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**HIV and the Multifaceted Deregulation of Interleukin-12 Through Vpr-Gr, CD14, TLR4 expression
and SAPK Signalling**

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HIV and the multifaceted deregulation of interleukin-12 through
Vpr-GR, CD14, TLR4 expression and SAPK signalling

By

Fiona M. Frappier

THESIS
Submitted to the School of Graduate Studies in partial fulfillment of the requirements
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Department of Biochemistry, Microbiology & Immunology
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ABSTRACT

Infection with HIV is characterized by a progressive loss of cell mediated immunity and altered cytokine production. The decreased production of the cytokine IL-12 is pivotal in the multi-faceted immune pathogenesis of HIV infection. The objective of this study was to understand the mechanisms of IL-12 production and HIV-mediated inhibition of IL-12 in primary human monocytes. The HIV-mediated effect on LPS-induced IL-12 p40 was investigated through the evaluation of 1) the stress activated protein kinase (SAPK) pathway, 2) HIV-Vpr interactions with the glucocorticoid receptor (GR), and 3) the expression of LPS receptors CD14 and TLR4. Our results indicate that HIV may utilize the SAPK pathway through p38 and JNK to inhibit LPS-induced IL-12 p40 nuclear factors Sp-1, Ets-2, NFκB, and AP-1 binding to the promoter, leading to decreased promoter activity, mRNA expression and IL-12 p40 protein production. HIV viral tropism, extracellular Vpr, and intracellular Vpr expressed through a retroviral system were further evaluated for their effects on upstream CD14, TLR4 and GR expression in the presence and absence of LPS stimulation. *In vitro* HIV infection resulted in an increase in CD14 expression and a decrease in TLR4 expression both with and without LPS stimulation, while there was no effect on GR expression. The C-terminal Vpr peptide containing the GR binding motif inhibited LPS-induced IL-12 p40 in a GR dependent manner. Vpr further enhanced the GR ligand, dexamethasone, mediated inhibition of IL-12 p40 production. The addition of extracellular Vpr resulted in increased CD14 and GR expression and decreased TLR4 expression following LPS stimulation. Intracellular expression of Vpr inhibited LPS-induced IL-12 p40 and TLR4 receptor expression in a GR dependent

mechanism. Furthermore, intracellular expression of Vpr inhibited the CD14 receptor response to LPS while having no effect on GR. The receptor expression and response to intracellular Vpr expression were different in monocytes cultured within PBMC and monocytes cultured in isolation. Overall this study furthers our understanding of HIV-mediated inhibition of IL-12 p40 and highlights a new potential therapeutic target for development to restore IL-12 p40 by targeting the GR binding motif of Vpr.

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DEDICATION

This thesis is dedicated to the memory of Dennis Frappier, who encouraged me to pursue a career in science and inspired me to be the best that I can be.

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LIST OF ABBREVIATIONS

| | |
|-------|------------------------------------|
| Ab | Antibody |
| ACTH | Andrenocorticotropic hormone |
| ADP | Adenosine diphosphate |
| AIDS | Acquired immunodeficiency syndrome |
| ANOVA | Analysis of variance |
| AP-1 | Activator protein 1 |
| APC | Antigen presenting cell |
| ATP | Adenine triphosphate |
| BCA | Bicinchoninic acid |
| BSA | Bovine serum albumin |
| Bp | Base pair |
| CCR5 | Chemokine (C-C-motif) receptor 5 |
| CD | Cluster of differentiation |
| cDNA | Complementary DNA |
| C/EBP | CCAAT enhancer binding protein |
| CMI | Cell mediated immunity |
| CNS | Central nervous system |
| CRH | Corticotropin-releasing hormone |
| CTL | Cytotoxic T lymphocyte |
| CXCR4 | Chemokine (C-X-C motif) receptor 4 |
| DC | Dendritic Cell |
| DMSO | Dimethyl sulfoxide |

| | |
|------------------|--|
| DNA | Deoxyribonucleic acid |
| ECL | Enhanced Chemiluminescence |
| ELISA | Enzyme-linked immunosorbent assay |
| EMSA | Electrophoretic mobility shift assay |
| ERK | Extracellular-related kinase |
| ERK ₁ | ERK inhibitor, PD98059 |
| FCS | Fetal calf serum |
| FITC | Fluorescein isothiocyanate |
| Ga-12 | GATA sequence in the IL-12 promoter |
| GALV | Gibbon ape leukemia virus |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| GC | Glucocorticoid |
| GM-CSF | Granulocyte macrophage colony stimulating factor |
| GR | Glucocorticoid receptor |
| GRE | Glucocorticoid response element |
| HAART | Highly active antiretroviral therapy |
| HIV | Human immunodeficiency virus |
| HPA | Hypothalamic pituitary adrenal |
| HPLC | High performance liquid chromatography |
| HRP | Horseradish peroxidase |
| Hsp | Heat shock protein |
| ICSBP | Interferon consensus sequence binding protein |
| IFN | Interferon |

| | |
|------------------|--|
| Ig | Immunoglobulin |
| IKK | I κ B kinase |
| IL | Interleukin |
| IRAK | IL-1 receptor associated kinase |
| IRF | Interferon regulatory factor |
| Jak | Janus associated kinase |
| JNK | c-jun N-terminal kinase |
| JNK _i | JNK inhibitor, SP600125 |
| Kb | Kilobase |
| LBP | LPS binding protein |
| LOS | Lipooligosaccharide |
| LPS | Lipopolysaccharide |
| LTA | Lipoteichoic acid |
| LTR | Long terminal repeat |
| mAb | Monoclonal antibody |
| MAPK | Mitogen activated protein kinase |
| MFI | Mean fluorescent intensity |
| MDM | Monocyte-derived macrophages |
| MEK | MAP kinase kinase |
| MHC | Major histocompatibility complex |
| MIP | Macrophage inflammatory protein |
| MKP-1 | Mitogen-activated protein kinase phosphatase-1 |
| MO | Monocyte |

| | |
|------------------|---|
| MOI | Multiplicity of infection |
| NARTI | Nucleoside analog reverse transcriptase inhibitor |
| NF | Nuclear factor |
| NF κ B | Nuclear factor κ B |
| NIH | National Institute of Health |
| NK | Natural killer |
| NNRTI | Non-nucleoside reverse transcription inhibitor |
| NRE | Negative regulatory element |
| NtART | Nucleotide analog reverse transcription inhibitor |
| OPD | Orthophenylenediamine-HCl |
| p38 _i | P38 inhibitor, SB 203580 |
| PAGE | Polyacrylamide gel electrophoresis |
| PAMP | Pathogen-associated molecular pattern |
| PARP | Poly-(ADP-ribose)-polymerase |
| PBMC | Peripheral blood mononuclear cell |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PE | Phycoerythrin |
| PHA | Phytohemagglutinin |
| PI3K | Phosphatidylinositol 3 kinase |
| PIC | Preintegration complex |
| PKC | Protein kinase C |
| qPCR | Quantitative polymerase chain reaction |

| | |
|--------------------|--|
| RLU | Relative luciferase units |
| RNA | Ribonucleic acid |
| RT-PCR | Reverse transcription-polymerase chain reaction |
| SAPK | Stress activated protein kinase |
| SH2 | Src-homology 2 domain |
| SIV | Simian immunodeficiency virus |
| Stat | Signal transducer and activator of transcription |
| TB | Tuberculosis |
| TCID ₅₀ | Tissue culture infectious dose 50 |
| TGF | Transforming growth factor |
| Th1 | Type 1 T helper cells |
| Th2 | Type 2 T helper cells |
| TIR | Toll/IL-1 receptor |
| TLR | Toll-like receptor |
| TMB | Tetramethylbenzidine |
| TNF | Tumor necrosis factor |
| TRAF6 | TNF receptor associated factor 6 |
| WHO | World Health Organisation |

I - INTRODUCTION

A. HIV Overview

Infection with human immunodeficiency virus (HIV) is one of the most serious pandemics of the 21st century. Currently, 39.5 million people are estimated to be living with HIV and 22 million people have died since monitoring began in 1981 (UNAIDS, WHO). An unprecedented effort has been made by international scientific and health care communities to address the societal issues caused by this virus. Great strides have been made in the understanding of the virus, its epidemiology, prevalence and spread of infection. The World Health Organization (WHO), in collaboration with many national public health agencies, has mapped the prevalence of infection in nearly all the countries on the globe (Figure 1). Addressing the spread of this virus has, and continues, to require international collaboration among health care professionals, scientists, community workers and politicians (1). The virus was identified by Montagnier and Barré-Sinoussi in 1983 for which they won the Nobel Prize in Medicine in 2008 (2, 3). HIV-1 is a single-stranded, positive sense, enveloped RNA retrovirus from the Lentivirus genus. Since its identification, there has been great progress in treatment options available.

1. Drug classes currently available

The first class of therapeutic drugs discovered were nucleoside analogue reverse transcriptase inhibitors (NARTI) (4) and nucleotide analogue reverse transcription inhibitors (NtART) (5). These are competitive substrate inhibitors, that act as analogues of the naturally occurring deoxynucleotides needed to synthesize the viral DNA. The NtART lacks the 3'-hydroxyl group on the deoxyribose moiety which is required by the

Adult prevalence (%)

■ 15.0% - 28.0%

■ 5.0% - <15.0%

■ 1.0% - <5.0%

■ 0.5% - <1.0%

■ 0.1% - <0.5%

■ <0.1%

■ No data available



Figure 1: UNAIDS/WHO 2007 Global HIV Prevalance. The World Health Organization, in collaboration with many national public health agencies, has mapped the prevalence of infection in nearly all the countries on the globe. Currently, the global view of HIV infection approximates 39.5 million people are living with HIV. Image is reproduced from <http://www.who.int/hiv/topics/treatment/data/en/index.html> with permission from the WHO.

incoming nucleotide to create a phosphodiester bond. This lack of phosphodiester bond effectively ends viral replication through chain termination with a terminal nucleotide. In a similar fashion, NARTI terminates viral transcription by competing with nucleosides (4).

The next class of drugs discovered was the non-nucleoside reverse transcriptase inhibitors (NNRTI) which act as non-competitive inhibitors preventing transcription by interfering with the HIV viral DNA polymerase, known as the reverse transcriptase. The NNRTI acts by preventing the reverse transcription of the viral RNA genome into a double stranded DNA chain, thus preventing integration into the host genome (6). Recently, integrase inhibitors, which prevent HIV's retroviral DNA from integrating into the host cells genome, have become available (7).

The greatest revolution in HIV therapy occurred when the use of protease inhibitors was combined with the classes of drugs described above. The protease inhibitor drug class functions by preventing cleavage of nascent proteins, effectively preventing viral packaging (8). The combination of protease inhibitors with the other classes of drugs is referred to as highly active anti-retroviral therapy, or more commonly known as HAART. The potency of this combination arises from its ability to simultaneously attack the virus through several mechanisms.

Given the level of knowledge on the entry mechanism for HIV, a new class of drugs known as entry inhibitors has been developed. For HIV to enter a cell requires a binding of the viral glycoprotein-120 (gp120) to the host cells cluster of differentiation-4 (CD4) receptor and the binding of the viral glycoprotein-41 (gp 41) to a co-receptor (9). Several different co-receptors can be used and the tropism of the virus is defined by

the co-receptor it utilizes. In R5 tropic viruses, binding occurs through the chemokine C-C motif receptor 5 (CCR5) co-receptor. In X4 tropic viruses, binding occurs through the chemokine C-X-C motif receptor 4 (CXCR4) co-receptor (10-12). In addition, there are dual-tropic viruses which may use either co-receptor (13). The entry inhibitor drugs developed so far prevent the interaction between gp41 and CCR5 (14), drugs that would prevent interaction with CXCR4 remain elusive.

Despite the effectiveness of currently available drugs, there remain several problems in addressing viral infection. For instance, although these drugs are effective they eventually lose their potency due to the rapidly evolving nature of the virus through its error prone polymerase (15). The two main issues for the immune system are: the inability to clear the virus from the host once infected and, the immune deficiencies that arise as a result of HIV infection which can lead to serious and often lethal opportunistic infections (16). To further understand how these problems arise we must first understand how HIV affects the immune system.

B. HIV Immunopathogenesis

HIV transmission can occur through several methods: contact with contaminated blood, sexual contact and, by vertical transmission from mother to child (17-19). The primary target cell for infection is the CD4 T cell. Importantly, infection with HIV also leads to the establishment of many latently infected cells which do not actively replicate HIV. These latently infected cells create what is known as a viral reservoir. Given that current drugs rely on active viral replication they cannot address this viral reservoir (16). This is a key reason for the inability to clear the virus from the host despite drug therapy. The antigen presenting cells (APC) are the main viral

reservoir. The latent infection of APC is established primarily due to the inherent ability of the immune system to mount an effective immune response (20).

Infection with HIV compromises several of the host's immune functions. These include CD4 T cell depletion, ineffective antibody response, reduced cytotoxic lymphocyte (CTL) activity, deregulated cytokine production, as well as decreased response to mitogens and viral antigens (21). The immune dysfunction not only affects the response to HIV, but also prevents a normal response to other incoming pathogens. Together, this immune dysfunction leads to acquired immunodeficiency syndrome (AIDS) within approximately ten years of HIV infection (22). The mechanisms HIV utilizes to attack each of these fundamental host immune responses have been widely studied. Given the knowledge of these mechanisms a new therapeutic option based on the restoration of the immune response, referred to as immune based therapy (23, 24), would seem possible but has so far proven to be elusive.

1. HIV and Infection of Monocytes

i. Monocytes and the Establishment of HIV Infection: Monocytes and macrophages are important APC that HIV readily infects. Many investigations indicate that APC are the first cell type to become infected (25). Monocytes become infected to a lesser extent than macrophages (26, 27) . Monocytes do not divide readily and therefore infection is typically latent within these cells (28). Monocytes play a prominent role in the establishment of HIV-1 infection, virus dissemination, and development of viral reservoirs (25, 29). In addition, infected monocytes circulate freely in the blood and the lymphatic system until activated by a secondary signal which can lead to migration to other tissues, cellular activation, initiation of viral

replication and release and thus furthering the infection of the host (21). Monocytes are viral reservoirs, particularly in patients receiving HAART, preventing clearing of infection despite treatment (30-32). Infected monocytes can therefore act as a Trojan horse of infection, since HIV evades immune detection within the cell and drugs are unable to target this viral reservoir.

ii. Effect of HIV Infection on Monocyte Function: Monocyte function includes the phagocytosis and digestion of pathogens, and forms the bridge between innate and adaptive immunity through antigen presentation and the release of cytokines that activate cell mediated immunity (CMI) (33). Moreover monocytes are a source of cytokines and chemokines which affect viral replication and pathogenesis (26, 34). Infection with HIV affects monocyte function and leads to a progressive loss of CMI (21) and the deregulation of the production of the cytokines critical for CMI (35).

2. HIV Infection and Altered Cytokine Production

The immune system is regulated by a complicated network of immune cells and cytokines. Cytokines play an important role in immune homeostasis and activation. Monocytes can promote or impair adaptive immunity through cytokine production (36). These cytokines can lead to immune polarization of T cells, from Th0 into either T-helper cells type 1 (Th1) or type 2 (Th2) cells. Interleukin-12 (IL-12) production is the primary cytokine responsible for the development of Th1 cells. The production of IL-12 is influenced by other Th1 and Th2 cytokines.

In healthy cells, IL-12 activates the Th1 lymphocytes that in turn produce the Th1 cytokines such as IL-2, interferon- γ (IFN γ) and tumor necrosis factor- β (TNF- β). IFN- γ reciprocally activates macrophages and monocytes serving as a positive

feedback. This kind of regulation provides a fast and highly amplified response necessary for an efficient primary immune defense. This amplified response then requires a rapid negative feedback to prevent inflammatory pathogenesis. The Th1 cytokine IFN- γ ensures that this negative feedback takes place through the reduced production of Th2 cytokines (37, 38). The Th2 cytokines transforming growth factor (TGF- β), IL-4, IL-5, IL-10, and IL-13 are known for their anti-inflammatory effects as well as their ability to stimulate the humoral response (39).

In addition to the T helper types, Th1, Th2 there is a newly described Th17. The Th17 cells participate in protecting mucosal surfaces from bacteria and fungi by secreting defensins and recruiting scavenger cells. Th17 cells are induced by TGF β and IL-21 exposure and are characterized primarily by the secretion of IL-17.

Infection with HIV alters the immune systems network of immune cells and cytokines. During the course of HIV-1 infection, secretions of Th1 cytokines are generally decreased whereas productions of Th2 cytokines are increased. HIV-mediated cytokine deregulation include augmented production of Th2 cytokines (IL-4, IL-10, TGF- β) and expression of pro-inflammatory cytokines (TNF- α , IL-1 and IL-6) (35, 40-44). HIV infection also leads to deficiencies in Th1 cytokine activity including IFN- γ production (45-48). HIV infection *in vivo* is associated with an enhanced production of TNF- α (49-54), and impaired IL-12 production. IL-12 plays a key role in inducing Th1 proinflammatory cytokines such as TNF- α and IL-1 β (55, 56). IL-12 downregulates type 2 cytokines IL-10, TGF- β (57, 58), through a bi-directional negative feedback cytokine network.

C. HIV Infection and IL-12

IL-12 is an important cytokine in the multi-faceted immune pathogenesis mediated by HIV; both because HIV-mediate suppression of IL-12 and because IL-12 enhances the HIV immune response. IL-12 production has been shown to be deregulated in whole blood and peripheral blood mononuclear cells (PBMC) from HIV seropositive donors and during *in vitro* HIV-infection of primary PBMC and primary monocytes (44, 45, 59-76). HIV infection also results in the impairment of IFN- γ production from accessory and effector cells (77). Since IFN- γ is an important inducer of IL-12 this further reduces the IL-12 production. The production of IL-12 is inhibited by these HIV-induced effects and results in the loss of CMI response.

The reduction in IL-12 is especially important, since IL-12 has been shown to enhance the HIV immune response. HIV-infected PBMC have restored immune function following the addition of IL-12. Restored immune functions include: immune response to HIV peptides and mitogens with enhanced effector functions, increased IL-12 secretion and, increased proliferation of PBMC in response to HIV envelope protein gp120 (73, 78). PBMC and CD4 T cells respond to HIV p24 antigen through increased IFN- γ production and HIV specific CTL activity (79). IL-12 has been shown to enhance both of these responses (80). The addition of IL-12 results in increased IFN- γ production and suppression of IL-10 and TGF- β in PBMC from seropositive donors. IL-12 augments various functions of immune effector cells from HIV+ individuals including, natural killer (NK) lytic activity of PBMC to HIV antigens. This highlights IL-12 as a potential immune based therapy because of its capacity to restore the HIV-impaired CMI and its promotion of the HIV-specific adaptive immune response.

Unfortunately, although IL-12 has been used as a vaccine adjuvant, high levels of IL-12 are associated with several proinflammatory conditions such as endotoxin induced shock, multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease such as Crohn's disease and graft versus host disease (81, 82). IL-12 is a potent immune activator that, when present at appropriate levels, aids in mounting an immune response, but is unlikely to reduce HIV pathogenesis via systemic administration due to potential toxic effect (83, 84). To enable the effective development of a novel immune based therapy, we need to understand how IL-12 is synthesized, and how HIV prevents its production in order to identify appropriate targets for further development.

1. IL-12 Production and Function

IL-12 production can be induced in a T cell dependent or independent manner. IL-12 production can be induced through CD40 a co-stimulatory receptor on APC which is bound by CD40L on T helper cells to activate APC and downstream molecules. Pathogens can induce IL-12 production in a T-cell independent activation through pathogen associated molecular patterns (PAMPs).

IL-12 is produced by APC including monocytes, macrophages and dendritic cells (85, 86). An innate immune response, including increased of IL-12 production, is induced following binding of viral, fungal, protozoan, or bacterial antigens to cell surface proteins called Toll-like receptors (TLR) (87). The TLR on macrophages recognizes these pathogens through their PAMP and activates the innate immune response (88). More specifically IL-12 production can be induced by: bacterial cell wall components such as lipopolysaccharide (LPS) (detected by TLR4), bacterial lipoproteins and lipoteichoic acid (detected by TLR3), bacterial DNA containing CpG

(detected by TLR9), bacterial flagellin (detected by TLR5) and, double stranded RNA (detected by TLR3) (88). IL-12 is also produced following CD40 ligand (T cell) interaction with CD40 on the cell surface of APC (75, 76, 89).

IL-12 acts on NK and T cells through binding to its β_1 and β_2 receptors and initiates a Jak/Stat signalling cascade which results in IFN- γ production from these cells (90). IFN- γ creates a potent positive feedback loop when binding to its receptor on APC leading to further enhanced IL-12 production by APC (85). In which an initial stimulation of NK and T cells initiates a small production of IFN- γ which activated APC to produce a small amount of IL-12. The IL-12 then acts on the NK and T cells to initiate a greater production of IFN- γ , which in turn stimulate a greater production of IL-12. The interplay of IFN- γ and IL-12 priming and production depends on the inducer or microorganism investigated, but invariably leads to mutually enhanced response and cytokine production (85). Together IL-12 and IFN- γ initiate a type 1 pro-inflammatory cytokine cascade referred to as a Th1 effector response. IL-12 is a key factor in the induction of T-cell-dependent and -independent activation of macrophages. This signalling cascade results in further enhanced production of Th1 cytokines including IL-2, IFN- γ , TNF- β , additional IL-12 and the down-regulation of Th2 cytokines IL-4 and IL-10 (91).

IL-12 is critical to the control of various intracellular pathogens including *Leishmania major* (92, 93), *Toxoplasma gondii* (94, 95), *Listeria Monocytogenes*, *Trypanosoma cruzi* (96), *Cryptococcus neoformans* (97), *Mycobacteria tuberculosis* (98) and *Bacille Calmette-Guerin*. This has been demonstrated in mice deficient in IL-12 or its receptor (56, 99-103). *Cryptococcus*, *Toxoplasma* and *Mycobacteria* are well

known opportunistic pathogens in AIDS patients. The ultimate function of IL-12 is to enable the differentiation of Th0 T cells into Th1 T cells and drive the CMI response. The activation of Th1 cells by IL-12 leads to the generation of viral specific CTL and NK cells through the induction of IFN- γ (86, 104). Together, the interaction between T cells, NK cells and APC form CMI the primary mode of immune response to intracellular pathogens and the fundamental bridge between innate and adaptive immunity through antigen presentation.

2. IL-12 Family Monomers and Heterodimers

IL-12 itself is a p70 heterodimer composed of a beta subunit p40 from unrelated chromosomes 5q 31-33 and an alpha subunit p35 from 3q12-3q13-2 linked by disulfide bonds (105). Most investigations into IL-12 focus on the p40 molecule since it is produced in excess of the p70 heterodimer and is therefore easier to detect (87). The p35 subunit has received less attention, due in part to the fact that its production is ubiquitous in many cells, even in cells that do not produce IL-12 p70 (105).

The IL-12 family also includes the recently identified cytokines IL-23 and IL-27 which are related by both structure and function and are produced by monocytes, macrophages and dendritic cells (86). IL-23 is composed of the IL-12 p40 subunit and the IL-23 p19 subunit (106) and is associated with the generation of the Th17 response (107). Th17 cells are differentiated from activated CD4 T cells and unlike Th1 cells they produce IL-17 (108-110). Interaction between IL-23 and its receptor on Th17 cells can induce rapid proliferation of Th17 cells contributing to mucosal immunity. IL-27 is composed of IL-27 p28 and IL-27 EBI3 subunits (111). IL-27 plays an important function in regulating the activity of B and T lymphocytes. Both IL-27 and IL-12

induce IFN- γ production from T and NK cells and together they act synergistically (112).

The potential implication of decreased IL-12 p40 subunit production is reduced production and biological activities of the heterodimer IL-12 p70, resulting in reduced CMI. The IL-12 p40 subunit together with p19 forms the IL-23 heterodimer. Reduced IL-12 p40 production will decrease the availability of IL-23 which preferentially acts on effector and memory CD4 T cells to enhance function. In addition the IL-12 p40 subunit can form a homodimer IL-12 p80 which can act as an antagonist to IL-12 and IL-23 by competing for binding with their respective receptors.

The impaired NK cell, CD4+, and CD8+ T cell, effector functions which results from reduced IL-12 p40 production leads to greater susceptibility to infection and reduced capacity to clear infection. IL-12 p40 KO mice are susceptible to several pathogens including *Franciscella tularensis* (113), *Mycobacterium tuberculosis* (114), *Mycobacterium bovis BCG* (115), *Salmonella enteriditis* (116) and *Cryptococcus neoformans* (117). This susceptibility which is marked by increased mortality and pathogen burden is more pronounced in IL-12 p40 KO mice than IL-12 p35 KO mice and may be explained by IL-12 p40 role in IL-12 p70 and IL-23 heterodimers (118).

3. LPS Signalling Cascade

i LPS Induces IL-12: Our laboratory and others have previously shown that LPS is an important inducer of IL-12 production (85, 119-121). Furthermore, LPS is a quintessential immune mitogen used to evaluate the innate immune response. Therefore LPS is a useful research reagent for the evaluation of IL-12 production. The mechanism of LPS-induced IL-12 p40 production is not sufficiently characterized to enable the

understanding of HIV-mediated inhibition of LPS-induced IL-12. Further evaluation of this mechanism is therefore warranted.

ii LPS is Clinically Relevant: The potent mitogen LPS is a complex glycolipid found on the cell membrane of gram-negative bacteria. Several gram negative pathogens that are responsible for sexually transmitted disease including, *Hemophilus influenzae*, and coliform bacteria, can lead to circulating LPS. Patients with HIV/AIDS have chronic secondary infections in part due to impaired cellular immunity in response to LPS (122). Also, HIV viral shedding can be increased in patients with the gram-negative bacteria *Neisseria gonorrhoea* and *Chlamydia trachomatis* (123). Together, these observations demonstrate that further understanding of the LPS signalling cascade is clinically relevant.

iii LPS Signalling Cascade LPS is a potent immunostimulatory molecule recognized by monocytes/macrophages through TLR4 and CD14. Monocytes are stimulated by bacterial LPS following binding to the LPS binding protein (LBP) (124). This complex then interacts with the cell surface receptor CD14 (125) and accessory protein myeloid differentiation 2 (MD-2) to activate intracellular signalling through TLR4 (124). The TLR4 signals through the adapter protein myeloid differentiation factor 88 (MyD88), immediately downstream of the interleukin-1 receptor-associated kinase (IRAK) and tumor necrosis factor (TNF) receptor-associated factor-6 (TRAF6) leading to the activation of the mitogen activated protein kinase (MAPK) pathway (126) (Figure 2). The activity of MAPK is regulated through three different proteins: extracellular signal regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 kinase (p38) (127).

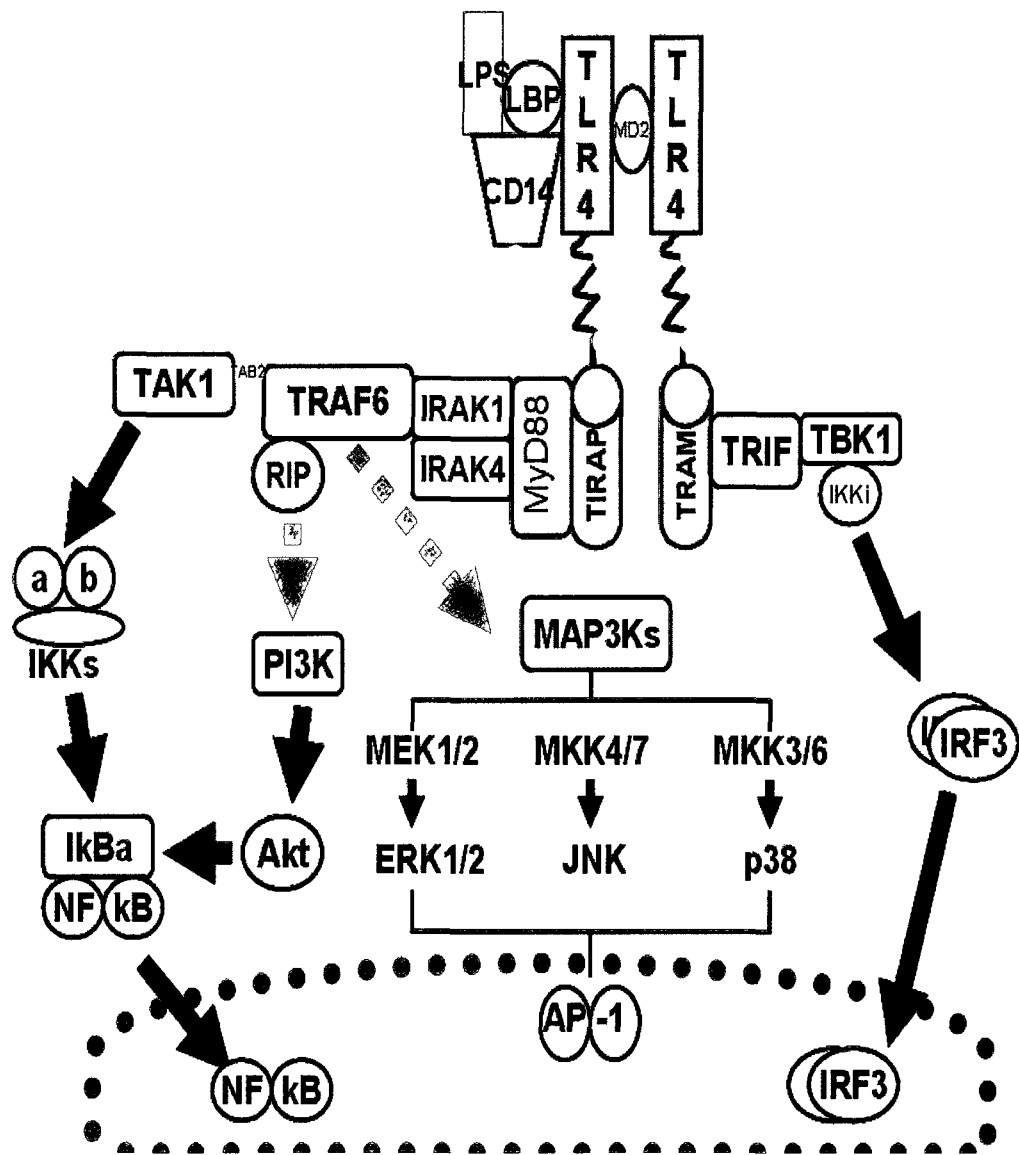


Figure 2: LPS Signalling Cascade. Bacterial LPS binds to the LPS binding protein (LBP). This complex then interacts with the cell surface receptor CD14 and accessory protein myeloid differentiation 2 (MD-2) to activate intracellular signalling through Toll-like receptor-4 (TLR4). The TLR4 signals through the adapter protein myeloid differentiation factor 88 (MyD88), immediately downstream of the interleukin-1 receptor-associated kinase (IRAK) and tumor necrosis factor (TNF) receptor-associated factor-6 (TRAF6) leading to the activation of the mitogen activated protein kinase (MAPK) pathway. The activity of MAPK is regulated through three different proteins: extracellular signal regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 kinase (p38).

