The impact of HIV-1 subtype C Envelope N-glycosylation on DC-SIGN meditated modulation of DC function to facilitate transmission or enhance viral pathogenesis

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Abstract

N-glycosylation plays an important role in Envelope (Env) function and may be involved in the modulation of the immune response to HIV-1 infection. In this study, we hypothesized that Env N-glycosylation may affect viral pathogenesis by influencing Env structure and function. Furthermore, we also postulated that differences in Env glycosylation could affect interactions between Env and DC-SIGN of dendritic cells (DCs), activating alternative signalling pathways which stimulate the release of different immune modulators. We generated pseudovirus of eighteen Env clones (PSVs) with variable number and position of potential N-glycan sites (PNGs) and compared their ability to infect TZM-bl cells, bind to Raji+ DC-SIGN cells, *trans*-infect TZM-bl cells when captured by either Raji-DC-SIGN cells or monocyte-derived dendritic cells (MDDCs) and modulate MDDC signaling by investigating the release of Interleukin-10 (IL-10) and other immune modulatory cytokines and MAPK activation. Entry efficiency, DC-SIGN binding and *trans*-infection varied widely across all clones. The level of IL-10 secreted by MDDCs in response to PSV stimulation varied 32-fold. The induction of IL-10 secretion by purified gp140 confirmed that Env was the viral component that stimulated the secretion of IL-10 via interaction with DC-SIGN and potentially other undefined receptors. PSV and purified gp140 stimulated MDDC signaling via ERK and JNK phosphorylation, while p38 was not activated. The addition of recombinant DC-SIGN lowered the levels of secreted IL-10 and ERK /JNK phosphorylation, suggesting that DC-SIGN plays a role in these responses. As Env mannosylation correlated with DC-SIGN binding, five highly conserved Env PNGs (241, 262, 386, 392, and 448) previously identified to carry high mannose type N-glycans and hence thought to be involved in DC-SIGN binding were deleted in two Env clones by site-directed mutagenesis to confirm their importance in Env function. The potential role of these PNGs in Env entry efficiency, DC-SIGN binding, *trans*-infection, induction of MDDC IL-10 secretion and activation of MAPK phosphorylation was determined. Deletion of these sites significantly affected the entry efficiency, DC-SIGN binding, *trans*-infection and MDDC IL-10 secretion, with one Env clone proving to be more sensitive to mutation than the other. This suggests that PNGs influence Env function in a clone-specific manner. As deletion of highly conserved PNGs abrogated Env function we used sequence analysis to identify PNGs involved in binding DC-SIGN and inducing MDDC IL-10 secretion. We grouped PSVs based on the presence or absence of specific PNGs in Env sequences and compared entry efficiency, DC-SIGN binding, *trans*-infection, stimulation of MDDC IL-10 secretion and induction of

MAPK phosphorylation. Three Env PNGs were significantly associated with entry efficiency (N356, N392, and N674), and three sites (N289, N356 and 674) were significantly associated with *trans*-infection while N674 also influenced DCSIGN binding. The majority of MDDC donors secreted higher levels of IL-10 when stimulated with PSVs that carried PNGS at N130 ($p = 0.0016$) and N332 ($p = 0.0039$) and lacked N674 ($p = 0.033$). When Envs were graded on whether they had 0, 1, 2 or 3 of the PNGs (e.g. -130, -332, +674; -130, +332 and +674, etc.) those that carried either one of the PNGs or the entire induction motif (N130+ N332+ N674-) significantly stimulated MDDCs to secrete higher levels of IL-10 than those that completely lacked the motif ($p = 0.0335$ and $p = 0.0304$, respectively). As the presence of N674 was linked to reduction in all functions of Env, it is likely that the presence of an N-glycan at this site affected Env structure and could skew the analysis. Excluding N674 indicated that the presence of PNGs at position 130 and 332 was sufficient to induce significantly higher IL-10 release than those that had either none or one of these sites ($p = 0.0053$). When we determined whether N130 and N332 were enriched in subtype C acute infection Envs, these sequences were not enriched with PNGs at either N130 or N332 compared to chronic infection viruses. However, when IL-10 levels were compared between MDDC donors stimulated with PSV of either acute or chronic infection clones, those from early infection significantly enhanced MDDC secretion of IL-10 ($p = 0.0039$). This suggests that even though PNGs at 130 and N332 could be involved in inducing MDDC IL-10 secretion, it is not the only requirement for enhanced stimulation. Although Env differentially activated ERK and JNK phosphorylation, ERK phosphorylation did not correlate with IL-10 secretion, suggesting that this MAPK signaling pathway was not solely responsible for triggering the release of MDDC IL-10 and other regulatory cytokines. PSVs also stimulated the release of TNFα, IL-1β, IL-6, IL-8, MIP-1a, and MIP-1b while having no effect on IL-12 levels. This suggests that HIV-1 binding to DCs in the genital tract could change the dynamics of DC immune responses, deregulating their cytokines secretion and destabilising the Th0 cell differentiation to facilitate viral survival and thus productive clinical infection. We therefore conclude that HIV-1 variants differentially stimulate MDDCs to release immunosuppressive IL-10 and that transmitted founders could be better at modulating immune responses in the genital tract compared to chronic infection variants.

List of abbreviations

°C	Degrees Celsius
AI	acute infection
ANOVA	analysis of variance
APC	antigen presenting cells
APOBEC3G	apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
CAPRISA	Centre for the AIDS Programme of Research in South Africa
CCR5	C-C chemokine receptor type 5
CD	cluster of differentiation
CHO	Chinese hamster ovary cell
CI	chronic infection
CLRs	C-type lectin receptors
c-Myc	cellular-myelocytomatosis oncogene
CMV	cytomegalovirus
CO_2	carbon dioxide
CRD	carbohydrate recognition domain
CTL	cytotoxic T lymphocytes
CVL	cervico-vaginal lavage
CXCR4	C-X-C chemokine receptor type 4
DC	dendritic cell
DCIR	DC immunoreceptor
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DMEM	Dulbecco's modified eagle's medium

DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
Endo H	Endo-β-N-acetylglucosaminidase H
Env	envelope
ER	endoplasmic reticulum
ERK	extracellular-signal-regulated kinases
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FGT	female genital tract
FMO	fluorescence minus one
Garcer	galactosyl ceramide
GM-CSF	granulocyte macrophage-colony stimulating factor
gp	glycoprotein
HAART	highly active anti-retroviral therapy
HEK	human embryonic kidney
HIV-1/2	Human immunodeficiency virus type 1/2
HIVR4P	HIV research for prevention conference
HKLM	heat killed Listeria monocytogenes
HM/H	high mannose/hybrid
hr(s)	hour(s)
ICAM-3	intracellular adhesion molecule
IFN-γ	Interferon gamma

IgG	Immunoglobulin G
IIDMM	Institute of Infectious Diseases and Molecular Medicine
IL	interleukin
IMC(s)	Infectious molecular clone(s)
JNK	c-jun NH2 terminal kinase
kb	kilobases
kDa	kiloDalton
LARG	Leukemia associated Rho-GTPase
LC	Langerhans cell
LCMV	lymphocytic choriomeningitis virus
LPS	lipopolysaccharide
LTR	Long terminal repeat
M	Molar
MAPK	mitogen-activated protein kinase
MCLR	mannose C type lectin receptor
MCP-1	monocyte chemoattractant protein 1
mDCs	myeloid DCs
MDDCs	monocyte derived dendritic cells
MEK	MAPK/ERK kinase (MEK)1/2
mg	milligrams
MgCl2	magnesium chloride
MHC	Major Histocompatibility complex
min	minute(s)
MIP	macrophage inflammatory protein
M	molar
MOI	multiplicity of infection

MR	mannose receptor
MVBs	multivesicular bodies
MW	molecular weight
Na_2EDTA	disodium ethylenediaminetatraacetate
NaCl	sodium chloride
NaHCO3	sodium bicarbonate
nAb	neutralizing antibodies
NF-κB	nuclear factor of Kappa B
NICD	National Institute of Communicable Disease
NK	natural killer
PAGE	polyacrylamide gel electrophoresis
PAMP	Pathogen associated molecular patterns
PBMCS	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pDCs	plasmacytoid dendritic cells
PE	phycoerythrin
PEI	polyethylenimine
Per-Cp-Cy	Peridinin Chlorophyll protein- Cyanine dye
PMA	phorbol myristyl acetate
PNGase F	peptide N-glycosidase F
PNGS	potential N-linked glycosylation site(s)
PRR	pattern recognition receptors
PSV	pseudotyped Envelope
Raf-1	v-raf-1 murine leukemia viral oncogene homologue 1
Ras	rat sarcoma virus oncogene

RLU	Relative light units
RNA	ribonucleic acid
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
SAMHD1	sterile a motif and HD-domain containing 1
SDM	site directed mutagenesis
SDS	sodium dodecyl sulphate
sec	second(s)
SEM	standard error of the mean
SGA	single genome amplification
SHIV	Simian-Human immunodeficiency virus
SIV	Simian immunodeficiency virus
TBS-T	Tris-buffered saline-tween
T reg	regulatory T cells
T/F	transmitted/ founder
Th	T helper
TLR	toll like receptors
TNF-α	tumour necrosis factor-α
TRIM 5 α	Tripartite motif 5α
U	unit(s)
WHO	world health organisation
WT	wild type
α	alpha
β	beta
Δ	delta

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Chapter 1 Literature review

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1.1 Introduction

Although the global burden of HIV-1 seems to have stabilized in terms of global prevalence, the total number of new cases continues to increase. Despite increased availability of antiretroviral therapy (ART), ART treatment remains accessible to less than half of the 37 million infected people. Only 30 – 40% of people living with HIV in low and middle income countries had access to ART in 2014 (WHO, 2014; Deblonde et al., 2015; UNAIDS, 2015). More so, poor ART adherence and associated drug resistance are critical issues which need to be addressed in the development of better treatment options. More options are needed to reach the 2030 WHO goal of ending AIDS or making the AIDS pandemic less of a global public health problem (UNAIDS, 2016). Sub-Saharan Africa has the highest prevalence of HIV and with women in some communities still unable to negotiate safe sex, the situation might take longer than expected to improve. Unfortunately, the pandemic affects mostly women of child-bearing age with 56 % of new infections in Sub-Saharan Africa in 2015 due to heterosexual transmission in young women (UNAIDS, 2016).

Despite successful programs for prevention of mother to child transmission (MTCT), about 240 000 children born to HIV positive mothers were diagnosed globally in 2013 (United Nations Children's Fund (UNICEF), 2013; UNICEF, 2014). This calls for enhanced efforts to find new therapeutic options to control infection and more importantly, the identification of novel interventions to prevent HIV transmission. New strategies to combine treatment with prevention are needed globally to control the growing pandemic (Hladik & Doncel, 2010).

Although the rate per coital act is said to be as low as 0,001%, heterosexual transmission still accounts for most HIV-1 infections (Royce A. Rachel et al., 1997; Wawer et al., 2005; Hladik & McElrath, 2008; Boily et al., 2009; Gray & Wawer, 2012). Developing effective strategies to reduce HIV-1 transmission requires proper understanding of the initial events that take place at the genital mucosa during transmission.

Attempts to decrease HIV transmission through a vaccine or microbicide are limited by an incomplete understanding of how HIV-1 penetrates the mucosal barriers and subsequently spreads to the lymphoid tissues. The virus and/or infected cells are believed to cross the epithelium shortly after exposure in the genital tract to infect target cell (Pope & Haase, 2003; Hladik & McElrath, 2008; Hladik & Hope, 2009; Haase, 2010; Hladik & Doncel, 2010)

However, the mechanism(s) by which the transmitted variants interact with host cells and the type of cells first encountered are still debated (Spira et al., 1996; Hu, Gardner & Miller, 2000; Zhang, Wietgrefe, et al., 2004; Hladik et al., 2007).

The virus seems to have multiple challenges to overcome in order to infect target cells especially in a healthy genital mucosa. In most cases, only a single or a few variants from the donor's quasispecies succeed in establishing infection (Keele et al., 2008; Abrahams et al., 2009; Keele & Estes, 2014), leading to a genetically homogenous viral population during the first weeks post infection. Variants that successfully establish infection might have biological properties that enable them to do so.

Dendritic cells (DCs) have been postulated to play a role in the establishment of infection as they possess C-type lectin receptors (CLR) like DC specific intracellular adhesion molecule (ICAM)-3 non-integrin (DC-SIGN) and CD4+ as well as co-receptors required for HIV infection (Hong et al., 2007; Liao et al., 2011; Shen, Kappes, et al., 2014). Upon HIV binding to DC-SIGN, DCs translocate to the lymph nodes where they then *trans*-infect CD4+ T cells facilitating systemic infection (Geijtenbeek, Torensma, et al., 2000; Geijtenbeek et al., 2002; Shen, Kappes, et al., 2014).

Previous studies have found that specific Env potential N-glycan sites (PNGs) interact with DC-SIGN (Hong et al., 2007, Liao et al., 2011) on DCs and that myeloid DCs facilitate HIV transmission (Shen et al., 2014). Furthermore, studies that showed that subtype C and A transmitted founder (TF) Env PNG numbers were lower than those from chronic infection (CI) (Derdeyn et al, 2004; Chohan et al., 2005) and that site-specific PNGs of TFs were enriched with high mannose (HM) N-glycans (Go et al., 2011), suggested that Env N-glycosylation could play a role in HIV transmission. The role of specific/combination of PNGs in interacting with cellular receptors at transmission is not fully understood.

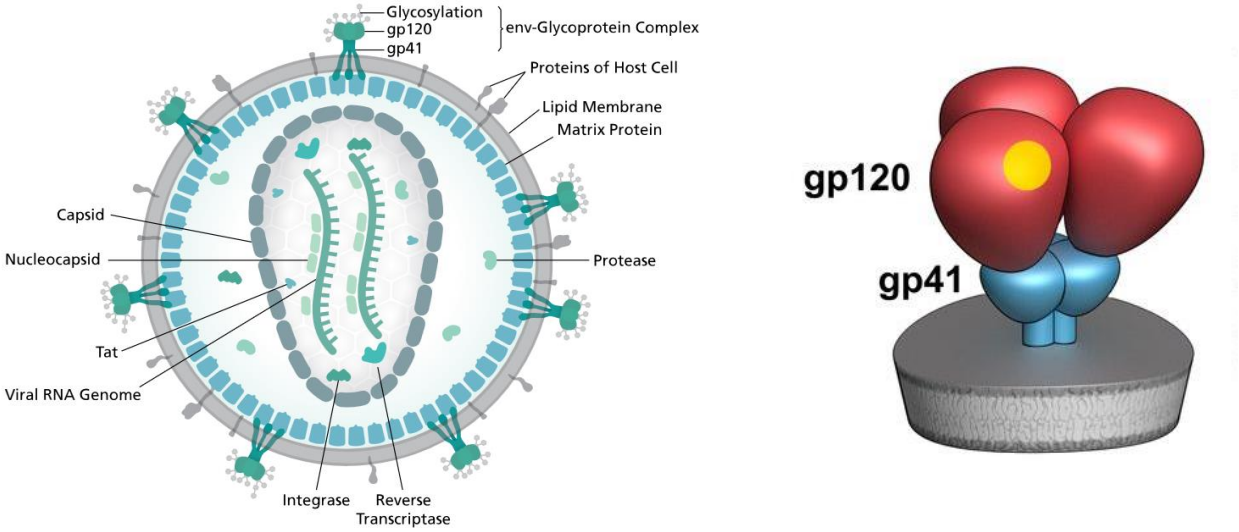
Moreover, interaction of Env with DC-SIGN on monocyte derived dendritic cells (MDDCs) was found to induce the secretion of IL-10, an immune suppressive cytokine (Shan et al., 2007). IL-10 production has also been found to enhance HIV infection (Sozzani et al., 1998; Stylianou et al., 1999) suggesting that HIV Env N-glycosylation could modulate DC function. A number of PNGs have recently been shown to form epitopes for neutralising antibodies (Scanlan et al., 2002; Moore et al., 2012; Pritchard, Spencer, et al., 2015), suggesting that the inclusion of Env N-glycans in vaccine immunogens could induce neutralising adaptive immune responses. However, if Env PNGs modulate DC function via binding DC-SIGN or other CLRs then this

could lead to unwanted immune responses that reduces vaccine efficacy. Understanding the role of specific/combination of Env PNGs in interacting with DC-SIGN and how this interaction modulates DC function to facilitate HIV transmission or disease progression will provide important information for the design of new strategies to prevent HIV transmission.

An overview of the structure of the HIV virus particle, the infection process, the role of DCs and mechanisms of infection and the role of Env and its N-glycosylation in HIV pathogenesis is explored in more detail in the next sections.

1.2 Structure of HIV

HIV-1 is a positive stranded RNA lentivirus of the family Retroviridae. The HIV genome consists of two identical single stranded RNA molecules, each 9.2 kb in length, which encode all the structural and regulatory proteins required for the life cycle of the virus. A total of 9 genes are encoded by the RNA. Three of these (*gag*, *pol* and *env*) are structural genes which encode for structural proteins; Gag (matrix, capsid and nucleocapsid), Pol (protease, reverse transcriptase) and Env proteins, all of which are found in all lentiviruses. The remaining six genes; *vif, vpr, tat, rev, vpu* and *nef* encode viral accessory proteins. The packaged RNA is tightly bound to the nucleocapsid protein, p7, a sub-protein of Gag. The enzymes required for virus replication such as reverse transcriptase, integrase and protease are encoded by the *pol* gene and together with the nucleocapsid are bound within the viral capsid. The capsid protein, p24, together with the matrix, p17, the p6 protein and the nucleocapsid are encoded by the *gag* gene. They are initially synthesized as the Gag-p55 precursor that is cleaved following processing into the matrix protein that stays bound to the viral membrane and the capsid which encloses the viral core (Mervis et al., 1988; Veronese et al., 1988; Goto, Nakai & Ikuta, 1998) (Figure 1.1). The outermost surface of the viral particle is composed of the spherical lipid bilayer membrane which is of host cell origin, and Env (Figure 1.1).



A. B.

Figure 1.1 Structure of HIV and Envelope on the surface of the virus. A) Adapted from Thomas Splettstoesser (www.scistyle.com), CC BY-SA 4.0, https://commons.wikimedia.org/w/index.php.curid=38751738

B) Quaternary structure of trimeric gp120 and gp41 in unliganded form on the surface of the virion (Audray K. Harris et al. 2013 J. Virol. ;87:7191-7196)(Harris et al., 2013).

1.3 Envelope

Env comprises of gp120 and gp41 generated by cleavage of gp160 encoded by the *env* gene. As gp120 and gp41 units are exposed at the viral surface and make first contact with host cells, they are considered important HIV-1 immunogens for the generation of protective immunity (Goto, Nakai & Ikuta, 1998; Sierra, Kupfer & Kaiser, 2005). However, thus far, vaccines designed to elicit an immune response to gp120 have failed due to high variability in *env* (Reviewed in Araujo and Almeida, 2013)(Araújo & Almeida, 2013). Env diversity is caused, most especially high replication rate of HIV, error prone reverse transcriptase (Araújo & Almeida, 2013) and the probably the pressure imposed by the large population size, but also by post translational modifications like N-glycosylation (Binley et al., 2002; Banerjee et al., 2009).

Env is one of the most heavily glycosylated proteins (Zhang, Gaschen, et al., 2004) and N-glycan modifications are important for interaction with receptors on target cells (Ly & Stamatatos, 2000; Hong et al., 2007; Liao et al., 2011) and may play other roles in infection (Wang et al., 2013), which are not yet known. N-glycans also mask conserved epitopes from the immune system, and are involved in switching to X4 tropism during the course of infection (Pollakis et al., 2001).

1.3.1 Env Biosynthesis

Env is synthesized from bicistronic vpu/env mRNA as an unprocessed gp160 polyprotein precursor in the rough endoplasmic retriculum (RER). Cellular signal peptidases then cleave and remove the RER signal peptide co-translationally but the hydrophobic transmembrane domain of the gp41 prevents its complete release from the cytoplasm into the lumen of the ER (Reviewed in Checkley, Lutge and Freed, 2011). This is followed by addition of high mannose (HM) N-linked and O-linked oligosaccharide side chains during translation (Leonard et al., 1990). Monomers of gp160 oligomerize into trimers (Poignard et al., 2001), although some dimers and tetramers are also formed, and translocate to the Golgi (Earl, Koenig & Moss, 1991). HM sugars are trimmed and complex type carbohydrates added as the Env passes through the Golgi to the trans-Golgi network (TGN) (Scanlan, Offer, et al., 2007). During this time, Vpu remains bound to CD4 receptors to down-regulate expression and to prevent it from binding prematurely to the newly oligomerized Env (Fujita, Omura & Silver, 1997; Schubert et al., 1998; Gomez et al., 2005; Hill et al., 2008). The gp160 is then proteolytically cleaved at a conserved Arg-X-Lys/Arg-Arg (R-E-K/R-R) motif by furin-like proteases to give rise to the gp120 surface unit (SU) and the gp41 trans-membrane (TM) protein (Moulard & Decroly, 2000; Binley et al., 2002). The two Env subunits, remain non-covalently associated, with 3 molecules each of gp120 and gp41 forming a heterotrimer (Freed, Myers & Risser, 1989; Kantanen, Leinikki & Kuismanen, 1995; Staropoli et al., 2000; Harris et al., 2013), which is transferred and incorporated into the plasma membrane (Willey et al., 1988) (Figure 1.1 B). Some of the gp120 is shed upon arrival at the membrane (Egan et al., 1996; Zhu et al., 2003; Yang et al., 2005) due to the weak non-covalent bonds between gp120 and gp41, leaving the virions with few Env spikes (Bachrach et al., 2005).

1.3.2 Env structure

Env consist of five variable regions (V1 – V5) interspaced by five conserved regions (C1-C5) (Willey et al., 1986) (Figure 1.2). Variants infecting a single donor can differ up to 20 % due to diversity within V1-V5 because of recombination, insertions and deletions and also point mutations introduced during reverse transcription. The variability is highest in the V1-V2 regions due to differences in length and number of PNGs which were suggested to change with disease state (Chohan et al., 2005; Sagar et al., 2006). Predominantly, C1, C2, C3 and the V3 regions are involved in interaction with CD4 receptor and co-receptors (Lasky et al., 1987). The V3 domain carries epitopes for PGT121 family, some of the most potent neutralizing

antibodies (nAbs) discovered thus far (Walker et al., 2011; Garces et al., 2014; Doores, 2015; Pritchard, Spencer, et al., 2015)

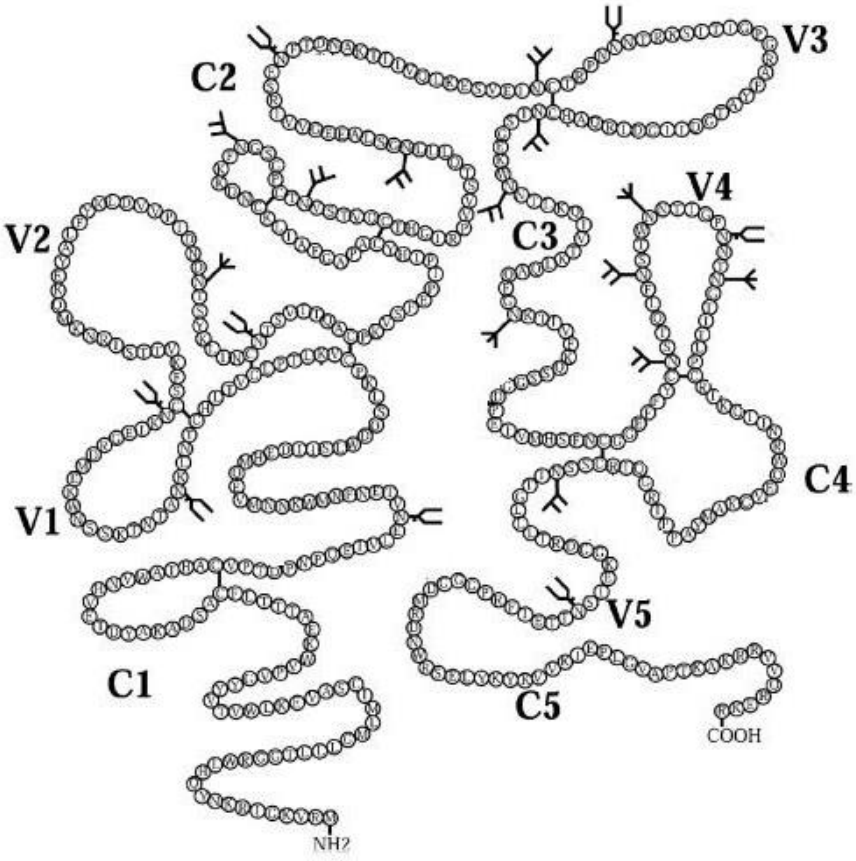


Figure 1.2 Schematic representation of gp120 structural features The amino acids are illustrated by connected circles and the five variable (V1 - V5) and five constant (C1 – C5) regions are indicated, separated by disulphide bridges shown by short connecting lines. N-glycans are indicated by ц for complex type N-glycans and ц for high mannose/ hybrid types whereas Ψ represent unknown glycan types (McCaffrey *et al.*, 2004, Leonard et al., 1990).

Env has about 20 cysteine residues that are covalently linked to form 10 disulphide bridges necessary for folding Env into its native structure (Leonard et al., 1990). Almost half of the molecular weight of gp120 is made up of N-glycans (Leonard et al., 1990), with about 20 – 35 N-linked glycans in gp120 and 3 – 5 in gp41. These N-linked glycans play a critical role in protecting the conserved epitopes on the protein backbone from immune recognition, folding of the protein and forming receptor binding sites (Montefiori, Robinson Jr. & Mitchell, 1988; Binley et al., 1998; Li, Chien Jr., et al., 2008; Raska et al., 2014). Based on the orientation in its trimeric form, gp120 consist of an inner and an outer domain linked by bridging sheets (Kwong et al., 1998, 2000).

Gp41 is required for trimerizeration of Env and mediates viral fusion with host cells during infection. It is organized into the ectodomain, TM and cytoplasmic tail (CT) (Freed, Myers & Risser, 1990). The ectodomain has a fusion peptide which is usually hidden within the gp120/gp41 core and only becomes exposed following interaction with the CD4 receptor and co-receptor and is inserted into the host cell membrane. The ectodomain also comprises of the heptad-repeat regions (HR1 and HR2) that form α-helical coiled coils, a polar region and a membrane proximal external region (MPER), whose interaction drives the fusion process (Freed, Myers & Risser, 1990; Munoz-Barroso et al., 1999). One of the driving aims of vaccine design is generating a form of Env expressed in high amounts that reflects the structure of native, functional trimers to elicit broadly neutralising immune responses. As gp41ectodomain is required for trimerisation, soluble gp140 was constructed lacking the TM either with or without cleavage sites and in some cases, stabilised by a disulphide bond. However, successful trimerisation required cleavage and correct N-glycosylation whereas the presence of a disulphide bond linking gp120 and gp41 resulted in mostly oligomeric Env (Schülke et al., 2002; Forsell, Schief & Wyatt, 2009; AlSalmi et al., 2015). Thus gp41 plays an important role in the formation of functional Env trimers.

1.3.3 Envelope processing

Disulphide bonds form as gp160 folds in the RER and it is generally accepted that native trimers and soluble gp120 carry nine canonical disulphide bonds whereas gp41 has only one (Julien et al., 2013; Lyumkis et al., 2013; Kwon et al., 2015). However, it has also been shown that recombinant gp120 and gp140 have aberrant disulphide bonding patterns based on the exchange of bonds between different cysteine residues during folding (Go, Zhang, et al., 2011; Ringe et al., 2015). Native trimers carry canonical disulphide linkages because the process of trimerisation excludes heterodimers that are incorrectly disulphide linked. As trimerisation also influences N-glycosylation, there is an interdependence between native structure, disulphide bonding and N-glycosylation of Env (Go et al., 2016).

The N-glycosylation of Env is very important for proper processing and folding into the correct conformation required for interaction with cellular receptors (Fenouillet et al., 1989; Fenouillet, Gluckman & Jones, 1994; Fenouillet & Jones, 1995; Land, Zonneveld & Braakman, 2003). It may also assist in the transport of the protein from the RER to the Golgi as well as cleavage of gp160 into gp120 and gp41 (Fenouillet & Jones, 1995). N-glycosylation involves the addition of a carbohydrate group to an Asparagine residue on the sequon N-X-S/T, S and T being Serine

and Threonine while X is any amino acid except Proline (Gavel & Heijne, 1990; Apweiler, Hermjakob & Sharon, 1999). The biosynthetic machinery of the host cell determines the composition and type of N-glycans added at each PNG (Go et al., 2013). This process starts in the ER, with the transfer of a pre-assembled oligosaccharide group (five mannose residues and two N-acetyl-glucosamine [GlcNAc] residues) linked to a membrane carrier lipid dolichol pyrophosphate to the Asparagine residue of the N-X-S/T sequon of the newly synthesized growing polypeptide by a series of glucosyltransferases (Yan & Lennarz, 2005; Kelleher & Gilmore, 2006). The oligosaccharide and dolichol complex is subsequently translocated to the lumen of the ER by the help of transmembrane lipid transporter enzymes, flippases (Helenius et al., 2002; Sanyal & Menon, 2009). Glycosyltransferases subsequently add mannose and glucose residues forming the Glc3Man9GlcNAc2 core (Helenius et al., 2002). Removal of three α-1,2 linked terminal glucose residues serve to ensure proper folding (Hebert, Garman & Molinari, 2005) followed by removal of mannose residues, which prepares the protein for proper folding and transport to the Golgi (Kornfeld & Kornfeld, 1985).

On arrival at the *cis*-Golgi, more mannose moieties are trimmed off to form the Man3GlcNAc2 molecule, which serves as a substrate for formation of complex or hybrid type N-glycans. The protein is then transported to the medial Golgi, where there is addition of GlcNAc to form hybrid N-glycans (Choi, Bobrowicz, et al., 2003; Nett et al., 2010). At times, an α 1-6 linked fucose residue is added to the oligosaccharide core at GlcNAc of the hybrid N-glycans formed of (Stein & Engleman, 1990; Crispin et al., 2006; Scanlan, Ritchie, et al., 2007). The process moves on to the *trans*-Golgi, where the N-acetyl glucosaminyl transferase (GnT) family of enzymes add more GlcNac moieties to the growing GlcNAcMan5GlcNAc to form complex N-glycans. Galactose and sialic acid are subsequently added at the terminals to form complex type N-glycans (Kim, Lee & Jeong, 2009). When fully processed, Env is heterogeneously N-glycosylated with a mixture of high mannose, hybrid and complex N-glycans and is transported to the plasma membrane for incorporation into the virus during budding.

1.3.4 Function of Env N-Glycans

1.3.4.1 Role of N-glycosylation in structure and function of Env

Monomeric gp120 and uncleaved gp140 oligomers have been shown to carry more processed, complex-type N-glycans than native trimers enriched with oligomannose (Bonomelli et al., in press; Pritchard, Vasiljevic, et al., 2015). However, N-glycan processing only happens after the Env has folded into its native structure and trimerisation has occurred. Therefore, addition of

oligomannose or more processed N-glycans is likely a consequence of whether Env forms monomers or trimers. Env proteins synthesized in the presence of tunicamycin, a drug that inhibits N-glycosylation, led to misfolded aggregates that were biologically inactive, confirming that N-glycans help fold Env into its functional conformation (Fenouillet, Gluckman & Jones, 1994; Land, Zonneveld & Braakman, 2003) (Montefiori, Robinson Jr. & Mitchell, 1988). PNGs at specific regions of Env were associated with DC-SIGN binding (Hong et al., 2007; Liao et al., 2011). Mutational studies suggested that N262 and N386 form conformational epitopes as deletion resulted in reduced binding to antibodies like b12 and 17b (Pritchard, Spencer, et al., 2015) and to loss of function in other studies (Wang et al., 2013; Mathys et al., 2014). Some PNGs help determine viral tropism as the PNG at N301 was suggested to prevent CXCR4 usage favouring R5 or dual tropism (Clevestig et al., 2006), while N334/335 was suggested to help in CCR5 interaction (Clevestig et al., 2006). Further, a PNG at position 616 in gp41 was essential for incorporation of Envs into viral particles for some clones but not for others (Mathys & Balzarini, 2014). Therefore, the role of PNGs in Env function is highly variable and differs from one variant to another.

Table 1.1 Potential N-glycan sites that play a role in Envelope phenotype

Presence of potential N-glycosylation site	Envelope Phenotype	Reference
N262 and N386	Env function	Wang *et al*., 2013; Mathys *et al*., 2014
N301	Favours R5 and dual tropism	Clevestig *et al*., 2006
N334/335	Involved in binding to CCR5	Clevestig *et al*., 2006
N616	Incorporation of Envs into viral particles	Mathys & Balzarini, 2014
N230, N295, N674	Loss of Env function	Mathys & Balzarini, 2014, 2014, 2015
N295, N332, N339, N386 and N392; N160 and N156 or N173;	Form epitopes for 2G12; PG9 and PG16; PGT127 and PGT128	Scanlan *et al*., 2002; Calarese,2003; McLellan *et al.*, 2011;

		Pejchal *et al*., 2011; Walker *et al*., 2011; Moore *et al*., 2012
N448 and N230; N301 and N386 N332	Escape from antibodies:	Li, Chien Jr., *et al*., 2008; Koch *et al*, 2003 Moore et al., 2012

1.3.4.2 N-glycosylation and pathogenicity

As N-glycosylation was shown to impact Env function, by extrapolation, it will also influence the replicative fitness of the virus and thus HIV pathogenicity. Deletion of some conserved PNGs like at N262 and N386 abrogated infectivity of these PSV mutants (Wang et al., 2013; Mathys et al., 2014) whereas PSVs produced in the presence of tunicamycin were non-infectious (Montefiori, Robinson Jr. & Mitchell, 1988). In some instances, deletion of PNGs lead to increased infectivity as they could sterically hinder Env native structure, and impair viral fitness. PNGs at N230, N295 (Mathys & Balzarini, 2015) and N674 reduced infectivity while present but enhanced replication once deleted (Mathys & Balzarini, 2014). PNGs could be important for the modulation of host responses to HIV infection as a switch in PNG position from N332 to N334 at six months post-infection helped subtype C Env escape neutralizing Ab recognition and thus enhanced virus fitness (Moore et al., 2012).

1.3.4.3 N-glycans protect important epitopes from immune recognition

Many studies have suggested that N-glycosylation helps the virus to escape immune pressure (Derdeyn et al., 2004; Chohan et al., 2005; Sagar et al., 2006) as they shield or mask conserved epitopes from neutralizing antibodies as shown when deglycosylation of Env led to enhanced neutralization (Binley et al., 1998; Koch et al., 2003; Wei et al., 2003; McCaffrey et al., 2004; Li, Cleveland, et al., 2008; Walker et al., 2011) Masking of epitopes recognized by broadly neutralizing antibodies might have contributed to the poor immunogenicity of most Env immunogens (Shan et al., 2007; Banerjee et al., 2009). Examples of these are N386, which was found to mask the b12 epitope by steric hindrance (in some subtypes) and N301 whose deletion enhanced CD4bs antibody neutralization of some Envs (JRFL, Yu-2 and HXB2)(Koch et al.,

2003). PNGs at N448 and N230 have also been shown to affect proteolytic cleavage of CD4 T cell epitopes preventing presentation and thus activation of CD4+ and CD8+ T cells (Li, Chien Jr., et al., 2008). Lastly, the reduced Env PNG number of transmitted founders compared to variants from chronic infection (Subtypes A and C) suggested that N-glycosylation could also be involved in the selection of variants for transmission (Derdeyn et al., 2004; Chohan et al., 2005; Sagar et al., 2006).

1.3.4.4 N-glycosylation enhances immune recognition

Despite protection of some epitopes by PNGs, some PNGs have however been found to be epitopes for broad and potent neutralizing antibodies. The 2G12 antibody recognizes an N-glycan epitope in the C2-C4 region, that includes PNGs at N295, N332, N339, N386 and N392 (Scanlan et al., 2002; Calarese, 2003). Most of these PNGs were profiled as high mannose type N-glycans and 2G12 was suggested to recognise α1-2 linked mannose residues (Scanlan et al., 2002; Calarese et al., 2003). Other classes of broadly neutralizing antibodies (bnAbs) including PG9 and PG16 that recognize high mannose N-glycans at N160 and N156 or N173 and PGT127 and PGT128 that recognize epitopes linked to PNGs at N301 and N332 in the V3 loop were identified (McLellan et al., 2011; Pejchal et al., 2011; Walker et al., 2011; Moore et al., 2012). These bnAbs recognize mainly Man8 and Man9 N-glycans and were found to neutralize a vast array of isolates across clades. Most recently, the mannose patch around N332 and the V3 loop of gp120 was described as the "supersite of immune vulnerability" as they were recognized by the PG128 class of bnAbs. The N-glycans around this area form glycan-glycan interactions or glycan-protein interactions that form a thick fence of N-glycans that prevent access of mannosidases, leading to poor/incomplete processing of the N-glycans. This leaves an oligomannose "patch" which cannot be processed to hybrid and complex N-glycans, and identifies them as non-self to the immune system. It was also found that some antibodies are able to extend their variable regions to penetrate the glycan shield around this area thus enabling binding to epitopes below the glycan shield (Pejchal et al., 2011; Pritchard, Spencer, et al., 2015).

1.3.4.5 N-glycosylation and receptor interactions.

N-glycosylation has been linked to the switch from R5 to X4 tropism (Clevestig et al., 2006), suggesting that N-glycans determine Env interactions with co-receptors on CD4 T cells. Furthermore, it has also been demonstrated that N-glycans interact with the DC CLR, DC-

SIGN (Hong et al., 2002). The DC-SIGN binding site overlapped with 2G12 binding, suggesting that DC-SIGN interaction with Env gp120 might involve PNGs N295, N301 and N386 (Hong et al., 2002). Furthermore, this same group and later Liao *et al.* (2011) identified PNGs by deletion mutagenesis named the DC-SIGN interactive domains which included N276, N295, N356, N386 and N392, as they were involved in binding to DC-SIGN. Liao *et al* did not test functionality of the deletion mutants and loss of PNGs could have abrogated Env function and thus likely structure, as in the case of N386 (Wang et al., 2013; Mathys et al., 2014). In addition, the interaction was suggested to favour mostly high mannose type N-glycans. Env expressed in cells that add mostly mannose type N-glycans were found to enhance the interaction between Env and DC-SIGN (Lin et al., 2003; Eggink et al., 2010; van Montfort et al., 2011). This suggested that high mannose type N-glycans were important to bind DC-SIGN. Interaction with DC-SIGN also leads to the release of the immune suppressor, IL-10 by MDDCs (Shan et al., 2007). DC-SIGN-initiated immunosuppressive responses raises the concern that poor immunogenicity of past vaccine candidates may be due to gp120 N-glycans. Therefore, further research into the effects of N-glycosylation on the regulation of the immune response by DCs could be very important for future vaccine design.

1.4 HIV transmission

1.4.1 Mechanisms of HIV transmission

Infection of HIV is a multi-step process, whereby the Env mediates attachment of the virus to the host cell by sequential binding to the CD4 receptor and then to co-receptors, CCR5 or CXCR4. This is followed by conformational changes that leads to fusion of the viral and host cell membranes and then transfer of the viral components into the host cell (Maddon et al., 1986; McDougal et al., 1986, 1991; Jones, Korte & Blumenthal, 1998; Sullivan et al., 1998).

Although HIV infection occurs via multiple routes: parenteral, intra-partum, homosexual and heterosexual intercourse, heterosexual transmission accounts for about 70% of HIV infections, despite the low per coital transmission rate (Wawer et al., 2005; Hladik & McElrath, 2008; Boily et al., 2009; Gray & Wawer, 2012).

Multiple mechanisms have been proposed to explain the low per coital infection rate of HIV. Firstly, physically protective barriers such as the genital mucosa and the stratified squamous epithelial (SSE) layer of cells are protection against incoming pathogens (Haase, 2011). There are also relatively few CD4+ T cells at the genital mucosa (Spira et al., 1996; Hu, Gardner &

Miller, 2000; Hladik et al., 2007) and are found mostly in the lamina propria, to which the virus has poor access. However, some authors hold that HIV directly infects T cells in the genital mucosa, resulting in systemic, productive infection (Zhang, Wietgrefe, et al., 2004).

There are also local immune responses from cells in the female genital tract (FGT) (Reis Machado et al., 2014) from DCs and other innate immune cells of monocyte lineage. DCs are expected to bind HIV via pathogen capture receptors including DC-SIGN and other C-type lectins expressed on the cell surface, undergo maturation and present antigens to CD4 T cells in the lymph nodes, triggering an adaptive immune response (Steinman, 1991; Banchereau & Steinman, 1998; Steinman & Hemmi, 2006). It has been suggested, however, that HIV is able to hijack this process, facilitating its transmission and sustained replication (Geijtenbeek, Kwon, et al., 2000; Kwon et al., 2002; Parrish et al., 2013; Shen, Kappes, et al., 2014)

1.4.2 Biological features of transmitted viruses

Highly homogenous variants are transmitted during mucosal HIV-1 transmission in healthy individuals (Wolfs et al., 1992; Zhang et al., 1993). Typically, heterosexual transmission has been characterized by a genetic bottleneck, whereby 80% of productive infections are initiated by a single variant despite the diverse quasi-species circulating in the infected donor (Keele et al., 2008; Abrahams et al., 2009; Keele & Estes, 2014). This was further confirmed in non-human primates models (Rhesus Macaques) inoculated with diverse SIVmac251 or SIVsmmE660 where only one or few variants established productive infection 1 - 5 weeks post inoculation (Keele & Derdeyn, 2009). This is unlike transmission in the presence of sexually transmitted infections (STI's), where the mucosal barrier is compromised and infection with more than one variant takes place (Haaland et al., 2009). Whether transmission of a single variant happens randomly or whether the transmitted founders (TFs) possess specific biological properties (Shaw & Hunter, 2012) that give them an advantage over those not transmitted has been a subject of research for some time now.

1.4.3 The role of Env in HIV transmission

1.4.3.1 Phenotypic properties of HIV-1 transmitted founder Envs

Previous studies have attempted to characterize TF Envs and the properties that could influence their likelihood of transmission (Table1.1). A common characteristic of TFs is their ability to use CCR5 for entry and it was previously suggested that R5 tropism was due to the availability of macrophages at the site of transmission. However, recent studies have suggested that TFs

are not macrophage-tropic, require high CCR5 density and are more sensitive to CCR5 inhibitors (Berger, Murphy & Farber, 1999; Parker et al., 2012; Ping et al., 2013). Furthermore, when Etemad et al. (2009) took the V1-V5 region from early and chronic variants and cloned it into an isogenic HIV backbone to generate chimeric infectious molecular clones (IMCs), the chronic infection variants replicated to higher titers in cells with lower CCR5 levels compared to those from early infection, suggesting that TF variants might require higher levels of CCR5 for infection (Etemad et al., 2009).

On the contrary, when 8 transmission pairs were evaluated for differences in co-receptor utilization, it was shown that both TFs and chronic infection viruses had a requirement for high CD4 and CCR5 levels and utilized alternative receptors similarly (Isaac-Beck et al., 2009).This was supported when Willen *et al.* (2011) compared the Env from 24 transmitted/founder viruses to 17 from chronic infection in a pseudotyped assay for their ability to utilize CCR5, CD4+ subset cell tropism, fusion kinetics and dendritic cell *trans*-infection. They found that the TFs were only marginally more sensitive to CD4 binding site antibodies than those from chronic infection. TFs had increased sensitivity to neutralizing antibodies (like b12 and VRC01) and pooled HIV hyper-immune immunoglobulin [HIVIG] (Wilen et al., 2011), or preferentially bound a subset of CD4+ T cells with increased α4β7 integrin levels (Arthos et al., 2008; Cicala, Arthos & Fauci, 2011; Nawaz et al., 2011). However when this was tested using a panel of SGA derived *env* clones from 20 acute and 20 chronic infection variants from different participants, there was no difference in α4β7 reactivity, utilisation of CD4 and CCR5 nor sensitivity to CD4 binding antibodies (Parrish et al., 2013). These variants were also suggested to replicate more efficiently in CD4+ T cells while others were found to have enhanced binding to DC-SIGN receptor relative to chronic infection clones (Parrish et al., 2013; Ping et al., 2013). These conflicting results (table 1.1) could suggest that different approaches that include the use of pseudoviruses and IMC's and transmitted founder and chronic samples from different participants might confound the final result.

Table 1.2 Phenotypic characterisation of transmitted founder HIV-1 variants.

Phenotype	Subtype	TF compared to chronic infection variants	Publication
Sensitivity to CD4 binding site Abs (b12, VRC01 and pooled HIV IgG	B	Increased	*Wilen *et al*., 2011; Parker *et al*., 2012; Parrish *et al*., 2013)
Sensitivity to neutralization by autologous Abs, but not heterologous	C	Increased	(Derdeyn et al., 2004)
Sensitivity to CCR5 inhibitor (Maraviroc)	C, B	Increased	#Parker *et al*., 2012; Ping *et al*., 2013)
Receptor tropism	C	More CD4 dependence, enhanced replication in CD4+ cells	Ping *et al*., 2013 Parrish *et al*., 2013
PNG at position 415 involved in escape from autologous Abs	B	PNG at 415 underrepresented in T/F	Gnanakaran *et al*., 2011
PNGs at N234, N442, N611 and N625 associated with sensitivity to Ab	C	Less represented in T/F	Ping *et al*., 2013
Replication in primary CD4+ T cells	B	Similar kinetics	Parrish *et al*., 2013
MDDC capture & enhanced interaction with vaginal myeloid DCs and transfer to CD4+ cells	B, C	Enhanced	de Witte, Nabatov & Geijtenbeek, 2008; Parrish *et al*., 2013; Shen, Kappes, et al., 2014
Concentration of Env particles per virus	B, C	Increased	(Parrish et al., 2013)
IFN resistance	B	More resistant	(Parrish et al., 2013)
Interaction with Langerin on LCs/infection of LCs		Both	(Kawamura et al., 2005; Hladik et al., 2007; de Witte, Nabatov & Geijtenbeek, 2008)
Interaction with α4β7	C	Increased No difference	(Arthos et al., 2008; Cicala, Arthos & Fauci, 2011; Nawaz et al., 2011) Parrish et al, 2013

#Parrish et al., 2013, used infectious molecular clones (IMC), not paired samples while Parker et al., 2012 samples were not paired but used IMC.

*Wilen et al., 2011, used pseudovirus, of paired samples from the same participant that were SGA derived.

1.4.3.2 Genotypic properties of early founder viruses

Genotypic properties identified in subtypes A, C and D early founder viruses included shorter variable loop length with fewer PNGs (Chohan et al., 2005; Sagar et al., 2006) but this was not apparent for subtype B TFs (Frost et al., 2005). PNG numbers increased during the course of infection with insertions/deletions and shifting of PNG positions likely due to escape from immune pressure as TFs have increased sensitivity to neutralizing antibodies (Derdeyn et al., 2004; Sagar et al., 2006). Whereas previous studies found that early founder viruses encode Env sequences closer to the ancestral sequence (Herbeck et al., 2006; Sagar et al., 2009), variants from chronic infection were generally more diverse. In subtype B variants, a Histidine or basic amino acid was more frequent at position 12 of the signal peptide of early founder variants, which enhanced Env expression, incorporation into viral particles and infectivity. Lack of or underrepresentation of a PNG at N234, N442, N611 and N625 (Ping et al., 2013) and N415 (Asmal et al., 2011; Gnanakaran et al., 2011) compared to chronic infection clones was also identified in subtype B TFs. Furthermore, PNGs at positions N241, N262, N386, N392 and N448 of subtypes B and C Envs were specifically enriched with high mannose type N-glycans in TFs, unlike Envs isolated at chronic infection (Go, Hewawasam, et al., 2011). These findings coupled with the change in number and reshuffling of PNGs and the importance of mannose N glycans in interaction with DC-SIGN (Lin et al., 2003; Eggink et al., 2010; van Montfort et al., 2011) led to the suggestion that Env N-glycosylation might play a role in the selective interaction of some variants with genital mucosal DCs for transmission and productive infection.

1.5 The Genital mucosa barrier

The vaginal epithelium is composed of many layers of SSE with a thickness ranging from 200 to 300 µm of tightly packed cells separating the mucus membrane from the lamina propria and an underlying vascular sub-mucosa. The mucus membrane, epithelium and lamina propria comprise the mucosa. SSE is made up of four zones: basal, squamous, granular and cornified layers (Miller & Shattock, 2003; Haynes & Shattock, 2008). The cells of the epithelium as well as the more fragile single cell layer epithelium of the endocervix, are held together by proteins that form desmosomes, tight junctions, and adherens junctions, whose permeability fluctuates depending on environmental changes (Pope & Haase, 2003).

Trauma during sexual intercourse and co-STI-infections could lead to tears in the epithelial layers that allow passage of HIV despite mucus covering the endo-cervical epithelium (Haaland et al., 2009). Ecto- and endo-cervical epithelial and endometrial mucosae have been suggested to be involved in HIV transmission through transcytosis of the virus across tight junctions. Some studies suggested that endocytosed virus was stored prior to transfer to target cells (Wu, Chen & Philips, 2003; de Witte, Bobardt, et al., 2007; Kinlock et al., 2014). It was also suggested that DCs and Langerhans cells (LCs) are interspersed within the vaginal lumen and the lamina propria (Figure 1.3). DCs and LCs extend their dendrites into the mucosa to sense pathogens, trap virus and can either return to the epithelium or travel with bound virus to the sub-epithelium to infect target cells (Hu, Gardner & Miller, 2000; Miller & Shattock, 2003; Miller et al., 2005). Studies in rhesus macaques showed that DCs and LCs were the predominant target cell type infected within 24 – 72 hours post intravaginal inoculation (Haase, 2011). Using vaginal tissues explants, DCs were also the major cell type infected following inoculation (Shen et al., 2014) although Zhang *et al.* (1999) detected viral RNA (vRNA) mostly in CD4+ T cells in the endocervix (Zhang et al., 1999) following inoculation. The latter study did indicate that breaks in the single epithelial layer lining the cervix might have compromised the genital tract barrier, leading to infection of CD4+ T cells present at higher numbers in this region (Zhang et al., 1999) which could explain why vRNA was detected in very few DCs and macrophages (Zhang et al., 1999). In non-human primates, foci of productively infected cells have been found in the endocervix early in infection, (Li, Estes, et al., 2009; Haase, 2010) although they did not specify if virus was captured by other cell types before delivery to CD4+ T cells.

Bobardt *et al.* (2007) further suggested that primary genital epithelial cells expressed galactosyl ceramide (GalCer) and proteoglycans such as chondroitin sulfate proteoglycans (CSPGs) and heparan sulfate proteoglycans (HSPGs) that promote HIV attachment to these primary epithelial cells (Bobardt et al., 2007; Magerus-Chatinet et al., 2007). Furthermore, it was suggested that epithelial cells in this area are capable of producing chemokines which help recruit plasmatoid (pDC) to the basolateral surface of the cervical epithelium (Li, Estes, et al., 2009; Nazli et al., 2010). Nazli *et al.* (2010) found, using genital and intestinal epithelial cell line models that HIV induced a chemokine concentration gradient, opening up tight junctions and allowing target cells from underlying layers to gain access to virus (Nazli et al., 2010) . Other *ex vivo* studies, using an R5 virus have also suggested that when the surface of epithelial

layers were exposed to virus, gp120 attracted DC-SIGN-expressing cells that made contact with the virus (Cavarelli et al., 2013).

1.6 Mechanism of Dendritic Cell mediated transmission

DCs and macrophages are frequently present at the sub-epithelium of the genital mucosa (Spira et al., 1996; Hu, Gardner & Miller, 2000; Hladik et al., 2007) and are permissive to HIV infection although with poor efficiency (Lee et al., 1999; Wu & KewalRamani, 2006). However, other studies have suggested that despite their limited susceptibility to infection (Smed-sörensen et al., 2005), DCs play a facilitating role by binding HIV in the genital mucosa and transferring virus to CD4+ T cells in the sub-epithelium or lamina propria, and/or translocate to the proximal lymph nodes and *trans*-infect CD4+ T cells (Curtis, Scharnowske & Watson, 1992; Geijtenbeek, Torensma, et al., 2000; Wu & KewalRamani, 2006) (Figure 1.3).

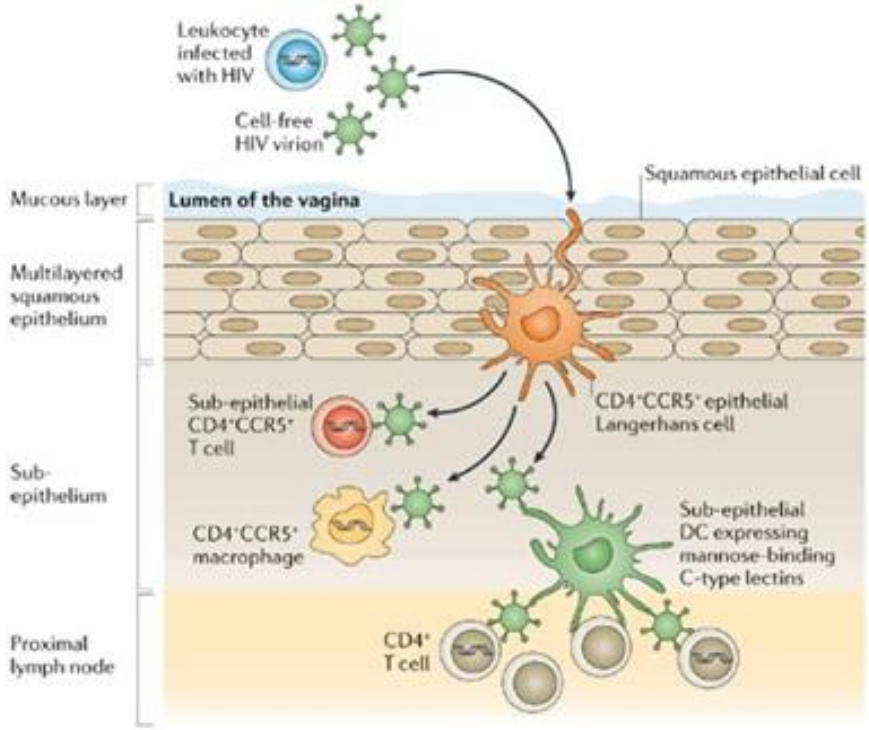


Figure 1.3 Structure and arrangement of genital tract tissues. Normal vaginal epithelium comprises a multi-cellular layer of stratified squamous epithelial cells. Intra-epithelial dendritic cells (DCs) or Langerhans cells (LCs) extend processes into the vaginal lumen, bind virus and infect CD4+ T cells in the sub-epithelium. Within hours after exposure, $CD4^+$ T cells in the sub-epithelium are infected as well as sub-epithelial DCs and macrophages expressing mannose-binding C-type lectins. Shortly thereafter, the virus can be found in nearby lymphoid tissues (Lederman, Offord & Hartley, 2006), Lederman *et al. Nature Reviews Immunology* **6**, 371–382 (May 2006) | doi:10.1038/nri1848

Alternatively, studies in non-human primate models suggested that DCs took up and protected virus until inflammation attracted increasing numbers of activated CD4+ T cells to the mucosa for local amplification before dissemination to lymphoid tissues (Hu, Gardner & Miller, 2000; Miller & Shattock, 2003; Miller et al., 2005; Hladik et al., 2007).

