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IL-23 and IL-27 Expression in Dentritic Cells and the Effects of HIV-1

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IL-23 AND IL-27 EXPRESSION IN DENDRITIC CELLS AND THE EFFECTS OF HIV-1

Andréane Chénier

Thesis Submitted to the Faculty of Graduate and Postdoctoral Studies
in partial fulfillment of the Requirements for the
MSc degree in Microbiology and Immunology

Biochemistry, Microbiology and Immunolgoy
Faculty of Medicine
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ISBN: 978-0-494-50865-7
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ISBN: 978-0-494-50865-7

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LIST OF ABBREVIATIONS

DCs: Dendritic cells

iDCs: immature DCs

mDCs: myeloid DCs

pDCs: plasmacytoid DCs

CCR: Chemokine (CC motif) receptor

CXCR: chemokine (CXC motif) receptor

APC: antigen presenting cell

MDDCs: Monocyte-derived Dendritic cells

LPS: Lipopolysaccharide

CD: Cluster of Differentiation

IL: Interleukin

IL-12: Interleukin-12

IL-23: Interleukin-23

IL-27: Interleukin-27

IL-6: Interleukin-6

EBI3: Epstein Barr virus-induced gene 3

pTat : retroviral Tat wt

rTat: recombinant Tat

Tat wt: Tat wild type

HIV-1: Human Immunodeficiency virus type 1

HAD: HIV-associated dementia

HAART: highly active antiretroviral therapy

CNS: central nervous system

TLR: Toll-like receptor

IFN: Interferon

IRF: IFN Regulatory Factor

MyD88: Myeloid Differentiation Factor 88

NF-κB: Nuclear Factor kappa B

MHC: major histocompatibility complex

ER: endoplasmic reticulum

TAP: transporter associated with antigen presentation

CTLs: cytotoxic $CD8^+$ T lymphocyte

HLA: Human leukocyte antigen

IL-12Rβ1: IL-12 receptor β1 chain

IL-12Rβ2: IL-12 receptor β2 chain

MAPK: mitogen-activated protein kinase

PI3K: phosphatidylinositol 3-kinase

mTOR: mammalian target of the rapamycin

S6K1: 70 kDa ribosomal S6 kinase 1

CMV: cytomegalovirus

JAK: Janus Kinase

STAT: Signal Transducers and Activator of Transcription

TGF-β1: Transforming growth factor β1

TCCR/WSX-1: T cell cytokine receptor

G-CSF: granulocyte colony-stimulating factor

LIF: leukemia inhibitory factor

OSM: oncostatin M

SOCS3: Suppressor of Cytokine Signalling 3

DC-SIGN: DC-specific intercellular adhesion molecule 3 (ICAM3)-grabbing non-integrin

TAR: transactivation response

PBS: phosphate buffered saline

FCS: fetal calf serum

PBMCs: Peripheral blood mononuclear cells

GM-CSF: Granulocyte macrophage-colony stimulating factor

7AAD: 7-amino-actinomycin-D

RPMI: Roswell Park Memorial Institute medium

DMEM: Dulbecco modified Eagle's minimal essential medium

RT-PCR: reverse transcription polymerase chain reaction

NTP: nucleoside triphosphate

ATP: adenosine triphosphate

TTP: thymidine triphosphate

GTP: guanosine triphosphate

CTP: cytidine triphosphate

DEPC: Diethylpyrocarbonate

FITC: Fluorescein isothiocyanate

PE: Phycoerythrin

PC5: phycoerythrin-cyanine 5

ABSTRACT

Dendritic cells (DCs) are potent antigen presenting cells whose role in eliciting immune responses in the context of human immunodeficiency virus type-1 (HIV-1) infection remains unclear. The objective of this project was to determine if HIV-1 modulates DC function by downregulating the production of IL-23 and IL-27, cytokines important in the generation of cellular immune responses. Dendritic cells were isolated from the epidermal and dermal skin layers resulting from elective abdominoplasties by CD1a and CD1c microbeads. Circulating DCs were isolated from peripheral blood by CD1c microbeads. As an experimental model, monocyte-derived DCs (MDDCs) were generated from freshly isolated monocytes which were cultured for 6 days with IL-4 and GM-CSF. The phenotypes of these 4 DC populations were compared. To determine the effects of an endogenous source of HIV-1 Tat on cytokine expression, MDDCs were infected with a pLXIN (ptat) construct containing the HIV-1 tat wt gene or the empty vector for 24h, before a 4h LPS (1μg/mL) stimulation. To determine the effects of an exogenous source of Tat, which is known to be secreted by infected cells, DCs were pre-treated with recombinant Tat protein (rTat) for 1h before LPS stimulation. Alternatively, DCs were cultured in the presence of HIV-1 dual-tropic strain 92HT593 for 24h before LPS stimulation. IL-23p19 and IL-27EBI3 and p28 mRNA expression were evaluated by quantitative real-time RT-PCR (qRT-PCR) and reported as relative expression levels. DCs are readily infected with the ptat, as shown by RT-PCR. The presence of endogenous Tat resulted in a decrease in IL-23p19, IL-27 EBI3 and p28 mRNA

expression. Incubation of DCs with rTat similarly decreased IL-23p19, IL-27 EBI3 and p28 mRNA expression. When cultured in the presence of HIV-$1_{92HT593}$, a similar downregulation in IL-23p19 and IL-27EBI3 was observed, with no significant effect on p28 expression. In conclusion, the presence of HIV-1 Tat protein whether from an endogenous or exogenous source significantly downregulated the expression of IL-23p19 and IL-27EBI3 and p28 mRNAs. HIV-1 similarly downregulated the gene expression of IL-23p19 and IL-27EBI3, but appeared to have no effect on IL-27p28 gene expression. HIV-1 disregulation of IL-27 subunits EBI3 and p28 and IL-23p19 may be a mechanism by which HIV-1 evades the infection-clearing immune response. Understanding the function of cytokines expressed and secreted by DCs to initiate T cell polarization may lead to better understanding of HIV-1 pathogenesis and the development of novel therapies for HIV infection.

ACKNOWLEDGEMENTS

Many people have helped me along the way and I would like to take this opportunity to thank them;

- *Dr. Jonathan Angel*, for supervision, guidance and funding,
- *Dr. Ashok Kumar*, for advice in the culturing of dendritic cells, troubleshooting, and putting research into perspective,
- *Dr. Karen Copeland*, for providing guidance, advice, and a friendly ear when needed,
- Dr. Lakshmi Krishnan, for guidance and advice,
- *Agatha Komsic*, for being a friend and sounding board, always ready to inject practicality into the everyday insanity of research, and help with real-time PCR,
- *Charlene Young*, for being a friend and training in the uses of flow cytometry, and for knowing everything there is to know in the Angel lab,
- *Katrina Gee*, for too much advice to mention, in particular with dendritic cell culture,

Thanks to all the members of the Angel, Copeland and MacPherson labs, and the staff at the Ottawa Health Research Institute for making working enjoyable, and last, but not least, to the Ontario HIV Treatment Network for providing me with funding through their studentship award program and the University of Ottawa for awarding me the Excellence Award.

1. INTRODUCTION

1.1 DENDRITIC CELLS

Dendritic cells (DCs) are cells of the innate immune system that bridge the gap between the innate and adaptive immune responses. DCs are very potent antigen-presenting cells (APC)[1] and are essential in the successful clearance of infections, and the establishment of immunological memory.

DCs have two different origins; they are of myeloid or lymphoid origin. There are 3 different types of DCs. Two are of myeloid origin, epidermal DCs and dermal DCs and one of lymphoid origin are plasmacytoid DCs[1, 2]. Epidermal DCs are also known as Langerhans cells. Plasmacytoid dendritic cells are generally considered to be mainly IFN-α and IFN-β producing cells, rather than APCs[3, 4]. Each of these three main subpopulations has its own specific phenotype[2] that is determined by the microenvironment of the peripheral tissue in which it resides. Precursors for myeloid DCs (mDCs) disseminate from the bone marrow, through the blood to the peripheral tissues[1]. While DCs are in the periphery, they are in their immature phenotype (iDCs) and scan tissues and extracellular environments for the presence of antigen. After encountering antigen, iDCs internalize and process them, an event that is critical in their life cycle. At this point, these cells exit the tissue via the afferent lymph, and migrate to lymph nodes[1]. Migration is enabled by the CCR7 receptor, which responds to CCL19/21, and allows for the migration of DC from the periphery to the nearest lymphoid tissue, be it lymph node or spleen[5].

The migration and maturation of DCs are processes that necessarily occur together. Once DCs reach the lymph nodes, they activate T cells, and receive activation signals in return through the engagement of co-receptors and the secretion of cytokines; these two processes are necessary to produce a mature DC. The nature of the cytokines secreted by DCs dictates the differentiation pathway, T_H1, T_H2, T_H17 or T_{reg}, of circulating T cells.

1.2 DENDRITIC CELL STIMULATION

Bacterial products, such as lipopolysaccharide (LPS) and CpG motifs[6], viral RNA and DNA, proteins associated with necrosis (like exposed heat-shock proteins)[7], are known to activate DCs through lectin-domain scavenger receptors and Toll-like receptors (TLRs)[8, 9]. TLRs recognize microbial structures in the earliest phase of the host defenses and elicit the expression of immune and inflammatory genes. The response is tailored by the stimulated TLR to eradicate the invading pathogen. TLR3, TLR7, TLR8 and TLR9 all recognize different types of viral nucleic acids in endosomal compartments and trigger a signaling cascade that results in IFN type I production[10]. TLR1, TLR2 and TLR6 recognize lipopeptides, TLR5 recognizes Flagellin and TLR11 recognizes uropathogenic bacteria and all stimulated TLRs result in the production of inflammatory cytokines[11].

LPS is a fundamental element of Gram negative bacterial outer membranes[12] and signals through TLR4 and CD14 in a MyD88-dependant pathway to active the NF-κB transcriptional pathway. This will result in cytokine production such as IL-12, IL-23, and IL-27[13-17].

Dendritic cell stimulation by antigen engenders their migration and maturation, with DCs exiting the tissue and homing to the secondary lymphoid organs to present the antigen to resident T cells.

1.3 Antigen presentation

One outcome of DC-T cell interactions, also known as the immunological synapse, is T cell activation and proliferation. Naïve T cells do not traffic throughout the body, but rather make a daily circuit of the secondary lymphoid organs, sampling APCs for their cognate ligand[18]. Activated DCs are considered the only APCs capable of priming them[19]. DCs have been reported to acquire antigen by endocytosis, macropinocytosis, and phagocytosis[20, 21]. Antigenic peptides are presented in the context of both major histocompatibility complex (MHC) I and II receptor molecules[1] and antigenic lipids are presented in the CD1 family of molecules[22].

MHCI molecules present antigenic peptides that have been produced in the proteasome. The MHCI complex is assembled in the endoplasmic reticulum (ER), where it associates with β_2-microglobulin. Professional APCs have modified proteasomes that are more efficient at antigenic peptide processing[23]. The peptides then enter the ER through the transporter associated with antigen presentation (TAP) complex[24], are loaded onto MHCI molecules and transported to the plasma membrane for display[18, 25]. Presentation of antigen in the context of MHCI is recognized by cytotoxic $CD8^+$ T lymphocyte (CTLs) and CTLs ligating their cognate receptors along with co-stimulation allows for the generation of effector cells that directly kill of an infected cell. This CTL response is the major

adaptive immune response for the clearance of virally infected cells[25]. The CTL response in known to be dysregulated in human immunodeficiency virus type 1 (HIV-1) infection and has been attributed to impaired maturation and T cell dysfunction[26, 27].

In contrast to the ubiquitous presence of MHCI, the MHCII molecule is restricted to APCs[28], T cells[29, 30] and thymic epithelial cells[31], though it can be induced in other cell types in inflammatory conditions. The mechanism for MHCII presentation differs from that of MHCI presentation. The MHCII complex is trafficked from the ER to lysosomes in the endocytic pathway. Once there, cysteine proteases process the complex to prepare for antigenic presentation[32]. Lysosomal proteases generate antigenic peptides from proteins taken up by endocytosis that are loaded onto MHCII molecules[23]. The peptide:MHCII complex can then stimulate $CD4^+$ T cells[19, 33]. Once these antigen-MHC associations are formed, the endocytic capability of DCs decreases in order to allow for the accumulation of MHC complexes on the cell surface for presentation[34].

Cross-presentation occurs when peptides from an exogenous source are presented in the context of MHCI, rather than the through the MHCII pathway. There are 2 distinct pathways that are utilized in cross-presentation. The phagosomal pathway is TAP- and proteasome-dependant. The antigen is internalized in phagosomes or in macropinocitosomes, and the antigen transferred to the cytosol, where it can come in contact with proteasomes for antigenic peptide generation. The peptides can then be transported into the ER

for display. A fraction of phagosomes have been found to contain some of the antigen-presentation machinery acquired from the ER, and may be responsible for the antigenic transport in and out of the "ER-phagosomes"[35-37].

Some antigen, like viral proteins or viral-like particles, can be cross-presented by the vacuolar pathway which is TAP- and proteasome-independent[38, 39] but cathepsin S-dependant[40]. Cathepsin S is known to be active in a neutral pH environment, and can cleave peptides to be stably loaded onto MHCI complexes in the vacuole. The loaded MHCI can then be trafficked back to the membrane for presentation[25].

Closely related to the MHC family of receptors are the receptors of the CD1 family. Like MHC molecules, they are found on the cell surface when associated to β_2-microglobulin. These molecules present lipids, both self and foreign antigen, to the TCR for immune recognition [22]. Where MHCI and II sample cytosolic and endosomal antigen, CD1 antigen presentation follows a different route. The association with β_2-microglobulin occurs in the ER, travels to the Golgi where it is targeted for surface expression. Newly synthesized and β_2-microglobulin-associated CD1a-d acquire self-lipids, and are secreted to the surface[41].

