MΦs to

HIV infection (18, 19). The percentage of tissue resident MΦs infected with HIV can range anywhere from 1-50% (17, 20-24).

Mos and MΦs have important immune functions including antigen presentation, co-activation of B-lymphocyte cells (B-cells) and T-cells, antimicrobial functions (i.e.: phagocytosis and chemotaxis), anti-tumour activities, and cytokine and chemokine production (25). Cell-mediated MΦ immune responses are essential for the clearance of various bacterial, fungal, and parasitic infections. HIV impairs many of these functions in Mos and MΦs, leading to increased secretion of proinflammatory cytokines, suppressed production of IL-12, and impaired anti-microbial functions, contributing to chronic immune activation, and increased susceptibility to reactivation of infections and opportunistic infections.

HIV infection of Mos and MΦs has been shown to impair cytokine secretion, phagocytosis, chemotaxis, intracellular pathogen killing, and increase the production of neurotoxins (reviewed by Kedzierska (25)). HIV-infected MΦs have altered cytokine/chemokine secretion profiles (25). Exposure of MΦs to HIV has been shown to sensitize these cells to toll-like receptor (TLR)-agonists, resulting in increased production of tumour necrosis factor alpha (TNFα), a proinflammatory cytokine (26). Proinflammatory cytokines, like TNF-α can enhance HIV viral transcription through nuclear factor-kappa B (NF-κB)-dependent mechanisms (27). Increased chemokine secretion also enhances the recruitment of immune cells promoting viral dissemination. DCs have been shown to promote viral dissemination to CD4 T-cells by binding HIV particles to the dendritic cell-specific intracellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) surface receptor (28). Thus, when DCs encounter CD4 T-cells in the lymph nodes during antigen

presentation, the viral particle can be transmitted to these CD4 T-cells (28). Chronic inflammation also continually activates CD8 T-cells, eventually leading to replicative senescence, further exacerbating disease outcome (29). Additionally, HIV-infected MΦs can cross the blood-brain barrier and secrete proinflammatory cytokines, lipid mediators, reactive oxygen species, and excitotoxins, creating a neurotoxic environment contributing to the development of neuro-cognitive disorders (30-34). Overall, HIV-infected MΦs are a significant contributor to chronic immune activation.

Although MΦs and Mos are significant contributors to chronic immune activation, they are also important mediators of anti-viral responses. MΦs and Mos secrete cytokines that trigger innate and adaptive anti-viral responses. In MΦs, viral infections trigger the induction of type-1 interferons (IFN)-α/β which activates interferon regulator factor (IRF)-3 and IRF-7 (35). This leads to the induction of IFN-response genes which are important in interfering with viral replication and spread (35). Activated Mos and MΦs can secrete interleukin (IL)-15 and IL-12 which can induce anti-viral responses from natural killer cells (NK cells) and T-helper 1 (Th1) cells, respectively (36, 37). IL-12 also promotes cytotoxic anti-viral responses from CD8 T-cells and NK cells (38, 39). HIV-infected monocyte-derived macrophages (MDMs), and Mos exposed to HIV-glycoprotein-120 (gp120) have also been shown to up-regulate CCR5-agonists macrophage inflammatory protein (MIP)-1α, MIP-1β, and regulated on activation, normal t-expressed and secreted (RANTES) (40-42). Similarly, in human Mos, MIP-1α, and MIP-1β are induced in response to *in vitro* HIV infection (40). Increased production of MIP-1α, MIP-1β, and RANTES inhibits fusion of HIV-envelop (Env) with CCR5 (12), thus inhibiting viral entry, and viral dissemination.

Thus, cytokine and chemokine production by Mos and MΦs may contribute to the inhibition of viral spread of CCR5-tropic HIV infection.

HIV-infected MΦs serve as an important model for studying immune dysregulation because they produce cytokines that mediate anti-viral responses. Unlike studies of cytokine levels in the serum of HIV-infected patients, MΦs can be used as a model to estimate the cytokine levels present in tissue microenvironments and to investigate the cytokine profiles that would be induced by opportunistic infections. Additionally, the use of MΦs as a study model can give insight into 1) what cytokines are impaired in MΦ reservoirs, 2) why immune recovery is incomplete in HIV patients on HAART, and 3) why HIV-infected individuals have increased susceptibility to opportunistic infections. Further understanding of how HIV impairs MΦ function and how impaired MΦ functions contribute to chronic immune activation and poor viral clearance will be important in developing more effective therapeutics to improve HIV disease outcome.

Cytokines and chemokines in HIV infection:

Studying cytokine responses in HIV infection is challenging because it is difficult to differentiate whether an elicited immune response is dampening the effects of HIV infection or worsening disease outcome. Several cytokines are dysregulated in acute and chronic HIV infection (summarized in Table 1 and 2). Proinflammatory chemokines, such as IFNα, MIP-1α, and MIP-1β can recruit immune cells to sites of infection which can enhance viral spread (43). Additionally, many cytokines, including TNFα, IL-6, IL-15, and IFNγ have been shown to increase viral replication (27, 44-46). Increased TNFα, IL-6, IL-18, and IL-7 have also been correlated with decreased CD4 T-cell counts in HIV infection (47). In contrast,

Table 1: Cytokines dysregulated in acute HIV infection

Cytokine	Location	Increased/ Decreased	References
IL-12	Peripheral lymph node (LN), gut-associaed lymphoid tissue (GALT), genital tract	Increased	(48-50)
IL-23	Mos, myeloid conventional DCs	Increased	(51)
IL-15	Plasma	Increased	(52)
IL-7	Plasma	Increased	(53, 54)
IL-10	Serum, plasma, genital tract	Increased	(49, 53, 55, 56)
IL-2	Plasma	Increased	(53)
MIP-1α	Peripheral LN, GALT	Increased	(48)
MIP-1β	Peripheral LN, GALT	Increased	(48, 57)
RANTES	Peripheral LN, GALT	Increased	(48, 57)
TNFα	Peripheral LN, GALT, serum, plasma, Mos	Increased	(48, 52, 53, 55, 56, 58, 59)
Transforming growth factor (TGF)-β	Mos	Increased	(60)
Monocyte chemotactic protein (MCP)-1	Plasma	Increased	(52)
IL-1β	Peripheral LN, GALT, serum, plasma, Mos	Increased	(48, 49, 53, 55, 58, 59, 61)
IL-1α	Plasma	Increased	(53)
IFNα	Serum	Increased	(52)
IFNγ	Serum/plasma	Increased	(52, 55, 61)
IFNγ-Induced Protein (IP)-10	Plasma	Increased	(52, 53)
IL-6	Plasma, genital tract, Mos and tissue MΦs	Increased	(49, 52, 53, 56, 60)
IL-8	Plasma, Mos and tissue MΦs	Increased	(49, 52, 53, 60)
IL-18	Plasma	Increased	(52)

Table 2: Cytokines dysregulated in chronic HIV infection

Cytokine	Location	Increased/ Decreased	References
IL-12	Serum, peripheral blood mononuclear cells (PBMCs), whole blood	Decreased	(62-67)
IL-27	Serum	Trend for decreased	(68)
IL-15	Serum	Decreased	(67, 69)
IL-7	Plasma, DC in LN	Increased	(54, 70-73)
IL-10	CD4 T-cells, Serum, Plasma	Increased	(74-76)
IL-2	PBMCs, CD4 T-cells, CD8-Tcells	Decreased	(76-78)
IL-2	Plasma	Increased	(74)
IP-10	Serum	Increased	(67)
IL-4	CD4 T-cells	Increased	(76)
IFNγ	Plasma, PBMCs, CD8 T-cells	Increased	(76, 78-80)
IFNγ	PBMCs, CD4 T-cells	Decreased	(76, 77)
MIP-1α	CD8 T-cells, whole blood, plasma	Increased	(81-83)
MIP-1β	CD8 T-cells, whole blood, plasma	Increased	(81-83)
RANTES	Whole blood, plasma, renal interstitial and glomerular tissues	Increased	(75, 82-84)
IL-6	Serum, cervicovaginal washings, plasma, PBMCs	Increased	(85, 86)
TNFα	Serum, cervicovaginal washings	Increased	(67, 79, 85, 87, 88)
IL-1	Plasma	Increased	(79)
IL-1α	Serum	Increased	(88)
IL-3	Plasma	Increased	(79)
IL-4	Plasma	Increased	(79, 80)
TNFβ	Plasma	Increased	(79)
TGFβ	Plasma, brain, PBMCs, mononuclear kidney cells	Increased	(79, 80, 89-91)
IL-5	PBMCs	Decreased	(77)

other cytokines, including IL-15, IL-12, and IL-27 can enhance anti-viral responses (62, 92-106). However, cytokines mediating anti-viral responses or enhancing disease progression are not mutually exclusive. For example, IL-15 has been shown to enhance simian immunodeficiency virus (SIV)-specific CD8 T-cell responses in SIV-infected macaques, but has also been correlated with disease progression, higher viral set-points, and increased viral replication (44, 107). Cytokines could be beneficial as a therapy to improve HIV disease outcome, however, the relationship between HIV, cytokine production, and disease outcome are poorly described.

The role of IL-12 in HIV infection:

Th1 cytokines, IL-12 and IFNγ are increased during early/acute infection (48-50), and decline in chronic infection (39, 63-65, 67, 108, 109). Chehimi and colleagues were the first to discover decreased IL-12 production in the serum of HIV-infected individuals relative to healthy individuals (110). This was supported by Trinchieri et al's observations, demonstrating that HIV sero-positive individuals had poor induction of IL-12 in response to bacterial stimuli (111). Subsequent studies have also shown decreased IL-12 production in the serum (67, 112) and culture supernatants of activated PBMCs (63-65, 108, 110), Mos/MΦs (113), and DCs (114) from HIV-infected patients. HIV accessory and regulatory proteins have also been shown to inhibit IL-12 production. For example, HIV-viral protein R (Vpr) inhibits IL-12 production in DCs (115) and IL-12 p35 expression in Mos and MΦs (116). Similarly HIV-trans-activator of transcription (Tat) has been shown to inhibit IL-12 induction from PBMCs stimulated with *Staphylococcus aureus Cowan* 1 strain (117). Since

HIV inhibits IL-12 production, and IL-12 is important in mediating cell-mediated immunity, restoration of IL-12 production in HIV infected individuals may improve disease outcome.

IL-12 regulates cell-mediated immunity through the induction of IFNγ production from Th1 cells, and the induction of proliferation, IFNγ production and cytolytic activity in CD8 T-cells and NK cells (95). IL-12 also inhibits the production of T-helper 2 (Th2) cytokines, IL-10, and IL-4 (118), which are elevated during chronic HIV infection (74-76, 109). IL-12 administration has been shown to improve HIV-specific cell-mediated immunity (62) and NK cell cytotoxic activity (105), in PBMCs of HIV-infected patients exposed *ex vivo* to HIV-Env antigen. These studies have drawn attention to the potential benefit of using IL-12 as an adjuvant in deoxyribonucleic acid (DNA) vaccination to enhance cell-mediated immune responses.

Several studies have investigated the potential of IL-12 as an adjuvant in DNA vaccination with HIV/SIV-antigens in mice and rhesus macaques (96, 97, 100, 102-104, 119, 120). IL-12-deficient mice immunized with HIV-gp120 DNA have impaired cytotoxic T-lymphocyte (CTL) immune responses and antigen-specific IFNγ responses (121, 122). Exogenous addition of IL-12 in IL-12-deficient mice restores cell-mediated immune responses, indicating that IL-12 is essential in mediating HIV-specific CTL responses (121). IL-12 has been shown to enhance antigen-specific IFNγ production (97, 100, 102, 103), granzyme-B production (97), and proliferative responses (103) and increase antigen-specific effector memory T-cell function in mice/rhesus macaques immunized with HIV/SIV-antigens (96, 103, 104). Therefore, not only is IL-12 essential for mediating cell-mediated immunity, IL-12 also has therapeutic potential to enhance cell-mediated immunity.

IL-12 therapy in early/acute and chronic SIV has been investigated in rhesus macaques (123-125). Administration of IL-12 in rhesus macaques acutely-infected with SIV resulted in increased survival rates, lower viral loads, and increased antigen-specific CD8 T-cells and memory T-cell responses (125). Additionally, HIV-exposed sero-negative women have increased production of IL-12 from immature DCs than HIV-infected sero-positive women, suggesting higher induction of IL-12 from antigen presenting cells may contribute to HIV resistance and viral control (126). Recovery of IL-12 production from activated DCs from HIV-infected individuals on HAART has been correlated with CD4 T-cell recovery (127, 128). These studies demonstrate a pivotal role for IL-12 in the induction of HIV-specific immunity. However, in contrast, chronically SIV-infected rhesus macaques are not responsive to IL-12-mediated enhancements of cell-mediated immune responses (129), or decreases in viral load (124). This discrepancy between IL-12 therapy in acute and chronic SIV/HIV infection might be due to the progressive impairment of the immune system, and suggests there may be a threshold to the effectiveness of using cytokines as a therapy in HIV-infected individuals.

Two other IL-12 family cytokines, IL-23 and IL-27, have been poorly investigated in HIV infection. There is increasing interest in their potential value as therapeutics in HIV infection due to their roles in T-cell regulation. The following sections will review the biological properties of IL-23 and IL-27, the implications of these cytokines in HIV infection, and the signalling pathways regulating their expression.

Interleukin-23:

Biological properties of IL-23:

Oppmann discovered IL-23 in 2000, when it was observed that the IL-12 subunit p40 could form a covalent disulfide bond with a new subunit, p19 (130). The structure of IL-23 is shown in Figure 1. This new cytokine was demonstrated to have similar as well as distinct functions from IL-12 (130). IL-23 p19 has been shown to be expressed in human and murine Mos, DCs, and murine Th1 cells, with activated MΦs and DCs being the predominant producers of IL-23 (130). IL-23 production has been shown to be induced with TLR-2, TLR-3, TLR-4, TLR-7/8, TLR-9, and CD40 agonist stimulation (131-136). The IL-23 receptor is composed of IL-12RβI and IL-23R (Figure 1) (137). IL-23R does not bind IL-12, however both the IL-23 and IL-12 receptor signalling results in the activation of janus kinase (JAK)/ signal transducer and activator of transcription (STAT)s signalling molecules (137). IL-23 signalling has been shown to activate JAK-2, tyrosine kinase (TKY)-2, STAT-1, STAT-3, STAT-4 and STAT-5 (130, 137). IL-23-induced STAT-3 activation is essential for the maintenance of T-helper 17 (Th17) cells (138).

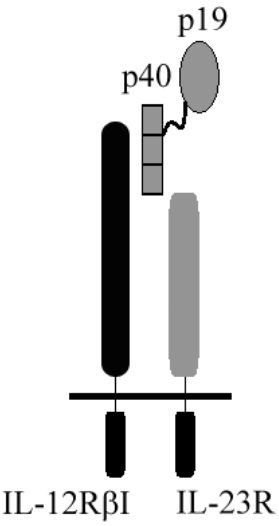


Figure 1: Structure of IL-23 and its receptor.

IL-23R subunit of the IL-23 receptor is expressed in human T-cells, NK cells, monocytes, and DCs, and activated murine MΦs (137). IL-23, like IL-12, induces the production of IFNγ from memory T-cells, but in contrast to IL-12, IL-23 has a distinct role in the induction of IL-17 production from Th17 and memory T-cells (139-141).

IL-23 is essential for the maintenance and functions of Th17 cells (142). However, the cytokines required for the commitment of naive CD4 T-cells to differentiate into Th17 cells is controversial. In mice, TGFβ and IL-6 are required for *in vivo* production of Th17 cells (143-147). In humans, TGFβ plus IL-6 or TGFβ plus IL-21 could not induce Th17 cell differentiation in some studies (148, 149), whereas others have shown TGFβ in combination with IL-6, IL-21, or IL-1β could induce Th17 cell differentiation (150, 151). Th17 cell differentiation has also been observed in the absence of TGFβ. For example, IL-1β plus IL-6, or IL-1β plus IL-23 has been shown to induce Th17 cell polarization (149, 152). The IL-23R subunit of the IL-23 receptor is not expressed on naive CD4 T-cells (137); however, TGFβ, IL-6, and IL-21 have been shown to induce IL-23R expression (138, 153, 154). Furthermore, IL-23 p19-deficient mice cannot produce Th17 cells *in vivo*, indicating IL-23 is critical for the development of Th17 cells (155).

Potential role for IL-23 in HIV infection:

IL-23-induced Th17 cell responses are important for the clearance of infection and maintaining mucosal integrity of the gut. IL-23-induced Th17 responses result in the induction of cytokines, and recruitment of neutrophils that are essential for the clearance of extracellular fungal and bacterial pathogens, including *Klebsiella pneumoniae, Staphylococcus aureus, Citrobacter rodentium, Candida albicans,* and *Pseudomonas*

aeroginosa (Reviewed by Yannam et al (156)). This is important in the context of HIV infection because infected individuals are more susceptible to opportunistic infections.

IL-23-induced IL-17 production has been shown to enhance the integrity of epithelial barriers through the induction of claudin-1 and -2, which increases tight-junction formation (157), and through the induction of mucin gene expression, which increases mucus secretion from epithelial cells (158). IL-17 has also been shown to induce antimicrobial peptide, β-defensins (159). Similarly, Th17-derived IL-17A, IL-17F, and IL-22 were able to induce the production of antimicrobial peptides, including β-defensin, from keratinocytes, in an additive and synergistic manner when co-expressed (160). These studies suggest that IL-23-induced Th17 responses may play an important role in maintaining the integrity of the intestine, through the induction of antimicrobial peptides and mucus secretion, and the maintenance of tight junctions. Therefore, IL-23 expression in HIV infection may be essential to maintain the integrity of the intestinal barrier, and suppress microbial translocation.

Moreover, IL-23 can enhance anti-viral immune responses. DC's expressing higher levels of IL-23 have been shown to have a greater propensity to prime CD8 T-cells with HIV-antigens (101). As well, IL-23 has been shown to induce IFNγ production and proliferation in activated and memory T-cells (130). Thus, IL-23 may play an important role in the induction of HIV-specific immune responses.

Microbial translocation and systemic immune activation has been observed in HIV infection, and persists in HIV patients on cART. Depletion of Th17 cells in the circulation and gastrointestinal tract of HIV-infected individuals (66, 161-165) and SIV-infected rhesus and pigtailed macaques has been observed (166-168). Interestingly, normal levels of Th17 frequencies or IL-17 expression have been observed in the gastrointestinal tract and blood of

HIV-infected long term non-progressors and elite-controllers, and in SIV-infected African green monkeys and sooty mangabeys, which do not develop AIDS (165, 167, 169-171). This suggests that restoration of Th17 cells in HIV-infected individuals may be correlated with improved disease outcome. It has been demonstrated that Th17 cell counts are positively correlated with CD4 T-cell counts (163, 165) and inversely proportional with microbial translocation (161) and plasma viremia in HIV infection (165, 168). Treatment with cART in HIV/SIV infection has been shown to increase Th17 cell frequencies (161, 163, 166, 172); however, responsiveness is variable (161). Ndhlovu has claimed that complete suppression of viremia is needed to restore IL-17 to normal levels in HIV infection (173). Thus, viral reservoirs and IL-23 expression may contribute to the variable recoveries of Th17 cells in the gut in HIV patients on therapy.

It remains unclear whether IL-23 production is affected by HIV, and whether IL-23 production influences HIV disease progression. To date, there are only two studies that have investigated the effects of HIV on IL-23 production. Lee and colleagues observed decreased basal levels of IL-23 mRNA expression in adherent cells from PBMCs of severely-immunodeficient HIV-infected patients on HAART who had achieved increased CD4 T-cell counts (174). The authors suggested that the decrease in IL-23 production may be associated with increased susceptibility of HIV-infected individuals to opportunistic infections (174). In contrast, Louis et al demonstrated that LPS/IFNγ-induced IL-23 production from Mos and DCs were increased in acutely-infected HIV patients (51). These authors also observed that IL-12 production was increased in Mos and DCs of these patients (51), which is consistent with other studies that have observed an increase in IL-12/23 p40 production in acute HIV infection (48-50).

Since IL-12 and IL-23 both share the p40 subunit, it is possible that IL-23 production is altered in a similar manner as IL-12 in HIV infection. For example, IL-23 production may also be increased in acute infection, and decreased in chronic infection. Enhanced production of IL-23 in acute HIV infection may act as a feedback response to the loss of Th17 cells in the gastrointestinal tract, and may enhance cell mediated immunity. However, decreased production of IL-23 in chronic HIV infection may inhibit Th17 differentiation and impair the mucosal integrity of the gut in HIV-infected individuals, resulting in increased systemic immune activation. Additionally, decreased production of IL-23 may increase the susceptibility of infected individuals to opportunistic infections. Thus, it is important to investigate how HIV infection influences IL-23 production, and this was the subject of my investigation.

Interleukin 27:

Biological properties of IL-27:

IL-27 was characterized in 2002 as a heterodimeric cytokine composed of Epstein Barr virus-induced gene-3 (EBI3) and p28, which shares homology with the IL-12 subunits p40 and p35, respectively (94). The structure of IL-27 is shown in Figure 2. IL-27 is produced predominantly by activated Mos, MΦs, and DCs (94). Stimulation with TLR-3, TLR-4, TLR-7/8, TLR-9, CD40, IL-1βR, and IFNR agonists have all been shown to induce IL-27 secretion (94, 175-179). The IL-27 receptor (IL-27R) is composed of IL-27Rα/WSX-1/T-cell cytokine receptor (TCCR) and glycoprotein-130 (gp130) (Figure 2) (94, 180). Gp-130 is part of the IL-6 receptor family, and is shared among other cytokines receptors including IL-6, IL-11, LIF, OM, CT-1, and CNFT (181). WSX-1 is structurally similar to

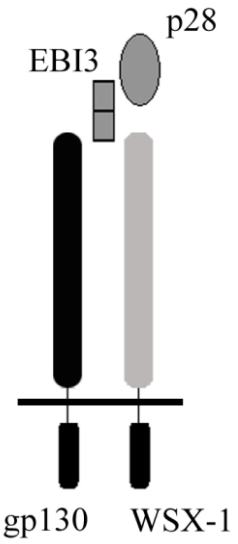


Figure 2: Structure of IL-27 and its receptor

gp130, and was first identified through a homology search of gp130 cDNA (182). The IL-27R is expressed on myeloid and lymphoid lineages of hemopoietic cells, stem cells, keratinocytes, and vascular endothelium (183-186). IL-27 signalling results in JAK /STAT activation. Specifically, IL-27 has been shown to activate JAK-1, JAK-2, TYK-2, STAT-1, STAT-2, STAT-3, and STAT-5, and to a lesser extent STAT-4 (187-189).

STAT-1 activation plays an indispensible role in IL-27 signalling (187, 188, 190). IL-27 induced T-bet activation and up-regulation of IL-12Rβ2 are suppressed in STAT-1-deficient mice (188). As a result, STAT-1-deficient mice have impaired synergistic enhancement of IFNγ in response to IL-27 and IL-12 stimulation, independent of IL-27-induced CD4 T-cell proliferation (188). Additionally, STAT-1 activation is critical for IL-27-mediated down-regulation of GATA binding protein-3 (GATA-3) (187). GATA-3 is a transcription factor important in Th2 cell differentiation (191), and can inhibit Th1 cell development (192, 193). Thus, IL-27-induced inhibition of GATA-3 further enhances Th1 cell differentiation. IL-27 has also been shown to inhibit Th17 cell differentiation through

STAT-1- and T-bet-dependent mechanisms (190). Overall, IL-27 enhances Th1 polarization at the expense of Th2 and Th17 cells.

IL-27 can induce proinflammatory and anti-inflammatory functions through the regulation of T-cell responses. When IL-27 was first discovered, it was shown to synergize with IL-12 to enhance Th1 polarization, and IFNγ production, while having no effect on the production of Th2 cytokines (94). IL-27 was also shown to synergize with IL-2 or IL-12 to enhance IFNγ secretion from NK cells (94). Furthermore, IL-27 regulates other T-cells subtypes as well. For example, IL-27 can inhibit T-regulatory (Treg) cells (194), and Th17 cells (195, 196), and induce CD8 T-cell (99, 106), type-1 T-regulatory (Tr1) cells (197), and functional T-follicular helper cells (198). Thus, IL-27 may be useful as a therapeutic to manipulate T-cell function in HIV infection.

Potential role for IL-27 in HIV infection:

Cell-mediated immune responses are severely impaired in HIV infection. The production of Th1 cytokines, IL-12 and IFNγ, are decreased in chronic HIV infection (62-65, 67, 76, 77, 118), whereas the production of Th2 cytokines, IL-4 and IL-10, are increased in chronic HIV infection (74-76, 109). HIV exposed sero-negative individuals are able to generate robust Th1 responses (109), suggesting effective Th1 responses are pivotal to effective HIV immunity. IL-27's ability to inhibit Th2 responses, and promote Th1 responses may be useful to enhance anti-HIV immune function. IL-27 has also been shown to induce proliferation, IFNγ secretion, and granzyme-B production in murine and human naive CD8 T cells (99, 106). DCs expressing higher levels of IL-27 are more efficient in cross-presenting HIV-antigen to CD8 T-cells and can induce IFNγ and granzyme-B production from these

primed cells (101). Thus, IL-27 may have the potential to enhance HIV-specific anti-viral responses and cell-mediated immunity. Additionally, since IL-27 shares similar functions to IL-12 and is capable of inducing IL-12, it may also be effective in enhancing HIV-specific DNA vaccination, and lowering viral set-points in HIV-infected individuals.

T-regulatory (Treg) cells play an important role in maintaining intestinal homeostasis by regulating T-cell effector functions and maintaining immune tolerance at the mucosal barrier (199). HIV-infected patients have increased intestinal permeability, and microbial translocation from the gut, which is one of the main causes of increased systemic immune activation in HIV infection (200). Shaw et al recently demonstrated that the frequencies of Treg cells in HIV infection are positively correlated with increased immune activation in the rectal mucosa of patients (201). Higher immune activation and disease severity has also been correlated with increased relative frequencies of Treg cells in the blood of HIV-infected patients (162, 202). Although the exact role of Treg cells in the course HIV disease progression remains poorly understood, IL-27 therapy may be beneficial in decreasing the relative frequency of Treg cells in HIV infection. The inhibition of Treg cells in the gut may help restore the Treg/Th17 cell balance, improving the mucosal integrity of the gut, and decreasing systemic immune activation.

Fakruddin et al discovered the ability of IL-27 to inhibit HIV replication in CD4 T-cells, MΦs, and PBMCs (203). It was later observed that IL-27 inhibited HIV-replication indirectly, through the induction of IFNα in CD4 T-cells, and IFNα and IFNβ in PBMCs, Mos, and MΦs (204). Activation of type-1 IFNα and IFNβ resulted in increased expression of apolipoprotein B mRNA editing, enzyme-catalytic, polypeptide-like (APOBEC)-3A and APOBEC3G, respectively (204). IL-27's ability to inhibit HIV replication was specific,

demonstrated by the fact that other IL-12 family cytokines, IL-12 and IL-23, did not inhibit HIV replication (204). Additionally, IL-27's inhibition of HIV was independent of viral entry since CD4, CXCR4, and CCR5 expression was not affected by IL-27 administration (204). These findings suggest that IL-27 may be a promising candidate to inhibit HIV replication.

Overall, IL-27 is a potential candidate to suppress HIV replication, enhance cell-mediated immunity, and inhibit Treg cells. However, it is not clear whether HIV influences the production of IL-27. To date, there is only one study that has investigated the levels of IL-27 production in HIV infection (68). Guzzo et al observed a trend for decreased IL-27 production in the serum of HIV-infected individuals (68). However, one limitation of their study is that serum levels of cytokines are not representative of the cytokine levels observed in tissues, or induced by TLR-stimulation during an infection. In order to overcome this limitation, I sought to investigate whether *in vitro* HIV-infection could impair IL-27 production in LPS-stimulated MΦs.

TLR signalling:

The TLR super-family is an important group of receptors responsible for mediating innate and adaptive immunity. In humans the TLR super-family includes 10 TLRs (TLR-1 to -10) and the IL-1 receptor (IL-1R) (205). TLRs and the IL-1R are type-1 integral membrane glycoproteins (205), sharing significant homology in their cytoplasmic tails (206) and contain distinct extracellular/endosomal domains recognizing specific, conserved, microbial ligands (207). The cytoplasmic tail of TLRs contains conserved TLR/IL-1R homology (TIR) domains (206), which interact with TIR domains on adaptor proteins (208). To date, five different adapter molecules containing TIR domains have been identified, namely myeloid

differentiation primary response protein 88 (MyD88), TIR domain containing adapter protein (TIRAP), TIR domain containing adapter protein inducing IFNβ (TRIF), TRIF-related adapter molecule (TRAM), and B-cell adapter for phosphoinositide-3-kinase (PI3K) (BCAP) (Figure 3) (209, 210).

