

The Effect of HIV-1 and Accessory Proteins on Monocyte Derived Dendritic Cell
Maturation and Function.

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Abstract

Dendritic cells (DCs) are specialized members of the innate immune system that are responsible for the initiation of primary adaptive immune responses whose purpose is to resolve infection and inflammation. During most viral infections, mature dendritic cells present critical viral antigens to naïve T-cells within secondary lymphoid organs, resulting in the generation of an antigen-specific adaptive immune response and clearance of the virus. During infection with HIV-1 however, the virus is not cleared and a chronic systemic infection develops characterized by immune dysfunction, CD4⁺ T-cell depletion, systemic inflammation, and opportunistic infections. A growing body of evidence indicates that HIV-1 subversion of DCs contributes to both HIV-1 pathologies and viral dissemination. A number of similar effects by accessory HIV-1 peptides on DC physiology have also been reported. *In vitro* studies demonstrate that HIV-1 inhibits DC maturation and function. *Ex vivo* studies on the other hand describe partially mature, dysfunctional DCs collecting in secondary lymphoid organs. *In vitro* studies examining the effects of HIV-1-Tat and HIV-1-Vpr have described opposing effects on DC maturation. Therefore we undertook experiments to comprehensively describe the effects of HIV-1 and the Tat and Vpr accessory peptides on DC maturation and function.

To understand the contributions of individual viral proteins to DC dysfunction we infected DCs with a dual tropic HIV-1 and examined phenotypic and functional changes after maturation with inflammatory cytokines. Following this we examined the influence of exogenous and endogenous HIV-1-Tat and HIV-1-Vpr on MDDC maturation and function using recombinant proteins and deletion mutant lab adapted HIV-1 strains.

Live dual tropic HIV-1 was found to selectively inhibit aspects of phenotypic maturation as well as antigen capture and presentation functions. MDDC MAPK responsiveness to bacterial LPS remained intact however. Exogenous accessory HIV-1 Tat and Vpr did not affect MDDC phenotype but inhibited dextran endocytosis and viral peptide presentation. HIV-1-gp120 increased iMDDC maturation while blunting cytokine induced decreases in MDDC antigen capture abilities. The deletion of HIV-1-Tat did not affect MDDC phenotype, but was found to affect antigen capture decreases by R5 tropic HIV-1_{BaL}. Deletion of HIV-1-Vpr likewise did not affect MDDC phenotype, however it was found to be influential in HIV-1 induced decreases in MDDC antigen presentation to autologous T-cells. These accumulated results indicate that HIV-1 subverts DC maturation and function through whole virus effects and individual accessory peptide influences.

Understanding the mechanisms of DC dysfunction in HIV infection may provide some insight into infection prevention strategies and therapies leading to adaptive immune system activation and viral clearance.

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List of Abbreviations

AIDS	Acquired Immunodeficiency Syndrome
AT-2	Aldrithiol-2
AP-1	Activator protein 1
APC	Antigen presenting cell
APOBEC3G	Apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G
AMP	Adenosine monophosphate
ART	Antiretroviral treatment
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CaCl ₂	Calcium Chloride
cAMP	Cyclic Adenosine monophosphate
CARD	Caspase activation and recruitment domain
CCL	Chemokine ligand
CCR	C-C Chemokine receptor
CDC42	Cell division control protein 42
CD	Cluster of Differentiation
CEF	Cytomegalovirus, Epstein-Barr and Influenza viruses
CFSE	Carboxyfluorescein succinimidyl ester
CLR	C-type lectin receptors

CLEC7A	C-type lectin domain family 7, member A
CLEC9A	C-type lectin domain family 9, member A
CLEC12A	C-type lectin domain family 12, member A
CLEC4C	C-type lectin domain family 4, member C
CLEC4E	C-type lectin domain family 4, member E
CO ₂	Carbon dioxide
CpG	Cytosine-phosphate-Guanine
CXCL2	C-X-C motif chemokine ligand 2
CXCR-4	C-X-C motif chemokine receptor type 4
°C	Degrees Centigrade
DC	Dendritic Cells
DCIR	Dendritic cell immunoreceptor
DC-LAMP	Dendritic cell Lysosome-associated membrane glycoprotein
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
E.coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EP	E-prostanoid receptors
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase

EtBr	Ethidium bromide
FADD	Fas-associated death domain
FasL	Fas ligand
FCS	Fetal calf serum
fDC	Follicular dendritic cell
FITC	Fluorescein isothiocyanate
g	Gravity
GM-CSF	Granulocyte-macrophage colony-stimulating factor
gp41	Glycoprotein 41
gp120	Glycoprotein 120
gp130	Glycoprotein 130
gp160	Glycoprotein 160
GTP	Guanosine triphosphate
h	Hours
HAART	Highly active antiretroviral treatment
HCL	Hydrochloric acid
HLA	Human leukocyte antigen
HIV-1	Human immunodeficiency virus-1
HEK	Human embryonic kidney
HEPES	4-(2)-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase
ICAM-1	Intercellular Adhesion Molecule 1
iDC	Immature dendritic cell
IFN	Interferon

IFN α	Interferon alpha
IFN $\alpha\beta$	Interferon beta
IFN γ	Interferon gamma
Ig	Immunoglobulin
IgG	Immunoglobulin G
IL	Interleukin
IL-1R	Interleukin-1 receptor
IL-6R	Interleukin-6 receptor
iMDDC	Immature monocyte derived dendritic cell
IPS-1	Interferon-beta promoter stimulator 1
IRAK	Interleukin-1 receptor-associated kinase
IRF	Interferon regulatory factor
ITAM	Immunoreceptor tyrosine-based activation motifs
JAK	Janus kinases
JNK	c-Jun N-terminal kinases
Kb	Kilobase
LARG	Leukemia-associated Rho guanine-nucleotide exchange factor
LB	Luria Broth
LC	Langerhans cell
Lck	Lymphocyte-specific protein tyrosine kinase
LDL	Low density lipoprotein
LFA-1	Lymphocyte function-associated antigen 1
LGP2	Laboratory of Genetics and Physiology 2
LMP	Low molecular weight protein

LPS	Lipopolysaccharide
LSP1	Lymphocyte-specific protein 1
LTR	Long Terminal Repeat
MAPK	Mitogen activated protein kinase
MDA5	Melanoma Differentiation-Associated protein 5
mDC	Mature dendritic cell
MDDC	Monocyte derived dendritic cell
MDP	Muramyl dipeptide
Meso-DAP	Meso-diaminopimelic acid
MgCl ₂	Magnesium Chloride
MHC	Major histocompatibility complex
min	Minutes
MIP1 α	Macrophage inflammatory protein 1 alpha
MIP1 β	Macrophage inflammatory protein 1 beta
MGL	Macrophage galactose-type lectin
μ g	Microgram
mg	Milligram
ml	Millilitre
μ l	Microlitre
μ m	Micrometer
M.O.I.	Multiplicity of Infection
mRNA	Messenger ribonucleic acid
M-Tropic	Macrophage tropic
MyD88	Myeloid differentiation primary response gene (88) proteins

NaCl	Sodium Chloride
NaF	Sodium Fluoride
NALP	NACHT, LRR and PYD domains-containing protein
Nef	Negative regulatory factor
NFAT	Nuclear factor of activated T-cells
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHL	Non-Hodgkin's Lymphomas
NHP	Non-human primates
NIH	National Institutes of Health
NK	Natural Killer
NLR	NOD-Like receptor
NLRP	Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain containing family
nm	Nanometre
nM	Nanomolar
NOD	Nucleotide-binding oligomerization domain
OD	Optical density
PAK	p21-Activated Kinases
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PB	Polybrene
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell

PE	Phytoerythrin
PGE ₂	Prostaglandin E-2
PHA	Phytohemagglutinin
PI3K	Phosphoinositide 3-kinase
PIC	Preintegration complex
PKC	Protein Kinase C
PKR	Double stranded(ds)RNA activated serine/threonine protein kinase
PLC	Phospholipase C
PRR	Pattern recognition receptor
RANTES	Regulated and normal T cell expressed and secreted
Ras	Rat sarcoma
Rev	Regulator of viral expression/splicing control
RHoA	Ras homolog gene family, member A
RHoB	Ras homolog gene family, member B
RNA	Ribonucleic acid
rNef	Recombinant HIV-1-Nef
RIG	Retinoic acid-inducible gene
rhGM-CSF	Recombinant human Granulocyte Macrophage Colony Stimulating Factor
rhIL-4	Recombinant human Interleukin 4
RIP2	Receptor interacting protein-2
RLR	RIG-like receptors
ROCK1	Rho-associated, coiled-coil containing protein kinase 1
ROS	Reactive oxygen species

RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RT-PCR	Reverse transcriptase polymerase chain reaction
S	Seconds
SAPK	Stress activated protein kinase
SDS	Sodium dodecyl sulfate
SIV	Simian immunodeficiency virus
SIV-Vpx	Simian immunodeficiency virus Viral Protein X
SMAD	Sma and Mad Related Family
SOE	Splice overlap extension
STAT	Signal Transducer and Activator of Transcription
Stbl2	Stable-2
Syk	Spleen tyrosine kinases
TAE	Tris base, acetic acid and EDTA
TAP	Transporter associated with antigen processing
Tat	Transactivator of transcription
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline plus Tween 20
TCR	T-cell receptors
TGF- β	Transforming growth factor beta
Th1	T-helper-1
Th2	T-helper-2
THP-1	Human acute monocytic leukemia cell line
TIR	Toll/Interleukin-1 receptor

TLR	Toll-like receptor
TMB	Tetramethylbenzidine
TNF	Tumour necrosis factor
TNF α	Tumour necrosis factor alpha
TNF-R	Tumour Necrosis Factor-receptor
TRADD	Tumor necrosis factor receptor type 1-associated DEATH domain protein
TRAF	Tumour Necrosis Factor receptor-associated factor
TRAIL	Tumour Necrosis Factor-related apoptosis-inducing ligand
T-reg	Regulatory T-cell
TREX1	Three prime repair exonuclease 1
TRIS	Tris(hydroxymethyl)aminomethane
tRNA	Transfer-Ribonucleic acid
T-tropic	T-cell tropic
U	Units
V	Volts
Vif	Viral infectivity factor
Vpr	Viral protein R
Vpu	Viral protein U
VSV	Vesicular stomatitis virus

1.0 Introduction

Inflammation is a highly regulated physiological defence response to tissue injury, irritants, and infections [1-3]. Its progression and regulation are dependent on a series of coordinated interactions between components of the innate and adaptive immune systems [1-3]. Dendritic cells (DCs) are specialized members of the innate immune system that are responsible for the initiation of primary adaptive immune responses [4]. Diminished DC function can contribute to impaired adaptive immune responses resulting in poor resolution of pathologies and infections [4].

1.1 Dendritic cells

DCs are phagocytic members of the innate immune system that mediate interactions between the innate and adaptive immune systems [4]. The chief function of DCs is to collect information from the anatomic periphery and present their findings for interpretation and direction by the adaptive immune system [4]. They are the only type of phagocytic cells capable of inducing primary adaptive immune responses [5]. DCs are also responsible for the induction of adaptive immune system memory [6-8] and tolerance [9,10]. Impairment of DC maturation and function can result in impaired adaptive immune system direction and function and impaired memory T-cell differentiation [11]. DC progenitors in humans are derived from 2 distinct bone marrow CD34⁺ hematopoietic stem cell (HSC) populations [12,13]. Depending on the progenitor population, DC precursors released into the circulation either home to tissues, or remain in circulation where they take up protective roles [14-17].

1.1.1 Myeloid precursor derived dendritic cells

Myeloid DC precursor populations can be divided into CD14⁺ CD11c⁺ CD1⁻ monocytes and CD14⁻ CD11c⁺ CD1⁺ cells [18-20]. After migration from the circulation into tissues, these cells differentiate in response to chemical signals within their environments into distinct resident immature DC (iDC) populations [17-20].

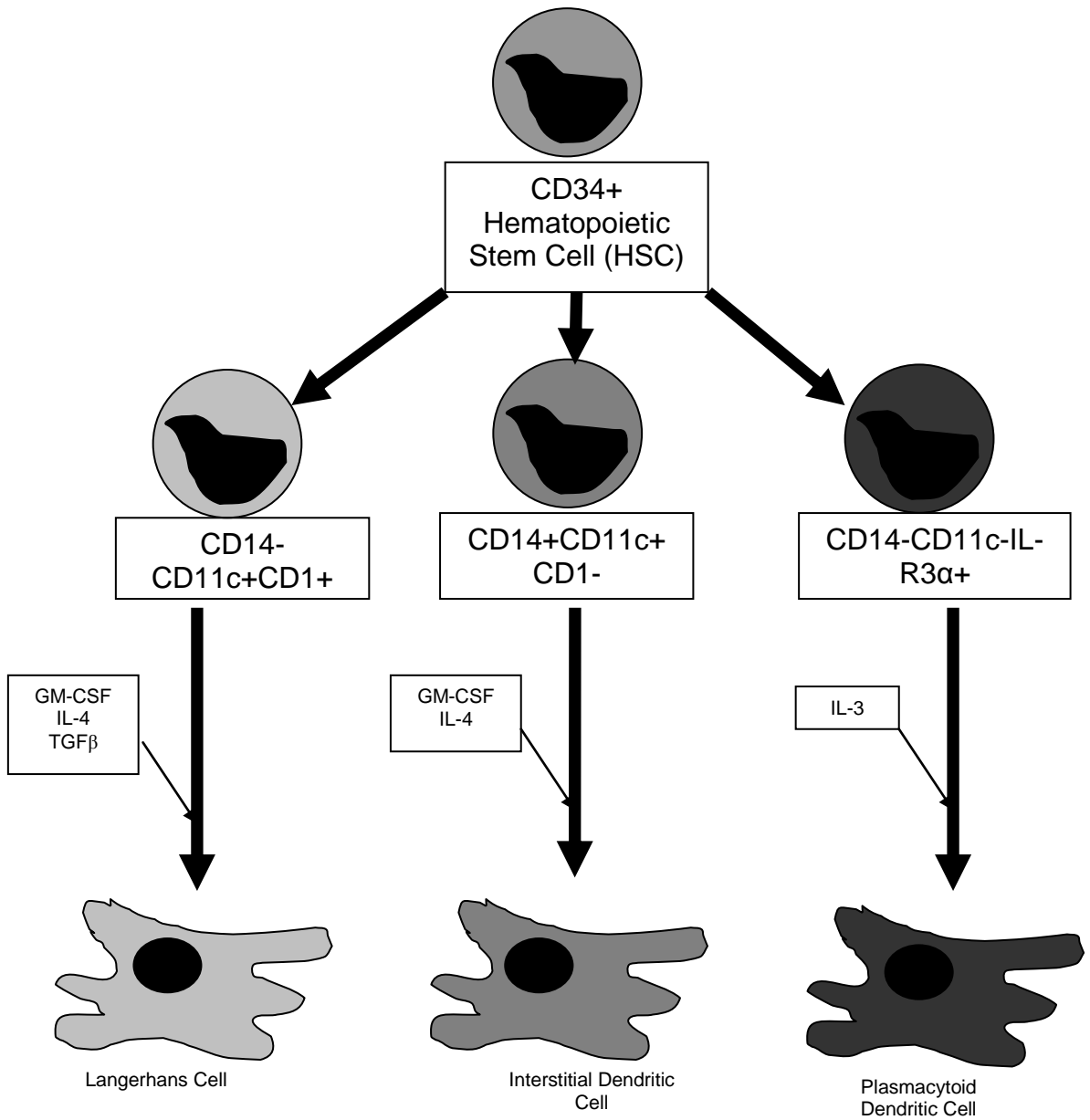
When stimulated with Granulocyte-macrophage colony-stimulating factor (GM-CSF), and interleukin 4 (IL-4), CD14⁺ CD11c⁺ CD1⁻ monocytes differentiate into immature interstitial dendritic cells [15,18]. These highly phagocytic immature interstitial DCs take up residence within a variety of non-lymphoid tissues where they survey their environments for infectious material and tissue abnormalities [4]. Interstitial DCs have been identified in virtually every tissue in the human body which has led to the identification of distinct dermis DCs [21], splenic marginal DCs [22], T-cell zone interdigitating DCs [23], germinal centre DCs [24], thymic DCs [25], kidney DCs [26], and liver DCs [27]. In any case, after antigen recognition, iDCs begin the process of maturation in preparation for antigen presentation to the adaptive immune system [4].

Precursor CD14⁻ CD11c⁺ CD1⁺ cells stimulated with GM-CSF, IL-4, and transforming growth factor beta (TGF-β), differentiate into a specialized subset of immature DCs called Langerhans cells (LCs) [28]. LCs are highly phagocytic cells that reside in the epithelia and visceral mucosa, and are often the first DC line of defence between the human body and the external environment [28]. They are named for their expression of langerin (CD207), a unique surface molecule that binds extracellular carbohydrate-associated ligands for internalization and degradation in Birbeck granules [29]. Like iDCs, after antigen recognition, LCs mature and move to secondary lymphoid tissues for antigen presentation [30].

1.1.2 Plasmacytoid Dendritic Cells

Stimulation of a third CD14⁻ CD11c⁻ IL-3Rα⁺ population of DC precursors with interleukin 3 (IL-3) gives rise to immature lymphoid DCs identified as plasmacytoid DCs (pDC) [13]. Instead of homing to tissues, these DC precursors take up a protective role in the circulatory and lymphatic systems [31]. Their primary function is antiviral defence via the secretion of type 1 interferons (IFN) [32].

Figure 1: Dendritic cell subset differentiation from CD34+ hematopoietic stem cells.



1.2 Pattern recognition receptors

Immature DCs recognize antigens using a large variety of molecular structures called pattern recognition receptors (PRRs) [33-35]. These membrane bound and cytosolic receptors recognize structural proteins expressed by non-mammalian cells called pathogen-associated molecular patterns (PAMPs) [33-35], various self stress proteins [36], acquired non-self antigens [37], and apoptotic bodies [38]. PRRs are strategically positioned on the outer plasma membrane of DCs [33,34], within intracellular vesicles [33] and in their cytosol [35]. Activation of several different PRRs simultaneously allows for diverse innate responses to a large variety of physiological perturbations and pathogens that will optimally direct the adaptive immune system to best resolve the problem [40,41].

Some of the better understood DC-expressed PRRs include Toll-like receptors, C-type Lectins, Scavenger Receptors, Nucleotide Oligomerization Domain (NOD) Receptors, and RIG-like receptors.

1.2.1 Toll-like receptors

Toll-like receptors (TLRs) are transmembrane proteins located on the cell surface and within intracellular vesicles where they are positioned to recognize extracellular and intracellular microbial structures respectively [33].

TLRs found on the cell surface include TLR1, TLR2, TLR4, TLR5, and TLR6. Homo and heterodimerization occur during ligation with structures typically associated with extracellular pathogens as well as some superficial viral structures [42-44]. TLR3, TLR7, TLR8, and TLR9 within intracellular vesicular compartments on the other hand detect viral genetic material and unmethylated CpG bacterial DNA [45-47].

TLR ligation (with the exception of TLR3) results in the interaction of their cytoplasmic Toll/IL-1 receptor (TIR) domains with associated adaptor protein Myeloid differentiation primary response gene (88) (MyD88) proteins [48]. MyD88 recruits cellular substrates and subsequently activates inflammatory transcription programs via Mitogen activated protein kinase (MAPK) and Phosphoinositide 3-kinase (PI3K) pathways, and Nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) activation [49]. Intracellular pathway activation leads to the induction of type 1 IFNs [31,50], TNFα [50,51], IL-1β [51], IL-6 [52], IL-8 [53], IL-12 [51,53], the chemokine receptor ligands MIP1α, MIP1β, RANTES [49,53], and expression of CD40, CD80, CD83, CD86, and MHC II molecules [54].

1.2.2 C-Type lectin receptors

C-type lectin receptors (CLR) are a large group of DC transmembrane and soluble PRRs that recognize acquired nonself antigens and carbohydrate molecular patterns common to infectious pathogens [34]. CLRs are grouped into 2 different categories: the mannose receptors [55] and the asialoglycoprotein receptor family [56].

1.2.2.1 The Mannose Receptors

Mannose receptors expressed on macrophages and DCs are identified as the Mannose receptor (CD206) [55] and DEC205 (CD205) [57]. They recognize repeated mannose carbohydrates on microbes [55] and facilitate the endocytosis of extracellular antigenic material which is processed through the HLA-DR antigen presentation system [58].

The downstream signalling partners of the mannose receptors are Cell division control protein 42 (CDC42) [59], Ras homolog gene family member B (RHoB) [59], p21-Activated Kinases (PAKs) [60], and Rho-associated, coiled-coil containing protein kinase 1 (ROCK1) [61]. Signalling via these pathways leads to the activation of cytoskeletal elements involved in endocytosis, vesicular trafficking, polarization, cytokinesis, migration, and differentiation [59-61].

1.2.2.2 The asialoglycoprotein receptor family

The asialoglycoprotein receptor family includes a large number of receptors broken down into 3 main families: the classical asialoglycoprotein receptor family [56], the dectin 1 subfamily [62], and the DCIR subfamily [63].

The classical asialoglycoprotein receptor family includes Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) [64], Langerin (CD207) [65], and the macrophage galactose-type lectin (MGL) [66].

DC-SIGN ligand binding and endocytosis is a predominantly immunosuppressive event involving signalling through the (Lymphocyte-specific protein-1) LSP1 [67], LARG [68], RHoA [68], and Rat sarcoma (Ras) proteins [69]. DC-SIGN binding can eventuate in antigen presentation [64], increased TLR-induced IL-10 production [69], regulatory T-cell differentiation [69], T_H1 cell differentiation inhibition [70], and TLR signal inhibition [71].

Langerin binding induces ligand internalization and shuttling to Birbeck granules for degradation and classical presentation [65]. This has been observed to be important for processing HIV-1 [72] and *M.leprae* [73]. Signalling after langerin ligation is poorly understood, however MyD88 has been observed to be required for a response to *C. albicans*, but not for contact hypersensitivity [74].

MGL has been observed to be an important phagocytic receptor for Filoviruses [75] and *S. mansoni* [76]. Associated signalling has been observed to involve ERK NF- κ B and an increase in antigen presentation [77].

1.2.2.3 The Dectin 1 subfamily

The Dectin 1 subfamily members expressed by DCs includes Dectin-1 [78], the lectin-type oxidized LDL receptor 1 (LOX-1) [79], Dectin 1/C-type lectin domain family (CLEC) members CLEC1 [80], CLEC2 [80], CLEC7A [81], CLEC9A [82], and CLEC12A [83].

Upon binding, their ligands are endocytosed and associated Spleen tyrosine kinases (Syk) are phosphorylated by receptor associated cytosolic immunoreceptor tyrosine-based activation motifs (ITAM) [84]. Dectin ligated induced Syk activation can then induce increases in reactive oxygen species (ROS) production and inflammatory cytokine production by NF- κ B [85]. However Syk activation of AP-1 can also increase IL-10 production [86]. CLEC9A ligation on the other hand, has been observed to induce TNF α production [87] and antigen cross presentation [88] and CLEC12A ligation has been observed to be involved in TLR4 activity inhibition leading to lowered IL-12 production [83].

1.2.2.4 The DCIR subfamily

The DCIR subfamily includes Dectin 2, [89] CLEC4A (DCIR) [90], CLEC4C [91], and CLEC4E [92]. Despite poorly understood signalling pathways, some outcomes of ligand binding have been elucidated. Dectin 2 ligation has been observed to cause increases in TNF α and IL-6 production [89] as well as Cysteinyl leukotriene synthesis [93]. DCIR

ligation on the other hand, has been observed to inhibit TLR8 induced TNF α and IL-12 production [90] and TLR9 induction of TNF α and IFN α [94]. CLEC4C ligation has been observed to cause inhibition of TLR9 induced type 1 IFN production [95]. However, CLEC4E activation can induce TNF α and IL-10 secretion as well as CXCL2 production [96].

1.2.3 Scavenger receptors

Scavenger receptors are phagocytic PRRs expressed on the plasma membrane of phagocytes including DCs that recognize modified low density lipoprotein (LDL) [97] and microbial diacylglycerides [98]. After ligand recognition the antigen is endocytosed and processed with classical and cross antigen processing machinery [99]. Scavenger receptor signalling in DCs has not been extensively studied; however PI3K in THP-1 cells [100] and MAPK activity have been reported to be activated through these receptors in macrophages [101].

1.2.4 Nucleotide Oligomerization Domain (NOD) receptors

Cytoplasmic NOD-Like receptors (NLRs) activate various proinflammatory cellular substrates after oligomerization with microbes [102] or self stress proteins [103]. Family members include Nucleotide-binding oligomerization domains (NOD) 1 [104] and 2 [105] and The Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain containing (NLRP) family [106].

NOD1 recognizes meso-diaminopimelic acid (meso-DAP) [104] and NOD2 recognizes bacterial intracellular muramyl dipeptide (MDP) [105]. NLRPs have 14 family

members that are activated by a variety of microbial proteins [106]. Mutations in these proteins are observed to cause multiple inflammatory disorders [107-109].

After ligand binding, NALP and NOD-associated-receptor interacting protein-2 (RIP2) [110] are activated which allows for NF- κ B phosphorylation leading to IL-1 β production [111] and caspase activation [112].

1.2.5 RIG-like receptors Nucleotide Oligomerization Domain (NOD) receptors

RIG-like receptors (RLRs) are cytoplasmic PRRs that recognize viral RNA [113]. Family members include (Retinoic acid-inducible gene) RIG-1, Melanoma Differentiation-Associated protein 5 (Mda5), and Laboratory of Genetics and Physiology 2 (LGP2) [114]. RLR engagement results in recruitment of the adaptor proteins Helicase [115] and Caspase activation and recruitment domain (CARD) proteins [116] as well as Interferon-beta promoter stimulator 1 (IPS-1) [117] and TNF receptor-associated factor 3 (TRAF3) [118] leading to the the induction NF- κ B [115,116], MAPKs [119], PI3K [119] and IRF activation [117,119].

1.3 Chemokine receptors expressed by dendritic cells

Anatomical distribution of DCs in stromal tissues and secondary lymphoid organs after maturation is dependent on their expression of seven-transmembrane protein receptors called chemokine receptors [120].

After their ligation with chemokines secreted from cells in distinct anatomical locations, receptor-associated Rho-GTPases are activated causing increases in cellular cyclic AMP (cAMP) [121]. This in turn activates NF κ B via the ERK MAPK and Protein Kinase C (PKC) pathways which activate cell transcription programs that induce

cytoskeletal rearrangements and lamelopodia formation [122]. These cellular extensions allow for DCs to traffic towards increased chemokine concentrations [122]. Without proper chemokine receptor function, DCs are unable to migrate to either the stroma or secondary lymphoid tissues for antigen capture and presentation respectively [123].

Immature DCs express the chemokine receptors CCR1, CCR5, and CCR6. During maturation, DCs decrease their expression of these molecules and increase their expression of CCR7 [120,123,124].

Ligation of CCR1 with the chemokine ligands (CCL) CCL3, CCL4, and CCL5 can induce iDC movement along chemokine gradients [124]. CCR1 engagement with CCL5 has also been observed to result in increased secretion of inflammatory mediators by iDCs [125].

Actin remodelling also occurs after CCR5 engages CCL3-5 [124] and when CCR6 engages CCL20 [126]. During the course of maturation, DCs decrease CCR1, CCR5 and CCR6 expression and increase CCR7 expression [124]. When CCR7 ligates with CCL19 or CCL21, actin remodelling and lamellipodia formation mediate DC chemotaxis to the T-cell regions of secondary lymphoid tissues [127].

1.4 Dendritic cell cytokines

DCs produce [128] and respond to [123,124,127] a number of different cytokines in response to antigenic insults. The nature and identities of the secreted cytokines depends on compliment of activated PRRs, and the cytokine environment of the DCs.

1.4.1 Interferons

Viral antigen ligation with vesicular DC TLRs initiates cellular antiviral responses and the secretion of type 1 interferons (IFN) (IFN α and IFN β) via Interferon regulatory factors (IRF) [129]. Type 1 IFNs exert their effects through paracrine means and include activation of double stranded(ds)RNA activated serine/threonine protein kinase (PKR) [130], increased MHC I expression [131], and stimulation and sequestration of Th1 cell activity [132] in the absence of cellular proliferation [133]. Their extracellular secretion engages type 1 IFN cytokine receptors on neighbouring cells which induces their transcription of antiviral genes via Janus kinases and Signal Transducer and Activator of Transcription mechanisms (JAK/STAT) [134]. IFN receptor ligation induces dimerization and creation of STAT docking sites by JAK mediated receptor phosphorylation [134]. STATs then bind to these docking sites and phosphorylate each other leading to their dimerization and translocation to the nucleus where they induce IRF transcription [134].

1.4.2 Tumor Necrosis Factor (TNF) α

TNF α is secreted by DCs [135]. It is synthesized as secreted or membrane bound nonglycosylated type 2 transmembrane protein [136]. Activation of the soluble form of the cytokine requires cleavage into 17 kDa peptide chains by a membrane-associated metalloproteinase [136] that trimerize into the active secreted 51 kDa form [137]. Secreted TNF α binds to TNF-receptors (TNF-RI and TNF-RII) which recruit RIP and (Tumour necrosis factor receptor type 1-associated DEATH domain protein) TRADD adaptor proteins to the cytoplasmic TNF-R death domains [138]. These proteins then recruit TNF receptor-associated factor 2 (TRAF2) and Fas-associated death domain (FADD) [139]. TRAF2 recruitment results in nuclear factor kappa B (NF- κ B) and AP-1 activation [140]

which induce proinflammatory gene expression in a large number of cell types [141-143]. Locally, TNF α effects include increased expression of recruiting chemokines [144], selectins [145], and integrins [146] by endothelial cells for recruitment of leukocytes to sites of inflammation [147], and activation of phagocytic cells [148] and maturation of APCs [149]. However, if the infection is persistent and TNF α is secreted in large quantities, this can result in a number of physiological disturbances including fever [150], serum protein secretion [151], cachexia [150], vascular muscle tone loss [152], and septic shock [150].

1.4.3 Interleukin-1

IL-1 is secreted by DCs [153] in α and β forms that function synergistically with TNF α [154]. IL-1 α is active both as membrane bound and secreted forms while IL-1 β is only active in secreted form [155]. They share approximately 30% homology, however despite this both forms bind Interleukin-1 receptor (IL-1R) I and II [156]. These receptors signal through a cytosolic TIR domain and an associated interleukin-1 receptor-associated kinase 4 (IRAK4) which facilitates NF κ B [157], MAPK [158], and PKC activation [159]. Like TNF α , IL-1 β recruits leukocytes to sites of inflammation and stimulates local and systemic pro-inflammatory cellular responses [160]. Like TNF α , IL-1 can induce systemic shock [161]. IL-1 β also induces DCs to produce IL-12 [162] and can activate procaspase-3 [163] which has been observed to contribute to apoptosis [164] and brain development [165].

1.4.4 Interleukin-12

IL-12 is secreted mainly by DCs and is an important cytokine for T-cell activation [166]. After binding its heterodimeric receptors IL-12R- β 1/2, JAK/STAT signalling [167] pathways induce increased CD8+T-cell cytotoxicity [168], increased Natural Killer (NK) activity [169], and increased interferon gamma (IFN- γ) secretion from CD4+ T-cells [170], which are the primary directors of adaptive immune responses [171-173].

1.4.5 Interleukin-6

IL-6 secreted by DCs [174] signals through the IL-6 receptor (IL-6R) which involves receptor dimerization and association with glycoprotein 130 (gp130) accessory molecules at the plasma membrane [175]. Similar to the mechanism of action of IL-12, IL-6 activates both proinflammatory gene expression and cellular substrates contributing to DC maturation [176], B-cell antibody production [177], T-cell activation [178], and acute phase protein synthesis [179] by the liver via JAK/STAT and ERK MAPK signalling pathways [180]. It can also induce immune system modulation [181].

1.4.6 Prostaglandin E-2

Prostaglandin E-2 (PGE₂) is a member of the prostaglandin family secreted by stromal cells [182] in inflamed areas that contributes to DC maturation [183] and immunomodulation [184]. On DCs, PGE₂ exerts its effects through 4 G-protein-coupled PGE₂ E-prostanoid receptors (EP1-4) which can result in activation of phospholipase C (PLC) [185], adenylate cyclase [186], STAT signalling [187], IRFs [187] and inhibition of the ERK MAPK [188]. Consequences on DC biology after PGE₂ binding induces several proinflammatory outcomes including increased expression of CCR7 [189], increased DC

maturation [183], increased IL-12 production [190], and increased IL-23 production [191]. However, PGE₂ binding with DCs has also been observed to cause increased IL-10 secretion [186], inhibited antigen presentation [192], and decreased IL-23 production [188].

In addition to effects on DCs, PGE₂ can inhibit lymphocyte migration [193], proliferation [194], activity [195], and to aid in the induction of Th2 [196] and Th17 immune responses [197]. It can also exert effects on smooth muscle tone [198] vasodilation [199], bronchodilation [200], gastric acid production [201], gastric mucous production [202], homeostatic temperature regulation [203], and fever [204].

1.5 Antigen processing and presentation

Evolutionary pressure [205] has necessitated the development of an adaptive immune system comprised of 2 T-lymphocyte populations and a B-lymphocyte population [206,207]. In addition to increased antigen specificity and diversity, a defining characteristic of the adaptive immune system is an increased magnitude of immune response over time [208,209]. These qualities make this system a powerful immunological tool for resolving pathologies. However, uncontrolled adaptive immune system activation can result in deleterious physiological effects and death [210-212]. To prevent unmitigated adaptive immune responses, initial innate immune system input is required before adaptive immune system activation can proceed [213-215]. The more limited antigen recognition repertoire of the innate immune system prevents self antigen recognition [216] and unmitigated activation of the adaptive immune system [217].

As described earlier, using PRRs and highly developed antigen processing machinery, DCs identify and process antigens for presentation to the adaptive immune

system. DCs have 2 antigen processing pathways at their disposal for processing extracellular and intracellular antigens [218,219].

1.5.1 Antigen processing

To resolve extracellular infections, iDCs in the anatomic periphery use PRRs to identify and endocytose antigenic PAMP-associated antigens into early endosomes [33-35,219]. Within these membranous vesicles, the acidic environment and proteolytic enzymes begin denaturation and degradation of internalized materials [220]. Endosomes next fuse with intracellular membrane bound lysosomes, which are proteolytic enzyme dense intracellular vesicles [221]. This results in the production of digested peptides of optimal length for binding to the open ended peptide binding clefts of MHC II molecules (15-30 amino acids) [222].

At the same time, MHC II molecules bound with invariant chains are synthesized in the endoplasmic reticulum (ER) and transported through the golgi apparatus [223]. From there, they are sent in membrane bound vesicles to fuse with the late endosomal vesicles containing the digested microbial peptides [224]. After fusion, the invariant chains are removed from the MHC II molecules by proteolytic enzymes and HLA-DM molecules and they are replaced with the digested microbial peptides [225]. This stabilized tripeptide complex is then transported to the cell surface for presentation to the T-cell receptors (TCRs) of CD4⁺ T-cells [225].

To resolve intracellular infections, DCs identify pathogens using cytosolic and vesicular PRRs [33,35]. These can be viral [37], bacterial [33], or abnormal quantities of self peptides [36]. After identification, the offending protein is ubiquitinated and degraded by the proteasome [226]. Under normal conditions the proteasome exists as a 700kDa

structure and functions as a housekeeping agent for the cell, degrading misfolded or damaged proteins [227]. After DC stimulation by IFN γ , 2 additional catalytic subunits: low molecular weight protein 2 (LMP-2) and LMP-7 are bound to the proteasome forming the immunoproteasome [227]. These modifications allow for the generation of peptides of an appropriate length for binding to MHC I presentation clefts (8-11 amino acids) [228]. Peptides generated in this manner are translocated to the endoplasmic reticulum [229] with the assistance of the ATP dependent ER membrane transporter associated with antigen processing (TAP) proteins [230] which are also stimulated by IFN- γ [231]. The TAP proteins are optimized for the transport of peptides 6 to 30 amino acids long with either basic or hydrophobic carboxy terminals [228], are also bound to unoccupied MHC I by tapasin molecules [232]. Following their introduction into the ER, these peptides bind unoccupied MHC I molecules bound to the activated TAP proteins [232]. These complexes then move through the ER and the Golgi complex to the cell surface in vesicles for surface expression and surveillance by CD8⁺ T-cells [229].

1.5.2 Antigen presentation

1.5.2.1 Effects of antigen recognition and processing on dendritic cell phenotype

Antigen recognition and processing induces DC maturation. This results in changes in DC phenotype including, but not limited to decreased expression of PRRs [233], CCR1, CCR5, and CCR6, [120] and increased expression of CCR7 [120,234], MHC molecules, CD80, CD86, CD83, CD40, CD58 [234], and Intercellular Adhesion Molecule 1 (ICAM-1) molecules [235]. DCs also change in morphology from an amorphous shape to one with many dendritic processes [234]. These changes optimize mature DCs (mDCs) for antigen presentation to T-cells in secondary lymphoid tissues [234].

1.5.2.2 The Immunological Synapse in T-cell activation

Under the direction of increased CCR7 expression, mDCs enter the T-cell areas of secondary lymphoid tissues and engage resident naïve and memory T-cells [234]. The actual physical interaction between DCs and T-cells is called the immunological synapse [236].

Within the immunological synapse, T-cell CD4 and CD8 molecules bind to the peptide bound β 2-region of MHC II [237] and α 3 region of the MHC I molecules respectively [237]. Also within the synapse, costimulator CD80 and CD86 molecules expressed by mDCs bind CD28 on the T-cells providing the second signal for adaptive immune activation [238]. Next, TCR associated lymphocyte-specific protein tyrosine kinase (Lck), phosphorylates the cytosolic ITAMS of the accessory TCR associated CD3 and ζ - transmembrane proteins [239]. This creates docking sites for cellular substrates that initiate Calcium/Calcineurin [240], PKC [241], and MAPK pathways [242] within the T-cells. These activate the transcription factors Nuclear factor of activated T-cells (NFAT) [243], NF- κ B [240], and AP-1 [242] which together induce the transcription the T-cell growth factor IL-2 [244].

After CD40/CD40L ligation, MAPK activation of NF κ B induces the transcription of IL-12 within the presenting DC [245]. IL-12 induces increased IFN γ secretion from nearby and engaged T-cells via JAK/STAT [246] and MAPK pathways [247] regulated by Calcineurin signalling pathways [248].

Increased IFN γ production by CD4⁺ T-cells can increase DC expression of B-7 molecules, MHC molecules [249], and production of ROS likely via JAK/STAT mechanisms [250]. It can also increase CD8⁺ T-cell numbers and cytolytic activity during

viral infections [251]. Other important cytokines that are secreted by T-cells within the synapse are IL-10 and Transforming growth factor-beta (TGF- β). Interleukin 10 signals via JAK/STAT [252] and inhibit DC and adaptive immune system activation via MAPK [253] and PI3K pathway inhibition [254]. On the other hand, TGF- β signals through a type 2 TGF-beta receptor which recruits and phosphorylates a type 1 TGF-beta receptor [255]. Following this, receptor-regulated Sma and Mad Related Family (SMADs) proteins are recruited and phosphorylated at the cell surface [255]. These then translocate to the nucleus where they can induce a number of opposing pro and anti-inflammatory effects which are cell type dependent. These include: DC [256] and LC differentiation [257], decreased DC expression of CCR7 [258], inhibited DC B-7 molecule expression [257], inhibited antigen presentation [257], and inhibited DC migration [258].

Non-signalling molecules surround these molecular ligations which provide for synapse stability ensuring sufficient signal duration to induce adaptive immune system activation. The adhesion molecules CD58 and ICAM-1 anchored to the DC plasma membrane ligate with CD2 and Lymphocyte function-associated antigen 1 (LFA-1) respectively [260].

1.5.2.3 T-cell differentiation, migration, and responses to antigen presentation

After sufficient DC stimulation, CD4⁺ and CD8⁺ T-cells differentiate into memory and effector T-cells [261]. Central memory CD4⁺ and CD8⁺ T-cells remain in secondary lymphoid tissues where they are maintained to respond quickly to reencountered antigens [261]. Effector and effector memory CD4⁺ T-cells migrate to inflamed areas [261] where they engage CD40 expressing members of the innate system such as macrophages. Through this interaction they direct their immune functions via increased secretion of IFN- γ

secretion [262]. Effector CD8⁺ T-cells also migrate to inflamed areas, however rather than instruct innate immune system cells, they lyse cells expressing their corresponding MHC I bound peptide with perforin and Fas ligand (FasL) induced apoptosis [263].

1.5.2.4 Alternate antigen presentation pathways

A third non-classical antigen presentation pathway results in DC presentation of cytosolic linear peptide antigens to CD4⁺ T-cells on MHC II molecules [264] or presentation of exogenous antigens by MHC I complexes [265]. Presentation of endogenous peptides by MHC II is thought to involve endogenous protein translocation to the endosomal pathway compartments for presentation on MHC II molecules [264]. On the other hand, cross presentation of exogenous antigens on MHC I molecules likely involves ER-phagosome fusion events [265]. The proposed mechanisms may account in part for the development of virus-specific B-cell isotype switching [266] and tumor-specific cytotoxic T-cells [267].

Yet another specialized pathway of antigen processing that has been identified in DCs is engagement of langerin with mannose and fucose on extracellular pathogens [65,268]. After endocytosis and subsequent degradation in Birbeck granules, linear antigens are then presented to T-cells via classical and cross presentation pathways [269] however this has been disputed. [270].

1.6 HIV-1 Immunopathogenesis

1.6.1 Brief history of HIV-1/AIDS

In 1981, otherwise healthy young homosexual men in the United States began presenting with abnormally aggressive Kaposi's sarcoma (KS) [271], Non-Hodgkin's Lymphomas (NHL) [272], unusually resistant *Pneumocystis carinii* pneumonia (PCP) lung infections, and depressed lymphocyte counts [273]. Similar pathologies also began appearing in heterosexual drug users and haemophiliacs in Europe [274] and Haiti in 1982 [275]. In 1982, the commonly observed immune suppression and evidence supporting infectious agent involvement, led to a collective designation of the disease states as Acquired Immunodeficiency Syndrome (AIDS) [276].

In 1983, The Human Immunodeficiency Virus Type 1 (HIV-1) was identified as the causative agent of AIDS [277,278]. Since its discovery, over 60 million individuals have been infected, almost half of whom have died due to HIV-1 disease complications [279]. More than 50% of infected individuals receive inadequate antiviral treatment [279] however, gender bias in many endemic countries leaves women and children particularly vulnerable to HIV-1 infection [280]. Consequently, women presently represent greater than 50% of all individuals living with HIV-1 [280] and HIV-1 is now the leading cause of mortality for women of child bearing age worldwide [281]. It has also been estimated that if current prevention and treatment strategies do not improve, by 2031 worldwide HIV-1 infection and disease treatment will cost in excess of 35 billion dollars [282].

1.6.2 Clinical HIV-1 Infection and Disease Progression

The course of HIV-1 infection is divided into acute infection, clinical latency, and clinical AIDS [283]. Acute infection lasts 2 to 8 weeks after initial infection [283]. During

this stage, high levels of virus are detected in the plasma [284] and secondary lymphoid tissue infection is established [285]. Influenza or mononucleosis like symptoms including fever, lymphedema, elevated T-cell number, fatigue, and malaise often manifests [283]. During this stage of infection, viral levels decrease as the immune system exerts control over the infection with HIV-1-specific CD8⁺ T-cells [286] and humoral antibodies [287].

The clinical latency phase lasts 2 to 10 years [288]. During this phase of the infection, the virus actively replicates to a set point in lymphoid tissues while depleting circulating CD4⁺ T-cells [289] and destroying lymph node architecture [290]. Also despite maintenance of CD8⁺ T-cells number, their secretion of IFN- γ and cytolytic abilities are impaired [291].