MAPK are an evolutionarily conserved phosphorylation enzyme family that act through the phosphorylation of the amino acids serine and threonine (127). The main downstream effector kinases are ERK composed of p44 ERK1 and p42 ERK2 enzymes (ERK1/2) (128, 129). The stress activated protein kinase (SAPK) family are sometimes included in the larger MAPK family although they are differentiated by their primary response to cellular stresses (130). The two effector kinases are p38 which is composed of five isoforms p38 α , p38 β , p38 δ , p38 ϵ , p38 γ (131) and JNK which is composed of three isoforms p46 JNK1, p54 JNK2 and p49 JNK3 (130).

iv. HIV, Kinases and IL-12: Kinase signalling cascades connect cell surface receptors to their intracellular targets by inducing the phosphorylation of transcription factors and co-activators in the cytoplasm leading to their translocation into the nucleus where they bind to their respective promoter sites and initiate transcription. The MAPK/SAPK pathways have been connected to each of the fundamental cellular processes including cell growth, differentiation, cell death, and to the production of various cytokines (127).

In certain cellular contexts, the kinase phosphorylation through p38, JNK and ERK has been implicated in the production of IL-12 p40 (121). These findings have been system specific and some inconsistencies remain (132). Explaining these inconsistencies may provide further insight into the involvement of the MAPK pathways in the production of IL-12 p40.

HIV infection of the human acute monocytic leukemia cell line, THP-1, and primary adherence derived monocytes suppresses LPS-induced phosphorylation of p38 and JNK. This may present a likely mechanism for HIV mediated inhibition of IL-12

p40 production (120). Further understanding of this pathway may help to elucidate the mechanism of HIV-mediated inhibition of LPS-induced IL-12 p40 production.

v. HIV and the LPS Receptor CD14: In addition to decreased kinase activity, a lower expression of monocyte CD14 has been associated with a higher viral load and a greater degree of infection of macrophages (133). This may suggest that monocytes with a low level of CD14 may be more susceptible to HIV infection and may enhance viral replication (134, 135). The observation that CD14 expression is low during persistent infection despite a normal monocyte count (136, 137) may indicate an HIV-mediated mechanism to suppress the LPS-induced immune response. To further our understanding of HIV-mediated immune deregulation in response to LPS we must understand its effect on the expression of CD14. This may highlight a key mechanism of HIV-mediated immune dysfunction and merits further investigation.

4. Transcriptional Control of IL-12 p40

The protein kinases are responsible for signal transduction to activate transcription factor binding to targeted promoter sites leading to gene activation. The human IL-12 p40 promoter has at least eight putative transcription factor binding sites, including C/EBP, AP-1, Sp-1, NF κ B, PU.1, IRF-1, NF-IL-6 and Ets-2 (85) and one repressor site, Ga-12 (138) (see Figure 3). The previous work in our laboratory has shown that *in vitro* HIV infection specifically affects the LPS-induced binding of nuclear factors to four of the IL-12 p40 promoter sites : NF κ B, Sp-1, AP-1 and, Ets-2 (85, 120, 121, 139).

The nuclear factors associated with binding to these four sites have been identified. Activation of the nuclear factor binding to the NF κ B is dependant on the

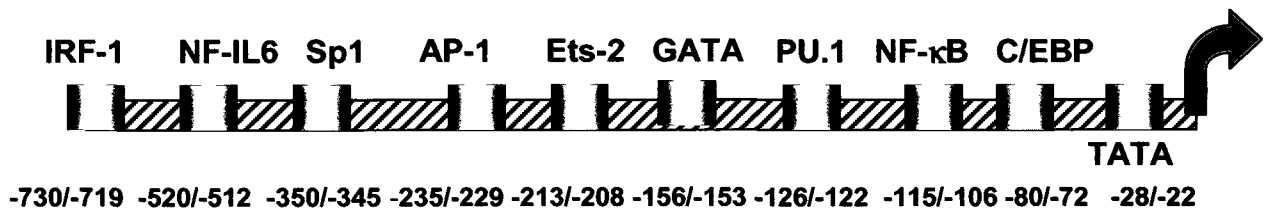


Figure 3: IL-12 p40 Putative Nuclear Factor Binding Sites. The IL-12 p40 promoter is composed of nine promoter sites their locations are indicated in this figure relative to the TATA box start site. Four of these sites have been implicated by *in vitro* HIV infection of isolated monocytes to have irregular binding in response to LPS stimulation. These sites include NFκB, Sp-1, AP-1 and Ets-2 promoter regions.

phosphorylation of the inhibitor of kappa B α ($\text{I}\kappa\text{B}\alpha$) subunit by the $\text{I}\kappa\text{B}$ kinase (IKK) (140). The Sp1 family proteins are ubiquitously expressed and the binding sites are present in numerous promoters (141). The Sp1 promoter site is bound by the zinc-finger transcription factors Sp1 and Sp3. The Sp-1 transcription factor family member has been shown to repress promoter activation (142). AP-1 is considered to be a family of transcription factors composed of jun isoforms (v-jun, c-jun, junB, junD) which can homo- or hetero- dimerize with fos proteins (v-fos, c-fos, fosB, Fra1, Fra2) or activated transcription factors (ATF-2, ATF-3, B-ATF) (119). JNK activation and other protein kinase pathways can lead to the phosphorylation of jun and increased transcription of nuclear factor binding to the promoter (127). The Ets-2 promoter site, can be a repressor site, and can be bound by IRF-1 and interferon consensus binding protein (ICSBP) (143).

Several of these nuclear factors have been shown to have impaired LPS-induced nuclear factor binding in HIV-infected monocytes (120). The nuclear factor binding to the promoter is likely altered through dysfunctional activation as the expression of the nuclear factors was shown to be unchanged in these HIV-infected monocytes (120), while the kinase activity of p38 and JNK was inhibited. This would suggest that HIV-mediated suppression of nuclear factor binding to the promoter occurs through the inhibition of kinases.

D. HIV and HIV-Vpr

1. HIV Transcription

The HIV genome consists of nine open reading frames encoding three structural proteins Gag, Pol and Env that are incorporated into the viral particle; and six

regulatory proteins Tat, Vif, Rev, Nef, Vpr and Vpu that contribute to viral functions and immunopathogenesis. HIV transcription in resting CD4⁺T cells and macrophages differs for at least two major reasons. Macrophages do not die upon HIV infection (they become viral reservoirs) and they have a pre-existing pool of transcription factors that may allow HIV to undergo continuous low level gene transcription. In conjunction with HIV regulatory proteins such as HIV Tat and Vpr viral transcription is enabled in monocytes. While in CD4 T cells, HIV transcription is dependant on extracellular stimulation to induce translocation of transcription factors to the nucleus (144, 145).

The HIV-induced deregulation of monocyte/macrophage activation and differentiation contribute to HIV and AIDS pathogenesis (146). A better understanding of the importance of macrophages to HIV pathogenesis may lead to an approach that could enable eradication of HIV. Further evidence of the direct impact of HIV infection on monocytes in systemic infection, or monocytes within infected PBMC culture, would contribute to clarifying the effects of cell specific viral infections (147).

2. Vpr Function

HIV-1 Vpr (p14 protein) is a 96 amino acid, 14 kDa non-structural viral protein (148). Vpr is associated with virus particles and is available to host cells prior to *de novo* synthesis and productive infection (149). As such, Vpr is one of the first viral proteins encountered by the host cell (150). Measurable quantities of free Vpr protein are present in the blood of HIV infected patients and can be endocytosed by APC (151). Vpr peptide has the ability to cross both the cytoplasmic and nuclear membranes (152) through protein transduction (153, 154). During viral transcription, Vpr is synthesized as a late HIV protein, and is associated with viral particles (155). Vpr is a highly

conserved (156) pleotropic protein which shares no strong homology with any other known protein. Vpr is conserved among the primate lentiviral HIV-1, HIV-2 and simian immunodeficiency virus and thus plays an important role in the virus life cycle *in vivo* (157). As a virion associated molecule Vpr has been implicated in the early events during infection and immune activation (158). Vpr is capable of deregulating the cell cycle with arrests at the G2/M phase, altering host gene expression, enhancing the nuclear transport of the pre-integration complex and inducing apoptosis (159) (160). Together this makes Vpr an attractive target for prevention and therapeutic interventions.

Several investigations have also shown Vpr to be necessary for optimal infection of monocytes, macrophages and other non-dividing cells (161-163). These studies surmise, Vpr is not essential to viral replication in cell lines, while its presence enhances replication in primary cell cultures, and Vpr is essential for *in vivo* infection in humans (157, 158). Furthermore, defective or mutated Vpr has been associated with long-term non-progressors (164-166). This exhibits Vpr's role in establishing viral reservoirs and *in vivo* pathogenesis (167).

Vpr can contribute to viral replication by blocking cell proliferation in G2 phase of the life cycle in infected cells (160, 168, 169). Although the importance of this function is minimal in proliferating cells (167, 170) it is essential in macrophages and other non-dividing cells for efficient replication (161, 171). The mechanism of this function is not defined, but may be through Vpr-dependant nuclear import of the viral pre-integration complex (PIC) in macrophages (172, 173). The promotion of nuclear import of the viral DNA in macrophages through the PIC may be essential for HIV

replication (174-177). Several studies support Vpr to be particularly important for HIV-1 infection of macrophages (153, 161, 178) due in part to Vpr-dependant nuclear translocation (173). Alternatively there may be a block in replication in macrophages which requires the presence of Vpr for efficient replication (179, 180) and may be dependant on Vpr-induced G2 arrest (181).

In addition to the importance of cell cycle for HIV replication there are also impacts for anti-HIV drugs (182-184), as the cell cycle arrest in G2 can impact certain anti-HIV drug function (185, 186), specifically by decreasing sensitivity to NARTI (e.g. AZT and d4T) and to a NNRTI (e.g. nevirapine) (182).

The presence of Vpr is crucial not only for translocation of viral DNA into the nucleus, but also for subsequent integration of viral DNA in non-dividing cells (187). Interaction between Vpr and HIV genome is known to transactivate the proviral LTR (188, 189), increasing *in vitro* replication (190). In particular, promoter driven expression of Vpr is critically required to increase transcription of Gag and Nef (191). Vpr also increases fidelity of reverse transcription (192, 193).

Vpr readily induces apoptosis (194, 195), although Vpr has demonstrated both pro-apoptotic and anti-apoptotic roles (196, 197). Vpr-mediated immune dysregulation is independent of Vpr-induced apoptosis (198).

Through binding to the cortisol receptor GR, Vpr may further the pathogenic HPA axis effect to the CMI response. Vpr has been shown to prevent LPS-induced IL-12 production (198) and merits further investigation. GR-Vpr interaction affect GR function since extracellularly administered Vpr can enhance dexamethasone suppression of IL-12 p70 in PBMC (198). Both N-terminal and C-terminal synthetic

HIV-Vpr protein activates JNK, NFκB and AP-1 in primary monocytes (159, 199), which is demonstrated herein to promote IL-12 production. Mirani *et al.* has shown that Vpr decreased TLR4 promoter activity (198). If Vpr proves to affect cell surface expression of TLR4 it may present a novel mechanism for the Vpr mediated decrease of IL-12 production.

3. Vpr Interactions with the Glucocorticoid Receptor

Vpr acts as a potent co-activator of the glucocorticoid receptor (GR) and increases the sensitivity of host target tissues to host glucocorticoids (GC) by 3-20 fold (200). Vpr binds to the GR through the LXXLL domain (201) motif between the 64-68 amino acids of Vpr (198, 202, 203) and this domain is conserved in HIV infected individuals (204). Vpr-GR transactivates the HIV-1 long terminal repeat (LTR) through glucocorticoid responsive elements (GRE), and this transactivation is blocked by the GR inhibitor Ru 486 in both X4 and R5 tropic viruses (205).

In association with GR, Vpr can recruit co-activating protein Poly (ADP-ribose) polymerase family member 1 (PARP-1) (206). Vpr-GR-PARP-1 forms a complex and prevents nuclear localization which is necessary for Vpr to suppress NFκB (206). The GR-PARP-1 complex does not associate following ligation through human glucocorticoids, indicating a gain of function attributed to viral Vpr (202, 206). Overall, Vpr protein may be important for IL-12 production through its effect on GR function.

E. Human Glucocorticoids and Glucocorticoid Receptor

The innate immune system stimulates controlled production and timely release of glucocorticoids; the inhibitory action of glucocorticoids prevents the negative impact of an excessive localized inflammatory process (207). Th1 and Th2 cytokines act in

conjunction with glucocorticoids to determine the final outcome of the initial inflammatory and immune response. GR is also an important regulator of macrophage function (208-210).

Activation of the GR on mononuclear cells directly affects innate and adaptive immunity (211, 212) by suppressing expression of cytokines, chemokines and adhesion molecules (213). Endogenous glucocorticoids are protective against cytokine-induced pathology (214) and have been shown to inhibit TNF production (215-217), IL-1 β gene expression (218) and MHC class II antigen expression by macrophages (215-217). Endogenous glucocorticoid responses suppress cellular immune responses and bias the response in the direction of humoral immunity by inhibiting Th1 cytokine production (219, 220) and enhancing Th2 cytokine responses (221-225). The modulation by glucocorticoids was further demonstrated with the GR antagonists, mifepristone, which can enhance LPS induced Th1 cytokine release (226).

As the main Hypothalamus-pituitary-adrenal (HPA) effector, glucocorticoids act via binding to cytoplasmic GR (207). The immunosuppressive role of the glucocorticoid binding to GR has been attributed to GR-protein interaction with transcription factors (202, 227). GR is a ubiquitous intracellular receptor protein and GR responsive genes represent 10-20 % of the human genome by the indirect and the direct influence of the ligand-activated GR (228). The activated GR translocates to the nucleus and acts as a ligand-dependant transcription factor that modulates the expression of glucocorticoid responsive genes (229) or modifies the activity of other transcription factors such as NF κ B and AP-1 (200, 202). Upon binding with glucocorticoids, the GR dissociates from a hetero-oligomer of the cytoplasmic heat

shock proteins Hsp 90, Hsp 70 and Hsp 50 (230). The GR then translocates into the nucleus through the nuclear pore, via an active process, to exert its effect by binding either to the GRE or to NF κ B (228) and AP-1 (230, 231). Therefore glucocorticoids are important regulators of cytokine production and act through binding to the GR.

F. Immune Homeostasis: HPA and Infection

Glucocorticoids are potent anti-inflammatory agents and are the primary immune regulatory steroid of the hypothalamic-pituitary-adrenal (HPA) axis (232). Data now indicate that cytokines produced by the immune system can have profound effects on the neuroendocrine system, in particular the HPA axis. HPA activation by cytokines (via the release of glucocorticoids such as cortisol), in turn, has been found to play a critical role in restraining and shaping immune responses (221).

Evidence suggests that HIV infection is associated with a progressive reduction in type 1 cytokines IL-2 and IL-12 and an increase in type 2 cytokines IL-4, IL-6 and IL-10 (40). These changes in the immune response may be due to an alteration in the immune-endocrine feedback loop, which is modulated by the HPA axis and the glucocorticoid, cortisol (233). HIV infected patients have altered glucocorticoid-regulated responses including hypercortisolism (234-239) and cortisol resistance (235, 239, 240).

Th1 and Th2 cytokine production is modulated differently by glucocorticoids (221, 241). Glucocorticoids promote a Th2 response by inhibiting IFN- γ production, and stimulating IL-4, IL-10 and TGF- β secretion (223, 242) and reducing Th1 cytokine production, including IL-12 (221). In addition, IL-10 was shown to sensitize monocytes to the pharmacological cortisol mimic, dexamethasone, contributing to glucocorticoid

mediated inhibition of Th1 cytokines (220, 243). GR agonist binding inhibits Th1 cytokines. In addition, the expression of GR is induced in response to infection leading to a protective effect against excessive inflammation (244-247), through suppression of Th1 cytokines.

It has been suggested that HIV may utilize the glucocorticoid pathway to alter Th1 cytokine production (198, 221). The mechanism for HIV deregulation of Th1/Th2 cytokines is unknown, but may be dependant on Vpr-GR interactions leading to altered GR expression or to altered glucocorticoid-GR response to stimulation.

1. HIV and the Hypothalamus Pituitary Adrenal Axis

Glucocorticoid production is initiated through the HPA. Infection with HIV is associated with activation of the HPA axis and increases the production of the glucocorticoid family stress hormones including plasma and urinary cortisol in both the early and late stages of the disease (248-251). Glucocorticoids, such as cortisol, act on APCs by binding to ubiquitously expressed cytosolic/nuclear GR (252). Since the stress hormone cortisol is often over-produced in patients with HIV, it may contribute to HIV immune pathogenesis in addition to leading to metabolic problems. Many symptoms associated with HIV infection including reduced CMI (21), hypercortisolemic states (253), myopathy (254), dyslipidemia (255, 256), and insulin resistance (257, 258) are similar to those associated with excess glucocorticoid production (251, 259).

The paraventricular nucleus of the hypothalamus secretes corticotrophin-releasing hormone (CRH) which regulates the secretion of adrenocorticotrophic hormone (ACTH) from the anterior lobe of the pituitary gland. ACTH stimulates glucocorticoid production,

mainly cortisol from the adrenal cortices. Glucocorticoids in turn initiate negative feedback signalling to suppress CRH and ACTH production (232).

Patients with HIV infection exhibit a state of glucocorticoid hypersensitivity with increased plasma glucocorticoid levels (238, 260). HIV neuroendocrine imbalances/disturbances include, increased ACTH and basal cortisol (238, 260), blunted ACTH and cortisol response to stress (261) or to a CRH challenge (262, 263). Infection with HIV is also associated with increased plasma and urinary cortisol concentrations (237, 250, 260). Increased cortisol levels lead to a suppression of Th1 cytokines and an increase in Th2 cytokines (223) and can result in deficient CMI in response to pathogens (232, 264).

Antigen presenting cells release IL-12 following exposure to extracellular pathogens, leading to the release of other Th1 cytokines and the development of Th1 polarized T cells. In the absence of IL-12, the cell-mediated immune response is impaired. Therefore, understanding the regulation of IL-12 production and the mechanism of its inhibition by HIV is of therapeutic interest. To further our understanding of HIV-mediated inhibition of IL-12, we have undertaken a study of the multi-faceted pathways of regulation to highlight key targets for future development of a novel immune based therapy. Overall, this research is directed at understanding impaired CMI resulting from HIV infection. The focus of the project is to determine the mechanism of IL-12 down-regulation which occurs following HIV-1 infection (119, 120).

G. Rationale

In vitro studies have demonstrated that exogenous IL-12 improves the immune responsiveness of T lymphocytes and NK cells from HIV-infected individuals, thus identifying a role for IL-12 deficiency in HIV-1 pathogenesis and suggesting a potential therapeutic role of IL-12 against opportunistic pathogens as well as to HIV-1 infection itself (60, 265-267). However, initial studies on the administration of recombinant human IL-12 have not provided promising results (83, 84). Infection with HIV-1 elicits a broad range of host responses, many of which may interfere with regulatory pathways of LPS-induced IL-12 gene expression (59, 119, 120), such as functional MAPK/SAPK phosphorylation and nuclear factor binding to the IL-12 p40 promoter. In addition to the effect of HIV-1 on the immune system and IL-12 expression, the virus affects many metabolic pathways, including the production of the glucocorticoid family of stress hormones. Glucocorticoids act on APCs by binding to ubiquitously expressed cytosolic/nuclear GR (252). The ligand-associated GR can repress Th1 cytokines (213), including IL-12 (221). The expression of GR in HIV infected cells and Vpr treated cells is likely critical to understanding this pathway. Expression of CD14 and TLR4 is critical for LPS-induced IL-12 p40 production (86) and may be affected by HIV and/or Vpr.

H. Hypothesis

We hypothesise that HIV, through Vpr, may act as a glucocorticoid-mimic through ligation to GR and tolerize the cell to subsequent immune induction by altering upstream CD14 and TLR4 cell signalling receptor expression. We hypothesise these changes will result in decreased IL-12 p40 production.

I. Specific Aims

1. To evaluate the downstream signalling of TLR4, through MAPK/SAPK following LPS stimulation and its impact on IL-12 p40 production.
2. To determine the impact of MAPK/SAPK signalling on nuclear factor binding to NFκB, AP-1, Sp-1 and Ets-2 on the IL-12 p40 promoter.
3. To evaluate the impact of *in vitro* HIV on the expression of upstream signalling receptors CD14, TLR4 and GR.
4. To characterize the role of extracellular Vpr peptide on LPS-induced CD14, TLR4, and GR expression.
5. To quantify the effect of whole virus and extracellular Vpr peptide on IL-12 p40 transcription.
6. To determine the role of intracellular Vpr expression of LPS-induced CD14, TLR4 and GR expression.
7. To quantify the impact of intracellular Vpr on IL-12 p40 production and IL-12 family IL-23 and IL-27 production.

II - Materials and Methods

A. Primary Cells and Cell Lines

1. THP-1

THP-1 cells are a promonocytic cell line isolated from a patient with acute monocytic leukemia obtained from American Type Culture Collection (Manassas, VA). The cells were cultured in RPMI 1640 (Gibco Invitrogen corporation, Grand Island NY) and supplemented with 10% fetal calf serum (Gibco) and 100 ug/ml Penicillin 100 ug/ml Streptomycin (Gibco) and 2 mM L-glutamine. Cells were maintained in suspension at 500,000 cells per milliliter (ml) at 37°C, 5% CO₂. Cell stocks were made by freezing down 10x10⁶ cells in cryovials with 10% DMSO overnight at -80°C in an isopropanol bath. Cells were stored long-term in liquid nitrogen tanks. Quantification and viability of cells was assessed with a hemocytometer and trypan blue staining (120).

2. Peripheral Blood Mononuclear Cells

Blood from healthy volunteers was layered over Ficoll-PaqueTM PLUS (GE Healthcare/Amersham Biosciences, Baie d'Urfe, QC) in a ratio of 2:1 and centrifuged for 30 minutes at 1500 rotations per minute (rpm). Peripheral blood mononuclear cells (PBMC) were isolated from the gradient and washed twice in Dulbecco's phosphate-buffered saline (PBS) (Gibco) and resuspended at 2x10⁶ cells/ml in RPMI 1640 (Gibco) and supplemented with 5% Human AB Serum (Sera Care Life Sciences Inc, Oceanside, CA) and 100 units/ml Penicillin 100 ug/ml Streptomycin (Gibco) and 2 mM L-glutamine.

3. Adherence Derived Monocytes

PBMC were cultured in T75 cm² flasks and monocytes were left to adhere for 90 minutes (min), following which isolated monocytes were cultured at 1×10^6 cells/ml overnight with 5% Human AB Serum (Sera Care Life Sciences Inc, Oceanside, CA) and 100 ug/ml Penicillin 100 ug/ml Streptomycin (Gibco) and 2 mM L-glutamine before beginning experimentation (120).

4. Negative Bead Isolation of Monocytes

White blood cells were isolated from PBMC from healthy donors with Ficoll-PaqueTM PLUS as described above. Monocytes were purified by a negative bead selection system by magnetic cell sorting with the human monocyte isolation kit II (MiltenyBiotech, Bergisch Gladbach, Germany) with antibodies specific for CD3 (T cells), CD19 (B cells), CD7 (T cells), CD16 (NK and dendritic cells), CD56 (NK cells), CD123 (B and T cells) and glycoporphin A (Basophils) and isolated by autoMACS according to manufacturer's protocol. Cells were cultured in suspension in non-stick 6 well plates (Falcon, Franklin Lakes, NJ) in media as described above.

B. Reagents

1. p38 Inhibitor (p38_i)

SB203580 (4-(4'-fluorophenyl)-2-(4'-methylsulfinylphenyl))-5-(4'-pyridyl) imidazole is HPLC purified to 99.5% (Calbiochem, EMD Bioscience La Jolla, CA) and stocks were made in anhydrous dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St-Louis, MO). P38 inhibition with SB203580 is the most potent commercially available pyridine imidazole inhibitor of p38; inhibiting α , β , β_2 isoforms (but not γ and δ isoforms) by competing with the substrate ATP with no significant effects on ERK or JNK MAPK

subgroups (268). Cells were treated with a range of 0-100 μ M to determine a dose response.

2. JNK Inhibitor (JNK_i)

SP600125 (anthrax (1,9-cd) pyrazol 6 (2H)-one 1,9-pyrazoloanthrone) is HPLC purified to 99.5% (Sigma-Aldrich) and stocks were made in anhydrous DMSO. The inhibition of JNK by SP600125 anthrapyrazolone acts by reversible ATP competition inhibitor of JNK2, with no effect to ERK1 or p38 (269). Cells were treated with a range of 0-100 μ M to determine a dose response. Prior to the availability of SP600125 dexamethasone was used as a JNK inhibitor (121).

3. ERK Inhibitor (ERK_i)

PD 98059 (2'-Amino-3'-methoxyflavone) is HPLC purified to 99.2% (Calbiochem) and stocks were made in anhydrous DMSO. PD 98059 is a flavone that acts by selectively blocking the phosphorylation activation of upstream MEK1 (but not MEK2) without affecting the activity of other serine threonine protein kinases including p38 and JNK (270). Cells were treated with a range of 0-100 μ M to determine a dose response.

All inhibitors were prepared by serial dilutions in culture media and the final inhibitor concentrations were normalized to a 1/500 DMSO to media ratio to control for solvent effects. Cells were pretreated with inhibitors for 1 hour prior to lipopolysaccharide (LPS) stimulation and inhibitors remained in culture during LPS stimulation.

4. Lipopolysaccharide

LPS derived from *Salmonella enteridis* (Sigma-Aldrich) is a mitogen found on the outer membrane of gram-negative bacteria and elicits a strong immune response (121, 271, 272). Cells treated with a final concentration of 1 ug/ml of LPS in culture media for 24 hours were designated LPS stimulated.

5. Pharmacological Glucocorticoid Mimic

Dexamethasone (9 α -fluoro-16 α -methylprednisolone) (Sigma-Aldrich) is a potent highly stable synthetic glucocorticoid and cortisol mimic that can be used pharmacologically, purified to greater than 97% for cell culture. The pharmacological mimic, dexamethasone can act more potently than the natural glucocorticoids, corticosterone and deoxycorticosterone (273). Stocks were made with DMSO and working solutions were dissolved in culture media. Cells were treated with a range of 0.01-1000 nM to determine a dose response, prepared by serial dilution in culture media.

6. Glucocorticoid Receptor Inhibitor

Ru486 purified to greater than 98% for cell culture (Sigma-Aldrich) is also known as mifepristone. Ru486 acts as a competitive receptor antagonist. At the progesterone receptor, its relative binding affinity at the glucocorticoid receptor is more than three times that of dexamethasone and more than ten times that of cortisol (273). Stocks were made in DMSO and working solution diluted with PBS. Cells were treated with 7 uM of Ru486 to inhibit the downstream glucocorticoid receptor signalling.

7. Vpr Peptide

A C-terminal Vpr peptide fragment (52 to 96 amino acids) which encompasses the GR binding motif between amino acids 64-68, and a control N-terminal Vpr peptide (1-45 a.a) were synthesized by Genemed Synthesis Inc (San Francisco, California, USA) and generously donated by Dr. Ashok Kumar (Children's Hospital of Eastern Ontario, Ottawa, ON) (159). The peptides were purified by reverse phase HPLC (>95%) and confirmed by electrospray mass spectrometry and were as follows: C-terminal DTWAGVEAI IRILQQLFI HFRIGCRHSR IGVTRQRRAR NGASRS and N-terminal MEQAPEDQGP QREPYNWTL ELLEELKSEA VRHFPRIWLH NLGQH. Because of the high propensity of Vpr peptides to bind to other proteins, cells (1×10^6 cells/ml) were treated with peptides in an isotonic buffer (13 mM HEPES, 2.4% glucose, 68 mM NaCl, 1.3 mM KCl, 4 mM Na₂HPO₄, 0.7 mM KH₂PO₄, pH 7.2) for 30 minutes at 4°C followed by the addition of fresh medium. Cells were treated with a range of 5-500 pM of Vpr to determine a dose response.

C. HIV Propagation and *In Vitro* HIV Infection:

The dual-tropic HIV-1 clinical isolate, HIV_{cs204}, was obtained from Dr. F. Diaz-Mitoma (Children's Hospital of Eastern Ontario, Ottawa, ON) (119). This virus isolate was propagated in the THP-1 cell line (American Type Culture Collection; Manassas, VA) and tissue culture infectious dose 50% (TCID₅₀) determinations were made for the virus stock in PBMC (119, 120). The X4-tropic laboratory strain HIV_{IIIB} was obtained from Dr. Robert Gallo, and the R5-Tropic laboratory strain HIV_{ADA} was obtained from Dr. Howard Gendelman through the AIDS Research and Reference Reagent Program,

Division of AIDS, National Institutes of Health AIDS research (Germantown, MD), propagated and the TCID₅₀ was determined in PBMC.

1. *In vitro* HIV Infection

Ten million PBMC cultured in suspension in polypropylene tubes (Falcon, Franklin Lakes, NJ) were stimulated with 2.5 ug/ml of phytohemagglutinin (PHA) (Sigma-Aldrich) and 20 units/ml of IL-2 (Sigma-Aldrich) for 48 hours (119, 120). Cells were then washed with PBS and treated with 2 ug/ml of polybrene (Sigma-Aldrich) for 1 hour in fresh media. Subsequently the cells were washed, re-cultured in fresh media followed by infection with cell-free HIV-1 at a multiplicity of infection (MOI) of 0.002 for 4 hours before being washed and cultured in fresh media. This time point was considered to be the zero time point in the infection assays and cultures were maintained for 4 days. In order to determine HIV-1 infection, cell-free supernatants were taken at 0, 24, 48, 72 and 96 hours post-infection and virus replication was assessed by quantifying HIV-1 p24 production by ELISA (NIH AIDS) according to the NIH protocol (119, 120).