TIRAP interacts with the plasma membrane through its phosphatidylinositol 4,5-bisphosphate (PIP_2) domain (Figure 3) (211). When the TLR is activated, TIRAP interacts with MyD88 delivering it to the TLR, facilitating signal transduction (211). Similarly, TRAM is necessary for TLR4-induced TRIF-dependent signalling (Figure 3); however, TLR3-induced TRIF-dependent signalling occurs independently of TRAM (212). BCAP is one of the most recently discovered TIR-containing adapter molecules (210). Troutman et al have shown that MyD88 is required for BCAP to interact with the TLR, and is essential for mediating the activation of the PI3K/ protein kinase B (AKT) pathway (Figure 3) (210).

Most TLR-induced immune responses have been linked to either MyD88 and/or TRIF-dependent signalling pathways. The activation of the MyD88 and TRIF pathways leads to the recruitment and activation of various adaptor proteins including IL-1R associated kinase (IRAK) and TNF-associated factor (TRAF) signalling molecules which leads to the activation of TGFβ activated kinase 1 (TAK-1) and other end-points (Reviewed extensively (205, 209, 213-215)). Although these signalling pathways discussed above are common to the activation of most TLR-induced responses, downstream in the signalling cascade, the signalling pathways diverge and begin to activate more specific genes. The PI3K, protein kinase C (PKC), and mitogen activated protein kinase (MAPK) pathways are some of the most studied pathways involved in regulating specific immune responses. Understanding the

Figure 3: TLR-signalling and cross-talk between pathways tightly regulating immune responses and cytokine production.

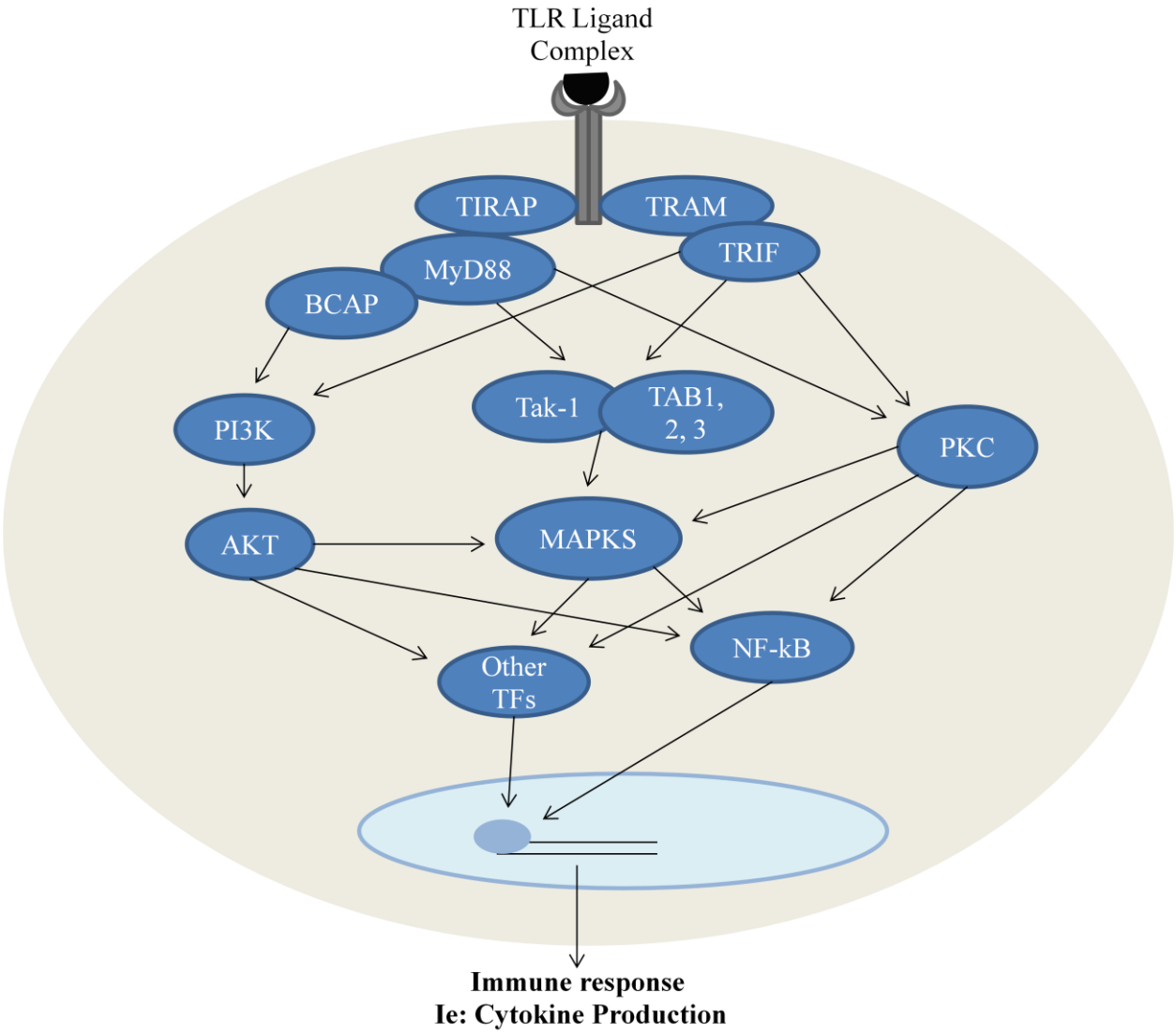

TLR Ligand
Complex
TIRAP
TRAM
TRIF
MyD88
BCAP
PI3K
Tak-1
TAB1,
2, 3
PKC
AKT
MAPKS
NF-kB
Other
TFs
Immune response
Ie: Cytokine Production

FIGURE 3

role of these signalling molecules in regulating specific gene expression and cytokine production will be important in developing strategies to restore immune function impaired in disease.

The PI3K pathway:

PI3K is a heterodimeric signalling molecule composed of the p85-regulatory and the p110-catalytic subunits (216). AKT is a downstream target of PI3K signalling (217), and its activity is often measure to indirectly determine the activity of PI3K. Merely a few months ago, a new TIR-containing adapter protein BCAP, was found to mediate PI3K activation in a MyD88-dependent fashion (Figure 3) (210). This is in agreement with another study that has shown MyD88 to be required for the activation of the PI3K pathway downstream of TLR-5 stimulation in colonic epithelial cells (218). However, the authors did not investigate whether BCAP could also interact with TRIF, and mediate PI3K activation. Other studies have shown that PI3K can also interact with TRIF (219). It is possible that BCAP may also link the TRIF-dependent pathway to PI3K activation. Overall, the exact mechanism of PI3K activation downstream of TLR-signalling is not clearly defined; however, both MyD88- and TRIF-dependent mechanisms are involved.

The PKC pathway:

There are three classes of PKC isoforms, namely the classical PKCs (cPKCs) including PKC α, βI, βII, and γ, the novel PKCs (nPKCs) including PKC ε, ϕ,δ, η/L, and the atypical PKCs (aPKCs) including PKC ζ and ι/λ. Activation of all three classes of PKCs require adenosine triphosphate (ATP), release of the pseudosubstrate from the catalytic

domain, translocation of the PKC to a membrane, interaction with phosphatidylserine, and phosphorylation of the hydrophobic motif, activation loop, and turn motif (220). The three classes of PKC isoforms are classified based on additional co-factors required for their activation. cPKCs also need to bind Ca^{+2} and phorbol esters, such as diacylglyceride for activation, whereas nPKCs require phorbol esters but not Ca^{+2} for activation, and aPKCs do not require additional cofactors (220).

Although the exact mechanism by which TLR-stimulation leads to the activation of PKCs is not defined, it appears that both the MyD88- and TRIF-dependent pathways can play a role (Figure 3). Specific PKC isoforms have been shown to activate specific immune responses downstream of TLR-signalling. For example, Johnson and colleagues demonstrated that PKCα but not PKCβ was involved in the positive regulation of IRF-3 downstream of TRIF-dependent TLR-3 signalling (221). PKCα has also been shown to physically interact with the TLR-2/MyD88 complex in murine bone-marrow-derived DCs (222). Similarly, PKCε has been shown to be activated through MyD88-dependent mechanisms (223).

The MAPK pathway:

The activation the p38, c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinases (ERK) MAPK pathways downstream of TLR-signalling also occurs through MyD88 and TRIF-dependent mechanisms. MyD88 and TRIF-dependent activation of TAK-1 leads to the recruitment of TAK-binding protein 1 (TAB1), TAB2 and TAB3 (224). The TAK/TAB complex can then activates NF-κB and the p38, JNK, and ERK MAPK signalling pathways, which are essential for cytokine production (Figure 3) (225).

The activation of TAK-1 leads to the activation of mitogen activated protein kinase kinases (MKK) (225). The p38 MAPK family consists of at least four isoforms, namely α, β, δ, and γ (226). Both Mos and MΦs predominantly express p38α and do not express detectable levels of p38β or p38γ (227). Mos and MΦs also express p38δ; however, monocytes have lower expression of this isoforms (227). The p38 MAPK pathway is activated through MKK-3, MKK-4, and MKK-7 (228).

JNK MAPK on the other hand has three isoforms, namely JNK-1, JNK-2, and JNK-3 (229). JNK-1 and JNK-2 are constitutively expressed, whereas JNK-3 is expressed predominantly in neuronal cells (229). JNK MAPKs are activated through MKK-4 and MKK-7 (228).

Two isoforms of ERK have been identified namely ERK-1 and ERK-2, also called p44/42 MAPKs (228). ERK-1/2 are activated downstream of MKK-1 and MKK-2, also known as extracellular signal-regulated kinase kinase (MEK)-1 and MEK-2, respectively (228).

The activation of the p38, JNK, and ERK MAPKs requires the dual phosphorylation of the Threonine-X-Tyrosine activation motifs (where X represents distinct amino acids for each MAPK) (230).

Cross-talk between signalling pathways:

Although the regulation of MAPKs and NF-κB have been clearly defined to occur downstream of the activation of the TAK-1/TAB complex, the PI3K and PKC pathways have also been implicated in the activation of MAPKs and NF-κB (Figure 3).

PI3K/AKT pathway has been implicated in the negative regulation of NF-κB activation in LPS-stimulated THP-1 cells (231), while others have demonstrated a positive role for PI3K in the regulation of NF-κB in Hep2G cells, 3T3 fibroblasts, and human Mos (232-234). PI3K/AKT knockdown has also been shown to inhibit LPS-induced JNK activation without affecting ERK or p38 MAPK activation in MDMs and DCs (235). In contrast, the constitutive activation of AKT has been shown to positively regulate NF-κB downstream of p38 MAPK activation (233).

PKCα/β have been implicated in the positive regulation of p38, JNK, and ERK MAPK and NF-κB activation downstream of LPS-stimulation in neutrophils (236). JNK and p38 MAPK, and NF-κB have also been demonstrated to be regulated by other PKCs specific isoforms downstream of TLR-signalling (222, 237-240).

Additionally, TLR-stimulation also induces inhibitory signals. For example, Shan et al demonstrated that the p38 MAPK pathway could negatively regulate ERK MAPK/NF-κB-dependent increases in pro-asthmatic symptoms, downstream of TLR-4 signalling (241).

These studies demonstrate that there is a significant amount of cross-talk between the PI3K, PKC, MAPK, and NF-κB pathways. Figure 3 depicts a general model for TLR signal transduction. However, there are likely several more intermediates involved in these signalling pathways. Notably, it is possible that the PI3K and PKC pathways may regulate the TAK1/TAB complex or the MKKs prior to the activation/inhibition of specific MAPKs.

The activation of PI3K, PKC, and MAPKs pathways downstream of TLR-signalling can differentially regulate various genes, likely contributing to the tight regulation of immune responses. These signalling molecules have been implicated in the signalling of

TLR-induced IL-23 and IL-27 which will be discussed below. These signalling molecules likely play an important role in HIV immune dysfunction.

Signalling pathways regulating TLR-induced IL-23 production:

The signalling pathways regulating IL-23 production have predominantly been investigated downstream of lipopolysaccharide (LPS)/TLR-4 activation in DCs, MΦs, or Mos. Since IL-12 was discovered before IL-23, the regulation of IL-12/23 p40 has been studied more extensively than the regulation of IL-23 p19 and IL-23.

The ERK MAPK pathway has been shown to negatively regulate IL-12/23 p40 expression post-stimulation with LPS in murine MΦs (242-244) and human MΦs (245) and post-stimulation with *Mycobacterium tuberculosis* in MDMs (246). In contrast, others have demonstrated ERK to positively regulate, or have no effect on LPS-induced IL-12/23 p40 expression (247-249).

The p38 MAPK pathway has also been extensively studied in the regulation of IL-12/23 p40 expression. Various groups have demonstrated a role for p38 MAPK in the positive regulation of p40 in response to LPS in murine MΦs (247, 249), THP-1 cells (250), primary human Mos (250, 251), PBMCs (251), human myeloid DCs (248), and human MΦs (245). Additionally, the p38 MAPK pathway has been shown to positively regulate p40 post-stimulation with CpG in murine MΦs (252), human DCs (253), and MDMs (254), post-stimulation with serum amyloid A in THP-1 cells and pMos (255), and post-stimulation with *M. tuberculosis* in MDMs (256). In contrast, a negative role for p38 MAPK in the regulation of IL-12/23 p40 expression in human MΦs post-stimulation with various TLR agonists (LPS, CpG, flagella, and lipid A) has also been observed (257).

The regulation of IL-12/23 p40 expression by the JNK and PI3K pathways has been more controversial. For example, IL-12/23 p40 expression has been shown to be positively and negatively regulated by the JNK pathway in LPS-stimulated human Mos (250, 258). Utsugi and colleagues demonstrated that JNK-1 and JNK-2 differentially regulate IL-12/23 p40 expression in THP-1 MΦs (259). Specifically, JNK-1 was shown to positively regulate IL-12/23 p40 expression, whereas JNK-2 was shown to negatively regulate IL-12/23 p40 expression in response to LPS-stimulation (259). These discrepancies may be attributed to the different model systems and activation protocols used in these studies.

The regulation of IL-12/23 p40 by the PI3K pathway seems to be highly dependent on the cell type and stimulus used for induction. For example, in human MΦs, the PI3K pathway negatively regulates IL-12/23 p40 production in response to *M. tuberculosis* (predominantly a TLR-2 agonist) stimulation (246) but positively regulates IL-12/23 p40 production in response to LPS (TLR-4 agonist) stimulation (259). Similarly, in human primary Mos, PI3K positively regulates IL-12/23 p40 production post-stimulation with *Francisella tularensis* (TLR-2 agonist) (260), and negatively regulates IL-12/23 p40 production post-stimulation with CpG (TLR-9 agonist) (261).

PKCs have also been investigated in the regulation of IL-12/23 p40 secretion. PKCα has been shown to positively regulate p40 post-stimulation with TLR-2, TLR-3, and TLR-4 agonists in murine bone-marrow-derived DCs (222) and PKCδ has been implicated in the positive regulation of p40 in murine MΦ and DCs post-stimulation with LPS and LPS/IFNγ (262). In human DCs, the cPKCs have been shown to positively regulate p40 production in response to flagellin, IL-1β, and FSL-1(222), but not in response to Poly (I:C) or LPS (222, 263).

Similar to p40 expression, IL-23 p19 expression has been shown to be positively regulated by the p38 MAPK pathway in MDMs (256), and human microglial cells (264) in response to *M. tuberculosis* and LPS, respectively. The p38 MAPK pathway has also been implicated in the positive regulation of IL-23 secretion in human Mos (132), MΦs (256) and myeloid DCs (133, 265).

The JNK and ERK MAPK pathways were also shown to positively regulate p19 production in murine MΦs stimulated with Theiler's virus (131), and IL-23 production in TLR-3/4-stimulated human myeloid DCs (133). In contrast, Jackson demonstrated ERK and JNK MAPK inhibition to have no effect on IFNγ/LPS-induced IL-23 production from human myeloid DCs (265). Similarly, La Sala demonstrated JNK MAPK, but not ERK MAPK to positively regulated IL-23 production in human Mo stimulated with C5a, a G_i-protein-coupled receptor agonist (266). These discrepancies may be explained by the differential regulation of p40 and p19 expression by the JNK and ERK pathways.

The PI3K pathway has also been shown to positively regulate *F. tularensis*-induced p19 and C5a-induced IL-23 production in primary Mo (260, 266).

There is only one study to my knowledge that has investigated the role of PKCs in the regulation of IL-23 production. Johnson et al demonstrated that inhibition of cPKCs in human DCs did not affect p19 mRNA expression or IL-23 secretion in response to LPS- or Poly(I:C)-stimulation (263). Thus, the PKC pathway has not been implicated in the regulation of IL-23.

Overall there is a strong consensus that the p38 MAPK pathway positively regulates p40 and p19 mRNA expression, and IL-23 secretion in TLR-stimulated myeloid cells. The PI3K pathway was also shown to positively regulate p19 expression and IL-23 secretion

(260, 266). The ERK pathway has been shown to negatively and positively regulate p40 expression (242-248), and positively regulate p19 expression (131), leading to discrepancies in whether ERK regulates IL-23 production (133, 265, 266). Similarly, the p40 subunit is differentially regulated by JNK-1 and JNK-2 (259), whereas, JNK has been implicated in the positive regulation of p19 (131). Thus, the effect of inhibiting JNK on the production of IL-23 remains controversial (133, 265, 266). However, in contrast to the regulation of p40, the ERK and JNK MAPK and PI3K pathways have yet to be shown to negatively regulate p19 or IL-23 production, suggesting p19 may play a more dominant role over p40 in the regulation of IL-23 production. However, further studies investigating the production of p40, p19 and IL-23 production simultaneously are needed.

Signalling pathways regulating TLR-induced IL-27 production:

There is very limited knowledge of the signalling pathways regulating IL-27 production. Zhang et al has demonstrated that p38 MAPK activation inhibits activator protein (AP)-1/c-Fos binding to the p28 promoter, resulting in reduced IL-27 production in response to *M. tuberculosis* in MΦs (267). IL-27 p28 has also been shown to be regulated by TRIF-dependent activation of IRF-3 (268), and through MyD88- and NF-κB-dependent mechanisms (269). IL-27 EBI3 has been shown to be activated through MyD88-dependent p50-p65 NF-κB activation (270). In astrocytes, LPS-induced IL-27 has been shown to be positively regulated by p38 MAPK and NF-κB pathways and not ERK pathway (271). Similarly in myeloid DCs, IFNγ/LPS-induced IL-27 secretion was shown to be positively regulated by the p38 and JNK MAPK pathways but not the ERK MAPK or PI3K pathways (265). There is only one study to date that has investigated the role of PKCs in regulating IL-

27 production. Johnson et al demonstrated that inhibition of the cPKCs impairs IRF-3 binding to the p28 promoter, and that PKCα knock-out mice have impaired IL-27 production in bone-marrow-derived DCs (263).

Overall, there have been few comparable studies that can give a general consensus of the pathways regulating IL-27 production. The p38 MAPK pathway has been positively and negatively implicated in the regulation of IL-27 production (265, 267), whereas the ERK pathway has been shown to have no effect on IL-27 production in two studies (265, 271). Additionally, the JNK and PKCα pathways have also been implicated in the positive regulation of IL-27 (263, 265). Further studies are needed to confirm whether the regulation of IL-27 is conserved between different myeloid cells, and in response to different TLR-stimulants.

Rationale:

The IL-12 family of cytokines are important cytokines linking innate and adaptive immunity. IL-12 production is inhibited in chronic HIV infection, and IL-12 administration in acute SIV infected rhesus macaques has been associated with improved disease outcome. IL-23 and IL-27 are also members of the IL-12 family of cytokines and are structurally similar to IL-12. IL-23 and IL-27 have important roles regulating T-cell responses. Additionally, IL-27 has been shown to inhibit HIV replication. IL-27 and IL-23 may be useful as a therapy to improve T-cell regulation, and decrease chronic immune activation in HIV infection. However, the effect of HIV infection on the production of IL-23 and IL-27 is not known. Furthermore, the signalling pathways regulating the production of IL-23 and IL-27 in human monocytes and macrophages remains poorly understood. IL-23 and IL-27

production can be induced by LPS in Mos and MΦs. LPS-stimulation results in the activation of various signalling pathways including the MAPKs, PI3K, PKC pathways. Therefore, it was important to determine if *in vitro* HIV infection of MDMs influences LPS-induced production IL-23 and IL-27 and the signalling pathways by which HIV may influence the production of these cytokines.

Hypothesis:

HIV inhibits LPS-induced IL-23 and IL-27 production in human MDMs by suppressing the activation of signalling pathways regulating their expression.

Objectives:

1. Determine whether HIV influences LPS-induced or basal levels of IL-23/IL-27 production in human MDMs
2. Determine the signalling pathways regulating LPS-induced IL-23 and IL-27 production in human MDMs
3. Determine whether HIV inhibits the activation of the signalling pathways regulating LPS-induced IL-27 production
4. Determine whether cPKCs or PKCε regulate LPS-induced IL-23 or IL-27 production in THP-1 cells, primary Mos, or MDMs

CHAPTER II: MATERIALS AND METHODS

Cell culture and reagents:

All cells were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin, and 100 μg/mL streptomycin unless otherwise stated (Gibco/Invitrogen, Grand Island, NY). THP-1 promonocytic cells were obtained from American Type Culture Collection (Manassa, VA). THP-1 cells stably transfected with CD14 cDNA sequences (CD14-THP-1 cells) were obtained from Dr. Richard Ulevitch (The Scripps Research Institute, La Jolla, CA) (272). Blood was obtained from healthy donors using a protocol approved by The Ottawa Hospital, Research Ethics Board. Written informed consent was obtained from all donors. PBMCs were isolated from whole blood using a density gradient over Ficoll-Hypaque (Pharmacia Biotech, Piscataway, NJ). Primary Mos were isolated from PBMCs using autoMACS negative selection (Miltenyi Biotec, Auburn, CA) as per the manufacturer's instruction. In order to produce MDMs, 5 million PBMCs were plated in 12-well dishes and were allowed to adhere at 37^{o}C for 3 h in serum-free media. Non-adherent cells were washed off with serum free-media, and adherent Mos were differentiated into macrophages by culturing the cells in complete media supplemented with 1 μg/mL macrophage-colony-stimulating factor (M-CSF) (R&D systems, Minneapolis, MN) for 7 days.

Pharmacological inhibitors were used to specifically inhibit the p38 MAPK (SB203580), JNK MAPK (SP600125), ERK MAPK (PD98059), PI3K (LY294002), cPKC/PKCε (PKCβ) and PKCε (PKCε) pathways (Calbiochem, San Diego, CA). LY294002 is a competitive ATP inhibitor of PI3K, having no effect on the activity of MAPKs, and

PKCs (273). SB203580 is a pyridinyl imidazole, competitive ATP inhibitor, used to specifically inhibit p38 MAPK activity and does not affect the activity of other kinases including ERK and JNK MAPK (274, 275). SP600125 is a reversible ATP inhibitor which is 300 fold more selective for JNK MAPK than p38, and ERK MAPK (276). PD98059 is a reversible, cell permeable inhibitor for MKK-1/MEK-1 and MKK-2/MEK-2 which results in the inhibition of ERK MAPK activation (277-279). The PKCβ inhibitor was used to inhibit all of the cPKCs (βI IC_{50} = 21 nM, βII IC_{50} = 5 nM, α IC_{50} =331 nM and γ IC_{50} >1 μM) and PKCε (IC_{50} = 2.8 μM) (Calbiochem, San Diego, CA). The PKCε inhibitor is a translocation inhibitor specific for PKCε (Calbiochem, San Diego, CA).

MDMs, primary Mos, or THP-1 cells were treated with inhibitor for 2 h, at the indicated concentrations, prior to induction of IL-27 and IL-23 production with 1 μg/mL LPS (Sigma-Aldrich, St. Louis, MO). Expression of IL-27 EBI3, and IL-27 p28 and IL-12/23 p40 and IL-23 p19 mRNA was evaluated by quantitative real time polymerase chain reaction (qRT-PCR) 4 h post-stimulation with 1 μg/mL of LPS as described below. IL-27, IL-23, and IL-12/23 p40 secretion was evaluated by enzyme linked immunosorbant assay (ELISA) and IL-27 EBI3 protein expression was evaluated by western blot 24 h post-stimulation with LPS as detailed below.

Cell viability was assessed by propidium iodide (Sigma-Aldrich, St. Louis, MO) staining and flow cytometry after 2 h of incubation with the inhibitors, and 24 h after LPS-stimulation, as previously described (280). Treatment of cells with LY294002, SB203580, SP600125, PD98059 and PKCε, inhibitors at the maximum dose of 50 μM had no effect on cell viability (data not shown). Similarly, treatment of cells with the PKCβ inhibitor at the maximum dose of 10 μM had no effect on cell viability (data not shown).

Flow cytometry:

MDMs were characterized by measuring various surface receptors by flow cytometry. Briefly, MDMs were stained for 15 min in the dark with phycoerythrin (PE)-conjugated antibodies recognizing CD11a, CD11b, CD11c, CD80, CD83, CD86, CD14, CD16, or HLA-DR (BD Pharmingen, Franklin Lakes, NJ). Cells were washed and surface expression was analyzed by measuring fluorescence on a FACSCanto flow cytometer and using FACSDiva software (BD Biosciences, Franklin Lakes, NJ). Surface fluorescence was normalized to unstained cells and percent fluorescence was obtained using FACSDiva software. Histograms were created using WinMDI version 2.8 software (J. Trotter, Scripps Institute, San Diego, CA).

HIV stocks, infection, and p24 ELISA:

Dual tropic HIV clinical isolate, CS204, was a gift from Dr. F. Diaz-Mitoma (Children's Hospital of Eastern Ontario, Ottawa, Canada) (281). HIV_{CS204} stocks were grown in CD14 THP-1 cells. Mock viral stocks were prepared from supernatants from uninfected CD14 THP-1 cells.

The concentration of HIV present in cell-free viral stocks was assessed indirectly by measuring p24 protein concentration by ELISA. Aliquots of the mock and viral stocks were inactivated for 2 h at 37^{o}C, in 1% Triton X-100 (Sigma-Aldrich, St. Louis, MO), and concentration was assessed by ELISA using the HIV-1 $p24^{CA}$ antigen capture assay kit, following the manufacturer's instructions (AIDS & Cancer Virus Program, National Cancer Institute, Fredrick, MD).

MDMs were infected with viral stocks of dual tropic HIV_{CS204} supernatants containing 30,000 pg of HIV-p24 protein, supplemented with 8 μg/mL of polybrene (Sigma-Aldrich, St. Louis, MO) for 2 h. Mock-treated MDMs were treated in parallel with the HIV-infected MDMs, using mock viral stocks collected on the same day as the viral stock, and using the same volume. Media was changed and collected for p24 ELISA every 3 to 4 days. Following infection, MDMs were thoroughly washed with PBS and cultured for up to 15 days in complete media. Productive infection of MDMs was confirmed by p24 ELISA, as described above.

RNA extraction and qRT-PCR:

MDMs, primary Mos, or THP-1 cells were untreated or stimulated with 1 μg/mL LPS for 4 h. Total RNA was extracted from cell pellets using the RNeasy Plus Mini Kits (Qiagen, Germantown, MD). Total RNA was reverse transcribed into cDNA using the high capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA). cDNA was analyzed by qRT-PCR on the 7500 RT-PCR System (Applied Biosystems, Carlsbad, CA), using the TaqMan Universal Master Mix, and primers specific for β-actin (ACTB Hs99999903_mL), IL-27 EBI3 (EBI3 HS00194957_mL), IL-27 p28 (IL-27A Hs00377366_mL), IL-12/23 p40 (IL-12B Hs00233688_mL) and IL-23 p19 (IL-23A Hs00372324_mL) (Applied Biosystems, Carlsbad, CA). As per the manufacturer's instructions, qRT-PCR parameters were set as follows: Step 1 (1 cycle): 2 min at 50^{o}C, Step 2 (1 cycle): 10 min at 95^{o}C, and Step 3 (40 cycles): 15 sec at 95^{o}C, and 1 min at 60^{o}C (Applied Biosystems, Carlsbad, CA). The 7500 SDS software (Applied Biosystems, Carlsbad, CA) was used to calculate the cycle threshold (Ct). IL-27 p28, and EBI3 and IL-12/23 p40 and IL-23 p19 gene expression was normalized

to β-actin gene expression, and the dCt method was used to calculate fold change relative to uninfected and unstimulated cells.