1.6.1 DCs and their contribution to HIV infection

DCs are located in all peripheral tissues and function in immune surveillance as professional antigen presenting cells (APCs). They possess multiple pattern recognition receptors (PRR) [toll like receptors (TLR) and CLRs] for capturing antigens, including HIV. They are able to initiate both innate and adaptive immune responses upon pathogen encounter, and modulate the immune response. Upon antigen capture, they traffic to secondary lymphoid tissues to present antigens to naïve T cells to initiate an adaptive immune response (Banchereau & Steinman, 1998; Banchereau et al., 2000; Pulendran, 2001). Once they recognize a pathogen, DCs mature and induce the expression of pro-inflammatory cytokines (Janeway & Medzhitov, 2002; Iwasaki & Medzhitov, 2004; Medzhitov, 2007)(Huppa JB and Davis MM, 2003) during antigen (Ag) presentation to T cells via MHC-T cell receptor (TCR) complexes. They also up-regulate co-stimulatory molecules to enhance T cell priming and produce cytokines to initiate and maintain the adaptive immune response (Banchereau & Steinman, 1998; Banchereau et al., 2000). During Ag presentation, an immunological synapse forms that HIV uses as a "virological/infectious synapse" that aids efficient transfer of virus to CD4+ T cells (McDonald, 2003). *In vitro* studies found this method led to enhanced infection levels (Cameron et al., 1992; Kwon et al., 2002; Wu & KewalRamani, 2006; Piguet & Steinman, 2007; Reyes-Rodriguez, Reuter & McDonald, 2016).

1.6.1.1 DC subtypes and roles in HIV transmission

DCs include myeloid (mDCs), pDCs and LCs. Although mDCs and pDCs are involved in HIV infection the role of LCs is still debated (Kawamura, 2000; Kawamura et al., 2005; de Witte, Nabatov, et al., 2007). Although both are involved in HIV transmission, mDCs which are located in mucosal surfaces are more susceptible to HIV infection than pDCs (located mostly in tissues but also in blood). pDCs play more often an antiviral role as they secrete antiviral interferons (Granelli-Piperno et al., 2004; Smed-sörensen et al., 2005).

As LCs are located in the sub-epithelium (Figure 1.3) and extend their processes to sample incoming pathogens, they are among the first cell types to encounter HIV (Spira et al., 1996;

Hu, Gardner & Miller, 2000). They capture the virus using langerin, a CLR and target virus to the Birbeck granules, a special organelle/compartment for degradation (de Witte, Nabatov, et al., 2007). Since they also possess CD4 and CCR5, some authors argue that LCs become infected when high viral loads lead to saturation of langerin. It has been suggested that this could be one mechanism to explain why TFs are R5 tropic as LCs do not express CXCR4 (Sozzani et al., 2000; Kawamura et al., 2005; de Witte, Nabatov & Geijtenbeek, 2008). Other studies suggest LCs are not infected by HIV but instead bind virus in the vaginal lumen and infect CD4+ T cells in the sub-mucosa (Kawamura et al., 2005; Hladik et al., 2007) because of their strategic location (de Witte, Nabatov & Geijtenbeek, 2008). Studies conducted *in vitro* on mDCs, pDCs and MDDCs suggested that DCs are infected with HIV as well as sequester infectious virus in endosomal compartments for transfer to CD4+ T cells (Loré et al., 2005; Nobile et al., 2005). The consistency between findings using mDCs, pDCs and MDDCs suggests that these cells have similar properties and morphology. This is important as mDCs and pDCs are difficult to isolate and most experiments utilise MDDCs differentiated from CD14+ monocytes to mDCs (Sallusto & Lanzavecchia, 1994).

1.6.1.2 Cis- and *trans*-infection

1.6.1.2.1 Cis- infection

DCs facilitate HIV transmission and have been found to play a critical role in dissemination at local mucosal sites and systemic spread (Wu & KewalRamani, 2006; Piguet & Steinman, 2007; Rinaldo, 2013; Ahmed et al., 2015). All subsets of DCs are poorly infected most likely due to low levels of CD4 and CCR5 expression (Lee et al., 1999). MDDCs express 10- to 100-fold lower levels compared to CD4+ T cells (Wu & KewalRamani, 2006). Furthermore, DCs have interferon (IFN) dependent and independent restriction factors like apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G (APOBEC3G), a Cytidine deaminase human enzyme which introduces G-A mutations in the HIV genome. APOBEC3G is found in DCs and CD4+ T cells and was upregulated after SIV inoculation in a macaque study (Pido-Lopez et al., 2007). Other HIV restriction factors include Sterile A motif and HD-domain containing 1 (SAMHDI) which has RNAse activity and hydrolyzes deoxyribonucleotides in cells, thus limiting nucleotide availability for reverse transcription (Pion et al., 2006), Tripartite motif 5α (TRIM5α) and tetherin. These limit viral replication in DC subsets, macrophages and sometimes T cells (Pion et al., 2006; Huthoff & Towers, 2008). SAMHDI is specific for monocytes, mDCs and macrophages and is inhibited by Vpx (Laguette et al., 2011), although

inhibition is reduced by high SAMHDI levels (Bloch et al., 2014). Poor replication in DCs however could still produce sufficient virus (*de novo* virus) to infect T cells in *trans*. In addition, apart from helping virus dissemination, viral uptake by DCs could lead to latent viral reservoirs (Kwon et al., 2002; Reyes-Rodriguez, Reuter & McDonald, 2016).

1.6.1..2.2 CLR dependent *trans*-infection

The binding and transfer of HIV to T cells by DCs without being infected themselves is termed *trans*-infection and involves cell to cell contact (Geijtenbeek, Torensma, et al., 2000; Kwon et al., 2002). *Trans*-infection was suggested to play a key role in virus spread and dissemination as the virus needs to reach the lymphoid tissues to replicate to high levels to establish systematic infection (Geijtenbeek, Kwon, et al., 2000). When T cells were co-cultured with HIV-1 pulsed DCs, infection was higher than when T cells were directly infected with the same amount of virus (Cameron et al., 1992). Curtis *et al*. (1992) found that the binding and transfer of HIV by DCs was via interaction with gp120 in a CD4 independent mechanism, but via an unidentified CLR (Curtis, Scharnowske & Watson, 1992). Further studies by Geijtenbeek *et al.* (2000) found that HIV gp120 bound MDDCs through a mannose binding CLR (MCLR), DC-SIGN (Geijtenbeek, Kwon, et al., 2000; Geijtenbeek, Torensma, et al., 2000). It was suggested that DCs take up the virus, sequester and protect it in an infectious form before transferring to T cells across an infectious synapse (Geijtenbeek, Kwon, et al., 2000; Kwon et al., 2002). Once virus is taken up into the endosomal compartment, Env stays bound to DC-SIGN, which helps recycle the virus to the surface upon encounter with a T cell. The Env-DC-SIGN complex is concentrated at sites in contact with the T cells, which then recruit CD4 and co-receptors forming the infectious/virological synapse (Figure 1.4 A). Hence, both DC-SIGN and T cell receptors are required for formation of the adhesive junction between the two cells (McDonald, 2003). Adhesion molecules: ICAM-1, ICAM-3 and LFA-1, enhance the strength of the synapse and ensure efficient transfer of the virus to the T cells.

Trans-infection was initially demonstrated in MDDCs, but was also later shown in mucosal and dermal DCs, which also express DC-SIGN. However, the fate of virus taken up by DCs remains debatable (Gummuluru, KewalRamani & Emerman, 2002; Wu & KewalRamani, 2006). While some studies suggested that immature DCs can retain infectious virus for up to six days following exposure (Figure 1.4, A) (Geijtenbeek, Kwon, et al., 2000; Kwon et al., 2002; McDonald, 2003), other studies found that virus taken up by MDDCs is degraded within 24 hours so that infection of CD4+ T cells is by *de novo* virus (Turville et al., 2004, 2008)

(Figure 1.4, C). Turville *et al.* (2004) then suggested a "two phase" mechanism of DC-mediated HIV transfer. The first phase occurs within 24 hours when endosomally recycled virus are transferred to T cells (Figure 1.4, A) and the second phase involves the transfer of *de novo* R5 viruses after 24 hours (Turville et al., 2004). This was supported by studies conducted with mDCs and pDCs (Loré et al., 2005; Nobile et al., 2005).

Other studies found that following binding of Env to DC-SIGN on DCs, some of the virus was taken up into multivesicular bodies (MVB) (Figure 1.4 B) rich in tetraspannins (CD81). These variants were translocated by formation of DC vacuoles (Gummuluru, KewalRamani & Emerman, 2002) that budded into T cells upon contact with DCs in a gp120 independent manner, transferring the virus by exocytosis. The virus taken up into MVB escapes degradation and survives in acidic, non-lysosomal compartments and binding to DC-SIGN helps in the uptake process (Gummuluru, KewalRamani & Emerman, 2002)..

DCs express other CLRs able to bind HIV and *trans*-infect T cells, including Mannose receptor (MR) and DC immunoreceptor (DCIR) (Turville et al., 2001; Lambert et al., 2008).

Irrespective of the mechanism, transfer of HIV to CD4+ T cells by DCs is in conflict with its role as immune sentinels (Steinman, 1991, 2000; Banchereau & Steinman, 1998). Instead, DCs are hijacked by HIV which subverts their function to get access to T cell rich areas. How this DC-SIGN-bound HIV escapes processing and presentation is not fully understood.

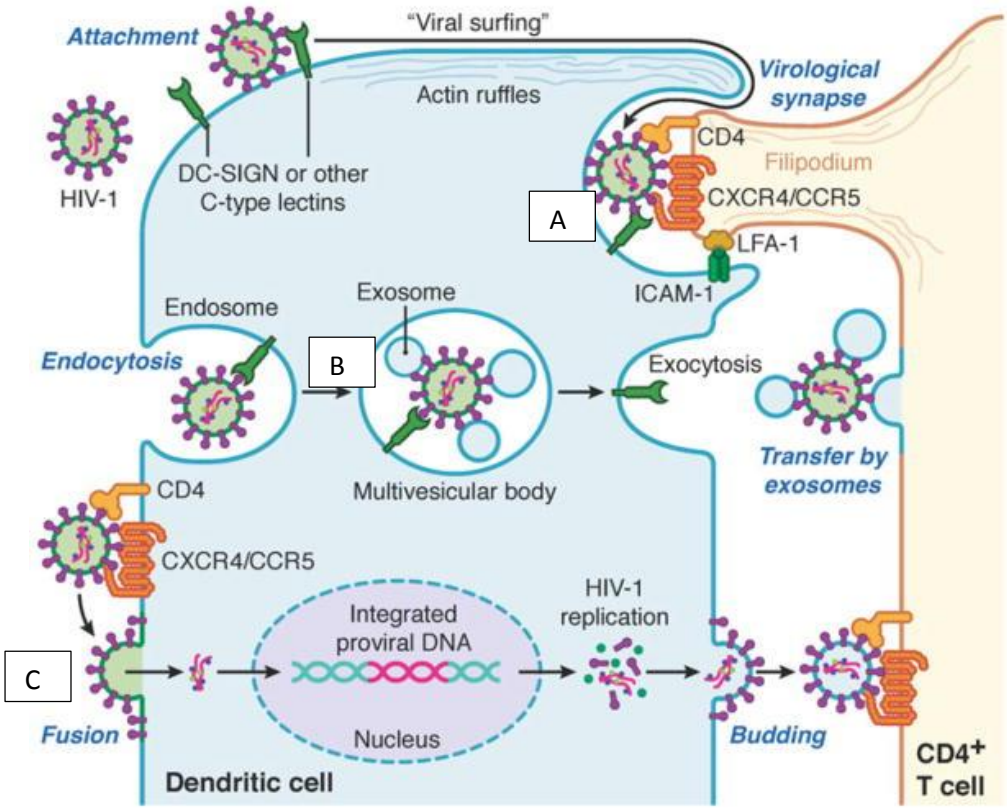


Figure 1.4 Mechanisms of DC-mediated HIV-1 transmissions. Incoming HIV-1 can be transmitted from DCs to $CD4^+$ T cells by three distinct mechanisms. A*: trans*-infection via the virological synapse: HIV-1 binds to DC-SIGN on the DC surface and taken up into endosomal compartments. When DCs make contact with $CD4^+$ T cells, a virological synapse is formed stabilized by interactions between ICAM-1 and LFA-1. The $CD4^+$ T cell extends a filipodium, by actin cytoskeleton rearrangements, into the pocket on the DC surface to capture the HIV-1 while concentrating its CD4 and co-receptors at the surface. B: *trans*-infection via the exosome secretory pathway where endocytosed virus form multi-vesicular bodies and is released in association with exosomes to infect nearby $CD4^+$ T cells. C: *cis*-infection where following binding to DC-SIGN and CD4/co-receptors, HIV-1 enters DCs by fusion with CD4 and co-receptors and replicate to generate *de novo* infectious HIV-1 that infect nearby $CD4^+$ T cells (Coleman, St Gelais & Wu, 2013) Adv. Exp. Med. Biol. 2013; 762: 109–130. doi: 10.1007/978-1-4614-4433-6_4

1.6.1.3 CLR-independent transfer of HIV by DCS

Besides CLR-mediated DC transfer of virus to T cells, there are other Env-CLR independent attachment factors on some DCs subsets. These include Syndecan-3, a DC-specific heparin sulfate (HS) proteoglycan that binds to Env via charged basic interactions and stabilizes virus-DC interaction to favour *cis*-infection (de Witte, Bobardt, et al., 2007). Glycosylphospholipids (GSLs) are Env independent methods of virus transfer by MDDCs. Trisialotetrahexosylganglioside (GM3) (and also GM1) are incorporated into virions as it buds from the plasma membrane of host cells following replication (Izquierdo-Useros, Lorizate,

Contreras, et al., 2012). Gangliosides are highly sialylated GSLs, with terminal α-2, 3 sialic-acid residues that bind to CD169, a sialoadhesin (also called Siglec-1) on mature MDDCs (Puryear et al., 2012) that enable transfer of captured virus to T cells. CD169 capture of HIV has two mechanisms: 1) a gp120-independent one which uses GSLs on mature MDDCs and 2) a gp120-dependent mechanism observed in macrophages (Puryear et al., 2012; Kijewski & Gummurulu, 2015). Galactosyl ceramide (GalCer) is another receptor expressed on immature MDDCs (iMDDCs) and primary DCs isolated from human blood, mucosal tissue and epithelial cells. GalCers capture HIV-1 through gp41 and mediate transfer of virus to CD4+ cells (Magerus-Chatinet et al., 2007).

1.7 DC-SIGN-mediated regulation of DC immune responses

To facilitate their survival pathogens have evolved to use DC-SIGN or other CLRs to activate a DC-specific signaling mechanism to sabotage TLR-dependent responses (Geijtenbeek et al., 2003; Van Kooyk, Appelmelk & Geijtenbeek, 2003). HIV-stimulated DC-SIGN-mediated signaling in conjunction with TLR8 activation, recruited pTEF (transcription elongation factor) to the NF-KB promoter, which led to full-length HIV transcripts (Gringhuis et al., 2010). Furthermore, HIV-DC-SIGN stimulated signaling increased Rho-GTPase activity required for the formation of DC-SIGN-mediated virological synapses (Hodges et al., 2007). The immunological consequences of HIV-induced DC signalling are not fully understood.

It has been suggested that pathogens or pathogenic products that induce IL-10 expression could to subvert DC function to favour immune suppression and thus pathogen survival (Geijtenbeek et al., 2003; van Kooyk & Geijtenbeek, 2003; Geijtenbeek & Gringhuis, 2009). The interaction of DC MCLR with gp120 was suggested to induce the secretion of immunosuppressive cytokines like IL-10 (Shan et al., 2007), while impairing DC maturation and IL-12 secretion (Fantuzzi et al., 2004; Granelli-Piperno et al., 2004). This was accompanied by impaired up-regulation of co-stimulatory and MHC class II molecules. Env-DC-SIGN induced IL-10 secretion resulted from activation and phosphorylation of ERK (Shan et al., 2007) while DC-SIGN-mediated modulation of the TLR-dependent activation of NF-kB for inflammatory gene expression also enhanced IL-10 expression (Geijtenbeek & Gringhuis, 2009). Understanding how HIV-1 modulates DC-SIGN-meditated modulation of DC IL-10 production and ERK phosphorylation is important for the development of a vaccine that does not result in immune suppression (Shan et al., 2007; Banerjee et al., 2009).

Enhanced IL-10 production was due to DC-SIGN-mediated activation of NF-KB via Raf-1 (Gringhuis et al., 2007). Raf-1 activation was influenced by the type of carbohydrate on the PAMP/pathogen as mannose-containing pathogens induced responses different from those that carried fucose (Gringhuis et al., 2009). MAPKs are well known downstream effectors of Raf-1 (Wellbrock, Karasarides & Marais, 2004) (Figure 1.6), but Raf-1 activation by ManLAM in the absence of other TLR ligands did not lead to ERK activation (Gringhuis et al., 2007). However, other DC-SIGN ligands have been found to activate ERK. Capparos *et al*. (2006) found that ERK was phosphorylated in response to an anti-DC-SIGN antibody (MR-1) binding to DC-SIGN of MDDCs, ThP-1 and Jurkat cells (Caparros et al., 2006). Furthermore, studies by Shreffler *et al.* (2006) found that ligation of DC-SIGN by *Schistosoma mansoni* egg antigen led to activation of ERK. Zhao *et al* (2013) also found that the ligation of DC-SIGN by E2 of hepatitis C Env activated ERK. Moreover, Hsu *et al* (2010) found that a Bermuda grass protein, BG60 also activated ERK together with Raf-1 following binding to DC-SIGN (Caparros et al., 2006; Shreffler et al., 2006; Hsu et al., 2010; Zhao et al., 2013). Further studies by Gringhuis *et al*. (2009) found that DC-SIGN ligation led to the activation of Raf-1 kinase and to the modulation of TLR induced production of cytokines (Gringhuis et al., 2007; Geijtenbeek & Gringhuis, 2009) as well as acetylation of the p65 subunit of NF-κB. Modulation of TLR activation of inflammatory cytokine expression depends on the type of carbohydrate on the PAMP/ligand. While HIV and *Mycobacterium tuberculosis*, which carry mostly mannose type N-glycans induced Raf-1, *Helicobacter pylori*, which carry Fucose did not induce Raf-1 phosphorylation (Geijtenbeek & Gringhuis, 2009; Gringhuis et al., 2009), although both pathogens lead to enhanced IL-10 expression. As Shan *et al.* (2007) also found that HIV-1 Env mannose type N-glycans activated MDDC ERK leading to IL-10 production, (Shan et al., 2007) signaling downstream of Raf-1 could differ depending on variation in Env N-glycosylation. Taken together, these studies suggest that PAMPs with different carbohydrates may differentially influence the activation of signaling pathways via binding DC-SIGN and modulating TLR-mediated signaling via Raf-1. The impact of differential HIV-1 Env N-glycosylation on mitogen activated protein kinases (MAPK) activation is not known.

Dysregulation of DC-SIGN-activated immune responses was also shown to tilt the Th1/Th2 balance in favour of Th2 and regulatory T cell phenotypes (Clerici & Shearer, 1993; Clerici et al., 1993; Chehimi et al., 1994), but the impact of N-glycosylation on Th0 polarization is not well understood. Given that activation of DC-SIGN signaling could impact adaptive immune responses to vaccine immunogens, we investigated the impact of Env PNGs on the release of

cytokines by MDDCs. In view of the role of carbohydrates in DC DC-SIGN-mediated-signaling, we hypothesized that HIV Env N-glycosylation may have a role in modulating DC responses given that DC-SIGN binds gp120 N-glycans.

1.8 Cytokines regulate DC function

Cytokines are soluble mediators, which together with other inflammatory mediators like chemokines represent the effector phase of the innate immune response. They are secreted following ligand binding to pattern recognition receptors (PRR) such as TLRs and CLRs on DCs. Cytokine production is considered very important in HIV infection as cytokines produced by the innate immune system determines the strength of an adaptive immune response (Borish & Steinke, 2003). The types of cytokines produced following PAMP signaling depend on the pathogen so that the appropriate immune responses are initiated. PRRs are differentially distributed on inflammatory cells so that immune cells recognize different classes of PAMPs and induce similar but not identical patterns of soluble mediators. The response to pathogens thus varies according to the PAMP (Trinchieri, 2003), but also the PRR triggered. Both the innate and the adaptive immune responses regulate each other through the cytokines they produce, which sets the stage for pathogen clearance.

1.8.1 Cytokine storm in HIV infection

In HIV infection, inflammation meant to initiate an effective host immune response is often exploited by the virus (Deeks, Tracy & Douek, 2013). Inflammatory mediators cause the influx of immune cells to sites of infection, which contrary to fighting infection provide target cells for robust replication (Hladik & McElrath, 2008; Li, Estes, et al., 2009). Cytokine deregulation has been found in plasma of HIV infected patients: concentrations of pro-inflammatory cytokines, IL-8, IL-1 and TNF-α, and pleiotropic cytokines such as IL-10 and IL-6 were increased, while there was a decrease in IL-2, IL-12 and IFN-γ (Breen et al., 1990; Matsumoto et al., 1993; Chehimi et al., 1994; Daftarian et al., 1995; Taoufik et al., 1997; Marshall et al., 1999; Stylianou et al., 1999; Jennes et al., 2004; Roberts et al., 2010; Borges et al., 2014, 2015). Cytokine deregulation intensified with disease progression and was partially resolved with antiretroviral (ARV) treatment, suggesting a relationship between viral replication and inflammation (Stylianou et al., 1999). Further research found that combinations of certain cytokines enhanced HIV replication and disease progression (Breen, 2002), while HIV infection resulted in a switch from a Th1 to Th2 response (Clerici & Shearer, 1993; Buisson et al., 2009), with the cells of monocyte lineage and APCs being the main producers of IL-10

(Borghi et al., 1995; Ji et al., 2005). Deregulation was initially thought to be caused by replication of the virus in APCs (Poli et al., 1990; Hewson et al., 1999). However, later studies found that the mere interaction of gp120 N-glycans with DC-SIGN was sufficient to induce IL-10 secretion by MDDCs (Borghi et al., 1995; Shan et al., 2007) although other cell types may also contribute to the "cytokine storm". IL-10 release was accompanied by poor maturation of DCs and lack of up-regulation of co-stimulatory markers.

Buisson and colleagues further found that treatment of MDDCs with HIV-1 not only enhanced IL-10 secretion, but also inhibited the release of IL-12 and their capacity to stimulate T cell proliferation. HIV-induced IL-10 suppressive effects on DCs could thus have negative consequences on T cell responses to HIV infection (Buisson et al., 2009).

1.8.1.1 Anti-inflammatory cytokine IL-10

IL-10 is produced by multiple cell types including T cells, B cells, DCs and macrophages (Brockman et al., 2009; Hedrich & Bream, 2010). It is an anti-inflammatory cytokine that down-regulates the expression of inflammatory cytokines (IL-12, IL-1α, IL-1β, IL-18, TNF-α, IL-6, MCP-1, IL-8, IP10 and MIP-2α). It influences the outcome of Signal 1 and Signal 2 (Figure 1.5) by regulating MHC class II and co-stimulatory molecules in both an autocrine (Moore et al., 2001; Couper, Blount & Riley, 2008) and paracrine manner which can limit cellular immunity. It also inhibits the production of mononuclear phagocytes and NK cells and also alters Th1 and Th2 cytokine production (Clerici & Shearer, 1993; Buisson et al., 2009; Alter et al., 2010).

It is a multifunctional cytokine with both protective and regulatory roles. It protects against excessive tissue damage due to Th1, CD8+ and NK cell responses, especially in infections like *Toxoplasma*, *Trepanosoma*, *Mycobacterium* and some *Plasmodium* spp. that cause excessive production of IFN-γ and TNF-α (Moore et al., 2001). However, in viral and other intracellular bacterial infections, its inhibitory and anti-inflammatory functions/effects tend to suppress the innate and adaptive responses of DCs, macrophages and T cells respectively, inducing immune tolerance and thus survival of the pathogens and establishment of infection (de Waal Malefyt, Haanen, et al., 1991; Taoufik et al., 1997; Moore et al., 2001). This was recently demonstrated in experimental mouse models of Lymphocytic choriomeningitis virus infection (LCMV) and other persistent viral infections, where inhibition of IL-10 using either anti-IL-10 antibodies or

blocking IL-10 receptors led to clearance of persistent infection (Brooks et al., 2006; Ejrnaes et al., 2006; Wilson & Brooks, 2011).

1.8.1.2 Role of IL-10 in HIV infection

In HIV infection, there is increased serum IL-10 levels, which increase with disease progression, impair IL-12 production and tilt the Th1/Th2 polarization in favour of Th2 phenotypes (Clerici & Shearer, 1993; Taoufik et al., 1997; Stylianou et al., 1999). IL-10 production occurred very early in infection (Stylianou et al., 1999; Norris et al., 2006; Liu et al., 2014), increased with viral load suggesting that viral replication was the cause of IL-10 production (Stylianou et al., 1999). Further investigations found that IL-10 was not only induced by virus but that gp120 also influenced IL-10 secretion (Ameglio et al., 1994; Borghi et al., 1995). Additionally, HIV-induced IL-10 production leads to DC dysfunction in HIV infected subjects as IL-10 and IL-4 specific antibodies could reverse the Th1- Th2 switch in HIV infected lymphocytes from HIV infected donors (Clerici et al., 1993). Cells from healthy donors also became dysfunctional when exposed to HIV or gp120 (Borghi et al., 1995; Fantuzzi et al., 2004; Granelli-Piperno et al., 2004; Chougnet & Gessani, 2006; Shan et al., 2007; Alter et al., 2010).. However, Shan *et al*. (2007) found that only some gp120 variants stimulated MDDC IL-10 secretion independently of MDDC donor. They also suggested that gp120 N-glycans, specifically the mannose type glycans contributed significantly to the induction of IL-10 production in healthy MDDCs through their interaction with mannose CLRs (Shan et al., 2007). This was shown when removal of mannose residues significantly reduced gp120-stimulated IL-10 secretion and prevented loss of DC maturation and function (Shan et al., 2007; Banerjee et al., 2009, 2012).

1.8.1.3 IL-12 production and its role in infection

Bioactive IL-12p70, commonly known as IL-12 is a heterodimer of IL-12p40 and IL-12p35 subunits (Abdi, 2002). Not only is the secretion of the heterodimer highly modulated, the subunits are encoded by genes on separate chromosomes that are independently regulated at transcriptional and post-translational levels (Abdi, 2002). Therefore, the expression of IL-12 is carefully regulated and influenced by multiple factors (Carra, Gerosa & Trinchieri, 2000). It has been shown that the production of IL-12p70 and IL-12p40 by cells is significantly correlated, suggesting that secretion of IL-12p40 could be a surrogate marker for the heterodimer (Muller-berghaus et al., 2005). However, IL-12p40 can dimerise with itself to

form IL-12p80 and with IL-12p19 to form IL-12p23, with each playing a different role in immune responses. IL-12p40 and IL-12p80 were found to act as agonists of IL-12 by competing for binding to the IL-12R and preventing IFN-γ expression and thus inhibiting the initiation of a Th1 response in both murine and human cell models. However, knock-out experiments showed that mice lacking IL-12p35 but not IL-12p40 were resistant to infection and the authors suggested that IL-12p40 might form heterodimers with other molecules that initiate Th1 responses (Hölscher, 2004). It is also involved in stimulating the migration of DCs to the lymphoid tissues (Khader et al., 2006). Therefore, it has been suggested that the function of IL-12p40 is much more complex and that it might also act independently of IL-12 (Piccotti et al., 1997; Hölscher, 2004). The predominant function of IL-12 is to regulate the adaptive immune response. IL-12, IL-10 and the other regulatory cytokines play a very important role in the development of naıve CD4+ T cells into either Th1, Th2 cells or regulatory T cell subsets, respectively, which are essential for an effective adaptive immune response (de Waal Malefyt, Haanen, et al., 1991; Fiorentino et al., 1991; D'Andrea et al., 1993; Chehimi et al., 1994; Trinchieri, 1994, 2003; Biron & Gazzinelli, 1995; Marshall et al., 1999). IL-12 production by DCs enhances Th1 cell phenotype which then produces more IL-12 and IFN-γ (Figure 1.5d). Together with IFN-γ, IL-12 enhances phagocytic and cytotoxic activity of T cells, NK and macrophages while impairing Th2 differentiation and production of related cytokines like IL-4, IL-5 and IL-13 (Clerici et al., 1993; Marshall et al., 1999; Hamza, Barnett & Li, 2010). IL-12 is thus the main cytokine that regulates Th1 differentiation and preferentially induces T cell differentiation and expansion.

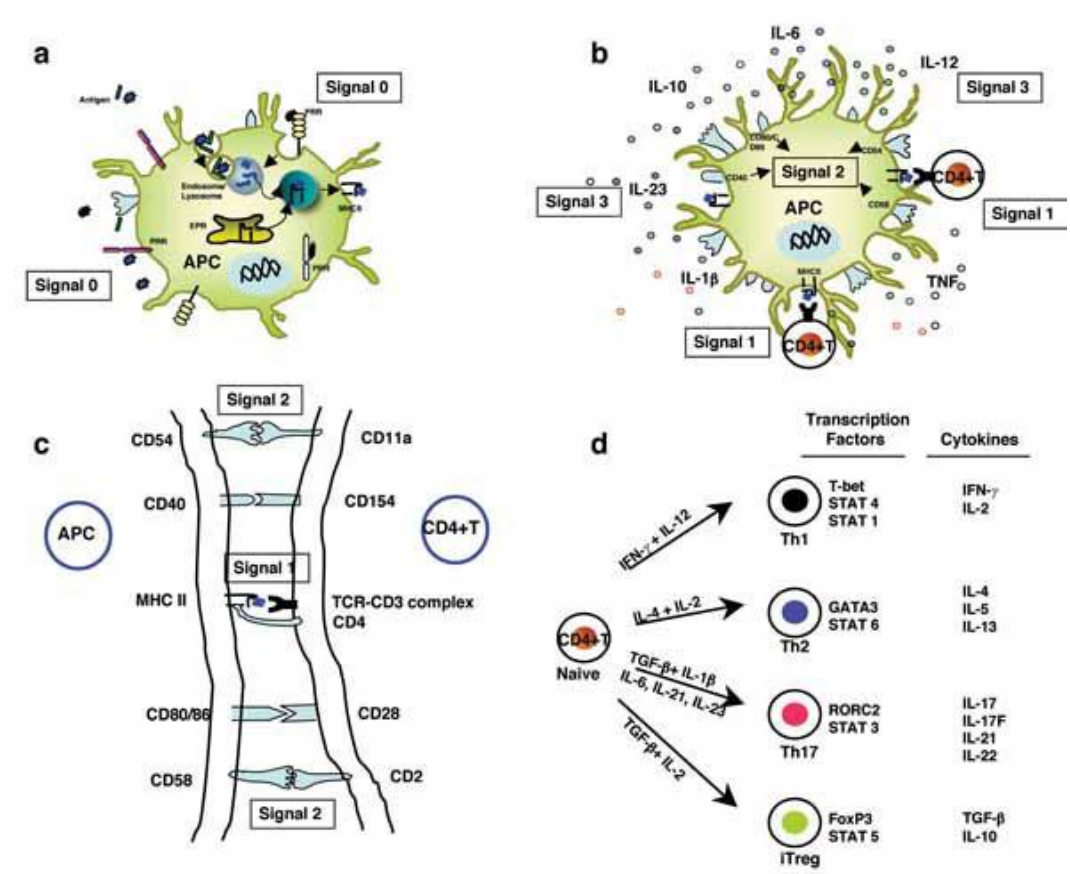


Figure 1.5 Dendritic cell (DC) stimulation and initiation of T cell polarization and differentiation into various subsets guided by the appropriate cytokine environment. a) Following the recognition of pathogen-associated molecular patterns (PAMPs) by PRRs on immature DCs, they mature (Signal 0) by up-regulation of maturation markers like MHC II, CD80 and CD86. Pathogen is internalized into the endosomes/lysosomes and is processed into microbial peptides. b) Antigenic peptides are transferred and bound to MHC II molecules and transported to the cell surface where they are presented in complex with the MHC II to T cell receptor (TCR) of naïve T cells (Signal 1). c) Mature and activated DCs express high levels of MHC, co-stimulatory (CD40, CD80 and CD86) and adhesion molecules (CD54 and CD58) (Signal 2) that helps to stabilize the immunological synapse, and secrete high amounts of cytokines IL-12 IL-10, IL-1 α, IL-1β, IL-6, IL-23, IL-4, IL-5, IL,13 and TNF-α and chemokines MIP1 α, MIP 1β, MCP-1, MIP3 α, IL-8 (Signal 3). d) The cytokines released by DCs play a very important role in inflammation and differentiation of CD4+ T cells into the right phenotypes or subset as Th1, Th2, Th17 and induced Tregs (iTregs). From (Ludewig et al., 2001; Aimanianda et al., 2009), Trends Pharmacol Sci 2009, 30:287-295.

1.8.1.4. Pro-inflammatory cytokines

IL-1, IL-6, IL-8 and TNFα together with IL-12 are pro-inflammatory cytokines predominantly produced by activated cells of the monocyte/macrophages lineage, including DCs, when exposed to inflammatory stimuli (Kedzierska & Crowe, 2001). They can also be produced by other cell types including B cells, T cells, fibroblasts and endothelial cells in response to viral and bacterial infection.

HIV-1 infection deregulates pro-inflammatory and anti-inflammatory cytokines (Breen et al., 1990; Chehimi et al., 1994; Daftarian et al., 1995; Taoufik et al., 1997; Marshall et al., 1999; Narimatsu, Wolday & Patterson, 2005; Roberts et al., 2010; Shah et al., 2011; Borges et al., 2014, 2015) by lowering the expression of Th1 cytokines such as IL-2, IL-12 and IFN-γ and increasing IL-10, IL-1, IL-6, IL-8 and TNF-α. It has been shown that TNF-α, IL-1 and IL-6 are suppressed by IL-10 under normal non-HIV conditions (Moore et al., 2001) whereas HIV infection seems to be associated with increased levels of inflammatory cytokines. Bebell *et al*. (2008) and Roberts *et al*., (2010, 2012) found elevated levels of IL-12, IL-1α, IL-1β, IL-6, TNF-α, and IL-8 in plasma and CVL of HIV infected women. A study by Stacey *et al.* (2009) which followed acutely infected HIV patients longitudinally also found up-regulation of these pro-inflammatory cytokines in plasma, with levels increasing over time (Stacey et al., 2009). These very cytokines have also been shown to enhance the risk of HIV acquisition in high risk women (Masson et al., 2015). IL-1β is one of the cytokines whose levels are indicative of infection and is highly correlated with HIV acquisition risk (Sturm-Ramirez et al., 2000; Masson et al., 2014, 2015; Liebenberg et al., 2016) However, these studies focussed on CVL and plasma of HIV-1 infected women and could thus not identify the source of increased inflammatory responses.

It has been shown that HIV interaction with DCs enhances infection of co-cultured CD4+ cells (Cameron et al., 1992), suggesting that DCs could play a very important role in HIV transmission. Due to the paucity of DCs *in vivo* MDDCs are commonly used in *in vitro* experiments (Romani et al., 1994) to identify how HIV interacts with DCs and subverts their normal immune responses. MDDCs infected with HIV failed to mature while stimulating the release of IL-10, suggesting a role for immunosuppression in HIV-1 infection (Granelli-Piperno et al., 2004; Shan et al., 2007). There is thus, a disconnect between *in vivo* and *in vitro* studies where the one suggests that HIV-1 stimulates inflammation which results in IL-10 levels increasing to prevent tissue damage (de Waal Malefyt, Abrams, et al., 1991) and the other suggesting that in DCs, HIV-1 binding to DC-SIGN induces IL-10 expression that, instead of down-regulating inflammatory cytokines, skews Th0 to Th2, facilitating HIV-1 survival in the female genital tract (Gringhuis et al., 2007). Both approaches have limitations: MDDCs might not be ideal models to study DC immune responses and results must be carefully considered before extrapolation to *in vivo* HIV-1 infection (Wu & KewalRamani, 2006) and *in vivo* studies are limited in their ability to identify cell types and the mechanism involved in DC immune modulation.

1.9 Signal activation and transduction following interaction of PAMPs with DC receptors including DC-SIGN.

The binding of extracellular ligand to specific transmembrane receptors leads to the conversion of extracellular stimuli to intracellular signaling pathways, which leads to inducible cellular processes such as transcription, translation, proliferation, differentiation and even apoptosis (Shaul & Seger, 2007).

TLRs play a very important role in the initiation of the immune response to pathogens, usually by two major regulatory programs: 1) a universal one activated by nearly all TLRs triggered by a wide range of ligands and 2) ligand-specific responses triggered by TLRs specific to individual microbial agents (Akira & Kiyoshi, 2004; Kawai T & Akira, 2005; Kawai & Akira, 2007). Once TLRs are triggered by PAMPs, intracellular signaling pathways are activated that lead to the induction of pathogen-tailored immune responses (Kawai & Akira, 2007). For NF-κB to be activated, inhibitor of kappa kinase (IKK) is degraded leaving NF-κB to translocate to the nucleus where it binds to enhancers or promoters of gene targets (Silverman & Maniatis, 2001; Janeway & Medzhitov, 2002).

Although Raf-1 phosphorylation leads to enhanced IL-10 expression and is necessary for DC-SIGN mediated modulation of TLR signaling, TLR activation is required before DC-SIGN activation can lead to NF-κB activation. A series of kinases downstream of Raf-1 are involved in NF-kB activation including phosphatidylinositol-3 kinase, Akt, and the most characterized and evolutionary conserved, the Ras-Raf-MEK/MAPK kinase pathway (Figure 1.6), responsible for the regulation of cell growth and differentiation in mammalian cells.

1.10 Mitogen Activated Protein Kinases

MAPKs are evolutionarily conserved protein kinases and central mediators that convey signal from cell surfaces to the nucleus. They are present in nearly all cell types and are called Serine/Threonine kinases after the amino acids that are phosphorylated during activation. Although they are the main components of signal transduction, they also phosphorylate other substrates including transcription factors such as c-Myc (Myelocytomatosis Viral Oncogene) and AP-1 (activator protein 1), which are both involved in cell growth, transformation and differentiation. In some cases, MAPKs amplify signals instead of just transmitting them and can also integrate signals from different receptors (Shaul & Seger, 2007; Kim & Choi, 2010).

MAPKs play critical roles in key cellular activities like proliferation, differentiation, survival and are also implicated in a variety of human disorders including cancers, and neurodegenerative diseases (Johnson & Lapadat, 2002; Zhang, Wei , Tu Liu, 2002; Kim & Choi, 2010). Given that components of the MAPK signaling pathway act differentially in many human diseases, a proper understanding of this pathway in response to HIV could provide valuable information for the design of vaccines that elicit the most protective immune responses against viral infection. Three major groups of MAP kinases have been well characterized in mammalian cells and include the extracellular regulated kinases (ERK), the c-Jun NH2 terminal kinases (JNK) and the p38 MAP kinases (Zhang, Wei , Tu Liu, 2002). Each of these groups have a tripeptide motif, Thr-X Tyr, with each MAPK differing by X. X is Glutamine, Glycine and Proline for ERK, p38 and JNK respectively. The activation of these kinases is by dual phosphorylation of the Thr and Tyr units of the tripeptide motif that transmits signal via mitogen activated protein kinase kinase kinase (MKKK), mitogen activated protein kinase kinase (MKK) and MAPK cascade (or Ras-Raf-ERK) in response to different ligands, although there is considerable cross talk between them (Johnson & Lapadat, 2002; Wellbrock, Karasarides & Marais, 2004), Figure 1.6).

P38 MAP kinases were first identified for their role in inflammation by regulating pro-inflammatory cytokine biosynthesis. Both JNK and p38 are activated by the presence of mitogen but JNK is also activated by cellular stress and the presence of inflammatory cytokines. JNK together with ERK have been suggested to prevent maturation of DCs (Tian, Zhang & Cohen, 2000). Activation of these MAP kinases leads to instructive signals that activate transcription factors present in the cytoplasm and the nucleus of DCs leading to the expression of target genes and then the appropriate biological response. Activation of the MAPKs occurs within minutes and their signals are transient (Shaul & Seger, 2007; Kim & Choi, 2010; Sancho & Reis e Sousa, 2012). Whereas ERK is mostly activated in response to pathogens, JNK and p38 are mostly activated in response to stress and inflammatory cytokines (Chang & Karin, 2001).

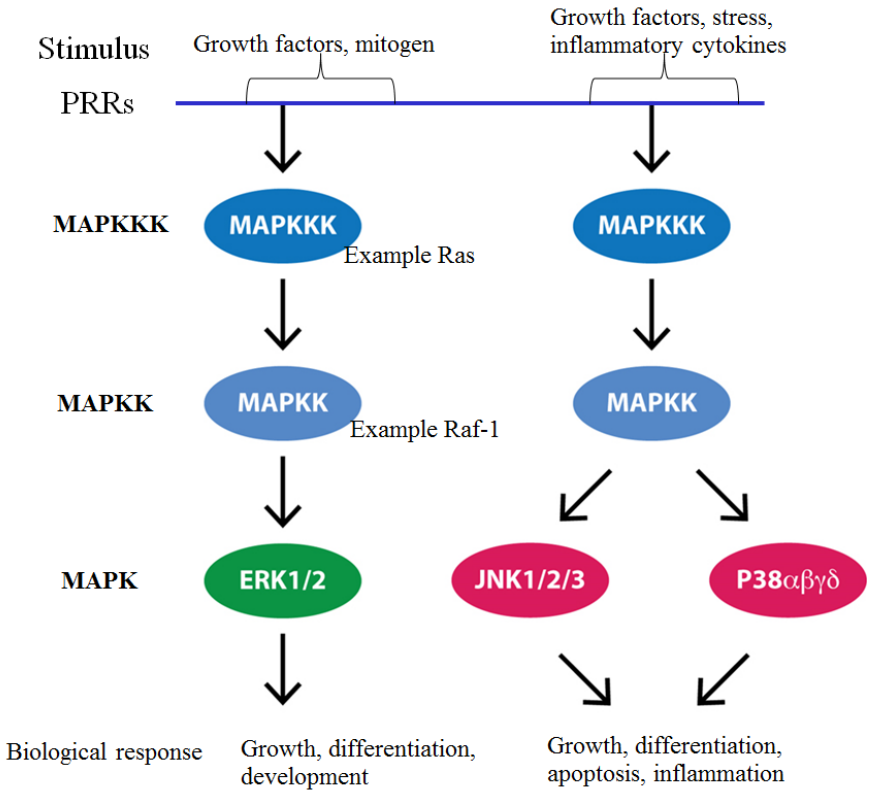


Figure 1.6 MAPK signalling pathways. Following activation of the PRR by either a mitogen (pathogen or pathogenic product) or growth factors, the receptors trigger a signal cascade in which Mitogen activated protein kinase kinase kinase (MAPKKK, e.g. Ras) is activated. This MAPKKK in turn activates a Mitogen activated protein kinase kinase (MAPKK, e.g. Raf-1 in the case of HIV-1 and MANLAM) which then activates Mitogen activated protein kinase (MAPK, e.g. ERK, JNK and p38). The MAPKs activate transcription factors such as NF-KB that then switches on genes involved in cell growth, inflammation, development, differentiation or apoptosis (Adapted from (Roberts & Der, 2007; Dzamko et al., 2014)

Concluding remarks

Current data shows that HIV-1 binds to DC-SIGN on DCs resulting in *trans*-infection of target cells, dissemination and systemic spread. DC-SIGN is a PRR, an antigen capture receptor that should, upon binding HIV, trigger DCs to process and present antigens to T cells to initiate an adaptive immune response via TCR, co-stimulation and secretion of cytokines and other soluble mediators. HIV and other pathogens instead use DC-SIGN to subvert DC function, preventing DC immune responses and enhancing their survival. How they do this has been a subject of debate.

It has been suggested that impaired maturation of DCs and deregulated secretion of cytokines and chemokines are the major mechanisms of sabotage. Triggering PRRs on the surface of DCs activate a series of intracellular signaling pathways which culminate in activation of signaling

kinases and transcription factors. Activation of the NF-kB transcription factor can either result in the secretion of soluble mediators that clear infection or factors that promote pathogen survival. NF-kB activation has been found to activate the LTR of HIV and thus directly enhance viral replication. Mannose-carrying PAMPS and not fucosylated motifs activate Raf-1 which modulates TLR induced activation of DCs. As the type of carbohydrate influences activation of signaling kinases and kinases differentially modulate cytokine secretion by DCs, we hypothesised that Env N-glycosylation could play a role in modulating DC immune responses via ERK. Furthermore, PNGs of Env have been found to vary with disease state of HIV-1 and mannose type N-glycans that bind DC-SIGN are enriched at specific sites of TF Envs, suggesting that immune modulation during transmission might be advantageous for HIV survival.

By providing insight into the molecular mechanism of how Env N-glycans impact DC immune responses, we might better understand the design of effective immunogens that do not suppress or skew neutralising immune responses but instead activate them.

1.11 Aim and objectives

Aim: The aim of this project is to determine the impact of HIV Envelope N-glycosylation on dendritic cell immune responses.

Rationale

Heterosexual transmission of HIV-1 across a healthy genital mucosa has a very low probability due to the protective mucosal barrier, leading to transmission of a single virus variant in 80% of cases. It has been suggested that Dendritic cells (DCs) are the first cell type to interact with HIV-1 following transmission. HIV-1 binds to DC's DC-SIGN via its high mannose type N-glycans and stimulates the release of IL-10 a regulatory cytokine linked to immune suppression. Transmitted founder Envs have differing N-glycan profiles than those from chronic infection, suggesting that Env N-glycans could be important for HIV transmission. We thus hypothesise that the N-glycans of transmitted founder Envs interact with DC-SIGN in such a way as to enhance DC IL-10 secretion, leading to deregulation of DC function and thus providing some variants with a survival advantage.

1.11.1 Objectives:

1. Characterise the N-glycosylation of subtype C Envelope and determine the role of specific N-glycans and types of N-glycans in Envelope function
2. Determine the role of subtype C HIV-1 Envelope N-glycosylation in DC-SIGN-mediated modulation of Dendritic Cell cytokine release and how this impacts pathogenesis.
3. Understand the impact of Envelope N-glycosylation on activation of signaling pathways that modulate Dendritic Cell function

Chapter 2: Material and Methods

2.1 Biosafety statement

This project was approved by the Faculty of Sciences Biological Safety Committee, University of Cape Town (approval number: FSREC 024-2014) for the use of hazardous biological agents and/or genetically modified organisms. Cell lines used here were maintained and used under Biosafety Level II conditions, while primary cell work which included generating monocyte derived dendritic cells (MDDCs) from donor PBMCs and experiments using HIV pseudovirus (PSV) were carried out under Biosafety Level II infrastructure with Biosafety Level III practices (BSL II+) in the departments of Molecular and Cell Biology, University of Cape Town. Some work was carried out in Dr. Fauci's laboratory of Immunoregulation, NIAID, NIH,-Bethesda, USA**.**

2.1.1 Consent statement

PBMCs were collected from healthy donors through a NIH Department of Transfusion Medicine protocol that was approved by the Institutional Review Board of the National Institute of Allergy and Infectious Diseases, National Institutes of Health. A written informed consent was provided by study participants and/or their legal guardians before donating blood.

2.2 Study Cohort

This study will utilize reagents generated from the CAPRISA Acute Infection study, Durban, South Africa. This cohort is a valuable tool for the study of HIV pathogenesis as acute infection was identified within three months of infection with longitudinal follow-up for up to 4 years. This project will focus on 4 study participants (CAP45, CAP206, CAP210 and CAP239) enrolled into the study between 2 and 4 weeks post-infection.

2.3 Plasmids

Eighteen functional full-length *envelope* (*env*) clones from five HIV positive women sampled longitudinally at two weeks post-infection (wpi) and 2-3 years post-infection (ypi) were used for this study. These envelopes were cloned into pcDNA/his-topo or pTarget mammalian expression vectors under the control of a CMV promoter (Gifts from Penny Moore, NICD, Carolyn Williamson, UCT and Liliwe Shuping, UCT). Four of the five women were infected with a single variant at 2 wpi as determined by single genome amplification (SGA) and five Env clones represented the transmitted founder (C1, C3, C7, C12 and C15) whereas thirteen Envs were isolated during the chronic stage of infection (C2 C3, C4, C5, C6, C8, C9, C10,

C11, C12, C14, C16, C17 C18). One individual was infected with more than one variant at 2 wpi from whom clones C3, C4, C5, C6 were isolated.

2.4 Cell culture

Human embryonic kidney (HEK) 293T (ATCC) and TZM-bl cells (NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH from Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc) (Platt et al., 1998) are adherent cell lines that were maintained in complete growth medium made up of Dulbecco modified Eagle high glucose medium (DMEM) (Lonza, BE12-604E, Whitehead Scientific) supplemented with 10% fetal bovine serum (PAA, Biocom Biotech) and 1 U/ml penicillin and 1µg/ml streptomycin (Lonza, Whitehead Scientific). TZM-bl cells are a HeLa cell line engineered to stably express CD4 and CCR5 and CXCR4 co-receptors and the luciferase gene under the control of the HIV promoter (LTR). Raji cells and a DC-SIGN stably expressing variant (Raji-DC-SIGN) cells (Cat No. 9944 and Cat No. 9945; NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH from Drs. Li Wu and Vineet N. KewalRamani) (Wu et al., 2004). These are non-adherent cells that were maintained in RPMI supplemented with 10% fetal bovine serum. All cells were cultured in a humidified incubator at 37^{o}C with 5% CO_2 and were tested for mycoplasma infection using the Fluorescent Hoechst DNA stain (Mowles, 1990) every three months.

2.4.1 Transfections

HEK 293T cells were plated at 2 x 10^5 cells/ml (2mls per six well tissue culture plate) and grown O/N to 40-60% confluence. Plasmid DNA at a ratio of 1:3 of DNA: Polyethylenimine (PEI) (1mg/ml) transfection reagent was mixed in serum free DMEM and incubated for 20 min at room temperature (RT). Fresh complete medium was added to the cells and the DNA-PEI mix was added drop-wise. The cells were returned to the CO_2 incubator for 48 hrs.

2.4.2 Env expression

Env (6 µg) were transfected as outlined above. Once the 48 hr incubation is completed, the medium is removed and 300µl RIPA buffer (10mM Tris buffer pH 7.5, 2mM Na2EDTA pH 8, 150mM NaCl2, 1% Triton X-100 detergent, with PMSF protease inhibitor added at 100µM) was added and incubated on ice for 10 min before transferring to an eppendorf and centrifuging at 14000 rpm for 5 min to remove cell debris. Supernatants are stored at -20^{o}C and protein concentration was determined using a Bradford assay, with a standard curve constructed using bovine serum albumin (BSA) (A2153, Sigma) according to manufacturer's instructions

2.5 N-glycosylation analysis of Env

Growth mediums (1 ml) from HEK 293T cell transfections were diluted with 1 ml of binding buffer (20mM morpholineethanesulfonic acid, 130mM NaCl, 10mM $CaCl_2$) at 4ºC. For each Env, 30µl of *Galanthus nivalis* agarose beads (Sigma-Aldrich®) was added and binding of the Env protein to the lectin agarose beads was carried out overnight at 4ºC with gentle mixing on a rolling apparatus. The agarose beads were centrifuged at 14000 rpm for 2 min and the supernatant removed. The beads were washed twice with PBS (Whitehead Scientific) at 4˚C, supplemented 0.9mM $CaCl_2$ and 0.49mM $MgCl_2$ and then treated with either endo-β-N-acetylglucosaminidase H (Endo-H) (0.5 Units(U) in buffer containing 50mM sodium acetate (pH 5.5), 0.1% sodium dodecyl sulfate, and 0.1% bovine serum albumin at 37°C, peptide N-glycosidase F (PNGase-F) (0.5 U) or Neuraminidase (0.5 U) according to the manufacturer's recommendations (New England Biolabs®) and then analysed by SDS-PAGE and Western blotting to compare electrophoretic mobility shifts.

HIV-1 IIIB gp120 recombinant protein (Cat # 11784) was used as positive control and endoglycosidase digestion controls in these experiments. The HIV-1 IIIB gp120 reagent was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: HIV-1 IIIB gp120 Recombinant Protein from ImmunoDX, LLC."

Once the Envs were visualized by autoradiography, the molecular weights (MW) of the bands were determined using their migration distances. Images of the autoradiography films were captured by a molecular imager, Chemi Doc™ XRS (Biorad) and Image lab ™ software was used to generate a standard curve of log MW vs relative migration distance (R_f) of the bands in the MW ladder. The R_f was measured from the top of the gel to the centre of the experimental bands. The MW of glycosidase treated and untreated Env bands were then calculated automatically by the software. Differences in N-glycosylation were determined by comparing the migration of the bands after Endo-H and PNGase-F treatment to those of the untreated samples (Lin et al, 2003).

2.6 Pseudovirus production

To produce pseudovirus, HEK 293T cells were transfected as in the Envelope expression above except that 2.5µg of *env* DNA and 5µg of the viral backbone pSG3ΔEnv were used. Pseudovirus was collected after 48 hours (hrs), filtered through a 0.22µm pore size filter, FBS concentration was adjusted to 20% and stored in single-use aliquots at -70°C.

2.6.1 Pseudovirus titration

To determine the titre of pseudovirus, a TZM-bl infectivity assay was used. TZM-bl cells, a Hela cell line genetically engineered to express CD4 and co-receptors CCR5 and CXCR4 along with a luciferase gene under the control of the HIV LTR were used. The luciferase reporter under the control of the LTR is activated upon infection with HIV due to transactivation by Tat (Montefiori, in press; Wei et al., 2003). Cells were seeded at a density of 10^4 cell per well in 200ul total volume of medium in a 96 well TC plate 18 hrs prior to infection. Two fold serially diluted pseudovirus (diluted in another 96 well plate before addition to TZM-bl cells) was added used to infect the TZM-bl cell. Triplicate wells of TZM-bl cells were infected with pSG3ΔEnv to control for the effect of other viral proteins and another triplicate of TZM-bl cells were left uninfected to serve as background luciferase of cells. Infectivity was allowed to carry on for 48 hrs following infection and the luciferase assay (BriteGlo® Luciferase Assay System; lysis buffer/substrate; Promega PRE263C-B, PRE264C-X) was used to determine the infectivity of the pseudovirus. The results were readout on a luminometer (Turner Biosystems® Modulus Microplate). Relative luciferase light units were plotted against the dilution factors and a linear regression analysis was used to relate the dilution factor to folds above background (cell infected with pSG3). The dilution factor that an infectivity relative ligh unit (RLU) gave 50x above background titre was adopted for MDDC stimulation following optimisation.

2.6.2 p24 ELISA

In order to determine the concentration of pseudovirus (ng/ml), a chemiluminescent in-house modified p24 ELISA using Aalto Bio reagents (Capture antibody; D7230, Recombinant protein for standard; AG6054, conjugate antibody BC1071) and the TROPIX® detection system Sapphire II (T1025, CDP-Star®, Applied Biosystems or life technologies) was carried out. High bind assay plates (Porvair, Whitehead Scientific) were coated with 100µl of sheep anti HIV-1 p24 gag antibody (D7230, Aalto Bio reagents) diluted to 3µg/ml in coating buffer (100m M $NaHCO_3$ pH 8.5) and incubated overnight at room temperature.

Unbound antibody was removed and the wells were washed three times with 200µl 1x TBS (50mM Tris, 150mM NaCl, pH 7.5). Plates were blocked with 5% bovine serum albumin (PAA, Biocom Biotech) in TBS for 1 - 2 hrs at room temperature and thereafter stored at -20°C until needed. Pseudovirus was inactivated in 1% Empigen-TBS, and serial dilutions (100µl) were added to the antibody-coated plates that were washed four times to remove the bovine serum albumin. A p24 protein; (AG6054, Aalto Bio reagents) was serially diluted in 1% Empigen-TBS and used to construct a standard curve.