D. Generation of Vpr Expressing Retrovirus

1. Vpr Cloning

The p89.6 vector (catalog number 3552) was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Ronald G. Collman, MD . Vpr was cloned from the p89.6 vector containing the entire HIV genome (subtype B) in a pUC 19 backbone, using primers with BamHI restriction sites at the 5' and 3' ends by PCR. The resulting Vpr cDNA was then ligated into the pLXIN retroviral vector expression system (Clonetech, Palom CA). The oligonucleotides used for PCR

sense primer were 5' ACT TGG ACA GGA GTG GAAGC 3' and anti-sense primer 5' GCT TCC ACT CCT GTC CAA G 3'. Thirty cycles of PCR were performed under the following conditions with Pfu polymerase: Denature 94°C for 30 sec, anneal for 1 min at 55°C followed by an extension at 72°C for 7 min. The pLXIN-Vpr plasmid was then confirmed by restriction enzyme digest and sequencing by the OHRI Sequence Facility (Ottawa, ON).

2. Vpr Retrovirus Packaging

A polytropic mammalian retroviral packaging cell line, PT67 was purchased from BD Biosciences Clontech (Mississauga, ON). PT67 cells were designed for producing high titer retrovirus that can efficiently infect target cells by entering via the surface receptors GALV (Pit 1) and RAM1 (Pit 2). To transfect the PT67 cells with the Vpr-pLXIN clone or the empty control pLXIN, 1×10^6 cells/ml were plated in a 6 well dish (Falcon) one day prior to transfection and transduced with 5 ug of DNA plus 10 ul of Superfect reagent (Qiagen) in 0.7 ml of serum free RPMI according to the manufacturer's protocol. After 24 hours, the culture medium was replaced with fresh complete RPMI and stably transfected cells were selected culturing with 800 ug/ml of G418. Seven days post-transfection, RNA was purified for determining the integration of the Vpr gene by PCR (forward Vpr primer 5' GCC TCG ATC CTC CCT TTA TC 3'; Reverse Vpr primer 5'-CGG CAA TAT GGT GGA AAA ATC 3'). In order to determine Vpr expression cellular mRNA was isolated and converted to DNA by RT-PCR. The presence of Vpr specific mRNA was determined by PCR. To collect Vpr or control retrovirus containing supernatants, 10×10^6 cells in 10 ml were grown in T75 cm² flasks with 600 ug/ml of G418 overnight until 70-90% confluency. The

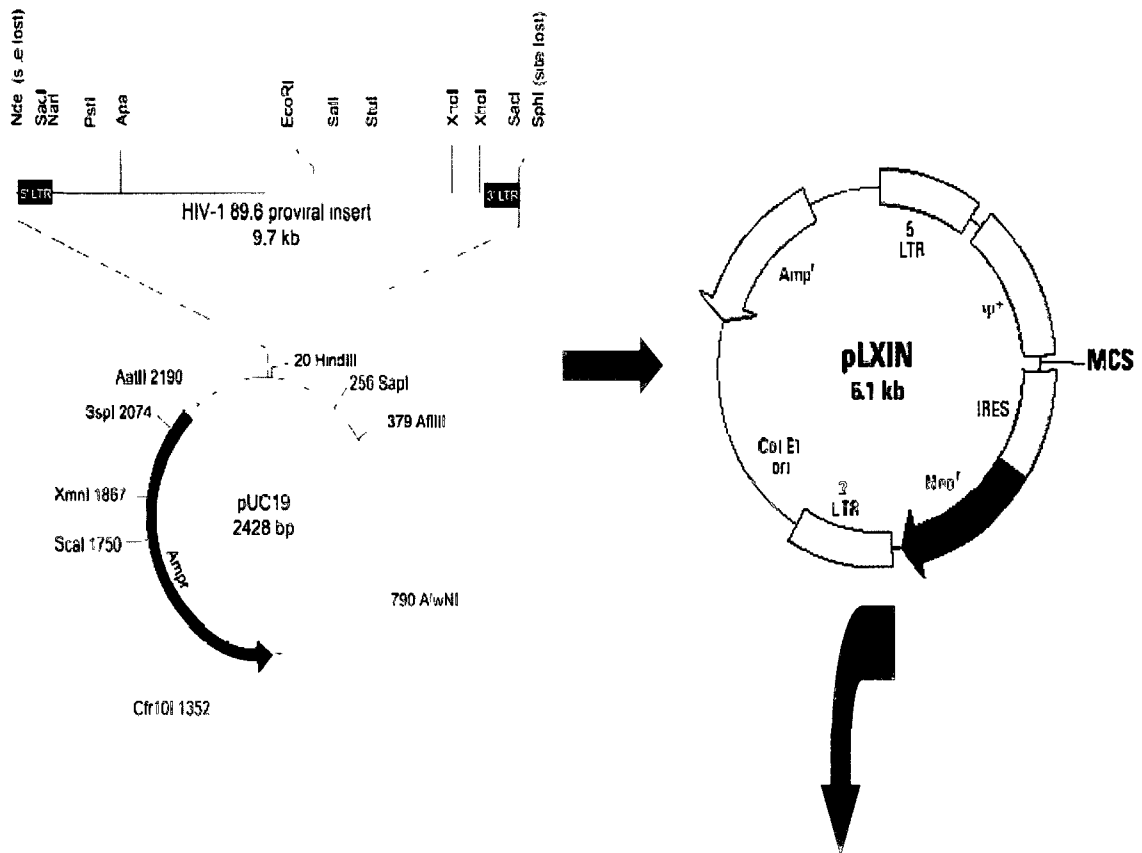
supernatants were collected, centrifuged to remove contaminating cells, pooled to create a large reference stock and stored at -80°C . The viral titers of the Vpr and pLXIN control viruses were determined in NIH 3T3 cells by serial dilution of virus and selection with G418 according to manufacturer's protocol (BD Biosciences Clontech).

3. Infection with Vpr Expressing Retrovirus

The expression of Vpr mRNA and Vpr protein in infected cells was confirmed by PCR and immunoblotting, respectively. Infection of monocytes and PBMC with Vpr retrovirus was performed at MOI of 1 at 37°C . Cells were cultured in 3 ml of pLXIN-Vpr or pLXIN retrovirus containing supernatant collected from the PT67 packaging cell line in the presence of 3 $\mu\text{g}/\text{ml}$ of polybrene for 16 hours and infected a second time under the identical conditions for an additional 16 hours. Cells were then washed and resuspended in fresh media. This time point was considered to be the zero time point of the infection assay.

4. Western Blotting of Vpr Expression

To confirm expression of Vpr protein from retrovirus-infected cells were collected, washed twice in ice-cold PBS, and lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 $\mu\text{g}/\text{ml}$ each aprotinin, leupeptin, pepstatin A, 1 mM Na_3VO_4 , 1 mM NaF). Total cell lysates were stored at -80°C until further use. Fifty μg of cell lysate were separated by SDS-PAGE, transferred to PVDF membrane (Immobilon-P, Millipore, Bedford, MA), blocked for one hour and blotted with primary antibodies against Vpr (donated by Dr. Eric Cohen) at 1:5000 dilution overnight. Membranes were washed in TBST buffer (20 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.05% Tween-20) followed by incubation with



Packaging Cell
 (produces viral proteins from stably integrated genes)

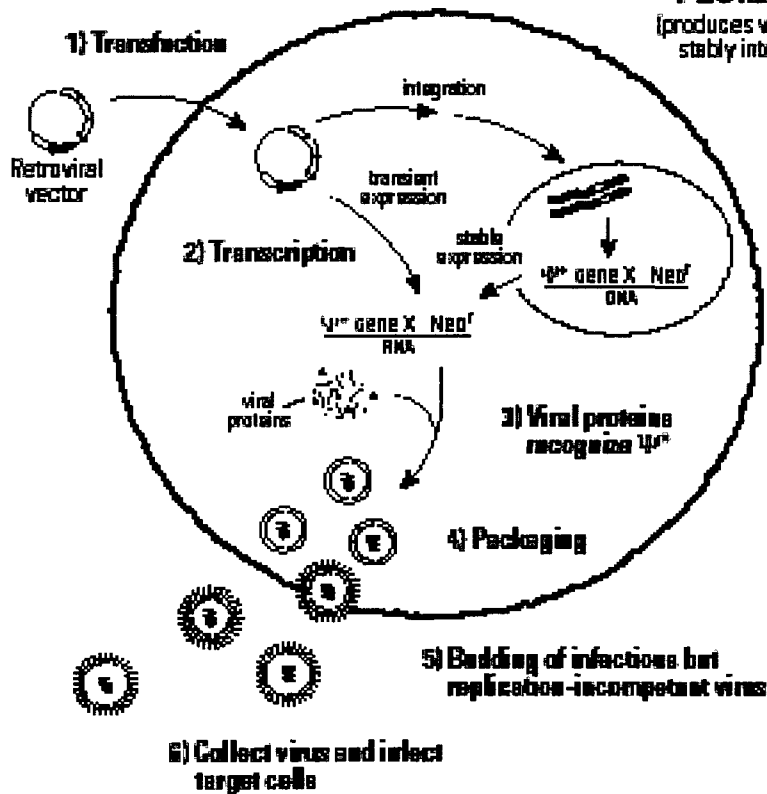


Figure 4: The Retroviral Expression System. Vpr was cloned from the p89.6 vector containing the HIV genome using primers with BamHI restriction sites at the 5' and 3' ends by PCR. The resulting Vpr cDNA was then ligated into the pLXIN retroviral vector expression system (Clonotech, Palom Ca). A polytropic mammalian retroviral packaging cell line, PT67 was purchased from BD Biosciences Clonotech (Mississauga, Ontario, Canada) and was transfected with either the Vpr-pLXIN clone or the empty control pLXIN vector. The Vpr or control retrovirus was collected from the PT67 supernatants, pooled and titered to create a reference stock. Infection of monocytes and PBMC with the retrovirus was performed at an MOI of 1 at 37°C. Images are reproduced from the Retroviral Gene Transfer and Expression User Manual with permission.

anti-rabbit-HRP secondary antibodies (Bio-Rad Laboratories, Mississauga, Ontario) at 0.2 ug/ml for 1 hour at room temperature. After washing, bands were detected by ECL chemiluminescence using LumigenTM TMA-6 Substrate (GE Healthcare UK Limited, Little Chalfont, Buckinghamshire, UK), and visualized using the Chemi genius Bio imaging system and analysed with GeneSnap software.

E. Molecular Assays

1. Electrophoretic Mobility Shift Assays for NFκB, AP-1, Sp-1 and Ets-2 Activation

i. Nuclear Extract Preparation: Prepared as described in (120), briefly inducible nuclear factor binding activity was assessed at the putative transcription factor binding sites found in the human IL-12 p40 promoter of adherence derived monocytes following stimulation with or without 1 ug/ml LPS. In parallel, 5×10^6 cells were pretreated with a MAPK inhibitors (MAPK_i) (10 uM SB203580/p38_i, PD98059/ERK_i and 1 nM for dexamethasone/JNK_i) for one hour before LPS stimulation. Following 2 hour LPS stimulation, cells were washed with ice-cold PBS, and lysed in hypotonic lysis buffer. The nuclei were then resuspended in a high-salt buffer to strip DNA binding proteins from nuclear DNA, followed by addition of a low-salt buffer to neutralize the osmolarity of the nuclear protein-containing solution. Cells were harvested in Tris-EDTA-saline buffer (pH 7.8) and centrifuged at 200 g for 5 min at 4°C. The cells were lysed for 10 min at 4°C with Buffer A [10 mM HEPES, 10 mM KCL, 1.5 mM MgCl₂, 0.5 mM DTT and 0.5 mM PMSF (pH 7.9)] containing 0.1% Nonidet P-40. The lysates were centrifuged at 14,000 g for 10 min at 4°C. The pellet containing the nuclei was suspended in Buffer B (20 mM HEPES, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, and 25% glycerol) at 4°C for 15 min. Both Buffers A and B contained the

proteolytic inhibitors including DTT, PMSF and spermidine, and 5 ug/ml each of aprotonin, leupeptin and pepstatin. The supernatant containing the nuclear proteins was collected and frozen at -80°C. The protein concentration was determined after thawing by bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology/Thermo Fisher Scientific Inc, Rockford, IL).

Oligonucleotide probes for $\gamma^{32}\text{P}$ labeling representing the transcription factor binding sites of interest (NF κ B: 5' ACG AAC TTC TTG AAA TTC CCC CAG AAG GTT TT 3'; AP-1: 5' TCC TTC CTT ATT CCC CAC CCA 3'; Ets-2: AAA GTC TCC TCT TAG TTC ATT 3'; Sp-1: 5' GTC TGA CCG CCC CTT GGC 3') and their respective complementary sequences were synthesized by the University of Ottawa Biotechnology Research Institute. The oligonucleotides were resuspended in TEN buffer (10 mM Tris-CL pH 8.0, 1 mM EDTA, 100 mM NaCl), and complementary strands were mixed in an equimolar ratio, denatured at 95°C and annealed by gradually equilibrating to room temperature overnight. The $\gamma^{32}\text{P}$ labeling reaction was activated using T4 polynucleotide kinase and unincorporated nucleotides were removed by MicroSpin G-50 Columns (Amersham). The end-labelled probes were then precipitated, washed in ethanol and resuspended in ddH₂O.

ii. Electrophoretic Mobility Shift Assays: Nuclear proteins (5 ug) were mixed for 20 min at room temperature with either $\gamma^{32}\text{P}$ -labelled NF κ B, AP-1, Sp-1 or Ets-2 oligonucleotide probes, and the complexes were subjected to non-denaturing 5% PAGE polyacrylamide gels for 4 hours. The oligonucleotide probes contain sequences corresponding to the NF κ B, AP-1, Sp-1 and Ets-2 sites in the IL-12 p40 promoter. To illustrate specificity of nuclear factor binding for NF κ B, AP-1, Sp-1 and Ets-2 probes,

parallel EMSA reactions were incubated with 50 fold excess of cold unlabelled probe. The gels were dried and exposed on phosphorous screens for 3 days and the image was detected and analyzed by Storm Imager (120).

2. Luciferase IL-12 p40 Promoter Activity

The functional role of transcription factor binding sites in LPS-induced IL-12 p40 promoter activation was investigated. The promoter reporter construct made with a wild-type IL-12 p40 promoter was delivered to the cells using an adenoviral vector delivery system [courtesy of Dr. Robin Parks (120)]. Luciferase activity of the wild-type promoter was measured in response to LPS in the presence or absence of the individual MAPK inhibitors. Monocytes or THP-1 cells (2×10^6 cells/2ml) were cultured overnight prior to infection with recombinant adenovirus vectors containing the wild-type IL-12 p40 promoter/luciferase vectors at an MOI of 50 (120). After 24 hours, cells were stimulated with LPS (1 ug/ml) for an additional 48 hours, determined in preliminary experiments to be the optimal concentration for luciferase detection from the LPS-stimulated wild-type promoter. Cells were lysed and assayed for luciferase activity, using the Enhanced Firefly Luciferase Assay Kit (BD Biosciences/Pharmingen; Mississauga, ON) according to manufacturers' instructions. The relative light units among the different experimental conditions was normalized to the total protein content of cellular extracts and termed relative luciferase units. This was done by subtracting the background reading from the luminometer and multiplying the reading by the protein content correction factor (average ug protein/ sample ug protein).

3. Real-Time PCR of IL-12 p40 Expression

Primary monocytes (5×10^6) were collected from parallel cultures and stimulated for 4 hours with LPS in the presence or absence of MAPK inhibitors (MAPK_i) (10 μ M SB203580/p38_i, PD98059/ERK_i, SP600125/JNK_i). Peak IL-12 p40 mRNA expression has been previously shown to occur four hours after LPS stimulation (119), thus this time point was chosen for evaluation. Total RNA was extracted with the RNeasy kit (Qiagen Inc., Valencia, CA) and IL-12 p40 mRNA levels were determined by real-time PCR amplification using TaqMan One-Step RT-PCR Master Mix Reagents (Applied Biosystems, Branchburg, NJ) with a Joe-IL-12 p40 primer set IL-12 p40 Forward sequence 5' AACATAACAAGACCCCGTCTCTCTATA 3', Reverse sequence 5' CAGCTGGGACCACAAGCAT 3' Taqman®.

The samples were normalized to GAPDH measured with a FAM-GAPDH primer 5' AAAAAATTTAAAATTAGCCAGGAGTGGTGGC 3' Taqman® GAPDH control reagent (Applied Biosystems). Reactions were carried out using 50 ng of total monocyte RNA as described in the ABI Prism 7000 real time PCR protocol one step Taqman® under the following conditions: 48°C for 30 min, 95°C for 10 min, and 40 cycles of 95°C for 10 sec and 60°C for 30 sec and 72°C for 45 sec. Samples were detected and analysed using the ABI Prism 7000. The relative copy number was calculated with respect to a relative standard curve. The standard curve was prepared by serial dilution of a high expression reference sample. The study design was analyzed with ABI Prism 7000 sequence detection system (SDS) software which prepared the standard curve based on the cycle at which input standard reference crossed the

threshold (C_T). The target sample is normalized to GAPDH with a correction factor (average/sample GAPDH).

F. Immunoassays

1. Enzyme-Linked Immunosorbent Assay of IL-12 p40 Production

Inhibitors of MAPK were used to investigate the role of these signalling pathways in LPS induced IL-12 p40 expression in stimulated THP-1 cells and primary monocytes. Purified monocytes ($5 \times 10^6/5\text{ml}$) were incubated in T25 cm^2 flasks (Falcon BD Biosciences, Franklin Lakes, NJ). THP-1 cells ($2 \times 10^5/0.2\text{ml}$) were cultured in 12 well plates (Falcon BD Biosciences). Cells were left untreated or stimulated with LPS (1 $\mu\text{g}/\text{ml}$) for 24 hours in the presence or absence of increasing concentrations of inhibitors of JNK, p38, and ERK 1/2 MAPK activity (0-100 μM SB203580, 0-100 μM PD98059, and 0-100 μM SP600125 respectively). Culture supernatants were collected and the concentration of IL-12 p40 protein was quantified using a commercially available IL-12 p40 ELISA (R&D Systems). To ensure cell death was not a factor in altered IL-12 secretion, cell viability was assessed using trypan blue exclusion dye. Supernatants were collected and cellular debris was removed by centrifugation. Samples were stored at -20°C until analysis by ELISA. The IL-12 p40 ELISA were performed according to manufacturer's directions.

2. Cytokine Analysis of Vpr-Retroviral Infected Cells

At time zero after infection with either the pLIXN control virus or Vpr-retrovirus, PBMC were stimulated with LPS for 24 hours and cell free supernatants were collected and compared to their unstimulated counterparts for IL-12 p40, IL-23 and IL-27 protein

production. IL-12 p40 and IL-23 (eBioscience) were analysed using commercially available ELISA kits and IL-27 was analysed using an in-house ELISA.

3. IL-27 ELISA

Briefly, each well of 96 well plates was coated with 20 ug of monoclonal anti-human IL-27 antibody (R&D Systems) in coating buffer (0.04 M Na₂CO₃ pH 9.6) overnight at 4°C. Wells were then washed 4 times with 0.05% Tween-20 in PBS and blocked with 10% FCS in PBS for 2 hours at room temperature. Each well was washed again and 100 ul sample or standard was added to their respective wells and incubated overnight at 4°C. The standard curve was made with recombinant human IL-27 (R&D Systems) in 10% FCS-PBS in serial dilutions ranging from, 0 pg/ml to 1000 pg/ml. Each well was washed again and 5 ug biotinylated secondary anti-human IL-27 antibody (R&D Systems) in 10% FCS-PBS was added for 2 hours. Wells were washed and 10 ng streptavidin peroxidase in 10% FCS-PBS was added and incubated at room temperature for 30 minutes. Wells were then washed again and OPD substrate (o-phenylenediamine dihydrochloride), hydrogen peroxide 30% (W/W) solution, 0.1 M citric acid, 0.2 M Na₂HPO₄) was added for 30 minutes in the dark. The reaction was stopped with 1N HCL and absorbance was read at 450 nm. The relative expression was calculated by dividing the LPS induced cytokine production by its unstimulated levels for each sample, the average was then graphed with the standard error of the mean.

4. Flow Cytometry

The PBMC were subjected to flow cytometric analysis with gating on the live monocyte cell population as assessed by the forward and side scatter patterns. The expression of different receptors (eg. CD14, GR etc) was assayed by either one or two

colour flow cytometry. Measurement of the total (intracellular and surface) receptor expression was performed as follows: 1×10^6 cells were permeabilized with the Fix&Perm cell permeabilization kit (Caltag Laboratories, Burlingame, CA) according to the manufacturer's protocol and resuspended in 100 μ l of PBS. Following antibody titration, cells were labeled with 5 μ l of fluorochrome-conjugated monoclonal antibodies FITC-conjugated anti-CD14 monoclonal antibody (Beckman Coulter, Fullerton CA), PE-conjugated anti-TLR4 monoclonal antibody (Imgenex Corp, San Diego CA) or FITC-conjugated anti-GR monoclonal antibody (ABD Serotec, Oxford, UK) for 30 min at room temperature in the dark prior to flow cytometry analysis. Autofluorescence and isotype matched controls for GR and TLR4 were used as a negative control. The commercially available TLR4 isotype control was complemented with an additional control of unlabeled TLR4 antibody (Imgenex Corp.) titrated against the PE labeled to create baseline fluorescence. Results were assessed on an EPICS ALTRA HyPerSort FACS System (Beckman Coulter) after gating on monocytes based on their forward and side scatter properties and confirming gating by FITC-conjugated anti-CD14 and PE-conjugated anti-CD40 of untreated cells (Beckman Coulter). Analysis was performed using Expo 32 software (Applied Cytometry Systems, Sacramento, CA) and was based on a minimum of 30,000 events. Values are expressed as the mean fluorescence intensity as analyzed using FCS express V.2 software package (Beckman Coulter).

G. Statistical Analysis

Means were compared by student t-test or ANOVA with Dunnett's post-test with the GraphPadPrism version 3 software program. The ANOVA analysis was performed

when evaluating multiple concentrations and the student t-test was performed when comparing two independent conditions. The exact p value has been included for each figure. The results were expressed as mean \pm standard error of the mean (SEM).

III - RESULTS

A. The role of p38, JNK and ERK on IL-12 p40 protein production in LPS-stimulated primary monocytes. It has been previously demonstrated that the infection of primary monocytes with HIV prevents LPS-induced phosphorylation of p38 and JNK, but not ERK (120). These experiments set out to determine the impact of p38 and JNK on LPS-induced IL-12 production. Primary monocytes were collected by adherence and 2×10^5 cells were used for each condition. Cells were pre-treated for 1 hour with increasing doses of p38 inhibitor (p38_i; SB203580), ERK inhibitor (ERK_i; PD98059), JNK inhibitor (JNK_i; SP600125) or untreated. Following pre-treatment, the cells were stimulated with 1 ug/ml LPS for 24 hours. Cell-free supernatants were collected and IL-12 p40 production was determined by ELISA. Unstimulated primary monocytes did not produce detectable IL-12 p40 (data not shown). Stimulation with LPS induced 477 ± 102 pg/ml of IL-12 p40 (Figure 5 A). Inhibition of p38 significantly decreased IL-12 p40 protein production (157 ± 39 pg/ml with 100 uM SB203580) (Figure 5 A). Inhibition of ERK phosphorylation had no effect on IL-12 p40 production (Figure 5 B). Inhibition of JNK resulted in a significant decrease in IL-12 p40 with as little as 1 nM of the inhibitor dexamethasone (281 ± 44 pg/ml) (Figure 5 C). Inhibition of JNK resulted in a significant decrease in IL-12 p40 with as little as 10 uM of the inhibitor SP600125 (260 ± 82 pg/ml) (Figure 5 D). This data suggests that p38 and JNK, but not ERK signalling are important for LPS-induced IL-12 p40 production.

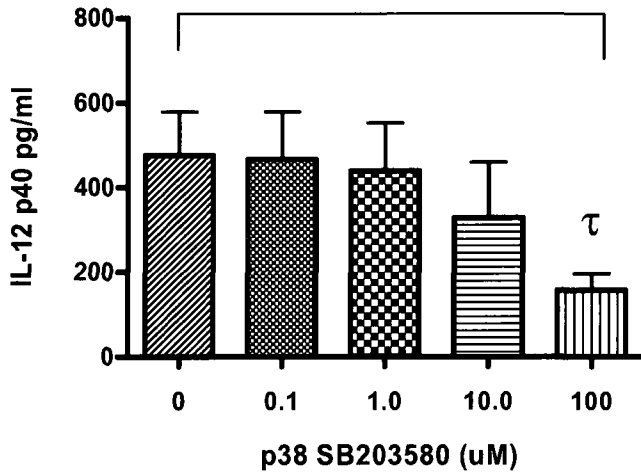
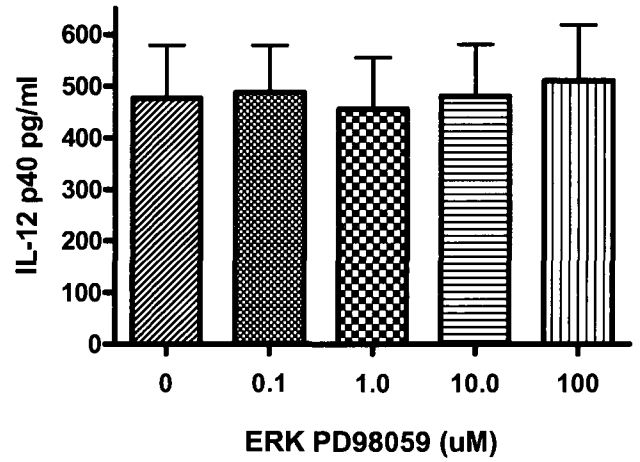
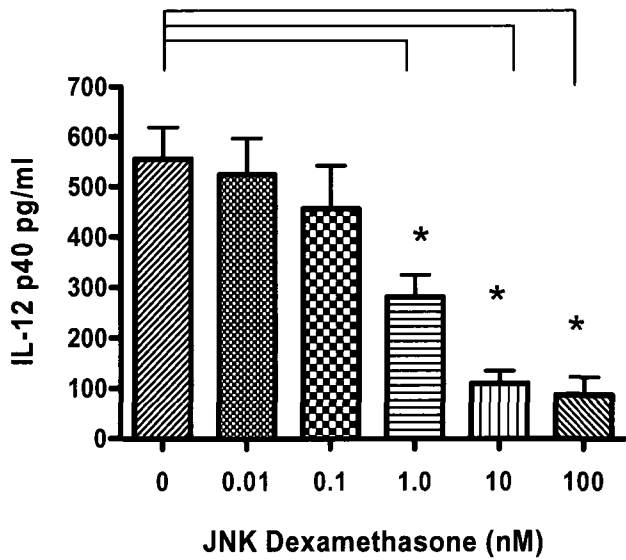
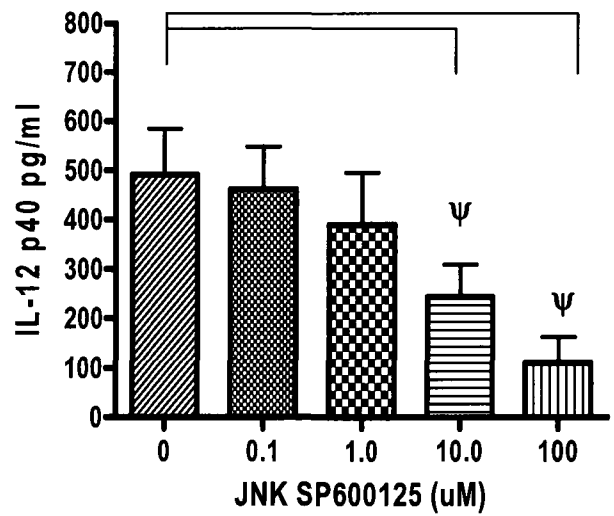
A**B****C****D**

Figure 5: Inhibition of p38 and JNK resulted in a decrease in IL-12 p40 protein production. Primary adherence derived monocytes (2×10^5) were stimulated with LPS in the presence or absence of increasing concentrations of SAPK/MAPK inhibitors. IL-12 p40 protein concentrations were measured in pg/ml by ELISA. **A)** Cells were treated with increasing concentrations of the p38 kinase inhibitor SB203580. Decreased IL-12 p40 production is observed at 10 μ M, and becomes statistically significant at 100 μ M (τ , $p=0.0025$). **B)** Cells were treated with increasing concentrations of the ERK kinase inhibitor PD98059, with no effect. **C)** Cells were treated with the JNK kinase inhibitor dexamethasone. Statistically significant decreases in IL-12 p40 production can be seen from 1 and 100 nM concentrations (*, $p=0.0001$). **D)** Cells were treated with the JNK kinase inhibitor SP600125. Statistically significant decreases in IL-12 p40 production can be seen at both 10 and 100 μ M concentrations (ψ , $p=0.001$). Statistical significance was determined by 2-way ANOVA with Dunnett's post-test, $n=3$. Error bars represent SEM.

B. The role of p38, JNK and ERK on IL-12 p40 mRNA expression in LPS stimulated primary monocytes cultured in isolation. Since p38 and JNK inhibition were shown to decrease IL-12 p40 protein production, the mechanism of this decrease was further investigated. To accomplish this, 5×10^6 primary adherence derived monocytes were pre-treated for 1 hour with 10 μ M of p38_i (SB203580), 10 μ M of JNK_i (SP600125) inhibition or 10 μ M of ERK_i (PD98059). Cells were then collected and mRNA was isolated for quantitative PCR (qPCR). The results were normalized to GAPDH production and quantification of IL-12 p40 was analyzed by quantitative PCR relative to a standard curve. Unstimulated monocytes had undetectable levels IL-12 p40 mRNA (data not shown). Inhibition of p38 or JNK with 10 μ M of SB203580 or SP600125 respectively resulted in a decrease in IL-12 p40 mRNA production (53 ± 6 and 55 ± 9 respectively) compared to LPS stimulation alone (98 ± 14) (Figure 6). ERK inhibition did not alter LPS-induced IL-12 p40 expression as expected since no effect was seen with IL-12 p40 protein production. Inhibition of either p38 or JNK kinase results in decreased LPS-induced IL-12 p40 mRNA in primary adherence-derived monocytes.