IL-12/23 p40, IL-23, and IL-27 ELISA:

MDMs, primary Mos or THP-1 cells were stimulated with 1 μg/mL of LPS for 24 h followed by the assessment of IL-27, IL-23, and IL-12/23 p40 secretion by ELISA. Supernatants from uninfected cells were added directly to coated ELISA plates, whereas mock-treated or HIV-infected MDMs were pre-incubated for 2 h at 37^{o}C in a final concentration of 1% Triton X-100 to inactivate HIV. All ELISAs were done in Costar high binding 96 well ELISA plates (Corning Incorporated, Corning, NY), and washed with 0.05% Tween 20 in phosphate buffered saline (PBS) between steps. All ELISAs were developed using 3,3',5,5'-tetramethylbenzibidine (TMB) one component horse radish peroxidase (HRP) microwell substrate solution and 450 nm liquid stop solution for TMB microwell substrates (BioFX Laboratories, Owings Mills, MD).

IL-12/23 p40 protein secretion was measured by ELISA using two different monoclonal antibodies recognizing distinct epitopes. ELISA plates were coated overnight at 4^{o}C, with 4 μg/mL of mouse monoclonal anti-human IL-12 p40 antibody (Invitrogen, Burlington ON, CA), in ELISA coating buffer (eBioscience, San Diego, CA). Plates were washed as described above and blocked for 2 h with 10% FCS in PBS. Culture supernatants were added and incubated overnight at 4^{o}C. Recombinant human IL-12 p40 was used to prepare standards (R&D Systems, Minneapolis, MN). Plates were incubated for 2 h with biotinylated secondary mouse monoclonal anti-human IL-12 p40 antibody (Biosource, Camarillo, CA), at a final concentration of 200 ng/mL in 10% FCS in PBS, in order to detect

IL-12 p40. Plates were incubated for 30 min with streptavidin peroxisdase (Jackson ImmunoResearch, West Grove, PA), diluted 1 in1000 in 10% FCS in PBS, to detect the secondary antibody. Plates were developed as described above.

IL-23 was quantified by ELISA using commercial reagents from the Ready-Set-Go IL-23 ELISA kit (eBiosciences, San Diego, CA). Incubation times, temperatures, and diluents were the same as described for the IL-12/23 p40 ELISA. Briefly, ELISA plates were coated overnight with 2 μg/mL monoclonal anti-human IL-23 p19 antibody diluted in ELISA coating buffer (eBioscience, San Diego, CA). Samples and standards were added. Standards were prepared with recombinant IL-23 (p19/p40) from the Ready-Set-Go IL-23 ELISA kit (eBiosciences, San Diego, CA). IL-23 was detected with 2 μg/mL biotinylated anti-human IL-12 p40/70 antibody (eBiosciences, San Diego, CA). Plates were incubated for 30 min with streptavidin peroxisdase (Jackson ImmunoResearch, West Grove, PA), and developed as described for the IL-12/23 p40 ELISA.

IL-27 production was measured by the IL-27 ELISA (R&D systems, Minneapolis, MN) as per the manufacturer's instruction. Briefly, ELISA plates were coated overnight at room temperature with 0.4 μg/mL of capture antibody diluted in PBS. Plates were blocked with 1% bovine serum albumin (BSA) for 2 h. Subsequently, standards and samples were incubated in plates overnight at room temperature. Recombinant human IL-27 provided in the kit was used as a standard. Plates were incubated for 2 h at room temperature with 200 ng/mL biotinylated detection antibody diluted in 1% BSA in PBS followed by incubation for 20 min with streptavidin-HRP diluted 1 in 200 in 1% BSA in PBS. Plates were developed as described above.

Immunoblotting:

Lysed cells were subjected to 10% denaturing SDS PAGE followed by transfer to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). Blots were probed with antibodies specific for phospho-Akt (1/500), Akt-1 (1/500), phospho-p38 MAPK (1/1000), p38 MAPK (1/1000), (Cell Signalling Technology, Danvers, MA), phospho-ERK (1/500), ERK-2 (1/500), phospho-JNK (1/250), JNK-1 (1/500), PKCα (1/1000), PKCγ (1/1000), PKCβII (1/1000) (Santa Cruz Biotechnology, Santa Cruz, CA) IL-27 (1/500) (R&D systems, Minneapolis, MN), and/or GAPDH (1/10000) (Sigma-Aldrich, St. Louis, MO). All primary antibodies were diluted with 2.5% BSA in TBST. Donkey anti-goat IgG-HRP (Santa Cruz Biotechnologies, Santa Cruz, CA), Goat anti-rabbit IgG HRP conjugate, and goat anti-mouse IgG HRP conjugate secondary antibodies were diluted with 5% milk in TBST (BioRad, Hercules, CA). ECL (Amersham Biosciences, Uppsala, Swedan) was used to develop membranes. Densitometry was performed using Gene Tools vs 3.06 (SynGene, Beacon House, Cambridge, England).

Transient transfection:

THP-1 cells were transfected with either PKCα, PKCγ, or PKCβII silencing RNA (siRNA) (Santa Cruz Biotechnologies, Santa Cruz, CA) using a 40:4 (picomole:microlitre) Santa Cruz siRNA transfection reagent (PKCα) (Santa Cruz Biotechnologies, Santa Cruz, CA) or 40:1 (picomole:microlitre) Lipofectamine^{TM} 2000 (Invitrogen, Grand Island, NY) (PKCγ and βII). The transfection reagent and siRNA were incubated for 30 mins at room temperature in 100 μL antibiotic/serum free media, and added to cells in antibiotic/ serum free media for 5 h. After 5 h, cells were resuspended in complete media. After 19 h or 43 h,

LPS was added for 24 h to determine the effect of PKC knockdown on IL-27 production. Successful knock-down of PKCα, PKCγ, or PKCβII was confirmed by western blot, as described above.

Statistical analysis:

Mock-treated versus HIV-infected samples were compared using the paired student t-test. Cells treated with pharmacological inhibitors were analyzed using ANOVA and post-hoc Dunnett test, using untreated, LPS-stimulated cells as the control.

CHAPTER III: RESULTS

3.1 *In vitro* HIV infection inhibits LPS-induced IL-23 and IL-27 production in MDMs:

MDM characterization:

Mos were differentiated into MDMs with M-CSF over 7 days and were assessed for surface expression of CD11a, CD11b, CD11c, CD80, CD83, CD86, CD14, CD16, and HLA-DR by flow cytometry (Appendix A, Figure A1). Leukocyte adhesion surface receptors CD11a, CD11b, and CD11c were highly expressed on MDMs (Figure A1 A-C). Co-stimulatory molecules CD80 and CD86 were also expressed on MDMs (Figure A1 D and F). In contrast, the well known DC maturation marker CD83 (282) was not significantly expressed in these cells (Figure A1 E). CD14 is widely used as a MΦ marker (283) and was expressed in nearly 100% of cells (Figure A1 G). CD16 was only expressed in approximately 15% of MDMs, which is consistent with phenotypes previously reported for blood-derived Mos/MΦs (Figure A1 H) (284). Finally, the MHC class II receptor, HLA-DR was also highly expressed on these MDMs consistent the phenotype for antigen presenting cells (Figure A1 I).

MDMs were productively infected with HIV:

MDMs were infected with dual tropic HIV-1_{CS204} supernatants containing 30,000 pg of HIV-p24 protein as described in the materials and methods (281). In order to confirm productive infection of MDMs with HIV, supernatants were analyzed by ELISA to measure HIV-1 p24 secretion. Immediately after infection, p24 in the culture supernatant was nearly zero in HIV-infected and mock-treated MDMs (Figure 4). Minimal amounts of p24 in HIV-

Figure 4: MDMs were productively infected with HIV. MDMs from healthy donors were treated with mock supernatants or infected for 2 h with HIV_{CS204} supernatants containing 30,000 pg of HIV-p24 protein. MDMs were washed and cultured in complete media for up to 7 days. Culture supernatants collected on days 0, 4, and 7 post-infection were analyzed for HIV-1 p24 secretion by ELISA (n=7).

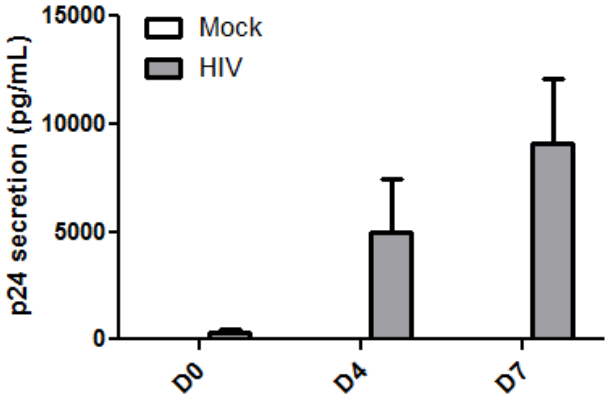


FIGURE 4

infected MDMs may be accounted for by residual p24 protein present from the HIV stocks used to infect the MDMs. Levels of p24 increased in a time-dependent manner from 4 to 7 days post-infection with HIV. In contrast, mock-treated MDMs had undetectable levels of p24 protein in supernatants, indicating that MDMs were productively infected with HIV.

In vitro HIV infection of MDMs does not affect basal IL-23 production:

HIV has been shown to induce the production of cytokines through TLR-7/8-dependent mechanisms (285-287). It was unknown whether HIV influences basal levels of IL-23 secretion in MDMs. To investigate whether *in vitro* HIV infection influences IL-23 production in MDMs, cell lysates were analyzed for IL-12/23 p40 and IL-23 p19 mRNA expression by qRT-PCR, and culture supernatants were used to determine IL-12/23 p40 and IL-23 secretion by ELISA.

There was a trend for increased basal p40 mRNA expression in HIV-infected MDMs compared to controls on days 5 and 8 post-infection (Figure 5A). The expression of p19 mRNA was variable, but not significantly different between mock-treated and HIV-infected MDMs (Figure 5B).

There was a trend for increased basal IL-12/23 p40 secretion in HIV-infected MDMs relative to controls on day 5 post-infection ($p=0.08$); however, the same was not true on day 8 and 15 post-infection (Figure 5C). Basal IL-23 secretion in mock-treated MDMs was less variable than IL-12/23 p40 secretion and averaged approximately 8 pg/mL on days 5, 8, and 15 post-infection (Figure 5D). Basal IL-23 secretion was similar in HIV-infected MDMs compared to untreated MDMs, on days 5 and 8 post-infection, averaging approximately 9 pg/mL. However, on day 15 post-infection, the two donors analyzed had much higher levels

Figure 5: *In vitro* HIV-infection for 5, 8, or 15 days does not induce IL-12/23 p40 or IL-23 p19 mRNA expression or IL-12/23 p40 or IL-23 secretion in MDMs. MDMs from healthy donors were infected with HIV_{CS204} supernatants containing 30,000 pg of HIV-p24 protein. Cells were cultured for the indicated number of days prior to harvesting for the determination of IL-23. A) p40 mRNA expression (D5: n=6 D8: n=5, D15: n=1) and B) p19 mRNA expression (D5: n=8, D8: n=5 and D15: n=4) by qRT-PCR, and collecting supernatants for the quantification of C) IL-12/23 p40 secretion (D5: n=12, D8: n=6, and D15: n=2) and D) IL-23 secretion (D5: n4, D8: n=3, and D15: n=2) by ELISA. mRNA fold change was calculated relative to mock-treated MDMs harvested on the same day.

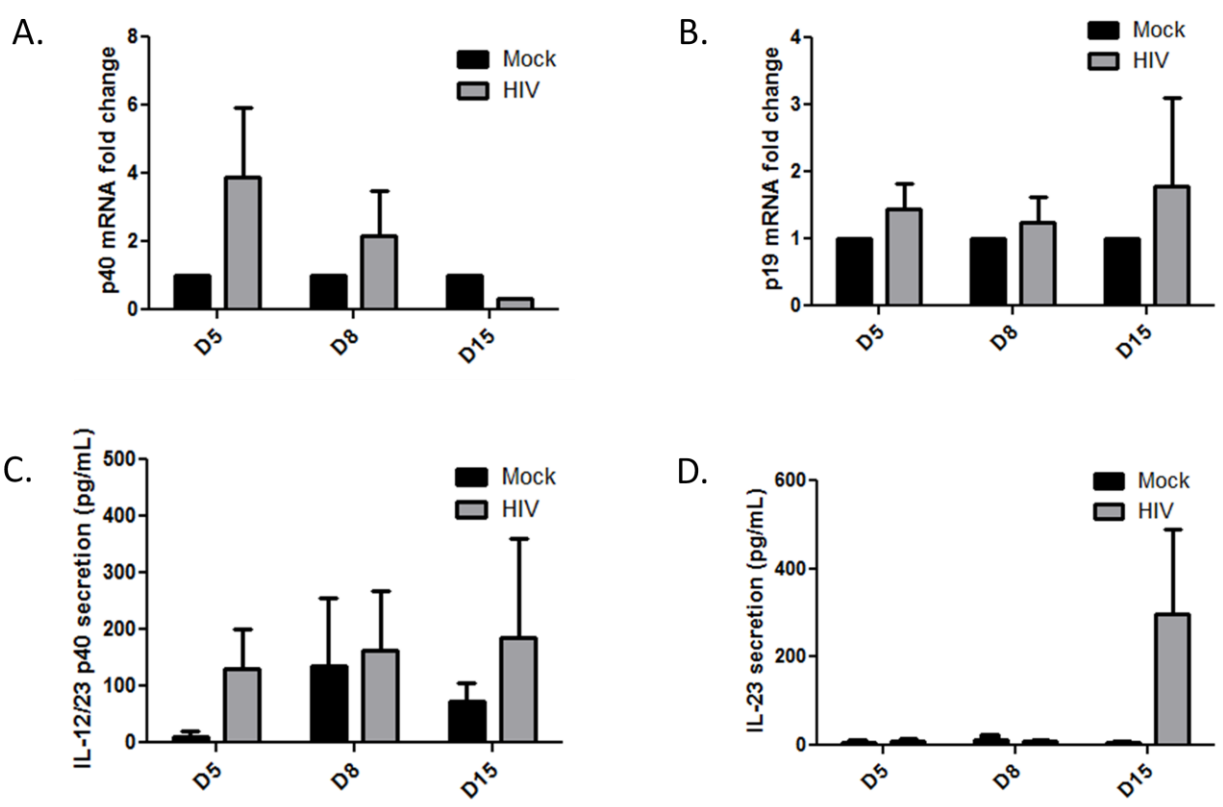

A.
p40 mRNA fold change
0
2
4
6
8
Mock
HIV
D5
D8
D15
B.
p19 mRNA fold change
0
1
2
3
4
Mock
HIV
D5
D8
D15
C.
IL-12/23 p40 secretion (pg/mL)
0
100
200
300
400
500
Mock
HIV
D5
D8
D15
D.
IL-23 secretion (pg/mL)
0
200
400
600
Mock
HIV
D5
D8
D15

FIGURE 5

of IL-23 secretion in HIV-infected MDMs than mock-treated MDMs (Donor 1: 6.5 pg/mL in mock versus 102 pg/mL in HIV-infected MDMs; Donor 2: 4.5 pg/mL in mock versus 492 pg/mL in HIV-infected MDMs).

Overall, HIV did not significantly affect basal expression of p40 and p19 mRNA expression, or IL-12/23 p40 secretion in MDMs at any time following infection with HIV. Similarly, basal levels of IL-23 secretion were unaffected by HIV-infection on days 5 and 8 post-infection. However, HIV infection may enhance IL-23 secretion later in the course of infection.

In vitro HIV infection of MDMs does not affect basal IL-27 production:

Similar to IL-23, it was unknown whether HIV influences basal IL-27 production in human MDMs. MDMs were infected with HIV_{CS204} supernatants containing 30,000 pg of HIV-p24 protein, and were cultured for up to 15 days. On days 5, 8, and 15 post-infection, IL-27 EBI3 and p28 mRNA expression in cell lysates were evaluated by qRT-PCR, and IL-27 secretion from MDMs was evaluated by ELISA.

There was a trend for increased p28 mRNA expression in HIV-infected MDMs relative to mock-treated MDMs on day 8 post-infection ($p=0.06$); however, p28 mRNA expression was not significantly different between mock-treated and HIV-infected MDMs on days 5 and 15 post-infection (Figure 6A). The p28 mRNA expression ranged from 0.1 to 41 fold in HIV-infected MDMs relative to controls on days 5, 8, and 15 post-infection. EBI3 mRNA expression was less variable ranging from 0.3 to 2.7 fold in HIV-infected MDMs relative to controls (Figure 6B). In contrast, EBI3 mRNA expression was decreased in HIV-

Figure 6: *In vitro* HIV infection did not significantly affect EBI3 or p28 mRNA expression and IL-27 secretion in MDMs. MDMs from healthy donors infected *in vitro* with HIV_{CS204} supernatants containing 30,000 pg of HIV-p24 protein were cultured for the indicated number of days prior to the collection of cells for the determination of IL-27 A) p28 mRNA expression (D5, D8 and D15: n=5) and B) EBI3 mRNA expression (D5 and D8: n=5 and D15: n=4) by qRT-PCR, and supernatants for the quantification of C) IL-27 secretion by ELISA (D5: n=11, D8: n=4, and D15: n=3). mRNA fold change was calculated relative to mock-treated MDMs harvested on the same day. Statistical analysis: Paired student t-test. **$p \leq 0.01$.

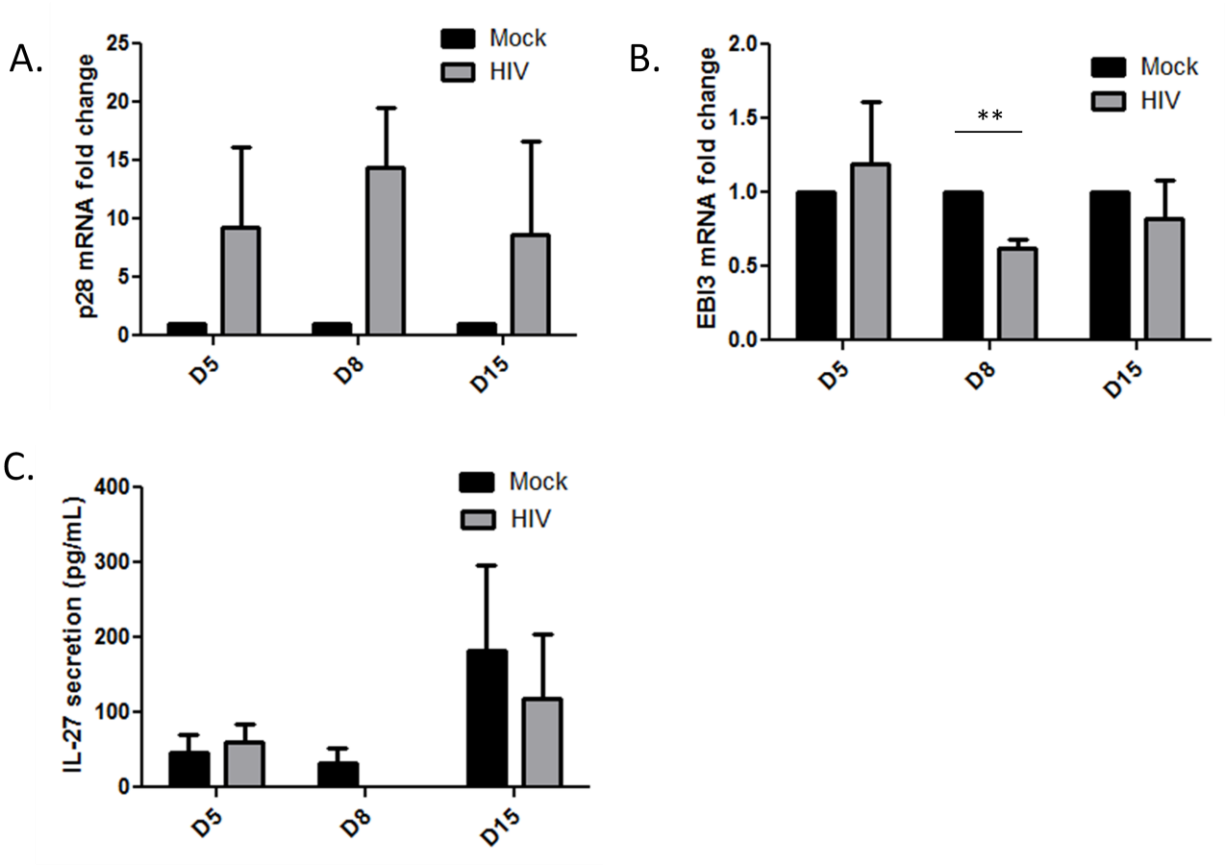

A.
p28 mRNA fold change
25
20
15
10
5
0
Mock
HIV
D5
D8
D15
B.
EBI3 mRNA fold change
2.0
1.5
1.0
0.5
0.0
**
Mock
HIV
D5
D8
D15
C.
IL-27 secretion (pg/mL)
400
300
200
100
0
Mock
HIV
D5
D8
D15

FIGURE 6

infected MDMs from all donors, 8 days post-infection compared to controls (p=0.003). However, EBI3 mRNA expression was not significantly different 5 or 15 days of post-infection with HIV relative to controls (Figure 6B).

Basal levels of IL-27 secretion were variable between donors, but were not significantly different between HIV and mock-treated MDMs (Figure 6C). Overall, these results suggest that HIV-infection does not significantly affect basal levels of IL-27 production in human MΦs.

In vitro HIV infection inhibits p40 and p19 mRNA expression and IL-12/23 p40 and IL-23 secretion in LPS-stimulated MDMs:

IL-23 secretion can be induced in MDMs by LPS-stimulation (288). Since a significant amount of LPS can be detected in the plasma of HIV-infected individuals (200), I sought to investigate whether IL-23 production is affected by HIV in LPS-stimulated MDMs. MDMs were infected with HIV_{CS204} supernatants containing 30,000 pg of HIV-p24 protein, and treated with LPS for 4 h to assess IL-12/23 p40 and IL-23 p19 mRNA expression in cell lysates, or 24 h to assess MDM IL-12/23 p40 and IL-23 secretion into culture supernatants. Cells and supernatants were both harvested on days 5, 8, and 15 post-infection.

LPS-stimulation of mock-treated MDMs increased p40, and p19 mRNA expression and IL-12/23 p40 and IL-23 secretion (Figure 7A-D) relative to unstimulated mock-treated MDMs (Figure 5A-D).

LPS-induced p40 mRNA expression was decreased in 100% of LPS-stimulated HIV-infected MDMs cultures analyzed on days 5, 8, and 15 post-infection compared to controls (Figure 7A). HIV infection inhibited LPS-induced p40 mRNA expression by an average of

Figure 7: *In vitro* HIV-infection inhibits LPS-induced IL-12/23 p40 and IL-23 p19 mRNA expression, and IL-12/23 p40 and IL-23 secretion in MDMs. MDMs from healthy donors were infected with HIV_{CS204} supernatants containing 30,000 pg of HIV-p24 protein. MDMs were cultured for the indicated number of days prior to harvesting cells and supernatants. MDMs were treated with LPS for 4 h prior to collecting cells for the determination of IL-23 A) p40 mRNA expression (D5: n=6, D8: n=5, and D15: n=2) and B) p19 mRNA expression (D5: n=8, D8: n=5 and D15: n=4) by qRT-PCR (A-B). mRNA fold change was calculated relative to unstimulated, mock-treated MDMs from the same donor harvested on the same day (shown in Figure 5A-B). MDMs were stimulated on days 4, 7, and 15 post-infection with LPS for 24 h prior to the collection of supernatants for the quantification of C) IL-12/23 p40 secretion (D5: n=12, D8: n=6 and D15: n=2) and D) IL-23 secretion (D5: n=4, D8 and D15: n=3) by ELISA. Statistical analysis: Paired student t-test. *$p \leq 0.05$, ***$p \leq 0.001$.

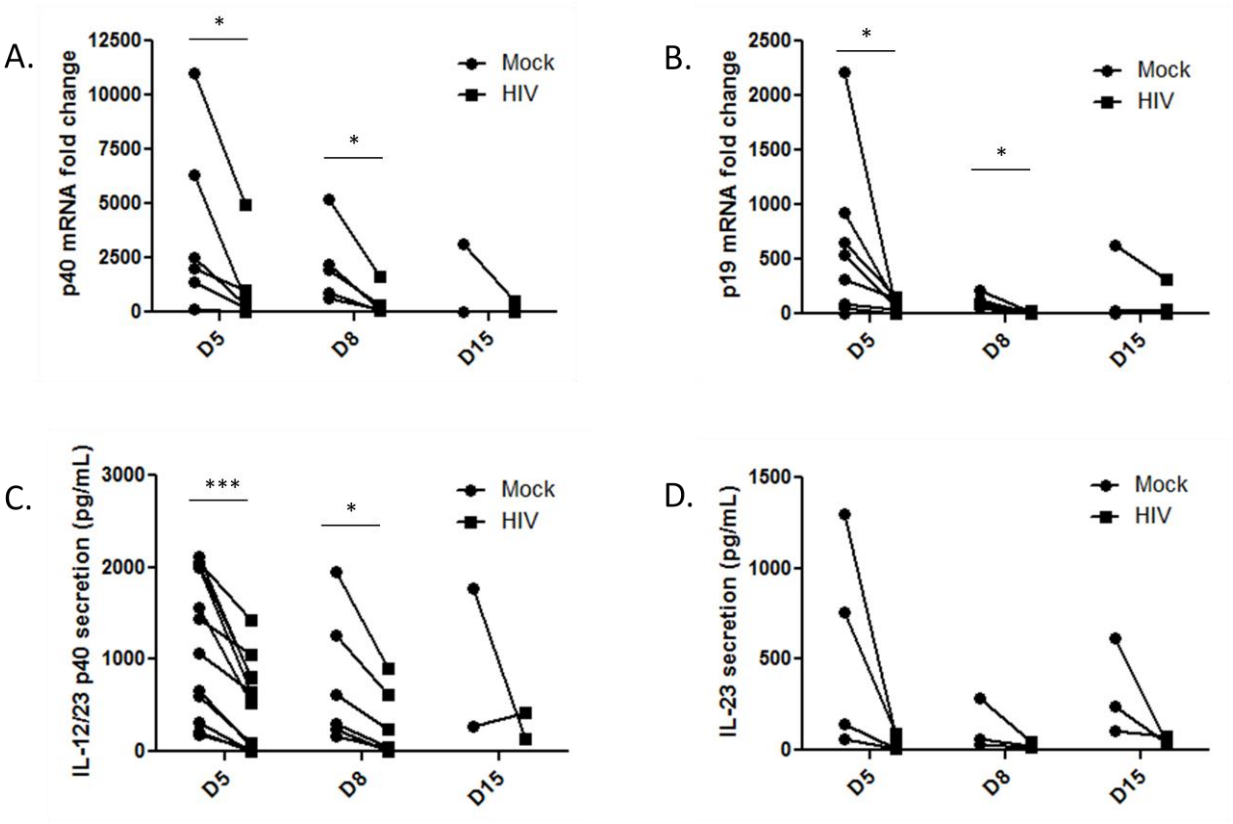

A.
p40 mRNA fold change
12500
10000
7500
5000
2500
0
*
*
Mock
HIV
D5
D8
D15
B.
p19 mRNA fold change
2500
2000
1500
1000
500
0
*
*
Mock
HIV
D5
D8
D15
C.
IL-12/23 p40 secretion (pg/mL)
3000
2000
1000
0
***
*
Mock
HIV
D5
D8
D15
D.
IL-23 secretion (pg/mL)
1500
1000
500
0
Mock
HIV
D5
D8
D15

FIGURE 7

80% relative to controls on days 5, 8, and 15 post-infection. LPS-induced p19 mRNA expression was significantly inhibited by HIV on days 5 and 8 post-infection compared to mock-treated cells (D5: p=0.04; D8: p=0.03) (Figure 7B). However, on day 15 post-infection, LPS-induced p19 mRNA expression in HIV-infected MDMs was increased in 2 of 4 donors, and decreased in 2 of 4 donors, relative to mock-treated MDMs (Figure 7B).

There was a significant decrease of 70% (~580 pg/mL) in LPS-induced IL-12/23 p40 secretion in HIV-infected MDMs on days 5 and 8 post-infection (D5: p=0.0002; D8: p=0.02) (Figure 7C). However, on day 15 post-infection, the effect of HIV on LPS-induced IL-12/23 p40 secretion was variable between two donors (Figure 7C). One donor exhibited significant inhibition of IL-12/23 p40 secretion whereas, the other donor showed increased IL-12/23 p40 secretion in LPS-stimulated HIV-infected MDMs compared to uninfected MDMs (Figure 7C).

Finally, LPS-induced IL-23 secretion was also inhibited in 100% of MDM cultures infected with HIV compared to mock-treated controls on days 5, 8, and 15 post-infection (Figure 7D). There was an average decrease of 75% in LPS-induced IL-23 secretion in HIV-infected MDMs compared to controls.

