Molecular mechanism involved in HIV-Tat mediated inhibition of LPS-induced IL-23 and IL-27 production in human macrophages

By

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Abstract

Monocyte-derived macrophages (MDMs) from HIV-infected patients and MDMs infected *in vitro* with HIV manifest inhibition of various cytokines including IL 12. Recently, IL-27 was shown to inhibit HIV replication in macrophages. Whether HIV infection or HIV regulatory proteins such as tat, impact IL-23 or IL-27 production in macrophages remains unknown. I have demonstrated that intracellular HIV-tat expression as well as HIV-tat basic domain peptides inhibited LPS-induced IL-23 and IL-27 proteins and their subunits in MDMs.

First I investigated the signalling pathways involved in the regulation of LPS-induced IL-23 and IL-27 production in MDMs infected with control pLXIN retrovirus-infected MDMs. The p38 MAPK, SHP-1 and PI3K signalling molecules positively regulated LPS-induced IL-23 expression. In contrast, Src kinases and JNK MAPK negatively regulated LPS-induced IL-23 production. On the other hand, LPS-induced IL-27 production was positively regulated by the PI3K, p38 MAPKs and SHP-1 and Src kinases. Src kinases positively regulated LPS-induced IL-27 production whereas Src kinases and JNK negatively regulated LPS-induced IL-23 production.

HIV-Tat significantly inhibited p38 MAPK and PI3K which were implicated in HIV-Tat-mediated inhibition of LPS-induced IL-23 and IL-27 production. Even though HIV-Tat inhibited ERK and JNK MAPK activation, these kinases were not involved in HIV-Tat-mediated inhibition of LPS-induced IL-23 and IL-27 production.

While SHP-1 regulated LPS-induced IL-23 and IL-27 production, HIV-Tat did not inhibit SHP-1 and therefore were not involved in HIV-Tat-mediated inhibition of LPS-induced IL-23 and IL-27 production. HIV-Tat did not inhibit Src kinases and hence were not involved in HIV-Tat-mediated inhibition of LPS-induced IL-27 production. Furthermore, HIV-Tat did not inhibit the

expression of upstream TLR4-activated signaling molecules including TRAF3, TRIF, MyD88, IRAK1, IRAK3, IRAK4, TRAF-1, TRAF-2, cIAP-1, cIAP-2 and, xIAP.

These results suggest association of IL-23 and IL-27 inhibition by HIV with decreased HIV-specific immune responses, and increased viral replication. These results further suggest novel strategies to improve cellular immune responses and inhibition of HIV replication.

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List of abbreviations

AIDS: Acquired Immunodeficiency Syndrome

AKT: Protein Kinase B

AP1: Activating protein-1

APC: antigen presenting cell

APOBEC: Apolipoprotein B mRNA Editing, Enzyme-Catalytic, Polypeptide-like

ART: Antiretroviral Therapy

ATP: Adenosine Triphosphate

BCAP: B-cell Adapter for PI3K

B-cells: B-Lymphocyte Cells

bp: Base pairs

BSA: Bovine Serum Albumin

CCR4: CC-chemokine receptor 4

CCR5: CC-Chemokine Receptor-5

CD: Cluster of Differentiation

cDNA: complementary DNA

CLRs: C-type lectin like receptors

CO_2: carbon dioxide

CTL: Cytotoxic T-Lymphocyte

CX3CR1: CX3C chemokine receptor 1

CXCR4: CXC-Chemokine Receptor -4

Cys: cysteine

DCs: Dendritic Cells

DNA: Deoxyribonucleic Acid

EBI3: Epstein Barr Virus-Induced Gene-3

ECL: enhanced chemiluminescence

EDTA: ethylene diamine tetra-acetic acid

ELISA: Enzyme Linked Immunosorbant Assay

Env: Envelop

ERK: Extracellular Signal-Regulated Kinase

FBS: fetal bovine serum

GALT: Gut-Associated Lymphoid Tissue

GATA-1: GATA Binding Protein-1

GATA-3: GATA Binding Protein-3

gp120: Glycoprotein-120

gp130: Glycoprotein-130

HAART: Highly Active Antiretroviral Therapy

HIV: Human Immunodeficiency Virus

hr: Hour

HRP: Horse Radish Peroxidase

HSPGs : Hepran Sulphate Proteoglygans

IFN: Interferon

IL: Interleukin

IL-12R: IL-12 Receptor

IL-1R: IL-1 Receptor

IL-23R: IL-23 Receptor

IL-27R: IL-27 Receptor

IRAK: IL-1R-Associated Kinases

IRF: Interferon Regulator Factor

JAK: Janus Kinase

JNK: c-Jun N-Terminal Kinase

Kb: kilobase

kDa: kilodalton

LBP: LPS binding protein

LN: Lymph Node

LPS: Lipopolysaccharide

LRP: Lipoprotein Receptor-related Protein

LTR: Long Terminal Repeat

M: Molar

mAb: Monoclonal antibody/antibodies

MAPK: Mitogen Activated Protein Kinase

MAPKK: MAPK kinase

MAPKKK: MAPK Kinase kinase

M-CSF: Macrophage Colony-Stimulating Factor

MDMs: Monocyte-Derived Macrophages

Me/me: moth-eaten mice

MEK: Extracellular Signal-Regulated Kinase Kinase

MHC II: major histocompatibility complex II

MKK: Mitogen Activated Protein Kinase Kinase

mRNA: Messenger Ribonucleic Acid

M-tropic: Macrophage-Tropic

MyD88: Myeloid Differentiation Primary Response Gene 88

NF-κB: Nuclear Factor-Kappa B

NK cells: Natural Killer cells

NO: nitric oxide

PBMCs: Peripheral Blood Mononuclear Cells

PBS: Phosphate Buffered Saline

PCR: polymerase chain reaction

PI3K: Phosphoinositide-3-Kinase

PIP2: Phosphatidylinositol 4,5-Bisphosphate

PRRs: pattern recognition receptors

PTK: protein tyrosine kinase

PTP: protein tyrosine phosphatase

PVDF: polyvinylidene difluoride

qRT- PCR: Quantitative Real Time Polymerase Chain Reaction

RNA: Ribonucleic acid

RT-PCR: Reverse transcriptase polymerase chain reaction

SD: standard deviation

Ser: serine

SH2: Src-homology domains 2

SHP-1: SH2 domain containing phosphatase-1

SHP-2: SH2 domain containing phosphatase-2

siRNA: Small Interfering RNA

SIV: Simian Immunodeficiency Virus

SP: specificity protein

ssRNA: Single-Stranded RNA

STAT: Signal Transducer and Activator of Transcription

T reg: T regulatory cell

TAB1: TAK-Binding Protein 1

TAR: trans activation response

Tat: Trans-Activator of Transcription

TBST: Tris buffered saline and Tween-20

TCCR: T-cell Cytokine Receptor

T-cells: T-lymphocyte Cells

TGF: Transforming Growth Factor

Th cells: T-helper cells

Th1: T-helper 1

Th2: T-helper 2

Th17: T-helper 17

TIR: Toll/IL-1 Receptor

TIRAP: TIR Domain Containing Adapter Protein

TIRAP: Toll-interleukin 1 recetor adaptor protein

TKY: Tyrosine Kinase

TLR: toll-like receptor

TNF: Tumor Necrosis Factor

Tr1: Type-1 T-Regulatory

TRAFs: TNF-Associated Factors

TRAM: TRIF-Related Adapter Molecule

Treg: T-Regulatory

TRIF: TIR-domain-containing adapter-inducing interferon-β

T-tropic: T-cell Tropic

UTR: un-translated region

Vpr: Viral Protein R

WSX-1: IL-27 receptor chain

WT: wild-type

Chapter 1: Introduction

1.1 Human immunodeficiency virus infection:

The human immunodeficiency virus (HIV) is a lentivirus that causes acquired immunodeficiency syndrome (AIDS) (1). In 2012, it was estimated that 35.3 million people worldwide were living with HIV and that 1.6 million people died of AIDS related illnesses in that year. In 2012, the population living with HIV in Canada was estimated to be 59,000 to 85,000 (2).

HIV infects CD4+ T cells, dendritic cells (DC), monocytes and macrophages (3),which lead to severe immunological and neurological disorders. Two of the most fundamental characteristic changes identified in HIV infection are the depletion of CD4+ T-cells and the increase of viral loads (4). These two characteristics are able to amplify each other, creating a immune dysfunction. HIV infection can be divided into several phases: acute infection followed by the chronic infection and ultimately, the symptoms of AIDS. AIDS is characterized by chronic immune activation and viral persistence (5). HIV-infected patients who respond effectively to antiretroviral therapy (ART) have increased CD4+ T-cell counts and decreased viremia. Even though chronic immune activation is reduced in HIV-infected patients on ART, these individuals have high levels of immune activation markers compared to uninfected individuals (6,7). It has been reported that chronic immune activation is a leading cause of premature aging and increased susceptibility to age-related diseases such as dementia, cancer, and cardiovascular disease (8).

A massive depletion of CD4+ T cells is a characteristic of the final stages of HIV infection (AIDS). AIDS arises after several years of untreated chronic HIV infection, where CD4+ T cell counts reach a critical level of 200 CD4+ T cells /µl. This final stage of HIV infection makes the patient extremely vulnerable to rare opportunistic infections that would not otherwise be dangerous to an immunocompetent individual. Examples of such infections are oral candidiasis, mycobacterial infections and reactivation of herpes simplex and varicella virus. The extreme immunocompromised phenotype of AIDS also allows tumour cell and oncogenic virus replication to go unchecked. Cancers such as Kaposi's sarcoma, caused by the Human Herpes Virus 8 (HHV8), lymphoma associated with Epstein-Barr virus (EBV) and cervical and anal carcinoma associated with human papilloma virus are examples of risks a patient with AIDS faces. Ultimately, the massive compromise of CD4+ T cell immunity in AIDS patients is extremely dangerous and can often be fatal due to opportunistic infections and/or uncontrolled malignancies.

1.1.2 Transmission of HIV and Reverse transcription:

Generally the main routes of HIV infection are mediated by homosexual and heterosexual intercourse through the rectal and cervicovaginal mucosa (9), the use of unsafe needles by drug users, transfusion by contaminated blood products, and perinatal transmission from infected mother to infant. In addition, HIV can be transmitted either *in utero* or during breast-feeding (10). While HIV infection is mediated via body fluids, HIV infects specially CD4+ T cells, dendritic cells (DC), monocytes and macrophages by fusion and endocytosis (3). The productive infection of target cells is initiated following interaction of HIV gp120 with CCR5 or CXCR4 co-receptors expressed on the surface of CD4+ T cells to form a CD4-gp120-co-receptor ternary complex (11). The formation of this complex leads to a conformational change in HIV protein gp41, resulting in the insertion of the highly hydrophobic N-terminal “fusion peptides” of the gp41 molecules into the plasma membrane of host cell (12–14).

In situ or *ex vivo* studies have shown that Langerhans cells (LCs) and DCs are infected by HIV and consequently transmit HIV to the host autologues CD4 T cells (15–19). After the virus gains entry, DCs migrate to the local lymphoid structures thus disseminating the virus from mucosal sites to the secondary lymphoid tissues. In this way, LC and DC can transmit virus to activated CD4+ cells (20).

After the virus enters and uncoats in T cells and macrophages, the viral genome is released into the cytoplasm. This step is associated with the start of reverse transcription. The reverse transcription of the viral genome into cDNA is catalyzed by the viral reverse transcriptase enzyme in the cytoplasm of the host cell; however, some evidence suggests that it may be completed in the nucleus (21). Subsequently, viral cDNA is introduced into the nucleus as a component of the pre-integration complex (PIC) (22). After nuclear import, HIV-1 retroviral integrase (IN) catalyses the insertion of viral cDNA into a host cell chromosome (23). Once HIV cDNA is integrated into the host genome, the transcription is regulated by the long terminal repeats (LTR) located upstream and downstream of the viral coding sequences (24,25). In order to have efficient transcript elongation, a 5'end TAR (Tat associated region) hairpin element is required to bind to the HIV-Tat protein (24,25). HIV-Tat protein enhances the efficiency of viral transcription and elongation of their transcripts (26). Following completion of transcription, the assembly of infectious virus particles starts by full-length RNA transcripts being packaged inside a nucleoprotein complex (21). This nucleoprotein complex is enveloped by the membrane and then exits the cell by budding from the plasma membrane (21) (Fig 1).

Figure 1: A schematic representation of the HIV lifecycle

The HIV envelope protein (Env) binds CD4 and, following a conformational change, either the CCR5 (R5 strains) or CXCR4 (X4 strains) chemokine receptor. Entry into host cells occurs by fusion of the virus and host cell membranes. Uncoating of the virus capsid releases the pre-integration complex and reverse transcription begins, generating both linear and circular double-stranded viral complementary DNA. The linear form integrates into the genome of the host cell. Transcription generates both spliced and unspliced viral genomic transcripts, which are transported from the nucleus to the cytoplasm. Viral proteins such as Tat, Nef, Vpr, Vpu, Rev are then translated and transported to the plasma membrane, where progeny virions are released from the infected cell by budding. Highly active antiretroviral therapy comprises a cocktail of fusion inhibitors, reverse transcriptase (RT) inhibitors and protease inhibitors, and the points in the lifecycle in which each of these inhibitor classes function are shown.

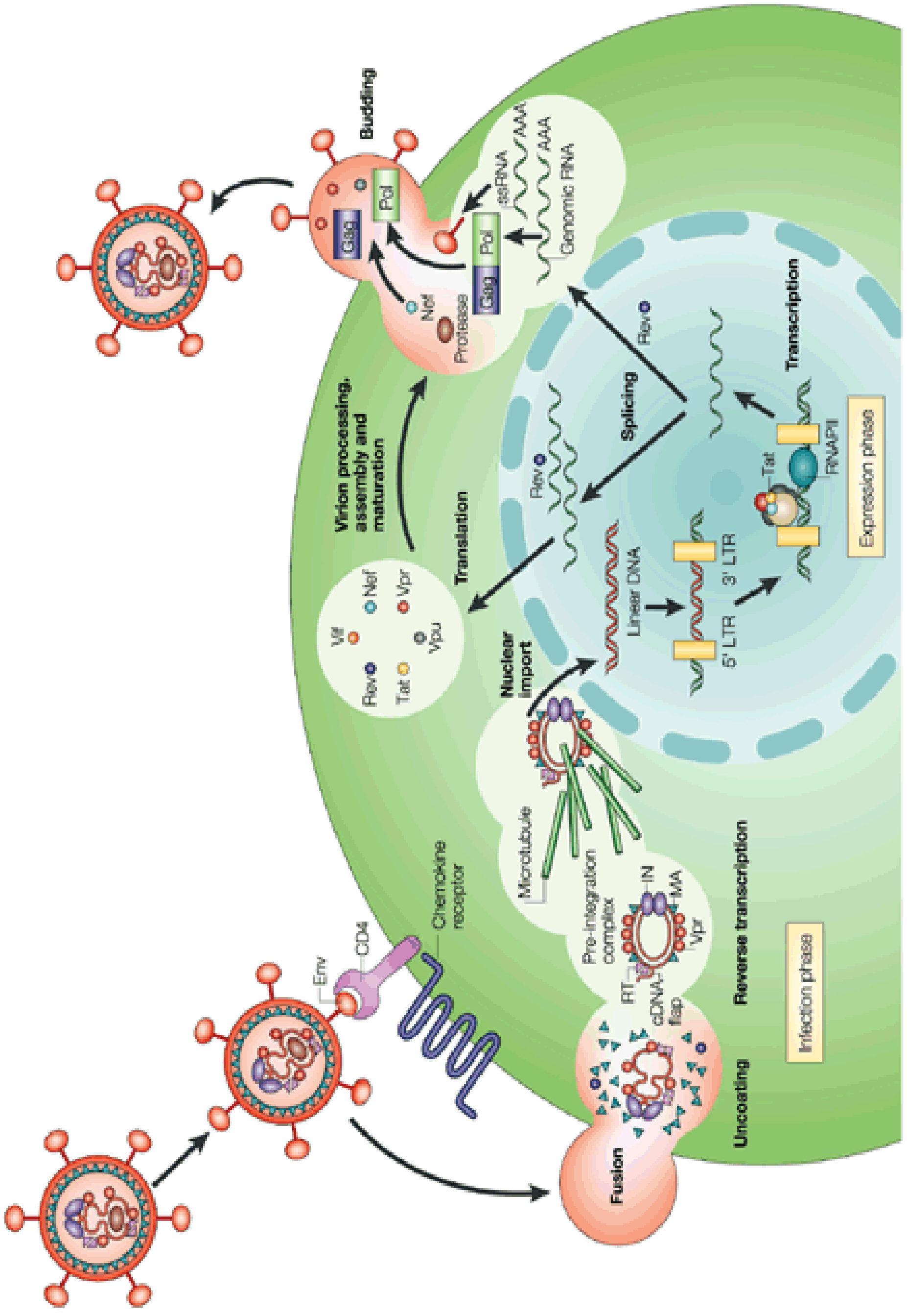


Figure 1

Han *et al. Nature Reviews Microbiology* **4**, 95–106 (February 2007)

1.1.3 HIV structure and genome organization:

HIV identified in 1983, is a single stranded, positive-sense RNA virus belonging to the lentivirus genus of the Retroviridaea family (27). The virion consists of two positive sense copies of the genome. The envelop glycoprotein is composed of gp41 hetrotrimers and gp120 glycoprotein. The HIV genome is roughly 10 kb in size and encodes 16 functional genes, which transcribe over 30 mRNAs (28). The genome of HIV encodes two regulatory proteins, namely Tat and Rev, and four accessory proteins, vif (viral infectivity factor), Vpr (viral protein r), Vpu (viral protein u), and Nef (negatif factor) (29). Tat is the main HIV protein involved in HIV transcription. The Rev protein interacts with a structure named RRE (Rev responsive element) positioned in the *env* gene (29). This interaction allows mono and unspliced mRNA to cross the nuclear membrane to enter the cytoplasm. Then the mRNA are translated into proteins and encapsidated in the nascent viral particles (29). Like Tat and Rev, Nef is also an early protein which is produced following HIV infection. This protein enhances virion infectivity, decreases expression of cell surface CD4 and MHC class I molecules. Nef is also important for proviral DNA synthesis and is essential for cell to cell viral transmission. Vif protein is involved in the final step of the nucleoprotein core packaging (30). The Vpr affects localization of viral nucleic acids and virus repliction in nondividing cells such as monocyte-derived macrophages (MDMs) (31), whereas the Vpu protein increases virion release and degrades CD4 in the endoplasmic reticulum (32). The genomes of all retrovirus have three main open reading frames that encode the viral structural and enzymatic proteins: *gag, pol* and *env*. These three essential structural proteins are encapsulated by the lipid envelope. The *gag* gene encodes structural proteins: matrix protein (MA, p17), nucleocapsid protein (NC, p7), and capsid protein (CA, p24).

In the following sections, I will discuss HIV-Tat and its role in HIV immunopathogenesis as my research project focussed on the effect of HIV-Tat on IL-23 and IL-27 production in MDMs.

1.2.1 HIV-Tat and its function:

HIV encodes a transcriptional activator nuclear protein, which is well known as trans-activator of transcription (Tat) (33). Unlike other transactivators, Tat also binds to the HIV proviral DNA (34) and the DNA from host cells (35). Following cDNA synthesis, HIV-Tat is the earliest protein to be translated from the proviral DNA in the infected cell (36). Furthermore, HIV-Tat may also play a significant role in transcription complex (TC) assembly at the pre-initiation step (37). HIV-Tat modifies the conformation of chromatin structure at the proviral integration site for optimized viral transcription (37–39). To accomplish this, HIV-Tat binds to the secondary hairpin structure created at the 5' end of HIV RNA transcripts – which is known as a trans activation response (TAR) element. TAR activates through cyclin T1 and also bridges the Tat domain with the TAR loop (37–39). HIV-Tat helps to recruit transcriptional complexes such as enzymes with histone and acetyl transferase factor (HAT and FAT), which alter chromatin structure and enhance transcription (40). Tat bound to viral RNA through TAR elements recruits positive transcriptional elongation factor (pTEFb) complex through cyclin T1 (39,41,42). Recruitment of pTEFb by Tat regulates the activity of RNA polymerase II (RNAPII) and results in enhanced viral transcription (43,44). Even though recruitment of the pTEFb complex is important, Tat promotes transcription from the HIV-LTR in several other ways. Tat mediates Sp1 activation by enhancing its phosphorylation and several binding sites for Sp1 are present in the HIV-LTR (45,46). Tat also interacts with the host cell acetylation process and is acetylated itself at Lys50 by p300/CBP and at Lys28 by PCAF (47). This process causes Tat to dissociate from the pTEFb complex. This leads pTEFb to be transferred to the elongating polymerase complex. As a result, it can continue to promote carboxyl-terminal domain (CTD) phosphorylation and also frees Tat to initiate a new round of transcription (48,49).

1.2.2 Role of HIV-Tat in HIV pathogenesis:

HIV-Tat has a number of other functions in addition to transactivation of HIV transcription. HIV-Tat is recognized as a major immunomodulator in AIDS pathogenesis (50). Kashanchi et al (1994) reported that reverse transcription can occur in the absence of a functional Tat gene. However, the accumulation of proviral DNA intermediates with non-functional Tat is dramatically reduced (51). This finding showed the importance of HIV-Tat during reverse transcription and also suggests that without HIV-Tat, optimal reverse transcription cannot take place during HIV infection. HIV-Tat accumulates in the nucleus of HIV-infected cells and is secreted into the extracellular environment, mainly from CD4+T cells. It accumulates at the plasma membrane in CD4+ T cells due to its specific binding to phosphatidylinositol-4,5-bisphosphate (PI(4,5)P2) (52,53). This interaction is mediated by a specific motif in the basic domain of Tat. This recognizes a single PI(4,5)P(2) molecule and is stabilized by the insertion of Tat tryptophan side chain (53).

Tat is actively released from HIV-infected cells into the microenvironment and circulates throughout the body (54–56). Free Tat protein is detectable in tissues and in the plasma of HIV-infected individuals at a concentration of 4 - 550nM (56–61). The supernatants of *in vitro* HIV-infected monocytes contain Tat protein at a concentration of 10-100 pM range (60,62,63). Thus extracellular Tat interacts with a variety of uninfected cells, leading to the various biological effects resulting in immune dysfunction which contribute to AIDS pathogenesis.

Circulating HIV-Tat mediates immune dysfunction and pathogenic effects via particular interaction with surface receptors including CD26 (64), CXCR4 (57,65), hepran sulphate proteoglygans (HSPGs) (66), lipoprotein receptor-related protein (LRP) (67), and the VEGF receptor KDR/flk-1 (68). Many different cell types including DCs, macrophages, CD8 T cells, B-cells, and monocytes scavenge free Tat by endocytosis (69–71). Once in the cytosol, Tat can affect post-translational modification of host cell proteins (72,73), activate cell signalling pathways, induce protein degradation and also alter surface receptor molecules (74).

Tat has also been implicated in the pathophysiology of the neurocognitive deficits related with HIV infection. HIV-Tat is actively released from the infected cells and interacts with the surface receptors of uninfected cells in the brain leading to cellular dysfunction (36). In addition, Tat taken up by cells activates several host genes. Tat is highly potent and has a distinctive ability to travel along neuronal pathways. However, its production is not impacted by the use of ART once the proviral DNA has been formed (36).

It has been shown that synthetic Tat protein significantly upregulated the surface expression of CXCR4 in erythroid cells (75). In their study, they showed that Tat-mediated up-regulation of surface expression of CXCR4 mRNA and protein levels were increased in erythroid cells. Their data suggested that Tat protein plays an important role in haematopoietic gene regulation and there is a possible connection of HIV-Tat-induced anaemia (75).

1.2.3 HIV-Tat and immune dysfunction:

In addition to playing a major role in activating viral transcription, HIV-Tat is associated with immune dysfunction that is crucially involved in HIV pathogenesis. Immune-suppression is one of the major features of AIDS and Tat plays a key role in this suppression by triggering macrophages (76,77). HIV-Tat causes alterations of cytokine and chemokine production, which eventually leads to immunosuppression in HIV-infected patients. Tat alters the expression of several receptors including MHC1 (78), β2-microglobulin (79), and CD25 (the alpha chain of IL-2 receptor) (80). Tat not only kills CD4+ T cells, but also increases immunosuppression in uninfected T cells by enhancing TNF-α production (81). Since TNF-α has antiproliferative effects and high level of immunosuppressive activities, its increased production in the context of HIV-Tat promotes generation of suppressor regulatory T cells (81). It has been reported that the basic region of Tat binds to the cell membrane of lymphocytes and exhibits a cytotoxic activity (82). Their data suggested that potential cytotoxicity of HIV-Tat on immune T cells led to their depletion and the immunosuppressed phenotype of HIV-infected patients (82).

Tat not only causes cytotoxic depletion of lymphocytes, but also detrimentally effects calcium-mediated events in immune cells leading to the immune dysfunction. HIV-Tat inhibits some DCs functions by competing with L-type calcium channels expressed by these cells (83). Several groups have observed that L-type calcium channels are expressed by NK cells responsible for killing cancer cells. Additionally, they identified that L-type calcium channels are the basic molecular target of Tat on NK cells. Blocking of these channels by Tat results in lack of degranulation, and ultimately leads to impairment of NK mediated cytotoxicity (84).

Tat also downregulates MHC-1 expression on HIV-infected cells by suppressing the activity of a major MHC class I gene promoter (78) leading to the loss of CTL activity (85).

Furthermore, in macrophages, HIV-Tat modulates the activity of nitric oxide synthase (iNOS) gene – causing dysfunction of the macrophage respiratory burst mechanism. This further weakens the host's first line of defence against the pathogens (86).

An additional mechanism by which Tat plays a role in HIV pathogenesis is by increasing expression of HIV surface coreceptors such as CXCR4 and CCR5 on CD4 T cells, resulting in enhanced susceptibility of cells to HIV infection (87). HIV-Tat is also able to mimic the chemokine receptor which leads to migration of monocytes / macrophages towards infected cells and assists in the activation of infection (88). Katrina Gee et al has shown that extracellular and intracellular HIV-Tat induced IL-10 expression in normal human monocytes and promonocytic THP-1 cells (89,90). Furthermore, recent evidence showed that HIV-Tat peptide interacts with TLR4-MD2 to promote the immunosuppressive cytokine IL-10 and proinflammatory cytokines (TNF-α) in mouse monocytes/macrophages (91).

1.2.4 Structure of HIV-Tat:

HIV-Tat is a small 101 amino acid polypeptide which is a dominant transcriptional activator of viral gene expression and acts to enhance HIV long terminal repeat-specific transcription (37,92)(37). Tat consists of two exons: 1-72 amino acid (AA) encoded in exon 1 and the residue 73-101 encoded by the second exon(93).

HIV-Tat protein sequence can be divided into six well-described regions (Figure 2). The first amino-terminal region (1-21AA) is involved in the release of Tat protein from infected cells (94). Upon acidification below pH 6.0, Tat inserts into membranes such as monolayers or lipid vesicles. This insertion step relies on Tat single Trp-11 which is strictly required for translocation (95).

The second region (22-37 AA) is the cysteine rich region. This region contains 7 conserved cysteine residues at amino acid positions 22, 25, 27, 30, 31, 34, and 37. These cysteines participate in zinc ion binding while associated with the p-TEFb complex (96). Mutations within this region, except at position 31, result in a loss of HIV-Tat-mediated transcription (97).

The third region (38-48 AA) is the core region. It consists of the ^{41} "KGLGISYG"^{48} amino acid sequence. The amino acid sequence of AA45 to 48 (^{45}ISYG^{48}) is the minimal region necessary for transcriptional transactivation (98).

The fourth region of Tat (49-57 AA) is basic. It is arginine-lysine-rich (49-57) and has a well known sequence of "RKKRRQRRR", which is necessary for the nuclear localization and nucleic acid binding (99,100). Additionally, the arginine-lysine rich basic region (48-60AA) has been reported to facilitate membrane transduction (101) assisting in HIV reverse transcription (102) and augments integrin receptor binding through α5β1 and αvβ3 (103). It is also required for interaction of extracellular Tat with heparan sulfate proteoglycans (HSPGs) at the cell surface before endocytosis of the protein and Tat protein translocation from the cytosol to the nucleus (104,105). This basic region is also required for binding to the TAR element (98). This region has been shown to alter many post-translational modifications such as acetylation (106), ubiquitination (107), and methylation (108,109). It has been shown that HIV-Tat peptide (basic region) interacts with TLR4-MD2 to promote the immunosuppressive cytokine IL-10 and proinflammatory cytokines (TNF-α) in mouse monocytes/macrophages (91).

A glutamine rich region from residue 60 to 72 is the fifth region of the Tat protein. This region is involved in enhancement of tubulin polymerization by directly trigging the mitroclndial pathway to induce Tat mediated apoptosis in T cells (110).

The sixth region (73-86 AA) is a C-terminal region that contains the RGD domain found on extracellular matrix molecules (69,111). This RGD domain allows Tat to adhere to the cells expressing integins α5β1 and α5β3 (69,99,112,113). This region is also able to modify cytoskeleton structure, (114) alters normal organization of primary rodent neurons and astrocytes (115,116) (Fig 2).

Fig 2: The amino acid sequence of human immunodeficiency virus 1 (HIV-1) Tat protein

Acidic N-terminal Region (residues 1 - 21)

- 13 amino acids with amphipathic characteristics

Cysteine Rich Region (residues 22 - 37)

- contain 7 cysteines (highly conserved between HIV-1 isolates)

Core Region (residues 38 - 48)

- contains RKGLGI motif (conserved between HIV-1, HIV-2 and SIV)

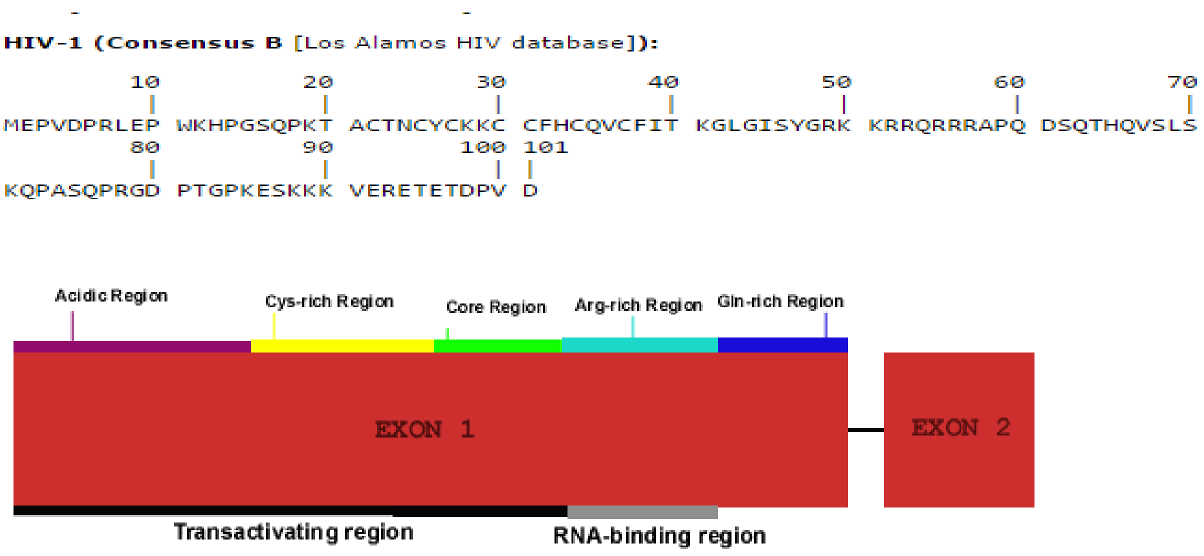
Basic Region (residues 49 - 72)

- contains RKKRRQRRR motif

Cell Adhesion C-terminal Regions (residues 73 - 101)

- contains RGD motif (cell adhesion signal for binding to cellular integrins)

The amino acid sequence of human HIV-1 Tat protein



```
HIV-1 (Consensus B [Los Alamos HIV database]):
        10         20         30         40         50         60         70
         |          |          |          |          |          |          |
MEPVDPRLEP WKHPGSQPKT ACTNCYCKKC CFHCQVCFIT KGLGISYGRK KRRQRRRAPQ DSQTHQVSLS
        80         90        100 101
         |          |          |  |
KQPASQPRGD PTGPKESKKK VERETETDPV D
```

Synthetic Tat peptide sequence

MEPV DPRL EPWK HPG SQPK TACT NCYC	Tat 1 Peptide (1-27)	Acidic region
QPK TACT NCYC KKCC FHCQ VCFI TKGL	Tat 2 peptide (17-43)	Cysteine rich region
HCQ VCFI TKGL GISY GRKK RRQR RRAP	Tat 3 peptide (33-59)	Core+ Basic region
RKK RRQR RRAP QDSQ THQV SLSK QPAS	Tat 4 peptide (49-75)	Basic+ Glutamine rich region
HQV SLSK QPAS QPRG DPTG PKES KKKV	Tat 5 peptide (65- 91)	Glutamine rich region + C-terminal region
PTG PKES KKKV ERET ETDP VDQ	Tat 6 peptide (81-102):	C-terminal region + Exon-2

Figure

Modified from http://www.bioafrica.net/proteomics/TATprot.html

1.3.1 HIV infection in Macrophages:

The main targets of HIV are CD4+ T cells and macrophages (116,117). Macrophages are differentiated immune cells, which play a key role in cleaning of cellular debris and pathogens by phagocytosis. In addition they also work as antigen presenting cells, which present antigen peptides to CD4+ T cells through MHC II (117,118). Due to this interaction between macrophages and CD4+ T cells, macrophages transmit HIV particles to CD4+ T cells (118,119). Furthermore, HIV-infected macrophages discharge soluble cytotoxic factors capable of causing apoptosis of bystander cells such as CD8+ and CD4+ T cells (120,121). The hallmark of HIV infection is the progressive depletion of CD4+ T cells. However macrophages are resistant to the cytopathic effect of HIV (122,123). Since macrophages are a key reservoir in HIV infection, they act as a source of virus production for the HIV-infected individuals (124). In addition, macrophages are virtually present in all organs of the body and hence can spread HIV all throughout the body (125).

1.3.2 HIV entry into macrophages:

HIV enters the target cells following interaction of viral gp120 with CD4 receptors, which are present on both T cells and macrophages (126,127). The second step involves fusion of HIV envelope with the host cell membrane and this process is supported by either CXCR4 (T cell strain or X4) or CCR5 (R5 strains) or both co-receptors (R5X4) (128,129). R5 and X4 viruses are able to replicate in both T cells and macrophages but their efficiency of replication is different between these cell types because of different cellular environment. Furthermore, the viral progeny from T cells and macrophages can be similar but they may contain different sets of host proteins integrated within their viral particle depending on the cell type from which they were generated. In addition to CCR5 and CXCR4, other chemokine receptors including CCR2b, ChemR1 and CCR3 are also used by M-tropic and dual tropic viral strains (130,131). Interestingly HIV-Tat induces CXCR4, CCR5, and CCR3 expression in peripheral blood mononuclear cells (PBMCs). The induction levels of CCR5 and CXCR4 are associated with Tat-enhanced infectivity of M- and T-tropic viruses, respectively (87).

1.4.1 HIV infection and cytokines production:

Cytokines are important immunological mediators of immune response. In response to an infection, cells generate proinflammatory cytokines such as type 1 interferon (IFN), TNF-α, IL-15, IL-6, and IFN-γ which have been shown to up-regulate viral replication (132–134). Many cytokines and chemokines including IFN-α, IL-15, TNF-α, IP-10, MCP-1, IL-6, IL-8, IL-10, and IL-18 have been detected in an HIV-infected individual's plasma at an early stage of HIV infection (135). It has been reported that increased TNF-α, IL-6, IL-18, and IL-7 correlated with decreased CD4+ T-cell counts in HIV infection (136). Other cytokines such as IL-15, IL-12, and IL-27 can increase anti-viral responses (137–139). In contrast, no change or down regulation of IL-2, IL-4, IL-6, or TNF-α production was observed in HIV-infected individual's plasma (140–142). Additionally, alveolar macrophages from HIV-infected individuals exhibited decreased TNFα production in response to TLR-4 stimulation (143). Nevertheless, cytokines mediating anti-viral responses or increasing disease progression are not mutually exclusive. For example, in SIV-infected macaques, IL-15 has been reported to increase simian immunodeficiency virus (SIV)-specific CD8+ T-cell responses; however, it has also been correlated with higher viral set-points, disease progression, and increased viral replication (133,142).