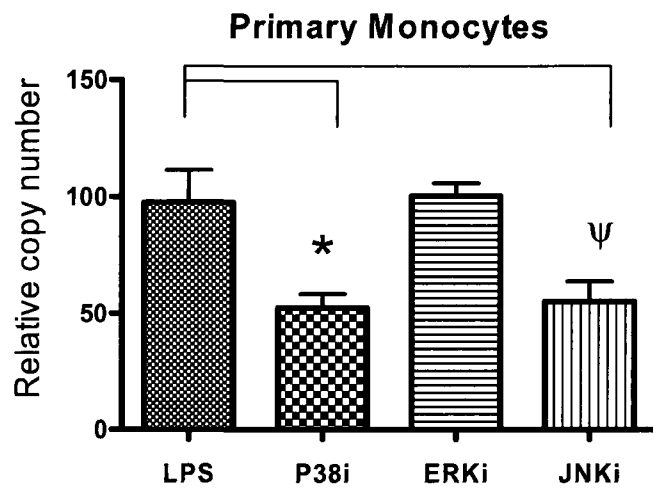
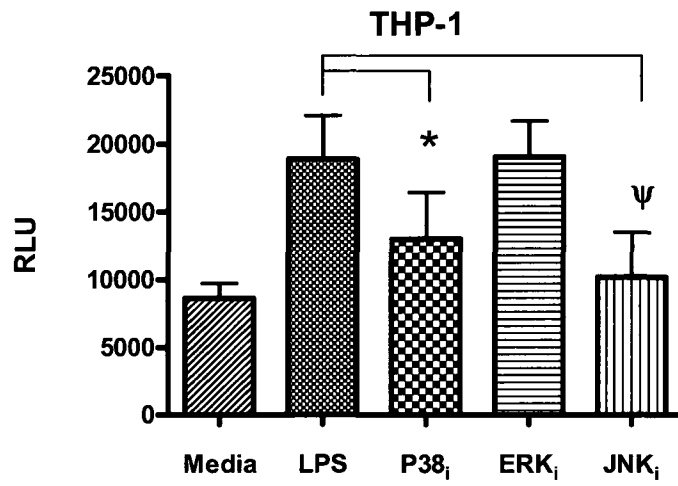


Figure 6: Inhibition of p38 and JNK resulted in a decrease in IL-12 p40 mRNA expression. Primary adherence derived monocytes (5×10^6 cells) were stimulated with LPS in the presence or absence of SAPK/MAPK (10 μ M) inhibitors: (p38_i (SB203580); ERK_i (PD98059); JNK_i (SP600125)). Samples were normalized to GAPDH. Both the p38 (*, $p=0.0160$) and JNK inhibitors (ψ , $p=0.0124$) decreased mRNA expression in LPS stimulated cells as compared to LPS stimulation alone. Statistical significance was determined by student t-test, $n=3$. Error bars represent SEM.

C. The role of p38, JNK and ERK on IL-12 p40 promoter activity in LPS stimulated primary monocytes. Given that p38 and JNK inhibited IL-12 p40 mRNA expression, our subsequent objective was to determine if this occurs through the repression of the IL-12 p40 promoter. To analyze promoter activity, a 3.3 kb luciferase-tagged IL-12 p40 construct was created and assembled into a recombinant adenoviral vector. Both THP-1 cells and primary monocytes were studied. The monocyte cell line, THP-1 was chosen for its ease of adenoviral infection and promoter activity analysis. Cells (2×10^6) were infected with the adenoviral luciferase construct (MOI 50) and stimulated with LPS for 48 hours. LPS induced an increase in promoter activity relative to unstimulated cells ($18,900 \pm 3200$ Relative Luciferase Units (RLU) vs. $8,600 \pm 1100$ RLU). This activation was inhibited by p38_i and JNK_i ($13,000 \pm 3400$ RLU and $10,200 \pm 3300$ RLU respectively) but was not affected by ERK_i. (Figure 7 A).

Adherence derived primary monocytes (2×10^6 cells) were infected with the adenoviral luciferase construct (MOI 50). Cells were stimulated with LPS for 48 hours to induce promoter activity. LPS induced IL-12 p40 promoter activity relative to unstimulated cells (327 ± 42 RLU vs. 159 ± 20 RLU respectively) (Figure 7 B). Promoter activity decreased with p38_i and JNK_i inhibition (254 ± 31 RLU and 223 ± 39 RLU, respectively). These results indicate that inhibition of p38 and JNK reduce LPS-induced activation of the IL-12 p40 promoter, while ERK has no effect in either THP-1 or primary monocytes.

A



B

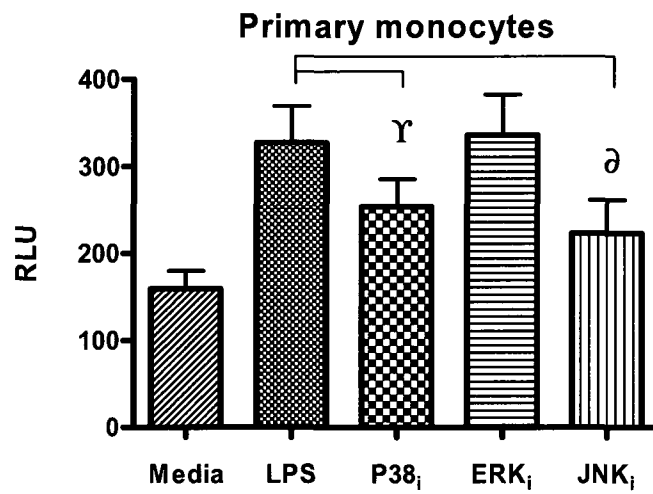
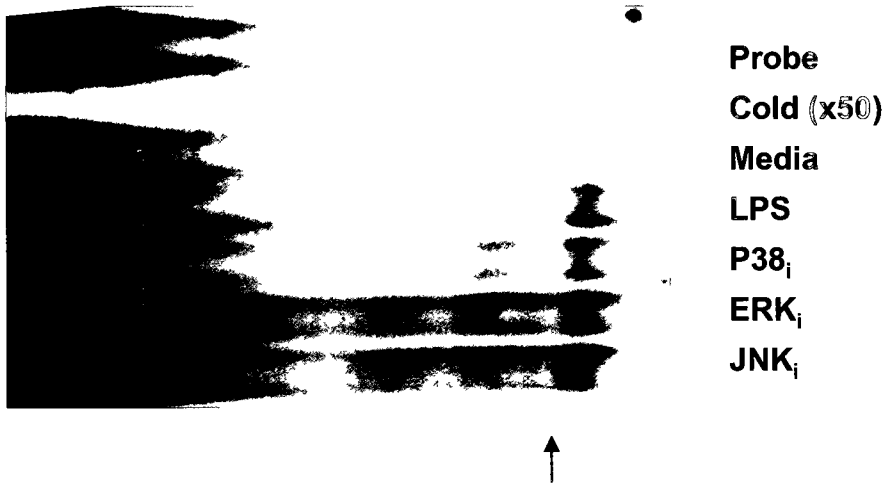


Figure 7: Inhibition of p38 and JNK resulted in decreased IL-12 p40 promoter activity. **A)** THP-1 cells (2×10^6) and **B)** primary adherence derived monocytes (2×10^6) were infected with an adenoviral luciferase construct, containing a tagged IL-12 p40 promoter. Cells were cultured with LPS in the presence or absence of SAPK/MAPK inhibitors (p38, 10 μ M of SB203580; ERK, 10 μ M of PD98059; JNK, 10 μ M of SP600125). Relative luciferase units were normalized to the total protein content of cellular extracts. Primary monocyte infection with adenovirus is relatively inefficient compared to the THP-1 cell line and this is reflected in the relatively small induction of the promoter activity (RLU) in primary cells. Decreased IL-12 p40 promoter activity by inhibition of p38 and JNK was statistically significant in primary monocytes $n=8$ (Υ , $p=0.0051$; ∂ , $p=0.0009$ respectively) and THP-1 cells (*, $p=0.0159$; ψ , $p=0.0369$ respectively), $n=3$ by student t-test. Error bars represent SEM.

D. The role of p38, JNK and ERK on nuclear factor binding to NFκB, AP-1, Sp-1 and Ets-2 promoter sites. The IL-12 p40 promoter is composed of 9 promoter sites (274), four of which have been shown to be deregulated with *in vitro* HIV infection of primary adherence derived monocytes: NFκB, AP-1, Sp-1 and Ets-2 (120). Transcription factors have been shown to be activated by p38, ERK and JNK signalling cascades through phosphorylation (127). Given that p38_i and JNK_i affect IL-12 p40 promoter activity, our objective was to determine if this occurs by disruption of nuclear factor binding to the IL-12 p40 promoter. Five million adherence derived monocytes were pre-treated with the p38_i (10 uM of SB203580), the JNK_i (1 nM dexamethasone), or the ERK_i (10 uM PD 98059) followed by 2 hours of stimulation with LPS. Inhibitor effects were determined by comparing band shift observed with an electrophoretic mobility shift assay. Binding to the NFκB is enhanced following LPS stimulation as compared to unstimulated cells. Inhibition of either p38, ERK or JNK had little effect on LPS-induced NFκB binding (Figure 8 A). Nuclear factor binding to the Sp-1 site is the most prominent in unstimulated cells. Stimulation with LPS results in decreased Sp-1 binding. Both p38_i and JNK_i increased Sp-1 binding relative to LPS alone, while ERK_i seemed to have no effect. (Figure 8 B). Nuclear factor binding to the AP-1 site is reduced by LPS stimulation. JNK_i and to a lesser extent, p38_i and ERK_i increase AP-1 binding relative to LPS stimulation alone (Figure 9 A). Stimulation with LPS induced binding to the Ets-2 site compared to unstimulated cells. P38_i, ERK_i and to a lesser extent, JNK_i prevented LPS induced binding to the Ets-2 site (Figure 9 B). These data indicate that inhibition of kinases affect nuclear factor binding to the IL-12 p40 promoter.

A. NFκB



B. Sp-1

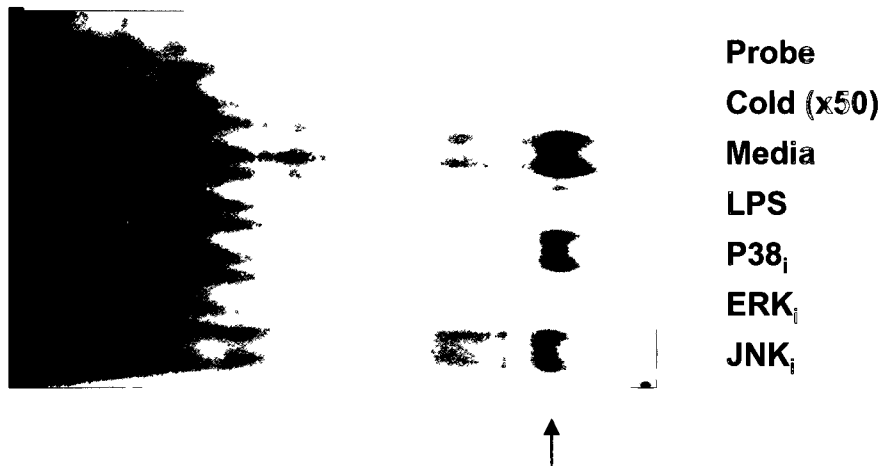
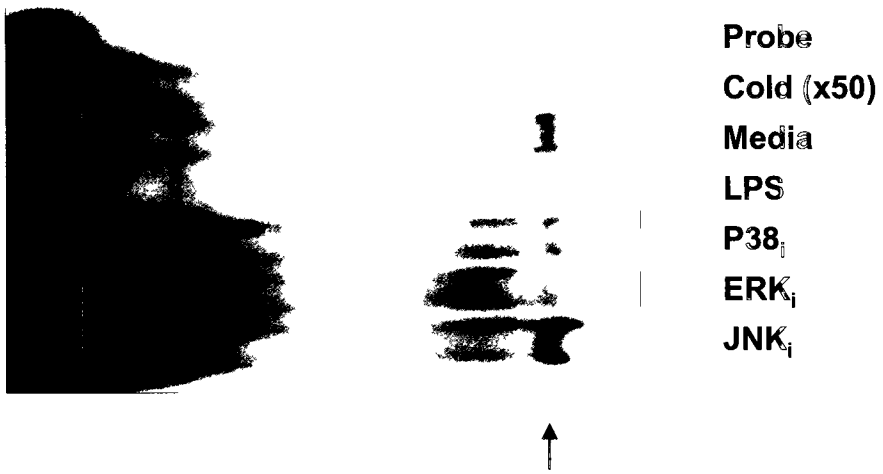


Figure 8: Inhibition of JNK and p38 altered nuclear factor binding to NFκB and

Sp-1 promoter sites. Isolated primary monocytes were stimulated with LPS for two hours in the presence and absence of SAPK/MAPK inhibitors (p38_i, 10 μM of SB203580; ERK_i, 10 μM of PD98059; JNK_i, 1 nM of dexamethasone) prior to nuclear extraction. Electrophoretic mobility shift assays were performed for nuclear factor binding to NFκB (**A**) and Sp-1 (**B**) sites in the IL-12 p40 promoter. Images shown are representative of three independent experiments. Arrows indicate the position of the nuclear factor complex.

A. AP-1



B. Ets-2

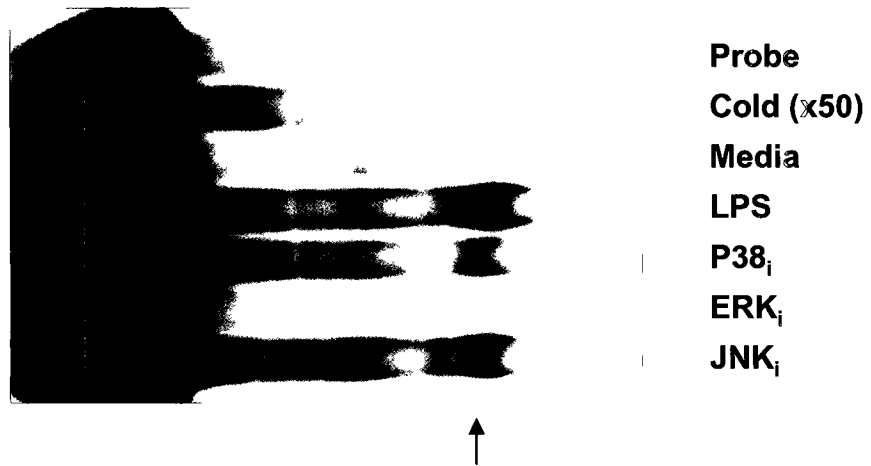


Figure 9: Inhibition of JNK and p38 altered nuclear factor binding to AP-1 and Ets-2 promoter sites. Isolated primary monocytes were stimulated with LPS for two hours prior to nuclear extraction, in the presence and absence of SAPK/MAPK inhibitors (p38_i, 10 μ M of SB203580; ERK_i, 10 μ M of PD98059; JNK_i, 1 nM of dexamethasone). Electrophoretic mobility shift assay was performed for nuclear factor binding to the AP-1 (**A**) and Ets-2 (**B**) sites in the IL-12 p40 promoter. Images shown are representative of three independent experiments. Arrows indicate the position of the nuclear factor complex.

IV – RESULTS

Following the implication of the LPS kinase signalling cascade in the production of IL-12 p40, the expression of the upstream LPS receptors CD14 and TLR4 was measured with and without HIV. In addition, the implication that the HIV protein Vpr plays a role IL-12 production by Mirani *et al.* was further investigated (198). To this end, Vpr-mediated inhibition of IL-12 p40 was determined and Vpr-mediated effect on the upstream signalling receptors CD14 and TLR4. The expression of the Vpr co-receptor GR was also evaluated in the context of both HIV infection and Vpr treatment.

A. *In vitro* infection of PBMC altered CD14, TLR4 and GR expression on gated monocytes in a PBMC culture. Suppression of IL-12 is an important mechanism of HIV cell-mediated immune pathogenesis (35, 73). IL-12 p40 production is dependant on several upstream signalling receptors including TLR4, CD14 and GR (86, 221). Our objective was to determine if CD14, TLR4 or GR expression were altered by *in vitro* infection of PBMC with HIV. To investigate this effect, cells were infected, or mock infected, with the laboratory X4 tropic strain HIV-1_{IIIB}, the dual-tropic clinical isolate HIV-1_{cs204}, or with the R5 tropic strain HIV-1_{ADA}. Four days post-infection, PBMC were fixed, permeabilized and labeled with FITC-conjugated anti-CD14 mAb, PE-conjugated anti-TLR4 mAb, or FITC-conjugated anti-GR mAb. Cells were then analyzed by flow cytometry. Monocytes were gated based on size and granularity, and this was confirmed by both CD40 and CD14 expression.

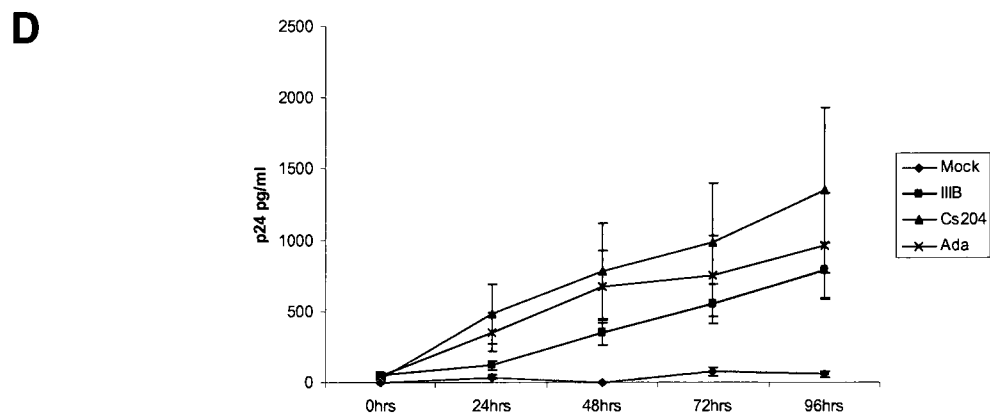
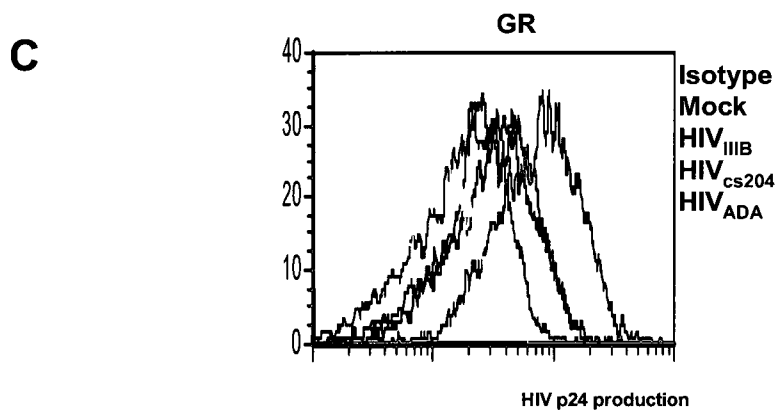
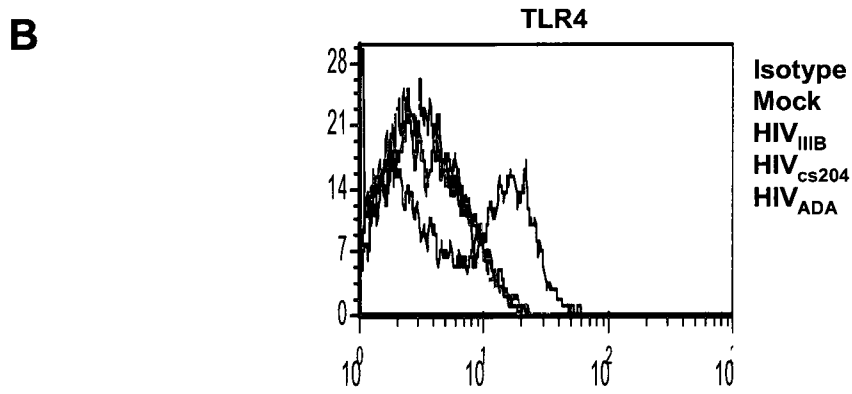
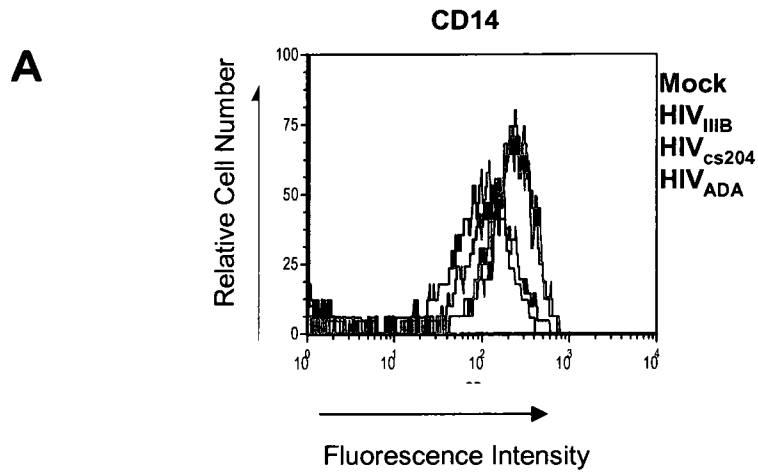


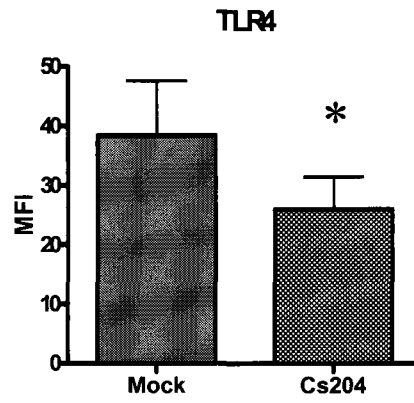
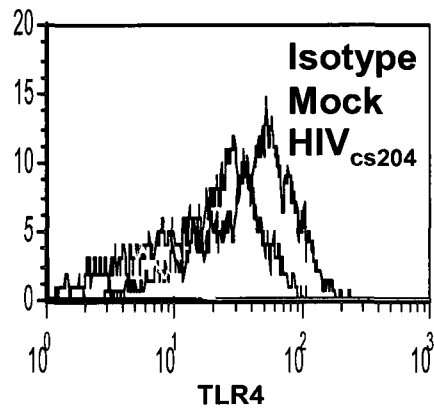
Figure 10: *In vitro* HIV infection of PBMC altered CD14, TLR4 and GR expression on monocytes. PBMC were either mock infected or infected with the laboratory X4 tropic strain HIV-1_{IIIB}, the dual-tropic clinical isolate HIV-1_{cs204}, or with the R5 tropic strain HIV-1_{ADA}. Cells were labeled with **A)** FITC-conjugated anti-CD14 mAb, **B)** PE-conjugated anti-TLR4 mAb or **C)** FITC-conjugated anti-GR mAb, and receptor expression was assessed by flow cytometry. The CD14 expression following HIV-1_{cs204} or HIV-1_{ADA} infection was increased, when compared to mock infection. The TLR4 receptor expression was decreased and GR expression was increased following infection with all three viruses, as compared to mock infection. **D)** *In vitro* HIV infection of PBMC was monitored by p24 production. Data shown is representative of three independent experiments.

The analysis of CD14 expression indicated an increase in CD14 following HIV-1_{cs204} or HIV-1_{ADA} infection, when compared to mock infection. HIV-1_{III B} infection, however, did not alter CD14 expression on gated-monocytes within the infected culture (Figure 10 A). The analysis of CD14 expression indicated potential tropism effects. A notable decrease in TLR4 receptor expression was observed following infection with all three viruses, as compared to control (Figure 10 B). Infection with all virus strains also increased GR expression relative to control cells, particularly those infected with HIV-1_{cs204} (Figure 10 C).

B. The effect of HIV on LPS-induced regulation of CD14, TLR4 and GR.

Since cellular responses to secondary immune stimulation are important in understanding the mechanism of HIV pathogenesis, we investigated how receptor expression patterns change in response to secondary stimulation. PBMC cultures were infected or mock infected with HIV-1_{cs204} then stimulated with LPS for 24 hours. Cells were then fixed, permeabilized and labeled with FITC-conjugated anti-CD14 mAb, PE-conjugated anti-TLR4 mAb, or FITC-conjugated anti-GR mAb, followed by flow cytometric analysis. Infection of cells with HIV-1_{cs204} significantly reduced the expression of TLR4 on monocytes in LPS-stimulated PBMC culture relative to LPS-stimulated mock infection (26±5 mean fluorescence intensity (MFI), 39±9 MFI, respectively) (Figure 11 A). HIV-1_{cs204} was also found to significantly increase CD14 expression as compared to LPS-stimulated mock infection (125±35 MFI, 22±2 MFI, respectively) (Figure 11 B). GR expression was unaffected by LPS in HIV-1_{cs204} infected cells (Figure 12 A). *In vitro* HIV-1_{cs204} infection of PBMC samples was monitored by p24 production (Figure 12 B).

A



B

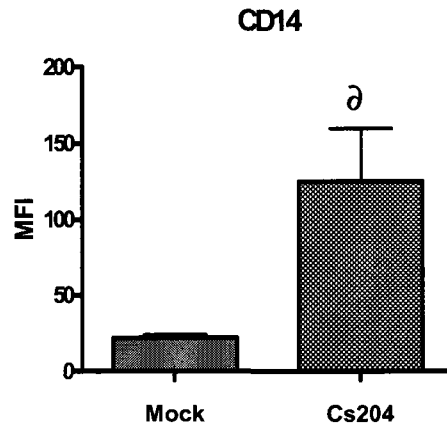
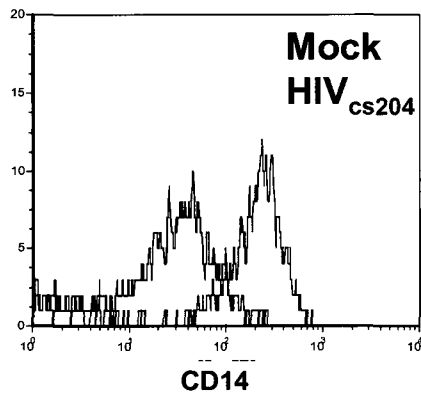


Figure 11: Infection with HIV_{cs204} decreased TLR4 and increased CD14 expression in LPS-stimulated monocytes. PBMC were either mock infected or infected with the dual tropic strain HIV_{cs204}, and stimulated with LPS for 24 hours **A)** PE-conjugated anti-TLR4 receptor expression was measured on monocytes following LPS stimulation (*, p=0.048). **B)** FITC-conjugated anti-CD14 receptor expression was measured on monocytes following LPS stimulation (∂, p=0.047), n=3, statistical significance was determined by student t-test. Error bars represent SEM.

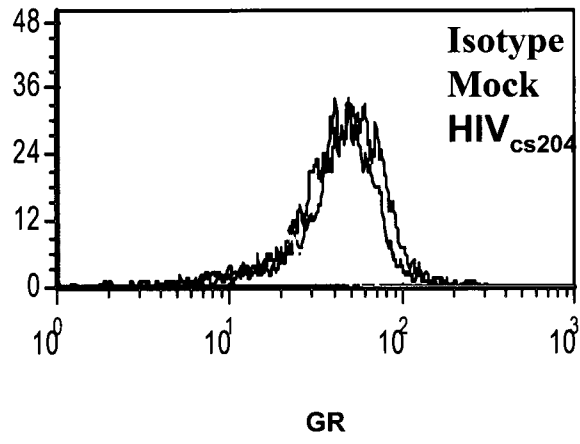
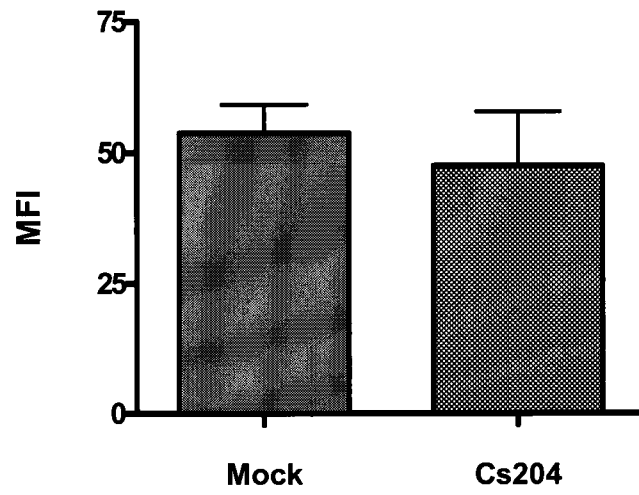
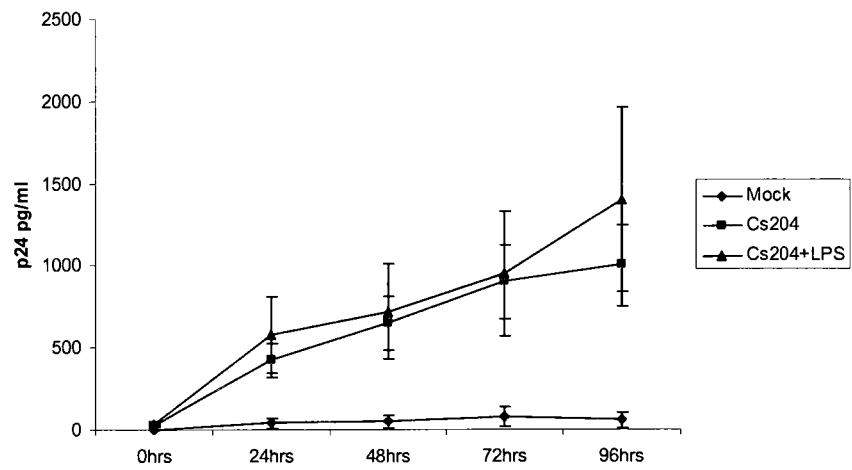
A**GR****HIV p24 production****B**

Figure 12: GR expression remained unchanged in HIV_{cs204} infected monocytes stimulated with LPS. A) PBMC were either mock infected or infected with HIV_{cs204}, and stimulated with LPS for 24 hours. GR expression was measured following LPS stimulation, n=3. Error bars represent SEM. **B)** In vitro HIV infection of PBMC was monitored by p24 production.