Overall, my results suggest that HIV inhibits LPS-induced IL-23 production through the inhibition of p40 and p19 mRNA expression, and IL-12/23 p40 secretion in human MΦs.

In vitro HIV infection inhibits EBI3 and p28 mRNA expression, and IL-27 secretion in LPS-stimulated MDMs:

LPS was used as a stimulus to induce IL-27 secretion since it is increased in the serum of HIV-infected patients, and is believed to contribute to chronic immune activation (200). MDMs were infected with dual tropic HIV_{CS204}. Both cell lysates and supernatants

were collected on days 5, 8, and 15 post-infection. Before harvesting cells, MDMs were stimulated with LPS for 4 h in order to evaluate LPS-induction of IL-27 EBI3 and p28 mRNA expression in cell lysates. MDMs were stimulated with LPS on days 4, 7, and 14, for 24 h and supernatants were evaluated for IL-27 secretion by ELISA.

LPS-stimulation of mock-treated MDMs increased p28, and EBI3 mRNA expression and IL-27 secretion (Figure 8A-C) relative to unstimulated mock-treated MDMs (Figure 6A-C).

LPS-induced EBI3 and p28 mRNA expression was decreased in HIV-infected MDMs compared to mock-treated controls (Figure 8A-B). IL-27 p28 mRNA expression was decreased in HIV-infected MDMs compared to mock-treated MDMs 5 days post-infection (D5: $p=0.04$) (Figure 8A). There was an average decrease of approximately 30% and 65% in LPS-induced p28 mRNA expression 8 and 15 days post-infection with HIV, respectively (Figure 8A). Although all donors had decreased LPS-induced p28 mRNA expression in *in vitro* HIV-infected MDMs relative to controls, due to donor variability, there was only a trend for decreased p28 mRNA expression in HIV-infected MDMs, 8 and 15 days post-infection (D8: $p=0.07$; D15: $p=0.27$) (Figure 8A). EBI3 mRNA expression was decreased by approximately 50% in LPS-stimulated HIV-infected MDMs compared to controls on days 5 and 8 post-infection (D5: $p=0.0008$; D8: $p=0.014$). However, there was only a trend for decreased EBI3 mRNA expression in LPS-stimulated HIV-infected MDMs on day 15 post-infection (D15: $p=0.26$), despite the fact that each donor had at least a 20% decrease in EBI3 mRNA expression in HIV-infected MDMs relative to controls (Figure 8B).

In accordance with the inhibition of LPS-induced p28 and EBI3 mRNA expression by HIV (Figure 8A-B), LPS-induced IL-27 secretion was decreased in 100% of MDM cultures infected with HIV relative to mock-treated MDMs (Figure 8C). HIV-infected

Figure 8: *In vitro* HIV-infection inhibits LPS-induced IL-27 p28 and EBI3 mRNA expression, and IL-27 secretion in MDMs. MDMs from healthy donors were infected with HIV_{CS204} supernatants containing 30,000 pg of HIV-p24 protein. MDMs were cultured for the indicated number of days prior to harvesting cells and supernatants. MDMs were treated with LPS for 4 h prior to collecting cells for the determination of IL-27 A) p28 mRNA expression (D5, D8, and D15: n=5) and B) EBI3 mRNA expression (D5 and D8: n=5 and D15: n=4) by qRT-PCR. mRNA fold change was calculated relative to unstimulated, mock-treated MDMs from the same donor harvested on the same day (shown in Figure 6A-B). C) MDMs were stimulated on days 4, 7, and 14 post-infection with LPS for 24 h prior to the collection of supernatants for the quantification of IL-27 secretion by ELISA (D5: n=11, D8: n=4, and D15: n=4). Statistical analysis: Paired student t-test. *$p \leq 0.05$, **$p \leq 0.01$.

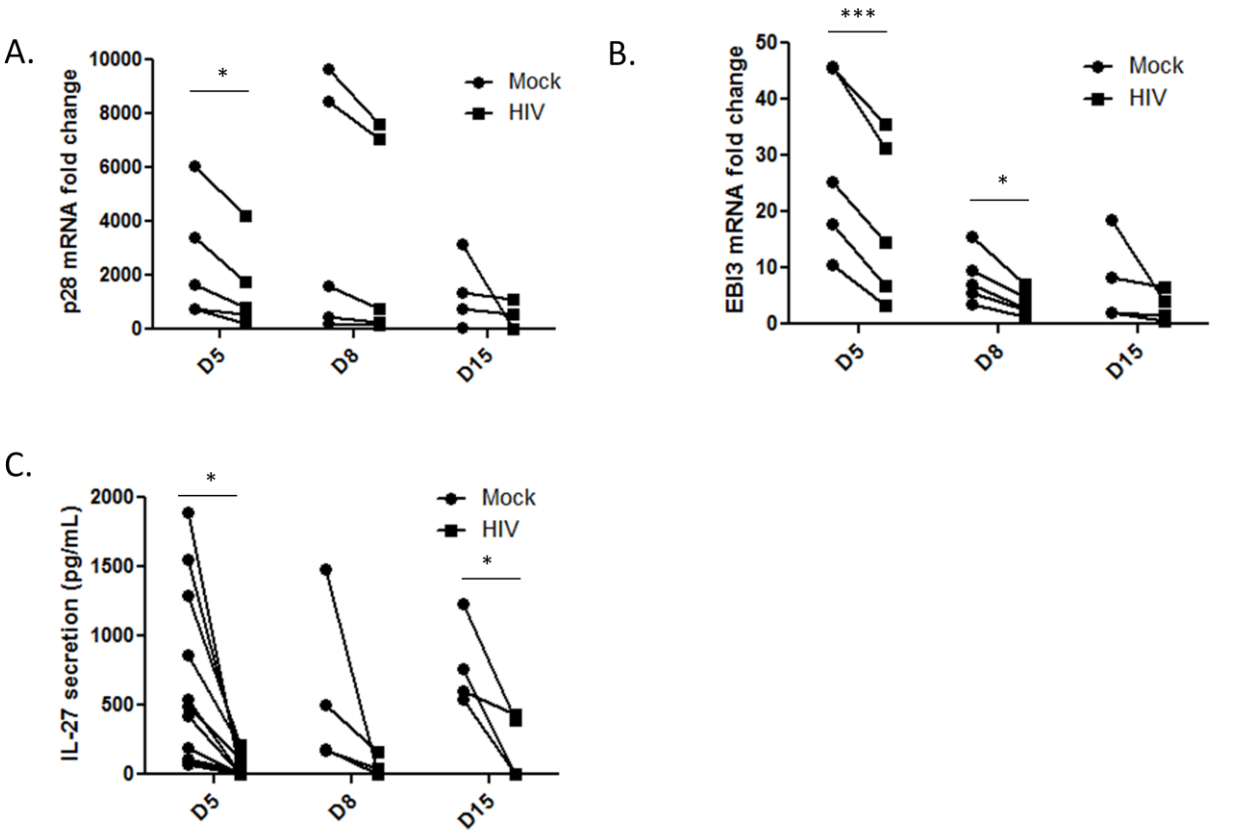

A.
p28 mRNA fold change
10000
8000
6000
4000
2000
0
*
Mock
HIV
D5
D8
D15
B.
EBI3 mRNA fold change
50
40
30
20
10
0
***
*
Mock
HIV
D5
D8
D15
C.
IL-27 secretion (pg/mL)
2000
1500
1000
500
0
*
*
Mock
HIV
D5
D8
D15

FIGURE 8

MDMs secreted an average of 75% less IL-27 compared to controls from the same donor on days 5, 8, and 15 post-infection. These results suggest that HIV inhibits LPS-induced EBI3 and p28 mRNA expression, and IL-27 secretion in human MΦs.

3.2 The pathways regulating LPS-induced IL-23 and IL-27 production, and the impairment of p38 and JNK MAPK activation by HIV in MDMs:

LPS-induced IL-23 secretion from MDMs on Day 1 post-differentiation is variable between donors:

It was of interest to determine how HIV inhibits LPS-induced IL-27 and IL-23 production in MDMs. However, the molecular mechanisms and signalling pathways regulating LPS-induced IL-23 and IL-27 expression in human MΦs are poorly understood and had to be investigated prior to understanding how HIV inhibits LPS-induced IL-23 and IL-27 expression. Although various studies have investigated the signalling pathways regulating TLR-induced IL-23 and IL-27 in other cell types including DCs, and THP-1 cells, whether the same signalling pathways regulate LPS-induced IL-23 and IL-27 in MDMs remains unknown.

To investigate the pathways regulating LPS-induced IL-23 production, MDMs were treated with pharmacological inhibitors for the PI3K, p38, JNK, and ERK pathways, and prior to LPS stimulation. The production of IL-23 was assessed by qRT-PCR and ELISA.

After conducting experiments with MDMs from 10 donors, it was noticed that MDMs derived from only 40% of donors were secreting IL-23 in response to LPS-stimulation (Figure 9). Notably, all of these donors secreted IL-12/23 p40 and IL-27 in response to LPS (data not shown). Since IL-23 production from these MDMs in response to

Figure 9: LPS-induced IL-23 responders and non-responders in MDMs. MDMS from 10 donors were treated with LPS for 24 h, 1 day post-differentiation of monocytes into MDMs. Supernatants were assessed for IL-23 secretion by ELISA. Donors were grouped into non-responders (N) (n=6) and responders (R) (n=4) to LPS. Statistical analysis: Paired student t-test. * $p<0.05$, NS=not significant.

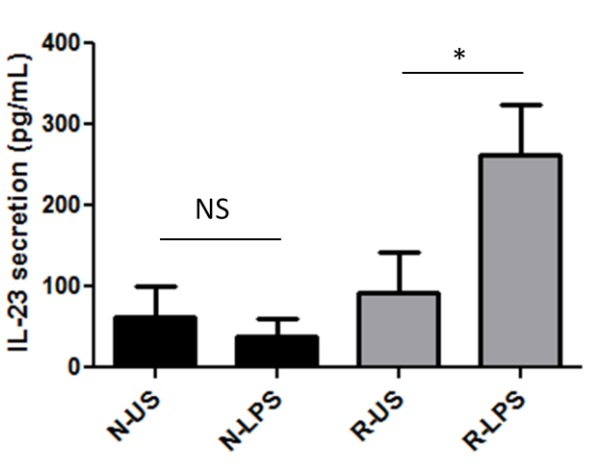


FIGURE 9

LPS was not consistently observed, the signalling pathways regulating LPS-induced IL-23 production in MDMs were not investigated in subsequent studies.

The PI3K pathway positively regulates LPS-induced p28 and EBI3 mRNA expression, EBI3 protein expression, and IL-27 secretion in MDMs:

In order to investigate the mechanism by which HIV inhibits LPS-induced IL-27 production in MDMs, the signalling pathways regulating IL-27 in MDMs were determined. The p38 and JNK MAPK, and PI3K pathways have been implicated in the regulation of LPS-induced IL-27 in astrocytes or myeloid DCs (265, 271). Whether these pathways also regulate LPS-induced IL-27 production in MDMs was not known. Additionally, previous studies have not addressed which subunits of IL-27 are regulated by these pathways in myeloid DCs, and astrocytes (265, 271). This is of particular interest, since HIV inhibits both LPS-induced EBI3 and p28 mRNA expression. In order to elucidate the signalling pathways regulating LPS-induced IL-27 production, MDMs were treated with pharmacological inhibitors for the PI3K (LY294002), p38 (SB203580), JNK (S600125), and ERK (PD98059) MAPK pathways prior to LPS-stimulation. IL-27 production was assessed by qRT-PCR, western blot, and ELISA.

Inhibition of the PI3K pathway by LY294002 inhibited both LPS-induced EBI3 and p28 mRNA expression in a dose-dependent manner (Figure 10A-B). PI3K inhibition decreased LPS-induced EBI3 mRNA expression by approximately 80% at 25 μM, and 90% at 50 μM doses of inhibitor (Figure 10A). Similarly, p28 mRNA expression was decreased by 30% at 25 μM, and 80% at 50 μM doses of the PI3K inhibitor compared to cells stimulated with LPS alone as a control (Figure 10B).

Figure 10: The PI3K pathway positively regulates LPS-induced IL-27 EBI3 and p28 mRNA expression, EBI3 protein expression, and IL-27 secretion in MDMs. MDMs from healthy donors were treated with the PI3K inhibitor, LY294002, for 2 h prior to LPS-stimulation (A-E). A-B) Cell lysates from MDMs stimulated with LPS for 4 h were analyzed for IL-27 EBI3 and p28 mRNA expression by qRT-PCR (n=3). mRNA fold change was calculated relative to untreated, and unstimulated MDMs from the same donor. C-D) In order to quantify EBI3 protein expression and IL-27 secretion, MDMs were stimulated with LPS for 24 h. C) Cell lysates were assessed for EBI3 protein expression by western blot. One representative western blot is shown. Densitometry was used to normalize EBI3 protein expression to the GAPDH loading control (n=4). D) IL-27 secretion from MDMs into culture supernatants was assessed by ELISA (n=5). E) In order to assess the biological activity of the inhibitor, after incubation with the inhibitor, MDMs were treated with LPS for 15 min and cell lysates were assessed for p-AKT and AKT protein expression by western blot. Statistical analysis: ANOVA post-hoc Dunnett test comparing all samples to untreated, LPS-stimulated MDMs. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

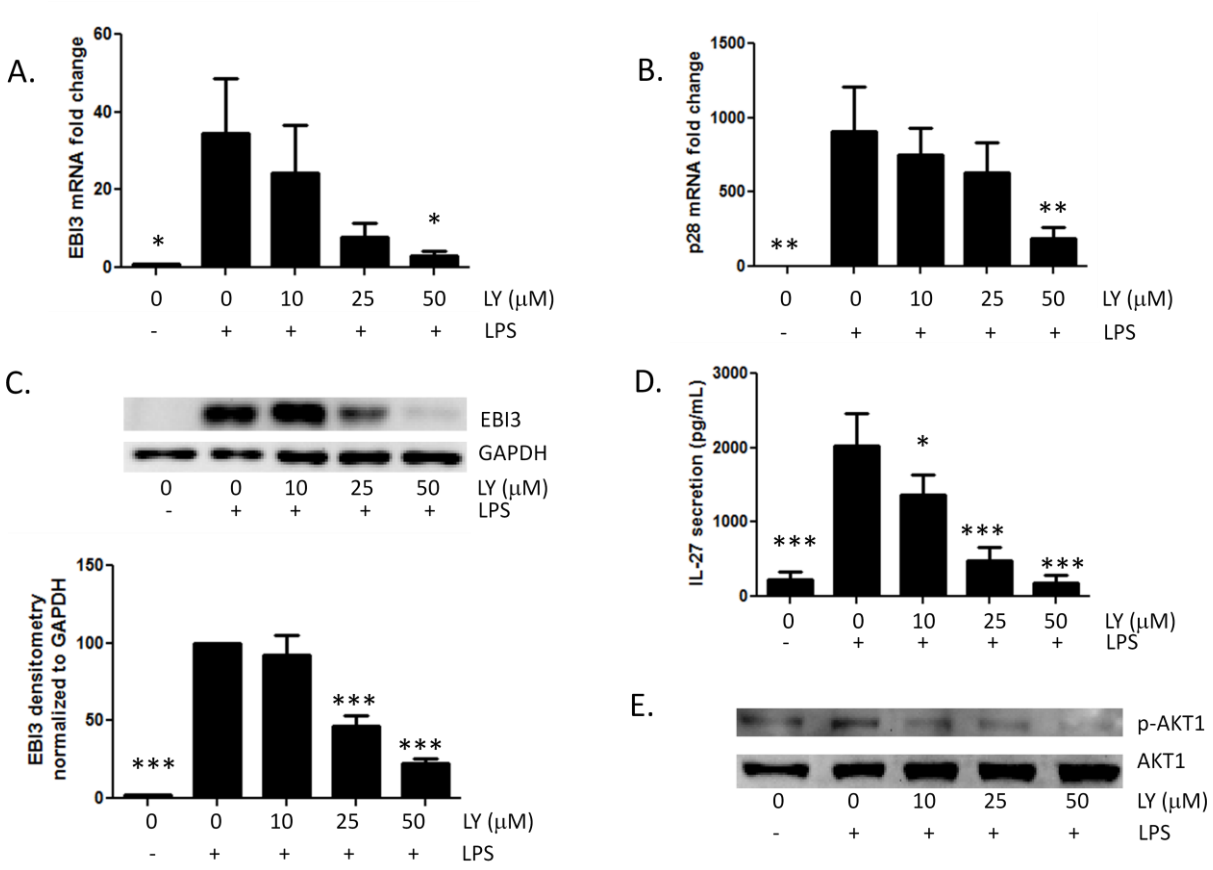

A.
EBI3 mRNA fold change
60
40
20
0
*
*
0 0 10 25 50 LY (μM)
- + + + + LPS
B.
p28 mRNA fold change
1500
1000
500
0
**
**
0 0 10 25 50 LY (μM)
- + + + + LPS
C.
EBI3
GAPDH
0 0 10 25 50 LY (μM)
- + + + + LPS
EBI3 densitometry normalized to GAPDH
150
100
50
0
***
***
***
0 0 10 25 50 LY (μM)
- + + + + LPS
D.
IL-27 secretion (pg/mL)
3000
2000
1000
0
***
*
***
***
0 0 10 25 50 LY (μM)
- + + + + LPS
E.
p-AKT1
AKT1
0 0 10 25 50 LY (μM)
- + + + + LPS

FIGURE 10

Consistent with EBI3 mRNA expression, EBI3 protein expression, as determined by western blot analysis, was also decreased significantly in response to inhibition of the PI3K pathway (Figure 10C). There was an average decrease of 55% and 80% in LPS-induced EBI3 protein expression at 25 and 50 μM doses of inhibitor, respectively, compared to the untreated, LPS-stimulated control (Figure 10C).

Finally, LPS-induced IL-27 secretion was also decreased in a dose-dependent manner in response to the PI3K inhibitor (Figure 10D). All doses of the inhibitor used (10, 25, 50 μM) resulted in significantly decreased IL-27 secretion relative to the untreated, LPS-stimulated cells alone (Figure 10D).

In order to validate the activity of the PI3K inhibitor, LPS-induced phosphorylation of AKT was assessed by western blot analysis (Figure 10E). Basal levels of AKT phosphorylation were high, and LPS-stimulation increased AKT phosphorylation. Treatment of MDMs with LY294002 at all doses significantly decreased LPS-induced AKT-phosphorylation to nearly undetectable levels (Figure 10E).

These results suggest the PI3K pathway positively regulates LPS-induced EBI3 and p28 mRNA expression, EBI3 protein expression and IL-27 secretion in MDMs.

The p38 MAPK pathway positively regulates LPS-induced EBI3 mRNA and protein expression and IL-27 secretion, but not p28 mRNA expression:

The p38 MAPK inhibitor SB203580 was used to assess whether the p38 MAPK pathway regulates LPS-induced IL-27 in MDMs. MDMs treated for 2 h with SB203580 and stimulated for 4 h with LPS were assessed for IL-27 EBI3 and p28 mRNA expression by qRT-PCR. MDMs treated with SB203580 for 2 h and stimulated with LPS for 24 h were

used to assess EBI3 protein expression in cell lysates by western blot, and IL-27 secretion into supernatants by ELISA. The biological activity of SB203580 was assessed by measuring the phosphorylation of p38 MAPK relative to total-p38 MAPK expression by western blot.

Inhibition of the p38 MAPK pathway significantly decreased LPS-induced EBI3 mRNA expression by 50% and 75% at doses of 25 and 50 μM of SB203580, respectively, compared to the untreated, LPS-stimulated cells (Figure 11A). In contrast, p28 mRNA expression was unaffected by SB203580 (Figure 11B).

As expected, LPS-induced EBI3 protein expression was also significantly inhibited by SB203580. EBI3 protein expression was inhibited by 35% and 50% at concentrations of 25 and 50 μM of SB203580, respectively, compared to untreated, LPS-stimulated cells.

LPS-induced IL-27 production was also significantly inhibited in a dose-dependent manner by the p38 MAPK inhibitor. SB203580 inhibited IL-27 secretion by 40% at a concentration of 25 μM, and 70% at a concentration of 50 μM of inhibitor relative to untreated, LPS-stimulated cells (Figure 11C-D).

Treatment of MDMs with the SB203580, p38 MAPK inhibitor resulted in the inhibition of LPS-induced p38 MAPK phosphorylation, demonstrating the biological activity of the inhibitor (Figure 11E).

Overall, these results demonstrate that the p38 MAPK pathway positively regulates LPS-induced IL-27 secretion through the regulation of EBI3 mRNA and protein expression, but not p28 mRNA expression.

Figure 11: The p38 MAPK pathway positively regulates LPS-induced IL-27 production through EBI3 mRNA and protein expression, but not through p28 mRNA expression. MDMs from healthy donors were treated with the p38 MAPK inhibitor, SB203580, for 2 h prior to LPS-stimulation (A-E). A-B) Cell lysates from MDMs stimulated with LPS for 4 h were analyzed for IL-27 EBI3 and p28 mRNA expression by qRT-PCR (n=3). mRNA fold change was calculated relative to untreated and unstimulated MDMs from the same donor. C-D) In order to quantify EBI3 protein expression and IL-27 secretion, MDMs were stimulated with LPS for 24 h. C) Cell lysates were assessed for EBI3 protein expression by western blot. One representative western blot is shown. Densitometry was used to normalize EBI3 protein expression to the GAPDH loading control (n=3). D) IL-27 secretion from MDMs into culture supernatants was assessed by ELISA (n=5). E) In order to assess the biological activity of the inhibitor, after incubation with the inhibitor, MDMs were treated with LPS for 15 min and cell lysates were assessed for p-p38 and p38 MAPK protein expression by western blot. Statistical analysis: ANOVA post-hoc Dunnett test comparing all samples to untreated, LPS-stimulated MDMs. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

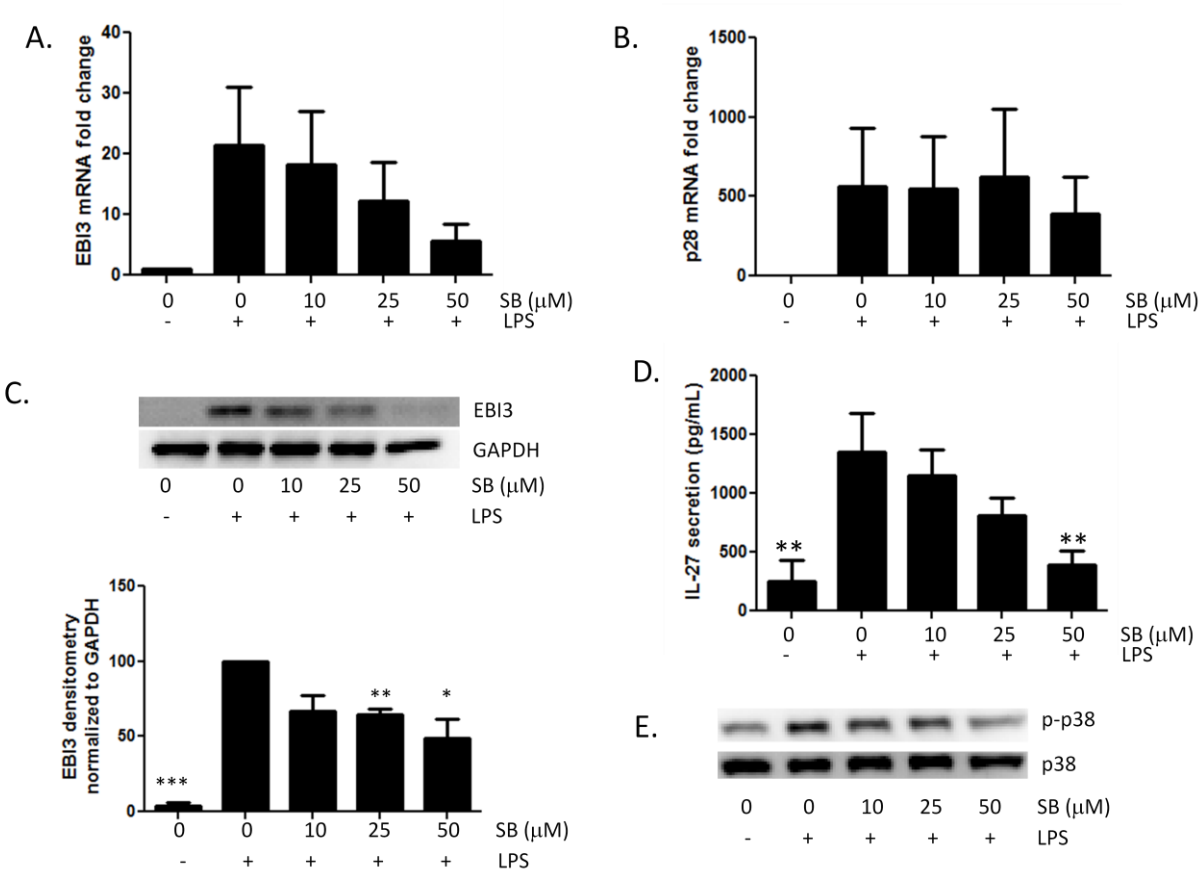

A.
EBI3 mRNA fold change
40
30
20
10
0
0 0 10 25 50 SB (μM)
- + + + + LPS
B.
p28 mRNA fold change
1500
1000
500
0
0 0 10 25 50 SB (μM)
- + + + + LPS
C.
EBI3
GAPDH
0 0 10 25 50 SB (μM)
- + + + + LPS
EBI3 densitometry normalized to GAPDH
150
100
50
0
***
**
*
0 0 10 25 50 SB (μM)
- + + + + LPS
D.
IL-27 secretion (pg/mL)
2000
1500
1000
500
0
**
**
0 0 10 25 50 SB (μM)
- + + + + LPS
E.
p-p38
p38
0 0 10 25 50 SB (μM)
- + + + + LPS

FIGURE 11

The JNK MAPK pathway positively regulates LPS-induced p28 mRNA expression, EBI3 protein expression, and IL-27 secretion but not EBI3 mRNA expression:

The role of the JNK MAPK pathway in the regulation of LPS-induced IL-27 production was determined by pre-treating MDMs with the SP600125 JNK inhibitor. Inhibition of JNK MAPK pathway by SP600125 did not affect LPS-induced EBI3 mRNA expression (Figure 12A). However, SP600125 significantly inhibited LPS-induced p28 mRNA expression (Figure 12B). SP600125 reduced LPS-induced p28 mRNA expression by 60% at a concentration of 25 μM, and 80% at a concentration of 50 μM, compared to LPS-stimulated cells (Figure 12B).

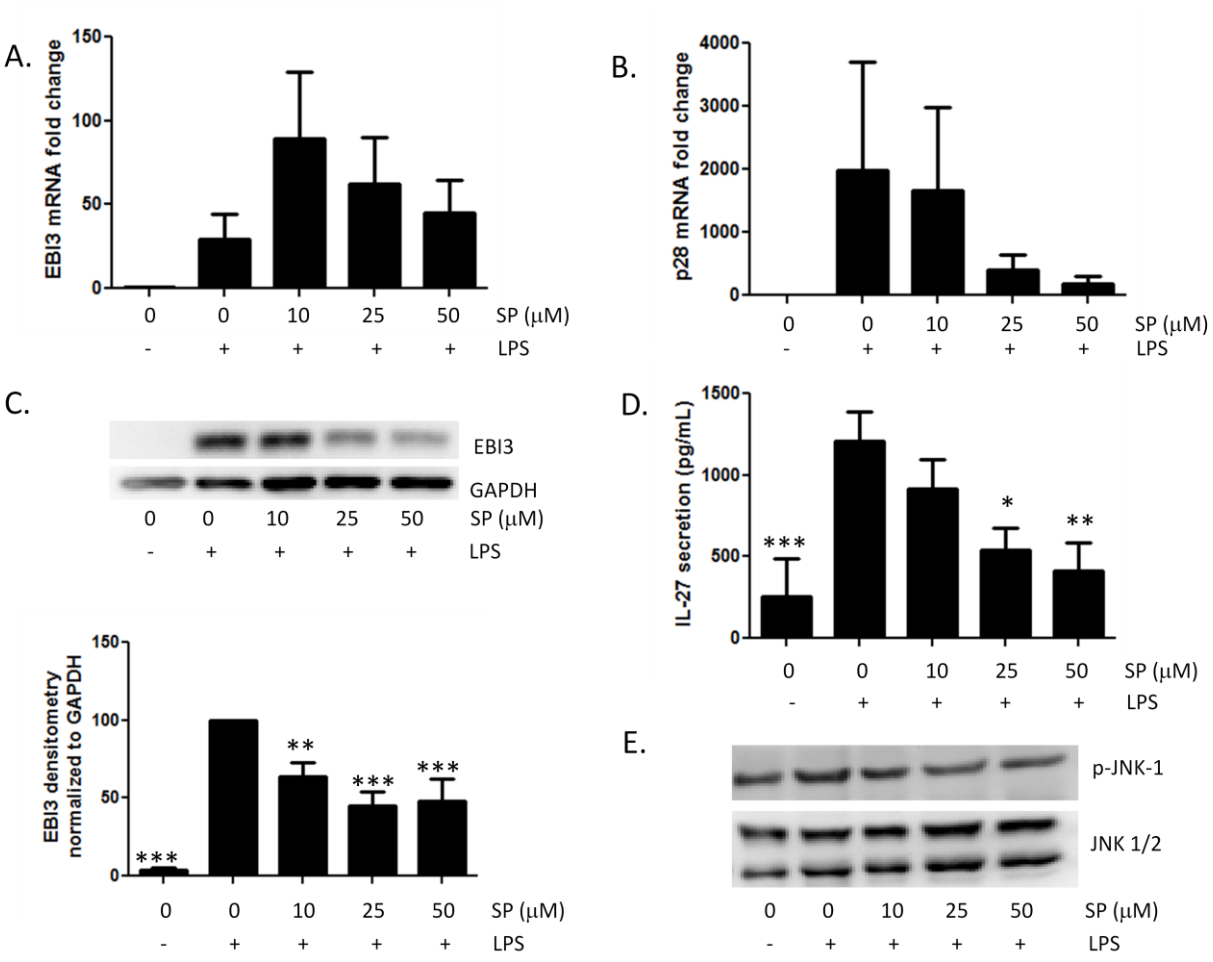
In contrast to EBI3 mRNA expression, SP600125 inhibited LPS-induced EBI3 protein expression by 35%, 55%, and 50% at concentrations of 10, 25, and 50 μM, respectively (Figure 12C).