1.4.2 IL-12 family of cytokines and their role in HIV infection:

IL-12 has various biological functions and significantly bridges innate and adaptive immunity by promoting a Th1 response (144). Th1 cells make IFN-γ, IL-2, TNF-β, which activate B cells and macrophages and induces cell-mediated immunity (145). IL-12 is particularly important to host resistance against intracellular pathogens. The activation of macrophages through induction by IFN-γ from T cells and NK cells enhances cell-mediated immune responses (144). IL-12 also acts to reduce Th2 cytokines, including IL-10 and IL-4 in allergen-specific human CD4+ T lymphocytes (146), which are up regulated throughout chronic HIV infection (147–149). During early/acute HIV infection, Th1 cytokines, IL-12 and IFN-γ production are increased (150,151). The levels of these cytokines decreases with disease progression towards chronic infection (152–155). Moreover, HIV-infected individuals have been shown to have decreased IL-12 production in serum (155), supernatants activated PBMCs with phytohaemagglutinin and LPS (154,156,157), monocyte/macrophages (158), and DCs(159).

An increased activation of macrophages in HIV-infected individuals on HAART has been linked to increased microbial translocation from the gut (160). Thus, macrophages from HIV-infected individuals are being constantly exposed to bacterial products including LPS/endotoxins. Macrophages constitute an important source of immunoregulatory cytokines such as IL-12 family of cytokines particularly following LPS stimulation. It is well established that macrophages from HIV-infected individuals and macrophages infected *in vitro* with HIV exhibit reduced ability to secrete various cytokines including IL-12 (148,152,156,160,161). Thus, dysregulation of macrophage function in HIV infection is an important factor in the incomplete immune recovery in HIV infected patients on HAART. Since IL-12 family of cytokines are critical mediators of innate and adaptive immune responses (162), it is important to understand whether HIV or its accessory proteins influence other members of IL-12 family cytokines such as IL-23 and IL-27.

One of the HIV accessory proteins, Vpr, has been shown to decrease IL-12 production in DCs (163) and IL-12p35 cytokine levels in monocytes and macrophages (164). There is only one study that showed inhibitory effects of HIV-Tat on IL-12 production in human PBMCs stimulated with *Staphylococcus aureus* Cowan 1 strain (SAC) (165).

Monocytes and macrophages play an important role in a variety of immunoregulatory, phagocytic, and secretory functions. Macrophages are a major source of many cytokines including IL-12 family of cytokines such as IL-12, IL-23 and IL-27 (166). The role of IL-23 and IL-27 in HIV infection has yet to be elucidated. There is increasing attention to their possible value as immunomodulatory thearapies in HIV infection because of their important function in T-cell regulation and cell signaling transduction pathways.

These observations have drawn attention to the possible benefit of giving IL-12 as an adjuvant in DNA vaccination to enhance cell-mediated immune responses. The potential of IL-12 as an adjuvant in DNA vaccination with HIV/SIV-antigens has been investigated in mice and rhesus macaques. (167–172). The IL-12-deficient mice vaccinated with HIV-gp120 DNA responded inadequately by producing low levels of antigen-specific IFN-γ, and impaired cytotoxic T-lymphocyte (CTL) immune responses (173,174). Interestingly, IL-12- deficient mice exhibited restored cell-mediated immune responses, following administration of exogenous IL-12 indicating that IL-12 plays an important role in HIV-specific CTL responses (173). Their data suggested that the CTL-promoting function of IL-12 is IFN-γ-mediated. IL-12 has been observed to enhance antigen-specific IFN-γ production (168,169,174,175), proliferative responses (175), granzyme-B production (168) and enhance antigen-specific effector memory T-cell function in mice/rhesus macaques vaccinated with HIV/SIV-antigens (167,170,175). Thus, IL-12 is important for cell-mediated immunity and also has therapeutic potential to enhance cell-mediated immunity in HIV infection.

Taken together, IL-12 can act as an immunomodulatory therapy by enhancing cell-mediated immunity. In contrast to the findings discussed above, it has been shown by other groups that chronically SIV-infected rhesus macaques do not respond to IL-12 therapy because the IL-12 did not lead to inhibition of SIV replication (176) or the viral loads were found to remain unchanged (177). Two other members of the IL-12 family of cytokines namely IL-23 and IL-27 have been characterized and are discussed below (178,179).

1.5.1 Interleukin-23:

IL-23 is composed of two subunits, IL12p40 and IL23p19 whereas IL-12-p40 is shared between IL-12 and IL-23. IL-23p19 is 70% homologous with IL12p35 (180,181) (Fig 3). Unlike IL-12p35, IL-23p19 is not ubiquitously present and is expressed only in immune cells like T cells and APCs (180). In order to form a biologically active IL-23, both subunits IL12p40 and IL23p19 have to be nearby in the same cell (180). The IL-23 receptor comprises of IL12Rβ1, which is expressed on CD4+ T cells and the IL23R-α chain. IL-23 receptor signalling involves the activation of janus kinase (JAK) signaling pathways. It also activates the (STAT)s transcription signalling molecules (182) including STAT-1, STAT-3, STAT-4, STAT-5 JAK-2, and tyrosine kinase (TKY)-2 (178,183). STAT-3 activation by IL-23 is necessary for the maintenance of the Th17 cell population (184).

IL-23 receptor is greatly expressed on memory T cells but not on naive T and effector T cells (185). This suggests that IL-23 does not induce Th1 differentiation in a manner similar to IL-

12. Furthermore, IL-23R is present on APC including monocytes, macrophages, and DCs (183). IL-23 induces the production of pro-inflammatory cytokines including IL-1β and TNF-α, which helps to sustains the Th17 cell population (186). Th17 response is considered as a pro-inflammatory response and is characterized by the production of IL-26, IL-22, IL-21 and IL-17 (187). IL-17 predominantly induces the expression of CCR6, which brings neutrophils to the infection site to promote innate immune responses (188). Similar to IL-12, IL-23 is also considered as a pro-inflammatory cytokine because of its distinctive property to activate the Th17 cellular response.

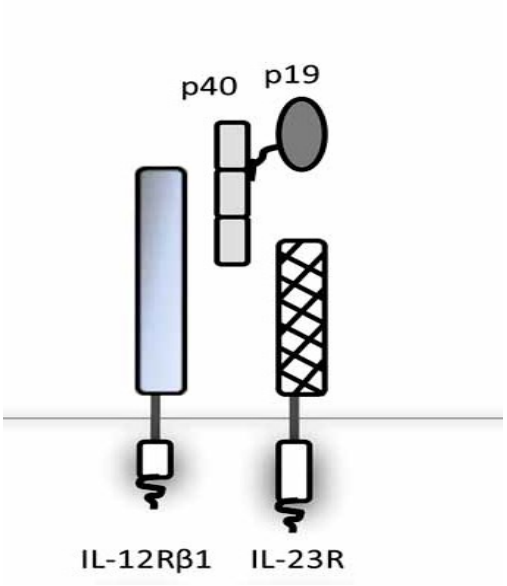


Fig. 3: IL-23 and its receptor.

1.5.2 The role of IL-23 in HIV infection:

IL-23-induced Th17 cells are essential for removal of infection and maintenance of mucosal integrity of the gut. IL-23-induced Th17 responses result in the induction of various cytokines and recruitment of neutrophils, which are important for the removal of bacterial pathogens and extracellular fungi such as *Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeroginosa, Citrobacter rodentium, and Candida albicans* (189). IL-23-induced IL-17 production is known to increase the integrity of epithelial barriers via the initiation of claudin-1 and -2, which help to enhance tight-junction formation in the intestine (190). IL-17 has been shown to increase antimicrobial peptide β-defensins in human airway epithelium (191). Similarly, Th17-derived IL-17A and IL-17F were capable of producing antimicrobial β-defensins from keratinocytes (192). The above findings imply that IL-23-induced Th17 responses may help to maintain the integrity of the intestine via mucus secretion, induction of antimicrobial peptides and the maintenance of tight junctions. IL-23 is also known to induce IFN-γ production and activate memory T-cells (181). Thus, IL-23 production in HIV infection may be important to uphold the integrity of the intestinal barrier suppress microbial translocation and play a key role in the induction of HIV-mediated immune responses.

IL-23 is highly expressed in DCs and has been shown to prime CD8+ T cells against HIV antigens (193). During HIV infection, systemic immune activation and microbial translocation have been observed in patients. It is known that in HIV-infected individuals, Th17 cells were depleted in the gut associated lymphoid tissue. The production of IL-17 has been measured in the gastrointestinal tract and blood of HIV-infected long term nonprogressors (LTNP) and typical-progressors (TP) (194–196). Th17 cells were increased in LTNP and were inversely correlated with HIV-RNA suggesting greater number of Th17 cells in LTNP led to a more preserved immune response against bacterial infection leading to lower immune activation and slower disease outcome (194). Further, HIV/SIV-infected individuals treated with combination antiretroviral therapy (cART) have been observed to have increased Th17 cell populations (197–199); however, inconsistent responsiveness has been observed (197). One group pointed out that in order to restore IL-17 in HIV infection to normal levels, the total suppression of viremia is required (200). Therefore, IL-23 production and viral reservoirs may play a role in variable recoveries of Th17 cells in HIV-infected individuals' guts. However, it is uncertain whether IL-23 is affected by HIV infection or whether IL-23 manipulates disease progression remains unknown.

Currently, very little is known about the effect of HIV on IL-23 production. HIV-infected individuals with severe disease receiving ART exhibited low IL-23 mRNA basal levels in their PBMCs. It was suggested that IL-23 downregulation may be correlated with increased vulnerability to opportunistic infections (201). However, another group showed enhanced LPS/IFN-γ-induced IL-23 production from acute HIV-infected individual's DCs and monocytes (202).

The involvement of the HIV-Tat protein in the regulation of proinflammatory cytokines has been studied in human monocytic cells. There is one report that suggests the involvement of HIV-Tat protein in the inhibition of IL-12 production in PBMCs. Specifically, the role of HIV-Tat in modulation of IL-23 production has not been investigated. Therefore the main focus of the study described herein is to investigate HIV-Tat in modulation of LPS-induced IL-23 production in human MDMs.

1.6.1 Interleukin-27:

IL-27, a member of the IL-12 family of cytokines, is a heterodimeric cytokine composed of two subunits, namely EBI3 and p28 that are homologous to IL-12p40 and IL-12p35 proteins respectively (203) (Fig 4). The EBI3 subunit is expressed only in APCs such as macrophages and DCs (204). Upon stimulation through TLR-2, 4 and 9, EBI3 is induced via activation of NFκB and PU1 transcription factors on its promoter region through the MyD-88 signalling pathway (204,205). Like IL-23p19, IL-27p28 is also expressed in activated monocytes, macrophages and DCs (206). The activation of TLR4 by LPS induces IL-27p28 expression (205).

The two subunits of IL-27 are regulated differently; however, both subunits are necessary to be present in the same cell to secrete biologically active IL-27 (203). IL-27 is considered to be both pro- as well as anti-inflammatory cytokine (203).The IL-27 receptor is composed of IL-27Rα/WSX-1/T-cell cytokine receptor (TCCR) and glycoprotein-130 (gp130) (206,207). Naive T cells express lower WSX-1 surface levels compared to activated T cells (207). WSX-1 knockout mice have been shown to have lower levels of IFN-γ early on following infection with *Leishmania major*; whereas, normal levels were restored via secondary infection IL-27 is known to enhance IL-12-induced IFN-g production via induction of IL-12R-beta-2. These observations suggested that WSX-1 is important for the initial mounting of Th1 responses (208,209). Furthermore, IL-27 may have a unique pro-inflammatory function in addition to its anti-inflammatory properties. Furthermore, WSX-1 knockout mice infected with intracellular microbes failed to down-regulate their adaptive immune response and suffered from a lethal CD4+ T cell-dependent inflammatory disease (210).

IL-27 is required for inhibition of Th17 response through the activation of STAT1, which down regulates RORγ transcription factor in these cells to avoid Th17 secretion including IL-17

(211). Recently, IL-27 has been identified as an important factor to induce IL-10 immunosuppresive cytokine (212). These observations suggest that IL-27 works as an anti-inflammatory mediator to reduce an existing pro-inflammatory responses (213). As a consequence, IL-27 biological activity is in direct opposition with IL-23 biological activity.

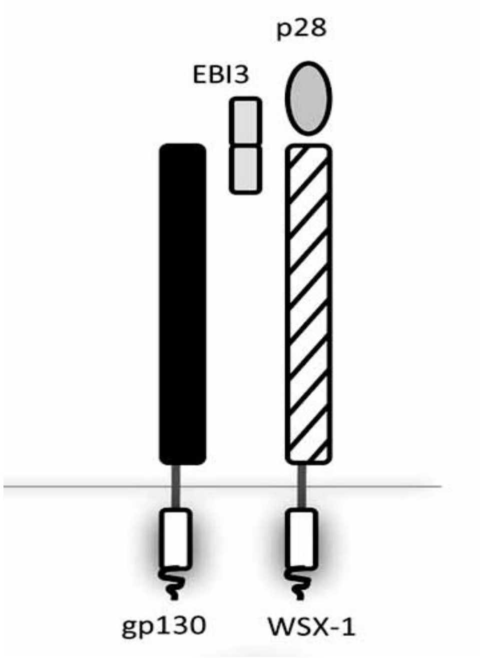


Fig. 4: IL-27 and its receptor

1.6.2 The role of IL-27 in HIV infection:

During HIV infection, cell-mediated immune responses are severely impaired. In chronic HIV infection, the production of Th1 cytokines such as IL-12 and IFN-γ are decreased (146,148,152,153,160). In contrast, Th2 cytokines including IL-4 and IL-10 are enhanced during chronic HIV infection (147–149). An *in vitro* study conducted with HIV-exposed sero-negative patient's PBMCs showed that their immune cells were capable of producing a strong Th1 responses (149). These findings suggest that efficient Th1 responses are essential for HIV-specific immunity.

IL-27's capability to inhibit Th2 responses and promote Th1 responses may be useful to improve anti-HIV immune function. One study demonstrated suppressed circulating IL-27 production in HIV-infected individuals (214). IL-27 has also been demonstrated in naive CD8+ human and murine T cells to induce proliferation, IFN-γ production, and granzyme-B production (139,215). DCs produce high levels of IL-27, present HIV-antigen to the CD8+ T cells and induce granzyme-B production and IFN-γ (193). Therefore, IL-27 may enhance HIV-specific anti-viral responses and cell-mediated immunity. Therefore, due to the biological functional similarities between IL-12 and IL-27, similar to IL-12, IL-27 may be helpful in enhancing efficacy of DNA vaccination HIV specific immunity and reducing viremia in HIV-infected patients.

T-regulatory (Treg) cells maintain intestinal homeostasis by regulating T-cell effecter activities and sustaining immune privilege at the mucosal barrier (216). In the gut, microbial translocation and intestinal permeability are up-regulated in HIV-infected individuals. This may be one of the factors causing increased systemic immune activation in HIV-infected patients (217). Additionally, in the rectal mucosa of HIV patients, Treg cell frequencies were positively correlated with increased immune activation (218). Furthermore, elevated immune activation and severity of disease has also been correlated with upregulated relative frequencies of Treg in the blood of HIV-infected individuals (219,220). The role of the IL-27 in the regulation of Th17/ Treg expression has been investigated in unexplained early recurrent miscarriage patients. IL-27 was shown to inhibit IL-17 production and at the same time upregulated IL-10 in unexplained early recurrent miscarriage patients (221). In addition, the expression of IL-17 was decreased and Treg populations increased in the spleens of CIA mice treated with IL-27-Fc compared with the CIA control mice (222). While the precise function of Treg cells in HIV disease progression remains inadequately understood, administration of IL-27 as a therapy to the HIV infected individuals may be helpful in reducing the relative frequency of Treg cells. Thus, inhibition of Treg cells in the gut may help in re-establishing the Treg/Th17 cell balance, which may help improve mucosal integrity in the gut and decrease immune activation.

HIV is modulated by an array of host cellular factors. Recent evidence suggests that IL-27 inhibits SPTBN1 (spectrin β nonerythrocyte 1), one of the host restriction factors necessary for HIV-1 infection in macrophages (223). Additionally, IL-27 inhibits the expression of SPTBN1 through the TAK-1-mediated MAPK signaling pathway (223). The above findings suggest that the critical host component factor, SPTBN1, can be targeted to inhibit HIV replication in one of the key HIV-1 reservoirs – the macrophages. IL-27 was also shown to inhibit HIV replication in monocytes and CD4+T cells from PBMC (224). Moreover, IL-27 inhibited HIV-replication indirectly via the induction of IFN-α in CD4+ T cells and IFN-α and IFN-β in monocytes, macrophages, and PBMCs (225). IFN-α and IFN-β activation resulted in increased expression of apolipoprotein B mRNA editing enzyme-catalytic polypeptide-like (APOBEC)-3A and APOBEC3G.

IL-12 and IL-23 have not been reported to inhibit HIV replication (225). However, IL-27's unique ability to inhibit HIV replication has been well documented. Furthermore, IL-27 inhibition of HIV appears to be independent of viral entry because CD4, CCR5 and CXCR4 expression was not affected by IL-27 (225). Inhibition of HIV replication by IL-27 suggests that IL-27 could be used as a promising anti-HIV therapy.

In general, IL-27 increases cell-mediated immunity, inhibits host cellular factors and suppresses Treg cells. Presently, combinatorial antiretroviral therapy (ART) is generally prescribed to suppress HIV infection (226,227). ART has made significant contributions to increasing the life span of HIV-infected individuals (227). However, ART is incapable of completely eliminating HIV from infected individuals since the levels of virus quickly rebound if antiviral treatment is stopped (228,229). Since growth kinetics of HIV differs in T cells and macrophages, various novel approaches are required to entirely eliminate HIV-1 from infected patients. Currently, there is little evidence to show the level of IL-27 production in HIV infection. There is also no evidence available in the literature regarding the effect of HIV or accessory proteins such as Tat on IL-27 production. Recently, Shifawn O'Hara (previous master student) demonstrated that *in vitro* HIV infection can inhibit LPS-induced IL-27 production in MDMs. It is possible that one of the accessory proteins of HIV such as Tat may inhibit LPS-induced IL-27 production. Therefore, one of my research objective was to study the effect of HIV-Tat in the modulation of LPS induced IL-27 production in human macrophages.

1.7.1 Toll-Like Receptor Signalling:

It has been shown that TLRs mediate a key role in innate detection of microbes that leads to activation of adaptive immunity (230). The production of cytokines involves the activation of TLR signaling pathways (230). TLR signaling pathway activation is initiated by extracellular ligand attachment through cell membrane receptors (231). Recognition of pathogens is mediated by transmembrane molecules identified as a pattern recognition receptors (PRRs), which play a significant role in stimulation of both innate and acquired immunity (230). Presently, 13 human TLRs have been discovered to be expressed on a range of cell types such as macrophages, endothelial cells, DCs, lymphocytes and epithelial cells (231,232). The majority of TLRs are expressed on the cell membrane (TLR1, 2, 4, 5, 6, 10 and 11) while the rest of the TLRs (TLR-3, 7, 8, and 9) are expressed on intracellular membranes (231). TLRs are expressed on immune cells as homodimers or heterodimers and each receptor pair is accountable for detection of a diverse range of microbial ligands (231).

Figure 5: LPS/TLR4 activated signaling pathways

The MyD88-dependent pathway. MyD88 activates IRAKs/ TRAF6 as well as the transcription factors NFκB, AP-1 and IRF-5 further downstream. These transcription factors induce expression of proinflammatory cytokine genes.

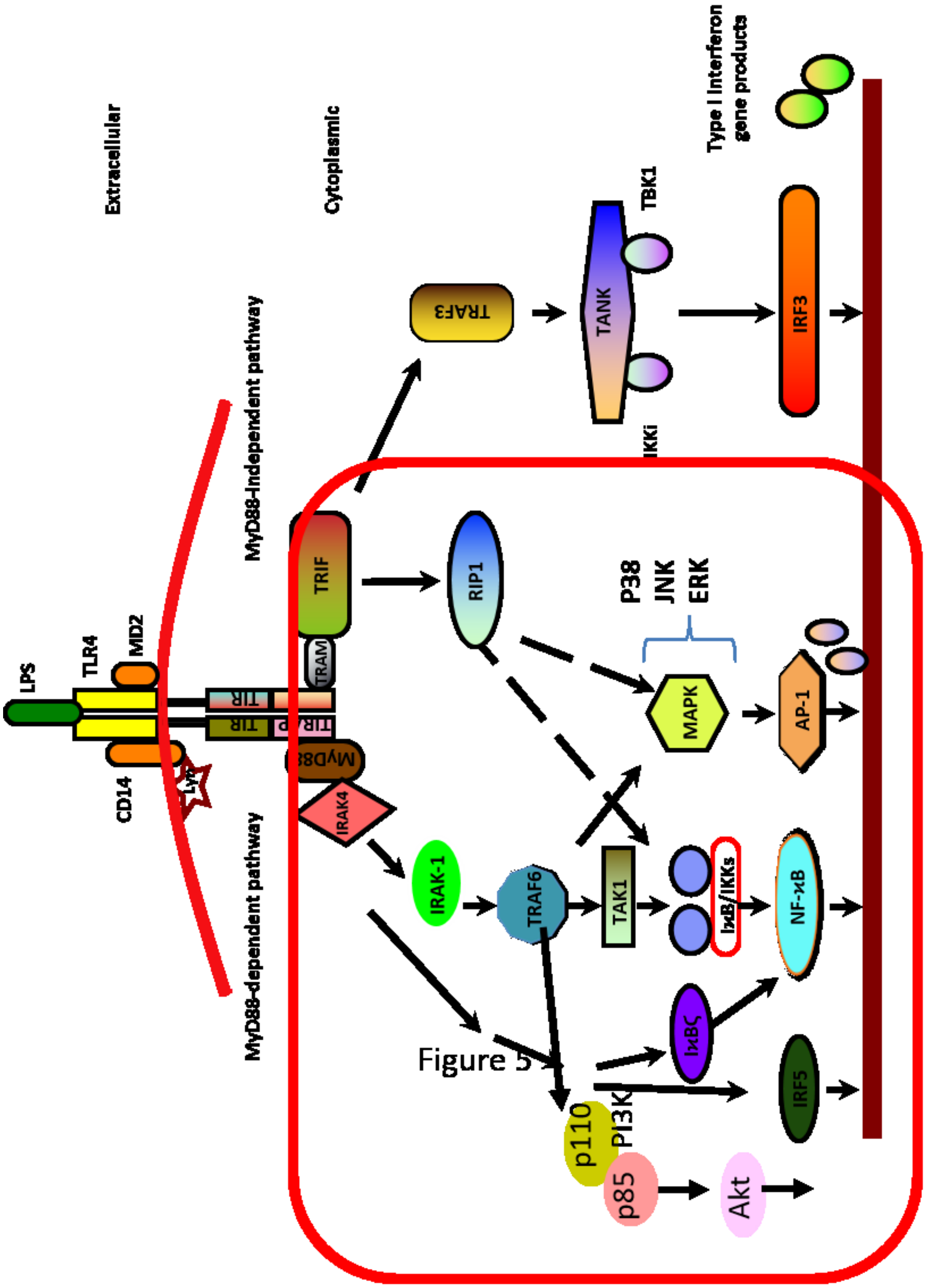


Figure 5

Modified from Y.-C. Lu et al. *Cytokine* 42 (2008) 145–151

TLR4 binds LPS from gram-negative bacteria, TLR2 detects peptidoglycans from gram-positive bacteria, TLR5 recognises flagellin and TLR9 detects foreign genomic DNA (233,234). However, viral recognition, binding of dsRNA and ssRNA are detected by TLR3 and TLR7/8 (235,236). Microbial ligand attachment to the TLRs activates a wide range of pro- and anti-inflammatory cytokines (231). The TLRs cytoplasmic tail contains conserved TLR/IL-1R homology (TIR) domains (237), which cooperate with TIR domains on adaptor proteins (238). The TIR domain is required for signal transduction during interaction with TLR adaptor proteins (239). Presently five TLR adaptor proteins have been recognized, namely myeloid differentiation factor 88 (MyD88), Toll-interleukin 1 receptor adaptor protein (TIRAP), TIR-domain-containing adapter-inducing interferon-β (TRIF), the B-cell adapter for phosphoinositide-3-kinase (PI3K) (BCAP), and TRIF-related adaptor molecule (TRAM) (240,241). The binding of TIR-TIR on the TLR causes recruitment of Interleukin-1 receptor associated kinase (IRAK) proteins and TNF receptor associated factor 6 (TRAF6) to the complex, which further triggers downstream signaling pathways.

The TLR-induced immune responses have been related to either MyD88 and/or TRIF-dependent signalling pathways (242). Activation of MyD88-dependent signaling pathway promotes proinflammatory cytokine release including TNF-α and IL-12 from B cells (243). Activation of TLR4-MyD88 dependent pathways leads to MAPKs activation triggering activation of downstream transcription factors including AP-1 and NFκB, which are essential for the induction of IL-12 family of cytokines including IL-12, IL-23 and IL-27 (244). On the other hand, the TLR4-TRIF dependent pathway induces type-1 interferon genes by activating IRF3 transcription factor (242). Studying the role of TLR molecules, the regulation of gene expression, and the production of cytokines in response to pathogenic invasion will be strategically useful to restore the impaired immune function in the context of HIV infection.

1.7.2 PI3Ks and MAPKs:

Upon LPS binding to the CD14 protein, the TLR4 signaling molecules promote dimerization and subsequent activation of wide range of transduction pathways eventually leading to the induction of genes encoding both pro- and anti-inflammatory cytokines (242). Among others, TLR4 activates two main pathways: phosphoinositide-3- kinase (PI3K) pathway and the mitogen activated protein kinase (MAPK) pathway (245). PI3K is a heterodimeric signalling molecule composed of the p85-regulatory and the p110-catalytic subunits (246). The downstream target of the PI3K signalling molecule is AKT (247) and the activity of AKT is measured to determine indirectly activity of PI3K. A newly identified TLR adaptor protein, BCAP, has been found to mediate through the MyD88-dependent PI3K activation pathway (241). It has been shown that PI3K can interact with TRIF through TLR3 or TLR4 (248). BCAP may also associate with the TRIF-dependent pathway to activate PI3K (241).

In general the MAP family is involved in regulating cell growth, cell survival, neuronal response, differentiation, and the immune response (249). The canonical activating signaling pathway for MAPKs is a triple kinase module, where a MAPK kinase kinase (MKKK) phoshorylates a MAPK kinase (MKK) which in turn activates a terminal MAPK during a dual phoshorylation mechanism on a tyrosine and threonine residue (250). Upstream of this canonical pathway, it has been identified that Ras and Rho families of GTPases mediate the signal pathway from receptor complexes at the membrane (250).

Four main MAPK pathways have been identified: extracellular-signal-regulated kinase 5 (ERK-5 also known as Big MAPK-1 (BMK-1)), extracellular-signal-regulated kinase (ERK), c-

Jun-N-terminal Kinase (JNK) and p38 pathways (250,251). Moreover, each MAPKs are expressed in different isoforms: ERK1, ERK2, JNK1, JNK2, JNK3, p38α, p38β, p38γ and p38δ (250). Upon MAPK activation, the molecules of ERK1/2 separate from anchor proteins and translocate to various organelles, including 50-70% to the nucleus (252). ERK1/2 is important for its activation of Elk1 transcription factor involved in the induction of immediate early genes essential for proliferation and differentiation (252).

Similarly, p38 is also important for the proliferation and differentiation of hematopoietic cells (253). p38α activates transcription factors GATA-1, and C/EBP and it has been observed that p38 is important to maintain neutrophils at the site of infection (253). Further, p38 mediates activin-A-mediated differentiation in other myeloid cells (253). Therefore, both ERK1/2 and p38 play an important role in cell survival and differentiation.

The JNK pathway is involved in the production of many proinflammatory cytokines. For example, JNK has been shown to activate c-Jun and ATF2 transcription factors, and JNK-knockout cells demonstrate significantly reduced levels of TNF-α (254). Taken together, the MAPKs get activated upon TLR ligand binding and have been implicated in cytokine production.(254). Downstream TLR activation of p38, JNK and ERK MAPKs also happens through MyD88 and TRIF-dependent pathways. The activation of MyD88 and TRIF-dependent TAK-1 triggers the recruitment of TAK-binding protein 1 (TAB1), TAB2 and TAB3 (254). The complex of TAK/TAB is able to activate NF-κB, followed by p38, JNK, and ERK MAPK signalling pathways, which are important for cytokine production. The LPS/TLR signalling pathway is depicted in Fig 5.

1.7.3 MAPK-mediated regulation and cross-talk between TLR molecules:

There is contrasting evidence describing the PI3K/AKT-mediated regulation of NF-κB. Upon LPS-stimulation in THP-1 cells, the PI3K/AKT signaling pathway has been shown to negatively regulate NF-κB activation (255), whereas other researcher has identified PI3K as a positive regulator of NF-κB in human monocytes, 3T3 fibroblasts and Hep2G cells (256–258). The siRNA knockdown of PI3K/AKT in MDMs and DCs inhibited LPS-induced JNK activation without affecting the activation of ERK or p38 MAPK (259). On the contrary, constitutive AKT activation was reported to positively regulate NF-κB downstream of p38 activation (257). TLR-stimulation can also induce inhibitory signals. For example, p38 could negatively regulate ERK MAPK/NF-κB-dependent increases in proasthmatic symptoms (260). It has been revealed that there is a considerable amount of cross-talk among the PI3K, MAPK and NF-κB signaling pathways. Also, it is possible there are several other TLR interactions involved in signaling pathways. PI3K and MAPKs activation can diversely regulate a range of genes, which may contribute to the balance and regulation of immune responses. These TLR molecules likely play a key role in HIV immune dysfunction. IL-23 and IL-27 cytokine production have been shown to be impacted by TLR signaling molecules, which will be discussed in the following section.

1.7.4 Regulation of TLR-induced IL-23 production:

LPS induced TLR4 signaling pathways regulating IL-23 production have been mainly studied in monocytes, macrophages and DCs. The regulation of IL-12/23 p40 has been investigated more extensively. However, since IL-23 is a newly discovered cytokine compared to IL-12, the regulation of IL-23 p19 and specifically IL-23 protein is not well understood.

In response to LPS stimulation, ERK MAPK was shown to negatively regulate IL-12/23 p40 expression in human macrophages (261) murine macrophages (262–264) and in macrophages post-stimulation with *Mycobacterium tuberculosis* (265). On the contrary, some researchers demonstrated a positive regulation or no effect on the expression levels of LPS-induced IL-12/23 p40 by ERK activation (266–268).

Similar to the ERK MAPK, p38 MAPK has been widely investigated in the regulation of IL-12/23 p40 expression. In response to LPS, p38 MAPK positively regulated IL-12/23 p40 in murine macrophages (266–268), human monocytes (269,270), THP-1 cells (269), human myeloid DCs (267), human macrophages (261) and PBMCs (270). Furthermore, post-stimulation with CpG, the p38 MAPK positively regulated IL-12 p40 in murine and human macrophages (271)(272), human DCs (273), post-stimulation with endogenous serum amyloid A in THP-1 cells and human monocytes (274) and *M. tuberculosis* post-stimulation in human macrophages (275). Another contrasting study showed that upon stimulation with various TLR agonists including LPS, CpG, flagella, and lipid A, p38 MAPK negatively regulated IL-12/23 p40 expression in human macrophages (276). These findings suggest that involvement of p38 MAPK in the regulation of IL-12/23p40 expression perhaps depend upon the type and state of cell activation.

Similar to ERK and p38, the role of JNK and PI3K pathways in the regulation of IL-12/23 p40 expression are controversial. For instance, in response to LPS stimulation, JNK pathway positively and negatively regulated IL-12/23p40 expression in human monocytes (269,277). Another group showed that in THP-1 cell derived macrophages, IL-12/23 p40 expression is differentially regulated by JNK-1 and JNK-2 (278). In response to LPS-stimulation, JNK-1 is generally thought to positively regulate IL-12/23 p40 expression, whereas JNK-2 negatively regulates IL-12/23 p40 expression (278). These contradictions may be due to different cellular models and the different protocols used in the activation of cells.

The regulation of the PI3K pathway in IL-12/23 p40 induction greatly depends on the stimulus and the type of cells used. For instance, IL-12/23 p40 production was negatively regulated by the PI3K pathway in response to *M. tuberculosis* (TLR-2 agonist) stimulation (265), whereas a positive regulation was observed in response to LPS (TLR-4 agonist) stimulation (278). Likewise, IL-12/23 p40 production was positively regulated by the PI3K post-stimulation with *Francisella tularensis* (TLR-2 agonist) in human monocytes (279) and negative regulation was observed post-stimulation with CpG (TLR-9 agonist) (280).

IL-23 protein and IL-23 p19 expression has also been observed to be positively regulated by the p38 MAPK in human microglial cells (281) and in macrophages (275) upon stimulation with *M. tuberculosis* and LPS, respectively. Furthermore, IL-23 secretion is also positively regulated by the p38 MAPK pathway in macrophages (275), human monocytes (282) and myeloid DCs (283,284).

IL-23 p19 production has also been observed to be positively regulated by the JNK and ERK MAPK pathways following stimulation with Theiler's virus in murine macrophages (285) and in response to TLR-3/4 stimulation in human myeloid DCs (283). Another group observed that the inhibition of ERK and JNK MAPK signaling pathways did not affect IFNγ/LPS-induced production of IL-23 in human myeloid DCs (284). Another group observed that the JNK MAPK, but not the ERK MAPK pathway, positively regulated IL-23 production in human monocytes following stimulation with C5a (Gi-protein- coupled receptor agonist) (286). These contradictory data may perhaps be explained by the differential regulation of JNK and ERK pathways on IL-12/23 p40 and IL-23 p19 expression.

The role of PI3K regulation in IL-23 expression has been poorly described. It has been observed in primary monocytes stimulated with *F. tularensis,* that the PI3K pathway positively regulated p19 and IL-23 production (279,286).

Overall, there is a strong consensus that IL-12/23 p40, IL-23 p19 mRNA expression and IL-23 secretion are positively regulated by the p38 MAPK pathway in TLR-stimulated myeloid cells. IL-23p19 mRNA expression and IL-23 production have also been shown to be positively regulated by the PI3K pathway (279,286). However, ERK has been observed to negatively as well as positively regulate the expression of IL-12/23 p40 (262,267) and positively regulate the expression of IL-23 p19 mRNA(285), demonstrating the discrepancies in the ERK regulation of IL-23 production (283,284,286). JNK-1 and JNK-2 differentially regulate the IL-12/23p40 subunit (278), while IL-23p19 has been implicated in positive regulation by the JNK pathway (285). Therefore, the inhibition of JNK pathway in regulation of IL-23 production remains controversial (283,284,286). Nevertheless, in contrast to the IL-12/23p40 regulation, IL-23 p19 expression and IL-23 production have been shown to be negatively regulated by the ERK and JNK MAPK pathways, suggesting that, in the regulation of IL-23 production, IL-23 p19 could play a more central role over IL-12/23p40. Still, more studies need to be conducted to investigate the main mechanism of regulating IL-12/23 p40, IL-23 p19 and IL-23 production in response to the pathogens binding to the TLRs specifically in TLR4.

1.7.5 Regulation of TLR-induced IL-27 production:

The signalling pathways that regulate the production of IL-27 are not well described. One of the studies showed that p38 activation inhibits activator protein (AP)-1/c-Fos binding to the p28 promoter, resulting in decreased IL-27 production in response to *M. tuberculosis* in macrophages (287). IL-27 p28 is regulated by the activation of the TRIF-dependent IRF-3 (288) and MyD88-

and NF-κB-dependent mechanisms (289). Whereas EBI3 was activated through the MyD88-dependent p50-p65 NF-κB mechanisms (290), p38 MAPK and NF-κB pathways have been shown to positively regulate LPS-induced IL-27 production in astrocytes, but not through the ERK pathway (291). The p38 and JNK MAPK pathways have been shown to positive regulate IFNγ/LPS-induced IL-27 secretion in myeloid DCs, but not through the ERK MAPK or PI3K pathways (284).

In summary, similar to IL-23, regulation of IL-27 and its subunits depend on the type and state of cell activation. IL-27 production has been shown to be regulated positively and negatively by the p38 MAPK pathway (284,286), where the ERK pathway has been observed to have no effect on IL-27 production (284,291). Further, JNK has also been shown to positively regulate IL-27 production (284). More evidence is required to verify whether IL-27 regulation is conserved among responses to different TLR-stimulants and in different myeloid cells.