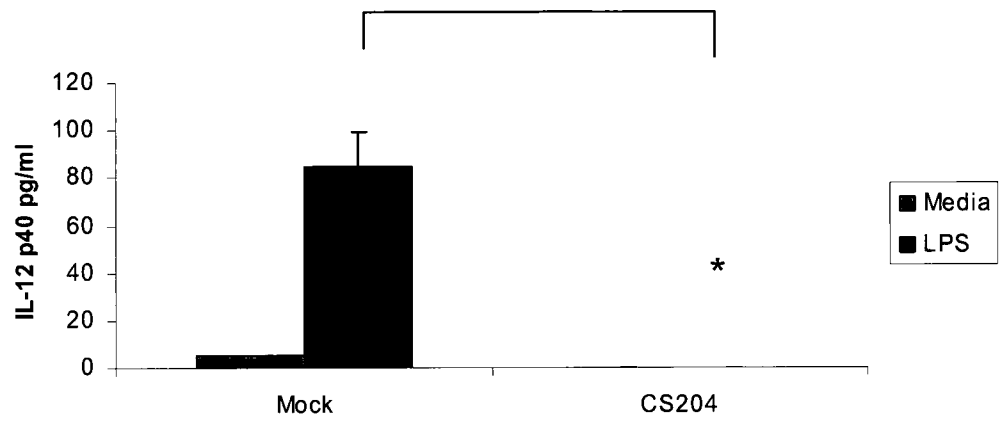


Figure 13: LPS did not enhance IL-12 p40 in HIV infection of PBMC. PBMC were either infected or mock infected with HIV_{cs204} and stimulated with LPS for 24 hours. Cell-free supernatants were collected and analyzed for IL-12 p40 by ELISA. HIV_{cs204} significantly inhibited LPS-induced IL-12 p40 production in PBMC, n=3 (*, p=0.027) by student t-test. Error bars represent SEM.

C. The effect of HIV infection on IL-12 p40 production in PBMC.

Since LPS signalling receptors were shown to be modulated by HIV infection, the downstream effect on IL-12 p40 production was examined. To do this, IL-12 p40 production was evaluated in HIV-1_{cs204} infected cells with and without secondary LPS stimulation. Following 24 hours of LPS stimulation, PBMC culture supernatants were collected and analyzed by ELISA. As expected, LPS enhanced IL-12 p40 production in uninfected cells (85 ± 15 pg/ml). In cells infected with HIV_{cs204} IL-12 p40 was undetectable even after LPS stimulation (Figure 13).

D. C-terminal Vpr peptide inhibited LPS stimulated IL-12 p40 production in PBMC. Since HIV prevents LPS induced IL-12 p40 production, further investigation of this mechanism was undertaken. Given that Vpr binds to GR (201), and we demonstrated that the ligation of GR by dexamethasone (see Figure 5) leads to a decrease in LPS-induced IL-12 p40 production, Vpr was chosen as a likely target for further investigation. Two peptides were tested in this experiment: a C-terminal peptide fragment (52-96 amino acids) which encompasses the GR binding motif between 64-68 amino acids, and an N-terminal Vpr peptide (1-45 amino acids) to control for general peptide effects. Isolated PBMC were treated with a range of C-terminal peptide 0.5-500 nM to determine its effect on LPS induced IL-12 p40 production. The C-terminal peptide significantly inhibited LPS stimulation of IL-12 p40 in a dose dependant manner, with a decrease in IL-12 p40 observed at concentrations as low as 5 nM as compared to LPS stimulation alone (352 ± 88 pg/ml and 190 ± 39 pg/ml respectively) (Figure 14). Treatment of cells with 100 nM of N-terminal Vpr peptide leads to a modest decrease in IL-12 p40 protein although not statistically significant (Figure 14).

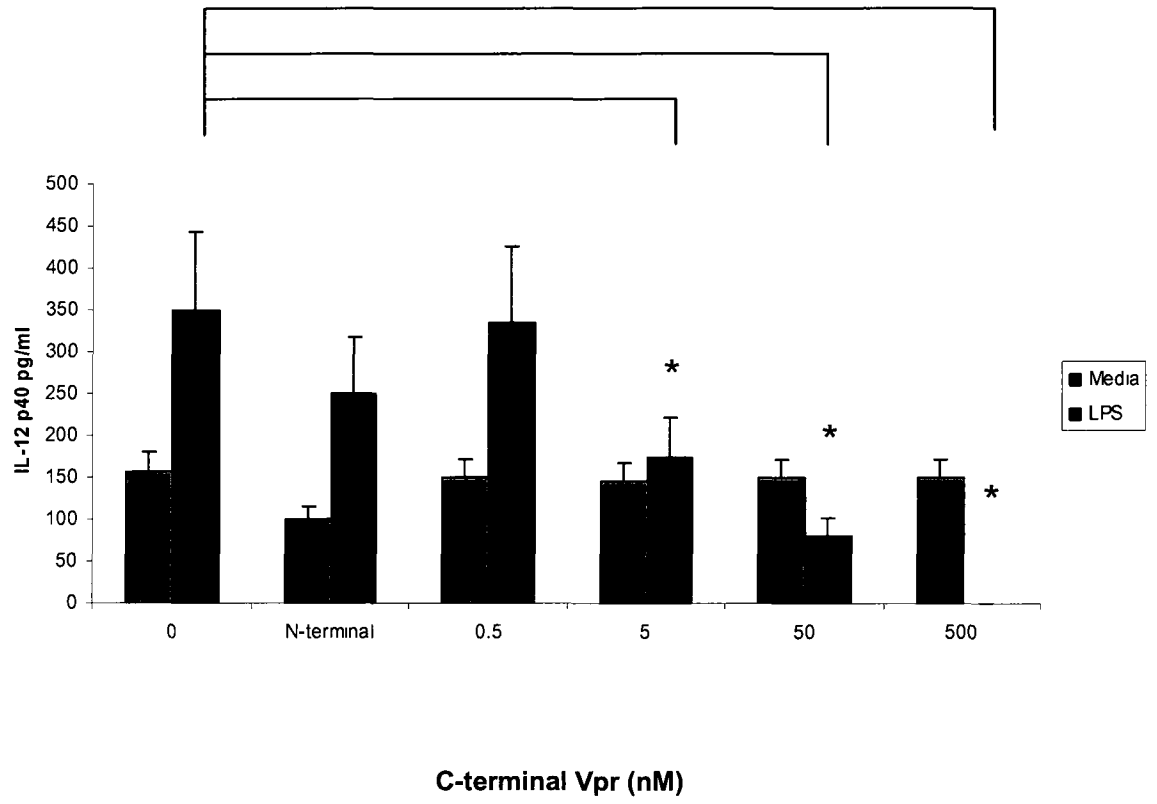


Figure 14: C-terminal Vpr peptide inhibited LPS-stimulated IL-12 p40 production in PBMC. Cells were pre-treated with C-terminal (0.5-500 nM) or N-terminal (100 nM) Vpr peptides, for 30 minutes, before stimulation with LPS. Cell-free supernatants were analysed for IL-12 p40 protein production by ELISA. C-terminal Vpr (5-500 nM) mediated inhibition of IL-12 p40 was statistically significant as compared to untreated LPS stimulated control, n=4. Statistical significance was determined by ANOVA (*, p=0.0001) with Dunnett's post-test. Error bars represent SEM.

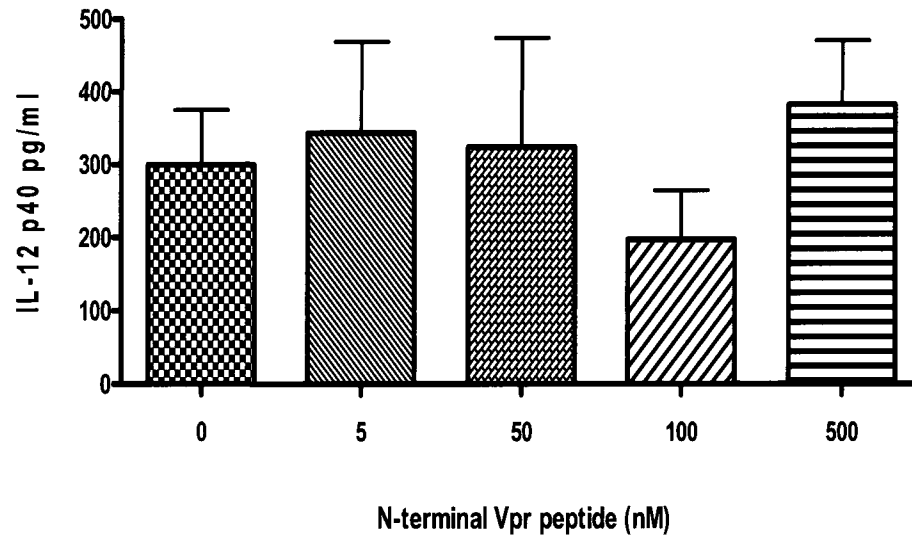


Figure 15: The control N-terminal Vpr peptide does not affect LPS-stimulated IL-12 p40 production in PBMC. Cells were pre-treated with N-terminal Vpr peptides (0.5-500 pM) for 30 minutes before stimulation with LPS for 24 hours. Cell-free supernatants were analysed for IL-12 p40 protein production by ELISA. N-terminal Vpr peptide did not alter IL-12 p40 production n=4. Error bars represent SEM.

A dose response curve reveals that cells were unaffected by the N-terminal peptide with concentrations up to 500 nM (Figure 15).

E. C-terminal Vpr-mediated inhibition of LPS-stimulated IL-12 p40 production was prevented by GR inhibition. The Vpr cellular receptor binding partner is the GR (201) and preventing GR signalling has been shown to prevent some Vpr-mediated function (198). In this study, the effect of the inhibition of GR signalling on exogenous addition of C-terminal Vpr was investigated. Isolated PBMC were pre-treated with the GR inhibitor (GR₁), RU486, for one hour prior to the addition of Vpr peptide. Cells were then stimulated with LPS, or left as unstimulated controls. As previously demonstrated (Figure 14) cells treated with C-terminal Vpr had decreased IL-12 p40 production, compared to PBMC with LPS alone (100±SEM pg/ml, 300±SEM pg/ml respectively). When cells were pretreated with GR₁ the IL-12 p40 production was restored (250±SEM pg/ml) (Figure 16).

F. Vpr enhances dexamethasone inhibition of LPS-induced IL-12 p40 production. Infection with HIV leads to a deregulation of the HPA axis and ultimately an increase in systemic cortisol (238). The pharmacological cortisol mimic, dexamethasone, and Vpr both inhibit IL-12 p40, but it was not known if there is an additive effect on cytokine production when both are present. Our objective was to confirm that dexamethasone can inhibit IL-12 p40 production in PBMC and to determine if Vpr enhances this effect. Isolated PBMC were treated with increasing concentrations of dexamethasone (0.01-1000 nM) and subsequently stimulated with LPS. Dexamethasone was found to inhibit IL-12 p40 production relative to controls, in a dose dependant manner (Figure 17 A). To determine if there was an additive, or

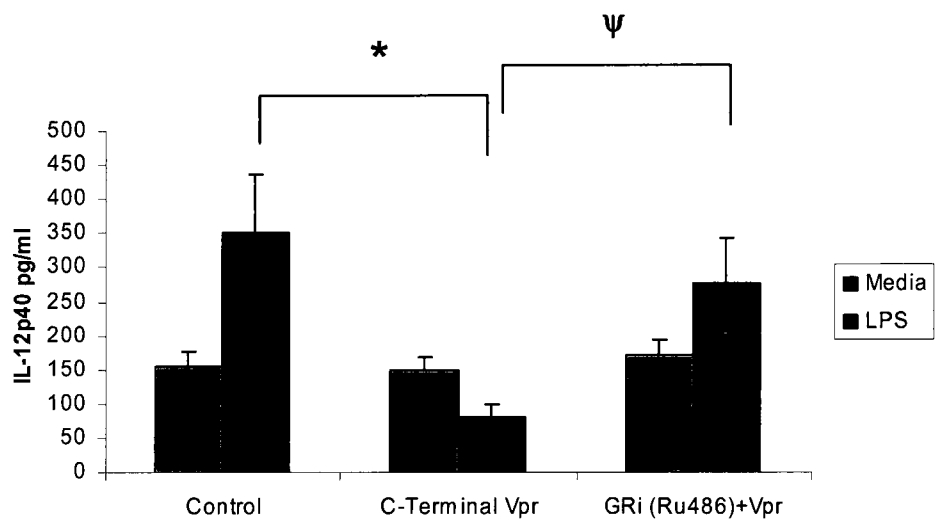
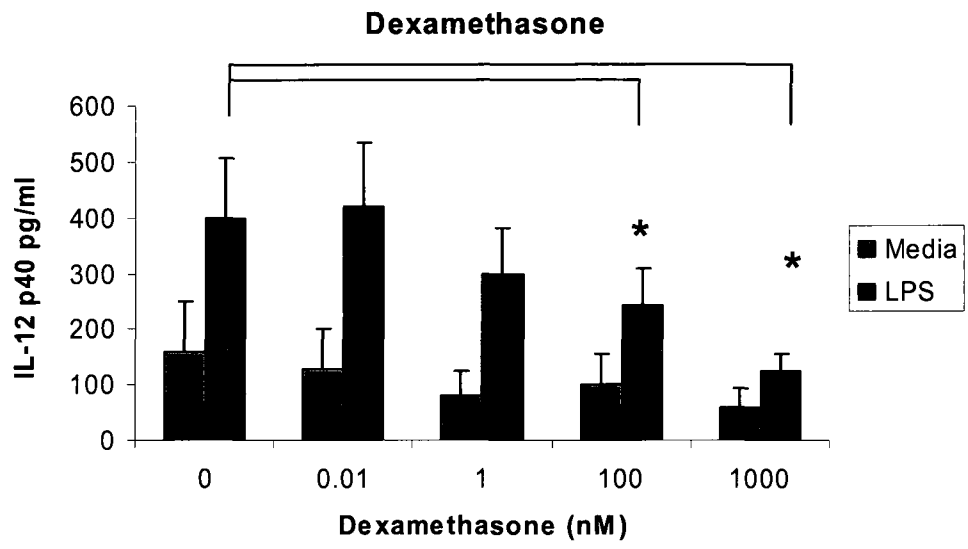


Figure 16: C-terminal Vpr mediated inhibition of LPS-stimulated IL-12 p40 production is prevented by inhibiting GR signalling. Cells were pre-treated (or left untreated) with the GR_i, Ru486 (7 uM) for 1 hour, before the addition of C-terminal Vpr (50 nM) for 30 min. Cells were then stimulated with LPS for 24 hours. Cell- free supernatants were collected and IL-12 p40 production was analyzed by ELISA. C-terminal Vpr inhibited IL-12 p40 production when compared to LPS stimulation alone (*, p=0.0205). Pre-treating the cells with the GR_i prior to Vpr rescued IL-12 p40 production PBMC, n=4 (∂, p=0.0319). Error bars represent SEM and statistical significance was determined by student t-test.

A



B

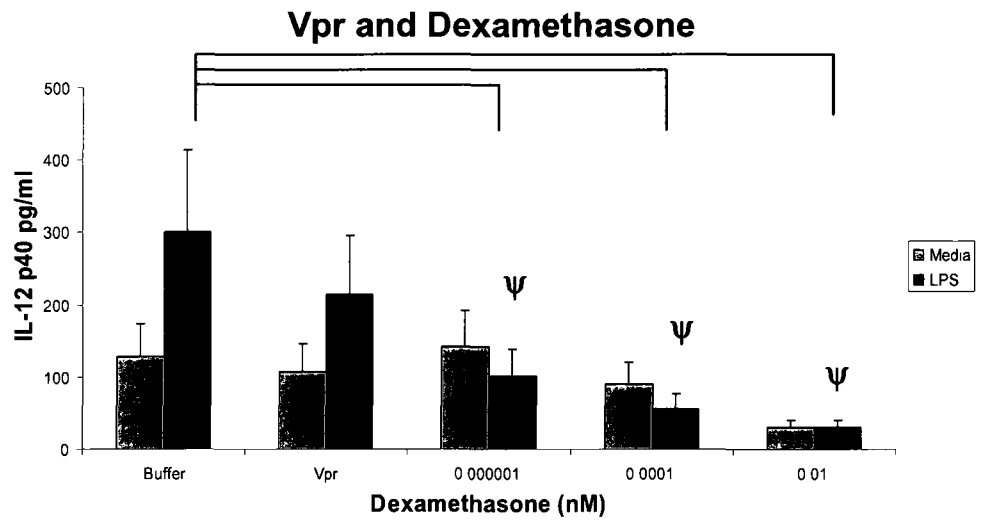


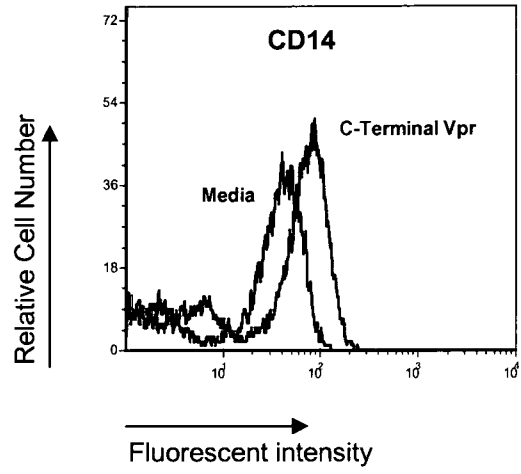
Figure 17: Dexamethasone inhibited LPS-stimulated IL-12 p40 production and enhances Vpr-mediated inhibition of IL-12 p40. **A)** PBMC were cultured with or without dexamethasone (0.01-1000 nM) for one hour prior to LPS stimulation 24 hours. Cell-free supernatants were collected and IL-12 p40 production was analyzed by ELISA. Dexamethasone demonstrated a dose dependant inhibition of IL-12 p40 in PBMC (*, $p=0.0087$). **B)** PBMC were treated with 5 nM of C-terminal Vpr peptide for 30 minutes, followed by dexamethasone treatment for 1 hour (0.000 001-0.01 nM). Cells were then stimulated with LPS for 24 hours. Cell-free supernatants were collected and IL-12 p40 production was analyzed by ELISA. C-terminal Vpr enhanced dexamethasone effect on IL-12 p40 production (ψ , $p=0.0004$). Statistical significance was determined by ANOVA with Dunnett's post-test. Error bars represent SEM, $n=4$.

enhanced effect between dexamethasone and C-terminal Vpr, PBMC were pre-treated with 5 nM of C-terminal Vpr for 30 min prior to dexamethasone treatment (0.000 001-0.01 nM). Cells treated with both dexamethasone and C-terminal VPR had decreased production of IL-12 p40 when compared to dexamethasone alone (Figure 17 B).

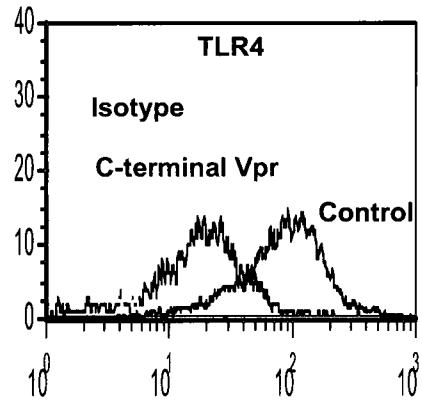
G. C-terminal Vpr peptide altered CD14, TLR4 and GR expression.

Our earlier experiments demonstrated altered CD14, TLR4 and GR expression by infection with HIV. Given the Vpr-mediated effect on LPS-induced IL-12 p40 production, the objective of these experiments was to investigate the effect of Vpr on CD14, TLR4 and GR in LPS-stimulated PBMC. To investigate this phenomenon PBMC were treated with 50 nM C-terminal Vpr and then stimulated with LPS for 24 hours. Cells were then collected, fixed, permeabilized and labeled with FITC-conjugated anti-CD14 mAb, PE-conjugated anti-TLR4 mAb and FITC-conjugated anti-GR mAb. Cells were then analyzed for total receptor expression. The expression of CD14 and GR was found to be increased by C-terminal Vpr when compared to LPS-stimulated control cells (Figure 18 A, C). TLR4 expression was found to be decreased by C-terminal Vpr when compared to LPS-stimulated control cells (Figure 18 B).

A



B



C

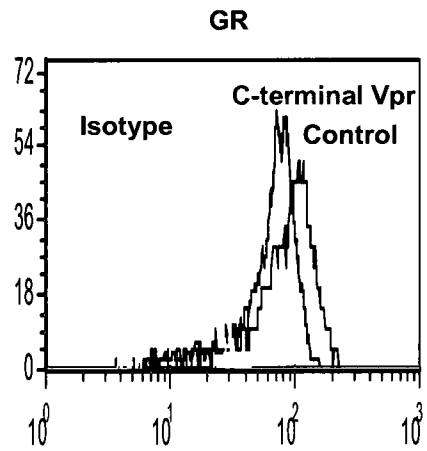


Figure 18: C-terminal Vpr peptide altered CD14, TLR4 and GR expression in LPS-stimulated PBMC. Cells were pre-treated with C-terminal Vpr peptides (50 nM) for 30 min, then stimulated with LPS for 24 hours and compared to LPS-stimulated control. Cells were labeled with **A)** FITC-conjugated anti-CD14 mAb, **B)** PE-conjugated anti-TLR4 mAb or **C)** FITC-conjugated anti-GR mAb, and receptor expression was assessed by flow cytometry. Data shown is representative of three independent experiments.

H. C-terminal Vpr-induced increases in GR expression while GR_i prevents these effects. GR expression is regulated by a positive feedback loop following ligand binding (227). Our objective was to investigate if C-terminal Vpr can initiate this positive feedback loop through GR expression. PBMC were treated (or untreated) with GR_i (Ru486) or dexamethasone for one hour, followed by the addition of C-terminal Vpr peptide (30 min) and stimulated with LPS (24 hours). GR expression was then analyzed by flow cytometry in the presence and absence of Vpr. As shown previously, Vpr increased GR expression in LPS stimulated cells when compared to LPS stimulation alone. Vpr-mediated an increase in GR expression in LPS-stimulated cells treated with dexamethasone. Treatment with GR_i prevented the Vpr-mediated effect (Figure 19). The changes to GR expression by various ligands and signalling inhibitors are of particular interest when considering the concomitant effects they have on IL-12 p40 expression. These results suggest a role for GR expression in IL-12 p40 production.

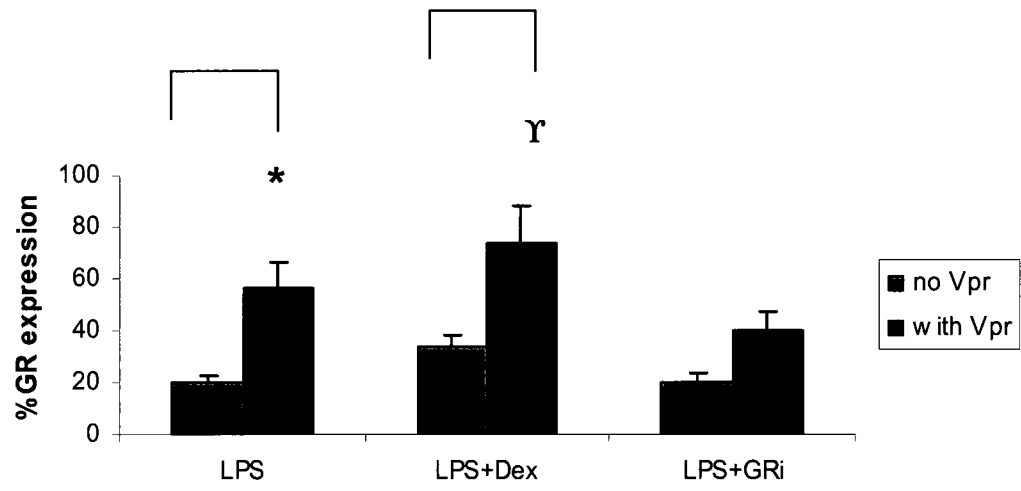


Figure 19: Vpr-induced GR expression while the GR_i prevented Vpr-mediated effects. PBMC were treated (or untreated) with C-terminal Vpr peptides (50 nM) for 30 min, then stimulated with LPS for 24 hours (LPS). Cells were also either pre-treated with 10 pM Dexamethasone (LPS+Dex) or 7 uM GR_i (LPS+GR_i) followed by the addition of Vpr peptide. Cells were then labeled with FITC-conjugated anti-GR mAb, and receptor expression was assessed by flow cytometry. Vpr-mediated increased GR expression was observed which was prevented by GR_i, n=3, (*, p=0.0378; †, p=0.0256) by student t-test.

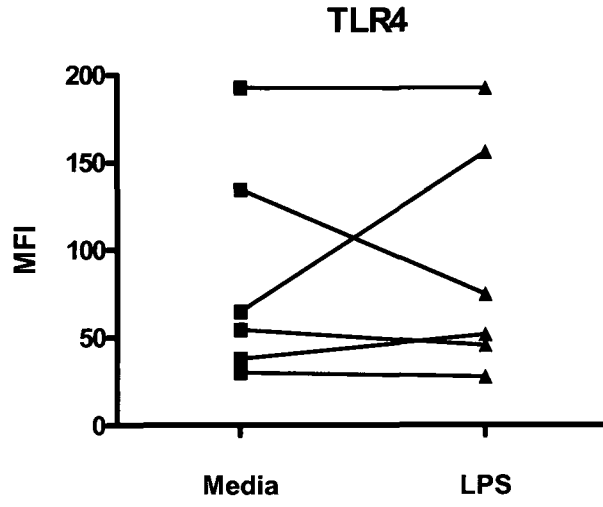
V - RESULTS

The previous chapter evaluated the effects of HIV and extracellular Vpr peptide on IL-12 p40 and upstream signalling receptors. Because intracellular expression of Vpr is more physiologically relevant, a Vpr retroviral expression system was developed. This system was used to further evaluate the Vpr-mediated effects on LPS-induced IL-12 p40 production and receptor expression.

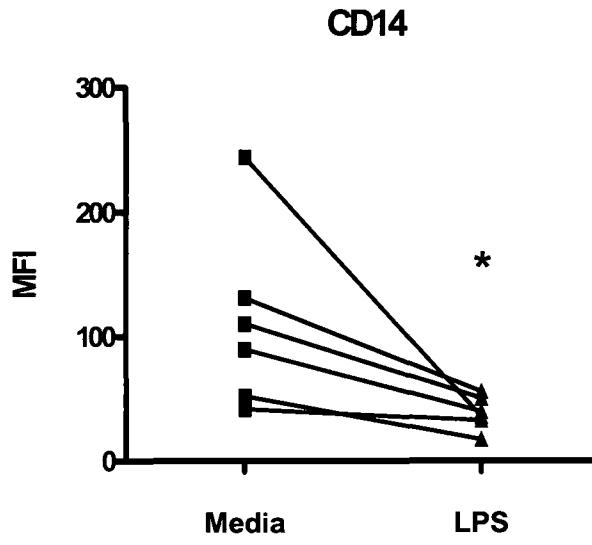
A. LPS altered CD14, GR and TLR4 expression on monocytes in PBMC culture compared to unstimulated cells. Since the retroviral expression system requires several days of culture, and culture itself can affect receptor expression, a new baseline of expression levels had to be established. In addition to this, further receptor analysis was done to examine the change in expression following LPS stimulation. Cells were isolated and cultured in suspension for 48 hours, followed by LPS stimulation (or control) for 24 hours. PBMC were then collected, fixed, permeabilized and labeled with FITC-conjugated anti-CD14 mAb, PE-conjugated anti-TLR4 mAb or FITC-conjugated anti-GR mAb. Gated monocytes were then analyzed by flow cytometry. Analysis of LPS-induced TLR4 expression demonstrated minimal change (Figure 20 A). LPS stimulation reduced CD14 expression when compared to control cells (MFI=104±26 vs. MFI=36±5 respectively) (Figure 20 B). LPS stimulation did not induce a change in GR expression (Figure 20 C).

B. LPS-induced production of IL-23, IL-27 and IL-12 p40 protein production in PBMC culture. To eventually characterize the effects of Vpr on IL-12 family cytokines, cytokine production was first measured in response to LPS stimulation of

A



B



C

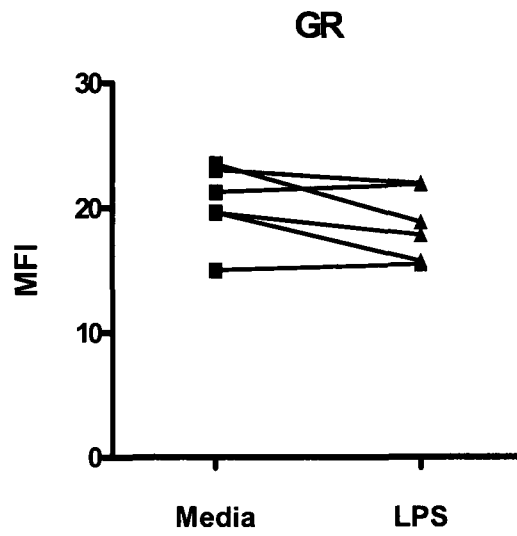
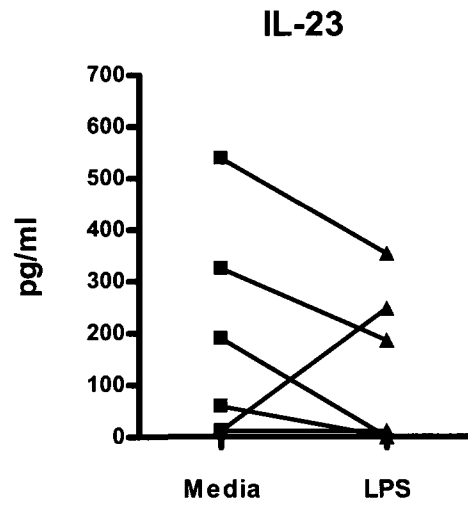
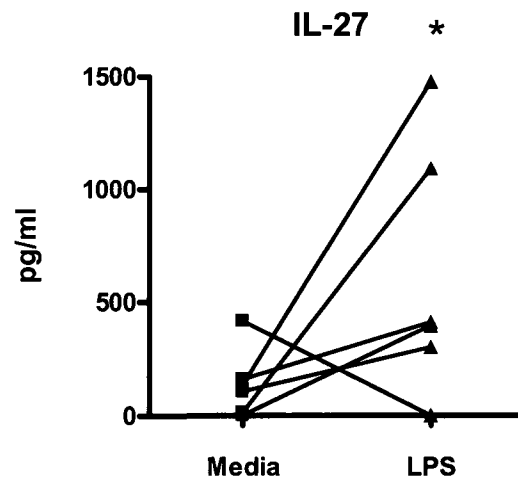


Figure 20: LPS stimulation reduced CD14 expression on gated monocytes in PBMC culture. PBMC were cultured for 48 hours and LPS-stimulated (or unstimulated) for 24 hours. Cells were then collected, fixed, permeabilized and labeled with **A)** FITC-conjugated anti-TLR4 mAb, **B)** PE-conjugated anti-CD14 mAb, **C)** FITC-conjugated anti-GR mAb. Receptor expression was analyzed by flow cytometry, on a gated monocyte population. Data was measured by mean fluorescent intensity, and statistical significance was determined by student t-test, n=6, (*, p=0.0255).