As DCs mature, MHCI and II levels rise sharply. In contrast, CD1 levels remain fairly constant throughout because the internalization rate will not be altered in any dramatic fashion[42]. CD1 will also efficiently present its lipid antigen regardless of the maturation state of the dendritic cell[43].

1.4 Inducing Tolerance versus Immunity

Because DCs are responsible for presenting antigen, and therefore inducing immune protection, they may be responsible for inducing self-tolerance. When DCs pick up their antigen from the periphery, some self-antigen is also being presented[44]. Central tolerance, the clearance of self-reactive T cells as they are being produced, occurs in the thymus and is mediated by thymic DC-induced apoptosis[45]. Thymic DCs differ from other DC populations, in that they live and die in the thymus, without migration. They are generated from a thymic precursor, and their sole function seems to be the induction of self-tolerance[46].

The need for induction of peripheral tolerance becomes more marked when it is considered that all self-antigen cannot be presented in thymic tissues. Recent evidence suggests that state of maturation/activation of DCs is responsible for the induction of tolerance rather than immune responses[47, 48]. Activated DCs, those activated by danger signals, provide immune responses, while quiescent DCs will induce tolerance[49].

1.5 Dendritic Cell Phenotypes

HLA ABC is one gene product from the MHCI region of the chromosome, and HLA DR is a gene product generated from the MHCII region of the chromosome. Both of these molecules are expressed in DCs[1]. CD1a, CD1c and CD1d are molecules of the CD1 family of lipid antigen presenting molecules, and are considered DC markers[22, 42, 50].

CD80 and CD86 are costimulatory molecules that are found on activated monocytes and on iDCs, but at a low levels[51, 52]. These two molecules bind CD28

on T cells, which will result in IL-2 production, and T cell proliferation. In contrast, CD80 and CD86 can also bind CD152, for which they have a higher affinity. Signalling through this complex will inhibit T cell proliferation.

CD83 is a DC marker that is present only on activated DCs[53, 54], and its unidentified ligands are expressed on monocytes, some T cell subsets and DCs. Its function is as yet unclear, but a few reports indicate an immunosuppressive effect of the soluble extracellular receptor on DC-dependant T cell activation[55]. CD38 is an ectoenzyme linked to lymphocytic activation, capable of intracellular signalling and is important in the release of Ca^{2+}. It is present on monocytes and iDCs, and upregulated on DC maturation. It has been reported that CD38 signalling occurs through NFκB, and is important in CD83 upregulation and IL-12 secretion[53]. This factor may also be involved in the signalling and secretion of IL-23 and IL-27.

CD11c is a scavenger receptor, and while it is present on monocytes, high expression of this cell surface molecule is considered a DC trait. CD14 is a receptor involved in TLR4 signalling and is considered a monocyte marker, though its expression is not restricted to this cell type. Conversely, its expression is low in DCs[1].

CCR5 is an inflammatory chemokine receptor expressed on monocytes and iDCs. Its expression is downregulated on mature DCs as DCs lose their ability to respond to inflammatory chemokines and acquire the lymphoid tissue homing chemokine receptors. This is one of many chemokine receptors required

for DCs to extravasate from the blood to the peripheral tissue, and for extravasation from the peripheral tissue to the afferent lymph[2].

1.6 The Interleukin-12 Family of cytokines

Interleukin-12 (IL-12) is a 70kDa heterodimeric protein, composed of two subunits, p40 and p35. It is mainly produced by DCs and monocytic cells in response to intracellular pathogens including fungi, virus and intracellular bacteria[56]. IL-12 is produced early in the immune response to invading pathogens by ligation of TLRs or CD40 in the context of antigen presentation and binds to its receptor composed of IL-12Rβ1 and IL-12Rβ2 chains[57, 58] to result in interferon-γ (IFN-γ) production from natural killer cells and T cells and in the skewing of naïve $CD4^+$ T cells to the T_H1 phenotype[56, 59]. IL-12 is critical to the elimination of intracellular parasitic and bacterial pathogens and some viral infections[15, 60-62].

The simultaneous expression and production of p35 and p40 is essential to the secretion of the biologically active IL-12p70 molecule, and yet the molecules are not regulated in the same fashion. IL-12p35 is constitutively produced, while the expression of the p40 product must be induced by stimulation. The p40 gene product is induced via the MAP kinase pathway. In the context of HIV-1 infection, it has been previously reported by our group that HIV-1 disrupts MAP kinase activity and the subsequent binding of nuclear factors to the p40 promoter. This results in decreased IL-12p40 production[15, 63]. Two novel interleukins, IL-23 and IL-27, which share structural similarities with IL-12, have now been associated with the helper T cell differentiation pathway.

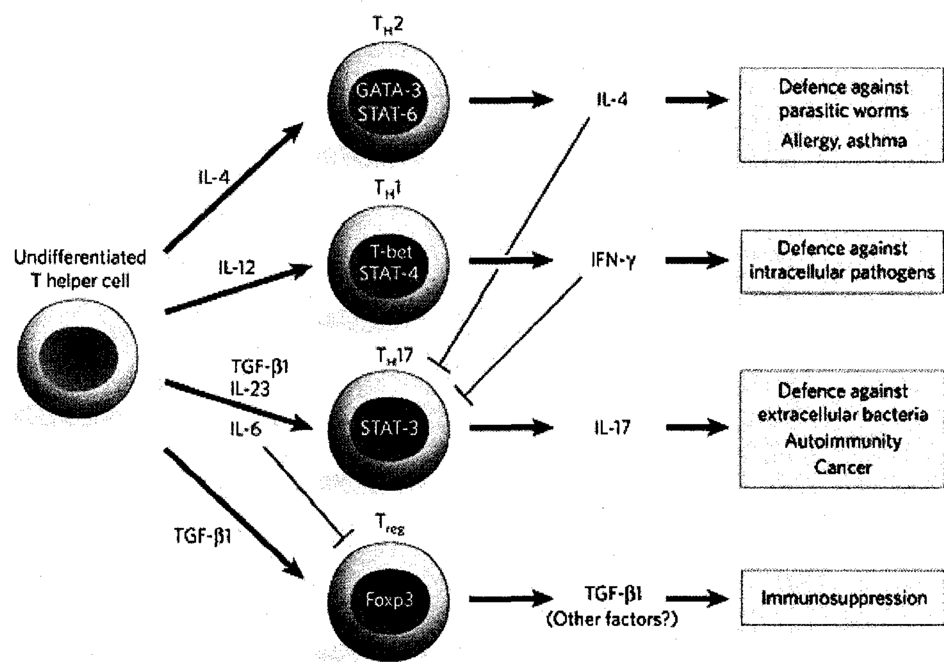
1.7 INTERLEUKIN-23

IL-23 was discovered by a computational analysis for IL-6 paralogs. IL-23 is formed from the same p40 subunit as IL-12 and a different p35-like subunit called p19 linked through a disulfide bond[64]. Like IL-12, it is primarily secreted by monocytes, macrophages and DCs. Because it shares the p40 subunit with IL-12, the receptor has the IL12Rβ1 chain but is associated with the IL-23R molecule[58, 65]. The expression of IL-23p40 and p19 subunits has been reported, in macrophages, to be regulated by the phosphatidylinositol 3-kinase (PI3K), mammalian target of the rapamycin/70 kDa ribosomal S6 kinase 1 (mTOR/S6K1) and p38 mitogen-activated protein kinase (MAPK). It has been linked to autoimmune disease in the brain through overactivation of macrophages. IL-23 has been shown to induce IFN-γ and IL-12 production by DCs and in a CMV plasmid system, IL-23 induced longer-lasting $CD4^+$ and $CD8^+$ T cell immune responses than IL-12[66]. IL-23 induced the proliferation and expression of IFN-γ by T cells, preferentially expanding the memory T cell subset[64]. This contrasts with IL-12, which preferentially induces proliferation in naïve T cells. IL-23 is known to induce the activation of Jak2, STAT1, -3 -4, -5[67]. It's gene expression is regulated by c-Rel, a member of the NFκB family[68], similarly to IL-12[15]. IL-23, like IL-12 is important in the bridging of innate and adaptive responses and the host's ability to eliminate pathogens[62, 69-71]. Impaired cytokine signalling at the time of antigenic peptide presentation by DCs can result in improper responses by the adaptive immune system.

IL-23 has been shown to induce IL-17 production from a specific type of Th cell called Th17 T cells[72] and has been implicated in autoimmune disorders[73, 74] and in the negative regulation of allergic asthma[75]. It has also been shown to induce the expression of IL-22 by Th17 cells in a dermal inflammation model. Normally, IL-17 is involved in the resistance to pathogens like *Toxoplasma gondii*[76] and *Klebsiella pneumoniae*[61, 77]. However, it has also been linked to several models of autoimmunity, including rheumatoid arthritis[78], multiple sclerosis, systemic lupus erythematosus[79], inflammatory bowel disease[80], and cystic fibrosis[81]. While *in vitro* Th17 generation requires only IL-6 and transforming growth factor β1 (TGF-β1), IL-23 is required *in vivo* for Th17 cell function (see figure1), and recent evidence suggest that IL-21, IL-23 and TGF-β1, without IL-6, could induce IL-17 expression[82]. Interestingly, IL-23 was shown to be a potent inducer of antitumor immunity when administered systemically[83], though this effect is controversial[84, 85].

Figure 1 – Polarization Pathways of Naïve T cells

The fate of naïve $CD4^+$ T cells is determined by the cytokine signalling that occurs at the time of their activation. When IL-4 is present, the Th2 T cell phenotype develops and the Th2 cell also produces IL-4, a cytokine critical to protection against parasites. Production of IL-4 suppresses the development of Th17 cells. If IL-12 is present, the Th1 phenotype is produced and the Th1 cell goes on to produce IFN-γ which is important to the successful clearance of intracellular pathogens. IFN-γ also suppresses Th17 cell differentiation. The presence of TGF-β1, IL-23 and IL-6 will produce a Th17 cell, and this Th17 cell will secrete IL-17, which is important to defense against extracellular bacteria and the anti-tumour response. Presence of TGF-β1 alone will produce a regulatory T cell capable of itself secreting TGF-β1, along with other factors, and is important to controlling the immune response. The presence of IL-6 will suppress the regulatory T cell developpement. Reprinted by permission from Macmillan Publishers Ltd: Nature[86] © 2006



T_H2
GATA-3
STAT-6
IL-4
Defence against
parasitic worms
Allergy, asthma
IL-4
T_H1
Undifferentiated
T helper cell
IL-12
T-bet
STAT-4
IFN-γ
Defence against
intracellular pathogens
T_H17
TGF-β1
IL-23
IL-6
STAT-3
IL-17
Defence against
extracellular bacteria
Autoimmunity
Cancer
T_{reg}
TGF-β1
Foxp3
TGF-β1
(Other factors?)
Immunosuppression

1.8 INTERLEUKIN-27

IL-27 was identified in 2002 by Pflanz *et al*[87] by computational screening for IL-12p35 subunit and IL-6 family paralogs. Like IL-12, IL-27 is a heterodimeric protein, composed of a p40-like subunit called Epstein-Barr virus-induced gene 3 (EBI3), and a p35-like subunit, p28 [88]. The IL-27 receptor is composed of two chains; gp130, a receptor chain common to other cytokine receptors of the IL-6 family like IL-6, IL-11, OSM, G-CSF, and LIF[89], and T cell cytokine receptor (TCCR/WSX-1), a type 1 cytokine receptor[90]. This receptor is found at the highest expression levels on resting NK cells, resting NKT cells, regulatory T cells, effector T cells and memory T cells[91], and at lower levels is found on monocytes, B lymphocytes, mast cells and endothelial cells[90]. The expression of p28 has been reported to be regulated by the Toll/IL-1R-containing adaptor inducing IFN-beta and its associated IFN regulatory factor (IRF) 3 transcription factor in human DCs[17]. In macrophages, the expression of the p28 gene has been shown to be regulated by the MyD88 and IFN-γ pathways[92]. EBI3 expression is induced on TLR ligation by MyD88 and NFκB activation.[93]

Upon receptor ligation, the Jak/Stat signalling pathway is induced through activation of Jak1, Jak2, STAT1, 2, 3, 4 and 5[88, 94, 95]. Activation of STAT1 induces T-bet expression and the subsequent upregulation of the IL-12 receptor chain β2 important in the generation of a Th1 response (IL-12Rβ2)[96]. STAT1 activation also suppresses the expression of GATA-3, a crucial transcription factor in the T_H2 differentiation pathway that has been shown to directly inhibit $CD4^+$ T_H1 cell functions[97].

Figure 2 – IL-27 activates different pathways to obtain opposing results

In the initial secretion of IL-27 at the initiation of the immune response, IL-27 will signal through its receptor, WSX1/gp130, activate Jak1, Jak2 and Tyk2, which activate STAT3 to induce proliferation in the target cell.