End stage HIV-1 infection is reached when high plasma viral titres indicate a failure of the immune system to contain the infection [283]. CD4⁺ T-cell numbers are drastically reduced [283] and CD8⁺ T-cells become anergic and apoptotic [292]. The depleted immune system is no longer able to combat normally manageable infections and pathologies and a number of different serious disease conditions manifest including chronic fever, night sweats, diarrhea, opportunistic infections, cancers, and neurological disorders [283].

Approximately 80% of infected individuals develop late stage disease symptoms 8-10 years after infection if untreated [293]. Rapid progressors that make up 10% of infected individuals manifest late stage disease symptoms in 2-3 years without treatment [294]. Long term survivors make up 5% of infected individuals and present with lowered CD4⁺ T-cell numbers but no late stage diseases [295]. Long term nonprogressors (5%) experience no CD4⁺ T-cell loss or HIV-1 related pathology [296].

1.6.3 HIV-1 structure

HIV-1 is a lentivirus within the Retroviridae family. The virus is approximately 100nm in diameter with a host-derived phospholipid envelope [299]. A shell of inner p17 matrix proteins that maintain virus shape are attached to the transmembrane gp41 and surface gp120 glycoproteins which bind and fuse with target cells during infection [299]. Within the p17 shell, approximately 2000 copies of the structural p24 capsid protein surround 2 identical linear, positive strand RNA molecules bound to p7 nucleocapsid proteins [299].

1.6.4 The HIV-1 genome

The 9.3 Kb HIV-1 RNA molecules code for the retroviral gag, pol, and env genes, and additional vif, vpu, rev, nef, tat, and vpr genes in alternate reading frames [299]. The HIV-1 gag gene codes for the p18, p24, p7, and p15 viral structural proteins and the viral enzymes protease (p10), reverse transcriptase (p66/65), and integrase (p22) are coded for by the HIV-1-pol gene [299]. HIV-1-env encodes glycoprotein gp160, which is cleaved into the gp41 and gp120 envelope structural proteins [299]. Additional viral genes include viral infectivity factor (vif), viral protein U (vpu), viral protein R (vpr), regulator of viral expression/splicing control (rev), negative regulatory factor (nef), and the transactivator of transcription (tat) protein which encode for viral proteins that facilitate viral infectivity and disease progression [299].

1.6.5 HIV-1 cellular infectious life cycle

HIV-1-gp120 first binds CD4 surface molecules on expressing cells [300]. This causes a conformational change in the structure of associated transmembrane gp41 which

binds to CCR5 in the case of macrophage-tropic (R5-tropic) virus strains [301] or CXCR4 in the case of T-cell -tropic (X4) virus strains [302]. The viral envelope then fuses with the target cell membrane and the viral capsid is released into the cytoplasm and uncoated releasing HIV-1 RNA [301,302] and accessory molecules reverse transcriptase [303], integrase [304], protease [305], ribonuclease [306], HIV-1-Vpr [307], and cellular tRNA^{lys3} [308] molecules found within mature virions.

Once in the cytosol HIV-1 RNA is reverse transcribed by viral reverse transcriptase using tRNA^{lys3} template molecules into HIV-1 proviral DNA [309]. Integrase and vpr form the preintegration complex (PIC) with reverse transcribed HIV-1-DNA, which is shuttled into the nucleus and integrated into host DNA [310]. HIV-1-Vpr also arrests the cell cycle increasing the likelihood of proviral integration [311]. At the same time, viral ribonuclease degrades parent HIV-1 RNA strands after reverse transcription and in doing so, hides viral genetic material from cytosolic PRRs that might otherwise detect the infection [312]. A period of latent infection is then entered until stimulation of proviral DNA transcription [313].

At both the 5' and 3' ends of the proviral DNA, Long Terminal Repeat (LTR) regions provide binding sites for NFκB and NFAT whose stimulation is sensitive to external signals [314]. Proviral transcripts are spliced into over 25 different mRNAs which are themselves spliced by HIV-1-Rev and translated into viral polyproteins [315]. These are cleaved into smaller proteins by aspartyl HIV-1 protease and used for virion construction [305]. With the aid of newly translated HIV-1-Tat, the entire HIV-1 genome is then transcribed into *de novo* HIV-1 RNA [316]. Complete viruses are then assembled at the cellular membrane where they bud from the infected cell surface and a new infection cycle begins [317].

1.7 HIV-1 infection and dendritic cells

Heterosexual intercourse is the primary route of new HIV-1 infections [318]. The location of DCs throughout the vaginal [319], rectal [320], and oral mucosae [321] makes them likely target cells of HIV-1 during primary infection.

During most viral infections, mature dendritic cells present viral antigens to naïve T-cells within secondary lymphoid organs, resulting in the generation of an antigen-specific adaptive immune response and clearance of the virus [4]. During infection with HIV-1 however, the virus is not cleared and a chronic systemic infection develops characterized by immune dysfunction, CD4⁺ T-cell depletion, systemic inflammation, and opportunistic infections [283].

The effects of HIV-1 on T-cells are well documented contributors to HIV-1 related immune dysfunction and disease pathology [283]. However, a growing body of evidence now indicates that HIV-1 subversion of DC function contributes to HIV-1 related immune dysfunction and associated pathologies.

1.7.1 In vivo effects of HIV-1 on DCs

Due to ethical constraints and the nature of HIV-1 infection and disease, *in vivo* experimentation examining initial infection events is mostly limited to studies examining simian immunodeficiency virus (SIV) infection of macaques and Asian non-human primates (NHP). SIV is a slow retrovirus related to HIV-1 [322] that causes a progressive model of chronic immune activation and eventual immune system depletion similar to that of HIV-1 in humans [323]. SIV also affects simian immune cell populations in ways similar to those of HIV-1 in humans making this model a valuable tool that can be used to study *in vivo* immune-cell specific retroviral infection [323]. Conclusions drawn from these models

can be used in conjunction with human *ex vivo* and *in vitro* models to characterize HIV-1 infection in humans; however this should be done with caution since both the virus and animals affected are distinct from both HIV-1 and humans respectively.

1.7.2 The effects of SIV Infection of DCs

Similar to HIV-1 infection in humans, when mucosal membranes are intact, SIV transmission *in vivo* generally requires a number of viral exposures [324]. Viral infection rates increase when mucosal membranes and epidermal surfaces are compromised [325,326]. Eventual SIV transmission and systemic infection is believed to be due in part to alterations in DC number, phenotype, and function [327].

Also like HIV-1 infection in humans, SIV infection rate of simian mDCs is much lower than that of CD4⁺ T-cells [328]. Despite this their numbers and functions are also observed to be altered during SIV infection [328].

During acute SIV infection, baseline DC numbers increase and localized populations increase in size, particularly in secondary lymphoid tissues [328,329]. As infection progresses, pDC numbers in the lymph tissues decline due to direct viral killing of infected cells [330,331], Fas ligand induced DC apoptotic death [332] and accumulation in gut tissues [333].

In addition to changes in numbers, DC phenotype [328] and function are also affected [329-331] during different stages of SIV infection. Increases in expression of CD40, CD80, CD86 [331], and B7-H1 molecules [334] on mDCs are observed during acute SIV infection. This may be due in part to SIV-induced pDC bystander activation of mDCs as observed during HIV-1 infection [335]. During chronic infection these changes ablate and partially mature myeloid DCs become the predominant myeloid DC phenotype

[336]. Like in chronic HIV-1 infection in humans, these mDCs collect in secondary lymphoid tissues [336] where they may contribute regulatory T-cell induction [337]. However, both losses [338] and imbalanced T-reg populations [339] have been reported during SIV infection, leaving SIV effects on DC-T-reg interactions unresolved.

1.7.3 Ex vivo effects of HIV-1 on DCs

For the most part, the effects of chronic HIV-1 infection on DCs in humans are limited to *ex vivo* studies involving the examination phenotype and function of DCs taken from chronically infected individuals. While there has been evidence of DC hyperfunctionality during primary HIV-1 infection [340], this is in disagreement with much of the *ex vivo* and *in vitro* research whose discussion follows. In light of the limited number of *in vivo* studies involving human HIV-1 infection, *ex vivo* HIV-1 effects on DCs are described.

With the exception of follicular DCs (fDC), HIV-1 has been observed to productively infect the 3 remaining subcategories of DCs: Langerhans cells, myeloid DCs, and plasmacytoid DCs [341-343]. FDCs are not classical DCs in derivation or antigen presentation function. However, during HIV-1 infection, destruction of lymph tissue during viral infection [290] and attached infectious virions can compromise their function and contribute to T-cell infection [344] and B-cell dysfunction [345].

Integrative, productive HIV-1 infection of susceptible DCs occurs at low levels compared to that of T-cells [342]. The observed low infectivity of dendritic cells by HIV-1 has been attributed to DC maturation blocks [346], low levels of HIV-1 receptor and coreceptor expression [347], the characteristic ability of dendritic cells to degrade attached virions [348], and intrinsic host resistance factors like apolipoprotein B mRNA-editing

enzyme-catalytic polypeptide-like 3G (APOBEC3G) [349], the ubiquitin-editing enzyme A20 [350], and the three prime repair exonuclease 1 (TREX1) [351].

Without antiretroviral treatment (ART), chronically infected individuals suffer from reduced numbers of all subcategories of DCs [352,353]. The mechanisms of DC population reduction are incompletely understood. Systemic inflammation is thought to partially contribute to trafficking of infected DCs to lymphoid tissues, where they are maintained in a partially activated state [336]. In the case of fDCs, the destruction of lymph node architecture [290] may be responsible in part for reductions in their numbers.

DC phenotype and function have also been observed to be affected by HIV-1 infection. Myeloid DCs from acutely infected individuals have been found to express decreased levels of CD80 and CD86, and to collect in secondary lymphoid organs in a semi activated state [354]. Lowered CD83 expression by splenic DCs from HIV-infected individuals has also been observed [355]. These phenotypic disturbances may contribute to the *ex vivo* observation that DCs from chronically infected individuals are inhibited in their ability to stimulate allogeneic [356] and autologous T-cells [357].

Plasmacytoid DCs from HIV-1 infected individuals have been observed to be compromised in phenotype, number, migration, and function. Specifically, CCR7 expression by mature pDCs from infected individuals has been observed to be impaired [358]. This is in disagreement with a report describing normal expression of CD83 by pDCs in HIV-1 exposed intravenous drug users [359]. While it would appear that HIV-1 induces partial phenotypic pDC maturation *in vivo*, that the drug users were not infected indicate that chronic and acute responses to HIV-1 infection by pDCs may differ. Indeed, diminished type 1 interferon production [360], abnormal migration to lymph tissues [361,362], inhibited NK stimulating abilities [363], impaired antigen presentation [364],

and decreased numbers of pDCs [365] during chronic HIV-1 infection, have all been observed. By contrast however, enhanced pDC activity appears to be correlated with lack of disease progression in elite controllers [366] and partial pDC numbers and functions may be restored upon successful anti-retroviral therapy however [367]. However these abnormalities may be persistent even despite treatment [368].

1.7.4 *In vitro* effects of HIV-1 on DCs

Examinations of early HIV-1 infection events using isolated human DCs or monocyte derived dendritic cells (MDDC) have been carried out using *in vitro* experimental systems.

Similar to *ex vivo* observations, pDCs [369], myeloid DCs [370] and, LCs [72], are all susceptible to *in vitro* HIV-1 infection [492,493]. After viral infection, mature MDDC expression of the maturation markers CD83, MHC II, and Dendritic cell Lysosome-associated membrane glycoprotein (DC-LAMP) are inhibited [370]. HIV-1 infected mDCs also have an impaired ability to stimulate allogeneic T-cells and induce increases in IL-10 secretion from HIV-1 infected DC co-cultures [370]. Also, it has been found that unless MDDCs are coinfecting with (SIV Viral Protein X) SIV-Vpx, they do not mature after HIV-1 infection [371].

MDDCs have also been shown to maintain infectious virions on their surface and in endocytic vesicles after incubation with live HIV-1 [372]. In T-cell coculture, MDDC [372] and exosome associated viruses have been observed to be much more infectious than free virions [373]. This is accomplished through the formation and maintenance of an *in vitro* phenomenon called the infectious or viral synapse [374]. While neither heavily studied, nor yet observed *in vivo*, early observations suggest DCs physically transfer HIV-1 to CD4+ T-

cells through a cell to cell interface. Specifically, DC-SIGN-bound-HIV-1 is transferred to CD4 molecules on T-cells through DC-T-cell interfaces [374] that are maintained by ICAM-1 and LFA-1 interactions [375]. Synapse formation may also assist in viral dissemination to vulnerable CD4⁺ T-cells via exosome transfer [373] and membrane extension viral transfer [376]. These observations suggest DCs not only fail to recognize and process HIV-1, but that they may also facilitate viral dissemination.

However, other reports have described *in vitro* HIV-1 infection of DC increases mDC maturation [377]. These findings are contrary to the majority of studies detailing inhibitory or non-effects of HIV-1 *in vitro* on mDC maturation [370,371]. However, their results are in agreement with the partial maturation observed in bystander mDCs by HIV-1 activated pDCs [335]. They are also consistent with an *in vitro* examination of DC infection susceptibility [342], and *ex vivo* descriptions of partially matured mDCs from HIV-1 infected individuals [336,340]

While the effects of HIV-1 on LC maturation are not well studied, one study has demonstrated a maturing effect of HIV-1 on LCs [377]. On the other hand, LC mediated viral dissemination has been more thoroughly investigated. Similar to MDDCs, LCs have been observed to facilitate infection of vulnerable CD4⁺ T-cells by cis [378] and trans-infection [379]. Langerhans cells are resistant to HIV-1 infection due to their expression of the C-type lectin langerin, which binds HIV-1 resulting in its endocytosis and degradation in associated Birbeck granules [72]. However, with a sufficiently high viral titre, this protection is abrogated and productive infection can occur [72].

In vitro HIV-1 infection has also been observed to have a number of often contradictory effects on pDCs. Both increases [335] and decreases [380] in secretion of type 1 interferons has been observed from pDCs after incubation with HIV-1 *in vitro*. They

also have been observed to secrete TNF α [335], CCL5, CCL3 and IFN α after CD40 ligation and HIV-1 infection [381], and to secrete IFN γ , CCL3, and CCL4 in response to aldrithiol-2-inactivated HIV-1 [382]. Furthermore, pDCs activated by HIV-1 have been observed to induce increased NK activity [383], myeloid DC maturation [335], and to contribute to TNF-related apoptosis-inducing ligand (TRAIL) expression and apoptosis on CD4⁺ T-cells [384]. PDCs have also been observed to transfer HIV-1 to antigen-specific CD4⁺ T-cells [385], potentially contributing to viral dissemination *in vivo*.

1.7.5 The effects of HIV-1 peptides on DCs.

The study of the effects of individual HIV-1 proteins on DC biology is particularly important. Since DCs are relatively refractory to HIV-1 infection [342], yet DCs from HIV-1 infected individuals are compromised in function [336,337], HIV-1 effects may not be solely the result of productive cellular infection. Several HIV-1 peptides have been observed to have effects on DCs: Nef, gp120, Vpu, Tat, and Vpr.

To begin, the viral HIV-1-Nef protein has been observed to have a number of effects on DC maturation and function. Firstly, recombinant HIV-1-Nef (rNef) can alter the cellular phenotype of DCs. Specifically rNef has been observed to increase CD1a, CD40, CD80, CD86, CD83, HLA-DR, CXCR4, expression while inhibiting the expression of HLA-ABC [386]. It has also been observed to inhibit mannose-receptor mediated endocytosis while also increasing DC secretion of inflammatory cytokines (IL-1 β , IL-12, IL-15, TNF α) and chemokines (MIP-1 α , MIP-1 β , IL-8) and increasing their T-cell stimulating capacity [386]. Other observed effects of rNef on DCs is dysfunctional DC/NK cross-talk [387] and increased CD8⁺ T-cell apoptosis [388].

Endogenous Nef has also been observed to affect DC physiology. For instance, adenovirus-delivered HIV-1-Nef is reported to induce cytokine, chemokine, and T-cell activation by MDDCs in the absence of phenotypic maturation marker expression [389]. Others however have reported no effect on DC maturation or physiology by adenoviral [390] or retrovirally delivered HIV-1-Nef [391]. By contrast an HIV-1-Nef-expressing vaccinia virus has been observed to impair MHC I molecule expression by DCs leading to impaired antigen presentation to CD8+ T-cells [392]. Likewise when delivered with a Vesicular stomatitis virus (VSV) expressing HIV-1-Nef, DC MHC I and CD1a molecule expression were downregulated [393]. Finally, HIV-1-Nef has been observed to induce tetherin [394] and DC-SIGN [395] expression which may be partially responsible for the observation that HIV-1-Nef increases DC-mediated virus transfer to CD4+ T-cells using deletion mutants [396]. Thus HIV-1-Nef has a number of effects on DCs that could be critical to the immunopathogenesis of HIV-1.

Like HIV-Nef, HIV-1-gp120 has also been observed to have a number of effects on DCs. Firstly, HIV-gp120 has been observed to induce increased expression of B-7, CD83, CD40, and HLA DR molecules while inhibiting endocytosis reductions, antigen presentation and IL-12 secretion normally associated with a mature DC phenotype [397]. HIV-1-gp120 has also been observed to elicit IL-10 secretion from iDCs [398] and to modulate co-stimulator molecule expression [399]. However, data has been published describing a lack of effect of HIV-1-gp120 on DC maturation and function [400].

PDCs have also been observed to react to HIV-1-gp120 by secreting large quantities of type 1 IFNs, CCL2, CCL3, and CCL4 in typical antiviral fashion [382]. Opposing observations of inhibited type 1 IFN secretion and by pDCs have also been reported however [401].

By contrast the effects of HIV-1-Vpu on DCs are limited to MDDCs. The observed effects of HIV-1-Vpu incubation are impaired MDDC lipid presentation as a result of defective CD1a expression [402].

Recombinant exogenous HIV-1-Tat has been observed to induce the maturation of mDCs and MDDCs phenotypically and functionally. Specifically, exogenous HIV-1-Tat can induce DC upregulation of CD40, CD83, CD86 and MHC molecules [403,404]. MDDCs exposed to HIV-1-Tat also secrete TNF- α and IL-12 and of MIP-1 α , MIP-1 β which may increase the number of target cells in infected areas [404]. HIV-1-Tat can also induce IFN γ from autologous T-cells both in vitro and in vivo [404] and improve DC antigen presentation [404]. However, immature DC infection with Adenovirus expressing HIV-1-Tat has been reported to induce an IFN gene induction from MDDCs without phenotypic or functional maturation [405]. Thus extra- and intracellular HIV-1-Tat have differing effects on DCs.

Like the previously discussed peptides, HIV-1-Vpr has a variety of effects on DCs. To begin, inhibited pDC type 1 IFN secretion and NK cell stimulation were observed after incubating pDCs with recombinant HIV-1-Vpr [406].

DCs of myeloid origin respond to HIV-1-Vpr in a similar fashion. Recombinant HIV-1-Vpr is observed to inhibit MDDC phenotypic and functional maturation measured by costimulator expression and antigen presentation [407]. Similarly, intracellular HIV-1-Vpr can decrease transcription of B-7 molecules and CD83 while at the same time decreasing IL-12 production and increasing IL-10 secretion [408]. Cytosolic HIV-1-Vpr was also reported to increase Fas ligand mediated apoptosis of cocultured CD8⁺ T-cells [409] and to induce macrophage and monocyte secretion of IL-6 [410] which can decrease DC activity [181].

1.8 Summary

HIV-1 and HIV-1 derived peptides have been observed to have a number of *in vivo*, *ex vivo*, and *in vitro* effects on DC biology. Most of the reported effects describe DC dysfunction that aids in viral dissemination. However, a clear linkage between live virus effects and those of its accessory proteins on DC maturation and function is needed. Therefore, further studies describing the effects of HIV-1 on DC biology are of critical importance to prevent new infections and to manage existing infections.

1.9 Hypothesis

The overall hypothesis of this thesis is that HIV-1 inhibits DC phenotypic and functional maturation and induces their dysfunction through whole viral infection and distinct effects of the viral proteins HIV-1-Tat, HIV-1-Vpr, and HIV-1-gp120.

1.10 Rationale

The proposed project is aimed at understanding the effects of HIV-1 on MDDC maturation and function. Despite their resistance to productive viral infection, reduced numbers and impaired function of DCs are observed during HIV-1 infection that can be partially restored by antiretroviral therapy. *In vitro* HIV-1 infection similarly affects MDDCs. The accessory peptide HIV-1-Vpr can also inhibit *in vitro* MDDC maturation and function. By contrast, the viral accessory peptide HIV-1-Tat can induce MDDC maturation. Since DCs are refractory to HIV-1 infection, it is important that we elucidate the mechanism(s) behind HIV-1 induced inhibited DC maturation and function. The focus of this thesis research was to study the effect of HIV-1 infection on DCs and the effects HIV-1-Tat and HIV-1-Vpr can have on DC maturation and function.

1.11 Specific Aims

Aim 1: To determine the *in vitro* effect of HIV-1_{CS204} infection on MDDC maturation and function.

Aim 2: To determine the *in vitro* effect of the HIV-1-Tat, HIV-1-Vpr, and HIV-1-gp120 peptides on MDDC maturation and function.

Aim 3: To determine the *in vitro* effect of HIV-1-Tat and HIV-1-Vpr gene deletion on the effects of HIV-1 effects on MDDC maturation and function.

2.0 Materials and Methods

2.1.1 *Cell preparation and culture*

Ethically approved donated whole blood from healthy donors was layered at a ratio of 2:1 on Ficoll Paque™ PREMIUM (GE-Healthcare Bio-Sciences, Mississauga, ON) and centrifuged for 30 min at 443 x g with no brake. Buffy coats containing peripheral blood mononuclear cells (PBMC) were aspirated with Pasteur pipettes (Sigma-Aldrich Canada, Oakville, ON), washed twice with Phosphate Buffered Saline (PBS) (Thermo Fisher Scientific, Ottawa, ON), and centrifuged at 443 x g for 10 min. Monocytes were isolated from PBMC pellets with anti-CD14-coated microbeads according to the manufacturer instructions (Miltenyi Biotec, Mississauga, ON) and maintained in complete Roswell Park Memorial Institute medium (RPMI) media (RPMI 1640 medium containing L-glutamine, supplemented with 10% fetal calf serum (FCS), 100 µg/ml streptomycin, and 100 U/ml penicillin; Invitrogen, Burlington, ON) in 12 well tissue culture plates (Becton Dickinson and Company, Franklin Lakes, NJ) at 1×10^6 cells/ml at 37°C and 5% CO₂.

2.1.2 *Generation of monocyte derived dendritic cells*

Monocytes were differentiated into immature monocyte derived dendritic cells (iMDDC) as previously described [539]. Isolated monocytes were incubated in complete RPMI media supplemented with 500 U/ml recombinant human Interleukin 4 (rhIL-4) and 1000 U/ml recombinant human Granulocyte Macrophage Colony Stimulating Factor (rhGM-CSF) (R&D Systems, Burlington, ON) in 12 well tissue culture plates at 1×10^6 cells/ml at 37°C and 5% CO₂ for 24 h.

2.1.3 Maturation of monocyte derived dendritic cells

To induce maturation, iMDDCs in complete RPMI media at a density of 1×10^6 cells/ml were incubated with 1000 U/ml tumour necrosis factor alpha (TNF α), 10 ng/ml interleukin 1 beta (IL-1 β), 10 ng/ml interleukin 6 (IL-6), and 1 μ M/ml prostaglandin E2 (PGE $_2$) (R&D Systems, Burlington, ON) for 48 h at 37°C and 5% CO $_2$ in 12 well tissue culture plates [411].

2.2 Viral Stocks

Five different viral strains were used in this study: a dual tropic HIV-1_{CS204} (a gift from Dr. Francisco Diaz-Mitoma at the Children's Hospital of Eastern Ontario, Ottawa, ON), a pUC18 inserted T-tropic lab adapted HIV-1_{NL4-3} (The AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: pNL4-3 (Figure 1) from Dr. Malcolm Martin, Germantown, MD), a pUC19 inserted T-tropic lab adapted HIV-1_{Dvpr} lacking HIV-1-Vpr derived from the HIV-1_{NL4-3} (NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: p210-19 (Δ Vpr) (Figure 2) from Dr Ronald Desrosiers, Germantown, MD), a pXf3 inserted M-tropic lab adapted HIV-1_{BaL} (The AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: pWTBaL (Figure 3) from Dr. Bryan R. Cullen, Germantown, MD), and a pHXB2gpt derived M-tropic lab adapted HIV-1_{Mtat} strain with an TAG stop codon in the Tat gene promoter region (the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: pMtat(-) (Figure 4) from Dr. Reza Sadaie.) Germantown, MD).

Figure 2: pNL4-3 plasmid map: The full length T-tropic HIV-1_{NL4-3} pNL4-3 plasmid used to study the effects of HIV-1-Vpr deletion on MDCC maturation and function.

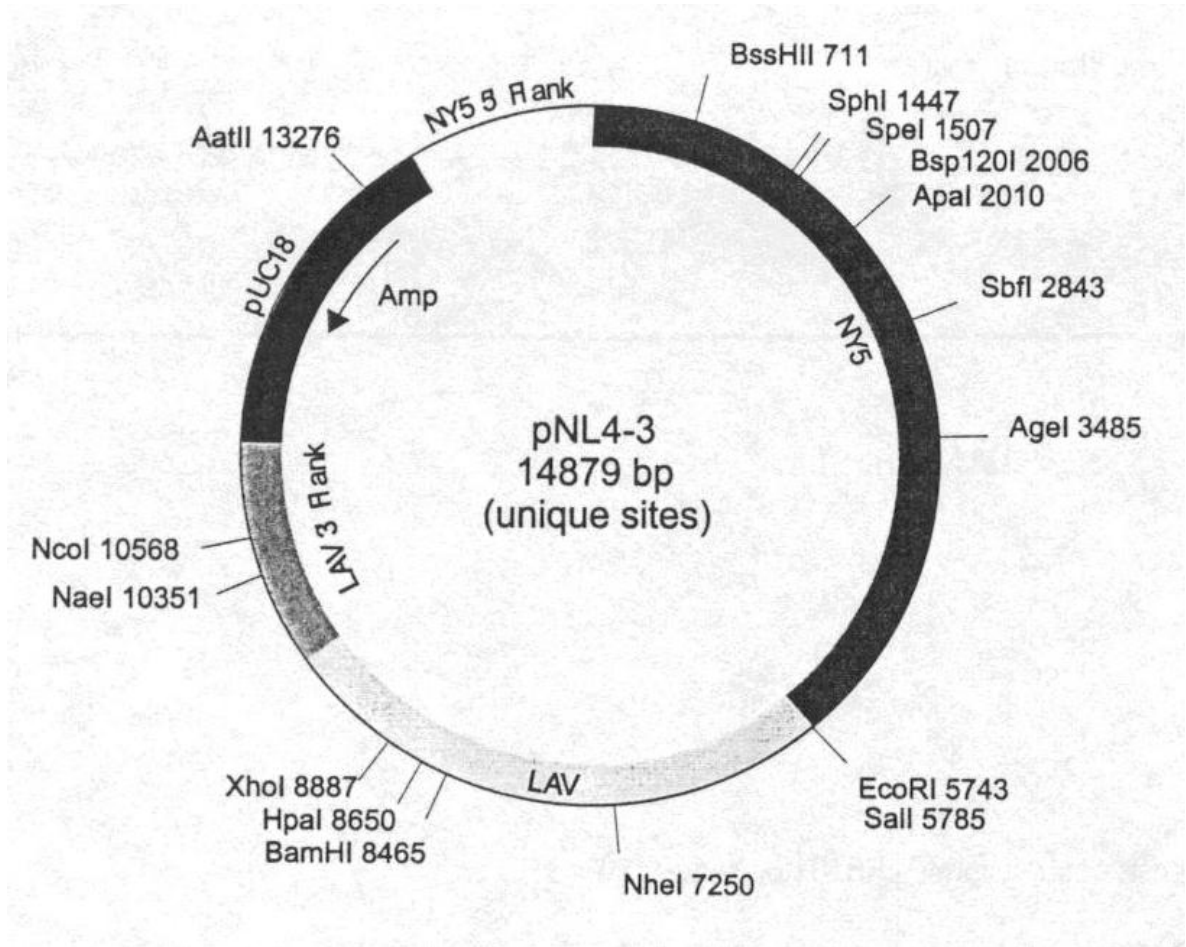
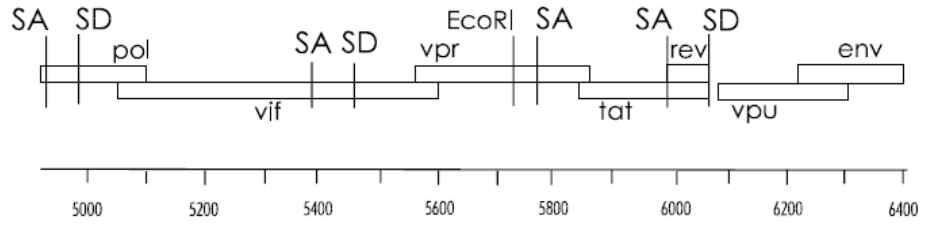


Figure 3: p210-19 (Δ Vpr) plasmid map: The T-tropic HIV-1_{NL4-3(-Vpr)} p210-19 (Δ Vpr) plasmid used to study the effects of HIV-1-Vpr deletion on MDCC maturation and function. Splice overlap extension (SOE) polymerase chain reaction (PCR) was used to delete the Vpr gene from the full length viral genome.



(p210-19)



Figure 4: pWTBaL plasmid map: The M-tropic HIV-1_{BaL} pWTBaL plasmid used to study the effects of HIV-1-Tat deletion on MDDC maturation and function.

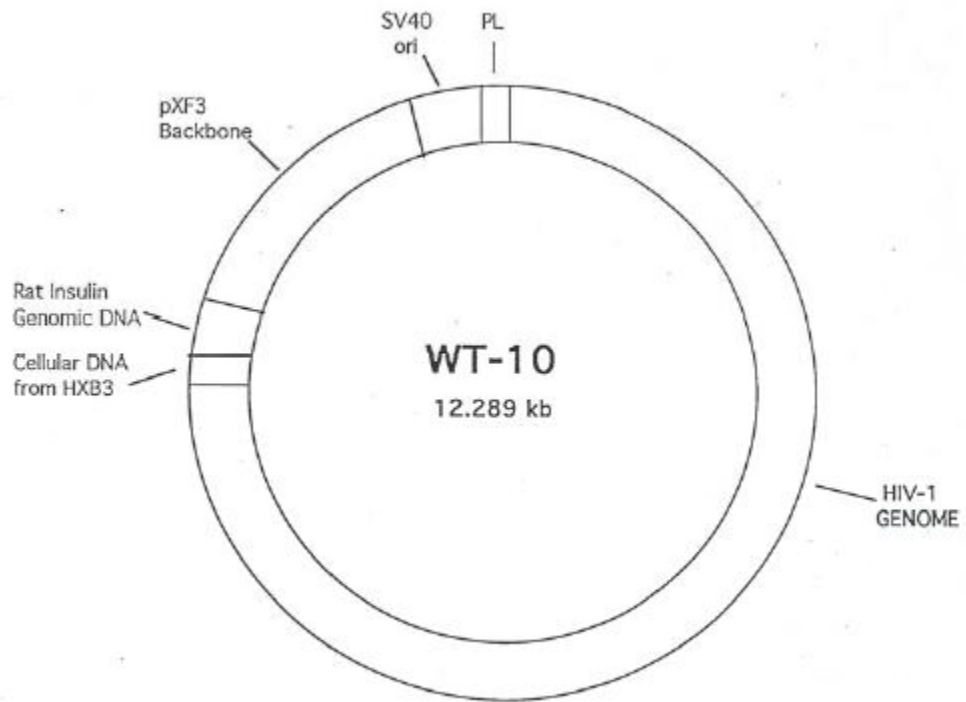
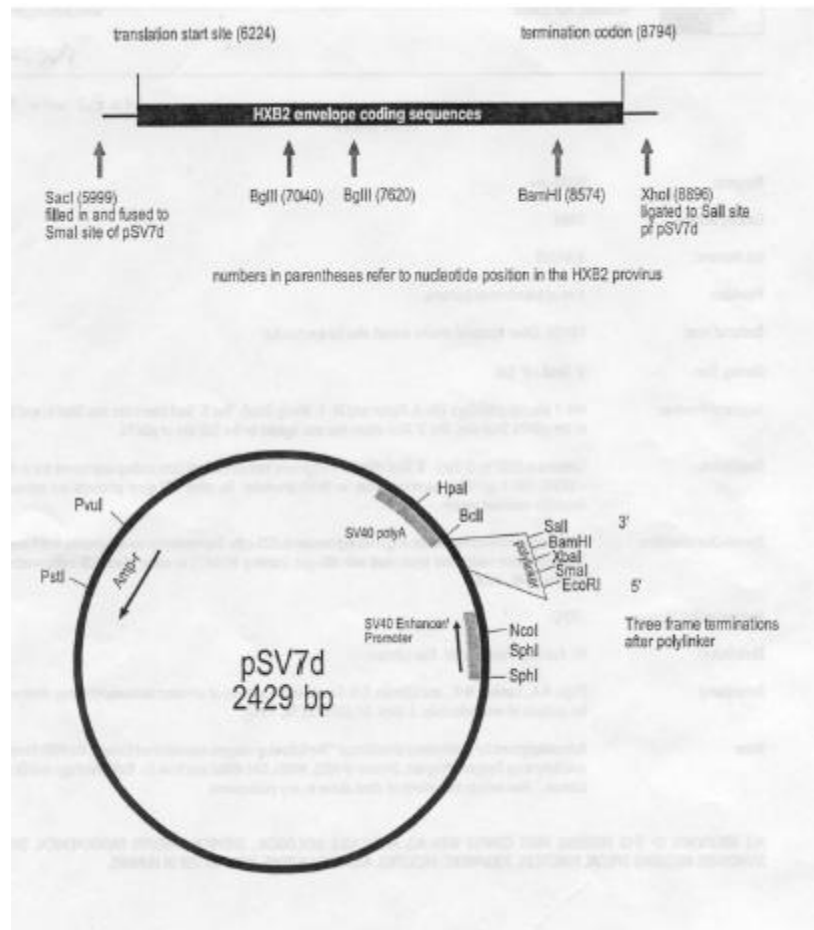


Figure 5: pMtat(-)plasmid map: The M-tropic HIV-1_{HXB3(-tat)} pMtat(-)plasmid used to study the effects of HIV-1-Tat deletion on MDDC maturation and function. Site directed mutagenesis was used to substitute a nonsense mutation TGA stop codon in place of the ATG initiator codon.



2.2.1 Propagation of HIV-1_{CS204}

PBMCs isolated from whole blood as previously described, were suspended in complete RPMI media supplemented with 50 U/ml IL-2 (Invitrogen, Burlington, ON), and 2.5 µg Phytohaemagglutinin (PHA) (Invitrogen, Burlington, ON) in two cultures of 8×10^7 cells at a density of 2×10^6 cells/ml for 48 h at 37°C and 5% CO₂. The PBMCs were then incubated with 50 µg/ml polybrene (PB) (Millipore, Etobicoke, ON) for 3 h at 37°C and 5% CO₂, washed in PBS and then centrifuged for 10 min at 443 x g. PBMCs were resuspended in 1 ml of either concentrated HIV-1_{CS204} or complete RPMI media (mock) for 2 h at 37°C and 5% CO₂. The cultures were increased in volume to 40 ml with complete RPMI media supplemented with 50 U/ml IL-2 and 2.5 µg Phytohemagglutinin (PHA) at 37°C and 5% CO₂ and maintained at 2×10^6 cells/ml with blasting allogenic PBMCs every 3-5 days. Every 7 days, samples from each culture were removed using a serological pipette (Eppendorf Canada, Mississauga, ON) and stored at -80°C for later p24 Enzyme-linked immunosorbent assay (ELISA) and Multiplicity of Infection (M.O.I.) analyses.

After 3 weeks the cultures were centrifuged for 10 min at 443 x g to remove particulate matter and sterile filtered using non-pyrogenic, low-protein binding 45 µm syringe filters (Thermo Fisher Scientific, Ottawa, ON). They were then aliquoted and stored in 1ml cryovials (Becton Dickinson and Company, Franklin Lake, NJ) at -80°C. The p24 ELISA and M.O.I. analyses were conducted according to instructions from the NIH HIV-1 p24^{CA} Antigen Capture Assay Kit and NIH p24 ELISA (National Cancer Institute, Frederick, MD, U.S.A.).

2.2.2 Propagation of lab adapted HIV-1 Virus Stocks from Plasmids

2.2.3 Preparation of competent *Escherichia coli*

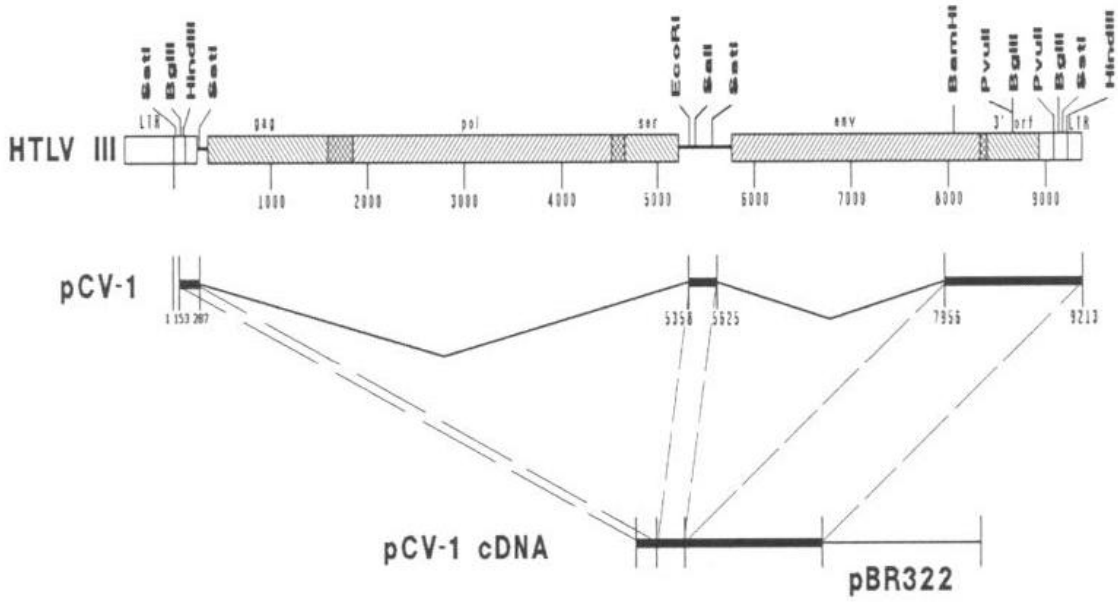
Stbl2 *Escherichia coli* (E.coli.) (a gift from Scott Sugden, The Ottawa Hospital Research Institute, Ottawa, ON) cultures were propagated in 250 ml volumes of 40% Luria Broth (LB) (Luria Broth base (Sigma-Aldrich Canada, Oakville, ON)) in 1000 ml Erlenmeyer flasks (Thermo Fisher Scientific Canada, Ottawa, ON). The cultures were incubated in a Thermo Forma MAxQ Shaker (Thermo Fisher Scientific, Ottawa, ON) at 37°C until the bacterial growth reached an optical density (OD) of 0.6 read at 600 nm on a SpectraMax 190 plate reader (Molecular Devices, Toronto, ON) and SpfWin software (Command Technology Products, McMinnville, OR USA).

The bacterial cultures were centrifuged at 5,000 RPM for 15 minutes in a JA-14 rotor at 4°C using a Beckman J-25i Medium Speed Centrifuge (Beckman Coulter Canada, Mississauga, ON). The bacteria pellets were then resuspended in 250ml chilled MgCl₂ (Sigma-Aldrich Canada, Oakville, ON) and incubated on ice for 5 min. After this, the suspensions were centrifuged at 5,000 RPM for 15 min at 4°C in a JA-14 rotor at 4°C using a Beckman J-25i Medium Speed Centrifuge and the bacterial pellets were resuspended in 250 ml of chilled CaCl₂ (Sigma-Aldrich Canada, Oakville, ON). The suspensions were incubated for 20 min on ice and then centrifuged at 5,000 RPM in a JA-14 rotor for 10 min at 4°C using a Beckman J-25i Medium Speed Centrifuge. The bacterial pellet was then resuspended in chilled CaCl₂ + 15% Glycerol (Sigma-Aldrich Canada, Oakville, ON) and 1ml aliquots were flash frozen in 95% methanol (Sigma-Aldrich, Oakville, ON) on dry ice (Ottawa Hospital, Ottawa, ON) and stored at -80°C.

2.2.4 Transformation of competent *Stbl2 E.coli*.

Competent *Stbl2 E.coli* were thawed on ice and 200 µl aliquots were pipetted into chilled 1.5ml eppendorf tubes (Thermo Fisher ScientificCanada, Ottawa, ON) incubated on ice. Next, 50 ng of pNL4-3, p210-19, pBaL, pMtat, pCV1 (Figure 5) (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: pCV-1 from Dr. Flossie Wong-Staal, Germantown, MD), pXF3 (Cedarlane Laboratories, Burlington, ON), pUC18, pUC19 (Thermo Fisher Scientific Canada, Ottawa, ON), were added to separate 200 µl volumes of bacteria. The mixtures were incubated on ice for 30 min and then placed in a 42°C water bath for 90 s followed by an second incubation on ice for 2 min. The bacterial cultures were then centrifuged for 1 min at 8000 rpm for 5 min in a 5415 Eppendorf Micocentrifuge (Eppendorf Canada, Mississauga, ON) and resuspended in 200µl LB.

Figure 6: pCV1 plasmid: HIV-1Mtat(-) transfected HEK cells were cotransfected with the HIV-1-Tat encoding pCV1 plasmid to allow for virus transcription and translation for MDDC infection.



2.2.5 Selection of transformed bacteria

Using proper aseptic techniques, the transformed bacteria were streaked onto Agar plates (12.5g LB and 7.5g Agar (Sigma-Aldrich Canada, Oakville, ON), 500ml H₂O) supplemented with appropriate selective antibiotics: Ampicillin (50µg/ml) (Sigma-Aldrich Canada, Oakville, ON) (NL4-3, p210-19, pWT/BaL, pMtat(-), pUC18, pUC19) (Cedarlane Laboratories, Burlington, ON) (pXF3) or Tetracycline (50µg/ml) (Sigma-Aldrich Canada, Oakville, ON) (pCV1) and incubated at 37°C for 16 h.

Single bacterial colonies growing on the selective Agar were selected and used to seed 5 ml of LB supplemented with 50 µg/ml of appropriate selective antibiotics. These cultures were incubated in a Thermo Forma MAXQ Shaker at 37°C for 8h and then expanded by 50x in LB supplemented with 50 µg/ml of appropriate antibiotics. The bacterial cultures were incubated again in a Thermo Forma MAXQ Shaker at 37°C, until the bacterial cultures reached an optical density (OD) of 0.6 read at 600nm on a SpectraMax 190 plate reader.