A



B



C

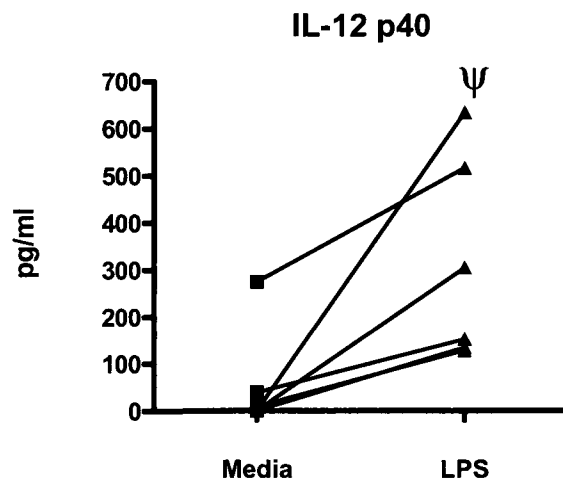


Figure 21: LPS stimulation induced IL-27 and IL-12 p40, but not IL-23 production in PBMC culture. PBMC were cultured for 48 hours, then stimulated with LPS for 24 hours. Cell-free supernatants were collected and analyzed by ELISA, n=6, (*, p=0.044; ψ , p=0.03) . Statistical significance was determined by student t-test.

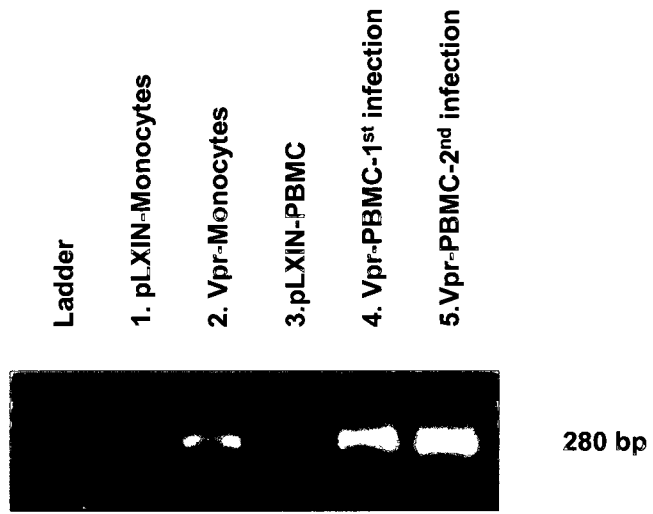
PBMC. Cells were cultured for 2 days and stimulated with LPS for 24 hours. Cell free supernatants were then analysed by ELISA. LPS stimulation did not induce IL-23 production in PBMC (Figure 21 A). LPS stimulation induced IL-27 production relative to control (612 ± 226 pg/ml vs. 137 ± 62 pg/ml) (Figure 21 B). As expected, LPS stimulation also induced IL-12 p40 production in this culture system as compared to control (233 ± 59 pg/ml vs. 72 ± 34 pg/ml) (Figure 21 C).

C. Intracellular Vpr expression: Development of a retroviral vector. Vpr has been measured extracellularly in the circulating serum of some patients (154) although the majority of Vpr is expressed intracellularly (275). To better understand the potential physiological impact of intracellular Vpr, a retroviral expression system was constructed (Figure 22 A). The integration of pLXIN-Vpr in the transfected PT67 cells was verified by RT-PCR analysis (280 bp fragment) of the RNA from PT67 cells 7 days after transfection (Figure 22 A). The ability of the virus to infect other cell types (monocytes and PBMC) was also verified by RT-PCR (Figure 22 A). The intracellular expression of Vpr protein in PT67 cells and PBMC was determined by Western blot analysis (14 kDa) (Figure 22 B).

D. Retrovirus alone induced TLR4 expression on monocytes in response to LPS stimulation, while Vpr prevented this induction. TLR4 expression is critical to initiate the LPS-induced cellular response. TLR4 expression was measured following infection with retroviral vectors in the presence and absence of LPS stimulation. PBMC were infected with pLXIN- or Vpr- retrovirus, and were subsequently stimulated (or unstimulated) with LPS for 24 hours. Gated monocytes were analyzed for TLR4 expression (Figure 23 A). Prior to LPS stimulation, both pLXIN and Vpr expressing

Vpr PCR

A



B

Western

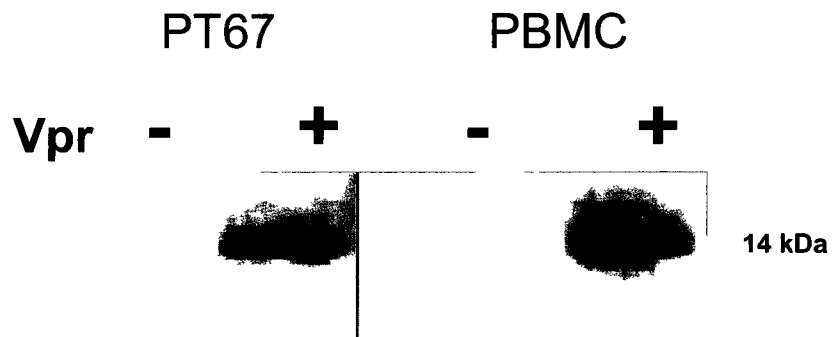


Figure 22: Intracellular Vpr expression: Development of a retroviral vector Vpr. Vpr from the HIV plasmid p89.6 was cloned into a pLXIN vector and packaged into retrovirus by PT67 cells. Viral infectivity was confirmed in THP-1 cells, monocytes and PBMC by RT-PCR. **A)** Verification of the 280 Vpr fragment by RT-PCR: Lane 1. PT67 integrated stock; lane 2. Vp-infected THP-1; lane 3. Vpr-infected monocytes; lane 4. Vpr-infected PBMC. **B)** Vpr protein expression was determined by Western blot in integrated PT67 cells and in infected PBMC.

cells have similar expression of TLR4 (MFI=71±22 and MFI=73±16). In response to LPS stimulation, however, only cells infected with the pLXIN retrovirus show enhanced expression of TLR4 (MFI=120±40) (Figure 23 B). The change in expression following LPS stimulation is demonstrated in Figure 23 C, where the expression of TLR4 was increased by 2±0.5 fold in pLXIN infected cells whereas in Vpr infected cells the relative change was 0.8±0.1 in response to LPS stimulation. Overall, Vpr alters the TLR4 expression in response to LPS, by preventing the upregulation of TLR4 following LPS stimulation. These results also demonstrate that retrovirus itself enhances the LPS-induced TLR4 expression (Figure 23 A) when compared to uninfected LPS stimulated cells (Figure 20 A).

E. Vpr mediated TLR4 expression in response to LPS stimulation, through GR.

The LPS-induced production of IL-12 p40 was prevented by Vpr and restored with the GR inhibitor Ru486. While Vpr prevented TLR4 expression, it remained to be determined if this effect was GR-mediated. To examine this, the pLXIN- and Vpr-retrovirus infected PBMC were pre-treated with 7 uM of GR₁ (Ru486) prior to LPS stimulation. Cells were processed as before and TLR4 was analyzed by flow cytometry. The GR₁ restored the retroviral-induced TLR4 response to LPS stimulation seen in cells infected with Vpr-retrovirus (Figure 24 A, B, C). The Vpr-mediated inhibition of LPS-induced TLR4 expression is GR dependent. It should be noted that although the GR₁ restored LPS-induced TLR4 expression in Vpr expressing cells, the GR₁ prevented LPS-induced TLR4 expression in pLXIN treated cells. The GR inhibition can effect both Vpr expressing and non-expressing cells although with differing effects in the expression of TLR4 in response to LPS.

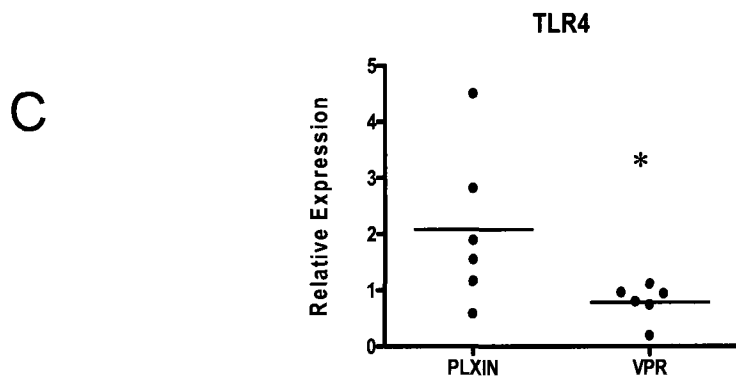
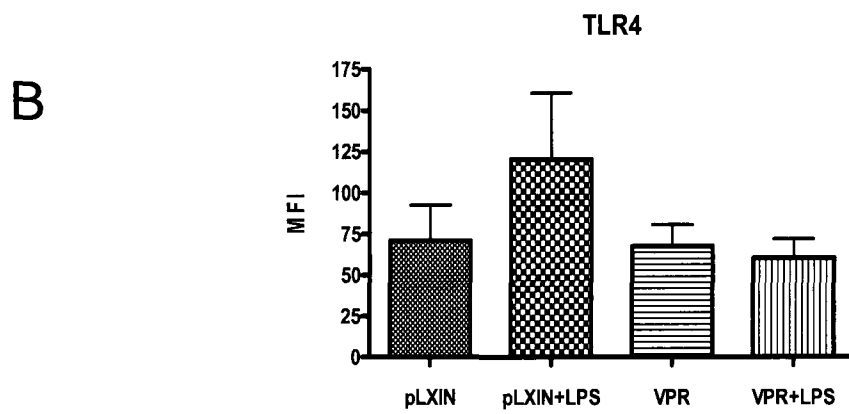
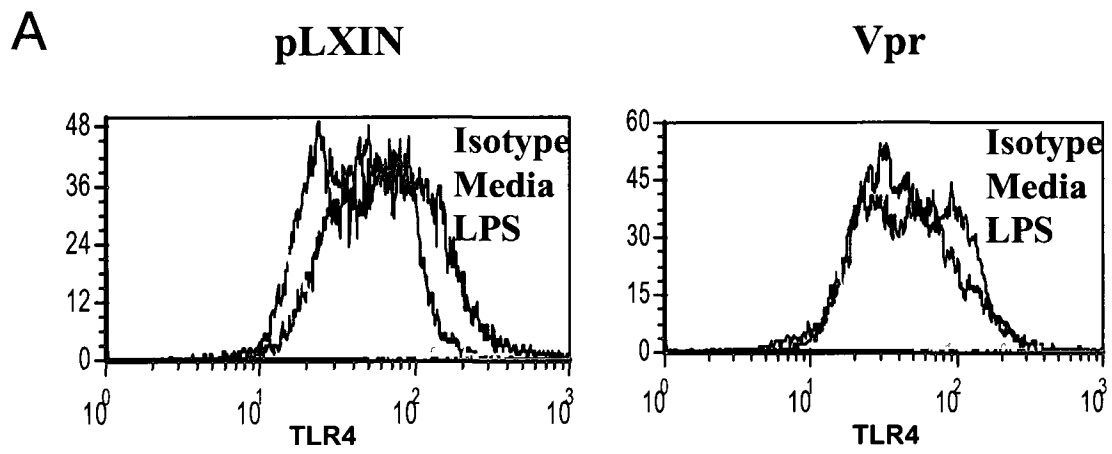


Figure 23: Vpr suppressed LPS-induced expression of TLR4 on monocytes. PBMC were infected with either pLXIN or Vpr-pLXIN retrovirus. TLR4 expression on gated monocytes was analyzed by flow cytometry, in the presence and absence of LPS-stimulation. **A)** Histogram overlay showing increased TLR4 expression in response to LPS-stimulation in pLXIN, but not Vpr infected cells **B)** Average mean fluorescence intensity, quantifying TLR4 receptor expression **C)** Relative expression of TLR4 in LPS stimulated relative to unstimulated cells, n=6 (*, p=0.043). Error represents SEM. Statistical significance was evaluated by student t-test.

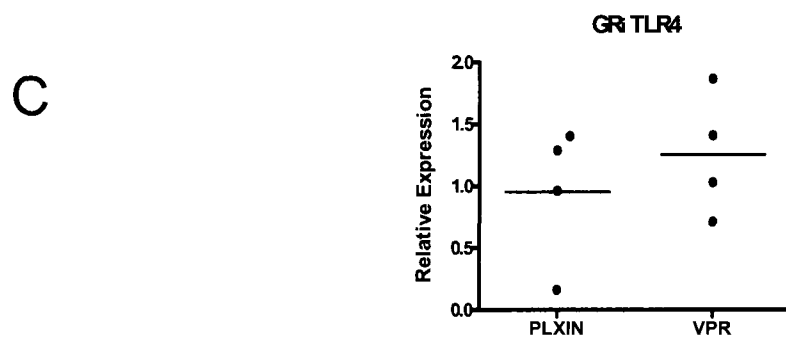
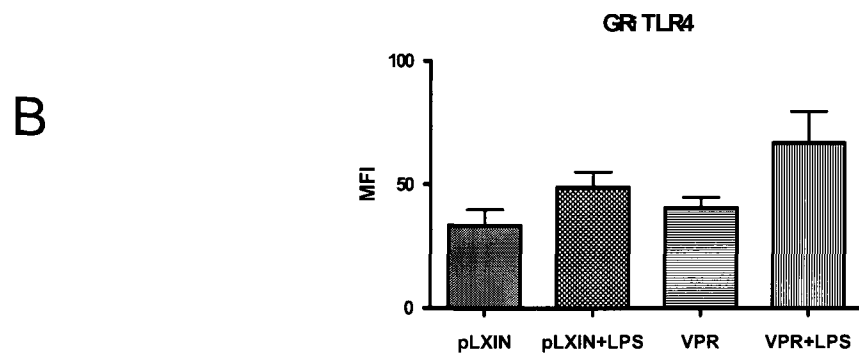
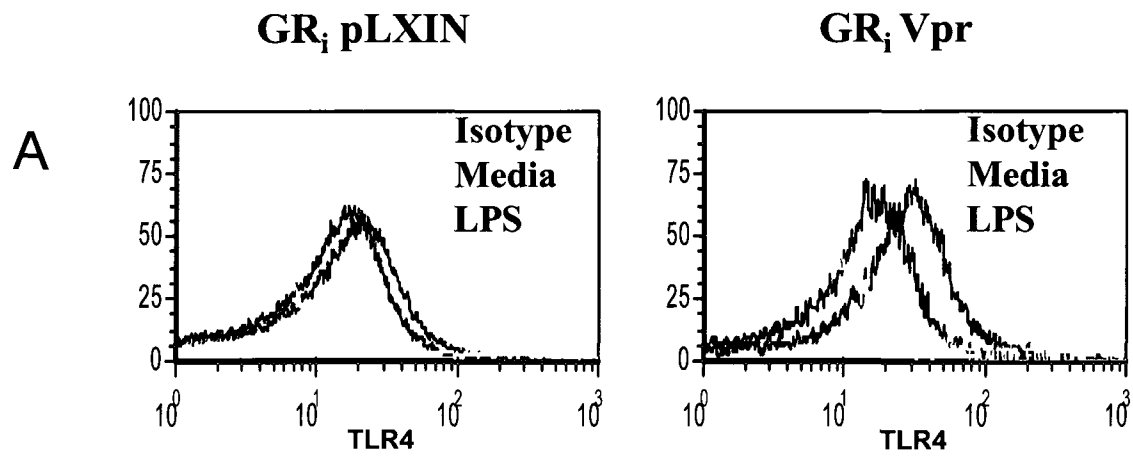
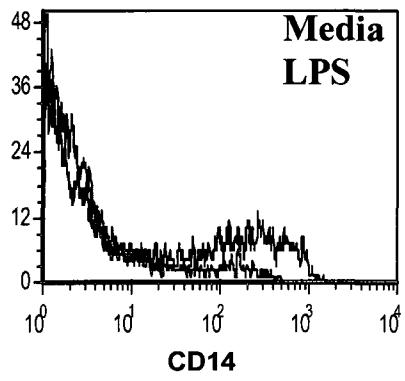


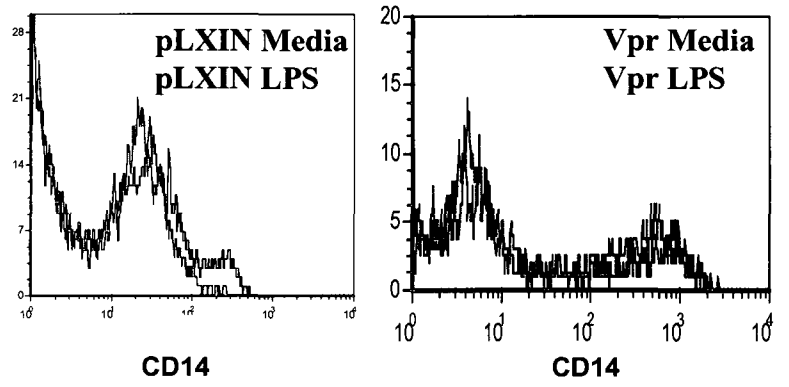
Figure 24: Inhibition of GR signalling prevented Vpr-mediated suppression of LPS-induced TLR4 expression. PBMC were infected with either pLXIN or Vpr-pLXIN retrovirus. Vpr or pLXIN cells were pretreated with the glucocorticoid receptor inhibitor, Ru486 (7 uM) prior to LPS stimulation for 24 hours. Cells were then collected, fixed and permeabilized and receptor expression was analyzed by flow cytometry. TLR4 expression on gated monocytes was analyzed by flow cytometry. **A)** Histogram overlay, showing restored LPS-induced increase in TLR4 in GR_i treated Vpr expressing cells. **B)** Average mean fluorescence intensity, quantifying TLR4 receptor expression **C)** Relative expression of TLR4 in LPS stimulated relative to unstimulated cells, n=4. Error represents SEM.

F. Expression of CD14 on pLXIN-retrovirus and Vpr-retrovirus infected cells, following LPS stimulation. LPS signalling is dependant on CD14. The objective of these experiments was to determine if Vpr had any effect on the expression of CD14 in LPS-stimulated or unstimulated cells. In uninfected PBMC, cultured in parallel to retrovirally infected PBMC, CD14 expression was significantly decreased in response to LPS as compared to unstimulated controls (MFI=36±5 vs. MFI=104±26) (Figure 25 A, C). In cells infected with pLIXN retrovirus the decrease in CD14 following LPS stimulation remains present. Cells infected with Vpr seem to express less CD14 compared to pLIXN-infected cells (MFI=64±21 vs. MFI=98±22) (Figure 25 B, D). No further decrease in CD14 expression was noted following LPS stimulation, in Vpr infected cells (Figure 25 D). This lack of response is important, since it is characteristically seen in uninfected cells (Figure 25 A, C). Interestingly the MFI pattern is comparable between uninfected and pLIXN infected PBMC, while the histogram analysis indicates a different phenomenon. In uninfected cells, a single cell population is observed while in pLIXN infected cells two CD14⁺ cell populations are observed.

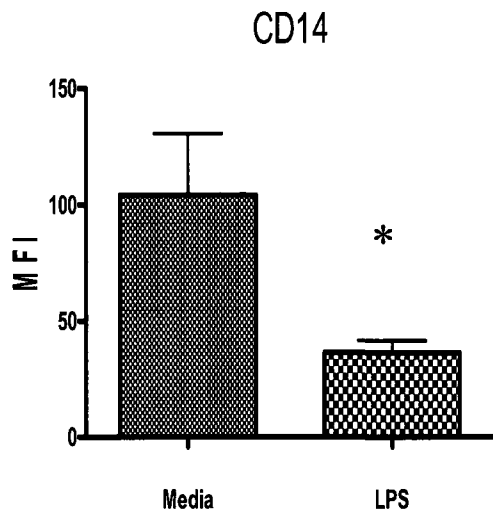
A



B



C



D

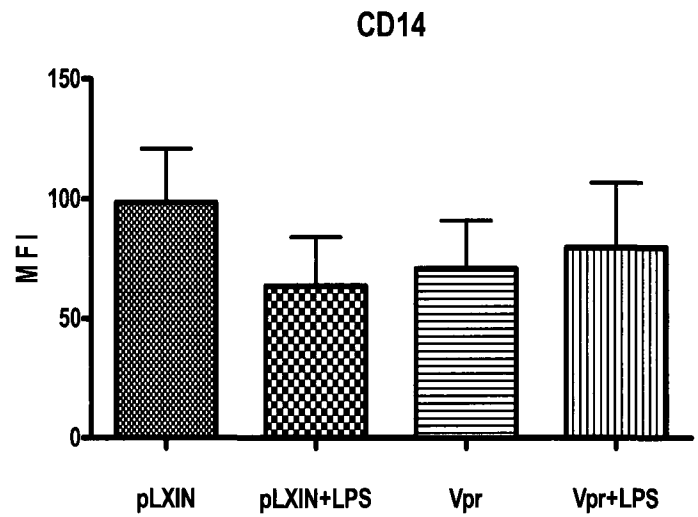


Figure 25: Stimulation with LPS decreased CD14 expression and Vpr prevents this cellular process. PBMC were cultured for 48 hours prior to stimulation with LPS for 24 hours. Cells were analyzed by **(A)** flow cytometry and **(C)** MFI was assessed, n=5, (*, p=0.01) by student t-test. PBMC were infected with the retrovirus control (pLXIN) or Vpr-pLXIN prior to LPS stimulation for 24 hours. Cells were then collected, fixed, permeabilized and labeled with FITC-conjugated anti-CD14 mAb. Receptor expression was analyzed by **(B)** flow cytometry and **(D)** mean MFI was assessed, n=5, n.s. by student t-test.

G. LPS stimulation resulted in no change in expression of GR in retrovirus infected cells. Changes in expression of GR have been linked to many cellular processes. GR is also the only receptor known to ligate Vpr. Our objective in this experiment was to determine if Vpr affected GR expression in LPS stimulated PBMC. Cells were infected with pLXIN or Vpr, stimulated with LPS, and analyzed by flow cytometry. In both histogram overlay analysis and average MFI, no significant change was seen in GR expression (Figure 26).

H. The effect of intracellular expression of Vpr on IL-12 p40 protein production.

We previously demonstrated that extracellular addition of Vpr decreases LPS-induced IL-12 p40. This experiment evaluated the effects of intracellular expression of Vpr on the production of LPS-induced IL-12 p40. Cell-free supernatants from uninfected, pLXIN- or Vpr- infected cells were collected following 24 hours of LPS stimulation for analysis by ELISA. The cells infected with pLXIN produced an average 70 pg/ml of IL-12 p40 following LPS stimulation, compared to nearly undetectable levels in unstimulated cells. Vpr expressing cells did not produce detectable levels of IL-12 p40 following LPS stimulation (Figure 27).

I. The effect of intracellular expression of Vpr on IL-23 and IL-27 protein production.

Intracellular expression of Vpr prevented LPS induced IL-12 p40 production. Our objective in this experiment was to determine if the other IL-12 family cytokines IL-23 and IL-27 protein production were similarly affected. PBMC were infected with pLXIN or Vpr and stimulated with LPS (or unstimulated) for 24 hours. The cell-free supernatants were collected and protein production was analyzed by ELISA. LPS

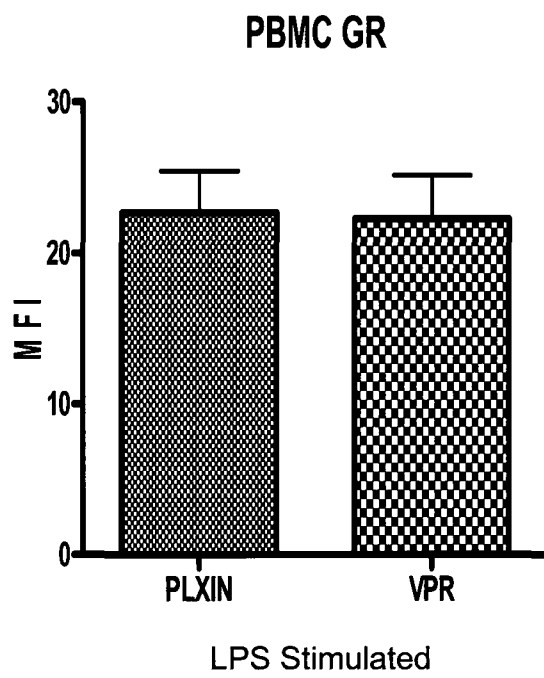
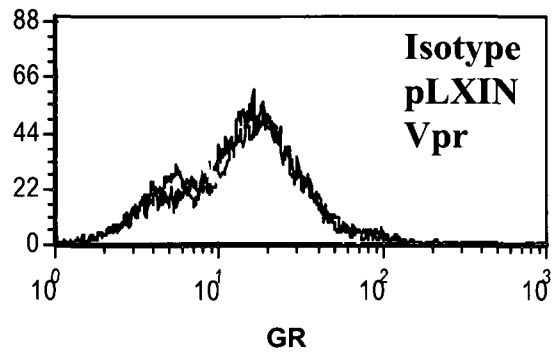


Figure 26: Vpr-retrovirus did not alter expression of GR. PBMC were infected with the retrovirus control containing the empty pLXIN vector or the Vpr-pLXIN and infection prior to LPS stimulation for 24 hours. Cells were then collected, fixed and permeabilized and GR was labeled with a FITC-conjugated anti-GR and receptor expression was analyzed by flow cytometry. Analysis shown as histogram overlay and average MFI, n=6.

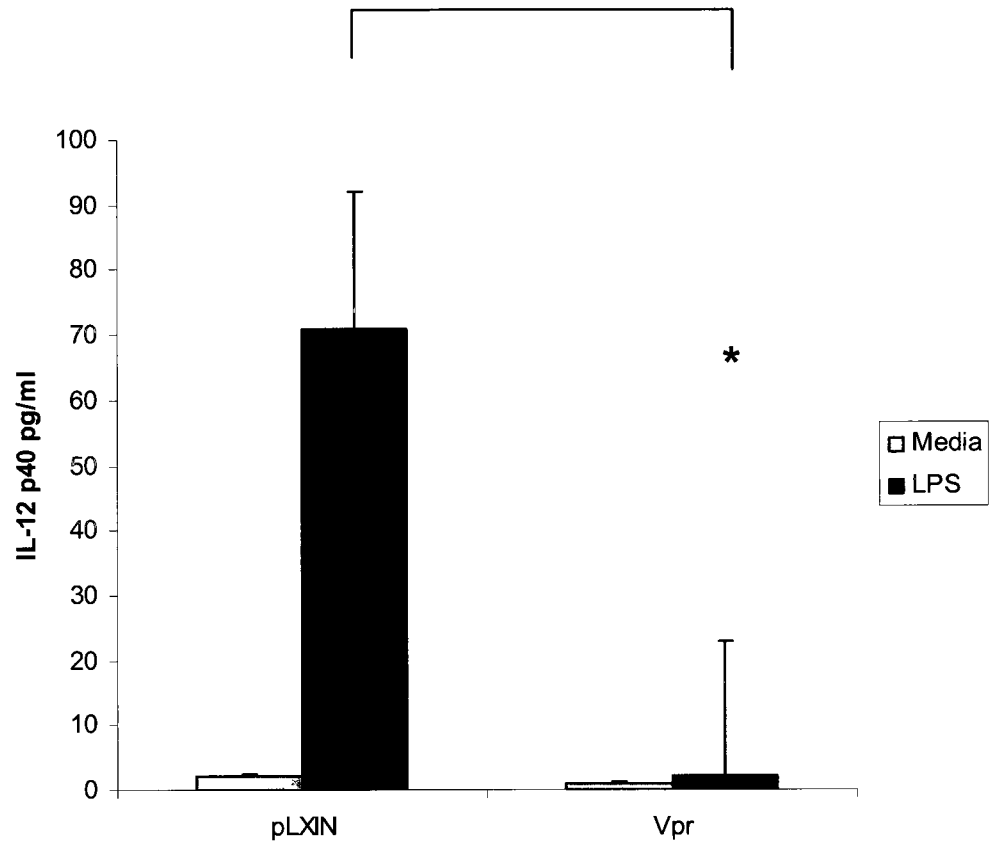
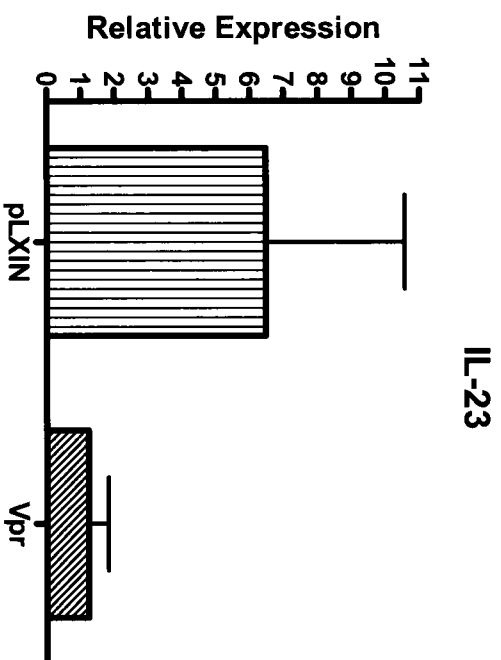


Figure 27: Vpr retrovirus inhibited IL-12 p40 protein production. PBMC were infected with the retrovirus control containing the empty pLXIN vector or the Vpr-pLXIN, prior to LPS stimulation for 24 hours. Cell-free supernatants were collected and IL-12 p40 was measured by ELISA. n=5, (*, p=0.023) by student t-test.

A



B

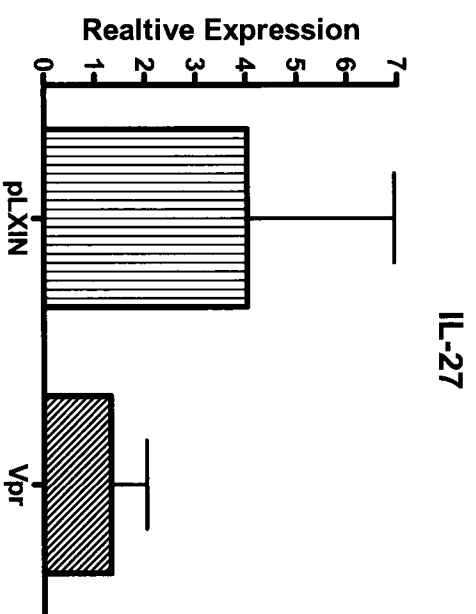


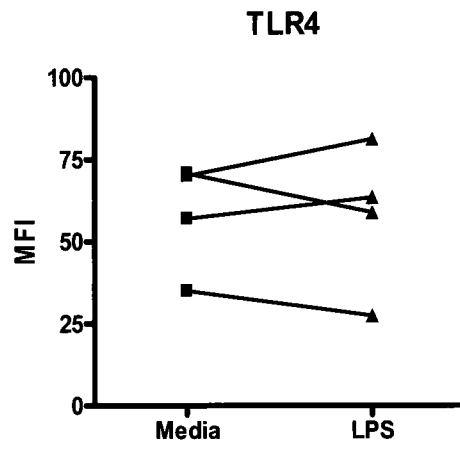
Figure 28: Vpr-retrovirus decreased LPS-stimulated IL-23 and IL-27 production.