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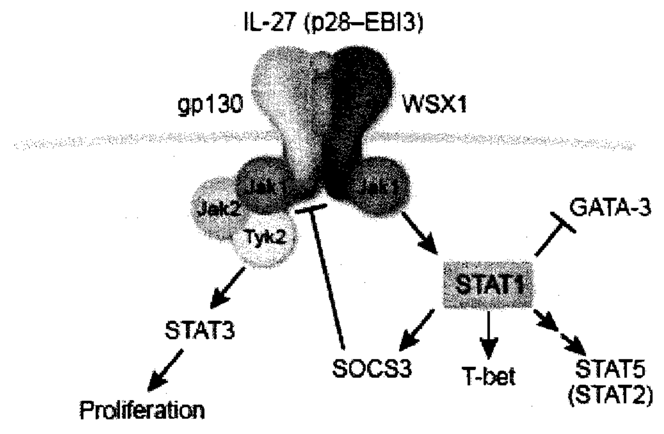

IL-27 (p28–EBI3)
gp130
WSX1
GATA-3
STAT1
STAT3
Proliferation
SOCS3
T-bet
STAT5
(STAT2)

IL-27 has been implicated in the suppression of IL-2 production by activating Suppressor of Cytokine Signalling 3 (SOCS3) in a negative feedback mechanism[99].The T cell growth factor IL-2 is the major cytokine that is produced by Th0 cells. Once there is commitment to a T_H polarization pathway, the expression of IL-2 declines to be replaced by IL-12 (T_H1 commitment) or IL-4 (T_H2 commitment) [100]. Deficiencies in IL-27 signalling, whether from knockout[101-103] of the receptor or the cytokine, have resulted in exaggerated and lethal T cell-mediated inflammation[101, 104-106].

IL-27 has been reported to induce T cell proliferation and augment the production of IFN-γ by Th1 cells[87, 94, 96, 107, 108]. This secretion of IFN-γ is critical to the successful clearance of many pathogens, including *Listeria monocytogenes* and *T. gondii*. It has also been reported as being critical in the regulation of IL-17-mediated inflammation in the brain via activation of STAT1 by suppressing Th17 cell development[101], and to the suppression of T cell hyperproliferation induced by IL-6[106].

1.9 Dendritic Cells & Human Immunodeficiency Virus-1

The main sites of HIV transmission are the mucosal surfaces, and DCs are among the first cells to be targeted[109, 110]. DCs express, among many others, the chemokine receptors CCR5, CXCR4 and low levels of CD4. The CD4 receptor is the main receptor for viral attachment in viral entry, and the strain of HIV-1 determines which coreceptor will be used; for an R5 (macrophage tropic) strain, the CCR5 coreceptor is used and in an X4 (T cell tropic) strain, CXCR4 is the coreceptor[111]. Varying levels of these receptors can be found on different DC

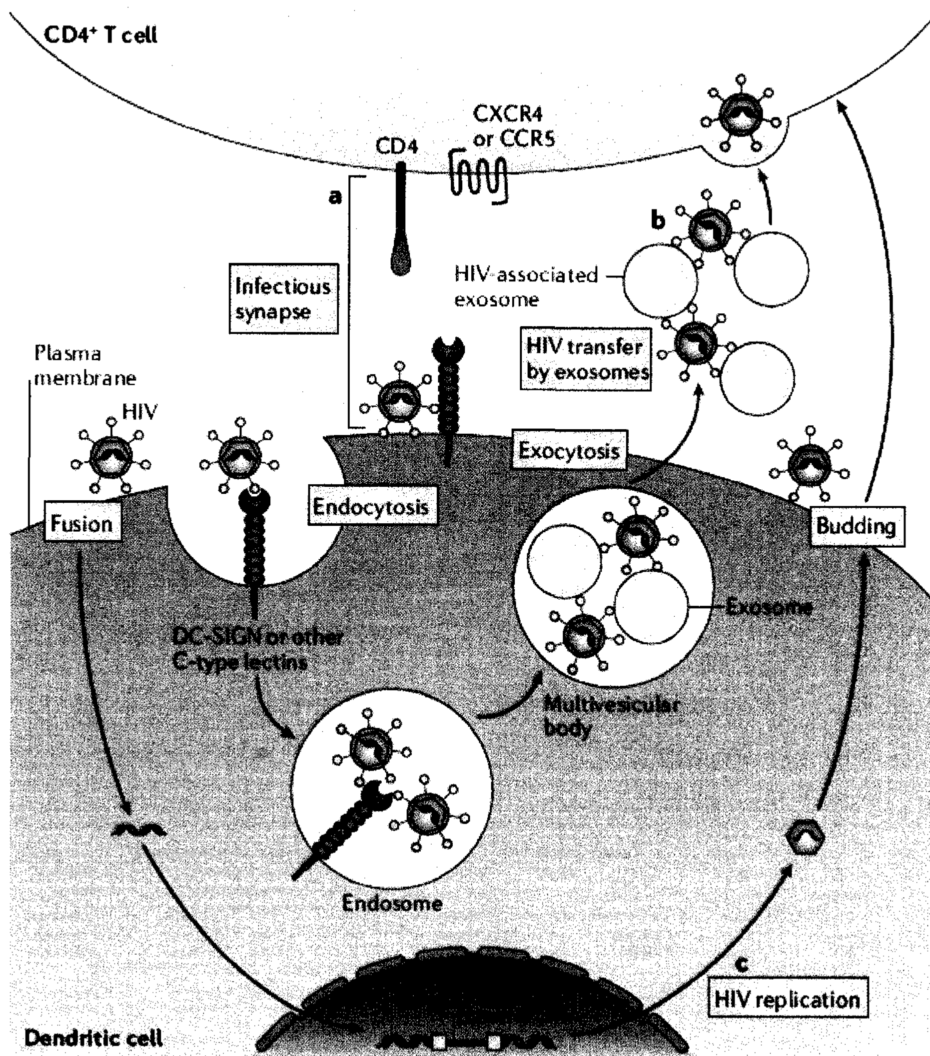
subsets, and myeloid DCs are more easily infected with R5 strains of HIV-1. The frequency of HIV-1 infection in DCs is 10 to 100 times less *in vivo* than in $CD4^+$ T cells, the main target of HIV-1 infection[112]. DCs are not readily infected, as only 1-3% of cells become productively infected[113], but can interact with HIV-1.

There are a few mechanisms by which it is suggested that DCs may transmit the virus to T cells (see figure 3). DCs carrying HIV bound to DC-SIGN have been suggested as a mechanism for the spread of HIV to T cells at the immunological synapse[114-117]. Some studies show infected DCs as being the source of infection of T cells, while others suggest that HIV is internalized bound to lectin receptors. Regardless of the mode of transfer, DCs seem to be an important vehicle for transport of HIV from the periphery to the lymphoid organs, a T cell rich environment[109].

DCs from HIV-infected individuals have been shown to have decreased ability to induce a primary immune response, whether by reduced antigen presentation capabilities[118], or by a cytokine environment that is not conducive to DC maturation. Studies have been performed showing reduced DC numbers in the blood of HIV-infected individuals[119-121]. A correlation has been established where active replication of the virus corresponds to lower blood DC levels, both pDC and mDC, and that these lower numbers of pDC are correlated to disease progression and opportunistic infection development[122].

Figure 3 – Mechanisms of DC-mediated HIV transmission

Two types of mechanisms for HIV transmission have been proposed. The trans-infection of T cells by DCs occurs in two pathways (a, b). The cis-infection pathway is shown in (c). a) DCs transfer captured HIV to CD4 T cells at the infectious synapse through cell-cell junctions. DC-specific intercellular adhesion molecule 3 (ICAM3)-grabbing non-integrin (DC-SIGN) is involved in the infectious synapse formation. b) HIV endocytosed into exosomes can be released and transmitted to the target CD4 T cell by exosomal membrane binding and fusion. c) HIV can directly productively infect DCs to release newly produced virions. CCR5, CC-chemokine receptor 5; CXCR4, CXC-chemokine receptor 4. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews, Immunology[111] © 2006


CD4⁺ T cell
CXCR4
or CCR5
CD4
a
b
Infectious
synapse
HIV-associated
exosome
Plasma
membrane
HIV
HIV transfer
by exosomes
Exocytosis
Fusion
Endocytosis
Budding
Exosome
DC-SIGN or other
C-type lectins
Multivesicular
body
Endosome
c
HIV replication
Dendritic cell

DCs from HIV-infected individuals have a decreased ability to stimulate T cells to proliferate[123] which may be caused by a reported decrease in the expression of costimulatory molecules CD80 and CD86 on the DC surface membrane[124]. HIV can induce the maturation of DCs, both epidermal DCs and monocyte-derived DCs,[125] however, other studies reported that the cytokines secreted by these DCs have been altered when compared to healthy individuals. One reported a decrease in IL-12 production, a cytokine important to effective Th1 development[126]. One reported increased IL-10 production, an immune-suppressive cytokine, and a reduced capacity to induce T cell proliferation when compared to healthy controls[110].

The HIV protein Tat has been shown to induce the production of chemoattractants in DCs to attract activated T cells and macrophages, the targets of HIV-1[127, 128]. One of the first genes expressed during HIV replication is the transcriptional activator Tat protein [127, 129], a 14kDa polypeptide. It binds to the transactivation response (TAR) element at the 5' end of the HIV-1 transcript, and promotes the initiation and elongation of the HIV-1 transcript. Interfering with the TAR/Tat interaction is sufficient to inhibit transcriptional activation of HIV-1 gene expression[130].

Tat is known to affect uninfected cells. The secretion of full-length Tat proteins by HIV-infected cells has been found in culture media. It is known to be internalized by many different cell types and affect their cellular functions[131, 132]. One study reported that different concentrations of Tat affected Kaposi's Sarcoma cells (KS cells) differently. When recombinant Tat was added a low

concentrations (0.1ng-1ng/mL), it stimulated KS cell growth, but had little effect on HIV replication. When the concentration was increased to greater than 100ng/mL, the replication of HIV was enhanced[131]. Another reported that biologically active Tat protein was effectively taken up by mature DCs. This is despite the downregulation of the ability to take up antigen by phagocytosis and macropinocytosis as DCs mature. Uptake of active Tat in this model induced the upregulation of MHC and costimulatory molecules and secretion of IL-12, TNFα and β-chemokines when compared to inactivated Tat protein[132].

Little is known about the effects of HIV-1 on IL-23 or IL-27, or of the effects of IL-23 and IL-27 on HIV-1. HIV-1 replication of both the X4 and R5 viral strains was inhibited when human papilloma virus-like particles induced IL-27 production in PBMCs, or added to $CD4^+$ T cell or macrophage cultures[133]. And in the context of HIV-1 infection, previous work has shown the p40 subunit of IL-23 to be downregulated through disruption of the MAPK signalling pathway and of nuclear factors binding[15, 63] and serum levels of IL-23 and IFN-γ in HIV-infected patients were decreased[134].

The role of HIV-1 and the HIV Tat protein have not been investigated in the context of IL-23 and IL-27 expression by DCs. Because DCs are at the site of infection, have varying phenotypes and are important in the initiation of proper cell-mediated immunity necessary to the successful clearance of virally infected cells, the presence of HIV and HIV Tat protein may affect the expression of IL-23 and IL-27 by DCs. Moreover, these effects may differ among the different DC populations.

1.10 Hypothesis

The presence of HIV-1, and the presence of the accessory protein Tat, will alter the expression of cytokines IL-23 and IL-27 in DCs.

1.11 Specific Aims

Aim 1 – To isolate and characterize human dendritic cells from various sources by flow cytometry

Aim 2 – To characterize the expression of IL-23 and IL-27 by DCs in response to LPS and the effects of HIV-1 and HIV-1 Tat protein on IL-23 and IL-27 expression

2. Materials and Methods

2.1 Dendritic Cell Isolations

2.1.1 Epidermal and Dermal Dendritic Cells

Skin tissue was obtained with the informed consent of donors undergoing elective abdominoplasties at the Ottawa Hospital. To isolate tissue-resident epidermal and dermal dendritic cells, the methodology was adapted from techniques previously described[50, 135] with some modifications. Skin tissue was obtained at the time of removal and placed in cold phosphate buffered saline (PBS) (Invitrogen, Burlington, Ontario). Tissue processing occurred within hours. Fatty tissues were removed and skin tissues were cut into pieces approximately 3-5mm by 3-5mm. Tissues were placed in 1.2U/mL of dispase II (Invitrogen) in RPMI (Invitrogen) supplemented with 10% fetal calf serum (FCS) (HyClone, Logan, Utah), 10mM L-glutamine (Sigma-Aldrich, Oakville, Ontario) and 10mM streptomycin (Sigma-Aldrich) overnight at 37°C rather than 4°C. The next day, the epidermal section was mechanically separated from the dermal sections using tweezers. The epidermal sections were then placed in trypsin (Invitrogen) 2.5μg/mL and 0.05U/mL of DNase I (Sigma-Aldrich) for 30 minutes. Aggregates were then filtered using a 70μm Nylon cell strainer (BD Falcon, Mississauga, ON Canada) and cells washed twice in PBS, then resuspended at 1 x 10^6 cells/mL. Cells were then selected for the expression of CD1a and CD1c by magnetic bead separation[50] using the AutoMACS system (Miltenyi Biotech, Auburn, CA) rather than by Ficoll/NaCl density gradient centrifugation[135].

To isolate dermal dendritic cells, dermal sheets obtained after the epidermal separation were placed in RPMI supplemented with 10% FCS, 10mM L-glutamine and 10mM streptomycin for 2 days at 37°C. Cells that had migrated out were then selected for magnetic bead separation by CD1a and CD1c (Miltenyi Biotech), adapted from the technique describe for epidermal DCs[50]. Cells were then analysed by flow cytometry for the expression of the following surface molecules; CD38, CD80, CD86, CD83, MHC I and MHC II, CCR5 and CD1d and using the following antibodies; CD38-PE, CD83-PC5, HLA-ABC-FITC, HLA-DR-PE (Beckman Coulter, Fullerton, CA) CD80-FITC, CD86-PC5, CCR5-FITC and CD1d-PE (BD Pharmingen, Mississauga, Ontario).

2.1.2 Circulating Dendritic Cells

Circulating DCs were isolated according to the manufacturer's protocol (Miltenyi Biotech DC isolation kit). Peripheral blood was obtained from healthy donors by venipuncture. Peripheral blood mononuclear cells (PBMCs) were obtained by density gradient centrifugation using Ficoll-Hypaque (GE Healthcare, Mississauga, Ontario) at 1600 RPMS without brake. PBMCs were then washed twice in PBS (Invitrogen) then subjected to CD19-depletion, followed by CD1c magnetic bead separation by AutoMACS (Miltenyi Biotech).