2.2.6 Plasmid purification

Viral plasmids were isolated and purified using a QIAGEN Maxiprep kit (QIAGEN, Mississauga, ON). Inoculated LB cultures were centrifuged in a Beckman J-25i Medium Speed Centrifuge at 5000 rpm for 15 min at 4°C. The bacterial pellets were resuspended in 10ml of Buffer P1 to which 10 ml of P2 Buffer was added. The mixtures were mixed by inversion and incubated for 5 min at room temperature. Chilled Buffer P3 (10ml) was then added to the suspensions which were gently mixed by inversion and incubated on ice for 20 min. After centrifugation in a Beckman J-25i Medium Speed Centrifuge at 12000 rpm for 30 min at 4°C, the supernatants were added to equilibrated

Qiagen-tip 500 columns and allowed to flow through. The columns were then washed twice with 30ml of Buffer QC and the DNA was eluted into clean centrifuge tubes by adding 2 consecutive 5ml volumes of QF Buffer to each column. A volume of 7 ml of room temperature isopropanol (Sigma-Aldrich Canada, Oakville, ON) was then added to each elutate to precipitate DNA and the mixtures were centrifuged in a Beckman J-25i Medium Speed Centrifuge at 12000 rpm for 5 min at 4°C. The supernatants were discarded and the pelleted DNA was centrifuged in a Beckman J-25i Medium Speed Centrifuge at 12000 rpm for 5 min at 4°C to dry the DNA. Remaining liquid was removed using a Pasteur pipette and the DNA was air dried for 5 min at room temperature. The DNA was then resuspended in 500µl TE buffer (10mM tris(hydroxymethyl)aminomethane (TRIS) and 1mM ethylenediaminetetraacetic acid (EDTA) at pH of 8.0) in a 1.5ml eppendorf tube and stored at -20°C.

2.2.7 Transfection of cells

293T Human embryonic kidney (HEK) cells were grown to 90% confluency in T-75 filter capped flasks in complete DMEM. Mixtures of 20 µg of pNL4-3 (Figure 1), p210-19 (Figure 2), pBaL (Figure 3), pMtat (Figure 4), and pCV1 (Figure 5) or pUC18, or pU19 plasmids (Addgene Caimbridge, MA) or pXF3 plasmids (Sigma-Aldrich Canada, Oakville, ON), 500µl 2 M CaCl₂ (Thermo Fisher Scientific, Ottawa, ON) and 500µl 2 M HEPES buffered saline (HBS) (Thermo Fisher Scientific, Ottawa, ON) were prepared and incubated at room temperature for 45 min. They were then added in an even and dropwise fashion to separate HEK cell cultures which were incubated at 37°C and 5% CO₂ for 16 h. HEK cells were then gently washed with warm PBS and supplemented with 10 ml of fresh complete Dulbecco's Modified Eagle's Medium (DMEM) and the cultures were

reincubated at 37°C and 5% CO₂ for 24 h. The media was then aspirated, clarified by centrifugation at 443 x g for 10 min, filtered with a non-pyogenic, low-protein-binding 45µm filter and stored at -80°C in 1 ml cryovials. The p24 ELISA and M.O.I. analyses were conducted according to instructions from the NIH HIV-1 p24^{CA} Antigen Capture Assay Kit and NIH p24 ELISA kit.

2.2.8 NIH p24 ELISA

The p24 content of cultures was measured according to instructions from the NIH HIV-1 p24^{CA} Antigen Capture Assay Kit and NIH p24 ELISA plates, and an ELx50 automatic plate washer (Bio-tek Instruments Inc., Winooski, VT, U.S.A.). Infected cells were centrifuged at 1600 rpm for 10min and lysed in 1/10 volume of Triton-X-100 (Fisher Scientific Canada Limited, Ottawa, ON) and incubated for 1 h at 37°C and 5%CO₂. Serial dilutions of standard were prepared and 100ul of both standards and each sample was then pipetted into the plate according to NIH instructions. The plate was then sealed and incubated for 2h. Next the plate was washed and incubated for 1h at 37°C and 5%CO₂ with 100ul of primary p24 anti-HIV (MN) p24 antibody at a titer of 1:150, washed and then incubated for 1h with 100ul of goat anti-rabbit IgG (H+L)-HRP antibody at a titer of 1:100, all at 37°C and 5%CO₂. After washing, 100ul of TMB Peroxidase Substrate was pipetted into all wells for 30 min at room temperature before addition of 100ul of 1N HCL to all wells to stop the reaction. Plate contents were read at 450nm with a reference of 650nm on a SpectraMax 190 plate reader (Molecular Devices, Toronto, ON) and SpfWin (Command Technology Products, McMinnville, OR USA).

2.3 Viral titrations

2.3.1 HIV-1 stock titration

Infectious viral titers of the mock solutions and the HIV-1 stock solutions were assessed using serial dilutions of both the mock and viral stocks within the central 21 wells of 96 well plates (Becton Dickinson and Company, Franklin Lakes, NJ). PBMCs isolated from whole blood as previously described, were suspended in complete RPMI media supplemented with 50 U/ml IL-2, and 2.5 µg PHA for 48 h at 37°C and 5% CO₂. The blasting PBMCs were then incubated with 3 µg/ml PB for 3 h at 37°C and 5% CO₂ and washed with warm PBS. Next, the PBMCs were centrifuged at 443 x g for 10 min and the pelleted cells were suspended in complete RPMI media supplemented with 50 U/ml IL-2, and 2.5 µg PHA at 4x10⁶ cells/ml. Aliquots of mock and viral titration stocks were then thawed in a 37°C water bath and 150 µl of neat mock or infectious supernatants were added to the leftmost wells. The remaining stocks were then diluted 1:12 in complete RPMI media and 150 µl of these were added in rows to the empty central plate wells, serially diluting by 25% while moving from left to right. Using a micropipette (Eppendorf Canada, Mississauga, ON), 50 µl of resuspended blasting donor PBMCs were then added to all inner wells and 200 µl of PBS was added to the outer wells. The plate was then covered and incubated at 37°C and 5% CO₂. On day 4, 125µl of each culture was removed and discarded and replaced with 150µl of complete RPMI media supplemented with 50 U/ml IL-2, and 2.5 µg PHA. On day 7, the cultures were harvested and lysed with 20 ul Triton X-100 (Sigma-Aldrich Canada, Oakville, ON) and evaluated for p24 content and infectious viral particle number according to instructions from the NIH HIV-1 p24^{CA} Antigen Capture Assay Kit and NIH p24 ELISA and Reed and Münch statistical analysis.

2.4 Viral infections

2.4.1 HIV-1_{CS204} infection of monocyte derived dendritic cells

Immature MDDCs suspended in complete RPMI media supplemented with rhIL-4 (500U/ml) and of rhGM-CSF (1000U/ml) were incubated with live dual tropic HIV-1_{CS204} (a gift from Dr. Francisco Diaz-Mitoma at the Children's Hospital of Eastern Ontario, Ottawa, ON) using a multiplicity of infection of 1 for 24 h at 37°C and 5% CO₂. MDDCs were then washed twice in PBS and suspended in complete RPMI media supplemented with rhIL-4 (500U/ml) and of rhGM-CSF (1000U/ml) in 12 well tissue culture plates at a density of 1x10⁶ cells/ml at 37°C and 5%CO₂. HIV-1 infection was evaluated 3 days post infection using *Alu*-nested PCR detection and an NIH HIV-1 p24^{CA} Antigen Capture Assay Kit and NIH p24 ELISA.

2.4.2 Lab adapted HIV-1 infection of monocyte derived dendritic cells

Immature MDDCs were incubated with 30 ng/ml of p24 of live HIV-1_{NL4-3}, HIV-1_{NL4-3-Vpr} (Dvpr), HIV-1_{pWT/BaL}, or HIV-1_{pWT/BaL-Tat} (Mtat), or with equivalent volumes of supernatants from the pUC18 (pNL4-3 mock control) or pXF3 (pWT/BaL mock control) transfections for 24 h at 37°C and 5% CO₂. After 24 h, MDDCs were washed twice in PBS and suspended in complete RPMI media supplemented with rhIL-4 (500U/ml) and of rhGM-CSF (1000U/ml) in 12 well tissue culture plates at a density of 1x10⁶ cells/ml at 37°C and 5%CO₂. Virus infection was evaluated 3 days post infection using *Alu*-nested PCR detection and nested HIV-1-p24 mRNA detection.

2.5 MDDCs and HIV-1 peptides

2.5.1 Incubation of MDDCs with HIV-1-Tat

Prior to iMDDC incubation, the HIV-1-Tat peptide (NIH AIDS Research and Reference Reagent Program, Germantown, MD) was endotoxin depleted using an Lipopolysaccharide (LPS) depletion column (Sigma-Aldrich Canada, Oakville, ON) according to manufacturers instructions at 4°C and in the dark. The HIV-1-Tat peptide concentration was then determined by Bradford assay and 50 ng/ml of HIV-1-Tat was incubated with iMDDCs for 24 h at 37°C and 5% CO₂. MDDCs were then washed twice in PBS and suspended in complete RPMI media supplemented with rhIL-4 (500U/ml) and of rhGM-CSF (1000U/ml) in 12 well tissue culture plates at a density of 1x10⁶ cells/ml at 37°C and 5% CO₂.

2.5.2 Incubation of MDDCs with HIV-1-Vpr

MDDCs differentiated from primary monocytes as previously described were incubated with 10 pg/ml HIV-1-Vpr (a gift from Dr. Ashok Kumar (Invitrogen, Burlington, ON)), for 24 h at 37°C and 5% CO₂. MDDCs were then washed twice in PBS and suspended in complete RPMI media supplemented with rhIL-4 (500U/ml) and of rhGM-CSF (1000U/ml) in 12 well tissue culture plates at a density of 1x10⁶ cells/ml at 37°C and 5% CO₂.

2.5.3 Incubation of MDDCs with HIV-1-gp120

MDDCs differentiated from primary monocytes as previously described were incubated with 3 µg/ml HIV-1-gp120 (a gift from Dr. Ashok Kumar (Protein Sciences Corporation, Meriden, Connecticut, U.S.A.) for 24 h at 37°C and 5% CO₂. MDDCs were

then washed twice in PBS and suspended in complete RPMI media supplemented with rhIL-4 (500U/ml) and of rhGM-CSF (1000U/ml) in 12 well tissue culture plates at a density of 1×10^6 cells/ml at 37°C and 5% CO₂.

2.6 Detection of HIV-1 DNA, RNA, and p24 protein

2.6.1 Infected cell DNA purification

DNA was isolated from 1×10^6 MDDCs using the QIAGEN DNeasy Blood and Tissue kit (Qiagen, Mississauga, ON, Canada). MDDCs were gently scraped, washed in PBS and centrifuged at 443 x g for 10 min. MDDC pellets were resuspended in 200 µl of PBS supplemented with 20 µl of proteinase K and 200 µl of buffer AL. The mixture was incubated for 10 minutes at 56°C. After this, 200 µl of 100% ethanol (Sigma-Aldrich Canada, Oakville, ON) was added to the sample and the mixture was applied to a DNeasy spin column which was centrifuged for 1 min at 14000 rpm in an Eppendorf Micocentrifuge (Eppendorf Canada, Mississauga, ON). The flow through and collection tube were discarded and the column was moved to a new collection tube. Using a micropipette, 500 µl of Buffer AW1 was then applied to the column and the column was centrifuged at 14,000 rpm for 1 minute in an Eppendorf Micocentrifuge. The flow through and collection tube were discarded and 500 µl of Buffer AW2 was added to the DNeasy spin column which was then placed in a new collection tube and centrifuged at 14,000 rpm for 1 minute in an Eppendorf Micocentrifuge. The column was then placed in a new 1.5 eppendorf tube and DNA was eluted by applying 100 µl of buffer AE on the DNeasy column membrane and centrifuging the column at 14000 rpm for 1 minute in an Eppendorf Micocentrifuge.

2.6.2 Polymerase Chain Reaction (PCR)

Viral infection was confirmed by Alu-nested PCR amplification adapted from previous work. Two outward-facing Alu primers (300 nM) (Invitrogen, Burlington, ON) that anneal within conserved regions of the Alu repeat element in conjunction with an HIV-1 LTR specific primer (300 nM) with an extended lambda phage-specific heel sequence at the 5' end of the oligonucleotide (L-M667) (Invitrogen, Burlington, ON) to amplify integrated HIV-1 DNA were used as previously described [412].

The first-round PCR cycle conditions consisted of: a denaturation step of 7 min at 94°C and 12 cycles of amplification (94°C for 1 min, 59°C for 1 min, and 72°C for 1 min) using Taq PCR Mastermix (Qiagen, Mississauga, ON). During the second round of PCR, 1/10 of the first-round PCR products were amplified using a lambda specific primer (Lambda T) (300nM) and an LTR primer (AA55M) (300nM) (Invitrogen, Burlington, ON). Second-round PCR cycle conditions consisted of a denaturation step of 7 min at 94°C and then 30 amplification cycles (94°C for 1 min, 59°C for 1 min, and 72°C for 1 min) in Taq PCR Mastermix using an Eppendorf Mastercycler ep 543X instrument (Eppendorf, Mississauga Canada). The β -actin PCR products from step 1 and the HIV-1 PCR products from step 2 were visualized on a 1% Agarose gel (Invitrogen, Burlington, ON) stained with Ethidium bromide (EtBr) (Thermo Fisher Scientific, Ottawa, ON).

Primers:

L-M667 – ATGCCACGTAAGCGAACTCTGGCTAACTAGGGAACCCACTG

Alu 1 – TCCCAGCTACTGGGGAGGCTGAGG

Alu 2 – GCCTCCCAAAGTGCTGGGATTACAG

LambdaT – ATGCCACGTAAGCGAACT

AA55M – GCTAGAGATTTTCCACACTGACTAA

Actin 1 ACCGAGCGCGGCTACAG

Actin 2 CTTAATGTCACGCACGCACGATTTC

2.6.3 Isolation and purification infected cell mRNA

RNA was isolated from 1×10^6 MDDCs using the QIAGEN RNeasy kit (Qiagen, Mississauga, ON, Canada). MDDCs were gently scraped, washed in PBS and centrifuged at $443 \times g$ for 10 min. They were then lysed in 350 μ l of RLT buffer and the homogenized using a QIAshredder (Qiagen, Mississauga, ON). Next, 350 μ l of 70% ethanol (Sigma-Aldrich Canada, Oakville, ON) was added to the homogenized RNA and the mixture was added to an RNeasy spin column which was then centrifuged for 15 s at 14,000 rpm in an Eppendorf Microcentrifuge. The flow through was discarded and 700 μ l RW1 buffer was added to the RNeasy spin column and it was centrifuged for 15s at 14,000 rpm in an Eppendorf Microcentrifuge. After discarding the flow through, 500 μ l of RPE Buffer was added to the RNeasy column which was centrifuged for 15s at 14,000 rpm in an Eppendorf Microcentrifuge. This was followed by another wash with 500 μ l of RPE buffer for 2 min at 14,000 rpm in an Eppendorf Microcentrifuge. The flow through and collection tubes were discarded and the RNeasy column was placed in a new collection tube. RNA was eluted by applying 30 μ l of RNase-free water directly to the spin column membrane which was then centrifuged for 1 min at 14,000 rpm in an Eppendorf Microcentrifuge.

2.6.4 Reverse transcription of β -actin and HIV-1 mRNA

The isolated RNA was reverse transcribed using the OneStep RT-PCR kit (Qiagen, Mississauga, Ontario, Canada). The master mix components (RNase-free water, 5x

QIAGEN OneStep RT-PCR Buffer, dNTP Mix, Primer A, Primer B, QIAGEN OneStep RT-PCR Enzyme Mix, and RNase inhibitor) were thawed and prepared: 1 x OneStep RT-PCR Buffer, 400 μ M dNTPs, and 0.6 μ M primers in a final volume of 50 μ l. Template RNA (2 μ g) and RNase-free water were then added for a total volume of 50 μ l per reaction. The first-round PCR cycle conditions consisted of: a denaturation step of 1 min at 94°C and 30 cycles of amplification (denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min).

2.6.5 Nested PCR detection of β -actin and HIV-1-gag DNA

Next, 5 μ l of first-round PCR products were added to 25 μ l of Taq PCR Master Mix (Qiagen, Mississauga, ON) and 2 primers that anneal within outer regions of the HIV-1 gag element (300 nM) (Invitrogen, Burlington, ON) for a total volume of 50 μ l per reaction. PCR cycle conditions consisted of: a denaturation step of 5 min at 94°C and then 24 amplification cycles (denaturation at 94°C for 30s, annealing at 41°C for 30s, and extension at 72°C 30s) in Taq PCR Mastermix using an Eppendorf Mastercycler ep 543X instrument.

Next, 1/10 of the second-round PCR products were amplified using 2 primers that anneal within inner regions of the HIV-1-gag element (300 nM) (Invitrogen, Burlington, ON). Second-round PCR cycle conditions consisted of: a denaturation step of 5 min at 94°C and then 24 amplification cycles (denaturation at 94°C for 30 s, annealing at 41°C for 30 s, and extension at 72°C for 30 s) in Taq PCR Mastermix using an Eppendorf Mastercycler ep 543X instrument. The β -actin PCR products from step 2 and the HIV-1-p24 PCR products from step 3 were visualized on a 1% Agarose gel stained with EtBr.

Primers

JA4 GAAGGCTTTCAGCCCAGAAG gag (1319-1338)

JA5 ACCATCAATGAGGAAGCTGC gag (1446-1465)

JA6 TATTTGTTTCCTGAAGGGTAC gag (1577-1558)

JA7 TCTCCTACTGGGATAGGTGG gag (1615-1596)

Actin 1 ACCGAGCGCGGCTACAG

Actin 2 CTTAATGTCACGCACGCACGATTTC

2.6.6 NIH p24 ELISA

The p24 content of cultures was measured according to instructions from the NIH HIV-1 p24^{CA} Antigen Capture Assay Kit and NIH p24 ELISA plates, and using an ELx50 automatic plate washer (Bio-tek Instruments Inc., Winooski, VT, U.S.A.). Infected cells were centrifuged at 443 x g for 10 min and lysed in 1/10 volume of Triton-X-100 (Fisher Scientific Canada Limited, Ottawa, ON). The lysed samples were incubated for 1 h at 37°C and 5% CO₂. Serial dilutions of standard were prepared and 100 ul of both standards and each sample were then pipetted into the plate according to NIH instructions. The plate was then sealed and incubated for 2 h at 37°C. Next, the plate was washed and incubated for 1 h at 37°C and 5% CO₂ with 100 ul of primary p24 anti-HIV (MN) p24 antibody at a titer of 1:150. The plate was then washed and samples were incubated with 100 ul of goat anti-rabbit immunoglobulin G (IgG) (H+L)-HRP antibody at a titer of 1:100 for 1 h at 37°C and 5% CO₂. The plate was washed and 100 µl of Tetramethylbenzidine (TMB) Peroxidase Substrate (Mandel Scientific Company Inc., Guelph, ON) was pipetted into all wells. The plate was incubated for 30 min at room temperature and 100 µl of 1 N HCL (Thermo Fisher Scientific, Ottawa, ON) was added to all wells to stop the reactions. Plate contents were

read at 450 nm with a reference of 650 nm on a SpectraMax 190 plate reader (Molecular Devices, Toronto, ON) and SpfWin (Command Technology Products, McMinnville, OR USA).

2.7 Phenotyping

2.7.1 Monocyte phenotype

Monocytes isolated as previously described were incubated with saturating concentrations of Fluorescein isothiocyanate (FITC)-conjugated anti CD14, DC-SIGN, CD80, CD86, CCR5, CCR7, MHC I, or MHC II antibodies, phycoerythrin (PE)-conjugated anti MHC I antibodies or isotype controls (Beckman-Coulter Canada Inc., Mississauga, ON) in 5 ml polypropylene round bottom tubes (Becton Dickinson and Company, Franklin Lakes, NJ). Surface expression was measured using a Coulter Epics Altra flow cytometer (Beckman-Coulter Canada Inc., Mississauga, ON) and analyzed with FCS Express 2.00 software (De Novo Software, Los Angeles, CA).

2.7.2 Monocyte derived dendritic cell phenotype

Monocytes in complete RPMI media supplemented with 500 U/ml rhIL-4 and 1000U/ml rhGM-CSF for 24 h at 37°C and 5%CO₂ isolated from healthy donors as described above were incubated with saturating concentrations of Fluorescein isothiocyanate (FITC)-conjugated anti CD14, DC-SIGN, CD80, CD86, CCR5, CCR7, MHC II antibodies, phycoerythrin (PE)-conjugated anti MHC I antibodies or isotype controls in 5 ml polypropylene round bottom tubes. Surface expression was measured using a Coulter Epics Altra flow cytometer and analyzed with FCS Express 2.00 software.

2.7.3 Mature monocyte derived dendritic cell phenotype

Immature MDDCs incubated with 1000 U/ml TNF α , 10 ng/ml IL-1 β , 10 ng/ml IL-6, and 1 μ M/ml PGE₂ for 48 h at 37°C and 5%CO₂ were incubated with saturating concentrations of Fluorescein isothiocyanate (FITC)-conjugated anti CD14, DC-SIGN, CD80, CD86, CCR5, CCR7, MHC I, or MHC II antibodies, or phycoerythrin (PE)-conjugated anti MHC I antibodies or isotype controls in 5 ml polypropylene round bottom tubes. Surface expression was measured using a Coulter Epics Altra flow cytometer and analyzed with FCS Express 2.00 software.

2.8 FITC-Dextran endocytosis assay

MDDCs were differentiated and infected or incubated as described above and 250,000 cells were incubated in 5 ml polypropylene round bottom tubes with 1 mg of FITC-conjugated dextran (Sigma-Aldrich Canada, Oakville, ON) in the dark for 1 h on ice or at 37°C and 5% CO₂. Cells were then washed twice in PBS and subjected to flow cytometric analysis of mean fluorescence intensity (MFI) using FCS Express 2.00 software.

2.9 Monocyte derived dendritic cell co-culture

MDDCs, differentiated and infected as above, were pulsed for 3 h with 3 μ g/ml of a CEF peptide pool containing 23 HLA-ABC-restricted T-cell epitopes from human Cytomegalovirus, Epstein-Barr and Influenza viruses (CEF) (Anaspec Inc., Fremont, CA USA). MDDCs were then washed in PBS and resuspended in rhIL-4 and rhGM-CSF supplemented complete RPMI media at 7×10^7 cells/ml.

The negative fraction obtained from the monocyte isolation (to serve as the pool of autologous T-cells) was suspended at 1×10^7 cells/ml in 5mM CellTrace™

Carboxyfluorescein succinimidyl ester (CFSE) in 0.1% bovine serum albumin (BSA) (Sigma-Aldrich Canada, Oakville, ON) in PBS for 10 min at 37°C and 5%CO₂ in 15ml polypropylene conical tubes in the dark. The cells were then washed in cold PBS, incubated for 5 min on ice, pelleted by centrifugation for 10 min at 443 x g and suspended at 1x10⁶ cells/ml in complete media.

In a 96 well plate (Becton Dickinson and Company, Franklin Lakes, NJ), 250,000 CFSE-labelled autologous cells from the negative fraction and 25,000 dendritic cells from each condition were cocultured together in the dark for 7 days at 37°C and 5%CO₂. A negative control culture containing colchicine (100ng/ml) (Sigma-Aldrich Canada, Oakville, ON) was also included. Entire cocultures were then transferred to 5ml polypropylene round bottom tubes and stained with PE-conjugated anti-CD8 antibodies (R&D Systems, Burlington, ON). CD8⁺ T-cell proliferation was measured by flow cytometric analysis (CFSE dilution). Only those cultures that proliferated in response to the CEF antigen pool beyond the level of media controls were further evaluated for the influence of HIV on proliferation.

2.10 Preparation of cell lysates and immunoblot analysis

Changes in the phosphorylation and the total protein expression of the ERK, c-Jun N-terminal/Stress activated protein kinases (JNK/SAP), and p38 proteins in response to LPS after HIV-1 infection were measured using immunoblot analysis as previously described [413]. HIV-1 infected or uninfected MDDCs were centrifuged for 10min at 443 x g, washed in PBS, and resuspended in complete media at 1x10⁶ cells/ml. Next they were incubated in the presence or absence of 2µg/µl LPS (*E.coli*, 0111:B4, Sigma-Aldrich Canada, Oakville, ON) for 1h at 37°C and 5%CO₂. Cells were then collected by

centrifugation at 443 x g for 10 min, washed in PBS and then lysed on ice using 250µl lysis buffer (0.05M 4-(2)-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.15 NaCl, 10% glycerol, 1% Triton-x-100, 7.5×10^{-4} M MgCl₂, 0.1M NaF, and 0.001M ethylene glycol tetraacetic acid (EGTA) (pH 7.7)) (Fisher Scientific Canada Limited, Ottawa, ON). After boiling the samples with 4x treatment buffer (8% SDS, 10% 2-mercaptoethanol, 30% glycerol, 0.008% bromophenol blue, 0.25 M Tris HCl) (Fisher Scientific Canada Limited, Ottawa, ON) for 10 min, 40µg of total protein of each lysate was added to each well of an 8% Sodium dodecyl sulfate (SDS) polyacrylamide gel and subjected to electrophoresis for 2h at 90V using a wet tank blotting system (Biorad Laboratories Inc., Burlington, ON). The proteins were electrophoretically transferred to nitrocellulose sheets (Protran® , Bioscience, Schleicher and Schuell, Mandel, ON) at 15V for 45min on a semidry electrophoretic transfer machine (Biorad Laboratories Inc., Burlington, ON) and blocked with Amersham™ ECL Advance Blocking agent (GE-Healthcare Bio-Sciences, Mississauga, ON) for 24h.

The membranes were incubated at 4°C with the primary phosphorylated anti-p38, JNK/SAPK, or ERK1/2 and β-actin antibodies (Product #'s 9215S, 9251S, 99101S, and 4967 respectively, Cell Signaling Technologies, New England Biolabs Limited, Toronto, ON) at a titre of 1:500 in Amersham™ ECL Advance Blocking agent in 1x Tris Buffered Saline (TBS) (Fisher Scientific Canada Limited, Ottawa, ON) plus Tween 20 (Fisher Scientific Canada Limited, Ottawa, ON) (TBST) for 24h. The membranes were washed in TBST and incubated with secondary antibodies covalently bound to horseradish peroxidase (HRP) (Santa Cruz Biotechnology) at a titre of 1:1000 in Amersham™ ECL Advance Blocking agent (GE Healthcare Life Sciences, Baie d'Urfe, QC) in TBST at 4°C for 24h. The membranes were washed in TBST and signals were detected using

Supersignal HRP substrate (Pierce Chemical Company, Brockville, ON) and AlphaEase FC 6.0.0 software (Cell Biosciences, Santa Clara, CA, U.S.A.).

2.11 Statistical analyses

Data were analyzed using paired t-tests or the Wilcoxon rank test when appropriate for identification of statistically significant differences ($p \leq 0.05$ was considered significant) between experimental groups using Sigma Plot 8.0 (Systat Software Inc., Chicago, IL, USA).

3.0 Results

3.1 Differentiation of monocyte derived dendritic cells from primary monocytes

3.1.1 Characterization of monocytes

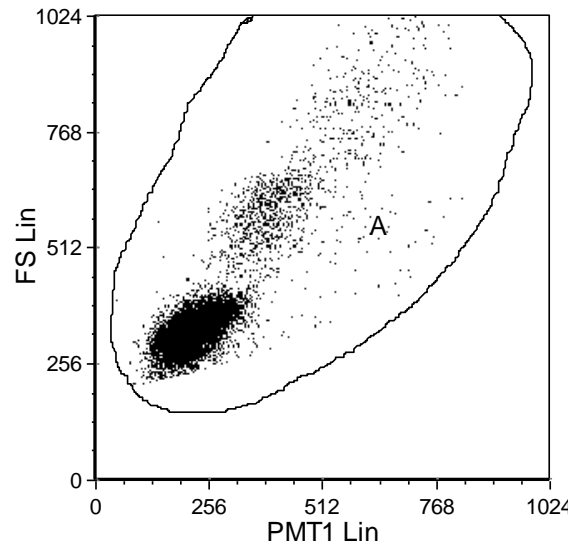
To confirm the purity and phenotype of monocytes isolated from the blood of healthy individuals, flow cytometry was used to examine their expression of CD14, DC-SIGN, CD83, CD80, CD86, CD40, CCR7, MHC I, and MHC II. Live cells were gated based on forward scatter and side scatter patterns (Figure 6A, gate A).

Isolated cells consistently stained over 90% positive for the monocytic marker CD14 (Figure 6B). They also expressed high surface levels of CD14, CD40, and MHC I and low levels of surface Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN/CD209), CD83, CD80, CD86, and MHC II (Figure 6B). These staining patterns are consistent with monocyte phenotype staining reported in the literature [411].

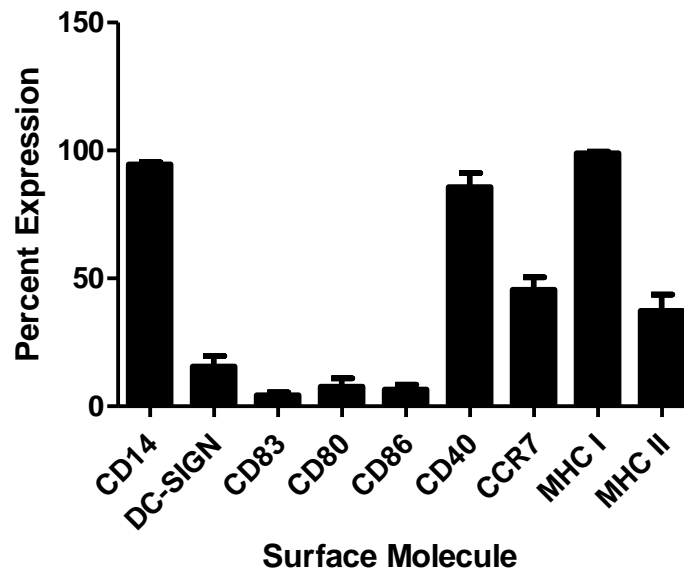
Figure 7: Monocyte phenotype:

Monocytes were isolated from PBMCs from 3 healthy donors. Expression of CD14, DC-SIGN, CD83, CD80, CD86, CD40, CCR7, MHC I, and MHC II on A) live cells (gate A) as measured by flow cytometry on B) monocytes were observed at expected levels. Results are expressed as means \pm standard deviation of percent of total cells expressing each surface molecule. (n = 3).

A



B



3.1.2 Characterization of immature monocyte derived dendritic cells

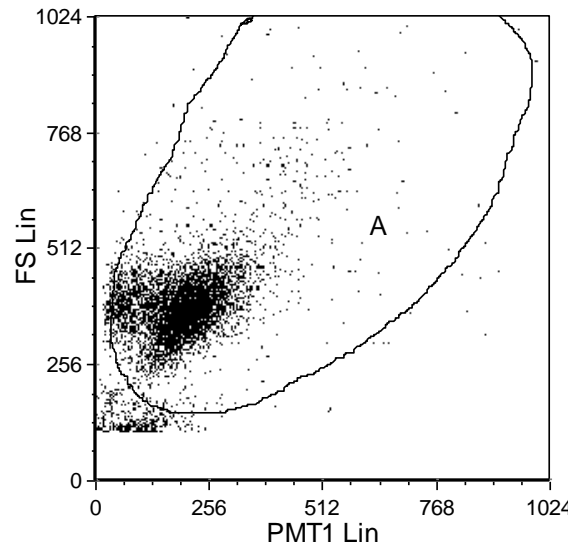
To confirm the purity and phenotype of iMDDCs, flow cytometry was used to examine their expression of CD14, DC-SIGN, CD83, CD80, CD86, CD40, CCR7, MHC I, and MHC II. Live cells were gated based on forward scatter and side scatter patterns (Figure 7A, gate A).

Compared to monocytes, the gated cells expressed low levels of CD14 and high levels of DC-SIGN (Figure 7B). They also expressed higher levels of surface CD83, CD80, CD86, CD40, MHC I, and MHC II and low levels of CCR7 (Figure 7B). Results are expressed as means \pm standard deviation (n=3) and were consistent with other published reports describing MDDC phenotype [411].

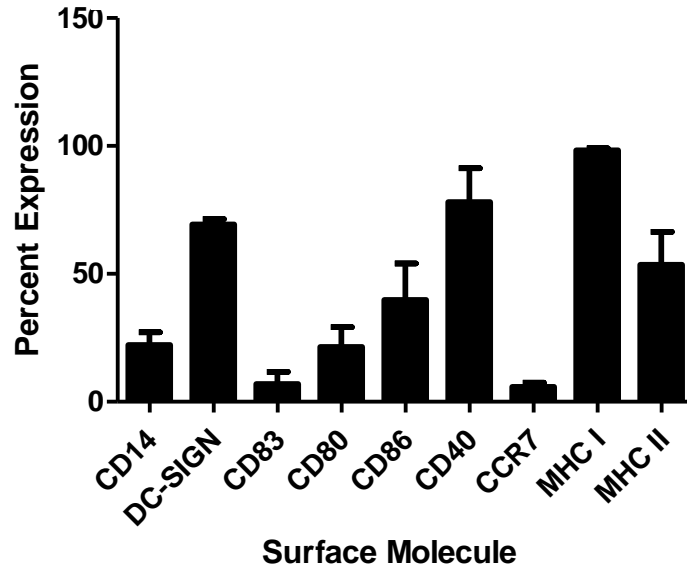
Figure 8: Immature MDDC phenotype

PBMC isolated monocytes from 3 healthy donors were differentiated into iMDDCs by incubation with IL-4 and GM-CSF for 24 h at 37°C and 5% CO₂. Expression of CD14, DC-SIGN, CD83, CD80, CD86, CD40, CCR7, MHC I, and MHC II on A) live cells (gate A) as measured by flow cytometry on B) iMDDCs were observed at expected levels. Results are expressed as means ± standard deviation of percent of total cells expressing each surface molecule. (n= 3).

A



B



3.1.3 Characterization of mature monocyte derived dendritic cells

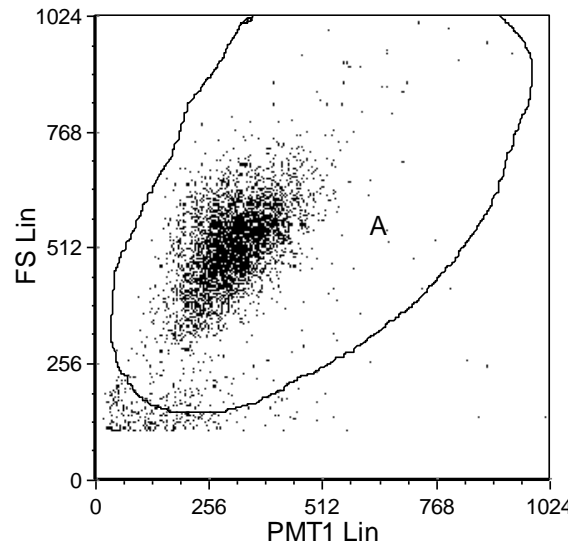
To confirm the purity and phenotype of mMDDCs, flow cytometry was used to examine their expression of CD14, DC-SIGN, CD83, CD80, CD86, CD40, CCR7, MHC I, and MHC II. Live cells were gated based on forward scatter and side scatter patterns (Figure 8A, gate A).

After incubation with inflammatory cytokines, mature MDDCs were found to express low levels of CD14, and DC-SIGN, and high levels of CD80, CD83, CD86, CD40, CCR7, MHC I, and MHC II (Figure 8B). Results are expressed as means \pm standard deviation (n=3) and were consistent with other published reports describing MDDC phenotype [411].

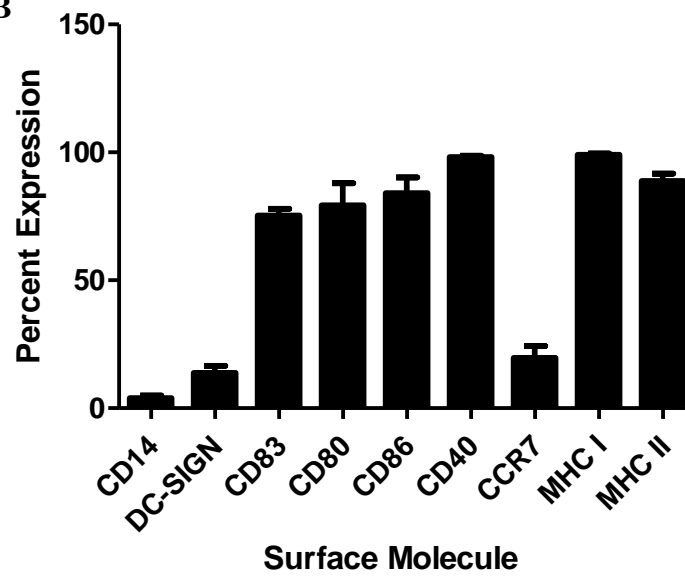
Figure 9: mature MDDC phenotype

Monocytes isolated from PBMCs from 3 healthy donors were differentiated into iMDDCs by incubation with IL-4 and GM-CSF for 24 h at 37°C and 5% CO₂ and then matured into TNF α , IL-1 β , IL-6, and for 48 h at 37°C and 5% CO₂. Expression of CD14, DC-SIGN, CD83, CD80, CD86, CD40, CCR7, MHC I, and MHC II on A) live cells (gate A) as measured by flow cytometry on B) mMDDCs were observed at expected levels. Results are expressed as means \pm standard deviation of percent of total cells expressing each surface molecule. (n= 3).

A



B



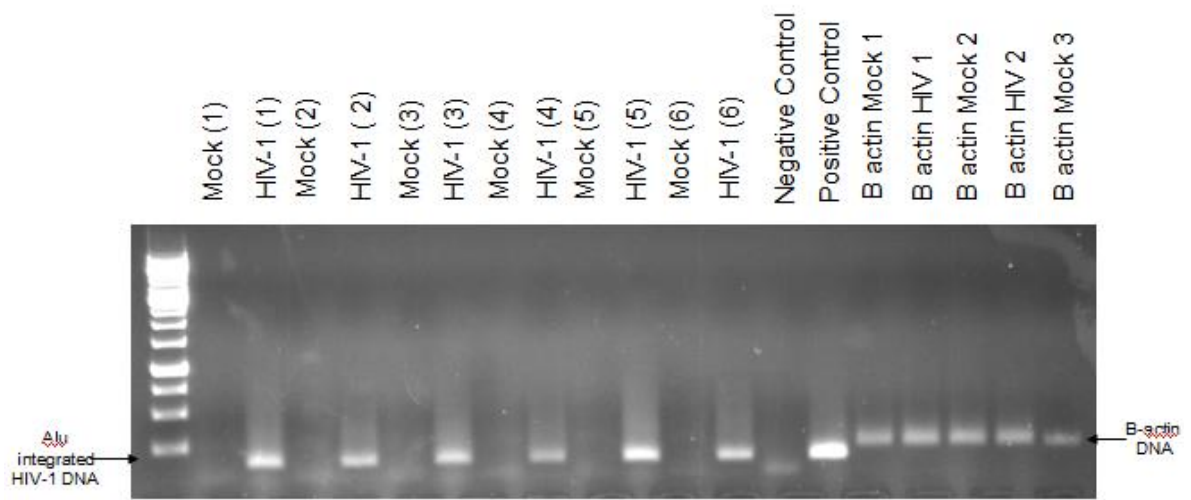
3.2 Characterization of HIV-1_{CS204} infection of iMDDCs

3.2.1 HIV infection of iMDDCs: evidence of integrated HIV-1 DNA

Nested *Alu* PCR was used to detect the presence of integrated HIV-1_{CS204} DNA in iMDDCs. After a 24 h incubation with HIV-1_{CS204} and 48 h of culture, integrated HIV-1_{CS204} DNA was consistently detected in HIV-1_{CS204} infected cultures (Figure 9). There was no detectable HIV-1_{CS204} DNA in the mock infected cultures over the same period of time (Figure 9). Water was used as a negative control. Beta-actin primers and DNA isolated from HIV_{CS204} infected PBMCs served as positive controls for detection of host DNA and integrated HIV-1 DNA respectively (Figure 9).

Figure 10: Detection of proviral HIV-1_{CS204} DNA by nested *Alu* PCR.

DNA isolated from iMDDCs was amplified in 2 steps in Taq PCR Mastermix using an Eppendorf Mastercycler ep 543X instrument. During the first round of replication, two outward-facing *Alu* primers (300 nM) and an HIV-1 LTR specific primer (300 nM) were used. During the second round of replication, 1/10 of these first-round PCR products were amplified using a Lambda T (300 nM) and an LTR primer (300 nM). Beta-actin and DNA isolated from HIV_{CS204} infected PBMC served as positive controls for detection of host DNA and integrated HIV-1 DNA respectively. Water was used as a negative control. Samples were visualized on an EtBr stained 1% Agarose, Tris base, acetic acid and EDTA (TAE) gel. Numbers in parentheses within lane legends represent respective infection experiments.



3.3 The effect of HIV-1_(CS204) infection on the maturation and function of MDDCs

To determine the effect of HIV-1_{CS204} infection on MDDC maturation, surface phenotype, carbohydrate endocytosis, antigen presentation, and MAPK response to LPS were investigated.

3.3.1 HIV-1_{CS204} infection inhibits MDDC maturation

HIV-1 has been observed to have a variety of effects on dendritic cell maturation both *ex vivo* and *in vitro*. While HIV-1 has been reported to induce dendritic cell maturation *in vitro* [377], there is considerably more evidence to suggest that HIV-1 does not induce normal maturation [355-358, 370,371]. Since one measure of dendritic cell maturation is the surface expression of distinct surface molecules, we first determined if HIV-1 infection influences the cell surface phenotype of MDDC during the course of maturation.

Flow cytometry was used to examine the effect of HIV-1_{CS204} infection on MDDC expression of CD14, DC-SIGN, CD80, CD83, CD86, CD40, CCR7, MHC I, and MHC II.

After incubation of iMDDCs with HIV-1_{CS204} for 24 h and 48 h of culture, there were no changes in the expression of CD14 (Figures 10A and 10B), CD80 (Figures 10E and 10F), CD86 (Figures 10G and 10H), CD83 (Figures 10I and 10J), CD40 (Figures 10K and 10L), CCR7 (Figures 10M and 10N), MHC I (Figures 10O and 10P), or MHC II (Figures 10Q and 10R) indicating that HIV-1_{CS204} itself was not capable of inducing DC maturation. There was, however, an increase in DC-SIGN expression following HIV-1_{CS204} infection (Figure 10B).