After addition of samples and p24 standards in triplicate to the appropriate wells, the plates were incubated for 2.5-3 hrs, before unbound p24 protein was removed and the wells washed four times with 200 µl TBS. An alkaline phosphatase conjugated mouse anti-HIV-1 p24 antibody; (EH12AP, Aalto Bio reagents) was diluted to 1 in 16 000 in TBS- 0.1% Tween-20 (TBS-T) containing 20% sheep serum (PAA, Biocom Biotech) and 2% bovine serum albumin and 100µl was added to each well. The plates were incubated for 1 hr at room temperature, protected from light. Unbound antibody was removed and the wells washed with TBS-T eight times and then twice more with 1x TROPIX® buffer (200mM Tris, 10mM $MgCl_2$, pH 8.9). The detection reagent was diluted four times with TROPIX buffer and 50µl was added to each well. Luminescence was read using a plate reader (Turner Biosystems, ANATECH Instruments). Relative light units (RLU) of pseudovirion samples were converted to p24 concentration (ng/ml) using the standard curve constructed from the standard protein used and a non-linear regression analysis plot.

2.6.3 Entry efficiency assay

To determine the entry efficiency of the pseudovirions stocks, TZM-bl cells were used. TZM-bl cells were seeded at 10^4 per well in a 96 well plate 18 hrs before, were infected with 5-fold serial dilutions of the pseudovirus when the cell monolayers were 50 - 60% confluent. All pseudovirion stocks were normalised to 100ng/ml p24 (pre-determined by p24 ELISA Aalto, Bio-reagents), prior to serial dilution. Half of the growth medium was removed from each well and replaced with 100µl of titrated pseudovirus in triplicate (100ng/ml, 50ng/ml, 25ng/ml and 12.5ng/mls). Triplicate wells of TZM-bl cells were infected with pSG3ΔEnv to control for the effect of other viral proteins and another triplicate of TZM-bl cells were left uninfected to serve as background luciferase of cells. Pseudovirus single round infection was carried out for 48 hours at 37°C and infectivity was determined by the luciferase reporter assay (BriteGlo® Luciferase Assay System; lysis buffer/substrate; Promega PRE263C-B,PRE264C-X) where luciferase activity is measured using a luminometer (Turner Biosystems® Modulus Microplate). Background luciferase readings from cells not infected with virus or wells transfected with pSG3ΔEnv was subtracted from sample readings. For all comparisons between clones, RLU readings measured using a titer of 100 ng/ml of PSV was used as this measurement fell within the linear range of the curve.

2.7 DC-SIGN binding assay

A DC-SIGN binding assay was adapted from Alexandre *at al.* (2012). Briefly, 100ng (in 100µl) of pseudovirus (predetermined by p24 ELISA) was added to Raji and Raji DC-SIGN cells seeded in a 96 well plate at a density of 1 x 10^5 cells per well in 100µl RPMI growth medium in triplicates (total volume 200µl per well). Pseudovirus was allowed to bind to the cells for 2.5 hrs at 37°C in a humidified CO_2 incubator and cells were then washed four times by centrifugation (2500 rpm) with RPMI to remove all unbound virus, then re-suspended in 130µl of lysis buffer (1% Empigen-TBS). A 100µl volume of cell lysate was used to assay by p24 ELISA to determine the amount of pseudovirus (ng/ml) that bound to the DC-SIGN expressing cells.

2.8 *Trans*-infection

In this assay, 100ng/ml of virus (determined by p24 ELISA) was added to and incubated with 10^5 Raji/DC-SIGN cells and allowed to bind for 2.5 hr in a 37ºC incubator. Unbound virus was removed with four washes as in the binding assay above before the Raji/ Raji-DC-SIGN cells bound to virus was added to TZM-bl cells seeded the day before at a density of 10^4 cells per well of a 96 well TC dish. Virus was allowed to infect TZM-bl cells for 48 hr and the PSV entry was measured by luciferase reporter assay in a luminometer (Turner Biosystems® Modulus Microplate reader) as outlined in the infectivity assay. Direct infection was also carried out whereby 100ng/ml of PSV was used to infect TZM-bl cells without prior binding to Raji/Raji-DC-SIGN cells. *Trans*-infection RLU was normalised to RLU of direct infection to control for the effect of varying entry efficiency across the Env clones.

2.9 Generation of mutants

A modified Quikchange® Site-Directed mutagenesis (SDM) protocol (Stratagene, La Jolla,) was used to generate Env mutants lacking specific PNGs and soluble gp140. Complimentary primers carrying the desired mutations were designed using Dnaman software and restriction enzymes sites were introduced through silent mutation analyses using WATCUT (http://watcut.uwaterloo.ca). All primers were at least 45 nucleotides long with a minimum of 15 bases on either side of the mutated nucleotides. PCR annealing was carried out between 55°C - 58°C and the cycling conditions were as follows: 94°C for 3 min followed by 20 cycles of 94°C for 30 sec, annealing for 30 sec, and elongation 72°C for 12 min, followed by a final annealing step at 72°C for 20 min.

SDM PCR products were verified on 1% agarose electrophoretic gels prior to digestion with 20 U of *Dpn*I (Thermo Scientific). This enzyme recognises methylated DNA and will digest template DNA but leave the SDM PCR product intact. D*pn*I digested DNA was checked using a 1% agarose gel alongside an aliquot of the undigested sample before competent *E.coli* (JM109, Promega) were transformed, spread on Luria agar (LB, 1.5% w/v agar) plates and left to incubate at 37ºC overnight (O/N). Colonies were picked, grown in 5ml Luria broth (1% NaCl, 1% tryptone, 0.5% yeast extract) overnight with carbenocillin disodium (100 µg/ml) and plasmid extracted using the PureYield™ Plasmid Miniprep System (Cat # A1222 Promega) according to the manufacturer's instructions. Colonies were screened by digestion using the restriction site added during SDM and positive clones were cultured in 50ml Luria broth overnight before plasmid DNA was extracted using the PureYield™ Plasmid Midiprep kit (Cat # A2495, Promega). Introduction of the mutation was confirmed by sequencing (DNA Sequencing Unit, Stellenbosch University, South Africa).

2.10 Recombinant envelope protein preparation

2.10.1. Codon optimization, expression and purification

Env coding sequences of CAP88, C12, C13 and C14 were commercially synthesized (Mutagenex, Piscataway, NJ), codon-optimized for expression in mammalian cells (DNA2.0, Menlo Park, CA) and the endogenous Env signal peptide was replaced with that of the tissue plasminogen activating protein (TPA) to improve the yield . The sequences of each envelope protein following synthesis from +1 to the gp120-gp41 junction were cloned into a mammalian expression vector downstream of a synthetic TPA leader sequence. Non-adherent Chinese hamster ovary cells (CHO-S cells) (Invitrogen, Grand Island, NY) were transfected using the FreeStyleTM MAX expression medium (Gibco, 12651-014) according to the manufacturer's instructions in a 37ºC incubator on a rotator. CHO cells were used to maximize expression since Go *et al.* (2011) proposed that Env N- glycosylation was similar when expressed in CHO and HEK 293 cells (Go, Hewawasam, et al., 2011). Cell culture medium was harvested 72 hrs post transfection by centrifugation at 2000 rpm for 10 mins to remove any cells then filtered through a 0.22um filter (Millipore). The supernatants were then stored at -20ºC if not used immediately. They were thawed in a 37ºC water bath and passed over a column of *Galanthus nivalis* lectin sepharose (Vector Labs, Burlingame, CA) for lectin affinity chromatography. *Galanthus nivalis* sepharose was diluted 5 fold with unliganded sepharose 4B (GE Healthcare; 17-0120-01) to minimize avidity binding effects. Following column washes, gp120 was eluted

with 20mM Glycine-HCl, pH 2.0, 150mM NaCl, 500mM α-methyl-mannopyranoside (Sigma, St. Louis, MO) and collected in 5ml fractions directly into 1M Tris-HCl, pH 8.0. SDS PAGE and Western blotting was used to identify peak fractions which were pooled and then concentrated with a stirred cell concentrator (Millipore, Billerica, MA). The protein was then dialysed exhaustively against HEPES, pH 7.4, 150mM NaCl and the proteins were quantitated by absorbance at O.D. λ280 (extinction coefficient 1.1). The values were then confirmed by measurement with a bicinchoninic acid protein assay (Thermo Scientific, Rockford, IL). This was done at the laboratory of Immunoregulation, NAID, NIH-Bethesda under the supervision of Dr. Arthos James.

2.10.2 Endotoxin removal (Triton extraction) from gp140 protein and DC-SIGN protein

To remove endotoxins from the purified protein, gp140 aliquots were slowly thawed on ice. Triton (10%) was added to the protein (final 1% triton concentration), gently mixed by inverting the tube and incubated for 5 min at 37ºC. The mixture was centrifuged at 14000 rpm for 1 minute. The protein precipitate (upper layer) was removed, transferred into a new tube and the extraction repeated 3-4 times. Protein was then dialysed extensively in 1x PBS or 1x Hang's buffered saline (HBS) for 48 hrs, changing buffer after the first 6 hrs, and then after 24 hrs.

2.11 Generation of monocyte derived dendritic cells (MDDCs)

Peripheral blood monocytes (PBMCs) were obtained from healthy blood donor buffy coats from the Department of Transfusion Medicine, NIH, Bethesda or the Western Cape Province blood bank, Cape Town. PBMCs were isolated by density gradient centrifugation using Ficoll-Hypaque (1077, Sigma-Aldrich). Monocytes were isolated from the PBMCs by positive selection using CD14+ coated beads (130-050-201 Miltenyi Biotec, USA or Biochom Biotech, S.A) according to the manufacturer's instructions with the following exception: Beads were pre-washed to remove azide (Appendix II) and 5µl of washed beads were used (instead of 20µl as recommended by the manufacturer) per 10^7 PBMCs. In some experiments, monocytes were isolated by plastic adherence in 100cm dishes. Elutriated monocytes were also sometimes obtained from the department of Transfusion medicine, NIH, Bethesda. Elutriated monocytes (and PBMCs when isolated by plastic adherence) were adhered to TC plates (100cm dishes for PBMCs and six-well TC plates for elutriated monocytes) in serum free medium for two hours at 37ºC, 5% CO_2. Non-adherent cells were washed off using pre-warmed 1x PBS (10010049,

Gibco; without Ca^2 or Mg^2) then rinsed with RPMI and differentiation medium (RPMI with 2% human serum AB, 100µg/ml penicillin /streptomycin, 2mM L-glutamine, 10mM HEPES), was added. Differentiation was done in the presence of 1000 U/ml of recombinant human GM-CSF (PHC2005, Life Technologies) and 500 U/ml of recombinant human IL-4 (PHC0045, Life Technologies) for six days with recombinant cytokines supplemented every other day.

2.11.1 Flow cytometry analysis of MDDCs

Before MDDC stimulation with pseudovirus or with Env protein, an aliquot of MDDCs were stained for surface expression of surface markers to confirm differentiation and maturation status. Antibodies including CD83-APC (BD Cat # 551073), CD86-BV450 (BD Cat # 560359) and CD209-FITC (BD Cat # 561764) were used for MDDCs while monocyte purity was checked with CD14-PE, CD3-FITC CD4-PE and, IgG-PE for the isotype control.

For the surface phenotype check, 2 $x10^5$ cells were used per donor and staining was done in V-bottom 96 well plates. Cells were washed by centrifugation, re-suspended in 100µl staining buffer (1x PBS containing 0.1% FBS) and 2µl of the required fluorophore conjugated antibody (as recommended by the manufacturer, < 0.25µg per million cells) was added and incubated at 4^oC for 30 min. Samples were then washed 3x with 200µl of wash buffer by centrifugation before re-suspension in 1x PBS and fixed with 2% paraformaldehyde. Flow cytometry acquisition of MDDCs or monocytes was done using a FACS Canto II, (BD USA) and analysed by Flowjo software (TreeStar Inc, USA)

2.11.2 Stimulation of MDDCs

After six days of differentiation, MDDCs were lifted by pipetting and transferred into 50 mL polypropylene tubes. The plate was washed with pre-warmed 1x PBS without calcium or magnesium (10010049, Gibco) and added to the tube. Cells were centrifuged at 1200 rpm for 10 min to pellet cells and washed twice with warm 1x PBS.MDDCs were washed to remove all differentiation medium with warm 1x PBS, re-suspended at 4 x 10^6 cells/ml and stimulated in a 48 or 96 well tissue culture dish (Coster) at a final concentration of 2 x 10^6/ml with either pre-titered PSV (50 fold background) or purified gp140 at a concentration of 2µg/ml. Stimulation was done in culture medium consisting of RPMI containing 2% human AB, serum 1% Penicillin/Streptomycin, 1% non-essential amino acids, 1% sodium pyruvate and 2mM L-glutamine for 24 hrs. Following stimulation for 24 hrs, the plates were centrifuged at 2000 rpm for 10 min to pellet any cells in suspension before the supernatants were harvested. The culture

supernatants were stored in single-use aliquots at -80^{o}C until cytokine analysis by Luminex assay, which read on a Bio-plex 200 array system platform (Bio-rad).

2.11.3 Quantification of Cytokines in culture supernatants

2.11.3.1 Luminex Multiplex assay

The amount of each cytokine secreted into culture supernatants was determined using a multiplex cytokine assay, Luminex, according to the manufacturer's instructions with some changes. This assay quantifies the cytokines released by MDDCs into the culture supernatant based on Luminex suspension array technology. The principle is similar to sandwich ELISA but uses cytokine-specific antibody-immobilized magnetic beads to capture cytokines in culture supernatants. In summary, antibody-coated microsphere beads were sonicated for about 30 secs before pipetting to avoid clumping. Samples were assayed without dilution due to very low levels of some cytokines. A total of 25µl of each sample was added to the well containing antibody coated beads and incubated for 2 hr at ambient temperature (RT) away from light in a Millipore multiscreen plate (Millipore, Billerica, MA). The liquid was then aspirated using a BioPlex Pro II plate washer (Bio-rad, Hercules, CA), and the plates were washed twice with 200µl of assay buffer (provided with kit). The beads were then re-suspended in 25µl of assay buffer and 25µl of biotinylated secondary antibody was added, incubated for 30 min at RT away from light. The plates were again washed twice with PBS, the beads re-suspended in 25µl of assay buffer, and 25µl of streptavidin-phycoerythrin conjugates (SA-PE, Millipore, MA) was added. The median fluorescence intensity (MFI) of these beads was recorded (a minimum of 62 beads were required for each read) with a Luminex 200 platform (Bio-rad) and analysed with Bioplex Manager Software (Version 6.0, Bio-Rad) using a 5P regression algorithm. In order to quantify the level of cytokines, standards were used and MFI was plotted against protein concentration for the standard protein. Linear regression was used to determine the concentration of cytokines in each experimental sample using the standard concentration. Cytokines analysed included IL-10, IL-12, IL-6, IL-8, IL-1β, TNF-α, MIP1-α and MIP-1β and MIP-3α.

2.11.4. MDDC Stimulation and of MAP kinases detection

For intracellular detection of MAPK, MDDCs were re-suspended in incomplete medium (serum-free RPMI medium with 100µg/ml Penicillin/Streptomycin and 2mM L-glutamine) at a concentration of 10^6 per ml in round bottom polypropylene tubes, with 1ml per tube and rested for 24 hrs in a 37^{o}C humidified incubator. Following resting, MDDCs were centrifuged

at 2000 rpm to remove medium and re-suspended in stimulation medium (2% human AB serum) at a density of 5 x 10^6/ml, taking into consideration the volume of PSV required to obtain a final concentration of 1% serum medium for stimulation This was maintained at 37^{o}C for the required time. Lipopolysaccharide (LPS) at a concentration of 1 µg/million cells was included as a positive control. To enhance signal, IL-1β at 10ng/ml and TNF-α at 25ng/ml were added to the LPS in some experiments. Mock-treated cells not exposed to PSV or gp140 was also included. Mock-treated cells included 1) MDDCs stimulated with viral-like particles generated by transfection of HEK 293T cells with only the pSGΔEnv backbone to determine the effects of viral proteins other than Env, 2) culture medium of HEK 293T cells seeded during PSV preparation but not transfected to control for effects of the culture medium. In the case of when recombinant protein was used for stimulation, 1% complete medium was used as a negative control.

2.11.4.1 Intracellular staining and flow cytometric detection of MAPKs

Initially sufficient MDDCs were prepared for stimulation for three time periods (5, 15 and 30 min), but 15 mins had the least background and subsequent stimulations were done for 15 mins. PSV or gp140 were added and stimulation carried out in a 37^{o}C incubator for the required time and subsequently an equal volume of 4% paraformaldehyde (PFA) was added to the sample to fix the cells and stop further stimulation. Fixation was done for 10 - 12 min at RT. Cells were then centrifuged to remove medium and PFA and permeabilised by adding cold BD perm buffer III (BD Cat# 558050) or 90% methanol (kept at -20^{o}C before use). Permeabilisation was done on ice for 30 min after which cells were centrifuged to remove perm solution and stained in 50µl of half strength perm buffer. Fluorescent-conjugated antibodies were added for detection of phosphorylated MAPK using BV421-pERK1/2 (pT202/pY204, BD Cat # 562981, clone 20A, monoclonal), PE-pJNK (pT183/pY185, BD Cat # 562480, Clone N9-66, monoclonal), PerCp-Cy-p38 (pT180/pY182 BD Cat # 560406, Clone 36/p38) at RT away from light. Following staining, samples were washed 3x using half strength perm buffer III and re-suspended in half strength perm buffer III with 2% PFA for analysis on a flow cytometer (FACScanto II, B D, USA). Flow data was analysed using Flowjo software (Treestar Inc, USA). Fixed and stained cells were either analysed immediately or after overnight storage at 4^{o}C. Results from single colour -stained and unstained mouse kappa beads and stimulated MDDCs stained with single colours were used to calculate compensations. Cell doublets were excluded using forward scatter-area versus forward scatter-height parameters.

For inhibition experiments, using DC-SIGN protein or anti-DC-SIGN monoclonal Ab, PSVs were pre-incubated with 2µg/ml DC-SIGN protein in the presence of 2mM $Ca^{2+}$and allowed to rock at RT for 1 hr, then incubated for one additional hour at 37^{o}C before being used to stimulate MDDCs. For inhibition of PSV-CD4 interaction, sCD4 (Cat # 7356, ARP) or VRC01 (Cat # 12033, ARP) was added to PSV and incubated on ice for 1 hr before being used to stimulate MDDCs. When Leu3A antibody was used to block CD4 binding [Leu 3A(SK3), BD Biosciences, San Jose, CA], Leu3A was added to the MDDCs, incubated for 2 hr at 4^{o}C, and excess antibody removed by washing before stimulation with PSV.

2.11.4.2 Western blot detection of phosphorylated MAPKs

For the western blot experiments, 1 x 10^6 cells were used for each experimental condition. Cells were centrifuged immediately following stimulation at 2000 rpm to remove stimulation medium, then 2x Ripa lysis buffer for primary cells was added [40mM HEPES (pH 7.6), 300mM NaCl, 2mM EGTA, 1% NP-40, 100M phenylarsine oxide, 100mM NaF, 2mM Na3VO4, 2 mM Pefabloc, 20mM iodoacetamide, and 2mM phenylmethylsulfonyl fluoride]. Lysis was done on ice for 10 min followed by sonication for 10 sec, and then cell lysates were transferred into eppendorfs and centrifuged at 14000 rpm for 10 min. Supernatants were collected and stored at -20^{o}C.

Concentrations of cell lysates were determined by Bradford assay according to the manufacturer's instructions and equal amounts of lysates from each experimental condition were added to 5x protein sample buffer (100mM TRIS-HCl pH 6.8, 5% (w/v) SDS, 20% (v/v) glycerol, 2% β-mercaptoethanol and 0.1% (w/v) bromophenol blue) and heated at 100^{o}C for 5 min. Samples were then centrifuged for 1 min and then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions at 25mA/gel. The separated proteins were transferred to an Immobilon polyvinylidene difluoride membrane (PVDF; Millipore, Bedford-MA) for 1 hr at 100 V. After blocking non-specific protein binding sites with 4% skimmed milk in 1x TBS-T pH 7.6 (50 mM Tris-Cl, 150 mM NaCl, 0.1% Tween-20) at RT for 1 hr, protein on the membranes were probed with primary antibodies (rabbit anti-human MAPKs) to phosphorylated ERK and JNK [Phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204)(Cat #9101) and Phospho-SAP/JNK (Thr183/Tyr185) (Cat # 9251)] diluted 1: 1000 in blocking buffer at 4^{o}C overnight. Membranes were washed 3 times with TBS-T before incubation with horseradish peroxidase-conjugated anti-rabbit secondary antibodies (diluted 1: 2000 in blocking buffer) for 1 hr at RT. Membranes were washed 3 times with TBS-

T and detection was performed using the Super signal West Pico Chemiluminescent system (Pierce, Rockford, IL). Membranes were stripped of the primary antibody in stripping buffer (62.5mM Tris-HCl pH 6.7, 100mM β-Mercaptoethanol, 2% SDS). Stripping was done for 30 min at 50°C with occasional agitation, membranes were washed with 1x TBS-T and re-probed with antibodies against phosphorylated and unphosphorylated MAPKs: p44/42 MAPK (ERK1/2) for total ERK (Cat # 9102) and SAPK/JNK for total JNK (Cat # 9252) to normalise the level of MAPK phosphorylation between different samples and these were normalized to the negative controls included; mock treated MDDCs for that donor as outlined in section **2.11.4** above.

2.12 Statistical analysis

Analysis of data obtained was done using GraphPad PRISM (version 5). Mann-Whitney test was used to compare unpaired events and Wilcoxon matched test for pairwise comparison. Comparison of mutants with WT was done using ANOVA with Bonferoni post-test. Each experiment done in triplicate was repeated independently 2–3 times for comparison of mutants to WT, and 2-5 times for phenotypic characterisation of Env clones. Figure legends indicate the number of biological repeats carried out. When we identified PNGs associated with entry efficiency, DC-SIGN binding, *trans*-infection and IL-10 secretion the median or mean (depending on extent of variation) of the biological repeats were compared between the two groups using a Mann-Whitney test. Due to high inter-donor variation between experiments, and to identify Env PNGs most likely to stimulate IL-10 release irrespective of donor, we compared IL-10 levels from different MDDC donors using a Mann Whitney test. Statistical significance was indicated as *, ** and *** for p values less than 0.05, 0.01 and 0.001.

Chapter 3: The role of N-glycosylation in HIV-1 subtype C Envelope function

3.1 Introduction

Heterosexual contact remains the predominant route of HIV-1 acquisition despite the apparent low efficiency of transmission (Wawer et al., 2005; Boily et al., 2009; Patel et al., 2014). As Env orchestrates the first interaction with host cells during infection and is the main target for neutralizing antibodies (nAb) (Earl et al., 1994; Pantophlet & Burton, 2006), it is one of the chief targets for vaccine design.

Almost half of the molecular weight of gp120 is made up of N-glycans forming a dense array of carbohydrates that covers the protein backbone (Leonard et al., 1990; Fenouillet & Jones, 1995). Gp120 has an average of 25 potential N-glycan sites (PNGs) (Leonard et al., 1990) and these N-glycans help in the proper folding (Li et al., 1993; Fenouillet, Gluckman & Jones, 1994) and processing of Env (Doores et al., 2010; Pritchard, Spencer, et al., 2015). Inhibition of N-glycosylation was shown to impair function and/or lead to reduced infectivity (Montefiori, Robinson Jr. & Mitchell, 1988; Ratner, Heyden & Dedera, 1991; Land & Braakman, 2001) while other studies reported that PNGS at N156, N160, N181, N197, N289 and N448 in subtypes BC and N262 in both BC and C clades were required for structure and function, although this was not true for all subtypes (Wang et al., 2013; Mathys et al., 2014). Loss, gain and shifting of PNG positions occur during HIV disease progression and can influence viral pathogenicity (Derdeyn et al., 2004; Chohan et al., 2005; Sagar et al., 2006). This is not only by directly altering Env structure, but also by altering the processing of Env (Pritchard, Spencer, et al., 2015).

Despite reliance on N-glycans for structure and function, Env can remain unaffected by a number of PNG changes that contribute to immune evasion. N-glycans shield epitopes from immune recognition (Binley et al., 1998; Wei et al., 2003; McCaffrey et al., 2004; Scanlan, Offer, et al., 2007) and the shifting nature of Env N-glycans is a major challenge to vaccine design. Moore *et al*. (2012) showed that the shift of an Env PNG from positions 332 to 334 at six months post-infection was linked to escape from broadly neutralizing antibodies (bnAb) (Moore et al., 2012). This was supported by studies that showed that deletion of variable loop N-glycosylation sites or enzymatic removal of N-glycans improved antibody recognition of Env (Binley et al., 1998; Koch et al., 2003; Banerjee et al., 2009; Ma et al., 2011). However, paradoxically the type and extent of N-glycosylation can also enhance the immunogenicity of Env. Li *et al*. (2008) suggested that N197 enhanced sensitivity to neutralization (Li, Cleveland, et al., 2008; Wang et al., 2013). It was also reported that N-glycans helped in the formation of

properly processed trimers which allowed antibodies to bind better to CD4 binding sites (BS) and to CD4 inducible BS (Kong et al., 2010; Raska et al., 2010). Furthermore, some N-glycans at N295, N332 and N392 were found to constitute or support the proper conformation of epitopes for bnAb like 2G12, PG9 and PG16 (Sanders et al., 2002; Scanlan et al., 2002; Calarese et al., 2003; McLellan et al., 2011). Also, more recently, it was suggested that the "mannose patch" which includes N332 and other PNGs, form part of the epitopes of some of the most potent bnAbs like the PGT121 and PGT138. These epitopes are stable in the face of sequence variation and this mannose patch has thus been termed the "supersite of immune vulnerability" (Doores et al., 2010; Pejchal et al., 2011; Walker et al., 2011; Garces et al., 2014; Pritchard, Spencer, et al., 2015).

Apart from influencing Env entry efficiency, tropism and Ab neutralization (Pollakis et al., 2001; Clevestig et al., 2006; François & Balzarini, 2011; Wang et al., 2013; Raska et al., 2014), N-glycosylation has also been suggested to play a role in HIV transmission (Go, Hewawasam, et al., 2011; Shen, Raska, et al., 2014). Identification of genotypic and phenotypic traits common to transmitted founder variants (TFs) is essential to inform the design of novel HIV-1 preventive therapy. Micro-abrasions or ulcerations resulting from sexual intercourse-related trauma or sexually transmitted infections, thinning of the genital mucosa by hormonal contraceptives or inflammatory responses to altered vaginal microbiota could facilitate virus access to CD4+ T cells, dendritic cells (DCs), macrophages and Langerhans cells (LCs) resulting in productive infection by multiple variants (Selhorst et al., 2016; Abrahams et al., 2009; Haaland et al., 2009). However, as a single TF variant is responsible for initiating productive infection in 80% of infections, in the absence of predisposing conditions, it is possible that the virus is able to overcome the mucosal barrier due to advantageous phenotypic traits that could include binding to DCs via Env N-glycans, high infectivity or replication efficiency, and binding to the integrin $\alpha 4\beta 7$ (Selhorst et al., 2016; Geijtenbeek, Kwon, et al., 2000; Arthos et al., 2008; Cicala, Arthos & Fauci, 2011; Nawaz et al., 2011; Wilen et al., 2011; Parrish et al., 2013; Shen, Kappes, et al., 2014). Genotypic features of TFs have included shortening of variable loops and reduction in the number of potential N-glycans (PNGs) for subtypes A and C (Derdeyn et al., 2004; Chohan et al., 2005; Sagar et al., 2006) compared to variants from chronic stages of infection. Previous research has suggested that subtle differences exist in how gp120 interacts with DC-SIGN due to the length of the V1/V2 loop and the charge of the V3 loop (Nabatov et al., 2006). As shortening of the variable loop and changes in the number/position of PNGs has been observed in TFs, it is possible that successful

HIV-1 transmission requires either specific Env PNGs or a precise combination of N-glycans for optimum interaction with receptors on target cells.

DCs and LCs are said to extend their dendrites through an intact mucosa into the lumen to trap HIV-1 prior to translocation to lymphoid tissues and may result in degradation or *trans*-infection of CD4+ cells (Hu et al., 2000; Spira et al., 1996). Env binds to DCs via the DC-SIGN receptor and other C-type lectin receptors (CLR) (Geijtenbeek, Kwon, et al., 2000; Turville et al., 2001, 2004). Gp120 expressed in cell lines that enhanced the level of high mannose (HM) N-glycans bound better to DC-SIGN (Lin et al., 2003; Montfort et al., 2011), suggesting that DC-SIGN interacts more favourably with HM type N-glycans. However, other studies found that a flexible combination of both HM and complex type N-glycans were required for Env interaction with DC-SIGN. Studies by Hong *et al*. (2007) and Liao *et al*. (2011) suggested that Env PNGs at N275, N295 N351, N386, and N392 in subtype B (JR-CSF) and recombinant CRF-07 BC were involved in interaction with DC-SIGN and termed them "DC-SIGN interactive sites" (Hong et al., 2007; Liao et al., 2011). Go *et al*. (2011) found that N-glycans at positions N241, N262, N386, N392 and N448 were predominantly HM/hybrid (HM/H) in subtype C TF Envs (Go, Hewawasam, et al., 2011), suggesting that not only were the positions of some PNGs conserved but also the type of N-glycan at each site. As Env HM residues are important for binding to DC-SIGN (Lin et al., 2003; van Montfort et al., 2011), we hypothesised that sites with HM residues could be involved in interacting with DC-SIGN.

Therefore, the aim of this project was to understand the importance of N-glycosylation on Env-mediated PSV entry into TZM-bl cells, binding to DC-SIGN and *trans*-infection of CD4+ cells. Although N275, N295 and N351 were suggested to play a role in binding to DC-SIGN in subtype B and recombinant CRF-07BC (Hong et al., 2007; Liao et al., 2011), these sites were not present in our subtype C clonal sequences and were thus excluded from the analysis. As DC-SIGN was found to interact more favourably with HM type N-glycans (Lin et al., 2003; Eggink et al., 2010; Montfort et al., 2011) and TF subtype C Env PNGs at N241, N262, N386, N392 and N448 preferentially carried HM/H type N-glycans (Go, Hewawasam, et al., 2011), we deleted these PNGs by site directed mutagenesis (SDM) and studied the impact of each on Env function. Moreover, to identify other PNGs that could also be involved in Env function, we used sequence analysis to determine whether the presence or absence of PNGs within the constant regions were associated with changes in entry efficiency, binding to DC-SIGN and *trans*-infection.

3.2 Aim and Objectives

Aim: Characterise the N-glycosylation of subtype C Envelope and determine the role in Envelope function.

Rationale:

Env N-glycosylation is essential for correct folding and processing and deletion of specific PNGs have resulted in loss of function and viral infectivity (Li et al., 1993; Fenouillet, Gluckman & Jones, 1994; Wang et al., 2013; Pritchard, Spencer, et al., 2015). However, the number and position of PNGs shift over the course of infection to enable the virus to evade nAb responses (Derdeyn et al., 2004; Chohan et al., 2005; Sagar et al., 2006). Therefore, viral survival depends on a delicate balance between immune escape and maintaining Env function. Subtype C TF Envs tend to be more compact with fewer PNGs, suggesting that they could differ phenotypically from variants that emerge during chronic infection (Derdeyn et al., 2004; Chohan et al., 2005; Sagar et al., 2006). This difference could provide TFs with a functional advantage during transmission and targeting these features in vaccine design could prevent HIV-1 infection. However, loss and gain of PNGs in response to a vaccine-elicited immune response could also enhance viral infectivity and it is thus important to understand how changes in N-glycosylation influence Env function.

3.2.1 Specific Objectives:

1. Determine whether differentially N-glycosylated Env pseudovirus (PSV) differ in entry efficiency, binding to DC-SIGN and *trans*-infection of CD4+ cells using both Raji-DC-SIGN cells and monocyte-derived dendritic cells (MDDCs).
2. Identify specific Env PNGs involved in PSV entry efficiency, binding to DC-SIGN and *trans*-infection

3.3 Results

3.3.1 PNG frequency analysis

Env generally has about 24 PNGs in gp120 and five in gp41 (Leonard et al., 1990). Despite earlier studies focussing on the role of Env N-glycosylation in HIV-1 pathogenesis, the impact of specific PNGs and how they might act in concert to influence Env function are not fully understood. This study thus aimed to determine how specific PNGs and overall HM/H N-glycosylation influenced subtype C Env entry efficiency, binding to DC-SIGN and *trans*-infection of CD4+ cells. Eighteen Env SGA-derived amplicons were cloned and used for genotypic and phenotypic analysis. The number of PNGs (range: 25 – 33) and frequency of PNGs at specific N-glycan sites varied between our clones (Figure 3.3.1; Table 3.3.1). Consistent with previous studies (Derdeyn et al., 2004; Chohan et al., 2005) , the number of PNGs increased significantly over the first 2-3 years post-infection ($p < 0.009$). The caveat of this analysis was that multiple clones were compared from only 5 participants and further sampling is needed to confirm the observed correlation (Figure 3.3.1). However, comparing multiple chronic infection clones to a single TF indicates that an increase in PNGs is not restricted to only a single variant.

To confirm that our clone sequences were representative of the larger cohort we compared the frequency of the PNGs of our 18 clones to 36 phylogenetically distinct, consensus sequences from the same cohort. We found an overall significant association ($p < 0.0088$, $r = 0.70$) between our clones and that of the other cohort sequences but the variation between the two groups indicates some sampling bias. Only PNGs at position 241 and 262 were 100 % conserved in our cohort sequences and this was supported by frequency analysis of 589 random subtype C sequences from the Los Alamos database and published data (Moore et al., 2012; Pritchard, Spencer, et al., 2015). PNG frequency in the cohort sequences correlated with those from the subtype C database ($p = 0.0002$, $r = 0.94$) whereas PNG frequency in our clonal sequences did not always correlate with those in the population ($r = 0.5$), likely due to the small sample size of our clonal sequences. However, those that were conserved and those that were infrequent were consistent between the two alignments. N674 seemed to be under negative selection, confirming previous data (Moore et al., 2012; Pritchard, Spencer, et al., 2015).

Table 3.3.1 Characterisation of Envelop clones and frequency of potential N-glycan sites (PNGs) deleted by site-directed mutagenesis.

Participant ID	**ID**	**Time post infection (wks)**	**No. of PNGs**	**Position of N-Glycan sites**									
				N241	N262	N448	N386	N392	N130	N289	332	N356	N674
CAP45	C1	2	29	+	+	-	-	-	-	+	-	+	+
	C2	108	28	+	+	-	-	-	-	+	+	+	+
CAP177	C3	2	24	+	+	+	-	+	-	-	-	-	-
	C4	173	33	+	+	+	-	+	+	+	-	-	+
	C5	173	31	+	+	+	-	+	+	+	-	-	-
	C6	173	35	+	+	+	-	+	+	+	-	-	+
CAP206	C7	4	25	+	+	-	-	-	+	-	+	-	-
	C8	173	28	+	+	-	-	-	+	+	+	-	-
	C9	173	27	+	+	-	+	-	+	+	+	-	-
	C10	173	27	+	+	-	+	-	+	+	+	-	-
	C11	173	26	+	+	-	-	-	+	+	+	-	-
CAP210	C12	2	33	+	+	+	+	+	+	+	+	+	-
	C13	80	32	+	+	-	+	-	+	+	+	+	+
	C14	80	33	+	+	-	+	-	+	+	+	+	+
CAP 239	C15	5	29	+	+	+	+	+	+	-	+	+	-
	C16	173	31	+	+	+	+	+	-	-	-	+	-
	C17	173	32	+	+	+	+	+	+	-	-	+	-
	C18	173	30	+	+	+	+	+	-	-	-	+	-
Clonal frequency[a]				100	100	50	89	50	72	67	55	50	33
Study cohort frequency [b]				100	100	58.33	91.67	69.44	55.56	80.56	72.22	72.22	16.66
Population frequency[c]				99.5	100	71.5	85.56	71.5	54.5	83.5	80.5	87.9	24.5
Published frequency[d]				97	99	72	89	70	54	81	77/75	74	ND

[a]Frequency of PNGs in the 18 functional clonal sequences used in this study
[b]Frequency of PNGs in 36 sequences from the same cohort
[c]Frequency of PNGs in 589 Subtype C sequences from the Los Alamos database (www.Los Alamos.com)
[d]Published subtype C population frequency from analysis of 4000 sequences and 1371 sequences (Pritchard *et al.*, (2015) and Moore *et al.*, (2012)/ND: Not done.

3.3.2 N-glycosylation analysis

Mannose type N-glycans have been found to help in the folding of Env into conformations required for recognition by some bnAb epitopes (Sanders et al., 2002; Scanlan et al., 2002; McLellan et al., 2011), binding to DC-SIGN on MDDCs and DC-SIGN-expressing cell lines (Lin et al., 2003; Shan et al., 2007; van Montfort et al., 2011) and infectivity (Montefiori, Robinson Jr. & Mitchell, 1988). As N-glycosite analyses of our 18 Env clones using the Los Alamos tool (www.lanl.gov) indicated high variation in number (24-33) and frequency of PNGs (50 - 100%) (Figure 3.3.1), we determined if this translated into varying ratios of HM/H to complex type N-glycans. We truncated the gp160 of 9/18 Env clones by introducing a stop codon before the transmembrane (TM) domain of gp41. Based on previous publications, the soluble gp140 (gp140) construct would be N-glycosylated, cleaved into gp120 and truncated gp41 and form soluble trimers that would be directly secreted into the culture medium of HEK293T cells. However, it has also been suggested that cleaved gp140 forms unstable trimers that are stabilised by deletion of the cleavage site and introduction of a disulphide bond, covalently tethering gp41 to gp120 (Binley et al., 2000; Herrera et al., 2005; Pancera et al., 2005; Moore et al., 2006; Chung et al., 2014). Deletion of the cleavage site has been shown to prevent folding of gp120 into its native conformation and introduction of a disulphide bond affected Env function (Binley et al., 2002; Abrahamyan et al., 2003) and we elected not to include these modifications in our gp140 constructs.

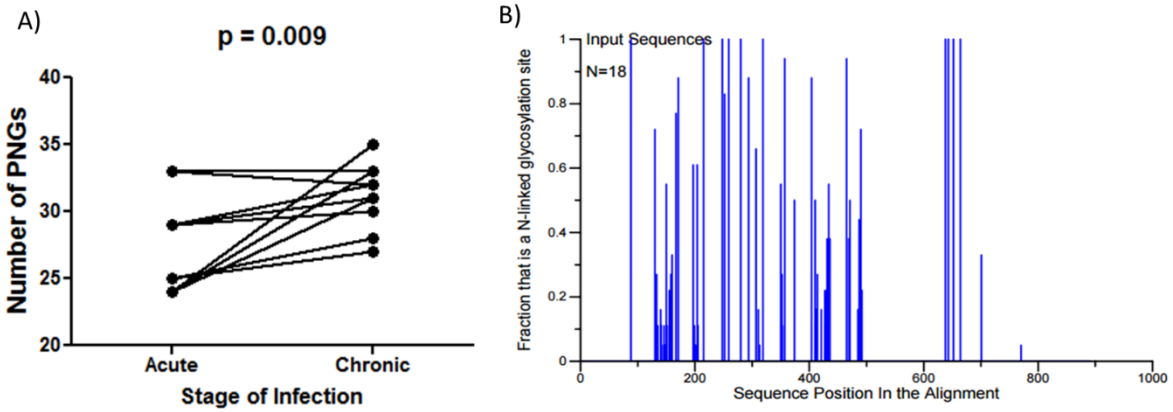


Figure 3.3.1 Characterisation of potential N-glycan sites in eighteen functional Env clones. BioEdit was used to align Env sequences and the alignment was analysed by N-glycosite (www.los Alamos.com). A) Env clones were grouped according to time of sampling (Acute infection: 2-5 weeks post-infection; Chronic infection: 80-173 weeks post-infection) and the number of potential N-glycan sites (PNGs) was compared between the two groups using a paired t test (Prism 5.0). B) N-glycosite analysis showing the position of the PNGs in Env on the x-axis and the y-axis indicates the

frequency of the PNGs in the 18 Env sequences. Each blue bar indicates a unique PNG found in one or more sequences.

Endoglycosidase digestion with enzymes that either cleave only HM/H type N-glycans (EndoH) or all types of N-glycans (PNGaseF) is a useful technique to determine the relative composition of HM/H to complex type N-glycans associated with Env (Raska et al., 2010). After transient transfection of HEK293T cells, gp140 was harvested using lectin affinity chromatography and digested with EndoH and PNGaseF and the change in electrophoretic mobility of the bands were compared to undigested controls. The expression levels of C1 and C2 were too low to purify gp140 from the culture medium (Data by Ms Liliwe Shuping; Appendix 1, Figure A1) and these clones were thus excluded from the analysis.

Only undigested (U) C13, C14, C15 and C16 gp140 indicated two bands representing uncleaved (UNC) gp140 and gp120 assumed to derive from fully N-glycosylated and cleaved gp140 trimers (Figure 3.3.2). The average apparent molecular weight of undigested gp120 was 131 kDa, similar to the purified gp120 positive control (132 kDa) (Table 3.3.2) with a standard deviation (SD) of 10.43, indicating high heterogeneity in N-glycosylation across the 4 clones. However, bands corresponding to gp120 were not apparent in the untreated samples of most of the clones and thus bands representing gp140 were used to compare the ratio of HM/H: complex type N-glycans between the clones. Cleavage of gp160 occurs after N-glycosylation and trimerisation *en route* to the plasma membrane and thus uncleaved gp140 could represent the fully N-glycosylated native form of gp120 (Checkley, Luttge & Freed, 2011). The variation in the average apparent molecular weight of undigested UNC gp140, 144 kDa (SD: 10.33), was very similar to that of fully glycosylated gp120 suggesting similarly high heterogeneity in N-glycosylation of gp140 and gp120 across the 9 clones.

The absence of two clear bands for gp140 and gp120 was likely due to overloading, weak cleavage of gp140 resulting in low levels of gp120 and/or poor resolution of gp140 and gp120 with similar molecular weights. After PNGaseF treatment, deglycosylated gp140 and gp120 bands became apparent for all of the clones, confirming that a fraction of all gp140 clones was cleaved into gp120. The secretion of UNC gp140 into the culture medium supports the finding by Moore *et al*. (2006) that gp160 reaches the cell surface and is incorporated into virus particles (Moore et al., 2006).

The difference in MW between untreated and PNGaseF-digested UNC gp140 is the weight due to N-glycosylation. Similarly, the difference in MW between untreated and EndoH-digested UNC gp140 indicates the weight contributed by HM/H N-glycans. The ratio of HM/H: total N-glycans provides a measure of mannosylation (%) of UNC gp140 (Table 3.3.2; Figure 3.3.3). The control, deglycosylated purified gp120, had the apparent MW of 57.9 kDa, higher than the predicted MW of 53.32 kDa calculated using the Compute pI/Mw tool available at SIB Expasy Bioinformatics Resources Portal (https://www.expasy.org/) (Table 3.3.2). EndoH digestion of the purified gp120 positive control resulted in an electrophoretic mobility shift which corresponded to a change in molecular weight of 132.2kDa to 105.1 kDa, suggesting that 36.57% of the overall molecular weight of gp120 is due to HM/H. Similar to the purified gp120 control, the average apparent MW of PNGaseF-treated UNC gp140 differed by nearly 4 kDa to the predicted mass of unglycosylated gp140 (Table 3.3.2).

The level of gp140 mannosylation varied between clones with HM/H N-glycans contributing 23.43 – 42.9% to the total molecular weight of all clones except C3 with only 6.8% mannosylation (Figure 3.3.3). Comparison between the number of Env PNGs and UNC gp140 mannosylation showed a significant association ($p = 0.017$, $r = 0.78$), suggesting that as the number of PNGs increase, so does the level of mannosylation. This supports the suggestion that increased number of PNGs could lead to denser N-glycosylation which would sterically hinder Man9GlcNac trimming, increasing the level of HM/H associated with Env (Bonomelli et al., 2011; Pritchard, Spencer, et al., 2015). Mannosylation of UNC gp140 was less than what was previously reported for virion-associated gp120 (56 – 79% oligomannose) (Bonomelli et al., 2011; Doores et al., 2010; Go et al., 2015; Pritchard, Harvey, et al., 2015; Pritchard, Vasiljevic, et al., 2015) but similar to what Go *et al.* found when they analysed subtype C gp140 (20 – 30%) (Go, Hewawasam, et al., 2011). To determine whether gp120 derived from gp140 cleavage was more similar to virion-associated gp120, we determined the level of mannosylation of gp120 from C13, C14, C15 and C16.

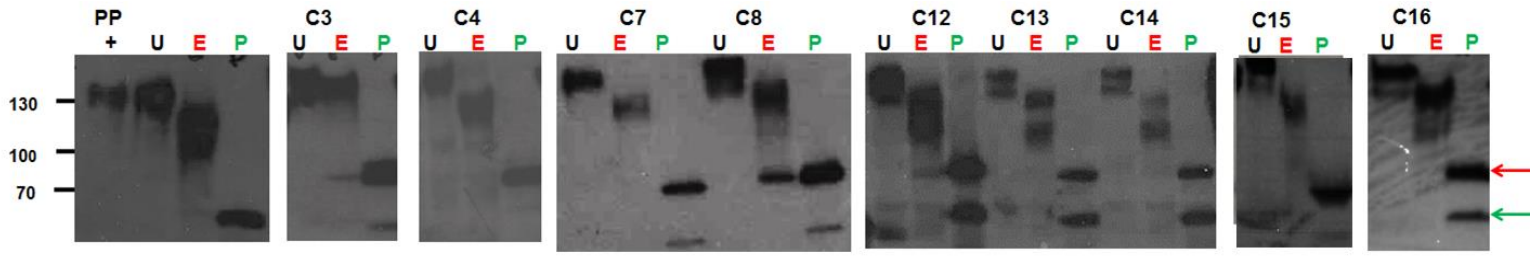


Figure 3.3.2 N-glycosylation of soluble gp140 constructs. N-glycan processing of nine gp140 clones (C3, C4, C7, C8 and C12-C16), were determined by endoglycosidase treatment following transient expression in 293T and partial purification using *Gallanthus nivalis* lectin agarose. Western blotting for each clone was done to compare the electrophoretic mobility shift of fully N-glycosylated, partially N-glycosylated and fully deglycosylated gp140 and gp120. Representative Western blots of at least three independent repeats of undigested (U), Endo H- (E) and PNGaseF-digested (P) gp140 were compared to digested purified gp120 (PP)(AIDS Reagent Programme; Catalogue # 11784). The Western blots were aligned according to the molecular weight marker included on all gels (not shown) so that N-glycosylated, demannosylated and deglycosylated gp120 and gp140 were juxtaposed across all clones. The MW of each band was calculated according to the MW standard and the relative migration of digested bands was determined relative to undigested controls using Image lab^{TM} software on a Chemi Doc^{Tm} Bio-rad imaging (visualizing) system. The relative positions of deglycosylated gp140 and deglycosylated gp120 are shown by red and green arrows, respectively. + indicates purified gp120 (PP) (AIDS Reagent Programme; cat # 11784) as a loading control for Env.

The MW of deglycosylated gp120, 56 kDa (+/- 2) was similar to the expected average gp120 MW, 57 kDa (+/-0.8) with low heterogeneity between clones (Table 3.2.2). Mannosylation of gp120 ranged from 37 to 50 %, higher than that of gp140 but still lower than previously reported for virion-associated gp120 (Bonomelli et al., 2011). Mannosylation of UNC gp140 correlated with that of gp120, suggesting that if gp140 carried higher levels of HM/H then so did gp120, although this correlation did not reach significance ($p = 0.0833$; $r = 1.0$), most likely due to the low sample number ($n = 4$).

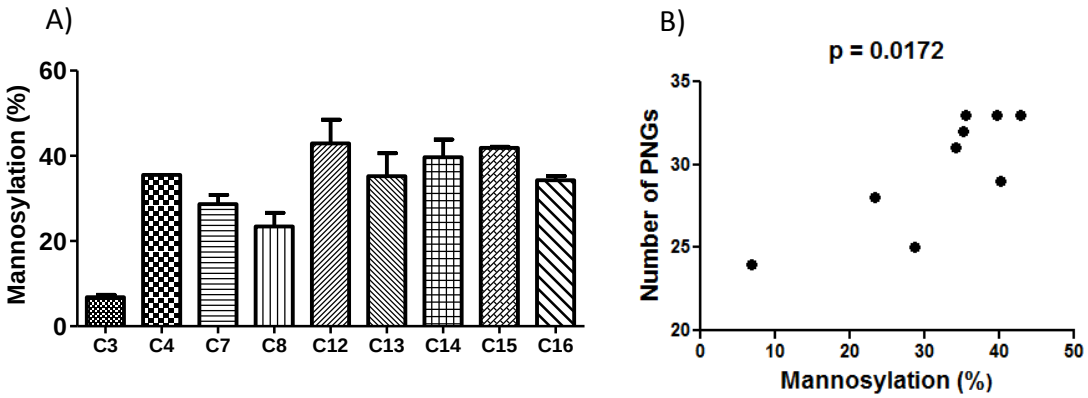


Figure 3.3.3 Mannosylation of soluble gp140 and correlation with number of Env PNGs. Molecular weight comparison of EndoH- and PNGaseF-digested gp140 relative to undigested gp140 was used to determine the relative level of high mannose/hybrid N-glycans. A) Level of mannosylation (%) was calculated using the equation: (MW of untreated gp140 - EndoH-treated gp140) / (MW of untreated gp140 - PNGaseF-treated gp140) X 100. The data above is an average of two biological repeats with error bars representing standard error of the mean (SEM). B) The correlation between number of potential N-glycan sites (PNGs) and gp140 mannosylation was determined using the Spearman Test (GrapPad Prism 5.0).

Table 3.3.2 Endoglycosidase analysis of undigested, Endo H and PNGase F digested gp140 to determine relative levels of high mannose/hybrid type N-glycans.

Clone ID	No. of PNGs	Apparent MW (kDa) of undigested		Apparent MW (kDa) of EndoH-digested		PNGase F-digested gp140		PNGase F-digested gp120		*MW of N-glycans (kDa)		Level of HM/H (%)
		gp140	gp120	gp140	gp120	#Predicted MW (kDa)	^Apparent MW (kDa)	#Predicted MW (kDa)	^Apparent MW (kDa)	HM/H MW (kDa)	Total N-glycans	
Purified gp120		N/A	132.2	N/A	105.1	N/A	N/A	53.32	57.9	27.1	74.1	36,57
C3	24	149	N/A	143.3	N/A	75	73.1	55.05	57.6	5.6	75.8	7.4
C4	33	146	N/A	119.6	N/A	77	72.4	57.11	56.0	26	73.2	35.52
C7	25	130	N/A	112.2	96.8	75.93	72	56.16	53.3	17.92	58.12	30.83
C8	28	131	N/A	116.2	95.8	76.56	76.2	56.78	55.8	14.5	54.5	26.61
C12	33	159	N/A	128	108	77.34	76.8	57.58	60.8	30.5	81.7	37.33
[a]C13	32	157	139.3	131.8	101.3	76.62	71.5	57.0	57.8	25.6	85.9	29.8
[a]C14	33	157	140.1	126.2	99.2	76.81	71.1	56.9	57.6	30.4	85.5	35.55
[a]C15	29	135	119.2	109.0	87.4	76.0	72.0	56.56	56.1	30.0	63.3	42.11
[a]C16	31	146	125	122.6	98.8	78.0	76	57.85	53.3	23.2	698	33.24
[b]Average	30	146	127.26	123.22	98.19	76.58	73.4	56.68	56.62	22.64	141.78	31.51

The data in this table represents one of two independent experiments shown in Figure 3.3.3 to indicate how values were derived using molecular weight of N-glycosylated, demannosylated and deglycosylated gp140.

Predicted Molecular Weight: ProtParam calculator on Expasy programme

^ Apparent Molecular Weight: Derived from standard MW marker using Chemi Doc^{Tm} Bio-rad imaging system and Image lab software

* Calculations:

HM/H MW = Apparent MW of undigested – Apparent MW of Endo H-digested

Total N-glycan MW = Apparent MW of undigested – Apparent MW of PNGase F-digested

Level of HM/H= [(HM/H MW)/ (Total N-glycan MW) x 100]

[a]Only 4/9 clones had bands corresponding to N-glycosylated gp120.

[b]Average of clones excluding purified gp120

Purified gp120 (Aids Reagent Programme cat # 11784) Accession number: AF189159 was used as a positive control.

3.3.3 Entry efficiency of Env clones

N-glycosylation was previously shown to influence infectivity but not replication (Montefiori et al, 1988). As mannosylation levels as well as the number and position of PNGs varied across the 18 Envs, we determined whether this variation influenced the efficiency with which PSVs could enter CD4+ CCR5+ CXCR4+ cells.

There was high variation in entry efficiency, ranging from 3.5 - 100% (Figure 3.3.4). C3, with the lowest level of oligomannose moieties was also the weakest enterer, followed by C2 which was very poorly expressed as determined by Western blotting (Data by Ms Liliwe Shuping; Appendix I, Figure A1). There was no statistically significant correlation between mannosylation and entry efficiency ($p = 0.25$, $r = 0.37$). This could suggest that entry efficiency is not dependent on the type of N-glycans associated with Env.

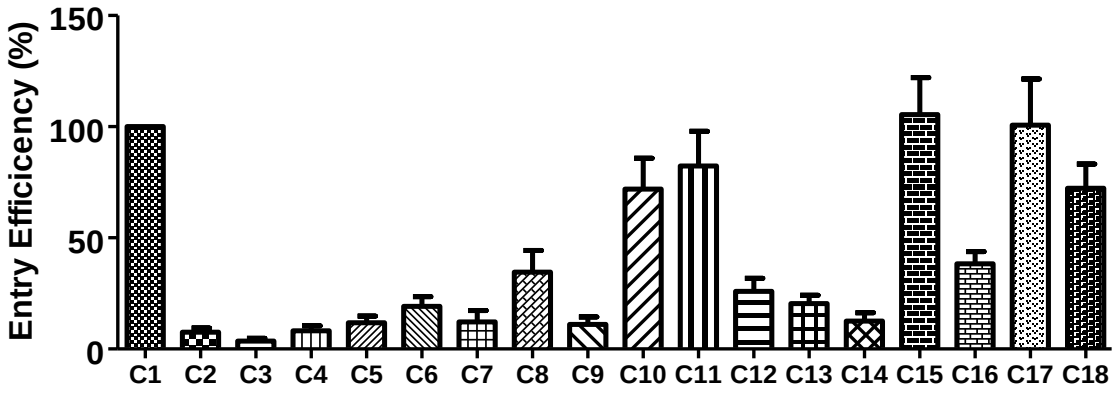


Figure 3.3.4 Pseudovirion entry efficiency. Pseudotyped Envs (PSV) equivalent to 100 ng p24 were compared for their ability to enter TZM-bl cells. Entry was monitored through the activation of a luciferase reporter gene under the control of the LTR. Four biological repeats were carried out in triplicate and each repeat was normalised to C1 to control for inter-experimental variation. The bar graphs are means of the four biological repeats with error bars showing standard error of the mean.

3.3.4 Binding to DC-SIGN

DC-SIGN binding was also variable with 2.3 - 11.5% PSV captured by Raji-DC-SIGN cells relative to input virus or 1 - 3% when normalised to C1 (Figure 3.3.5). To make sure the PSVs were captured due to DC-SIGN-Env interactions and not through non-specific binding, PSVs were also added to Raji cells not expressing DC-SIGN and PSVs lacking Env (PSG3ΔEnv only) was used as a control. No binding was observed (Data not shown), confirming that Env-DC-SIGN interactions are required for PSV binding to Raji cell. There was no correlation between binding to DC-SIGN and entry efficiency. This was expected since Raji cells do not express CD4 and TZM-bl cells but express DC-SIGN. However, there was a significant

association between binding to DC-SIGN and the level of mannosylation ($p = 0.0369$, $r = 0.71$) (Figure 3.3.6) which supported previous findings by Lin *et al.* (2003) that DC-SIGN-Env interactions depend on Env HM/H N-glycans (Lin et al., 2003; Montfort et al., 2011). There were, however, instances where this relationship was not supported such as C14 which had high levels of HM/H but did not bind to DC-SIGN better than C4 that was less mannosylated. Liao *et al*. (2011) suggested that a flexible combination of HM/H and complex-type N-glycans form the optimum binding sites for DC-SIGN (Liao et al., 2011). It is thus possible that DC-SIGN binding is via HM/H but that the clone-specific arrangement of complex N-glycans within the 3-dimensional structure could influence the binding efficiency.