Cells were then analysed by flow cytometry for the expression of the surface molecules CD38, CD80, CD86, CD83, MHC I and MHC II, CCR5 and CD1d and using the following antibodies; CD38-PE, CD83-PC5, HLA-ABC-FITC, HLA-DR-PE (Coulter) CD80-FITC, CD86-PC5, CCR5-FITC and CD1d-PE (BD Pharmingen).

2.1.3 Monocyte-derived Dendritic Cells

Monocytes were obtained from PBMCs by negative selection according to the manufacturer's protocol (monocyte isolation kit II, Miltenyi Biotec). Monocytes were labelled with a biotin-conjugated cocktail of antibodies to remove T cells (CD3, CD7), granulocytes (CD16), NK cells (CD56), B cells (CD19), plasmacytoid DCs (CD123) and erythroid cells (CD235a). Cells were then counted and resuspended at 1 x 10^6 cells/mL in RPMI supplemented with 10% FCS, 10mM L-glutamine and 10mM streptomycin, 20ng/mL of IL-4 and 10ng/mL of GM-CSF (R&D Systems, Minneapolis, MN). Cells were allowed to adhere, and cultured for 6 days at 37°C with 5% CO_2 in a humidified atmosphere. After the 6 day culture, cells were washed from the supernatants and assayed by flow cytometry for the expression of the surface molecules CD1a, CD1c, CD11c, CD38, CD80, CD86, CD83, MHC I and MHC II, CCR5 and CD1d and using the following antibodies; CD1a-PE, CD1c-FITC, CD11c-PC5, CD38-PE, CD83-PC5, HLA-ABC-FITC, HLA-DR-PE (Coulter) CD80-FITC, CD86-PC5, CCR5-FITC and CD1d-PE (BD Pharmingen).

2.2 Flow cytometry

All flow cytometry was performed on a Coulter Altra flow cytometer (Beckman Coulter). Cells were stained with fluorochrome-labelled monoclonal antibodies to the surface molecules, CD1c-(Miltenyi Biotech), CD38, HLA-ABC, HLA-DR, CD83, CD1a, CD11c (Coulter) CD80, CD1d, CD86, CCR5 (BD Pharmingen) and the intracellular DNA-binding dye 7-amino-actinomycin-D (7AAD) (Calbiochem, San Diego CA). Events were captured using the Expo32

analysis software, and analysed using the FCS Express analysis software and WinMDI version 2.9.

2.3 Retroviral expression of Tat wt

Endogenous expression of the HIV-1 Tat wild type (Tat wt) protein was achieved using the retroviral vector pLXIN expressing the tat wt gene sequence (pTat). The tat wt sequence was inserted into the vector, and was transfected into the packaging cell line PT67 (BD Biosciences, Mississauga, Ontario) as previously described[136]. PT67s were cultured in DMEM medium (Invitrogen) containing 10% FCS, 10mM L-glutamine and 10mM streptomycin in T-75 culture flasks (Falcon). Supernatants containing the pLXIN empty vector or pTat were collected after 24h cell passaging. Cells were passaged 4 times, and all supernatants were combined, before being frozen in 3mL aliquots at -80°C. The retroviral supernatants were quick thawed in a 37°C water bath before use, and any excess discarded.

For the infection, $2x10^6$ DCs were incubated with 3mL of pTat or pLXIN-containing supernatants for 24h. The media-controlled cells were incubated with 3mL of DMEM supplemented with 10% FCS, 10mM L-glutamine and 10mM streptomycin. Mock infection was done with conditioned media collected from PT67 containing no retroviral vector cultured in DMEM media supplemented with 10% FCS, 10mM L-glutamine and 10mM streptomycin for 24h.

The presence and absence of the tat wt gene was assayed by RT-PCR using primers for the tat wt sequence:

Fwd: 5' TCGACCCGCGTCTGGAAC 3'

Rev: 5' AAGCAAACCTGGCAGTGGAAG 3'

The PCR product was then run on a 1.5% agarose gel prepared with TAE buffer, and ethidium bromide used as the detection method.

2.4 Recombinant Tat Preparation

Recombinant Tat protein (rTat) was obtained from Applied Biosciences. To ensure that the recombinant Tat was endotoxin free, the Tat protein preparation was treated with Polymyxin B-coated agarose beads (Sigma) as previously described[136]. For treatment of DCs, rTat was used to pre-treat $2x10^6$ cells for 1h. The rTat was added to the cultures in a dose response experiment ranging from 10ng to 1000ng.

2.5 Human Immunodeficiency Virus-1 Cultures

For the HIV-1 cultures, $2x10^6$ DCs were exposed to the dual-tropic strain of HIV-$1_{92HT593}$ (1 to 10 ng/mL of p24) obtained from the NIH (NIH AIDS Research and Reference Reagents Program, Germantown, MD) for 24h. Alternatively, mock infection was performed using viral preparations that were heat-killed at 65°C for 3h. No cytokine or polybrene was added.

2.6 LPS Stimulation and RNA extraction

All cell stimulations were performed with LPS (Sigma) for 4 hours at a concentration of 1µg/mL. Subsequently, RNA was extracted using the RNeasy Mini Kit (Qiagen, Mississauga, Ontario) according to the manufacturer's

instructions. With the HIV-1 experiments, the QiaShredder columns (Qiagen) were also used in place of a 28-gage needle to disrupt cellular membranes.

2.7 RT-PCR

1000ng of RNA was used in each RT reaction in a total 60µL volume. To make a dNTP mixture, ATP, GTP, CTP and TTP (Invitrogen) were added in equal parts. The reaction mix was performed as follows: 2µL random primers (Invitrogen), 2µL dNTPs and 40µL (DEPC H_2O/RNA) was mixed, then denatured at 65°C for 5min, followed by rapid cooling on ice for 2min. After a quickspin, 8µL of 5X buffer (Invitrogen), 4 µL of 0.1M dTT (Invitrogen), 2 µL of RNase Inhibitor (Invitrogen) and 2 µL of Superscript II (Invitrogen) was added to the reaction mixture.

The IL-23p19, IL-27EBI3 and S18 genes were assayed using the SYBR Green systems from Applied Biosciences as per the manufacturer's instructions, using the following primer sequences;

IL-23p19 Fwd: 5' CGTCTCCTTCTCCGCTTC 3'

IL-23p19 Rev: 5' ATCTGAGTGCCATCCTTGAG 3'

IL-27EBI3 Fwd: 5' CCGCCTGCTCCAAACTCC 3'

IL-27EBI3 Rev: 5' GCTTGATGATGTGCTCTGTTATG 3'

S18 Fwd: 5' CTGCCATTAAGGGTGTGG 3'

S18 Rev: 5' TCCATCCTTTACATCCTTCTG 3'

These primers were designed using the Beacon 4.0 software to evaluate binding, secondary structures and GC content. The IL-27p28 subunit was assayed using the TaqMan probe assay (Applied Biosystems, Roche), as per the manufacturer's instructions. The primer sequences were not provided.

Each real-time PCR was performed with a standard curve, which consisted of a ten-fold serial dilution of the gene standard with duplicates of each dilution point, ranging from 5×10^{-11}g to 5×10^{-15}g. PCRs with standard curve R-squared values of less than 0.94 were discarded and repeated. Samples with comparable PCR efficiencies as determined by the standard curve were compared. Each sample was processed as a triplicate with 400ng of template per reaction for the SYBR Green assays and 100ng of template for the TaqMan probe assay, with the average Ct value evaluated.

To quantitate the gene expression levels, the relative expression $\Delta\Delta C_T$ method of calculation was employed[137].

$$\Delta C_T = C_T \text{ target} - C_T \text{ reference}$$

$$\Delta\Delta C_T = \Delta C_T \text{ test sample} - \Delta C_T \text{ calibrator sample}$$

The target refers to the gene of interest and the reference is the housekeeping gene, S18, a ribosomal protein. Values were discarded when they fell below the detection levels for the negative controls, or outside the range of the standard curve. Thus, changes in gene expression in each treatment are evaluated in relation to the levels induced by LPS, which is used as the calibrator sample, and are expressed as fold change from LPS-induced gene expression.

2.8 Statistical Analysis

The statistical significance of differences in cytokine expression by DCs with after exposure to rTat and HIV-1 was analyzed using Kruskal-Wallis one-way ANOVA. In addition, the multiple comparisons to the control group were determined by Dunn's Method or Holm-Sidak Method. The statistical significance of the cytokine expression by DCs with pTat was analyzed by the Students *t*-test.

3. RESULTS

3.1 DENDRITIC CELL ISOLATION

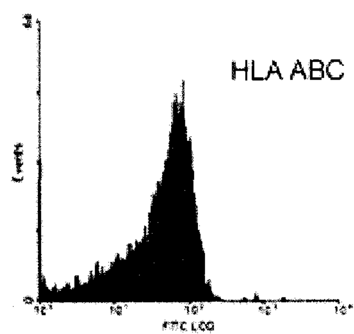
The first objective of this project was to isolate and characterize human DCs from different sources, and to characterize their expression of different surface markers by flow cytometry. Cells from different human tissues were isolated, and flow cytometry was used to phenotype them. Negative signals have been subtracted from all signals displayed on the single parameter histograms.

3.1.1 EPIDERMAL DENDRITIC CELLS

Skin tissues were obtained from plastic surgery and extra tissues removed. After overnight dispase II digest, the epidermal tissue was mechanically separated from the dermal tissue. The epidermal tissue was then digested with trypsin and DNase to obtain a single cell suspension, and then the cells were washed and bead isolated using magnetic microbeads specific for CD1a and CD1c. Cells that were isolated from the epidermal skin layer are consistent with the phenotype associated with immature epidermal DCs[50, 135] in that they were positive for HLA ABC and HLA DR and DC marker CD1d^{low} as seen in Figure 4. They had low CCR5 expression, a chemokine receptor that is downregulated in mature DCs as they prepare to exit the tissue, and low expression of both CD83 and CD38. The absence of strong CD80 and CD86 signals confirms that there were not mature.

Figure 4– Epidermal dendritic cell phenotype

Dendritic cells isolated from the epidermal layer of skin by overnight dispase II digestion and CD1a/CD1c microbeads by positive selection. Phenotype is determined by flow cytometry using HLA ABC-FITC (MHCI molecule), HLA DR-PE (MHCII molecule), CD83-PC5 (DC maturation marker), CCR5-FITC (chemokine receptor), CD1d-PE (DC marker), CD80-FITC (maturation marker), CD38-PE (immune cell activation marker) and CD86-PC5 (maturation marker).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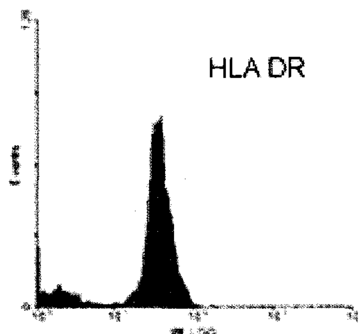
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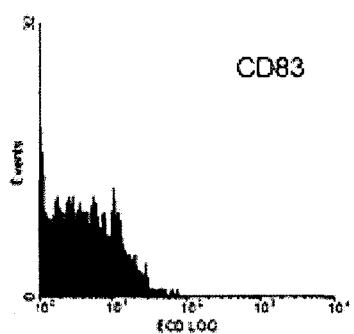
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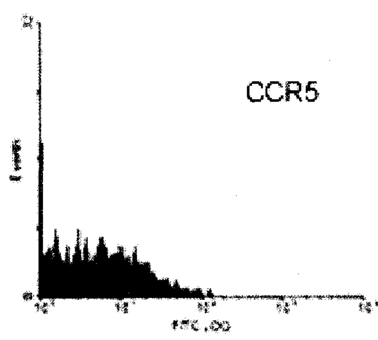
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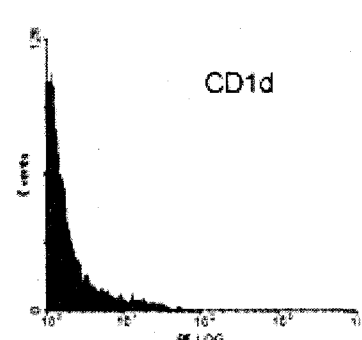
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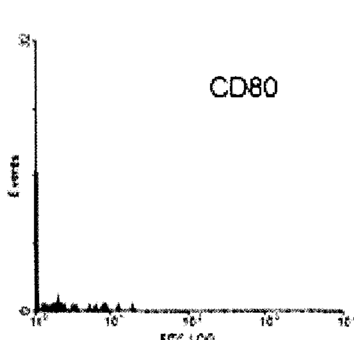
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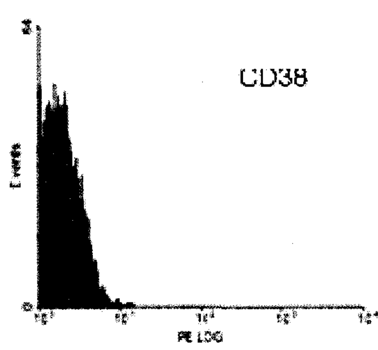
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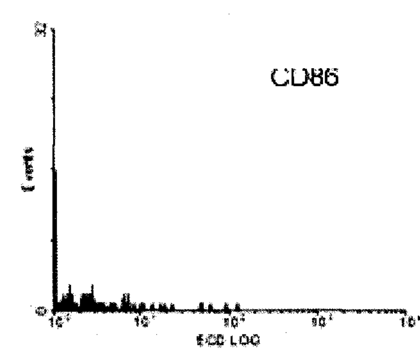

CD86

3.1.2 Dermal Dendritic Cells

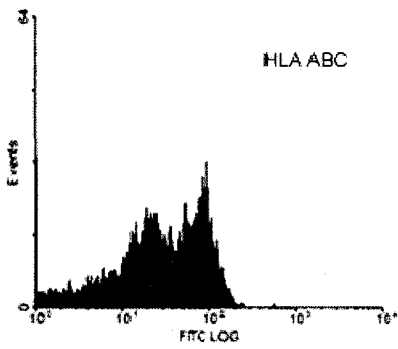
Skin tissues were obtained from plastic surgery and extra tissues removed. After overnight dispase II digest, the epidermal tissue was mechanically separated from the dermal tissue. The dermal tissues were then returned to the incubator for 2 days to allow for the migration of DCs out of the tissues. The cells were collected from the supernatant and bead isolated using magnetic microbeads specific for CD1a and CD1c. Cells isolated from the dermal layer of the skin have a phenotype that is consistent with that of dermal DCs[50, 135]. As seen in Figure 5, they are CD1d^{low}, HLA ABC^{+} and HLA DR^{+}. The expression of CCR5 is low. Similar to epidermal DCs, there are early signs of activation as indicated by low CD83 expression, but no upregulation of CD80, CD86, or CD38 to indicate a fully mature phenotype.