Rationale:

Both *in vitro* HIV-infected MDMs and MDMs from HIV-infected individuals produce reduced levels of various cytokines including IL-12 (161,292). However, the effect of HIV-infection on IL-23 and IL-27 production in monocytic cells remains unknown. The preliminary studies conducted in our laboratory have suggested that HIV infection does not affect the expression of basal levels of IL-23 and IL-27 and their subsets. However, *in vitro* infection of MDMs with HIV was shown to significantly inhibit LPS-induced expression of IL-23 and IL-27 and their subunits. The role of HIV-accessory/regulatory protein in the regulation of IL-23 and IL-27 is also not known. While the involvement of the HIV-Tat protein in the regulation of pro inflammatory cytokines has been studied in human monocytic cells, only one report suggests the involvement of HIV-Tat protein in the inhibition of IL-12 production in PBMCs. More specifically, the role of HIV-Tat or any other HIV-accessory/regulatory protein in modulation of LPS-induced IL-23 and IL-27 production in general and specifically in human MDMs has not been investigated. Moreover, the impact of HIV-Tat on activation of LPS-activated cell signalling pathways regulating the production of IL-12 family of cytokines in MDMs is unknown.

Hypothesis:

HIV-Tat inhibits IL-23 and IL-27 production in human monocyte derived macrophages through interference with distinct LPS/TLR4-activated signal transduction pathways.

Objectives:

1. To evaluate the effect of intracellular and extracellular (peptide) HIV-Tat on LPS induced IL-23 and IL-27 production in human MDMs

a. Expression of biologically active HIV-Tat in stably transfected PT67 cells and productive infection of MDMs with HIV-Tat and pLXIN retroviruses

b. To evaluate the effect of intracellular HIV-Tat on LPS induced IL-23 and IL-27 production in human MDMs

c. To evaluate the effect of extracellular HIV-Tat on LPS induced IL-23 and IL-27 production in human MDMs

2. To examine the signalling pathways involved in intracellular HIV-Tat mediated inhibition of LPS-induced IL-23 and IL-27 production in human MDMs

3. To examine whether HIV-Tat impacts upstream MyD88-dependent and MyD88-independent TLR4 signaling molecules in LPS-activated MDMs

Chapter 2: Materials and methods

Transfection of PT 67 cells with pTat or pLXIN:

Retrovirus gene transfer technique was used to introduce HIV-Tat gene into the packaging -PT67 cells (BD Biosciences, Mississauga, Canada) genome(293) essentially as described in the Retroviral gene transfer expression user manual (BD Biosciences). PT 67 cells are derived from a mouse fibroblast cell line designed for production of high-titer retrovirus (294) . These cells were used to transfect either pTat or pLXIN-empty vector.

pLXIN vector (BD Biosciences) is a bicistronic retroviral vector intended for retroviral gene expression and delivery(295).

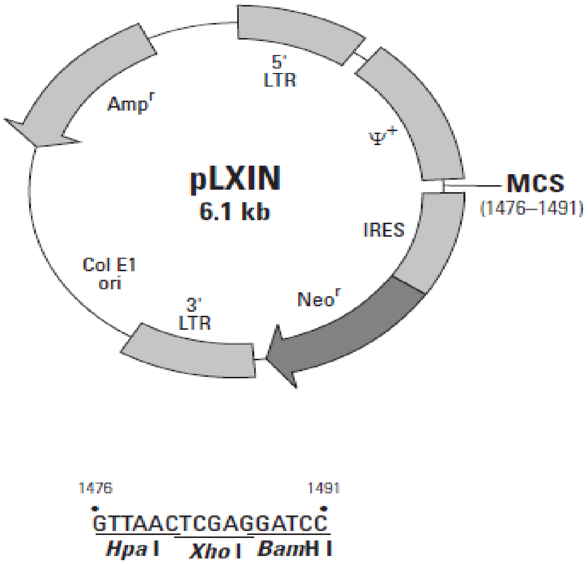


Fig 6: Retroviral Gene Transfer and Expression User Manual

Cloning of Tat gene:

Exon 1 of Tat was inserted into pLXIN as follows: Exon 1 of HIV-Tat was amplified by PCR from pSV2tat72 (NIH AIDS research programme Germantown, MD, USA) using the following primers:

sense: 5'-TTGGAGGCCTAGGCTTTTG-3'
antisense: 5'-TGTAGGTAGTTTGTCCAATTATGTCA-3'

Two EcoRI restriction sites were inserted at each terminus by employing the pGEM-T Easy Vector System (Promega Madison USA). ECOR1 digested Tat amplicons were ligated into an EcoRI sites of the retroviral backbone pLXIN vector (BD Biosciences). The pTat plasmid was obtained from Dr. Jonathan Angel's lab (The Ottawa Hospital Research Institute).

$1x10^5$ PT 67 cells were cultured in 6 well plates (Thermoscientific, Ottawa, ON) prior to trasfection. The transfection reagent; Fugene 6 (Roch Basel,Switzerland) as recommended by the supplier was incubated with 4 µg of pTat or 4 µg of pLXIN for 30 min. The supernatant from PT 67 cells was replaced with 1 ml of serum free media and then trasfection mixture was added to the cells and incubated overnight. The following day, the culture supernatant was replaced with 3 ml of the Iscove's Modified DMEM 1X media(Wisent, St. Bruno, Quebec) supplemented with 10% FBS (PAA, Etobicoke, Ontario) and 100 units/mL of penicillin/gentamicin (Gibco-Invitrogen).

Selecting Stable HIV-Tat/pLXIN Virus-Producing PT 67 Cell Line:

Transfected $1x10^{5}$ PT 67 cells were cultured in the 6 well plates containing 4 ml of complete medium with varying amounts of G418 (BD Biosciences) ranging from 0, 50, 100, 200, 400, 800, and 1600 µg/ml. Cells were incubated for 14 days and media was replaced every 4 days. To choose stable PT67 transfectants, the lowest concentration of G418 was used that caused cell death in 7 days and killed all the untransfected PT 67 cells within two weeks. In the final step, the stably transfected PT67/ pTat or PT67/ pLXIN cells were selected using 800 µg/ml G418 antibiotic. Once a stable PT 67 cell culture was established, cells were expanded in T75 tissue culture flasks. Once cells reached a 80% confluency, the cells were frozen with freezing medium containing 10% DMSO (Sigma-Aldrich, Oakville, Ontario, Canada) at 1x10^6 cell density and stored in liquid nitrogen.

Collection of HIV-Tat/pLXIN virus containing supernatant:

Once stably generated HIV-Tat containing cells reach 100% confluence, the supernatant containing virus was collected by centrifugation at 500 x g for 10 min. The supernatants thus collected at different time points were pooled. The virus supernatant was aliquoted in a desired volume to avoid multiple free-thaw cycles and stored at –80°C. Cells were passaged a maximum of 4 times.

Determination of Viral Titer:

One day prior to beginning this procedure, 0.5x10^5 NIH 3T3 cells (NIH AIDS research programme) were cultured in 6 well plates. The virus containing supernatant was collected from stably generated packaging PT67 cells constituted the viral stock. The 10 fold serial dilutions of HIV-Tat or pLXIN virus supernatant were prepared in complete medium with the presence of 4 µg/ml polybrene and 800 µg/ml G418. NIH 3T3 cells were infected with serially diluted HIV-Tat or pLXIN virus. The media was changed every three days. At day 14, supernatants were removed and the cell layer was stained with violet blue. The number of colonies in each dilution was counted. The viral titer of HIV-Tat or pLXIN corresponds to the number of colonies observed at the uppermost dilution multiplied by the dilution factor.

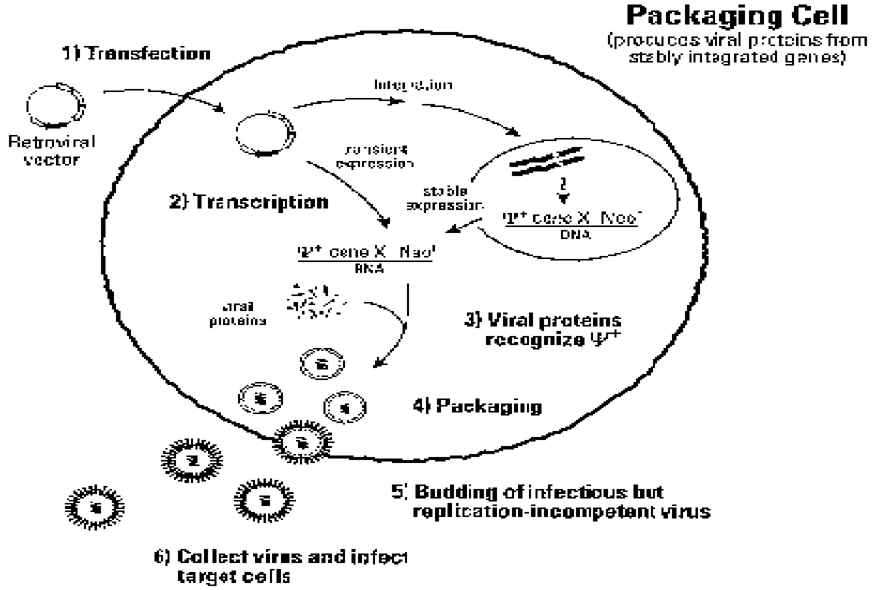


Fig 7: Retroviral Gene Transfer and Expression User Manual

Confirmation of Biological activity of HIV-Tat by Beta Glo assay:

The biological activity of stably generated HIV-Tat virions was measured by Beta Glo assay (Promega). TZMB-1 cells (NIH AIDS research programme) are derived from the HeLa epithelial cell line and express CCR5 and CD4 as transfected genes. These cells contain inducible luciferase and ß-gal genes under the control of the HIV-1 LTR. In a 96 well plate, 7000 TZMB-1 cells were plated. The following day, HIV-Tat or pLXIN virus was added and further incubated for 48 hours. Next, virus supernatant was aspirated and replaced with 50 µl of complete medium followed by equal volume of Beta Glo reagent. The plate was incubated for 30 minutes at the room temperature and measured by luminescence (BioTec-synergy, Winooski, Unite states).

Cell culture and reagents:

All cells were cultured in Modified DMEM 1X media (Wisent) supplemented with 10% fetal bovine serum (FBS) (PAA, Etobicoke, Ontario), 10 units/mL of penicillin/gentamicin (Gibco-Invitrogen). The cells were maintained in a humidified environment at 37°C, 5% CO2/air mixture incubator.

Blood was obtained from healthy donors according to a protocol approved by The Ottawa General Hospital Research Ethics Review Committee. All the donors provided written informed consent.

To generate MDMs, PBMCs form healthy donors were isolated by density gradient over Ficoll-Hypaque. PBMCs (4×10^6 cells) were cultured in serum free media in 12 well plates and incubated at 37ºC for 3 hrs. Cells were washed with serum free media to get rid of non-adherent cells and adherent monocytes were differentiated into MDMs by culturing with the complete media in presence of 10 ng/ml of Macrophage-Colony-Stimulating Factor (M-CSF) (R&D Systems, Minneapolis, MN USA) for 6 days. The media supplemented with M-CSF was replaced every 2 days. Previously our laboratory confirmed that MDMs contained 98% CD14+ cells and had the following phenotypes CD14+, CD16+, CD80+, CD11a+, CD11b+, CD11c+, HLA-DR+ and CD83- as determined by flow cytometry analysis.

Infection of MDMs with pLXIN/HIV-Tat retrovirus:

MDMs were infected with 1 ml of supernatant containing 10^3 titer of HIV-Tat virus, supplemented with 4 µg/ml polybrene (Sigma-Aldrich) for 2 hours followed by 3 rounds of infection. Control pLXIN virus was treated in parallel with the HIV-Tat, using the similar viral titer stocks collected on the same day as the HIV-Tat viral stock. Cells were thoroughly washed twice with PBS and incubated for another 48 hours with complete media.

Cytokine measurement by ELISA:

Culture supernatants levels of TNF-α, IL-10, IL-23 and IL-27 were measured by enzyme-linked immunosorbent assay (ELISA)) as recommended by the supplier and as described below:

IL-10, IL-23, IL-27 and TNF- α protein measurement by ELISA:

To measure cytokine production, retrovirus infected HIV-Tat or control pLXIN MDMs were treated with an indicated concentration of either, LY294002 (PI3K/Akt inhibitor), SP600125 (JNK inhibitor), SB203580 (p38 inhibitor), PD 098059 (ERK inhibitor), sodium stobogluconate (SHP-1 inhibitor) , or SU6656 (Src inhibitor) (purchased all from Calbiochem, La Jolla, CA) for 2 hours followed by 1 µg/mL of LPS and then cells were cultured for another 24 hours. Culture supernatants were collected by centrifugation at 596 RCF(x g) for 5 min. Culture supernatants were assayed by the IL-23 ELISA DuoSet, IL-27 ELISA DuoSet (both from R&D systems), IL-10 (BD biosciences, Mississauga, ON), and TNF-α (R&D systems) as per the manufacturer's instruction. Costar high binding 96 well ELISA plates (Corning Incorporated, Corning, NY) were coated with the following capture antibodies: 4 µg/mL of anti IL-23 p19 antibody, 0.8 µg/mL of anti IL-27 antibody, 4 µl/ml of anti IL-10 and 4 µg/mL of anti TNF-α antibody followed by overnight incubation. The following day, plates were washed with washing buffer [0.05% Tween 20 (Sigma) in PBS, pH=7.2-7.4] ,and blocked with 1% bovine serum albumin (BSA) for 2 hr. Serial two fold dilutions of standard was prepared as instructed by the manufacture. Subsequently, 100 µl/well of the samples and the standards were added and then incubated at 4°C. On the third day, the plates were washed, and then incubated for 2hr with biotinylated secondary antibody diluted in 1% BSA in PBS followed by 20 min incubation with streptavidin-HRP in 1% BSA in PBS. Subsequently, plates were washed with washing buffer and 100 µl/well of substrate solution was added. (BioFX Labs, Owing Mills, MD). In the final step, the reaction was stopped using 50 µl/well of Stop Solution (BioFX Labs, Owing Mills, MD). Absorbance was read at 450 nm using iMark Microplate reader (Biorad, Missisauga, Ontario) and data was processed using Micro Plate Manager 6 software.

Preparation of cell lysates:

To prepare total cell protein, retrovirus infected HIV-Tat or control pLXIN MDMs were treated with indicated concentrations of either, LY294002 (PI3K inhibitor), PD98053 (ERK inhibitor), SB203580 (p38 inhibitor), SP600125 (JNK inhibitor), sodium stobogluconate (SHP-1 inhibitor), or SU6656 (Src inhibitor) inhibitors for 2 hr. To detect the effect of HIV-Tat on TLR-4-activated kinases including PI3K, p38, JNK, ERK, and Src, infected MDMs were stimulated with 1 µg/mL of LPS for 15 min and TLR4-mediated upper signalling molecules including IAPs, IRAKs, SHP-1, MyD88, TRIF, and TRAFs were detected by stimulating infected MDMs with 1 µg/mL of LPS for 24 hr. Cell pellets were collected by centrifugation at 596 RCF(x g) for 5 min. Cell pellets were lysed with 30 µl of complete lysis buffer , and left to incubate for 45 min on ice. The lysis buffer consists of complete protease inhibitor tablet (Roche, Mississauga, Ontario), 150 mM NaCl (BDH, West Chester, PA) 20mM 41 Tris HCl (Sigma, St. Louis, MO), and 1 mM sodium orthovanadate (Sigma, St. Louis, MO). Cell lysates were spun down at 21100 RCF(x g) for 20 min at 4°C.

Western blot analysis:

Protein concentration was estimated by bradford assay with BSA standard (Calbiochem), using Protein Assay Dye Reagent Concentrate (Biorad). The absorbance was read at 595 nm using an iMark Microplate reader. (Biorad). The total estimated proteins 30µg were subjected to SDS-PAGE and transferred onto a Immun-blot-PVDF (Bio-Rad). Membranes were incubated with primary antibodies against phospho-Akt (1/250), Akt-1 (1/500), phospho-p38 MAPK (1/1000), p38 MAPK (1/1000), phospho-ERK (1/500), ERK-2 (1/500), phospho-JNK (1/500), JNK-1 (1/500), cIAP1 (1/500), cIAP2 (1/500), XIAP (1/500),TRAF1 (1/500), TRAF2 (1/500), TRAF6 (1/500), MyD88 (1/500), IRAK1 (1/500),IRAK2 (1/500), IRAK4 (1/500), TRIF (1/500), pSrc (1/500), SHP-1 (1/500) or Src (1/500) (All from Cell Signalling Technology, Danvers, MA), murine anti-Tat mAB (Immuno DX Woburn, MA, USA) at 4◦C overnight. All the primary antibodies were prepared with 2.5% BSA (Sigma) in TBST (Tris HCl (Sigma) and NaCl (Sigma) with Tween 20 (Sigma). The membranes were rinsed with the TBST buffer for 15 min three times. Goat anti-rabbit IgG HRP conjugate or goat anti-mouse IgG HRP conjugate was used as secondary antibodies diluted with the 5% skim milk in TBST and incubated for 1hr. The immunoblots were visualized using Amersham ECL (GE Healthcare, Buckinghamshire, UK) Western blotting detection reagent. The images were taken with the Chemigenius Bio-imaging system and the GeneSnap software (both from Syngene). The loading control was detected by stripping the membrane with striping buffer and re-probed to normalizing with the anti-GAP-DH (1/1000)

(Sigma Aldrich) or β actin (1/1000) (Cell Signaling Technology) antibodies. The Stripping buffer was prepared by adding 10 g SDS (Calbiochem, La Jolla, CA) and47.2g Tris HCl (Sigma, St. Louis, MO) in 500ml distilled water and adding 3.65ml of B-mercaptoethanol. (Sigma, St. Louis, MO).

RNA extraction and qRT-PCR:

Retrovirus-infected MDMs were stimulated with 1 µg/mL of LPS for 4 hr. Cell pellets were collected by centrifugation at 1600 rpm for 5 min. Total RNA was extracted using the RNeasy Plus Mini Kits (Qiagen, Mississauga, Ontario) according to the manufacturer's protocol. RNA concentration was measured by a spectrophotometer Nanodrop 2000C (Nanodrop Products, Wilmington, DE). Total RNA was reverse transcribed using the high capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA) according to the manufacturer's protocol and amplified for 2hr in a GeneAmp PCR System 2700 amplifier (Applied Biosystems, Carlsbad, CA) to yield cDNA. The PCR program was set up as follows: initial incubation for 10 min at 25°C; 2 hr at 37°C, and cycles of denaturation at 95°C for 15 seconds.

Thus prepared cDNA was used in Real-time PCR amplification. To carry out this process, cDNA was analyzed by qRT-PCR on the 7500 RT-PCR System (Applied Biosystems), using the TaqMan Universal Master Mix, and primers specific for β-actin (ACTB Hs99999903_mL), IL-12/23p40 (IL-12B Hs00233688_mL), IL-23p19 (IL-23A Hs00372324_mL), IL-27 EBI3 (EBI3 HS00194957_mL), and IL-27 p28 (IL-27A Hs00377366_mL) (purchased from Applied Biosystems). According to the manufacturer's protocol, qRT-PCR parameters were assigned as follows: Step 1 (1 cycle): 2 min at 50°C, Step 2 (1 cycle): 10 min at 95°C, and Step 3 (40 cycles): 15 seconds at 95°C, and 1 min at 60°C (Applied Biosystems). SDS software (Applied Biosystems) was compiled to calculate the cycle threshold (Ct). IL-12/23 p40, IL-23 p19, IL-27 p28, and EBI3 gene expression was normalized to β-actin gene expression, and the dCt values was used to calculate fold change relative to un stimulated cells.

Tat gene confirmation by PCR:

Stably transfected PT 67 HIV-Tat and pLXIN cell pellets were collected, followed by RNA extraction. The total RNA was transcribed into cDNA protocol similar to the one described for RNA extraction. The cDNA was amplified using AmpliTaq Gold PCR master mix (Applied Biosystems) and the Tat primers (**Eurofins Genomics, Alabama, USA).**

The Tat primer sequence is as follows:

Sense: 5` CTGCTGGAACCATGGAA 3`

anti-sense: 5`CGGCCGTAAGAGATACCT 3`

The PCR Program was set as follows: Step 1 (1 cycle): 2 min at 95°C, Step 2 (30 cycles): 30 sec at 95°C, 1 min at 58°C, 2 min at 72°C and Step 3 (1 cycle): 10 min at 72°C

The PCR products were run on 2% agarose gel electrophorosis with TAE buffer, and ethidium bromide was used as the detection method.

Treatment of macrophage with Tat peptides:

Six synthetic peptides representing the six domains of the full Tat protein were synthesized by Biomer Technology Pleasunton United States.

The amino acid sequences are as follows:

Tat 1 Peptide (1-27) - Acidic region:

MEPV DPRL EPWK HPG SQPK TACT NCYC

Tat 2 peptide (17-43) - Cysteine rich region:

QPK TACT NCYC KKCC FHCQ VCFI TKGL

Tat 3 peptide (33-59) -Core + Basic region:

HCQ VCFI TKGL GISY GRKK RRQR RRAP

Tat 4 peptide (49-57) -Basic + Glutamine rich region:

RKK RRQR RRAP QDSQ THQV SLSK QPAS

Tat 5 peptide (65- 91)- Glutamine rich region:

HQV SLSK QPAS QPRG DPTG PKES KKKV

Tat 6 peptide (81-102)- C-terminal region:

PTG PKES KKKV ERET ETDP VDQ

The peptides were reconstituted with sterile water at 1mg/ml concentration. MDMs were treated with 5, 10, 25, and 50 µg/ml concentration Tat peptides for 3hr followed by LPS 1 µg/ml stimulation for another 24 hr for ELISA or 4hr for RT-PCR. Supernatants were collected for ELISA and the cell pellets were collected to extract RNA for RT-PCR to measure the level of IL12p40, IL12p19, IL27p28 and EBI3 mRNA.

Apoptosis analysis by intracellular propidium iodide (PI) staining:

Retrovirus- infected MDMs were evaluated for cell death by PI staining. Cells were gently scraped and cell pellets collected. The cells were washed with PBS and fixed with 100% methanol for 15 min. Next, methanol was removed by washing with PBS and cells were incubated with 25 µl of 10 µg/ml RNase A, followed by staining with 25 µl of 1 mg/ml PI solution (Sigma-Aldrich) at 4°C for 1 hr. FACSCanto flow cytometer (BD Biosciences) was used to measure the DNA content and the data was analyzed using FACSDiva software. The subdiploid DNA peak (<2N

DNA), directly nearby to the G0/G1 peak (2N DNA), correspond to apoptotic cells and was calculated by histogram analyses. Cells with minimal light scatter were gated out from the analysis. PI histograms were acquired with WinMDI version 2.8 software (J. Trotter, Scripps Institute, San Diego, CA).

Statistical analyses:

Control pLXIN treated versus retrovirus infected samples were compared using GraphPad Prism 5. Cells treated with pharmacological inhibitors were analyzed using One-tailed paired test or Anova, followed by Tukey test. A *p* value of less than 0.05 was considered significant. * indicated $p<0.05$. Unless otherwise specified, plotted data correspond to the mean ± standard deviation of at least three samples.

Chapter 3: Results

3.1 Objective 1: To evaluate the effect of intracellular and extracellular (peptide) HIV-Tat on LPS induced IL-23 and IL-27 production in human MDMs

HIV-Tat protein has been detected in tissues (296,297) and plasma of HIV-infected individuals at a concentration of 4-550nM (57,59). The supernatants of *in vitro* HIV-infected monocytes contain Tat protein in the 10-100 pM range (298,299). There is evidence that HIV-Tat induces expression of cytokines in primary human monocytes such as IL-10 through activation of TLR-4 dependent pathways (89,300). HIV-Tat has also been shown to inhibit IL-12 production in human PBMCs stimulated with *Staphylococcus aureus* Cowan 1 strain (SAC) (165). IL-23 and IL-27 secretion can be induced in macrophages following LPS stimulation (301). However there is no current information whether HIV Tat influences either LPS-induced IL-23 or IL-27 production in human macrophages. To investigate the effect of intracellular HIV- Tat on LPS induced IL-23 and IL-27 production in human MDMs, HIV-Tat and pLXIN retroviruses were generated from PT67 cells.

3.1.1 Expression of biologically active HIV-Tat in stably transfected PT67 cells and productive infection of MDMs with HIV-Tat and pLXIN retroviruses

Retrovirus gene transfer technique was used to introduce stable, heritable genetic material into the genome of dividing cells (293). The viral *gag, pol* and *env* genes are important for viral formation and replication of stably integrated virions. These genes are expressed in the packaging cell line PT67 genome and produce replication incompentent virus particles (302). Thus, PT67 cells are excellent models for stable retrovirus production. Packaging signal ψ, transcription, processing elements and a cloning site for integration of the target gene are provided in the retroviral expression vector pLXIN (293).The PT 67 cells transfected with the pLXIN retroviral vector produce high-titer, replication-competent virus (293).

PT 67 cells were transfected with either HIV-Tat or emplty vector pLXIN as described in the material and methods section. To confirm the transcription efficiency, first of all I demonstrated that HIV-Tat is expressed in the stably transfected cells and the generated HIV- Tat retrovirus is present in PT67 cell supernatants by RT-PCR, Western blot analysis and Beta-Glo Assay. RT-PCR analysis in HIV-Tat-infected PT67 cells revealed the presence of HIV-Tat transcript (Fig. 8A upper panel). The HIV-Tat-infected PT67 cells also expressed HIV Tat protein as demonstrated by Western immunoblotting (Fig 8A middle panel). I also determined HIV-Tat present in PT67 cell supernatants was biologically active by employing Beta Glow Assay System kit and TZM-b1 cells expressing long terminal repeat-driven lacZ gene. If HIV-Tat is presents in the PT67 cell supernatants, it should transactivate the long terminal repeat-driven lacZ gene in TZM-b1 cells resulting in β-galactosidase production. My results clearly demonstrated that biologically active HIV-Tat is present in the stably generated HIV-Tat retrovirus-infected PT67 cell supernatants (Fig. 8A lower panel).

Subsequently, I examined whether MDMs can be infected by stably generated HIV-Tat and pLXIN retroviruses. For this, MDMs were infected with either the HIV-Tat or pLXIN retroviruses obtained from stably transfected PT67 cells for 48 hrs. MDMs infected with HIV-Tat or pLXIN were assayed for Tat expression by RT-PCR. The results show that MDMs infected with HIV-Tat retroviruses expressed HIV-Tat transcript (Fig 8B upper panel). The presence of biologically active HIV-Tat in the retrovirus infected MDMs supernatants was shown by Beta Glo assay as described above. The biologically active HIV-Tat was shown to be present in the HIV-Tat retrovirus-infected MDMs supernatants (Fig 8B lower panel).

The supernatants from HIV-Tat infected MDMs contained infectious viral particles as demonstrated by plaque assay (Fig 8C). For this, NIH3T3 cells were exposed to the supernatants obtained from HIV-Tat-infected MDMs for 14 days after which the plaques observed were counted. After multiplying with the dilution factor, the number of HIV-Tat or pLXIN viruses/ml was calculated (Fig 8C). The MDMs culture supernatants contained 100 HIV-Tat viruses per ml and 180 pLXIN viruses per ml.

To determine if infection of MDMs with either HIV-Tat or pLXIN control retroviruses caused apoptosis, MDMs infected with the retroviruses for 3 days were evaluated for apoptosis by flow cytometry with intracellular PI staining as described earlier by our laboratory (303). The results show that less than 5% cell death was seen in both HIV Tat and pLXIN retrovirus-infected MDMs (Fig 9).

Figure 8: Expression of biologically active HIV-Tat in stably transfected PT67 cells

A.

Upper panel: PT 67 cells were transfected with either pTat or emplty vector pLXIN. The cell pellets were assayed by RT-PCR for Tat expression.

Middle panel: The cell lysate was assayed for Tat expression by Western blot analysis. Images shown are representative for at least three experiments with similar results.

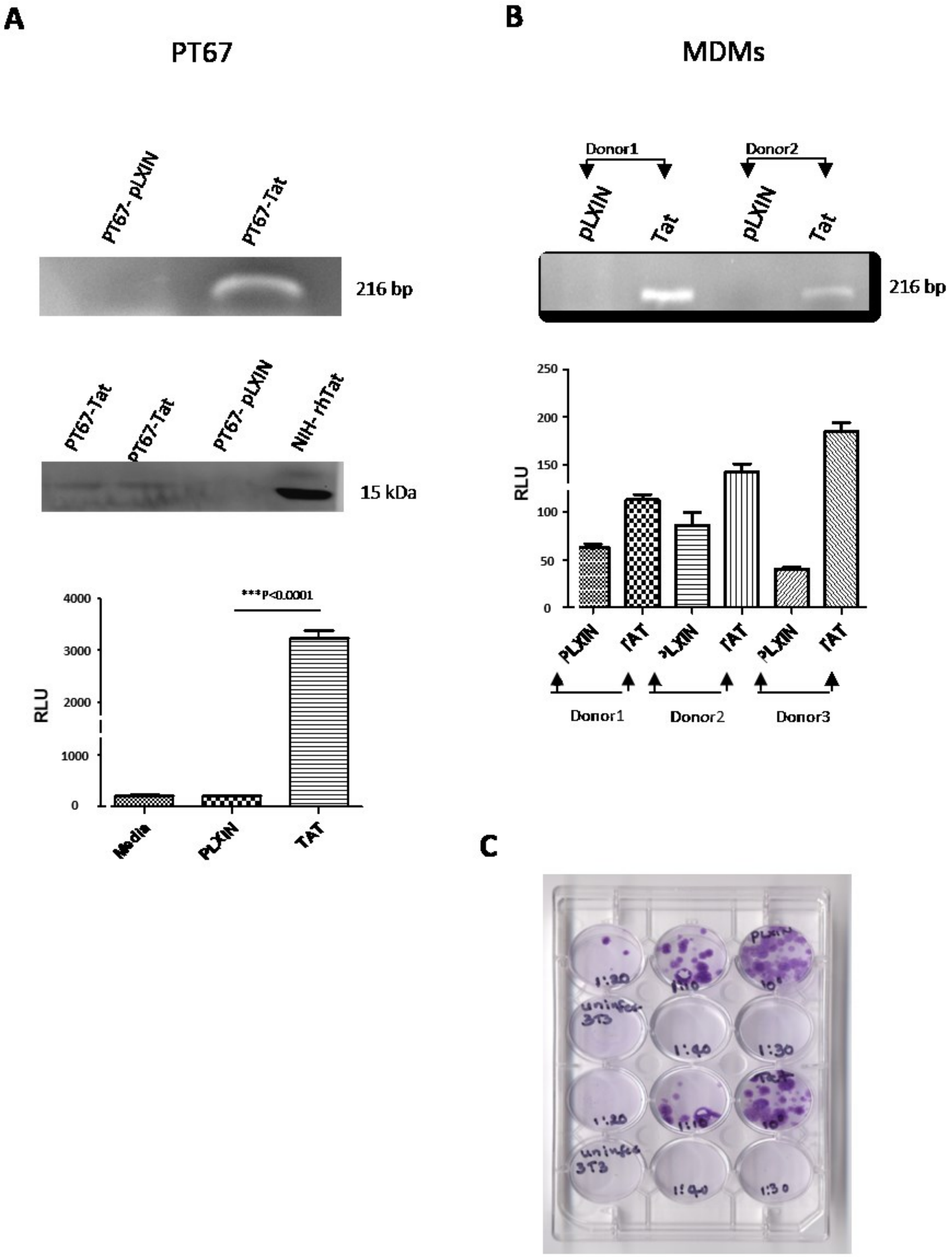
Lower panel: The virus containing supernatants were used to infect TZMB-1 cells and biological activity measured by Beta-Glo Assay.

B.

Upper panel: MDMs were infected with HIV-Tat or pLXIN retrovirus and cell pellets were analyzed by RT-PCR

Middle panel: The virus containing supernatants were used to infect TZMB-1cells to assess biological activity as measured by Beta Glo activity.

Lower panel: NIH 3T3 cells were infected with the virus containing supernatant. The virus titter was measured by the plaque assay. The image shown is representative for three experiments with similar results.


A
PT67
PT67- pLXIN
PT67-Tat
216 bp
PT67-Tat
PT67-Tat
PT67- pLXIN
NIH- rhTat
15 kDa
***P<0.0001
4000
3000
2000
1000
0
RLU
Media
PLXIN
TAT
B
MDMs
Donor1
Donor2
pLXIN
Tat
pLXIN
Tat
216 bp
250
200
150
100
50
0
RLU
PLXIN
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uninfec 3T3
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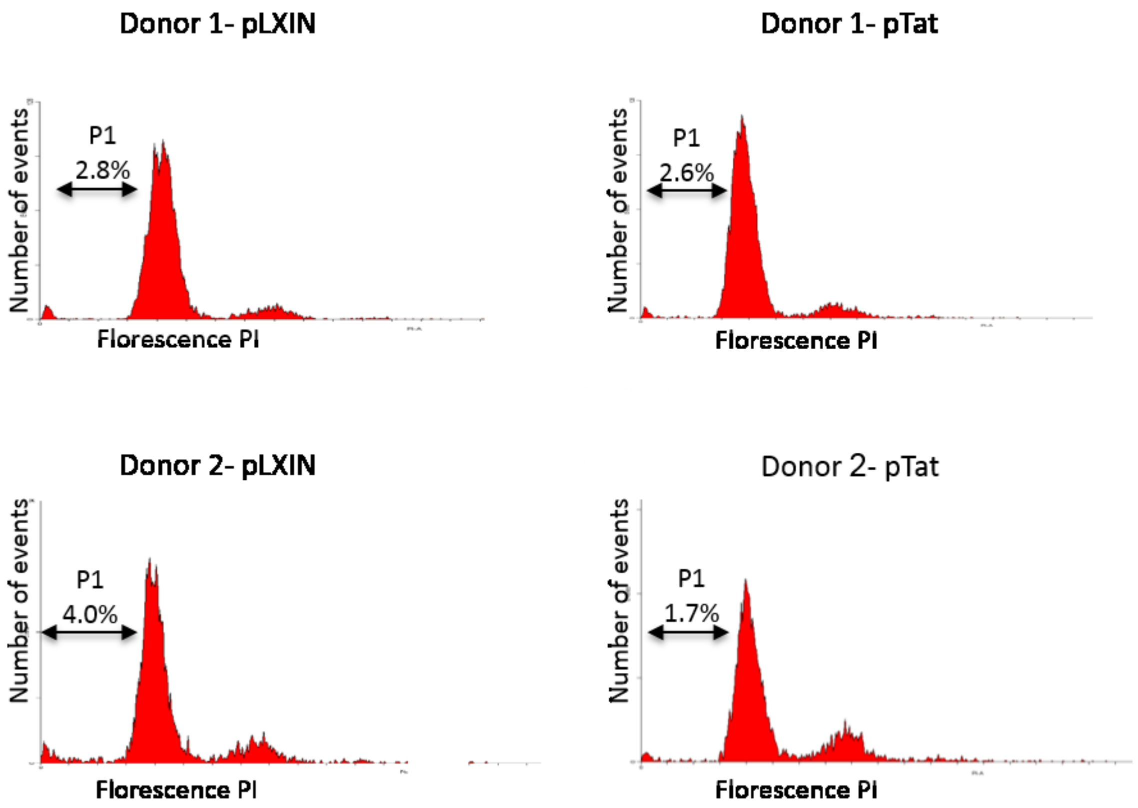
Tat
1:20
1:10
10
uninfec 3T3
1:40
1:30

Figure 8

Figure 9: HIV-Tat or pLXIN-infected MDMs do not show apoptosis

MDMs from Donor 1 and 2 were infected with either HIV-Tat or pLXIN retroviruses for 3 days following which MDMs were analyzed for apoptosis by flow cytometry with intracellular PI staining. The numbers represent percentage apoptotic cells with subdiploid DNA content.