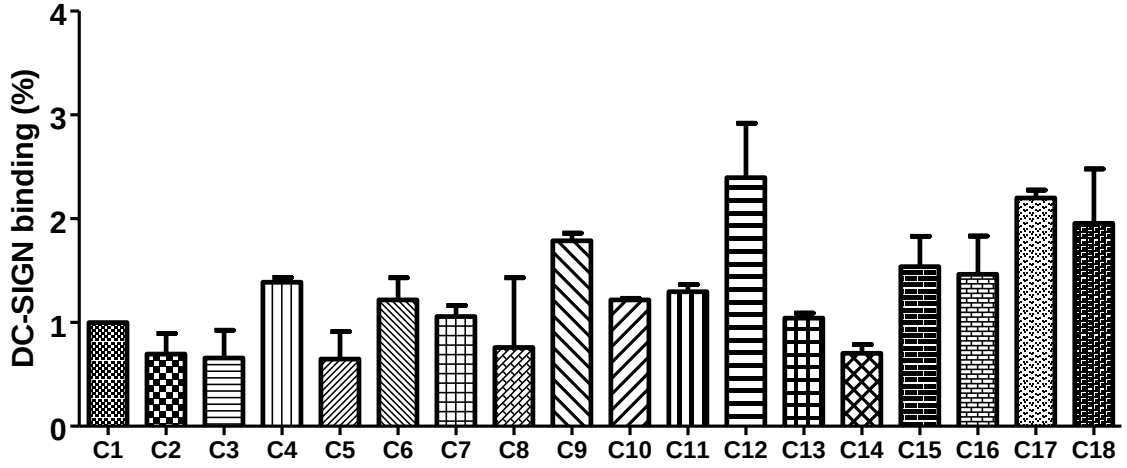


Figure 3.3.5 Pseudovirion binding to DC-SIGN on Raji-DC-SIGN cells. Pseudovirus (PSVs) (10ng total p24) binding to DC-SIGN was compared by capturing PSVs using Raji-DC-SIGN cells and measuring the amount of PSV bound to DC-SIGN by p24 ELISA and normalised to input PSV. To compare data between biological repeats, percentage PSV captured was normalized to a single clone (C1). Mean and standard error of the mean from two biological repeats are indicated.

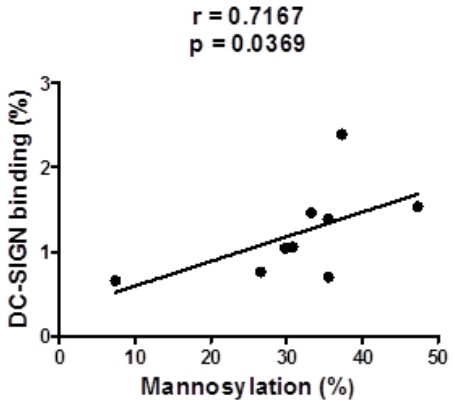


Figure 3.3.6 Correlation of DC-SIGN binding with level of Env mannosylation. Percentage PSV captured by DC-SIGN relative to input virus from two biological repeats was normalized to C1 and Spearman correlation analysis (GraphPad Prism 5.0) was used to determine whether there was a relationship between percentage Env mannosylation and DC-SIGN binding of nine PSV clones.

3.3.5 *Trans*-infection

Env binds to DC-SIGN on MDDCs facilitating *trans*-infection of CD4+ T cells, but the role this interaction plays in HIV transmission and pathogenesis *in vivo* is debated (Geijtenbeek, Kwon, et al., 2000; Turville et al., 2001; McDonald, 2003, 2010; de Witte, Nabatov & Geijtenbeek, 2008). Both *in vitro* and *ex-vivo,* studies have shown that MDDCs and DCs can bind and transfer virus to target cells via DC-SIGN and other C-type lectins (Geijtenbeek, Kwon, et al., 2000; Kwon et al., 2002; Shan et al., 2007; Shen, Kappes, et al., 2014). Non-CLR-mediated (heparin sulphate proteoglycans, CD169, GarCer) mechanisms of HIV *trans*-infection have also been reported for MDDCs (Saphire et al., 2001; Magerus-Chatinet et al., 2007; Izquierdo-Useros, Lorizate, Puertas, et al., 2012; Puryear et al., 2013). To determine whether *trans*-infection of PSVs was influenced by cell type and/or receptor identity, we investigated the *trans*-infection of our differentially N-glycosylated Envs using both Raji-DC-SIGN cells (Figures 3.3.7 and 3.3.8) and MDDCs (Figure 3.3.9). PSVs were also added to TZM-bl cells to determine the ability of the input virus to directly enter the reporter cell line without DC-SIGN-mediated *trans*-infection. This provided a measure of the entry efficiency of each PSV stock. An equal quantity of PSV was added to DC-SIGN+ cells (Raji-DC-SIGN and MDDCs) before cells were washed and mixed with TZM-bl cells to measure the amount of virus transferred to infect CD4+ cells.

3.3.5.1 Raji-DC-SIGN-meditated *trans*-infection

Trans-infection of TZM-bl cells using Raji-DC-SIGN cells for capture varied widely across PSVs: 3.3 to 160% relative to C1 (48-fold) (Figure 3.3.7 A). Since *trans*-infection consists of two steps, first binding to DC-SIGN and then entry of target cells, PSVs with high entry efficiency should have high *trans*-infection efficiency. There was a significant correlation between *trans*-infection and entry efficiency ($p = 0.0001$, $r = 0.95$), confirming this relationship (Figure 3.3.8 A). To determine whether the apparent variation in *trans*-infection of TZM-bl cells was entirely due to differences in PSV entry efficiency, *trans*-infection values were normalized by the entry efficiency of the input PSVs. Dividing the *trans*-infection values by the entry efficiency of each PSV did not have a consistent effect across all clones as variation was reduced (e.g. C1 vs C3), not affected (e.g. C10 vs C11) and increased (e.g. C15 vs C18) (Figure 3.3.7 B). However, normalising with entry efficiency lowered the overall variation in *trans*-infection 12-fold (i.e. from 48-fold to 4-fold variation across clones) and weakened the relationship between *trans*-infection and entry efficiency ($p = 0.0138$, $r = 0.6$), suggesting that entry efficiency was an important determining factor (Figure 3.3.7 B and Figure 3.3.8 B). DC-SIGN binding was also an important determining factor as there was a significant association between DC-SIGN binding and Raji-DC-SIGN-mediated *trans*-infection ($p = 0.0088$, $r = 0.6$) and this relationship was strengthened when *trans*-infection was normalised by entry efficiency ($p = 0.0022$, $r = 0.7$) (Figure 3.3.8 C and D). These findings confirm that both Env entry efficiency and DC-SIGN binding are important for *trans*-infection.

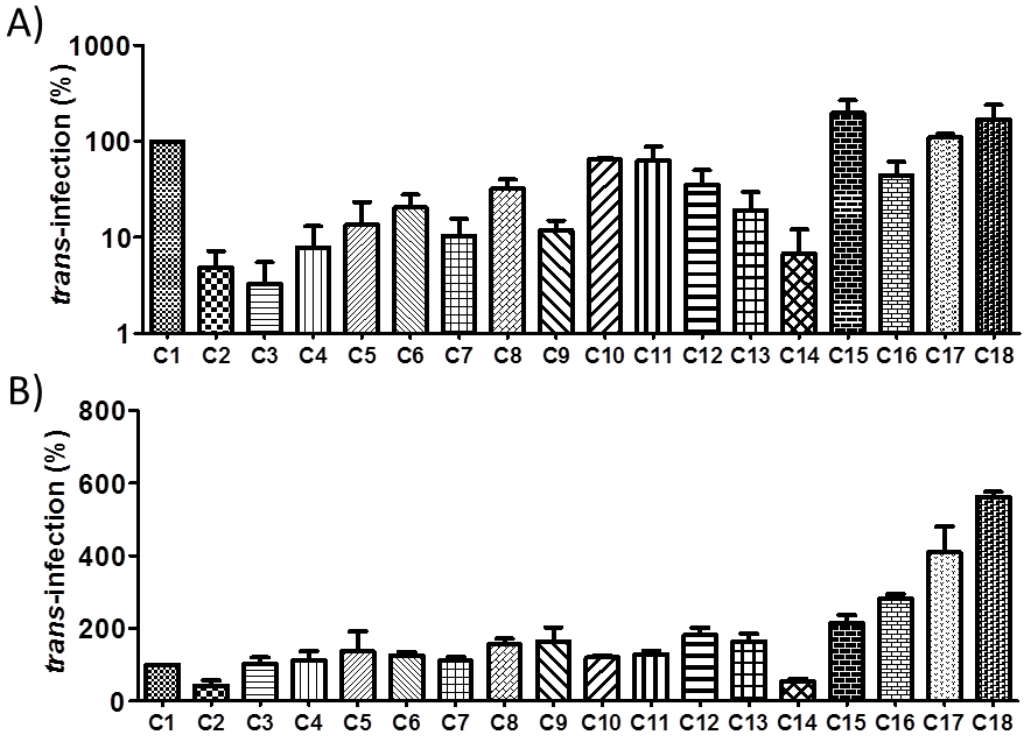


Figure 3.3.7 Raji-DC-SIGN-mediated *trans*-infection of CD4+ cells. Pseudovirus equivalent to 100 ng p24 was added directly to TZM-bl cells to measure entry efficiency or first captured by Raji-DC-SIGN cells before adding to TZM-bl cells to measure *tran*s-infection. Entry of TZM-bl cells was measured by luciferase reporter assay. A) The raw RLU values of each PSV DC-SIGN meditated *trans*-infection were expressed as a relative percentage of a single clone C1 or B) were first normalized to entry efficiency and then expressed as a percentage relative to C1. Experiments were done in triplicates and the average of two independent repeats with standard error of the mean are indicated.

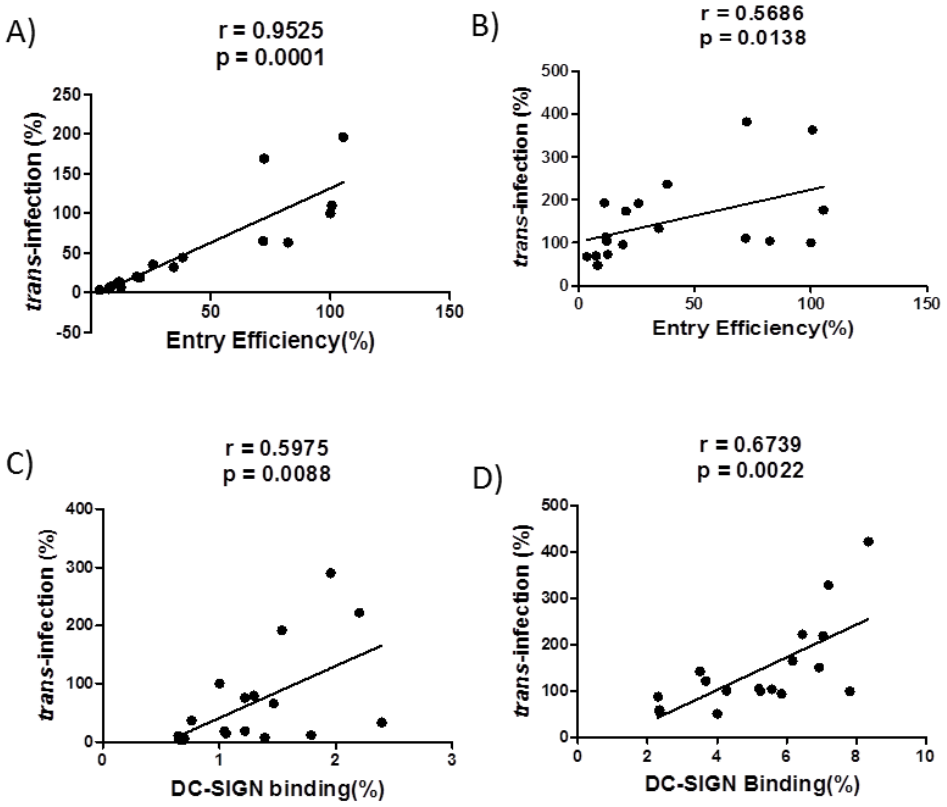


Figure 3.3.8: The relative role of Env entry efficiency and DC-SIGN binding in Raji-DC-SIGN-mediated *trans*-infection. Following Raji-DC-SIGN-mediated *trans*-infection, the results were normalized to C1 and an association with entry efficiency (A, B) and DC-SIGN binding (C, D) was determined using Spearman correlation analysis. To determine if entry efficiency is an important determining factor of *trans*-infection, *trans*-infection values were first divided by PSV entry efficiency before normalisation to C1 (B, D).

3.3.5.2 MDDC-mediated *trans*-infection

DC-SIGN, mannose C-type lectin receptors (MCLRs), CD4, CCR5 and CXCR4 have been documented to play a role in DC enhancement of HIV infection (Turville et al., 2002, 2008; Lambert et al., 2008). Other non-CLR receptor mechanisms have also been found to contribute to HIV *trans*-infection (Saphire et al., 2001; Magerus-Chatinet et al., 2007; Izquierdo-Useros, Lorizate, Puertas, et al., 2012; Puryear et al., 2012). MDDCs are physiologically close to myeloid dendritic cells and express CLRs and other virus capture receptors which have been suggested to aid in *trans*-infection. We thus determined whether MDDC-mediated TZM-bl *trans*-infection was similar to when using Raji-DC-SIGN for PSV capture. *Trans*-infection using MDDCs varied between 6 – 100% (16-fold) between clones and was therefore, not as variable as observed for Raji-DC-SIGN capture. Furthermore, the ranking of PSVs from high

to low *trans*-infection efficiency was also altered (Figure 3.3.9 A). Despite these differences, there was a significant correlation between *trans*-infection by both cell types ($p = 0.0001$) (Figure 3.3.10 C),

When *trans*-infection efficiency was normalized to entry efficiency, the variation between clones was reduced from 16-fold to 2.5-fold similar to Raji-DC-SIGN-mediated *trans*-infection although the effect on specific clones differed (e.g. C15 vs C18) (Figure 3.3.9 B). There was a significant correlation between MDDC-mediated *trans*-infection and entry efficiency ($p = 0.0001$, $r = 0.8535$) but once normalized to entry efficiency this relationship lost significance ($p < 0.56$) (Figure 3.3.10 A & B). This suggests that the variation in MDDC-mediated *trans*-infection was largely due to differences in TZM-bl entry. Furthermore, contrary to capture using MDDCs, in some experiments direct infection of TZM-bl cells was less efficient than Raji-DC-SIGN mediated *trans*-infection, suggesting that pre-exposure to Raji-DC-SIGN enhanced TZM-bl entry (Appendix I, Figure A2). There was a significant correlation between MDDC *trans*-infection and Raji-DC-SIGN mediated *trans*-infection (Figure 3.3.10 C).

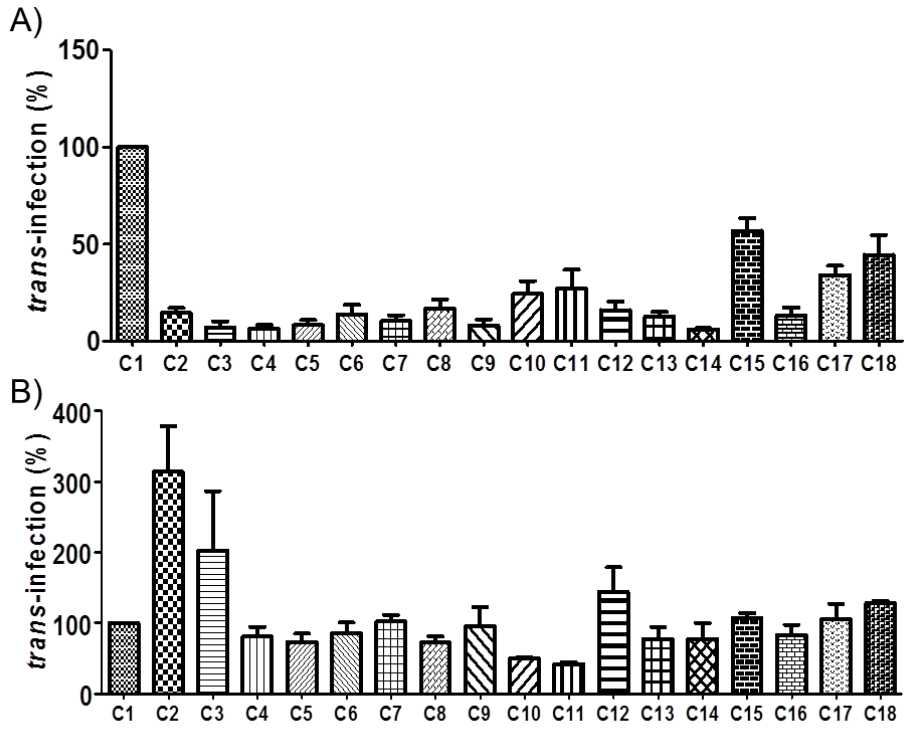


Figure 3.3.9 MDDC-mediated *trans*-infection of CD4+ cells. PSV equivalent to 100 ng p24 were captured for 2.5 hrs by MDDCs and used to infect TZM-bl cells. The relative luciferase units from MDDC PSV *trans*-infection relative light units (RLUs) were normalized to a single clone (% of C1) for each repeat (A) or normalized to entry efficiency and then as a percentage of C1 (B). Bar graphs are means and error bars represent standard error of the mean (+/- SEM) for three biological repeats done in triplicate.

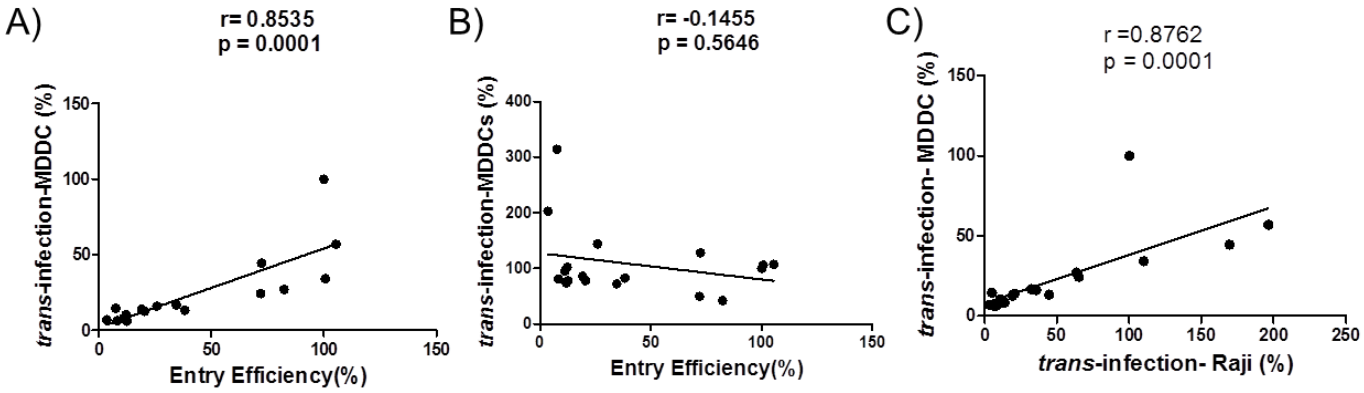


Figure 3.3.10: The role of Env entry efficiency in MDDC-mediated *trans*-infection and its similarity to Raji-DC-SIGN-mediated *trans*-infection. The association of MDDC-mediated *trans*-infection with entry efficiency (A,B) and Raji-DC-SIGN-mediated *trans*-infection (C) was determined using Spearman correlation analysis (GraphPad Prism 5.0). Following MDDC-mediated *trans*-infection, the RLU were normalized to C1 (A,C) or divided by Env entry efficiency first before normalization to C1 (B) to determine whether entry efficiency is an important determining factor of MDDC mediated *trans*-infection.

Trans-infection did not correlate significantly with Env mannosylation when using Raji-DC-SIGN cells and MDDCs for PSV capture despite the significant positive correlation between DC-SIGN binding and Env mannosylation and DC-SIGN binding and Raji-DC-SIGN-mediated *trans*-infection. This is possibly because of the strong dependence of successful *trans*-infection on entry efficiency which over-shadows the importance of DC-SIGN binding capacity. There is also the avidity effect: the ability of DC-SIGN-bound virus to dissociate from the receptor when added to the TZM-bl cells. They could be so that tightly bound PSVs and might not easily be transferred to TZM-bl cells. However, high DC-SIGN binding was associated with high trans-infection efficiency and it is thus unlikely that the mannosylation is influencing *trans*-infection by the avidity effect. An alternative explanation could be that successful *trans*-infection is more dependent on Env entry efficiency than DC-SIGN binding and Env entry is not influenced by mannosylation.

3.3.6 Role of specific PNGs in Env function

3.3.6.1 Impact of PNG deletions on Env function

3.3.6.1.1 Impact of PNG deletions on entry efficiency

HIV-1 Env PNGs have been found to impact interaction with target cell surface receptors (Pollakis et al., 2001; Clevestig et al., 2006; Li, Cleveland, et al., 2008; François & Balzarini, 2011; Wang et al., 2013; Mathys & Balzarini, 2014). To gain further insights into which specific PNGs are involved in entry efficiency, binding to DC-SIGN and *trans*-infection, PNGs previously suggested to be involved in DC-SIGN binding were deleted by SDM (Hong et al., 2007; Liao et al., 2011). Furthermore, Go *et al*. (2011) showed that PNGs at N241, N262, N386, N392 and N448 of subtype B and C TFs were enriched for HM/H N-glycans. As DC-SIGN binding has been suggested to favour mostly mannose type N-glycans (Lin et al., 2003; Shan et al., 2007; van Montfort et al., 2011), these sites were mutated and their impact on the function of C15 and C16 measured. These PNGs were conserved in C15 and C16 and the PSVs of these clones bound DC-SIGN with similar efficiency (1.5%) (Figure 3.3.5). However, C15 and C16 differed in entry efficiency (100 vs 40%, respectively) (Figure 3.3.4), percentage mannosylation (47 vs 33%, respectively) (Table 3.3.2), Raji-DC-SIGN mediated *trans*-infection (196 vs 44%, respectively) (Figure 3.3.7) and MDDC-mediated *trans*-infection (56 vs 13%, respectively) (Figure 3.3.9). The Asparagine of the NXS/T sequon was replaced by Glutamine at N241, N262, N386, N392, N448 in C15 and C16 to generate a total of 20 mutants

constituting single, double and triple mutants in various combinations of PNGs. The entry efficiency, binding to DC-SIGN and *trans*-infection of the deletion mutants were compared to WT.

C15 N262Q and C16 N392Q were unable to infect TZM-bl cells (Figure 3.3.11). Other studies have shown that deletion of the PNG at N262 lead to complete loss of infectivity (François & Balzarini, 2011; Wang et al., 2013; Mathys et al., 2014) and it was suggested that deletion resulted in misfolding of Env (Doores, 2015). C16 N262Q and C15 N386Q had only 10% residual function while deletion of PNGs at N241 and N448 were the only mutations that did not significantly alter the entry efficiency of C16 even when both were absent, contrary to what was observed for C15 (Figure 3.3.11). The differential effect of PNG deletion on the entry efficiency of the two clones suggests that loss and gain of some N-glycans affect Env function in a clone-dependent manner.

A) B)

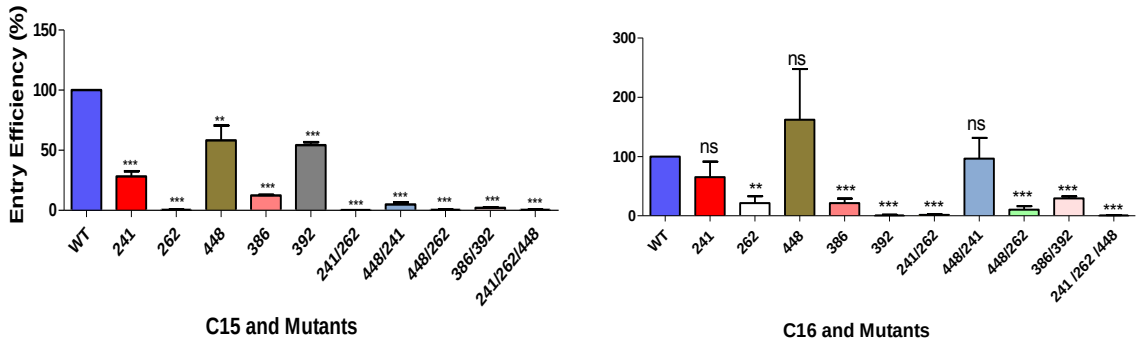


Figure 3.3.11: Impact of Env PNG deletion on PSV entry efficiency. N-Q mutants of C15 (A) and C16 (B) were pseudotyped and compared to WT for their ability to infect TZM-bl cells. Entry was measured by the luciferase reporter assay and the RLU values are indicated relative to WT. Bars indicate the mean of two independent experiments and the error bars indicate standard error of the mean. Statistical analysis was done using ANOVA. *, **and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively relative to WT, ns was not significant

PNGs at N241 and N262 in the C2 region of gp120 are 100% conserved in most subtypes including subtype C. The high conservation of these PNGs suggests that they are probably important for protein structure or function. Indeed, that deletion of PNGs at N241 and N262 in a previous study lead to reduced binding of by CD4-binding site antibodies b12 and 17b

(Pritchard, Spencer, et al., 2015). However, like in the case of C16 N262Q the same deletion in BaL (N262A) did not render PSVs non-infectious (Pritchard, Spencer, et al., 2015).

N386 and N392 are in the C3 region and together with N241 and N262 were profiled to preferentially carry oligomannose N-glycans in subtype B and C TFs (Go, Hewawasam, et al., 2011). Pritchard *et al.* (2015) reported that loss of certain PNGs in this region resulted in disruption of Env structure and function. Structural modelling of the N-glycan shield in this region showed that the Asparagine Cα-atoms of N386 and N392 were positioned about 12 Å (Angstroms) apart, leading to glycan-glycan interactions that could prevent mannosidase II from gaining access for oligomannose trimming. They suggested that due to bystander processing effects, deletion of one PNG could provide 1,2 α-mannosidase with access to a high mannose N-glycan, leading to trimming and the addition of complex carbohydrates which could then alter Env structure (Pritchard, Spencer, et al., 2015). Although we showed that total Env mannosylation was not associated with PSV entry efficiency, the presence or absence of either a HM/H or complex-type N-glycan at specific PNGs could be important for Env structure and/or function.

3.3.6.1.2 Impact of PNG deletions on binding to DC-SIGN

Deletion of N-glycans at positions N241, N262, N448, N386 and N392 led to significant reduction in binding to DC-SIGN for the C15 mutant, concomitant with loss of entry efficiency. C16 N241, N262 and N392 also had significantly reduced binding to DC-SIGN although deletion of N448 and N386 had no effect. PNG deletions impacted DC-SIGN binding of C15 and C16 differently: the DC-SIGN binding efficiency of C15 mutants ranged from 20 to 90% and approximately 20 to 60% for C16, suggesting that C15 was more sensitive to N-Q mutation than the C16 (Figure 3.3.12). However, the impact of each PNG on DC-SIGN binding was difficult to deduce because entry efficiency was compromised in nearly all mutants. We could therefore not distinguish whether a PNG was important for interacting with DC-SIGN or essential for Env structural integrity.

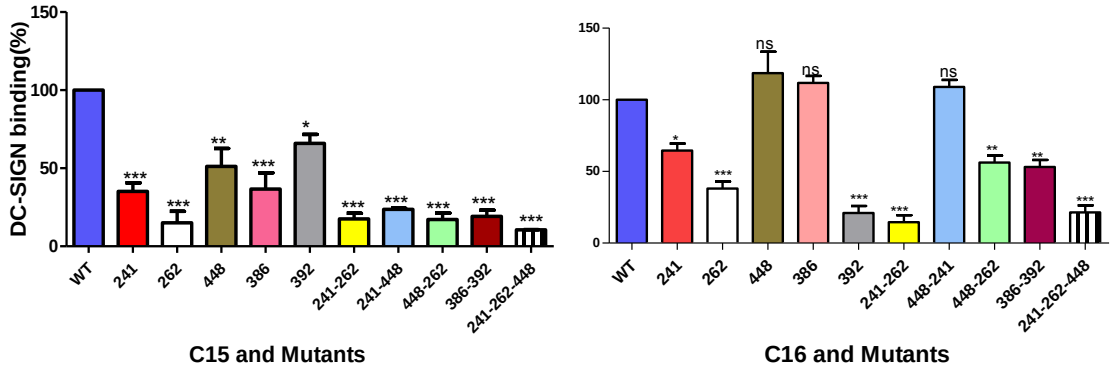


Figure 3.3.12: Impact of PNG deletion on Env-DC-SIGN interactions. The equivalent of 100 ng p24 PSV was added to Raji-DC-SIGN cells and allowed to bind for two and half hours before bound PSV was measured by p24 ELISA. Raji cells not expressing DC-SIGN were used as a control (Not shown). Percentage DC-SIGN binding relative to WT are indicated for C15 mutants (A) and C16 mutants (B). Bars indicate mean and SEM for two independent experiments. Results were compared to WT using one way ANOVA. *, **and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively relative to WT.

3.3.6.1.3 Impact of PNG deletion on trans-infection

As the *trans*-infection assay is dependent on TZM-bl entry, it was expected that the loss or reduction of entry efficiency of most PNG deletion mutants would result in poor *trans*-infection efficiency. The relationship between entry efficiency and *trans*-infection was apparent when deletion of C15 PNGs at N241, N392 and N448 reduced *trans*-infection to 7%, 23% and 39% respectively (Figure 3.3.13), with a concomitant decrease in entry efficiency to 29%, 50% and 60%, respectively (Figure 3.3.11). C16 N448 deletion mutant retained 100% entry efficiency, DC-SIGN binding and *trans*-infection capacity. However, although the entry efficiency of C16 N241 deletion mutant was not significantly reduced, its *trans*-infection was lowered. This is likely due to lowered DC-SIGN binding, suggesting that for C16, a PNG at position 241 could be important for DC-SIGN binding. This finding supports our data that *trans*-infection is significantly associated with both entry efficiency and DC-SIGN binding (Figure 3.3.8).

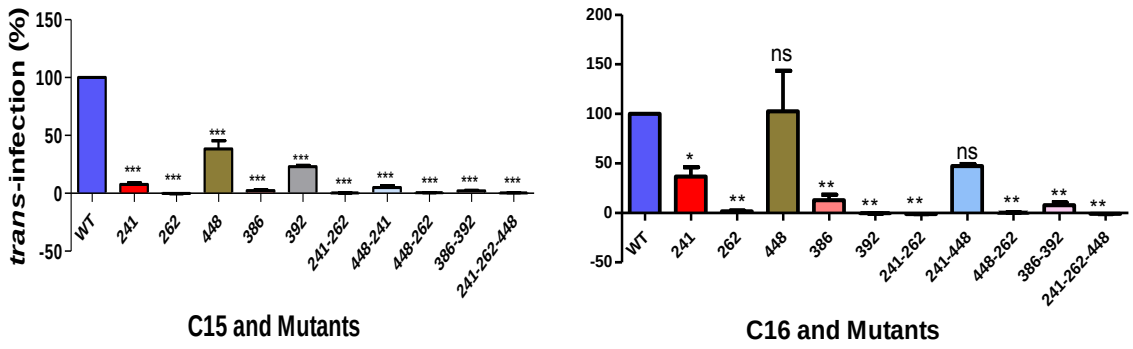


Figure 3.3.13 Impact of PNG deletions on *trans*-infection. PSV (100 ng p24) of PNG deletion mutants and WT were captured by Raji-DC-SIGN and added to TZM-bl cells and entry was measured after 48 hours using the luciferase reporter assay. The RLU values of the PNG mutants were normalised to WT. Bars represent means with SEM from two independent biological repeats. Statistical analysis was done using one way ANOVA and post-test. *, **and *** show degree of significance for $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively relative to WT.

3.3.6.2 Impact of presence or absence of specific PNGs on Envelope function

The impact of specific PNGs on Env function has been studied mostly in the context of PNG deletion (Wang et al., 2013; Mathys & Balzarini, 2014; Mathys et al., 2014). As deletion of PNGs impacted Env function, it remains unknown how the presence or absence of PNGs can influence DC-SIGN binding and *trans*-infection and thus HIV pathogenicity. We thus compared the phenotype of PSVs between groups that either carried or did not carry specific PNGs. In order to carry out accurate statistical sequence analysis on the Env clones, the following criteria had to be met: the frequency of the PNGs had to be between 33 – 67% and had to fall within the constant regions to unambiguously assign the correct position to each PNG. Envs were grouped based on the presence or absence of specific PNGs and compared phenotypically: entry efficiency, binding to DC-SIGN and *trans*-infection.

3.3.6.2.1 Impact of specific Env PNGs on entry efficiency

Statistical comparison of the entry efficiency of Env clones grouped based on the absence and presence of PNGs identified that the presence of N356 and N392 were significantly associated with enhanced entry (Figure 3.3.14). Of the five PNGs deleted, only the PNG at N392 was identified by both sequence analysis and PNG mutagenesis as essential for entry efficiency. N448 was not identified through statistical comparison, confirming deletion analysis that this site was not essential for entry efficiency. Identifying the impact of N392 and N448 in Env

entry efficiency by both statistical analysis and mutagenesis, in part, validates the two approaches. The remaining three PNGs deleted at N241, N262 and N386 could not be included in the analysis as they were > 67 % conserved across all sequences.

Wang *et al*. (2013) found an increase in infectivity when N448 was deleted in subtypes A, B and BC despite deletion resulting in reduced gp120 incorporation. N448 was 100% present in the Wang *et al*. study whereas the PNG was present in only 50% of our sequences and 72% in the subtype C database. This could suggest that either N448 could be important for entry efficiency in a clone dependent manner, influenced by HIV-1 subtype or play a role in an alternative function of Env**.**

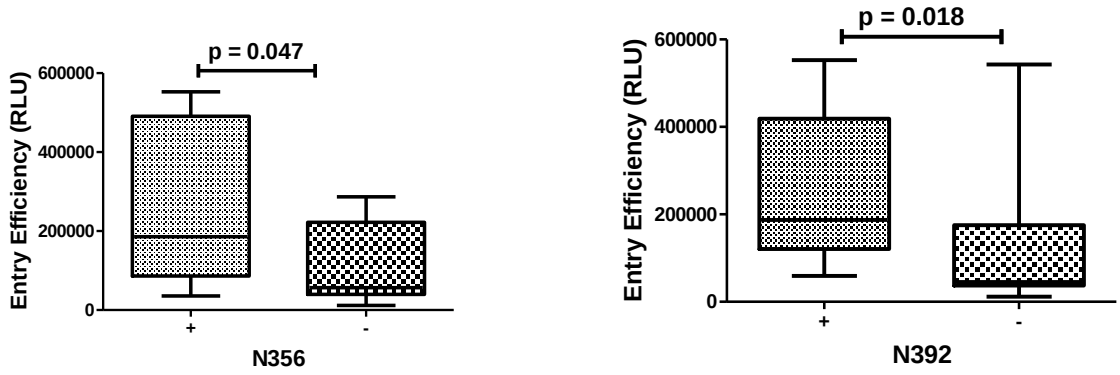


Figure 3.3.14 Identification of Env PNGs involved in PSV entry efficiency. Entry efficiency (RLU) was compared between Env clones grouped based on the presence or absence of PNGs in the constant regions with a frequency between 33 – 67 %. Results indicate mean RLU values from at least four biological repeats compared using a Mann-Whitney test, two tailed comparison.

PSVs lacking a PNG at N674 had higher DC-SIGN binding than those which carried a PNG at this position and this association almost reached statistical significance ($p < 0.067$) (Figure 3.3.15). This supported previous findings that deletion of the PNG at N674 resulted in enhanced virus capture (DC-SIGN binding) by 30% (Mathys & Balzarini, 2014), suggesting that a PNG at this site might hinder Env binding to DC-SIGN. Furthermore, as only 33% of the sequences encoded N674 it is likely under negative selection.

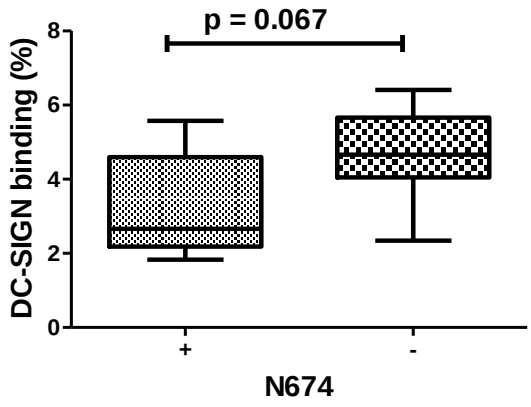


Figure 3.3.15 Identification of Env PNGs involved in binding to DC-SIGN. Envs were grouped based on the presence or absence of PNGs in the constant regions that varied between 33 – 67% and compared for binding to DC-SIGN using Raji-DC-SIGN cells. Means from two biological repeats were compared using the Mann-Whitney test.

3.3.6.2. Impact of specific Env PNGs on PSV *trans*-infection

Possession of a PNG at N392 and absence of one at N674 were significantly associated with enhanced *trans*-infection (Figure 3.3.15) when Raji-DC-SIGN cells were used for PSV capture. When MDDCs from healthy human donors were used for PSV capture, a PNG at N356 and absence of PNGs at N289 and N674 were associated with enhanced *trans*-infection although did not reach statistical significance probably due to variability in donor responses from different human donors (data not shown). More donors tested could confirm the importance of PNGs at N289, N356 and N674 in MDDC-mediated *trans*-infection. The fact that different PNGs were identified to influence MDDC-mediated *trans*- infection and Raji-DC-SIGN-mediated *trans*- infection could also be due to differences between Raji-DC-SIGN cells and MDDCs such as the expression of other receptors on MDDCs that could play a role in HIV *trans*-infection as well as the concentration of DC-SIGN on the surface of the cells (Pohlmann et al., 2001).

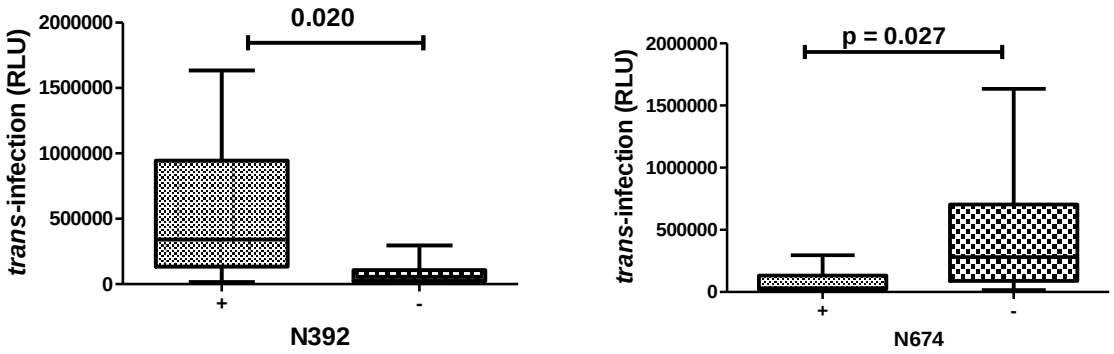


Figure 3.3.15 Identification of Env PNGs that influences PSV *trans*-infection using Raji-DC-SIGN cells for capture. PSVs were captured by Raji-DC-SIGN cells before adding to TZM-bl cells as described in materials and methods and *trans*-infection (RLU) values from all Env clones were compared between groups that carried or lacked PNGs in the constant regions varying in frequency from 33 – 67%. Results show medians from two biological repeats compared using the Mann-Whitney test.

Table 3.3.3 N-glycan sites associated with changes in Env entry efficiency, and *trans*-infection

Phenotype	Position of PNGs		
	356	392	674
Entry efficiency	+(0.018)	+(0.047)	
Raji-DC-SIGN mediated *trans*-infection		+(0.020)	-(0.027)

-/+ indicates negative and positive associations, respectively
p values are indicated in brackets and were determined using Mann-Whitney t-test**.**

3.4 Discussion.

As Envelope mediates the first step in HIV infection and is highly immunogenic, it is the favoured target for vaccine design. However, one of the stumbling blocks to identifying an effective vaccine candidate is the high genetic diversity of Env because an immune response against one variant might not result in an immune response to all. Env N-glycosylation is an important factor in immune evasion as it not only hides epitopes, it is highly immunogenic resulting in the production of decoy non-neutralising antibodies and bnAbs (Sanders et al., 2002; Calarese, 2003; Pejchal et al., 2011). N-glycans have also been found to play an important role in processing and folding of Env into its native conformation essential for interaction with receptors and co-receptors on target cells (Li et al., 1993; Pollakis et al., 2001; Clevestig et al., 2006). It has also been suggested that the type of glycan present on Env influences biological properties especially the immunogenicity of this protein (Go et al., 2015) and that Env N-glycosylation could be important for HIV transmission (Go, Hewawasam, et al., 2011; Shen, Raska, et al., 2014). With the recent findings that N-glycans form epitopes for potent bnAbs (McLellan et al., 2011; Moore et al., 2012; Kong et al., 2013; Pritchard, Spencer, et al., 2015), one of the key aims in HIV vaccine research is the design of immunogens with an N-glycosylation profile that represents that of transmitted founder-associated, native functional trimers (Sattentau, 2013). In this study, we investigated the impact of N-glycosylation on Env entry efficiency, binding to DC-SIGN and *trans*-infection to provide information on the PNGs involved in Env function.

To study the impact of the type of N-glycans on Env function, mannosylation of 9/18 functional clones were determined by comparing the molecular weights of fully N-glycosylated, demannosylated and deglycosylated gp140. Contrary to oligomannose-enriched cell-associated gp140 (Ms Liliwe Shuping; Data not shown), all clones carried both HM/H and complex type N-glycans and cleavage (e.g. furin) was observed for all PNGaseF-treated clones. Interestingly, uncleaved gp140 was present in the culture medium of all clones and EndoH treatment deglycosylated C3, C8 and C12, suggesting that not only was cleavage inefficient but also that a fraction of gp140 carried only HM/H. Furthermore, based on PNGaseF treatment which resolved both gp140 and gp120, the apparent amount of gp140 released into the culture medium exceeded that of gp120 for nearly all clones except C12, C13 and C14. Therefore, a band representing gp120 was not apparent in most undigested controls most likely due to inefficient cleavage of gp140 leading to low levels of fully processed gp120 as seen in a previous study (Binley et al., 2002). As a band corresponding to glycosylated gp120 was absent for most

clones, bands corresponding to gp140 were used to compare mannosylation between clones. We hypothesised that gp140 N-glycosylation could represent that of native Env as uncleaved gp140 form trimers with compact, native-like structures (Kovacs et al., 2014).

Mannosylation of gp140 varied from 23 – 43%, except for C3 with only 7% HM/H. There was no statistical correlation between mannosylation and Env entry efficiency, suggesting that the level of HM/H did not influence PSV entry. It has been suggested that Env entry efficiency depends on the structure and/or presence and location of specific N-glycans and not the type of carbohydrate (Wang et al., 2013; Mathys et al., 2014). Furthermore, the use of mutant cell lines to produce virus that resulted in the addition of mainly Man5GlcNac residues did not influence infectivity (Montefiori, Robinson Jr. & Mitchell, 1988; Eggink et al., 2010), while inhibition of all N-glycosylation lowered infectivity most likely due to misfolding of Env (Montefiori, Robinson Jr. & Mitchell, 1988). However, due to the level of gp140 mannosylation it is also possible that the N-glycan profile is not representative of PSV-associated Env trimers (Beddows et al., 2006).

It was shown that PSV- and virus-associated gp120 carried mainly oligomannose (Table 3.4.1) and the authors suggest that this is an intrinsic feature of HIV-1 Env (Bonomelli et al, 2011; Doores et al., 2010). Conversely, monomeric gp120 carried more complex-type N-glycans and it was suggested that trimer formation precludes HM trimming and the addition of complex-type carbohydrates (Bonomelli et al., 2011). However, Crooks *et al*. (2011) proposed that oligomannose-rich Env represented "junk" gp120/gp160 due to aberrant processing pathways and did not represent native functional trimers (Crooks et al., 2011). Irrespective of whether Env trimer oligomannose levels are due to native or aberrant processing pathways, it was recently confirmed that membrane-associated Env had much higher levels of oligomannose N-glycans than soluble gp140 (Go et al, 2015). Our results were consistent with these studies that showed monomeric gp140 carried 23 – 50% HM across most strains (Doores, 2015; Go et al., 2015; Pritchard, Vasiljevic, et al., 2015), suggesting that our gp140 did not form trimers and thus did not represent PSV functional Env. However, gel filtration of our purified gp140 indicated monomers, dimers and trimers (Dr Van Ryk, Data not shown), confirming that mixed oligomerisation had occurred as previously reported (Beddows et al., 2006). But whether these dimers and trimers were functional, was not shown. EndoH digestion deglycosylated some gp140, suggesting that a proportion of gp140 carried only HM/H. This fraction could represent

uncleaved gp140 trimers enriched with oligomannose and the ratio of gp140 oligomers to monomers could influence the overall apparent mannosylation of each clone.

Table 3.4.1 Abundance of released N-linked glycans obtained from recombinant (monomeric), pseudoviral, and viral gp120

Gp120 source	Cell type	Man5-9GlcNAc2 (%)	Mannose content relative to rgp120
Recombinant monomer	293T	29	1.0
Pseudovirus (pSG3Denv:pSVIII JRCSF, 2:1)	293T	98	3.4
Pseudovirus (pSG3Denv:pSVIII JRCSF, 10:1)	293T	85	2.9
Supernatant (pSG3Denv:pSVIII JRCSF, 10:1)	293T	73	2.5
Virus (pLAI-JRCSF env)	293T	56	1.9
Virus JRCSF (clade B)	PBMC	79	2.7
Virus 92RW009 (clade A)	PBMC	64	2.2
Virus 93IN905 (clade C)	PBMC	62	2.1

Edited from Bonomelli et al., (2011)

Table 3.4.2 Mannosylation of gp140 and gp120 of nine Env clones compared to positive control

gp120 source	Cell type	gp140		gp120	
		HM/H (%)	HM/H relative to rgp120	HM/H (%)	HM/H relative to rgp120
Recombinant monomer (rgp120)(IIB_LAI)	CHO	N/A	N/A	36.6	1
C3	293T	6.8	0.2	N/A	N/A
C4	293T	35.5	1.0	N/A	N/A
C7	293T	28.7	0.8	N/A	N/A
C8	293T	23.4	0.6	N/A	N/A
C12	293T	42.9	1.2	N/A	N/A
C13	293T	35.2	1.0	46.6	1.3
C14	293T	39.7	1.1	49.6	1.4
C15	293T	40.3	1.1	50.4	1.4
C16	293T	34.3	0.9	36.5	1.0

If gp160 cleavage is required for functional trimers (McCune et al., 1998), trimerisation sterically hinders trimming of oligomannose moieties (Bonomelli et al., 2011) and uncleaved gp140 trimers carry higher levels of complex-type N-glycans (Pritchard, Vasiljevic, et al., 2015), we hypothesised that glycosylated gp120 of C13, C14, C15 and C16 were likely derived from fully processed trimers and thus enriched with oligomannose when compared to gp140. Comparison of the HM/H levels of gp140 to a gp120 positive control indicated that percentage

mannosylation were very similar differing 0.6- to 1.2-fold, except for C3 which had very low levels of HM/H (Table 3.4.2). Mannosylation of gp120 ranged from 37 to 50%, marginally higher than that of gp140 and the positive control (1.0 – 1.4-fold) but still much lower than virion- or PSV-associated Env (1.9 – 3.4-fold) (Table 3.4.1), suggesting that the source of gp120 was monomeric cleaved gp140. The presence of only monomers was surprising as gp140 constructs with intact cleavage sites have been shown to form oligomers including trimers although with poor stability (Jeffs et al., 2004; Beddows et al., 2006). However, oligomerisation varied according to Env clone, suggesting that the extent of trimerisation and thus mannosylation could be variant specific (Jeffs et al., 2004; Beddows et al., 2006). Mannosylation of virus-associated gp120 from different variants ranged from 56 – 79% (Table 3.4.1). As some Envs have been shown to trimerise much more efficiently than others and studies have only characterised one or two variants at a time, Env mannosylation levels, whether monomeric or virus-associated trimers, might be more heterogeneous than previously reported (Bonomelli et al., in press; Jeffs et al., 2004; Beddows et al., 2006; Go et al., 2015).

Furthermore, other factors have also been shown to influence Env N-glycosylation including the cell type used to express the recombinant protein, cleavage efficiency of gp160 and level of Env expression (Table 3.4.1) (Bonomelli et al., 2011; Binley et al., 2002; Raska et al., 2010). We showed that expression of Env in the absence of pSG3ΔEnv, the pseudovirion backbone vector, was much lower than when pseudovirus was being prepared although relative expression remained the same (Appendix I Figure A1). Therefore, N-glycosylation analysis, preferably using mass spectrometry comparing virion-associated gp120 to gp140 is required of all eighteen clones to confirm whether gp140 mannosylation represents PSV-associated functional Env and thus determine whether mannosylation plays a role in Env entry efficiency.

However, PSV mannosylation could be similar to that of recombinant gp140 as we found a statistically significant correlation between PSV DC-SIGN binding and gp140 mannosylation, supporting previous findings that DC-SIGN interactions with Env favours mannose type N-glycans (Geijtenbeek, Torensma, et al., 2000; Lin et al., 2003; Montfort et al., 2011). Furthermore, both Raji-DC-SIGN and MDDCs were found to transfer PSVs to TZM-bl cells, confirming previous findings by Geijtenbeek *et al*. (2000) (Geijtenbeek, Torensma, et al., 2000). A correlation was found between DC-SIGN binding and DC-SIGN-mediated *trans*-infection further confirming studies by Geijtenbeek *et al.* (2000) that DC-SIGN is involved in *trans*-infection of target cells. This correlation was however not significant when MDDCs were

used for capture probably because MDDCs express not only DC-SIGN but other CLRs and non-CLRs (heparin sulphate proteoglycans, CD169, GarCer) which are also involved in HIV *trans*-infection (Saphire et al., 2001; Turville et al., 2002; Magerus-Chatinet et al., 2007; Lambert et al., 2008; Izquierdo-Useros, Lorizate, Puertas, et al., 2012; Puryear et al., 2012). Eggink *et al*. (2010) proposed that HIV-1 LAI, enriched with HM residues, bound weakly to iMDDCs due to the presence of receptors other than DC-SIGN that recognised Env complex glycans (Eggink et al., 2010).

Despite the lack of correlation between DC-SIGN binding and MDDC-mediated *trans*-infection, there was a highly significant correlation between the two *trans*-infection methods using Raji and MDDCs ($p < 0.0001$, $r = 0.8762$), suggesting that the overall mechanism was similar for both cell types. *Trans*-infection does not only depend on binding to MDDCS via DC-SIGN (and other receptors), it is also highly dependent on Env entry efficiency. There was a highly significant correlation between both DC-SIGN-mediated and MDDC-mediated *trans*-infection with entry efficiency confirming the role of Env entry in *trans*-infection. However, MDDC-mediated *trans*-infection seemed more sensitive to changes in Env entry efficiency than when Raji-DC-SIGN cells were used for capture and *trans*-infection was more efficient at infecting TZM-bl cells than direct infection in the absence of Raji-DC-SIGN cell capture. This suggested that binding to Raji-DC-SIGN and not MDDCs, enhanced the entry efficiency of Env, confirming previous suggestions that DC-SIGN binding may lead to *trans*-enhancement of HIV infection (Appendix I, Figure A2) (Cameron et al., 1992; Kwon et al., 2002; Reyes-Rodriguez, Reuter & McDonald, 2016). Previous studies have suggested a change in Env conformation or increased affinity following interaction with DC-SIGN that rendered the virus more infectious by stabilising the gp120-CD4 complex (Geijtenbeek, Kwon, et al., 2000; Kwon et al., 2002; Hijazi et al., 2011).

The differences in *trans*-infection of TZM-bl cells by the two cell types could be due to multiple factors. The concentration of DC-SIGN on the cell surface is different between MDDCs and Raji-DC-SIGN cells (data not shown) and *trans*-infection is highly dependent on DC-SIGN expression levels (Pöhlmann et al., 2001). Low levels of DC-SIGN were also shown in another study to enhance CD4+ cell *trans*-infection possibly because steric hindrance due to high levels of DC-SIGN interfered with gp120 binding to CD4 (Hijazi et al., 2011). DC-SIGN binds with high affinity to gp120 (Curtis, Scharnowske & Watson, 1992) and thus higher concentrations of DC-SIGN at the cell surface might prevent easy transfer to CD4 (Eggink et al., 2010).

However, another study suggested that enhanced DC-SIGN expression would favour HIV *trans*-infection because *trans*-infection efficiency was shown to increase with an increasing number of cell surface DC-SIGN molecules until a plateau was reached (Pöhlmann et al., 2001). MDDCs do not only express DC-SIGN, but also other CLRs and non-CLRs that could all play a role in capture/transferring PSV to TZM-bl cells and thus influence PSV dissociation from MDDCs, CD4 binding due to steric hindrance and stabilisation of the CD4-gp120 complex. The mechanism whereby DC-SIGN might enhance HIV-1 *trans*-infection might also depend on the strain of virus. DC-SIGN enhanced *trans*-infection of CD4-independent viruses by concentrating virus at the cell surface and also by stabilising the CD4-gp120 complex (Hijazi et al., 2011). Therefore, studies into the nuanced variation between the capture of PSVs using Raji-DC-SIGN cells and MDDCs could provide a novel understanding of the molecular mechanism of HIV-1 *trans*-infection.

Variable loop length and N-glycosylation have been suggested to influence the interaction of Env with DC-SIGN on MDDCs (Nabatov et al., 2006) and given that PNG number and variable loop length increases over the course of infection (Chohan et al., 2005), DC-SIGN could play a role in selection of variants during transmission and disease progression. Supporting this suggestion was our finding that TFs had a significantly lower number of PNGs than matched chronic infection Envs ($p = 0.009$), confirming previous findings (Derdeyn et al., 2004; Chohan et al., 2005). Comparison of the number of Env PNGs with gp140 mannosylation showed a significant association ($p = 0.017$), suggesting that as the number of PNGs increase, so does the level of mannosylation. Furthermore, Env mannosylation levels correlated with DC-SIGN binding, which was significantly associated with Raji-DC-SIGN-mediated *trans*-infection. Therefore, based on these findings, TF Envs carry fewer PNGs should be less mannosylated, and by extrapolation, bind more weakly to DC-SIGN and have less efficient *trans*-infection than variants in chronic infection. However, it seems that specific PNGs and not only Env N-glycan content influences DC-SIGN mediated *trans*-infection as this association was not significant for MDDC mediated *trans*-infection. This would suggest that Env mannosylation is not the only determinant of HIV-1 transmission via DC-SIGN binding.