3.1.3 Circulating Dendritic Cells

Whole blood was isolated by venipuncture from healthy individuals, and PBMCs separated out from whole blood by Ficoll-Hypaque density gradient. Cells isolated from PBMCs using the CD1c magnetic microbead possess the markers expected from circulating DCs[1]. As observed in Figure 6, they are HLA ABC^{+}, HLA DR^{+} and CD1d^{+}. The expression of CD1d is the highest observed within the isolated populations. These cells are also CCR5 positive. They do not express CD80, CD83 or CD86, and have high CD38 expression.

Figure 5– Dermal dendritic cell phenotype

Dendritic cells isolated from the dermal layer of skin by extratissue migration and CD1a/CD1c microbeads by positive selection. Phenotype is determined by flow cytometry using HLA ABC-FITC (MHCI molecule), HLA DR-PE (MHCII molecule), CD83-PC5 (DC maturation marker), CCR5-FITC (chemokine receptor), CD1d-PE (DC marker), CD80-FITC (maturation marker), CD38-PE (immune cell activation marker) and CD86-PC5 (maturation marker).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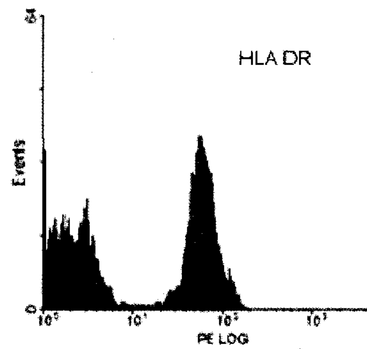
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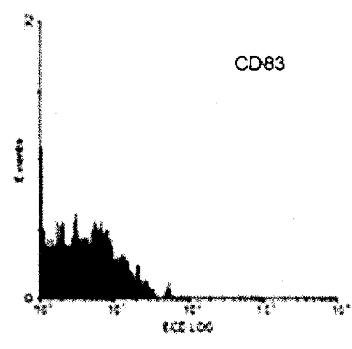
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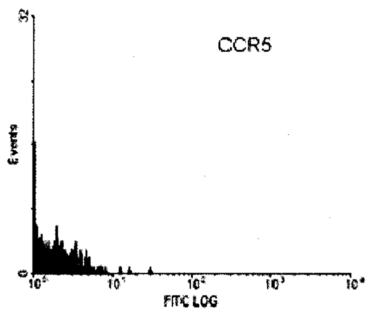
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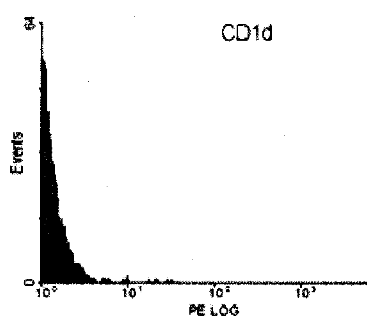
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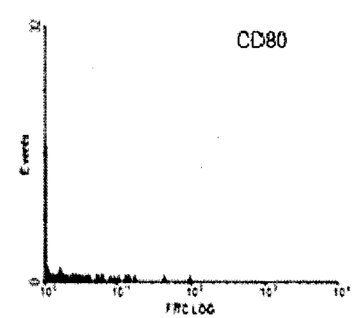
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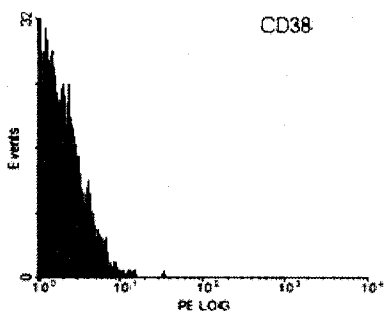
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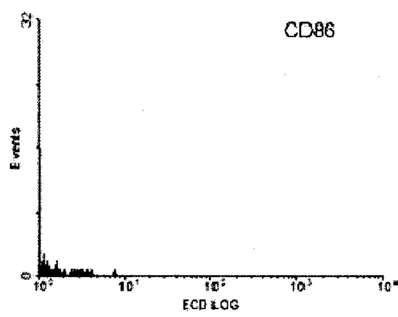
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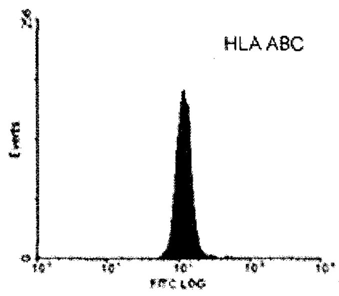
Figure 6 – Blood-derived dendritic cell phenotype

Dendritic cells isolated from blood by CD1c microbeads by positive selection. Phenotype is determined by flow cytometry using HLA ABC-FITC (MHCI molecule), HLA DR-PE (MHCII molecule), CD83-PC5 (DC maturation marker), CCR5-FITC (chemokine receptor), CD1d-PE (DC marker), CD80-FITC (maturation marker), CD38-PE (immune cell activation marker) and CD86-PC5 (maturation marker).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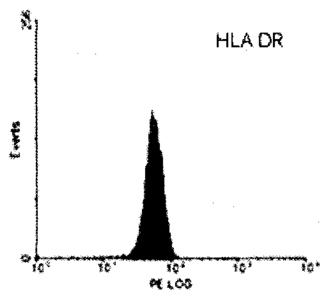
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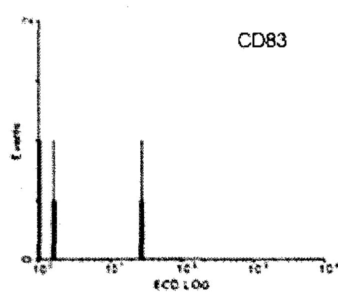
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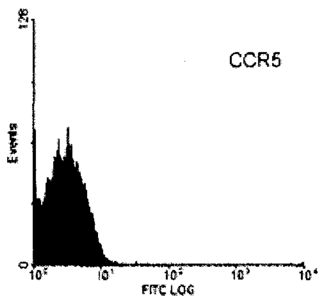
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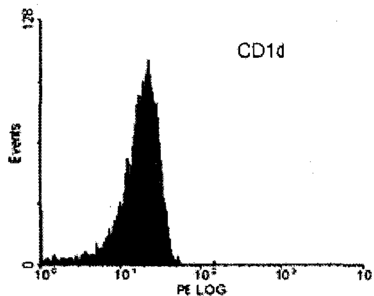
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Events
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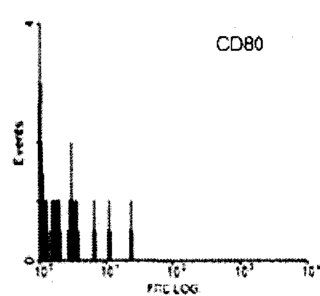
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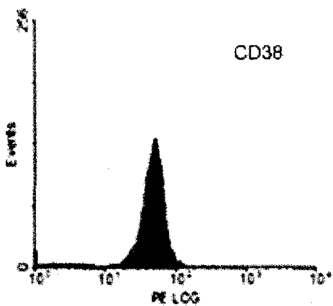
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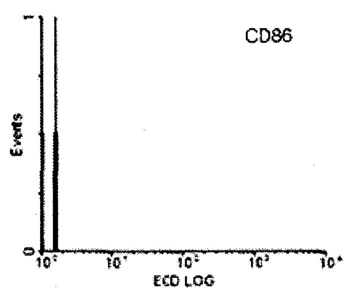

CD86
Events
ECD LOG

The isolation methods for epidermal and dermal DCs yield cells that have a similar phenotype, and while they display a slight activation phenotype as indicated by CD38 and CD83 expression, the expression of these markers is low. Blood-derived DCs have not upregulated the costimulation molecules CD80, CD86 and CD83. They also display a higher CD38 expression than epidermal and dermal DCs. All three isolation methods yield cells that display phenotypes consistent with a DC phenotype.

3.1.4 Monocyte-Derived Dendritic Cell generation

Monocyte-derived DCs are an experimental model that is used to study myeloid DCs. While being artificial, studies have compared mDDCs to tissue resident DCs and found them to be comparable in both phenotype and function[1, 2]. Figure 7 illustrates that the DCs derived from primary monocyte have a range of receptor expression that will vary from person to person. What is also illustrated is that the DC markers that are absent or have very low expression on monocytes have been upregulated on iDCs and that the ranges on monocytes and on iDCs are mutually exclusive.

The expression of HLA ABC remained unchanged during the differentiation period. However, in the case of HLA DR, the isolated monocytes expressed low levels of this marker, not exceeding 16.02%, while the iDCs displayed a range of 72.5%to 95.21% expression on the cell surface.

Table 1 – Monocyte to immature dendritic cell phenotype

Marker	Monocytes (on isolation) % expression range	iDCs (after 6 days in GM-CSF and IL-4) % expression range
HLA ABC	98.73-99.74	99.55-99.88
HLA DR	0.5-16.02	72.5-95.21
CD83	0.2-1.1	0.1-0.14
CD80	0.53-9.96	26.04-47.39
CD86	4.29-9.11	12.78-26.04
CD38	1.12-9.1	13.7-53.71
CD1c	1.41-2.1	49.35-78.22
CD1a	0.3-1.09	18.19-69.41
CD1d	0.2-1.02	3.07-27
CCR5	84.3-93.95	0.33-28.3
CD14	93.95-94.71	0.84-0.92
CD11c	13.47-60.4	90.2-97.6

Expression of cell surface markers on the day of isolation of monocytes and expression of cell surface markers on immature dendritic cells (iDCs) after 6 days in culture with 10ng/mL of IL-4 and 10ng/mL of GM-CSF. Results obtained by flow cytometry, using HLA ABC-FITC (MHCI molecule), HLA DR-PE (MHCII molecule), CD83-PC5 (DC maturation marker), CCR5-FITC (chemokine receptor), CD1a-PE (DC lipid antigen molecule), CD1c-FITC (DC lipid antigen molecule) CD1d-PE (DC marker),CD14-FITC (monocyte LPS receptor) CD80-FITC (maturation marker), CD38-PE (immune cell activation marker), CD11c (DC marker) and CD86-PC5 (maturation marker).

The expression of CD80 and CD86 was below 10% for monocytes, and was upregulated after differentiation to 26.04-47.39% for CD80 and 12.78-26.04 % for CD86 in iDCs. The expression of CD83 was not detected on either cell type. The expression of CD38, also an activation marker, was below 10% on monocytes, and expression increased in iDCs to a range of 13.7-53.71%. The expression of DC markers of the CD1 family was not observed in monocytes, but was increased to 49.35-78.22% for CD1c, 18.19-69.41% for CD1a, and 3-27% for CD1d. The expression of CCR5 on monocytes was evaluated to be greater than the expression levels in iDCs. Also, CD14 expression in monocytes was evaluated to be 93.95- 94.71%, and after differentiation, diminished to 0.84-0.92% of cells. The expression of CD11c on monocytes of 13.47-60.4% was increased to 90.2-97.6% evaluated after differentiation, and this high expression is considered a DC trait.

The different markers that have been evaluated indicate that the isolated cells are resting monocytes. After the 6 days of culture in differentiation media, the cells have adopted a phenotype that is consistent with that of immature DCs, and are consistent with the phenotype of tissue-isolated DCs.

As shown in Figure 8, all markers compared, with the exception of CD80 and CD86 on epidermal and dermal DCs, were present on all populations to some extent.

Table 2 – Relative comparison of immature dendritic cell populations

Surface Marker	Epidermal DCs	Dermal DCs	Blood-derived DCs	MDDC Day 6
HLA ABC	++	++	++	++
HLA DR	+	+	++	++
CD83	++	++	±	+
CD80	±	±	++	+
CD86	±	±	-	+
CD38	+	+	++	+
CCR5	-	-	+	-
CD1d	±	±	++	±

The expression level of each marker was compared on the different DCs populations as compared expression levels. All markers were present to some extent. Markers CD1a, CD1c and CD14 were not compared as they were not assessed on all populations. ± negligible expression – low expression + moderate expression ++ high expression

3.2 Dendritic Cell Response To Lipopolysaccharide

When stimulated by LPS, DCs start the maturation process that would involve downregulation of their antigen pickup capabilities, and upregulate their migratory and stimulatory functions. These functions can be observed by flow cytometry by targeting costimulatory and antigen presenting molecules to ensure that the *in vitro* generated model of iDCs can respond to LPS as expected.

The capacity of monocyte-derived iDCs to respond to LPS stimulation is illustrated in Figure 9. Immature DCs were treated with either a media control, or with LPS.