Donor 1- pLXIN

Donor 1- pTat

Donor 2- pLXIN

Donor 2- pTat

Figure 9

3.1.2. To evaluate the effect of intracellular HIV-Tat on LPS induced IL-23 and IL-27 production in human MDMs

To investigate the effect of intracellular HIV-Tat on LPS-induced IL-23 and IL-27 production in human MDMs, MDMs were infected with the stably generated HIV-Tat or pLXIN retrovirus for 48 hrs followed by stimulation with LPS (1 µg/ml). Cell culture supernatants were collected 24 hr after LPS stimulation and analysed by ELISA for IL-23 production. Similarly, cell pellets were collected 4 hr after LPS stimulation and RNA was analyzed by qRT-PCR for IL-12/23 p40 and IL-23 p19 mRNA expression. The results show that LPS stimulation of pLXIN-infected MDMs significantly increased IL-23 production (Fig 10A) and IL12p40 and p19 mRNA expression (Fig 10B). However, HIV-Tat-infected MDMs exhibited significantly decreased LPS-induced IL-23 production (~ 70% inhibition), IL12p40 mRNA (> 65% inhibition), and p19 mRNA expression (~50% inhibition).

Similar to IL-23, I determined whether IL-27 production was also affected by intracellular HIV-Tat in LPS-stimulated MDMs. The results show that LPS stimulation of pLXIN-infected MDMs significantly induced IL-27 production (Fig 11A) and p28, and EBI3 mRNA expression (Fig 11B). However, HIV-Tat-infected MDMs showed significantly decreased LPS-induced IL-27 production (~ 65%), p28 mRNA (> 65%) and EBI3 mRNA expression (~ 60%). To determine if intracellular expression of HIV Tat selectively inhibited LPS-induced IL-23 and IL-27 production, I analyzed IL-10 and TNF-α production by ELISA in HIV-Tat-infected MDMs culture supernatants collected 24 hr after LPS stimulation. Interestingly, HIV-Tat did not affect LPS-induced IL-10 and TNF-α production in MDMs (Fig 10C and 11C lower panels). Overall, intracellular HIV-Tat significantly inhibited LPS-induced IL-23 and IL-27 production without affecting IL-10 and TNF-α production in MDMs.

Figure 10: Intracellular HIV-Tat inhibits LPS induced IL-23 production in human MDMs

A. MDMs were infected with HIV-Tat or pLXIN retroviruses for 48 hrs followed by 24 hr stimulation with LPS (1 μg/ml). Cell culture supernatant was assayed for IL-23 production by ELISA. N= 14 different donors.

B. MDMs were infected with the HIV-Tat or pLXIN retroviruses for 48 hrs followed by 4 hr stimulation with LPS (1 μg/ml). mRNA was analyzed by qRT-PCR for IL-23 p19 expression. N= 4 different donors.

C. MDMs were infected with HIV-Tat or pLXIN retroviruses for 48 hrs followed by 24 hr stimulation with LPS (1 μg/ml). Cell culture supernatant was assayed for IL-10 production by ELISA. N=6

D. MDMs were infected with the HIV-Tat or pLXIN retroviruses for 48 hrs followed by 4 hr stimulation with LPS (1 μg/ml). mRNA was analyzed by qRT-PCR for IL-12/23 p40 expression. N= 4 different donors.

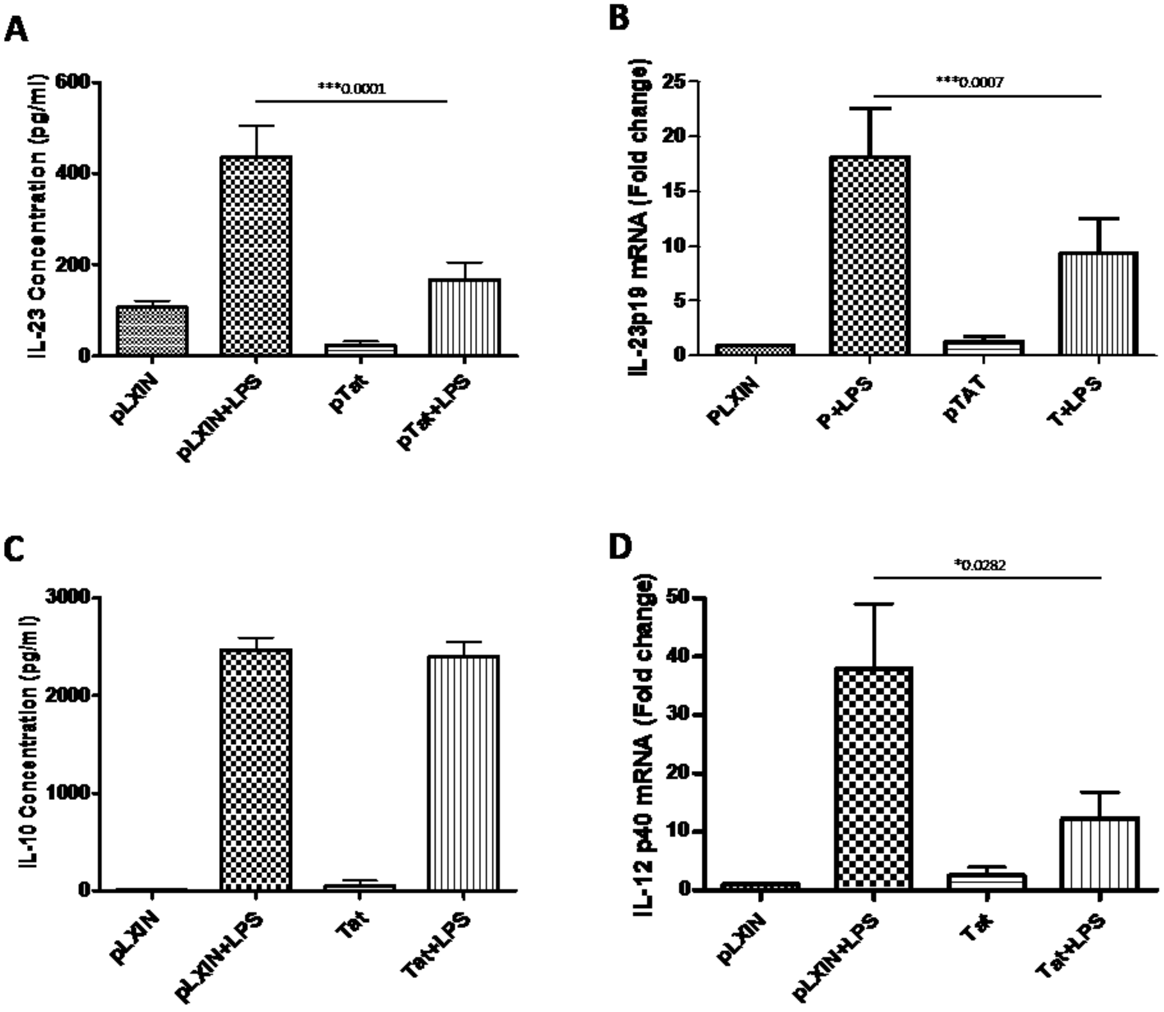

A
IL-23 Concentration (pg/ml)
600
400
200
0
***0.0001
pLXIN
pLXIN+LPS
pTat
pTat+LPS
B
IL-23p19 mRNA (Fold change)
25
20
15
10
5
0
***0.0007
PLXIN
P+LPS
pTAT
T+LPS
C
IL-10 Concentration (pg/ml)
3000
2000
1000
0
pLXIN
pLXIN+LPS
Tat
Tat+LPS
D
IL-12 p40 mRNA (Fold change)
50
40
30
20
10
0
*0.0282
pLXIN
pLXIN+LPS
Tat
Tat+LPS

Figure 10

Figure 11: Intracellular HIV-Tat inhibits LPS induced IL-27 production in human MDMs

A. MDMs were infected with HIV-Tat or pLXIN retroviruses for 48 hrs followed by 24 hr stimulation with LPS (1 μg/ml). Cell culture supernatants were assayed for IL-27 production by ELISA. N= 13 different donors.

B. MDMs were infected with the stably generated HIV-Tat or pLXIN retroviruses for 48 hrs followed by 4 hr stimulation with LPS (1 μg/ml). mRNA was analyzed by qRT-PCR for p28, N= 4 different donors.

C. MDMs were infected with HIV-Tat or pLXIN retroviruses for 48 hrs followed by 24 hr stimulation with LPS (1 μg/ml). Cell culture supernatants were assayed for TNF-α by ELISA. N=6

D. MDMs were infected with the stably generated HIV-Tat or pLXIN retroviruses for 48 hrs followed by 4 hr stimulation with LPS (1 μg/ml). mRNA was analyzed by qRT-PCR for EBI3 expression. N= 4 different donors.

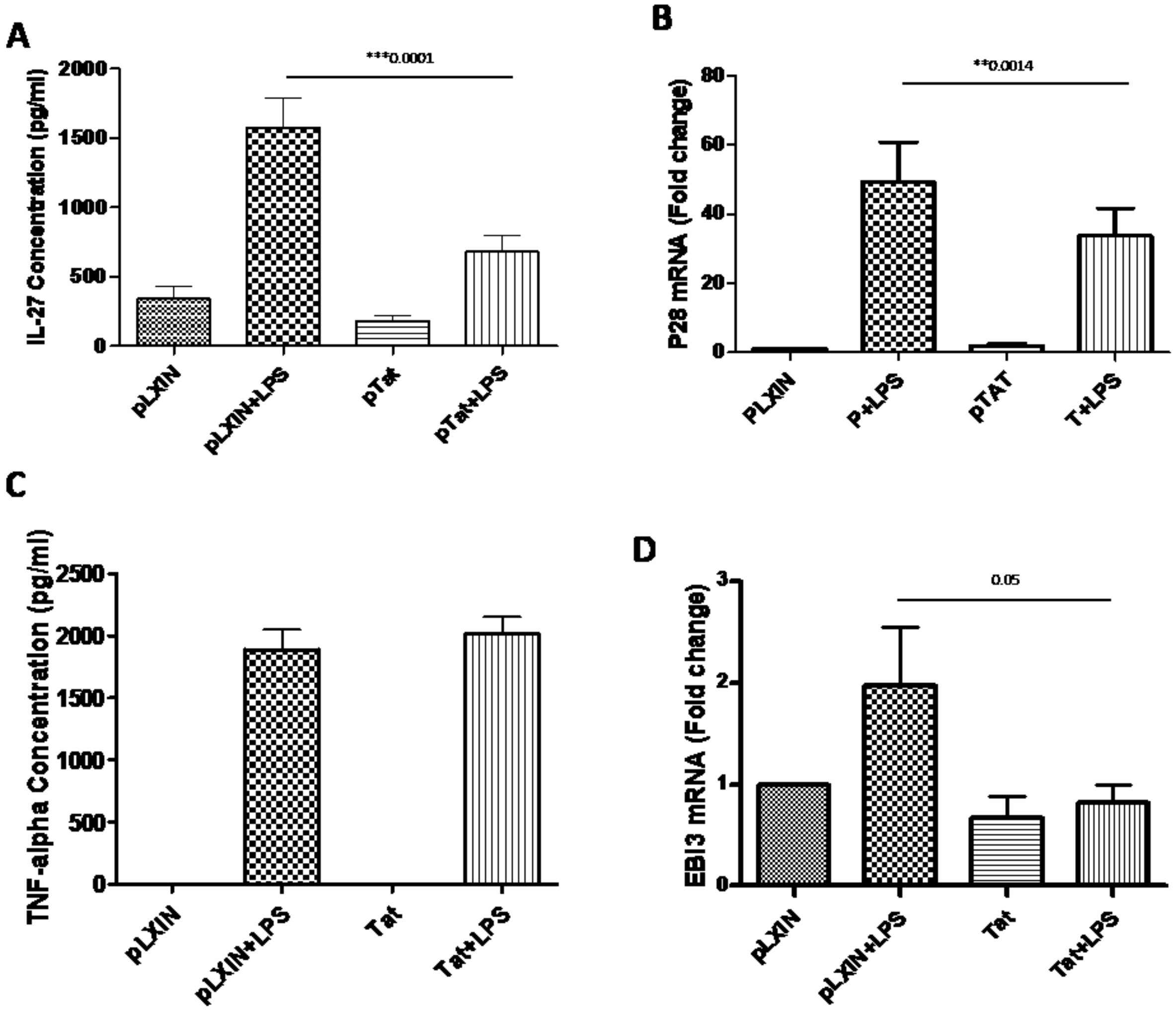

A
***0.0001
IL-27 Concentration (pg/ml)
2000
1500
1000
500
0
pLXIN
pLXIN+LPS
pTat
pTat+LPS
B
**0.0014
P28 mRNA (Fold change)
80
60
40
20
0
PLXIN
P+LPS
pTAT
T+LPS
C
TNF-alpha Concentration (pg/ml)
2500
2000
1500
1000
500
0
pLXIN
pLXIN+LPS
Tat
Tat+LPS
D
0.05
EBI3 mRNA (Fold change)
3
2
1
0
pLXIN
pLXIN+LPS
Tat
Tat+LPS

Figure 11

3.1.3. To evaluate the effect of extracellular HIV-Tat peptides on LPS induced IL-23 and IL-27 production in human MDMs

It has been reported that the first exon (1-72 AA) possesses full transactivation functions of HIV-Tat (304,305). The arginine-lysine rich basic region (48-60 AA) has been shown to facilitate membrane transduction (101), assist HIV-1 reverse transcription (102) and augment integrin receptor binding (69). The core region (38-48AA) is required for transcriptional transactivation (306). To elucidate whether extracellular HIV-Tat has similar effects on human macrophages on LPS-induced IL-23 and IL-27 production, as observed with intracellular HIV-Tat, this effect was examined by treating macrophages with 6 synthetic peptides representing the six domains of the full Tat protein for three hr (Fig. 2) followed by stimulation with LPS (1 μg/ml). Cell culture supernatants, collected 24 hr after LPS stimulation, and were assayed by ELISA for IL-23 and IL-27 production. Cell pellets were also collected 4 hr after LPS stimulation and RNA was analyzed by the qRT-PCR for IL-12/23 p40, IL-23 p19 mRNA and p28, and EBI3 mRNA expression. The results show that MDMs treated with peptide 1 (1-27 AA), peptide 2 (17-43 AA), peptide 4 (49-75 AA), and peptide 5 (65-91 AA) did not affect LPS-induced IL-23 (Fig 10 A,B,D,E,F) or IL-27 production (Fig 12 A,B,D,E,F) when compared to LPS-stimulated MDMs alone. Interestingly, Tat peptide #3 (33-59 AA) which encompasses the core and basic regions of HIV-Tat protein significantly inhibited LPS-induced IL-23 (Fig 12C) and IL-27 production (Fig 14 C) in a dose-dependent manner. Correspondingly, Tat peptide #3 also significantly inhibited LPS-induced IL-12/23p40, IL-23p19 (Fig 13) and p28 and EBI3 mRNAs expression (Fig 15) in dose-dependent manner. Interestingly, HIV-Tat peptide #6 (81-102 AA) also inhibited LPS-induced IL-23 production (Fig. 12F). The amino acid sequence of all the six peptides used in this study is shown in materials and methods section. Overall my results suggest that intracellular and extracellular HIV-Tat significantly inhibited LPS-induced IL-23 and IL-27 in human MDMs.

Figure 12: Extracellular HIV-Tat peptide #3 and 6 inhibits LPS induced IL-23 production in human MDMs

MDMs were treated with 6 synthetic peptides #1 - 6 (A-F) representing the six domains of the full HIV-Tat proteins for 3 hr followed by 24 hr stimulation with LPS (1 μg/ml). Cell culture supernatants were assayed for IL-23 production by ELISA. N=4 different donors.

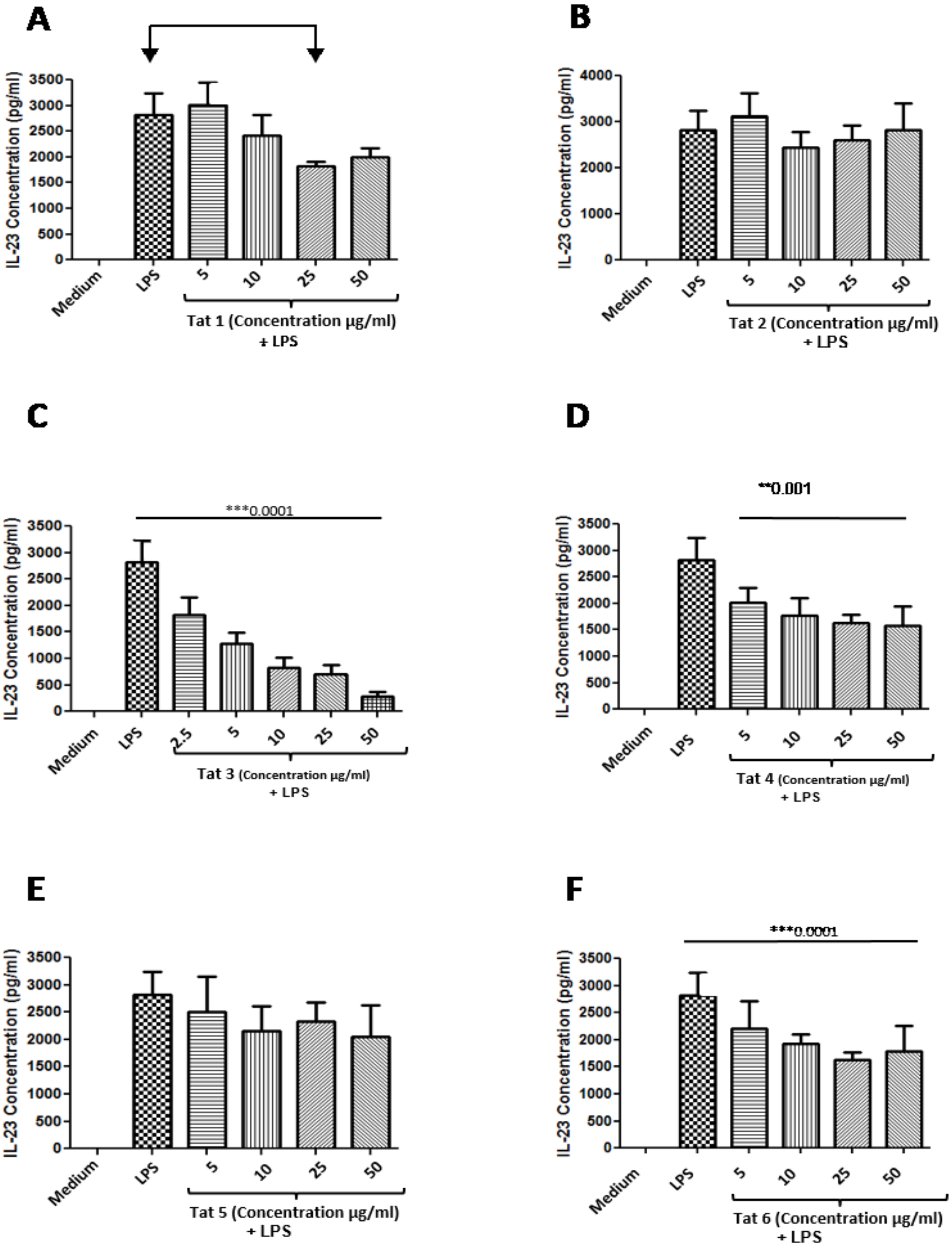

A
IL-23 Concentration (pg/ml)
0
500
1000
1500
2000
2500
3000
3500
Medium
LPS
5
10
25
50
Tat 1 (Concentration µg/ml)
+ LPS
B
IL-23 Concentration (pg/ml)
0
1000
2000
3000
4000
Medium
LPS
5
10
25
50
Tat 2 (Concentration µg/ml)
+ LPS
C
***0.0001
IL-23 Concentration (pg/ml)
0
500
1000
1500
2000
2500
3000
3500
Medium
LPS
2.5
5
10
25
50
Tat 3 (Concentration µg/ml)
+ LPS
D
**0.001
IL-23 Concentration (pg/ml)
0
500
1000
1500
2000
2500
3000
3500
Medium
LPS
5
10
25
50
Tat 4 (Concentration µg/ml)
+ LPS
E
IL-23 Concentration (pg/ml)
0
500
1000
1500
2000
2500
3000
3500
Medium
LPS
5
10
25
50
Tat 5 (Concentration µg/ml)
+ LPS
F
***0.0001
IL-23 Concentration (pg/ml)
0
500
1000
1500
2000
2500
3000
3500
Medium
LPS
5
10
25
50
Tat 6 (Concentration µg/ml)
+ LPS

Figure 12

Figure 13: Extracellular HIV-Tat peptide #3 inhibits LPS induced IL-23 mRNA expression in human MDMs

MDMs were treated with Tat peptide # 3 for 3 hr followed by LPS stimulation for 4 hr. RNA was analyzed by qRT-PCR for IL-12/23 p40 and IL-23p19 expression. N=4 different donors.

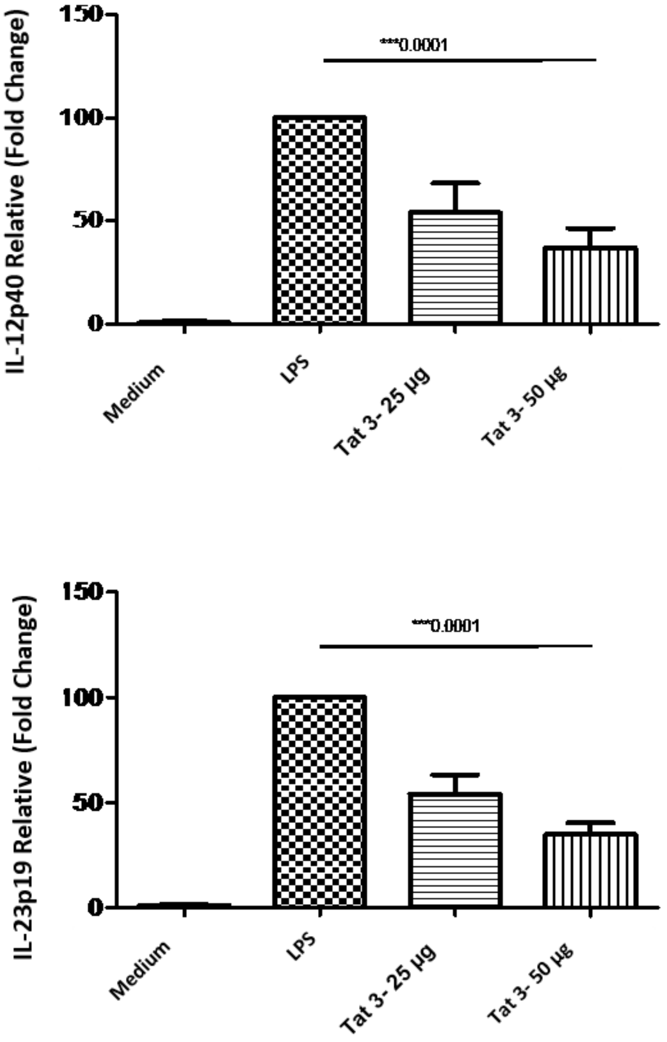

150
100
50
0
IL-12p40 Relative (Fold Change)
***0.0001
Medium
LPS
Tat 3- 25 µg
Tat 3- 50 µg
150
100
50
0
IL-23p19 Relative (Fold Change)
***0.0001
Medium
LPS
Tat 3- 25 µg
Tat 3- 50 µg

Figure 13

Figure 14: Extracellular HIV-Tat peptide #3 inhibits LPS induced IL-27 production in human MDMs

MDMs were treated with 6 synthetic peptides #1 - 6 (A-F) representing the six domains of the full HIV-Tat proteins for 3 hr followed by 24 hr stimulation with LPS (1 µg/ml). Cell culture supernatants were assayed for IL-27 production by ELISA. N=4 different donors.

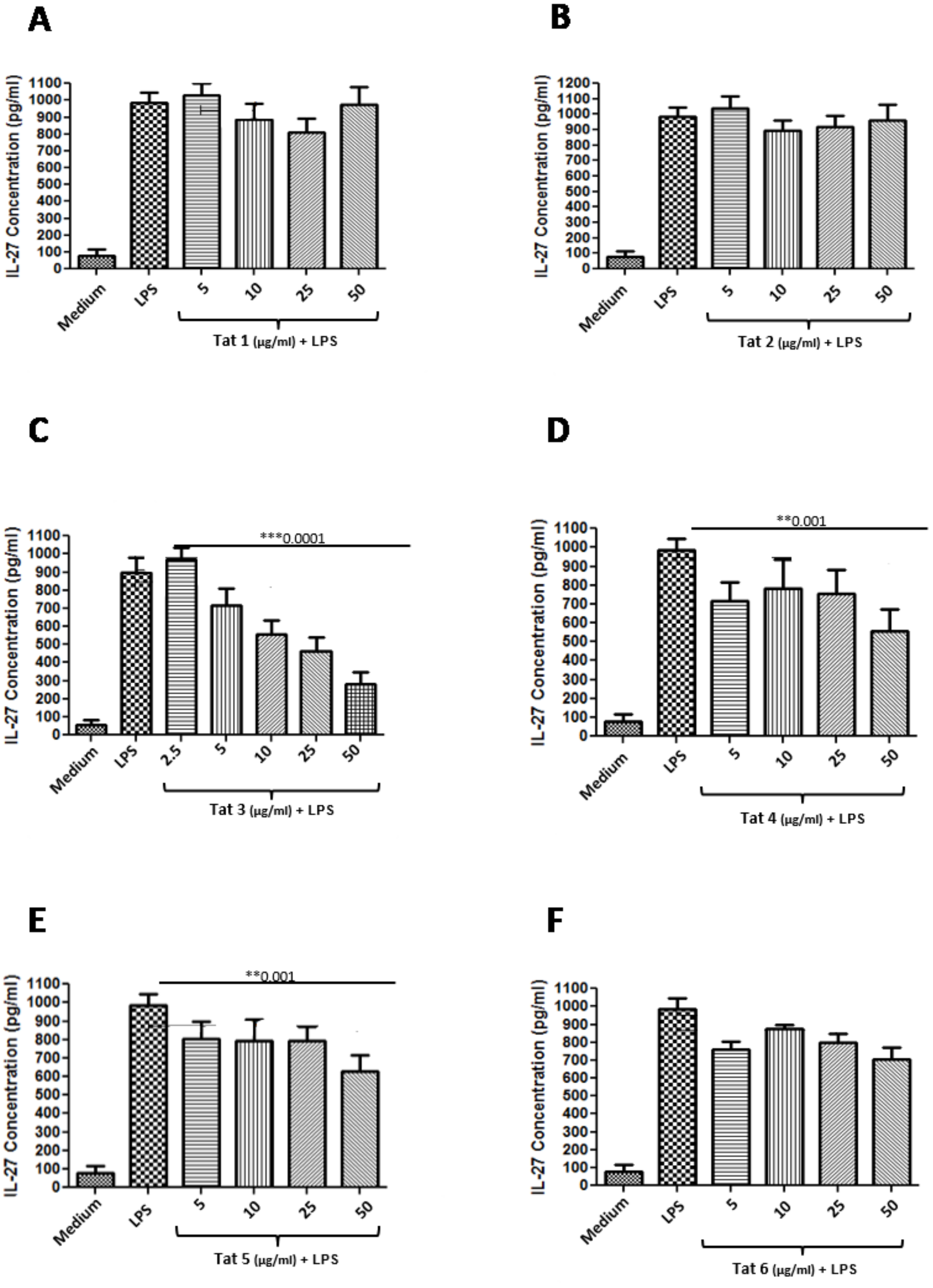

A
IL-27 Concentration (pg/ml)
0 100 200 300 400 500 600 700 800 900 1000 1100
Medium
LPS
5
10
25
50
Tat 1 (µg/ml) + LPS
B
IL-27 Concentration (pg/ml)
0 100 200 300 400 500 600 700 800 900 1000 1100 1200
Medium
LPS
5
10
25
50
Tat 2 (µg/ml) + LPS
C
IL-27 Concentration (pg/ml)
0 100 200 300 400 500 600 700 800 900 1000 1100
***0.0001
Medium
LPS
2.5
5
10
25
50
Tat 3 (µg/ml) + LPS
D
IL-27 Concentration (pg/ml)
0 100 200 300 400 500 600 700 800 900 1000 1100
**0.001
Medium
LPS
5
10
25
50
Tat 4 (µg/ml) + LPS
E
IL-27 Concentration (pg/ml)
0 100 200 300 400 500 600 700 800 900 1000 1100
**0.001
Medium
LPS
5
10
25
50
Tat 5 (µg/ml) + LPS
F
IL-27 Concentration (pg/ml)
0 100 200 300 400 500 600 700 800 900 1000 1100
Medium
LPS
5
10
25
50
Tat 6 (µg/ml) + LPS

Figure 14

Figure 15: Extracellular HIV-Tat inhibits LPS induced IL-27 mRNA expression in human MDMs

MDMs were treated with Tat peptide # 3 for 3 hr followed by LPS stimulation for 4 hr. RNA was analyzed by qRT-PCR for p28 and EBI3 expression. N=4 different donors.

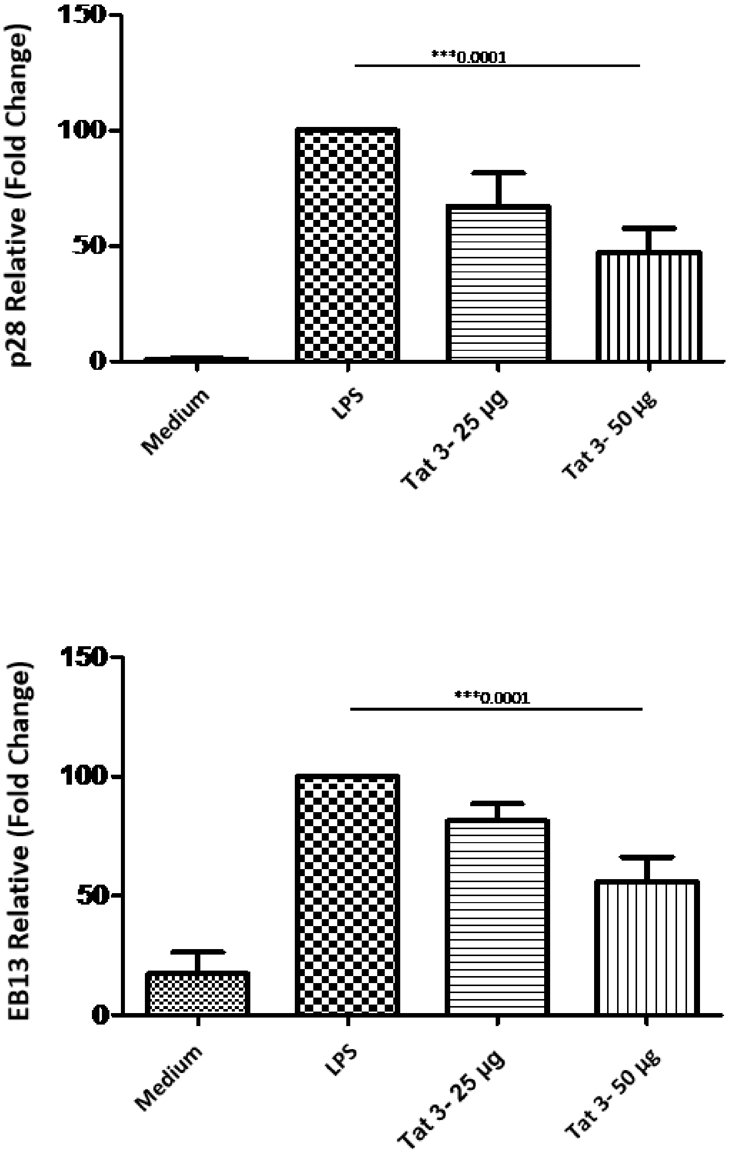

150
100
50
0
p28 Relative (Fold Change)
***0.0001
Medium
LPS
Tat 3- 25 µg
Tat 3- 50 µg
150
100
50
0
EB13 Relative (Fold Change)
***0.0001
Medium
LPS
Tat 3- 25 µg
Tat 3- 50 µg

Figure 15

3.2 Objective 2: To elucidate the signalling pathways involved in HIV-Tat mediated inhibition of LPS-induced IL-23 and IL-27 production in human MDMs

Several studies have demonstrated that TLR ligands induce IL-23 and IL-27 production in DCs and THP-1 cells (307–309). Since intracellular and extracellular HIV-Tat significantly inhibited LPS induced IL-23 and IL-27 mRNA expression and protein production in MDMs, it was of interest to determine the molecular pathways governing HIV-Tat-mediated inhibition of TLR4 signalling leading to the inhibition of IL-23 and IL-27 production in MDMs. The molecular mechanism and the TLR4 signalling molecules involved in LPS-induced IL-23 and IL-27 production in MDMs are poorly understood and hence were investigated in the setting of human MDMs infected with pLXIN retrovirus as a control. In this study, I specifically investigated the effect of HIV-Tat on TLR-4 activated kinases such as PI3K, p38, JNK, ERK, Src, and SHP-1 by employing their specific pharmacological inhibitors: LY294002 (PI3K/Akt inhibitor), SP600125 (JNK inhibitor), SB203580 (p38 inhibitor), PD 098059 (ERK inhibitor), SU6656 (Src inhibitor), and. sodium stibogluconate (SS, SHP-1 inhibitor).

3.2.1. HIV-Tat-mediated inhibition of LPS-induced IL-23 is regulated by p38 MAPK phosphorylation

Since intracellular and extracellular HIV-Tat inhibited both LPS-induced IL-23 protein and p19 and p40 mRNA expression, it is reasonable to hypothesize that HIV-Tat inhibits LPS-induced IL-23 production by modulating the TLR-4 activated signalling pathway in MDMs. I first investigated the role of MAPKs namely p38, ERK, and JNK MAPKs in HIV-Tat -mediated inhibition of LPS-induced IL-23 production. To study the role of the p38 pathway, I employed SB203580 – a p38 specific inhibitor. The biological activity of SB203580 was confirmed by pre-treating pLXIN-infected MDMs with SB203580 for 2 hr followed by 15 min of LPS stimulation. LPS-stimulation enhanced p38 phosphorylation and treatment with SB203580 decreased p38-phosphorylation in a dose-dependent manner (Fig 16A).

To examine the involvement of p38 MAPK in LPS-induced IL-23 production, pLXIN-infected MDMs were treated with SB203580 for 2 hr followed by 24 hr of LPS stimulation. The supernatants were assayed for the IL-23 production by ELISA. The results show that SB203580 significantly inhibited LPS-induced IL-23 production (Fig 16B).

To determine whether HIV-Tat-mediated inhibition of LPS-induced IL-23 production is regulated via inhibition of LPS-induced p38 phosphoralation, HIV-Tat or pLXIN-infected MDMs were stimulated with LPS for 15 min followed by analysis for p38 phosphorylation by Western immunoblotting. The basal levels of p38 phosphorylation were detected in pLXIN-infected MDMs that were not affected in Tat-infected MDMs. LPS-stimulation increased p38 phosphorylation in pLXIN-infected MDMs which was also significantly decreased in HIV-Tat-infected MDMs (Fig 16C, upper and lower panel).

Since HIV-Tat inhibits LPS-induced p38 phosphorylation, and SB203580 inhibits LPS-induced IL-23 production, it was of interest to determine if p38 inhibition mediated by HIV-Tat and SB203580 will co-operate with each other and further enhance inhibition of LPS-induced IL-23 production. For this, pLXIN or HIV-Tat-infected MDMs were stimulated with LPS in the presence or absence of SB203580. As expected, I observed ~70% inhibition of LPS-induced IL-23 production in HIV-Tat-infected MDMs compared to the pLXIN-infected MDMs (Fig 17). SB203580 at 25 and 50 μM concentration further inhibited LPS-induced IL-23 production in HIV-Tat-infected MDMs to almost basal levels (>90%) compared to the LPS-stimulated pLXIN infected cells (Fig 17). In summary, HIV-Tat is a potent inhibitor of p38 phosphorylation and along with SB203580, further enhanced inhibition of LPS-induced IL-23 production in MDMs. These results suggest that HIV-Tat decreases LPS-induced IL-23 production via inhibition of p38 MAPK phosphorylation.

Figure 16: HIV-Tat-mediated inhibition of LPS-induced IL-23 production is regulated by p38 MAPK

A. The biological activity of p38 was measured by treatment of pLXIN-infected MDMs with SB203580 for 2 hr followed by 15 min LPS stimulation and assessed for p38 phosphorylation by Western blot analysis. The image shown is representative of three experiments with similar results.

B. **P38 regulates LPS-induced IL-23 production:** pLXIN-infected MDMs were treated with SB203580 for 2hr followed by 24 hr LPS stimulation. The supernatants were assayed for IL-23 production by ELISA. N=4 different donors.