Alternatively, specific PNGs could be important for interaction of Env with DC-SIGN (Hong et al., 2007; Liao et al., 2011) and with CD4 and co-receptors (Pollakis et al., 2001; Clevestig et al., 2006). PNGs at positions 241, 262, 386, 392 and 448 of C15 and C16 were deleted to determine the importance of these N-glycans to DC-SIGN binding given that C15 and C16

were matched Envs from the same participant, carried all 5 PNGs and had similar binding to DC-SIGN. Mutation of specific PNGs previously profiled as carrying HM residues in subtypes B and C TF Envs (Go, Hewawasam, et al., 2011) confirmed that N241, N262, N386 and N392 were very important for entry efficiency and most likely Env structure (François & Balzarini, 2011; Wang et al., 2013; Mathys et al., 2014; Pritchard, Spencer, et al., 2015). N448Q was found to enhance antigenicity by another study without affecting Env CD4 binding capacity (Kumar et al., 2011). N448 and N356 had also been suggested to be potentially important for CCR3 co-receptor interaction (Cenci et al., 2014), protection of the V3 loop from nAbs, while promoting processing and presentation of T helper epitopes (Li, Chien Jr., et al., 2008; Li, Xu, et al., 2009). N448 deletion was found to increase entry efficiency, decrease Env incorporation and lower the abundance of oligomannose type N-glycans (Wang et al., 2013; Pritchard, Spencer, et al., 2015). We found a non-significant decrease in infectivity in one PSV clone and a non-significant increase in entry of the other relative to WT. This suggests that N448 may be important for Env function but in a clone dependent manner. Although some PNGs influenced binding to DC-SIGN and *trans*-infection in a clone dependent manner, C15, a TF Env was on the whole more affected by N241, N262, N386, N392 and N448 deletions than its matched chronic infection clone. This could suggest that not only does the number of PNGs differ over the course of infection but also the role they play in Env function. Whether or not the increased sensitivity of the TF to N-glycan deletion is related to HIV-1 transmission requires further investigation using a larger sample size.

PNG deletion analysis did not identify N-glycans important for DC-SIGN binding and *trans*-infection as these mutants had either no or greatly reduced entry efficiencies. Further characterization of the Envs was done based on the presence or absence of PNGs that varied in frequency between 33 – 67 % in the constant regions. This method confirmed the results of the mutational analysis that the PNG at N392 was important for Env entry efficiency and showed that N392 might also influence Raji-DC-SIGN-mediated *trans*-infection. The impact that a PNG at N392 might have on Raji-DC-SIGN-mediated *trans*-infection, could be due to their influence on PSV entry efficiency. The lack of a PNG at position 674, located between heptad repeat 2 (HR2) and the TM domain was significantly associated with increased *trans*-infection by Raji-DC-SIGN cells. PSVs that tended to have higher DC-SIGN binding also tended not to carry a PNG at 674. It is possible that N-glycans located close to the membrane might sterically hinder proper gp120 interaction with DC-SIGN. Mathys and Balzarini (2014) reported that deletion of this PNG enhanced Env binding to sCD4 and caused a 30 % increase in DC-SIGN

binding as well as transmission efficiency. This same study found that N674 had a frequency of approximately 15 % in A2, CRF04-cpx and CRF14-BG HIV-1 subtypes whereas a PNG at N674 was present in 33 % of the 18 Env clones studied and 24.8 % in the subtype C database, suggesting that it is under negative selection.

Previously Parrish *et al*. (2013) showed that TFs are better at infectivity, DC-SIGN binding and *trans*-infection than chronic infection variants (Parrish et al., 2013) and another study suggested that viral fitness is an important determining factor in HIV transmission (Carlson et al., 2014). Although our sample size was too small to accurately compare TF (n = 5) to matched chronic infection controls (n = 13), our analysis suggests that specific PNGs could be important for transmission and not Env mannosylation. This study has identified PNGs that could be involved in HIV pathogenesis and screening a larger cohort can confirm the association between PNGs and Env function. This study has shown that PNGs can either enhance or compromise Env function, depending on their absence or presence. Escape from autologous antibodies could require the introduction of a PNG that comes with a fitness cost. It is possible that evasion from vaccine-induced bnAbs could rely on the introduction of an additional PNG with concomitant loss of the original PNG involved in escape from autologous antibodies. The loss of this PNG will enhance infectivity, resulting in a fitter virus and potentially increased disease progression.

Conclusion

In conclusion, we identified PNGs that could be important for Env entry efficiency and *trans*-infection of CD4+ cells using both mutational and sequence analysis. The effect of N-glycans on entry efficiency, DC-SIGN binding and *trans*-infection was highly clone-dependent. With the recent findings that N-glycans form epitopes for potent bnAbs (McLellan et al., 2011; Moore et al., 2012; Kong et al., 2013; Pritchard, Spencer, et al., 2015), one of the key aims in HIV vaccine research is the design of Env immunogens with an N-glycosylation profile that represents that of transmitted founder-associated, native functional trimers (Sattentau, 2013). Vaccines that include N-glycan-containing epitopes could generate bnAb that prevent productive clinical HIV infection but they could also drive the rapid loss or gain of PNGs due to selective pressure on the virus to escape neutralisation. These PNG variations could either drive the emergence of fitter and more pathogenic variants or abrogate viral infectivity. We identified N356 as a potential fitness determinant. Therefore, if an epitope carrying a PNG at this position was included in a vaccine, the need to escape immune recognition could drive the

loss of a PNG at this site, significantly decreasing the fitness of the virus. However, as previously shown for N332, evolution of neutralising antibody responses can occur via loss of other PNGs that may be associated with enhanced infectivity. Therefore, the impact of N-glycans on Env function and potentially HIV replication fitness must be tested in a range of phylogenetically distinct viruses to not only ensure the safe outcome of an Env N-glycan-targeted vaccine but also as a means to drive the emergence of less fit variants.

Chapter 4: The role of subtype C HIV-1 Envelope N-glycosylation in DC-SIGN-mediated modulation of Dendritic Cell cytokine release

4.1 Introduction

Productive HIV infection requires dissemination and systemic spread. As DCs have a migratory nature, they are able to carry the virus to the lymphoid organs (Banchereau & Steinman, 1998; Banchereau et al., 2000), where they can infect T cells in *trans.* Therefore, although DCs may not be the primary target for HIV infection, they may play a more important role in viral dissemination. A murine knock-out study showed that autocrine IL-10 inhibited DC immune responses by down-regulating the translocation of DCs to lymphoid tissues and by locally regulating DC IL-12 production in the draining lymph nodes (Demangel, Bertolino & Britton, 2002). Therefore, as IL-10 has been shown to slow the migration of DCs infected with *M.tuberculosis* to lymph nodes, this suggests that it might also play a very important role in autocrine modulation of DC responses to HIV infection.

IL-10 has been found to play a critical role in modulating immunopathology related to exacerbated immune responses (Couper, Blount & Riley, 2008) and dampens the development of Th1 responses while encouraging Th2 development (Fiorentino et al., 1991; Dong & Flavell, 2001). As it limits inflammation and Th1 cytokine activity in response to viruses and other intracellular pathogens (like Leishmania, *Mycobacterium avium*), IL-10 encourages the establishment of chronic infections. High expression levels of IL-10 in human viral infections like Hepatitis B and C (HBV and HCV) infections correlated with poor T cell immune responses, and hence a failure to control viral replication (Spellberg & Edwards, 2001; Couper, Blount & Riley, 2008; Flynn et al., 2011; Redford, Murray & O'Garra, 2011; Singh & Thirumalapura, 2014). Studies in mouse models of Lymphocytic Choriomeningitis virus (LCMV) have also shown the role of IL-10 in limiting the T cellular immune response, permitting the virus to establish infection. Interventional interference with IL-10 activity in this model enhanced viral clearance (Brooks et al., 2006; Ejrnaes et al., 2006).

IL-10 immune regulation involves the suppression of MHC class II and costimulatory molecule, B7-1/B7-2 expression and restricts proinflammatory cytokine production. This includes IL-1α, IL-1β, IL-6, IL-12, IL-18, and TNF-α and chemokines such as MCP-1, MCP-5, RANTES, IL-8, IP-10, and MIP-2. Furthermore, IL-10 has a negative feed-back loop where it inhibits DC chemokine production, preventing migration to lymph tissues for recruitment and induction of Th1 responses (Couper, Blount & Riley, 2008). Production of IL-10, IL-4, IL-5 and IL-13 by APCs drives naïve (Th0) T cells differentiation into Th2 cells, enhancing B cell activity against extracellular pathogens like parasites and certain bacteria. IL-10 has pleotropic

function and is also immunoregulatory; promoting differentiation of Th0 cells into regulatory T cells (Treg) whichs cause induction of tolerance (Espir et al., 2014). The delicate balance of pro- and anti-inflammatory cytokines can drive naïve T cell differentiation towards either, Th1, Th2, Th17, Th22 or T regulatory pathways through the secretion of required cytokines and chemokines like IL-1β, TNF-α, IL-6, IL-8, IL-12, MIP-1α, and MIP-1β. These are either important for enhancing the immune response, preventing immune related pathologies or stimulating tolerance to a pathogen in order to protect the host from tissue damage caused by an excessive immune response (Kitagishi et al., 2012). In persistent viral infections, these regulatory functions of DCs could contribute to the establishment of infection and also determine the disease severity as they dampen the immune response (de Waal Malefyt, Haanen, et al., 1991), reviewed in (Espir et al., 2014).

Due to the immune modulation function of some cytokines, such as IL-1β and TNF-α, they have been coined immune modulatory cytokines. TNF-α has been shown to enhance HIV replication in cell line models (Finnegan et al. 1996) especially under immunosuppressive conditions. Thus DCs do not only initiate an adaptive immune response, they also maintain and modulate immune responses through cytokine secretion. Understanding the interplay between the host immune response and the role of APCs, such as DCs, in viral pathgenesis would provide not only valuable insight into HIV transmission and persistence, but also contribute to the search for more improved treatment and prevention methods

IL-12 production by DCs and other APCs causes polarization of the Th0 cells to Th1 cells, which further produce more IL-12 and IFN-γ and causes up-regulation of both co-stimulatory and maturation markers of DCs (Trinchieri, Pflanz & Kastelein, 2003). IL-12 acts directly on T cells, enhancing proliferation, IFN-γ production and increasing cytolytic activity of both cell types (Clerici & Shearer, 1993; Kennedy et al., 1994; Wolf, Sieburth & Sypek, 1994; Kedzierska & Crowe, 2001; Trinchieri, 2003; Trinchieri, Pflanz & Kastelein, 2003; Watford et al., 2003; Hamza, Barnett & Li, 2010). IL-12 thus has a critical role in regulating the development of native CD4+ T cells into Th1, while impairing the Th2 pathway and production of associated cytokines (Dong & Flavell, 2001). IL-12 and IFN-γ act on macrophages and natural killer cells (NK) to enhance their phagocytic activity against viruses and other intracellular pathogens. Therefore, differentiation of Th0 into either Th1 or Th2 responses is modulated by the relative expression of IL-10 and IL-12.

In HIV infection, a dysregulation of cytokine production has also been reported, with impaired IL-12 secretion by PBMCs from HIV infected patients in response to stimulants (Clerici et al., 1993; Chehimi et al., 1994; Daftarian et al., 1995; Marshall et al., 1999; Bal et al., 2005), suggesting that there is impairment of the Th1 pathway (Marshall et al., 1999). IL-10 levels were found to be elevated in the plasma of HIV infected patients (Stylianou et al., 1999). While it was found that IL-10 increase was mostly associated with acute infections (AI) (Norris et al., 2006), other studies showed that its levels increased with disease progression and were partially resolved following HAART (Stylianou et al., 1999; Norris et al., 2006; Roberts et al., 2010).

While Brockman *et al*. (2009) found that all cell types except DCs produced higher levels of IL-10 in HIV positive subjects compared to negative subjects (Brockman et al., 2009), another study couldn't differentiate the source of IL-10 when HIV infected DCs were co-cultured with T cells (Granelli-Piperno et al., 2004). Most of these past studies presupposed that viral replication in the cells was the cause of IL-10 production. It took many more studies to find that gp120 induced IL-10 expression but whether T cells and/or APCs were the initial sources remained unclear (Ameglio et al., 1994; Schols & De Clercq, 1996; Taoufik et al., 1997; Mellado et al., 1998). Some of these studies nevertheless found that the increase in IL-10 levels in HIV infected individuals indirectly impaired T cell responses by suppressing APC production of IL-12 and IFN-γ production (Clerici et al., 1993; Chehimi et al., 1994; Daftarian et al., 1995; Herbein, Montaner & Gordon, 1996; Taoufik et al., 1997; Marshall et al., 1999). Further research later found that interaction between gp120 and mannose C-type lectin receptors (MCLR) on MDDCs was enough to induce IL-10 production in culture (Shan et al., 2007). Moreover, IL-10 expression was accompanied by poor MDDC maturation and impaired ability of these MDDCs to stimulate T cells in mixed leukocyte culture (Fantuzzi et al., 2004; Shan et al., 2007). This suggested that induction of DC IL-10 secretion in the female genital tract could be a potential mechanism whereby HIV-1 is able to modulate immune responses which favour transmission as well as disease progression.

However, longitudinal follow-up of HIV-infected participants showed that there was a distinct pattern of expression of cytokines over time following infection (Stacey et al., 2009). Initially, there was a sharp increase in IFN-γ and IL-15, then IP-10 followed by a protracted increase in TNF-α and MCP-1. This was followed by the pro-inflammatory IL-6, IL-8, IL-18 and IFN-γ and then IL-10 (Stacey et al., 2009), suggesting that HIV induced inflammation cytokines driving IL-10 expression. However, the authors indicated that they did not identify the source

of the IL-10. As IL-10 is expressed by a wide range of cells, its differential regulation depends on cell type and environment and this will results in the fine balance between effective immune responses and immunopathology (Saraiva & O'Garra, 2010). In this study we stimulated purified MDDCs with HIV-1 and gp140, which can induce the production of pro-inflammatory cytokines via CLRs, TLR2 and/or TLR4 (Thibault et al., 2009), and IL-10 expression by both TLRs and DC-SIGN binding to gp120 (Shan et al., 2007; Saraiva & O'Garra, 2010). As HIV infection is associated with inflammatory responses, we wanted to understand how IL-10, an immunesuppresor might influence this relationship. We hypothesised that instead of inflammation triggering IL-10 responses, HIV-1 binding to DC-SIGN induced IL-10 production and autocrine IL-10 deregulated DC anti-HIV activity locally during early HIV infection.

Due to the relationship between Env mannosylation and MCLR DC-SIGN mediated stimulation of IL-10 in MDDCs (Shan et al., 2007), and the specific N-glycan features associated with TF Env (Chohan et al. 2005; Derdeyn et al. 2004; Sagar et al. 2006; Go et al 20011), we hypothesised that either the number of PNGs, type of N-glycans and/or specific PNGs might influence the modulation of DC function.

4.2 Aim and Objectives

Aim: Understand how HIV-1 Subtype C Envelope N-glycosylation impacts dendritic cell immune response to favour viral transmission.

Rationale:

Dendritic cells are important players in tailoring the immune response to specific pathogens via the secretion of pro-inflammatory and immune-suppressive cytokines. DCs are one of the first cells to encounter HIV within the genital mucosa and thus should play an important role in preventing productive HIV-1 infection. However, HIV seems to be able to circumvent DC-mediated viral clearance potentially by preventing DC maturation and immune responses. Env N-glycans stimulate MDDC release of IL-10 which then in turn regulates MDDC function as well as the course of pathogen clearance. Thus, changes in IL-10 levels could play an important role in persistent HIV infection. As the number of PNGs has been found to change over the course of infection and mannose type N-glycans are involved in the interaction between DC-SIGN and gp120, N-glycosylation might facilitate HIV transmission by modulating immune responses in the genital tract. Therefore, this study hypothesises that Env N-glycosylation is involved in stimulating the differential secretion of cytokines by DCs.

4.2.1 Specific objectives:

1. Determine whether differentially N-glycosylated Envs differ in their ability to induce IL-10 secretion by MDDCs from healthy blood donors.
2. Determine if Env PNGs at specific positions impact the induction of IL-10 secretion by MDDCs.
3. Determine whether differentially N-glycosylated Envs influence MDDC secretion of immune modulators other than IL-10.

4.3 Results

4.3.1 Pseudovirus stimulation of MDDCs to secrete IL-10

Chapter 3 showed that entry efficiency, DC-SIGN binding and *trans*-infection of CD4+ cells varied across Env clones, suggesting a wide range in phenotypic properties. We therefore determined whether the clones also varied in their ability to stimulate MDDC secretion of cytokines which could suggest that Envs influence immune responses in the genital tract. Nine HIV-1 subtype C Envs (C1, C2, C7, C8, C12-C16) (Figure 3.2.1 & Table 3.2.1) were selected that varied in PNG number and location to determine whether HIV-1 subtype C Env N-glycosylation influenced MDDC secretion of cytokines following stimulation with pseudovirus (PSVs) and purified soluble Env (gp140).

When PSVs were used to stimulate MDDCs, IL-10 levels in the culture supernatants varied from 15-725 pg/ml (Figure 4.3.1) for 7 healthy donors. The majority of PSVs (C1, C2, C8, C13, C15 and C16) induced low levels of IL-10 secretion, 1.2- to 2-fold above the negative control. Only C7 and C12 induced significantly higher MDDC IL-10 secretion (9.5- and 9.1-fold, respectively) than the negative control ($p = 0.04$ and $p = 0.013$, respectively). C14 induced 6-fold higher IL-10 although this response did not reach significance ($p = 0.08$).

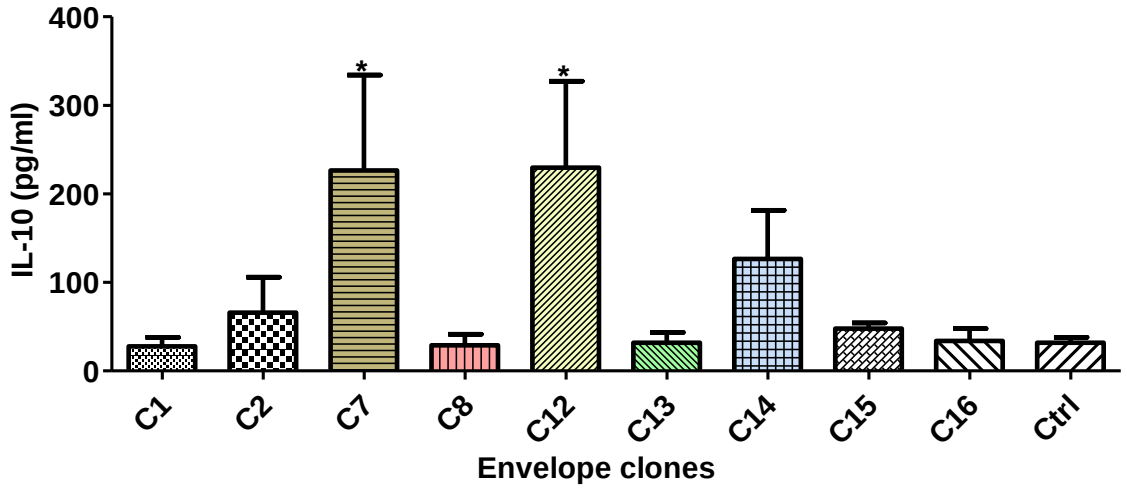


Figure 4.3.1 Pseudovirion stimulation of MDDC IL-10 release. PSVs from nine participants with varying PNG patterns were used to stimulate MDDCs from seven healthy blood donors and the release of IL-10 was measured by Luminex assay. PSVs generated using pSG3ΔEnv and empty vector was used as a negative control (Ctrl). Mann-Whitney test was used to compare IL-10 levels in the supernatants to the negative control. Bar graphs represent mean IL-10 (pg/ml) from 7 biological repeats with error bars showing +/-SEM (SEM). *, ** and *** represent p values less than 0.05, 0.01 and 0.001, respectively. MDDCs from the same donor were used to compare the induction of cytokine production between clones per biological repeat. However, significance was not upheld after adjusting for multiple comparisons.

4.3.2 Induction of MDDC IL-10 secretion by gp140

To confirm that Env was responsible for the variation in IL-10 secreted by MDDCs, gp140 was generated for three *env* clones (C12, C13 and C14) that differed at N448, N397 and N674 (Table 3.3.1), induced variable levels of IL-10 release (Figure 4.3.1) and had different binding efficiencies to DC-SIGN (Figure 3.3.5). When purified gp140 was used to stimulate MDDCs, all three clones differentially induced the release of IL-10 similar to PSVs. However, the hierarchy of clones was altered with C13 (6-fold) stimulating the release of more IL-10 than C14 (2.6-fold) albeit non-significantly (Figure 4.3.2). C12 and C13 induced significantly higher IL-10 levels than background with p values of 0.0001 and 0.047, respectively, while C14 did not have a statistically significant effect ($p = 0.117$). The difference in IL-10 levels induced by PSV and gp140 for C13 and C14 could be due to MDDC donor differences as secretion of IL-10 varied between donors (Figure 4.3.1). It is also possible that differences in cell line-specific N-glycosylation might have influenced whether PSVs and gp140 were similarly N-glycosylated. It has been shown that CHO and HEK293T cells N-glycosylate Env differently and altered Env N-glycan patterns of PSVs and gp140 could have impacted the stimulation of MDDC IL-10 release (Raska et al., 2010). Finally, gp140 expresion and purification resulted in monomers and dimers (data not shonw) and migh thus not represent natirve trimers of PSVs. Despite the variation between gp140 and PSVs, the release of IL-10 in response to purified gp140 confirmed that Env is likely partly responsible for inducing IL-10 secretion in MDDCs.

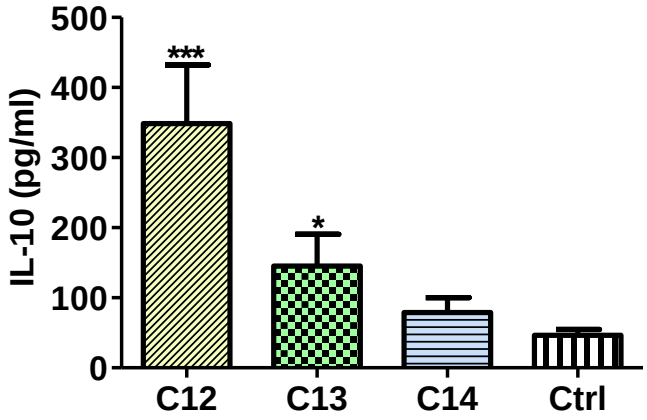


Figure 4.3.2 Purified soluble gp140 stimulation of MDDC IL-10 release. Env (gp140) was generated by the introduction of a stop codon before the transmembrane domain of three *env* clones (C12, C13 and C14). The mutants were transiently expressed in CHO cells and purified by affinity chromatography and size exclusion chromatography. Day six MDDCs were stimulated with purified C12-C14 gp140 and IL-10 released into the culture supernatant was measured by Luminex assay. Means of at least 5 biological repeats using different MDDC donors are shown with SEM. Mann-Whitney t-test was used to compare IL-10 levels in sgp140 stimulated samples to the negative control (no sgp140 added). P values were represented as *, ** and *** for values less than 0.05, 0.01 and 0.001, respectively.

4.3.3 Evaluation of DC-SIGN involvement in inducing MDDC IL-10 secretion

DCs express a range of PRR receptors on their surfaces including TLRs, CLRs and non-CLRs which have all been suggested to play a role in pathogen interaction with DCs. Although, CLRs and other non CLR receptors like Garcer and CD169 have been suggested to be involved in the transfer of HIV by MDDCs to T cells (Meng et al., 2002; Turville et al., 2002; Magerus-Chatinet et al., 2007; Lambert et al., 2008; Izquierdo-Useros, Lorizate, Puertas, et al., 2012), DC-SIGN has been suggested to be the main candidate (Geijtenbeek, Kwon, et al., 2000; Geijtenbeek, Torensma, et al., 2000; Geijtenbeek et al., 2002; Kwon et al., 2002). We therefore investigated whether IL-10 release was inhibited when PSVs were pre-incubated with recombinant DC-SIGN before stimulating MDDCs. The presence of recombinant DC-SIGN reduced IL-10 release by 65.25% for C12 when compared to the control without DC-SIGN pre-incubation ($p = 0.017$) (Figure 4.3.3), suggesting that DC-SIGN-Env interactions are important for MDDC IL-10 secretion. However, the inhibition was not as apparent for C13 as MDDC IL-10 secretion decreased by only 25.43%. C13 PSV induced MDDCs to release only 1.2-fold higher IL-10 compared to the background control. It is thus possible that as C13 PSV was not efficient at inducing MDDC to secrete IL-10, inhibition was difficult to detect.

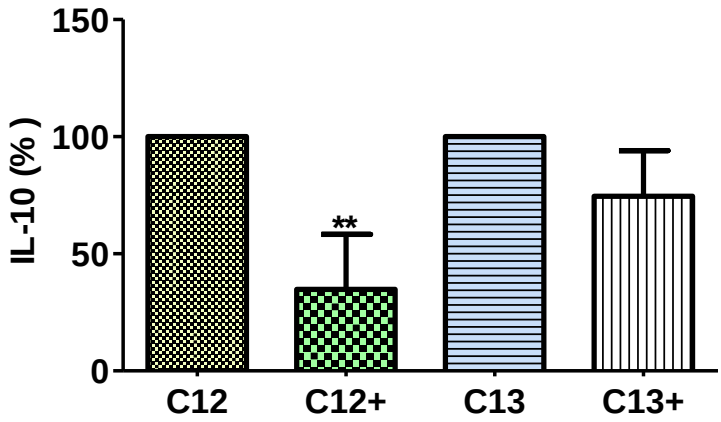
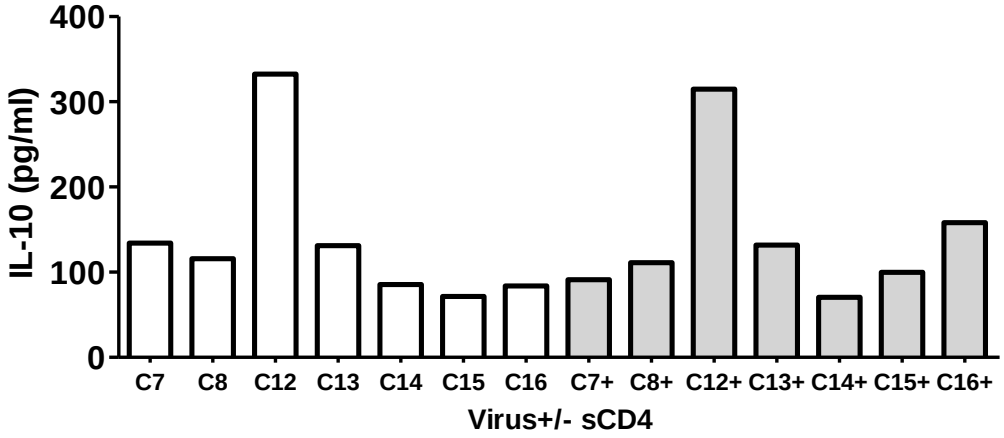


Figure 4.3.3 Pseudovirion stimulation of MDDC IL-10 release in the presence of recombinant DC-SIGN. Two PSV clones (C12 and C13) were pre-incubated with and without recombinant DC-SIGN to block DC-SIGN binding sites on Env prior to stimulation of MDDCs to release IL-10. The induction of IL-10 in the absence (denoted C12 & C13) and presence (C12+ and C13+) of recombinant DC-SIGN protein was measured in the culture supernatants by Luminex assay. Results shown are 3 biological repeats from three different donors expressed as a percentage of uninhibited PSVs and compared to the uninhibited control using one way ANOVA. *, ** and *** represent p values less than 0.05, 0.01 and 0.001, respectively.

4.3.4 Determine whether CD4 receptor is involved in induction of MDDC IL-10 secretion

As recombinant DC-SIGN did not completely inhibit IL-10 release and MDDCs express CD4, we blocked CD4-gp120 interactions by either pre-incubation of PSVs with recombinant soluble CD4 (sCD4) to block CD4 binding sites of Env or pre-incubation of MDDCs with Leu3A, an anti-CD4 monoclonal antibody to block CD4 receptors. sCD4 and Leu3a did not lower IL-10 levels, suggesting that Env-CD4 interactions are not involved in inducing MDDC IL-10 release (Figures 4.3.4 A and B). Although, only two donors were tested, one for sCD4 and the other for Leu3A inhibition, the analysis confirmed findings by previous groups and it is thus highly unlikely that the induction of MDDC IL-10 release is via CD4 (Ji et al., 2005; Shan et al., 2007).

A



B

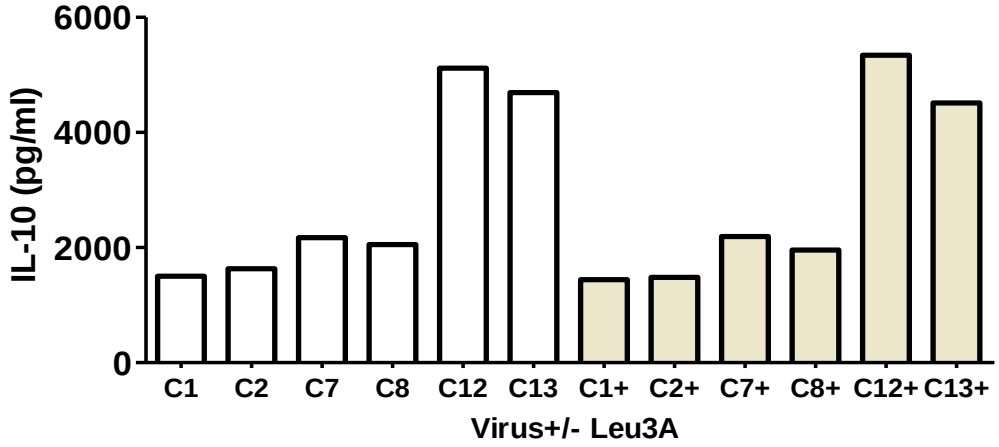


Figure 4.3.4 Stimulation of MDDC IL-10 release in the presence of CD4 receptor binding inhibitors. Env-CD4 interactions were blocked by pre-incubating A) PSV with sCD4 (+) and B) MDDCs with Leu3A (+) before stimulation of MDDC with C1, C2, C7, C8, C12 and C13 PSVs. IL-10 secretion was measured by Luminex assay and compared to PSVs not pre-incubated with inhibitors. A single donor was used for each inhibitor.

4.3.5 Importance of Env N-glycosylation in MDDC IL-10 release

4.3.5.1 Effect of Env PNG deletions on MDDC IL-10 release

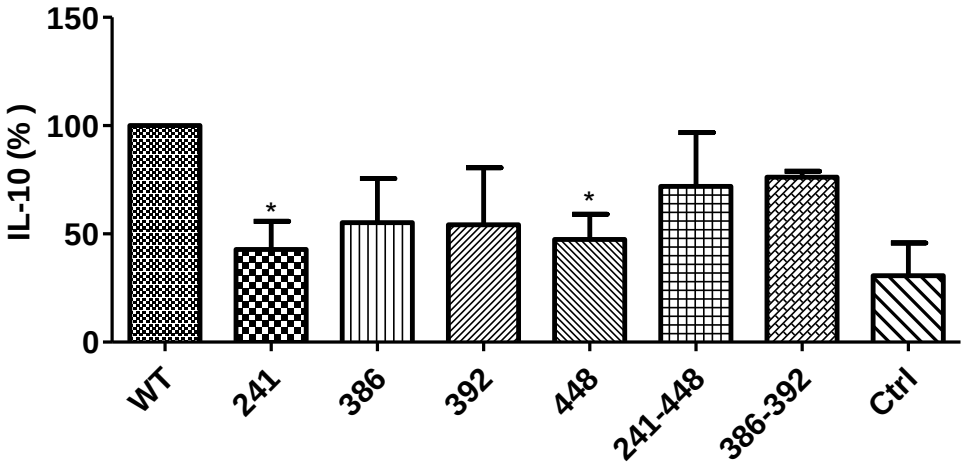
Next we determined whether the variation in IL-10 release in response to different Env clones was due to differences in Env PNGs. There was no significant relationship between MDDC IL-10 secretion in response to PSVs and PNG number or level of gp140 mannosylation (Data not shown). PNGs at N241, N262, N386, N392 and N448, previously identified to either

interact with DC-SIGN or shown to carry high mannose sugars (Hong et al., 2007; Go, Hewawasam, et al., 2011; Liao et al., 2011) were deleted in C15 and C16. These two clones carried all five PNGs but C15 had higher mannosylation than C16 (47% compared to 33%). These clones also differed in entry efficiency but they bound DC-SIGN *trans*-infected TZM-bl cells (Figures 3.2.5 3.2.7 and 3.2.9) and induced IL-10 release with similar efficiency (Figure 4.3.1).

C15 WT induced MDDC IL-10 secretion 1.5-fold higher than background in these donors and deletion of PNGs at N241 and N448 led to a statistically significant decrease ($p = 0.045$ and $p = 0.047$, respectively). Deletion of N386, N392, N241-448 and N386-392 also led to decreased IL-10 secretion relative to the WT but did not reach significance although this could be due to MDDC donor variation.

Similar to what was apparent in Chapter 3, deletion of the PNGs did not affect the ability of C16 to induce MDDC IL-10 secretion to the same extent as observed for C15 (Figure 4.3.5). IL-10 induction by the C16 mutants either remained the same as WT (N241 and N262) or increased non-significantly (N448, N386, N241-262, N241-448, N262-448, N386-392). However, the negative control induced nearly as much MDDC IL-10 secretion as WT, suggesting non-specific stimulation might have influenced the outcome of the experiment. As deletion of all the PNGs significantly reduced the ability of C15 to infect TZM-bl cells, these sites are likely to be essential for Env structure and function. This is corroborated by the high population frequency (Table 3.3.1) observed for these PNGs in subtype C sequences. Therefore, we were unable to conclude whether the presence of PNGs at N241, N262, N448, N386 and N392 are important determinants of MDDC IL-10 secretion as deletion seemed to disrupt the overall structural integrity of Env. Interestingly, there was no statistically significant correlation between IL-10 levels and PSV entry efficiency, DC-SIGN binding or *trans*-infection and despite the inability of C15 N386/N392 and C16 N241/N262 to enter TZM-bl cells (Figure 3.3.11); they were able to induce MDDCs to release IL-10. These findings suggest that activation of the signalling pathway leading to IL-10 release was not dependent on Env function.

A



B

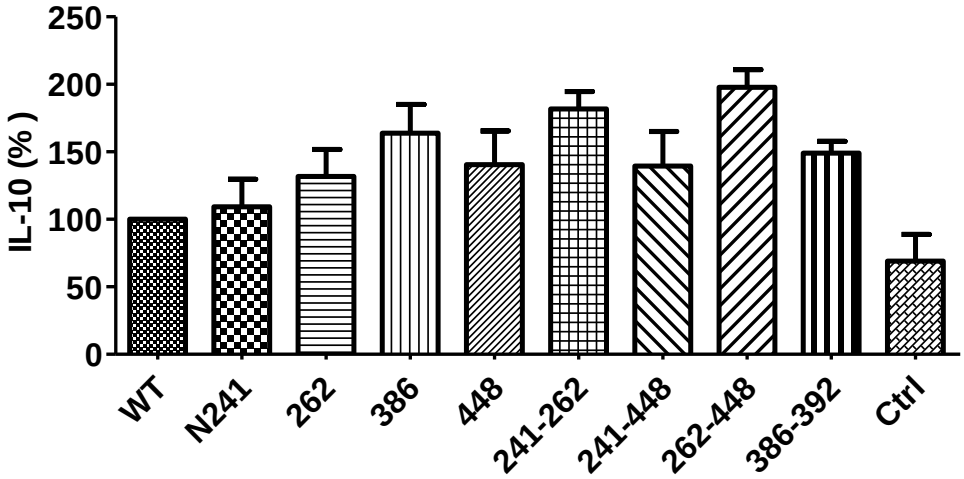


Figure 4.3.5 Impact of deletion of Env Potential N-Glycan sites (PNGs) on MDDC IL-10 release. Env PNGs at N241, N262, N448, N386 and N392 were deleted by site-directed mutagenesis of A) C15 and B) C16. PSVs of wild-type and mutants were compared for stimulation of MDDC IL-10 secretion to the negative control (Ctrl). PSVs generated using pSG3ΔEnv and empty vector was used as a negative control. The graph shows the mean of 3 biological repeats using 3 different donors plotted as percentage of WT (Error bars represent SEM). Means were compared using paired t test. *, ** and *** represent p values less than 0.05, 0.01 and 0.001, respectively.

4.3.5.2. Identification of Env PNGs that influence MDDC IL-10 release

The majority of sites deleted in C15 and C16 were highly conserved and thus their deletion led to reduced entry efficiency, suggesting that these sites were important for overall Env structure and function. In addition, Pritchard *et al*. (2015) showed that PNG deletions led to disruption

of bystander PNG processing (Pritchard, Spencer, et al., 2015). The secretion of IL-10 varied greatly across MDDC donors, with some secreting very low levels of cytokine irrespective of Env clone. Therefore, to identify Env PNGs associated with the ability to induce IL-10 secretion irrespective of donor, we compared PSVs that differed in the presence and absence of PNGs for all donors tested. Mann-Whitney test showed that the presence of PNGs at positions 130, 332 and the lack of a PNG at 674 had a significant impact on the induction of IL-10 release by MDDCs (Figure 4.3.6). When median or mean IL-10 levels were compared, statistical significance was lost most likely due to extreme variability between donors and small sample size (data not shown). However, even with these limitations, the trend was mainatained indicating that N130, N332 and N674 could be important in stimulating MDDCs to release IL-10.The frequency of the PNGs at position 130, 332 and 674 were 67%, 56% and 33%, respectively in our sequences and 55%, 77% and 25%, respectively in the subtype C population, suggesting positive selection at N130 and N332 and negative selection at N674.

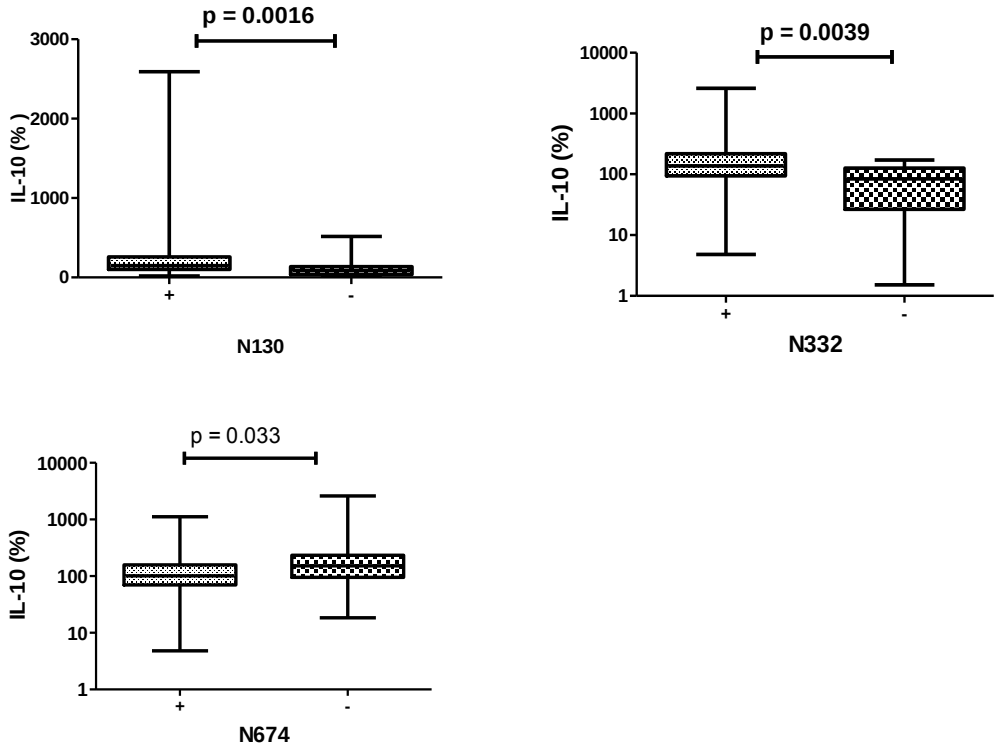


Figure 4.3.6 Identification of PNGs associated with IL-10 release irrespective of donor MDDC. Analysis of eighteen Env sequences identified PNGs that varied by 33 % - 67 % between sequences. Variable domains were excluded from the analysis. PSVs were grouped on whether the Env clones carried a specific PNG or not and the level of IL-10 secreted by each of seven MDDC donors was compared between the two groups using a Mann-Whitney test. In order to directly compare IL-10 levels between experiments with different donors, IL-10 levels were calculated as a percentage of background i.e. when no PSV was added. Significance was not upheld after adjusting for multiple comparisons.

Either the presence of a single PNG (or lack in the case of N674) or combination of N130+, N332+ and N674- N-glycans could be required to interact with DCs via DC-SIGN, triggering the release of IL-10. We hypothesized that if the potential IL-10 induction motif (N130+, N332+, and N674-) was essential for optimal MDDC stimulation then its presence would be significantly associated with increased MDDC IL-10 release irrespective of donor. MDDC IL-10 release was compared between PSVs that carried either 0, 1, 2 or 3 PNGs comprising the induction motif. PSVs that carried either 1 or all of the PNGs significantly induced the release of higher levels of IL-10 ($p = 0.0335$ and 0.030 respectively) compared to when the motif was absent or only 2/3 PNGs were present (Figure 4.3.7 A). This suggests that the presence of one of the PNGs might be sufficient to stimulate the release of IL-10 in most donor MDDCs.

The presence of N674 was significantly associated with reduced entry efficiency, disruption of DC-SIGN binding and a drop in *trans*-infection. This could suggest that an N-glycan at this position disrupted the structure and function of Env similar to the deletion of conserved PNGs in the previous experiments. It was previously shown that the introduction of a PNG at N674 facilitated escape from carbohydrate binding agents with concomitant reduced viral fitness (Mathys & Balzarini, 2014). Furthermore, as N674 is likely under negative selection with a frequency of only 25% in subtype C viruses we repeated the analysis excluding N674 (Figure 4.3.7 B). PSVs with PNGs at both N130 and N332 stimulated significantly higher IL-10 release than those that had either none or one of these sites ($p = 0.0053$). As a PNG at 674 could be influencing Env structure and function, site-directed mutagenesis of this site needs to be carried out to confirm these findings.

A B

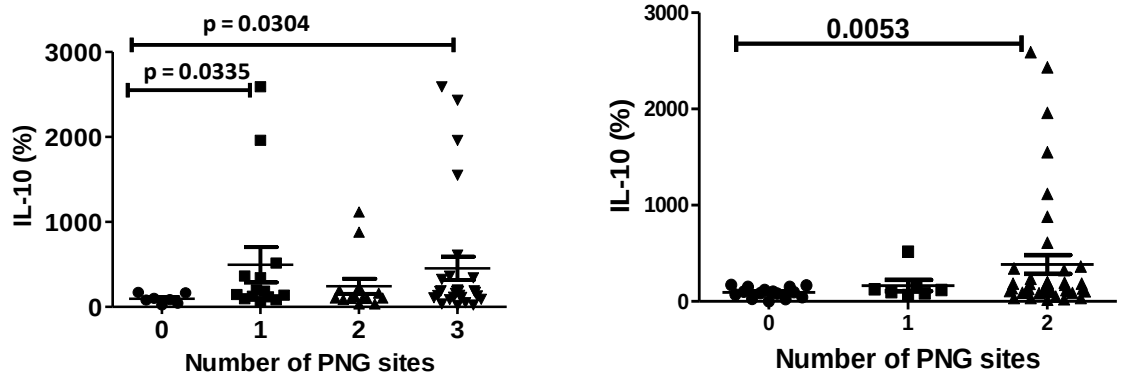
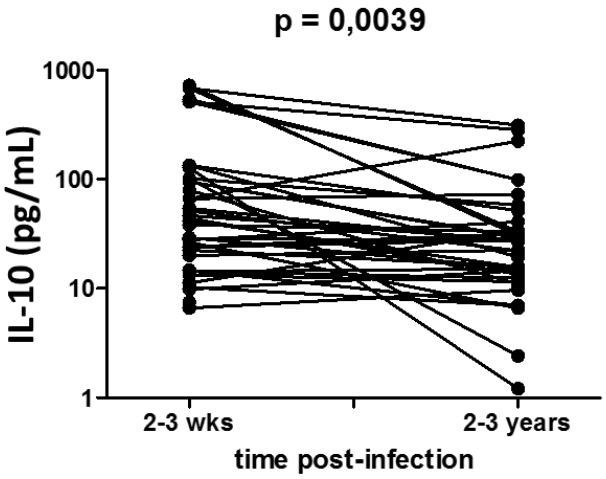


Figure 4.3.7 Evaluation of the relationship between PSV carrying specific PNGs (+N130, +N332 and –N674) and MDDC IL-10 secretion. Nine PSVs were grouped on whether Env had A) 0 (–N130, -N332, +N674), 1 (e.g. –N130, -N332, -N674), 2 (e.g. –N130, +N332, -N674) or 3 (+N130, +N332, -N674) and B) 0 (-N130, -N332), 1 (e.g.+N130, -N332) , or 2 (+N130, +N332) PNGs making up the potential IL-10 induction motif: PNGs at positions 130 and 332 and lack of a PNG at position 674. IL-10 values obtained from the stimulation of seven MDDC donors with each PSV were normalised to background and groups were compared using a Mann Whitney t-test with two tailed comparison. Significance was not upheld after adjusting for multiple comparisons.

4.3.6. Comparison of MDDC IL-10 induction between transmitted founder and chronic infection PSVs.

Previous studies of HIV transmitted variants indicated that subtypes C and A TF viruses tended to carry fewer PNGs with shorter variable loops when compared to those from chronic stages of infection. Viruses identified years post-infection tended to have increased number of PNGs with insertions and deletions contributing to shifting positions that facilitated escape from immune pressure (Derdeyn et al., 2004; Chohan et al., 2005; Sagar et al., 2006). These differences between viruses from acute and chronic stages of infection suggested that Env N-glycosylation might play an important role in transmission. We grouped PSVs based on time of sampling (2-3 wks and 2-3 years post-infection) and compared MDDC IL-10 secretion.

A



B

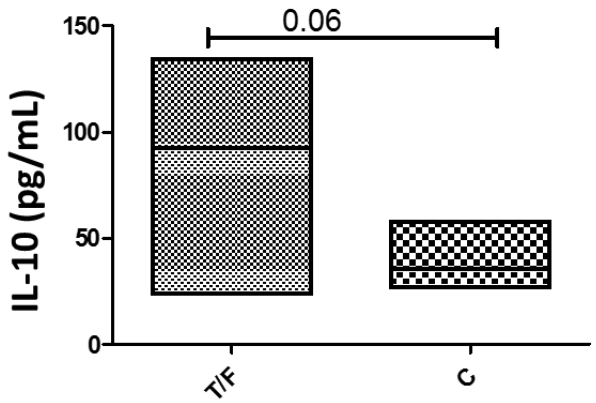


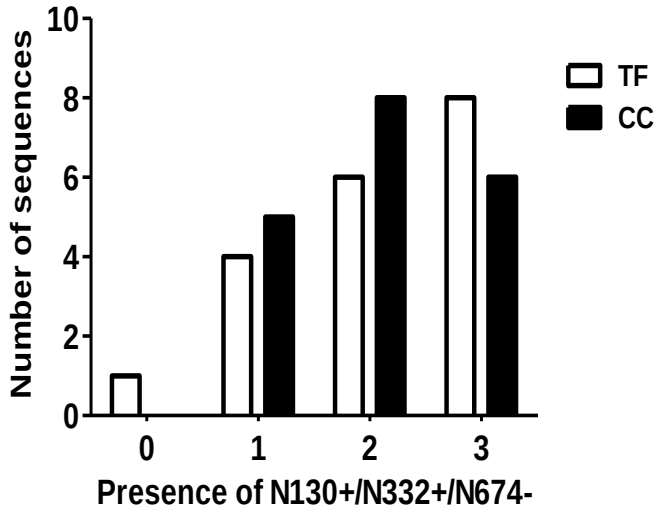
Figure 4.3.8 Comparison of multiple donor MDDC IL-10 secretion in response to stimulation by PSVs from acute and chronic stages of infection. PSVs were grouped on whether they were sampled 2-3 wks or 80-173 wks (2-3 years) post-infection. PSVs were used to stimulate up to seven donor MDDCs and IL-10 secretion was compared between matched acute and chronic infection pairs using a Wilcoxon matched pairs test, using A) all donors for each PSV and B) medians of IL-10 all donors stimulated with a single PSV. A) Each line connecting two points represents the data from one MDDC donor stimulated by one matched PSV pair, while for B), each bar represent median values of IL-10 released from all donors stimulated with either TF or chronic infection variants.

PSVs from acute infection stimulated significantly higher MDDC IL-10 secretion ($p = 0.0039$) than those from chronic infection irrespective of MDDC donor (Figure 4.3.8).

As N130+, N332+ and 674- were identified as associated with increased MDDC IL-10 release; we determined whether the potential IL-10 induction motif was enriched in subtype C acute

infection Envs. We hypothesised that acute infection variants, if enriched with the induction motif might stimulate the release of higher MDDC IL-10 than those from chronic infection thus dampening the immune response and facilitating HIV transmission. Comparison suggested that the putative IL-10 induction motif was marginally more frequent in sequences from acute infection than chronic infection (Figure 4.3.9). However, when N674 was excluded from the analysis, the frequency of both N130 and N332 in acute infection PSVs was only higher by one participant and acute infection variants tended to not carry either N130 or N332 compared to chronic infection viruses. It is thus possible that N130 and N332 are important for inducing MDDCs to release IL-10 but that these are not enriched in TFs. However, the sample size is too small to make this conclusion and further analysis is required.

A)



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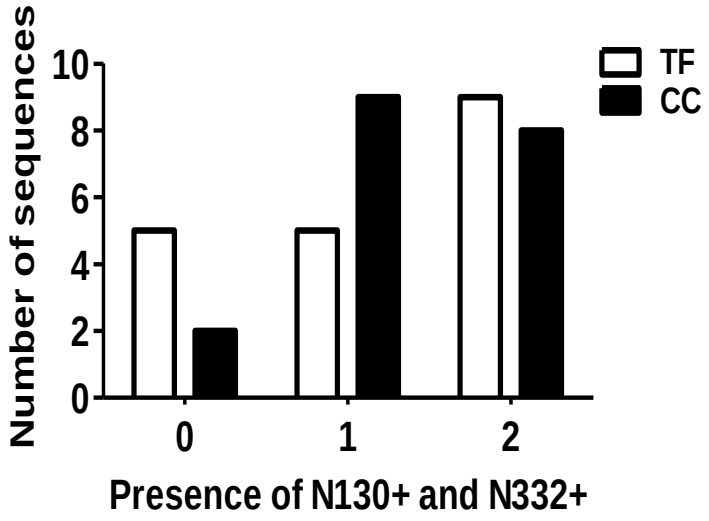


Figure 4.3.9 Comparison of the frequency of PNGs in sequences from acute and matched chronic infection controls. The frequency of the potential induction motif: A) +N130, +N332 and -N674 and B) +N130 and +N332 was compared between 18 sequences from acute infection (TF) and 18 matched sequences representing the consensus virus circulating during chronic infection (CC).

4.3.8 Influence of PSV stimulation on MDDC secretion of TNF-α, IL-6, IL-1β, IL-12, IL-8, MIP-1α and MIP-1β.

HIV-1 infection does not only deregulate IL-10 levels, but also other pro-inflammatory and anti-inflammatory cytokines (Breen et al., 1990; Chehimi et al., 1994; Daftarian et al., 1995; Taoufik et al., 1997; Marshall et al., 1999; Narimatsu, Wolday & Patterson, 2005; Roberts et al., 2010; Shah et al., 2011; Borges et al., 2014, 2015). IL-10 was initially said to inhibit the production of pro-inflammatory cytokines like TNF-α, IFN-γ, IL-1β, IL-6, IL-2 and IL-12 (Clerici & Shearer, 1993; Clerici et al., 1993; Borish & Steinke, 2003; Steinke & Borish, 2006; Villinger & Ansari, 2010). This was supported when Chehimi *et al*. (1994), Trinchieri *et al*. (2003) and Marshall *et al.* (1999) found that HIV infection was associated with increased lymphocytic IL-10 production while IL-12 expression was impaired (Chehimi et al., 1994; Marshall et al., 1999; Choi, Fallert, et al., 2003). However, whole blood from HIV-positive individuals showed deregulated IL-12 expression independent of IL-10 levels, suggesting that under certain conditions other cytokines might be influenced directly by HIV (Meyaard et al., 1997). Bebell *et al.* (2008) found that acutely HIV infected women had significantly higher IL-6, IL-10 and lL-12 levels in CVLs and higher plasma IL-1β, IL-8 and IL-10 compared to HIV negative controls, supporting the suggestion that HIV infection leads to a "cytokine storm"

(Bebell et al., 2008). Roberts *et al.* (2010) also confirmed this finding when they showed that IL-1α, IL-1β, IL-6, TNF-α, IL-8, Fractalkine, IP-10 and IL-10 were significantly elevated in plasma of HIV-1 acutely infected women (Roberts et al., 2010, 2012). To determine whether exposure to HIV-1 influenced the release of cytokines other than IL-10, we measured the release of TNF-α, IL-1β, IL-6, IL-8, IL-12, MIP-1α, and MIP-1β, by MDDCs in response to PSVs.

4.3.8.1 Impact of PSV stimulation on TNF-α secretion by MDDCs

Levels of TNF-α, a pro-inflammatory cytokine which stimulates the production of free radicals has been reported to increase during HIV infection, correlating with increased viral load set point and hence disease progression (Bahia & Silakari, 2010; Vaidya et al., 2014). TNF-α has also been found to enhance HIV infection in women with bacterial vaginosis (BV) (Sturm-Ramirez et al., 2000; De Jong et al., 2008) while high plasma levels were associated with increased risk of HIV acquisition (Roberts et al., 2012). TNF-α induced the production of other pro-inflammatory cytokines like IL-6 and IL-8 and also stimulated the production of IL-10 by monocytes (Platzer et al., 1995). While some studies have suggested TNFα inhibits HIV entry into tissue culture differentiated macrophages (Herbein, Montaner & Gordon, 1996; Lane et al., 1999), other studies found that it cooperated with IL-10 to enhance HIV replication (Poli et al., 1990; Finnegan et al., 1996). When MDDCs were stimulated with PSVs the differential release of TNF-α was similar to that of IL-10 with C7, C12 and C14 inducing significantly higher TNF-α secretion than the other clones when compared to the background control ($p = 0.0022$, $p = 0.0079$ and $p = 0.0087$, 6-, 7- and 3-fold, respectively) (Figure 4.3.10). However, C15 that did not stimulate MDDCs to release high levels of IL-10 induced significantly more TNF-α than the control. As C7, C12 and C14 both induced high levels of IL-10 and TNF-α, it is possible that the release of one cytokine induced the secretion of the other as suggested previously. However, IL-10 has been reported to repress TNF-α expression in activated macrophages (Zhang & An, 2007) by reduing myeloid differentiation primary response protein 88 (MyD88) expression and thus preventing NFκB activation in an LPS ch+allenged macrophage cell line (Dagvadorj et al., 2008). Furthermore, exogenous IL-10 inhibited TNF-α, IL-6 and IL-8 secretion by mouse macrophages and human epithelial cells exposed to Chlamydia (Yilma et al., 2011). Thus, TNF-α, like all pro-inflammtory cytokines initially induces IL-10 expression, but as part of its immune-suppressive function, IL-10 then inhibits TNF-α production to prevent immune-pathology (Couper, Blount & Riley, 2008). Our data

suggests that either HIV has deregulated the ability of IL-10 to inhibit TNF-α, TNF-α is enhancing the expression of IL-10 or HIV-1 is increasing the expression of both cytokines directly. These findings require further investigation on a larger sample size.