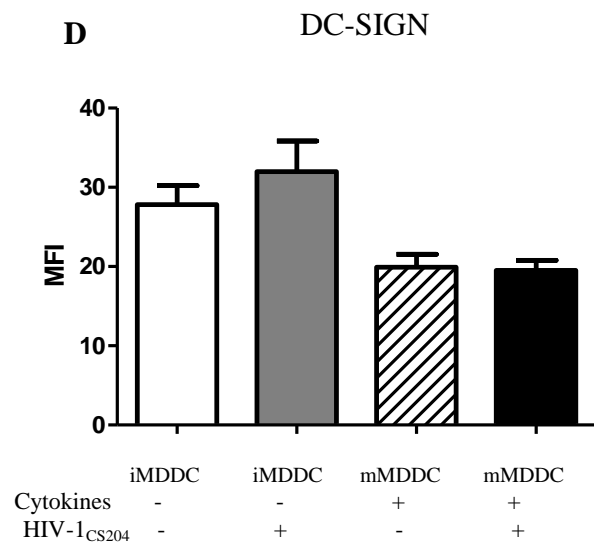
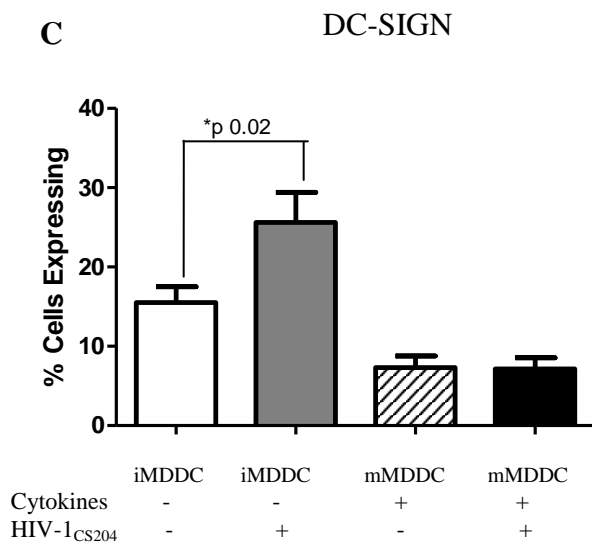
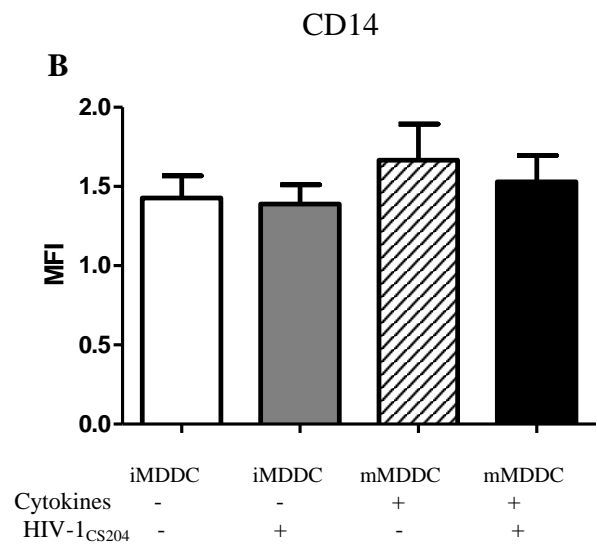
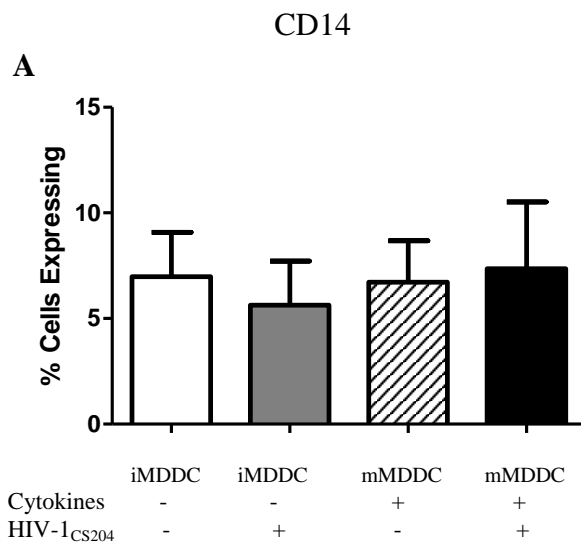
Immature MDDCs that were infected with HIV-1_{CS204} and then stimulated to mature, expressed lower levels of CCR7 (Figures 10K and 10L) and MHC II (Figure 10P)

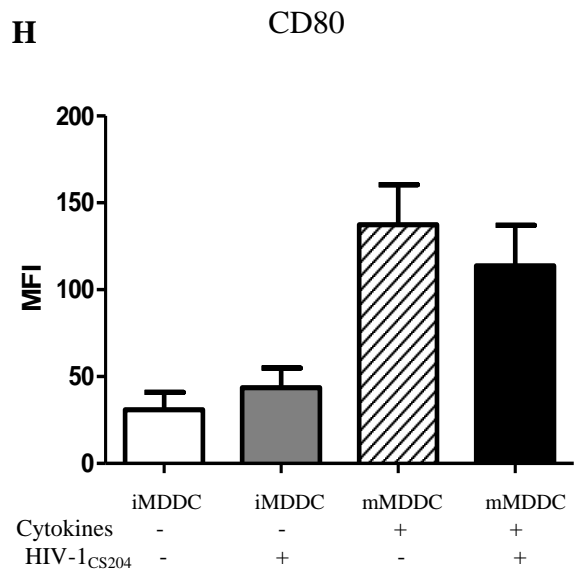
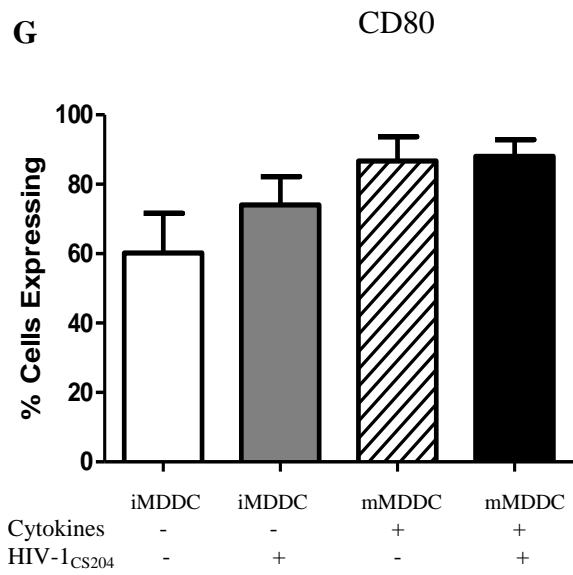
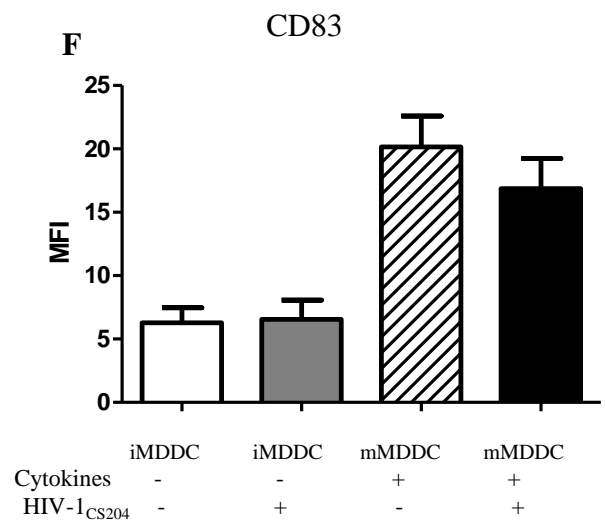
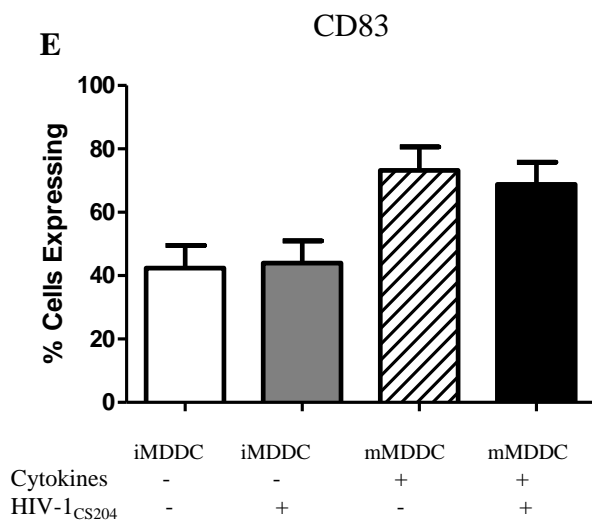
than that observed in uninfected cells, suggesting that HIV-1 inhibits the maturation of iMDDCs.

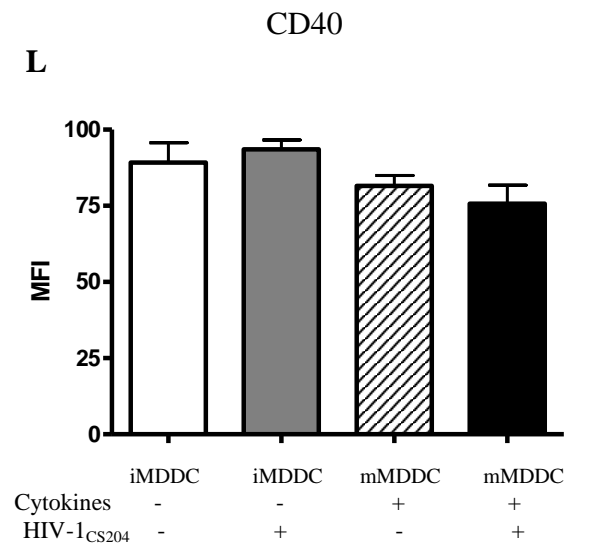
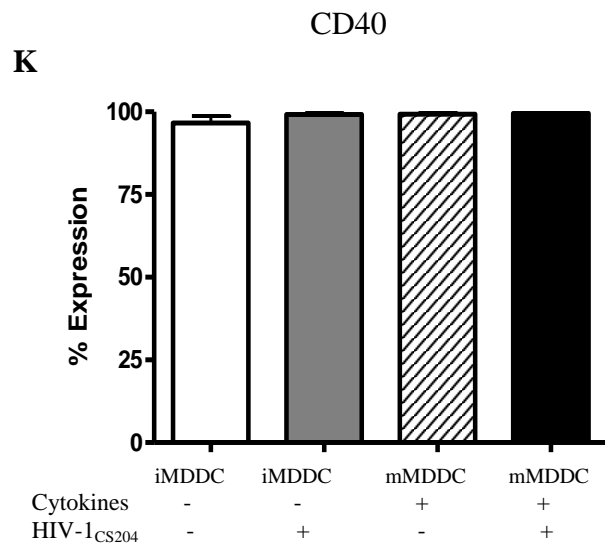
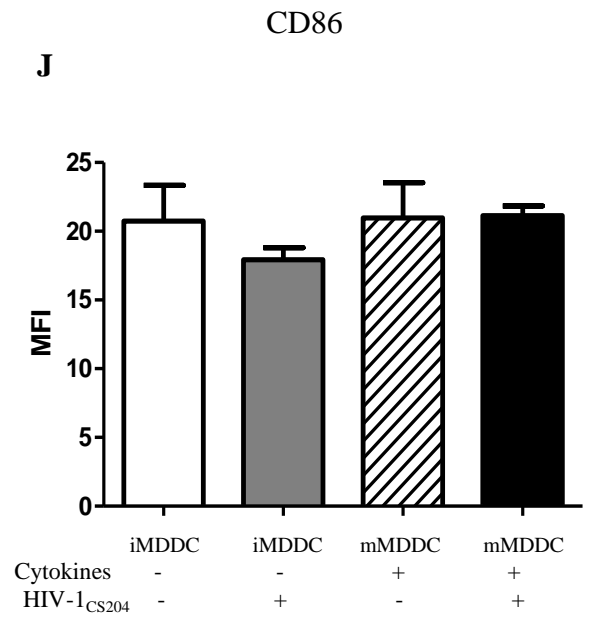
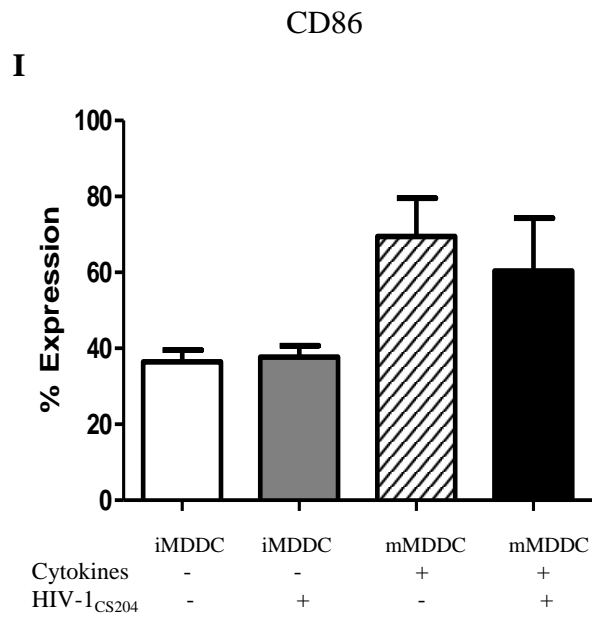
Figure 11: HIV-1 infection causes an increase in DC-SIGN expression on iMDDCs and decreases in CCR7 and MHC II expression on mMDDC

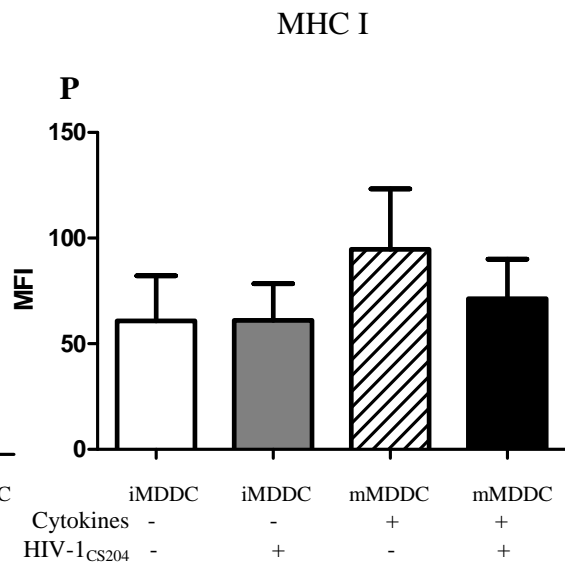
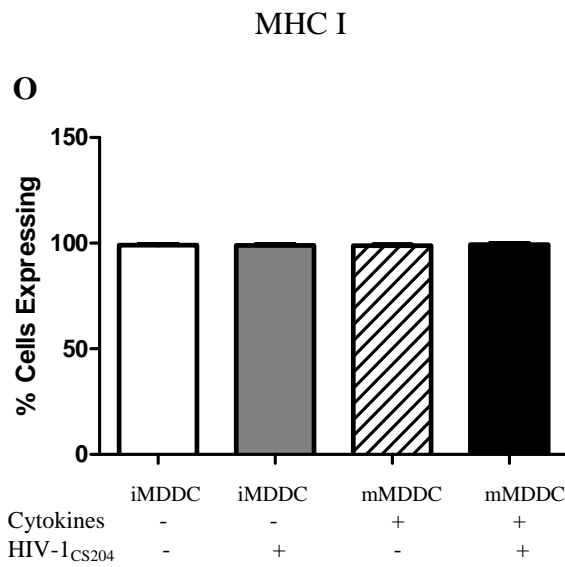
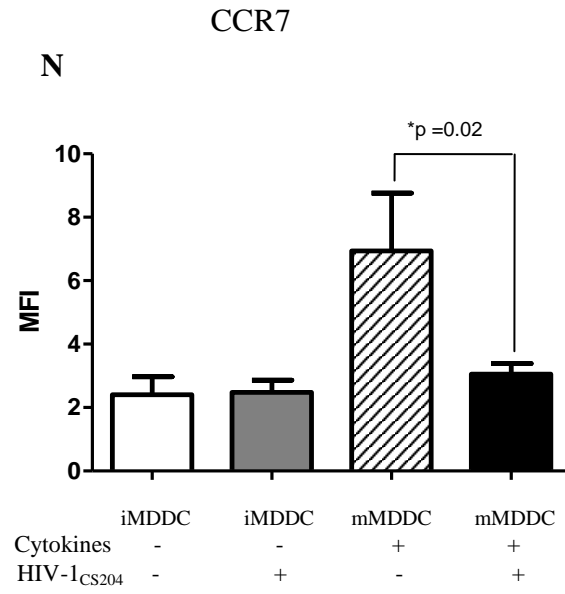
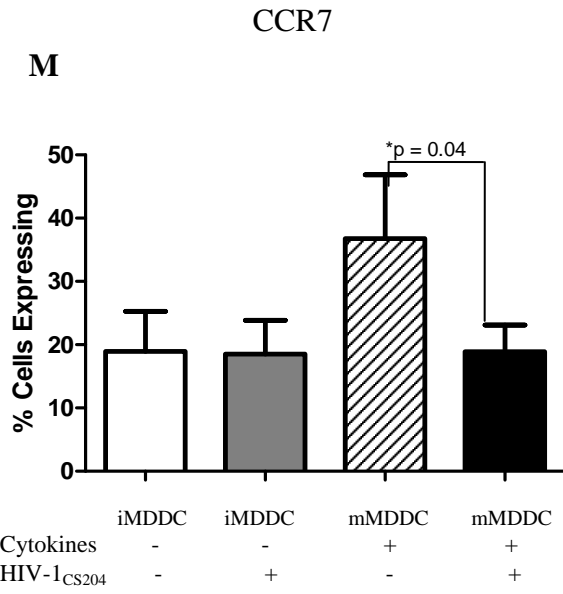
Immature MDDCs from 6 donors were incubated with a mock solution or a live dual tropic HIV-1_{CS204} (M.O.I. = 1) for 24h and incubated with or without TNF α , IL-1 β , IL-6, and PGE₂ for 48h at 37°C and 5% CO₂. Expression of CD14 (A and B), CD80 (E and F), CD86 (G and H), CD83 (I and J), CD40 (K and L), and MHC I (O and P) were unaffected by HIV-1_{CS204} infection. Expression of CCR7 on mMDDC (M) was found to be reduced after HIV-1_{CS204}. Surface expression of MHC II on mMDDCs was also found to be lower after HIV-1_{CS204} infection. Results are expressed as means \pm standard deviation.

*p=0.02; **p=0.04; ***p=0.02 by paired *t*-test (n=6).



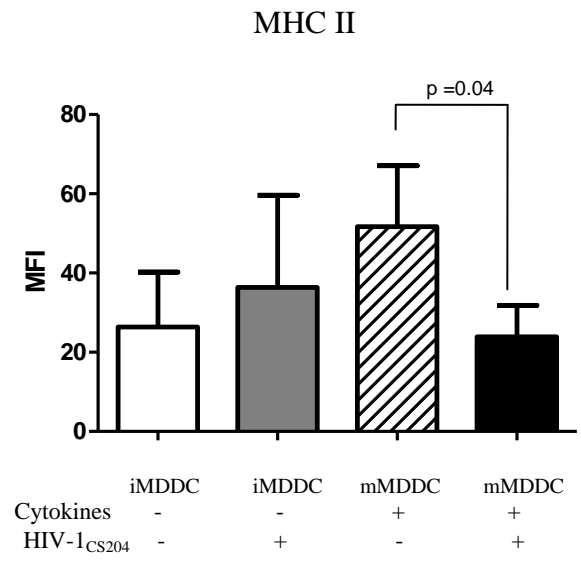
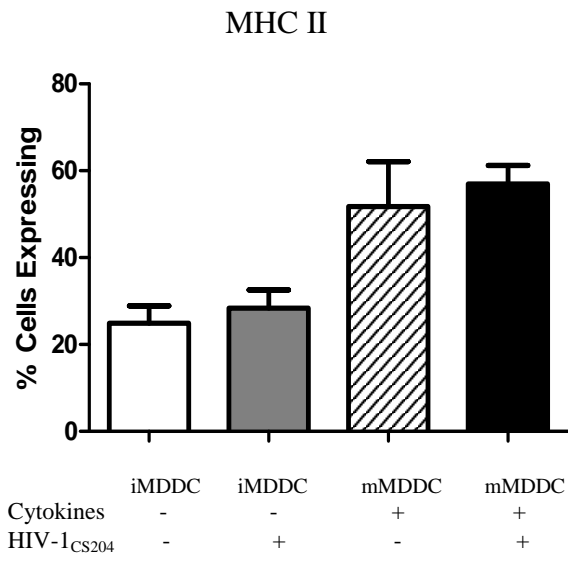






Q

R



3.3.2 HIV-1 infection blunts the maturation-induced downregulation of endocytosis

While a phenotypic analysis of MDDC can be used to partially identify the maturation status of an MDDC, determining the effects of HIV-1 on the functional character of MDDC over the course of maturation is required to elucidate a comprehensive picture of the effects of HIV-1 on MDDC maturation.

One critical function of dendritic cells is the uptake of antigen from the periphery for processing and presentation in lymphoid organs [4,5]. After endocytosing antigens, immature dendritic cells undergo maturation and move from the anatomic periphery to secondary lymphoid organs where their role becomes that of antigen presentation and not uptake [4,5]. As a measure of endocytic activity, and therefore the maturation state of MDDC, the effect of HIV-1 on dextran uptake was evaluated.

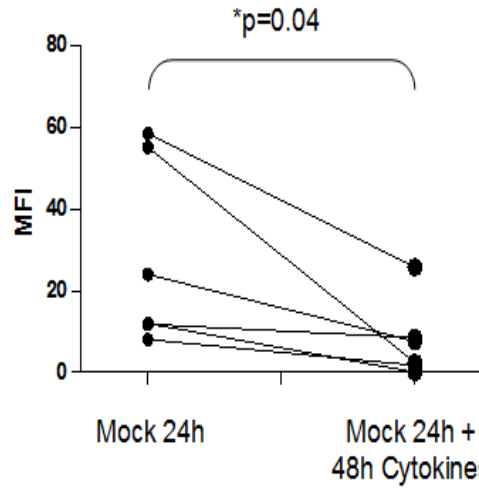
To determine the effect of HIV-1_{CS204} infection on MDDC antigen capture ability, we examined FITC-Dextran uptake by MDDCs after virus infection using flow cytometric analysis.

As expected, maturation of uninfected iMDDC resulted in a decrease in FITC-dextran uptake (Figure 11A). While HIV infection had no impact on the ability of iMDDC to take up dextran (Figure 11B), HIV-1 infection was associated with blunted downregulation of endocytosis by iMDDC (Figure 11C). HIV-1 infection therefore appeared to inhibit maturation reflected by the fact that HIV-1 infected DC partially retain their endocytic function.

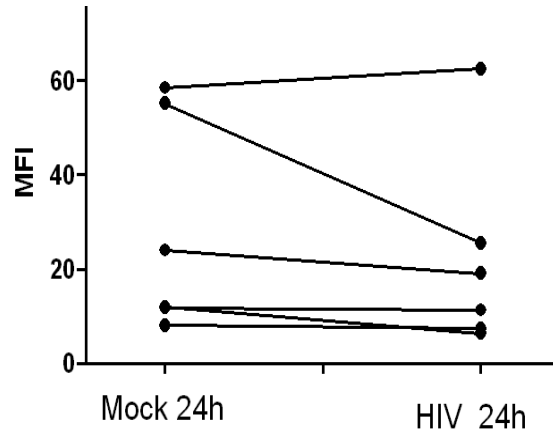
Figure 12: HIV-1 infection blunts the maturation-induced downregulation of endocytosis.

Immature MDDCs from 6 donors were incubated with a mock solution or a live dual tropic HIV-1_{CS204} for 24h washed and incubated with or without 1000 U/ml TNF α , 10 ng/ml IL-1 β , 10 ng/ml IL-6, and 1 μ M PGE₂ for 48 h at 37°C and 5% CO₂. They were then incubated with saturating concentrations of FITC-conjugated dextran for 1 h in the dark at 37°C and 5% CO₂ or on ice. A) Endocytosis of FITC-conjugated dextran was reduced in live-gated mMDDC compared to (B) iMDDCs. B) HIV-1_{CS204} infection of iMDDCs was not associated with any change in FITC-conjugated dextran compared to mock controls. C) HIV-1_{CS204} infection of iMDDCs was associated with blunted downregulation of endocytosis after the induction of maturation. Results are expressed as means \pm standard deviation. * $p < 0.05$ by paired *t*-test (n=6).

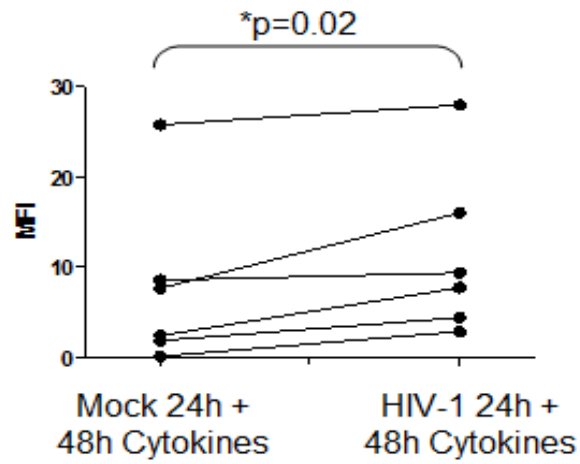
A



B



C



3.3.3 *HIV-1_{CS204} infection inhibits CEF antigen presentation by iMDDCs*

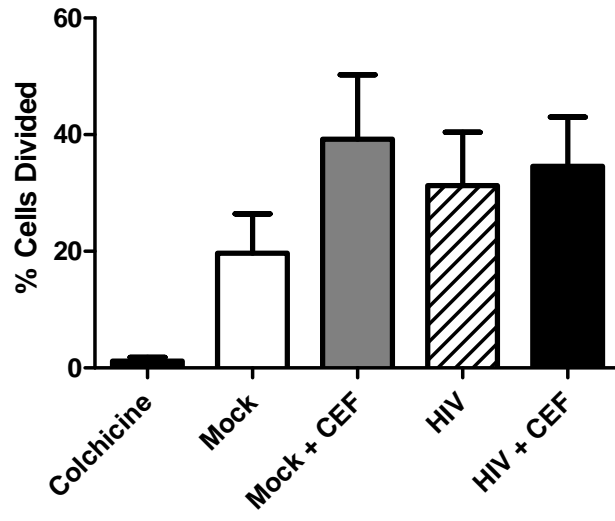
A primary function of dendritic cells is the presentation of antigens to naïve T-cells in peripheral lymphoid tissue [308]. The effect of HIV-1 infection on the ability of MDDC to present antigen to autologous CD8⁺ T-cells was determined by incubating HIV-1-infected MDDC with autologous PBMC in the presence of a CEF peptide pool comprised of 32 peptides, 8-12 amino acids in length, derived from human Cytomegalovirus, Epstein-Barr Virus and Influenza Virus as previously described [414].

After culturing CEF peptide-pulsed iMDDC with autologous PBMCs for 7 days, CD8⁺ T cells proliferated as expected (Figure 12). When iMDDC were infected with HIV-1 however, CD8⁺ T cell proliferation in response to the CEF peptide pool was not observed (Figure 12), suggesting that HIV-1 infection of DC prevented or interfered with antigen presentation.

Flow cytometry was used to investigate the effect of HIV-1_{CS204} infection on the ability of MDDCs to present antigen to autologous CFSE-stained CD8⁺ T-cells.

Figure 13: HIV-1_{CS204} infection inhibits CEF antigen presentation by iMDDCs

After a 24 h incubation with HIV-1_{CS204} and 48 h of culture, MDDCs from 6 donors were pulsed with a CEF peptide pool and then co-cultured with autologous CFSE stained PBMCs at a ratio of 1:10 for 7 days at 37°C and 5% CO₂ in the dark. While preincubation of uninfected iMDDCs induced the proliferation of autologous monocyte depleted PBMCs, HIV-1_{CS204} infection of iMDDCs impaired the presentation of a CEF peptide pool as measured by CFSE dilution. Results are expressed as means ± standard deviation. *p=0.02 by paired *t*-test (n=6).



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3.3.4 The effect of HIV-1_{CS204} on LPS induced MDDC MAP kinase activation

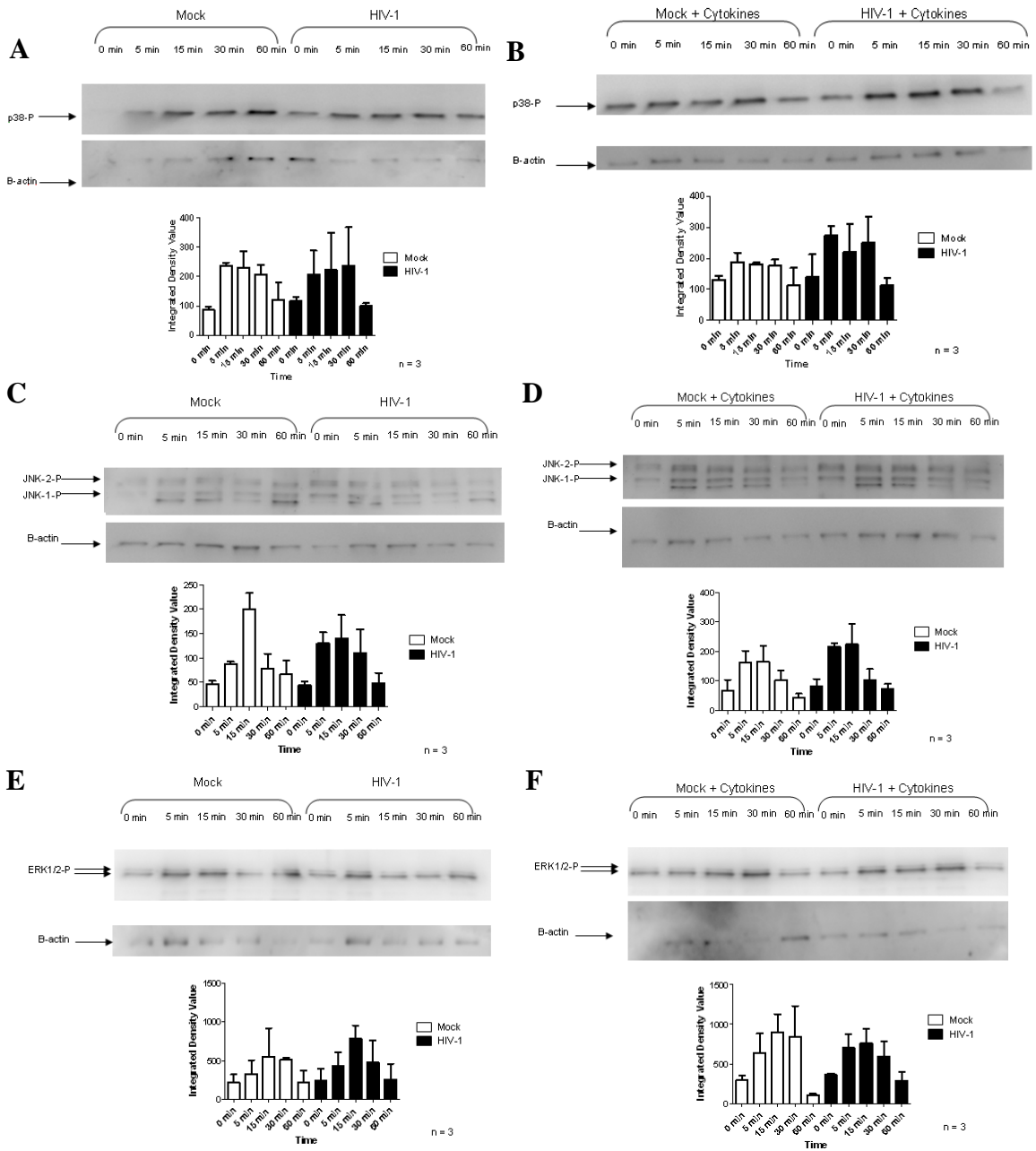
The p38, JNK, and ERK MAPKs have been found to be integral for the maturation and function of dendritic cells [253]. To further characterize the effect of HIV-1 on DC maturation, MAPK activation in response to LPS was evaluated before and after DC maturation with inflammatory cytokines.

To determine the effect of HIV-1 on LPS-dependent MAPK signalling in MDDCs, HIV-1_{CS204} and mock infected MDDCs were incubated in the presence or absence of LPS for 1 h and p38, JNK, and ERK MAPK phosphorylation were examined using immunoblot and AlphaEase FC 6.0.0 software analysis.

When iMDDC were infected with HIV-1_{CS204}, they exhibited similar patterns of LPS induced phosphorylation of p38, JNK, and ERK (Figures 13A, C, and E) to that observed in uninfected cells. Likewise, the patterns of MAPK phosphorylation observed after LPS stimulation of mMDDC were not affected by HIV-1-infection (Figures 13B, D, and F).

Figure 13: The effect of HIV-1_{CS204} on MAPK response to LPS by MDDCs

HIV-1 infected or uninfected iMDDC from 3 donors were incubated with or without TNF α , IL-1 β , IL-6, and PGE₂ for 48 h and then stimulated with LPS for 1 h. Changes in the phosphorylation of the ERK, JNK, and p38 proteins in response to LPS after HIV-1_{CS204} infection were measured using immunoblot and AlphaEase FC 6.0.0 software analyses. HIV-1_{CS204} infection did not affect LPS induced phosphorylation of p38 (A: iMDDC,B: mMDDC), JNK (C: iMDDC,D: mMDDC) or ERK1/2 (E: iMDDC,F: mMDDC). Densitometry analysis was performed on 3 individual experiments from using lysates from 3 separate donor cells and results are expressed as means \pm standard deviation.



3.4.2 Exogenous HIV-1-Tat does not affect MDDC Phenotype

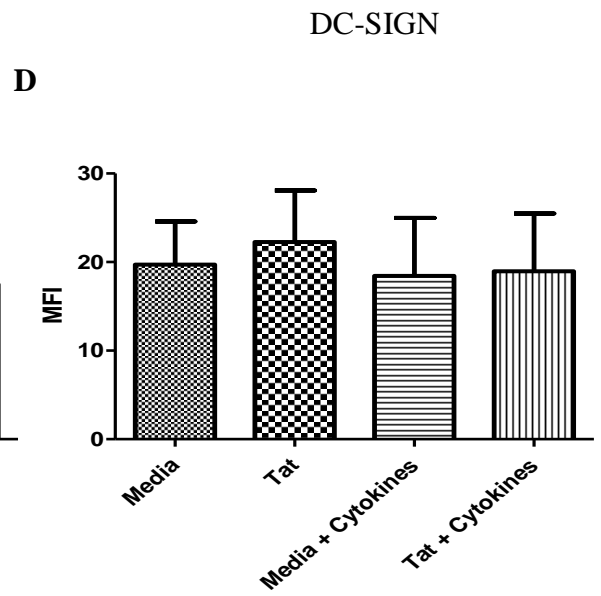
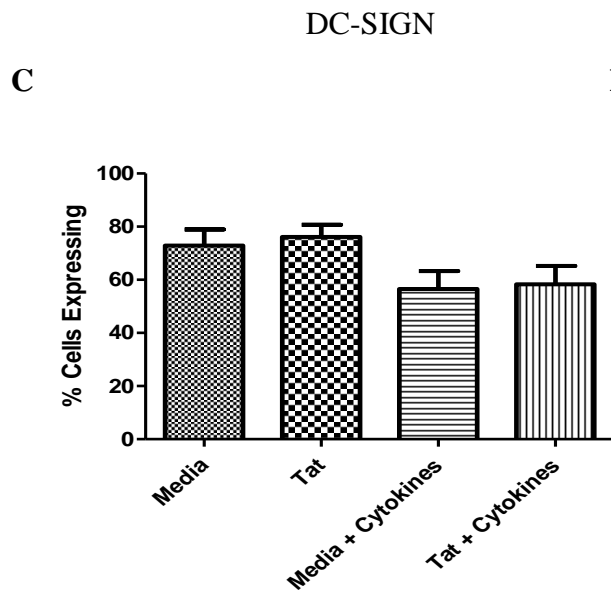
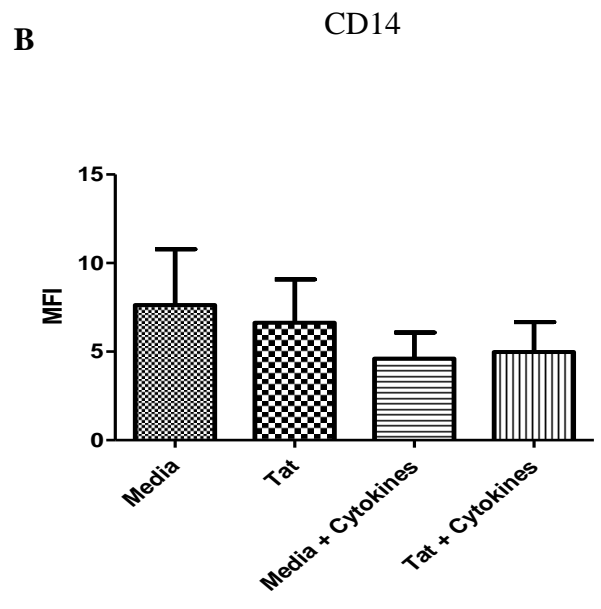
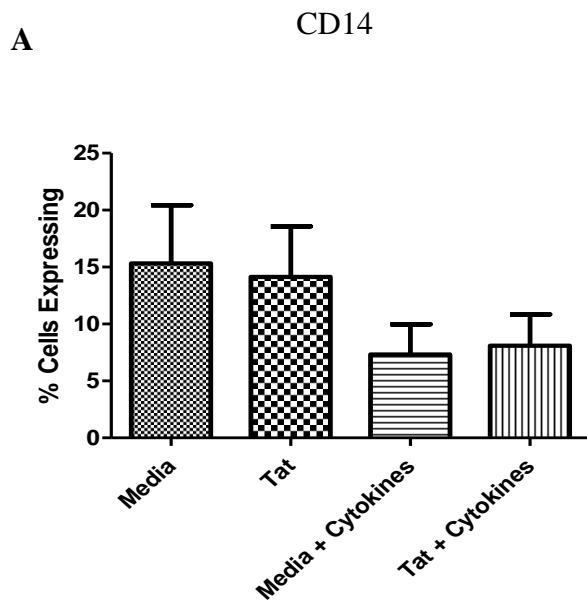
Exogenous HIV-1-Tat has been observed to induce the phenotypic and functional maturation of iMDDCs [403,404] and is found in the circulation of chronically infected individuals [415]. This is in contrast to the lack of phenotypic changes iMDDCs undergo after infection with HIV-1 [370]. Alterations in iDC phenotype during HIV-1 infection in the absence of integrated proviral infection are important to understand since DCs are refractory to HIV-1 infection [370,371] and because DC phenotype can be predictive of their function [4,5]. We expanded upon previous studies and began our investigation into the effects of exogenous Tat on iMDDC maturation with an examination of its effects on iMDDC phenotypic maturation. Flow cytometry was used to examine the effect of HIV-1-Tat on MDDC expression of CD14, DC-SIGN, CD80, CD83, CD86, CD40, CCR7, MHC I, and MHC II.

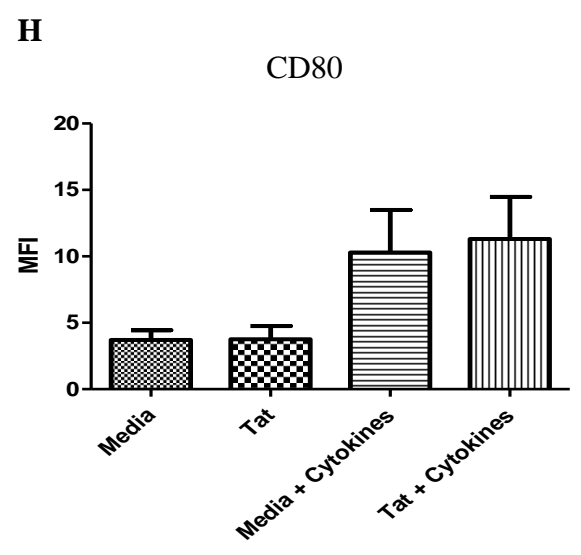
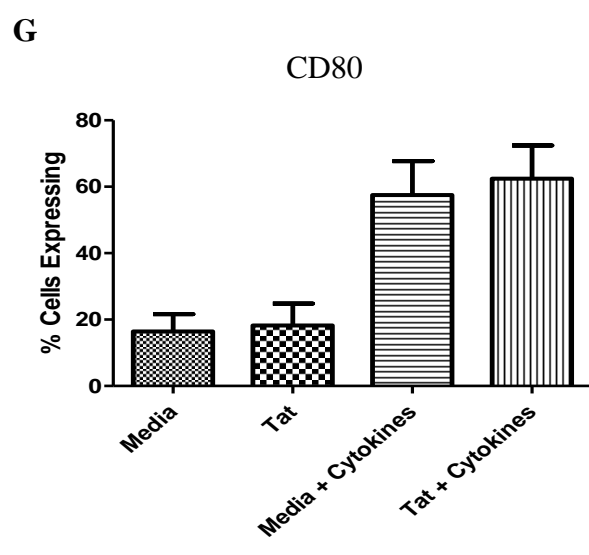
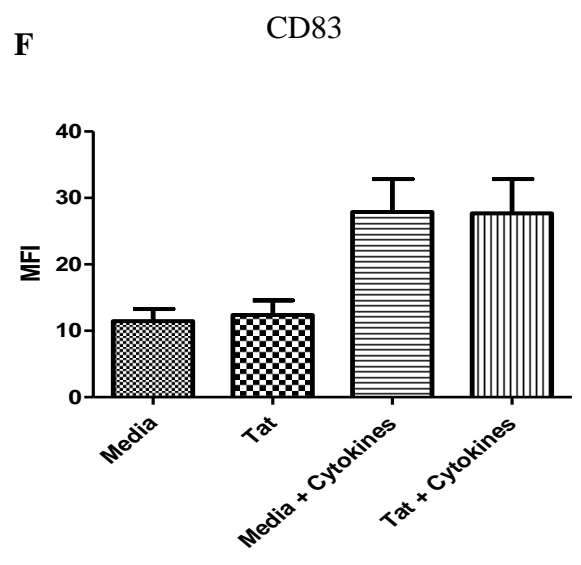
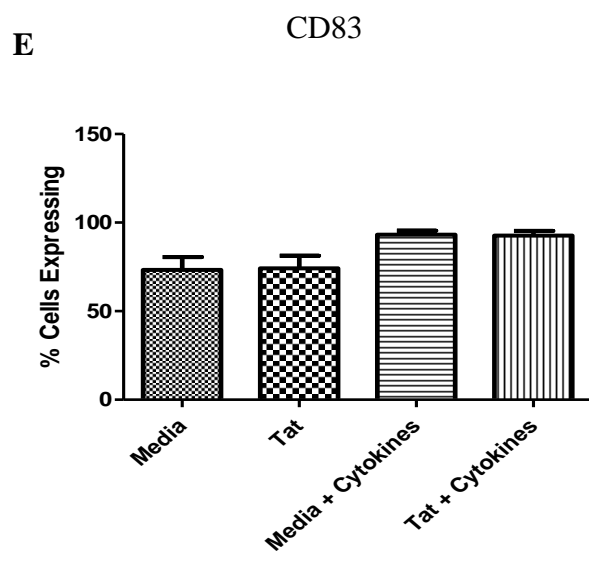
After incubation of iMDDC with Tat (50 ng/ml) for 24 h at and 48 h of culture, there were no changes in the expression of CD14, DC-SIGN, CD80, CD86, CD83, CD40, CCR7, MHC I, or MHC II (Figures 14A to 14R) indicating that HIV-1-Tat itself was not capable of inducing DC maturation.

Likewise, after iMDDC were incubated with Tat (50 ng/ml) and then stimulated to mature, there were no there was no changes in the expression of any of the surface molecules. These results suggest that exogenous Tat does not influence iMDDC phenotype or their cytokine-induced maturation.

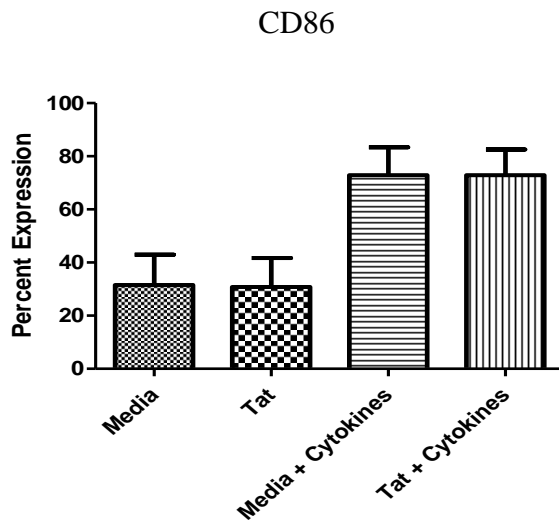
Figure 14: Exogenous HIV-1-Tat does not affect MDDC surface molecule expression and phenotypic maturation.

iMDDCs from 6 donors were incubated with endotoxin depleted Tat (50 ng/ml) for 24 h at 37°C and 5% CO₂ and incubated for 48 h at 37°C and 5% CO₂ with or without TNF α , IL-1 β , IL-6, and PGE₂. Expression of CD14 (14A,14B), DC-SIGN (14C,14D), CD80 (14E,14F), CD86 (14G,14H), CD83 (14I,14J), CD40 (14K,14L), CCR7 (14M,14N), MHC I (14O,14P), and MHC II (14Q,14R) were unaffected by Tat incubation as measured by flow cytometry. Results are expressed as means \pm standard deviation (n=6).