C. **HIV-Tat inhibits LPS-induced p38 MAPK phosphorylation:**HIV-Tat or pLXIN-infected MDMs were stimulated with LPS for 15 min followed by Western blot analysis (upper panel). Based on the densitometric analysis (Lower panel), the bar graph shows relative p38 phosphorylation as a mean ± SD from four experiments with different donors.

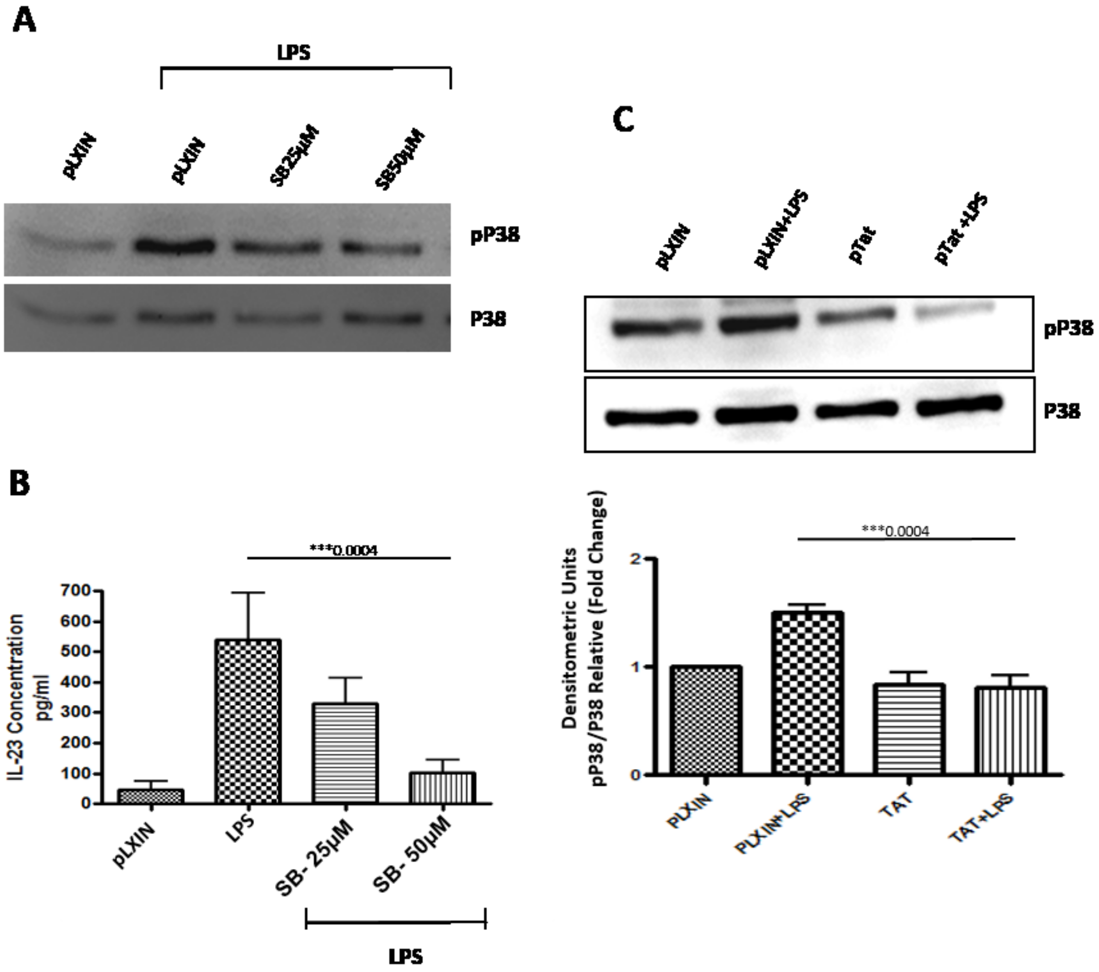

A
LPS
pLXIN
pLXIN
SB25µM
SB50µM
pP38
P38
B
***0.0004
IL-23 Concentration pg/ml
700
600
500
400
300
200
100
0
pLXIN
LPS
SB- 25µM
SB- 50µM
LPS
C
pLXIN
pLXIN+LPS
pTat
pTat +LPS
pP38
P38
Densitometric Units pP38/P38 Relative (Fold Change)
***0.0004
2
1
0
PLXIN
PLXIN+LPS
TAT
TAT+LPS

Figure 16

Figure 17: p38 MAPK inhibitor SB203580 and HIV-Tat additively inhibits LPS-induced IL-23 production in MDMs

pLXIN and Tat-infected MDMs were treated with SB203580 for 2 hr followed by LPS stimulation for 24 hr. The supernatants were assayed for IL-23 production by ELISA. N=4 different donors.

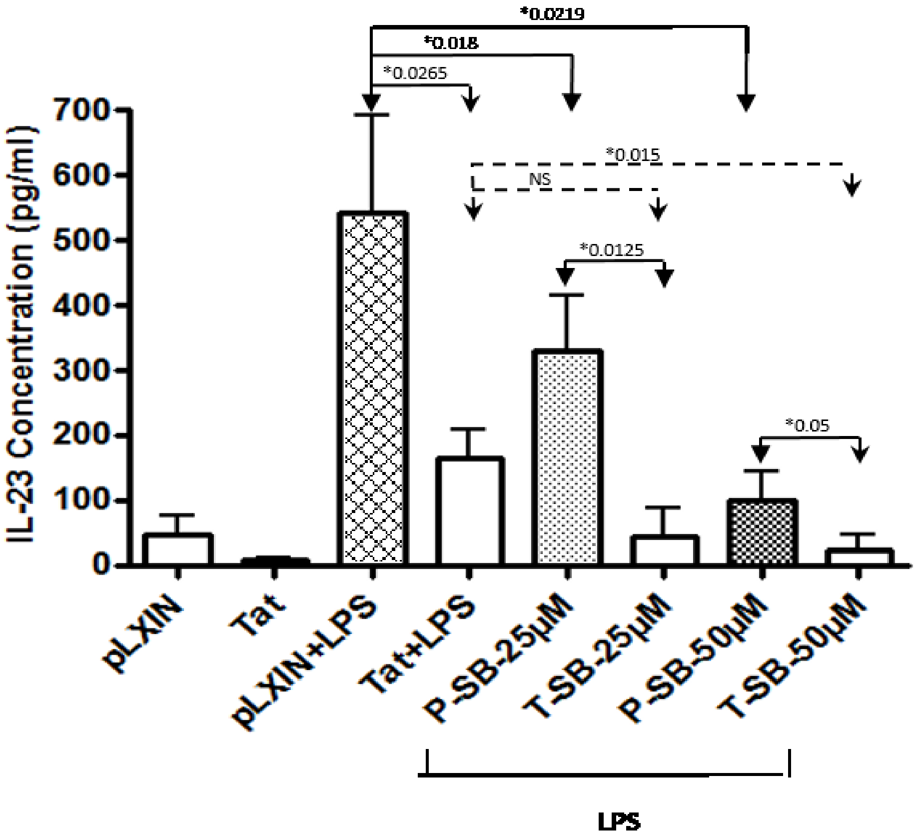

*0.0219
*0.018
*0.0265
*0.015
NS
*0.0125
*0.05
IL-23 Concentration (pg/ml)
700
600
500
400
300
200
100
0
pLXIN
Tat
pLXIN+LPS
Tat+LPS
P-SB-25µM
T-SB-25µM
P-SB-50µM
T-SB-50µM
LPS

Figure 17

3.2.1.2 ERK does not regulate LPS-induced IL-23 production in retrovirus infected MDMs

To investigate the role of ERK MAPK pathway, I used PD 098059, an ERK MAPK specific inhibitor. The biological activity of PD 098059 was confirmed following treatment of control pLXIN-infected MDMs with PD 098059 for 2 hr followed by 15 min of LPS stimulation. LPS-stimulation enhanced ERK phosphorylation and treatment with PD 098059 decreased ERK-phosphorylation in a dose-dependent manner (Fig 18A). To elucidate the involvement of ERK MAPK in LPS-induced IL-23 production, pLXIN-infected MDMs were treated with PD 098059 for 2 hr followed by LPS stimulation for 24 hrs and analyzed for IL-23 production. Even though PD 098059 inhibited ERK phosphoralation in a dose-dependent manner, ERK inhibitor did not affect LPS-induced IL-23 production in pLXIN retrovirus infected MDMs (Fig 18B).

To determine whether HIV Tat affected LPS-induced ERK phosphoralation, HIV-Tat or pLXIN-infected MDMs were stimulated with LPS for 15 min followed by analysis for ERK phosphorylation by Western immunoblotting. The basal levels of ERK phosphorylation were not statistically different in pLXIN-infected and Tat-infected MDMs. LPS-stimulation increased ERK phosphorylation in pLXIN-infected MDMs which was significantly decreased in HIV-Tat-infected MDMs (Fig 18C upper and lower panels).

The above results show that although HIV-Tat inhibits LPS-induced ERK phosphorylation, ERK does not regulate LPS-induced IL-23 production in pLXIN-infected MDMs. Therefore, it was of interest to determine if HIV-Tat will cooperate with the ERK inhibitor, PD 098059, to influence LPS-induced IL-23 production. For this pLXIN or HIV-Tat-infected MDMs were stimulated with LPS in the presence or absence of PD 098059. As expected HIV-Tat-infected MDMs showed decreased LPS-induced IL-23 production compared to the pLXIN-infected MDMs. However, PD 098059 at either 25 or 50 μM concentration did not inhibit LPS-induced IL-

23 production in HIV-Tat-infected MDMs compared to the untreated HIV-Tat-infected cells alone (Fig 19). These results suggest that although HIV-Tat significantly decreased ERK phosphoralation, HIV-Tat-mediated decrease in LPS-induced IL-23 production was not regulated by ERK.

Figure 18: HIV-Tat mediated inhibition of LPS-induced IL-23 production is not regulated by ERK MAPKs

A. The biological activity of ERK was measured by treatment of pLXIN-infected MDMs with PD 098059 for 2 hr followed by 15 min LPS stimulation and assessed for ERK phosphorylation by Western blot analysis. The image shown is representative of three experiments with similar results.

B. **ERK does not regulate LPS-induced IL-23 production in retrovirus infected MDMs**. pLXIN-infected MDMs were treated with PD 098059 for 2hr followed by 24 hr LPS stimulation. The supernatants were assayed for IL-23 production by ELISA. N=9 different donors

C. **HIV-Tat inhibits LPS-induced ERK phosphorylation**. HIV-Tat or pLXIN-infected MDMs were stimulated with LPS for 15 min followed by Western blot analysis (upper panel). Based on the densitometric analysis (Lower panel), the bar graph shows relative ERK phosphorylation as a mean ± SD from four experiments with different donors.

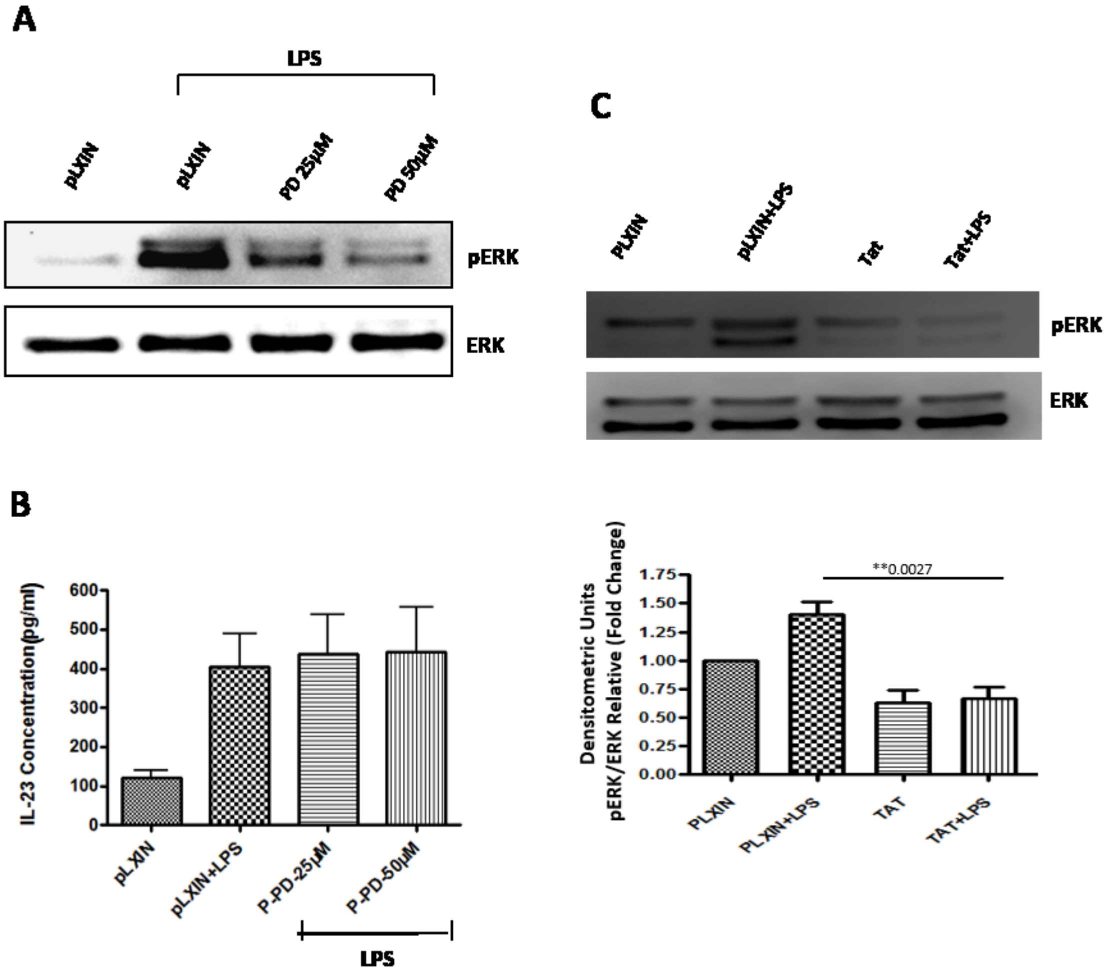

A
LPS
pLXIN
pLXIN
PD 25μM
PD 50μM
pERK
ERK
B
IL-23 Concentration(pg/ml)
600
500
400
300
200
100
0
pLXIN
pLXIN+LPS
P-PD-25μM
P-PD-50μM
LPS
C
PLXIN
pLXIN+LPS
Tat
Tat+LPS
pERK
ERK
Densitometric Units
pERK/ERK Relative (Fold Change)
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
**0.0027
PLXIN
PLXIN+LPS
TAT
TAT+LPS

Figure 18

Figure 19: ERK MAPK inhibitor PD 098059 either alone or in combination with HIV-Tat did not affect HIV-Tat-mediated inhibition of LPS-induced IL-23 production

pLXIN and Tat-infected MDMs were treated with PD 098059 for 2 hr followed by LPS stimulation for 24 hr . The supernatants were assayed for IL-23 production by ELISA. N=5 different donors.

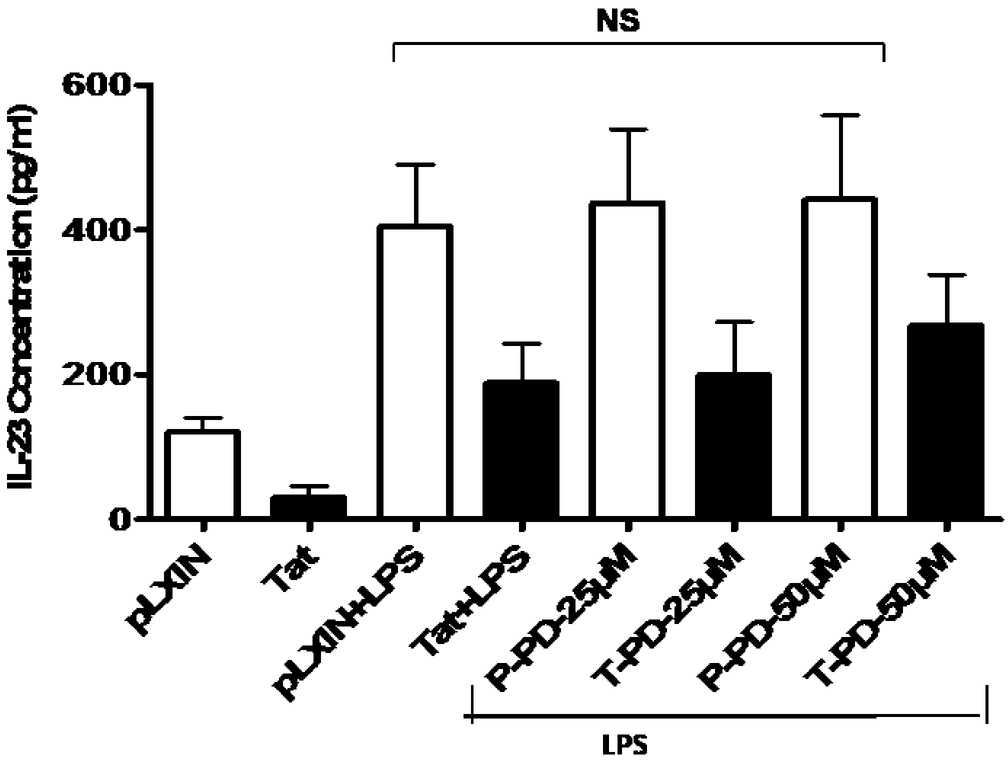

NS
IL-23 Concentration (pg/ml)
600
400
200
0
pLXIN
Tat
pLXIN+LPS
Tat+LPS
P-PD-25μM
T-PD-25μM
P-PD-50μM
T-PD-50μM
LPS

Figure 19

3.2.1.3 JNK MAPK negatively regulates LPS-induced IL-23 production in retrovirus infected MDMs

To investigate the JNK MAPK pathway, I employed SP600125, a JNK MAPK specific inhibitor. The biological activity of SP600125 was confirmed following treatment of control pLXIN-infected MDMs with SP600125 and LPS stimulation. LPS-stimulation enhanced JNK phosphorylation and treatment with SP600125 decreased JNK -phosphorylation in a dose-dependent manner (Fig 20A).

To study the involvement of JNK MAPK in LPS-induced IL-23 production, pLXIN-infected MDMs were treated with SP600125 for 2 hr followed by LPS stimulation. Unlike P38 and ERK MAPKs, JNK MAPK negatively regulated LPS-induced IL-23 production (Fig 20 B).

To determine the role of JNK in HIV-Tat-mediated inhibition of LPS-induced IL-23 production, I investigated the effect of HIV-Tat on LPS-induced JNK phosphoralation. HIV-Tat or pLXIN-infected MDMs were stimulated with LPS and analyzed for JNK phosphorylation by Western immunoblotting. The basal level of JNK phosphorylation was found to be similar in pLXIN-infected and HIV-Tat-infected MDMs. LPS-stimulation increased JNK phosphorylation in pLXIN-infected MDMs which was significantly decreased in HIV-Tat-infected MDMs (Fig 20C upper and lower panels).

Since HIV-Tat inhibits LPS-induced JNK phosphorylation and JNK negatively regulates LPS-induced IL-23 production in pLXIN-infected MDMs, I determined if JNK inhibition mediated by HIV-Tat and SP600125 will further upregulate LPS-induced IL-23 production. For this, pLXIN or HIV-Tat-infected MDMs were stimulated with LPS in the presence or absence of SP600125. The treatment with SP600125 enhanced LPS-induced IL-23 production in HIV-Tat-infected MDMs in a dose-dependent manner compared to the untreated HIV-Tat-infected cells alone (Fig 21). These results suggest that although HIV Tat significantly inhibited LPS-induced JNK phosphoralation, JNK inhibition enhanced LPS-induced IL-23 production in HIV-Tat-infected MDMs as well.

Figure 20: JNK MAPK does not regulate HIV-Tat mediated inhibition of LPS-induced IL-23 production

A. The biological activity of JNK was measured by treatment of pLXIN-infected MDMs with SP600125 for 2 hr followed by 15 min LPS stimulation and assessment for JNK phosphorylation by Western blot analysis. The image shown is representative of three experiments with similar results.

B. **JNK MAPK negatively regulates LPS-induced IL-23 production in pLXIN-infected MDMs**. pLXIN-infected MDMs were treated with SP600125 for 2 hr followed by 24 hr LPS stimulation. The supernatants were assayed for IL-23 production by ELISA. N=9 different donors.

C. **HIV-Tat inhibits LPS-induced JNK phosphorylation**. HIV-Tat or pLXIN-infected MDMs were stimulated with LPS for 15 min followed by Western blot analysis (upper panel). Based on the densitometric analysis (lower panel), the bar graph shows relative JNK phosphorylation as a mean ± SD from four experiments with different donors.

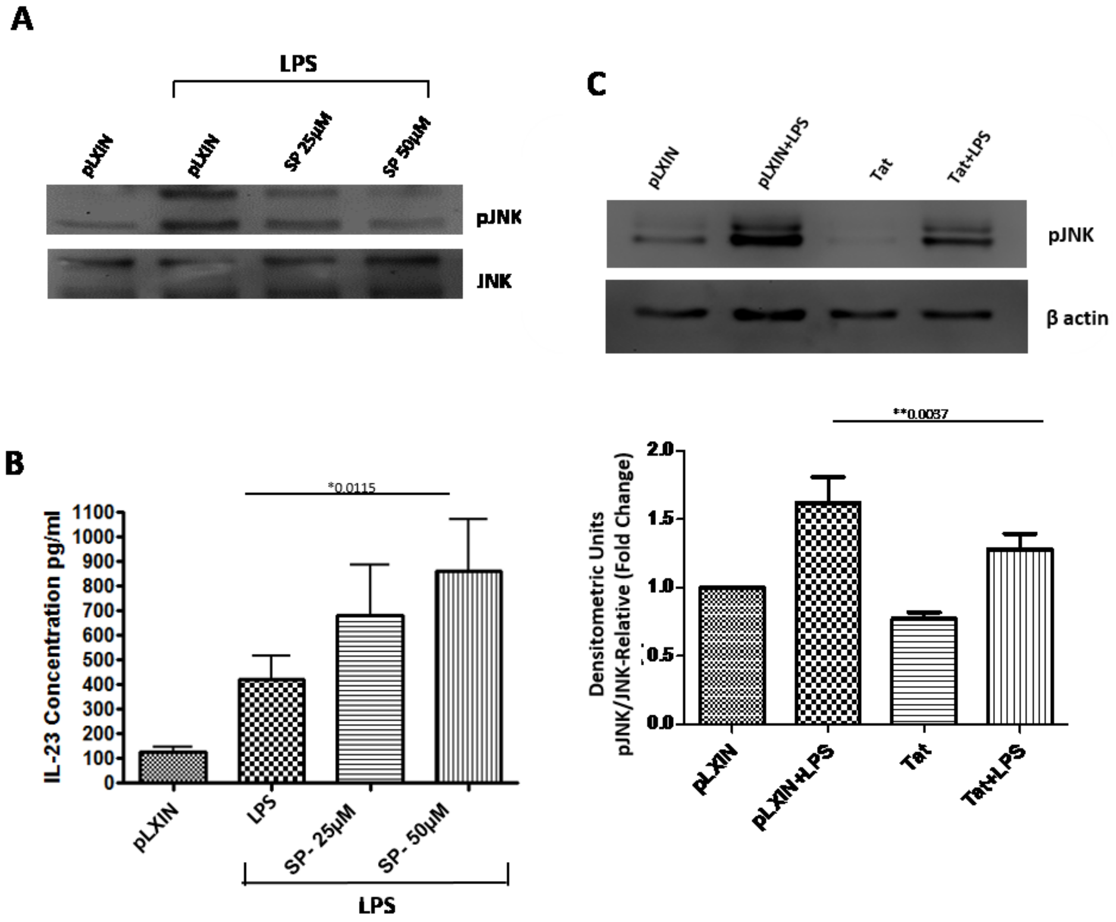
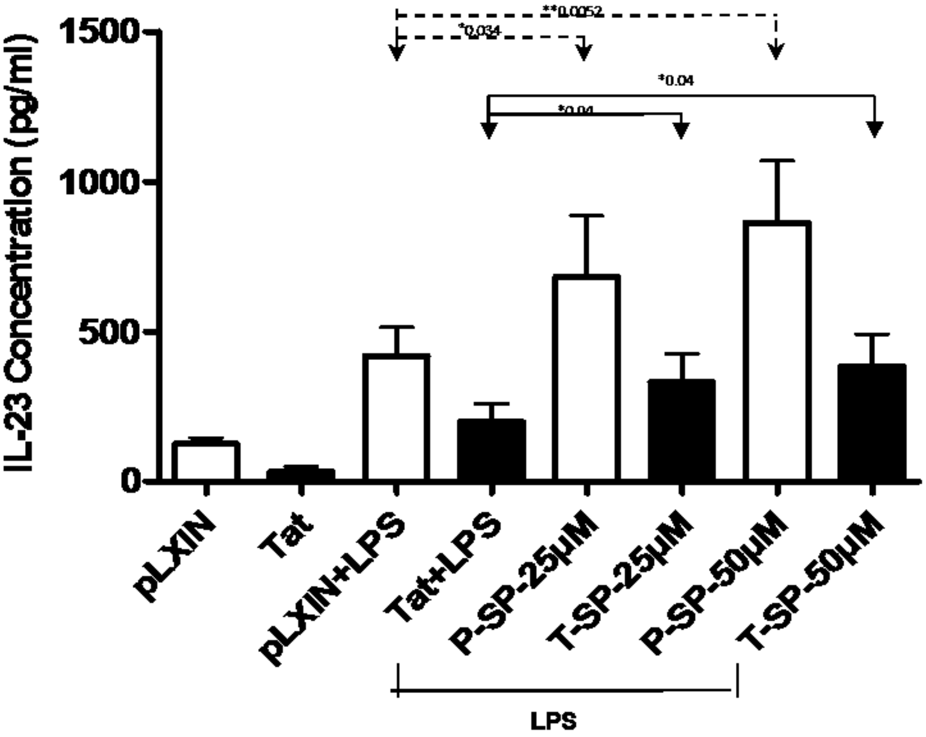

A
LPS
pLXIN
pLXIN
SP 25µM
SP 50µM
pJNK
JNK
B
*0.0115
IL-23 Concentration pg/ml
1100
1000
900
800
700
600
500
400
300
200
100
0
pLXIN
LPS
SP- 25µM
SP- 50µM
LPS
C
pLXIN
pLXIN+LPS
Tat
Tat+LPS
pJNK
β actin
**0.0037
Densitometric Units
pJNK/JNK-Relative (Fold Change)
2.0
1.5
1.0
0.5
0.0
pLXIN
pLXIN+LPS
Tat
Tat+LPS

Figure 20

Figure 21: JNK inhibitor SP600125 up-regulates LPS-induced IL-23 production in pLXIN and Tat-infected MDMs

pLXIN and HIV-Tat-infected MDMs were treated with SP600125 for 2 hr followed by LPS stimulation for 24 hr . The supernatants were assayed for IL-23 production by ELISA. N=8 different donors.



**0.0052
*0.034
*0.04
*0.04
IL-23 Concentration (pg/ml)
1500
1000
500
0
pLXIN
Tat
pLXIN+LPS
Tat+LPS
P-SP-25μM
T-SP-25μM
P-SP-50μM
T-SP-50μM
LPS

Figure 21

3.2.1.4 PI3K regulates HIV-Tat-mediated inhibition of LPS-induced IL-23 production

HIV-Tat may inhibit LPS-induced IL-23 production by modulating the pathways other than MAPKs such as the PI3K pathway. To determine the role of PI3K pathway, I employed LY294002, the PI3K specific inhibitor. The biological activity of LY294002 was confirmed by pre-treating pLXIN-infected MDMs with LY294002 followed by LPS stimulation. LPS-stimulation enhanced AKT phosphorylation and treatment with LY294002 decreased LPS-induced AKT-phosphorylation in a dose-dependent manner (Fig 22A).

To study the involvement of PI3K in LPS-induced IL-23 production, pLXIN-infected MDMs were treated with LY294002 followed by LPS stimulation. The results show that LY294002 significantly inhibited LPS-induced IL-23 production in a dose-dependent manner (Fig 22 B). To determine whether HIV-Tat-mediated inhibition of LPS-induced IL-23 production is regulated via inhibition of AKT phosphoralation, HIV-Tat or pLXIN-infected MDMs were stimulated with LPS for 15 min followed by analysis for AKT phosphorylation. LPS-stimulation increased AKT phosphorylation in pLXIN-infected MDMs which was significantly decreased in HIV-Tat-infected MDMs (Fig 22C).

Since HIV-Tat inhibits LPS-induced AKT phosphorylation and LY294002 inhibits LPS-induced IL-23 production, it was of interest to determine if PI3K inhibition mediated by HIV-Tat and LY294002 will co-operate to further enhance inhibition of LPS-induced IL-23 production. For this, pLXIN or HIV-Tat-infected MDMs were stimulated with LPS in the presence or absence of LY294002. As expected, I observed 75% inhibition of LPS-induced IL-23 production in HIV-Tat-infected MDMs compared to the pLXIN-infected MDMs (Fig 23). LY294002 at 50 μM concentration further inhibited LPS-induced IL-23 production in HIV-Tat-infected MDMs to almost basal levels (>95%) compared to the LPS-stimulated pLXIN or HIV-Tat-infected cells (Fig

23). In summary, HIV-Tat is a potent inhibitor of AKT phosphorylation and along with LY294002, it additively inhibits LPS-induced IL-23 production in MDMs. These results suggest that HIV-Tat decreases LPS-induced IL-23 production via inhibition of AKT phosphorylation.

Figure 22: **HIV-Tat-mediated inhibition of LPS-induced IL-23 is regulated by PI3K**

A. The biological activity of PI3K was measured by treatment of pLXIN-infected MDMs with LY294002 for 2 hr followed by 15 min LPS stimulation and assessed for AKT phosphorylation by Western blot analysis. The image shown is representative of three experiments with similar results.

B. **PI3K regulates LPS-induced IL-23 production**. pLXIN-infected MDMs were treated with LY294002 for 2hr followed by 24 hr LPS stimulation. The supernatants were assayed for IL-23 production by ELISA. N=5 different donors.

C. **HIV-Tat inhibits LPS-induced AKT phosphorylation**. HIV-Tat or pLXIN-infected MDMs were stimulated with LPS for 15 min followed by Western blot analysis (upper panel). Based on the densitometric analysis (lower panel), the bar graph shows relative AKT phosphorylation as a mean ± SD from four experiments with different donors.

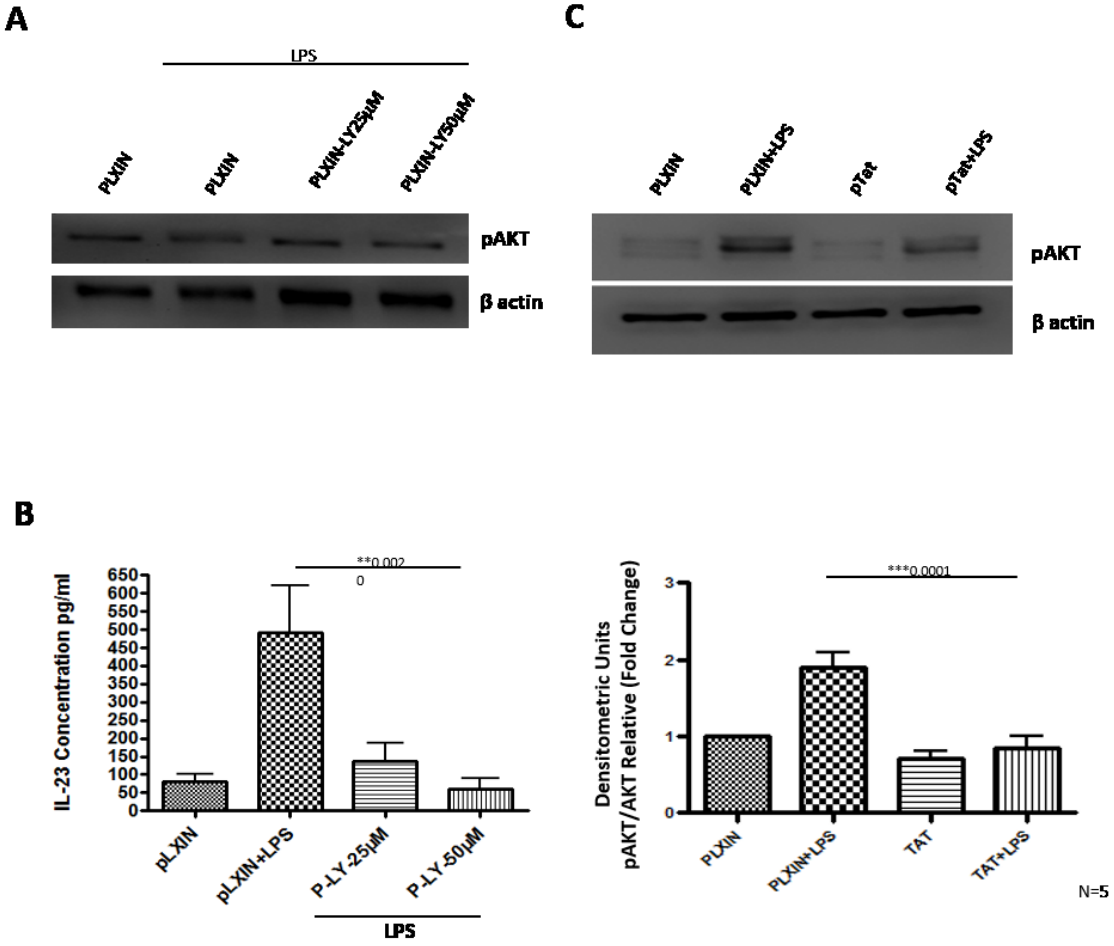

A
LPS
PLXIN
PLXIN
PLXIN-LY25µM
PLXIN-LY50µM
pAKT
β actin
C
PLXIN
PLXIN+LPS
pTat
pTat+LPS
pAKT
β actin
B
IL-23 Concentration pg/ml
650
600
550
500
450
400
350
300
250
200
150
100
50
0
**0.002
0
pLXIN
pLXIN+LPS
P-LY 25µM
P-LY 50µM
LPS
Densitometric Units
pAKT/AKT Relative (Fold Change)
3
2
1
0
***0.0001
PLXIN
PLXIN+LPS
TAT
TAT+LPS
N=5

Figure 22

Figure 23: PI3K inhibitor LY294002 further inhibits HIV-Tat mediated inhibition of IL-23 production in MDMs

pLXIN and Tat-infected MDMs were treated with LY294002 for 2 hr followed by LPS stimulation for 24 hr . The supernatants were assayed for IL-23 production by ELISA. N=5 different donors.

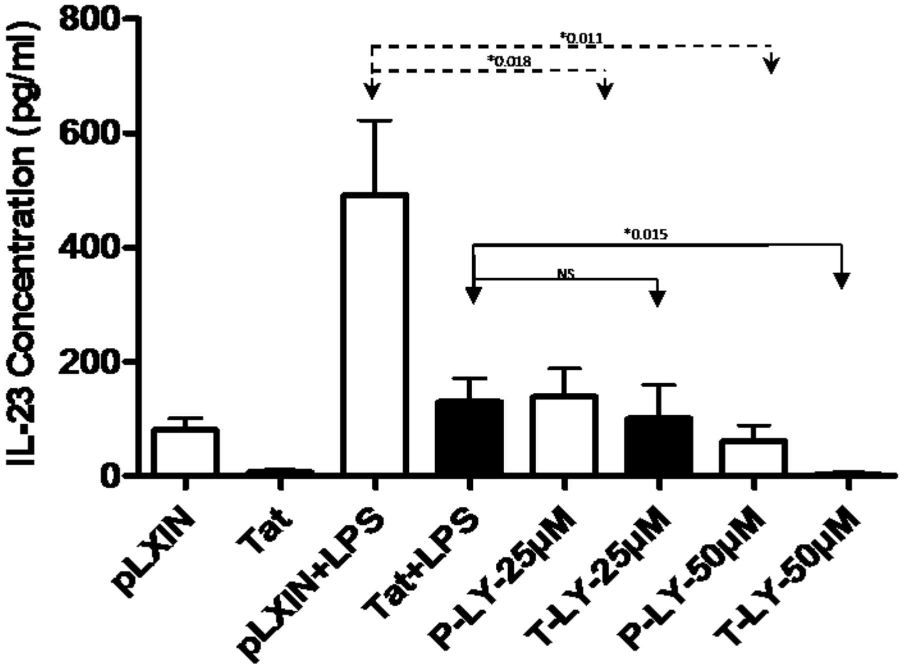

IL-23 Concentration (pg/ml)
800
600
400
200
0
*0.011
*0.018
*0.015
NS
pLXIN
Tat
pLXIN+LPS
Tat+LPS
P-LY-25µM
T-LY-25µM
P-LY-50µM
T-LY-50µM

Figure 23

3.2.1.5 Although Src negatively regulates LPS-induced IL-23 production, Src does not regulate HIV-Tat-mediated inhibition of LPS-induced IL-23 production in MDMs

Binding of LPS to TLR4 triggers tyrosine phosphorylation of various signalling molecules and activates protein tyrosine kinases (PTKs) including Src kinases. Since HIV-Tat inhibited LPS-induced IL-23 expression, I hypothesized that HIV-Tat interferes with TLR-4 activated PTKs leading to inhibition of LPS-induced IL-23 production. For this I used SU6656, the Src specific inhibitor. The biological activity of SU6656 was confirmed following treatment of pLXIN-infected MDMs with SU6656 and LPS stimulation. LPS-stimulation enhanced Src phosphorylation and treatment with SU6656 decreased LPS-induced Src-phosphorylation in a dose dependent manner (Fig 24 A). To study the role of Src, pLXIN- infected MDMs were treated with SU6656 followed by LPS stimulation and analyzed of IL-23 production. Like JNK, even though SU6656 inhibited Src phosphorylation, it enhanced LPS-induced IL-23 production in pLXIN-MDMs (Fig 24 B).