LPS-induced IL-27 secretion was also inhibited by SP600125, resulting in a 55% and 65% decrease in IL-27 secretion at doses of 25 and 50 μM of inhibitor, respectively (Figure 12D).

JNK phosphorylation was decreased in response to SP600125, demonstrating the biological activity of the JNK inhibitor (Figure 12E).

These results demonstrate that the JNK pathway positively regulates p28 mRNA expression, IL-27 secretion and EBI3 protein expression, but not EBI3 mRNA expression, in human MDMs.

Figure 12: The JNK MAPK pathway positively regulates LPS-induced p28 mRNA expression, EBI3 protein expression, and IL-27 secretion, but not EBI3 mRNA expression in MDMs. MDMs from healthy donors were treated with the JNK MAPK inhibitor, SP600125, for 2 h prior to LPS-stimulation (A-E). A-B) Cell lysates from MDMs stimulated with LPS for 4 h were analyzed for IL-27 EBI3 and p28 mRNA expression by qRT-PCR (n=3). mRNA fold change was calculated relative to untreated, and unstimulated MDMs from the same donor. C-D) MDMs were stimulated with LPS for 24 h in order to quantify EBI3 protein expression and IL-27 secretion. C) Cell lysates were assessed for EBI3 protein expression by western blot. One representative western blot is shown. Densitometry was used to normalize EBI3 protein expression to the GAPDH loading control (n=4). D) IL-27 secretion from MDMs into culture supernatants was assessed by ELISA (n=4). E) In order to assess the biological activity of the inhibitor, after incubation with the inhibitor, MDMs were treated with LPS for 15 min and cell lysates were assessed for p-JNK and JNK MAPK protein expression by western blot. Statistical analysis: ANOVA post-hoc Dunnett test comparing all samples to untreated, LPS-stimulated MDMs. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.


A.
EBI3 mRNA fold change
150
100
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0
0 0 10 25 50 SP (μM)
- + + + + LPS
B.
p28 mRNA fold change
4000
3000
2000
1000
0
0 0 10 25 50 SP (μM)
- + + + + LPS
C.
EBI3
GAPDH
0 0 10 25 50 SP (μM)
- + + + + LPS
EBI3 densitometry normalized to GAPDH
150
100
50
0
***
**
***
***
0 0 10 25 50 SP (μM)
- + + + + LPS
D.
IL-27 secretion (pg/mL)
1500
1000
500
0
***
*
**
0 0 10 25 50 SP (μM)
- + + + + LPS
E.
p-JNK-1
JNK 1/2
0 0 10 25 50 SP (μM)
- + + + + LPS

FIGURE 12

The ERK pathway does not regulate LPS-induced EBI3 or p28 mRNA expression, EBI3 protein expression, or IL-27 secretion in MDMs:

ERK MAPK pathway has been shown to not regulate TLR-induced IL-27 production in myeloid DCs and astrocytes (265, 271). However, it was unclear whether ERK MAPK regulates LPS-induced IL-27 production in human MDMs. The role of ERK MAPKs in the regulation of LPS-induced IL-27 production was determined by pre-treating MDMs with MEK-1/2 inhibitor PD98059. The inhibition of the ERK pathway, by PD98059, did not affect either p28 or EBI3 mRNA expression in LPS-stimulated MDMs (Figure 13A-B). EBI3 protein expression was also unchanged by pre-treatment of MDMs with PD98059, at any concentration, prior to LPS- stimulation (Figure 13C). Similarly, LPS-induced IL-27 secretion was also unaffected by ERK inhibition (Figure 13D)

LPS-stimulation resulted in a pronounced induction of ERK phosphorylation in MDMs. Pre-treatment of MDMs with PD98059 resulted in a clear dose-dependent decrease in ERK phosphorylation, demonstrating the biological activity of the inhibitor (Figure 13E). Taken together, these results indicate that LPS-induced IL-27 production in MDMs is not regulated by the ERK MAPK pathway.

In vitro HIV infection inhibits p38 and JNK MAPK phosphorylation in LPS-stimulated MDMs:

The above results suggest that the PI3K, p38 MAPK, and JNK MAPK pathways positively regulate LPS-induced IL-27 production in MDMs, and that *in vitro* infection with HIV inhibits LPS-induced IL-27 production in human MDMs. In order to elucidate the signalling pathways by which HIV inhibits LPS-induced IL-27 production in MDMs, the activation of the PI3K, p38 MAPK, and JNK MAPK pathways were investigated in HIV-

Figure 13: The ERK pathway does not regulate LPS-induced IL-27 production in MDMs. MDMs from healthy donors were treated with the ERK MAPK inhibitor, PD98059, for 2 h prior to LPS-stimulation (A-E). A-B) Cell lysates from MDMs stimulated with LPS for 4 h were analyzed for IL-27 EBI3 and p28 mRNA expression by qRT-PCR (n=3). mRNA fold change was calculated relative to unstimulated, untreated MDMs from the same donor. C-D) MDMs were stimulated with LPS for 24 h in order to quantify EBI3 protein expression and IL-27 secretion. C) Cell lysates were assessed for EBI3 protein expression by western blot. One representative western blot is shown. Densitometry was used to normalize EBI3 protein expression to the GAPDH loading control (n=5). D) IL-27 secretion from MDMs into culture supernatants was assessed by ELISA (n=4). E) In order to assess the biological activity of the inhibitor, after incubation with the inhibitor, MDMs were treated with LPS for 15 min and cell lysates were assessed for p-ERK and ERK MAPK protein expression by western blot. Statistical test: ANOVA post-hoc Dunnett test comparing all samples to untreated, LPS-stimulated MDMs. ***$p \leq 0.001$.

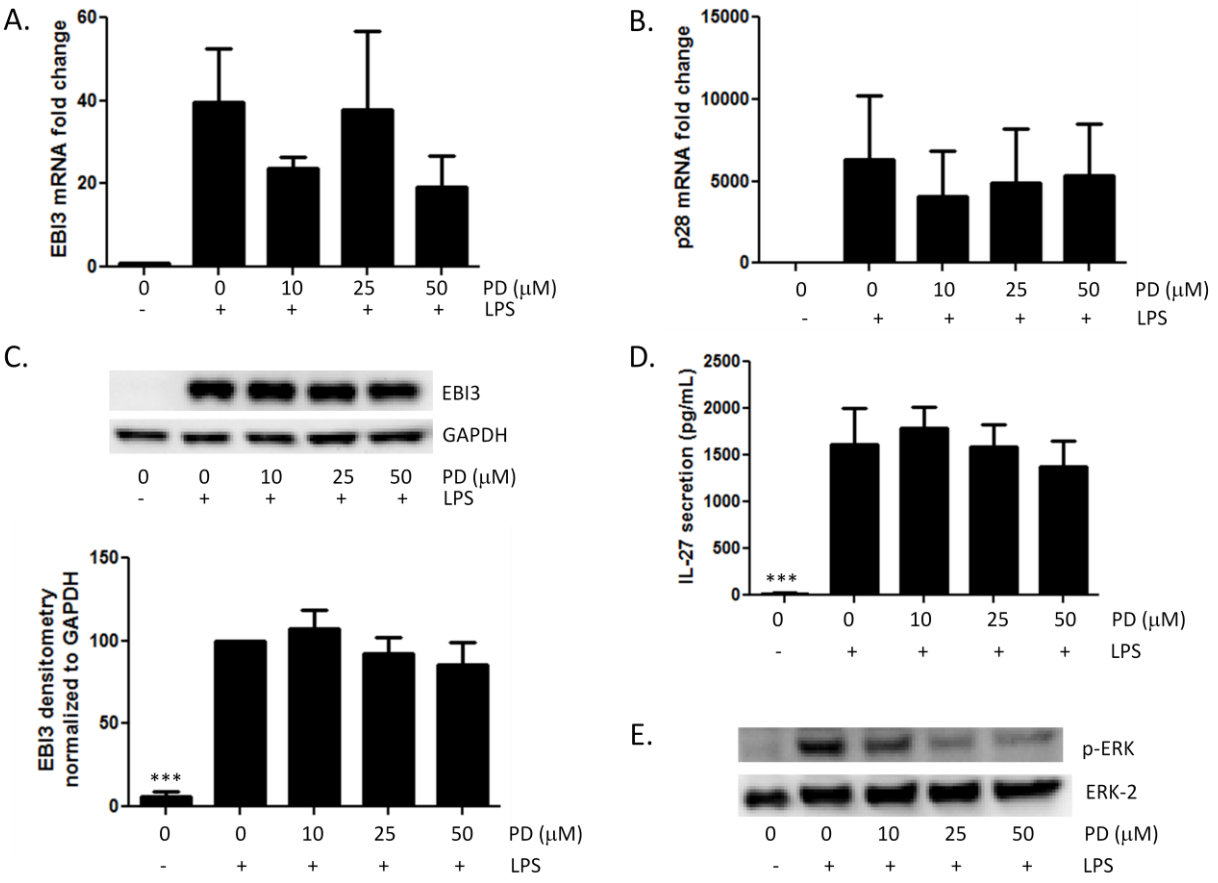

A.
EBI3 mRNA fold change
60
40
20
0
0 0 10 25 50 PD (μM)
- + + + + LPS
B.
p28 mRNA fold change
15000
10000
5000
0
0 0 10 25 50 PD (μM)
- + + + + LPS
C.
EBI3
GAPDH
0 0 10 25 50 PD (μM)
- + + + + LPS
EBI3 densitometry normalized to GAPDH
150
100
50
0
***
0 0 10 25 50 PD (μM)
- + + + + LPS
D.
IL-27 secretion (pg/mL)
2500
2000
1500
1000
500
0
***
0 0 10 25 50 PD (μM)
- + + + + LPS
E.
p-ERK
ERK-2
0 0 10 25 50 PD (μM)
- + + + + LPS

FIGURE 13

infected MDMs. Since HIV significantly inhibited LPS-induced p28 and EBI3 mRNA expression, and IL-27 secretion on day 5 post-infection (Figure 8), day 5 post-infection was used as a representative time point to investigate LPS-induced activation of the p38 MAPK, JNK MAPK, and PI3K pathways by immunoblotting.

LPS-induced p38 phosphorylation was significantly increased in mock and HIV-infected MDMs 30 min post-stimulation with LPS relative to unstimulated controls (Figure 14A). The phosphorylation of p38 MAPK was significantly decreased 30 min post-stimulation with LPS in HIV-infected MDMs compared to LPS-stimulated mock-treated MDMs, as demonstrated by an average decrease of 50% in p38 phosphorylation (Figure 14A). Basal levels of p38 phosphorylation were not significantly different between mock-treated and HIV-infected MDMs.

LPS-stimulation did not significantly increase JNK phosphorylation 30 min post-stimulation with LPS in mock-treated or HIV-infected MDMs (Figure 14B). Similarly basal levels of JNK phosphorylation were not significantly different between mock-treated and HIV-infected MDMs (Figure 14B). However, JNK phosphorylation was significantly decreased by 25% in LPS-stimulated HIV-infected MDMs compared to controls (Figure 14B).

LPS-stimulation for 30 min significantly increased AKT phosphorylation in HIV-infected MDMs ($p=0.03$), but did not significantly induce AKT phosphorylation in mock-treated MDMs (Figure 14C). Basal levels of AKT phosphorylation were not significantly different between mock-treated and HIV-infected MDMs. There was a trend for increased AKT phosphorylation in HIV-infected MDMs simulated for 30 min with LPS, compared to controls (Figure 14C).

Figure 14: LPS-induced p38 and JNK MAPK activation is inhibited in *in vitro* HIV-infected MDMs. MDMs from healthy donors were mock-treated or infected with HIV_{CS204} supernatants containing 30,000 pg of HIV-p24 protein. Cells were cultured for 5 days, and stimulated with LPS for the indicated times. Cell lysates were assessed for phosphorylated and total A) p38 MAPK B) JNK MAPK, and C) AKT protein expression by western blot. One representative western blot is shown. Densitometry was used to normalize phosphorylated A) p38 MAPK (n=3), B) JNK MAPK (n=4), and C) AKT (n=3) protein expression to their respective loading controls. Statistical analysis: Paired student t-test. * $p \leq 0.05$

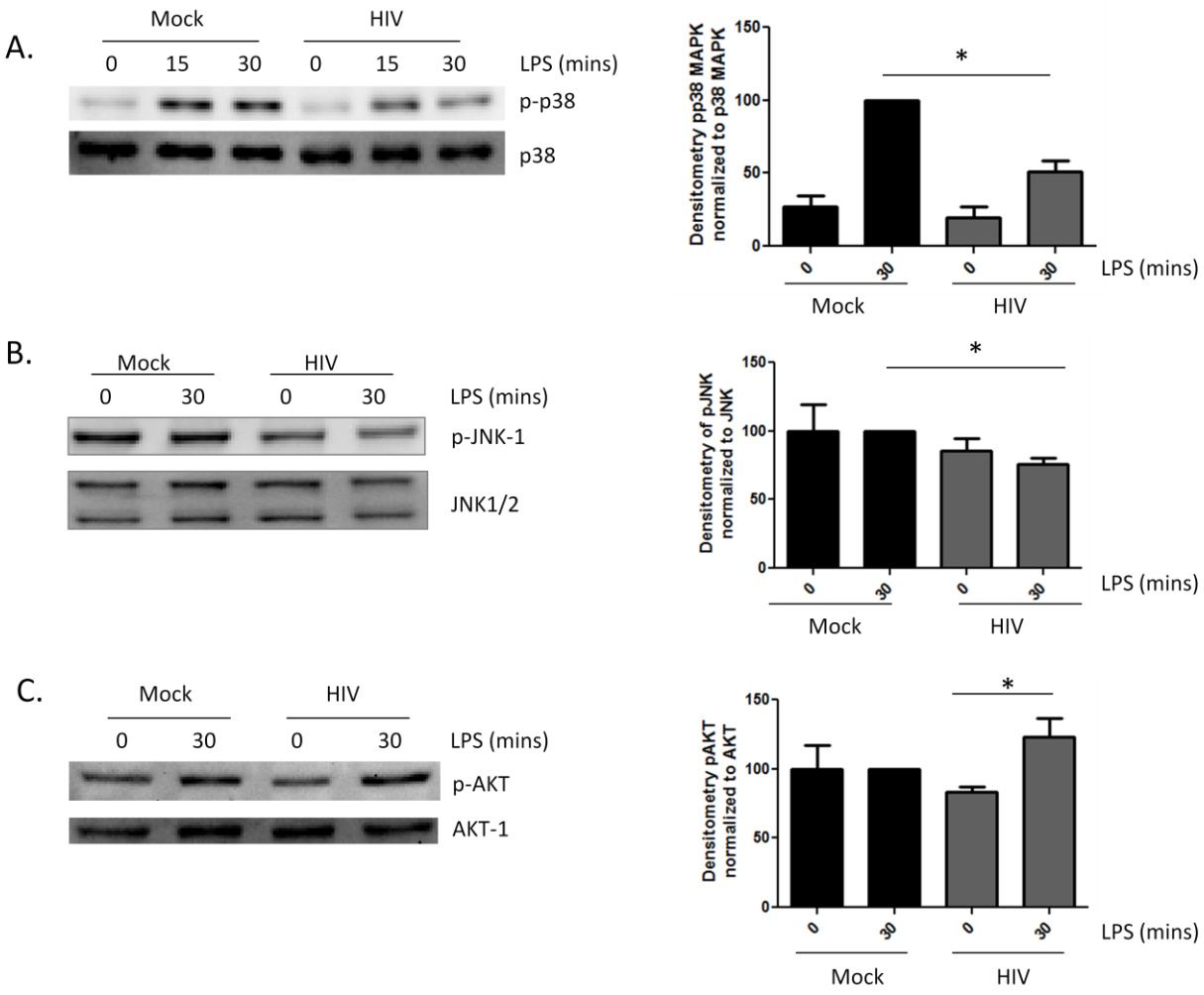

A.
Mock
HIV
0
15
30
0
15
30
LPS (mins)
p-p38
p38
Densitometry pp38 MAPK normalized to p38 MAPK
150
100
50
0
*
0
30
0
30
LPS (mins)
Mock
HIV
B.
Mock
HIV
0
30
0
30
LPS (mins)
p-JNK-1
JNK1/2
Densitometry of pJNK normalized to JNK
150
100
50
0
*
0
30
0
30
LPS (mins)
Mock
HIV
C.
Mock
HIV
0
30
0
30
LPS (mins)
p-AKT
AKT-1
Densitometry pAKT normalized to AKT
150
100
50
0
*
0
30
0
30
LPS (mins)
Mock
HIV

FIGURE 14

Overall, my results show that HIV inhibits p38 and JNK MAPK phosphorylation in human MDMs, whereas AKT phosphorylation is enhanced by LPS in HIV-infected MDMs. Since LPS-induced IL-27 production was shown to be positively regulated through the p38 MAPK, JNK MAPK, and PI3K pathways, these results suggest that HIV may inhibit LPS-induced IL-27 production in MDMs through the inhibition of p38 and JNK MAPK activation.

3.2 The regulation of LPS-induced IL-23 and IL-27 production by PKC isoforms in THP-1 cells, primary monocytes, and MDMs:

There are few studies that have investigated the involvement of PKCs in regulating LPS-induced IL-23 and IL-27. PKCα has been implicated in the positive regulation of IL-27 p28 mRNA expression in murine bone marrow derived MΦs (263), and PKCδ has been implicated in the positive regulation of IL-12/23 p40 in murine MΦs and DCs (262). However, the involvement of PKCs in IL-23 and IL-27 production in human Mos and MΦs has not been investigated. Thus in order to determine whether PKCs regulate LPS-induced IL-23 and IL-27 in human THP-1 cells, primary Mos, and MDMs, two PKC pharmacological inhibitors were employed. The inhibitors were namely, the PKCε translocation inhibitor and the PKCβ inhibitor, which is capable of inhibiting all the cPKCs, and PKCε (a nPKC). The specificity of the PKC inhibitors could not be determined by measuring PKC phosphorylation because of two reasons: 1) the phosphorylation of PKCs can be transient during activation (220) and 2) the PKC ε inhibitor does not specifically inhibit PKC phosphorylation.

THP-1 cells, primary human Mos, and MDMs were treated with the PKCε and PKCβ inhibitor for 2 h prior to LPS-stimulation. The expression of p19, p40, p28, and EBI3 mRNA was assessed by qRT-PCR after 4 h of LPS stimulation, and IL-12/23 p40, IL-23, and IL-27 secretion was assessed by ELISA after 24 h of LPS stimulation.

LPS-induced IL-23 is not regulated by the cPKCs or PKCε in THP-1 cells:

Inhibition of PKCε by its inhibitor did not affect LPS-induced p40 and p19 mRNA expression (Figure 15 A-B), or IL-12/23 p40 and IL-23 secretion in THP-1 cells (Figure 15 C- D). Similarly, the PKCβ inhibitor at concentrations of 2.5, 5, and 10 μM did not significantly affect LPS-induced p40 and p19 mRNA expression (Figure 16 A-B), or IL-12/23 p40 and IL-23 secretion (Figure 16 C-D). Overall, LPS-induced IL-23 production in THP-1 cells is not regulated by the cPKC or PKCε pathways.

The cPKC pathway positively regulates IL-12/23 p40 mRNA expression, and IL-12/23 p40, and IL-23 secretion in LPS-stimulated primary human Mos:

Similar to THP-1 cells, inhibition of PKCε did not affect LPS-induced p40 or p19 mRNA expression, or IL-12/23 p40 or IL-23 secretion in LPS-stimulated primary Mos (Figure 17A-D). However, inhibition with the PKCβ inhibitor resulted in significant inhibition of LPS-induced p40 mRNA expression (Figure 18A), and IL-12/23 p40, and IL-23 secretion (Figure 18C-D), without affecting LPS-induced p19 mRNA expression (Figure18B). Inhibition with the PKCβ inhibitor decreased LPS-induced p40 mRNA

Figure 15: PKCε does not regulate LPS-induced IL-12/23 p40 or p19 mRNA expression or IL-12/23 p40 or IL-23 secretion in THP-1 cells. THP-1 cells were cultured with the PKCε inhibitor for 2 h, prior to LPS-stimulation for 4 h (A-B), or 24 h (C-D). A) p40 mRNA expression (n=4) and B) p19 mRNA expression (n=4) in cell lysates were assessed by qRT-PC. mRNA fold change was calculated relative to unstimulated, untreated MDMs from the same donor. Supernatants were analyzed by ELISA to quantify C) IL-12/23 p40 (n=3) and D) IL-23 secretion (n=7) from THP-1 cells. Statistical test: ANOVA post-hoc Dunnett test comparing all samples to untreated, LPS-stimulated THP-1 cells. ***$p \leq 0.001$.

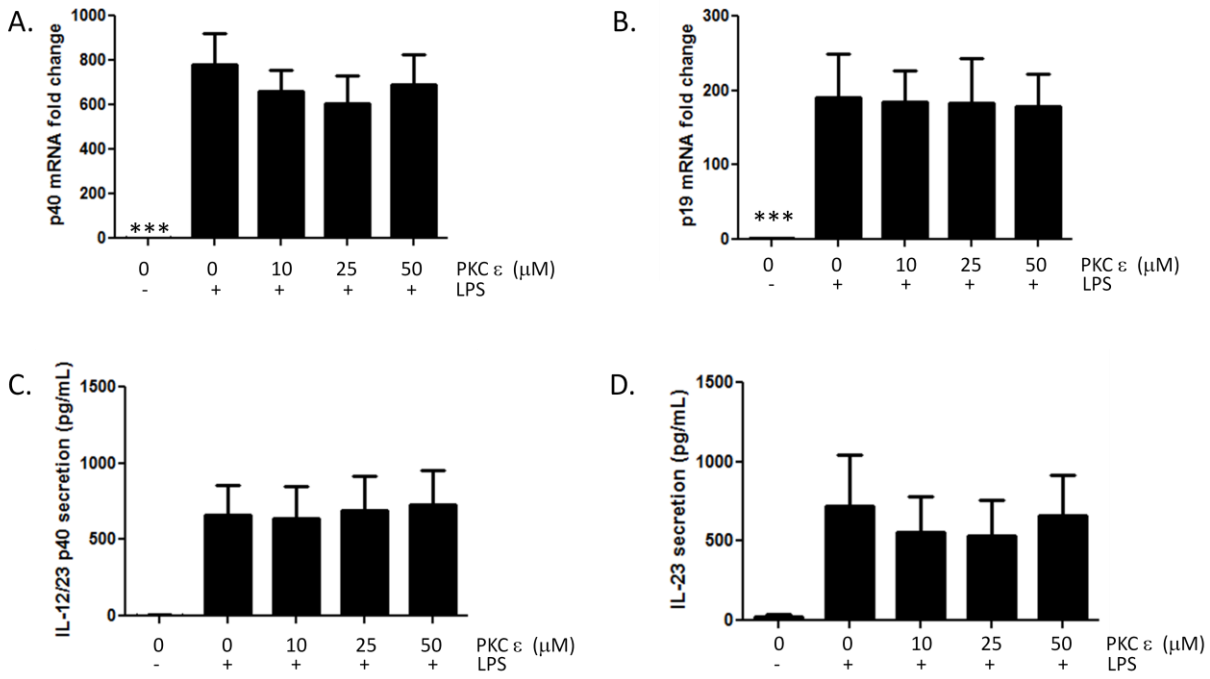
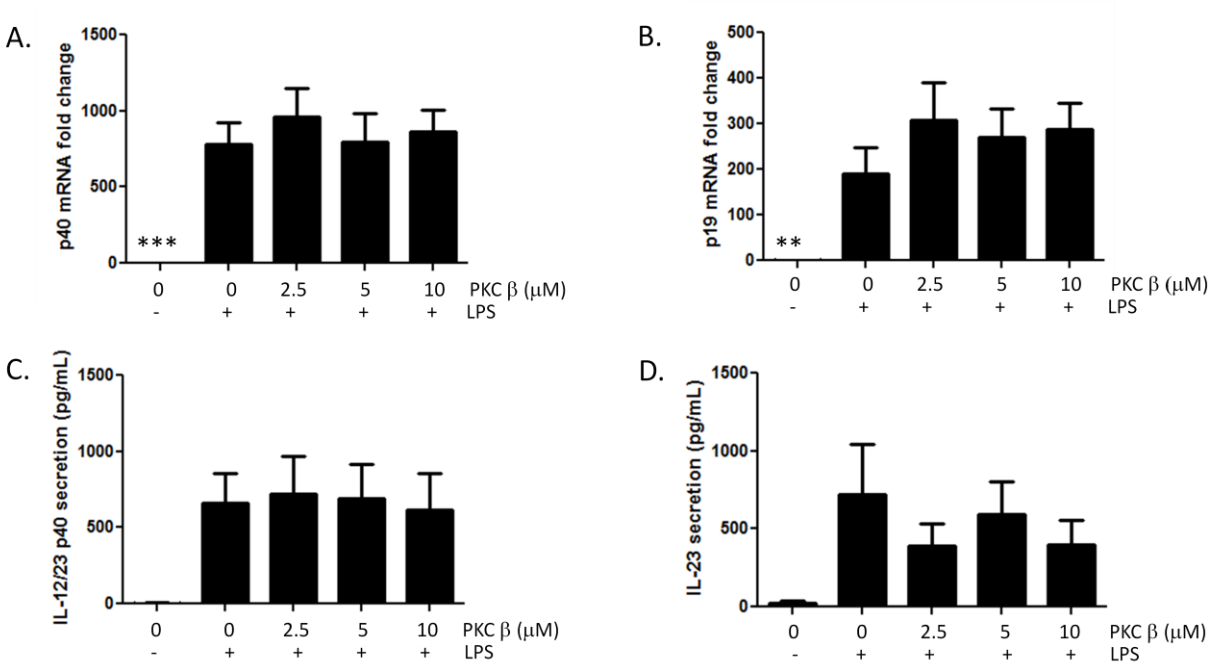

A.
p40 mRNA fold change
1000
800
600
400
200
0
***
0 0 10 25 50 PKC ε (μM)
- + + + + LPS
B.
p19 mRNA fold change
300
200
100
0
***
0 0 10 25 50 PKC ε (μM)
- + + + + LPS
C.
IL-12/23 p40 secretion (pg/mL)
1500
1000
500
0
0 0 10 25 50 PKC ε (μM)
- + + + + LPS
D.
IL-23 secretion (pg/mL)
1500
1000
500
0
0 0 10 25 50 PKC ε (μM)
- + + + + LPS

FIGURE 15

Figure 16: Classical PKCs do not regulate LPS-induced IL-12/23 p40 or IL-23 p19 mRNA expression or IL-12/23 p40 or IL-23 secretion in THP-1 cells. THP-1 cells were treated with the PKCβ inhibitor for 2 h, prior to LPS-stimulation for 4 h (A-B), or 24 h (C-D). A) p40 mRNA expression (n=4) and B) p19 mRNA expression (n=4) in cell lysates were assessed by qRT-PCR. mRNA fold change was calculated relative to unstimulated, untreated MDMs from the same donor. Supernatants were analyzed by ELISA to quantify C) IL-12/23 p40 (n=3) and D) IL-23 (n=7) secretion from THP-1 cells. Statistical test: ANOVA post-hoc Dunnett test comparing all samples to untreated, LPS-stimulated THP-1 cells. **$p \leq 0.01$, ***$p \leq 0.001$.