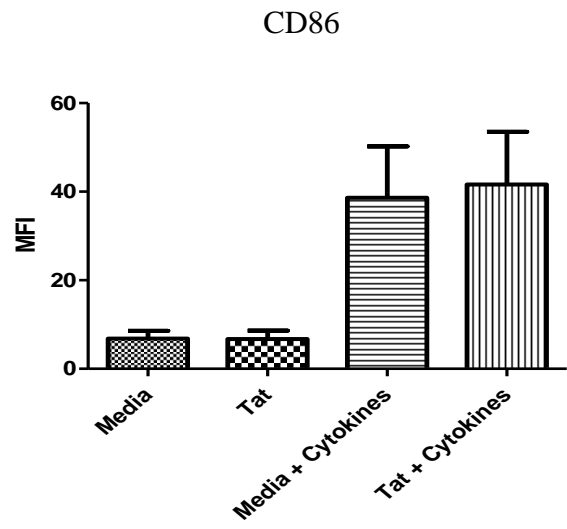




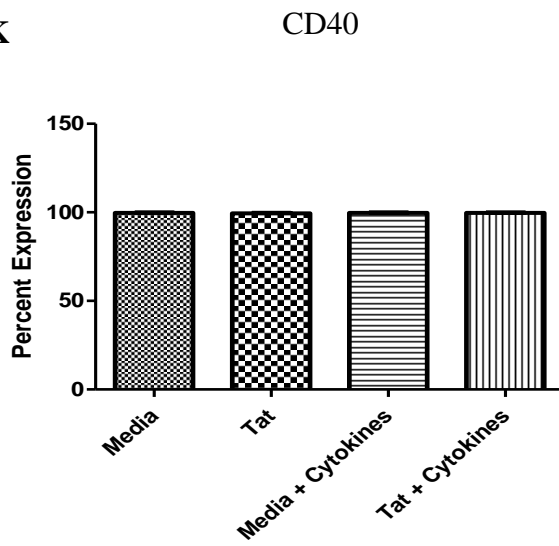
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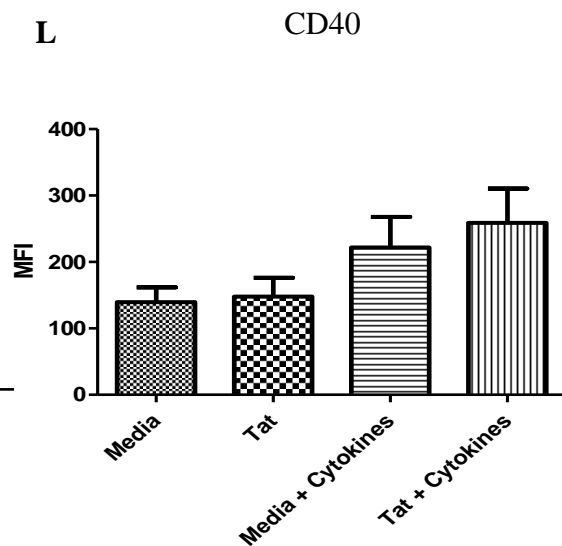
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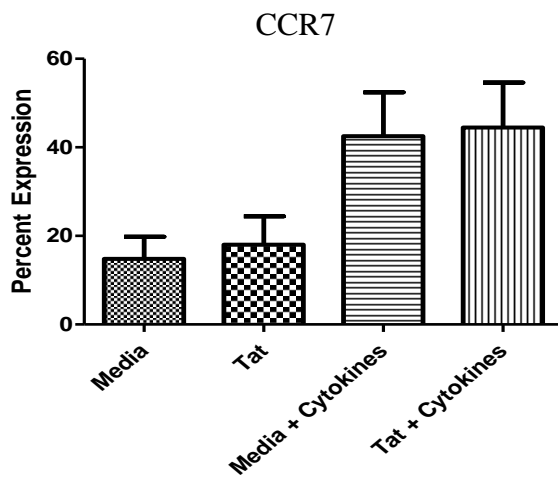
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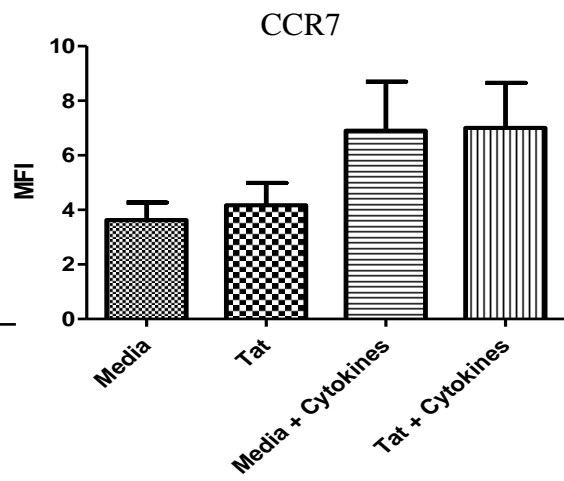
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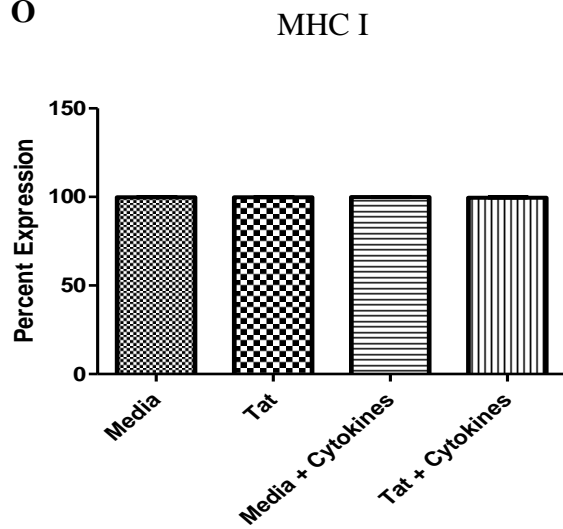
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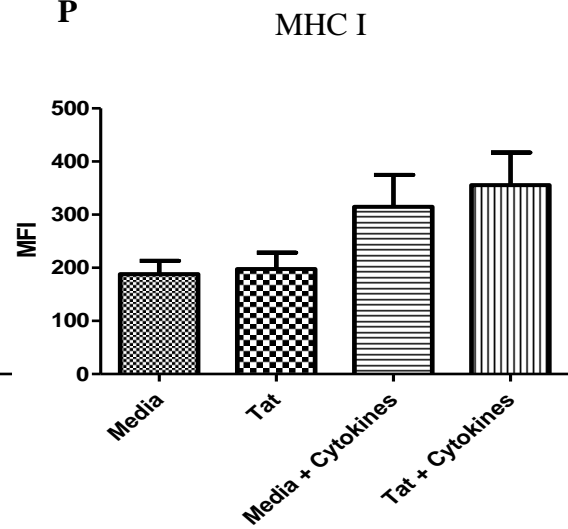
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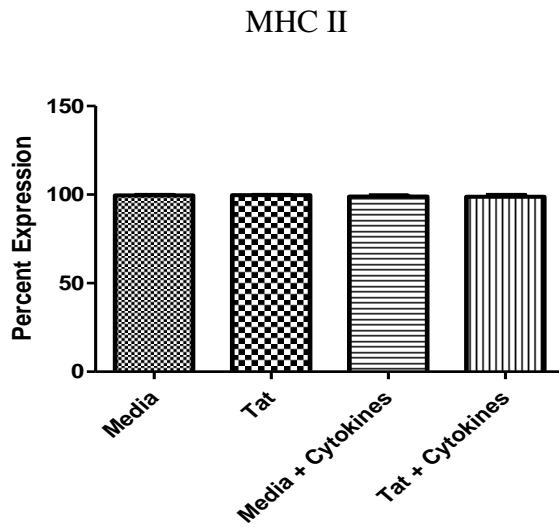
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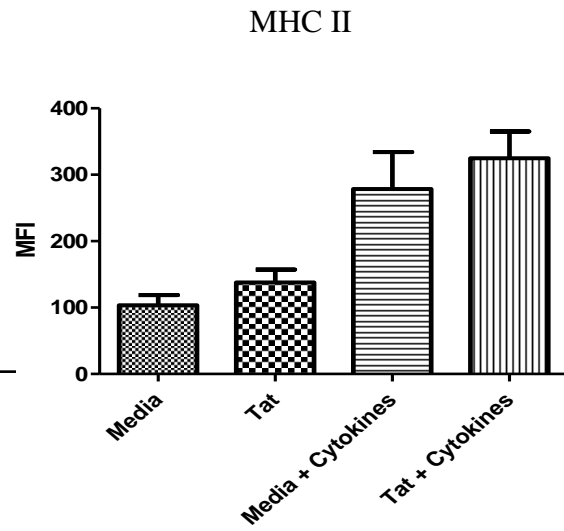
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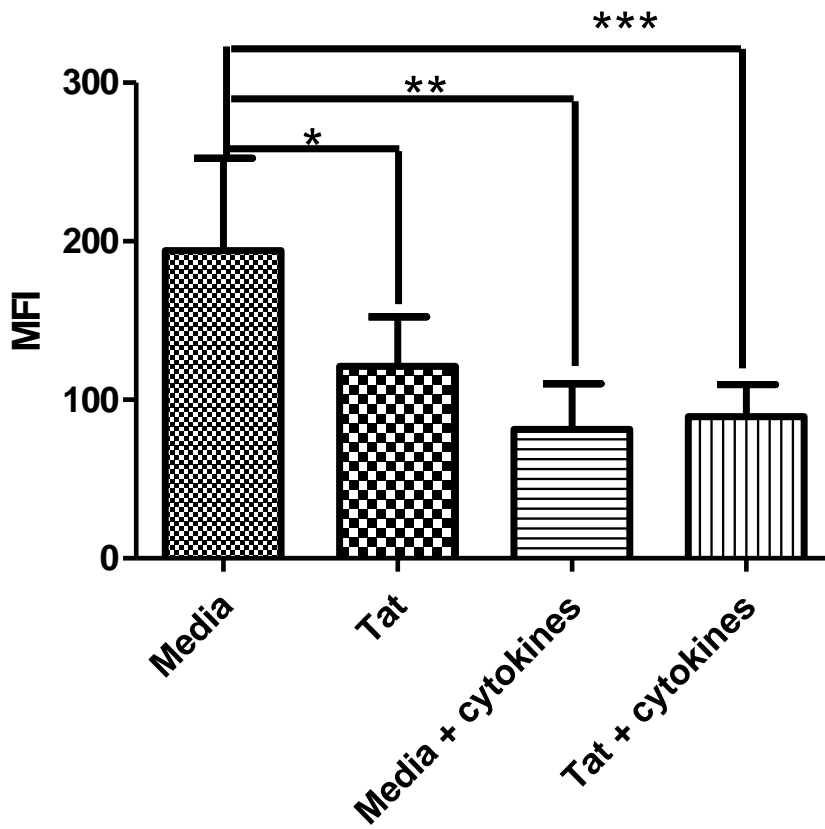
3.4.3 Exogenous HIV-1-Tat reduces iMDDC FITC-conjugated dextran endocytosis

As previously described, an important measure of iDC functional is their ability to efficiently capture antigens. During maturation, iDCs change from antigen capture to antigen presenting cell [4,5]. Exogenous Tat has been observed to induce iMDDC maturation [403,404] and is found in the circulation of infected individuals [415] where it could influence iDC maturation. Like in the case of phenotypic maturation, changes in DC function in the absence of productive HIV-1 infection could be important subversion methods employed by the virus to evade immune detection. Alterations in DC endocytic function during HIV-1 infection could also have negative effects on DC responses to antigens leading to inhibited adaptive immune system stimulation. Therefore, we investigated the effect of Tat incubation on MDDC antigen capture ability. We examined FITC-Dextran uptake by MDDCs after incubation with Tat and flow cytometric analysis.

Endocytosis of FITC-conjugated dextran by iMDDCs incubated with inflammatory cytokines was reduced (Figure 15). Tat incubation was also associated with decreased FITC-conjugated dextran endocytosis, but it did not further decrease FITC-conjugated dextran endocytosis of mMDDCs (Figure 15). Our results suggest that Tat can reduce iMDDC dextran endocytosis without altering their phenotype.

Figure 15: Exogenous HIV-1-Tat reduces iMDDC FITC-Conjugated Dextran endocytosis

iMDDCs from 6 donors were incubated with 50 ng/ml of endotoxin depleted Tat and then incubated for 48 h with or without TNF α , IL-1 β , IL-6, and PGE $_2$. Cells were then incubated with saturating concentrations of FITC-conjugated dextran for 1 h and endocytosis of dextran was examined using flow cytometric analysis of mean fluorescence intensity (MFI) using FCS Express 2.00 software. Endocytosis of FITC-conjugated dextran was reduced in mMDDC compared to iMDDC. Incubation of iMDDCs with 50 ng/ml Tat resulted in a similar decrease in FITC-conjugated dextran endocytosis. Tat incubation did not further decrease mMDDC FITC-Dextran endocytosis. Results are expressed as means \pm standard deviation. *p=0.03; **p=0.03; ***p=0.01 by paired *t*-test (n = 6).



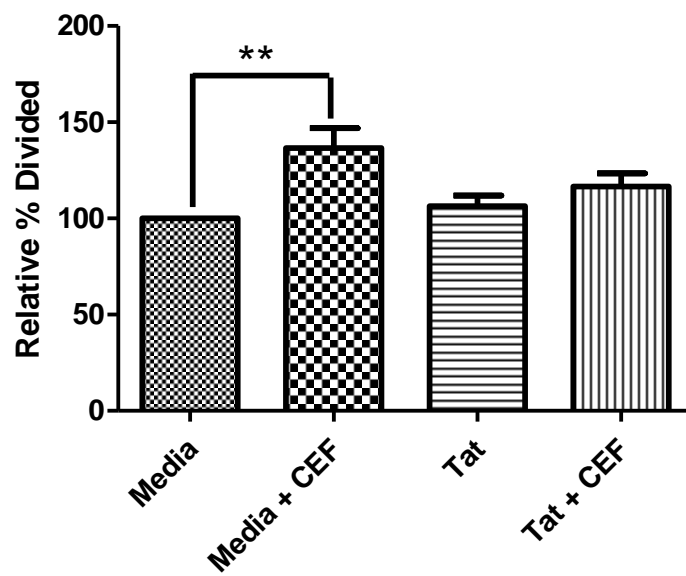
3.4.4 Exogenous HIV-1-Tat decreases CEF antigen presentation by iMDDCs

An important function of mature DCs is to present antigens to naïve T-cells in peripheral lymphoid tissues [4,5]. Also important within the context of HIV-1 infection is the ability of the virus to exert effects on cells in the absence of productive infection. Exogenous Tat has been previously observed to induce iMDDC maturation *in vitro* [403,404] and it is also found in the circulation of infected individuals [415] where it could alter iDC physiology. Therefore, it is important to understand the effects of this peptide on MDDC antigen presentation since alterations in this function could influence infection clearance and disease progression. Flow cytometry was used to investigate the effect of exogenous Tat on the ability of iMDDCs to present antigen to autologous CFSE-stained CD8⁺ T-cells.

After culturing CEF peptide-pulsed iMDDC with CFSE-stained autologous PBMCs for 7 days, CD8⁺ T cells proliferated as expected (Figure 16). However, when iMDDC were incubated with Tat, CD8⁺ T cell proliferation in response to the CEF peptide pool was inhibited (Figure 16). These results suggest that exogenous Tat interferes with antigen presentation.

Figure 16: Exogenous HIV-1-Tat decreases CEF antigen presentation by iMDDCs

After a 24 h incubation with Tat and 48 h of culture, iMDDCs from 6 donors were pulsed with a CEF peptide pool and then co-cultured with autologous CFSE stained PBMCs at a ratio of 1:10 for 7 days at 37°C and 5% CO₂ in the dark. Cells proliferating outside of the colchicine control were considered divided. While preincubation of uninfected iMDDCs induced the proliferation of autologous monocyte depleted PBMCs, 24h 50µg Tat incubation of iMDDCs impaired the presentation of a CEF peptide pool as measured by CFSE dilution. Results are expressed as means ± standard deviation. **p=0.001 by paired *t*-test (n=6).



3.4.5 Exogenous HIV-1-Vpr does not affect iMDDC phenotype or phenotypic maturation

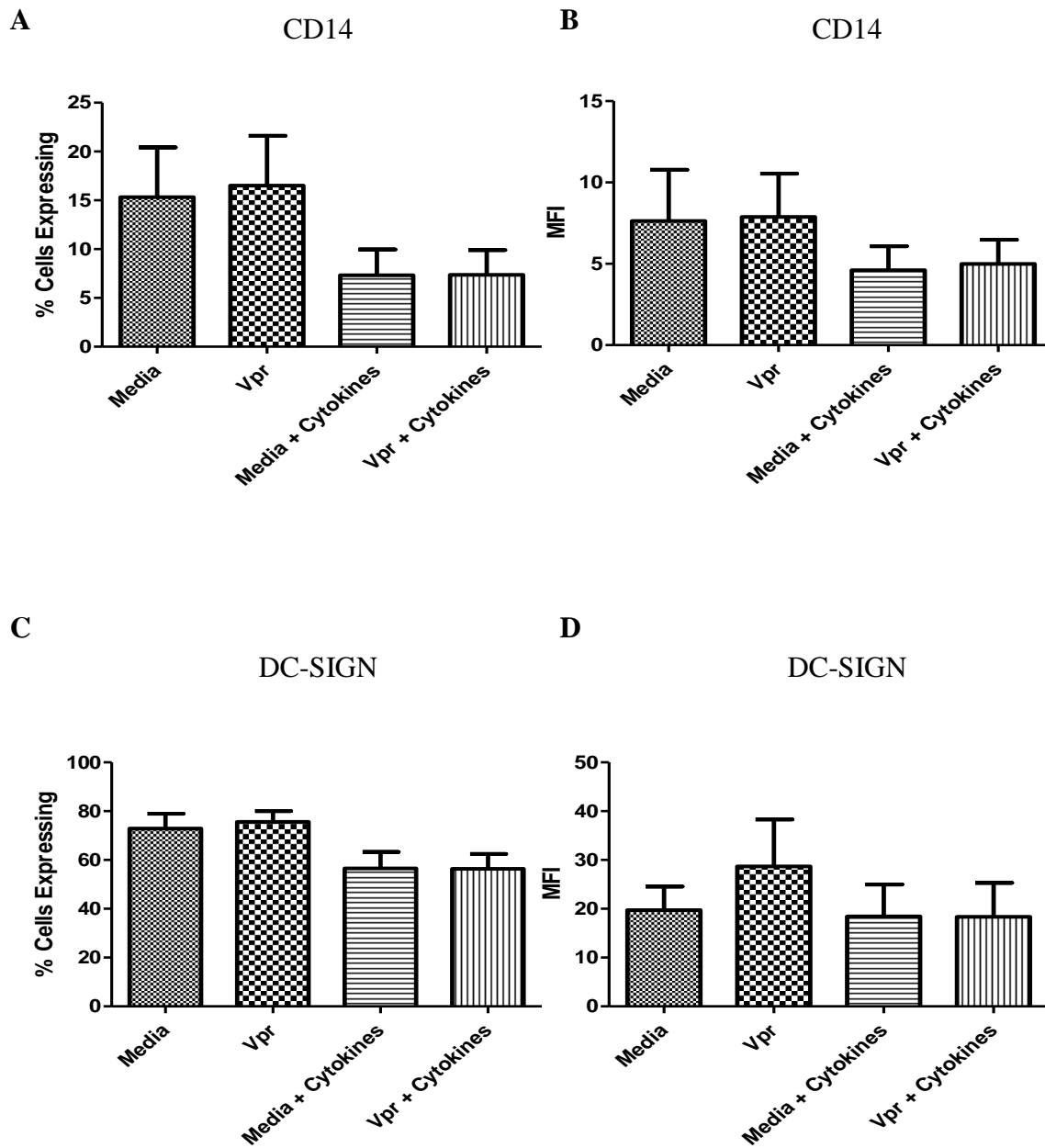
DC phenotype changes over the course of maturation from antigen capture cells to antigen presenting cells [4,5]. Previous experiments have shown that HIV-1 [370] and exogenous Vpr [407] can inhibit the maturation of iMDDCs. This peptide is also found in the circulation of infected individuals [416]. Alterations in iDC phenotype during HIV-1 infection without their productive infection are important to understand because DCs are refractory nature to HIV-1 infection [342]. Since phenotypic maturation is an important part of iMDDC maturation and may impact iDC function during viral infection, we expanded upon previous studies and investigated the effects of exogenous Vpr on iMDDC phenotypic and functional maturation. Flow cytometry was first used to examine the effect of Vpr incubation on MDDC expression of CD14, DC-SIGN, CD80, CD83, CD86, CD40, CCR7, MHC I, and MHC II.

After incubation with Vpr (10 ng/ml) for 24 h and 48 h of culture, there were no changes in the expression of CD14, DC-SIGN, CD80, CD86, CD83, CD40, CCR7, MHC I, or MHC II (Figures 17A to 17R) indicating that Vpr itself was not capable of inducing DC maturation.

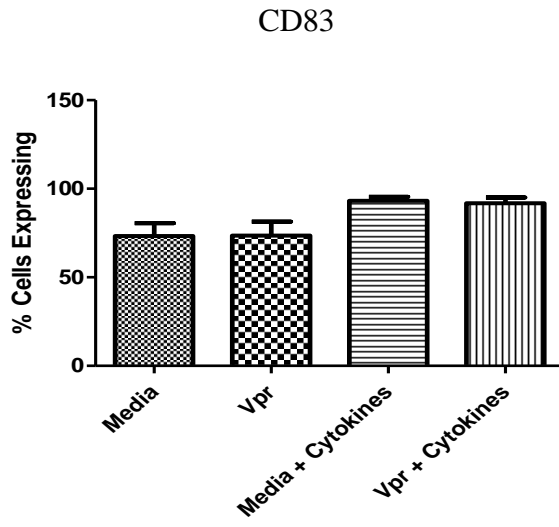
Likewise, after iMDDC were incubated with Vpr (10 ng/ml) and then stimulated to mature, there were no there was no changes in the expression of any of the surface molecules (Figure 17A to 17R). Exogenous Vpr therefore does not influence cytokine-induced MDDC maturation.

Figure 17: Exogenous HIV-1-Vpr does not affect iMDDC phenotype or phenotypic maturation

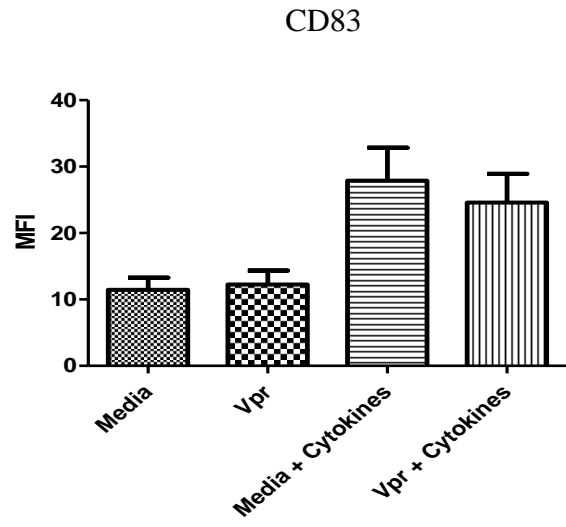
iMDDCs from 6 donors were incubated with exogenous Vpr (10 ng/ml) for 24 h at 37°C and 5% CO₂ and incubated for 48 h at 37°C and 5% CO₂ with or without TNF α , IL-1 β , IL-6, and PGE₂. Expression of CD14 (17A,17B), DC-SIGN (17C,17D), CD80 (17E,17F), CD86 (17G,17H), CD83 (17I,17J), CD40 (17K,17L), CCR7 (17M,17N), MHC I (17O,17P), and MHC II (17Q,17R) were unaffected by Vpr incubation as measured by flow cytometry. Results are expressed as means \pm standard deviation (n=6).



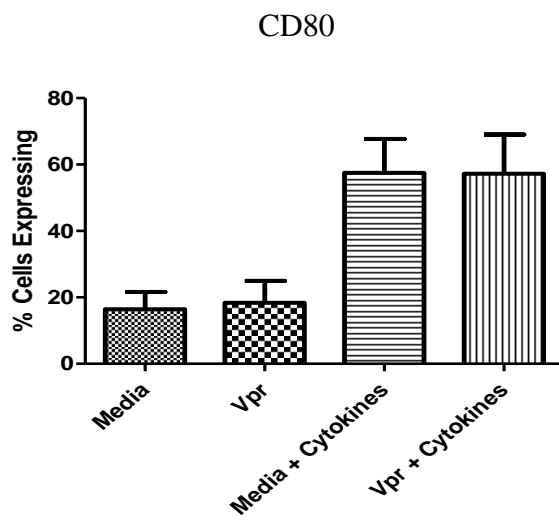
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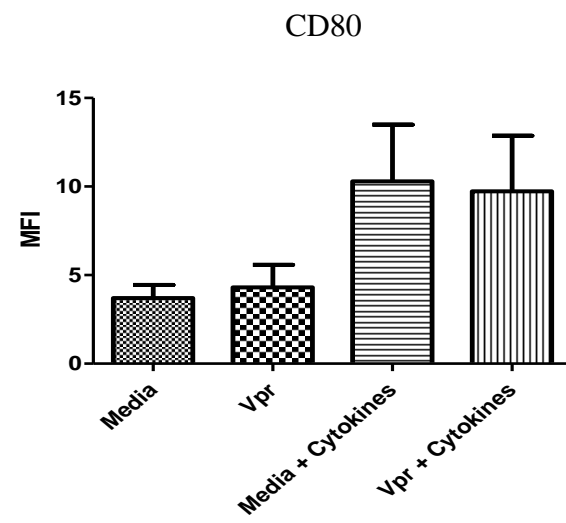
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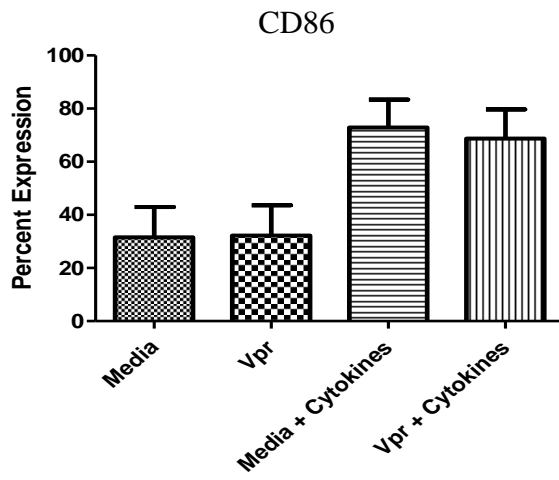
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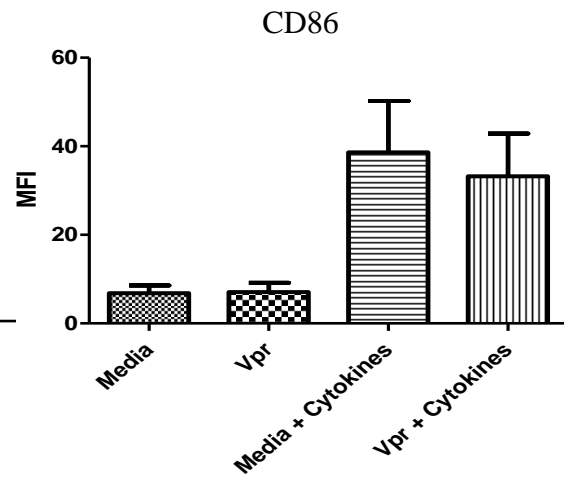
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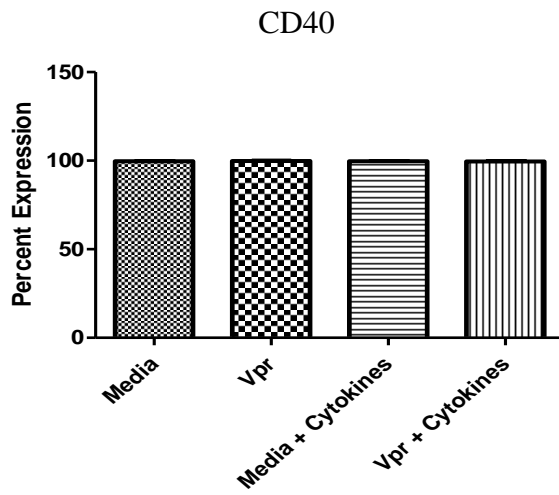
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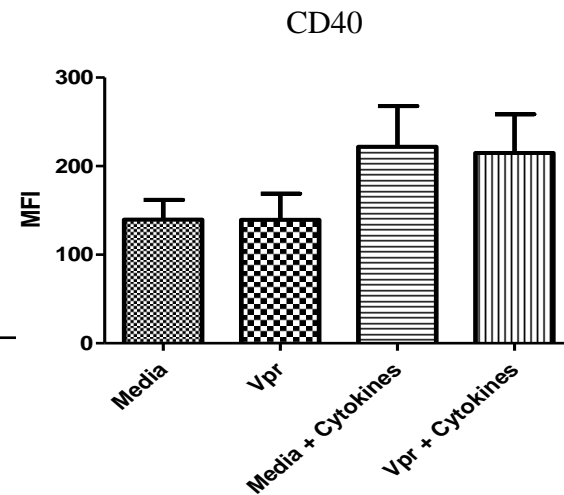
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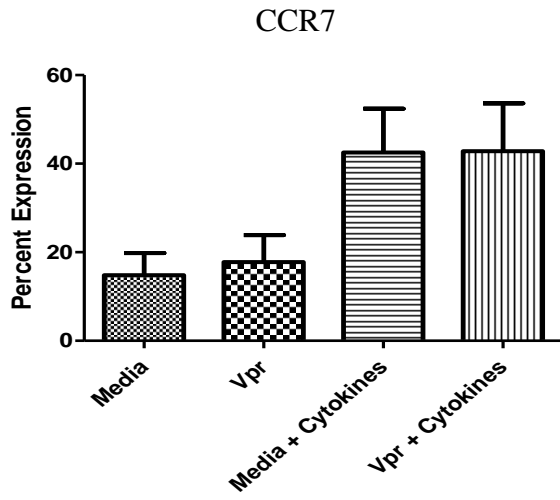
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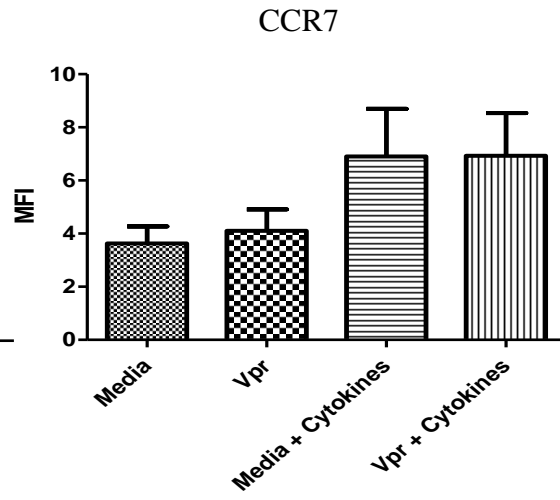
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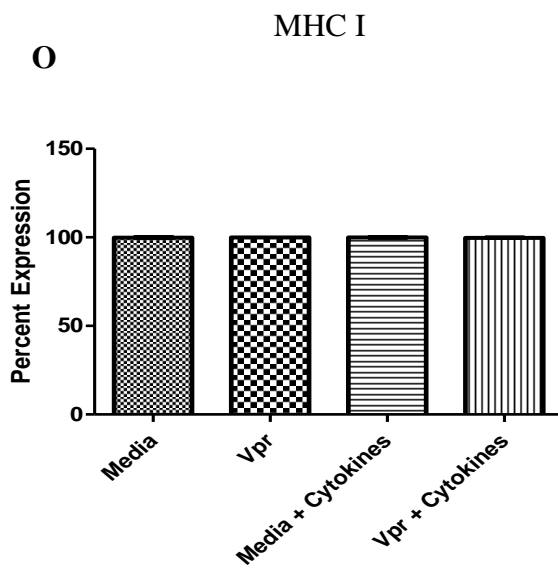
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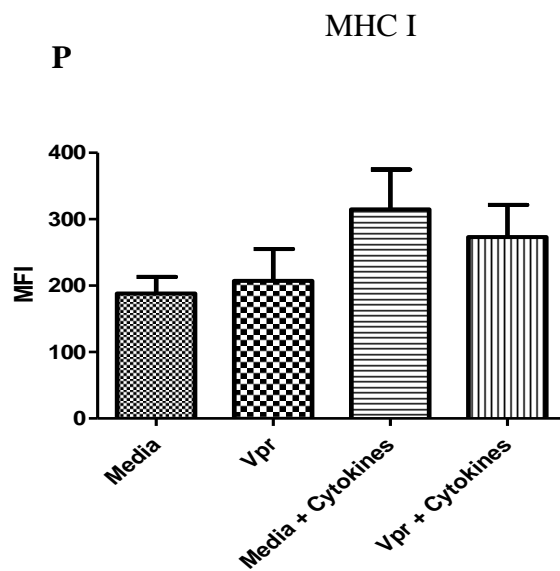
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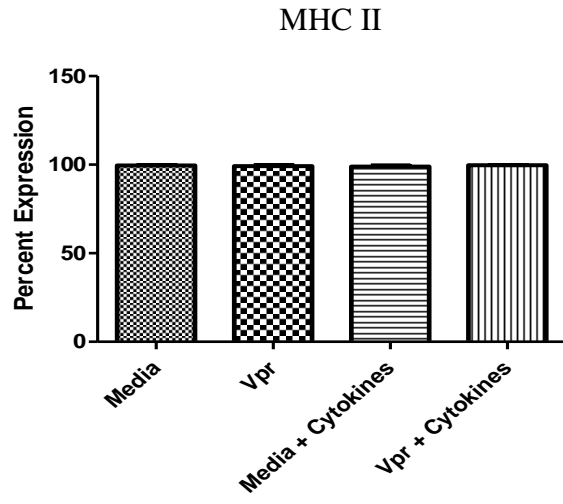
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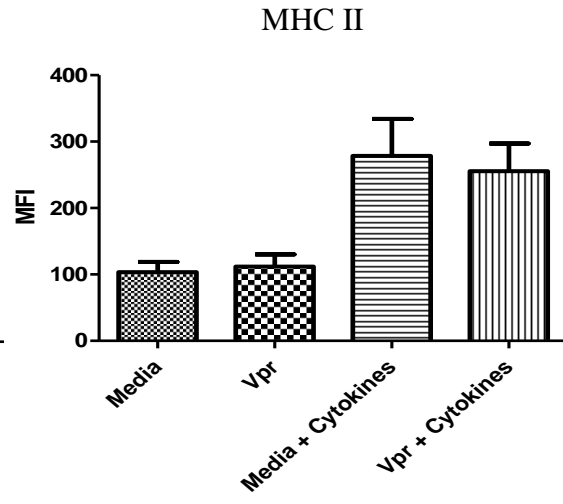
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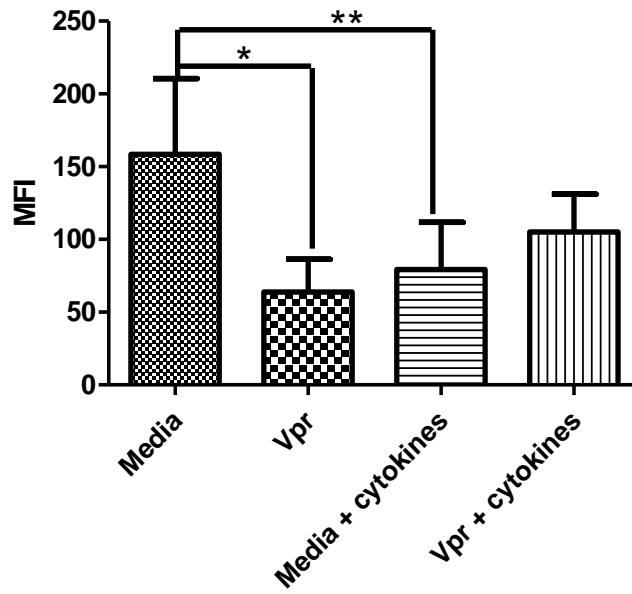
3.4.6 Exogenous HIV-1-Vpr decreases iMDDC endocytosis function and blunts inflammatory cytokine induced reductions in endocytosis function

During maturation, iDCs change from antigen capture to antigen presenting cell during maturation [4,5]. Exogenous Vpr has been found in the circulation of infected individuals [416] and has been observed to inhibit iMDDC maturation *in vitro* [407]. Changes in DC function without proviral integration are important methods used by the HIV-1 to increase infectivity and to evade immune detection. Alterations in DC endocytic function during HIV-1 infection could negatively affect adaptive immune responses. To further expand upon the known effects of Vpr on iMDDC maturation, we investigated the effect of Vpr incubation on MDDC FITC-Dextran uptake by flow cytometric analysis.

As expected, maturation of iMDDCs that were not incubated with Vpr resulted in a decrease in FITC-dextran uptake (Figure 18). Vpr incubation was also associated with decreased FITC-dextran endocytosis (Figure 18). However, iMDDC incubation with Vpr also blunted inflammatory cytokine decreases in FITC-dextran endocytosis by mMDDCs (Figure 18).

Figure 18: Exogenous HIV-1-Vpr decreases iMDDC endocytosis function and blunts decreases inflammatory cytokine induced reductions in endocytosis function

Immature MDDCs from 6 donors were incubated with of 10 ng/ml of exogenous Vpr and then incubated for 48 h with or without TNF α , IL-1 β , IL-6, and PGE $_2$. Cells were then incubated with saturating concentrations of FITC-conjugated dextran for 1 h and endocytosis of dextran was examined using flow cytometric analysis of mean fluorescence intensity (MFI) using FCS Express 2.00 software. Endocytosis of FITC-conjugated dextran was reduced in mMDDC compared to iMDDC. Incubation of iMDDCs with 10 ng/ml Vpr resulted in a similar decrease in FITC-conjugated dextran endocytosis. Vpr incubation also blunted inflammatory cytokine induced mMDDC FITC-dextran endocytosis. Results are expressed as means \pm standard deviation. *p=0.01; **p=0.02 by paired *t*-test (n=6).



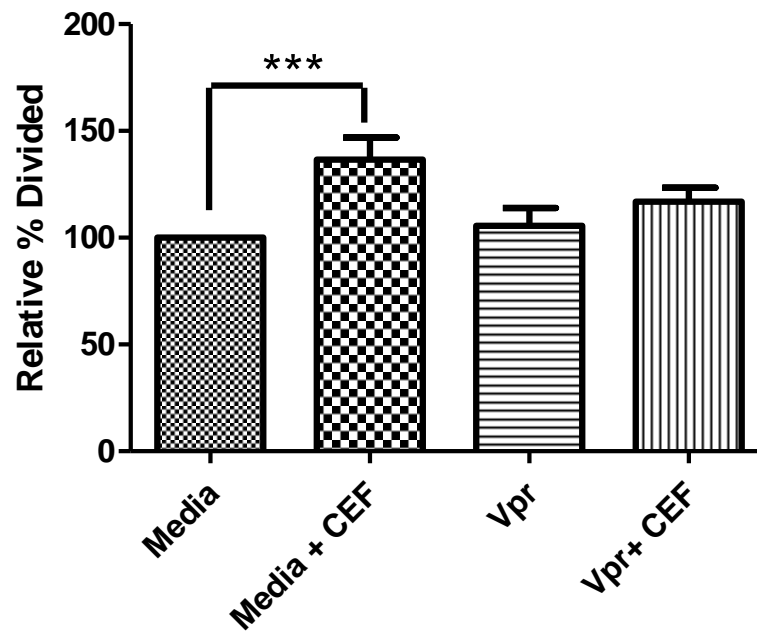
3.4.7 Exogenous HIV-1-Vpr inhibits CEF antigen presentation by MDDCs

As previously described, an important function of mature DCs is presentation of antigens to naïve T-cells in secondary lymphoid tissues. Exogenous Vpr has been observed to inhibit MDDC allogeneic T-cell activation *in vitro* [407] and is present in the circulation of infected individuals [416]. That Vpr can inhibit DC functions in the absence of proviral infection is an important mechanism through which HIV-1 can subvert the immune response of cells that are resistant to infection. It is important to understand the effects of this peptide on MDDC antigen presentation since alterations in this function could influence infection clearance and disease progression. Thus, we investigated the effects of exogenous Vpr on iMDDC antigen presentation. Flow cytometry was used to investigate the proliferation of cells responding to CEF as an indicator of antigen presentation by via autologous CFSE-stained CD8⁺ T-cells.

After culturing CEF peptide-pulsed iMDDC with CFSE-stained autologous PBMCs for 7 days, CD8⁺ T cells proliferated as expected (Figure 19). However, when iMDDC were incubated with Vpr, CD8⁺ T cell proliferation in response to the CEF peptide pool was inhibited (Figure 19). These results suggest that exogenous Vpr interferes with antigen presentation.

Figure 19: Exogenous HIV-1-Vpr inhibits CEF antigen presentation by MDDCs

After a 24 h incubation with Vpr and 48 h of culture, MDDCs from 6 donors were pulsed with a CEF peptide pool and then co-cultured with autologous CFSE stained PBMCs at a ratio of 1:10 for 7 days at 37°C and 5% CO₂ in the dark. While preincubation of uninfected iMDDCs induced the proliferation of autologous monocyte depleted PBMCs, Vpr incubation with iMDDCs impaired the presentation of a CEF peptide pool as measured by CFSE dilution. Results are expressed as means ± standard deviation or relative percent division. *p=0.001 by paired *t*-test (n=6).



3.4.8 Exogenous HIV-1-gp120 induce increases in CD83 and CD86 expression on iMDDC surface molecules and does not affect phenotypic maturation

Initial infection events are critical in determining the progression and outcome of HIV-1 infections [325-327]. During HIV-1 infection, initial virus-DC interactions in the anatomic periphery are thought to be some of the first events that may facilitate systemic viral infection [319]. Previous investigations have shown that exogenous HIV-1-gp120 can induce a variety of effects on MDDCs *in vitro*. Specifically it has been observed to induce partial phenotypic maturation [397], to inhibit functional maturation [397], to increase IL-10 secretion [398], to modulate costimulator molecule expression [399], and to have no observable effects on MDDCs [400]. Since DCs have been reported to be resistant to HIV-1 infection [342], initial viral interactions with DCs that alter cellular phenotype and function are important to understand. Therefore, the effects of gp120 incubation with DCs may provide insight into changes in DC physiology after initial contact with HIV-1. Thus, flow cytometry was used to examine the effect of exogenous gp120 incubation on MDDC expression of CD14, DC-SIGN, CD80, CD83, CD86, CD40, CCR7, MHC I, and MHC II.