To determine the role of Src in HIV-Tat-mediated inhibition of LPS-induced IL-23 production, HIV-Tat or pLXIN-infected MDMs were stimulated with LPS and assessed for Src phosphorylation (Fig 24 C). LPS-stimulation increased Src phosphorylation in pLXIN-infected MDMs which was not significantly decreased in HIV-Tat-infected MDMs.

Since HIV-Tat does not inhibit LPS-induced Src phosphorylation and SU6656 negatively regulates LPS-induced IL-23 production, I determined if Src inhibition mediated by SU6656 and HIV-Tat and will affect LPS-induced IL-23 production. For this, pLXIN or HIV-Tat-infected MDMs were stimulated with LPS in the presence or absence of the SU6656. As expected, HIV-Tat inhibited LPS-induced IL-23production. However, addition of SU6656 at 2.5µM and 5 µM to HIV-Tat-infected MDMs significantly enhanced LPS-induced IL-23 production in a dose-dependent manner compared to LPS-stimulated HIV-Tat-infected cells alone (Fig 24 D). However, there was no significant differences in LPS-induced IL-23 production in SU6656 treated pLXIN and HIV-Tat-infected MDMs. These results suggest that similar to JNK, although Src negatively regulates LPS-induced IL-23 production in both pLXIN and HIV-Tat-infected MDMs, HIV-Tat-mediated inhibition of LPS-induced IL-23 production was not regulated by Src.

Figure 24: Src negatively regulates LPS-induced IL-23 production, however Src does not regulate LPS-induced IL-23 production in MDMs

A. The biological activity of Src was measured by treatment of pLXIN-infected MDMs with SU6656 for 2 hr followed by 15 min LPS stimulation and assessed for Src phosphorylation by Western blot analysis. The image shown is representative of three experiments with similar results.

B. **Src negatively regulates LPS-induced IL-23 production**. pLXIN-infected MDMs were treated with SU6656 for 2hr followed by 24 hr LPS stimulation. The supernatants were assayed for IL-23 production by ELISA. N=6 different donors.

C. **HIV-Tat does not inhibit LPS-induced Src phosphorylation**. HIV-Tat or pLXIN-infected MDMs were stimulated with LPS for 15 min followed by Western blot.

D. **Src inhibitor SU6656 upregulates LPS-induced IL-23 production in pLXIN and HIV-Tat-infected MDMs**. pLXIN and Tat-infected MDMs were treated with the SU6656 for 2 hr followed by 24 hr LPS stimulation. The supernatants were assayed for IL-23 by ELISA .The graph represents six experiments with different donors.

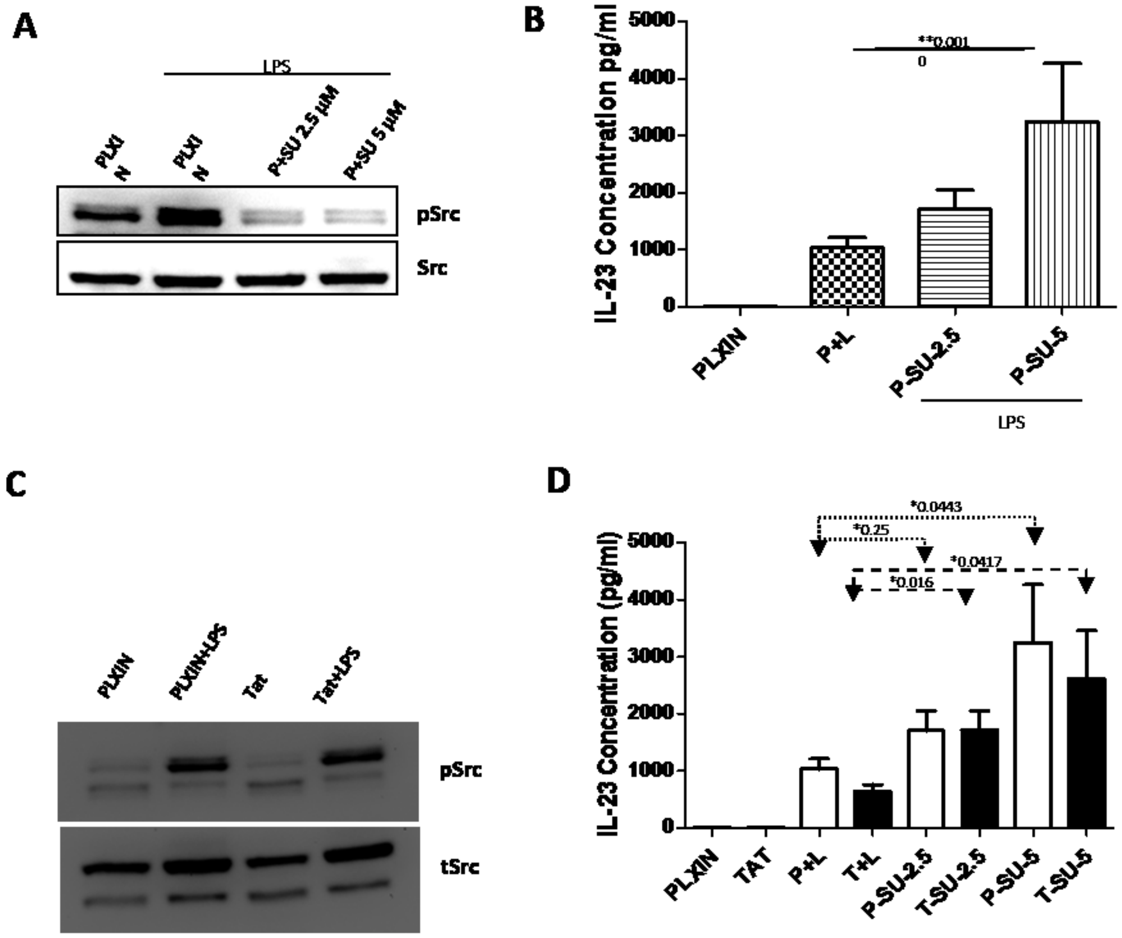

A
LPS
PLXI N
PLXI N
P+SU 2.5 µM
P+SU 5 µM
pSrc
Src
B
IL-23 Concentration pg/ml
5000
4000
3000
2000
1000
0
**0.001
0
PLXIN
P+L
P-SU-2.5
P-SU-5
LPS
C
PLXIN
PLXIN+LPS
Tat
Tat+LPS
pSrc
tSrc
D
IL-23 Concentration (pg/ml)
5000
4000
3000
2000
1000
0
*0.0443
*0.25
*0.0417
*0.016
PLXIN
TAT
P+L
T+L
P-SU-2.5
T-SU-2.5
P-SU-5
T-SU-5

Figure 24

3.2.1.6 Although SHP-1 regulates LPS-induced IL-23 production, it does not mediate HIV-Tat-mediated inhibition of LPS-induced IL-23 production in MDMs

Binding LPS to TLR4 triggers activation of phosphataises such as SHP-1. However, the role of SHP-1 in LPS-induced IL-23 production in general and in particular MDMs is not known. Since HIV-Tat inhibited LPS-induced IL-23 expression, I first determined the role of SHP-1 in LPS-induced IL-23 production in MDMs by employing sodium stibogluconate (SS), the SHP-1 specific inhibitor. The biological activity of SS was confirmed following treatment of pLXIN-infected MDMs with SS followed by LPS stimulation. LPS-stimulation enhanced SHP-1 activation and treatment with SS decreased SHP-1 activation in a dose-dependent manner in pLXIN-infected MDMs (Fig 25 A). To study the involvement of SHP-1 in LPS-induced IL-23 production, pLXIN-infected MDMs were treated with SS followed by LPS stimulation and analysis of IL-23 production. The results show that SS significantly inhibited LPS-induced IL-23 production (Fig 25 B).

Since SHP-1 regulated LPS-induced IL-23 production, I hypothesized that HIV-Tat may inhibit LPS-induced IL-23 production by inhibiting SHP-1 activation. To determine the effect of HIV Tat on LPS-induced SHP-1 activation, HIV-Tat or pLXIN-infected MDMs were stimulated with LPS followed by assessment of SHP-1 activation by Western blot analysis. The results show that HIV-Tat did not inhibit LPS-induced SHP-1 activation (Fig 25 C).

Since HIV-Tat does not inhibit LPS-induced SHP-1 activation but SHP-1 regulates LPS-induced IL-23 production, it was of interest to determine if SHP-1 inhibition by SS and HIV-Tat will co-operate to affect LPS-induced IL-23 production. For this, pLXIN or HIV-Tat-infected MDMs were stimulated with LPS in the presence or the absence of SS. HIV Tat inhibited LPS-induced IL-23 production as expected. However, addition of SS to HIV-Tat-infected MDMs did not further inhibit LPS-induced IL-23 production compared to the SS treated pLXIN-infected MDMs (Fig 25 D). In fact, there was no significant differences in IL-23 production in SS treated pLXIN and HIV-Tat-infected MDMs (Fig 25D). These results suggest that although SHP-1 regulates LPS-induced IL-23 production, it does not regulate HIV-Tat-mediated inhibition of LPS-induced IL-23 production in MDMs.

In summary, HIV-Tat inhibits LPS-induced IL-23 production through inhibition of PI3K and p38 MAPK signaling pathways. Although HIV-Tat inhibits ERK and JNK MAPK, these kinases do not regulate HIV-Tat mediated inhibition of LPS-induced IL-23 production. Interestingly, JNK MAPK and Src kinases negatively regulated LPS-induced IL-23 production in MDMs. Finally, although SHP-1 regulated LPS-induced IL-23 production it does not regulate HIV-Tat-mediated inhibition of LPS-induced IL-23 production in MDMs.

Figure 25: SHP-1 positively regulates LPS-induced IL-23 production however; SHP-1 does not regulate HIV-Tat mediated inhibition of LPS-induced IL-23 production

A. The biological activity of SHP-1 was measured by treatment of pLXIN-infected MDMs with SS for 2 hr followed by 24 hr LPS stimulation and assessed for SHP-1 activation by Western blot analysis. The image shown is representative of three experiments with similar results.

B. **SHP-1 positively regulate LPS-induced IL-23 production**. pLXIN-infected MDMs were treated with SS for 2hr followed by 24 hr LPS stimulation. The supernatants were assayed for IL-23 production by ELISA. N=7 different donors.

C. **HIV-Tat does not inhibit LPS-activated SHP-1**. HIV-Tat or pLXIN-infected MDMs were stimulated with LPS for 24 hr followed by Western blot.

D. **HIV-Tat does not further inhibit SS mediated inhibition of LPS-induced IL-23 production.** pLXIN and Tat-infected MDMs were treated with the SS for 2 hr followed by 24 hr LPS stimulation. The supernatants were assayed for IL-23 by ELISA. The graph represents seven experiments with different donors.

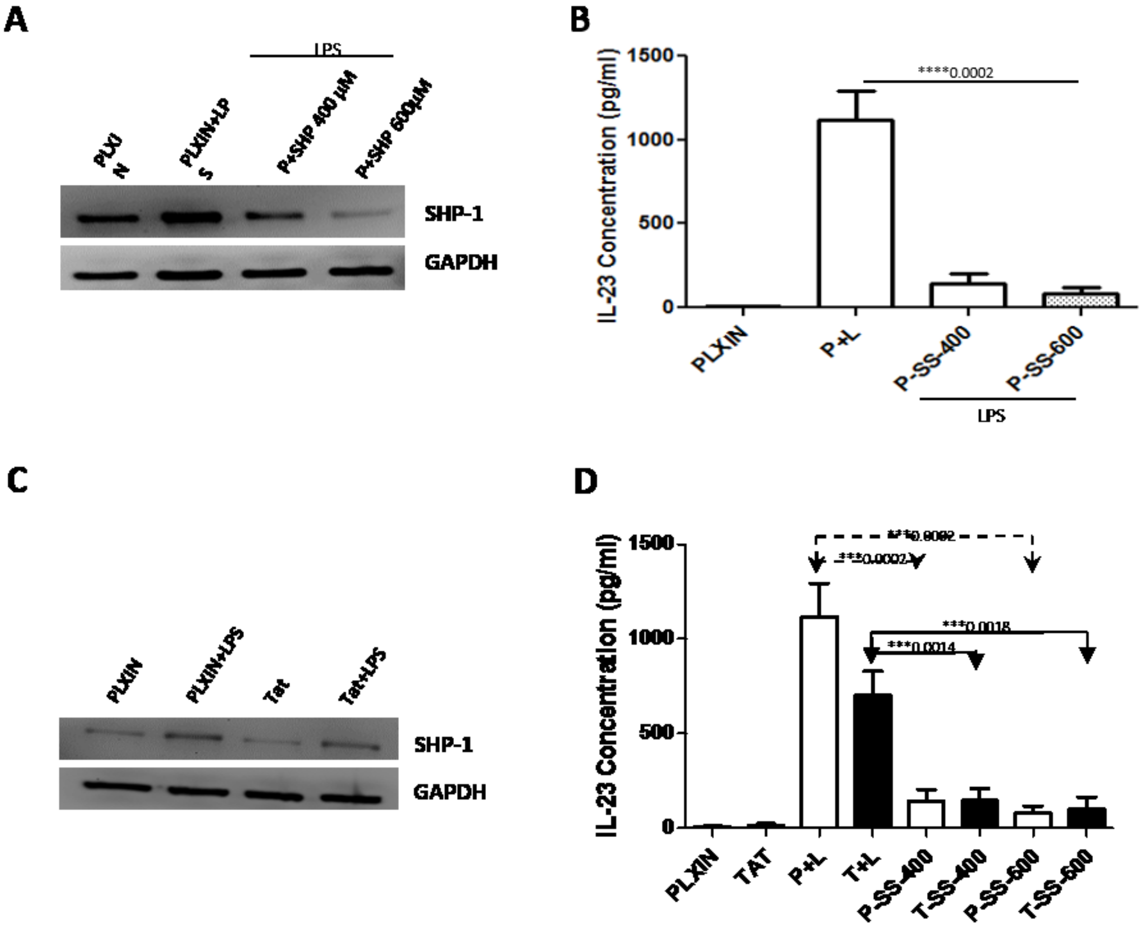

A
LPS
PLXI N
PLXIN+LP S
P+SHP 400 µM
P+SHP 600µM
SHP-1
GAPDH
B
IL-23 Concentration (pg/ml)
1500
1000
500
0
****0.0002
PLXIN
P+L
P-SS-400
P-SS-600
LPS
C
PLXIN
PLXIN+LPS
Tat
Tat+LPS
SHP-1
GAPDH
D
IL-23 Concentration (pg/ml)
1500
1000
500
0
***0.0002
***0.0002
***0.0018
***0.0014
PLXIN
TAT
P+L
T+L
P-SS-400
T-SS-400
P-SS-600
T-SS-600

Figure 25

3.2.2.1 Objective 2- Part 2: HIV-Tat-mediated inhibition of LPS-induced IL-27 is regulated by p38 MAPK phosphorylation

Since intracellular and extracellular HIV-Tat inhibited LPS-induced IL-27 protein and the p28 and EBI3 mRNA expression, I hypothesized that HIV-Tat inhibits LPS-induced IL-27 production by modulating the TLR-4 activated signalling pathway in MDMs. I first investigated the role of p38 MAPK pathway by employing SB203580, a p38 specific inhibitor. The biological activity of SB203580 was confirmed as shown in Fig 14A. To examine the involvement of p38 MAPK in LPS-induced IL-27 production, pLXIN-infected MDMs were treated with SB203580 for 2 hr followed by LPS stimulation for 24 hrs and analysis of IL-27 production. The results show that SB203580 significantly inhibited LPS-induced IL-27 production (Fig 26A).

To determine whether HIV-Tat-mediated inhibition of LPS-induced IL-27 production is mediated via inhibition of p38 MAPK phosphorylation, HIV-Tat or pLXIN-infected MDMs were stimulated with LPS for 15 min followed by analysis for p38 phosphorylation. LPS-stimulation increased p38 phosphorylation in pLXIN-infected MDMs which was also significantly decreased in HIV-Tat-infected MDMs (Fig 16C).

Since HIV-Tat inhibits LPS-induced p38 phosphorylation and SB203580 inhibits LPS-induced IL-27 production, it was of interest to determine if p38 inhibition mediated by HIV-Tat and SB203580 will co-operate to further enhance inhibition of LPS-induced IL-27 production. For this, pLXIN or HIV-Tat-infected MDMs were stimulated with LPS in the presence or the absence of SB203580. As expected I observed >65 % inhibition of LPS-induced IL-27 production in HIV-Tat-infected MDMs compared to the pLXIN-infected MDMs (Fig 26B). SB203580 at 25 and 50 µM concentration further inhibited LPS-induced IL-27 production in HIV Tat-infected MDMs to almost basal levels (>90%) compared to the LPS-stimulated pLXIN- infected cells (Fig 26B).

Overall, HIV-Tat is a potent inhibitor of p38 phosphorylation and it co-operated with SB203580 to inhibit LPS-induced IL-27 production.

Figure 26: HIV-Tat-mediated inhibition of LPS-induced IL-27 is regulated by p38 MAPK phosphorylation

A. **p38 regulates LPS-induced IL-27 production.** pLXIN-infected MDMs were treated with SB203580 for 2 hr followed by 24 hr LPS stimulation. The supernatants were assayed for IL-27 production by ELISA. N=9 different donors.

B. **SB203580 further inhibits HIV-Tat mediated inhibition of LPS-induced IL-27 production.** pLXIN and Tat-infected MDMs were treated with SB203580 for 2 hr followed by LPS stimulation 24 hr . The supernatants were assayed for IL-27 production by ELISA. N=9 different donors.

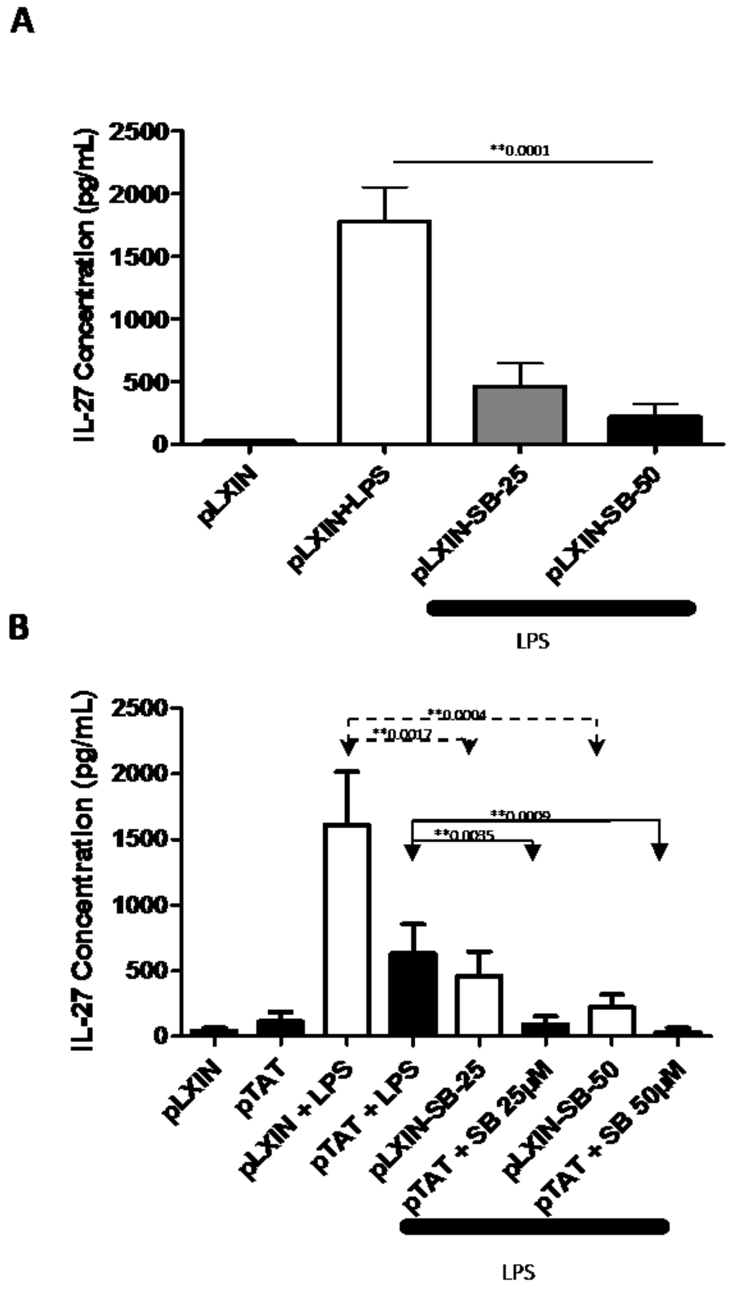

A
IL-27 Concentration (pg/mL)
2500
2000
1500
1000
500
0
**0.0001
pLXIN
pLXIN+LPS
pLXIN-SB-25
pLXIN-SB-50
LPS
B
IL-27 Concentration (pg/mL)
2500
2000
1500
1000
500
0
**0.0004
**0.0017
**0.0009
**0.0035
pLXIN
pTAT
pLXIN + LPS
pTAT + LPS
pLXIN-SB-25
pTAT + SB 25μM
pLXIN-SB-50
pTAT + SB 50μM
LPS

Figure 26

3.2.2.2 ERK and JNK MAPKs do not regulate LPS-induced IL-27 production in retrovirus-infected MDMs

To investigate the role of ERK and JNK MAPK pathways I used PD 098059 and SP600125, the ERK and JNK specific inhibitors respectively. The biological activity of PD 098059 and SP600125 was confirmed as shown in Fig 18A and 20A, respectively.

To elucidate the involvement of ERK and JNK MAPK in LPS-induced IL-27 production, pLXIN-infected MDMs were treated with PD 098059 or SP600125 followed by LPS stimulation for 24 hrs and analyzed for IL-27 production. Even though PD098059 and SP600125 inhibited ERK and JNK phosphoralation, respectively, these inhibitors did not affect LPS-induced IL-27 production in pLXIN infected MDMs (Fig 27A and 28A). I have previously shown that LPS-stimulation increased ERK and JNK phosphorylation in pLXIN-infected MDMs which was significantly decreased in HIV-Tat-infected MDMs (Fig 18C and 20 C).

The above results show that HIV-Tat inhibits LPS-induced ERK and JNK phosphorylation. However, ERK and JNK did not regulate LPS-induced IL-27 production in pLXIN retrovirus infected MDMs. I next determined if HIV-Tat will cooperate with ERK (PD98059) or JNK inhibitors (SP600125) to influence LPS-induced IL-27 production. For this, pLXIN or HIV-Tat-infected MDMs were stimulated with LPS in the presence or the absence of PD 098059 or SP600125. As expected HIV-Tat-infected MDMs showed decreased LPS-induced IL-27 production compared to the pLXIN-infected MDMs. However, PD 098059 and SP600125 at 25 and 50 μM concentrations did not inhibit LPS-induced IL-27 production in HIV-Tat-infected MDMs compared to untreated HIV-Tat-infected cells alone (Fig 27B and 28B). These results suggest that although HIV-Tat significantly decreased ERK and JNK phosphoralation, HIV-Tat-mediated decrease in LPS-induced IL-27 production was not regulated by ERK or JNK MAPKs.

Figure 27: ERK MAPK does not regulate HIV-Tat-mediated inhibition of LPS-induced IL-27 production

A. **ERK MAPK does not regulate LPS-induced IL-27 production.** pLXIN-infected MDMs were treated with PD 098059 for 2 hr followed by 24 hr LPS stimulation. The supernatants were assayed for IL-27 production by ELISA. N=7 different donors.

B. **PD 098059 does not affect HIV-Tat-mediated inhibition of LPS-induced IL-27 production.** pLXIN and Tat-infected MDMs were treated with PD 098059 for 2 hr followed by LPS stimulation for 24 hr . The supernatants were assayed for IL-27 production by ELISA. N=7 different donors.

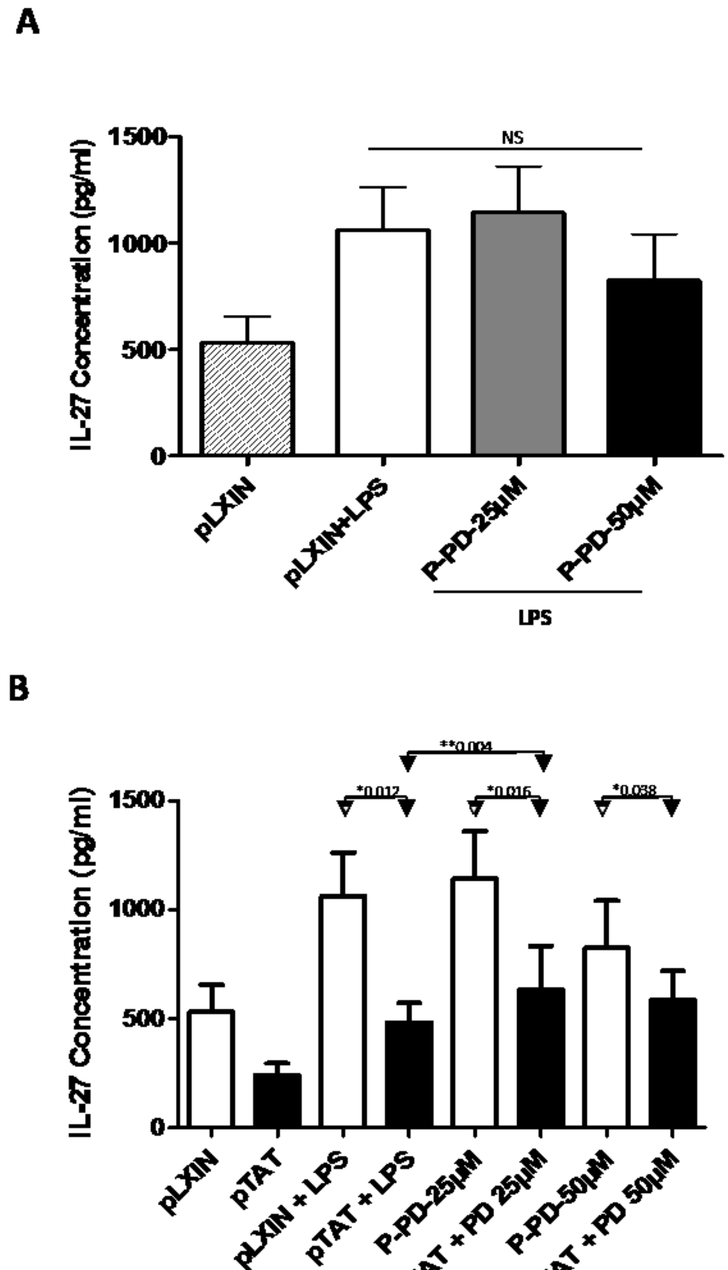

A
IL-27 Concentration (pg/ml)
1500
1000
500
0
NS
pLXIN
pLXIN+LPS
P-PD-25μM
P-PD-50μM
LPS
B
IL-27 Concentration (pg/ml)
1500
1000
500
0
**0.004
*0.012
*0.016
*0.038
pLXIN
pTAT
pLXIN + LPS
pTAT + LPS
P-PD-25μM
pTAT + PD 25μM
P-PD-50μM
pTAT + PD 50μM

Figure 27

Figure 28: HIV-Tat-mediated inhibition of LPS-induced IL-27 production is not regulated by JNK

A. **JNK MAPK does not regulate LPS-induced IL-27 production.** pLXIN-infected MDMs were treated with SP600125 for 2 hr followed by 24 hr LPS stimulation. The supernatants were assayed for IL-27 production by ELISA. N=7 different donors.

B. **SP600125 does not affect HIV-Tat-mediated inhibition of LPS-induced IL-27 production.** pLXIN and Tat-infected MDMs were treated with SP600125 for 2 hr followed by LPS stimulation for 24 hr . The supernatants were assayed for IL-27 production by ELISA. N=7 different donors.

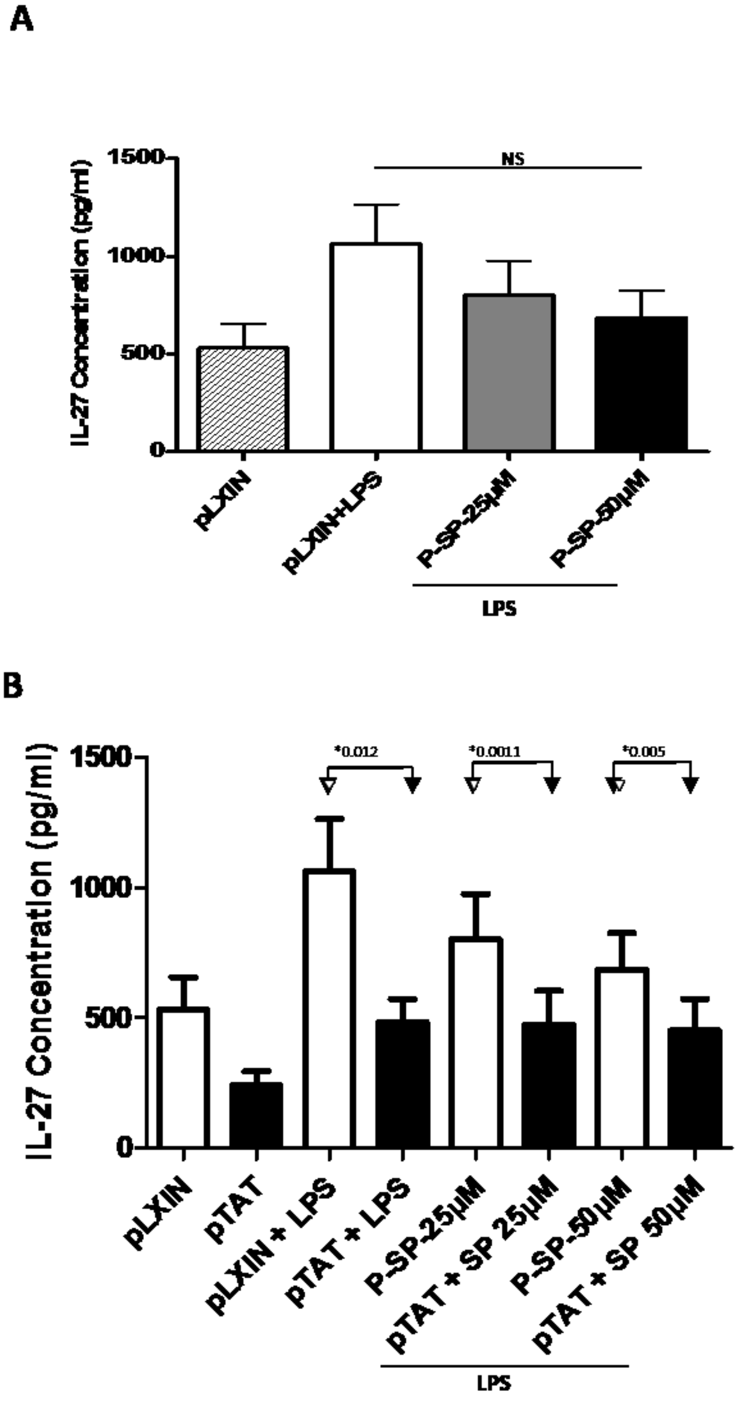

A
IL-27 Concentration (pg/ml)
1500
1000
500
0
NS
pLXIN
pLXIN+LPS
P-SP-25µM
P-SP-50µM
LPS
B
IL-27 Concentration (pg/ml)
1500
1000
500
0
*0.012
*0.0011
*0.005
pLXIN
pTAT
pLXIN + LPS
pTAT + LPS
P-SP-25µM
pTAT + SP 25µM
P-SP-50µM
pTAT + SP 50µM
LPS

Figure 28

3.2.2.3 HIV -Tat-mediated inhibition of LPS-induced IL- 27 production is regulated by PI3K

HIV-Tat may inhibit LPS-induced IL-27 production by modulating the TLR-4 activated PI3K signalling pathway in MDMs. To determine the role of PI3K pathway I employed LY294002, the PI3K specific inhibitor. The biological activity of LY294002 was confirmed as shown in Fig 22 A. To study the involvement of PI3K in LPS-induced IL-27 production, pLXIN-infected MDMs were treated with LY294002 followed by LPS stimulation and analyzed for IL-27 production. The results show that LY294002 significantly inhibited LPS-induced IL-27 production (Fig 29A). I have previously shown that LPS-stimulation increased AKT phosphorylation in pLXIN-infected MDMs which was significantly decreased in HIV-Tat-infected MDMs (Fig 29C).

Since HIV-Tat inhibits LPS-induced AKT phosphorylation and LY294002 inhibits LPS-induced IL-27 production, it was of interest to determine if PI3K inhibition mediated by HIV-Tat and LY294002 will co-operate to further inhibit LPS-induced IL-27 production. For this, pLXIN or HIV-Tat-infected MDMs were stimulated with LPS in the presence or absence of LY294002. As expected, I observed 60% inhibition of LPS-induced IL-27 production in HIV-Tat-infected MDMs compared to the pLXIN-infected MDMs (Fig 29A). LY294002 at 25 and 50 μM concentration further inhibited LPS-induced IL-27 production in HIV-Tat-infected MDMs to almost basal levels (>95%) compared to the LPS-stimulated pLXIN or HIV-Tat-infected cells (Fig 29B). In summary, HIV-Tat is a potent inhibitor of AKT phosphorylation and it co-operates with LY294002 to inhibit LPS-induced IL-27 production in MDMs.

Figure 29: HIV-Tat-mediated inhibition of LPS-induced IL-27 production is regulated by PI3K

A. **PI3K regulates LPS-induced IL-27 production in MDMs.** pLXIN-infected MDMs were treated with LY294002 for 2 hr followed by 24 hr LPS stimulation. The supernatants were assayed for IL-27 production by ELISA. N=6 different donors.

B. **LY294002 further inhibits HIV-Tat mediated inhibition of LPS-induced IL-23 production.** pLXIN and Tat-infected MDMs were treated with LY294002 for 2 hr followed by LPS stimulation for 24 hr . The supernatants were assayed for IL-27 production by ELISA. N=6 different donors.