A.
p40 mRNA fold change
1500
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***
0 0 2.5 5 10 PKC β (μM)
- + + + + LPS
B.
p19 mRNA fold change
500
400
300
200
100
0
**
0 0 2.5 5 10 PKC β (μM)
- + + + + LPS
C.
IL-12/23 p40 secretion (pg/mL)
1500
1000
500
0
0 0 2.5 5 10 PKC β (μM)
- + + + + LPS
D.
IL-23 secretion (pg/mL)
1500
1000
500
0
0 0 2.5 5 10 PKC β (μM)
- + + + + LPS

FIGURE 16

Figure 17: PKCε does not regulate LPS-induced IL-12/23 p40 or IL-23 p19 mRNA expression or IL-12/23 p40 or IL-23 secretion in primary human monocytes. Negatively selected primary monocytes from healthy donors were cultured with the PKCε inhibitor for 2 h, prior to LPS-stimulation for 4 h (A-B) or 24 h (C-D). A) p40 (n=4) and B) p19 (n=4) mRNA expression in cell lysates were assessed by qRT-PCR. mRNA fold change was calculated relative to unstimulated, untreated MDMs from the same donor. Supernatants were analyzed by ELISA to quantify C) IL-12/23 p40 (n=7) and D) IL-23 secretion (n=6) from monocytes. Statistical test: ANOVA post-hoc Dunnett test comparing all samples to untreated, LPS-stimulated monocytes. *$p \leq 0.05$.

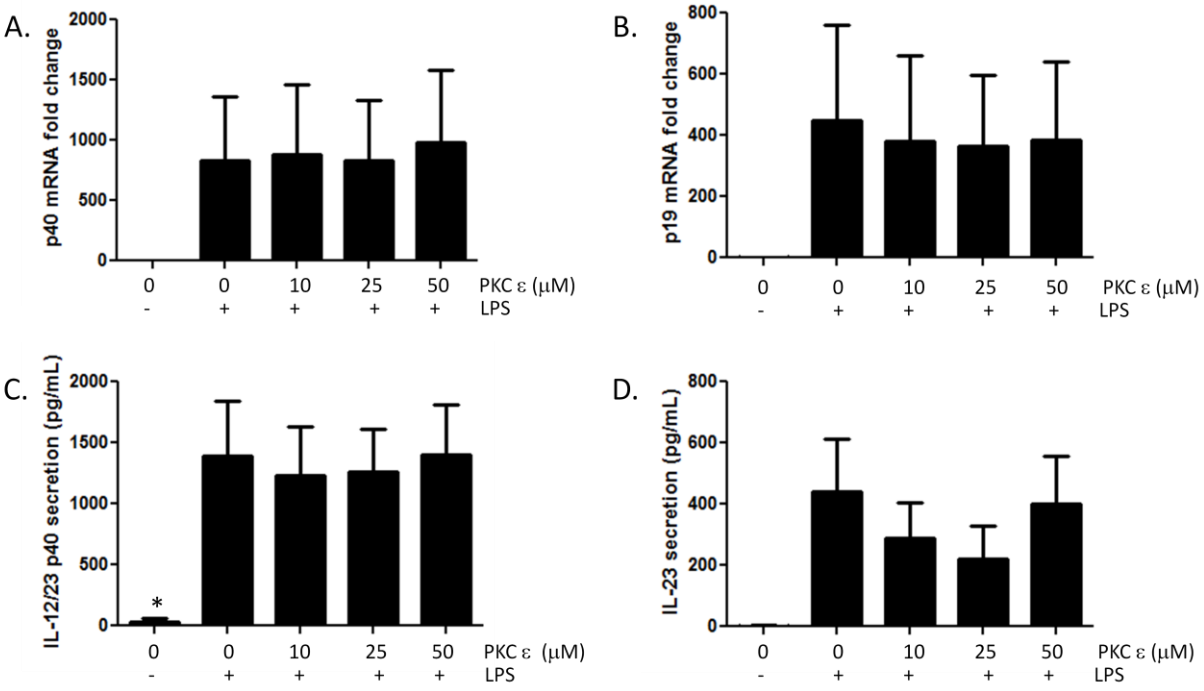
A.
p40 mRNA fold change
2000
1500
1000
500
0
0 0 10 25 50 PKC ε (μM)
- + + + + LPS
B.
p19 mRNA fold change
800
600
400
200
0
0 0 10 25 50 PKC ε (μM)
- + + + + LPS
C.
IL-12/23 p40 secretion (pg/mL)
2000
1500
1000
500
0
*
0 0 10 25 50 PKC ε (μM)
- + + + + LPS
D.
IL-23 secretion (pg/mL)
800
600
400
200
0
0 0 10 25 50 PKC ε (μM)
- + + + + LPS

FIGURE 17

Figure 18: Classical PKCs positively regulate LPS-induced IL-12/23 p40 mRNA expression and IL-12/23 p40 and IL-23 protein secretion in primary human monocytes. Negatively selected primary monocytes from healthy donors were cultured with the PKCβ inhibitor for 2 h, prior to LPS-stimulation for 4 h (A-B) or 24 h (C-D). A) p40 mRNA expression (5 μM: n=3, all others: n=4) and B) p19 mRNA expression (n=4) in cell lysates were assessed by qRT-PCR. mRNA fold change was calculated relative to unstimulated, untreated MDMs from the same donor. Supernatants were analyzed by ELISA to quantify C) IL-12/23 p40 secretion (1 μM: n=4, 2.5 μM: n=6, all others: n=7) and D) IL-23 secretion (1 μM: n=3, 2.5 μM: n=5, all others: n=6) from monocytes. Statistical test: ANOVA post-hoc Dunnett test comparing all samples to untreated, LPS-stimulated monocytes. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

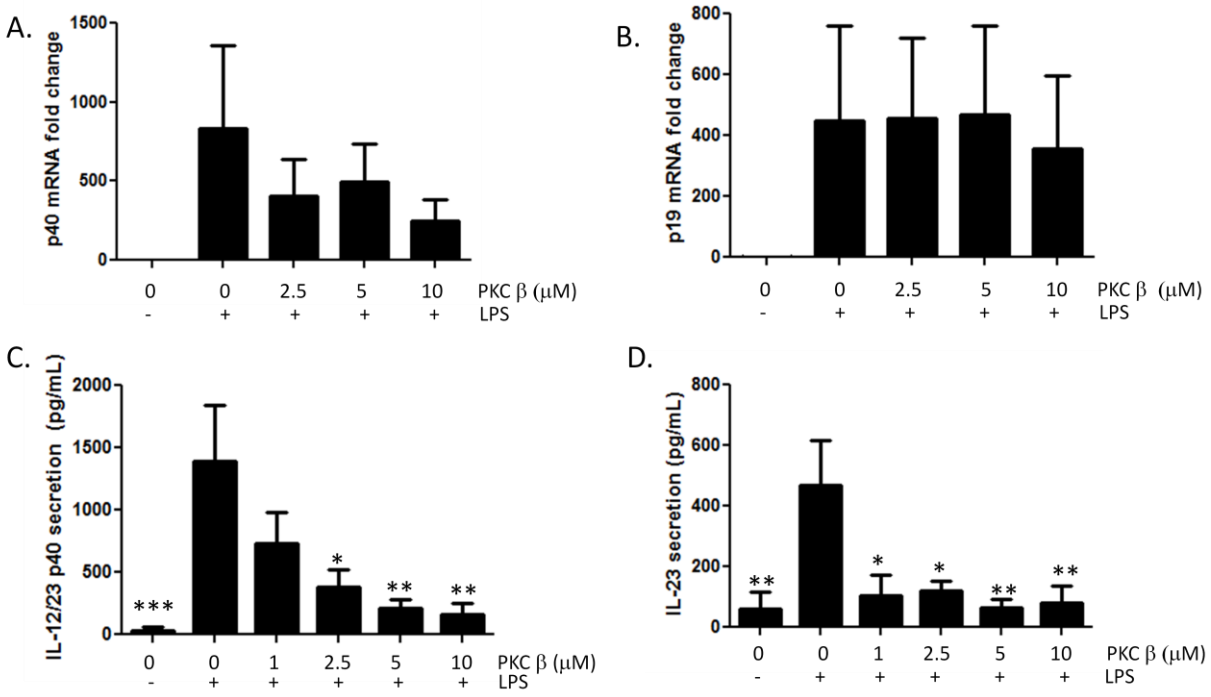
A.
p40 mRNA fold change
1500
1000
500
0
0 0 2.5 5 10 PKC β (μM)
- + + + + LPS
B.
p19 mRNA fold change
800
600
400
200
0
0 0 2.5 5 10 PKC β (μM)
- + + + + LPS
C.
IL-12/23 p40 secretion (pg/mL)
2000
1500
1000
500
0
***
*
**
**
0 0 1 2.5 5 10 PKC β (μM)
- + + + + + LPS
D.
IL-23 secretion (pg/mL)
800
600
400
200
0
**
*
*
**
**
0 0 1 2.5 5 10 PKC β (μM)
- + + + + + LPS

FIGURE 18

expression by 40% and 60% at doses of 5 and 10 μM of PKCβ inhibitor, respectively (Figure 18A). LPS-induced IL-12/23 p40 secretion was significantly decreased by 60%, 80%, and 90% at doses of 2.5, 5, and 10 μM of PKCβ inhibitor, respectively (Figure 18C). Similarly, LPS-induced IL-23 secretion was potently decreased by 65%, 80%, and 80% at doses of 2.5, 5, and 10 μM of PKCβ inhibitor, respectively (Figure 18D). Overall, these results suggest that one or more of the cPKC isoforms may positively regulate IL-23 production through p40 mRNA expression and secretion in LPS-stimulated primary human Mos.

The cPKC pathway positively regulates LPS-induced p28 mRNA expression, and IL-27 secretion in THP-1 cells:

LPS-induced IL-27 EBI3 and p28 mRNA expression, and IL-27 secretion were unaffected by the PKCε inhibitor (Figure 19A-C). Similarly, the PKCβ inhibitor did not affect EBI3 mRNA expression (Figure 20A). In contrast, the PKCβ inhibitor inhibited LPS-induced p28 mRNA expression (Figure 20B), and IL-27 secretion (Figure 20C) in a dose-dependent manner. IL-27 secretion was significantly decreased at the highest dose of PKCβ inhibitor used (10 μM) (Figure 20C). There was a 55% decrease in p28 mRNA expression, and 50% decrease in IL-27 secretion in THP-1 cells treated with the highest dose (10 μM) of the PKCβ inhibitor compared to the untreated LPS-stimulated THP-1 cells (Figure 20B and C). Since inhibition with the PKCε inhibitor had no effect IL-27 production, and inhibition with the PKCβ inhibitor decreased IL-27 secretion and p28 mRNA expression in LPS-stimulated THP-1 cells, these results suggest that one or more of the cPKC isoforms may positively regulate p28 mRNA expression and IL-27 secretion in THP-1 cells.

Figure 19: PKCε does not regulate LPS-induced IL-27 EBI3 or p28 mRNA expression or IL-27 secretion in THP-1 cells. THP-1 cells were treated with the PKCε inhibitor for 2 h prior to LPS-stimulation for 4 h (A-B), or 24 h (C). A) EBI3 mRNA expression (n=4) and B) p28 mRNA expression (25 μM: n=3, all others, n=4) in cell lysates were assessed by qRT-PCR. mRNA fold change was calculated relative to unstimulated, untreated MDMs from the same donor. C) Supernatants were analyzed by ELISA to quantify IL-27 secretion from THP-1 cells (n=9). Statistical test: ANOVA post-hoc Dunnett test comparing all samples to untreated, LPS-stimulated THP-1 cells. *$p \leq 0.05$.

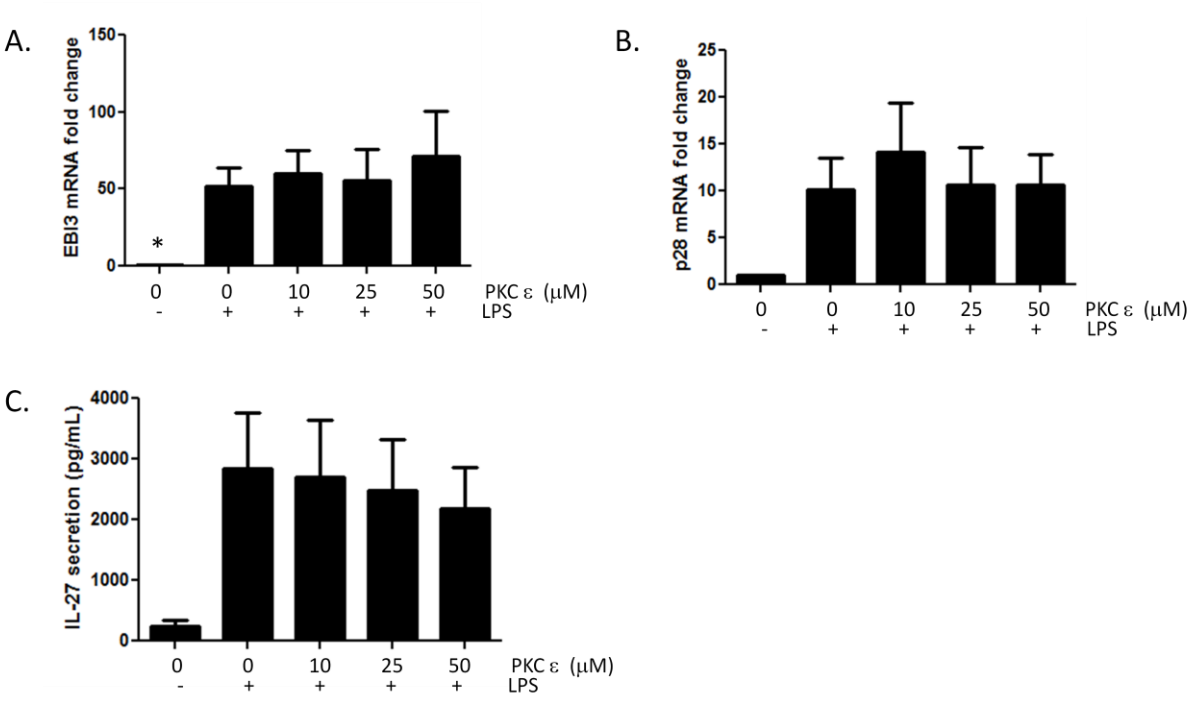
A.
EBI3 mRNA fold change
150
100
50
0
*
0 0 10 25 50 PKC ε (μM)
- + + + + LPS
B.
p28 mRNA fold change
25
20
15
10
5
0
0 0 10 25 50 PKC ε (μM)
- + + + + LPS
C.
IL-27 secretion (pg/mL)
4000
3000
2000
1000
0
0 0 10 25 50 PKC ε (μM)
- + + + + LPS

FIGURE 19

Figure 20: Classical PKCs regulate LPS-induced IL-27 p28 mRNA expression and IL-27 secretion in THP-1 cells. THP-1 cells were cultured with the PKCβ inhibitor for 2 h, prior to LPS-stimulation for 4 h (A-B), or 24 h (C). EBI3 (n=4) and B) p28 mRNA expression (n=4) in cell lysates were assessed by qRT-PCR. mRNA fold change was calculated relative to unstimulated, untreated MDMs from the same donor. C) Supernatants were analyzed by ELISA to quantify IL-27 secretion from THP-1 cells (n=4). Statistical test: ANOVA post-hoc Dunnett test comparing all samples to untreated, LPS-stimulated THP-1 cells. **p≤0.01, ***p≤0.001

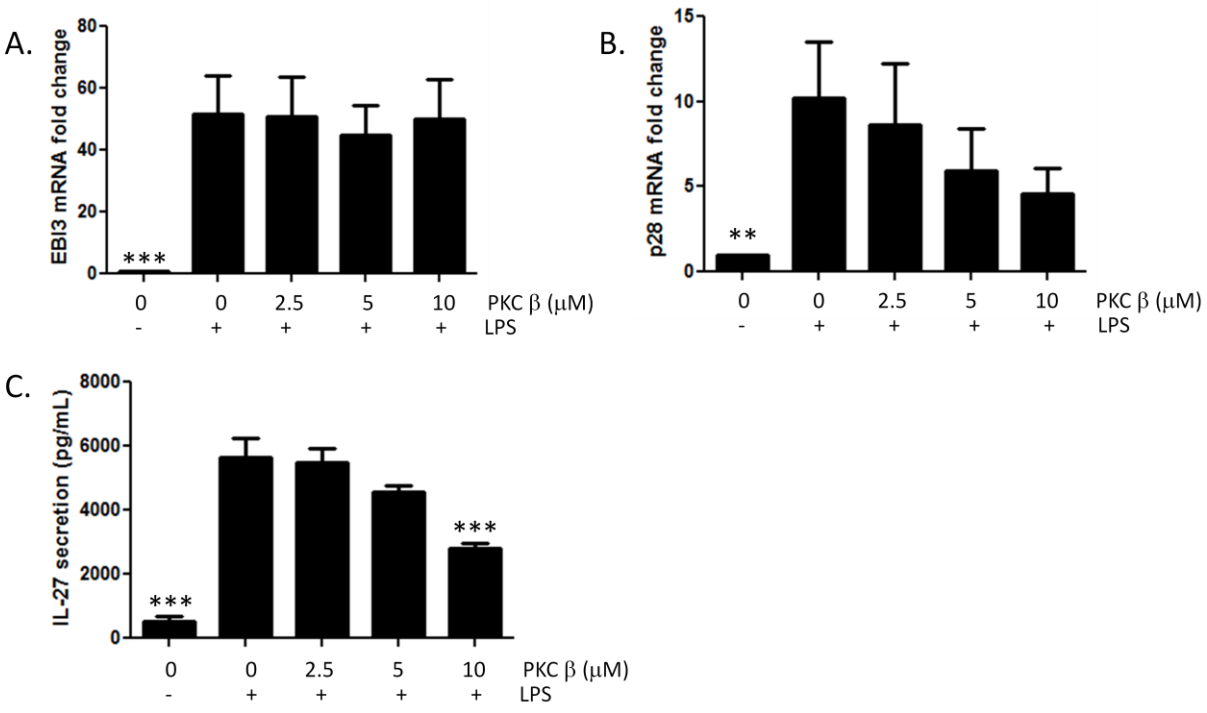
A.
EBI3 mRNA fold change
80
60
40
20
0
***
0 0 2.5 5 10 PKC β (μM)
- + + + + LPS
B.
p28 mRNA fold change
15
10
5
0
**
0 0 2.5 5 10 PKC β (μM)
- + + + + LPS
C.
IL-27 secretion (pg/mL)
8000
6000
4000
2000
0
***
***
0 0 2.5 5 10 PKC β (μM)
- + + + + LPS

FIGURE 20

The cPKC pathway positively regulates p28 mRNA expression and IL-27 secretion in LPS-stimulated primary human Mos:

Similar to the results obtained in THP-1 cells, PKCε inhibition did not affect LPS-induced EBI3 and p28 mRNA expression and IL-27 secretion in primary human Mos (Figure 21A-C). In contrast to the PKCε inhibitor, PKCβ inhibitor significantly inhibited LPS-induced p28 mRNA expression and IL-27 secretion in a dose-dependent manner, but did not affect LPS-induced EBI3 mRNA expression (Figure 22A-C). Concentrations of 2.5, 5, and 10 μM of the PKCβ inhibitor resulted in a 55%, 40%, and 65% decrease in LPS-induced p28 mRNA expression, respectively (Figure 22B). Similarly, LPS-induced IL-27 secretion was significantly inhibited by 20%, 30%, and 50% at doses of 2.5, 5, and 10 μM of the PKCβ inhibitor, respectively (Figure 22C). Overall, these results suggest that cPKCs positively regulate LPS-induced p28 mRNA expression and IL-27 secretion, but not EBI3 mRNA expression in primary human Mos.

The cPKC and PKCε pathways do not regulate LPS-induced IL-27 production in MDMs:

Since it was previously observed that IL-23 is not consistently produced in response to LPS in MDMs (Figure 9), I only investigated the role of PKCs in the regulation of LPS-induced IL-27 production in MDMs.

Treatment of MDMs with the PKCε (Figure 23A-C) and PKCβ inhibitor (Figure 24A-C) did not affect LPS-induced EBI3, and p28 mRNA expression, or IL-27 secretion in MDMs (Figure 23A-C; Figure 24A-C). Taken together, my results suggest that the cPKCs positively regulate LPS-induced IL-27 production in THP-1 cells and primary human Mos, but do not regulate LPS-induced IL-27 production in human MDMs.

Figure 21: PKCε does not regulate LPS-induced IL-27 EBI3 or p28 mRNA expression or IL-27 secretion in primary human monocytes. Negatively selected primary monocytes from healthy donors were cultured with the PKCε inhibitor for 2 h, prior to LPS-stimulation for 4 h (A-B) to 24 h (C). A) EBI3 mRNA expression (n=4) and B) p28 mRNA expression were quantified from cell lysates by qRT-PCR (n=3). mRNA fold change was calculated relative to unstimulated, untreated MDMs from the same donor. C) Supernatants were analyzed by ELISA to quantify IL-27 secretion from monocytes (n=5). Statistical test: ANOVA post-hoc Dunnett test comparing all samples to untreated, LPS-stimulated monocytes. **$p \leq 0.01$, ***$p \leq 0.001$.

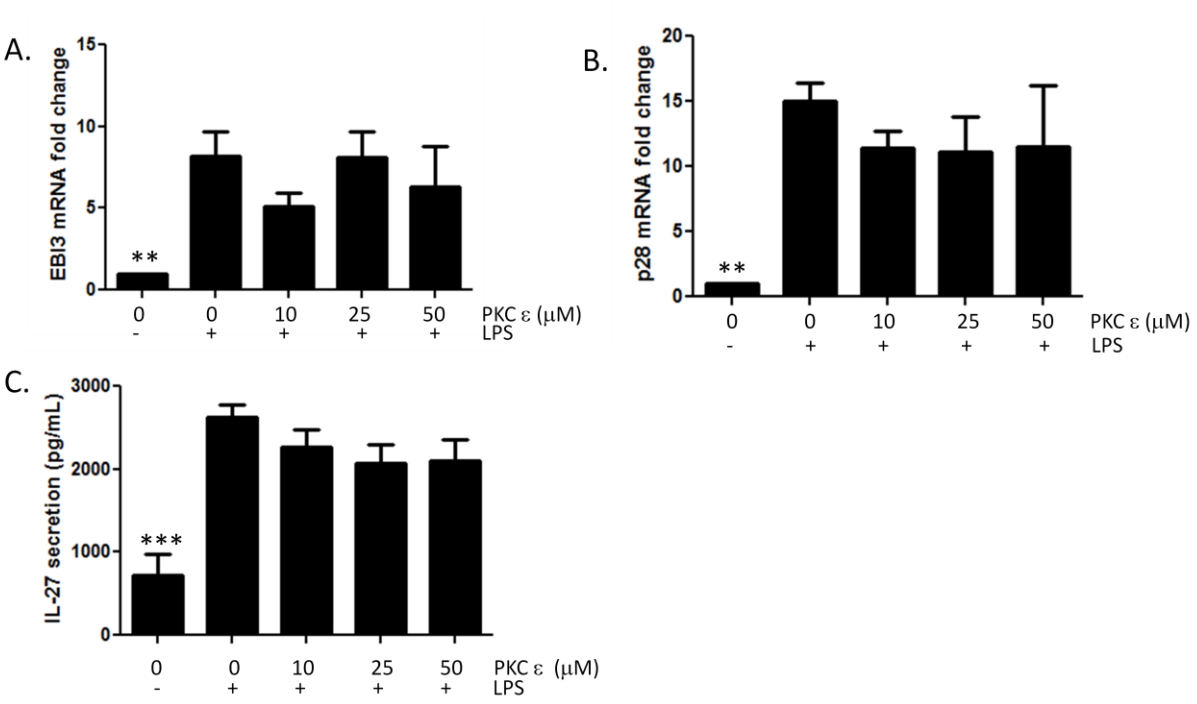
A.
EBI3 mRNA fold change
15
10
5
0
**
0
-
0
+
10
+
25
+
50
+
PKC ε (μM)
LPS
B.
p28 mRNA fold change
20
15
10
5
0
**
0
-
0
+
10
+
25
+
50
+
PKC ε (μM)
LPS
C.
IL-27 secretion (pg/mL)
3000
2000
1000
0
***
0
-
0
+
10
+
25
+
50
+
PKC ε (μM)
LPS

FIGURE 21

Figure 22: Classical PKCs regulate LPS-induced IL-27 p28 mRNA expression and IL-27 secretion in primary human monocytes. Negatively selected primary monocytes from healthy donors were cultured with the PKCβ inhibitor for 2 h, prior to LPS-stimulation for 4 h (A-B) or 24 h (C) A) EBI3 mRNA expression (n=4) and B) p28 mRNA expression (n=3) were quantified from cell lysates by qRT-PCR. mRNA fold change was calculated relative to unstimulated, untreated MDMs from the same donor. C) Supernatants were analyzed by ELISA to quantify IL-27 secretion from monocytes (2.5 μM n=4; all others n=5). Statistical test: ANOVA post-hoc Dunnett test comparing all samples to untreated, LPS-stimulated monocytes. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

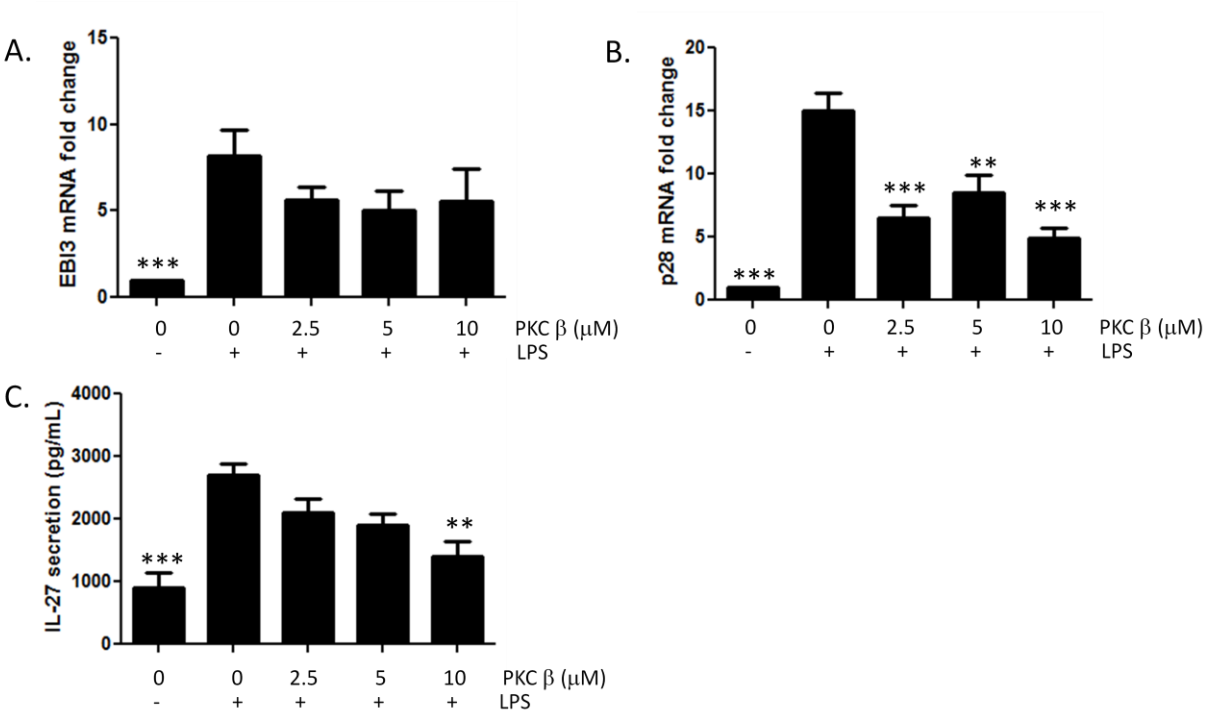
A.
EBI3 mRNA fold change
15
10
5
0
***
0
0
2.5
5
10
PKC β (μM)
-
+
+
+
+
LPS
B.
p28 mRNA fold change
20
15
10
5
0
***
***
**
***
0
0
2.5
5
10
PKC β (μM)
-
+
+
+
+
LPS
C.
IL-27 secretion (pg/mL)
4000
3000
2000
1000
0
***
**
0
0
2.5
5
10
PKC β (μM)
-
+
+
+
+
LPS

FIGURE 22

Figure 23: PKCε does not regulate LPS-induced IL-27 EBI3 or p28 mRNA expression or IL-27 secretion in MDMs. MDMs from healthy donors were treated with the PKCε inhibitor for 2 h prior to LPS-stimulation for 4 h (A-B) or 24 h (C). A) EBI3 mRNA expression (n=6) and B) p28 mRNA expression in cell lysates were quantified by qRT-PCR (n=6). mRNA fold change was calculated relative to unstimulated, untreated MDMs from the same donor. C) Supernatants were analyzed by ELISA to quantify IL-27 secretion from MDMs (n=4). Statistical test: ANOVA post-hoc Dunnett test comparing all samples to untreated, LPS-stimulated MDMs. *$p \leq 0.05$, **$p \leq 0.01$.