A shift to the right indicates an increase in signal intensity consistent with receptor upregulation on the cell surface. HLA ABC, HLA DR, CD80, CD83, CD38 and CD86 display a shift to greater signal intensity, showing receptor upregulation consistent with a mature DC phenotype.

The DC marker CD11c and DC lipid antigen presentation molecules CD1a, CD1c and CD1d do not vary in their expression levels. Also consistent with a maturation phenotype is the downregulation of CCR5.

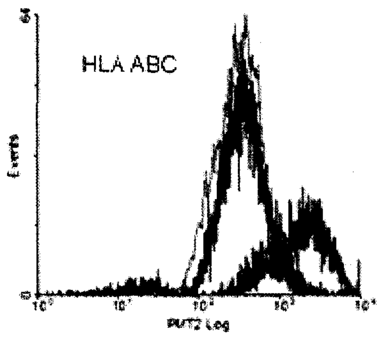
In conclusion, the monocyte-derived dendritic cells respond to LPS with increased expression levels of surface markers that are consistent with previously published reports of mature DC phenotypes[138-140].

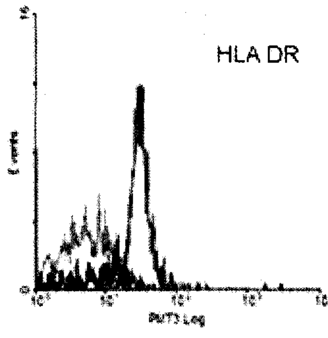
3.3 Interleukin-23 and Interleukin-27 Production in Dendritic cells

The second aim of this project was to evaluate the expression of IL-23 and IL-27 by DCs in response to LPS stimulation, and how HIV-1 and HIV-1 Tat protein affected this expression.

Figure 7 –Dendritic cell phenotype in response to LPS stimulation

Comparison of DCs at the time of final DC differentiation (red line), DCs after 48h in culture media (black line), or DCs in culture media supplemented with LPS (1µg/mL) (green line). Phenotype is determined by flow cytometry experiments using HLA ABC-FITC (MHCI molecule), HLA DR-PE (MHCII molecule), CD83-PC5 (DC maturation marker), CCR5-FITC (chemokine receptor), CD1a-PE (DC lipid antigen molecule), CD1c-FITC (DC lipid antigen molecule) CD1d-PE (DC marker), CD80-FITC (maturation marker), CD38-PE (immune cell activation marker), CD11c (DC marker) and CD86-PC5 (maturation marker).


HLA ABC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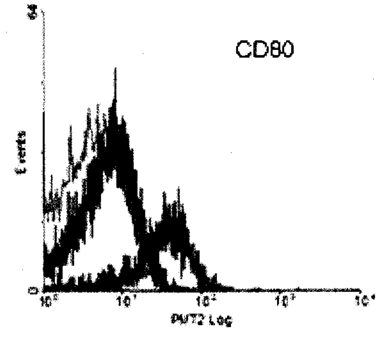
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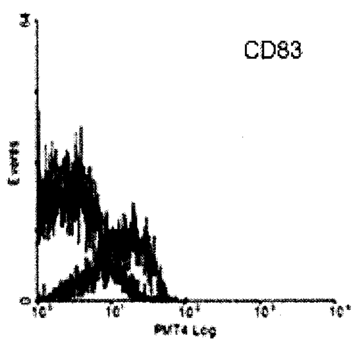
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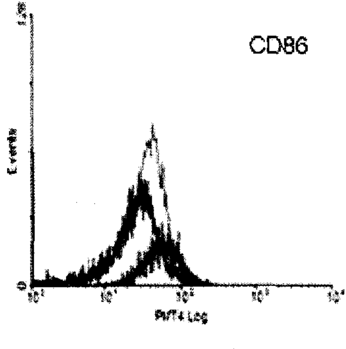
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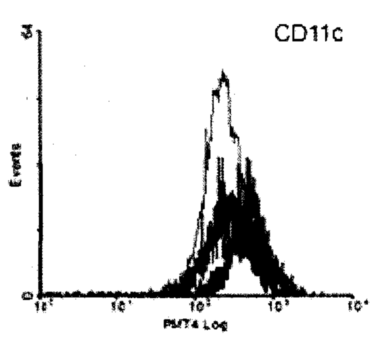
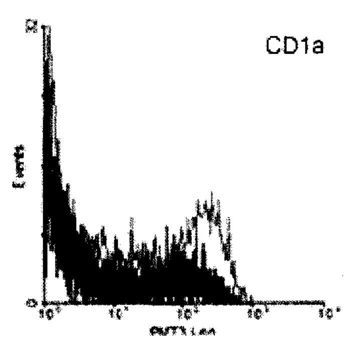

CD11c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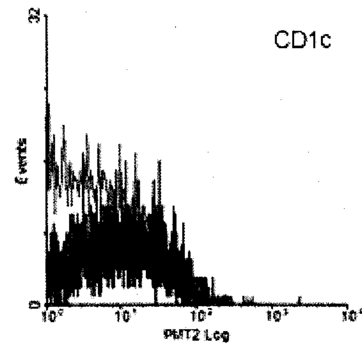
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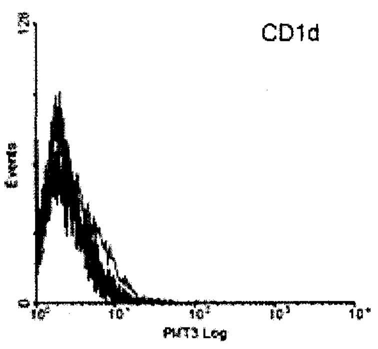
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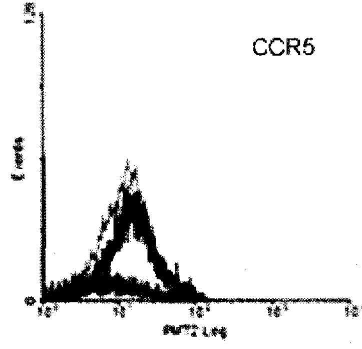
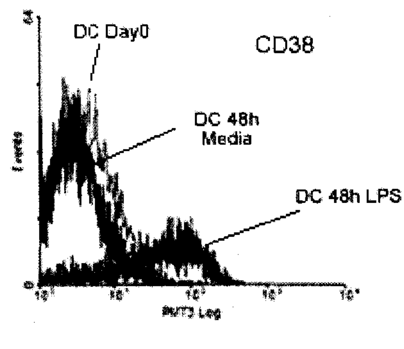

CCR5


DC Day0
CD38
DC 48h
Media
DC 48h LPS

3.3.1 INTERLEUKIN-23 AND INTERLEUKIN-27 RESPONSE TO LIPOPOLYSACCHARIDE

To assay the production of IL-23 and IL-27, which has been reported in DCs[17, 92, 141, 142], two million monocyte-derived DCs were stimulated with LPS at a concentration of 1µg/mL for 4 hours. The RNA was then extracted, RT performed, then real-time PCR was performed to assay for the expression of the p19 subunit of IL-23 and the EBI3 and p28 subunits of IL-27. The housekeeping gene S18, a ribosomal subunit, was used as the endogenous cellular control to control for the overall gene transcription.

To quantitate the gene expression levels, the relative expression $\Delta\Delta C_T$ method of calculation was employed[137]. Unstimulated DCs did not typically express detectable levels of IL-23 and IL-27 RNA. LPS-stimulated samples fell within the detectable limits of the standard curve of the assays for IL-23p19, IL-27EBI3 and IL-27p28. The LPS only treatment was set as the calibrator sample, and assigned a value of 1.

3.3.2 INTERLEUKIN-23 AND INTERLEUKIN-27 RESPONSE TO LIPOPOLYSACCHARIDE WITH PTAT

Two million monocyte-derived DCs were treated for a 24h period with either mock infected supernatants, or the pLXIN retroviral vector containing the Tat wt gene sequence (pTat). After 24h, the cells were treated with LPS at a concentration of 1µg/mL for 4 hours. The RNA was then extracted, RT performed, then real-time PCR was performed to assay for the expression of the p19 subunit of IL-23 and the EBI3 and p28 subunits of IL-27. The empty vector control treatment was not significantly different from the mock infection treatment. The presence of pTat was confirmed using RT-PCR, as shown in Figure 8.

Figure 8 – pTat is present and expressed in DCs

The presence of the pTat sequence was confirmed by RT-PCR. + indicates an unrelated positive control consisting of the p19 gene product, - is the negative media control. The sample presented is representative of all samples, all of which were positive for the wild type Tat mRNA sequence.

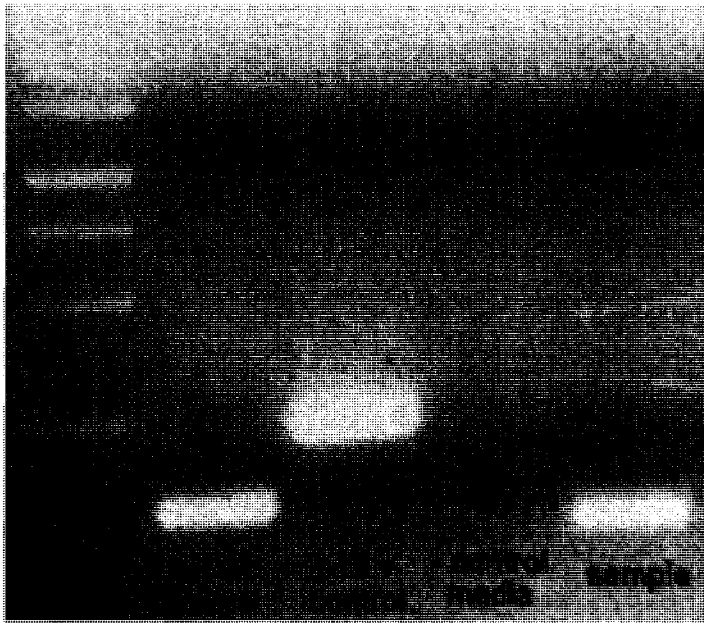
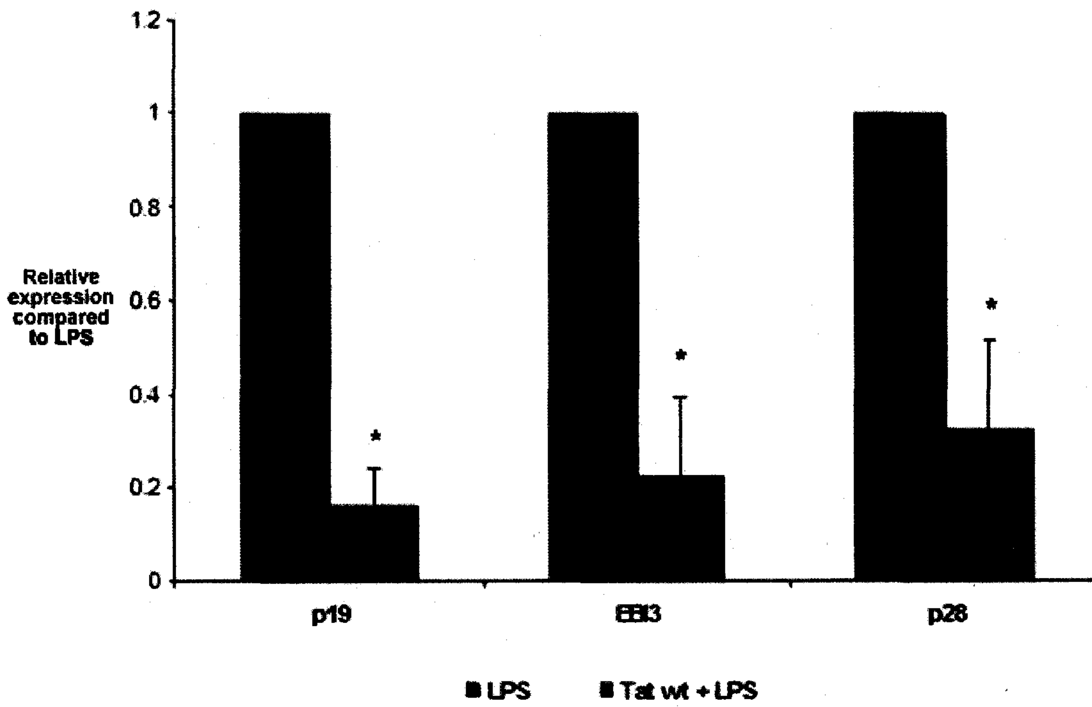

control
media
sample

Figure 9 – DC gene expression in the presence of endogenous expression of Tat

Mean gene expression of triplicates for IL-23p19, IL-27 EBI3 and p28 in response to a 4 hour LPS stimulation with or without the presence of pTat for 24 hours. Expression levels were assayed using relative real-time PCR and gene expression was normalized to S18, a ribosomal protein used as the housekeeping gene. Results are expressed as the fold change from expression levels induced by LPS stimulation, which acts as the calibrator (* means $P<0.05$). The statistical significance was evaluated by T-test using SigmaStat, with p values as follows: p19 ($P=0.017$), EBI3 ($P=0.008$) and p28 ($P=0.002$). n=6



1.2
1
0.8
0.6
0.4
0.2
0
Relative expression compared to LPS
*
*
*
p19
EBI3
p28
LPS
Tat wt + LPS

The expression level of IL-23 and IL-27 subunits on LPS stimulation was set at 1 and the expression levels of IL-23 and IL-27 subunits to LPS stimulation in all treatments were compared to this value and expressed as a relative change. As seen in figure 9, when treated with pTat, the expression level of the IL-23p19 gene was downregulated to 0.16±0.08 relative units and is statistically significant with a t-test value of p=0.017. The expression level to of the IL-27 EBI3 gene was downregulated to 0.22±0.18 relative units and was statistically significant with a t-test value of p=0.008. The expression level of the IL-27p28 gene was downregulated to 0.32±0.19 relative units and is statistically significant with a t-test value of p=0.002.