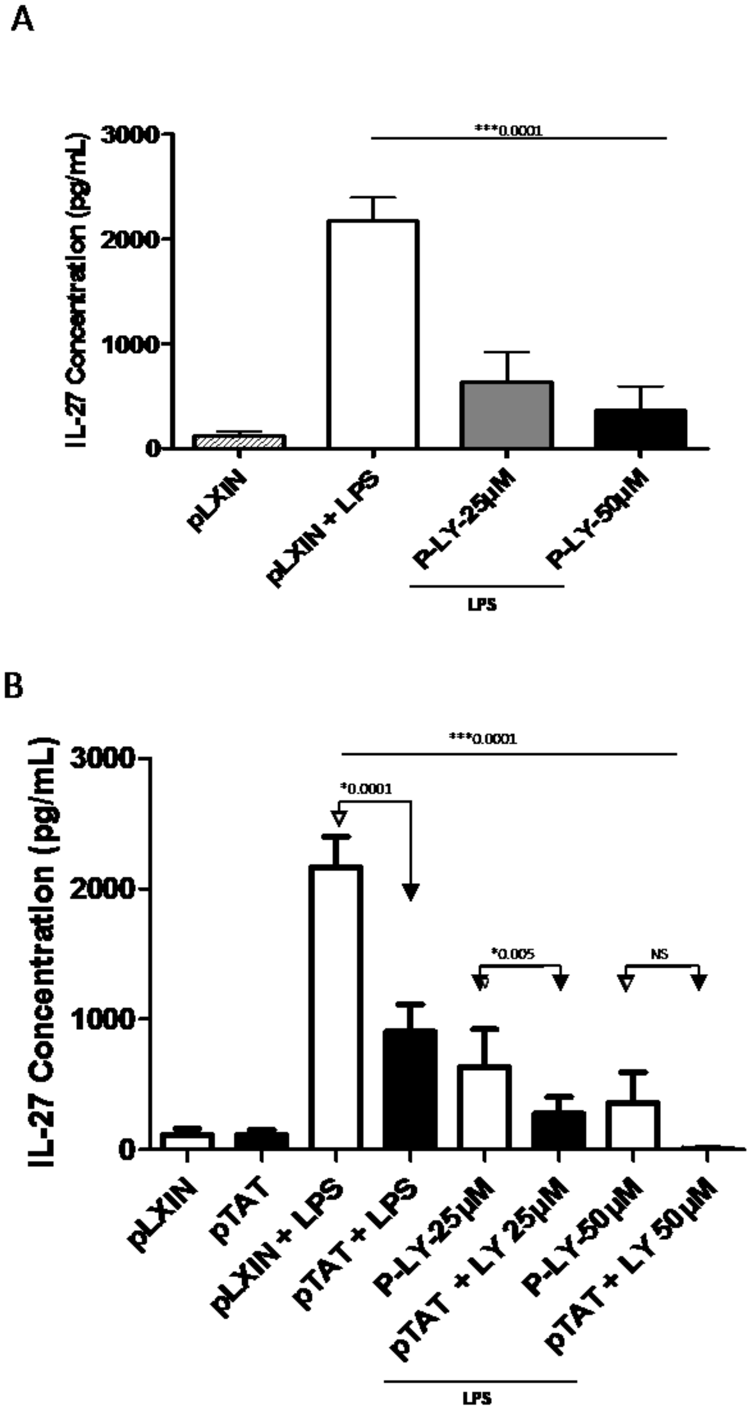

A
IL-27 Concentration (pg/mL)
3000
2000
1000
0
***0.0001
pLXIN
pLXIN + LPS
P-LY-25µM
P-LY-50µM
LPS
B
IL-27 Concentration (pg/mL)
3000
2000
1000
0
***0.0001
*0.0001
*0.005
NS
pLXIN
pTAT
pLXIN + LPS
pTAT + LPS
P-LY-25µM
pTAT + LY 25µM
P-LY-50µM
pTAT + LY 50µM
LPS

Figure 29

3.2.2.5 Although Src regulates LPS-induced IL-27 production, it does not regulate HIV-Tat-mediated inhibition of LPS-induced IL-27 production in MDMs

As mentioned earlier, TLR-4 activation triggers tyrosine phosphorylation of several PTKs including Src kinases. However, the role of Src kinases in LPS-induced IL-27 production in general and in particular MDMs is not known. Since HIV-Tat inhibited LPS-induced IL-27 expression, I hypothesized that HIV-Tat interferes with the TLR4-activated PTKs leading to inhibition of LPS-induced IL-27 production. For this, I used SU6656, the Src specific inhibitor. The biological activity of SU6656 was confirmed in Fig 24 A. To study the role of Src in LPS-induced IL-27 production, pLXIN-infected MDMs were treated with the SU6656 followed by LPS stimulation and analysis of IL-27 production. The results show that SU6656 significantly inhibited LPS-induced IL-27 secretion in a dose-dependent manner (Fig 30A). I have previously shown that LPS-stimulation increased Src phosphorylation in pLXIN-infected MDMs which was not significantly decreased in HIV-Tat-infected MDMs (Fig 24 C).

Since HIV-Tat does not inhibit LPS-induced Src phosphorylation and SU6656 positively regulates LPS-induced IL-27 production, I determined if Src inhibition mediated by SU6656 and HIV-Tat will co-operate to affect LPS-induced IL-27 production. For this, pLXIN or HIV-Tat-infected MDMs were stimulated with LPS in the presence or absence of SU6656. As expected, I observed ~60% inhibition of LPS-induced IL-27 production in HIV-Tat-infected MDMs compared to the control pLXIN-infected MDMs (Fig 30B). The treatment of pLXIN- infected MDMs with SU6656 at 2.5 and 5.0 μM concentrations reduced >75% LPS-induced IL-27 production compared to the untreated MDMs (Fig 30A). However, LPS-induced IL-27 production in pLXIN and HIV-Tat-infected MDMs in the presence of either 2.5 or 5.0 μM SU6656 was not found to be statistically different (Fig 30B). These results show that even though Src positively regulates LPS-induced IL-27 production, it does not impact HIV-Tat-mediated inhibition of LPS-induced IL-27 production in MDMs.

Figure 30: Although Src positively regulates LPS-induced IL-27 production, it does not affect HIV-Tat-mediated inhibition of LPS-induced IL-27 production

A. **Src regulates LPS-induced IL-27 production in MDMs.** pLXIN-infected MDMs were treated with SU6656 for 2 hr followed by 24 hr LPS stimulation. The supernatants were assayed for IL-27 production by ELISA. N=8 different donors.

B. **Src inhibitor does not affect HIV-Tat-mediated inhibition of LPS-induced IL-27 production.** pLXIN and Tat-infected MDMs were treated with SU6656 for 2 hr followed by LPS stimulation for 24 hr. The supernatants were assayed for IL-27 production by ELISA. N=8 different donors.

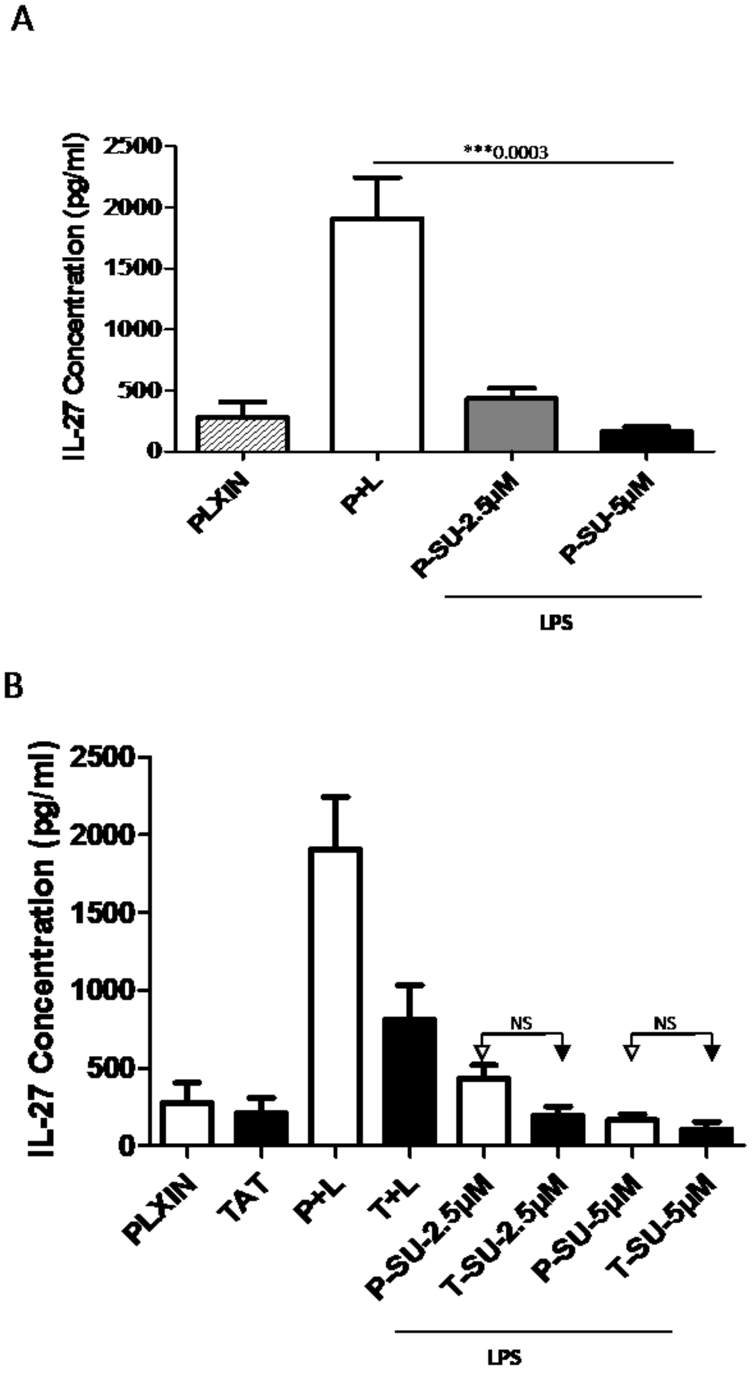

A
IL-27 Concentration (pg/ml)
2500
2000
1500
1000
500
0
***0.0003
PLXIN
P+L
P-SU-2.5µM
P-SU-5µM
LPS
B
IL-27 Concentration (pg/ml)
2500
2000
1500
1000
500
0
NS
NS
PLXIN
TAT
P+L
T+L
P-SU-2.5µM
T-SU-2.5µM
P-SU-5µM
T-SU-5µM
LPS

Figure 30

3.2.2.6 SHP-1 positively regulates LPS-induced IL-27 production, but it does not regulate HIV-Tat-mediated inhibition of LPS-induced IL-27 production in MDMs

As mentioned earlier, LPS induces the activation of phosphatases such as SHP-1(364). However, the role of SHP-1 in LPS-induced IL-27 production in general and in particular MDMs is not known. Since HIV-Tat inhibited LPS-induced IL-27 expression, I wished to understand the role of SHP-1 in LPS-induced IL-27 production in MDMs by employing sodium stibogluconate (SS), the SHP-1 specific inhibitor. The biological activity of SS was confirmed in Fig 25A. To study the involvement of SHP-1 in LPS-induced IL-27 production, pLXIN-infected MDMs were treated with SS followed by LPS stimulation and analysis of IL-27 production. The results show that SS significantly inhibited LPS-induced IL-27 production (Fig 31A). I have previously shown that HIV-Tat did not inhibit LPS-induced SHP-1 activation (Fig 25C).

Since HIV-Tat does not inhibit LPS-induced SHP-1 activation but SHP-1 regulates LPS-induced IL-27 production, it was of interest to determine if SHP-1 inhibition by SS and HIV-Tat will affect LPS-induced IL-27 production in MDMs. For this, pLXIN or HIV-Tat-infected MDMs were stimulated with LPS in the presence or the absence of SS. As expected HIV-Tat inhibited LPS-induced IL-27 production compared to the pLXIN-infected MDMs (Fig 31B). However, addition of SS to HIV-Tat-infected MDMs did not affect LPS-induced IL-27 production compared to the SS treated pLXIN-infected MDMs (Fig 31B). These results suggest that although SHP-1 regulates LPS-induced IL-27 production, it does not impact HIV-Tat-mediated inhibition of LPS-induced IL-27 production in MDMs.

In summary, HIV-Tat inhibits LPS-induced IL-27 production through inhibition of PI3K and p38 signaling pathways. Although HIV-Tat inhibits ERK and JNK MAPK activation, these kinases do not regulate HIV-Tat mediated inhibition of LPS-induced IL-27 production. Finally, although SHP-1and Src regulated LPS-induced IL-27 production, these kinases were not involved in HIV-Tat-mediated inhibition of LPS-induced IL-27 production in MDMs.

Figure 31: SHP-1 does not impact HIV-Tat mediated inhibition of LPS-induced IL-27 production in retrovirus infected MDMs

A. **SHP-1 positively regulates LPS-induced IL-27 production.** pLXIN-infected MDMs were treated with SS for 2 hr followed by 24 hr LPS stimulation. The supernatants were assayed for IL-27 production by ELISA. N=8 different donors.

B. **SHP-1 inhibitor does not affect HIV-Tat-mediated inhibition of LPS-induced IL-27 production.** SHP-1 inhibitor does not affect HIV-Tat-mediated inhibition of LPS-induced IL-27 production. pLXIN and Tat- infected MDMs were treated with SS for 2 hr followed by LPS stimulation for 24 hr. The supernatants were assayed for IL-27 production by ELISA. N=8 different donors.

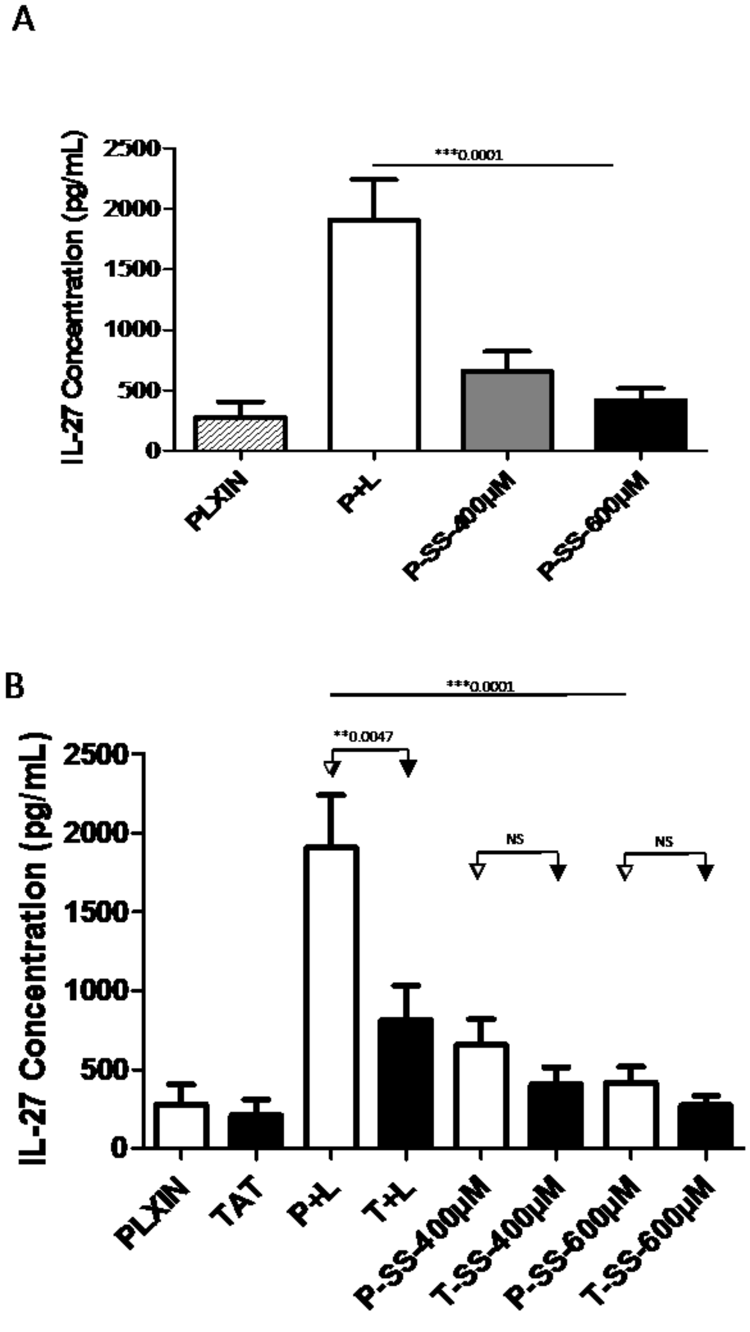

A
IL-27 Concentration (pg/mL)
2500
2000
1500
1000
500
0
***0.0001
PLXIN
P+L
P-SS-400µM
P-SS-600µM
B
IL-27 Concentration (pg/mL)
2500
2000
1500
1000
500
0
***0.0001
**0.0047
NS
NS
PLXIN
TAT
P+L
T+L
P-SS-400µM
T-SS-400µM
P-SS-600µM
T-SS-600µM

Figure 31

Objective 3: To examine whether HIV-Tat impacts upstream MyD88-dependent and MyD88-independent TLR4 signaling pathways in LPS-activated human macrophages

The above results show that HIV-Tat inhibits LPS-induced production of IL-23 and IL-27 through p38 MAPK and PI3K but not through the upstream Src and SHP-1 kinases. Since LPS stimulation recruits a series of adaptor molecules such as MyD88, IRAK4 and IRAK1 and consequently transduce the signal to TRAF-associated signaling complex (310–312) I hypothesized that HIV-Tat interferes not only with the downstream TLR4 signalling molecules but also with upstream signalling molecules leading to inhibition of LPS-induced IL-23 and IL-27 production. For this I stimulated pLXIN and HIV-Tat-infected MDMs with LPS (1 μg/ml) for 24 hr followed by analysis for the expression of IAPs, IRAKs, MyD88, and TRAF-1, 2, 3, 6 and TRIF by Western immunoblotting. The basal level of TRIF and TRAF were detected in both pLXIN and HIV-Tat-infected MDMs. LPS-stimulation increased levels of IRAK-1, IRAK-3, IAP-2 and TRAF-1; however, no change in their expression was observed in control pLXIN and HIV-Tat-infected MDM (Fig 32 and 33). Similar levels of TRAF-2, TRIF, TRAF-3, IRAK-4, MyD88, and IAP-1 were detected in unstimulated and stimulated pLXIN and HIV-Tat infected cells (Fig 32 and 33). Interestingly a reduced levels of TRAF-6 were detected in unstimulated as well as LPS-stimulated Tat retrovirus infected MDMs compared to the pLXIN-infected MDMs (Fig 35).

Taken together, these data suggests that HIV-Tat does not affect the expression of the MyD88-dependent and MyD88-independent pathway molecules including MyD88, IRAK1, IRAK3, IRAK4, TRAF-1, TRAF-2, cIAP-1, cIAP-2, xIAP and TRAF3 and TRIF. However, HIV-Tat reduced the expression of TRAF-6 in response to LPS stimulation in HIV-Tat-infected macrophages.

Figure 32: HIV-Tat does not affect expression of LPS-induced MyD88 independent TRIF, TRAF1 - 3, IRAK-1, and IRAK-3

HIV-Tat or pLXIN-infected MDMs were stimulated with LPS for 24 hr followed by Western blot. Cell lysates were analyzed for the expression of TRIF, TRAF1 - 3, IRAK-1 and IRAK-3 by Western immunoblotting. The images shown are representative for at least three experiments with similar results.

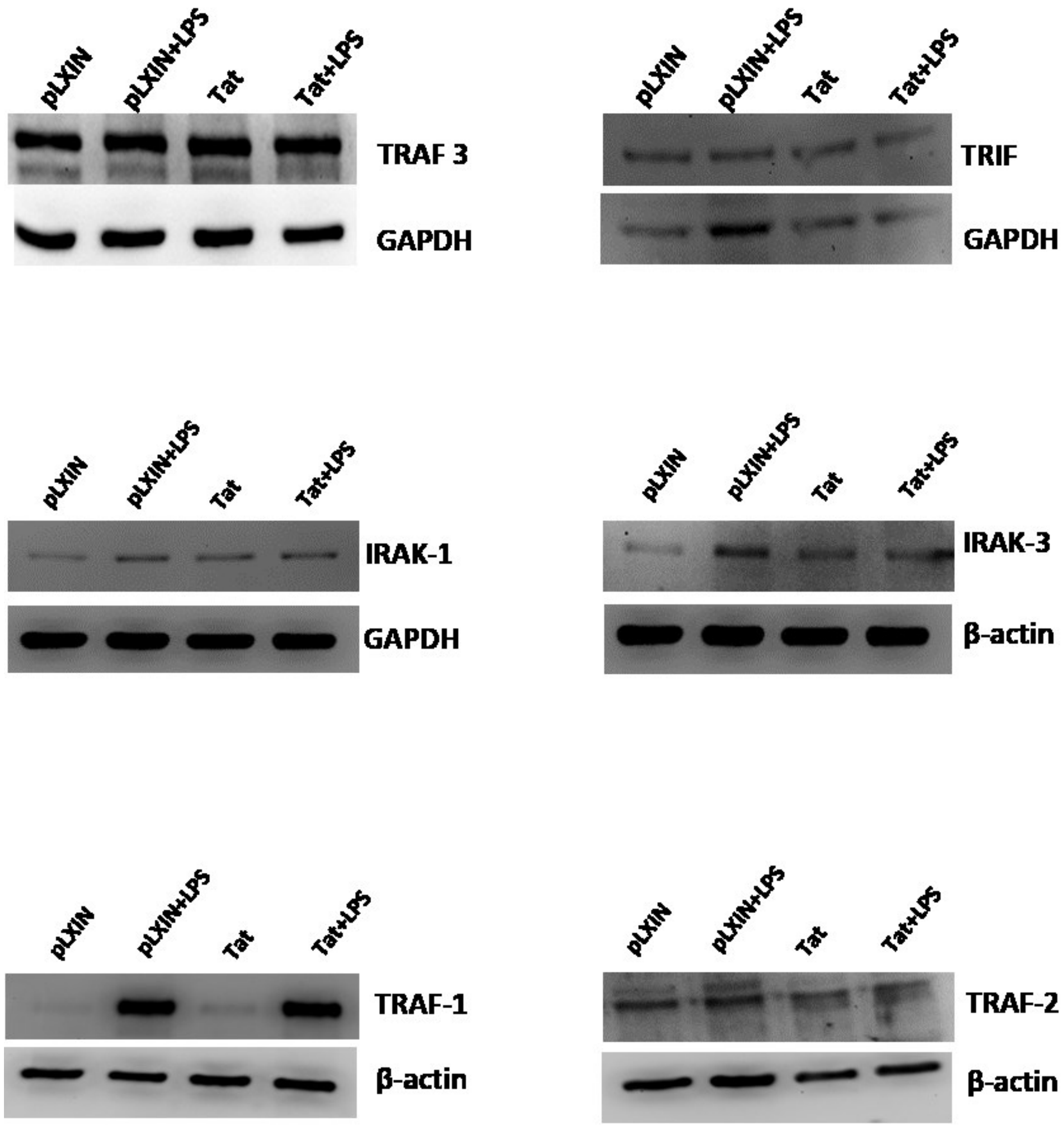

pLXIN
pLXIN+LPS
Tat
Tat+LPS
TRAF 3
GAPDH
pLXIN
pLXIN+LPS
Tat
Tat+LPS
TRIF
GAPDH
pLXIN
pLXIN+LPS
Tat
Tat+LPS
IRAK-1
GAPDH
pLXIN
pLXIN+LPS
Tat
Tat+LPS
IRAK-3
β-actin
pLXIN
pLXIN+LPS
Tat
Tat+LPS
TRAF-1
β-actin
pLXIN
pLXIN+LPS
Tat
Tat+LPS
TRAF-2
β-actin

Figure 32

Figure 33: HIV-Tat does not affect expression of LPS-induced IRAK-4, MyD88, cIAP1, cIAP2, and xIAP

HIV-Tat or pLXIN-infected MDMs were stimulated with LPS for 24 hr followed by Western blot. Cell lysates were analyzed for the expression of IRAK-4, MyD88, cIAP1, cIAP2, and xIAP by Western immunoblotting. The images shown are representative for at least three experiments with similar results.

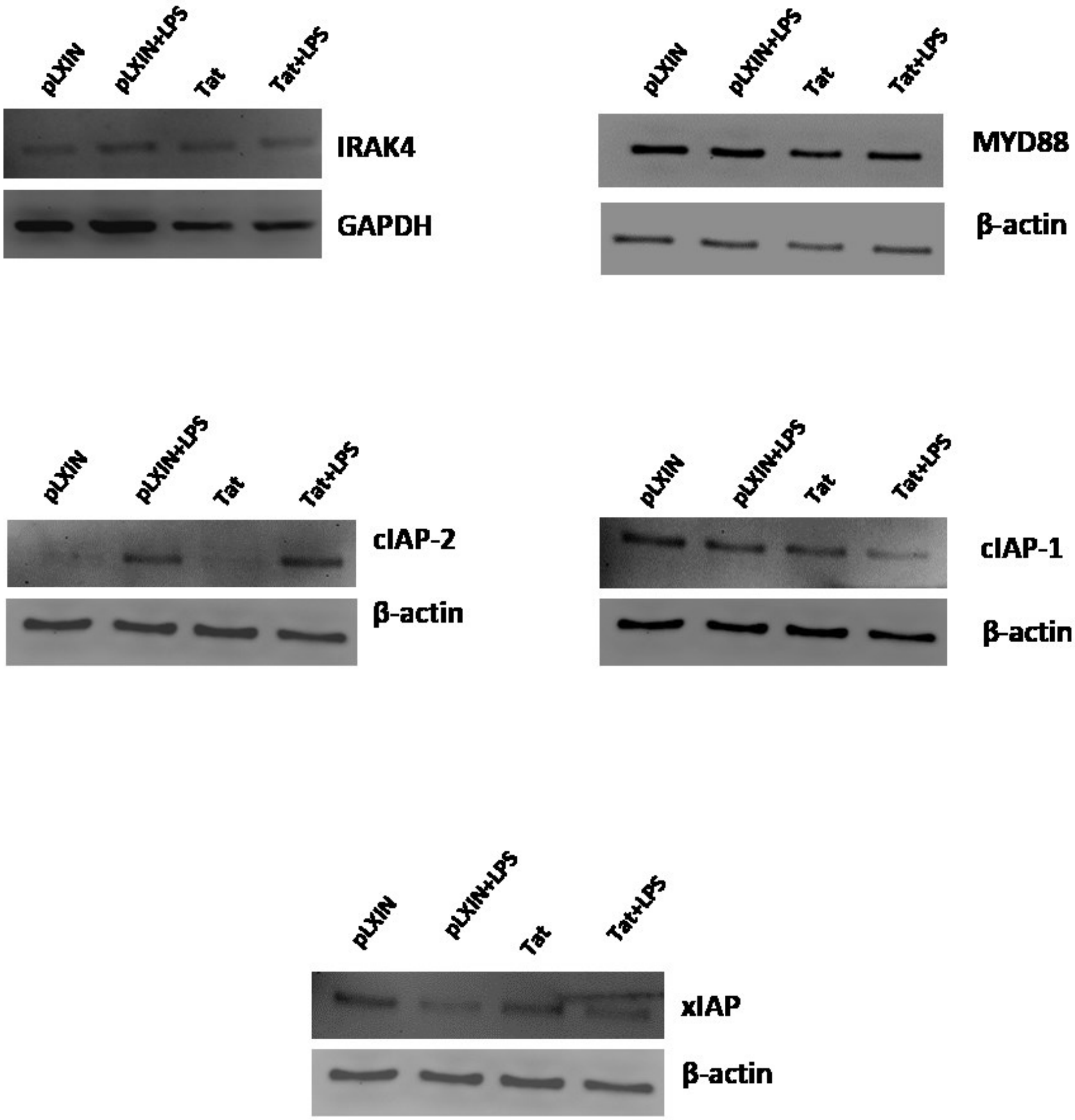

pLXIN
pLXIN+LPS
Tat
Tat+LPS
IRAK4
GAPDH
pLXIN
pLXIN+LPS
Tat
Tat+LPS
MYD88
β-actin
pLXIN
pLXIN+LPS
Tat
Tat+LPS
cIAP-2
β-actin
pLXIN
pLXIN+LPS
Tat
Tat+LPS
cIAP-1
β-actin
pLXIN
pLXIN+LPS
Tat
Tat+LPS
xIAP
β-actin

Figure 33

Figure 34: HIV-Tat impairs LPS-induced expression of TRAF-6 in retrovirus infected MDMs

pLXIN or HIV-Tat-infected MDMs were stimulated with LPS for 24hr and then cell lysates were analyzed for the expression of TRAF-6 by Western immunoblotting. The images shown are representative for at least three experiments with similar results.

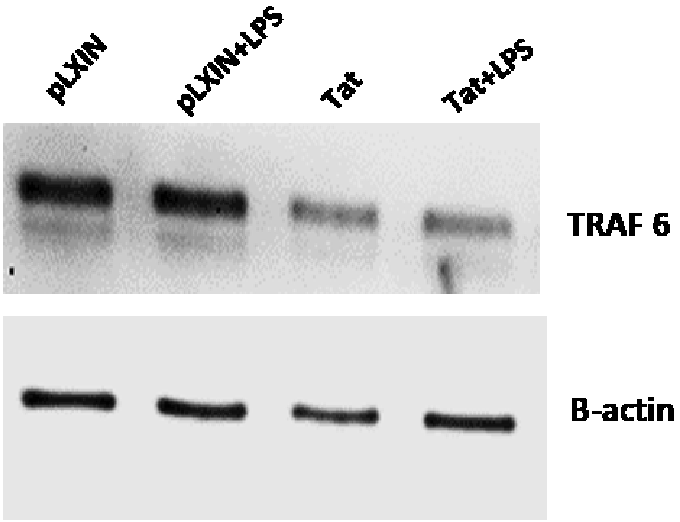


Figure 34

Chapter 4: Discussion

Macrophages are one of the first cells to become infected with HIV (313) and are resistant to the cytopathic effects of HIV (314). Macrophages are an important source of immunoregulatory cytokines such as IL-12 family of cytokines mainly following LPS stimulation. It is well established that macrophages from HIV-infected individuals and macrophages infected *in vitro* with HIV exhibit reduced ability to secrete various cytokines including IL-12 (137,148,152,156,160). Therefore, dysregulation of macrophage function in HIV infection is an important factor in the incomplete immune recovery in HIV infected individuals on HAART. While IL-12 family of cytokines are critical mediators of innate and adaptive immune responses (162), it is important to understand whether HIV or its regulatory proteins such as Tat influence other members of IL-12 family cytokines including IL-23 and IL-27. Our laboratory has previously shown that *in vitro* HIV infection of MDMs inhibited LPS-induced production of IL-23 and IL-27. Therefore, my focus of research was to investigate the effect of HIV-Tat on LPS-induced IL-23 and IL-27 production in human MDMs and its molecular mechanisms. I hypothesized that the HIV regulatory protein HIV-Tat may be involved in inhibiting LPS-induced IL-23 and IL-27 production.

Signalling pathways involved in HIV-Tat mediated inhibition of LPS-induced IL-27 production in MDMs:

To understand the signalling pathway by which HIV-Tat inhibited LPS-induced IL-27 expression I first demonstrated that LPS-induced IL-27 production is regulated by the PI3K, p38 MAPKs and SHP-1 and Src kinases in control retrovirus-infected MDMs. HIV-Tat potently inhibited p38 MAPK and PI3K which were implicated in HIV-Tat-mediated inhibition of LPS-induced IL-27 production. Although HIV-Tat inhibited ERK and JNK MAPK activation, these kinases were not involved in HIV-Tat-mediated inhibition of LPS-induced IL-27 production. While SHP-1 and Src kinases regulated LPS-induced IL-27 production, HIV-Tat did not inhibit these kinases and hence were not involved in HIV-Tat-mediated inhibition of LPS-induced IL-27 production. Furthermore, HIV-Tat did not inhibit the expression of upstream TLR4-activated signaling molecules including TRAF3, TRIF, MyD88, IRAK1, IRAK3, IRAK4, TRAF-1, TRAF-2, cIAP-1, cIAP-2 and, xIAP.

Decreased IL-27 production in HIV infection may influence HIV pathogenesis. IL-27 increases IL-12 receptor β1 subunit expression and synergistically enhances IL-12-induced IFN-γ production from Th1 cells (206). The capacity of DCs to cross-present HIV-antigens to CD8 T-cells is also associated with their ability to produce IL-27 (193). IL-27 has also been shown to inhibit HIV replication in macrophages and T cells (223,315). Therefore, HIV-mediated inhibition of IL-27 may decrease Th1 type cell-mediated immune responses and at the same time may result in increased HIV replication in infected individuals. There is considerable evidence that HIV-Tat can affect the physiology of antigen presenting cells (316–318). Thus it is possible that HIV-mediated inhibition of LPS-induced IL-27 production is regulated by HIV-Tat. My results demonstrate that lentivirally-encoded HIV-Tat significantly reduced LPS-induced IL-27 p28 and EBI3 mRNA expression and IL-27 secretion. This did not reflect a global inhibition of cytokine production as LPS-induced TNF-α secretion was unaffected by intracellular HIV-Tat, indicating a specific mechanism for Tat-mediated IL-27 suppression. Interestingly HIV-Tat infection did not appreciably increase IL-10 or TNF-α secretion in the absence of LPS stimulation, as described previously for recombinant Tat-treated monocytes (62,319,320). We have previously shown that intracellular HIV-Tat enhanced IL-10 production in primary human monocytes (89,90). However, in this study, I did not observed IL-10 upregulation in MDMs infected with HIV-Tat expressing lentiviruses. This discrepancy may be related to the different cell types used.

HIV-Tat is known to readily cross cell membranes (321) and the central basic domain has been implicated in mediating this transport and its subsequent translocation and accumulation in the nucleus (101). Although a peptide fragment corresponding to the basic domain of the full length-Tat peptide sequence strongly inhibited LPS-induced IL-27 expression other peptides which also contained the key basic sequence required for nuclear translocation showed little inhibitory activity, suggesting a key role for the sequences immediately flanking this region.

My results suggest that intracellular and extracellular HIV-Tat inhibit IL-27 expression by interfering with the TLR-4 signaling pathway. To understand how HIV-Tat inhibits LPS-induced IL-27 production, it is imperative to analyze the signaling pathways involved in the regulation of IL-27 production in human monocytic cells and in particular MDMs/macrophages. The signaling pathways regulating IL-27 production following LPS-stimulation of MDMs are not well understood. LPS-induced IL-27 mRNA in human astrocytes (291) and IFNγ/LPS-induced IL-27 production in myeloid DCs (284) was regulated by the p38 MAPK pathway. LPS/IFNγ-induced IL-27 production was; however, regulated by the JNK MAPK in myeloid DCs (284). These observations suggest that distinct signaling molecules may regulate IL-27 production depending on the cell type and the stimulus used. Therefore, I first demonstrated in the setting of human MDMs infected with control pLXIN retroviruses that LPS-induced IL-27 production was positively regulated by the PI3K, p38 MAPK, and upstream SHP-1 and Src kinases in control retrovirus-infected MDMs.

To understand the HIV-Tat impaired LPS-activated signaling pathways leading to the inhibition of LPS-induced IL-27 production, I have performed extensive analysis of LPS-activated signaling pathways inhibited by intracellular HIV-Tat in MDMs. Consistent with the findings of Yim et al 2009 (316), I observed inhibition of LPS-induced ERK phosphorylation in HIV-Tat-infected MDMs. In contrast to Yim's findings (316), I found that LPS-induced p38 and Akt phosphorylation was reduced in MDMs expressing intracellular HIV-Tat thereby implicating the contribution of p38 and PI3K towards Tat-mediated inhibition of LPS-induced IL-27 secretion. The reasons for the discrepancy of Tat-mediated effects on p38 and ERK MAPKs and PI3K in these two reports are not clear; however, it may relate to differences between treatment with exogenous Tat (Yim et al, 2009) and intracellular retroviruses expressing Tat used in this study. Additionally, the macrophage differentiation protocols used differed and this may have a polarizing effect on their response to HIV-Tat, as well as to LPS.

One of the mechanism involving in TLR4 signaling pathway is tyrosine phosphorylation, which helps to overturn phosphorylation of tyrosine residues to the key signaling proteins (322). The protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (SHP-1) maintain balance of tyrosine phosphorylation of various signaling molecules. The activity of SHP-1 and Src following LPS-stimulation is proximal in signal transduction (323). Thus it was of interest to investigate TLR4 upstream signal transduction pathways that may include SHP-1/Src activity in HIV-Tat mediated regulation of IL-27. It was shown that in murine bone-marrow derived macrophages, SHP-1 positively regulated LPS-induced IL-6 production (324). In addition, in activated microglia, Src kinase was demonstrated to induce TNF-α and IL-1β (325). In contrast, Meng et al.1997 showed that in triple knockout Hck/Fgr/Lyn murine macrophages, Src family kinases do not play a significant role in LPS-induced signaling (326). Additionally, it has been reported that SHP-1 and Src are involved in dendritic cell immunoreceptor (DCIR)-mediated signalling triggered by HIV (327). However, to the best of my knowledge, there is no report on the role of SHP-1 and Src regulation in LPS-induced IL-27 production. My data show that HIV-Tat did not inhibit Src phospharalation and SHP-1 activation. This suggested that SHP-1 and Src kinases regulate LPS-induced IL-27 production; however, HIV-Tat did not inhibit these kinases and hence, they were not involved in HIV-Tat-mediated inhibition of LPS-induced IL-27 production.