PBMC were infected with the retrovirus control containing the empty pLXIN vector or the Vpr-pLXIN, prior to LPS stimulation for 24 hours. Cell-free supernatants were collected and analyzed by ELISA. The relative expression was calculated by dividing the LPS-induced production by the cytokine production from unstimulated cells, n=6 n.s. by student t-test.

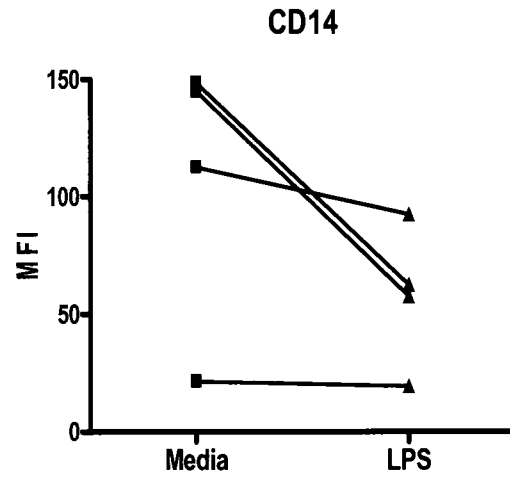
induced an average 6.5 ± 4 fold change in IL-23 expression in pLXIN expressing cells whereas, Vpr-infected cells showed little change in expression (1.2 ± 0.6 fold). LPS-induced an average 4 ± 3 fold increase in IL-27 expression in pLXIN infected cells while Vpr-infected cells showed little change in expression (1.3 ± 0.7 fold) (Figure 28). Vpr infection resulted in insignificant changes in IL-23 and IL-27 protein production.

J. LPS mediated CD14, GR and TLR4 expression on isolated monocytes. To further our understanding of CD14, TLR4 and GR receptor expression in a homogenous cell population, previous experiments were repeated on isolated monocytes. Monocytes were isolated by negative bead selection and cultured in suspension, since both trypsin and scraping to remove adherence derived monocytes affected to quality of the cell and receptor expression for analysis by flow cytometry. TLR4 expression was unaltered by LPS stimulation (Figure 29 A). Although not statistically significant, the expression of CD14 was decreased in response to LPS in isolated monocytes (MFI= 107 ± 30 vs. MFI= 58 ± 15) (Figure 29 B). Monocyte expression of GR remained unchanged in response to LPS stimulation (Figure 29 C).

A



B



C

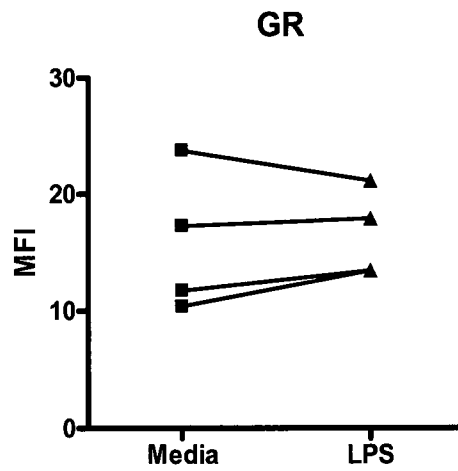


Figure 29: LPS mediated expression of TLR4, CD14 and GR in isolated monocytes. Monocytes were isolated by magnetic beads through negative selection and were cultured for 48 hours prior to stimulation with LPS for 24 hours. Cells were then collected, fixed, permeabilized and labeled with (A) PE-conjugated anti-TLR4 mAb, (B) FITC-conjugated anti-CD14 mAb or (C) FITC-conjugated anti-GR mAb. Receptor expression was analyzed by flow cytometry, n=4, n.s. by student t-test.

K. LPS induced changes in CD14 expression on monocytes infected with pLXIN or Vpr retroviruses. The CD14 expression is decreased in PBMC stimulated with LPS and this phenomenon is prevented by expression of Vpr. Our objective was to determine if CD14 expression was affected by Vpr in isolated monocytes. Monocytes were isolated by magnetic beads through negative selection and were infected with either pLXIN or Vpr retrovirus prior to stimulation with LPS for 24 hours. Following stimulation, cells were analyzed by flow cytometry. Although not statistically significant, CD14 expression was decreased following LPS stimulation (MFI=107±30 vs. MFI=58±15) (Figure 30 A). Cells infected with either retrovirus exhibited no change in CD14 receptor expression in response to LPS stimulation (Figure 30 B).

L. LPS induced no change in TLR4 and GR expression on monocytes infected with pLXIN or Vpr retrovirus. Our objective in this experiment was to further our understanding of the effect of Vpr in isolated monocytes by investigating the expression of TLR4 and GR with and without LPS stimulation. Monocytes were isolated by magnetic beads through negative selection and were infected with the retrovirus control containing the empty pLXIN vector or the Vpr-pLXIN, prior to LPS stimulation for 24 hours. Cells were analyzed as before by flow cytometry. TLR4 (Figure 31 A) and GR (Figure 31 B) expression remained unchanged, with or without LPS stimulation, in primary monocytes infected with either retrovirus. The lack of change in TLR4 expression is consistent with uninfected isolated monocytes and highlights the importance of analysis of TLR4 within a PBMC culture system. Overall TLR4 and GR expression are largely unaltered by Vpr in both unstimulated and LPS-stimulated isolated monocytes.

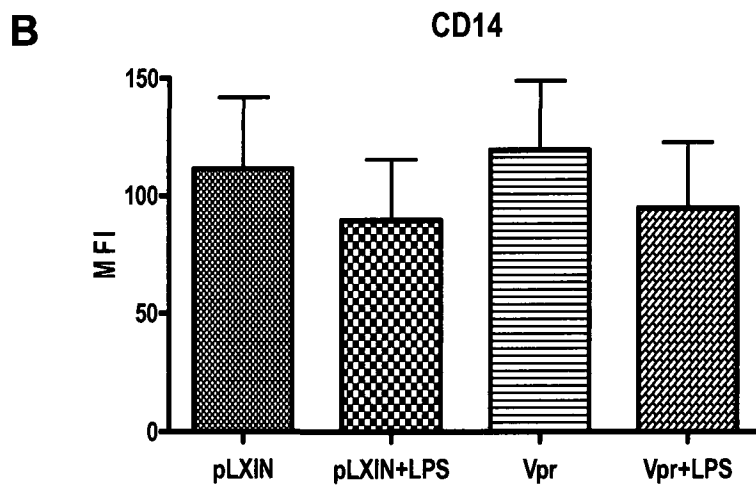
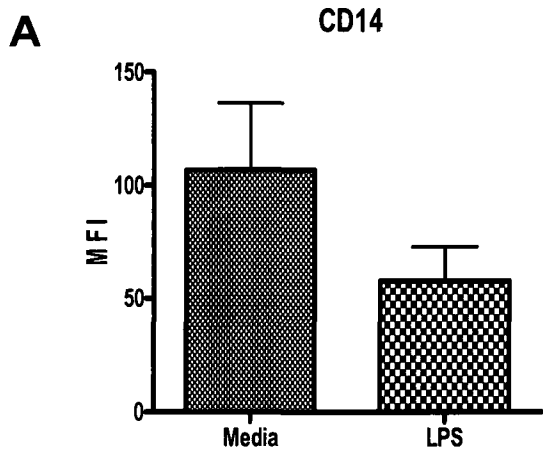


Figure 30: Vpr mediated CD14 expression in monocytes cultured in isolation.

Monocytes were isolated by magnetic beads through negative selection and were infected with the retrovirus control containing the empty pLXIN vector or the Vpr-pLXIN, prior to LPS stimulation for 24 hours. Cells were then collected, fixed, permeabilized, labeled and receptor expression was analyzed by flow cytometry. **A)** LPS caused a decrease in CD14 expression in uninfected monocytes cultured in isolation, n=4. **B)** The CD14 expression following LPS stimulation in macrophages infected with pLXIN or Vpr remained the same, n=4, n.s. by student t-test.

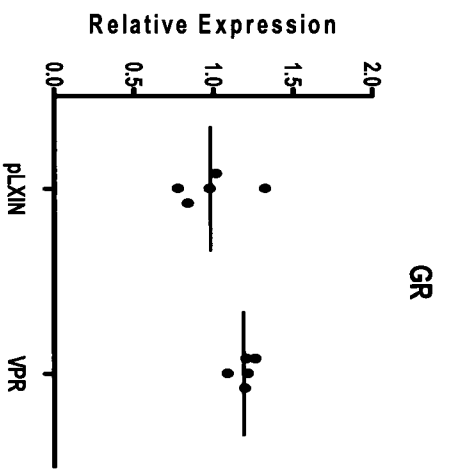
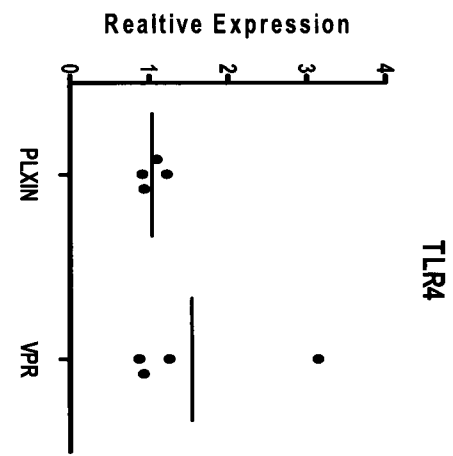
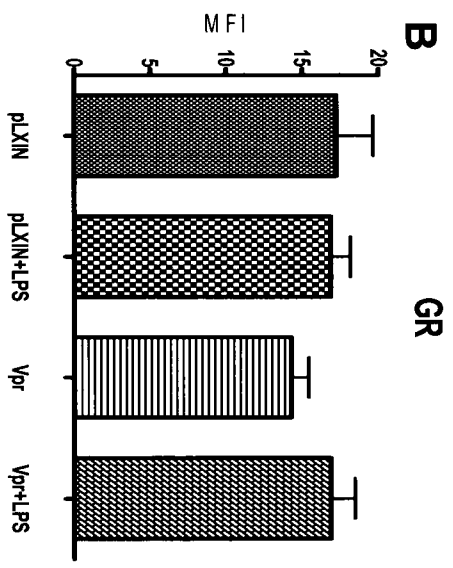
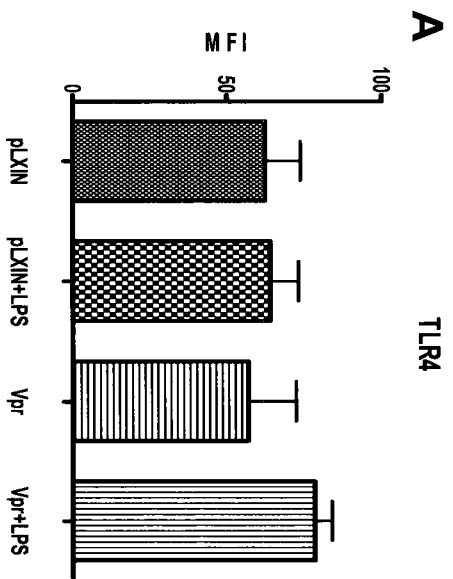


Figure 31: TLR4 and GR expression remain unchanged in response to LPS stimulation. Monocytes were isolated by magnetic beads through negative selection and were infected with the retrovirus control containing the empty pLXIN vector or the VPR-pLXIN and infection. Cells were then collected, fixed, permeabilized labeled and receptor expression was analyzed by flow cytometry. Receptor expression was measured with LPS stimulation for 24 hours. **A)** TLR4 expression following LPS stimulation in macrophages infected with pLXIN or Vpr remained the same, n=4, n.s. by student t-test. **B)** GR expression following LPS stimulation in macrophages infected with pLXIN or Vpr remained the same, n=4, n.s. by student t-test.

VI – DISCUSSION

IL-12 is a proinflammatory cytokine critical for maintaining CMI and the production of IL-12 declines as a result of HIV infection. The inadequate production of IL-12 prevents the generation of an efficient CMI response to HIV as well as to secondary opportunistic infections, such as tuberculosis and toxoplasmosis. Understanding the mechanism of HIV-mediated IL-12 downregulation may identify targets to which novel immune-based therapies could be developed.

HIV has been shown to directly inhibit the production of several key cytokines, including IL-12 (120). LPS-induced signalling via the SAPK pathway in particular is inhibited in HIV infected monocytes, and is characterized by decreased phosphorylation of p38 and JNK, leading to decreased gene activation. In addition, LPS-induced nuclear factor binding is altered in HIV-infected monocytes (285) at four of the nine putative IL-12 p40 promoter binding sites: NF κ B, AP-1, Sp-1 and Ets-2 (85, 120).

This thesis reports the results of studies which examine the mechanism(s) underlying HIV-mediated suppression of IL-12 p40 production including, the evaluation of upstream signal transduction events through p38 and JNK and upstream signalling receptors CD14, TLR4 and GR. The viral protein Vpr was also evaluated for its effect on receptor expression and IL-12 p40 production, given its potential to decrease IL-12 production. The overall hypothesis of this study was that HIV- and Vpr-mediated suppression of IL-12 p40 as a result of dysregulation of the key signalling pathways: CD14, TLR4 and GR expression, downstream kinase activity, nuclear factor binding, promoter activity, mRNA expression and ultimately protein production.

A. P38 and JNK are critical for LPS-induced IL-12 p40 promoter activity, mRNA expression, and protein production. It has previously been demonstrated in our laboratory that HIV inhibits the LPS-induced production of IL-12 p40, the activation of p38 and JNK and prevents nuclear factor binding to the IL-12 p40 promoter in adherence derived monocytes (119, 120). To better understand these observations, the effect of pharmacologically inhibiting p38, ERK and JNK on LPS-induced IL-12 p40 production was analyzed. In addition, analysis was done on the effect of inhibiting activation of p38 and JNK on nuclear factor binding to the AP-1, NF κ B, Ets-2 and Sp-1 sites of the IL-12 p40 promoter.

We demonstrated the novel finding that inhibition of p38 and JNK kinases leads to altered nuclear factor binding, decreased IL-12-p40 promoter activity, decreased mRNA production and decreased protein secretion in LPS-stimulated primary adherence derived monocytes. The inhibition of p38 and JNK decreased LPS-induced p40 protein production, while inhibition of ERK had no effect on p40 production. Both the p38 and the JNK inhibitors prevented LPS-induced nuclear factor binding to AP-1, Ets-2 and Sp-1 promoter sites, while there was little effect on binding to the NF κ B site in primary-adherence-derived monocytes. Taken together with our laboratory's previous results, these novel results further define the pathways that may be utilized by HIV to decrease IL-12 p40 production.

1. P38 and JNK Effects on Nuclear Factor Binding

In this study of adherence-derived-monocytes, we demonstrated that stimulation with LPS resulted in a decrease in binding to the Sp-1 site and that this response to LPS was prevented by the independent inhibition of p38 or JNK. That is there was a relative

increase in nuclear factor binding in the presence of these inhibitors. This is analogous to the previous observation in our laboratory where in the setting of HIV infection of adherence-derived-monocytes, binding at the Sp-1 site was found to be increased (285).

We further demonstrated that the LPS-induced nuclear factor binding to the Ets-2 site on the IL-12 p40 promoter was prevented by the inhibition of p38, ERK or JNK. This is comparable to the previous observation that the LPS-induced increase in nuclear factor binding to the Ets-2 site was prevented by infection with HIV (276).

LPS-induced nuclear factor binding to the NF κ B promoter site was not prevented by the inhibition of p38, ERK or JNK, whereas HIV infection appears to inhibit LPS induced NF κ B binding. The lack of change in nuclear factor binding with pharmacological inhibition of JNK also appears in contrast to our earlier work in THP-1 cells as discussed below.

We further demonstrated that LPS stimulation resulted in a decrease in nuclear factor binding to AP-1 and this decrease was prevented by the inhibition of JNK, while inhibition of p38 and ERK kinase activity had no such effect. Similarly, infection with HIV prevented the LPS-mediated effects on nuclear factor binding to the AP-1 site.

In conclusion, p38, ERK and JNK regulate nuclear factor binding to the IL-12 p40 promoter at several sites, and the inhibition of kinase phosphorylation may be a mechanism by which HIV infection leads to dysfunctional nuclear factor binding to the IL-12 p40 promoter, and a subsequent decreased protein production.

2. A Role for SAPK in Nuclear Factor Binding to IL-12 p40

Our findings complement the characterization of the SAPK/MAPK dependant activation of IL-12 p40 promoter transcription factors investigated in other systems (59,

120, 277). Studies in primary human monocytes and the pro-monocytic THP-1 cell line demonstrated the involvement of the SAPK/MAPK pathway in the LPS-induced activation of NF κ B and AP-1 transcription factors in the IL-12 p40 promoter (62, 120, 277-279).

However, other studies have shown conflicting results. In particular, AP-1 has been implicated in LPS-induced IL-12 p40 promoter activity in our laboratory (120), whereas deletion of the AP-1 site and upstream sites of the IL-12 p40 promoter in work by Ma *et al.* failed to abrogate promoter activity in bead isolated monocytes (121). Although the reasons for this apparent discrepancy are not clear, it appears that IL-12 p40 gene transcription may be dependent on the cell type studied and the nature of the external stimuli used.

An additional potential explanation for the inconsistencies is the proximity of the promoter regions to each other in the IL-12 p40 promoter. Nuclear factors may interact with other overlapping nuclear factors at neighbouring sites and therefore the length of the oligonucleotides used for analysis may contribute to the results observed. If the oligonucleotide used for analysis does not allow the appropriate interaction, nuclear factor binding may be prevented despite appropriate kinase activation, thus leading to differing reports with regard to promoter activity. Further analyses investigating this possibility could include the assessment of nuclear factor binding in relation to potential co-factors and oligonucleotide length.

3. The Regulation of Nuclear Factor Binding

Our laboratory has previously shown that LPS-induced binding to the NF κ B site on the IL-12 p40 promoter was prevented by JNK inhibition in THP-1 cells (285). Previous work by other researchers demonstrated similar findings (121). In contrast, this effect is not observed in our study in adherence-derived-primary-monocytes.

The lack of an effect in adherence-derived-monocytes may indicate a fundamental difference in nuclear factor activation in this cell type as compared to THP-1 cells or monocytes isolated by negative bead selection. Potential explanations for this difference include: 1) THP-1 cells are a promonocytic tumor cell line with several altered signalling pathways as compared to the primary-adherence-derived-monocytes; 2) the adherence derived monocytes are in a further state of maturation than THP-1 cells or negatively bead isolated monocytes; and 3) monocytes may be more sensitive to external stimulation than adherence-derived-monocytes.

The difference in sensitivity to external stimulation may be due to the difference in their function and the nature of their *in vivo* environment. There is increased mitogen stimulation in mucosal sites, where adherent tissue macrophages are located as compared to in the blood where monocytes are located. This may possibly explain the difference in the response to JNK inhibition on LPS-induced NF κ B binding in adherence-derived-monocytes (280, 281). Adherent tissue macrophages may require two signals, the first from LPS and the second from ATP (282, 283) whereas this may not be the case in monocytes in circulation.

4. The Role of SAPK in IL-12 p40 Production

There have been several reports regarding the involvement of p38, ERK and JNK in the production of IL-12 p40 in various primary human cells and cell lines. Overall these reports do not provide a clear description of the role of SAPK in IL-12 p40 production. For instance, Dobrva *et al.* demonstrated that p38 may have a negative regulatory role on IL-12 p40. Similarly, the deletion of the upstream p38 kinase, MKK3^{-/-}, in mice was shown to prevent production of IL-12 p40 (284). However, other demonstrated that inhibition of p38 had no role in LPS induced IL-12 p40 production in Thp-1 and primary bead-isolated-monocytes (121, 132). In addition, there are reports of ERK MAPK acting as a positive regulator of IL-12 p40 (285, 286), while others suggest it has only a minimal role as a negative regulator (62, 287, 288). These various observations are likely a result of the specifics of the different experiments.

The maturation of monocytes has been shown to lead to a greater capacity to produce IL-12 p40 following stimulation (86) in part due to production of IFN- γ , a strong positive inducer of IL-12 p40. Understanding the role of JNK in the production of IL-12 p40 is further complicated by the observation that JNK inhibition upregulates IFN- γ induced IL-12 p40 (335).

Interestingly the net effect of SB203580 (p38_i) on the production of IL-12 can also be positive or negative, depending on stimuli and cell population (121, 276). Depending on the setting, this may be in part due to the production of various cytokines such as IL-10 and IFN- γ (35, 289). In addition IL-10 can be induced by LPS in adherence-derived-monocytes and is known to have inhibitory effects on IL-12 p40 production (55). In LPS-stimulated IFN- γ -primed monocytes, inhibition of p38

enhances IL-12 production. This is in contrast to the inhibition of IL-12 observed if the cells are not primed with IFN- γ (290). It is likely that stimulation of cells with IFN- γ in addition to LPS may activate a distinct set of transcription factors required for IL-12 p40 gene activation. This is a further indication of the complexity of this process. Conflicting results in various studies may be due to the negative feedback regulation of IL-12 by immunoregulatory cytokines such as IL-10, as well as differences in cell types and the stimuli used to activate distinct signalling pathways.

5. Experimental Limitations

Both p38 and JNK play a role as positive regulators of IL-12 p40 production. The small sample size analyzed remains a limitation to this investigation. In addition to the modest luciferase activity measured in the primary monocytes. The controls available for the luciferase activity were limited due to the nature of the adenovirus luciferase construct. Both the monitoring of the biological activity and the specificity of the inhibitors may have further strengthened the observations. Further analysis of the specific nuclear factors affected by the MAPK inhibitors may be of interest for future work.

6. Further *In Vivo* Considerations

There are further aspects to consider in the context of HIV-mediated downregulation of IL-12 . It should be noted that in addition to the direct inhibition by HIV, a decrease in IL-12 may also result from declining CD4⁺ T cell numbers and function associated with disease progression. The lack of IFN γ production from CD4⁺ T cells may lead to a reduction in the positive IFN γ feedback loop, preventing optimal IL-12 production. Furthermore, IL-12 itself is necessary for the development and

activation of Th1 cells in response to infection. Without optimal IL-12 at the site of infection, the CD4⁺ T cell response will be further blunted. This cytokine imbalance may be exaggerated by the HIV-mediated production of Th2 cytokines such as IL-10 (40, 62), and TGF- β (62, 291) which negatively regulate the production of IL-12.

7. Proposed Molecular Mechanism

The research findings in this study demonstrate that the stimulation of primary monocytes with LPS induces a complex signalling pathway that results in IL-12 p40 production. The proposed mechanisms for the findings in this study indicate that inhibition of either p38 or JNK signalling prevents IL-12 p40 promoter activity, mRNA expression and protein production. The inhibition of p38, ERK or JNK also prevents LPS-induced nuclear factor binding to the four key promoter sites NF κ B, Sp-1, AP-1 and Ets-2. Therefore, phosphorylation of p38 and JNK are key to the production of IL-12 p40. The inactivation of the LPS-induced phosphorylation of p38 and JNK by infection with HIV presents a likely viral mechanism for IL-12 p40 inhibition.

B. HIV infection and Vpr peptide decreased LPS signalling receptors CD14 and TLR4 while increasing GR expression.

We further investigated the role of two receptors critical to LPS-stimulated IL-12 expression: the extracellular receptor CD14 which binds the LPS/LBP complex at the cell surface and the intracellular signalling receptor TLR4. In addition, we evaluated the glucocorticoid pathway by studying GR expression and ligand binding to the GR. The role of these receptors in HIV inhibition of IL-12 p40 was further investigated.

Infection of PBMC with an X4 tropic strain (HIV_{III B}) resulted in no change in CD14, reduced TLR4 expression and increased GR expression on gated monocytes. Both the dual-tropic strain, HIV_{cs204} and the R5 tropic, HIV_{ADA} lead to a reduction in TLR4 expression and an increase in GR and CD14 expression.

To understand the impact of HIV infection on monocytes receptor expression, further evaluation following LPS stimulation was undertaken. Compared to LPS-stimulated uninfected cells the LPS-stimulated HIV-infected cells had a reduced TLR4, an increased CD14 and no change in GR expression.

Altered receptor expression may represent a mechanism by which HIV alters LPS signalling thereby modulating IL-12 p40 production. A decrease in CD14 and TLR4 may prevent an appropriate response to LPS while the increase in GR expression may lead to increased signalling through the GR, which has been shown to have a negative effect on IL-12 expression (221).

1. Regulation of Receptor Expression

The changes in CD14 observed are further supported by the observations that CD14 expression is regulated by a number of factors, including the state of cell activation and the cytokines present in the microenvironment (292-296). Infection with HIV has been associated with upregulation of CD14 surface expression (135, 137) which is further supported by our findings with HIV_{cs204} and HIV_{ADA}. Infection with HIV_{III B} had no effect on CD14, indicating some tropism differences. Perhaps this indicates that infection of monocytes is required to affect CD14 expression, since HIV_{III B} primarily infects T-cells, and not monocytes.

Membrane CD14 is shed in response to immune activation or differentiation (297) leading to circulating CD14. Elevated levels of circulating CD14 have been identified in several conditions. These include patients with inflammatory conditions such as systemic lupus erythematosus (298), chronic active hepatitis (299), or septic shock (300), thus demonstrating that elevated membrane bound CD14 or circulating CD14 are important indicators in several disease states.

Ligand binding to the GR by dexamethasone has been reported to lead to an increase in GR expression (301). Ligand-dependant down regulation of the GR has been shown to limit hormone responsiveness, but the mechanisms involved in this process are poorly understood (302).

While the increase in GR expression was not seen in HIV_{cs204} infected cells when stimulated with LPS, reduced binding affinity to the GR may be occurring. Some studies have demonstrated changes in the GR in macrophages from HIV infected patients, which consists of decreased glucocorticoid affinity for GR and increased GR number (303). The glucocorticoid sensitivity is reduced, in part due to decreased binding affinity of the GR in HIV infection (304). This may be caused by HIV itself and/or the simultaneous presence of Th1 (IL-2) and Th2 (IL-4) cytokines (305), since proinflammatory cytokines can directly inhibit GR function (306).

In addition to the GR dysregulation, the regulation of glucocorticoids themselves affect viral susceptibility and decrease IL-12. Experimental conditions that diminish the production of glucocorticoid tend to increase susceptibility to viral infections in mice (214). The focus herein on HIV, IL-12 and glucocorticoids and the relation between the three in a unified response in PBMC may further the

understanding of this increased susceptibility. We therefore assessed the potential mechanisms of HIV mediated changes to CD14, TLR4 and GR in a PBMC culture. There are advantages in analyzing monocytes within a PBMC culture such as to the inclusion of interactions between cells and the effect of other cytokines, including IFN- γ (307). APCs require co-stimulation to mature and they are primed before they produce large amounts of IL-12, this function requires certain cellular pathways to be activated and this process may not be initiated when cells are studied in isolation. Our findings in monocytes within a PBMC culture was consistent with numerous previous reports in which LPS-induced IL-12 p40 production was decreased in HIV_{cs204} infected PBMC (62, 78, 119, 120). The investigation of receptor expression on monocytes cultured within the complex PBMC culture ultimately furthers our understanding of the interplay of these many factors.

2. Vpr and IL-12 p40 Production

C-terminal Vpr peptide, which binds to the GR, inhibited LPS induced IL-12 p40 production in PBMC in a dose dependent manner. This differs from one report indicating that Vpr-inhibited IL-12 p70 and IL-12 p35, but had no effect on IL-12 p40 production (198). However, the effect of HIV-Vpr on IL-12 p40 is not surprising, considering other GR ligands like dexamethasone have been shown to inhibit IL-12 p40 production. The C-terminal Vpr-mediated inhibition of IL-12 p40 was further demonstrated to be GR dependent as pre-treatment of the cells with a GR inhibitor, Ru486 (GR_i), prevented the Vpr-mediated effect on LPS-induced IL-12 p40. The prevention of Vpr-mediated effects with this GR_i has previously been demonstrated

(198, 201, 231), but this is the first report of the ability of GR_i to prevent Vpr-mediated inhibition of IL-12 p40 production.

This study also demonstrated the decreased expression of TLR4 on Vpr treated monocytes. These findings are supported by the previous observation that Vpr downregulates TLR4 promoter activity (198). Furthermore we demonstrated the Vpr effect on TLR4 to be GR dependent. Together, the Vpr-GR interactions decrease IL-12 p40 expression and likely affect cytokine production through upstream LPS signalling receptor TLR4 expression.

The inhibition of LPS-induced IL-12 p40 was further demonstrated with the cortisol mimic, dexamethasone. Together with Vpr, dexamethasone had an additive effect on LPS-induced IL-12 p40 production. The enhanced effect of dexamethasone on Vpr-mediated inhibition of IL-12 p40 is of particular interest considering the enhanced effect is likely occurring in patients with increased cortisol. Some viral infections, including infection with HIV are associated with increased plasma and urinary cortisol concentrations (232, 236). Increased cortisol levels lead to a suppression of Th1 cytokines and an increase in Th2 cytokines (221). The direct interaction of Vpr with GR is sensitive to changes in the concentration of dexamethasone likely as a result of binding to the same receptor. This may have important implications for HIV immunosuppression and disease progression (198, 259, 261).

The mechanism of Vpr-mediated inhibition of IL-12 p40 was further evaluated by investigating Vpr-mediated effects on receptor expression. Vpr treatment leads to upregulation of CD14 and GR in LPS-stimulated PBMC while TLR4 was decreased. Previous observations of the effect of other viral factors on receptor expression include

the increased expression of CD14 induced by HIV-Nef protein in LPS stimulated monocytes (136). Addition of gp120 can also lead to increased TLR4 protein expression (308). In addition to viral specific effects, the ligation of GR by dexamethasone alone has been reported to downregulate LPS induced CD14 expression (136). Our findings, in conjunction with these observations, further describe the viral-mediated receptor expression changes which may contribute to immune pathology.