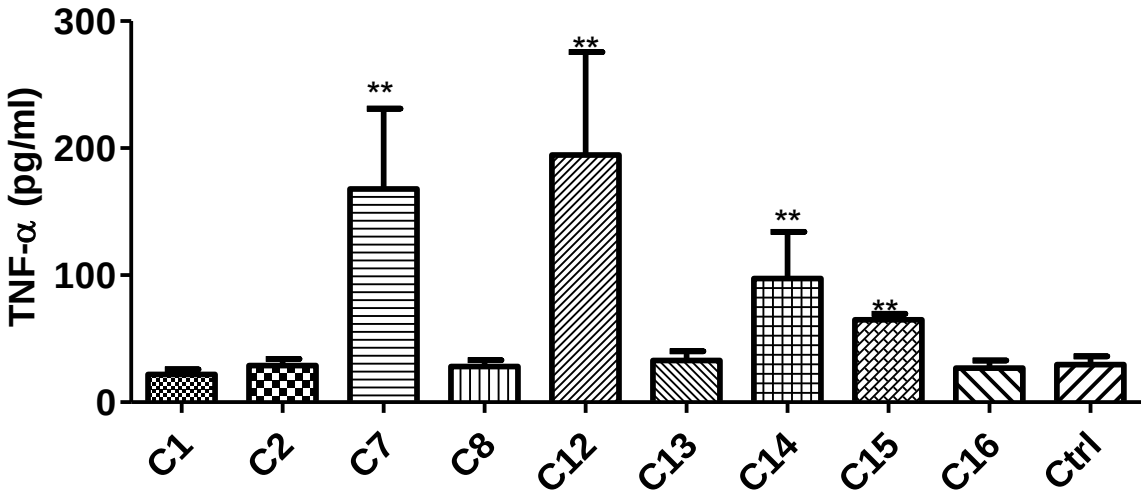


Figure 4.3.10 Impact of PSV stimulation on MDDC secretion of TNF-α. Day six MDDCs from six different donors were stimulated with PSV in culture and the release of TNF-α in the culture supernatant was measured by Luminex assay. Results plotted are means of TNF-α (pg/ml) of six biological repeats from six different donors and the error bars represent SEM. Mann-Whitney test was used to compare TNF-α induction between PSV clones and between PSV and background. PSVs generated using pSG3ΔEnv and empty vector was used the negative control (Ctrl). *, ** and *** represent p values less than 0.05, 0.01 and 0.001, respectively.

4.3.8.2 Impact of PSV stimulation on IL-6 secretion by MDDCs

IL-6 is a pleotropic cytokine that has been described as a double edged cytokine (Zhang & An, 2007). IL-6 is a special warning signal for the presence of pathogen. It has both pro- and anti-inflammatory properties with several lines of evidence to suggest that it plays a vital role during the transition from innate to acquired immunity (Tanaka, Narazaki & Kishimoto, 2014). IL-6 together with TGF-β participates in Th17 cell differentiation and these two cytokines are negatively regulated by IFN-γ, IL-2 and IL-27 (Ludewig et al., 2001). Besides its crucial role in B and T cell differentiation, IL-6 is also known to promote CD4+ T cell helper capacities through increased IL-21 production (Reviewed in (Deeks, Tracy & Douek, 2013), hence skewing Th0 differentiation towards Th2 and Th17 while inhibiting TGF-β driven differentiation of naïve CD4+ T-cells (Th0) towards regulatory T-cells. IL-6 serum levels were shown to be high in patients with high plasma HIV viremia and low CD4 counts (Breen et al.,

1990; Borges et al., 2014, 2015). There has been some contradiction to the role of IL-6 in HIV infection: while Poli *et al*. (1990) found that it synergizes with TNF-α to induce HIV replication, Rogez-Kreuz *et al.*(2005) showed that it enhanced the anti- HIV activity of IFN-τ in human macrophages (Poli et al., 1990; Rogez-Kreuz et al., 2005; Shah et al., 2011). In this study, C7, C12 and C14 PSVs stimulated MDDCs to release 8-, 9- and 4-fold increases in IL-6 secretion relative to background (Figure 4.3.11) (p = 0.03, p = 0.031 and p = 0.05, respectively). This was a similar trend to that of IL-10 and TNF-α secretion (Figures 4.3.1 and 4.3.10). The concomitant increase in IL-6, TNF-α and IL-10 in response to specific PSVs may induce chemokine expression which would in turn recruit target cells to the female genital tract, directly activate target cells and activate HIV replication via NF-κB (Liebenberg et al., 2016).

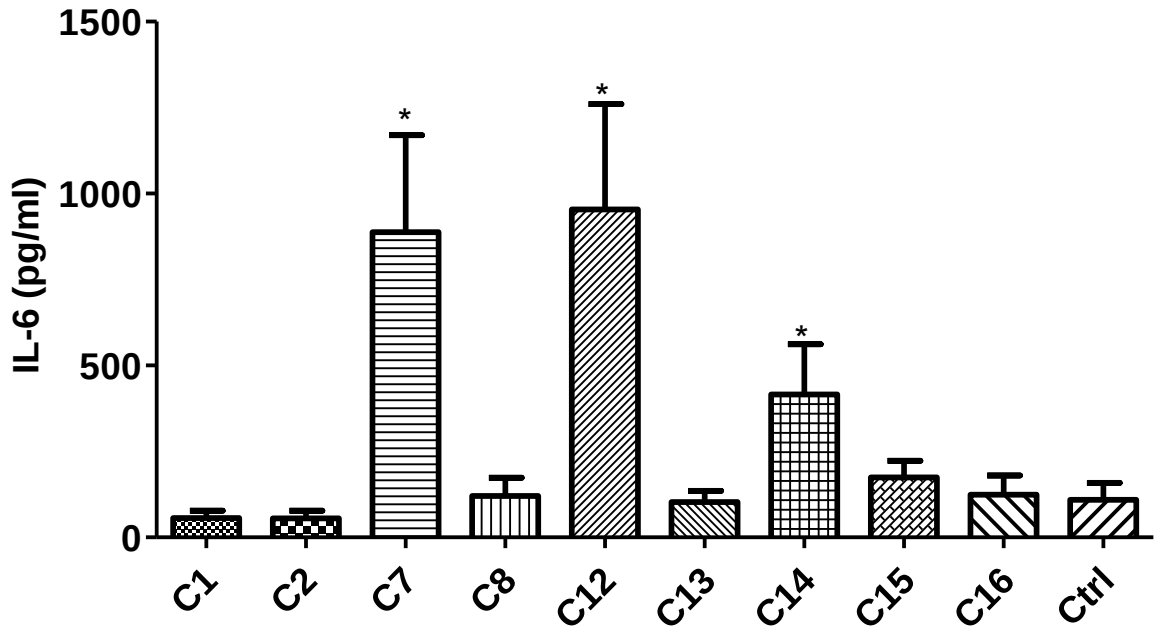


Figure 4.3.11 Impact of PSV stimulation on MDDC secretion of IL-6. Day six MDDCs were stimulated with PSVs for 24 hours in culture and IL-6 levels were measured in the culture supernatants by Luminex assay. Bar graph shows mean IL-6 concentration (pg/ml) from five independent donor MDDCs stimulated with different PSV preparations. Error bars indicate +/-SEM. The control (Ctrl) is the HIV pseudoviral backbone without Env (pSG3). Mann-Whitney test was used to compare IL-6 inductionbetween PSVs and control. * indicates p value less than 0.05.

4.3.8.3 Impact of PSV stimulation on IL-1β release by MDDCs

IL-1β is an important mediator of inflammation in many diseases. It has been shown that IL-1β synergises with IL-6 and IL-8 to upregulate the risk of HIV acquisition (Mlisana et al., 2012; Masson et al., 2015), athough these values were not significant in the Masson *et al.* study

(Masson et al., 2015). Levels of IL-1β together with IL-1α and IL-8 were high in BV which is associatd with increased risk of HIV infection (Sturm-Ramirez et al., 2000; Liebenberg et al., 2016). In a cross sectional study, higher plasma IL-1β was associated with lower CD4+ T cell levels in acutely HIV infected women (Bebell et al., 2008). It has been reported that binding of IL-1β to its receptors activates NF-kB that induces secretion of IL-6, TNF-α and IL-8 in mice models (Nambu and Nakae, 2010). IL-1β and other cytokine levels including TNF-α and IL-6 were elevated in the plasma of HIV infected subjects during early infection and in the supernatants of cells infected with HIV (Roberts et al., 2010). It was also suggested that infection of or exposure of macrophages to HIV induces secretion of these three cytokines, a trio which in turn increases HIV replication in these cells (reviewed in (Breen, 2002)), possibly by activating the NF-κB which in turn binds to the HIV promoter to directly enhance HIV replication.

High variation between MDDC donors and low IL-1β levels made it difficult to identify a significant increase in IL-1β in response to PSV (Figure 4.3.12). Apart from C8 and C13, stimulation by all PSVs lead to at least 2-fold increase in MDDC secretion of IL-1β compared to the negative control (Figure 4.3.12) although the difference did not reach statistical significance. The increase in IL-1β did not follow the pattern observed for IL-10, TNF-α and IL-6 and thus regulation of MDDC IL-1β release in the presence of HIV might differ to that of the other cytokines. However, the low levels of IL-1β released in response to PSV could be sufficient to enhance infection of target cells as suggested previously (Poli et al., 1990; Breen, 2002).

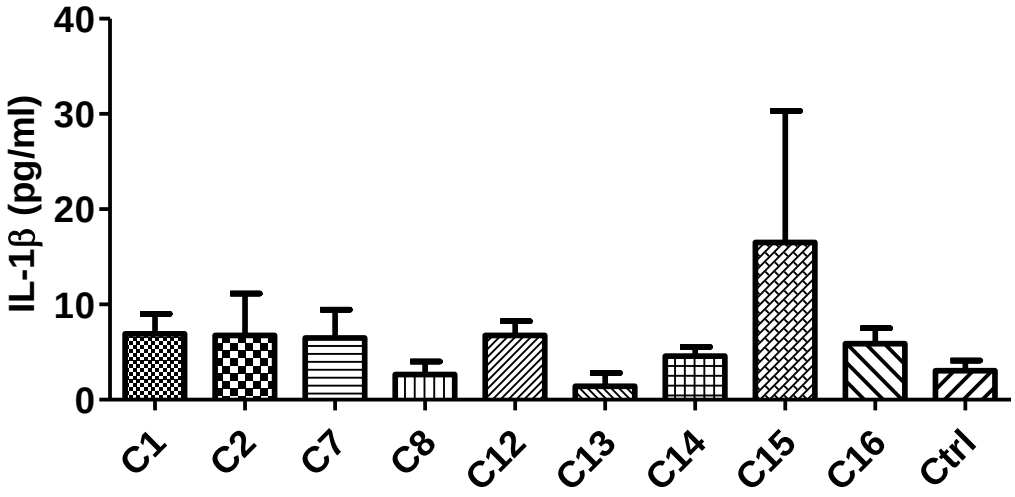


Figure 4.3.12 Impact of PSV stimulation on MDDC secretion of IL-1β. Day six MDDCs were stimulated in culture with PSVs for 24 hours after washing to remove differentiation medium and the secretion of IL-1β was measured in the culture supernatants by Luminex assay. Graph presents the mean IL-1β (pg/ml) of 3 biological repeats each representing a different donor. PSVs generated using pSG3ΔEnv and empty vector was used as a negative control (Ctrl). Error bars are +/-SEM. Mann Whitney test was used to compare PSV induced secretion of IL-1β between PSVs and Ctrl.

4.3.8.4 Impact of PSV stimulation on IL-12 secretion by MDDCs

IL-12 is a heterodimeric pro-inflammatory cytokine with immune regulatory properties. It has been identified as the master switch leading to the differentiation of Th0 cells to Th1 as well as regulating T cell proliferation and cytotoxicity. It regulates its own secretion by DCs and T cells and induces IFN-γ production by T cells while inhibiting the development of tolerance (Chehimi et al., 1994; Marshall et al., 1999; Villinger & Ansari, 2010). IL-12 comprises of IL-12p35 and IL-12p40 and a number of reviews have debated whether IL-12p40 dimers are agonists of IL-12 function (Ling et al., 1995; Holscher et al., 2001; Abdi, 2002; Hölscher, 2004; Khader et al., 2006; Abdi & Singh, 2015). Importantly, there was a positive correlation between the number of cells expressing IL-12 and IL-12p40, and we therefore used IL-12p40 as a surrogate marker of IL-12 expression and function (Muller-berghaus et al., 2005). When we measured MDDC IL-12 secretion after stimulation with PSVs, there was a slight increase in levels relative to background, suggesting that HIV-1 interactions with DCs resulted in very little or no IL-12 release (Figure 4.3.13). This finding corroborated previous findings (Roberts et al., 2010) that didn't identify increased levels of IL-12 in the plasma/CVLs of HIV-positive women. The pattern of IL-12 release did not match that of IL-10 secretion i.e. C7, C12 and C14 did not stimulate the secretion of higher IL-12 levels when compared to other clones. This could be due to PSVs directly preventing IL-12 release. Studies have shown that HIV and gp120 impair the secretion of IL-12 in response to stimuli. However, whether or not this is as a result of direct PSV stimulation or via PSV-mediated IL-10 secretion remains unclear (Chehimi et al., 1994; Daftarian et al., 1995; Taoufik et al., 1997; Marshall et al., 1999; Fantuzzi et al., 2004). Furthermore, we cannot exclude that IL-12p40 might be expressed differently from IL-12 in MDDCs in response to PSV. However, IL-12 levels are unlikely to be higher than that of its subunit even if IL-12p35 is expressed to high levels.

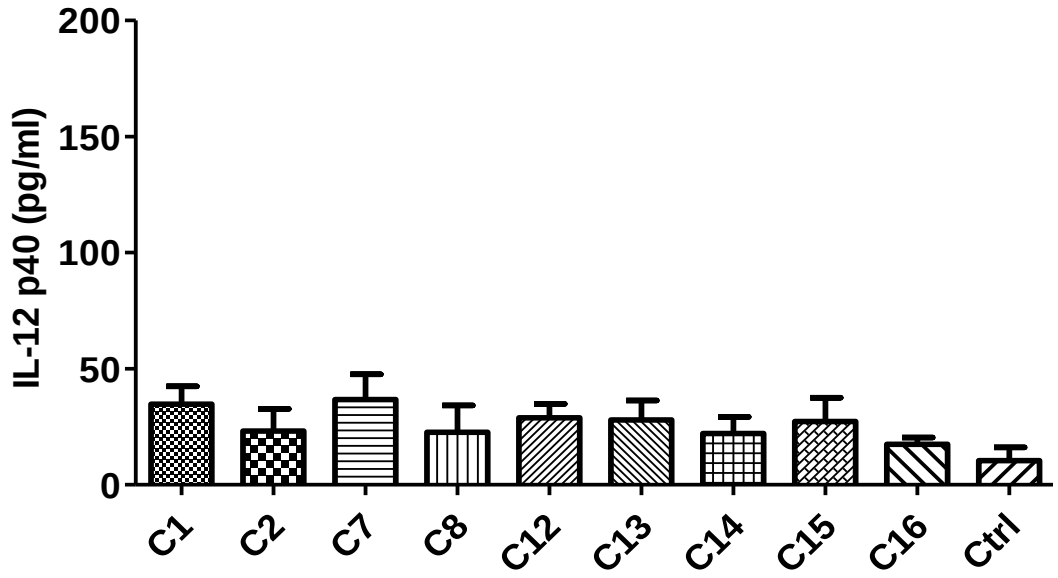
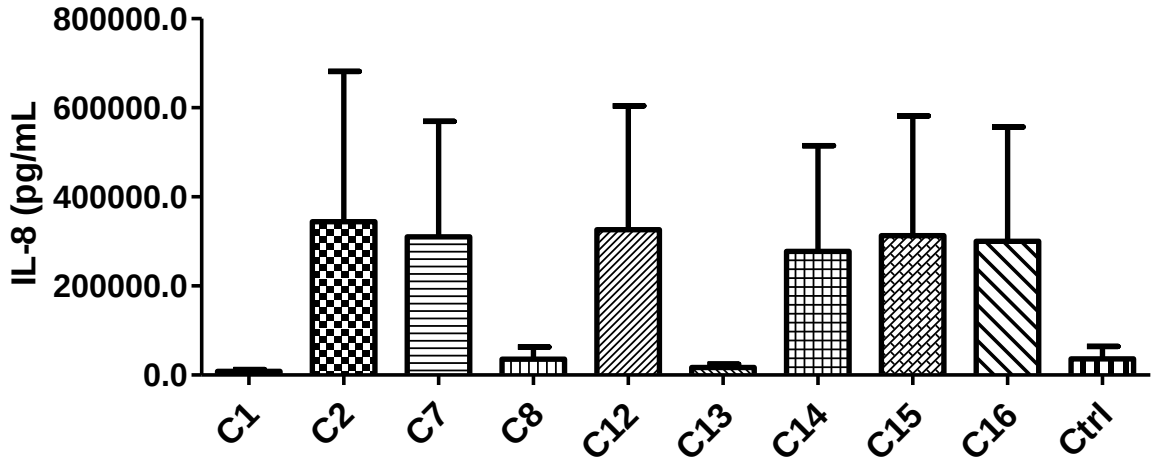


Figure 4.3.13 Impact of PSV stimulation on MDDC secretion of IL-12. Day six MDDCs were stimulated in culture with PSV for 24 hours and the secretion of IL-12 was measured in culture supernatants by Luminex assay. PSV generated using pSG3ΔEnv and empty vector was used as a negative control (Ctrl). Results show mean IL-12 (pg/ml) +/-SEM from five donors stimulated on different days with different virus preparations. Mann Whitney test showed no significant difference between PSV induced IL-12 secretion and Ctrl.

4.3.8.5 Impact of PSV stimulation of MDDCs on IL-8 secretion

IL-8 is a chemokine (CXC) released by a number of cells and it is responsible for attraction of neutrophils to sites of infection and enhancing phagocytosis (Koch et al., 1992; Matsumoto et al., 1993). It has been shown to increase several folds in the serum of HIV infected subjects compared to their uninfected controls (Matsumoto et al., 1993) and to enhance HIV replication in both lymphocytes and macrophages (Lane et al., 2001). Narimatsu *et al.* and Masson *et al.* and Naranhhai *et al.* also found that elevated levels of IL-8 in cervical tissue explants, CVLs and plasma increased HIV transmission risk (Narimatsu, Wolday & Patterson, 2005; Naranbhai et al., 2012; Masson et al., 2015). This could suggest that increased levels of IL-8 at sites of HIV transmission attracts an increased number of target cells to the female genital sub-mucosa which then enhances HIV productive infection (Lane et al., 2001; Narimatsu, Wolday & Patterson, 2005).

A



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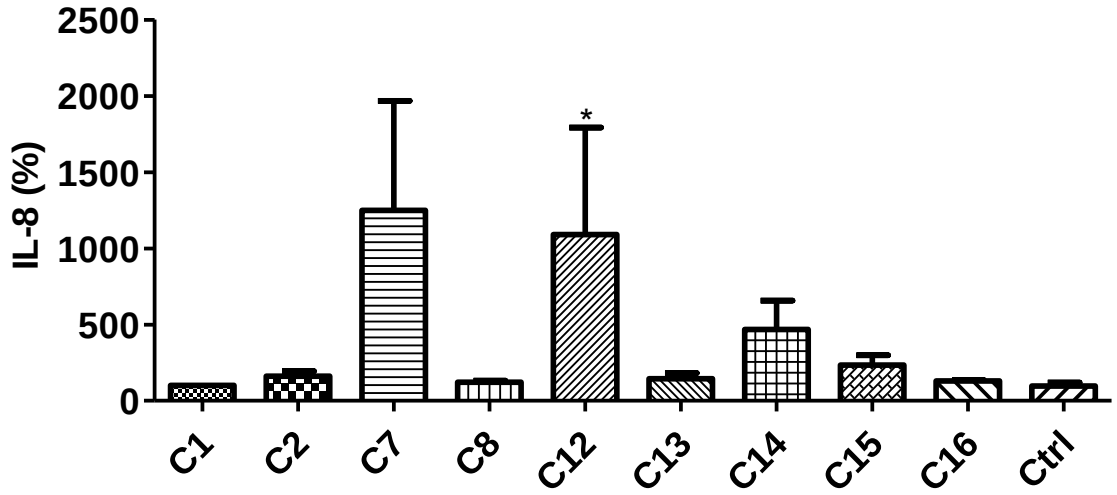


Figure 4.3.14 Impact of PSV on MDDC secretion of IL-8. Day six MDDCs were stimulated with PSVs and the secretion of IL-8 measured in culture supernatants by Luminex assay. Due to high MDDC donor variation, IL-8 values (pg/ml) A) were normalised to that of C1 (%) B). then compared for four independent experiments each using a different MDDC donor and PSV preparation. Error bars represent mean +/-SEM. PSVs generated using pSG3ΔEnv and empty vector was used as a negative control (Ctrl). Mann Whitney test showed no significant difference between PSV induced IL-12 secretion and Ctrl. * indicates p value less than 0.05.

This is supported by findings that higher relative concentrations of IL-8 in the genital tract compared to plasma was associated with HIV acquisition risk in women (Liebenberg et al., 2016). Soluble IL-8 levels in response to PSV stimulation varied greatly between donors and some donor MDDCs did not release IL-8 above background (Figure 4.3.14, A). To circumvent donor MDDC variability, IL-8 levels were calculated relative to an arbitrary clone, C1 per

donor (Figure 4.3.14 B) and the average percentages from five donors were compared between clones and the negative control. Similar to what was observed for IL-10, C7, C12 and C14 induced IL-8 secretion 13-, 11-, and 5-fold, respectively above the negative control. However, only C12 induced IL-8 secretion that reached statistical significance ($p = 0.05$) (Figure 4.3.14) likely due to variation in IL-8 secretion across donors. Once again, the relative differences in IL-10 levels across clones was reflected in IL-8 which could suggest that pro-inflammatory cytokine production in response to PSVs resulted in IL-10 upregulation to reduce tissue damage in response to inflammation. Alternatively, early studies showed that under normal circumstances, IL-10 inhibited IL-8 expression in neutrophils to prevent immunopathology (Wang et al., 1994) and limited the expression of most pro-inflammatory cytokines and chemokines (Moore et al., 2001; Couper, Blount & Riley, 2008) after LPS but not PHA stimulation (de Waal Malefyt, Abrams, et al., 1991). As IL-8 levels were increased similarly to IL-10, PSVs might have impaired IL-10's ability to inhibit pro-inflammatory cytokines.

4.3.8.6 Impact of PSV stimulation of MDDCs on MIP-1α and MIP-1β secretion

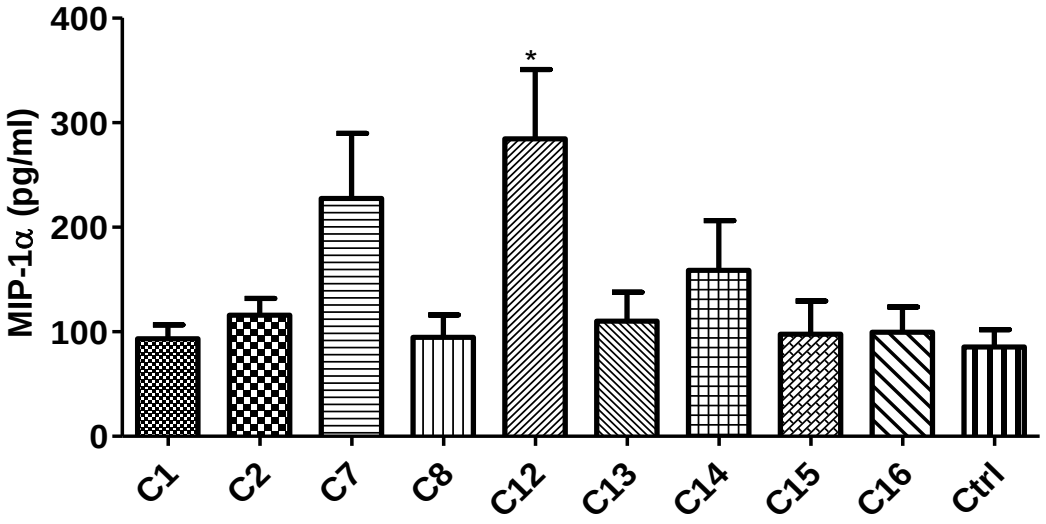
MIP1-α and MIP-1β are pro-inflammatory chemokines produced by pDCs in response to infection that attract CD4+ T cells to sites of infection (Li, Estes, et al., 2009). Peripheral blood cells from HIV positive subjects lacked the ability to secret MIP-1α and MIP-1β into culture supernatants despite high intracellular levels. However, the elevated intracellular levels of MIP-1α and MIP-1β in CD4+ T cells correlated with plasma viral load, suggesting that it played a role in HIV disease progression (Jennes et al., 2002, 2004). MIP-1α and MIP-1β levels were also shown to be elevated in CVLs of women with BV and associated with risk of HIV acquisition, with levels higher in women who later sero-converted than those who did not (Masson et al., 2015; Liebenberg et al., 2016).

Most of the PSVs stimulated the release of MIP-1α and MIP-1β only 1.5-fold above the background control. Although three PSVs (C7, C12 and C14) induced higher levels of MIP-1α and MIP-1β levels (2.7-, 3.3- & 2-fold and 4-, 5- & 2-fold respectively for MIP-1α and MIP-1β) compared to background (Figure 4.3.15 A & B), only C12 stimulation resulted in a significant increase ($p = 0.05$) for MIP-1α. However, when values were expressed relative to C1 to normalize for values from different donors, C7 and C12 induced significantly more MIP1-α and MIP1-β secretion than the controls(Figure 4.3.15 C & D).

IL-10 has been said to inhibit MIP1-α and MCP-1 in human blood monocytes and alveolar macrophages (Berkman et al., 1995), while Roberts *et al.* found elevated CVL MIP-1α

concentrations during early HIV infection which correlated with plasma viral load set (Roberts et al., 2012). Our data shows that in most donors PSVs did not stimulate MDDCs to release much MIP1-α, MIP1-β (Figure 4.3.15) and MCP-1 (data not shown) compared to the background control. However, C7, C12 and C14 induced higher levels of MIP-1α and MIP-1β consistent with what was observed for IL-10, TNF-α, IL-6 and IL-8. It is therefore possible that either PSV stimulation was responsible for the changes in the cytokines and chemokines apparent in this study or PSV induced an IL-10 autocrine regulation of DC cytokine release function.

A)



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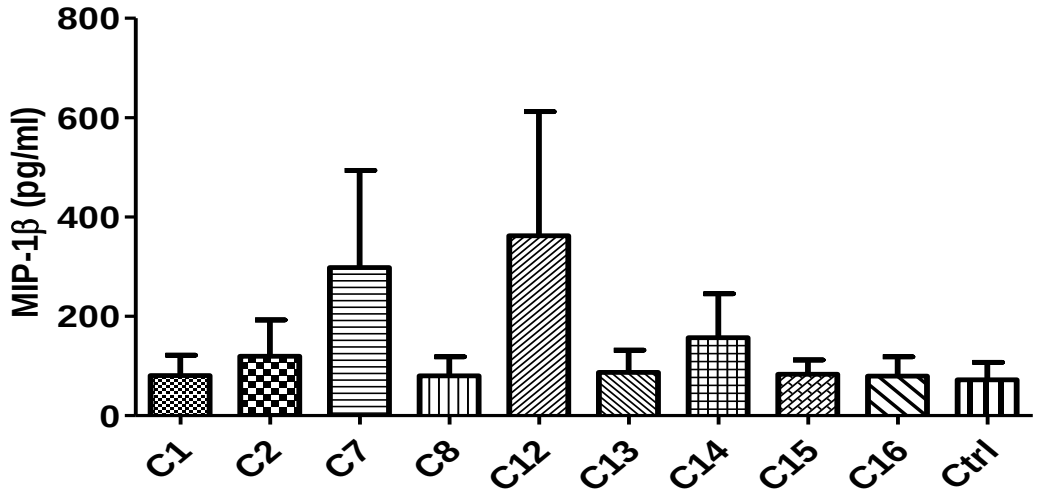

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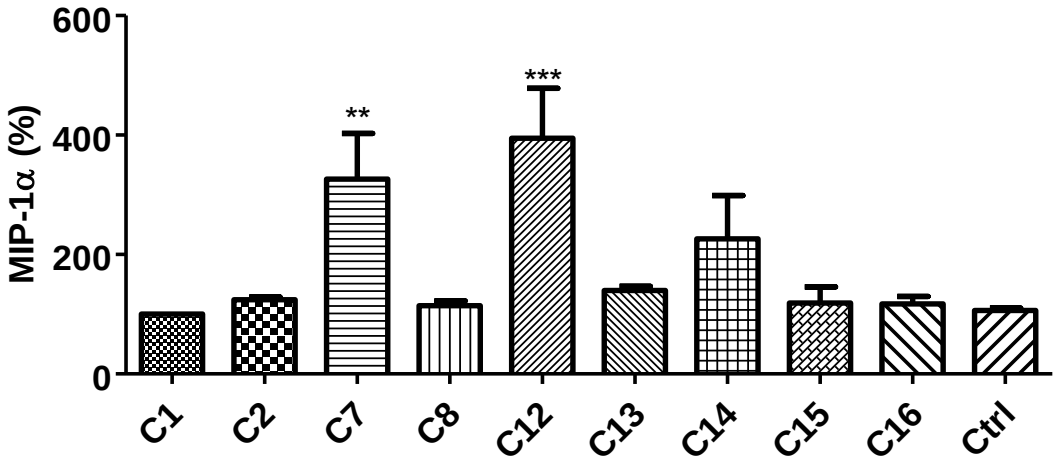


D)

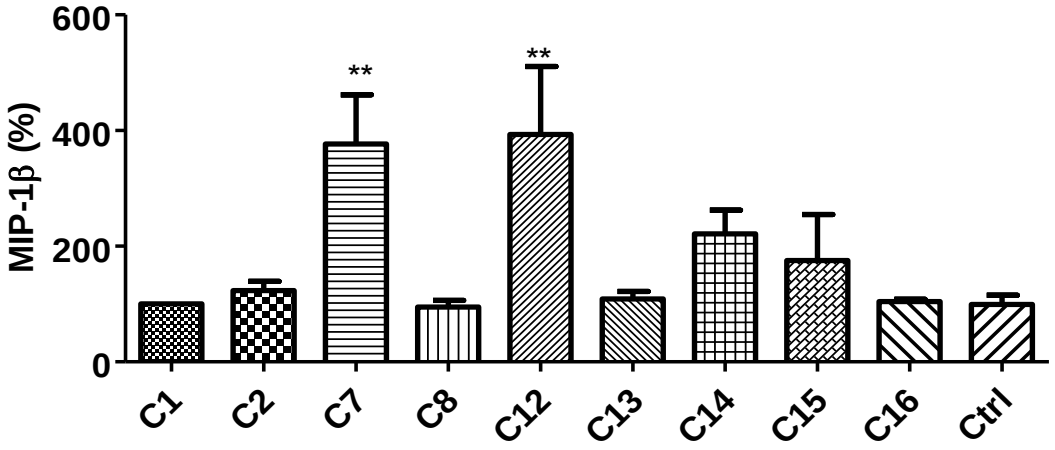


Figure 4.3.15 Impact of PSV MDDC stimulation on the release of A) MIP-1 α and B) MIP-1 β. Following differentiation MDDCs were stimulated with PSV in culture and the induction of A) MIP-1α and B) MIP-1β release by the PSV measured in culture supernatants by Luminex assay. The values of A) MIP-1α and B) MIP-1β (pg/ml) from three and four donors respectively, were expressed relative to C1 (C & D) to normalize cytokine variation between independent donor and the means +/- SEM are shown in bar graphs. ANOVA was used to calculate statistical differences between means from PSV stimulated donors and pSG3ΔEnv stimulated control. *, ** and *** represent p values of < 0.05, 0.01 and 0.001, respectively.

To summarize the effects of Env PSV on MDDC secretion of IL-10 and inflammatory cytokines, a hierarchical clustering of all cytokines was plotted. The plot showed the inter-relatedness between MDDC IL-10 secretion with that of the other cytokines and whether some Envs elicited the same cytokine profile. Cytokine profiles were highly heterogeneous with only

C7, C12 and C14 tending to stimulate the release of IL-10 together with TNF-α, IL-6, MIP-1α and MIP-1β (Figure 4.3.16).

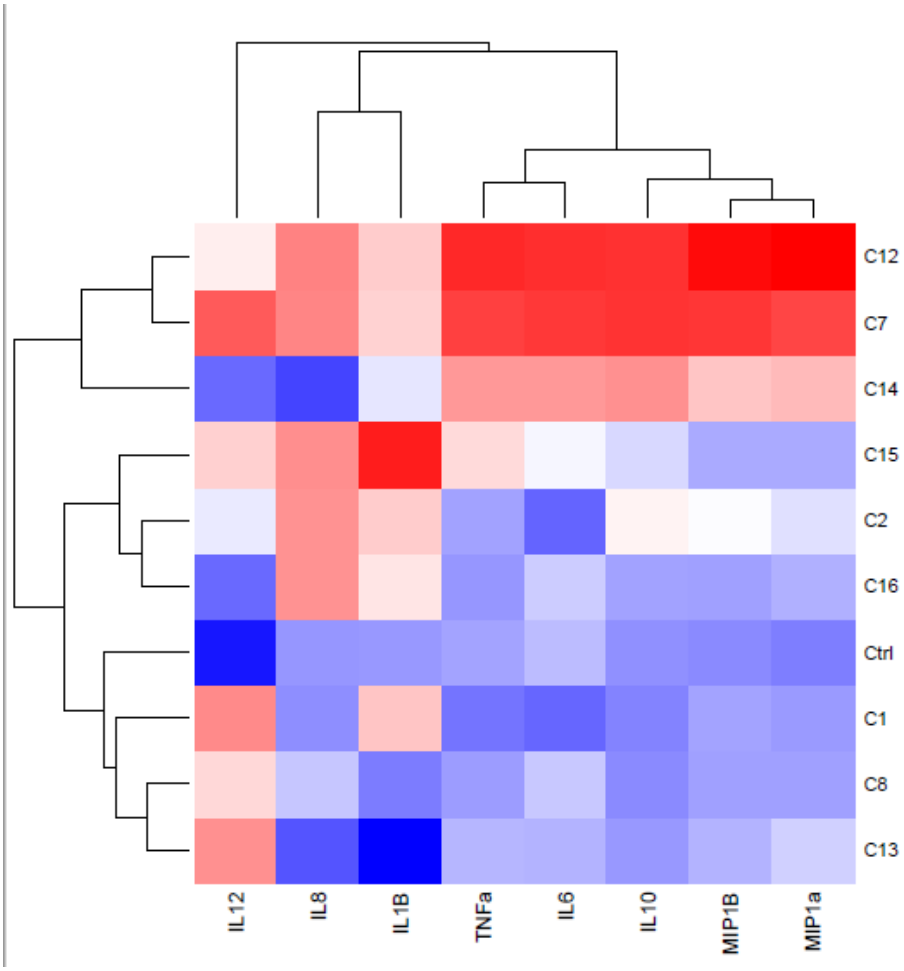


Figure 4.4.16: **Potential association between MDDC secretion of IL-10 and pro-inflammatory cytokines and chemokines**. IL-10, TNF-α, IL-6, MIP-1α, IL-8, IL12, IL-1β and MIP-1β levels were measured following stimulation of MDDCs with PSVs and were clustered by unsupervised hierarchical clustering (using R). C13, C8, C1, C16, C2, C15, C14, C7 and C12 are represented on the e y-axis and cytokines on the x-axis.

4.4 Discussion

An ideal environment for HIV-1 survival would include dampened phagocytic and cytotoxic activity with increased influx of immune cells to sites of infection. Viruses and intracellular pathogens are known to suppress or delay host cellular immune responses by controlling IL-

10 levels as IL-10 inhibits IL-12 production, allowing pathogens to establish infection or enhance persistence (Brooks et al., 2006; Finlay & McFadden, 2006; Hamza, Barnett & Li, 2010; Redford, Murray & O'Garra, 2011; Wilson & Brooks, 2011).

There is an apparent interdependent relationship between HIV-1 infection and inflammation as the one seems to exacerbate the other: inflammation is associated with increased risk of HIV-1 infection (Masson et al., 2015) and disease progression (Roberts et al., 2010, 2012) while HIV-1 replication and resultant immune dysfunction increases inflammatory responses. Inflammation is likely to enhance heterosexual HIV transmission by increasing the number of DCs, macrophages, and CD4+ T cells in the female genital tract, providing HIV-1 with target cells for replication (Miller & Shattock, 2003; Miller, 2007; Li, Estes, et al., 2009), reducing epithelial barrier integrity (Nazli et al., 2010) or directly promoting HIV-1 replicaiton (Osborn, Kunkel & Nabel, 1989). Therefore, understanding how HIV-1 manipulates inflammatory immune responses to favour its own survival will provide vital information for the design of HIV preventive, treatment and cure strategies.

IL-10 has been suggested to be the key cytokine involved in tissue repair during an inflammatory response as well as an initiator of Th2 type responses (Hazlett, Jiang & McClellan, 2014). While earlier studies stated that the principle function of IL-10 is to limit or terminate inflammatory responses, Trinchieri *et al.* pre-supposed that viral products may suppress IL-12 production directly (Trinchieri, 1997; Moore et al., 2001). Corinti *et al*., (2001) found that autocrine IL-10 decreased DC function and Aimaniandra *et al.* (2009) suggested the role IL-10 to enhance Tregs so as to impede maturation, IL-12 secretion and protective immune response (Corinti et al., 2001; Aimanianda et al., 2009). In Epstein Barr virus, IL-10 is not only produced to limit inflammation but it is produced very early to blunt immune response development (Redford, Murray & O'Garra, 2011). This could suggest that IL-10 can be produced together with or in the absence of IL-12 and/or other pro-inflamamtory cytokines following exposure of DCs to pathogen or pathogenic product. The fine balance of the cytokine profile produced by DCs determines the Th0 differentiation (Kennedy et al., 1994; Trinchieri, 1997; Romagnani, 2000; Watford et al., 2003). Other inflammatory cytokines like TNF-α, IL-6, IL-1β, IL-9 and IL-17 are also made by immature DCs, and these serve to expand other subsets of T cells like the Treg and Th17 which help either in the control of the immune response or immune homeostasis (Sakaguchi et al., 2009).

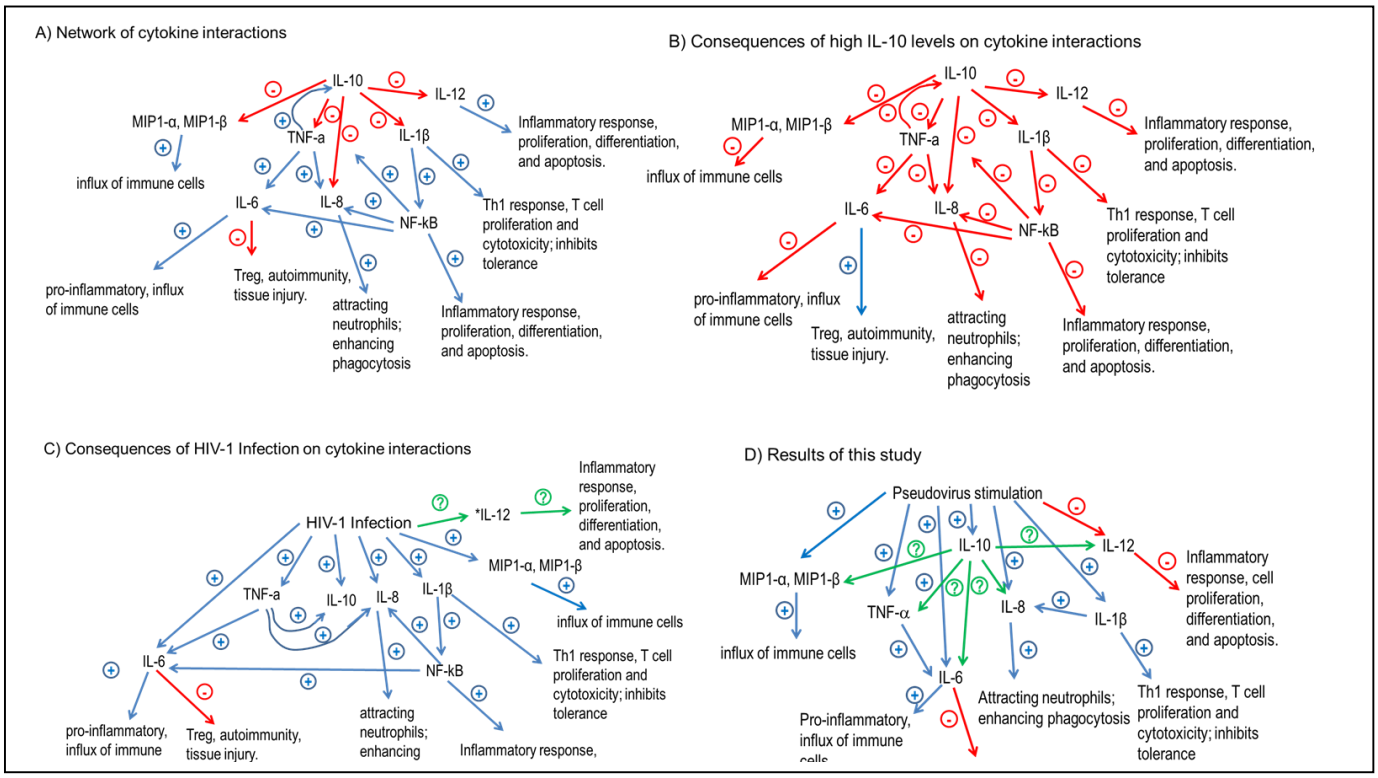


Figure 4.4.1 Variation in the cytokine interactions network focussed on the role of IL-10 as a modulator. The simplified diagrams represent only a subset of cytokine interactions specific to the immune modulators discussed in this study with the aim of highlighting the interconnectedness and complexity of the interactions. A) Literature discussed in this chapter was used to map out interactions between IL-10, IL-12, IL-1β, TNF-α, IL-8, IL-6, MIP-1α and MIP1β. Concentrations of cytokines were not taken into consideration nor were the outcome of Th0 differentiation into Th1 or Th2. B) Map of cytokine interactions if IL-10 levels were elevated. C) Studies on plasma, CVL and whole blood of HIV-positive individuals indicated that IL-10, IL-8, IL-6,TNF-α IL-1β, MIP1α and MIP1β levels were all increased compared to HIV-negative controls. When this data was included into Map A, inflammatory responses were increased. D) Map of the results of MDDC cytokine release in response to pseudovirus. The interactions and the consequences are nearly identical to that of Map C: inflammatory responses increased. Blue (+) and red (-) lines indicate increase/positive effect and decrease/negative effect, respectively. Green lines (?) indicate inconclusive findings. *Conflicting results on whether IL-12 levels were decreased or increased in HIV-infected samples. Figure based on literature from (Chehimi et al., 1994; Marshall et al., 1999; Choi et al., 2003; Meyaard et al, 1997; Bebell et al., 2008).

We found that stimulation of MDDCs with PSVs carrying Env with variable number, position and mannosylation of PNGs induced the secretion of varying levels of IL-10 for most of the donors tested, indicating that some Envs are better inducers than others. PSVs C7, C12 and C14 induced significantly greater IL-10 production than both the background control and the other Env clones (Figure 4.3.1), while other clones induced IL-10 secretion only slightly above background. As gp140 also induced IL-10 while recombinant DC-SIGN partially inhibited

PSV-stimulated MDDC IL-10 release, it is likely that gp120-DC-SIGN interactions were involved in the release of this cytokine (Figures 4.3.2 & 4.3.3). However, inhibition with recombinant DC-SIGN did not reach 100% suggesting that other receptors could also play a role as previously suggested (Turville et al., 2001; Shan et al., 2007; Lambert et al., 2008). Non-CLRs like Galcer, CD169 or heparan sulfate might also be involved as they capture HIV and *trans*-infect CD4+ T cells (de Witte, Bobardt, et al., 2007; Magerus-Chatinet et al., 2007; Izquierdo-Useros, Lorizate, Puertas, et al., 2012). Binding of HIV to TLR2 and TLR4 stimulated IL-10 expression, suggesting that TLR responses could also account for the release, in part of IL-10 (Figure 5.1.1) (Saraiva & O'Garra, 2010).

Env-DC-SIGN interactions are likely via high mannose as Shan *et al.* (2007) found that removal of mannose residues from gp120 abrogated their ability to induce MDDC IL-10 secretion (Shan et al., 2007). As the nine clones differed in number, position and level of mannosylation, we determined if there was a relationship between the level of gp140 mannosylation and the ability of PSVs to stimulate the release of IL-10. Even though C7, C12 and C14 were amongst the clones with the highest mannosylation and induction of IL-10 release, C13, one of the weaker IL-10 inducers also had approximately 40% mannosylation. There was thus no overall relationship between Env mannosylation and IL-10 secretion. This could be because sgp140 and PSVs are processed slightly differently, or Env activation of other receptors (e.g. TLR2 or TLR4) than DC-SIGN whose interaction with DC might not depend entirely on the number of high mannose residues could also contributes to induction of the IL-10 release seen.

As other studies have identified PNGs involved in DC-SIGN binding and/or carried high mannose residues, we deleted PNGs at positions 241, 262, 386, 392 and, 448 to determine whether these putative N-glycans played a role in inducing IL-10 secretion by MDDCs (Hong et al., 2002, 2007; Go, Hewawasam, et al., 2011; Liao et al., 2011). However, deletions of most PNGs had deleterious effects on Env function and are more likely essential for maintaining native Env structure rather than IL-10 secretion. In order to circumvent the risk of disrupting Env structure and function we took an alternative approach. PSVs were grouped based on the presence or absence of PNGs that varied between 33 - 67% within the cohort sequences and IL-10 secretion in response to PSVs of the two groups was compared. Each TF and matched chronic infection pair was used to stimulate MDDCs from the same donor multiple times. The variation between donors stimulated with the same PSVs ranged from 0.3 to 25-fold above

background, indicating that MDDC response to HIV was highly donor-specific. We thus compared the level of IL-10 released by each donor stimulated by Envs PSV that carried specific PNGs. Statistical analysis of IL-10 secretion data indicated that the presence of PNGs at N130, N332 and absence at N674 could be important for the induction of MDDC IL-10 release by PSVs irrespective of the donor. When IL-10 levels released by 7 donors stimulated by a single Env were averaged and a similar anlaysis was carried out, statistical significance was lost although the presence of PNGs at N130, N332 and absence at N674 were still associated with increased IL-10 levels. Therefore, the majority of MDDC donors are more likely to release IL-10 when stimulated with Envs that carry PNGs at N130 and N332 but not N674. As N674 was previously suggested to be important for Env structure and/or function, we excluded this PNG from the analysis. PSVs that carried both PNGs at N130 and N332 stimulated MDDCs to release higher levels of IL-10 than those that lacked all of the PNGs or only carried one of the two. This suggested that PNGs at position 130 and 332 of gp120 could interact with DC-SIGN in such a way as to stimulate the release of IL-10 by MDDCs. However, we cannot discount the importance of these N-glycans in binding to alternative DC receptors

As we had identified N130 and N332 as comprising a potential IL-10 inducing motif, we determined whether these PNGs were enriched in acute infection (AI) sequences. However, N130 and N332 were only marginally enriched in Envs from the acute stage of infection although this finding was possibly due to the small sample size of the study (18 matched AI vs 18 CI Env sequences). Furthermore, some sequences were sampled before 2 years post-infection and thus comparison of TF to later samples (more than 3 years) might detect a greater difference in the frequency of N130+ and N332+ between AI and chronic infection variants. To further investigate whether AI PSVs were better able to induce MDDC IL-10 secretion, matched PSVs were grouped on whether they were sampled 2-5 weeks or 80–173 weeks post-infection. Comparison of IL-10 levels in the culture supernatants of 7 MDDC donors after stimulation with PSVs showed that the AI clones induced significantly more MDDC IL-10 secretion than their matched CI pairs irrespective of MDDC donor. This could suggest a role of IL-10 in HIV transmission. Early IL-10 responses have been previously suggested to impair specific CD4+ T cell expansion, function and secondary recall in intracellular endosomal bacteria (Singh & Thirumalapura, 2014) and facilitated disease progression in HCV infection (Flynn et al., 2011). Norris *et al*. (2006) and Borghi *et al*. (1995) found IL-10 levels elevated in plasma at acute HIV infection(Borghi et al., 1995; Norris et al., 2006) whereas other studies showed that IL-10 levels increased but with disease progression (Roberts et al., 2010; 2012).

However, as this study focussed on nine matched Env pairs, a larger sample size must be evaluated to confirm the relationship between IL-10 release and HIV transmission.

IL-10 has been found to limit T cell, macrophage and NK activity via limiting MHC class II molecules, co-stimulatory molecules and changing the profile of pro-inflammatory and anti-inflammatory cytokines produced by APCs (de Waal Malefyt, Haanen, et al., 1991; Fiorentino et al., 1991; D'Andrea et al., 1993). Thus, to confirm whether PSV stimulation of MDDCs also resulted in differential release of other pro-inflammatory cytokines and chemokines, IL-12p40, IL-6, IL-8, IL-1β, MIP1α, MIP1β and TNF-α levels were measured in the supernatants of PSV-stimulated MDDCs. PSVs induced MDDCs to release very low levels of IL-12p40, suggesting that viral stimulation was not resulting in a Th1 response (Figures 4.3.13). This finding was similar to previous studies that found impaired IL-12 production with increased IL-10 levels (Shan et al., 2007) in MDDCs and some suggested that aberrant IL-12 expression was an indirect effect of IL-10 (Chehimi et al., 1994; Daftarian et al., 1995; Marshall et al., 1999) or failure to detect IL-12 using an anti-IL-12p40 detection system. It was also suggested that aberrant IL-12 expression was independent of IL-10 (Meyaard et al., 1997), although Bebell *et al*. (2008) found increased levels of both IL-10 and IL-12 in CVLs in acute HIV infection (Bebell et al., 2008). Despite contrary reports, PBMCs and MDDCs from HIV infected persons produced more IL-10 and less IL-12 than those from negative controls (Buisson et al., 2009) and autocrine IL-10 impaired IL-12 release and DC migration to lymph nodes in response to *M.* tuberculosis (Demangel, Bertolino & Britton, 2002).

This suggests that HIV-1 could prevent Th0 cell differentiation into Th1 subtype by favouring the expression of IL-10 over IL-12, halting an adaptive immune response and thus protecting the virus from immune clearance (Mosmann & Coffman, 1989; Pulendran, 2001). Brooks *et al.* (2008) suggested that blocking IL-10 enhanced the efficacy of a DNA vaccine to induce T cell responses to clear persistent for LCMV viral infection. This re-enforces the hypothesis that IL-10 impairs the Th1 pathway of the immune response which fights viral and intracellular pathogens (Brooks et al., 2006, 2008; Ejrnaes et al., 2006)**.**

Studies have suggested that IL-10 represses expression of TNF-α, IL-1β and IL-8 in activated macrophages (Zhang & An, 2007), suppresses the expression of IL-1α, IL-1β, IL-6, IL-8 and TNFα in human monocyte (de Waal Malefyt, Abrams, et al., 1991) and inhibits MIP-1-α, MIP-1-β and MCP-1 production by human blood monocytes (Berkman et al., 1995)., although this was contrary to and a cross sectional study (Roberts et al., 2010) .

When we investigated whether PSVs induced secretion of other immune modulators, we also found that the same PSVs (C7, C12 and C14) that induced MDDCs to release high levels of IL-10 also stimulated the release of higher concentrations of IL-6, IL-8, MIP1α, MIP1β and TNF-α than all other clones. Longitudinal and cross-sectional cohort studies of HIV infected women showed that HIV infection is associated with elevated levels of IL-1β, IL-6, IL-8, MIP-1-α, MIP-1-β and TNF-α, in CVL and plasma (Bebell et al., 2008; Stacey et al., 2009; Roberts et al., 2010) resulting in heightened inflammatory responses (Figure 4.4.1C). However, identification of increased IL-10 levels was done within the context of increased concentrations of these inflammatory cytokines. These studies, therefore suggested that HIV infection resulted in a robust inflammatory response that initiated the production of high levels of IL-10 (Stacey et al., 2009). Despite evidence that IL-10 reduces the expression of IL-6, IL-8 and TNF-α, the longitudinal study of Stacey *et al*. (2009) did not show a decrease in inflammatory cytokines over time in response to IL-10, highlighting the complexity of *in vivo* models (Stacey et al., 2009).

Further experiments in the presence of IL-10 inhibitors will be able to evaluate the relationship between IL-10 and the expression of IL-8, IL-6, TNF-α, MIP-1α, MIP-1β, and IL-1β by DCs in the presence of HIV or gp120. Despite opposing hypothesese as to whether IL-10 deregulates a Th1 response or inflammation leads to high IL-10 levels, both *in vivo* HIV-1 infection and *in vitro* PSV stimulation of MDDCs resulted in enhanced expression of the same cytokines. Therefore *in vitro* stimulation of MDDCs inflammation responses mimic the effect observed in HIV-infected individuals when they first come in contact with the virus. The overall outcome of the "cytokine storm" is increased inflammatory responses that could favour HIV transmission and survival by activating large numbers of target cells at sites of infection and promoting viral establishment probably by modualtion of the immune response by IL-10 together with the activation of the NF-KB.

N-glycans around the N332 and V3 loop of gp120 have been found to be very immunogenic and form epitopes for some of the most potent nAbs (Garces et al., 2014; Pritchard, Spencer, et al., 2015). Potentially, the PNG at N332 might be included in a vaccine cocktail to help the development of nAb response. However, this study has shown that the Env PNG at N332 could be involved in inducing MDDCs to release high levels of IL-10 and pro-inflammatory cytokines in a potentially deregulated Th1 response. An immunogen including N332 might enhance the development of nAbs, but this benefit could be counteracted by the action of

deregulated inflammatory signals. This study highlights the need to understand how HIV modulates the immune response to safeguard its survival to inform vaccine design.

In conclusion, our findings supported studies that looked at inflammatory responses in HIV infected women as PSV stimulated MDDCs to secrete increased levels of IL-10 and pro-inflammatory cytokines and chemokines. Inflammation was previously shown to increase the risk of infection although the cells responsible were not identified. Here we show that DC-mediated inflammation might be very important to HIV acquisition. Furthermore, acute infection PSVs stimulated higher levels of IL-10 than those from chronic infection, suggesting that it could play a role in HIV transmission. The proposed model therefore is that the secretion of IL-10, TNF-α, IL-6, IL-8, IL-1β, MIP-1 α and MIP-1β by DCs after binding HIV in the female genital tract attracts T cells, DCs and macrophages into the sub-mucosal, lamina propria and epithelium where they become infected. IL-10 could prevent DC maturation (Fantuzzi et al., 2004) and under these conditions enhance migration of DCs carrying virus to the lymph nodes where more CD4+ T cells are *trans*-infected. As IL-10 causes immunosuppression, a cytotoxic immune response may be impaired, while TNF-α, IL-6 and IL-8 may enhance the replication of the virus enough for effective dissemination and productive clinical infection (Israel et al., 1989; Finnegan et al., 1996; De Jong et al., 2008).

Chapter 5: The impact of Envelope N-glycosylation on activation of signaling pathways that modulate Dendritic Cell function.

5.1 Introduction

HIV Envelope (Env) N-glycosylation plays an important role in the host immune response to HIV infection (Binley et al., 1998; Scanlan et al., 2002; Calarese, 2003; Koch et al., 2003; Shan et al., 2007; Banerjee et al., 2009; McLellan et al., 2011; Moore et al., 2012). Like most pathogens or pathogen associated molecular patterns (PAMPs), Env N-glycans could alter immune responses by activating alternative signaling pathways in dendritic cells (DCs) (Geijtenbeek et al., 2003; Geijtenbeek & Gringhuis, 2009; Gringhuis et al., 2009). However, the mechanism whereby HIV-1 N-glycans could modulate DC signaling is not fully understood.