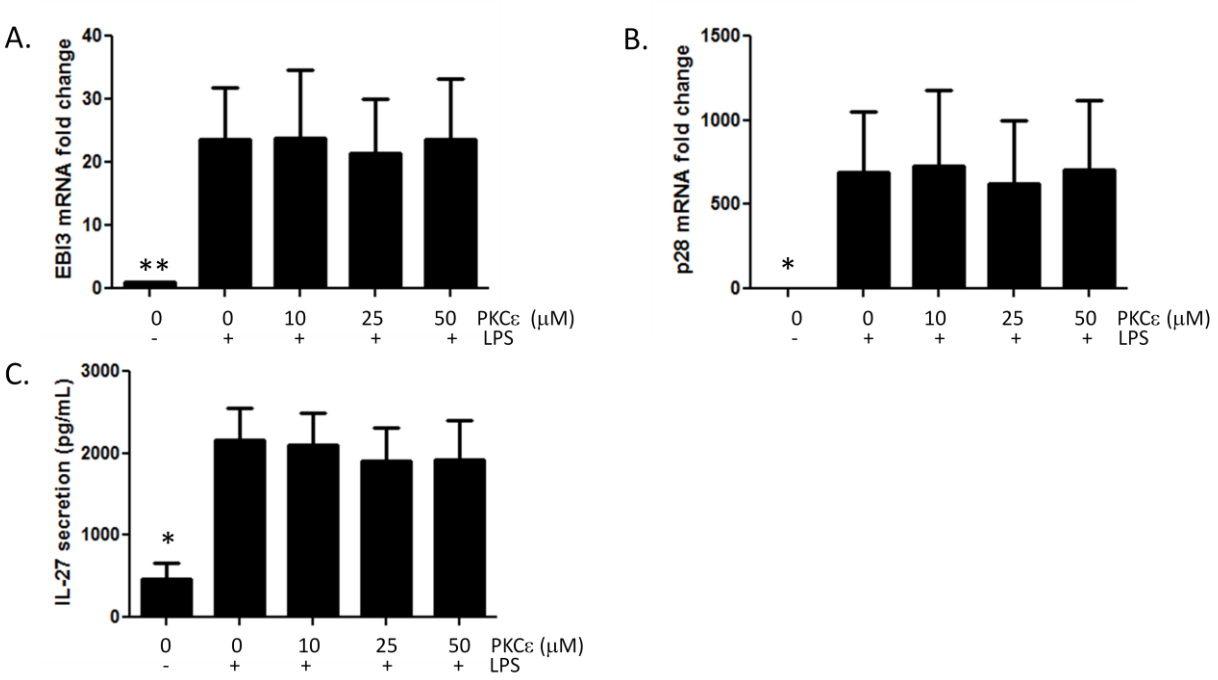
A.
EBI3 mRNA fold change
40
30
20
10
0
**
0 0 10 25 50 PKCε (μM)
- + + + + LPS
B.
p28 mRNA fold change
1500
1000
500
0
*
0 0 10 25 50 PKCε (μM)
- + + + + LPS
C.
IL-27 secretion (pg/mL)
3000
2000
1000
0
*
0 0 10 25 50 PKCε (μM)
- + + + + LPS

FIGURE 23

Figure 24: Classical PKCs do not regulate LPS-induced IL-27 EBI3 and p28 mRNA expression or IL-27 secretion in MDMs. MDMs from healthy donors were treated with the PKCβ inhibitor for 2 h prior to LPS-stimulation for 4 h (A-B) or 24 h (C). A) EBI3 (n=6) and B) p28 mRNA expression (n=6) from cell lysates were assessed by qRT-PCR. mRNA fold change was calculated relative to unstimulated, untreated MDMs from the same donor. C) Supernatants were analyzed by ELISA to quantify IL-27 secretion from MDMs (n=5). Statistical test: ANOVA post-hoc Dunnett test comparing all samples to untreated, LPS-stimulated MDMs. *$p \leq 0.05$, ***$p \leq 0.001$.

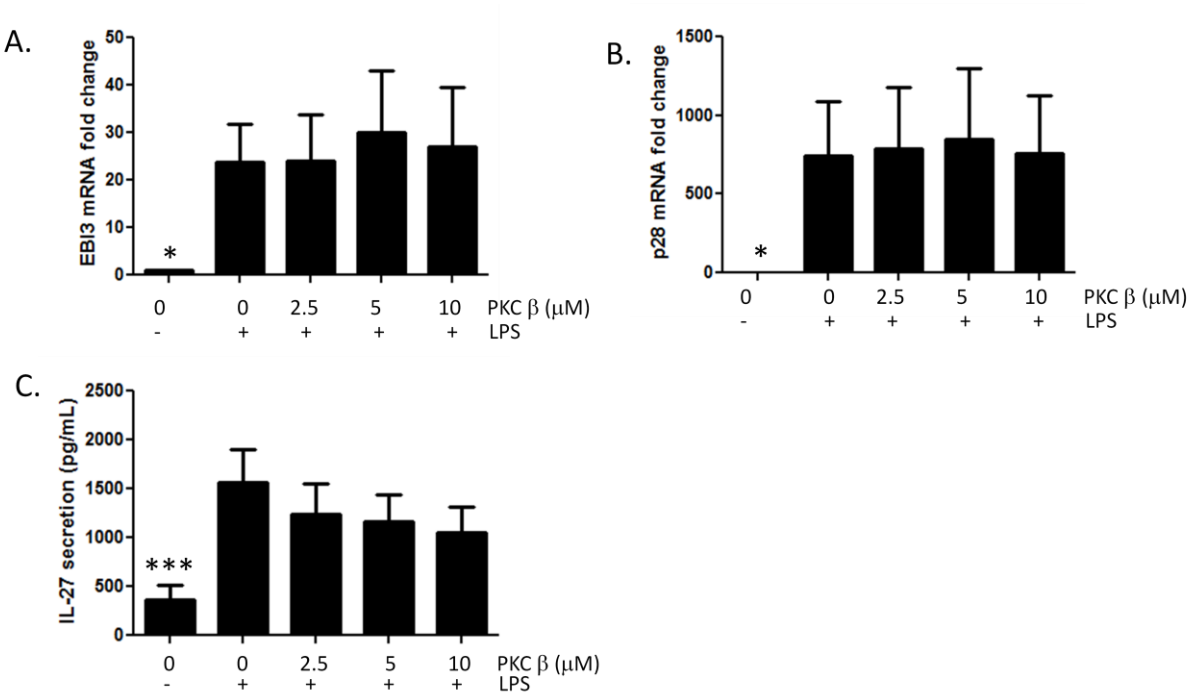
A.
EBI3 mRNA fold change
50
40
30
20
10
0
*
0 0 2.5 5 10 PKC β (μM)
- + + + + LPS
B.
p28 mRNA fold change
1500
1000
500
0
*
0 0 2.5 5 10 PKC β (μM)
- + + + + LPS
C.
IL-27 secretion (pg/mL)
2500
2000
1500
1000
500
0
***
0 0 2.5 5 10 PKC β (μM)
- + + + + LPS

FIGURE 24

siRNA knockdown of PKCβII, PKCα, and PKCγ does not inhibit IL-27 secretion in LPS-stimulated THP-1 cells:

In order to determine which cPKC isoforms were responsible for the regulation of IL-23 and IL-27 in THP-1 and primary Mos, knockdown of specific cPKC isoforms by siRNA was used. However, it is very difficult to significantly inhibit mRNA expression by siRNA in primary Mos since these cells begin to undergo spontaneous apoptosis within 24 h of culture and surviving cells differentiate into MΦ-like cells (289, 290). Since attempts to knockdown proteins by siRNA in these cells would not be representative of healthy Mos, the involvement of the cPKCs in the regulation of LPS-induced IL-23 and IL-27 production in primary human Mos was not further evaluated.

In order to determine which specific cPKC isoforms regulates LPS-induced p28 mRNA expression and IL-27 secretion in THP-1 cells, siRNA was employed. PKCβII (Figure 25A) and PKCα knockdown (Figure 25C) did not inhibit LPS-induced IL-27 secretion (Figure 25B and D). PKCγ knockdown with siRNA was more difficult to accomplish. PKCγ was only successfully knocked-down once (Figure 25E) out of seven experiments and in this one experiment LPS-induced IL-27 secretion was not inhibited (Figure 25F). PKCγ siRNA knockdown was attempted with four different transfection reagents (Lipofectamine, Santa Cruz Tranfection Reagent, Mirus, and Fugene 6), at different concentrations, and varying concentrations of siRNA, and was still unsuccessful (data not shown). Notably, PKCβII, PKCα, and PKCγ siRNA knockdown did not result in a 100% knockdown of the respective proteins (Figure 25A,C,and F), and treatment of THP-1 cells with PKCβ inhibitor only resulted in significant inhibition of LPS-induced IL-27 secretion at the highest dose of inhibitor used (10 μM) (Figure 20C). Notably, 10 μM of PKCβ inhibitor

Figure 25: PKCβII, PKCα, and PKCγ siRNA do not inhibit LPS-induced IL-27 secretion in THP-1 cells. THP-1 cells were treated with siRNA specific for PKCβII, PKCα, and PKCγ. Non-specific scrambled siRNA was used as the control. THP-1 cells were stimulated with LPS for 24 h, post the addition of siRNA for 24 h or 48 h, to assess IL-27 secretion from THP-1 cells. Protein knockdown in cell lysates was confirmed by western blot. One representative western blot showing A) PKCβII C) PKCα and E) PKCγ protein knockdown in THP-1 cells is shown. Culture supernatants were analyzed by ELISA to quantify IL-27 secretion from unstimulated and LPS-stimulated THP-1 cells in B) control and PKCβII siRNA treated cells (n=3), D) control and PKCα siRNA treated cells (n=4), and F) control and PKCγ siRNA treated cells (n=1). Statistical test: Paired student t-test, NS= Not significant.

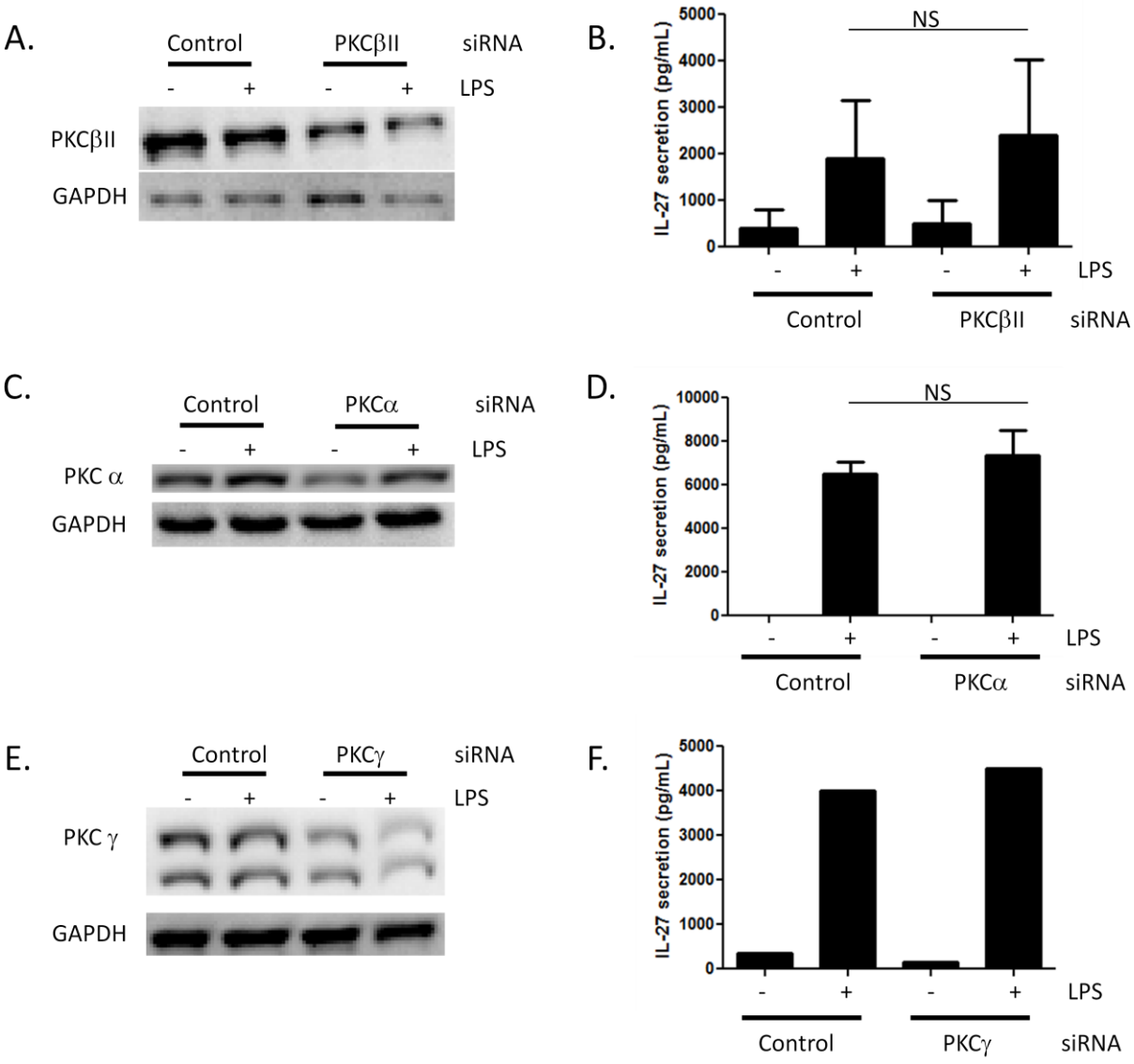
A.
Control
PKCβII
siRNA
- + - +
LPS
PKCβII
GAPDH
B.
NS
IL-27 secretion (pg/mL)
5000
4000
3000
2000
1000
0
- + - +
LPS
Control
PKCβII
siRNA
C.
Control
PKCα
siRNA
- + - +
LPS
PKC α
GAPDH
D.
NS
IL-27 secretion (pg/mL)
10000
8000
6000
4000
2000
0
- + - +
LPS
Control
PKCα
siRNA
E.
Control
PKCγ
siRNA
- + - +
LPS
PKC γ
GAPDH
F.
IL-27 secretion (pg/mL)
5000
4000
3000
2000
1000
0
- + - +
LPS
Control
PKCγ
siRNA

FIGURE 25

was at least 10 times greater than the IC_{50} (concentration that inhibits 50% of the PKC isoform) of all the cPKCs (Calbiochem, San Diego, CA). Overall these results were inconclusive in determining which cPKC isoform regulates p28 mRNA expression and IL-27 secretion in LPS-stimulated THP-1 cells.

CHAPTER IV: DISCUSSION

HIV-infected individuals have increased susceptibility to age related diseases due to chronic immune activation, caused by viral persistence and microbial translocation. There is an increasing need to develop better therapeutics to decrease immune activation in HIV infection. IL-23 and IL-27 are potential candidates for therapy to decrease chronic immune activation in HIV infection. IL-23 therapy could increase the number of Th17 cells, improving mucosal immunity and resistance to opportunistic infection, whereas IL-27 therapy could improve HIV-specific immune responses, and inhibit viral replication. However, very little is known about the effect of HIV on IL-23 and IL-27 production. Understanding how HIV influences IL-23 and IL-27 production will be important in developing effective strategies to increase IL-23 and IL-27 production in HIV infection.

I have shown that IL-23 and IL-27 expression was not affected following *in vitro* infection of MDMs with HIV. However, HIV inhibited LPS-induced IL-23 and IL-27 secretion through the inhibition of IL-23 p19 and p40 mRNA expression, IL-12/23 p40 protein secretion, and IL-27 p28 and EBI3 mRNA expression, respectively. Furthermore, LPS-induced IL-27 was positively regulated by the PI3K, p38 MAPK and JNK MAPK pathways in MDMs. *In vitro* HIV infection of MDMs was shown to inhibit the activation of p38 and JNK MAPK in LPS-stimulated MDMs suggesting this to be the mechanism by which HIV inhibits LPS-induced IL-27 production in MDMs.

I have also demonstrated that LPS-induced IL-27 and IL-23 are differentially regulated by the PKC pathway in THP-1 cells, primary Mos, and MΦs. The cPKCs positively regulated LPS-induced p40 mRNA expression, and secretion, and IL-23 secretion in primary Mos but not in THP-1 cells. Moreover, the cPKCs positively regulated IL-27

secretion through the regulation of p28 mRNA expression in LPS-stimulated THP-1 cells, and primary Mos but not in MDMs.

Effect of HIV on IL-23 and IL-27 production in MDMs:

IL-23 and IL-27 production has been observed in response to TLR-7/8 stimulation in MΦs and DCs (175, 179, 291, 292). Synthetic G-U rich HIV-encoded ssRNA has been shown to activate TLR-7 and TLR-8 in PBMCs, plasmacytoid DCs, and Mos to produce various cytokines (285, 286). Thus, it was of interest to determine whether *in vitro* HIV infection of MDMs could induce IL-23 or IL-27 production. My results show that *in vitro* HIV infection did not affect basal production of IL-23 or IL-27 or their subunits' mRNA expression in human MDMs. In agreement with my study, Brown et al has shown that the production of TNFα, IL-1, and IL-6 were not affected in human MΦs infected with HIV for 9 days (26). In the same study, the phosphorylation of IRF-3 and IRAK-1 were not induced in response to HIV-stimulation for 30 min to 24 h (26). TLR-activation generally leads to the activation of MyD88- and/or TRIF-dependent pathways. IRAK-1 is activated down-stream of MyD88-dependent signalling (293), whereas, IRF-3 is activated down-stream of TRIF-dependent and MyD88-independent pathways (294). These results suggest that HIV does not activate MyD88- or TRIF-dependent TLR-signalling in human MΦs. In contrast, Beignon et al has demonstrated that stimulation of plasmacytoid DCs with whole HIV induced IFNα via TLR-dependent mechanisms (287). These discrepancies in cytokine production may be attributed to the different cell model systems used for *in vitro* HIV infection. It is possible that stimulation of Mos/MΦ with HIV virions in this study, as opposed to synthetic HIV-ssRNA, may have allowed HIV to evade TLR-7/8 recognition and have no impact on IL-

23/IL-27 production in MDMs. Alternatively, prolonged exposure with HIV may have rendered MΦs tolerant to continuous TLR-7/8 stimulation. Thus my results reveal new questions: 1) Can synthetic HIV-ssRNA trigger IL-23 or IL-27 production? 2) Does HIV evade immune recognition from TLR-7/8 in MΦs? and finally 3) Does prolonged infection with HIV render MΦs tolerant to TLR-7/8 stimulation?

HIV infection has been shown to influence responsiveness of myeloid cells to TLR-stimulation (26, 295). For example, HIV infected MΦs produced significantly higher levels of TNFα, IL-6 and IL-1 in response to LPS (TLR-4), Poly (I:C) (TLR-3), CL097 (TLR-7/8), CL075 (TLR-8), and R387 (TLR-7) (26). In contrast, pre-treatment of murine MΦs with TLR-8 agonist (R848) or HIV ssRNA rendered these cells tolerant to restimulation with the same ligand resulting in decreased production of MIP-1β through the inhibition of IRAK-1 phosphorylation (295). Similarly, MΦs obtained from HIV-infected individuals exhibited suppressed induction of TNFα in response to TLR-4 stimulation (296). My results show for the first time that although HIV on its own did not alter IL-23 and IL-27 production, *in vitro* HIV-infection of MDMs inhibited LPS-induced IL-23 and IL-27 production. This suggests that HIV infection may have rendered MDMs tolerant to LPS-stimulation.

Inhibition of LPS-induced IL-23 production may influence HIV pathogenesis. IL-23 expression can have both pathogenic and protective functions. Over-expression of IL-23 has been correlated with various inflammatory diseases including rheumatoid arthritis (297), psoriasis (298), inflammatory bowel disease (299), and multiple sclerosis (155). Transgenic mice over-expressing p19 are characterized by systemic inflammation, cytokine dysregulation, and increased infiltration of lymphocytes, and MΦs in the skin, liver, pancreas, lungs, and digestive tract (300). Thus, the pathogenic role of IL-23 in autoimmune

diseases and inflammation raises concerns for the use of IL-23 as a therapy in HIV infections. In fact, inhibition of IL-23 production in HIV infection has been suggested to reduce chronic inflammation and immune activation (156).

IL-23 may also play a protective role in HIV infection. IL-23 has been shown to play an essential role in the clearance of various pathogens including *M. tuberculosis,* and *Toxoplasma gondii* (301, 302), which are common co-infections in HIV infection (303). Additionally, IL-23 is important for the maintenance of Th17 cells, and induction of IL-17 from Th17 cells (142). Th17 cells numbers and IL-17 production are decreased in the gut during HIV/SIV infection and have been correlated with increased microbial translocation (161, 167, 169, 304). Both the overall numbers and relative proportion of Th17 cells are decreased in HIV infection (162). Therefore inhibition of IL-23 may lead to decreased Th17 cell numbers which may contribute to increased microbial translocation and susceptibility to opportunistic infections in HIV-infected individuals. Furthermore, increased IL-23 production from DCs has been associated with increased cross-presentation of HIV-antigens to CD8 T-cells (101). Thus inhibition of IL-23 by HIV in infected human MΦs may decrease HIV-specific CD8 T-cell priming, leading to impaired immune responses. The multiple symptoms in HIV disease makes it difficult to elucidate the role of IL-23 in HIV disease especially since little is known about IL-23 expression through the course of disease. Although, my results show that IL-23 production is inhibited following *in vitro* HIV infection, further studies are needed to understand whether IL-23 production is altered in *in vivo* HIV infection through disease progression.

Guzzo et al has demonstrated a trend for decreased IL-27 secretion in the serum of HIV-infected individuals (68). Consistent with these results, I have shown that LPS-induced IL-27 production is significantly decreased in *in vitro* HIV infection. Decreased IL-27

production in HIV infection may influence HIV pathogenesis. IL-27 has been shown to increase IL-12βR1 expression, and synergistically increase IL-12-induced IFNγ production from Th1 cells (94). IL-27 production from DCs has been positively correlated with cross-presentation of HIV-antigens to CD8 T-cells (101). Furthermore, IL-27 has been shown to inhibit HIV replication (203, 204). Therefore, inhibition of IL-27 may inhibit Th1 and cell-mediated immune responses, and increase HIV replication in infected individuals. Thus, IL-27 therapy may be beneficial to improve HIV disease outcome.

IL-27 has also been shown to inhibit Treg and Th17 cell development (194-196). Interestingly, there is a reciprocal development between Treg and Th17 cells (143), and imbalances between the ratio of Treg and Th17 cells have been correlated with various diseases including HIV (305). Since IL-27 is capable of inhibiting both Th17 and Treg cells, it is important to investigate the effect of IL-27 on Th17/Treg developement in HIV infection.

Signalling pathways regulating LPS-induced IL-27 production in MDMs:

In order to elucidate the signalling mechanisms dysregulating LPS-induced IL-23 and IL-27 production in HIV-infected MDMs, I determined the signalling pathways involved in the regulation of LPS-induced IL-23 and IL-27 production in MDMs.

The differential ability of MΦs from different donors to produce IL-23 in response to LPS in this study was peculiar. Two types of MΦ polarizations have been described, namely, classical type-1 (M1) and type-2 (M2) MΦs (288, 306). M1-responses consist of high IL-12 and IL-23 expression, efficient antigen presentation capacity (288), and their ability to induce Th1 differentiation (306). M2 MΦs have been used as a more general term to

describe MΦs that are not M1 MΦs (306). Following this nomenclature, Verreck and colleagues defined M1 MΦs as those differentiated with granulocyte macrophage colony-stimulating factor (GM-CSF) (288) and M2 MΦs as those differentiated with M-CSF for 6 days (288). Although I generated MDMs following culture with M-CSF, MDMs from approximately 60% of donors did not produce IL-23; however, MDMs from all donors produced IL-12/23 p40 in response to LPS. However, when MDM were differentiated with M-CSF for 7 days and cultured for another 5, 8, or 15 days, IL-23 secretion and p40 and p19 mRNA expression was induced in response to LPS in all donors. Further investigations of these MDMs is needed in order to fully understand why IL-23 is produced in response to LPS in some donors, but not others at early time-points post-differentiation. Due to these discrepancies in IL-23 production from LPS-stimulated MDMs, I did not further investigate the pathways regulating LPS-induced IL-23 production.

I have demonstrated that IL-27 was positively regulated by the PI3K, p38 MAPK, and JNK MAPK pathways in LPS-stimulated human MDMs. The pathways regulating LPS-induced IL-27 production in MDMs are summarized in Figure 26. LPS-induced EBI3 and p28 mRNA expression and EBI3 protein expression were all inhibited by the PI3K inhibitor, LY294002. However, in a previous study the PI3K pathway was not implicated in the regulation of LPS/IFNγ-induced IL-27 production in myeloid DCs (265). This discrepancy may be attributed to the different cell types and stimuli used in my study.

The p38 MAPK pathway has been shown to positively regulate LPS-induced IL-27 mRNA in human astrocytes (271), and IFNγ/LPS-induced IL-27 secretion in myeloid DCs (265). However, Gorina et al did not specify whether IL-27 mRNA referred to EBI3 or p28 expression (271). Therefore, my results are the first study to demonstrate that the p38 MAPK

Figure 26: The signalling pathway regulating LPS-induced IL-27 production in human MDMs. The JNK MAPK pathway positively regulates LPS-induced IL-27 secretion through the regulation of p28 mRNA expression, and EBI3 protein expression, but not EBI3 mRNA expression. The PI3K pathway positively regulates IL-27 secretion in MDMs, through the regulation of p28 and EBI3 mRNA expression, and EBI3 protein expression. The p38 MAPK pathway positively regulates IL-27 secretion through the regulation of EBI3 mRNA and protein expression, but not p28 mRNA expression. *In vitro* HIV infection of MDMs inhibits JNK and p38 MAPK activity, and likely contributes to the inhibition of p28, and EBI3 mRNA expression, and IL-27 secretion observed in HIV infection MDMs.

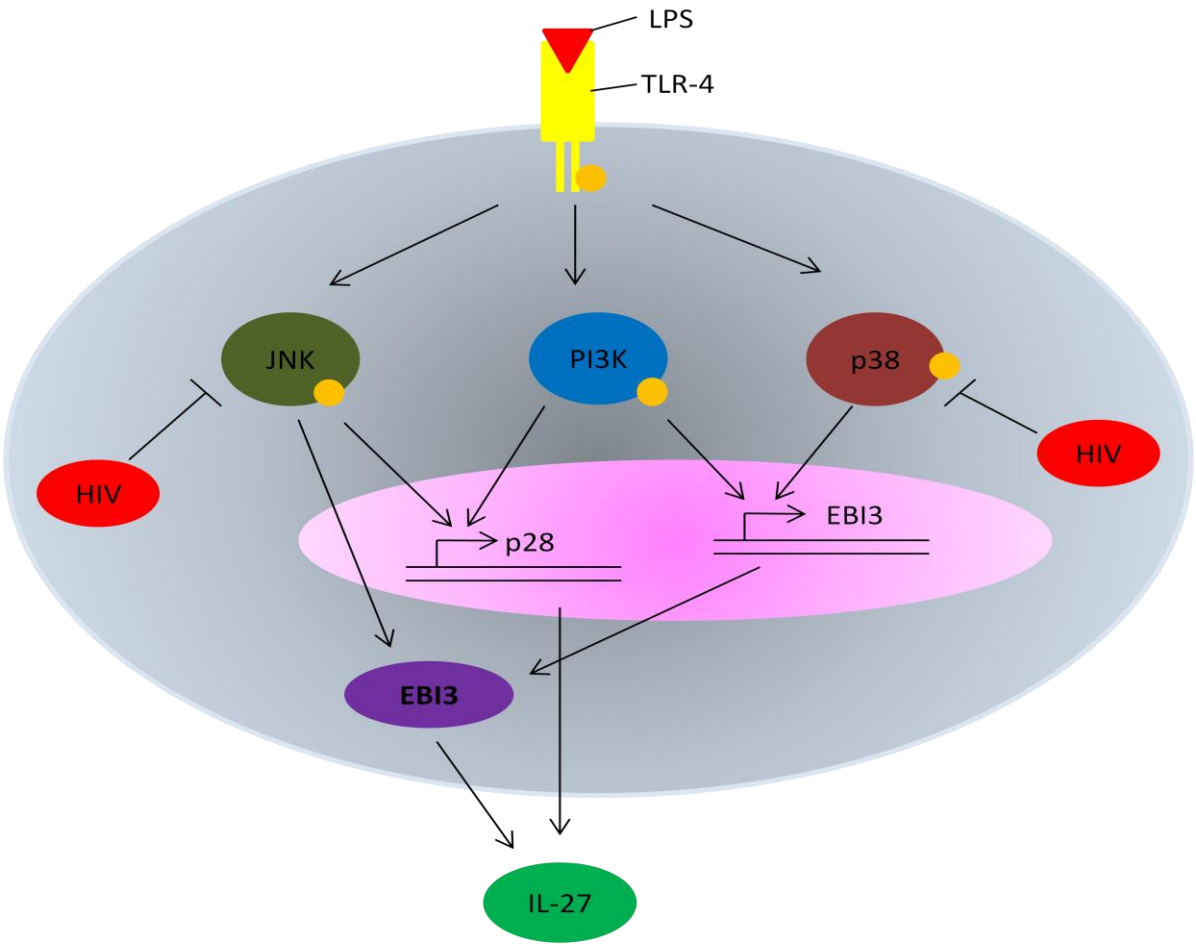

FIGURE 26

pathway positively regulates LPS-induced IL-27 production through the positive regulation of EBI3 mRNA and protein expression in human MDMs, and that the p38 MAPK pathway does not regulate LPS-induced p28 mRNA expression.