As HIV-Tat inhibited the phosphorylation of ERK and JNK MAPKs, I investigated the possibility that upstream mediators of the TLR4 signalling pathway may also be affected by Tat. When LPS binds to TLR4/CD14, a series of adaptor proteins are recruited including MyD88, IRAK4 and IRAK1. Surprisingly, my results show that the expression of most of the adaptor molecules linked to TLR4 signalling complex including MyD88, IRAK-4, IAP1/2, TRAF1, 2 and 3, SHP-1 and Src kinases were unaffected by intracellular HIV-Tat. These observations suggest that HIV-Tat specifically impaired the activity of an upstream signaling molecule involved in the activation of PI3K and /or MAPK pathway. The precise mechanism by which HIV-Tat impairs p38 and PI3K phosphorrlation remains to be investigated.

In one study MyD88-dependent signals, especially IRAK-1, MyD88 and NF-κB activation were shown to be reduced in alveolar macrophages from asymptomatic HIV-infected individuals while MyD88-independent signals including IFN regulatory factor-3 activation were unaffected (328). IL-27 EBI3 and p28 mRNA expression have both been demonstrated to be regulated by NF-κB (135,329). Thus, it is possible that HIV infection as well as HIV-Tat may inhibit LPS-induced IL-27 production through p38/PI3K-med iated decreased activation of NF-κB in MDMs.

The involvement of intracellular HIV-Tat or extracellular Tat on LPS-activated NFκB or its subunits is not known and needs to be investigated. It is also possible that IL-27 production or induction of IL-27 subunits p28 and EBI3 may involve transcription factors other than NFκB.

Therefore, the involvement of transcription factors involved in IL-27 p28 and EBI3 induction in LPS-stimulated MDMs by promoter analysis needs to be investigated. In addition, the inhibitory effect of Tat on such transcription factors involved in LPS-induced IL-27 p28 and EBI3 transcription in MDMs also remains to be investigated.

Signalling pathways involved in HIV-Tat mediated inhibition of LPS-induced IL-23 production in MDMs:

I have demonstrated that intracellular HIV-Tat and HIV-Tat peptides inhibited LPS-induced IL-23 production in MDMs. To study the signalling pathway by which HIV-Tat inhibited LPS-induced IL-23 expression, I first confirmed that LPS-induced IL-23 production is regulated by the PI3K, p38 MAPKs and SHP-1 in control retrovirus-infected MDMs. In contrast, Src kinases and JNK MAPK negatively regulated LPS-induced IL-23 production. HIV-Tat significantly inhibited p38 MAPK and PI3K which were implicated in HIV-Tat-mediated inhibition of LPS-induced IL-23 production. Even though HIV-Tat inhibited ERK and JNK MAPK activation, these kinases were not involved in HIV-Tat- mediated inhibition of LPS-induced IL-23 production. While SHP-1 regulated LPS-induced IL-23 production, HIV-Tat did not inhibit SHP-1 and therefore was not involved in HIV-Tat-mediated inhibition of LPS-induced IL-23 production. To the best of my knowledge, there is no other evidence to suggest HIV-Tat mediates inhibition of LPS-induced IL-23 production. However, there is only one study that demonstrated inhibition of IL-23 and decreased chronic inflammation and immune activation in HIV infection (189).

Shifawn O'Hara in our laboratory has previously shown that *in vitro* infection of MDMs with HIV significantly inhibited LPS-induced p19, IL-12p40, mRNA and IL-23 production in MDMs. There is evidence to suggest that HIV-Tat can affect the physiology of antigen presenting cells (316–318). Therefore, it is possible that HIV-mediated inhibition of LPS-induced IL-23 production is regulated by HIV-Tat. My results show for the first time that lentivirally-encoded HIV-Tat significantly inhibited LPS-induced p19, IL-12p40 mRNA transcription and IL-23 secretion in MDMs. LPS-induced TNF-α production was unaffected by intracellular HIV-Tat, indicating a specific mechanism for Tat-mediated IL-23 suppression. It has been shown that recombinant Tat-treated macrophages significantly increase IL-10 or TNF-α secretion in the absence of LPS stimulation (63,92) Interestingly; however, I did not see upregulation of IL-10 or TNF-α secretion in macrophages infected with HIV-Tat retroviruses. We have previously shown that intracellular HIV-Tat enhanced IL-10 production in primary human monocytes (89)(90). These discrepancies may relate to different cell types used in these studies.

My results also show that a peptide fragment corresponding to the basic domain of the full length-Tat peptide sequence (peptide #3) strongly inhibited LPS-induced IL-23 expression. Interestingly peptide # 1, 4 and 6 also inhibited LPS-induced IL-23 production albeit this inhibition was not dose-dependent and as dramatic as that observed with peptide #3 and could be non-specific. It is possible that in addition to the basic domain, other domains of Tat may be involved in the inhibition of LPS-induced IL-23 production. The precise mechanism by which the peptides #1, 3, 4 and 6 inhibit LPS-induced IL-23 production need to be further investigated.

Similar to the observations made with HIV-Tat mediated inhibition of LPS-induced IL-27 production, my results suggest that intracellular and extracellular HIV-Tat inhibited IL-23 production by interfering with the TLR-4 signaling pathway. To understand how HIV-Tat inhibits LPS-induced IL-23 production, I first investigated the signaling pathways involved in the regulation of IL-23 production in human MDMs/macrophages. The signalling pathways involved in IL-23 production in MDMs are poorly understood. IL-23 p19 expression has been shown to be positively regulated by the p38 MAPK in human microglial cells (281) and in macrophages (275) upon stimulation with *M. tuberculosis* and LPS. Additionally, IL-23 secretion was shown to be positively regulated by the p38 MAPK pathway in macrophages (275), human monocytes (282) and myeloid DCs (283,284). Since distinct signaling molecules may regulate IL-23 production depending on the cell type and the stimulus used, I therefore first demonstrated in the setting of human MDMs infected with control pLXIN retroviruses that LPS-induced IL-23 production was positively regulated by PI3K, p38 MAPK and upstream SHP-1 in control retrovirus-infected MDMs. Interestingly, LPS-induced IL-23 production in these cells was negatively regulated by Src kinase and JNK MAPK.

Protein tyrosine kinases (PTK) regulate a variety of cellular activities including cell growth, adhesion, differentiation and apoptosis (330). Src PTKs are composed of several conserved domains that include a myristoylated N-terminal tail, SH3 and SH2 domains, a tyrosine kinase domain and a short C-terminal tail (331–333). The Src family is comprised of nine members namely; Src, Fyn, Yes, Yrk, Blk, Fgr, Hck, Lck, and Lyn (334). Of the src kinases, Src, Fgr, Lyn and Hck are expressed in monocytes and macrophages (335). Hck and Lyn have been implicated in LPS signaling (336,337). Src kinase was shown to regulate LPS-induced TNF-α and IL-1β production in activated microglia. However, Hck-Fgr-Lyn triple knockout and normal littermate wild type murine macrophages responded similarly to LPS signaling (326) suggesting that Hck-Fgr-Lyn kinases may not play any role in LPS signaling. Therefore, it is possible that Src kinase may play a key role in LPS signaling. I have demonstrated that Src kinase negatively regulates LPS-induced IL-23 production whereas it positively regulates LPS-induced IL-27 production. The precise mechanism by which it differentially regulates IL-23 and IL-27 production in MDMs following LPS stimulation remains to be investigated. Since JNK MAPKs also negatively regulate

LPS-induced IL-23 production, it is possible that Src kinase activates a parallel Src-JNK pathway leading to a negative regulation of LPS-induced IL-23 production.

The protein tyrosine phosphatase (PTP), SH2 domain containing tyrosine phosphatase (SHP-1), is characterized by two tandem N-terminal SH2 domains (N-SH2 and C-SH2) – a single catalytic PTPase domain and a short C-terminal tail with two tyrosine and one serine phosphorylation sites (323,338). SHP-1 is primarily believed to be a negative regulator of signal transduction pathways in hematopoietic cells (339,340). For example, SHP-1 has been shown to negatively regulate the signalling pathways activated by T and B cell receptors (341,342) and several cytokine receptors such as CSF-1, IFN-γ, EPO, c-kit, IL-10 (343–347). Recently, SHP-1 was shown to positively regulate LPS-mediated IL-6 and IL-10 production in murine macrophages (348,349). However, the involvement of SHP-1 and PTKs in regulation of IL-12 family cytokines particularly murine as well as human IL-23 and IL-27 production remains poorly understood. Yulia Konarski in our laboratory has previously shown that LPS-induced IL-23 and IL-27 production in human monocytes and macrophages is positively regulated by SHP-1. In this study, I have demonstrated in the settings of control retrovirus infected human macrophages that LPS-induced IL-23 and IL-27 production is positively regulated by SHP-1 by employing SHP-1 specific inhibitor sodium stiboglucomate. The precise mechanism by which SHP-1 and Src interact leading to differential positive and negative induction of LPS-induced IL-27 and IL-23 production, respectively, in human MDMs needs to be further addressed.

The mechanism by which Src mediates LPS signalling has been studied. Lyn and Hck have been reported to be involved in LPS signalling. Lyn following association with CD14 is activated upon LPS-stimulation in macrophages (350). Src is present in an inactive state wherein Tyr527 in the c-terminus is phophorylated and binds the SH-2 domains. This interaction keeps src kinase in an inactive state. SHP-1 is known to interact with cytoplasmic tails of receptor chains and membrane associated proteins including Src (351). PTPs such as SHP-1 dephosphorylates Tyr527 and activates Src leading to the phosphorylation and activation of downstream signalling kinases (352). The Src kinase is known to phosphorylate and activate the focal adhesion proline-rich tyrosine kinase 2 (Pyk2) leading to IL-8 production in LPS-stimulated macrophages (353,354). Pyk2 interacts with Src-family kinases including Src and Fyn leading to downstream activation of MAPK and calcium-dependent signaling pathways (355–357).

My results also demonstrated that HIV-Tat impaired LPS-activated p38 and PI3K signalling pathways leading to the inhibition of LPS-induced IL-23 production. Yim et al 2009 demonstrated the effects of extracellular HIV-Tat protein (amino acid 1–86) in LPS-induced activation of MAPKs. Similar to my results, they showed that in human primary macrophages Tat peptide inhibited LPS-activated ERK1/2 phosphorylation. However, in contrast to my results, Yim et al did not observe inhibition of LPS-induced p38 MAPK activation by HIV-Tat peptide (316) . The reason for the different effect of HIV-Tat on p38, JNK and ERK MAPKs and PI3K in our studies and Yim et al is unclear; however, it may relate to differences between treatment with exogenous Tat (Yim et al 2009) and intracellular retroviruses expressing Tat used in this study. Additionally, the isolation and macrophage differentiation protocols used differed and this may have a polarizing effect on their response to HIV-Tat as well as to LPS.

As HIV-Tat inhibited the phosphorylation of ERK and JNK MAPKs, I investigated the possibility that upstream mediators of the TLR4 signalling pathway may also be affected by Tat. When LPS binds to TLR4/CD14, a series of adaptor proteins are recruited including MyD88, IRAK4 and IRAK1. Surprisingly our results show that the expression of most of the adaptor molecules linked to TLR4 signalling complex including MyD88, IRAK-4, IAP1/2, TRAF1, 2 and

3, SHP-1 and Src kinases were unaffected by intracellular HIV-Tat. These observations suggest that HIV-Tat specifically impaired the activity of an upstream signalling molecule involved in the activation of PI3K-MAPK pathway. (Fig 35)

In one study, MyD88-dependent signals, especially IRAK-1, MyD88, and NF-κB activation were shown to be reduced in alveolar macrophages from asymptomatic HIV-infected individuals while MyD88-independent signals including IFN regulatory factor-3 activation were unaffected (328). IL-12p40 and p19 mRNA expression have both been demonstrated to be regulated by NF-κB (135,329). Thus, it is possible that HIV infection as well as HIV-Tat may inhibit LPS-induced IL-23 production through decreased activation of NF-κB in MDMs. Further studies are needed to address the question of transcription factors involved in the regulation of IL-23p19, IL-23p40 transcription and IL-23 production. It may also be pertinent to determine how HIV-Tat will affect the transcription factors involved in the regulation of IL-23 and its subunits.

Decreased IL-23 production by human macrophages may influence HIV pathogenesis and disease progression during HIV infection. IL-23 has been reported to play an important role in the clearance of a range of pathogens such as *Toxoplasma gondii* and *M. tuberculosis,* (358,359), which are common co-infections in HIV (360). IL-23 is highly expressed in DCs and has been shown to prime CD8+ T cells against HIV antigens (193). Additionally, IL-23 inhibition has been shown to decrease chronic inflammation and immune activation in HIV infection (189). Therefore, inhibition of IL-23 in HIV infected human macrophages may lead to reduced priming of HIV-specific CD8+T-cells and thereby impaired immune responses. IL-23 has been shown to play a key role in the induction of Th17 cells and Th17 cytokines which are essential for removal of infection such as *Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeroginosa, Citrobacter rodentium, and Candida albicans,* recruitment of neutrophils, and maintenance of mucosal integrity of the gut (189). IL-23-induced IL-17 increases the integrity of epithelial barriers (190) and enhances antimicrobial peptide β-defensins expression in human airway epithelium (191) and keratinocytes (192). IL-23 is also known to induce IFN-γ production and activate memory T-cells (181). Moreover, Th17 cells were depleted in the gut associated lymphoid tissues of HIV-infected individuals and IL-17 was found to be higher in the gastrointestinal tract and blood of HIV-infected long term non-progressors (LTNP) and typical-progressors (TP) (194–196). Furthermore, HIV/SIV-infected individuals treated with combination antiretroviral therapy (cART) exhibited increased Th17 cell populations (197–199). Thus, decreased IL-23 production following TLR stimulation of macrophages during HIV infection may compromise the integrity of the intestinal barrier, enhance microbial translocation, reduce IFN-γ production by memory T cells and overall suppress the induction of HIV-mediated immune responses.

Figure 35: The Working Model for the HIV-Tat mediated inhibition of LPS-induced IL-23 and IL-27 production in human macrophages

HIV-Tat inhibits LPS-induced IL-23 and IL-27 production via inhibition of PI3K and p38. HIV-Tat also inhibits ERK and JNK activation; however, this does not reduce IL-23 and IL-27 production. While SHP-1 regulated LPS-induced IL-23 and IL-27 production, inhibit SHP-1 and therefore were not involved in HIV-Tat-mediated inhibition of LPS-induced IL-23 and IL-27 production. HIV-Tat did not inhibit Src kinases and hence were not involved in HIV-Tat-mediated inhibition of LPS-induced IL-27 production. HIV-Tat did not inhibit the expression of upstream TLR4-activated signaling molecules; however, HIV-Tat reduced the expression of TRAF 6.

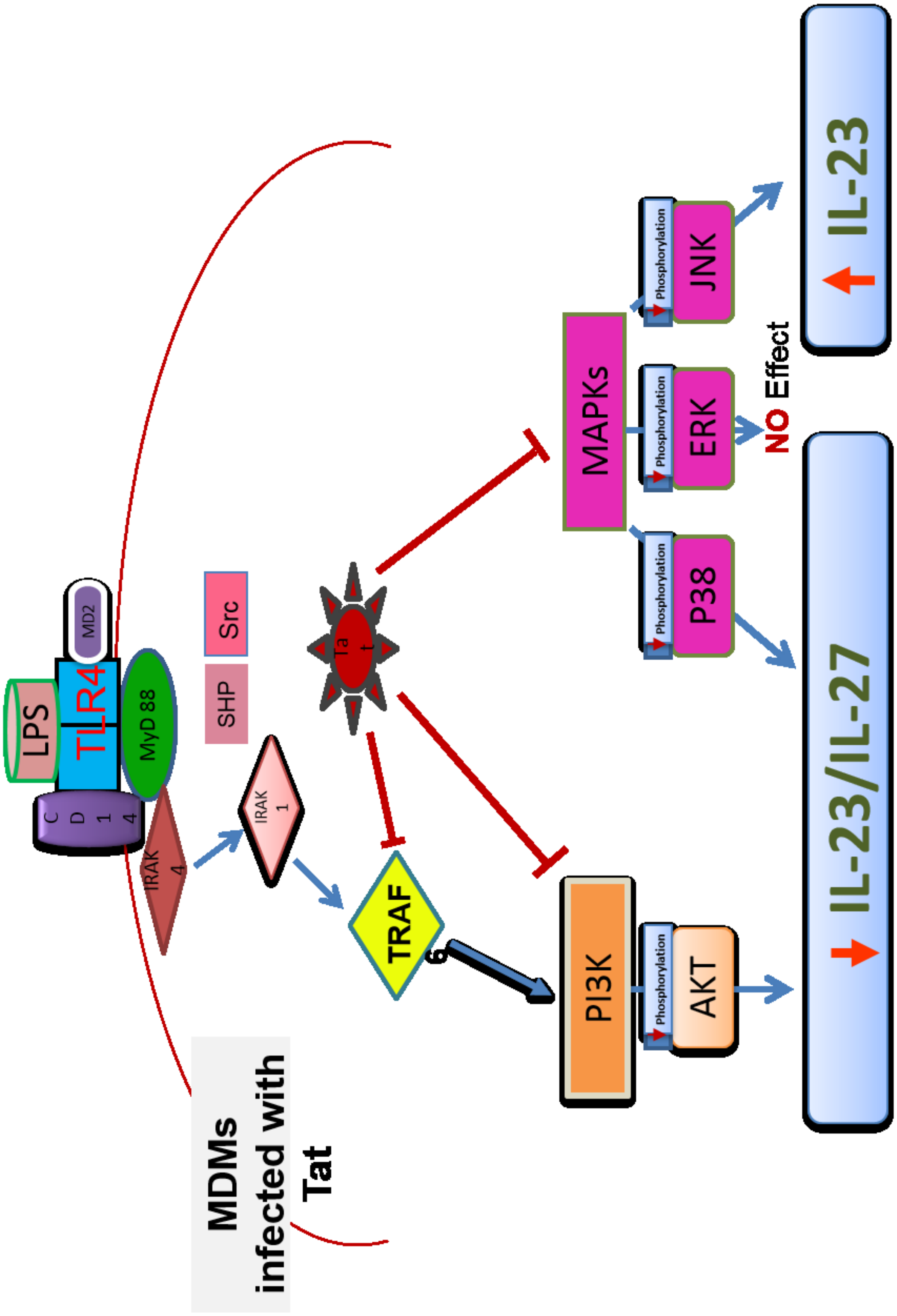

MDMs infected with Tat
LPS
TLR4
MD2
C D 1 4
MyD 88
IRAK 4
SHP
Src
IRAK 1
Tat
TRAF 6
PI3K
Phosphorylation
AKT
MAPKs
Phosphorylation
P38
Phosphorylation
ERK
Phosphorylation
JNK
NO Effect
IL-23/IL-27
IL-23

Figure 35

Chapter 5: Concluding Remarks and Future Studies

The focus of my research was to investigate the effect of HIV-Tat on LPS-induced IL-23 and IL-27 production in human MDMs and its molecular mechanisms. I investigated the effect of extracellular HIV-Tat peptides and intracellular HIV-Tat expression on LPS-induced expression of IL-23 and IL-27 and their subunits in MDMs. The results show intracellular HIV-Tat expression in human MDMs and exposure of MDMs to HIV-Tat peptide corresponding to the basic domain inhibited LPS-induced IL-23 and IL-27 proteins and their subunits. However, the effect of other HIV accessory proteins such as HIV Nef and Vpr on basal levels of IL-23 and IL-27 and LPS-induced IL-23 and IL-27 production remains to be understood.

I also determined the signalling pathways by which HIV-Tat regulates LPS-induced IL-23 and IL-27 expression in MDMs infected with control pLXIN retroviruses. The results show that p38 MAPK, SHP-1 and PI3K signalling molecules positively regulated LPS-induced IL-23 proteins and their subunits expression in MDMs. In contrast, Src kinases and JNK MAPK negatively regulated LPS-induced IL-23 production. It will be interesting to determine the precise mechanism by which Src kinases and JNK MAPKs negatively regulate LPS-induced IL-23 production. HIV-Tat significantly inhibited p38 MAPK and PI3K which were implicated in HIV-Tat-mediated inhibition of LPS-induced IL-23 production. Even though HIV-Tat inhibited ERK and JNK MAPK activation, these kinases were not involved in HIV-Tat-mediated inhibition of LPS-induced IL-23 production. The precise mechanism by which HIV-Tat impairs p38, ERK or JNK MAPK and PI3K phosphorylation is not known and needs to be investigated. Although basic domain of HIV-Tat was involved in inhibiting the phosphorylation of the above mentioned kinases, it is important to determine by mutational analysis the precise peptide fragment responsible for preventing their phosphorylation.

On the other hand, LPS-induced IL-27 production was positively regulated by the PI3K, p38 MAPKs and SHP-1 and Src kinases in control retrovirus-infected MDMs. Similar to IL-23, p38 MAPK and PI3K were implicated in HIV-Tat-mediated inhibition of LPS-induced IL-27 production. Although HIV-Tat inhibited ERK and JNK MAPK activation, these kinases were not involved in HIV-Tat-mediated inhibition of LPS-induced IL-27 production. While SHP-1 and Src kinases regulated LPS-induced IL-27 production, HIV-Tat did not inhibit these kinases and hence they were not involved in HIV-Tat-mediated inhibition of LPS-induced IL-27 production. Additionally, the precise mechanism by which SHP-1 and Src interact leading to differential positive and negative induction of LPS-induced IL-27 and IL-23 production, respectively, in human MDMs needs to be further addressed.

I also investigated the impact of HIV-Tat on the activation of upstream kinases involved in LPS-activated signalling pathway. The results show that HIV-Tat did not inhibit the expression of upstream TLR4-activated signaling molecules including TRAF3, TRIF, MyD88, IRAK1, IRAK3, IRAK4, TRAF-1, TRAF-2, cIAP-1, cIAP-2 and, xIAP. However, HIV-Tat did inhibit the activation of TRAF-6. It will be interesting to understand the role of TRAF-6 in IL-23 and IL-27 transcription and the precise mechanism by which HIV-Tat inhibits TRAF-6 activation.

I did not study the involvement of downstream transcription factors in the transcriptional regulation of IL-23 and IL-27 and their subunits. It will be of utmost importance to identify such transcription factors by promoter analysis and the impact of HIV-Tat on the activation of such transcription factors in IL-23 and IL-27 expression.

IL-23 and IL-27 play a critical role in the development and maintenance of protective cellular immune responses in infection with several intracellular pathogens including HIV/AIDS. IL-27 has been shown to inhibit HIV replication in several cell types including macrophages.

Decreased IL-23 and IL-27 production by human macrophages during HIV infection may influence HIV pathogenesis and disease progression by impaired HIV-specific immune responses on one hand and on the other hand may enhance HIV replication in macrophages. Thus, IL-23 and IL-27 inhibition by HIV and HIV-Tat in LPS-stimulated macrophages supports the hypothesis that IL-23 and/or IL-27 therapy may improve HIV disease outcome. During HIV-infection, restoration of IL-23 and IL-27 may decrease viral persistence through increased activation of HIV-specific T-cell responses. Thus, to improve HIV infection, future studies using IL-23 and/or IL-27 as a therapy should be conducted with the aim to improve the disease outcome.

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ACCOMPLISHMENTS:

- Sixteen years of professional experience in Molecular Immunology, Cellular Biology, Genetics and Biochemistry
- Cultured mammalian cells, human and animal origin, working with HIV-1 strains, HIV infected patient samples, In sterile technique and culturing and isolating pathogenic organisms
- Operational experience and knowledge in Confocal microscopy, flow cytometry, RealTime PCR, microArray
- Protein extraction, determining protein expression by western blot, ELISA, Flow cytometry
- Experience in genomic DNA/RNA extraction, transformation, siRNA transfection, cDNA synthesis, PCR and molecular cloning, quantifying mRNA expression levels using differential display PCR, Real Time PCR, Luciferase reporter assays and microarray
- Working with nanoparticles as a drug delivery system for HIV
- Experience in pharmaceutical drug testing
- Experience in animal studies and conducting research in peptide base HIV Vaccines
- Handled pathogenic and non pathogenic bacteria, filamentous fungus, and viruses in Level 2 and 3 containment facilities
- In-vitro toxicology testing:cell toxicity assay (alamarBlue), MTT assay
- Extensive experience in conducting scientific literature searches, scientific data, data presentation, data organization, and analysis
- Very good team player, having extensive teamwork experience in multicultural laboratory settings
- Strong organizational skills, with an ability to work in a multitask environment (handled multiple parallel drug development projects)
- Proven ability to achieve immediate and long-term goals and to meet operational deadlines

EDUCATION:

M.Sc. Cellular and Molecular Medicine — 2013 - 2015
University of Ottawa — Ottawa, Ontario

B.Sc. - Microbiology and Chemistry
University of Keleniya — Sri Lanka

CERTIFICATION:

1. Laboratory Safety
2. Bio-safety
3. Bio Safety Level III
4. Radiation Safety
5. Transportation of Dangerous Goods
6. Animal care
7.

WORK & RESEARCH EXPERIENCE:

Research Assistant — October – 2014 – Present

CHEO Research Institute — Ottawa, Ontario

NIRANJALA GAJANAYA

Research Assistant April 2012 –Dec 2012

University of Ottawa Ottawa, Ontario

- Vascular remodeling mechanisms during stroke recovery and in the pathogenesis of brain small vessel disease (in a laboratory rat model).

Research Technician May 2011 – March 2012

Public Health Agency of Canada Ottawa, Ontario

- Investigated new Pharmaceutical compounds against HIV
- Tested nanoparticles as a drug delivery system for HIV
- *In-vitro* toxicology testing, reviewing and synthesizing toxicological information

Research Assistant March – 2004 – April 2011

CHEO Research Institute Ottawa, Ontario

Key projects involved:

- Six years of research consisted of evaluating the role of the cell signaling pathway to the pharmaceutical drugs in different cells and its molecular mechanism. It involved genomics and proteomics methodologies, conducting, designing, and analyzing scientific data
- Worked within multi-disciplinary team, the research project involved testing a mucosal vaccine against HIV using cutting edge bioinformatics and mucosal vaccine delivery technology. Administrating vaccine intramuscularly, subcutaneous, Intraperitoneal, collection of sample of blood, saliva, stool, spleen, and intraepithelial lymphocytes
- Investigated the roles of HIV infection in regulation of IL-23 and IL-27 in LPS-induced primary human macrophages by cell signaling pathways and its molecular mechanisms
- Identified the inductive effects of an HIV protein called Tat on promoting IL-10 synthesis at the transcriptional level as well as the signaling pathways required to produce IL-10 in human monocytes

Research Technician June 2001 – Feb 2004

University of Ottawa, Faculty of Medicine Ottawa, Ontario

Evaluated efficacy determination of germicide activities using pathogenic organism:

- Determination of bactericidal, mycobactericidal, fungicidal and virucidal activities of the product for investigation of its effectiveness as a sanitizer using a suspension test method. The assays were conducted using different types of bacteria, spore bacteria, *Mycobacterium*, filamentous fungus and viruses

NIRANJALA GAJANAYA

Research Technician June 1997 - Dec 1997

Agriculture Canada

Ottawa, Ontario

- bacterial and fungal cultures, using long-term maintenance and preservation methods
- Experienced in methods of sterilization and aseptic techniques

EXTRA-CURRICULAR ACTIVITIES:

- Key main organizer Sri Lanka Sport Club-2014-2015
- Organizer badminton 2013 - Sri Lanka Badminton Club
- Captain Netball team - 2010-2013 Sri Lanka Canada Association
- Captain ladies cricket team - 2010- 2012 Sri Lanka Canada Association
- A team player - women badminton since 2005

SELECTED INTERNATIONAL JORNAL PUBLICATIONS:

- 1. **Niranjala Gajanayaka**, Shifawn O'Hara, Jason Fernandes, †, Kar Muthumani, Maya Kozlowski, Jonathan B. Angel†,¶ and Ashok Kumar, HIV-Tat inhibits LPS-induced IL-27 production through decreased activation of the p38 MAPK and PI3K pathways in human macrophages. Manuscript under preparation

- 2. **Niranjala Gajanayaka**, Shifawn O'Hara, Jason Fernandes, Kar Muthumani, Maya Kozlowski, Jonathan B. Angel,¶ and Ashok Kumar, HIV infection, intracellular and extracellular HIV Tat inhibit LPS-induced IL-27 production through decreased activation of the p38 and JNK MAPK and PI3K pathways in human macrophages- Manuscript under preparation

- 3. Lavigne, Carole; Slater, Kathryn; **Gajanayaka, Niranjala**; Duguay, Christian; Arnau Peyrotte, Erika; Fortier, Germaine; Simard, Martin; Kell, Arnold J; Barnes, Michael L; Thierry, Alain R, Influence of lipoplex surface charge on siRNA delivery: application to the *in vitro* downregulation of CXCR4 HIV-1 co-receptor. Expert Opinion on Biological Therapy, Volume 13, Number 7, September 2013 , pp. 973-985(13)

- 4. Maria A. Blahoianu, Ali A.R. Rahimi, **Niranjala Gajanayaka**, Maya Kozlowski, Jonathan B. Angel, Ashok Kumar, Engagement of CD14 Sensitizes Primary Monocytes to IFN-γ to Produce IL-12/23p40 and IL-23 Through p38 Mitogen-Activated Protein Kinase and Independent of the Janus Kinase/Signal Transducers and Activators of Transcription Signaling, Journal of Interferon & Cytokine Research, August 2013, 33 (8):434-445

- 5. Viraj J. Jasinghe, Erika Arnau Peyrotte, Adrienne F.A. Meyers, **Niranjala Gajanayaka**, Terry B. Ball, Paul Sandstrom, and Carole Lavigne. Human rElafin Inhibits HIV-1 Replication in Its Natural Target Cells. BioResearch Open Access Volume 2, Number 2, April 2013, DOI: 10.1089/biores.2012.027

- 6. Jonathan G. Boucher, Kelley A. Parato, Fiona Frappier, Peter Fairman, Aurelia Busca, Mansi Saxena, Maria A. Blahoianu, Wei Ma, **Niranjala Gajanayaka**, Robin J. Parks, Ashok Kumar, and Jonathan B. Angel. Disparate Regulation of LPS-Induced MAPK Signaling and IL-12p40 Expression Between Different Myeloid Cell Types with and without HIV Infection. *Viral Immunology*: 23(1), 17-28 (2010), doi:10.1089/vim.2009.0054

- 7. Blahoianu, Maria A. , Rahimi, Ali A. , Boucher, Jonathan, **Gajanayaka, Niranjala**, Angel, Jonathan B., Kumar Ashok. IFN-γ induces IL-23 expression in primary human monocytes via the p38 mitogen activated protein kinase independently of the JAK/STAT signalling Cytokine. ISSN: 10434666, 48, 99-100 (2009)

- 8. Gee, K., J.B. Angel, W. Ma, S. Mishra, Niranjala **Gajanayaka**, K. Parato, and A. Kumar. Intracellular HIV-tat expression induces IL-10 synthesis by serine 133 phosphorylation of the CREB-1 transcription factor through the activation of extracellular signal-related kinase 1/2 in human monocytic cells. *Journal of Biological Chemistry:* 281(42), 31647-58 (2006)

SELECTED INTERNATIONAL CONFERENCE ABSTRACT PRESENTATIONS:

- 1**. Niranjala Gajanayaka** , Jonathan B. Angel[†,¶] and Ashok Kumar

  HIV inhibits LPS-induced IL-27 production via HIV tat through the inhibition of TRAF-6, and consequent inhibition of PI3K and p38 and JNK MAPKs in human macrophages
  24th Annual Canadian Conference on HIV/AIDS Research – CAHR 2015, Toronto Ontario, Canada, Abstract BS27, April 30-May 3, 2015.

- 2. **Niranjala Gajanayaka,** Shifawn O'Hara, Jonathan B. Angel and Ashok Kumar
  HIV-Tat inhibits LPS-induced IL-27 production through decreased activation of the p38 MAPK and PI3K pathways in human macrophages
  CanCURE Annual Meeting November 20-21- 2014 Montréal (QC)

- 3. **Niranjala Gajanayaka**, Shifawn O'Hara, Maria Blahoianu, Maya Kozlowski, Jonathan B. Angel and Ashok Kumar
  HIV infection and intracellular HIV-Tat inhibit LPS-induced IL-23 and IL-27 production through decreased activation of the p38 and JNK MAPK and PI3K pathways in human macrophages

NIRANJALA GAJANAYA

20th international AIDS conference 20th - 25th July 2014 Melbourne Australia

- 4. Renée Spencer, **Niranjala Gajayanaka**, Sandra Stals, Carole Lavigne, Development of a cellular model for human prion surexpression, Poster, La Cité Collégiale, Ottawa, ON, April 27, 2012.

- 5. Nicolas Moisan, Arnold Kell, **Niranjala Gajayanaka**, Michael Barnes, and Carole Lavigne, Development and characterization of a novel PLGA-based nanosystem to transport siRNAs, Poster, La Cité Collégiale, Ottawa, ON, April 27, 2012.

- 6. Carole Lavigne, Arnold J. Kell, Alain R. Thierry, Kathryn Slater, Michael Barnes, Viraj J. Jasinghe, **Niranjala Gajanayaka**, Sandra Stals, SiRNA delivery using nanosystems to prevent HIV infection, 21st Annual Canadian Conference on HIV/AIDS Research – CAHR 2012, Montreal, QC, Canada, Abstract P003, April 19-22, 2012.

- 7. Carole Lavigne, Alain R. Thierry, Kathryn Slater, Viraj J. Jasinghe, **Niranjala Gajanayaka**, Arnold J. Kell, Michael Barnes, Sandra Stals, Interfering with HIV infection *in vitro* using siRNA delivered by a Neutraplex nanoformulation**,** Keystone Symposia on Molecular and Cellular Biology, Cell Biology of Virus Entry, Replication and Pathogenesis, Whistler, BC, Canada, Abstract 239, March 20-25, 2012.

- 8. Carole Lavigne, Arnold J, Kell, Alain R. Thierry, Michael L. Barnes, **Niranjala Gajanayaka**, Kathryn Slater, Nanoparticle-Mediated Delivery of siRNA targeting the HIV-1 Coreceptor CXCR4 in HeLa-Derived Cells. International Conference and Exhibition on Nanomedicine & Nanotechnology – Nanomedicine-2011 held July 27 – July 29, 2011 at University of Ottawa, Canada.

- 9. **Niranjala Gajanayaka**, Shifawn O'Hara, Ali A.R. Rahimi, Maria Blahoianu, Jonathan G. BoucherJonathan B. Angel, and Ashok Kumar 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. 18th Conference on Retroviruses and Opportunistic Infections (CROI 2011) held February 27-March 2, 2011 at Hynes Convention Center in Boston, MA

- 10. **Niranjala Gajanayaka**, Shifawn O'Hara, Jonathan B,. Angel, and Ashok Kumar, 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. OHTN Toronto ON 2010

- 11. Maria A Blahoianu, Ali A Rahimi, Jonathan,G Boucher, **Niranjala Gajanayaka**, Jonathan B Angel and Ashok Kumar. IFN-g Induces IL-23 Expression in primary human monocytes via the P38 MAPKS indepently of the JAK/STAT signaling. Tri society annual conference LISBON – 2009 Cellular and Cytokine Interactions in Health and Disease

- 12. Ali AR Rahimi**; Niranjala Gajanayaka;** Katrina Gee**;** Ashok Kumar. Regulation of the IL-12 Family Cytokines IL-23 and IL-27 in Response to HIV of Human Monocytic Cells. XVII International AIDS Conference Mexico City, Mexico, August 3-8, 2008

- 13. Ali A.R. Rahimi, **Niranjala Gajanayaka**, Jyoti Mishra, Ashok Kumar. P13K and P38 MAPK differentially regulate expression of IL-12 family cytokines IL-23 and IL-27 in LPS-induced Human Monocytic cells. Ontario HIV Treatment Network (OHTN) November 19 and 20, 2007 Toronto, Ontario, Canada

- 14. A. Alhetheel1, A. Benoit, K. Abdkader, D. Sirskyj, Y. Yakubtsov **Niranjala Gajanayaka**, and M. Kryworuchko Disruption of Cytokine Responsiveness by HIV: Molecular Mechanisms and Role in PathogenesisCIHR Institute of Infection and Immunity - New Investigator Forum in Toronto, April 14,2000