Pathogens that activate DCs do so by triggering a series of complex intracellular signals leading to DC maturation, up-regulation of co-stimulatory molecules (Banchereau & Steinman, 1998; Banchereau et al., 2000) and production of cytokines and chemokines. NF-kB and other transcription factors are also required for expression of inflammatory cytokines (Osorio & Reis e Sousa, 2011).

Earlier evidence has indicated an association between IL-10 and HIV infection. The level of IL-10 is elevated at the earliest time post-infection, increases with disease progression and is said to impair IL-12 production, hence dysregulating Th1/Th2 responses during HIV infection (Clerici & Shearer, 1993; Stylianou et al., 1999; Norris et al., 2006; Brockman et al., 2009). More specifically, Env mannose type N-glycans were found to stimulate MDDC IL-10 production (Shan et al., 2007) via DC-SIGN. Furthermore, as we have identified potential PNGs that could play a role in regulating MDDC IL-10 release and that TFs might induce MDDCs to release higher levels of IL-10 (Manuscript in preparation), we wanted to investigate if Env N-glycans activated alternative DC signaling pathways.

Although C-type lectin receptors (CLRs) are antigen capture receptors that help induce effective immune responses, pathogens like *Mycobacterium tuberculosis*, HIV and hepatitis C virus (HCV) subvert DC function when they interact with DC-SIGN (Geijtenbeek et al., 2003; van Kooyk & Geijtenbeek, 2003; Van Kooyk et al., 2004; van Vliet et al., 2007; Den Dunnen, Gringhuis & Geijtenbeek, 2009; Geijtenbeek & Gringhuis, 2009). Pathogen interaction with DC-SIGN has been reported to activate signaling via Raf-1. Raf-1 activation is central to modulating toll-like receptor (TLR) -mediated NF-κB activation and enhancing IL-10 production (Figure 5.1.1) (Geijtenbeek et al., 2003; Gringhuis et al., 2007, 2009; Den Dunnen, Gringhuis & Geijtenbeek, 2009). Raf-1 activation modulates the phosphorylation and

acetylation of the p65 subunit of NF-κB at Ser276. In response to *M. tuberculosis* infection, acetylation of NF-κB p65 leads to transcription of *Il-10*, *Il-6* and *Il-12* (Gringhuis et al., 2007, 2010; Sancho & Reis e Sousa, 2012). Although fucose-containing pathogens don't activate Raf-1, they also induce IL-10 while down-regulating the expression of IL-6 and IL-12 (Figure 1.4). It is possible that different PAMP-associated N-glycans can either activate Raf-1 or downstream effectors of Raf-1 (Geijtenbeek et al., 2003; Geijtenbeek & Gringhuis, 2009; Gringhuis et al., 2009, 2010), reviewed in (Sancho & Reis e Sousa, 2012). Mitogen activated protein kinases (MAPKs) are well established downstream effectors of the Raf-1 kinases in the Raf-Ras-MEK pathway (Wellbrock, Karasarides & Marais, 2004). As carbohydrate components of PAMPs influence Raf-1 activation, it is possible that carbohydrate content could also influence the MAPK kinases.

MAPKs that regulate some of the most critical functions (growth, differentiation and apoptosis) of the immune response and could play a role in the pathogenesis of a variety of human diseases (Johnson & Lapadat, 2002; Kim & Choi, 2010) (Shaul & Seger, 2007). The major groups of MAPKs include the extracellular regulated kinases (ERK), the c-Jun N-terminal kinase (JNK) [also known as the stress activated protein kinases (SAPK)] and p38 MAP kinases (Zhang, Wei , Tu Liu, 2002; Shaul & Seger, 2007). The relative importance of ERK, JNK and p38 in an immune response is dependent upon both the stimulating agonist and the target TLR. JNK and p38 are mainly stimulated by environmental stress and inflammatory cytokines (Wagner & Nebreda, 2009; Liu et al., 2016). The ERK pathway is involved in the control of cell growth, proliferation and cell survival in response to mitogens and growth factors (Lu & Xu, 2006; Boutros, Chevet & Metrakos, 2008). ERK activation leads to expression of cytokines necessary for the balance between cell expansion and survival (Lu & Xu, 2006; Shaul & Seger, 2007). Previous studies by Shrefler *et al*. (2006), Caparros *et al*. (2006) and Hsu *et al*. (2010) showed that DC-SIGN ligands activate ERK phosphorylation in DCs (Caparros et al., 2006; Shreffler et al., 2006; Hsu et al., 2010; Zhao et al., 2013). Hsu *et al*. (2010) and Zhao *et al*. (2013) found that the Bermuda grass protein, BG60 and E2 protein (one of the viral envelope glycoproteins of HCV) activated Raf-1 and ERK signalling (Hsu et al., 2010; Zhao et al., 2013) of MDDCs and THP1 cells via binding to DC-SIGN.

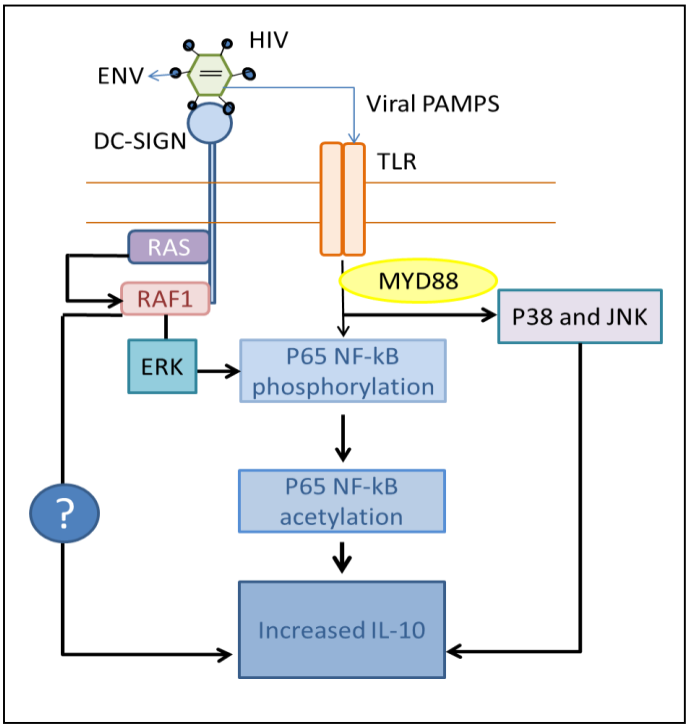


Figure 5.1.1 DC-SIGN modulation of TLR signaling. Signaling via DC-SIGN modulates TLR dependent signalling. Binding of HIV to DC-SIGN activates Raf-1 and modulates crosstalk between TLR and CLR-dependent activation of NF-KB, tipping the balance between immunopathology and innate immune response in its favour to establish infection. Simplified from (Hajishengallis & Lambris, 2011; Biasin et al., 2014).

Shan *et al.* (2007) found that Env mannose type N-glycans activated MDDC ERK to produce IL-10 and we showed that Env clones induced varying levels of IL-10 release, suggesting that some HIV variants might stimulate ERK better than others due to differences in Env N-glycosylation (Shan et al., 2007). The impact of differential N-glycosylation of HIV-1 gp120 on MAPK activation is not known. Given that some of these PNGs are important epitopes for broadly neutralising and potent Abs (Sanders et al., 2002; Scanlan et al., 2002; McLellan et al., 2011; Pejchal et al., 2011; Walker et al., 2011; Pritchard, Spencer, et al., 2015) and may be considered as components of HIV-1 vaccines, understanding their impact on DC CLR-mediated signaling could be very important to prevent harmful immune responses.

5.2 Aims and Objectives

Aim: Understand how HIV-1 Subtype C Envelope N-glycosylation impacts dendritic cell signaling.

Rationale:

Shan *et al*. (2007) showed that gp120 high mannose residues were involved in inducing DCs to secrete IL-10 and that not all Env clones were able to activate this pathway (Shan et al., 2007). Fucose-containing pathogens bind DC-SIGN but do not activate Raf-1 although they induce IL-10 secretion (Sancho and Reis e Sousa, 2012), suggesting that DC-SIGN-mediated signaling pathways are differentially activated depending on N-glycosylation of ligand. In Chapter 4 we showed that Envs stimulated MDDCs to release different levels of IL-10 and other cytokines. As N-glycan patterns differ between Envs, we hypothesise that differential N-glycosylation between Envs could activate alternative DC signaling pathways. This could result in the expression of different inflammatory cytokines that then shapes the immune response and potentially HIV-1 survival.

5.2.1 Specific Objectives:

1. Determine if differentially N-glycosylated Env PSV differ in their activation of the MAPK signaling pathway.
2. Determine if interactions between Env (gp140) and DC-SIGN are responsible for activating the MAPK signaling.
3. Determine the role of specific Env PNGs in the activation of MAPK signaling pathways.

5.3 Results

5.3.1 Optimisation of experimental conditions to determine the phosphorylation of ERK, JNK and p38 using flow cytometry

In chapters three and four, we showed that Env N-glycosylation could be important for Env structure and function, PSV entry efficiency, DC-SIGN binding, *trans*-infection and stimulating MDDCs to secrete IL-10. Next we determined if differences in Env PNGs impacted their ability to activate MAPK signaling as it is associated with MDDC release of IL-10. Phosphorylation and activation of the MAPK, ERK, leads to phosphorylation of the NF-KB p65 unit resulting in the activation of promoter regions for inflammatory cytokines genes (Dhillon et al., 2007). The ERK signaling pathway negatively regulates IL-12 production but enhances production of IL-10 and other inflammatory cytokines (Nakahara et al., 2006). Since we found that the production of IL-10, TNF-α, IL-6, IL-8 and IL-1β was enhanced by some PSVs and IL-12 was apparently not affected, we investigated whether Env with different PNG patterns differentially stimulated the phosphorylation of ERK. JNK and p38 were included in the study as they also differentially regulate MDDC cytokine production and p38 has been suggested to positively regulate DC maturation (Zhang & Kaplan, 2000; Kim et al., 2005).

5.3.1.1 Pseudovirion stimulation of MDDC MAPK phosphorylation

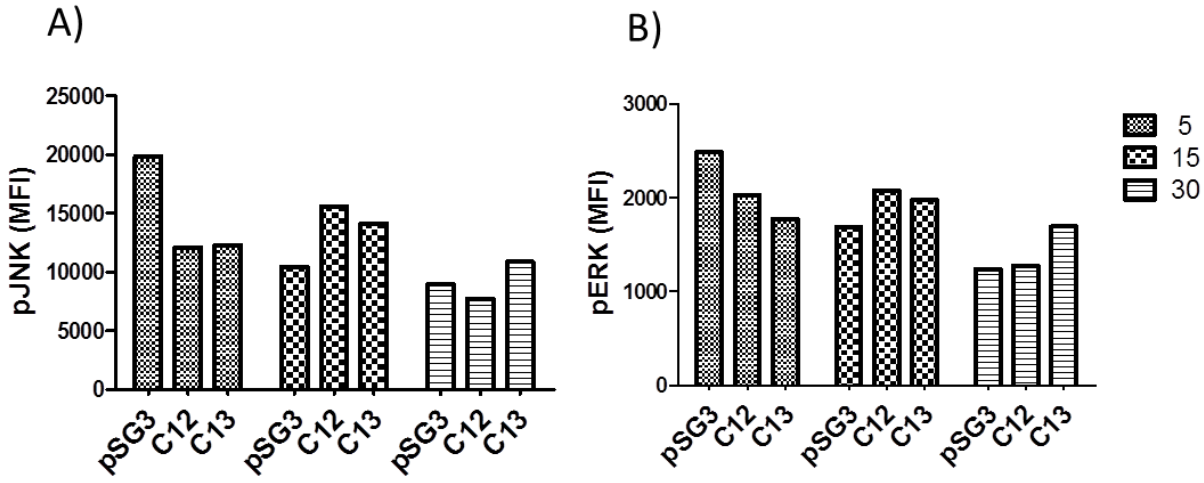


Figure 5.3.1 Flow cytometry analysis of MDDC ERK and JNK phosphorylation in response to stimulation with pseudovirus. Equal amount of pre-titered C12 and C13 PSV were added to day six MDDCs and incubated for 5, 15 and 30 minutes (min) at 37°C and the phosphorylation of JNK (pJNK) A) and ERK (pERK) B) was measured by flow cytometry. pSGΔEnv was used to control for the effect of other HIV-1 proteins on ERK and JNK phosphorylation (pSG3). Results were analysed by Flowjo software and the mean fluorescence intensities (MFIs) of the phosphorylated cells were plotted in GraphPad Prism 5.0. pJNK antibody was conjugated to phycoerythrin (PE) and pERK antibody to pacific blue (Pac Blue) fluorophores.

Hsu *et al.* (2010) was able to detect ERK phosphorylation following 5 and 15 min stimulation, Gringhuis *et al.* (2009) detected phosphorylated Raf-1 after 10 minutes of stimulation and Caparros *et al.* (2006) and Olsnes *et al.* (2011) detected pERK following 15 minutes of stimulation (Caparros et al., 2006; Gringhuis et al., 2009; Hsu et al., 2010; Olsnes, Olofsson & Aarstad, 2011) Therefore, phosphorylated ERK and JNK (pERK and pJNK) were detected by flow cytometry using fluorescent conjugated antibodies to phospho- ERK and JNK following stimulation of MDDCs for 5, 15 and 30 min with C12 and C13. PSVs lacking Env (pSGΔEnv control) were used to control for the effect that other viral proteins might have on MDDC stimulation. After 5 min stimulation, pSGΔEnv stimulated the phosphorylation of both ERK and JNK better than C12 and C13. After 15 min, there was a slight increase in pERK and pJNK in response to C12 and C13 relative to pSGΔEnv although this slight increase (1.2-fold) only remained apparent for C13 (1.4-fold) after 30 min. The relative pattern of phosphorylation of both ERK and JNK in response to C12 and C13 were similar at each time-point, suggesting that Env was activating the MAPKs. However, the high signal across all pSGΔEnv controls suggested that other viral proteins were also stimulating the phosphorylation of ERK and JNK especially at 5 minutes followed by a drop in activation over time (Figure 5.3.1). The reduced signal of the pSGΔEnv control at 15 and 30 min could also represent background 'noise', suggesting that Env-specific stimulation could be detected at these time points.

5.3.1.2 gp120 stimulation of MDDC MAPK phosphorylation

To eliminate the background signal, cell fixation was done immediately following stimulation to avoid further phosphorylation occurring during the staining process as well as to inactivate cellular phosphatases. Multiple factors present in the fetal calf serum used in the pseudovirus preparation may activate TLRs non-specifically, leading to non-specific phosphorylation of ERK. Therefore, MDDCs were stimulated with purified gp120 (gp120) to see if the absence of serum would reduce the background signal to detect Env-specific stimulation of MAP stimulation. We included LPS as a positive control and for a negative control; stimulation medium was added to the cells.

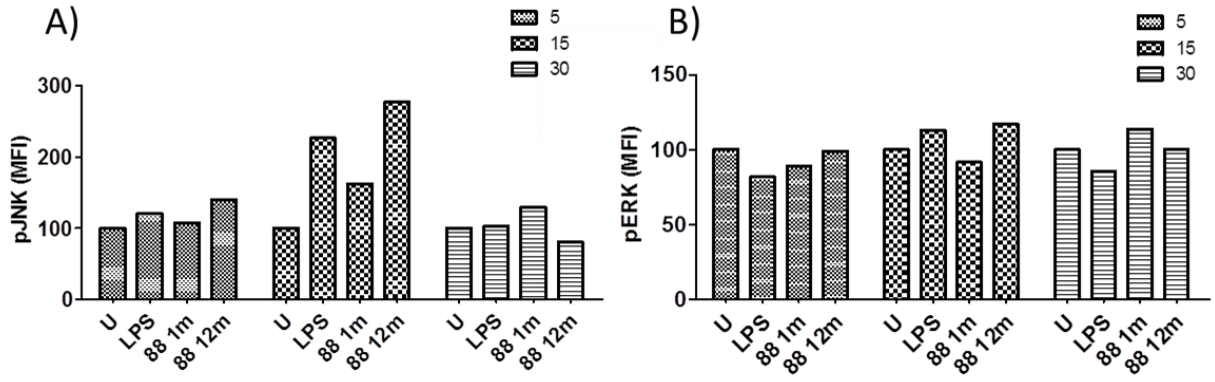


Figure 5.3.2 Determine the effect of purified gp120 on ERK and JNK phosphorylation over time. Day six MDDCs were washed with pre-warmed PBS and stimulated with 2 µg/mL of purified gp120 [CAP88 acute infection (AI) [isolated 1 month post infection, 88 1m and CAP88 chronic infection (CI), isolated 12 months post infection [88 12m] clones (Gift from Dr. J Arthos)] and the phosphorylation of A) JNK (pJNK) and B) ERK (pERK) was measured by flow cytometry. LPS at 500 ng/mL) was used as a positive control and culture medium was used as a negative, unstimulated control (U). Mean fluorescence intensity (MFI) was expressed as the percentage of unstimulated controls (U).

Five and thirty minute stimulation with gp120 did not eliminate background signal as the MFI of the unstimulated control was equal to MDDCs stimulated by LPS and gp120. LPS stimulated an approximately 2-fold increase in pJNK levels after 15 min of stimulation relative to the unstimulated control. Furthermore, pERK levels did not change in response to LPS which was contrary to the 5.5-fold and 1.4-fold increase above unstimulated controls previously reported (Emre et al., 2007; Olsnes, Olofsson & Aarstad, 2011). The variation between the studies could be due to different experimental conditions and varying basal phosphorylation of MAPKs between donors (Olsnes, Olofsson & Aarstad, 2011). After 15 min incubation with gp120 (CAP88 CI), pJNK levels increased approximately 2.5-fold, suggesting that activation had occurred at this time-point (Figure 5.3.2). However, this apparent increase was not observed for pERK and we thus tried to optimize the experiment further to reduce basal phosphorylation.

5.3.1.3 Effect of serum deprivation on MDDC ERK phosphorylation in response to PMA

Hamdorf *et al.* (2011) found high basal phosphorylation of ERK following differentiation of DCs from hematopoietic (CD34+) cells and they suggested that this basal activation was caused by IL-4 and GM-CSF in the differentiation medium (Caparros et al., 2006; Hamdorf et al., 2011). Furthermore, CSF has been shown to activate the MAPK pathways in mice (Smith et al., 2000). Studies by Caparros *et al.* (2006) deprived MDDCs of serum following

differentiation for 24 hours before stimulation to remove the effect of IL-4 and GM-CSF (Caparros et al., 2006). Therefore, after differentiation for 6 days, MDDCs were washed with pre-warmed PBS to remove all differentiation medium and were then rested O/N in serum-free medium before stimulation with phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C which results in phosphorylation of phosphotyrosine kinase and ERK (Chang et al., 2005). Irrespective of whether the MDDCs were rested or not the unstimulated control MFI did not change (735 Vs 763) whereas resting the MDDCs before PMA stimulation resulted in a 4-fold increase in MFI (2812 vs 633) (Figure 5.3.3). This could suggest that factors in the differentiation medium inhibited phosphorylation of ERK.

	Sample	Not rested (%)	Mean <PE>	Rested (%)	Mean <PE>
	Stimulated (PMA)	85.2	633	67	2812
	Unstimulated (Unstim) control	86.3	735	79.1	763

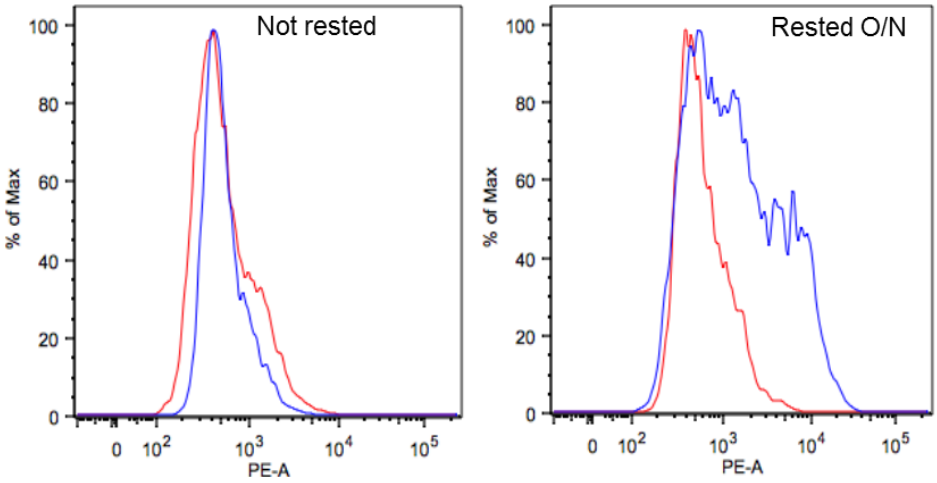


Figure 5.3.3 Phosphorylation of phosphotyrosine kinase after depriving MDDCs of serum. Day six MDDCs were either rested or not in serum-free medium for 24 hour before stimulation with phorbol 12-myristate 13-acetate (PMA) for 30 min and stained for phosphotyrosine-PE (pT-PE). Red histogram is unstimulated control and blue histogram indicates stimulated MDDCs from one donor. Each histogram was scaled up to the percentage of the maximum number of cells in each bin (denoted % of Max). Representative of two rested donors, one stimulated with PMA and the other stimulated with LPS and TNF-α+IL-1β.

5.3.1.4 Stimulation of rested MDDCs with LPS for varying lengths of time.

As resting MDDCs in serum-free medium led to phosphorylation of phosphotyrosine kinase in response to PMA, we stimulated rested MDDCs with LPS and determined pERK levels at different times. As LPS (500 ng/mL) stimulation did not result in robust stimulation of MAPK signal above that of background, IL-1β and TNF-α were included in these experiments to amplify the response. Levels of pERK increased with increasing time of stimulation with 30 minutes showing a 6.7-fold increase compared to unstimulated control (16000 vs 2365) (Figure 5.3.4). We then tested whether higher concentrations of LPS alone would also result in detectable increases in MAPK phosphorylation. We increased LPS concentration to 1 mg/ml and stimulated MDDCs for 5, 15 and 30 min to determine the best duration of stimulation (Figure 5.3.5). A fluorescent minus one (FMO) control was included to ensure that there was no spread of fluorescent signals from other channels. Auto florescence of unstained cells was negligible (15 – 96 for pERK and 181 – 400 for PE but greater than 600 most of the times for p38).

	Sample	Mean<Pac Blue>
	LPS + IL-1β +TNF-α 30 min	16000
	LPS + IL-1β +TNF-α 15 min	12263
	LPS + IL-1β + TNF-α 5 min	12866
	LPS + IL-1β + TNF-α 1 min	4358
	Unstimulated control	2365

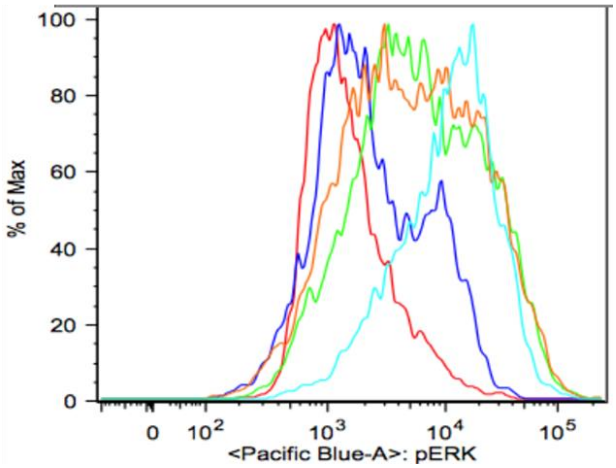


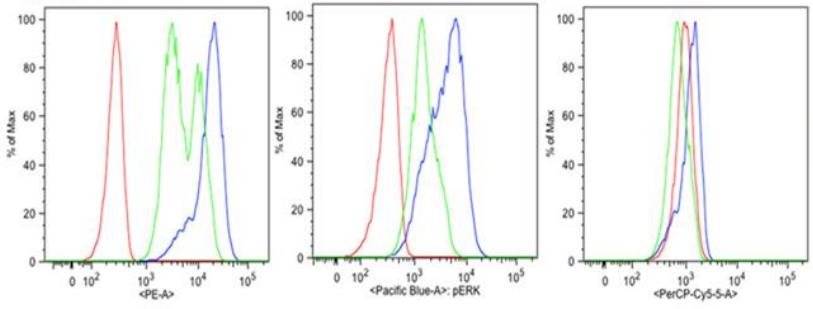
Figure 5.3.4 Phosphorylation of ERK after stimulating rested MDDCs with LPS, IL-1β and TNF-α. Day six MDDCs were either rested or not rested in serum-free medium for 24 hour before stimulation with LPS + IL-1β + TNF-α for various times and stained for pERK. The red histogram represents unstimulated controls, the blue histogram is 1 min stimulation, the green histogram is 5 min stimulation, the orange histogram indicates 15 min stimulation and the turquoise is 30 min stimulation. MFI is plotted and overlayed as the histograms.

The background was too high after 5 min of LPS stimulation to distinguish an increase in pJNK, pERK and p-p38 (Figure 5.3.5 A and D). However, after 15 mins of LPS stimulation MFI increased 2.5-fold for pJNK and 1.7-fold for pERK above the unstimulated control **(**Figure

5.3.5 B and D). Similarly, there was an increase in MFI for both pJNK and pERK after 30 min LPS stimulation. Although, there was an increase in MFI at 15 min (1.6-fold), LPS stimulation did not lead to phosphorylation of p38 at 30 min (Figure 5.3.5 B, C and D). The FMO control indicated that there was spill-over of pJNK-PE and pERK-PAC blue signals into the p-p38 channel, which might make it difficult to discriminate between positive versus negative signals for this p38 MAPK.

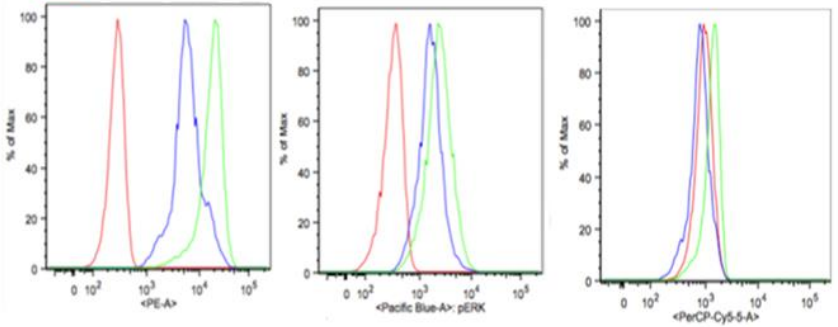
A)

	Sample	%	pJNK Mean <PE>	pERK Mean <Pac blue>	pP38 Mean <PerCP>
	LPS 5 mins	69.9	6835	1747	755
	Unstimulated 5 mins	65.4	19333	4964	1284
	FMO 5 mins	73.8	298	355	988



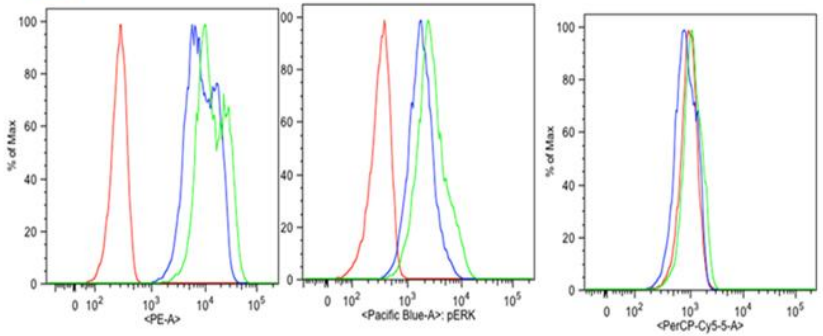
B)

	Sample	%	pJNK Mean <PE>	pERK Mean <Pac blue>	pP38 Mean <PerCP>
	LPS 15 mins	65.1	19094	2630	1319
	Unstimulated 15 mins	69.8	7688	1891	842
	FMO 15mins	73.8	290	345	988



C)

	Sample	%	pJNK Mean <PE>	pERK Mean <Pac blue>	pP38 Mean <PerCP>
	LPS 30 mins	60.2	17023	3394	1189
	Unstimulated 30 mins	57.2	10782	2036	903
	FMO 30mins	72.3	290	345	988



D)

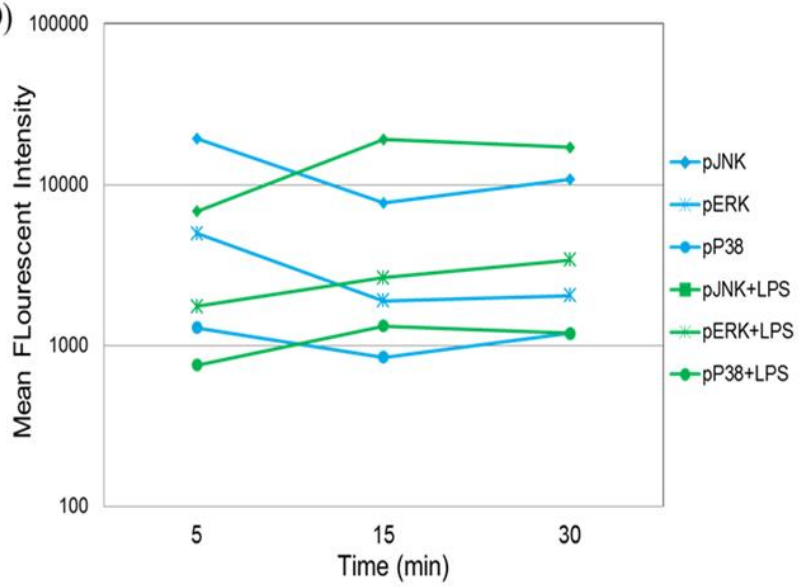


Figure 5.3.5 Optimising the duration of MDDC stimulation with LPS (1mg/mL). Day six MDDCs were washed and rested O/N in serum-free medium and stimulated with LPS at 1 mg/mL for A) 5, B) 15 and C) 30 min. Phosphorylation was measured by flow cytometry using antibodies to pJNK, pERK and pP38 conjugated to phycoerythrin (PE), pacific blue (Pac-Blue) and peridinin chlorophyll-a Protein-Cy5.5 (PerCP) fluorescent dyes, respectively. D) Mean florescent intensity (MFI) was plotted vs time for pJNK (diamond), pERK (star) and pP38 (circle) with (green lines) and without (blue lines) LPS.

5.3.2. Flow cytometry analysis of MDDC MAPK phosphorylation in response to pseudovirus

C12 and C13 PSVs were used to stimulate MDDCs and the effect of these Envs on phosphorylation of ERK, JNK and p38 were evaluated using flow cytometry. C12 and C13 were selected as they differed significantly in their ability to induce the secretion of IL-10 by MDDCs (Figure 4.3.1) (Manuscript in preparation) and also in their PNG profiles. Therefore, this study aimed to determine whether differences in genotype and phenotype influenced MDDC signaling evidenced by changes in JNK, ERK and p38 phosphorylation.

	Sample	%	pJNK Mean <PE>	pERK Mean <Pac blue>	pP38 Mean <perCP>
	C13 15 min	71.6	6197	3457	980
	C12 15 min	67.6	6118	3263	1113
	pSG3 15min	75.9	3785	1905	801
	LPS 15 min	75.8	8830	3346	1820
	Unstimulated 15 min	78.7	3294	2554	1007

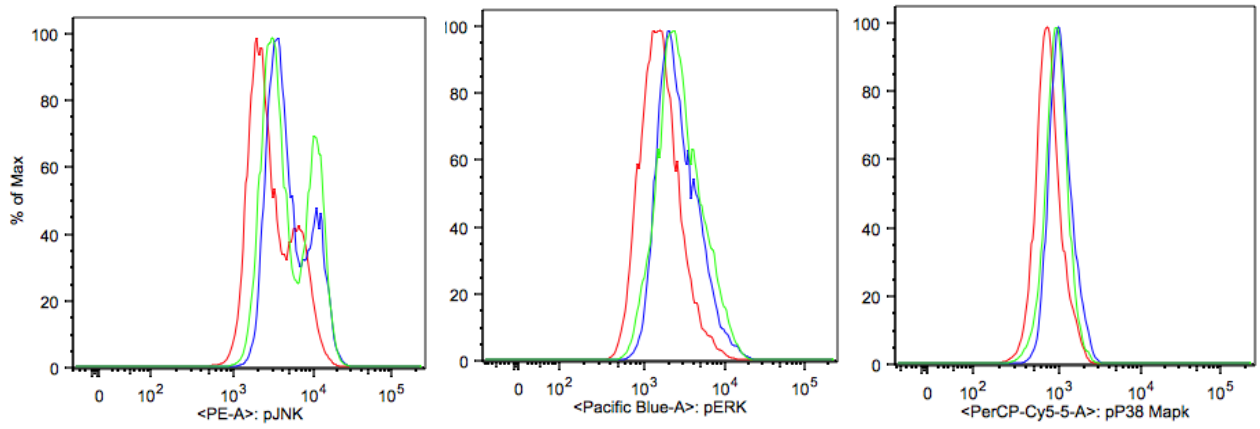


Figure 5.3.6 Impact of C12 and C13 on ERK, JNK and p38 phosphorylation. Day six MDDCs rested O/N in serum-free medium were stimulated for 15 min with C12 and C13 PSVs and the phosphorylation of ERK, JNK and p38 was measured by flow cytometry. Results are indicated as mean fluorescence intensity (MFI) and a representative of two experiments for pERK, pJNK and pP38 following 15 min of stimulation is shown. LPS was added as a positive control. The background control was HIV-1 backbone only (pSG3) to determine whether any increase in phosphorylation was due to the presence of other viral proteins present in the pseudovirus. Unstimulated control was MDCs to which conditioned medium was added.

The effects of both negative (unstimulated and pSG3) controls on MDDC stimulation were nearly identical for both pERK and pJNK and thus fold-increase in MFI for LPS and PSVs were calculated relative to the pSG3 control (Figure 5.3.6). LPS stimulated a 1.8-, 2.3- and 2.3-fold increase in MFI for pERK, pJNK and p-p38, respectively, indicating that signaling pathways involving activation of ERK, JNK and p38 were stimulated as expected. These results were similar to a previous study, which showed that LPS increased phosphorylation of pERK, JNK and p38 approximately 2-fold, 4-fold and 2.5-fold, respectively after 15 min stimulation of immature MDDCs (Fairman & Angel, 2012). C12 and C13 induced a 1.8-fold and 1.7-fold increase above the pSG3 control for pERK, respectively, and both PSVs stimulated a 1.6-fold increase in MFI for pJNK. This suggests that there was no difference between these two Envs in activation of ERK- and JNK-mediated signaling pathways. Despite an increase in p-p38 in response to C12 and C13 relative to the pSG3 control (1.4- and 1.2-fold), this apparent increase was lost when calculated relative to the unstimulated control (1-fold), suggesting that phosphorylation of p38 was not stimulated by PSV. However, the FMO control did suggest that spillover between channels could influence the detection of p-p38 by flow cytometry and we were thus unable to conclude whether p38 was activated in response to Env or not.

5.3.3 Western blot analysis of MDDC ERK phosphorylation in response to pseudovirus

To confirm our results obtained using flow cytometry, we also used western blotting (WB) to test MDDC ERK and JNK phosphorylation in response to C12 and C13 PSVs (Figure 5.3.7). However, pJNK was not detected using WB most likely due to poor antibody specificity. For pERK analysis, unstimulated and pSG3 controls were very similar and LPS stimulated a 2-fold increase in pERK above background similar to that observed with flow cytometry (1.8-fold), indicating that LPS activated ERK to the same extent **(**Figure 5.3.7). C12 and C13 were previously tested for MAPK phosphorylation by flow cytometry and the results of the WB were identical with a 1.8-fold increase in pERK MFI for both clones, confirming that C12 and C13 were both equally able to stimulate the activation of ERK-mediated signaling pathways. Overall, there was a good correlation between the results obtained using WB and flow cytometry.

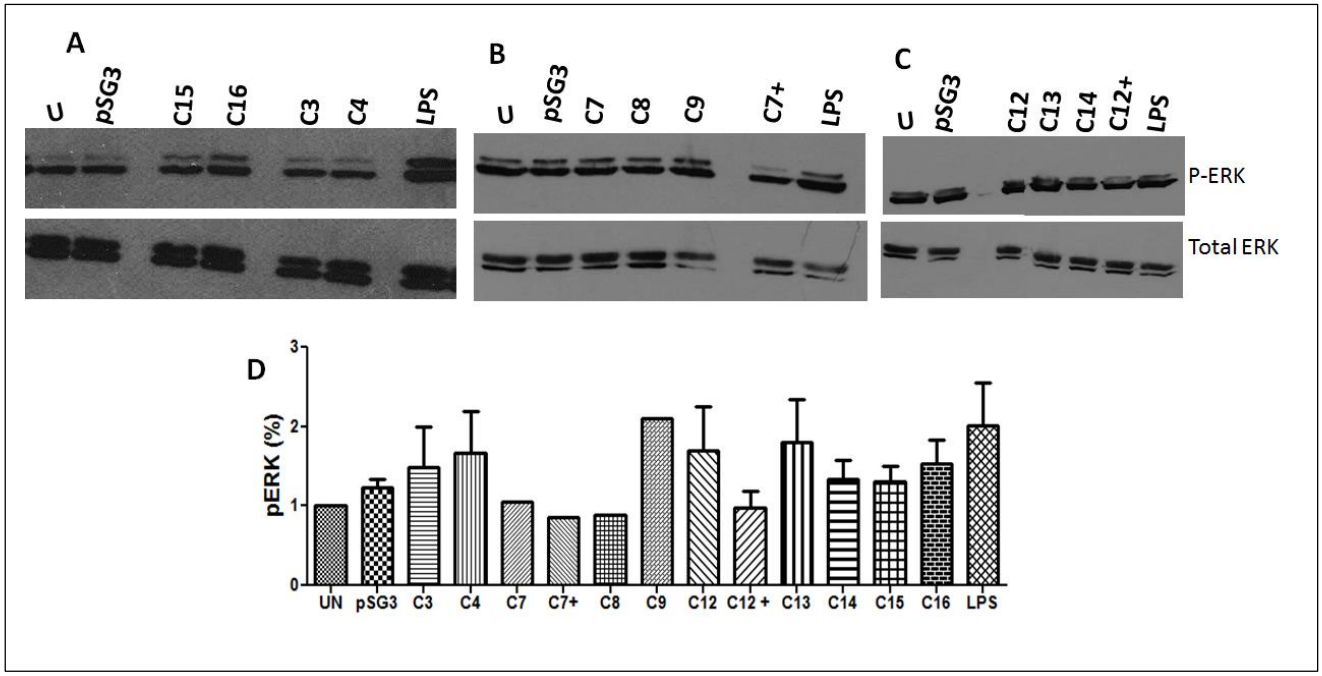


Figure 5.3.7 Western blot analyses of MDDC MAPK phosphorylation following stimulation with PSVs. Day six MDDCs were stimulated (after serum depriving O/N) with PSVs (C15, C16, C3, C4, C12, C13, C14, C7, C8 and C9) for 15 minutes, the cells were lysed and pERK measured by Western blotting using phosphor-p44/42 MAPKs and total ERK (ERK1/2) (Cell Signaling, Cat#9101/ #9102). C12 and C7 PSV were also used to stimulated MDDCs pre-incubated with anti-DC-SIGN Mab (Clone DCN46, ARP 7682) (denoted C7+ and C12+) to measure pERK with and without DC-SIGN inhibition. Representative Western blots of pERK and total ERK for two donors A), five donors B) and two donors C) are shown. Gene tool (Image J) was used to quantify band intensities and the ratios of pERK to total ERK densities were calculated relative to the unstimulated control for each donor. The mean pERK relative to Unstimulated controls was plotted in GraphPad prism 5.0 (D) with error bars indicating SEM. Mann Whitney test showed no statistically significant difference between pERK levels between different PSVs.

Due to limited access to a flow cytometry facility, other Env clones were compared for MAPK phosphorylation using WB to determine if ERK activation could be associated with clone-specific N-glycosylation, DC-SIGN binding and Env entry efficiency. ERK phosphorylation differed across the clones from 1- to 2-fold with C7 and C8 similar to the unstimulated control and C9 and LPS both stimulating a 2-fold increase in pERK (Figure 5.3.7). C7 and C9 had the same TZM-bl entry efficiency, confirming that Env function is not a prerequisite for activating signaling pathways. This was supported by the lack of correlation between ERK activation and Env entry efficiency. C3, C4, C15 and C16, were selected as they differed in mannosylation as well as number and position of PNGs. There was no difference in the phosphorylation of ERK when MDDCs were stimulated with C15, C16, C4 and C3 (Figure 5.3.7 A and D) even though C15 had more HM/H type N-glycans than C16 and C4 was more mannosylated than C3. Similarly, correlation analysis did not support a relationship between Env mannosylation and activation of ERK. However, the small sample size limits the extent of our conclusion.

5.3.4 Inhibition of MAPK phosphorylation by recombinant DC-SIGN

DC-SIGN binds HIV via Env N-glycans, is involved in *trans*-infecting CD4+ T cells and stimulating MDDCs to release IL-10 (Geijtenbeek, Kwon, et al., 2000; Kwon et al., 2002; Shan et al., 2007); (Figure 4.3.3; Manuscript in preparation). To investigate whether DC-SIGN was involved in MAPK phosphorylation in response to PSV stimulation, MDDCs were stimulated with C12 and C13 PSVs after inhibiting DC-SIGN interaction using recombinant DC-SIGN protein and pERK and pJNK were measured by flow cytometry. Pre-incubation of C12 with recombinant DC-SIGN lowered fluorescent signal by apparently 30.9% and 31.04% for ERK (2075 to 1438 MFI) and JNK (15579 to 10743 MFI), respectively indicating that preventing PSVs from binding MDDC DC-SIGN influenced MAPK phosphorylation levels (Figure 5.3.8). However, when background was subtracted from the C12 _DC-SIGN values, there seem to be a 100% inhibition of pERK and pJNK for C12, but considering the background variatons across donors it was compared to unstimulated control. Stimulation with C13 resulted in only a 12.9% reduction (data not shown), much lower than C12. The difference between C12 and C13 mimicked IL-10 secretion when C12 was more sensitive to inhibition by recombinant DC-SIGN than C13 (Figure 4.3.3). Complete inhibition of pERK and pJNK by recombinant DC-SIGN was not observed, suggesting that other receptors are also involved in MDDC MAPK signaling activation. However, LPS barely stimulated the phosphorylation of JNK and ERK (1.3-, and 1.2-fold, respectively), contrary to what was previously observed, suggesting that MDDC donor variation might have influenced the outcome of the experiment.

The data observed for flow cytometry analysis was confirmed using WB. When anti-DC-SIGN Mab (Clone DCN46, ARP 7682) was pre-incubated with MDDCs, there was a 42.7% reduction in ERK phosphorylation in response to C12 PSVs, (Figure 5.3.7), confirming that DC-SIGN-Env interactions are in part responsible for ERK activation. C7 PSVs were also pre-incubated with recombinant DC-SIGN which resulted in 18.6% decrease in ERK phosphorylation most likely because C7 was a poor activator of ERK phosphorylation (Figure 5.3.7). CD4 receptor is not involved in phosphorylation of pERK as addition of soluble CD4 to C7 PSVs before adding to the MDDCs had no effect on ERK phosphorylation (Appendix I, Figure A3). Despite, partial inhibition of ERK phosphorylation following DC-SIGN inhibition, there was no correlation between PSV DC-SIGN binding and pERK levels probably because of the small sample size.

	Sample	%	pJNK Mean <PE>	pERK Mean <Pac blue >	pP38 Mean <Per-CP>
	C12 + DC-SIGN 15 mins	74.7	10743	1438	773
	C12 15 mins	77.5	15579	2075	491
	LPS 15 min	79.6	14654	1778	429
	Unstimulated 15 min	81.6	11344	1537	641

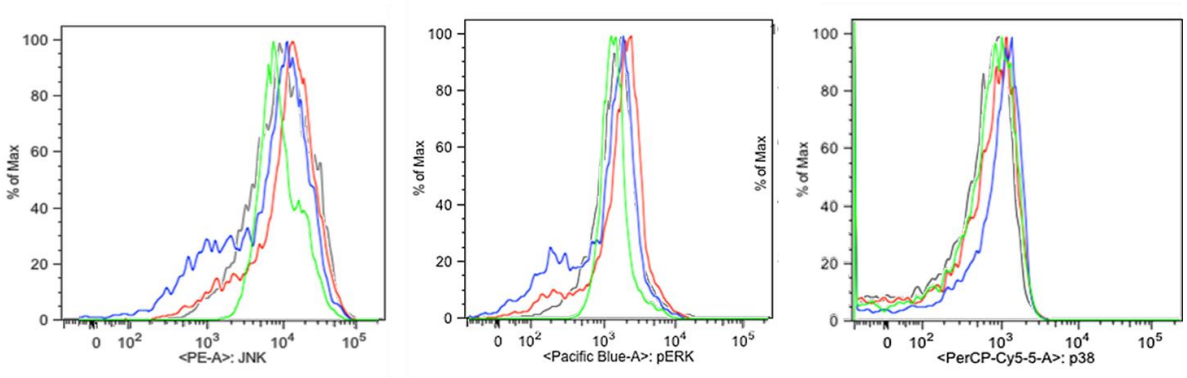


Figure 5.3.8 MAPK phosphorylation in the presence of soluble DC-SIGN. Day six MDDCs were stimulated with C12 pre-incubated with (blue line) and without (red line) recombinant DC-SIGN) for 15 minutes and pERK, pJNK and p-p38 measured by Flow cytometry. A representative results of two donors (from two independent experiments) stimulated with C12 is shown here with histograms generated by Flowjo analysis following stimulation with PSV. Controls included a medium only (unstimulated) control (green line) and LPS (black line).

5.3.5 Phosphorylation of ERK and JNK by Env protein

Env mediates first contact with target cells during HIV infection and is thus the preferred vaccine immunogen. As PSVs comprise not only Env but also other viral proteins, we tested purified soluble Env protein, gp140 to activate MAPK phosphorylation and flow cytometry was used to detect pERK and pJNK. C12 and C13 gp140 were used to stimulate MDDCs following triton extraction of all endotoxins. Similar to previous experiments LPS increased the MFI of pERK and pJNK by 1.7-fold and 2.6-fold compared to the unstimulated control. Compared to the unstimulated control, there was an increase in signal when C12 gp140 was used to stimulate MDDCs for 15 min (Figure 5.3.9) and the pattern was similar for both ERK (1.5-fold) and JNK (1.7-fold), suggesting that C12 gp140 was stimulating the phosphorylation of these two kinases. However, C13 gp140 did not seem to have the same effect as C12 on JNK (1.3-fold) and ERK (1.1-fold) although the difference between the two clones did not reach significance (Figure 5.3.9). C12 was also a much better inducer of MDDC IL-10 secretion than C13 so it is possible that there is an association between the activation of JNK and ERK and

IL-10 release for this clone. However, the increase in pERK and pJNK required for changes in expression of inflammatory cytokines is not known.

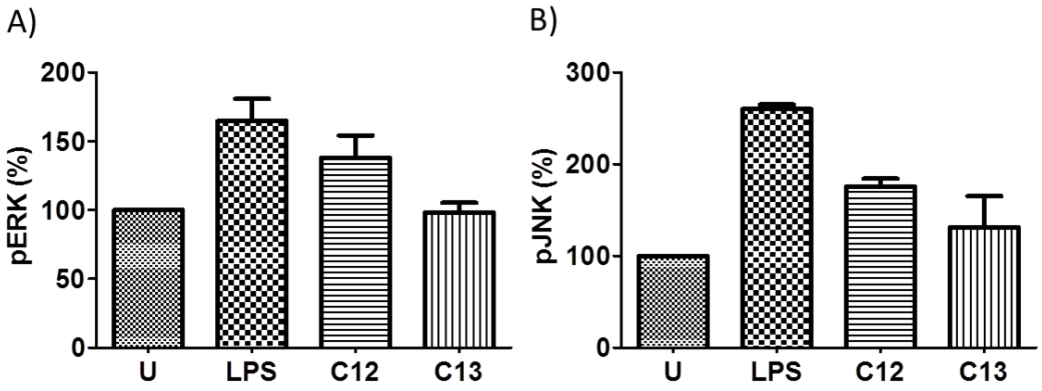


Figure 5.3.9 MAPK phosphorylation by purified gp140 protein. Day six MDDCs were rested O/N at 37ºC in serum free medium and stimulated with gp140 protein for 15 min before A) pERK or B) pJNK was measured by flow cytometry. The acquired flow cytometry data was analyzed using Flowjo software and the MFI from two biological repeats expressed as a percentage of the unstimulated control is shown with error bars indicating standard error of the mean (SEM). ANOVA showed no significant difference between C12 and C13.

5.3.6 Impact of specific Env N-glycans on the phosphorylation of ERK and JNK

We were unable to determine conclusively whether C12 and C13 differentially stimulated ERK and JNK phosphorylation as a result of their N-glycosylation differences as stimulation with PSVs and gp140 yielded differing results. Therefore, to determine if N-glycans are important for MAPK phosphorylation, we deleted PNGs at positions N241, N262, and N448 of Env and investigated whether or not the loss of these PNGs affected the ability of C15 and C16 PSVs to activate ERK and JNK. Previously, we showed that deletion of individual PNGs had varying effects on C15 and C16 function, suggesting that they influenced Env structure and function and that some clones were more sensitive to mutation than others. Deletion of the PNGs at N262 and N448 lead to a decrease and an increase in C16 entry efficiency, respectively (Figure 3.3.11). The C16 N262 PNG mutant decreased the MFI of pERK compared to WT whereas N448 PSV increased pERK and pJNK after 15 min stimulation (Figure 5.3.10 A and C). The change in pJNK in response to C16 N262 was not as apparent, but this is likely due to the low pJNK MFI induced by WT (Figure 5.3.10 A). Similar to the C16 mutants, when the PNG was

deleted at N262 of C15, stimulation of MDDCs with this mutant resulted in lower pERK and pJNK compared to the WT. Deletion of N262 abrogated the entry efficiency of C15 and overall, these findings suggest that structural integrity of Env is required for activating JNK- and ERK-mediated signaling pathways. This suggests that there are instances when non-functional Env (like C15 N262Q) is still able to activate signaling pathways of ERK and JNK although not as much as the functional Env. However, while deletion of the PNG at N241 significantly lowered entry efficiency of C15 it did not have a deleterious effect on the phosphorylation of ERK and JNK compared to WT (Figure 5.3.9 B and D), suggesting that both ERK and JNK phosphorylation was not influenced by the PNG at N241.

Previously we showed that PNGs at N130 and N332 of gp120 could be involved in inducing MDDCs to release IL-10. As this study hypothesized that activation of ERK-mediated signaling pathways could be involved in activating IL-10 expression and release, we wanted to determine whether N130 and N332 was also associated with phosphorylation of ERK When we compared pERK levels between PSVs with and without N130 and N332, there was no correlation between the PNGs and phosphorylation of ERK. This supported the lack of association between pERK levels and IL-10 secretion and suggests that although the ERK-mediated signaling pathway is differentially activated by Env clones, this does not translate into concomitant changes in IL-10 secretion. The lack of 100% inhibition by recombinant DC-SIGN could suggest that receptors other than DC-SIGN are also involved and that activation of alternative signaling pathways masks the relationship between MDDC stimulation of pERK and IL-10 secretion. This could confirm previous findings by van Vliet *at al*. (2007) that the interaction of PAMPs with DCs leads to activation at times of multiple receptors and the effect on transcription factor and inflammatory gene expression is the outcome of the integration of these signals (van Vliet et al., 2007).

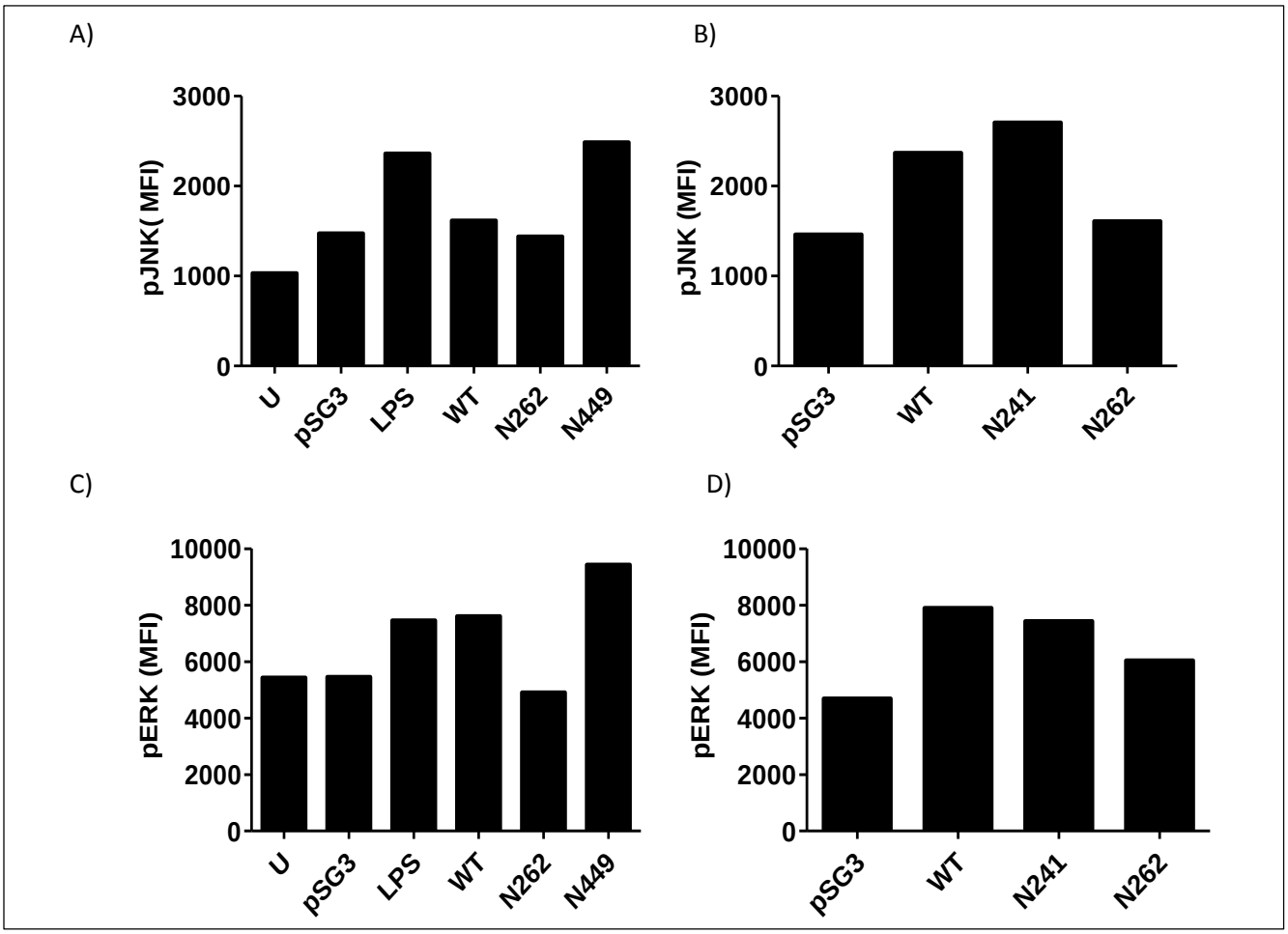


Figure 5.3.10 Impact of specific PNGs on the phosphorylation of MAPK in MDDCs. Day six MDDCs were stimulated with C16 (A and C) and C15 (B and D) and their mutant PSVs and the phosphorylation of JNK (A and B) and ERK (C and D) were measured by flow cytometry. PSG3 pseudovirus lacking Env was used as a negative control and LPS as a positive control for MAPK activation. Bar graphs are representative of two independent experiments.