In addition to the involvement of the p38 MAPK pathway, I demonstrated a positive role for the JNK MAPK pathway in the regulation of IL-27 production in LPS-stimulated MDMs through the positive regulation of p28 mRNA expression, and EBI3 protein expression. However, JNK did not regulate EBI3 mRNA expression. Consistent with these results, LPS/IFNγ-induced IL-27 production was shown to be positively regulated by the JNK MAPK pathway in myeloid DCs (265). The differential regulation of EBI3 protein and mRNA expression suggests that JNK may regulate post-transcriptional or post-translation modifications of EBI3. For example, JNK may affect mRNA stability, protein translation, intracellular protein degradation, or secretion of newly synthesized EBI3 protein. Further studies are needed to address these issues.

Signalling pathways dysregulating IL-27 production in LPS-stimulated HIV-infected MDMs:

Although it has been long appreciated that HIV infection impairs immune function in human cells, the exact signalling mechanisms that are impaired are poorly defined. Interestingly, I demonstrated that IL-12/23 p40, IL-23 p19, IL-27 EBI3, and IL-27 p28 mRNA expression were inhibited in HIV-infected MΦs following LPS-stimulation. Inhibition of IL-12/23 p40 production in HIV infection is consistent with previous studies (63, 65, 108, 281). Since all four subunits were inhibited, it is possible that HIV inhibited TLR-4 signalling in a broad fashion. In fact, Tachado et al has shown that MyD88-dependent

signals are reduced in alveolar MΦs from asymptomatic HIV-infected individuals (307). Specifically, IRAK, MyD88, and NF-κB activation were reduced in HIV-infected patients, while MyD88-independent signalling, including the activation of IRF-3, was unaffected (307). IL-12/23 p40, IL-23 p19, IL-27 EBI3 and IL-27 p28 mRNA expression have all been demonstrated to be regulated by NF-κB (247, 248, 260, 264, 269, 270, 281). Thus, it is possible that HIV inhibits LPS-induced IL-23 and IL-27 production by inhibiting MyD88-dependent pathway and NF-κB activation in MDMs.

After defining the PI3K, p38 MAPK and JNK MAPK signalling pathways as positive regulators of LPS-induced IL-27 production in MDMs, I sought to investigate whether HIV could impair their activation. The LPS-induced activation of the p38 and JNK MAPK pathways have been shown to be inhibited in THP-1 cells infected with HIV (281). Similar to these findings, I have shown that JNK and p38 MAPK phosphorylation is suppressed in *in vitro* HIV-infected human MDMs. This suggests that HIV likely inhibits LPS-induced IL-27 production through the inhibition of the JNK and p38 MAPK pathways (Figure 26). The JNK and p38 MAPK pathways regulate the expression of several genes, thus this study opens new questions as to how HIV specifically targets the expression of certain cytokines, while not affecting others. Additionally, there are likely other unknown mechanisms by which HIV inhibits LPS-induced IL-27 production in human MDMs.

Role of PKCs in LPS-induced IL-23 and IL-27 production in myeloid cells:

PKCs are important signalling molecules mediating immune function. Non-redundant roles for specific PKC isoforms have been identified (Reviewed in Table 1 Tan and Parker (308)). For example, PKCε knock-out mice have been characterized as having impaired

activation of MΦs (309), whereas PKCα knock-out mice have been characterized with a hyperproliferative T-cell phenotype (310). PKCs are dysregulated in various diseases including cancers (311), diabetes (312), and immune disorders (313). Various PKC inhibitors have been developed to treat disease, and have shown minimal toxicity in human clinical trials (314-319). Therefore, inhibition of PKCs is becoming a promising target for manipulating immune responses, and treating disease. However, to date there is limited knowledge of the involvement of PKCs in the regulation of TLR-induced IL-23 and IL-27 production in Mos and MΦs.

The role of PKCs in the regulation of IL-12/23 p40, IL-23 p19, and IL-23 secretion is poorly understood. Herein, I demonstrated for the first time that the cPKCs, but not PKCε positively regulate LPS-induced IL-12/23 p40 mRNA expression and protein secretion, and IL-23 secretion in primary human Mos. Interestingly, I also demonstrated that the cPKCs and PKCε do not regulate LPS-induced IL-23 production in THP-1 cells. The cPKCs have been implicated in the positive regulation of IL-12/23 p40 in human DCs (222), and PKCδ has been implicated in the positive regulation of IL-12/23 p40 in murine MΦs and DCs (262). However, to my knowledge, there is only one study that has investigated the role of PKCs in IL-23 production in DCs and demonstrated that cPKCs do not regulate LPS- or Poly (I:C)-induced p40, p19 and IL-23 production in human DCs (263). Thus, distinct PKC isoforms may differentially regulate IL-12/23 p40 and IL-23 production in different cell types.

LPS-induced IL-27 secretion and p28 mRNA expression was regulated by the cPKCs in primary human Mos and THP-1 cells but not in MDMs. Since PKCε inhibition had no effect on IL-27 production in any of the cells tested, this indicates that one or more of the cPKCs or non-specific effects of the inhibitor are responsible for the down-regulation of p28

mRNA expression and IL-27 secretion in THP-1 cells and primary Mos. To confirm the results of these inhibitors, I used siRNA to identify the cPKC isoform regulating LPS-induced IL-27 production. However, siRNA knockdown of cPKCs, PKCβII, PKCα, and PKCγ were inconclusive in determining which cPKC isoform regulates LPS-induced IL-27 production in THP-1 cells. Since the inhibition of the PKCs via siRNA was not 100% and the inhibition of IL-27 secretion was only about 50% at the highest dose of PKCβ inhibitor used, this study does not exclude the possibility that the cPKCs are involved in the regulation of LPS-induced IL-27 production in THP-1 cells. It is possible that complete inhibition of a particular cPKC isoform is needed to have any significant effect on the production of LPS-induced IL-27 production. Additionally, perhaps more than one of the cPKC isoforms are involved in the regulation of LPS-induced IL-27 production. These observations are similar to a study by Johnson et al which demonstrated that IL-27 p28 mRNA expression, and IL-27 secretion is regulated by PKCα-dependent activation of IRF-3, in murine and human DCs (263). It is possible that PKCα or another cPKC, may regulate LPS-induced p28 mRNA expression and IL-27 secretion through IRF-3-dependent mechanisms in primary Mos and THP-1 cells, as well.

Overall, my results suggest that cPKCs may regulate LPS-induced p40 and IL-23 production in primary Mos, but not in THP-1 cells. Additionally, the cPKC pathway was found to positively regulate p28 and IL-27 production in THP-1 cells, and primary Mos, but not MDMs. This study of PKC regulation of the IL-12 family of cytokines demonstrates the dynamic and differential regulation of cytokines in different cell types (summarized in Figure 27).

Figure 27: The cPKC pathway differentially regulates LPS-induced IL-23 and IL-27 in different myeloid cells. The cPKCs positively regulate p28 mRNA expression and IL-27 secretion in THP-1 cells, and primary Mos, but not MDMs. On the other hand, LPS-induced IL-23 secretion is positively regulated by cPKCs in primary Mos, through p40 mRNA expression in primary Mos, but not THP-1 cells. Additionally, inhibition of the PKCε pathway does not affect LPS-induced IL-23 or IL-27 production in any of the cell types tested.

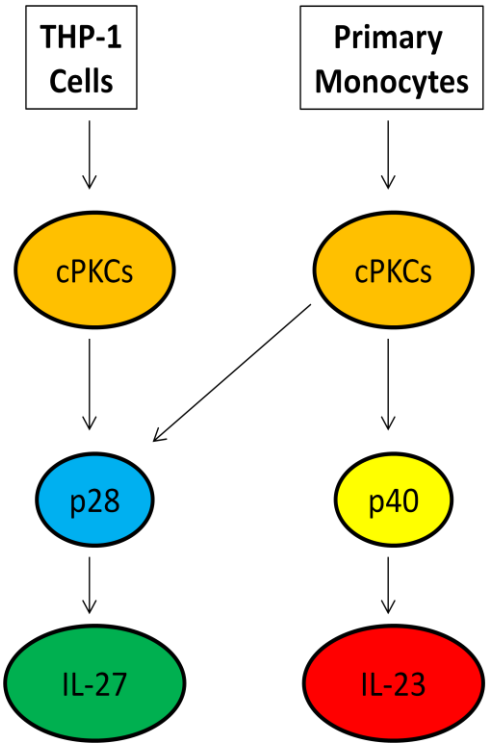

FIGURE 27

CHAPTER V: CONCLUDING REMARKS, AND FUTURE DIRECTIONS

Mos and MΦs serve as a reservoir for HIV infection which persists even in patients on HAART. Through the production of cytokines, Mos and MΦs can both enhance viral persistence, and viral-specific immune responses. MΦs and Mos are important producers of the IL-12 family of cytokines; however, the effect of HIV on the production of IL-23 and IL-27 in HIV-infected MΦs remains poorly understood. Herein, I have demonstrated that HIV cannot directly induce IL-23 or IL-27 production from resting MΦs, but that HIV inhibits the production of both IL-23 and IL-27 in LPS-stimulated MΦs. Specifically, I have demonstrated that HIV inhibits LPS-induced IL-12/23 p40 and IL-23 p19 and IL-27 EBI3 and IL-27 p28 mRNA expression in MDMs. This is the first study to show that HIV inhibits LPS-induced IL-23 and IL-27 production in HIV-infected human MΦs.

Cytokine production is altered in a dynamic manner through the course of HIV infection. In *in vivo* HIV infection, the sensitivity of TLRs to their agonists is dependent on the disease state and cell types (320). For example, Chang et al demonstrated that cytokine responses were variable between Mos, Mo-derived DCs, and in plasmacytoid DCs in response to various TLR agonists and throughout disease progression in HIV infection (320). Thus, in order to fully understand the expression of IL-23 and IL-27 production in HIV infection, their expression must be measured in dynamic models similar to Chang's study. Specifically, *in vivo* HIV/SIV models must be used to investigate IL-23 and IL-27 production in various compartments of the body, and cell types, in response to various stimuli, throughout disease progression.

Future studies should also encompass elucidating the specific HIV proteins dysregulating LPS-induced IL-23 and IL-27 production in human MDMs. Identification of HIV proteins dysregulating LPS-induced IL-23 and IL-27 would make HIV-protein neutralization a feasible approach to restore LPS-induced IL-23 and IL-27 responses.

The inhibition of IL-23 and IL-27 in HIV-infected LPS-stimulated MDMs supports the hypothesis that IL-23 and/or IL-27 therapy in HIV infection may improve disease outcome. Restoration of IL-23 and IL-27 in HIV infection may effectively decrease viral persistence through the induction of viral-specific T-cell responses, resulting in decreased immune activation. Therefore, future studies should also focus on the impact of using IL-23 and/or IL-27 as a therapy to improve HIV disease outcome.

The signalling mechanisms regulating LPS-induced IL-23 and IL-27 production in human MΦs are poorly understood, and this needed to be determined before the mechanisms by which HIV inhibits LPS-induced IL-23 and IL-27 production in MDMs could be investigated. This is the first study to investigate the regulation of LPS-induced IL-27 production in human MDMs. LPS-induced IL-27 production was positively regulated by the PI3K, p38 MAPK, and JNK MAPK pathways in human MDMs. However, the ERK pathway did not regulate LPS-induced IL-27 production. The IL-27 subunits EBI3 and p28 were shown to be regulated by different signalling pathways. IL-27 EBI3 mRNA expression was positively regulated by the PI3K, and p38 MAPK pathways, whereas EBI3 protein expression was positively regulated by the JNK MAPK pathway in addition to the PI3K, and p38 MAPK pathways. On the other hand, IL-27 p28 mRNA expression was positively regulated by the PI3K and JNK MAPK pathways, and not the p38 MAPK pathway.

Future studies should be directed at using alternative methods to confirm the involvement of the PI3K, p38 MAPK, and JNK MAPK pathways in the positive regulation

of LPS-induced IL-27 production in MDMs, to ensure that non-specific effects of the pharmacological inhibitors are not influencing LPS-induced IL-27 production. The use of siRNA, or dominant-negative knock-outs could be used for this purpose. Additionally, it will be of interest to determine whether JNK MAPK regulates post-transcriptional or post-translational modification of EBI3 which would be able to explain the differential regulation of LPS-induced EBI3 mRNA and protein expression by the JNK MAPK pathway.

Furthermore, it will be of interest to investigate why IL-23 is produced in response to LPS in MDMs from some donors, but not others at earlier time points post-differentiation, and why MDMs from all donors at later time points post-differentiation, secrete IL-23 in response to LPS.

In order to define the mechanism by which HIV inhibits LPS-induced IL-27 production in MDMS, the activation of the PI3K, p38 MAPK, and JNK MAPK pathways in response to LPS, were investigated. HIV infection was found to inhibit the phosphorylation of p38 and JNK MAPK in LPS-stimulated MDMs. Thus, this suggests that HIV may inhibit LPS-induced IL-27 production in MDMs through the inhibition of the p38 and JNK MAPK pathways. Additionally, this would suggest that other genes regulated by the p38 and JNK MAPK pathways may be affected in HIV-infected MDMs. It will be of interest to determine whether HIV specifically inhibits LPS-induced IL-23 and IL-27 production, or whether other cytokines regulated by the JNK and p38 MAPK pathways are also inhibited in HIV infection. Future studies should also investigate whether HIV can inhibit LPS-induced IL-27 production independently of the JNK and p38 MAPK pathways.

The role of PKCs in regulation of TLR-induced IL-23 and IL-27 production is poorly understood. Herein, I have shown that IL-23 and IL-27 are differentially regulated by the PKC pathways in different cell types in response to LPS. My results show that the cPKCs

positively regulate LPS-induced p40 mRNA expression and protein secretion, and IL-23 secretion in primary Mos, but not THP-1 cells. Additionally, I demonstrated that the cPKC pathway positively regulates LPS-induced p28 mRNA expression, and IL-27 secretion, in THP-1 cells and primary Mos, but not MDMs. Future studies should be directed at determining the cPKC isoform regulating LPS-induced IL-23 and IL-27 production in primary Mos. The PKCα/β specific inhibitor, Gö6976, could be used to further narrow down the PKC isoform involved in the regulation of LPS-induced IL-23 and IL-27. In order to identify the specific PKC isoforms regulating LPS-induced IL-23 and IL-27, dominant-negative knock-outs, small hairpin RNA, or PKC-isoform specific knock-out mice could be used.

CHAPTER VI: REFERENCES

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APPENDIX A

Figure A1: MDM characterization. Monocytes were isolated by adherence from PBMCs and cultured for 7 days in the presence of M-CSF. MDMs were washed and stained for 15 min with PE-conjugated antibodies recognizing A) CD11a, B) CD11b) C) CD11c, D) CD80, E) CD83, F) CD86, G) CD14, H) CD16, and I) HLA-DR. Cells were washed and analyzed by flow cytometry. The white peak represents background fluorescence from the unstained cells. The coloured peaks represent PE-fluorescence from the respective surface receptors. The percentages represent the number of cells expressing the respective surface receptor after normalizing for background fluorescence.

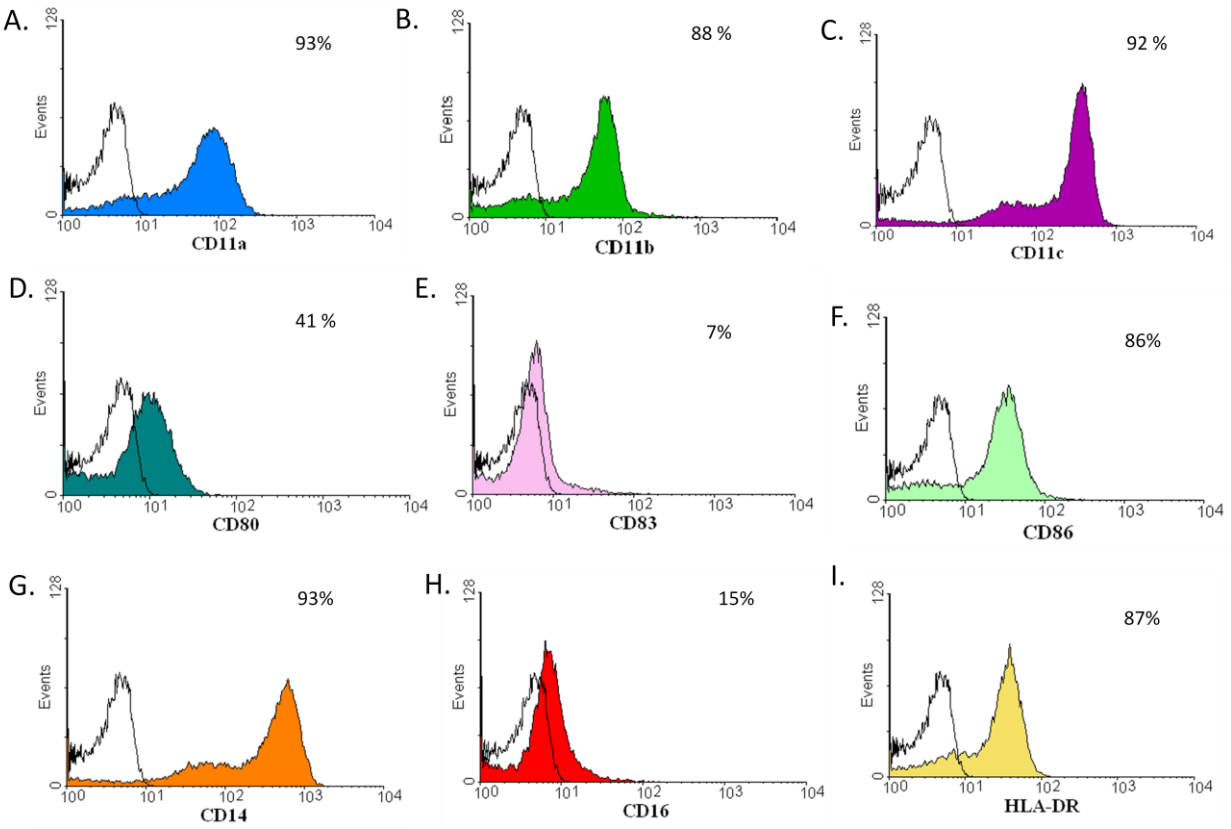
A.
93%
CD11a
B.
88 %
CD11b
C.
92 %
CD11c
D.
41 %
CD80
E.
7%
CD83
F.
86%
CD86
G.
93%
CD14
H.
15%
CD16
I.
87%
HLA-DR
Events
128
0
100
101
102
103
104

FIGURE A1

CIRRICULUM VITAE

Shifawn O'Hara

Education

Masters of Science, Microbiology and Immunology
University of Ottawa *Jan. 2010- present*

- Ontario Graduate Scholarship May 2011-Dec 2011 ($10,000), CGS-M NSERC Scholarship May 2010- Apr 2011 ($17,500/year), Excellence Scholarship (Tuition)
- Investigation of signalling pathways, including Protein Kinase C, regulating IL-23 and IL-27 cytokines in human primary monocytes and macrophages
- Investigation of the signalling pathways dysregulating LPS-induced IL-23 and IL-27 production HIV-infected macrophages
- Proficient in experimental techniques including western blotting, ELISA, RNA extraction, quantitative real time PCR, flow cytometry, confocal microscopy, transfection with siRNA and plasmid DNA, infection of cells with lentivirus, bacterial transformation, primer design, and culturing and differentiation of primary cells and cell lines
- Prepared manuscripts for publication
- Good understanding of statistical methods including the student t-test and ANOVA
- Experience working in a bio-safety level-3 laboratory
- Participated in an HIV work in progress and journal club
- Took non-compulsory French courses

Bachelor of Science, Biochemistry and Biotechnology, Cooperative Education
Carleton University *Sept. 2005-Dec. 2009*

- Co-op Student of the Year Award 2008
- General In-Course Scholarship ($3500, 2009), Deans' Honor List (2008-2009), Gary-Duck Scholarship ($3500, 2005), Reaching for the Top Queen Elizabeth Scholarship ($3500, 2005)
- CGPA 10.52/12, A-

Experience

Technical Officer
National Research Council of Canada *May 2012-present*
Ottawa, ON

- Characterization of activity-based protein profiling probes
- Characterization of differential protein activity between normal and hepatitis C virus infected hepatic cells

Teaching Assistant/Assistant d'enseignment
University of Ottawa *Jan.2011-April 2012*
Ottawa, ON
BCH 2333: Introduction to biochemistry (lecture) Jan-Apr 2012
BCH 2733: Introduction à la biochemie laboratoire; Jan-Apr 2011 and Jan-Apr 2012
BCH 3356: Molecular biology laboratory Sept-Dec 2011

BCH 2333: Introduction to Biochemistry laboratory; Jan-Apr 2011

- Prepared presentations on experimental theory, and supervised students conducting experiments
- Provided advice and guidance to students through online discussion forums, e-mails, and answering questions in class
- Marked lab reports, input marks into the course database, and provided students with advice to improve scientific writing and presentation skills
- Expanded my scientific vocabulary in French

Research Assistant/Summer Student
National Research Council of Canada (NRC) *May-Dec. 2009*
Ottawa, On

- Investigation of glycoprotein markers of *C.difficile* which could be used as a biomarker
- Glycoprotein detection using in-gel periodic acid staining
- Protein purification using 1D and 2D SDS PAGE, HPLC, acetone protein precipitation and isoelectric focusing
- Protein identification using nano-liquid chromatography tandem mass spectrometry (nLC-MS/MS), using Q-TOF, Ion trap and Orbit-trap instrumentation
- Finalist in the Annual National Research Council-Institute of Biological Sciences Sarang Narang Memorial Competition 2009
- Volunteered to continue project, as part of a directed studies course, through Carleton University (Sept.-Dec. 2009)

Research Assistant
Health Canada *May-Dec. 2008*
Ottawa, On

- Studied gene expression changes, cytokine secretion, and DNA damage in human lung and monocytic cells exposed to alpha particle radiation, associated with radon gas
- Developed strong laboratory techniques in immunofluorescent microscopy, total RNA isolation, preparation of Cyanine 3 labeled cDNA, microarray hybridization, microarray quantification, quantitative real time PCR, and mammalian tissue culture in bio-safety level 2 laboratory
- Assisted in the writing of manuscripts

Laboratory Research Assistant
Carleton University *May-Aug. 2007*
Ottawa, On

- Cloned the mutant gene of *N. crassa* into compatible vectors to ultimately transform the constructs into *N. crassa* and *S. pombe* and localize morphological changes.
- Proficient in various laboratory techniques including preparative and analytical agarose gel electrophoresis, DNA isolation, PCR, primer design, vector construct design, white/blue screening, restriction digests, transformations of bacteria, and transfection of eukaryotic cells, using electroporation and calcium phosphate techniques.

Skills Profile

- Excellent oral and written communication skills obtained from research positions and writing assignments in university
- Proficient in using internet browsers, Graph Pad Prism and MS Outlook, Word, PowerPoint Presentation, and Excel
- Competent in documenting, analyzing, interpreting and presenting results

- Good researching abilities and typing skills obtained from research assistant positions and completing assignments at university.
- Extraordinary multitasking skills and quick learning abilities obtained through involvement with cadets and demonstrated in research assistant positions
- Outstanding leadership and interpersonal skills developed through cadets
- Bilingual

Volunteer/Extracurricular Activities

University of Ottawa
Ottawa, ON

- Member of the Biochemistry, Microbiology, and Immunology Graduate Students' Association *(Oct. 2010-April 2011)*

CHEO Emergency Department *May 2010-June 2010*
Ottawa, On

- Facilitated the enrollment of patients in various clinical studies, by interacting with eligible patients, and obtaining informed consent

Carleton University *Ottawa, ON*

- Member of the student and employer advisory councils for co-operative education (*Apr. 2007-Dec. 2009*)
- Student ambassador for donor celebration and other events (*Mar. 2009-Dec. 2009*)
- Laboratory assistant in plant research laboratory (*Oct. 2007-Mar. 2008*)

Manuscripts

Chauhan V., Howland M., O'Hara S., Beaton L.A., Burn T.A., Stocki T.J., and Wilkins R.C. (2011). Proteomic profiling to identify potential biomarkers of alpha-particle radiation exposure in human lung epithelial cells. *Radioprotection* 46(6): S377-83.

Conferences

Keystone: Frontier in HIV Pathogenesis, Therapy and Eradication (X8)
Whistler, BC *March 26-31st, 2012*
HIV impairs TLR-4 induced IL-27 production via p38 and JNK MAPK activation in human macrophages
O'Hara S., Blahoianu M., Gajanayaka N., Angel J., and Kumar A.

- Poster Presentation
- Keystone Travel Scholarship

Ontario HIV Treatment Network (OHTN)
Toronto, ON *Nov. 14-15th, 2011*
HIV inhibition of IL-23 and IL-27 production in monocyte/macrophages and the signaling pathways involved in regulating their expression
O'Hara S. and Kumar A.

- Oral presentation
- OHTN Research Conference Scholarship

Toronto, ON *Nov.15-16th 2010*
HIV and its regulatory protein Tat inhibit IL-23 and IL-27 production in human monocyte-derived macrophages: the involvement of PI3K and p38 MAPK
Gajanayaka N., O'Hara S., Angel J.B., and Kumar A.

- Poster presentation
- OHTN Research Conference Scholarship

American Association of the Advancement of Science (AAAS): Forum on Science and Technology Policy
Washington, DC *May 5-6th, 2011*

- Was selected among other graduate students to attend
- Also attended the Allan Bromley Annual Memorial lecture
- Networked with key members of the science and technology policy community

Conference on Retroviruses and Opportunistic Infections (CROI)
Boston, MA *Feb.27-Mar. 2 2010*
HIV and its regulatory protein Tat inhibit IL-23 and IL-27 production in human monocyte-derived macrophages: the involvement of PI3K and p38 MAPK
Gajanayaka N., O'Hara S., Rahimi A., Blahoianu M., Boucher J.G., Angel J.B., and Kumar A.

- Poster presentation
- Young Investigator's Award

Health Canada Science Forum
Ottawa, ON
2009
Biological effects of radon gas exposure
Chauhan V., Howland M., O'Hara S., Kutzner B., Ferrarotto C., McNamee J., Bellier P., Stocki T.J., Beaton L.A., and Wilkins R.

- Co-author

Exploration of candidate biomarkers: radon exposure and genomic profiling
Howland M., O'Hara S., Malowany M., Williams A., McNamee J., Qutob S., Stocki T.J., Beaton L.A., Wilkins R., and Chauhan V.

- Co-author

Radiation Research
Savannah, GA *Oct 3-7th, 2009*
Effects of alpha particle exposure on gene expression in human pulmonary epithelial cells (A549)
Chauhan V., O'Hara S., Howland M., Qutob S., Malowany M., Williams A., McNamee J., Stocki T.J., Beaton L.A., and Wilkins R..

- Co-author

The effects of alpha radiation on DNA damage and chemokine secretion in human monocytic cells
Chauhan V., Howland M., O'Hara S., Kutzner B., Ferrarotto C., McNamee J., Bellier P., Stocki T.J., Beaton L.A., and Wilkins R.

- Co-author