We demonstrated that Vpr peptide decreased the critical upstream signalling molecules CD14 and TLR4 while increasing GR expression. Together, this may create a feedback loop which enhances Vpr's inhibitory effects on IL-12 p40. The GR is critical for Vpr to affect IL-12 p40 production and the increase in GR expression following Vpr or dexamethasone treatment may create a negative feedback loop leading to further inhibition of IL-12 p40 production. This may lead to the enhanced ability of Vpr to affect IL-12 production and may modify the glucocorticoid response through increased receptor expression. Together, Vpr and increased GR expression present a possible mechanism for additive or synergistic effects of cortisol on the Vpr-mediated decrease of IL-12 p40 production. LPS stimulation alone decreased GR expression and may present a model for a controlled immune response to invading pathogens. Further work is merited to investigate the phosphorylation state and binding affinity changes of receptors within this system.

In addition to the phosphorylation state of activated GR, the engagement of mitogen-activated protein kinase phosphatase-1 (MKP-1) by GR may be worth exploring, in particular since GR can mediate the inflammatory response in monocytes by selective inhibition of p38 SAPK through MKP-1. Specifically glucocorticoid

binding to the GR initiates MKP-1 production which inhibits downstream cytokine production by preventing p38 signalling (309). Vpr ligation to the GR may be able to affect the GR induction of MKP-1 and inhibition of p38 and merits further study.

3. Experimental Limitations

The limitations to this analysis include the lack of isogenic HIV viruses differing only in their ability to express Vpr to evaluate the Vpr specific effect of the virus. Unfortunately due to the restricted infection of primary monocytes without Vpr expression this experiment was not performed. In addition some of the effects observed were likely not only due to direct infection of monocytes, but also secondary effects of HIV infected PBMCs (e.g. CD4+ lymphocytes). Analyzing cell free media from infected cultures would further our understanding into these effects and may be of interest in future experiments. In the Vpr peptide experiments, the additive effect of Vpr on the inhibition of IL-12 p40 by dexamethasone could have benefited from investigating if dexamethasone enhanced the sensitivity of the cells to lower doses of Vpr peptide. In addition the N-terminal peptide exhibited some effect on IL-12 p40 although this was not found to be dose dependent. The analysis of the peptide did highlight the need to investigate the effect of intracellular full-length Vpr peptide in addition to the exogenous truncated Vpr.

The inhibition of IL-12 p40 is an important mechanism of HIV-mediated immune dysfunction. Vpr has been shown to act as a potent co-activator of the host GR. This study determined that Vpr alters IL-12 p40 protein and the upstream CD14, TLR4 and GR expression both in LPS-stimulated and unstimulated cells. We demonstrated that the Vpr-mediated decrease in IL-12 p40 was through the C-terminal peptide which

contains the GR binding motifs. Pre-treating the PBMC cell culture system with the GR inhibitor Ru486 prevented the Vpr-mediated decrease in IL-12 p40. Overall, our findings highlight the importance of receptor expression analysis and support the model of an inherent receptor feedback loop utilized by Vpr for viral advantage.

C. Intracellular expression of Vpr decreases the LPS response through CD14, TLR4 and GR leading to decreased IL-12 p40.

We also evaluated the effects of intracellular expressed Vpr using a retroviral-mediated expression system. This system provides a basis for comparing the effect of intracellular expression to our earlier study with extracellular expression of Vpr. The retroviral system was evaluated for its effect on LPS-mediated CD14, TLR4 and GR receptor expression on monocytes.

Monocytes demonstrated a consistent decrease in CD14 expression in response to LPS stimulation which was prevented by retroviral expression of Vpr. In pLXIN retroviral control infected PBMC the TLR4 expression on monocytes was enhanced following stimulation with LPS. The LPS-mediated increase of TLR4 was prevented by retroviral expression of Vpr. No significant GR receptor changes were observed.

The expression of TLR4 did not change in LPS-stimulated monocytes cultured in isolation. This was in contrast to monocytes cultured in PBMC, where LPS increased TLR4 expression in cells infected with the control retrovirus. This increase in TLR4 was prevented when the vector contained Vpr. Previous reports indicate that LPS stimulation decreases TLR4 expression (310). The increase in TLR4 expression following LPS stimulation can likely be attributed to the retrovirus itself. This LPS-induced increase in TLR4 may be indicative of either a decreased response to LPS or an

increase in immune response following initial viral priming. Through the increase in TLR4 expression, more receptors may be available to support the innate immune response, thus sensitising the innate response for an increased response.

The retrovirus itself also affects the LPS-induced decrease in CD14 expression in monocytes since the decrease in CD14 is not seen following infection with the pLXIN or Vpr expressing retrovirus in monocytes cultured in isolation. In these cells, the presence of Vpr resulted in an increase in LPS-induced expression of CD14. The consistent LPS-induced decreases in CD14 expression in control cells may be used as a measure of immune response. Treatment with either pLXIN or Vpr expressing retrovirus prevented the LPS-induced decrease in CD14 membrane expression. The net effect on the immune response may be a decrease of the LPS-induced response which may be functional for future stimulation. The decrease in LPS-induced IL-12 p40 production with Vpr likely indicates a decreased CD14 response to LPS stimulation.

Experimental Limitations

The Vpr-mediated effects were not limited to IL-12 p40, as Vpr was associated with a decrease in IL-23 and IL-27 production. The lack of statistical significance may be due to the small sample size. The limitations to this analysis include the parallel investigation of IL-12, IL-23 and IL-27. Although the design of this investigation had merit with regard to investigating the expression of these cytokines in the same cellular environment, the results indicate the need for tailored cell culture for each of these cytokines. Future experiments would benefit from analyzing these cytokines in culture systems designed for their respective optimal expression. In addition the effect of Vpr on the activity and function of these cytokines merits further investigation. A reduced

cytokine production may interfere with cytokine activity including the recruitment of cells to the site of infection and the differentiation of Th17 cells. In addition, future experiments may benefit from tagging Vpr expressing cells to enhance the flow cytometry analysis of Vpr-mediated changes in receptor expression.

Together, these results outline the need for further review of the immune system and its response to primary and secondary infections. This will further our understanding of these observations within the context of the immune response.

1. Intracellular Vpr Leads to Immune Tolerance or Immune Sensitisation

The changes in LPS-induced receptor expression following HIV-infection or Vpr expression are of particular interest when considered in the context of immune tolerance or immune sensitization (259, 311). Immune tolerance can be acquired or induced and refers to the immune systems' adaptation to external antigens, characterized specifically by non-reactivity following stimulation that otherwise would induce cell-mediated or humoral immunity (219, 303, 312). Immune tolerance can be induced by repeated administration of very large doses of antigen, or by doses below the threshold required for an immune response (58, 313).

Immune sensitization refers to sensitized cells or tissue which demonstrate significantly greater response to immune stimulation than in similar circumstances in the absence of sensitizing pre-treatment (314). Immune sensitization can also confer responsiveness to a specific antigen and/or hyperresponsiveness to nonspecific stimuli at lower concentrations than in nonsensitized cells (311).

Under normal physiological conditions immune homeostasis is a tightly regulated network that is able to maintain balance within the immune system. When

challenged with a foreign antigen, the immune network initiates specific responses that are aimed at restoring immune homeostasis. However, with certain infections, this balance is not maintained and the immune system either under or over responds to stimulation (315). The change in immune homeostasis may result in altered capacity to respond to secondary infection. Maintenance of immune homeostasis is therefore important for the protection against infection as well as autoimmune conditions. The mechanism of immune homeostasis varies between individuals in state of health, viral infections, bacterial infection and compounding secondary infections. Initial infection can lead to immune tolerance or can sensitize the immune system to secondary infection through activation of the immune response. The above descriptions provide the context for further discussion of our observations.

2. The Role of Retrovirus and Vpr in Immune Homeostasis

The pLXIN-control retrovirus may sensitize the immune response to LPS while Vpr may induce immune tolerance to LPS. Repeated stimulation with LPS alone has previously been demonstrated to lead to immune tolerance in macrophages exposed to low levels of LPS (313) and can result in decreased IL-12 production (316). The change in LPS response may affect CMI to secondary infection. During HIV infection, repetitive exposure to circulating LPS may be due to microbial translocation, or “leaky gut” as seen in late-stage infection, when the mucosal barrier of the gut can no longer function to contain gastrointestinal microorganisms, many of whom express LPS (122). This may create a situation of receptor deregulation which will affect the immune response to primary and secondary infection. The receptor deregulation may impair the mounting of an efficient immune response and lead to immune tolerance.

Many factors can lead to immune tolerance. Interestingly repeated exposure to LPS can not only lead to immune tolerance, but in addition repeated LPS stimulation can inhibit HIV replication in primary human macrophages (317). We demonstrate that Vpr may lead to immune tolerance through deregulation of TLR4 and GR expression. These Vpr effects may lead to LPS tolerance.

Since the GR-ligand, dexamethasone, has been shown to have immunosuppressive effects, ligation to the GR may result in a tolerance of the cell to LPS. The GR induced tolerance may be sensitive to changes in GR expression. For instance GR mediated inhibition of cytokine production may be sensitive to increases in GR expression.

Some data suggest that TLR4 signalling does not lead to an upregulation of HIV replication (318), while other research indicates that activation of membrane TLR4 by LPS increases activity of the HIV-LTR through the activation of NF κ B (319). Together these findings indicate that LPS can lead to multiple immune outcomes, including initiating the innate immune response, inducing both immune tolerance and immune sensitization, and may not only affect homeostasis, but may influence viral replication as well.

The work reported here suggests that HIV-1 infection and Vpr can affect the immune response through altered TLR4 expression and its expression in response to LPS stimulation. HIV infection is also associated with increased TLR6 and TLR7 expression and responsiveness, which may contribute to the innate immune dysfunction and activation (311).

LPS signalling can act as an environmental sensor for autophagy through binding to TLR4 (310). The autophagic response is a malleable static process which has been shown to be an important component of the innate immune response in the elimination of bacterial pathogens (320, 321). The TLR4 receptor expression and their response to LPS may lead to reduced immune responses. Our demonstration that TLR4 expression is altered by HIV and Vpr may have implications for the autophagic response to LPS.

Further understanding the mechanism of immune sensitization may add insight into the development of effective immune based therapies. Immune sensitization seems to result from the enhanced immune activation of an otherwise dormant cell to incoming stimulation by antigen or mitogen. A potential advantage of sensitization may be enhancement of the immune response to a second infection.

There may be multiple evolutionary reasons why immune tolerance would be advantageous following a primary viral infection on the other hand, there may also be multiple advantages for immune sensitization. Here we propose a new model incorporating CD14, TLR4 and GR which have all been independently demonstrated to be important markers of activation and critical for the immune response to LPS. In this study, receptor expression was measured and analyzed in cells under a number of conditions including exposure to Vpr and stimulation with LPS. Retroviral infection may lead to immune sensitization, whereas the expression of Vpr may lead to immune tolerance to LPS stimulation.

3. The Functional Significance of HIV and Vpr-Mediated Changes in Receptor Expression

HIV and Vpr-mediated immune tolerance via change in CD14, TLR4 and GR receptor expression has implications for susceptibility to secondary infections during HIV disease. *Mycobacterium tuberculosis* is the most frequent co-infection in humans infected with HIV-1, but little is known about mechanisms that favour co-infection (322), or the mechanisms that contribute to accelerated progression of *M. tuberculosis* infection from exposure to the bacterium to active tuberculosis (323-325). The compromised immune response may be linked to changes in CD14 and TLR4 receptor expression observed during HIV infection. Soluble CD14 expression in serum is elevated in TB and HIV patients (134) creating a micro-environment that facilitates entry of *M. tuberculosis* into macrophages through CD14 (326). In addition, TLR4 plays an important role in the pattern recognition of *M. tuberculosis*, and the presence of the Asp299Gly TLR4 polymorphism results in a greater risk of active tuberculosis (327). Furthermore, a greater reduction in CD4 T cells in HIV infected individuals with the Asp299GLy polymorphism is observed during active tuberculosis than in those without the polymorphism. Together, these findings indicate that TLR4 plays an important role in the innate immune response to *M. tuberculosis*. Overall, targeting Vpr may enable the restoration of receptor expression and eliminate the favorable environment for TB infection. Among other co-infections, *Neisseria gonorrhoeae* often occurs as a co-infection with HIV. Lipooligosaccharide (LOS) is a component of the gonococcal outer membrane that induces an innate immune response through engagement of TLR4. LOS expressed by various strains of *N. gonorrhoeae* induce

specific innate immune responses through TLR4 signalling and this response results in anti-HIV activity in human primary macrophages *in vitro* (328).

Retroviral infection may lead to immune sensitization, whereas the expression of Vpr may lead to immune tolerance to LPS stimulation through altered receptor expression. Together this adds context to the importance of receptor expression changes seen following Vpr expression within the cell.

D. Vpr as a Target for Therapy

Vpr increases virus replication in T cells and is necessary for the optimal infection of primary monocytes/macrophages and other non-dividing cells (25, 161). The ability of HIV to infect and replicate in non-dividing cells, such as monocytes, significantly contributes to the pathogenesis of AIDS and should be a target of further therapeutic developments. Efficient proviral integration requires Vpr and without Vpr, HIV is unable to infect monocytes and macrophages (329).

Targeting Vpr in a way that prevents infection of latent cells while enhancing the immune response through increasing production of IL-12 may be of particular benefit. In latently infected cells the Vpr binding to GR can activate the HIV LTR and GR_i (Ru486) prevents the transactivation of the HIV LTR by Vpr (205). The upregulation of GR may result in further cytokine deregulation (252, 302, 309). Therefore targeting Vpr may not only create advantages by increasing IL-12 production, but may also prevent viral replication.

Data suggests that glucocorticoids can overcome the restriction on HIV provirus formation and thereby increase the reservoir of virus producing cells (329). Despite these findings, utilizing glucocorticoid agents likely offers little immune advantage as

a therapeutic, since the use of glucocorticoids and their pharmacological mimics' results in immune suppression. Equally important is the fact that a functional human GR is required for health and therefore its complete blockade may not be a good therapeutic approach. Therefore preventing Vpr activity may present a better target for therapeutic development than using agents which inhibit glucocorticoid activity.

Targeting Vpr for therapeutic development may restore IL-12 production and lead to enhanced cell mediated immune function. Therapeutic use of IL-12 could enhance protective immune responses to various pathogens (330-332) as well as to tumors (333). Unfortunately the dosages required for therapeutic effects are toxic (83, 84). The IL-12 induced toxicities include, numerous necrotic lesions and apoptotic cells in lymphoid tissue and the effects on T-cell responses appear to be associated with cell death (315, 334).

The advantage of developing a target for the 64-68 amino acids of Vpr includes the potential ability to prevent Vpr binding to the GR and viral integration into dormant cells, while continuing to allow for the maintenance of normal GR function. In addition, targeting Vpr for therapeutic development presents a less toxic alternative than targeting host molecules such as glucocorticoids and GR, since preventing Vpr function is less likely to interfere with normal cellular functions. Studies of Vpr sequence homology indicate that the 64-68 amino acids are highly conserved in infected patients (204) providing a rationale for Vpr as a target for therapy since sequence conservation is rare with HIV's error prone polymerase. The inhibition of Vpr with GR_i allows for a normal TLR4 response to LPS and may dampen the immune response to incoming bacterial infections and reverse Vpr-mediated inhibition of IL-12 p40. Overall, targeting

the amino acids 64-68 of Vpr to restore IL-12 p40 production following HIV infection merits further investigations.

The results of our study further the understanding of the immune pathology caused by Vpr, through its ligation to the GR. This information provides further support for the study of Vpr as a therapeutic target.

E. Novelty of this Work

1. We demonstrated a mechanism by which HIV inhibits IL-12 through the inhibition of p38 and JNK leading to decreased IL-12 p40 mRNA expression and protein production in LPS-stimulated primary adherence derived monocytes.
2. We demonstrated that p38 or JNK inhibition prevented LPS-induced IL-12 p40 promoter activity, and demonstrated that the effect of inhibition of either of these kinases on nuclear factor binding is similar to that seen in active HIV infection.
3. We demonstrated that HIV upregulated CD14 and downregulated TLR4 while having no effect on GR expression in monocytes within LPS-stimulated PBMC.
4. We found that Vpr peptide upregulated CD14 and GR while decreasing TLR4 expression in monocytes within LPS-stimulated PBMC
5. We demonstrated that the C-terminal Vpr peptide inhibits LPS-induced IL-12 p40 through a GR-dependant mechanism.
6. We found that intracellular expression of Vpr through a retroviral delivery system increases CD14 and decreases TLR4 expression on monocytes and this Vpr mediated effect on TLR4 could be prevented by the GR inhibitor Ru486. We further demonstrated that Vpr and dexamethasone altered GR expression.
7. We demonstrated that Vpr decreased LPS-induced IL-23 and IL-27 production.

8. We found receptor expression on monocytes to differ in response to the Vpr retrovirus when cultured in isolation or in the context of PBMC.

F. Significance

Determining the signalling pathways utilized by HIV to impair IL-12 production in monocytes including the SAPK/MAPK and Vpr/GR pathways will further our understanding of the connection between the endocrine and immune systems. Understanding the role HIV and its protein Vpr play in the decrease of IL-12 production in response to secondary stimulation is critical to understanding how HIV affects the cellular immune response. The ability of HIV to utilize the endocrine system's inherent capacity to modulate the cytokine network merits further exploration. In addition, characterizing the cellular mechanisms by which HIV-1 inhibits IL-12 production will lead to a better understanding of how CMI responses are inhibited by HIV-1 infection, and potentially contribute to the development of novel immune-based therapies.

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335. Blahaoui, M. and Kumar, A. JNK inhibition by SP600125 upregulates IFN- γ induced IL-12 p40. University of Ottawa, Faculty of Medicine, Department of Microbiology and Immunology, graduate student poster day, April 2008.

VIII-CURRICULUM VITAE

FIONA MARGARET FRAPPIER, B.Sc, PhD candidate

I. SKILLS

- ❖ Analyse, synthesis and solve complex problems.
- ❖ Experience writing grant proposals and scientific abstracts.
- ❖ Award winning scientific presentations.
- ❖ Establish and maintain partnerships and lead multidisciplinary teams.
- ❖ Effective interpersonal relationships.
- ❖ Experience in implementing, monitoring and enforcing regulatory programs.
- ❖ Fluently bilingual in French and English, Canadian citizen

II. ACADEMIC BACKGROUND

Doctorate of Philosophy, (2010) University of Ottawa, ON
Biochemistry, Microbiology and Immunology
Thesis topic: Human Immunodeficiency Virus Mediated Inhibition of Interleukin-12.

Bachelor of Science (Honours), (2002) University of Ottawa, ON
Biochemistry

III. EXPERIENCE

PhD Student

Department of Biochemistry, Microbiology & Immunology, University of Ottawa,
Ottawa, ON
Molecular Medicine Program, Ottawa Health Research Institute, Ottawa, ON

- **Laboratory Skills:** Bacterial culture and aseptic techniques - Level II cell isolation and culture - Cloning of genes -Electromobility Shift Assay - Enzyme-Linked ImmunoSorbent Assay - Flow Cytometry - general lab maintenance - Luciferase-promoter assays- Level III certification – Phlebotomy - Polymerase chain reaction- Viral experience includes adenovirus-luciferase constructs, retrovirus-HIV gene constructs, Level III HIV (several strains); Western blots
- **Training** Provided the mandatory laboratory safety training to ensure compliance with regulatory requirements of other laboratory personnel. Laboratory skill training of new personnel. Reviewing oral and written presentations.
- **Research Design :** Developed and designed my project bringing a new area of expertise to our laboratory. Outlining projects for new students.
- **Regulatory certifications:** Biological Safety training Level I, Level II, Level III; Radiation Training (³²P specialisation); WHMIS; Health and safety training; First Aid

Public Health Research Assistant (2000)

Population Health, Public Health Branch, Health Canada, Ottawa, ON

- Initiated and developed the Hope and Health hypothesis
- Conducted a broad Literature review
- Wrote a summary report entitled *Hope and Health: a review of current knowledge in PsychoNeuroImmunology*; a summary report on the current knowledge regarding Psychoneuroimmunology and disease in support of policy development associated with the Wellness Initiative.

Teaching Assistant (2001-2005)

Department of Chemistry, University of Ottawa, Ottawa, ON

- Assisted and supervised weekly laboratory sessions
- Instruction and marking of weekly laboratory experimentation

Tutor (1999)

Peer Help Center, Academic Development Office, University of Ottawa, Ottawa, ON

- Support to students by providing expertise in work methods, stress management and organizational techniques.

IV. VOLUNTEER ACADEMIC SERVICE

President (2007)

Vice President – Communications (2005-2006)

Student Representative & Event Organizer (2003-2005)

Biochemistry, Microbiology & Immunology Graduate Student Association
University of Ottawa, ON

- Presenting and communicating with senior management including the Dean of the Faculty of Medicine, VP Academic, Department Chair and Program Directors.
- Vision planning
- Coordinate and chair monthly meetings
- Plan and execute social, academic and team building events
- Organising the elections, Annual General meeting, writing the Annual report

Editor-in-Chief (2007-2008)

Guest Editor-Contributor (2005-2007)

BMI Bulletin – Graduate Student Bi-Weekly Newsletter, University of Ottawa, ON

- Orchestrating the Bulletin released every two weeks distributed throughout the Department
- Contributing and coordinating a multidisciplinary team
- Recruit, edit and format all contributions
- Write the editorial and full length features

Student Council Representative

Department of Biochemistry, Microbiology & Immunology (2005-2009)

Faculty of Medicine Graduate Student Council (2007)

Faculty of Medicine Advisory Board (2006-2007)

Biochemistry, Microbiology & Immunology Program Council (2003-2007)

Faculty of Science (2001-2002), University of Ottawa, ON

- Communicate between interested parties on key issues

- Presenting to senior management

V. AWARDS & ACADEMIC SCHOLARSHIPS

Gold Poster Award (2007)

Canadian Institute of Health Research National Awards, Institute of Infection and Immunity, Winnipeg, Manitoba

Young Investigator Award (2003, 2008, 2009)

Conference on Retroviruses and Opportunistic Infections, Boston, Massachusetts

Best Graduate Presentation (2003, 2005)

Microbiology and Immunology, University of Ottawa, Ottawa, Ontario

VI. CONFERENCE PRESENTATIONS

Frappier, F., Boucher, J., O'Connor, A. M., Angel, J.B. (2009). Vpr-Mediated Inhibition of IL-12 p40 by Disruption of TLR4 through the Glucocorticoid Receptor. 15th Conference on Retroviral and Opportunistic Infections, Boston, Massachusetts.

Frappier, F., Boucher, J., O'Connor, A. M., Angel, J.B. (2008). HIV Mediated Disruption of TLR4 by HIV Protein Vpr Binding to the Glucocorticoid Receptor. Oral presentation in the basic science track, 16th Annual Canadian Conference on HIV/AIDS Research.

Frappier, F., Boucher, J., O'Connor, A. M., Angel, J.B. (2008). The Role of TLR 4 in HIV-Mediated Inhibition of IL-12. Keystone Symposia, Innate Immunity: Signalling Mechanism, Keystone, Colorado.

Frappier, F., Boucher, J., O'Connor, A. M., Angel, J.B. (2008). Vpr-Mediated Inhibition of IL-12 and Disruption of Upstream Signalling Receptors CD14, TLR4 and the Glucocorticoid Receptor. 15th Conference on Retroviral and Opportunistic Infections, Boston, Massachusetts.

Frappier, F., Young, C.D., Angel, J.B. (2007) HIV mediated decrease of IL-12 through interactions between the Glucocorticoid receptor, CD14 and TLR 4. 16th Annual Canadian Conference on HIV/AIDS Research.

Frappier F., J. B. Angel. (2006) The Role of Mitogen Activated Protein Kinases in Interleukin-12 Signalling. Ontario HIV Treatment Network Conference

Frappier, F., J.B. Angel (2006) HIV-mediated Inhibition of IL-12: The Role of Mitogen Activated Protein Kinase Signalling. The Canadian Federation of Biological Societies 50th Annual Meeting and the 4th Annual Northern Lights Fall Conference.

Frappier F., KA Chambers, JB Angel (2005) HIV Infection of Primary Monocytes Inhibits p38 and JNK Kinases, Leading to Dysfunctional Nuclear Factor Binding and Decreased IL-12-p40 Promoter Activity. 14th Annual Canadian Conference on HIV/AIDS Research.

Frappier F., K. A. Chambers, J.B. Angel. (2004) HIV Infection of Primary Monocytes Inhibits p38 and JNK Kinases leading to Downregulation of IL-12 p40. Ontario HIV Treatment Network

Frappier, F., K. A. Chambers, J.B. Angel (2004) HIV-Induced Alterations of MAPK and IL-12 p40 Promoter Activity Contributes to the Inhibition of IL-12 Synthesis. 13th Annual Canadian Conference on HIV/AIDS Research.

Frappier, F., K.A. Chambers, J.B. Angel (2003) HIV Inhibition of IL-12 Synthesis is Mediated by Altering MAPK Activity and Nuclear Factor Binding to the IL-12 p40 Promoter. Oral presentation in the basic science track 12th Annual Canadian Conference on HIV/AIDS Research.

Frappier, F., K.A. Chambers, J.B. Angel (2003) HIV Inhibition of IL-12 Synthesis is Mediated by Altering MAPK Activity and Nuclear Factor Binding to the IL-12 p40 Promoter. 10th Conference on Retroviral and Opportunistic Infections, Boston, Massachusetts.

VIX CONTRIBUTION OF COLLABORATORS

The research reported in this thesis was conducted exclusively by Fiona M. Frappier, with the following exceptions. Sequencing was performed by the Ottawa Health Research Institute sequencing facility. The construction of the recombinant adenovirus Vectors was performed by Dr. Robin Parks and his research technicians Robert Meulenbroek and Kathy Sargent at the Ottawa Health Research Institute. The construction of the pLXIN and Vpr retrovirus was prepared in collaboration with Jonathan Boucher, Suvreena Manhas and Allison O'Connor from Dr. Angel's Laboratory and Aurelia Busca from Dr. Ashok Kumar's Laboratory at the Children's Hospital of Eastern Ontario. The flow cytometry protocols were developed in collaboration with Charlene Young in the laboratory of Dr. Jonathan Angel. The preliminary data mentioned in the discussion regarding the use of the JNK inhibitor to assess the effect to IFN- γ was generated by Maria Blahoianu in the laboratory of Dr. Ashok Kumar.

APPENDIX I: LIST OF CHEMICALS

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

³²P-UTP (Amersham-Pharmacia Biotech, Baie d'Urfe, QC)

40% Acrylamide/bis-acrylamide (19:1) (Sigma-Aldrich, Oakville, ON)

5-bromo-4-chloro-3-indoyl- β -D-galactopyranoside (X-gal; Gibco/Invitrogen,
Burlington, ON)

Advantage cDNA PCR kit (BD Biosciences/Clonetech, Palom, CA)

Ammonium acetate (Sigma)

Ammonium sulfate (Sigma)

Bacteriological agar (Sigma)

BamHI restriction enzyme (Amersham)

BCA protein determination kit (Pierce, Rockfort, IL)

Bovine serum albumin (BSA; Sigma)

Caltag Fix-and-Perm kit (Cederlane, Hornby, ON)

FITC-LABELLED CD14 (Beckman Coulter, Fullerton, CA)

Dexamethasone (Sabex, Boucherville, QC)

DH5 α supercompetent cells (Gibco)

Diethylpyrocarbonate (DEPC; Sigma)

Dithiothreitol (DTT; Sigma)

EGTA (Sigma)

Enhanced firefly luciferase kit (BD Pharmingen)

Ethanol (Comercial Alcohols, Brampton, ON)

Ethidium bromide (Sigma)

Fetal bovine serum (FBS; Gibco)

Ficoll-Paque Plus (Amersham)

Fungizone (Bristol Myers Squibb, Montreal, QC)

G418 (BD Biosciences/Clontech)

GFX gel purification kit (Amersham)

Glycerol (Sigma)

Glycine (Sigma)

FITC-labeled GR (ABD Serotec, Oxford, UK)

Hepes (Sigma)

HIV-1 p24 antigen ELISA (NIH AIDS, Germantown, MD)

Human AB serum (Sera Care Life Sciences Inc. Oceanside, CA)

Human IL-12 ELISA (R&D Systems, Minneapolis, MN)

Human IL-23 ELISA (eBioscience)

Human Monocyte isolation kit II (MyltenyBiotech, Bergisch Gladbach, Germany)

Human type AB serum (Sigma)

Hybond-ECL nitrocellulose membrane (Amersham)

IL-2 (Sigma-Aldrich)

IL-27 sandwich antibody kit (R&D Systems)

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

Isoamyl Alcohol (Sigma)

Isopropanol (Sigma)

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

KCl (Sigma)

LB broth (Sigma)

Leupeptin (Sigma)

L-Glutamine (Sigma)

Lipopolysaccharide (LPS; Sigma)

Lumigen TMA-6 substrate (GE Healthcare UK Limited, Buckinghamshire, UK)

Methanol (Sigma)

MgCl₂ (Sigma)

Microspin G-50 columns (Amersham)

Na₃VO₄ (Sigma)

NaCl (Sigma)

NaF (Sigma)

NaOH (Sigma)

OPD substrate (Sigma)

P89.6 (NIH AIDS)

Paraformaldehyde (Sigma)

PD 98059 (Calbiochem, San Diego, CA)

Penicillin (Gibco)

Phenylmethanesulfonyl fluoride (PMSF; Sigma)

Phosphate-buffered saline (PBS; Gibco)

Phytohemagglutinin (PHA; Sigma-Aldrich)

pLXIN (BD Biosciences/Clonetechn)

Polybrene (Sigma-Aldrich)

Poly-L-Lysine (Sigma)

Proteinase K (Gibco)

PT67 (BD Biosciences/Clonetech)

Qiaprep Spin Miniprep kit (Qiagen, Mississauga, ON)

RNase inhibitor (Sigma)

RNase-free DNase (Sigma)

RNeasy RNA extraction kit (Qiagen)

RPMI-1640 medium (Gibco/Invitrogen)

Ru486 (Sigma-Aldrich)

SB203580 (Calbiochem)

SDS (Sigma)

SOC medium (Gibco)

Sodium acetate (Sigma)

Sodium deoxycholate (Sigma)

SP600125 (Calbiochem)

Streptomycin (Gibco)

Sucrose (Sigma)

Superfect reagent (Qiagen)

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

T4 polynucleotide kinase (Amersham)

Taqman FAM-GAPDH (Applied Biosystems)

TaqMan One-Step RT-PCR Master Mix Reagents (Applied Biosystems)

PE-LABELLED TLR4 (Imegenex Corp, San Diego, CA)

Tri-base (Sigma)

Tris-buffered phenol (Gibco)

Triton-X-100 (Sigma)

Trypsin (Sigma)

Trypsin-EDTA (Gibco)

Tween-20 (Sigma)