5.4 Discussion

Recombinant gp120 influenced the expression pattern of a number of surface DC proteins (Williams, Trout & Spector, 2002) and induced atypical maturation of DCs accompanied by functional changes (Fantuzzi et al., 2004). HIV-1 and *M. tuberculosis* have been shown to target DC-SIGN to suppress DC function and establish infection (Geijtenbeek et al., 2003; Cunningham, Harman & Donaghy, 2007; Den Dunnen, Gringhuis & Geijtenbeek, 2009; Gringhuis et al., 2009). The pathogenic products of *M. tuberculosis* and HIV-1 (ManLAM and gp120 mannose residues, respectively) induce Raf-1 phosphorylation and in the case of HIV-1, activated Raf-1 promoting LTR activation and formation of full length HIV transcript in DCs (Gringhuis et al., 2010). This suggests that signaling via Raf-1 might be important for pathogens that escape immune surveillance and subvert DC responses to establish infection. We hypothesized that to facilitate its transmission and survival, HIV-1 deregulates DC-mediated innate immunity by activation of signaling pathways that result in the aberrant expression of cytokines that favour viral replication.

Gringhuis *et al.* (2007) found that binding of ManLAM to DC-SIGN activated Raf-1 of the Ras-Raf-MEK-MAPK signaling pathway which resulted in NF-kB-stimulated expression of cytokines, including IL-10 (Gringhuis et al., 2007; Geijtenbeek & Gringhuis, 2009). As Env N-glycans interact with DC-SIGN, we determined if Env PNGs played a role in activating ERK, JNK and p38 (Johnson & Lapadat, 2002; Zhang, Wei , Tu Liu, 2002; Wellbrock, Karasarides & Marais, 2004). MAPKs have previously been targeted for immune-based therapies and could thus also be targeted for HIV-1 therapy (Johnson & Lapadat, 2002; Wellbrock, Karasarides & Marais, 2004). HIV-1 inhibits maturation of DCs; ERK phosphorylation, and to a lesser extent JNK phosphorylation, is said to inhibit maturation of DCs, whereas p38 phosphorylation is required for DC maturation (Nakahara et al., 2006). Therefore, other than stimulating the release of IL-10, Env could also modulate the maturation of DCs and thus the adaptive immune response.

PSVs were used to induce phosphorylation of the MAPKs and pERK, p-p38 and pJNK were detected using flow cytometry. Flow cytometry experiments were optimised to improve detection of pERK, pJNK and p-p38. We determined that the optimum stimulation time was 15 min and resting MDDCs in serum-free medium before stimulation with PSVs was required to obtain a signal above the background. Although, the fold-increase in signal above

background in response to LPS was quite low, this was comparable to some studies (Emre et al., 2007; Olsnes, Olofsson & Aarstad, 2011). In some experiments PSVs from many participants were used to stimulate the same donor MDDC, but most comparisons used different MDDC donors per participant pair due to the limited amount of MDDCs produced from some donor PBMCs. Although variation between donors was taken into account when MFI values from each donor were normalized to the unstimulated control, we cannot discount the possible effect that donor variation could have in these experiments.

HIV infection inhibited DC maturation (Fairman & Angel, 2012) and resulted in a transient increase in pERK levels and a sustained increase in p38 activation. Inhibition of p38 resulted in DCs unable to migrate along a chemokine gradient and the authors suggested that HIV-induced p38 activation facilitated DC-mediated CD4+ T cell *tran*s-infection (Wilflingseder et al., 2004). Phosphorylation of p38 above background was not observed for Env stimulation using flow cytometry despite increasing the length of stimulation, and buffer composition used for cell permeabilisation. Although, LPS is a well-known activator of p38, we were unable to show a robust response using flow cytometry. Previous studies showed that p38 was not activated in 30% DC donors in response to HIV (Wilflingseder et al., 2004) and that response to activators is cell-specific and varied according to physiological conditions (Zarubin & Han, 2005). It is thus possible that the lack of apparent p38 activation could be due to donor variability or as previously discussed, overlapping signals in the p38 channel. However, a previous study found that cross-linking DC-SIGN with an antibody lead to enhanced *il-10* transcription and phosphorylation of ERK1/2 and Akt but not of p38 (Caparros et al., 2006). This suggests that DC-SIGN ligands could trigger alternative signaling pathways that lead to the phosphorylation of ERK but not p38.

Flow cytometry and Western blotting indicated that C12 and C13 PSVs stimulated MDDC ERK and JNK phosphorylation equally. This was contrary to the observation that purified C12 gp140 stimulated phosphorylation of ERK and JNK better than C13 gp140. The ratio of functional: non-functional trimers could differ between PSVs and purified gp140 with the latter more likely to be mostly monomers (Go et al., 2015; Pritchard, Harvey, et al., 2015), suggesting that PSVs would represent a more physiologically relevant model. However, as C12 and C13 differed in mannosylation and PNG number and C12 also bound better to DC-SIGN, *trans*-infected TZM-bl cells more efficiently and induced significantly more IL-10 secretion than C13, it was tempting to speculate that C12 gp140 was a better inducer of ERK and JNK

phosphorylation because it was more heavily N-glycosylated and bound with higher efficiency to DC-SIGN. Furthermore, C12 represents a transmitted founder variant and C13 its chronic infection counterpart, suggesting that variants at transmission might be stronger activators of MDDC ERK signaling, leading to inhibition of DC maturation, immune deregulation and thus providing a fitness advantage. However, correlation analysis indicated that there was no relationship between ERK phosphorylation and Env mannosylation, entry efficiency, DC-SIGN binding and *trans*-infection.

Although, other viral proteins such as Tat have also been shown to activate the MAPK signaling pathway, these should not influence apparent differences between PSV clones as these proteins are identical in all pseudoviral preparations (Toschi et al., 2006). However, it is possible that the combined signal from gp120 and Tat might lower the sensitivity of the PSV induction assay so that small differences between C12 and C13 are less easily detected than when gp140 are compared. It was also shown that binding to CCR5 triggered signaling via Pyk2, p38 MAPK, and ERK1/2 signaling pathways (Wilflingseder et al., 2004), resulting in the activation of STAT3 involved in p38 MAPK–NF-κB pathways and IL-6 secretion in DCs. it is possible that signaling via CCR5, and not only DC-SIGN regulates MAPK phosphorylation (Cornò et al., 2014). The authors suggest that increased expression of IL-6 leads to constitutive activation of STAT3 which plays an important role in deregulation of DC immune function. However, DC-SIGN-gp120-induced IL-10 secretion down-regulated Nef-stimulated IL-6 expression, suggesting that cross talk between signaling pathways activated by gp120 and Nef could further complicate the comparison between PSVs and gp140 (Sarkar, Mitra & Chakrabarti, 2013). In this study, soluble CD4 did not inhibit PSV-stimulated ERK phosphorylation, suggesting that CCR5-mediated signalling was not playing a role in ERK phosphorylation.

Furthermore, DC-SIGN ligands stimulate alternative cellular responses: the peanut allergen (Ara H1) and Schistosoma egg antigen caused ERK phosphorylation (Shreffler et al., 2006) that lead to impaired IL-12 production and a Th2 biased immune response whereas Salp15 activated a Raf-1/MEK pathway that promoted TNF and IL-6 mRNA decay and decreased the production of IL-12 (Reviewed in (Sancho & Reis e Sousa, 2012). An anti-DC-SIGN antibody stimulated ERK and impaired IL-12 production (Caparros et al., 2006). It was also shown that HIV-1 activated Raf-1 but instead of ERK this lead to the activation of scaffold proteins (LSP1, CNK and KSR1) in the Ras-Raf-1/MEK signaling pathway (Gringhuis et al., 2009). Therefore,

it is possible that DC-SIGN-dependent Raf-1 activation by PSVs leads to a signaling cascade that bypasses ERK. The lack of correlation between ERK phosphorylation and Env mannosylation, entry efficiency, DC-SIGN binding and *trans*-infection is most likely due to the highly complicated nature of HIV-1 activation of MDDC signaling pathways.

It has been suggested that particular Env PNGs interact with DC-SIGN and therefore the presence or absence of specific N-glycans may be more important than overall levels of mannosylation for activating ERK-mediated pathways. Both Shan *et al.* (2007) and Nabatov *et al.* (2006) suggested that subtle differences in PAMPs might influence the interaction between DC-SIGN and its ligands (Nabatov et al., 2006; Shan et al., 2007) and thus DC signaling pathways. We had mutated Env at PNGs previously identified to play a role in DC-SIGN binding (Hong et al., 2007; Go, Hewawasam, et al., 2011; Liao et al., 2011), but found that deletion of most sites reduced PSV entry efficiency. In this study we tested PSVs with PNGs deleted at N241, N262, and N448 as these were not deleterious to all Envs and were shown to be enriched with high mannose type N-glycans (Go, Hewawasam, et al., 2011). Deletion of N262 in C16 reduced pERK levels and this corresponded to the drop in entry efficiency observed for the mutants. Similarly, deletion of N448 not only increased C16 entry efficiency, it also increased ERK phosphorylation. Together this would suggest that the native structure of Env is required for the activation of ERK-mediated pathways. However, deletion of N241 which reduced C15 entry efficiency had no effect on pERK levels, suggesting that poorly functional Env (50 - 60% decrease in entry efficiency compared to WT) can still activate DC signaling pathways. Although, deletion of specific PNGs lead to changes in ERK phosphorylation, we cannot conclude that N241, N262 and N448 are essential for activation of ERK-mediated signaling pathways as the deletions also affected Env function. An HIV vaccine needs to comprise highly immunogenic molecules irrespective of whether it retains function or not. Therefore, as PNG deletions influence ERK activation, Env immunogens comprising N-glycan motifs can thus be designed to elicit the most favourable DC-mediated immune responses for viral clearance.

ERK phosphorylation induced via DC-SIGN lead to activation and expression of IL-10 (Caparros et al., 2006; Shan et al., 2007), suggesting that those clones that induced higher levels of pERK should also induce the secretion of more IL-10. We did not find a statistically significant correlation between IL-10 secretion and ERK phosphorylation although C12, an efficient inducer of IL-10 also activated ERK. The lack of association between ERK

phosphorylation and IL-10 secretion could be due to the small sample size as we only had overlapping data for seven clones. It is also possible that Env binds to DC surface receptors other than DC-SIGN and thus inhibition studies were carried out to determine the importance of DC-SIGN-Env interactions on MAPK phosphorylation. Stimulation of MDDCs with C12, C13 and C7 PSVs pre-incubated with recombinant DC-SIGN only partially inhibited MAPK phosphorylation, confirming that other receptors besides DC-SIGN, and thus signaling pathways, were involved. However, pre-incubation with soluble CD4 had no effect on MAPK phosphorylation (Appendix I, figure A3), ruling out this receptor as a potential player. Gringhuis et al (2007) suggested that DC-SIGN cannot activate NF-kB p56-mediated expression of IL-10, IL-12 and IL-8 in the absence of pre TLR triggering (Gringhuis et al., 2007; Geijtenbeek & Gringhuis, 2009). Other PRRs such as CLRs like MR and DCIR of MDDCs have already been suggested to also play a role in binding HIV (Turville et al., 2001, 2002; Lambert et al., 2008). As stimulation of IL-12 production was poor, dectin 1 or dectin 2 might not be involved as these CLRs induce both IL-10 and IL-12 equally. Other non-CLR could also be important as they have been suggested to play a role in *trans*-infection (Saphire et al., 2001; Magerus-Chatinet et al., 2007; Izquierdo-Useros, Lorizate, Puertas, et al., 2012; Puryear et al., 2012; Izquierdo-Useros et al., 2014) The involvement of other receptors could also explain why there was no correlation between ERK phosphorylation and IL-10 secretion, mannosylation, entry efficiency, DC-SIGN binding and *trans*-infection.

Conclusion

We can conclude that Env triggers signal transduction in MDDCs that differentially activates and phosphorylates ERK and JNK. Some Env were better inducers than others, suggesting that vaccine design could utilise this approach to design highly immunogenic candidates. DC-SIGN is only one of the receptors involved in activating ERK and further study is required to identify any others. Changes in Env N-glycosylation seemed to be associated with ERK phosphorylation, although there was no clear relationship between overall levels of mannosylation or specific PNGs with ERK phosphorylation. Although PSVs differed in their ability to stimulate phosphorylation of ERK with C12 better at both IL10 and ERK phosphorylation, there was no association with their ability to induce IL-10 secretion. This is likely because PSVs activated more than one cross-linked signaling pathway so that IL-10 secretion was induced by more than one stimulus.

Chapter 6: Conclusion

Despite contradictory evidence, it is generally accepted that HIV infection is linked to aberrant cytokine expression and disruption of CD4+ T helper and CD8+ cytotoxic T cell function and that disease progression is mainly associated with a Th2 cytokine profile (Graziosi et al., 1994; Maggii et al., 1994; Sarih, Mabtaoui & Benslimane, 1996; Mellado et al., 1998). More recently, increased levels of genital tract inflammatory cytokines and chemokines such as MIP-1α, MIP-1β, and IP-10, were associated with an increased risk of HIV acquisition, further suggesting that female genital tract inflammation is important for HIV infection (Masson et al., 2015). Inflammation could attract target cells to sites of HIV infection, facilitating replication.

Modulation of immune responses by HIV could be mediated by Env N-glycans interacting with DC DC-SIGN (Hong et al., 2002, 2007; Lin et al., 2003; Liao et al., 2011; Montfort et al., 2011) and inducing the release of IL-10 (Ameglio et al., 1994; Shan et al., 2007) that culminates in a shift from Th1 to Th2 adaptive responses (Gessani et al., 1997; Mellado et al., 1998). IL-10, an anti-inflammatory cytokine plays a very important role in directing immune responses towards the Th2 route (Fiorentino et al., 1991; Fantuzzi et al., 2004; Singh & Thirumalapura, 2014). Furthermore, inhibition of IL-10/IL-10Rα interactions enhanced T cell activity against HIV Env although this effect was lost upon depletion of CD4 T cells (Landay et al., 1996). These findings led to the hypothesis that for productive clinical infection to occur increased IL-10 levels down regulate the maturation, but allow migration of DCs with infectious virus to lymph nodes, enhance cell-cell viral transfer via virological synapses and stimulate aberrant T cell differentiation and proliferation. When TF Envs were discovered to: 1) have fewer N-glycan sites (Derdeyn et al., 2004; Chohan et al., 2005; Sagar et al., 2006) 2) that were enriched with high mannose residues (Go, Hewawasam, et al., 2011), 3) mannose type N-glycans bound DC-SIGN (Lin et al., 2003; Montfort et al., 2011) and this interaction with DC-SIGN stimulated the release of IL-10 (Shan et al., 2007), we hypothesised that IL-10 could play an important role in HIV transmission and pathogenesis. However, these *in vitro* studies did not test whether inflammatory cytokines were also released by MDDCs in response to HIV stimulation. We found high levels of IL-8, IL-6, TNF-α, MIP-1α and MIP-1β secreted in response to PSVs. To understand how PSVs manipulated the release of DC cytokines, we determined whether N-glycosylation influenced Env's function, binding to DC-SIGN, ability to induce DC cytokines and activation of alternative MDDC signaling pathways.

We confirmed previous findings that the number of PNGs increased over the course of infection (Derdeyn et al., 2004; Chohan et al., 2005; Sagar et al., 2006), with sites carrying a heterogeneous mix of high mannose and complex-type N-glycans (Go et al., 2015). Although there was a positive correlation between PNG number and level of mannosylation the number of high mannose residues did not influence entry efficiency as reported in previous studies (Montefiori, Robinson Jr. & Mitchell, 1988; Binley et al., 2010; Eggink et al., 2010; van Montfort et al., 2011; Shen, Raska, et al., 2014). Mannosylation was associated with DC-SIGN binding (Lin et al., 2003; Montfort et al., 2011), and with DC-SIGN mediated *trans*-infection of TZM-bl cells, suggesting that the more high mannose residues, the better Env pseudovirus bound DC-SIGN and the more it was transferred to TZM-bl cells. The relationship was not apparent when MDDCs were used to *trans*-infect TZM-bl cells, probably because of the presence of other receptors on MDDCs which might have contributed to the process of *trans*-infection.

We deleted PNGs identified to be involved in binding DC-SIGN and PNGs of TF Envs enriched with high mannose residues (Hong et al., 2007; Go, Hewawasam, et al., 2011; Liao et al., 2011) in two Env clones. We found that deletion of conserved PNGs was deleterious and that the effect was Env-specific, with one clone more sensitive to loss of PNGs than the other. Due to the loss or significant reduction of function, we were unable to identify PNGs that were essential for interaction with DC-SIGN or *trans*-infection using site-directed mutagenesis. Comparing Env phenotype based on the presence or absence of specific PNGs in their sequences proved a better approach at identifying potential PNGs essential for Env function.

We confirmed that N392 was important for entry efficiency by both deletion and sequence analysis and N262 (which we identified by SDM) and N392 were important for Env structure (François & Balzarini, 2011; Wang et al., 2013; Mathys et al., 2014). Sequence analysis also showed that the presence of N356, and N392 were associated with enhanced Env entry efficiency, and N356, and lack of N674 for enhanced *trans*-infection. Moreover, a strong positive correlation was found between entry efficiency and *trans*-infection, supporting the rationale that the better the pseudovirus is able to enter TZM-bl cells, the better the *trans*-infection. As entry efficiency of Env seemed to play a determining role in *trans*-infection, we normalised Raji-DC-SIGN-mediated *trans*-infection to direct infection before correlation analysis with DC-SIGN binding. Raji-DC-SIGN-mediated *trans*-infection remained significantly correlated with DC-SIGN binding, further supporting studies by Geijtenbeek *et*

al. (2000) of the importance of DC-SIGN in *trans*-infection (Geijtenbeek, Torensma, et al., 2000).

Similar to Shan *et al*. (2007), this project found that the interaction of gp120 with MDDC- DC-SIGN induces IL-10 secretion and that levels of IL-10 released varied across Env clones. Despite the variation in IL-10 levels between Envs, there was no association with number of PNGs or Env mannosylation. This was surprising as PNG number and mannosylation correlated with DC-SIGN binding and blocking of DC-SIGN interaction using recombinant DC-SIGN protein inhibited MDDC IL-10 secretion. However, it was previously suggested that instead of Env mannosylation, differences in Env structure other than its N-glycosylation could also influence DC-SIGN-Env interaction (Nabatov et al., 2006) and could influence IL-10 release. Alternatively, DC-SIGN binds to complex-type N-glycans (Liao et al., 2011) and/or N-glycans have to be spaced correctly within Env native structure for binding to DC-SIGN (Hong et al., 2007; Liao et al., 2011) in a specific way as to trigger IL-10 release.

Further sequence analysis based on the presence or absence of specific PNGs identified that possession of PNGs at N130 and N332, with the latter forming part of the mannose patch close to the V3 loop were significantly more associated with Envs that induced more IL-10 secretion than those that did not, suggesting that PNGs at specific sites were important. Go *et al*. (2011) showed that N-glycans at N130 and N332 of subtype C Env were complex- and high mannose-type, respectively (Go, Hewawasam, et al., 2011; Pritchard, Spencer, et al., 2015) and thus the mechanism whereby they might influence Env N-glycan-DC-SIGN binding and MDDC IL-10 release remains unclear. Interestingly, N332 and other neighbouring PNGs have been found to be involved in immune escape in subtype C Envs (Moore et al., 2012; Krumm et al., 2016), suggesting that not only will the emergence of this N-glycan facilitate survival in the face of a neutralising immune response but could also stimulate the release of IL-10, and potentially other pro-inflamamtory cytokines.

N130 and N332 were found to be marginally more represented in TF Envs (though not statistically significant), suggesting a role of these N-glycans in early infection or HIV transmission. Moreover, Envs from acute infection tended to induce higher levels of MDDC IL-10 release than those isolated at chronic infection. This was contrary to the finding that IL-10 levels increased over the course of infection. However, cells other than DCs are known to produce IL-10 which could contribute to changing levels of plasma and CVL IL-10 according

to stage of HIV infection (Brockman et al., 2009; Hedrich & Bream, 2010; Saraiva & O'Garra, 2010; Rutz & Ouyang, 2011). As DCs are most likely the first cells to encounter HIV, TF Envs able to induce MDDC IL-10 might be advantageous for sustained survival in the female genital tract whereas cells other than DCs are the main producers of IL-10 later in infection.

Overall, we showed that TF Envs could carry PNGs that are involved in DC-mediated robust release of IL-10. Furthermore, as the pattern of secretion of IL-10 and inflammatory cytokines were the same, it is possible that their release are stimulated by the same cellular signaling pathway.

We showed that TNF-α, IL-6, IL-8, MIP-1α, and MIP-1β levels were elevated compared to unstimulated control, albeit not consistently across all clones. A recent cytokinomic study suggested that elevated levels of IL-6 and IL-10 could be prognostic markers of HIV-1 infection (Williams et al., 2013). When Li *et al.* (2009) inoculated macaques vaginally with SIV, MIP-1α, MIP-1β and IL-8 levels increased and was associated with an influx of CD4+ T cells to the site of infection and increased viral replication (Li, Estes, et al., 2009). C7, C12 and C14 PSVs were consistently better at stimulating MDDCs to secrete IL-10, IL-6, IL-8, TNF-α, MIP-1α and MIP-1β, although the effect of C14 was not as apparent for all cytokines. Interestingly, C7 and C12 are TF Envs suggesting that successful transmission could rely on inflammation as previously suggested (Figure 6.1). Further investigation of the impact of Env N-glycans on its interaction with DC and resultant cytokine production in a larger cohort could provide valuable direction to the design of appropriate N-glycan-containing immunogens.

In the absence of other predisposing inflammatory conditions there is paucity of HIV permissive cells in the genital mucosa. Immediately, after deposition of virus in the genital tract, secretion of immune regulators could elicit an inflammatory immune response that recruits target cells to the genital mucosa to establish viral replication. A model is therefore proposed that the secretion of TNF-α, IL-6, IL-8, MIP-1 α and MIP-1β by DCs after binding HIV attracts T cells, DCs and macrophages into the sub-mucosa, lamina propria and epithelium where they become infected, and DCs are induced by MIP-1α and MIP-1β to migrate to the lymphoid areas where CD4+ T cells are infected in *trans*. This results in high levels of inflammatory cytokines in the genital mucosa and rapid HIV dissemination. At the same time, as IL-10 causes immunosuppression of T cells by impairing IL-12 and the development of Th1 response, it is possible that the host cannot mount an effective cytotoxic immune response, while TNF-α, IL-6 and IL-8 enhances the replication of the virus for effective dissemination

and productive clinical infection (Israel et al., 1989; Finnegan et al., 1996; De Jong et al., 2008) (Figure 6.1). Unlike systemic inflammation, which could involve multiple cell types, we would also like to suggest that when TF Env binds DCs in the female genital mucosa, it results in IL-10 autocrine deregulation of DC maturation, and signaling. This localised, focused action thus subverts the function of DCs for viral survival.

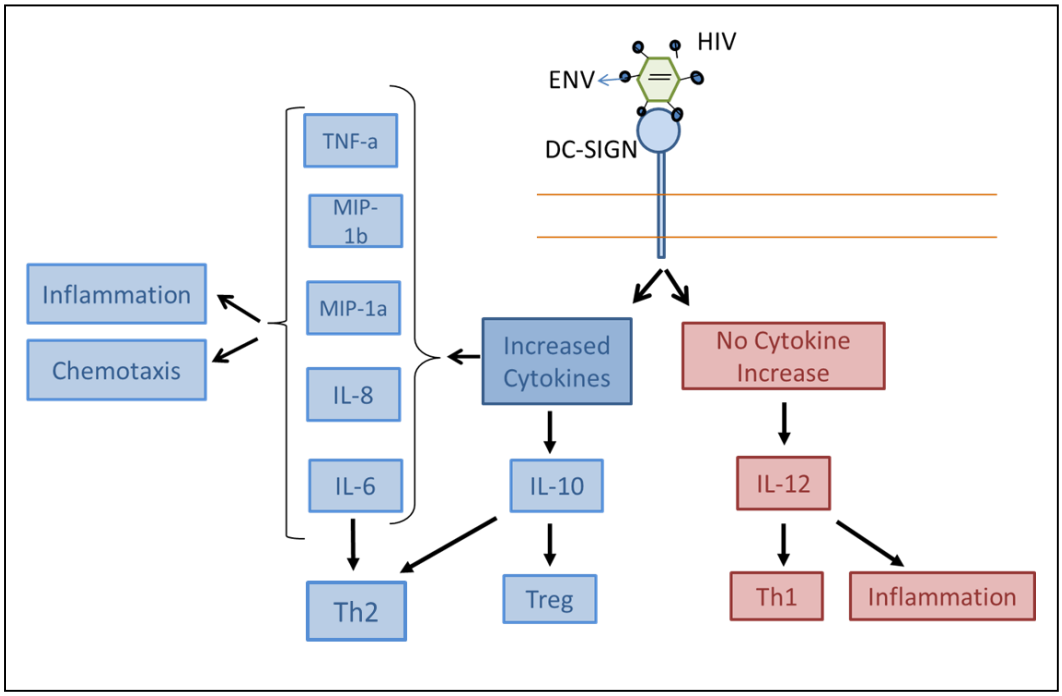


Figure 6.1 Model for HIV-1 deregulation of dendritic cell immune responses. When HIV Env binds DC-SIGN it does not culminate in an increase in IL-12 as expected to initiate a Th1 cytotoxic CD8+ T cell immune response for the clearance of infected cells. Instead it results in an increase of TNF-α, IL-10, IL-6, IL-8, MIP-1αand MIP-1β which stimulates the influx of immune cells including CD4+ T cells for viral replication and a Th0 and Treg response which promotes tolerance and humoral immunity, ineffective against viral infections.

Pathogens that use DC-SIGN have been found to subvert the immune response in DCs for their survival (Geijtenbeek et al., 2003; van Kooyk & Geijtenbeek, 2003; Van Kooyk et al., 2004; Den Dunnen, Gringhuis & Geijtenbeek, 2009). DC-SIGN has been suggested to modulate TLR induced activation of immune responses. Following DC-SIGN binding to Env, Raf-1 (Gringhuis et al., 2010) and MAPKs such as ERK are activated resulting in the production of IL-10 (Shan et al., 2007) via NF-kB activation in dendritic cells (Gringhuis et al., 2007).

We showed that interactions of PSV and gp140 with DC-SIGN of MDDCs stimulated phosphorylation of ERK and JNK. ERK phosphorylation varied across clones, suggesting that activation of ERK-modulated pathways differed according to Env. However, there was no relationship between MDDC IL-10 secretion and ERK activation. It is possible that binding of Env PAMPs to TLR2 and TLR4 of mDCs induce IL-10 secretion via activation of multiple effectors: ERK, NF-KB, TRIF/TRAF3 and MSK1/MSK2 (Saraiva and O'Garra, 2010) so it will be difficult to detect a clear association between ERK activation and IL-10 release in response to DC-SIGN only. Therefore, to study DC-SIGN-Env mediated cytokine regulation, TLR signaling must be inhibited.

In conclusion, Env interacts with MDDCs via DC-SIGN and those Env with high levels of mannosylation bind better to DC-SIGN with enhanced *trans*-infection efficiency than Envs with fewer high mannose residues. Env efficiency was a very important determinant of *trans*-infection, supporting recent evidence that viral fitness is very important for variant selection during HIV-1 transmission (Carlson et al., 2014). Binding to DC-SIGN and other receptors activated MAP kinases, leading to the secretion of IL-10 and other inflammatory cytokines but did not increase IL-12 expression. Sequence analysis suggested that PNGs at N130 and N332 could be important for IL-10 release although these sites were not associated with ERK phosphorylation. Overall, we can propose that PSV stimulation of IL-10 and other inflammatory cytokines but not IL-12 release suggests that binding to DC-SIGN is sufficient for the deregulation of a Th1 response required for viral clearance and that aberrant signaling might not only be via ERK but an alternative pathway(s).

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Appendices

Appendix I

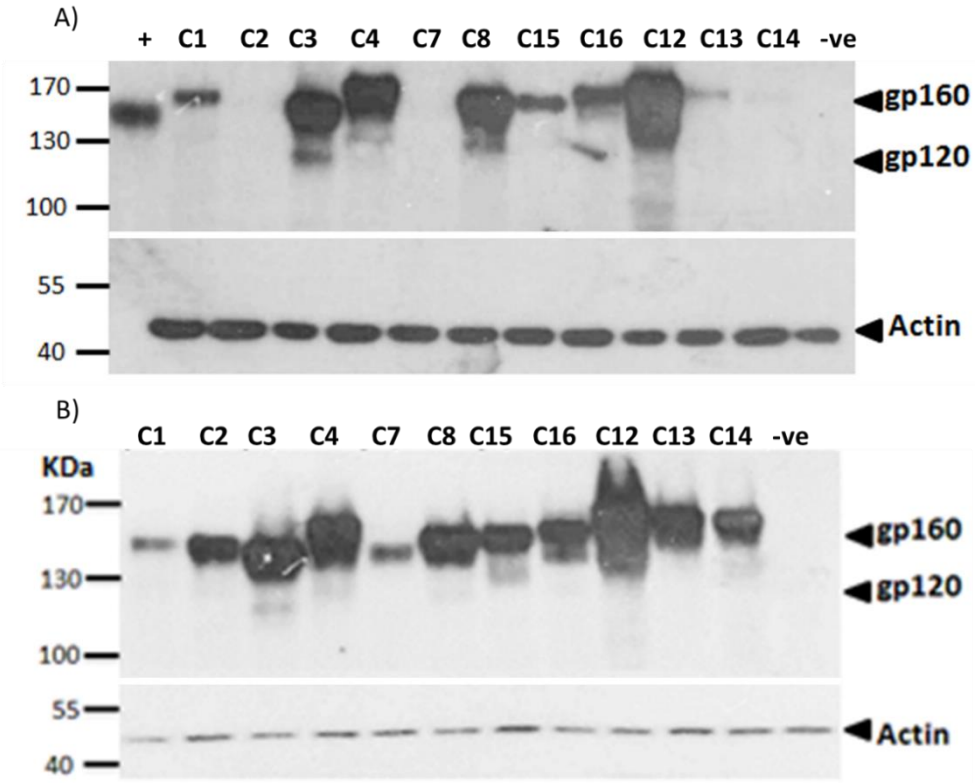

Figure A1 Expression of Env clones in the absence and presence of the viral backbone (pSG3ΔEnv). A) HEK 293T cells were transfected with Env plasmid DNA (5 µg) and 60 µg of total protein was analysed by SDS-PAGE and Western blotting. B) HEK 293T cells producing pseudovirus were lysed and 60 µg of total protein was analysed by SDS-PAGE and Western blotting. Anti-gp120 and anti-actin antibodies were used to detect gp120 and gp160 and the loading control protein β-actin, respectively. Env clones C1 – C4, C7, C8 and C12-C16 are indicated. Positions of the bands representing gp160, gp120 and actin were calculated using the standard curve of the molecular weight marker. The minus sign (-) represents cells transfected with an empty vector and the plus sign (+) represents 30 ng of recombinant purified HIV-1 gp120 (IIIB-CHO from the AIDS reagent programme). The molecular weight ladder is indicated in kilo-Daltons (KDa). A representative of three independent experiments is shown.

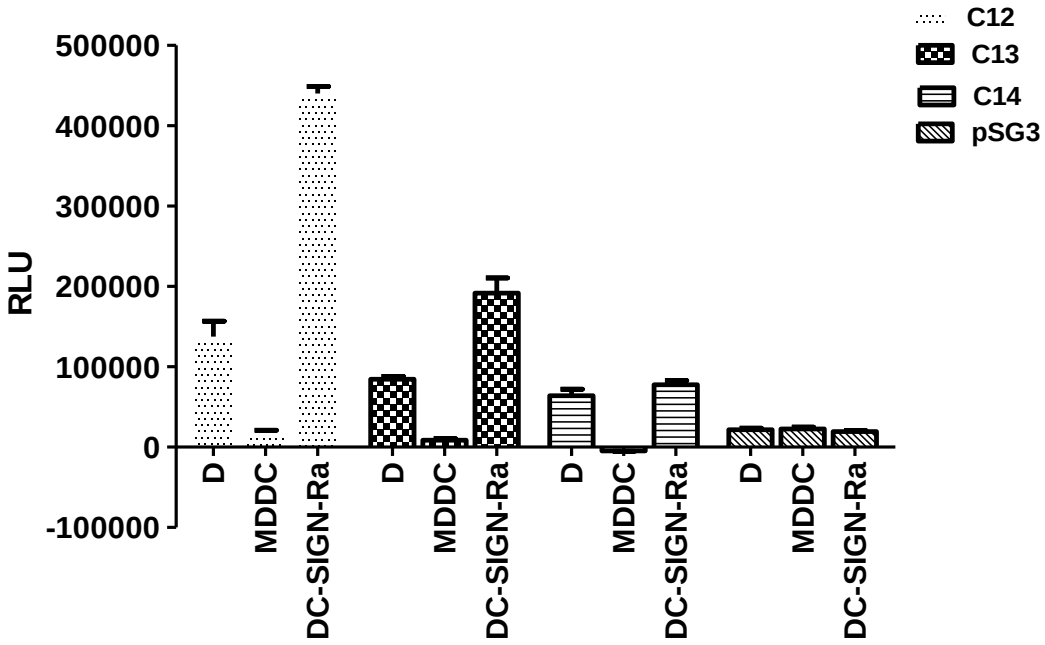

Figure A2 DC-SIGN mediated enhancement of *trans*-infection by Raji-DC-SIGN. Following *trans*-infection of TZM-bl cells using MDDC and Raji-DC-SIGN for capture of PSV at a concentration of 100 ng of p24 quantified by p24 ELISA, cell-associated luminescence (RLU) was compared between MDDC- and Raji-DC-SIGN-mediated *trans*-infection to direct infection of TZM-bl cells with same amount of PSV but without prior capture. Shown is a representative experiment for C12, C13 and C14 where D is direct infection of TZM-vl cells, MDDC refers to MDDC-mediated *trans*-infection and DC-SIGN-Ra indicates Raji-DC-SIGN-mediated *trans*-infection RLU values. pSG3 is the viral backbone without Env tested with the Env clones within the same experiment as negative control for Env.

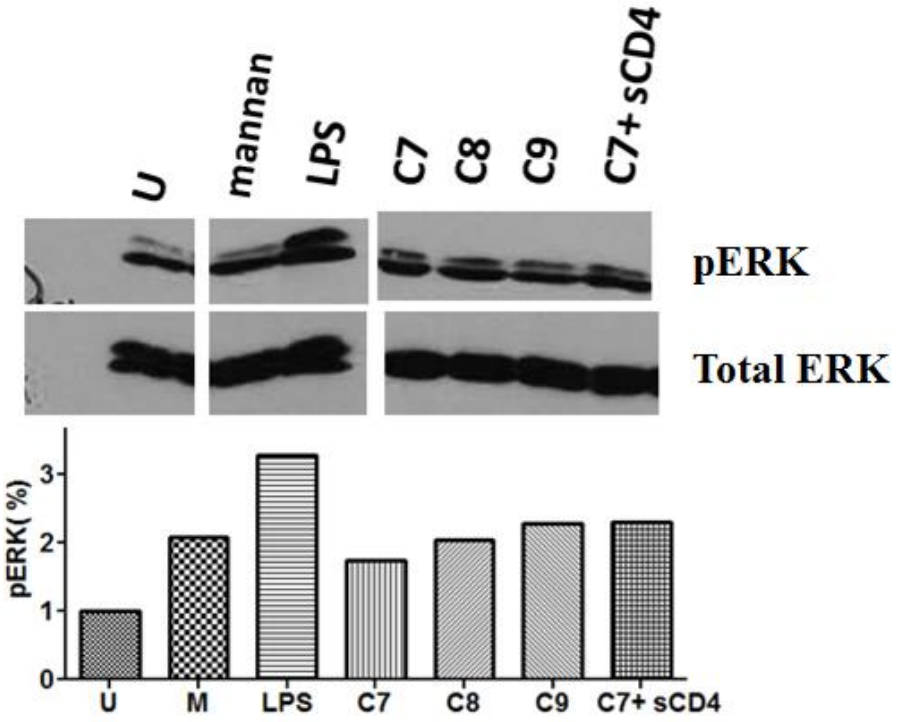

Figure A3. Determine the role of CD4 in ERK phosphorylation. Day six MDDCs were stimulated (after incubating in serum-free medium O/N) with PSVs (C7, C8 and C9) or Mannan (M) for 15 minutes before the cells were lysed and pERK levels were determined by Western blotting using phosphor-p44/42 MAPKs and total ERK (ERK1/2) antibodies (Cell Signaling, Cat#9101/ #9102). C7 was also pre-incubated with sCD4 (denoted C7+ sCD4) before stimulation to determine the effect of CD4 receptor inhibition on pERK levels. Mannan was used as a positive control for the activation of ERK via lectin receptors and the negative control (U) represents unstimulated MDDCs. Levels of pERK are indicated as the percentage densitometry of total ERK. This experiment represents a single experiment but was repeated for C12 and similar results were obtained (not shown).

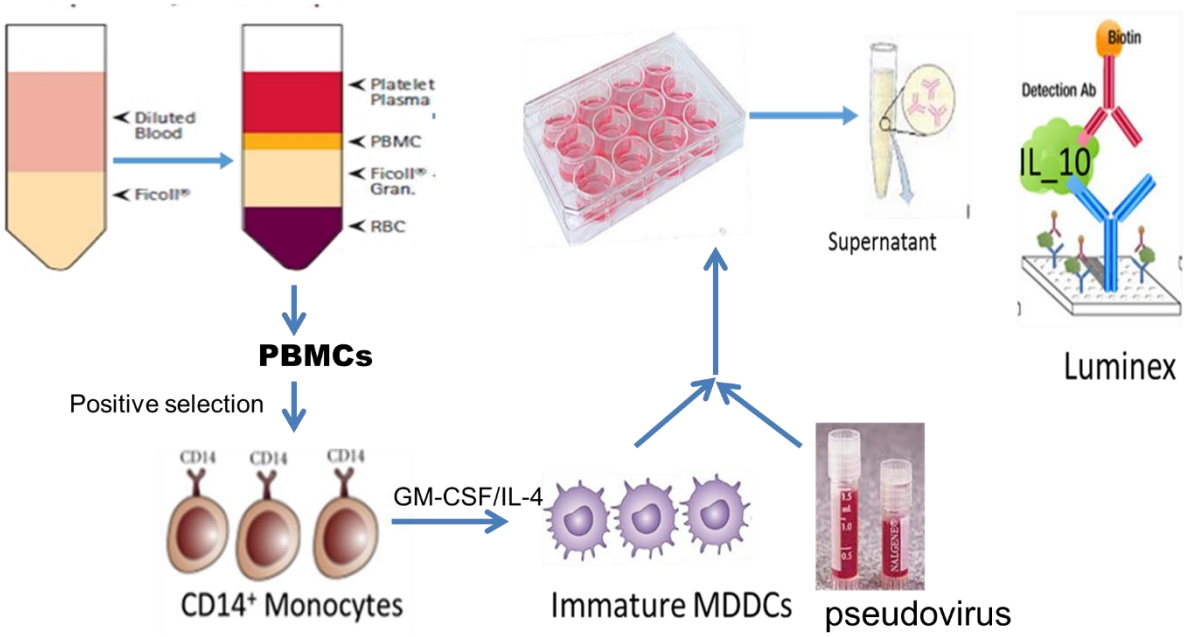

Figure A4 Overview of stimulation of MDDCs to secrete IL-10. PBMCs were isolated from whole blood by Ficoll gradient centrifugation and CD14+ cells were isolated by positive selection before differentiation by GM-CSF/IL-4 into immature monocyte derived dendritic cells. Cells were plated and stimulated with pseudovirus (or gp140) before supernatants were removed and Luminex was used to determine levels of IL-10.

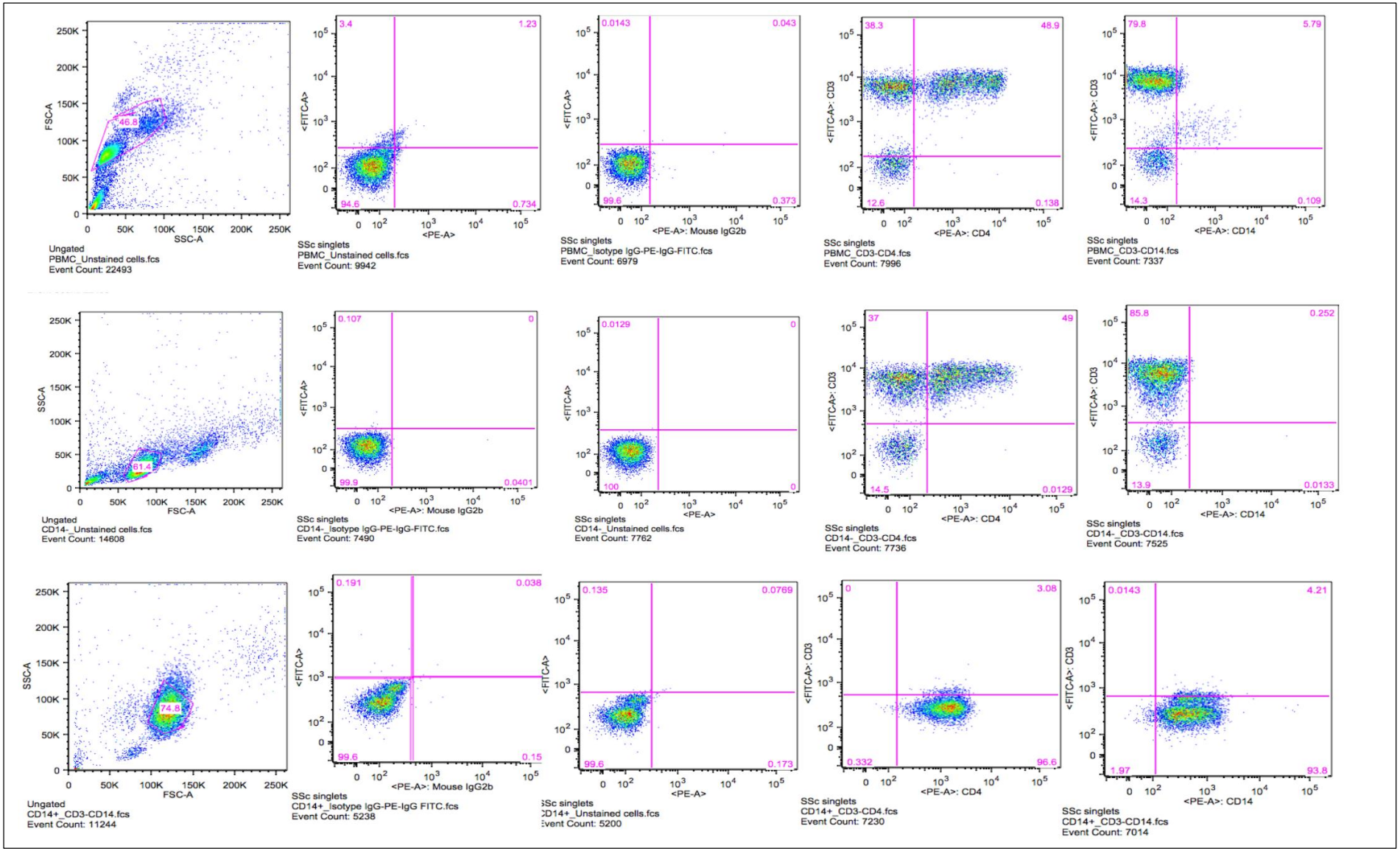
FSC-A
SSC-A
46.8
Ungated
PBMC_Unstained cells.fcs
Event Count: 22493
3.4
1.23
94.6
0.734
<FITC-A>
<PE-A>
SSc singlets
PBMC_Unstained cells.fcs
Event Count: 9942
0.0143
0.043
99.6
0.373
<PE-A>: Mouse IgG2b
SSc singlets
PBMC_Isotype IgG-PE-IgG-FITC.fcs
Event Count: 6979
38.3
48.9
12.6
0.138
<FITC-A>: CD3
<PE-A>: CD4
SSc singlets
PBMC_CD3-CD4.fcs
Event Count: 7996
79.8
5.79
14.3
0.109
<PE-A>: CD14
SSc singlets
PBMC_CD3-CD14.fcs
Event Count: 7337
SSC-A
FSC-A
61.4
Ungated
CD14-_Unstained cells.fcs
Event Count: 14608
0.107
0
99.9
0.0401
<PE-A>: Mouse IgG2b
SSc singlets
CD14-_Isotype IgG-PE-IgG-FITC.fcs
Event Count: 7490
0.0129
0
100
0
SSc singlets
CD14-_Unstained cells.fcs
Event Count: 7762
37
49
14.5
0.0129
SSc singlets
CD14-_CD3-CD4.fcs
Event Count: 7736
85.8
0.252
13.9
0.0133
SSc singlets
CD14-_CD3-CD14.fcs
Event Count: 7525
74.8
Ungated
CD14+_CD3-CD14.fcs
Event Count: 11244
0.191
0.038
99.6
0.15
SSc singlets
CD14+_Isotype IgG-PE-IgG FITC.fcs
Event Count: 5238
0.135
0.0769
99.6
0.173
SSc singlets
CD14+_Unstained cells.fcs
Event Count: 5200
0
3.08
0.332
96.6
SSc singlets
CD14+_CD3-CD4.fcs
Event Count: 7230
0.0143
4.21
1.97
93.8
SSc singlets
CD14+_CD3-CD14.fcs
Event Count: 7014

Figure A4 Surface staining of monocyte post isolation. Monocytes isolation from PBMC by positive selection, were surface stained to verify the purity and yield and to make sure there was not lymphocyte contamination. An aliquot of A) PBMCs, B) CD14- and C) CD14+ fractions were stained for expression of surface markers; CD4-PE, CD3-FITC and CD14-PE . A mouse Isotype control (IgG2b-PE) for CD14+ was added to exclude cell auto-flurorescence. Each population was sorted for forward and site scatter (FSC-A (x axis) and SSC-A (y-axis)) and the lymphocyte population, CD14- or CD14+ populations respectively for selected and stained for the CD3+, CD4 and CD14. Upper panel is PBMC before positive selection, where the y-axis is CD3-FITC and x-axis IgG-FITC isotype control, or CD4-PE or CD14-PE, middle panel is CD14 negative fraction post positive selection and the lower panel is CD14 positive fraction, where the y-axis is CD3-FITC and x-axis is CD4-PE or CD14-PE as indicated

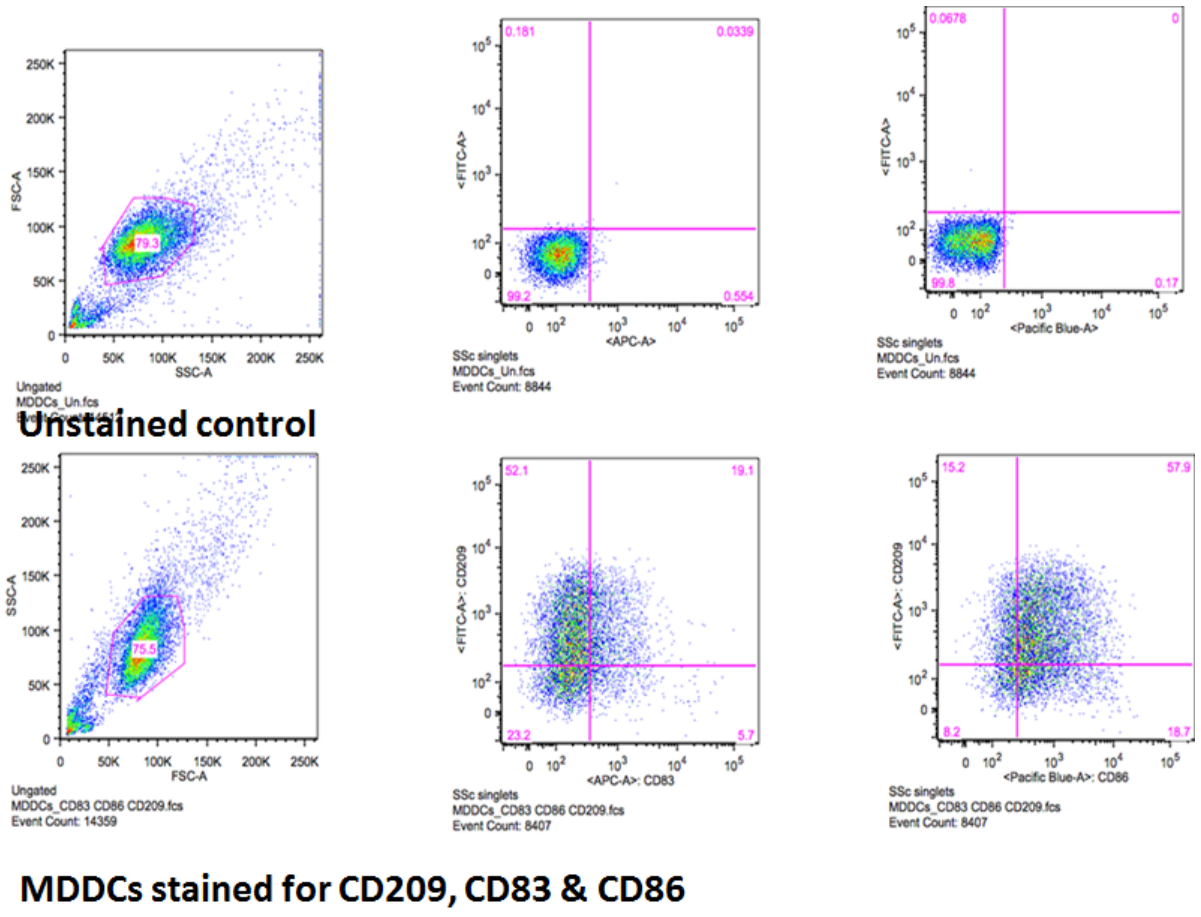

Figure A5. Surface staining for characterisation of differentiated MDDCs prior to stimulation. Day six MDDCs were surface stained for maturation marker CD83, co-stimulatory marker, CD86 and DC-SIGN (CD209) expression using monoclonal antibodies for these markers and acquired using BD Facs Canto II flow cytometry. Flow data was analysed using Flowjo software (Treestar, USA) and an example from one donor is shown. Following staining, FSC (y-axis) and SSC-A (x-axis) scatter, the MDDC population was analysed for the CD209-FITC (y-axis), CD86-Pacific blue (x-axis) and CD83-APC (x-axis). Upper panel represent unstained control showing negative for DC-SIGN receptor, CD209, while the lower panel represents a sample of MDDC stained which shows that DC-SIGN (CD209) was about 73% for this donor with some 23% activation as shown by the presence of CD83.

Table A1. Primers used for mutation of potential N-glycan sites by site-directed mutagenesis

C15 & C16	N-Q HXB2 position	Primer sequence	Silent mutation enzyme site introduced
C15	N241Q	**F 5'C AAT GGA GCA GGA CCG TGC ACT CAA GTC AGC ACA GTA CAA 3'** **R 5' TTG TAC TGT GCT GAC TTG AGT GCA CGG TCC TGC TCC ATT G 3'**	AlW44I
	N262Q	**F 5' ACT CAA CTA CTG TTA CAA GGT AGC TTA GCA GAA GAG GAT A 3'** **R 5' T ATC CTC TTC TGC TAA GCT ACC TTG TAA CAG TAG TTG AGT 3'**	Bpu1102I and BamHI in double digest
	N448Q	**F 5'ATA ACA TGT AAA TCA CAA ATC ACC GGA TTG CTC TTG ACA 3'** **R 5'TGT CAA GAG CAA TCC GGT GAT TTG TGA TTT ACA TGT TAT 3'**	BsaWI
C16	N241Q	**F 5' C AAT GGA ACA GGA CCG TGC ACT CAA GTC AGC ACA GTA CAA 3'** **R 5' TTG TAC TGT GCT GAC TTG AGT GCA CGG TCC TGT TCC ATT G 3'**	ApaLI
	N262Q	**F 5' ACT CAA CTA CTG TTA CAA GGT AGC TTA GCA GAA GAG GAT A 3'** **R 5'T ATC CTC TTC TGC TAA GCT ACC TTG TAA CAG TAG TTG AGT 3'**	Bpu1102I and BamHI double digest
	N448Q	**F 5' ATA ACA TGT CAA TCA CAA ATC ACC GGA TTG CTC TTG ACA 3'** **R 5' TGT CAA GAG CAA TCC GGT GAT TTG TGA TTG ACA TGT TAT**	BsaWI

Nucleotides mutated for N-Q conversion are in red and restriction enzyme sites introduced by silent sites are underlined**.**

Reagents (per reaction) for PCR	**Volume(µl)**
Buffer (5x)	10
dNTPs (10mM)	2
DMSO (100%)	1.5
For Primer (10uM)	2.5
Rev Primer (10uM)	2.5
High Fidelity DNA Polymerase(5U/µl)	0.5
dH_2O	26
DNA template (10ng/ul)	5

Appendix II

Preparation of MACs beads (130-050-201, **Miltenyi Biotec, USA)**

- It is essential to wash beads before use to remove Sodium Azide. This helps improve cell viability.
- Mount an LS column (130-042-401, Miltenyi biotec) on a MidiMACS Separator (130-042-302, Miltenyi biotec) (magnet) place on its Multi stand (130-042-303. Miltenyi biotec) as recommended by the manufacturer after taking out the plunger.
- Prepare LS column by pre-wetting with 3 ml of cold (stored at 4^{o}C) MACS buffer.
- Beads are gently re-suspended and the entire volume transferred using a plugged 2 ml pipette into the column (it is advisable to take note of the exact volume, about 2 -2.2 ml) to avoid losses following elution.
- Add resuspended beads to column and allow buffer to flow through the column.
- Rinse out original vial of beads with 3 mL wash buffer (MACs buffer) and add to the column.
- Washes 3x with 3 ml wash buffer with each wash allowed to go through the column completely before the next volume is added.
- Column is removed from the magnet and positioned over a 15 ml conical tube at least 5 cm away from the magnet. MACs buffer is added to the column (100µl less than the original volume of beads) and liquid is forced through the column with a plunger.
- 1 ml aliquots of cells are made in 1.5 mls flat-bottom Eppendorf tubes, labelled with date and expiry date and stored at 4^{o}C wrapped in aluminium foil to protect from light.

MACs buffer

1x PBS (10010049, Gibco)
1 % Human serum AB (Sigma Cat # H1513)
0.5 M EDTA
This is pH to 7.2 -7.4, then filtered through a 0.22µm filter and stored at 4^{o}C. Aliquots should be used within three months.

GM-CSF Preparation (Cat # PHC2013, BioSource, or life technology)

1 mg/mL stock is made by injecting sterile TC grade dH20 into the vial using a sterile syringe and needle

The 1 mg/ml stock has an activity of 15 x 10^6 IU/ml (international Units)

Aliquots of 33µl are made and stored frozen at -20ºC

Working stocks of 5 x 10^5 U/ml are made by adding 967 µl of 0.1 % human serum albumin (HAS) in PBS. Working stock can be stored at 4ºC. 2µl of this working stock can be added to 1 mL (dilution of 1:500) of MDDC culture medium to get a final 1000 U/ml, which gives 60ng/ml.

IL-4 (BioSource, PHC045), 100mg

725 µl of PBS (-Ca^2, -Mg^2) containing 0.1 % HAS is added to the entire volume (100mg) to give an activity of 1 x 10^6 IU/ml. Aliquots of 25 µl each are made and stored at -20ºC. Once each aliquot is thawed, it is stored at 4ºC and used at 1 in 10^4 dilutions with differentiation medium (see material and methods) an activity of 100 U/ml or concentration of 30 ng/ml.