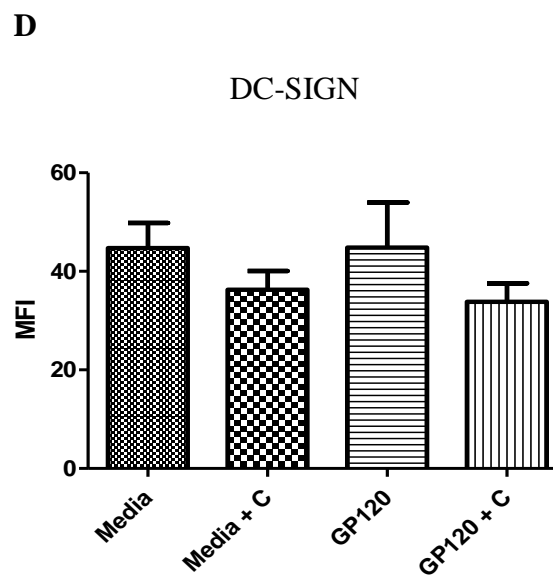
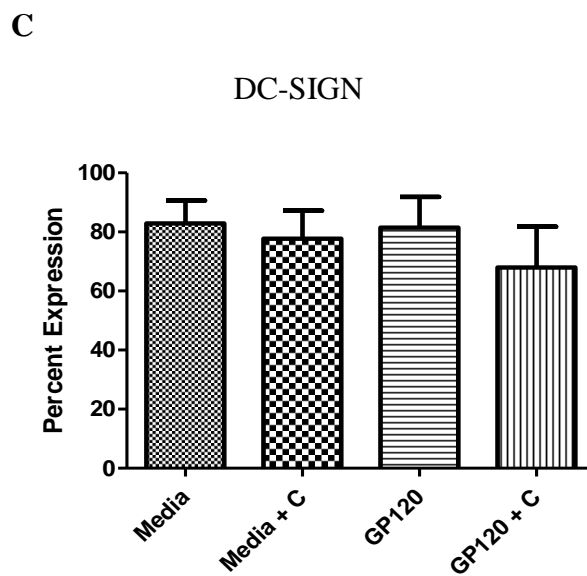
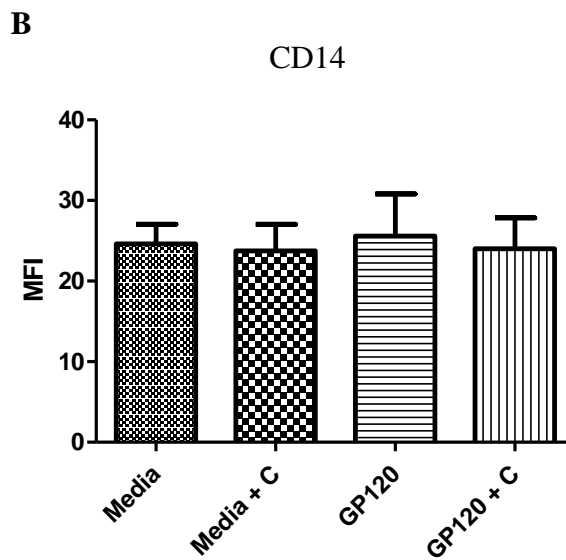
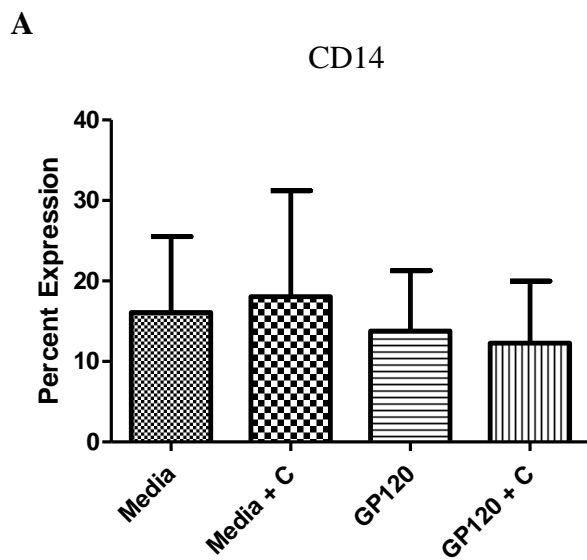
After incubation MDDCs with gp120 (3 µg/ml) for 24 h and 48 h of culture, there were no changes in the expression of CD14 (Figures 20A and 20B), DC-SIGN (Figures 20C and 20D), CD80 (Figures 20G and 20H), CD40 (Figures 20K and 20L), CCR7 (Figures 20M and 20N), MHC I (Figures 20O and 20P), or MHC II (Figures 20Q and 20R). However, incubation with gp120 caused an increase in CD83 (Figure 20E) and CD86 (Figure 20I and 20J) expression indicating that gp120 itself was capable of inducing a degree of MDDC maturation.

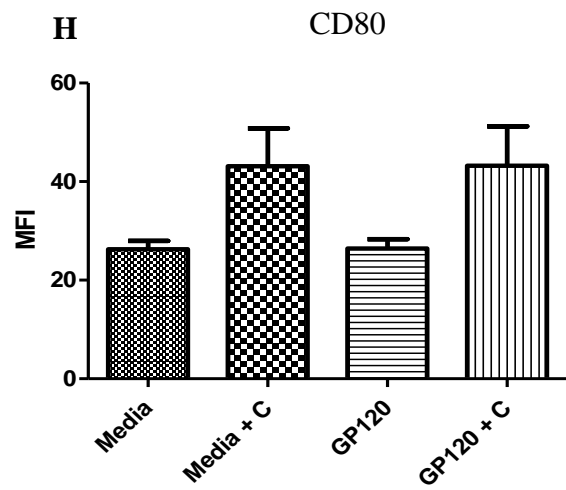
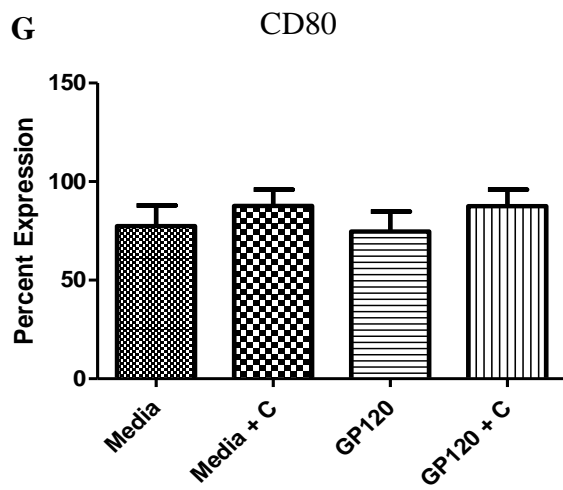
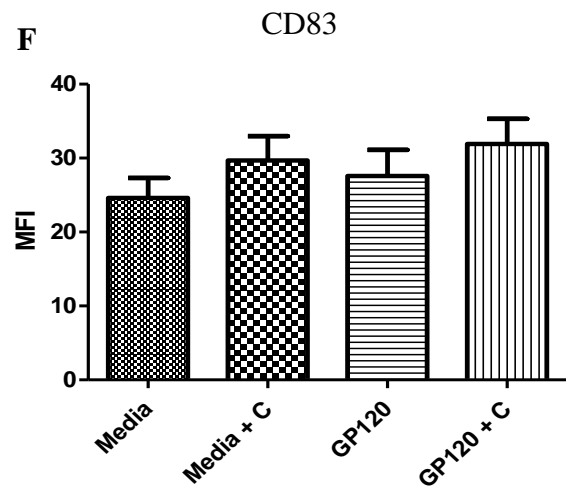
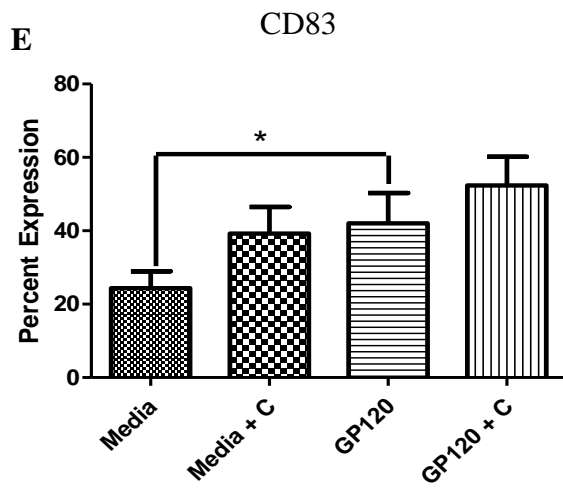
However, after iMDDC were incubated with gp120 (3 µg/ml) and then stimulated to mature, there were no there was no changes in the expression of any of the surface

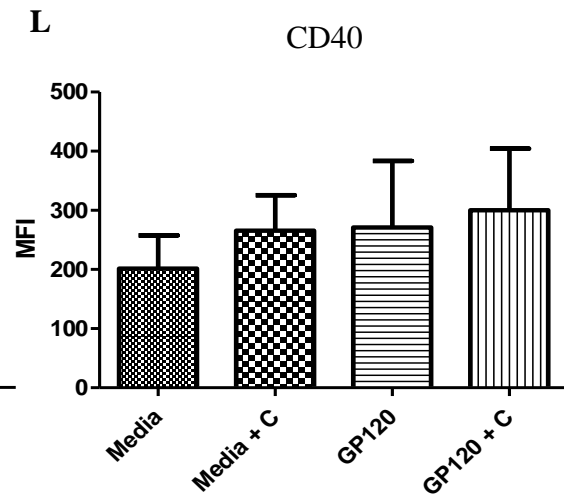
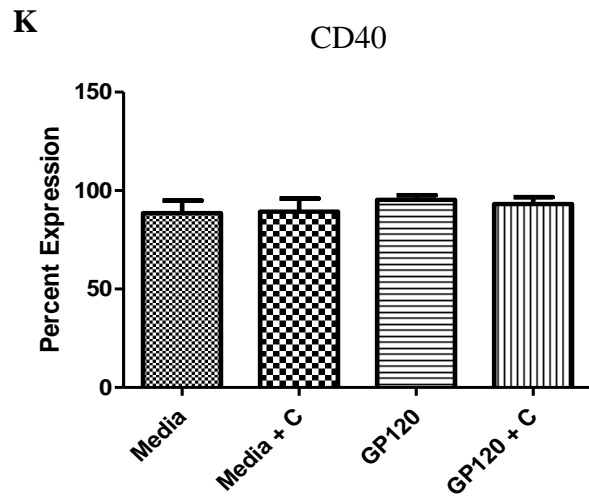
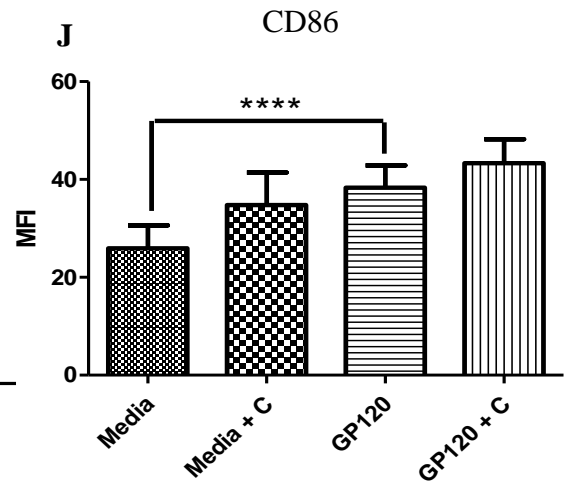
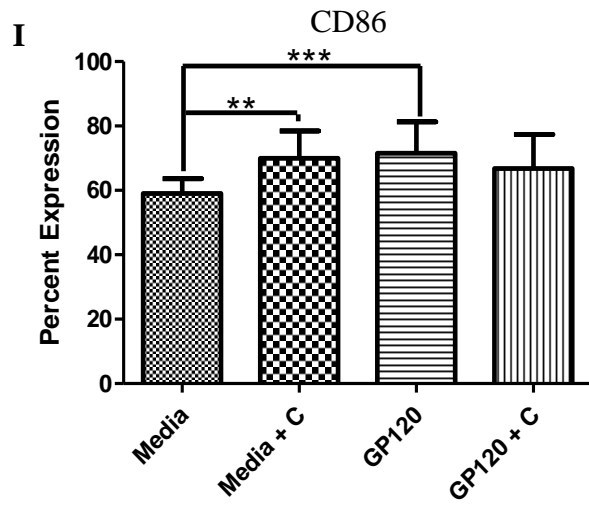
molecules (Figure 20A to 20R). Exogenous gp120 therefore does not influence cytokine-induced MDDC maturation.

Figure 20: Exogenous HIV-1-gp120 induce increases in CD83 and CD86 expression on iMDDC surface molecules and does not affect phenotypic maturation

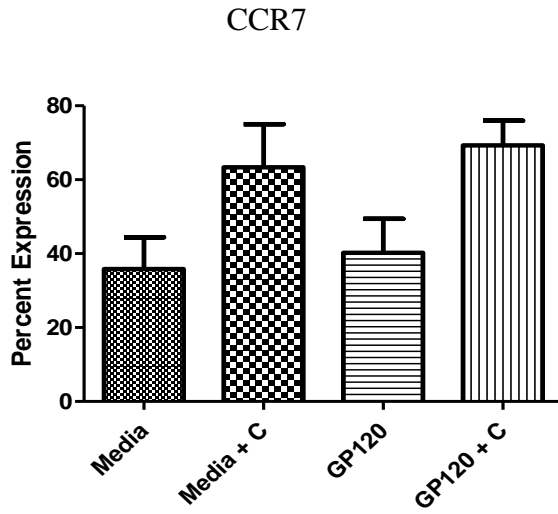
iMDDCs from 6 donors were incubated with gp120 (3 µg/ml) for 24 h at 37°C and 5% CO₂ and incubated for 48 h at 37°C and 5% CO₂ with or without TNFα, IL-1β, IL-6, and PGE₂. Expression of CD14 (20A,20B), DC-SIGN (20C,20D), CD80 (20G,20H), CD40 (20K,20L), CCR7 (20M,20N), MHC I (20O,20P), or MHC II (20Q,20R) were unaffected by exogenous gp120 when examined by flow cytometry. Expression of CD86 (20I,20J) and CD83 (20E) however, were increased after MDDC incubation with gp120 for 24h and cultured for 48h. Results are expressed as means ± standard deviation (n=4). *p=0.02; **p=0.03; ***p=0.06 ; ****p=0.05.



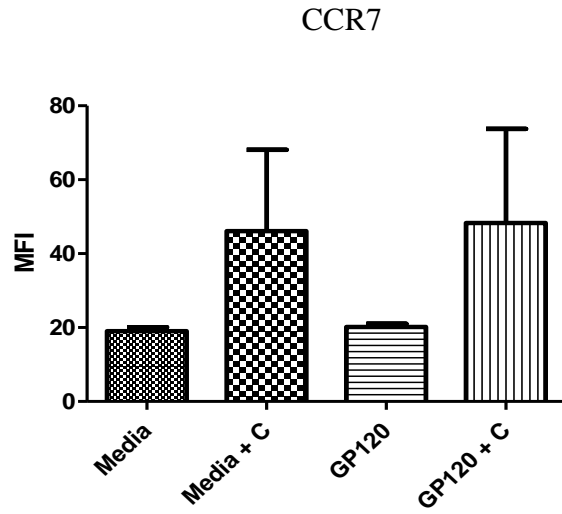




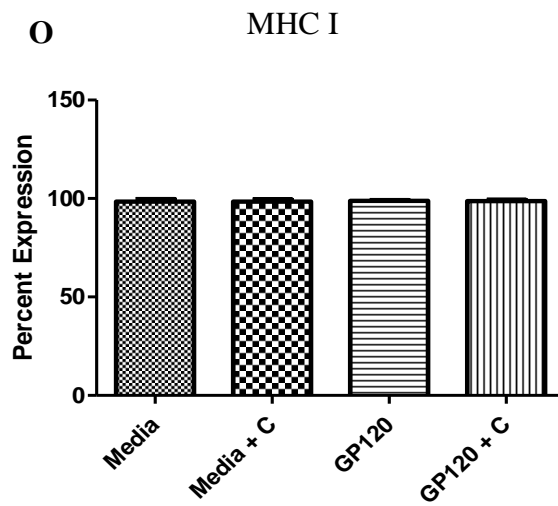
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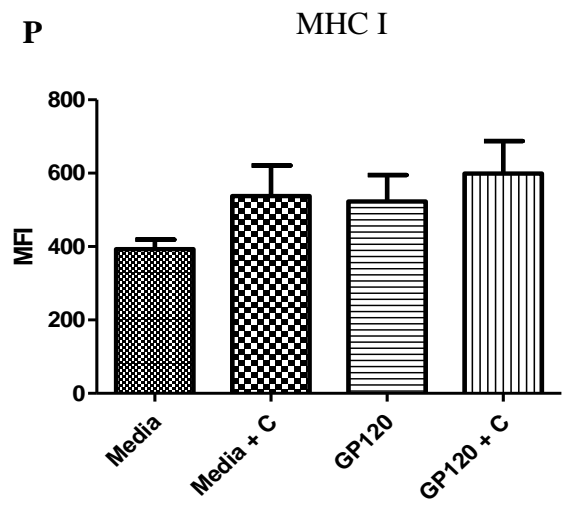
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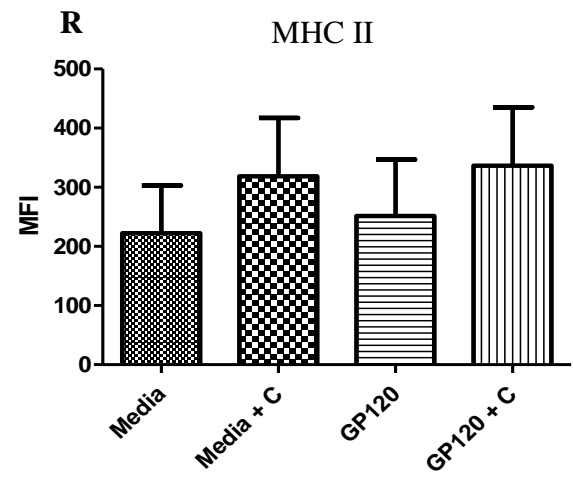
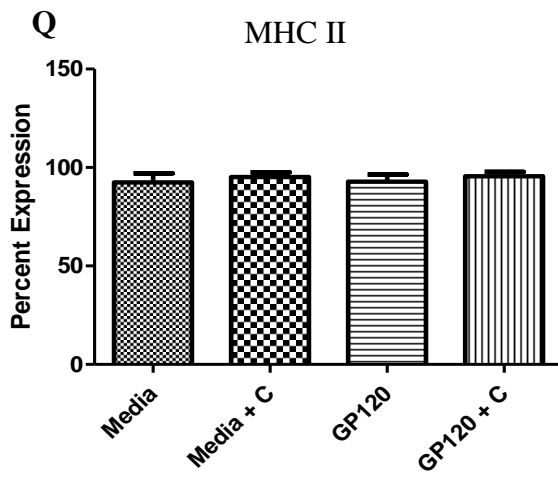


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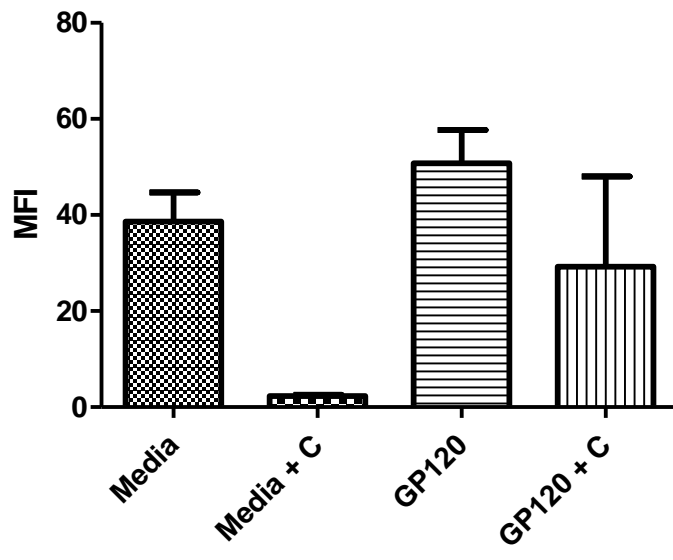
3.4.6 Exogenous HIV-1-gp120 blunts the maturation-induced downregulation of endocytosis

As described previously, a critical aspect of iDC function is endocytosis of antigens in the anatomic periphery for later presentation to naïve T-cells in secondary lymphoid tissues [4,5]. Viral effects that alter these functions in the absence of productive infection are important to understand given the refractory nature of DCs to HIV-1 infection [342]. The gp120 peptide has been observed to cause retention in endocytic function by mMDDCs and partial phenotypic maturation of iMDDCs [397]. It has also been reported to inhibit iMDDC phenotypic maturation [399] as well as increase IL-10 secretion [398], and have a negligible effect on MDDC phenotype and physiology [400]. Cell surface gp120 interactions with iDCs are thought to be some of the earliest and most critical events during primary infection [425,453]. We observed a similar retention in endocytic activity by mMDDCs after cytokine induced maturation after HIV-1 infection (Figures 10K, 10L, 10P, 11C, and 12). Changes in DC endocytic function during HIV-1 infection could compromise their ability to process antigens and may be a symptom of impaired antigen processing machinery. To determine the effect of gp120 on MDDC antigen capture, we examined FITC-Dextran uptake by MDDCs after gp120 incubation using flow cytometric analysis.

Maturation of uninfected iMDDC resulted in a decrease in FITC-dextran uptake (Figure 21). While gp120 incubation had no impact on the ability of iMDDC to take up dextran, gp120 incubation was associated with blunted downregulation of endocytosis by iMDDC (Figure 21). gp120 therefore appeared to inhibit maturation reflected by the fact that gp120 incubated MDDCs partially retain their endocytic function.

Figure 21: HIV-1-gp120 blunts the maturation-induced downregulation of endocytosis

iMDDCs from 2 individuals were incubated with 3 $\mu\text{g/ml}$ of gp120 and then incubated for 48 h with or without $\text{TNF}\alpha$, $\text{IL-1}\beta$, IL-6 , and PGE_2 . Cells were then incubated with saturating concentrations of FITC-conjugated dextran for 1 h and endocytosis of dextran was examined using flow cytometric analysis of mean fluorescence intensity (MFI) using FCS Express 2.00 software. Endocytosis of FITC-conjugated dextran was reduced in mMDDC compared to iMDDC. Incubation of iMDDCs with 3 $\mu\text{g/ml}$ gp120 did not result in any decrease in FITC-conjugated dextran endocytosis however, gp120 incubation blunted downregulation of endocytosis after the induction of maturation. Results are expressed as a means of 2 independent experiments (n=2).



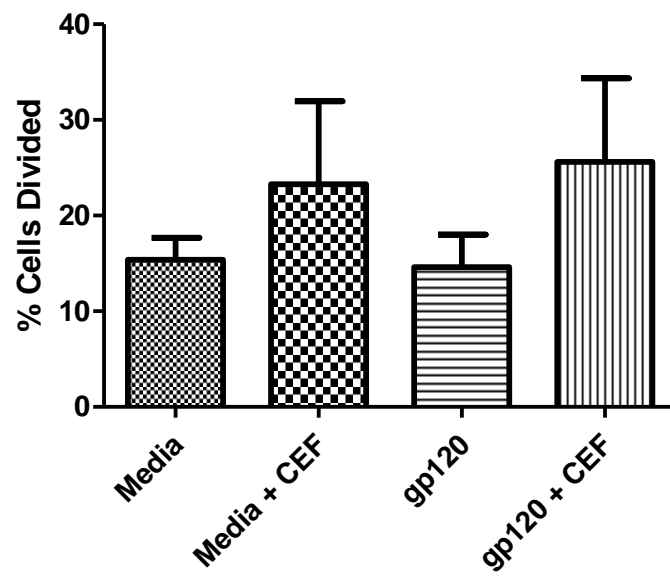
3.4.7 Exogenous HIV-1-gp120 does not inhibit CEF antigen presentation by iMDDCs

A critical function of mature DCs is antigen presentation to naïve T-cells in secondary lymphoid tissues [4,5]. HIV-1 has been observed to inhibit this function of DCs both *ex vivo* [356,357] and *in vitro* [370]. Also, the gp120 peptide has been observed to inhibit iMDDC MLR responses [397]. DCs have been observed to be refractory to HIV-1 infection [342] and gp120 interactions with iDCs are likely some of the first interactions that occur in the anatomic periphery [319]. Therefore, the effects of gp120 incubation with DCs may provide insight into changes in DC physiology after initial contact with HIV-1. Changes in DC antigen presentation during HIV-1 infection could compromise their ability to stimulate adaptive immune responses. This could result in increased infectivity and disease progression. Therefore, flow cytometry was used to investigate the effect of exogenous gp120 on the ability of MDDCs to present antigen to autologous CFSE-stained CD8⁺ T-cells.

After culturing CEF peptide-pulsed iMDDC with CFSE-stained autologous PBMCs for 7 days, CD8⁺ T cells proliferated as expected (Figure 22), however gp120 only marginally enhanced antigen presentation by iMDDCs (Figure 22). These results suggest that exogenous gp120 does not interfere with normal antigen presentation which is contrary to previous observations [397], but in agreement with others [400].

Figure 22: Exogenous HIV-1-gp120 does not inhibit CEF antigen presentation by iMDDCs

iMDDCs from 3 donors were incubated with gp120 for 24 h and 48 h with or without TNF α , IL-1 β , IL-6, and PGE $_2$. MDDCs were then pulsed with a CEF peptide pool and then co-cultured with autologous CFSE stained PBMCs at a ratio of 1:10 for 7 days at 37°C and 5% CO $_2$ in the dark. iMDDC incubation with gp120 did not inhibit CEF presentation to monocyte depleted autologous PBMCs. Results are expressed as means \pm standard deviation or relative percent division and analyzed by paired *t*-test (n=3).



3.5 The effect of HIV-1 gene deletion on HIV-1 effects on the maturation and function of MDDCs

As previously described, HIV-1 has been reported to have a variety of effects on DCs *in vitro* [370,371,377] and *ex vivo* [354-357,340]. Several of its accessory proteins have also been observed to have effects on DC physiology [386,397,403,407]. Previous experiments have also shown that HIV-1 gene deletions can have effects on HIV-1 iMDDC infection outcomes [389,405,408]. We observed inhibited iMDDC maturation after HIV-1 infection (Figures 10K, L, P, 11C, and 12) and what appeared to be iMDDC maturation after their incubation with exogenous Tat (Figures 15 and 16) and Vpr peptides (Figures 18 and 19). To more fully understand the effects of endogenous Tat and Vpr on iMDDC maturation and function, we investigated the effect HIV-1 mutants with deleted genes had on iMDDC phenotype and maturation.

To understand the effect of Vpr deletion on HIV-1 iMDDC infection, we used a full length pUC18 plasmid derived HIV-1_{NL4-3} virus, a pUC18 plasmid derived HIV-1-Vpr deleted HIV-1_{NL4-3} (HIV-1_{DVpr}) viral isolate and an empty pUC18 parent plasmid mock control. We also investigated the effect of Tat deletion on HIV-1 iMDDC infection using a full length pXF3 plasmid derived HIV-1_{BaL} virus, a pUC19 plasmid derived HIV-1-Tat deleted HIV-1_{HXB3} (HIV-1_{Mtat}) viral isolate and an empty pUC19 parent plasmid mock control.

Immature MDDCs were infected with a full length HIV-1 virus, a deletion mutant from the same parent virus, and a corresponding mock control for 24 h and then cultured with or without TNF α , IL-1 β , IL-6, and PGE₂ for 48 h. We investigated MDDC phenotype, carbohydrate endocytosis, and antigen presentation function after viral infection.

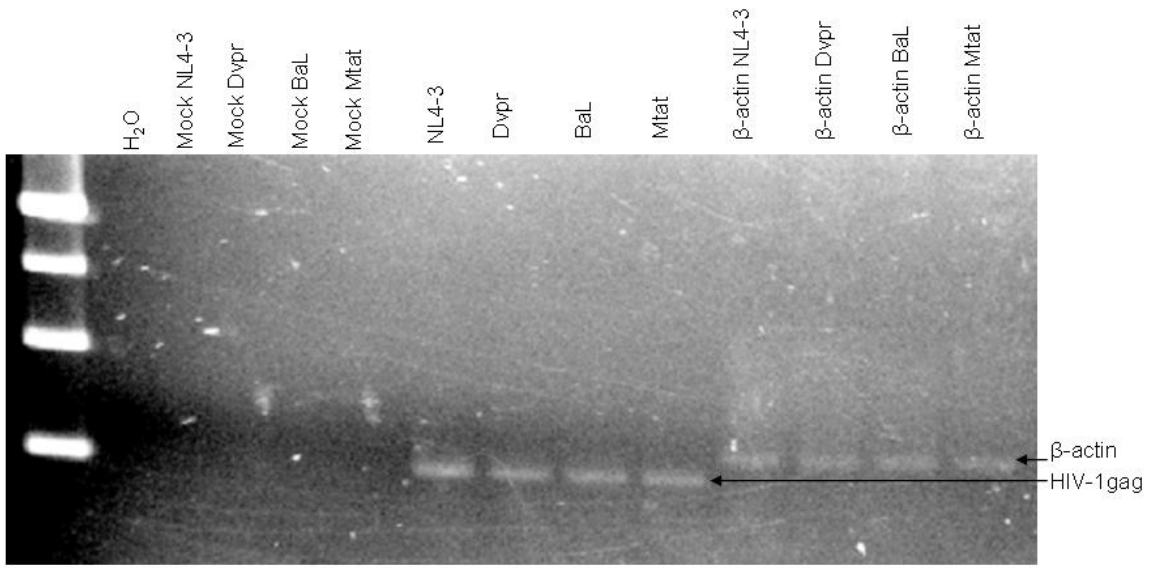
*3.5.1: Characterization of Lab Adapted HIV-1_{NL4-3}, HIV-1_{Dvpr} HIV-1_{BaL}, and HIV-1_{Mtat}
MDDC Infection: Evidence of HIV-1 RNA*

We were unable to detect the presence of integrated lab-adapted HIV-1 DNA from any of the full length, deletion mutant, or control infected iMDDC cultures with *Alu*-nested PCR. This suggested that proviral integration was not occurring. Nested HIV-1-gag PCR was then used to detect the presence of HIV-1_{NL4-3}, HIV-1_{Dvpr}, HIV-1_{BaL}, and HIV-1_{Mtat} mRNA in MDDCs.

After a 24 h viral infection and 48 h of culture, HIV-1gag mRNA was consistently detected in all virally infected cultures (Figure 23). There was also no detectable HIV-1gag mRNA in the mock infected cultures over the same period of time (Figure 23). Water was used as a negative control and beta-actin served as a positive control (Figure 23). Our results indicated that HIV-1-RNA was present in the virally infected cultures.

Figure 23: Detection of HIV-1_{NL4-3}, HIV-1_{Dvpr} HIV-1_{BaL}, and HIV-1_{Mtat}, mRNA by nested HIV-1gag PCR

iMDDCs were infected with a full length pUC19 plasmid derived HIV-1_{NL4-3} virus, a pUC19 plasmid derived HIV-1-Vpr deleted HIV-1_{NL4-3} (HIV-1_{Dvpr}) viral isolate, an empty pUC19 parent plasmid mock control, a full length pXF3 plasmid derived HIV-1_{BaL} virus, an empty pXF3 plasmid, a pUC19 plasmid derived HIV-1-Tat deleted HIV-1_{HXB3} (HIV-1_{Mtat}) viral isolate, or an empty pUC19 parent plasmid mock control for 24 h and then cultured for 48 h. Messenger RNA isolated from mock and virus infected MDDCs was reverse transcribed using an RT Enzyme and Buffer mix in an Eppendorf Mastercycler ep 543 X instrument. The resultant DNA was amplified in 2 steps in Taq PCR Mastermix using an Eppendorf Mastercycler ep 543X instrument. During the first round of DNA replication, 2 primers that anneal within outer regions of the HIV-1 gag element (300 nM) were used. During the second round of replication, 1/10 of the second-round PCR products were amplified using 2 primers that anneal within inner regions of the HIV-1 gag element (300 nM). Beta-actin mRNA served as positive control and water was used as a negative control. Samples were visualized on an EtBr stained 1% Agarose TAE gel.



3.5.2 Neither HIV-1_{NL4-3} nor HIV-1_{Dvpr} infection affects iMDDC phenotype or their phenotypic maturation

As previously described, DC phenotypic maturation is a critically important aspect of their antigen presenting function [4,5]. HIV-1 infection [370] and the Vpr accessory peptide have both been observed to inhibit DC phenotypic maturation [408]. To more fully understand the effect of endogenous Vpr on iMDDC physiology, we investigated the effect of HIV-1_{NL4-3} and HIV-1_{Dvpr} infection on their phenotypic maturation. Flow cytometry was used to examine the effect of HIV-1_{NL4-3} and HIV-1_{Dvpr} infection on MDDC expression of CD14, DC-SIGN, CD80, CD83, CD86, CD40, CCR7, MHC I, and MHC II.

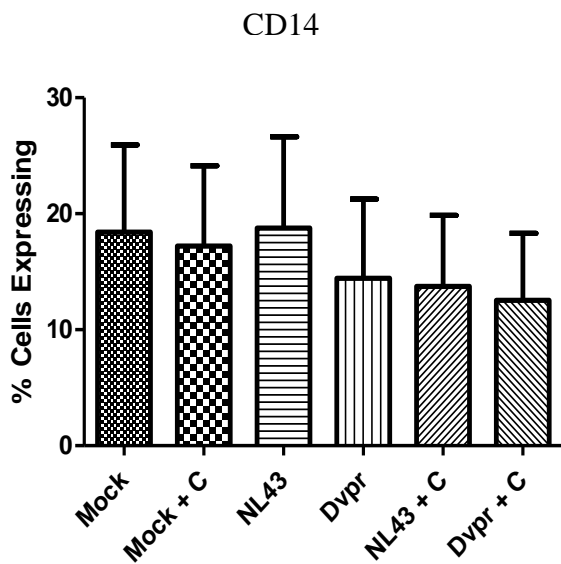
After incubation with HIV-1_{NL4-3} and HIV-1_{Dvpr} for 24 h and 48 h of culture, there were no changes in the expression of CD14, DC-SIGN, CD80, CD86, CD83, CD40, CCR7, MHC I, or MHC II (Figures 24A-24R), indicating that neither HIV-1_{NL4-3} nor HIV-1_{Dvpr} themselves were capable of inducing MDDC maturation.

The phenotype of iMDDCs that were stimulated to mature was also unaffected by viral (HIV-1_{NL4-3} or HIV-1_{Dvpr}) infection. This suggests that MDDC infection by the HIV-1_{NL4-3} and HIV-1_{Dvpr} viral strains does not affect iMDDC phenotype or their phenotypic maturation.

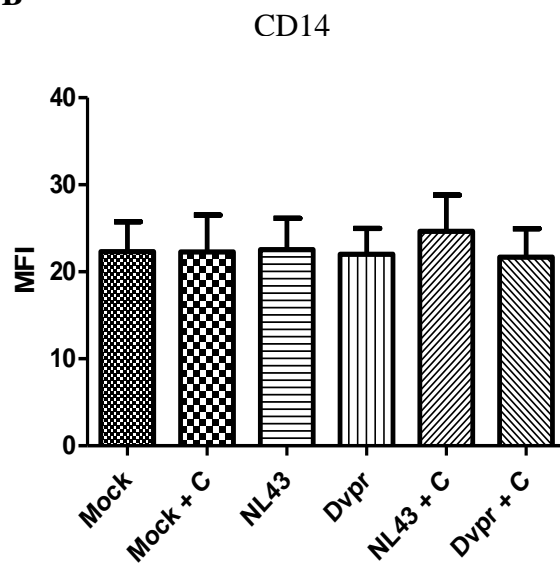
Figure 24: Neither HIV-1_{NL4-3} nor HIV-1_{Dvpr} infection of iMDDC effects surface molecule expression and phenotypic maturation

iMDDCs from 6 individuals were incubated with live HIV-1_{NL4-3}, HIV-1_{Dvpr}, or a mock control for 24 h and incubated for 48 h with or without TNF α , IL-1 β , IL-6, and PGE₂ to induce maturation. Expression of CD14, DC-SIGN, CD80, CD83, CD86, CD40, CCR7, MHC I, and MHC II molecules were unaffected by viral or mock infections (24A to 24R) as measured by flow cytometry. Results are expressed as means \pm standard deviation (n=6).

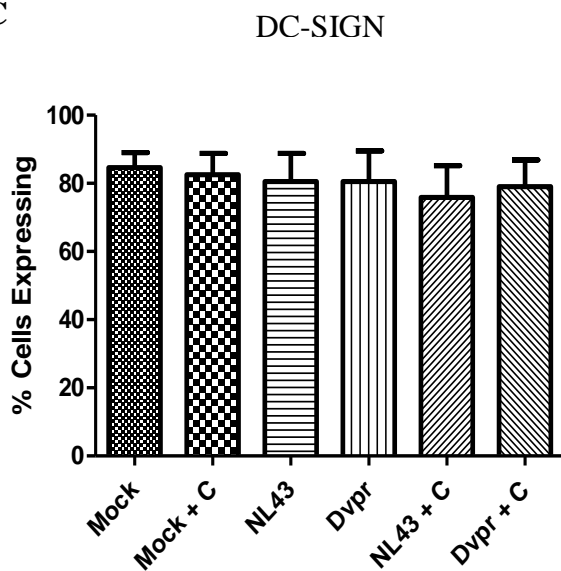
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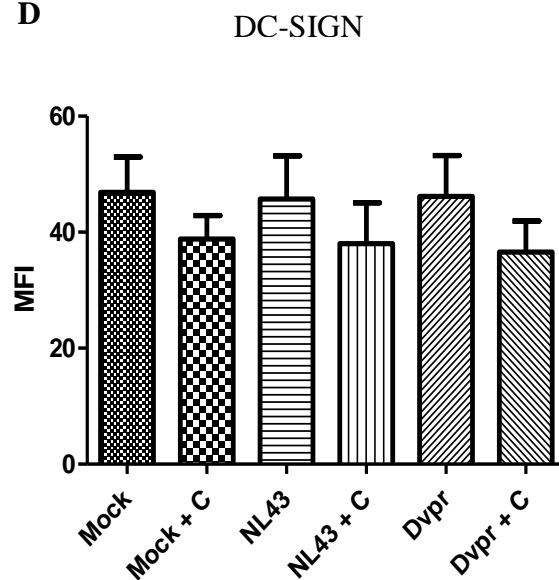
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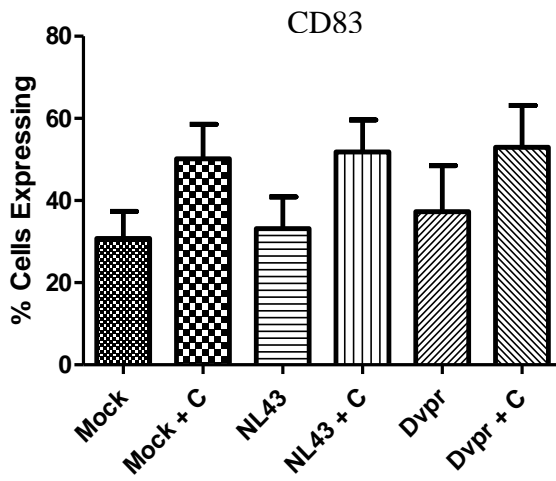
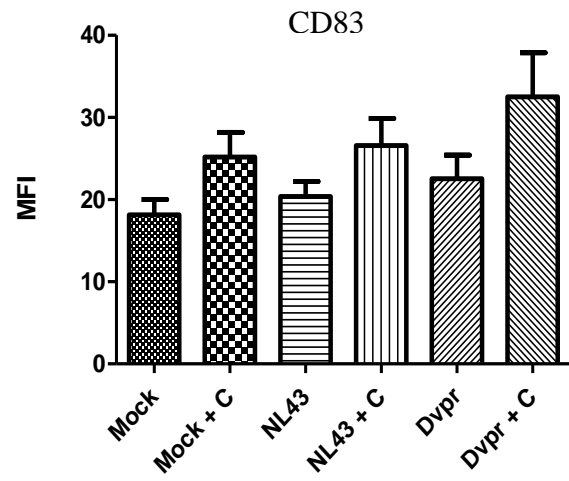
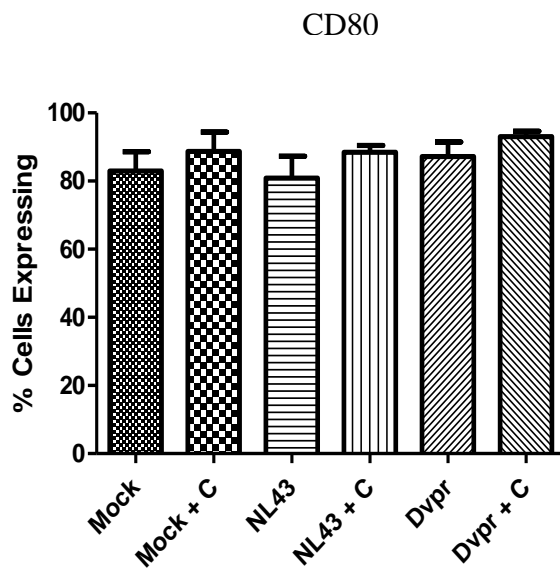
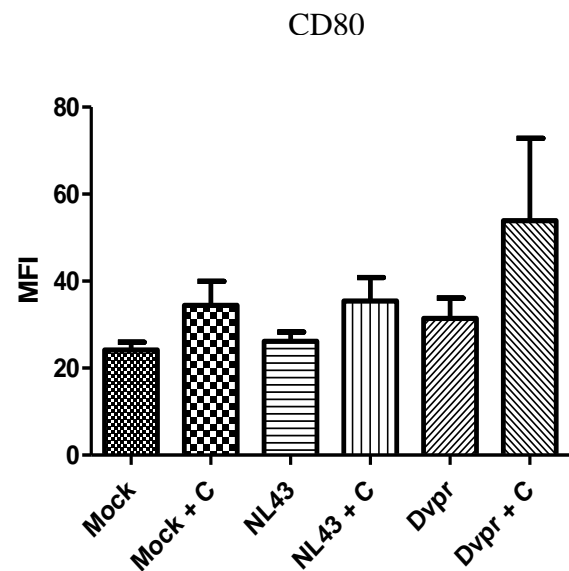