The addition of pTat to the culture media inhibited LPS-induced upregulation of the expression of all three genes.

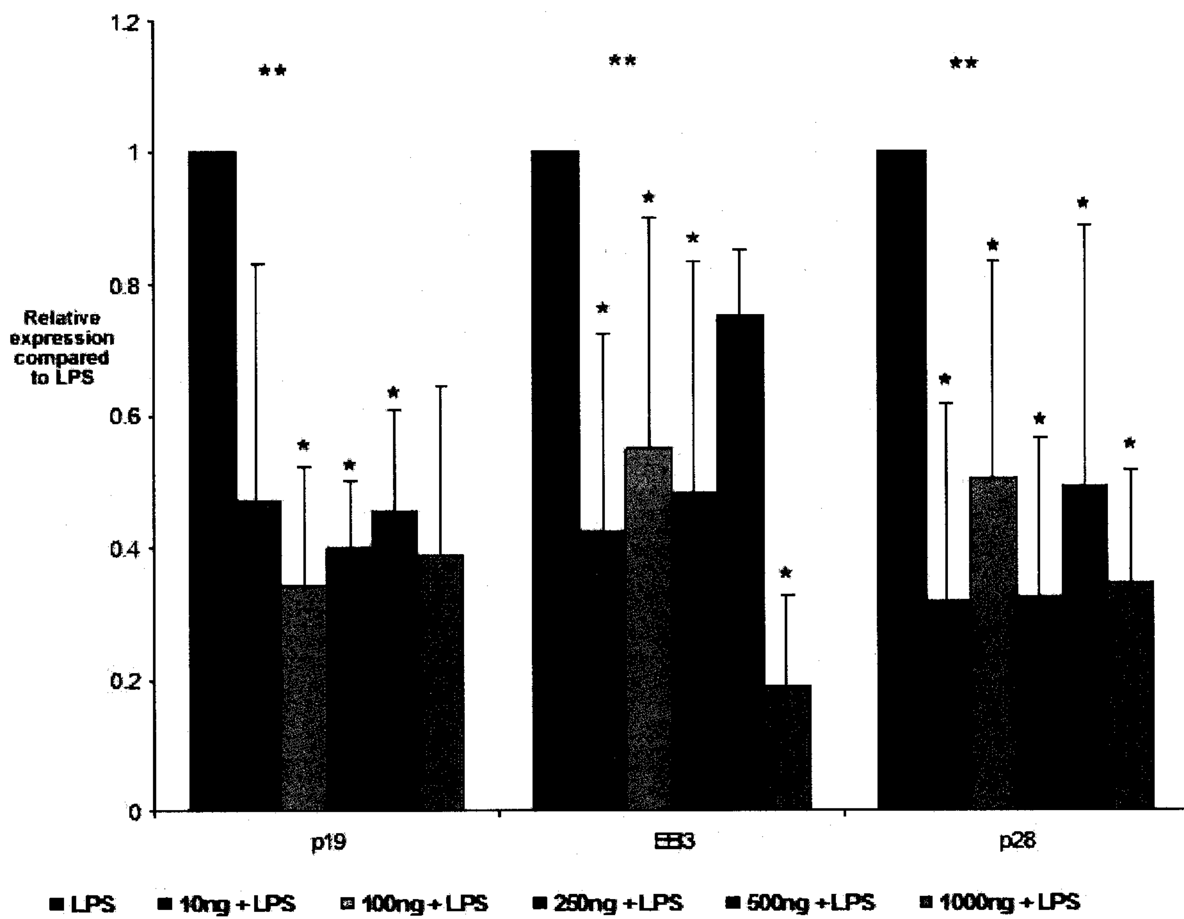
3.3.3 Interleukin-23 and Interleukin-27 Response to Lipopolysaccharide with rTat

Two million monocyte-derived DCs were pre-treated for 1h with increasing doses of rTat before being stimulated for 4h with LPS (1µg/mL). The RNA was then extracted, RT performed, then real-time PCR was performed to assay for the expression of the p19 subunit of IL-23 and the EBI3 and p28 subunits of IL-27.

The addition of rTat alone to the culture media did not stimulate cells to express any of the genes. The gene expression relative to the gene expression levels induced by LPS stimulation was set at 1. The relative expression of p19 decreased to 0.470±0.360 relative units when cells are pre-treated with 10ng/mL of rTat and no dose response was observed. The downregulation observed is statistically significant when evaluated by Kruskal-Wallis one-way ANOVA with a p value of 0.005.

Figure 10 – Mean gene expression in response to LPS with rTat pre-treatment

Mean gene expression levels of triplicates for IL-23p19, IL-27 EBI3 and p28 in response to a 4 hour LPS stimulation with rTat pre-treatment in a dose response experiment. Expression levels were assayed using relative real-time PCR and gene expression was normalized to S18, a ribosomal protein used as the housekeeping gene. Results are expressed as gene expression relative to the gene expression levels induced by LPS stimulation, which acts as the calibrator sample. The statistical significance was evaluated by ANOVA using SigmaStat, with p values as follows: ** p for p19 p=0.005, ** for EBI3 p=0.004 and ** for p28 p=0.006. The multiple comparison of the treatment with LPS stimulation versus LPS stimulation alone were performed using Dunn's Method and Holm-Sidak Method (*p<0.05). n=5



1.2
1
0.8
0.6
0.4
0.2
0
Relative expression compared to LPS
**
**
**
*
p19
EB3
p28
LPS
10ng + LPS
100ng + LPS
250ng + LPS
500ng + LPS
1000ng + LPS

The expression level of IL-23 and IL-27 subunits on LPS stimulation was set at 1 and the expression levels of IL-23 and IL-27 subunits to LPS stimulation in all treatments were compared to this value and expressed as a relative value. As seen in figure 10, the relative expression of IL-27 EBI3 decreases to 0.426±0.298 relative units when cells are pre-treated with 10ng/mL of rTat. There was no dose response observed. The downregulation observed on rTat treatment when compared to the LPS stimulation alone is statistically significant when evaluated by Kruskal-Wallis one-way ANOVA with a p value of 0.004.

The relative expression of IL-27p28 decreases to 0.320±0.298 relative units when cells are pre-treated with 10ng/mL of rTat, and there was no dose response observed. The downregulation observed on rTat treatment when compared to the LPS stimulation alone is statistically significant when evaluated by Kruskal-Wallis one-way ANOVA with a p value of 0.006.

The presence of rTat in the culture media is sufficient to induce a statistically significant downregulation of the expression of IL-23p19, IL-27EBI3 and IL-27p28.

3.3.4 Interleukin-23 and Interleukin-27 Response to Lipopolysaccharide In The Presence Of Human Immunodeficiency Virus-1

HIV-1 is known to affect the immune functions of different cell types, and our group has previously shown that HIV-1 infection downregulate IL-12p40 expression and production[15, 63]. The next experiment was to investigate whether HIV-1 would also affect the expression of IL-23p19 and IL-27EBI3 and p28 in DCs. Two million monocyte-derived DCs were DCs were exposed to HIV-1 in increasing amounts of HIV-1 (as measured by p24 concentration) for 24 hours before being stimulated for 4 hours with

LPS (1µg/mL). The RNA was then extracted, RT performed, then real-time PCR was performed to assay for the expression of the p19 subunit of IL-23 and the EBI3 and p28 subunits of IL-27. There was no statistically significant difference between the LPS stimulation of cells, and the LPS stimulation of cells with mock infection.

The expression level of IL-23 and IL-27 subunits from mock infected DCs with LPS stimulation was set at 1 and the expression levels of IL-23 and IL-27 subunits in all treatments were compared to this value and expressed as a relative value. As seen in figure 11, the relative expression of p19 decreased to an average of 0.396±0.424 relative units when cells were pre-treated with virus dosed at 1ng/mL of p24 and no dose response was observed. The downregulation observed is statistically significant when evaluated by Kruskal-Wallis one-way ANOVA with a p value of 0.005.

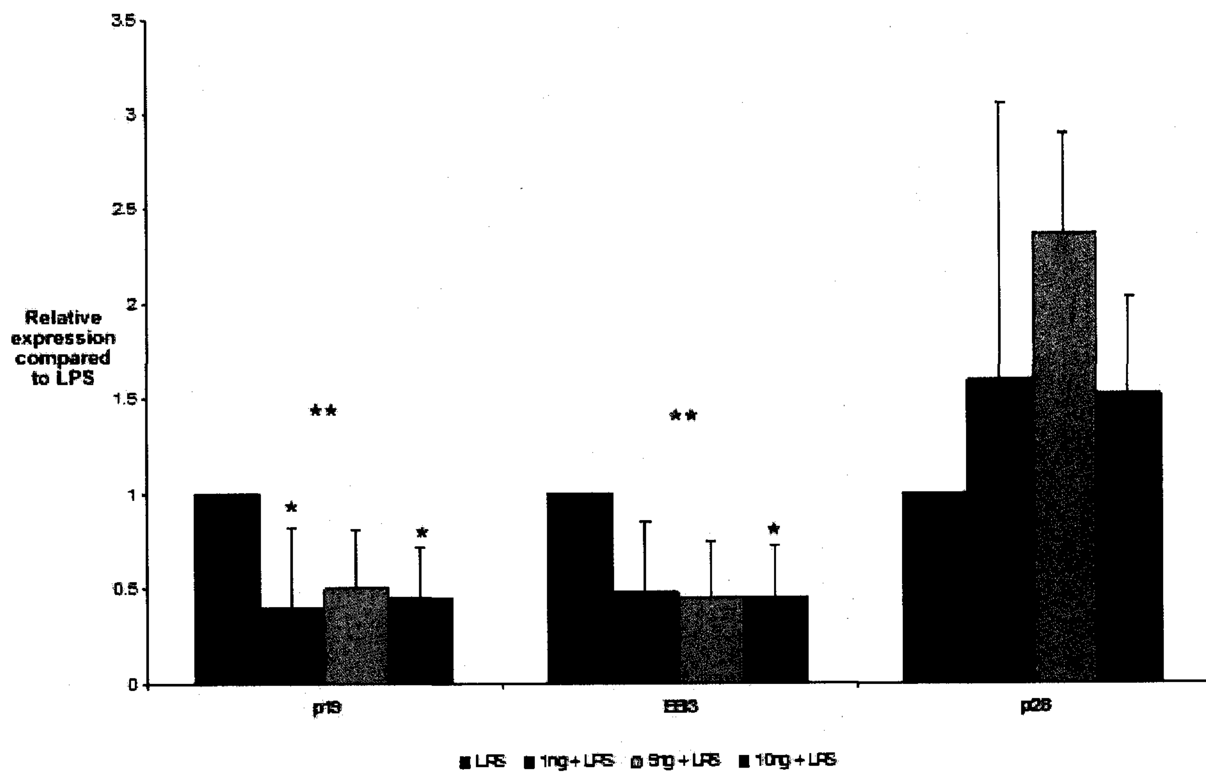
The relative expression of EBI3 decreased to an average of 0.477±0.373 when cells were pre-treated with virus dosed at 1ng/mL of p24 with no dose response observed. The downregulation observed is statistically significant when evaluated by Kruskal-Wallis one-way ANOVA with a p value of 0.032.

The relative expression of p28 was 1.598± 1.462 relative units when cells were pre-treated with virus dosed at 1ng/mL of p24. The gene expression was increased across the HIV treatments, but is not statistically significant by ANOVA or paired t-tests.

Overall, the presence of Tat, whether from an endogenous or exogenous source, caused the downregulation of the gene expression for IL-23p19, IL-27EBI3 and IL-27p28. The presence of HIV-1 had a similar downregulation effect for IL-23p19 and IL-27EBI3, but no significant change for IL-27p28.

Figure 11 – Mean gene expression in the presence of HIV-1 dual-tropic strain 92HT593

Mean relative gene expression for IL-23p19, IL-27 EBI3 and p28 in response to a 4 hour LPS stimulation in the presence of dual-tropic strain of HIV-1 92HT593 in a dose response experiment. Cells were exposed to HIV-1 for 24hours before stimulation. Expression levels were assayed using relative real-time PCR and gene expression was normalized to S18, a ribosomal protein used as the housekeeping gene. Results are expressed as relative expression levels compared to that induced by LPS stimulation alone, which acts as the calibrator sample. The statistical significance was evaluated by ANOVA using SigmaStat, with p values as follows: ** for p19 (p=0.005), ** for EBI3 (p=0.032). No statistical significance was observed in the case of p28 when compared to LPS alone. The multiple comparison of the treatment with LPS stimulation versus LPS stimulation alone were performed using Dunn's Method (*p<0.05). n=7


Relative expression compared to LPS
3.5
3
2.5
2
1.5
1
0.5
0
**
*
*
**
*
p19
EBI3
p28
LPS
1ng + LPS
5ng + LPS
10ng + LPS

3.4 SUMMARY

Dendritic cells were successfully isolated from epidermal and dermal skin tissues, and from peripheral blood and their phenotype consistent with that of previously published reports. The monocyte-derived DC population was successfully generated, and responded to LPS stimulus.

Upon pTat treatment, IL-23p19, IL-27 EBI3 and p28 DCs gene expression to LPS stimulation was significantly downregulated. A similar effect was observed with rTat pre-treatment. In the context of exposure to HIV-1, IL-23p19 and IL-27 EBI3 gene expression to LPS stimulation was significantly downregulated, but no effect was noted on IL-27p28 gene expression to LPS stimulation. No dose responses were noted.

4. DISCUSSION

4.1 DENDRITIC CELL ISOLATIONS

The DC phenotype is determined by the microenvironment in which the DC resides, and because these populations may have different responses to HIV, 4 different DC populations were generated. Human DCs can be isolated from the skin tissues, precursor DCs from blood, and can be generated *in vitro* from different cells. The literature that describes DC phenotypes is more commonly restricted to one type of DC, and many publications state that the studied DCs are positive for certain markers. Lymph node DC studies are done in mice models, which are genetically homogeneous.