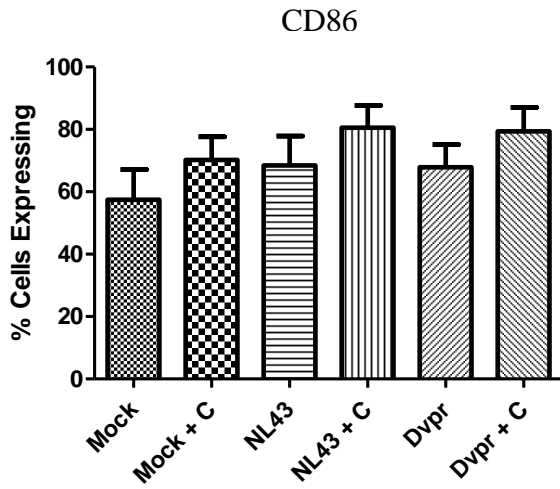
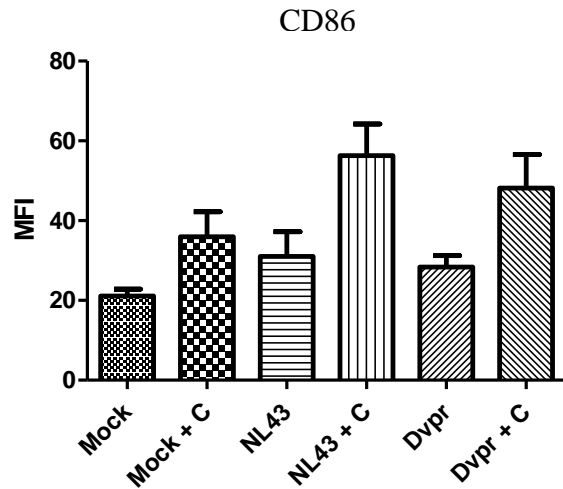
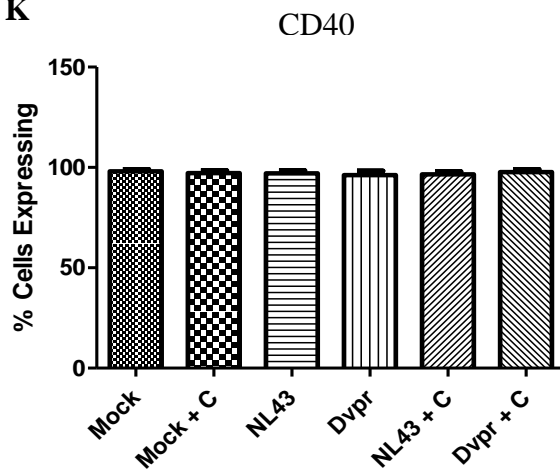
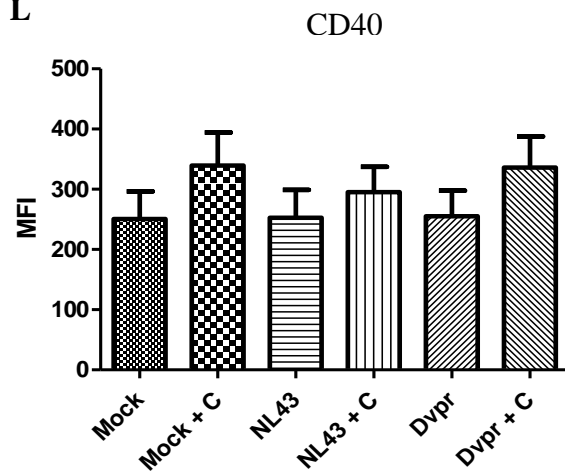
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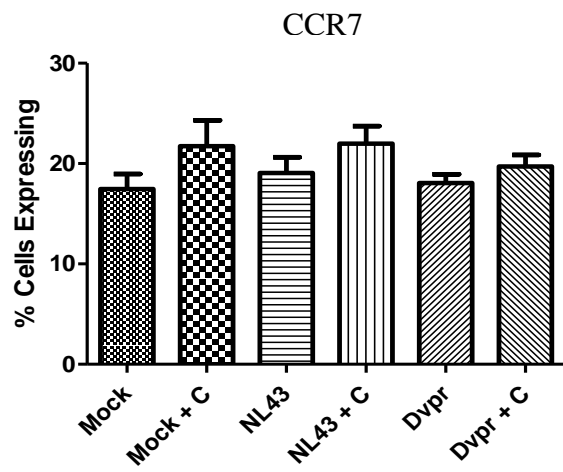
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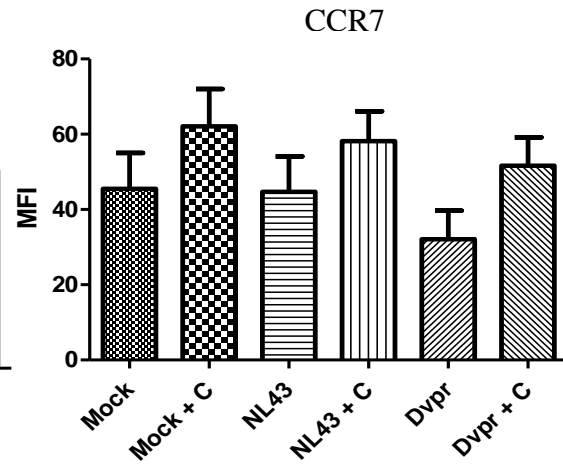
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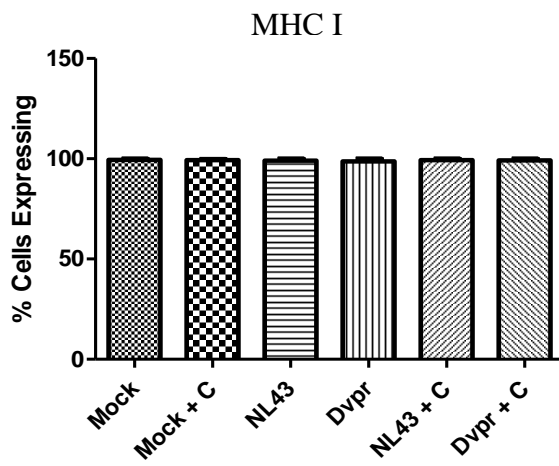
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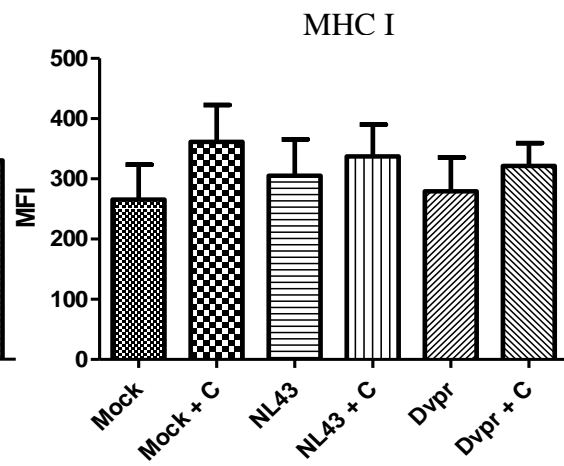
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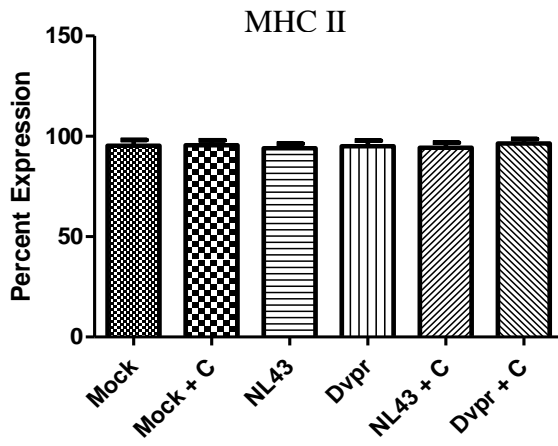
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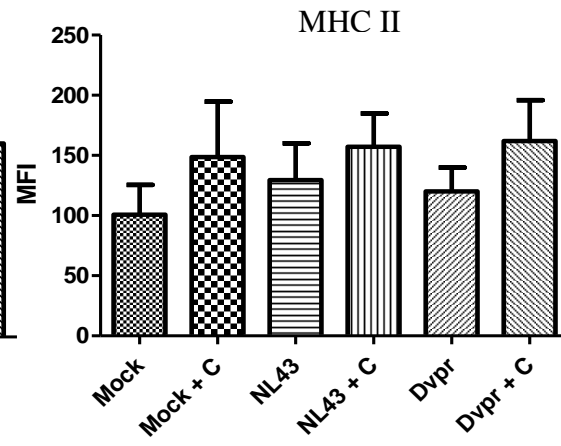
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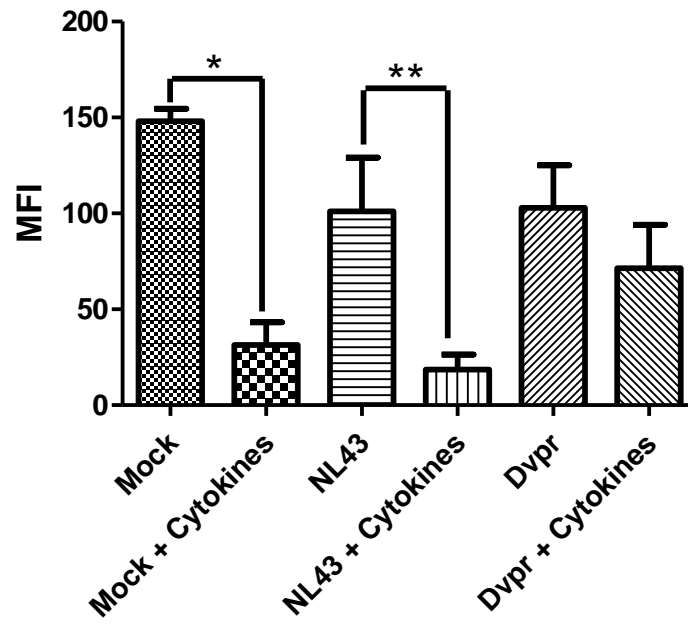
3.5.3 MDDC infection with full length HIV-1_{NL4-3} reduces MDDC endocytosis function

Capture of antigens in the anatomic periphery by iDCs is essential for the presentation of antigens [4,5]. Interference in this process may compromise mature DC antigen presentation in secondary lymphoid tissues [213]. HIV-1 has been observed to inhibit iMDDC maturation [370] and exogenous Vpr has been observed to alter iMDDC endocytosis processes by iMDDCs [407]. To understand the contribution endogenous Vpr makes during HIV-1-induced endocytosis retention we infected iMDDCs with either HIV-1_{NL4-3} or HIV-1_{Dvpr} and matured them or not and then examined FITC-dextran uptake by MDDCs after virus infection using flow cytometric analysis.

Maturation of uninfected iMDDC resulted in a decrease in FITC-dextran uptake (Figure 25). Infection with HIV-1_{NL4-3} MDDC infection also resulted in a decrease in FITC-dextran uptake (Figure 25). However, iMDDC infection with the HIV-1-Vpr deleted HIV-1_{NL4-3} virus (HIV-1_{Dvpr}) blunted this change (Figure 25). Therefore, HIV-1_{NL4-3} induced decreases in FITC-dextran endocytosis are partially dependent on the presence of the HIV-1 protein Vpr.

Figure 25: MDDC infection with full length HIV-1_{NL4-3} reduces MDDC endocytosis function

iMDDCs from 4 donors were infected with HIV-1_{NL4-3} and HIV-1_{Dvpr} and incubated for 48 h with or without TNF α , IL-1 β , IL-6, and PGE₂. Cells were then incubated with saturating concentrations of FITC-conjugated dextran for 1 h and endocytosis of dextran was examined using flow cytometric analysis of mean fluorescence intensity (MFI) using FCS Express 2.00 software. Results are expressed as means \pm standard deviation. *p=0.03; **p=0.005 by paired *t*-test (n=4).



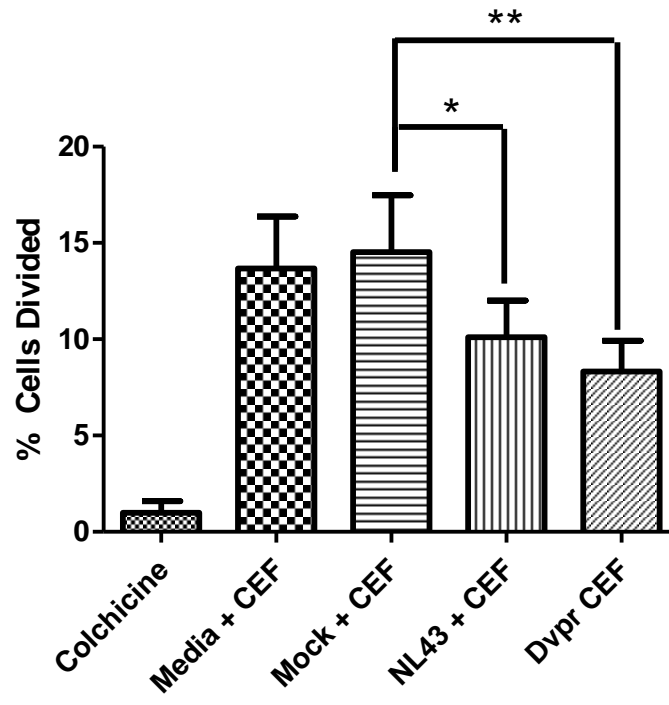
3.5.4 HIV-1_{NL4-3} and HIV-1_{Dvpr} decrease iMDDC CEF antigen presentation

Antigen presentation by mature DCs in secondary lymphoid organs is a critical function required for the clearance of infections and cancers [4,5]. Interruptions in this process can result in impaired immune responses and chronic illness progression like those observed in HIV-1 infection. DC antigen presentation has been observed to be inhibited by HIV-1 infection [356,357,370] and after incubation with the accessory Vpr peptide [407]. We expanded on previous studies to more completely understand the effects of endogenous Vpr on iMDDC antigen presentation using HIV-1_{NL4-3} or HIV-1_{Dvpr} infection of iMDDCs and a CEF peptide pool.

After culturing CEF peptide-pulsed iMDDC with CFSE-stained autologous monocyte depleted PBMCs for 7 days, CD8⁺ T cells proliferated as expected (Figure 26). When iMDDC were infected with either strain of HIV-1, CD8⁺ T cell proliferation in response to the CEF peptide pool was diminished (Figure 26), suggesting that HIV-1 infection of MDDC interfered with antigen presentation. Our results also indicate that HIV-1_{NL4-3} infection does not require Vpr to impair iMDDC CEF antigen presentation to autologous CD8⁺ T-cells.

Figure 26: HIV-1_{NL4-3} and HIV-1_{DVpr} decrease iMDDC CEF antigen presentation

After 24 h of incubation with HIV-1_{NL4-3} or HIV-1_{DVpr} and 48 h of culture, iMDDCs from 7 donors were pulsed with a CEF peptide pool and then co-cultured with autologous CFSE stained PBMCs at a ratio of 1:10 for 7 days at 37°C and 5% CO₂ in the dark. Infection of iMDDCs with both HIV-1_{NL4-3} and HIV-1_{DVpr} inhibited their presentation of a CEF peptide pool to autologous monocyte depleted PBMCs measured by CFSE dilution compared to mock infected controls. Cells proliferating outside of the colchicine control were considered divided. Results are expressed as means ± standard deviation of percent cells divided and examined by paired *t*-test. **p*=0.04; ***p*=0.03 (n=7).



3.6.2 Neither HIV-1_{BaL} nor HIV-1_{Mtat} infection affects iMDDC phenotype or their phenotypic maturation

As previously discussed, DC phenotype is critically important for efficient antigen capture and antigen presentation functions [4,5]. HIV-1 infection has been observed to inhibit iMDDC phenotypic maturation [370]. On the other hand exogenous Tat has been observed to induce DC phenotypic maturation [403,404]. However, endogenous Tat has been observed to induce an antiviral response from iMDDCs [405]. To more fully understand the effects of endogenous Tat on iMDDC physiology during HIV-1 infection, we investigated the effect of HIV-1_{BaL} and HIV-1_{Mtat} infection on their phenotypic maturation. Flow cytometry was used to examine the effect of HIV-1_{BaL} and HIV-1_{Mtat} infection on MDDC expression of CD14, DC-SIGN, CD80, CD83, CD86, CD40, CCR7, MHC I, and MHC II.

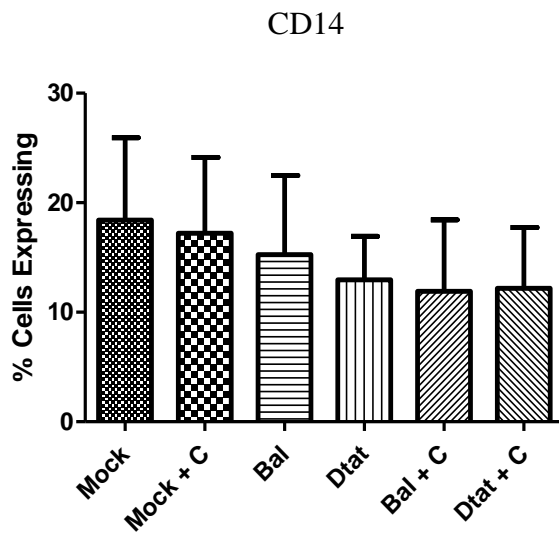
After incubation with HIV-1_{BaL} and HIV-1_{Mtat} or 24 h and 48 h of culture, there were no changes in the expression of any of the examined surface molecules: CD14, DC-SIGN, CD80, CD86, CD83, CD40, CCR7, MHC I, or MHC II indicating that neither HIV-1_{BaL} nor HIV-1_{Mtat} themselves were capable of inducing MDDC maturation (Figure 27A to 27R).

The maturation of HIV-1_{BaL} or HIV-1_{Mtat} infected iMDDC, was likewise unaffected by viral infection suggesting that iMDDC phenotypic maturation is unaffected by the HIV-1_{BaL} and HIV-1_{Mtat} viral strains.

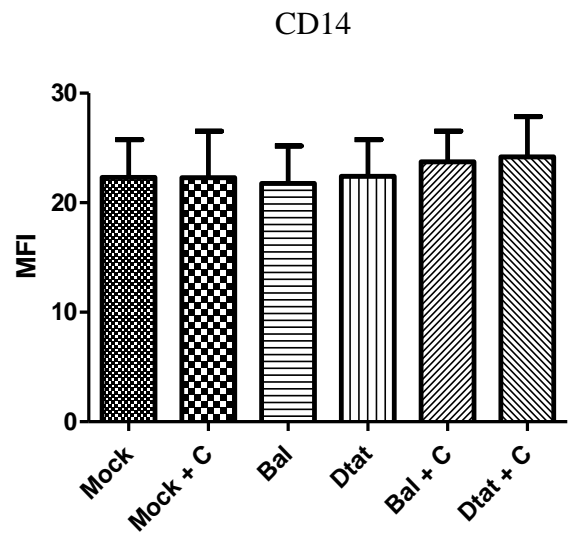
Figure 27: HIV-1_{BaL} and HIV-1_{Mtat} infection do not affect iMDDC surface molecule expression or phenotypic maturation

iMDDCs from 6 donors were infected with HIV-1_{BaL} or HIV-1_{Mtat} for 24 h and incubated for 48 h with or without TNF α , IL-1 β , IL-6, and PGE₂. Expression of CD14, DC-SIGN, CD80, CD83, CD86, CD40, CCR7, MHC I, and MHC II molecules was unaffected by viral or mock infections (27A to 27R) as measured by flow cytometry. Results are expressed as means \pm standard deviation (n=6).

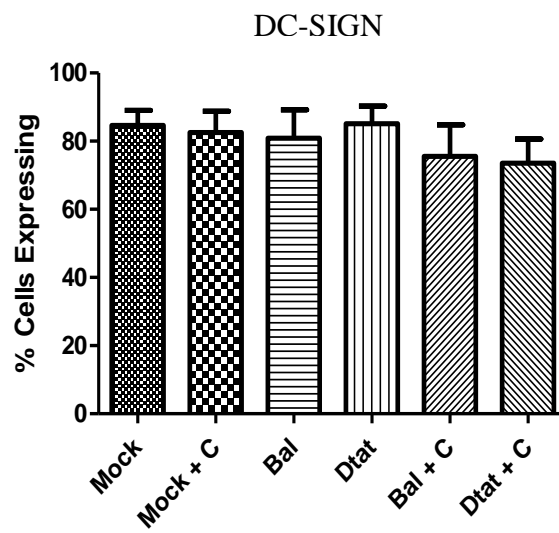
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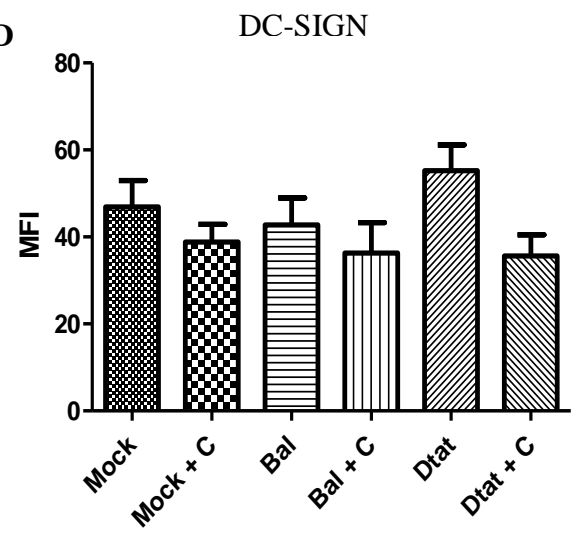
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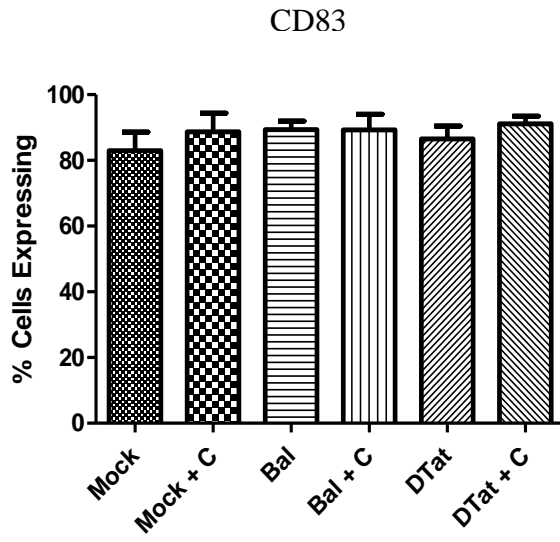
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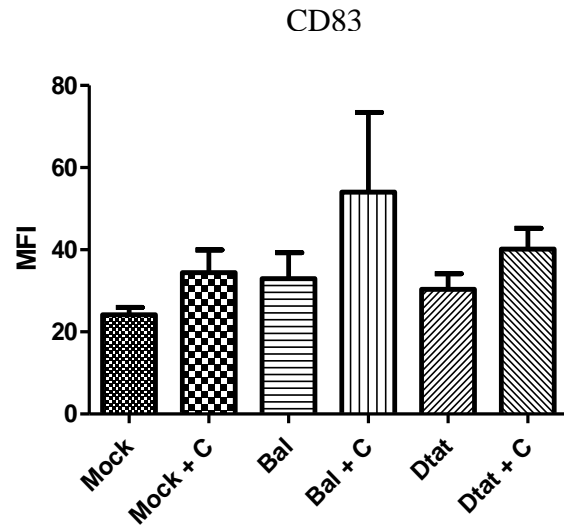
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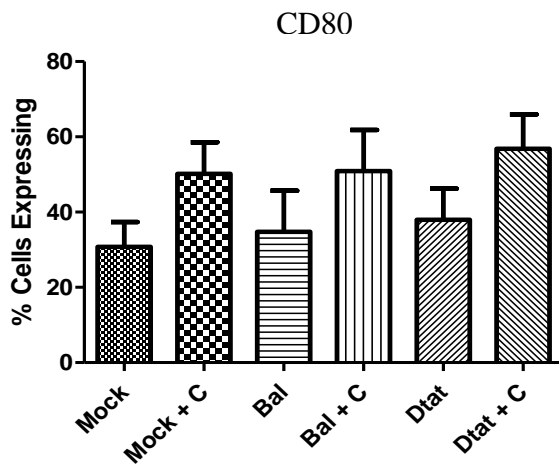
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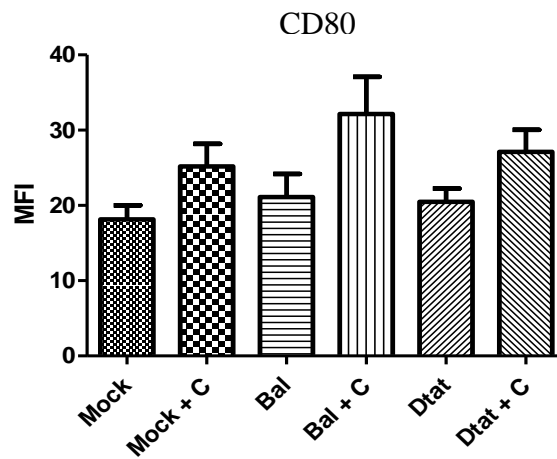
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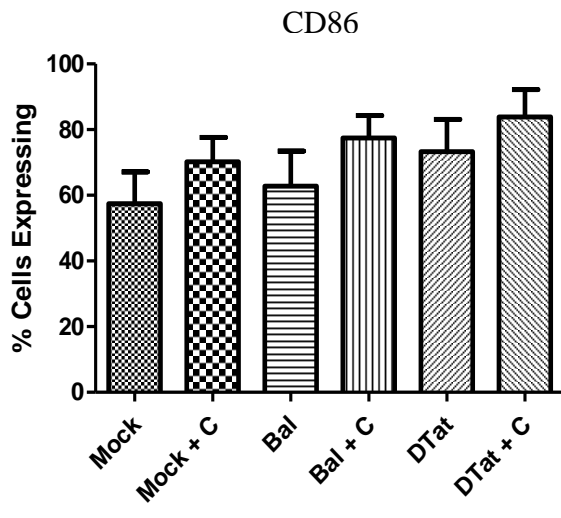
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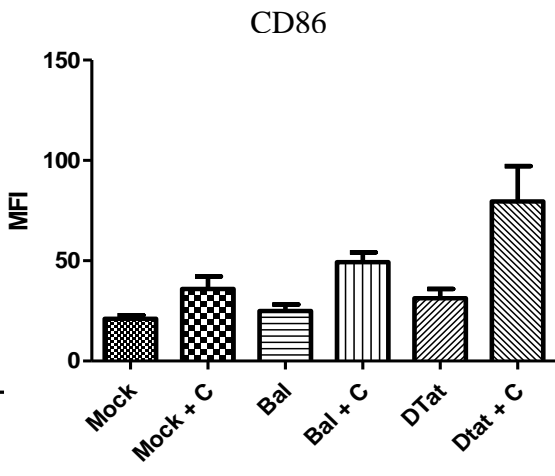
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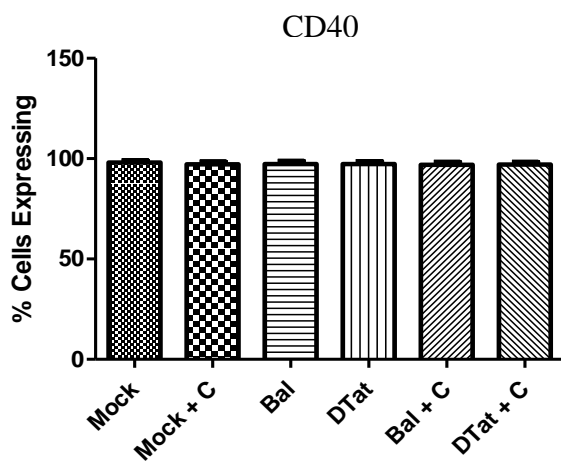
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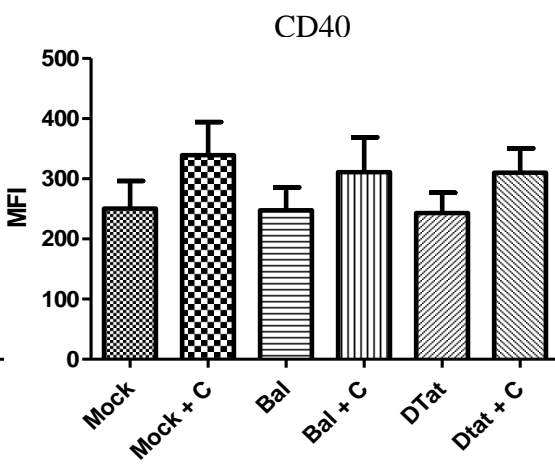
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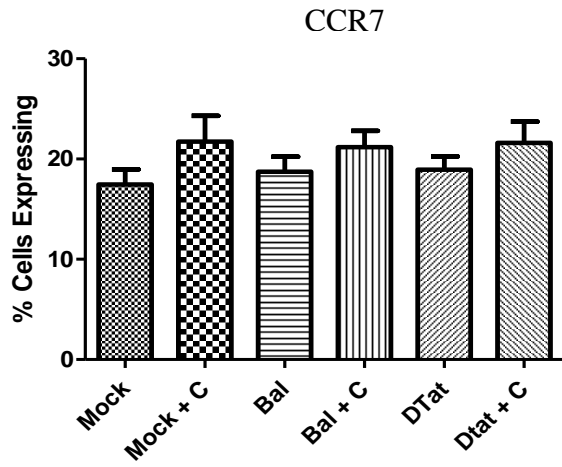
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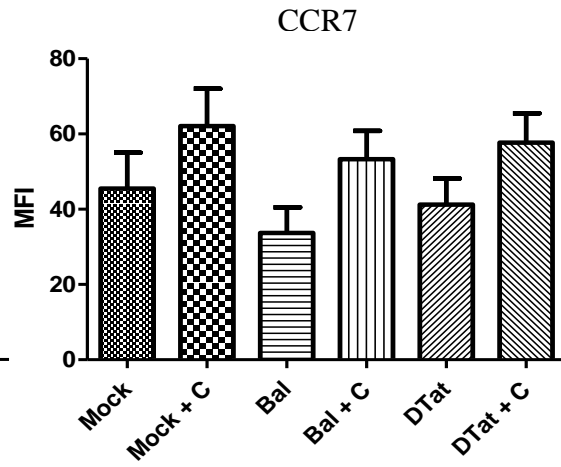
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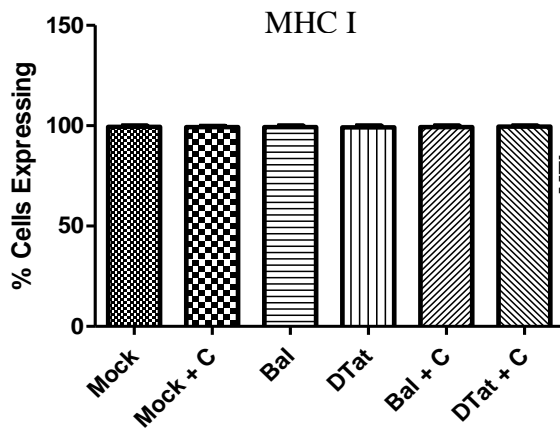
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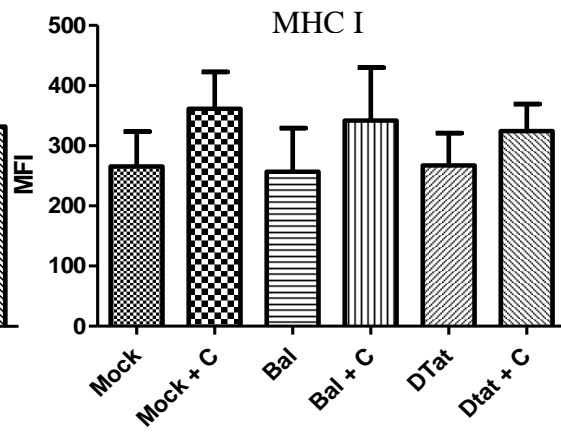
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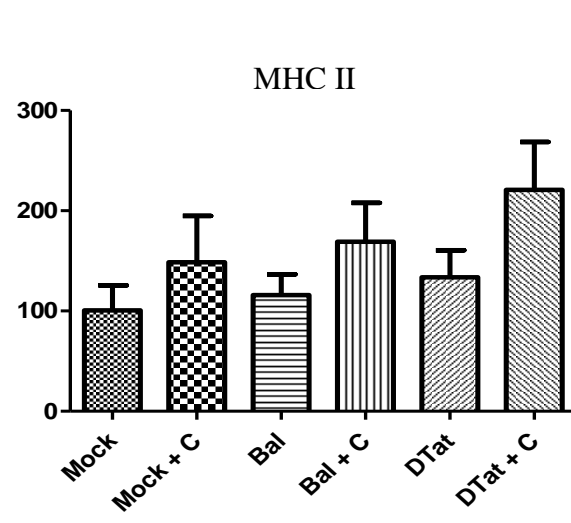
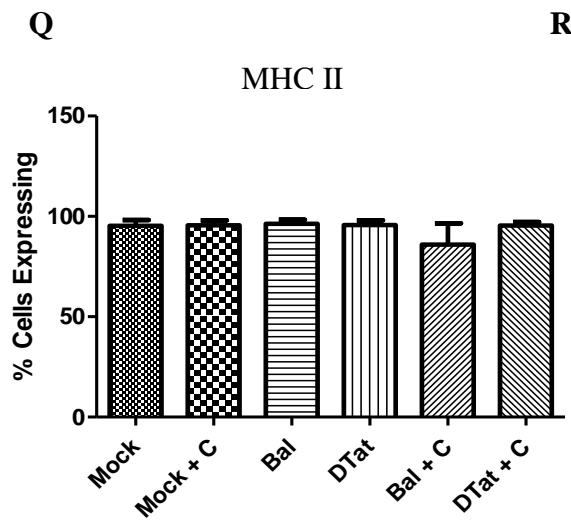


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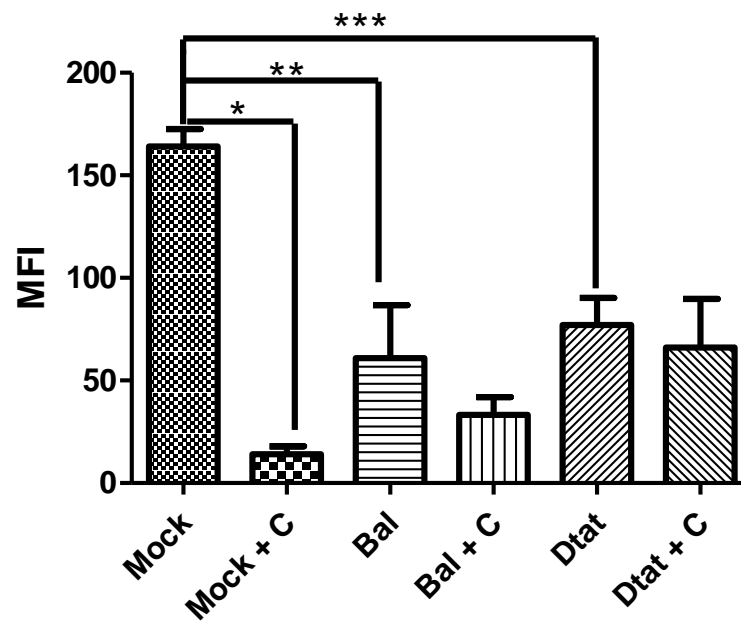
3.6.3 HIV-1_{BaL} and HIV-1_{Mtat} reduce MDDC endocytosis function

As previously discussed, a critical aspect of iMDDC function is their ability to capture antigens in the anatomic periphery for later presentation to the adaptive immune system. Interference in this process may compromise mature DC antigen presentation in secondary lymphoid tissues [213]. HIV-1 has been observed to inhibit iMDDC phenotypic maturation [370]. Also, we observed it to blunt cytokine induced decreases in dextran endocytosis by mMDDCs (Figure 11C). On the other hand, the other hand exogenous Tat has been observed to induce iMDDC maturation [403,404] while endogenous Tat induced an iMDDC antiviral response [405]. Thus it would appear that Tat induces opposing effects to those of the whole virus. To understand the contribution endogenous Tat makes during HIV-1 induced endocytosis retention we infected iMDDCs with either HIV-1_{BaL} or HIV-1_{Mtat} and examined MDDC FITC-dextran uptake with or without cytokine induced maturation.

Maturation of uninfected iMDDC resulted in a decrease in FITC-dextran uptake (Figure 28). Infection with both lab adapted virus strains also resulted in decreases in FITC-dextran uptake though to a lesser degree than that of cytokine-induced maturation (Figure 28). HIV-1_{BaL} infection also appeared to induce greater decreases in mMDDC FITC-dextran endocytosis compared to HIV-1_{Mtat} infection; however, these differences were not statistically significant. Thus it appears that endogenous Tat decreases dextran capture by HIV-1 infected MDDCs.

Figure 28: HIV-1_{BaL} and HIV-1_{Mtat} reduce MDDC endocytosis function

iMDDCs from 4 donors were infected with HIV-1_{BaL} or HIV-1_{Mtat} and incubated for 48 h with or without TNF α , IL-1 β , IL-6, and PGE₂. Cells were then incubated with saturating concentrations of FITC-conjugated dextran for 1 h and endocytosis of FITC-dextran was examined using flow cytometric analysis of mean fluorescence intensity (MFI) using FCS Express 2.00 software. HIV-1_{BaL} and HIV-1_{Mtat} iMDDC both reduced FITC-Dextran endocytosis. Results are expressed as means \pm standard deviation. *p=0.001; **p=0.01; ***p=0.001 by paired *t*-test (n=4).



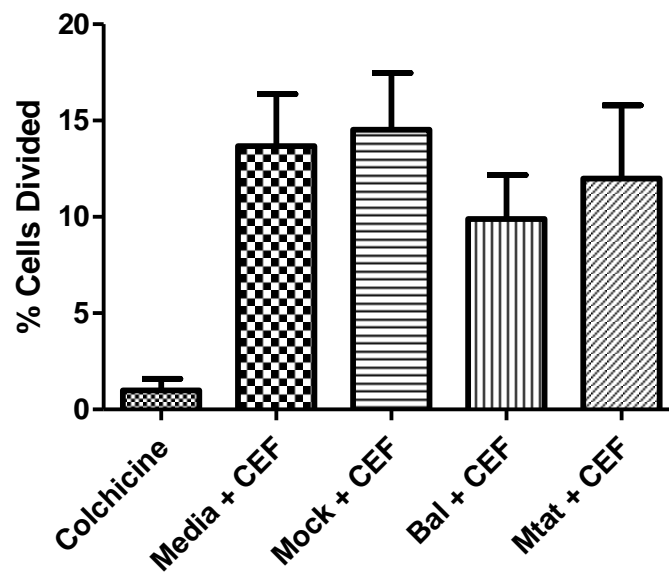
3.6.4 HIV-1_{BaL} and HIV-1_{Mtat} do not affect MDDC CEF antigen presentation to monocyte depleted autologous PBMCs

As described previously, antigen capture is an important function carried out by mDCs in peripheral lymphoid tissues [4,5]. Interruptions in this process can result in impaired immune responses and disease progression [213]. We observed inhibited antigen presentation by both HIV-1 (Figure 12) and exogenous Tat (Figure 16). The effects of Tat on MDDC physiology can vary depending on the location of the introduced peptide [403-405]. Therefore, to more fully understand the effect of endogenous Tat on iMDDC function, we investigated the effect Tat deletion had on HIV-1 infection effects on iMDDC antigen presentation. Flow cytometry was used to investigate the effect of HIV-1_{BaL} and HIV-1_{Mtat} infection on the ability of MDDCs to present antigen to autologous CFSE-stained CD8⁺ T-cells.

After culturing CEF peptide-pulsed iMDDC with CFSE-stained autologous PBMCs for 7 days, CD8⁺ T cells proliferated as expected (Figure 29). When iMDDC were infected with either HIV-1_{BaL} or HIV-1_{Mtat}, there were no observable changes in CD8⁺ T cell proliferation in response to the CEF peptide pool compared to mock infected controls (Figure 29). These results suggest that neither HIV-1_{BaL} nor HIV-1_{Mtat} infection of iMDDCs affect antigen presentation.

Figure 29: HIV-1_{BaL} and HIV-1_{Mtat} do not affect MDDC CEF antigen presentation to monocyte depleted autologous PBMCs

After a 24 h incubation with HIV-1_{BaL} or HIV-1_{Mtat} and 48 h of culture, MDDCs from 7 donors were pulsed with a CEF peptide pool and then co-cultured with autologous CFSE stained PBMCs at a ratio of 1:10 for 7 days at 37°C and 5% CO₂ in the dark. Infection of iMDDCs with both HIV-1_{BaL} and HIV-1_{Mtat} had no effect on iMDDC presentation of a CEF peptide pool to autologous monocyte depleted PBMCs measured by CFSE dilution compared to mock infected controls. Cells proliferating outside of the colchicine control were considered divided. Results are expressed as means ± standard deviation of percent cells divided and examined by paired *t*-test. (n=7).



4.0 Discussion

Mature DCs are responsible for the presentation of foreign antigens to T-cells in secondary lymphoid tissues [4,5]. Most viral infections are thought to stimulate immature DCs to mature through activation of TLRs [42-47]. In either case, after maturation, DCs present viral antigens to T-cells within the secondary lymph organs and initiate an adaptive immune response that results in clearance of the infection [4,5]. During HIV-1 infection however, the virus evades immune clearance and chronic, persistent infection results [288-293].

Initial investigations into the effects of HIV-1 on DC maturation and function revealed that DCs from HIV-1 infected individuals had impaired ability to stimulate autologous T-cell recall and proliferation [356]. Their ability to induce a mixed leukocyte reaction in co-culture was also compromised [357]. More recent examinations of the effects of HIV-1 on DCs have included additional analyses of the effects of HIV-1 on their maturation that support these initial investigations. Granelli-Piperno et al. found that HIV-1 infection of DCs did not induce their maturation as measured by CD83, MHC II, and DC-LAMP surface expression, but rather inhibited cytokine induced maturation of DCs [370]. While confirming previous reports that HIV-1 impairs the ability of DCs to stimulate allogeneic T-cells, they also observed an increase in IL-10 secretion from HIV-1 infected DC co-cultures that may contribute to the observed inhibition of T-cell stimulation by HIV-1 infected DCs [370]. Also in 2010, Manel et al published evidence that HIV-1 does not induce MDDC maturation [371]. Specifically, they found that MDDC maturation after HIV-1 infection required coinfection with SIV-Vpx protein.

While the majority of evidence suggests that the effect of HIV-1 on DCs is one of inhibition of maturation and induction of DC dysfunction, other groups have reported contrasting results. In 2006, Harman et al published findings detailing increases in myeloid DC maturation measured through increases in both costimulatory molecule mRNA and surface expression [377]. While their results describing increases in costimulatory molecule expression were contrary to the findings of the groups led by Granelli-Piperno and Manel, they were in agreement with the partial DC activation observed in *ex vivo* studies [336,337,340].

Other reports that describe HIV-1 induced maturation of DCs focus on highly virus-sensitive plasmacytoid DCs [358-368] which have immunologically and anatomically distinct characteristics from those of myeloid lineage [13,31,32]. The activation of pDCs by HIV-1 has also been reported to induce the maturation of bystander DCs of myeloid origin [335]. However, in this case it is not a direct effect of HIV-1.

Thus, despite some conflicting reports, it is generally accepted that HIV-1 negatively affects DC maturation and function. This is largely based on the effects of HIV-1 on the expression of cell surface markers associated with the state of DC maturation. Within this thesis, using a comprehensive set of experiments we confirmed that HIV-1 alters cell surface marker expression consistent with the inhibition of maturation and clearly link these changes with a number of aspects of DC function (endocytosis and antigen presentation). We also confirmed previously reported effects of the Tat , Vpr, and gp120 peptides on MDDC maturation and elucidated some novel effects of Vpr on MDDC physiology. Furthermore, during our investigations using deletion mutant viruses, we elucidated some novel effects of Vpr and Tat on MDDC maturation and further reinforced

the findings of previous reports describing viral tropism effects on MDDC functional maturation.

4.1 Determining the effect of HIV-1 on DC phenotypic and functional maturation in vitro

4.1.1 DC phenotype

Consistent with previous reports that described HIV-1 induced inhibition of DC maturation [370], we also found that HIV-1 inhibited the expression of several cell surface molecules associated with a mature phenotype. Specifically, it was observed that upregulation of CCR7 (Figures 10M and 10N) and MHC II (Figure 10R) was inhibited by HIV-1. The observed inhibition of MHC II expression in the presence of sustained costimulatory molecule expression after incubation with maturation inducing cytokines also complements previous *ex vivo* observations in which DCs expressing only select maturation markers were found to accumulate abnormally in the lymphoid tissues of HIV-1 infected individuals [336]. This lower MHC II molecule expression could result in impaired DC mediated presentation of exogenous antigens in both the periphery and in secondary lymphoid organs.

The significance of blunted CCR7 upregulation is unknown but may contribute to HIV-1 pathogenesis. While reduced CCR7 expression may not facilitate the dissemination of HIV-1 to naïve T-cells in secondary lymphoid tissue, it could delay the development of an effective adaptive immune response. Specifically, impaired expression of CCR7 by activated DCs in an inflammatory cytokine rich environment would allow for the maintenance of partially activated HIV-1 infected DCs in the anatomical periphery in the

presence of virus-susceptible resident effector T-cells and potentially increase HIV-1 infectivity.

4.1.2 Endocytosis

To complement the characterization of the effects of HIV-1 on cell surface molecule expression, we also investigated several functional aspects of mature DCs. Maturation of DCs is associated with decreases in endocytic activity [4,5] which was confirmed in our experimental system (Figure 11A). When DCs were infected with HIV-1, this inhibition of endocytosis was blunted (Figure 11C) demonstrating that HIV-1 infection inhibits functions associated with mature DCs in addition to its effects on surface marker expression.

These observations are supported by our earlier observations that MHC II expression is inhibited after HIV-1 infection (Figures 10Q and 6R) and the findings of Granelli-Piperno who likewise described HIV-1 inhibiting MDDC maturation [370]. Furthermore, our observations are consistent with reports describing inhibition of DC cathepsin and lysosome activity after HIV-1 infection [418] which could inhibit antigen processing after endocytosis.