Another layer of difficulty in analysing the literature is understanding that there are many different ways to generate human monocyte-derived DCs. There are many different differentiation mixtures that can be used, from starting with a myriad of IL-4 and GM-CSF combinations, to adding IL-1β, IL-6, and maturation mixes that include, IFN-γ, PGE_2, PolyI:C, LPS, CD40L and TNF-α, to name but a few. To add to the complexity, the original cell can be a monocyte that has been isolated by adherence, by CD14-positive selection or by CD14 negative selection. Then these different cells are differentiated for different timeframes, and matured for different timeframes. One of the questions that this project wanted to address was how different human DC populations compared to one another. This project also compares the *in vitro* model to the primary DC populations.

4.1.1 Epidermal and Dermal Dendritic Cells

Epidermal DCs were successfully isolated, and presented an immature phenotype. However, the presence of the $CD38^{low}$ and $CD83^{low}$ signal would suggest early signs of activation. This activation was not complete as they were $CD80^{-}$ and $CD86^{-}$. An activated, mature DC would have upregulated all four molecules. Dermal DCs were also successfully isolated from the dermal layer, and also displayed an immature DC phenotype. Like epidermal DCs, dermal DCs presented early signs of activation, being $CD38^{+}$ and $CD83^{+}$ and $CCR5^{low}$ but again were $CD80^{-}$ and $CD86^{-}$. The different HLA ABC and HLA DR intensities observed on these two DC populations may be linked to migration, and the activation that migration induces on DCs through the upregulation of these surface molecules[1]. The signs of activation have been attributed to the isolation method, which includes vigorous pipetting to ensure epidermal tissue dissociation and migration to isolated dermal cells[135].

Obtaining tissue was more difficult than anticipated. Obtaining the ethics approval from the Ottawa Hospital Ethics Board was a lengthy process in itself, and obtaining samples were also difficult. Patients were not informed of the study, would not give consent, or tissues would get discarded in the operating room before making it into the sample container.

4.1.2 Circulating Dendritic Cells

Circulating DCs were successfully isolated and are considered precursor DCs[2]. Isolated cells presented a phenotype that is consistent with DCs of immature phenotype being $CD80^{-}$, $CD86^{-}$ and $CD83^{-}$.

All three populations presented DC phenotypes, but the isolation methods could not generate the cell numbers required to perform the RT-PCR assays to examine the effects of HIV-1 and HIV-1 Tat protein on IL-23 and IL-27 expression in response to LPS stimulation. The isolation method from skin tissues yielded at most $10x10^6$ dermal and epidermal DCs combined and the isolation from peripheral blood yielded less than $2x10^6$ circulating DCs. These numbers were not sufficient to perform RT-PCR analysis, which required $24X10^6$ DCs across all treatments.

4.1.3 Monocyte-Derived Dendritic Cells

Isolated cells presented a monocyte phenotype as expected. They were HLA ABC^{high}, and were $CD14^+$, $CD11c^+$, HLA DR^{low}, $CD1c^-$, and $CD1a^-$. After the differentiation period of 6 days, cells were still HLA ABC^{high}, but were HLA DR^+, $CD11c^{high}$, $CD1c^+$ and $CD1a^+$. These are consistent with reported DC phenotypes[1, 2]. These cells could be generated in the required numbers to perform the assays and are the model studied.

4.1.4 Dendritic Cells Comparison

DCs have been reported to have different phenotypes dictated by the microenvironment in which they reside[1, 2, 10, 143]. This study is the first that compares the phenotypes directly to one another, as opposed to inferring them from different publications. The different populations expressed different levels of each marker when compared to one another. The most similar populations were the epidermal and dermal populations and these similarities could be explained by the similarity of their microenvironment. Both populations have migrated from

the blood stream, and completed their differentiation with all the necessary tissue signals. The isolation methods have induced incomplete maturation of dermal and epidermal DCs. The differences seen in blood-derived DCs are consistent with cells that have not migrated into their tissues and have not completed their differentiation. And lastly, the monocyte-derived DCs were obtained by differentiation of monocytes into a DC phenotype.

All populations express the same markers, but to different extents, which can be explained by their origin.

4.2 Dendritic Cell Response to Lipopolysaccharide

Improper maturation of DCs has been directly linked to improper T cell priming. Presentation of antigen in the absence of proper T cell stimulation signals has been shown to induce T cell tolerance rather than the induction of cell-mediated immunity. Virus-infected cells require Th1 polarization for proper clearance of infection. The maturation stimuli employed to mature DCs is directly linked to the Th polarization pathway they engender. CD40L, IFN-γ and LPS (this last considered a weak signal) are known to induce T cell polarization along the Th1 pathway, while maturation stimuli containing Prostaglandin E2 (PGE2) are known to induce differentiation along the Th2 pathway[111, 140, 144].

To assess whether iDCs could respond to LPS to confirm published results[1, 2], cells were treated for 48h with LPS. Antigen presentation molecules HLA ABC and HLA DR were upregulated, as were the costimulatory molecules CD80 and to a lesser extent CD86. CD38 and CD83 were also upregulated, and a slight upregulation in CD11c expression was noted, again consistent with a

mature phenotype. The downregulation of CCR5 observed was as previously described[1, 2], and consistent with DCs downregulating their inflammatory homing capabilities to upregulate lymphoid homing. Also as described was the lack of change in CD1 molecules. The phenotype observed was consistent with a mature DC phenotype[138-140].

When evaluated by relative real-time PCR, the gene expression levels of IL-23p19 and IL-27EBI3 and IL-27p28 without LPS stimulation were not consistently detectable. When LPS was added to the culture supernatants, the relative gene expression levels were consistently in the detectable range, indicating that the cells were responding to the LPS stimulation by increase gene expression of IL-23 and IL-27[17, 92, 141, 142].

4.3 The Effects of Tat and Human Immunodeficiency Virus-1 on Interleukin-23 and Interleukin-27

Tat is a protein that is produced within HIV infected T cells, released into the extracellular milieu to be picked up by neighbouring cells in acute infection and has been reported to affect DC maturation[132]. Because of these known characteristics, the effect of the endogenous production of Tat in a DC on the expression of IL-23 and IL-27 was assayed. The viral DNA vector pLXIN contains the tat wt gene (pTat) and the expressed gene sequence was detected by RT-PCR. When pTat was present in cells, the expression of IL-23 and IL-27 in response to LPS decreased significantly.

The effects of exogenous Tat protein (rTat) on the expression of IL-23 and IL-27 were assessed. When added, the expression of IL-23 and IL-27 also decreased significantly, to mimic the effect seen with pTat.

The expression of Tat by DCs has been shown to modulate IFN-inducible genes including STAT1 and interferon regulatory factor-7 (IRF7) without inducing the production of any interferons, and to induce the secretion of chemokines known to attract activated T cells, the preferred viral target[127]. It also known that the presentation of antigen to T cells in the absence of the proper stimulation signals given by cytokines can induce tolerance. In essence, DCs affected by Tat and HIV could attract T cells, but would not express IL-23 or IL-27 to activate and induce T cell proliferation. This could be an effective mechanism for both the transfer of HIV to its target cells, and a mechanism of immune evasion employed by the virus. This immune suppressive effect could be transmitted to uninfected DCs by the secretion of Tat by HIV-infected T cells into the extracellular milieu, and could suppress the immune response to other infections.

The maximal effect observed when rTat is added to the culture supernatant occurs at the lowest concentration used and a lack of dose response was observed. A report of the maximal effect of rTat on cell growth promotion peaks at 0.1-1ng/mL in a Kaposi Sarcoma cell line[131]. This concentration is lower than what was used in the DC cultures with recombinant Tat, and may explain the maximal effect seen at a concentration of 10ng/mL of rTat addition. Physiological levels of Tat in sera of HIV-infected individuals have been found to contain between 50-500ng/mL of secreted Tat[145]. The effect seen at the lower

concentrations of Tat may be more consistent with early infection when the activation of DCs would not be at its peak and the concentrations of Tat in the serum would be at its lowest.

In the presence of a dual tropic strain of HIV-1, one that can use both CCR5 and CXCR4 for viral entry in addition to CD4[111], both IL-23p19 and IL-27EBI3 are significantly downregulated. Previous reports have demonstrated that HIV-1 downregulates IL-12p40, the shared component with IL-23[15, 63]. This would effectively downregulate both IL-12 (p40p35) and IL-23 (p40p19), as the cytokine subunits must associate as heterodimers in order to be biologically active. Knocking out either subunit of each cytokine renders them biologically inactive[85, 146]. The effect on IL-27p28, or lack thereof, observed in the presence of whole virus differs from that which is observed with the isolated Tat protein. The difference could be attributed to other soluble HIV-1 factors such as gp120[126, 147, 148], nef [149-152] or vpr[153-155]. These proteins have all been reported to have many effects on the cellular functions of DCs. It must also be considered that the p28 subunit must be secreted with EBI3 to produce biologically active IL-27[9887]. Therefore, decreasing EBI3 is enough to prevent the secretion of IL-27.

DC maturation and T cell activation are processes that occur together. DCs receive activation signals in the periphery that dictate their maturation phenotype and the cytokines produced to activate T cells in the lymphoid tissues. There are three signals presented to T cells that dictate their fate. DCs first pick up and present antigens in the context of MHCI, MHCII or CD1, the first signal, then migrate from the tissue to the lymphoid organs via the afferent lymph. As

this occurs, they upregulate costimulatory molecules such as CD80 and CD86 to either stimulate or suppress the T cell response, constituting the second signal. Once DCs have engaged T cells at the immunological synapse, they secrete cytokines, the third signal. The type of cytokine that is secreted is programmed into DCs in the peripheral tissue according to the maturation signal received[111]. This in turn programs T cells along the proper Th polarization pathway. Interfering with the cytokine secretion field will affect the T cell proliferation outcome.

IL-27 expression has been shown to directly suppress viral replication[133], and to suppress Th2 differentiation through suppression of GATA-3[98]. Some of the benefits to the viral suppression of IL-27 expression may include greater viral replication as well as the enhanced proliferative capabilities in a greater proportion Th2-polarized $CD4^+$ T cells[97]. Th2 cells would also be inefficient in the clearance of a virally-infected cell, being more geared to humoral immunity. In addition, suppression of IL-27 increases the number of T cells that migrate through the blood-brain barrier to the CNS that may help explain the extent of neuronal injury observed in AIDS patients, including HIV-asociated dementia (HAD) and other AIDS-associated neurological pathologies. This suppression would also increase the number of Th17 cells that are produced, increasing the risk of autoimmunity[98, 101, 106].

One study reported that there was a significant increase in the presence of $CD4^+$ T cells in the brain in IL-27p28 knockout mice in the context of *T. gondii* infection[101], and HIV-1 dementia has been associated with an increase in the

number of HIV-1 infected $CD4^+$ T cells in the brain[156]. HAD was been reported to occur in 20-30% of patients with advanced HIV infection and low $CD4^+$ T cell counts in the early 1990s[157]. With the advent of highly active antiretroviral therapy (HAART), the incidence is believed to be lower, but new cases are being reported with HAD where the $CD4^+$ T cell count is higher than 200/µL in increasing numbers[158]. It must be noted that while the precise mechanism for neuronal injury is not known[159], it is believed to involve monocytic cells in the brain[160-163]. Examination of new information on what was once regarded as the immune privileged central nervous system (CNS), and the finding that a decrease in functional IL-27 can increase the localization of $CD4^+$ T cells to the brain could be an interesting avenue of inquiry. It is also interesting that p38 MAPK kinase activity, when engaged by gp120, will trigger neuronal cell death[163]. This kinase has been involved in the modulation of IL-12 in the context of HIV infection in monocytic cells[15].

A study also reported increased expression of IL-6, TNF and IL-17 in the absence of IL-27[99]. This could increase autoimmune phenomena caused by the improper cytokine secretions in the incorrect immune context, as well as providing another mechanism for immune evasion. IL-27 has been shown to inhibit Th2 polarization of T helper cells through the suppression of GATA-3, a critical transcription factor to successful Th2 polarization. This ability is significant, as Th2 polarized T cells have been reported to support greater HIV-1 viral replication [164].

The reduction of IL-23 by HIV would prevent the activation and expansion of effector memory T cells that may be able clear virally-infected cells. Early on in infection, the CTL response to HIV-infected cells is vigorous and does control viral replication. However, CTL become increasingly unresponsive to HIV, and eventually, HIV-infection rebounds with an increase in plasma viral levels[165]. IL-23 has also been involved in the maintenance of Th17 cell function involved in the clearance of *Mycobacterium* and *Klebsiella* infections. Decreasing IL-23 would prevent the proper clearance of these types of opportunistic infections.

When considering the effects of HIV and Tat on the DC functions, there may be some effects on the proper loading of epitopes onto the MHC machinery, which could also be involved in the processes of immune evasion. The Tat protein can affect the cellular machinery involved in antigen presentation[166]. When considering the effect of Tat in the inefficient cytokine production necessary to properly stimulate the T cell response to improper antigen presentation, a potential mechanism for immune evasion by induction of immune tolerance may be revealed.

5. Conclusions

The downregulation of IL-23 and IL-27 expression by DCs in response to LPS stimulation that is observed in the presence of HIV-1, exogenous Tat and endogenously expressed Tat may be a mechanism by which the virus can escape immune-mediated cell killing, and lead to disease progression. As the secreted Tat protein alone is enough to cause this downregulation, it may be a mechanism by which HIV-1 affects bystander cells, leading to a system that cannot properly respond to stimulation, and therefore lead to infections that would be cleared in an immune competent individual

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