Less reduction in MDDC endocytosis after HIV-1 infection could contribute to both viral infectivity and HIV-1 associated diseases. Increased MDDC endocytic function in spite of phenotypic maturation is likely a symptom of altered antigen processing machinery. Increased endocytosis of extracellular materials by DCs with inhibited cathepsin and lysosome activities [418] would allow for increased internalization and harbouring of infectious HIV-1 for later transfer to CD4+ T-cells. It could also conceivably

alter the endocytosis and processing of other infectious agents or cancerous cells facilitating the progression of HIV-1 associated diseases.

4.1.3 Antigen presentation

To further define the effects of HIV-1 on the functional aspects of mature DCs stimulated to undergo maturation, we evaluated antigen presentation as measured by autologous T-cell proliferation. To do so, HIV-1 infected (or uninfected) DCs were pulsed with a peptide pool derived from Cytomegalovirus, Epstein-Barr virus, and Influenza (CEF) virus and then co-cultured with CFSE stained autologous T-cells. Consistent with published reports [356,357,370], we found that HIV-1 infection of dendritic cells inhibited autologous T-cells proliferation (Figure 12). This impaired T-cell proliferation occurred despite the fact that HIV-1 had no effect on MHC I expression (Figures 10O and 10P). This indicates that the degree of MHC I expression does not appear to be a factor in the observed HIV-1 effects on T-cell proliferation. Compromised MDDC antigen presentation could contribute to HIV-1 disease progression by preventing adequate adaptive immune system responses to additional infectious agents and cancers.

4.1.4 LPS activation of HIV-1 infected DC MAPK pathways

Since a critical aspect of immature DC physiology is appropriate MAPK responses to pathogenic stimulation that trigger the maturation of DCs [49,77,101,253], we next investigated whether HIV-1 had any effect on LPS induced MAPK signaling. Interestingly, we found that HIV-1 infection had no effect on the p38, JNK, or ERK MAPK signaling pathways in immature DCs or *in vitro* matured DCs (Figures 13A to 13F). This was

consistent with our observations that HIV-1 did not affect CD14 expression on DCs (Figure 10A and 10B) which is necessary for TLR4 recognition of bacterial LPS [48].

Previous experiments examining MAPK activity in DCs revealed that HIV-1 itself was able to activate MAPK pathways, leading to increased expression of CCR7 [419]. MAPK pathways in T-lymphocytes and myeloid cells have also been observed to be affected by HIV-1 infection [420-422]. However our experiments examine the responsiveness of MDDC MAPKs after LPS stimulation, rather than the effect of the virus itself on MDDC MAPK activation.

Thus, in spite of other phenotypical and functional changes in MDDC physiology after HIV-1 infection, LPS MAPK responsiveness remained intact. A possible explanation for our findings is the recent report describing HIV-1 inducing increases in mDCs expression of TLR2 and TLR4 [423]. Yet this does not explain our observation that MAPK activation was not greater in magnitude in HIV-1 infected MDDCs than mock infected controls (Figures 13A to 13F). However, the stable expression levels of CD14 between MDDC experimental groups (Figures 10A and 10B), could limit TLR4 activation of MAPK signalling and account for our observations. Another possible limiting factor for MAPK activation may be increased activation levels of cellular phosphatases. This has been observed during HIV-1 infection in lymphocytes [424], and could limit TLR4 MAPK signalling. A final consideration is that it may be to the advantage of HIV-1 to leave MDDC LPS/MAPK signalling intact. It is well described that the LTR regions of integrated proviral DNA have promoter sites specific for NF- κ B [314]. Since LPS activation of MAPK pathways in MDDCs can induce NF- κ B activation [40,48,49], maintained MAPK responsiveness to LPS could allow for increased HIV-1 LTR activation. This could contribute to increased viral infectivity and productivity.

4.1.5 Conclusions

Within the present comprehensive set of experiments, not only have we confirmed that HIV-1 alters cell surface marker expression consistent with the inhibition of maturation, but for the first time, have clearly linked these changes with a number of aspects of DC function (endocytosis, antigen presentation). Our observations describing inhibited phenotypic and functional maturation are supported by published reports describing similar effects of HIV-1 on DC maturation [370], cathepsin activity [418], and antigen presentation [356,357,370]. That HIV-1 interferes with important aspects of DC function has implications in both HIV-1 pathogenesis, as it relates to the immunologic control of HIV replication, and in the immunodeficiency and risk of opportunistic infections associated with HIV disease.

4.2 Determining the effect of HIV-1-Tat, HIV-1-Vpr, and HIV-1-gp120 on DC phenotypic and functional maturation *in vitro*

As previously mentioned, productive infection of DCs from chronically infected individuals occurs less frequently than that of CD4 T-cells [342]. Despite this, DCs from infected individuals display altered *ex vivo* phenotype and antigen presentation abilities [336,340,356,357]. Possible explanations for these observations are circulating HIV-1 peptides found in the plasma of chronically infected individuals.

The viral peptides Tat, Vpr, and gp120 have been observed to alter DC phenotype and physiology and are found in the circulation of infected individuals [415,416,425]. For instance, exogenous Tat has been shown to have a maturing effect on MDDCs [403,404]. Vpr on the other hand has been observed to inhibit their maturation [407] while gp120 has

been observed to induce partial phenotypic maturation [397] and also to inhibit phenotypic and functional maturation [398,399].

Therefore, in light of our observations using whole virus and due to previous observations, we investigated the *in vitro* effects of these 3 peptides on the phenotypic and functional maturation of MDDCs.

4.2.1 DC phenotype

After incubation of MDDCs with Tat, we did not observe increases in costimulatory or MHC molecule (Figures 14E to 14H and 14O to 14R). Nor did we observe any increases in CD83, CD40, or CCR7 molecule expression (Figures 14I to 14N). These observations were in contrast to earlier reports showing increased expression of costimulatory and MHC molecules as well as increased MDDC secretion of IL-12, TNF α , and TNF β by exogenous Tat [403,404].

Since we used the same HIV-1_{IIB}-Tat peptide (BH10 isolate) as the previous investigations, we had expected to observe similar phenotypic effects on MDDCs. A potential explanation for our results is our use of the accelerated MDDC differentiation protocol. While not yet observed in MDDCs, monocytes can have both proinflammatory and immunosuppressive cytokine responses to Tat [426,427]. Therefore, it is possible that 2 day iMDDCs may respond differently to Tat than 5 day iMDDCs where IL-10 secretion could inhibit iMDDC phenotype maturation. Another possible explanation is alterations in Tat sequence. Despite coming from the same HIV-1 isolate, a single amino acid substitution could affect the biological activity of Tat as previously observed [428]. In this example a change from threonine to alanine near the arginine-rich nuclear localization domain contributed to differences in Tat-transactivation activities and overall viral

replication rates. Furthermore, Tat peptides from different clades have been shown to induce different cytokine profiles from primary monocytes [426,427]. Specifically HIV-1 Clade-B Tat was found to induce greater amounts of the inflammatory cytokines TNF- α and IL-6 than that of Tat from HIV-1 Clade C. HIV-1-Tat from Clade C also induced greater amounts of IL-10 from monocytes [426]. Furthermore, Spector et al found that a single change from cysteine to serine at position 31 significantly decreased IL-10 induction from monocytes after HIV-1 Clade B Tat incubation. Considering the mutability of HIV-1, it is plausible that changes in Tat amino acid sequence could induce different effects in iMDDCs than what has been previously observed with similar peptides.

Our observed effects of exogenous Vpr on MDDC maturation partially agreed with previous reports describing the effects of exogenous Vpr on DC phenotype and maturation. Like Muthumani et al [407], we did not observe any induction of MDDC maturation after their incubation with Vpr. By contrast however, we observed no inhibition in MDDC cytokine induced maturation after preincubation with exogenous Vpr (Figures 17A to 17R). Inconsistencies between our observations and those of Muthumani et al may be due to differences in Vpr peptide sequence and experimental methodologies. Muthumani et al used a Vpr peptide derived from the T-tropic subtype B HIV-1_{NL4-3} viral strain. We used a recombinant full length Vpr peptide whose primary sequence was based on the Indian subtype C HIV-1 M-tropic HIV-1_{93BIN905} virus strain. Vpr has been observed to differ in activity depending on the subtype [429]. Specifically, 10 differences in the amino acid profile of the Vpr molecules being compared (5 conservative and 5 non-conservative) were observed to endow Clade-B Vpr with greater LTR transactivation activities and Clade-C HIV-1-Vpr with greater apoptotic inducing characteristics [429]. Also, Vpr has been observed to differentially affect cells of myeloid origin. Specifically, Vpr can induce

monocyte apoptosis [430] but protect macrophages from it [431]. Thus, our Vpr peptide may induce different effects on MDDCs than the previously utilized strains and our shorter MDDC protocols could produce cells that differ in their response to Vpr than what has been observed previously.

Consistent with previous investigations [397], we observed an induction of partial iMDDC phenotypic maturation by gp120. Specifically, we observed increases in CD83 and CD86 expression by MDDCs after HIV-1-gp120 incubation (Figure 20E and 20I and 20J). We also observed increased MHC I expression, although the observed changes were not statistically significant (Figure 20P). Other investigators have observed inhibited iMDDC maturation [398], and generalized iMDDC activation and maturation by HIV-1-gp120 [399]. Differences between our results and those of other investigators may be the result of the use of HIV-1-gp120 from different viral strains which can vary in effect on DCs and monocytes depending on tropism and strain derivation. Specifically, we used a glycoprotein derived from HIV-1_{LAV} while Fantuzzi et al used gp120 derived from HIV-1_{IIIB} and HIV-1_{ADA} strains [397]. Our gp120 also differed from the HIV-1_{JR-FL}gp120 and HIV-1_{LAIG}gp120 peptides used by Shan et al [398]. While not completely explained, different effects by gp120 molecules on iDCs have been observed. Specifically, HIV-1-gp120 molecules that differ in strain and tropism can elicit differential IL-10 [398] and type 1 IFN secretion from iMDDCs [382] respectively. Reasons for the different effects we and other investigators have observed may lie in the cellular effects of the broad range of cellular ligands different HIV-1-gp120 molecules may bind. For instance, the gp120 glycoprotein can bind to a variety of surface molecules expressed by DCs including CD4 [300], DC-SIGN [432] and mannose C-type lectin receptors [433], resulting proinflammatory and immunosuppressive outcomes. Also, gp120 binding with CD4 on monocytes can induce their secretion of TNF α

[434]. While not yet observed in MDDCs, similar responses could explain in part our observed increases in MDDC phenotypic maturation. Ligation of DC-SIGN and mannose C-type lectin receptors with gp120 on the other hand can induce ERK MAPK pathway activation [398]. This could lead to secretion of IL-10 which can have immunosuppressive effects [370,398]. Depending on the possible combination of iMDDC ligands our gp120 glycoprotein bound during incubation, different outcomes may eventuate compared to those observed after the use of glycoprotein from different viral strains. This is further supported by the abrogated secretion of IL-12 by DCs after incubation with gp120 [397], which is in contrast to the DC secretion of IL-12 described by Williams et al [399] despite their use of the same gp120 molecule. Thus, even when using the same gp120 peptide, DC responses can vary. Therefore, while HIV-1-gp120 has observable effects on DCs, they vary between and within strains and tropisms.

Since DCs have been shown to be relatively refractory to HIV-1 infection [342], changes in their phenotype or function after their interactions with gp120 are very important. Initial infection may depend on these interactions facilitating viral infection. Increased CD83, CD86, and MHC I expression by DCs, could stimulate a degree of resident T-cell activation [4,5]. Increased numbers of vulnerable activated resident T-cells could contribute to the development of infectious foci [435]. Also, because the DCs were not fully matured by HIV-1-gp120 incubation, full T-cell activation and IFN γ secretion may be compromised [213]. This could result in inhibited DC IL-12 secretion and T-cell activation [436].

4.2.2 DC endocytosis

Since MDDC function changes during maturation, we next investigated the effects of these three HIV-1 peptides on MDDC endocytosis function.

After incubation with Tat, we observed decreases in MDDC dextran endocytosis similar to that of cytokine matured MDDCs (Figure 15). We also observed that Tat incubation did not alter maturation cytokine induced reductions in MDDC dextran endocytosis (Figure 15). Since a reduction in endocytosis is generally associated with increased functional maturation in MDDCs, our observations were consistent with previous reports describing Tat induced MDDC maturation [403,404].

Our observed decrease in MDDC endocytosis after incubation with Tat was not accompanied by phenotypic maturation (Figures 14A to 14R). Because previous reports have shown that Tat induces DC maturation, we were likely observing some degree of functional MDDC maturation. However, the absence of phenotypic maturation was curious.

Functionally mature DCs that do not express phenotypic markers of maturation would be unable to fulfill their primary role as antigen capture cells in the anatomic periphery. They may also be hampered in their ability to mature in response to phagocytosis of antigenic material. Also, since DCs are refractory to HIV-1 infection [342], that Tat could alter DC function without requiring productive DC infection is important. However, productive HIV-1 infection would likely have to occur before adequate amounts of circulating peptide could exert effects on DCs since it is not present in mature virions [299]. Once adequate amounts are in circulation, Tat could inhibit iDC endocytosis of antigens. Increases in viral infectivity and opportunistic disease progression would then result since primary adaptive immune responses to extracellular antigens would be compromised.

Consistent with the findings of Muthumani et al, we observed that Vpr reduced MDDC endocytosis of FITC-Conjugated Dextran (Figure 14). We also observed that cytokine induced reductions in MDDC endocytosis of FITC-Conjugated Dextran were unaffected by MDDC incubation with Vpr (Figure 18).

Since reductions in MDDC endocytic function are normally associated with maturation, our observations suggested that Vpr was inducing MDDC maturation. Previously observed Vpr induced reductions in endocytosis were due to reduced CD33 expression [407]. Although this molecule facilitates some DC endocytic functions [437], its downregulation may not have been evidence of MDDC maturation, since its downregulation was not accompanied by phenotypic maturation [407]. Similarly, our Vpr induced reductions in MDDC endocytosis of dextran may be due to a specific effect on a discrete cellular quantity, rather than MDDC physiology as a whole. However, since our experiments utilized dextran rather than apoptotic bodies and a Vpr protein distinct from those of the previous investigation, our results may have different mechanistic origins.

Selective reduced carbohydrate endocytosis by immature DCs in the anatomic periphery could spare a variety of pathogens, including HIV-1, from DC antigen processing machinery. Also, selective inhibition in DC endocytosis function may impair their ability to initiate maturation transcription programs in response to specific antigen endocytosis. These effects could result in progression of HIV-1 infection and other disease conditions whose elimination is dependent on initial DC endocytosis and eventual adaptive immune system intervention.

Our results examining the effects of gp120 on MDDC endocytosis were also consistent with previous observations [397]. After incubation with HIV-1-gp120, MDDC FITC-conjugated Dextran was not inhibited (Figure 21). However, inflammatory cytokine

reductions in FITC-conjugated Dextran endocytosis by MDDCs after HIV-1-gp120 incubated were blunted (Figure 21). Thus, HIV-1-gp120 induced some degree of partial phenotypic maturation and also altered MDDC function which supported previous observations with gp120. The inhibited MDDC maturation may be attributed in part to HIV-1-gp120 binding to MDDC DC-SIGN and mannose C-type lectins, whose ligation can result in IL-10 secretion due to immunosuppressive ERK MAPK activation [438]. An additional report detailed decreased endocytosis of FITC-conjugated dextran after iMDDCs were incubated with gp120 [398]. However our use of a different gp120 glycoprotein, can likely explain differences in experimental results.

Retained endocytic activity by mature DCs after inflammatory stimulation could allow for increased internalization of HIV-1 by DCs. If cathepsins and lysosomes are inhibited by HIV-1 [418], internalized virions could be harboured safely within intracellular vesicles. Since CCR7 expression was not inhibited by HIV-1-gp120 incubation, the phenotypically mature but functionally immature DC could still traffic to secondary lymphoid organs [127]. Once in the naïve T-cell zones of secondary lymphoid organs, phenotypically mature DCs could form synapses with CD4⁺ T-cells. This cell to cell contact could activate resident T-cells creating new viral targets [435], as well as facilitate synaptic and exosome mediated virus transmission [372-374].

4.2.3 DC antigen presentation

Since an important aspect of DC function after maturation is their change from antigen capture cell to antigen presenting cell, we examined the effects of these same peptides on MDDC presentation of a CEF peptide pool to autologous CD8⁺ T-cells.

Incubation of MDDCs with the Tat peptide resulted in a decrease in their ability to present CEF antigens to autologous CD8⁺ T-cells (Figure 16). This observation could be interpreted as MDDC maturation and would be consistent with previous reports describing similar Tat effects on DCs [403,404]. However, our observed losses in MDDC antigen presentation came in the absence of phenotypic maturation (Figures 14A to 14R).

Previous investigations and our examination of HIV-1-Tat effects on MDDC function support the notion that HIV-1-Tat induces MDDC maturation [403,404]. As explained previously, differences in Tat primary structure and experimental methodology may explain differences between our observations and those of other investigators. Functionally mature DCs that are phenotypically immature would be unable to form immunological synapses with nearby T-cells and capture antigens in the anatomic periphery. This could facilitate immune evasion of HIV-1 and other pathogens.

Like Tat incubation, incubation of MDDCs with Vpr inhibited their presentation of CEF antigens to autologous CD8⁺ T-cells (Figure 19). This agreed with the findings of Muthumani et al. [407]. However, unlike this report we did not observe any inhibition of B-7 molecule expression after induction of MDDC maturation (Figure 17E-17H). Decreased costimulatory molecule could contribute to decreased MDDC antigen presentation [213]. Therefore, the mechanism responsible for our observations may be different from that of Muthumani et al. As discussed earlier, our use of a different Vpr peptide and accelerated MDDC protocol may be responsible for our observations.

As previously mentioned, Vpr effects can vary according to strain derivation. That HIV-1-Vpr peptide has also been observed to induce IL-6 secretion from macrophages and monocytes [410] may help explain our observations. If DCs responded similarly, it is possible they would secrete IL-6. IL-6 alone can inhibit DC activity [181] and this could

negatively affect both antigen presentation as well as antigen capture (Figure 14). Thus, IL-6 could be influencing the antigen capture and endocytosis functions we observed during our experiments.

Our described effects of gp120 on antigen presentation were not consistent with published observations [397]. After incubation of MDDCs with gp120, we did not observe any decreases in antigen presentation to autologous T-cells (Figure 22). However, as described previously, gp120 from different viral strains and tropisms can induce different effects on MDDCs [398]. These phenomena may explain why our results differed from those of previous investigators. Phenotypically mature DCs with maintained endocytic abilities and intact antigen presentation abilities could facilitate intact virion transfer to CD4⁺ T-cell targets in secondary lymphoid tissues. While *in vitro* virological synapses have yet to be observed, partially mature DCs in secondary lymph tissues has been observed *in vivo* [336]. This, as well as previous observations of partial MDDC maturation after incubation with HIV-1-gp120 [397] agree with our observations and may provide some explanation for DC facilitation of HIV-1 pathology.

4.2.4 Conclusions

With respect to HIV-1 infection and disease progression, circulating peptides can induce changes in DC physiology in the absence of viral fusion or productive infection. This is an important mechanism through which HIV-1 can exert effects on infection resistant cells like DCs. The time frame during which these peptides would be available for DC ligation would also vary during the course of viral infection. During initial HIV-1 infection, circulating HIV-1-Tat and Vpr may not be present in appreciable amounts since their expression requires viral fusion events and productive viral infection. Therefore,

during initial infection, effects of gp120 ligation with DCs would be the primary means of immune evasion at the disposal of the virus.

The failure of inflammatory cytokines to induce MDDC maturation after gp120 incubation suggests that ligated DCs may be resistant to inflammation at the site of infection which could allow for a delay in maturation. This could result in a delay in virus presentation to the adaptive immune system, which could facilitate the establishment of infectious foci in the anatomic periphery in activated T-cells. Also, since HIV-1 has been observed to inhibit cathepsin activities, increased DC endocytosis after exposure to gp120 may not be a deterrent for infection spread. Instead, DCs could endocytose virions in spite of inflammation, which could then remain relatively intact due to inhibited cathepsin activities or other aspects of DC antigen processing machinery. In secondary lymphoid organs, intact virion transfer to vulnerable T-cells could then be facilitated by partially mature DCs harbouring infectious viral particles via cell to cell contact.

Following the establishment of systemic viral infection, circulating Tat and Vpr could then alter DC functions without inducing phenotypic maturation. Functionally mature, or inactive DCs would be unable to capture and present antigens necessary to induce adaptive immune responses. This could facilitate the viral infection process and compromise immune responses to associated opportunistic disease processes.

Thus, initial DC-gp120 contact may allow for a delay in DC maturation resulting in enhanced viral infectivity. Increased systemic infection could later interfere in DC function via circulating Tat and Vpr peptides, allowing for increased viral dissemination and opportunistic infection/pathology progression.

4.3 Determining the effect of HIV-1-Tat and HIV-1-Vpr deletion on DC phenotypic and functional maturation in vitro

To further delineate the effects of the Tat and Vpr peptides on MDDC maturation and function we infected them with Tat and Vpr deletion mutants. Through this we hoped to elucidate the contributions of Tat and Vpr to HIV-1 effects on MDDC maturation and function.

4.3.1 DC phenotype

To investigate the effects of Vpr deletion on HIV-1 MDDC infection, we used a full length T-Tropic HIV-1_{NL4-3} and a Vpr deleted HIV-1_{NL4-3} (HIV-1_{Dvpr}), both derived from a pUC18 plasmid. We did not observe any changes in MDDC phenotype or maturation after infection with either of the 2 viruses (Figures 24A-24R).

Likewise, when we infected MDDCs with either a full length pUC19 derived M-Tropic HIV-1_{BaL} or a Tat deleted HIV-1 (HIV-1_{Mtat}), we did not observe any changes in MDDC phenotype or maturation (Figures 27A-27R). There were also no differences between the effects of the deletion mutants and their respective parent strains (Figures 24A-24R and 27A-27R). These results differed from our observations examining the effects of live HIV-1_{CS204} on DC maturation (Figures 10M, 10N, and 10R) and the previously published report describing inhibited cytokine induced phenotypic maturation of iMDDCs by HIV-1 [493]. Specifically, HIV-1 infection has been observed to inhibit CD83, MHC II, and DC-LAMP expression by mature MDDCs [370].

Since productive HIV-1 infection can induce changes in MDDC phenotype before maturation [370] and after viral integration [371], it was important that MDDC infection during these experiments be characterized. We were unable to detect HIV-1 DNA in any

infected iMDDC cultures. However we did detect HIV-1 RNA in the virus infected cultures (Figure 23). Since we did not observe any phenotypic changes after HIV-1 infection, productive iMDDC infection was unlikely and input virus was the probable source of detected HIV-1-RNA.

Also, 3 of the 4 viral strains would have either Vpr peptides within their capsid [299] or Tat within their genomes [299]. Both of these have been observed to have detectable effects on MDDC phenotype and maturation when expressed endogenously within iMDDCs [408,405]. Therefore, the absence of detectable HIV-1 DNA and lack of effect from Vpr or Tat deletion makes it likely that fusion and proviral integration events were not occurring at levels sufficient to induce detectable changes in MDDC phenotype.

Another aspect of differing methodology that could affect HIV-1 infectivity is the culture system from which the viral supernatants were derived. Our clinical HIV-1_{CS204} isolate, was grown in primary PBMCs over the course of several weeks. By contrast, the lab adapted HIV-1 strains were grown in HEK cells over the course of several days. It has been shown that when HIV-1 buds from PBMCs, virions can incorporate cellular ICAM-1 into their envelopes which can ligate with DC LFA-1 [440]. This could prolong and improve cell mediated viral transfers. On the other hand, the virions budding from HEK cells would not incorporate cellular ICAM-1 into their envelopes [441], which could result less effective viral MDDC binding and thus lower infectivity.

The same could be said for the viral protein Nef. Even though endogenous Vpr has been shown to inhibit DC phenotypic and functional maturation, [408,409] the viruses used in one study to demonstrate this lacked the HIV-1-Nef gene in addition to Vpr [409]. HIV-1-Nef has been observed to have a variety of effects on DCs [386-396], and it can be found in mature HIV-1 virions [299]. Therefore, it is possible that the absence of this gene from

their viral constructs could result in different effects on DC maturation and function both in the presence and absence of viral fusion since lysing HEK cells in our system may release it into viral supernatants. Thus, our observations may be resultant of a system in which exogenous Vpr or Nef may be contributing to DC physiology rather than just a single viral component as previously described.

Assuming there is little productive infection or viral fusion events, MDDC interactions with HIV-1 are likely mediated by DC-SIGN ligation [442]. Although we did not see any increase in maturation marker expression which can be associated with gp120/DC ligation, there was inhibition of maturation observed functionally (described later) which is consistent with DC-SIGN associated cell signaling outcomes [68,69].

4.3.2 DC endocytosis

We next investigated the effects of Vpr and Tat gene deletion on MDDC function after HIV-1 infection. Neither the full length HIV-1_{NL4-3} nor the Vpr-deleted equivalent virus induced any decreases in FITC-Dextran endocytosis (Figure 25). However, the Vpr-deleted T-tropic viral strain blunted maturation associated decreased MDDC endocytic function (Figure 25). While not yet observed in DCs, these results agreed with a report describing IL-6 secretion by Vpr incubated macrophages and monocytes [410]. If iDCs responded similarly, an absence of Vpr could result in increased DC activity levels [181]. However, this explanation would be more likely if some degree of viral fusion or uncoating was occurring since the viral envelope proteins of the viral strains are identical. Leakage of HIV-1-Vpr from lysing HEK cells could be present in full-length viral supernatants and may also contribute to changes in iDC antigen capture abilities via induction of IL-6 [181] or decreases in CD33 expression [407]. Also as previously explained, the presence of Nef

in our virus cultures may also change the effects of Vpr deletion mutant effects on iMDDC phenotype and function.

By contrast, when MDDCs were infected with the M-tropic virus strains, there were significant reductions in FITC-Dextran endocytosis similar to those observed by MDDCs incubated with inflammatory cytokines (Figures 28). This was in the absence of MDDC phenotypic maturation (Figures 27A to 27R). Our results were supported by observations of partial DC [336,340] and full MDDC maturation [377] after HIV-1 infection.

A primary difference between the BaL and NL4-3 HIV-1 strains is their respective tropism. M-tropic HIV-1 strains may have different effects on MDDC maturation and function than T-tropic strains regardless of Tat expression. While not yet described in DCs, macrophage intracellular signalling has been shown to be differentially affected by differently tropic HIV-1 variants [443]. Specifically, calcium and the JNK and p38 MAPK signalling pathways were observed to be more highly activated by M-tropic viruses in macrophages [443]. These signalling pathways have been shown to be critically important in DC maturation [253,444]. Therefore, greater stimulation of the calcium and MAPK pathways via CCR5 tropic gp120 (rather than the T-tropic CXCR4 viruses) could induce MDDC maturation and explain our results.

Similar to our observed effects with the T-tropic HIV-1_{NL4-3} strains, the deleted accessory gene HIV-1-Tat appeared to contribute to iMDDC maturation (Figure 28). While not statistically significant, there was a visible difference between the levels of FITC-conjugated dextran endocytosis of the full length HIV-1_{BaL} and HIV-1_{Mtat}. Since the viral Tat peptide is not contained in mature virions [299], Tat peptides within HIV-1_{BaL} infected MDDC cultures would be integrated proviruses or peptide leakage from pHIV-1_{BaL} transfected HEK cell supernatants. It is possible that proviral integration was occurring at

levels that we could not detect with our *Alu*-nested PCR detection methods. In the event that proviral integration was occurring, Tat could be transcribed and translated to ultimately exert endogenous effects on MDDC maturation [405]. In the case of transfected HEK cell leakage, exogenous Tat would likewise be available to influence MDDC maturation as observed previously [403].

4.3.3 DC antigen presentation

After incubation with the lab adapted HIV-1 strains, both T-tropic HIV-1_{NL4-3} strains caused decreases in MDDC antigen presentation (Figure 26). These findings were consistent with our observations with the HIV-1_{CS204} clinical isolate and previous reports that HIV-1 decreases the ability of MDDCs to stimulate T-cells (356,357,370). There were also no differences in the effects of HIV-1_{NL4-3} and HIV-1_{DVpr} on MDDC antigen presentation (Figure 26). This means that Vpr may have less effect on MDDC antigen presentation than it does on endocytosis functions.

HIV-1_{BaL} infection also decreased iMDDC antigen presentation but these reductions were not statistically significant (Figure 29) and HIV-1_{Mtat} infection partially restored this function (Figure 29). Although not statistically significant, Tat from integrated proviruses or pHIV-1_{BaL} transfected HEK cell supernatants could induce some degree of MDDC maturation. This would make them less able to respond to CEF peptides. In the absence of Tat, iMDDCs would remain more sensitive to CEF peptides and thus, retain more antigen presenting abilities. This would agree with previous reports detailing a maturing effect of Tat on MDDCs [403,404].

4.3.4 Conclusions

These investigations suggest that viral tropism and the deletion of the accessory genes Tat and Vpr can cause different effects on MDDC maturation and function. However, the lower infectivity of these viral strains may have contributed to their inability to affect MDDC phenotype. Nevertheless, the viruses were still able to differentially alter cellular antigen capture and presenting machinery.

4.4 Overall conclusions

The overall theme of HIV-induced altered MDDC antigen processing machinery is consistent throughout the thesis and is also described in previous reports [336,337,340, 356,357, 370,418]. Our data describing individual HIV-1 peptides inhibiting the endocytosis of iDCs and antigen presentation also agrees with previous investigations [403,407].

Our observations of inhibited iMDDC maturation after HIV-1_{CS204} infection are similar to the results of Granelli-Piperno et al [370] who observed hindered DC-LAMP expression by mature MDDCs after HIV-1 infection. Manel et al observed proviral integration to be the stimulus for MDDC maturation after HIV-1 infection, which is contrary to our observation that integrative viral infection of MDDC can result in inhibited phenotypic maturation. However, unlike our use of a single dual tropic virus, they achieved integration using DC co-infection with the T-tropic HIV-1_{NL4-3} and the SIV-Vpx protein [371]. Since different HIV strains and their respective component peptides can vary in their effects on DCs, observational discrepancies are likely due to differences in experimental protocols and materials used by each group. In any case, HIV-1 interactions with iMDDCs

appear to alter their phenotypic and functional maturation. This data is complimented by our observations of HIV-1 peptide and lab adapted virus effects on MDCCs.

Since DCs are relatively refractory to infection, our observations of the effects of the M-tropic HIV-1_{BaL} and exogenous gp120 may be important during initial infection events. During initial HIV-1 infection, the founding virus is often an M-tropic virus [445]. If an M-tropic HIV-1 strain were to make contact with DCs in the anatomic periphery and alter their antigen capture (Figure 28) but not presentation functions (Figure 29), PRR internalized viruses may avoid lysosomal degradation [418]. This could also happen in the absence of productive DC infection since gp120 also altered MDCC endocytosis functions (Figure 21).

Also since M-tropic HIV-1 and the gp120 peptide interactions did not impede MDCC phenotypic maturation, depending on the virus strain, pDCs in the primary infection area could react to the virus and stimulate virus carrying myeloid DCs to traffic to secondary lymphoid tissues [335]. On the other hand, compromised CCR7 expression as we observed during our experiments with HIV-1_{CS204} could delay DC trafficking to lymph nodes for future interactions with T-cells. This could allow for establishment of infectious foci in the anatomic periphery which would facilitate local and eventual systemic HIV-1 infection.

The viral gp120 glycoprotein could also stimulate some degree of DC maturation which could also contribute to their maturation and future interactions with T-cells both locally and systemically. At the same time, if DC antigen processing machinery were compromised as we observed throughout our experiments with both viruses and peptides, intact virions may accompany DCs into the naïve T-cell zones of lymph tissues.

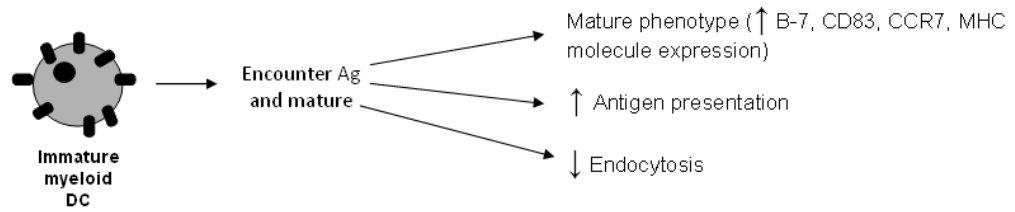
Once in secondary lymphoid tissue, partially mature DCs could form infectious synapses with T-cells using increased expression of DC-SIGN. Antigen presentation may be affected and this could lead to increased viral transfer and the inhibition of adaptive immune system activation. Viral tropism in lymph tissues may also change and become more T-tropic in target cell preference via mutation and selective pressures. This would aid in viral infection propagation since we observed T-tropic virus inhibition of antigen presentation.

After the establishment of a productive infection, the HIV-1 gp120, Tat, and Vpr peptides could enter circulation and inhibit DC maturation and function without requiring productive infection. This is particularly important since DCs have been described as refractory to HIV-1 infection. Also, the different effects of the respective peptides on MDDC physiology indicate that HIV-1 may employ different immune system subversion strategies during different stages of HIV-1 infection.

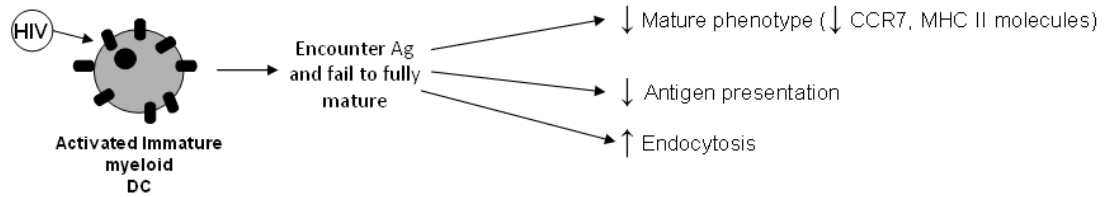
Thus, this report describes a number of novel effects HIV-1 and several accessory peptides can have on the maturation and function of iMDDCs (Figure 30). The pivotal role DCs have in orchestrating adaptive immune responses make these observations critically important for understanding HIV-1 infection events, infection prevention, and the development of a working HIV-1 vaccine.

Figure 30: Summarized *in vitro* effects of HIV-1 and HIV-1 regulatory peptides on MDDC maturation and function.

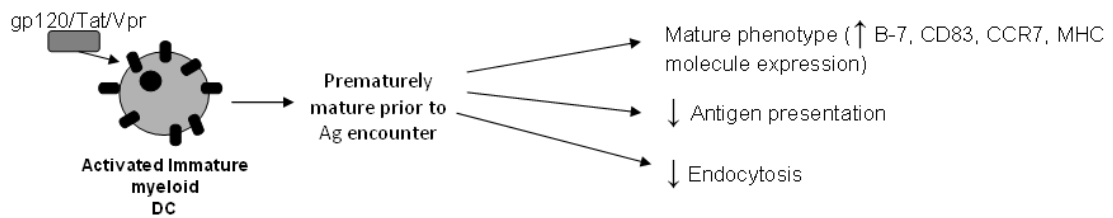
A) Healthy DC maturation



B) Impaired DC maturation following in vitro HIV infection



C) Premature DC maturation following incubation with HIV peptides



4.5 DC HIV-1 infection model

Our observed effects of HIV-1 and the associated gp120, Tat, and Vpr molecules provide new insight that can help explain the effects of HIV-1 on DC maturation and function that contribute to HIV-1 infection and disease pathogenesis. They also support previously published reports describing a number of mechanisms through which the virus has been observed to subvert and inhibit general DC function and maturation. Importantly, the new findings within this thesis contribute to the construction of an HIV-1 DC infection model that can provide insight into the various alterations in DC maturation and function observed both *in vitro* and *in vivo* during the course of untreated viral infection.

Since new HIV-1 infections are typically M-tropic and occur at mucous membranes, initial primary viral infection events would likely include M-tropic virus gp120 molecules ligating with DC surface molecules. Because DCs are observed to be relatively refractory to productive infection viral glycoprotein DC interactions that do not result in integrated proviral DNA would be among the first HIV-1 infection events.

In the absence of viral fusion events, immature mucosal DCs would likely bind incoming virions by external envelope gp120 molecules using surface CLR. An increased expression of DC-SIGN by iDCs in the primary infection area may also increase in response to HIV-1 which would facilitate DC-HIV-1 binding. Following CLR binding, a number of different cellular outcomes would eventuate.

One possible outcome would be increased iMDDC maturation after iDC incubation with HIV-1-gp120. This would also result in increased ERK MAPK activation and IL-10 secretion by gp120 ligated DCs has also been observed. Increased IL-10 secretion by DCs likely contributes to the retention in antigen capture abilities of mature DCs after maturation with inflammatory cytokines. HIV-1-gp120/iDC interactions would also result

in virion internalization by the iDCs after CLR ligation with gp120. Since HIV-1 has also been observed to inhibit cathepsin activities in DCs, internalized virions would be protected from lysosomal degradation and maintained within membranous vesicles rather than degraded by proteases and cathepsins. There they would be protected from additional immune surveillance and could safely traffic to secondary lymphoid organs after DC maturation for dissemination to CD4⁺ T-cells.

In the event that DCs are productively infected during primary HIV-1 infection, their maturation by inflammatory cytokines that would be produced by nearby pDCs. Lowered CCR7 expression would allow for maintenance of infected DCs in the anatomic periphery that could interact with local effector T-cells using normally expressed B-7, MHC I, CD40, and CD83 expression. Internalized virions could be transferred to these cells via the immunological synapse. Since the T-cells involved in the synapse would likely be activated by interactions with the partially mature DCs, they could become productively infected and begin producing *de novo* HIV-1 virions.

Another possible outcome of initial HIV-1 infection is the possibility that incoming M-tropic virions would inhibit iDC endocytosis as we observed with the HIV_{BaL} strain and iMDDCs. This could also impair endocytosis of other pathogens in the immediate area. In the event that HIV-1 had not yet inhibited DC cathepsin activities, the impaired endocytosis would protect HIV-1 from initial degradation and instead may maintain it safely associated with DC surface DC CLRs. Inhibited endocytosis of HIV-1 would also make increased numbers of virus particles available for pDC TLR sensing.

Incoming and newly budded HIV-1 would activate local pDCs to secrete inflammatory cytokines and type 1 interferons. These would mature bystander myeloid DCs harbouring infectious HIV-1 particles on their surface and within membranous

vesicles. Newly matured myeloid DCs would then traffic to secondary lymphoid organs where internalized or attached virions would likewise make their way to the lymph tissues using mature DCs as a vehicle.

Mature DCs entering the naïve T-cell zones of secondary lymphoid organs would then be able to synapse with and activate naïve T-cells. This would result in increased dissemination of internalized virions and progression of infection and disease processes. During this process the virus would also progressively mutate to an X4 tropism. This would alter viral effects on DCs and increase productive infection levels of vulnerable activated helper T-cells.

At this time, viral titres would begin to rise to levels sufficient to begin infecting larger numbers of iDCs. Before the dominant tropism is completely X4, dual tropic virions may begin to appear and exert effects on iDCs similar to those we observed during out *in vitro* experiments with the dual tropic HIV-1_{CS204}. Higher levels of DC-SIGN expression by iDCs would allow for greater HIV-1 attachment as well as a greater likelihood of iDC infection. Furthermore, the inflammatory environment of the chronic infection would initiate increased iDC maturation. However, with inhibited CCR7 and MHC II expression, both trafficking and antigen presentation would be inhibited. This would also allow for increased peripheral infections to develop.

Once the dominant viral tropism is X4, our observations with the HIV-1_{NL4-3} strains of HIV-1 indicate that antigen presentation in the secondary lymphoid organs and in the anatomic periphery would be inhibited. This would allow for further immune evasion by the virus and potentially allow for even greater levels of viral dissemination and productive infection of helper T-cells.

After productive infection of T-cells, HIV-1-Tat and HIV-1-Vpr would begin to enter circulation. Once these accessory peptides reach sufficient concentrations in the plasma, they would inhibit iDC antigen capture and presentation functions as we and some other investigators have observed. This could occur in the absence of phenotypic maturation and through this severely hinder the ability of iDCs to perform antigen capture functions in the anatomic periphery. This could allow further HIV-1 immune evasion and contribute to opportunistic infection progress as well as cancer progression.

Provided there is no ART, the anatomic distribution of DCs would begin to change. Partially activated DCs would likely begin to accumulate in secondary lymphoid organs, the spleen, and the gut mucosa like observed with SIV. Likewise there may also be a gradual decrease in DC number due to apoptosis.

The high viral titres that eventuate during chronic infection would be more able to overcome the refractory nature of DCs which now would be lesser in number and present in the areas of greatest viral titres. A higher rate of increased integrative DC HIV-1 infection could result in a greater number of DCs that are resistant to inflammatory cytokine maturation. This would be of particular importance within the highly inflamed environment and cytokine storm common in individuals suffering from untreated chronic HIV-1 infection. Impaired DC maturation and function would result in less antigen capture and presentation as well as keep mature DCs from migrating using CCR7 to naïve T-cells.

Finally, during the entire course of HIV-1 infection, due to the low fidelity of HIV-1 reverse transcriptase, viral mutations would occur that would result in incomplete virions and truncated genomes. Despite missing viral genes that are critical for virus life cycle completion and disease pathologies, a number of different viral effects could still take place and affect HIV-1 infection processes via DC subversion.

In the case of HIV-1 Vpr, our observations show that its absence may abrogate some of the inhibition of antigen presentation by T-tropic HIV-1 infected DCs. Since Vpr has been observed to induce IL-6 production from macrophages, and monocytes, it is possible that a virus missing Vpr may not induce IL-6 from an infected DC. This could enhance DC maturation and their ability to stimulate Th1 adaptive immune responses. Lowered IL-6 secretion by DCs and resultant increases in DC Th1 activation could encourage adaptive immune activation that would generate additional activated CD4⁺ T cell targets throughout the body. It would also increase systemic inflammation.

By contrast, virions lacking Tat would stimulate DCs less than whole virus as demonstrated by our data describing Tat deletion and M-tropic viruses and previous observations detailing maturing effects of Tat on iDCs. This could result in delayed DC migration to lymph nodes for adaptive immune system activation. It would also facilitate greater endocytosis of HIV-1. If Tat is not the viral component that inhibits cathepsins, decreased maturation in this case would provide protection of viruses from immune surveillance. Less DC maturation would also result in less DC migration and thus less initial dissemination. Delays in DC maturation and trafficking could also result in greater establishment of infectious foci that later contribute to systemic infection. Finally, there is also the potential for pseudo species interactions between deletion mutants similar to what has been observed in poliovirus infections. These could expedite additional infectious and disease processes of which we are thus far unaware.

Thus, our observations provide insights that facilitate the theoretical construction of an HIV-1 infection model within which DC subversion plays a critical role in infection establishment and progression. Because of the complexity and the often times contradictory effects of even single viral components on DC maturation and function, further

investigation is required to fully understand and elucidate the effects of whole virus and its individual components on DC physiology. Understanding HIV-1 disease processes and the contribution of DCs to viral dissemination are critically important for the prevention and control of HIV-1 infection.

5.0 Future Directions

The findings in these studies describe a number of salient effects HIV-1 and its accessory proteins have on DC maturation and function. However, much remains to be understood. Since DC populations in addition to myeloid derived DCs contribute to HIV-1 immunity and disease processes, it is important that the entire scope of DCs within the human body and their contributions to the disease state are accounted for.

Similar experiments using primary or laboratory derived Langerhans cells in an *in vitro* epidermal model would be essential for understanding the effects of these viruses and proteins during initial infection events.

Likewise using primary or *in vitro* Flt3 ligand differentiated pDCs [446] could provide valuable insight concerning the role of pDCs in HIV-1 infection and disease progression.

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