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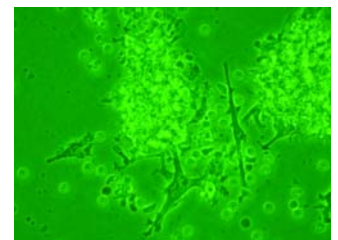
TRANSFECTION OF BABOON DENDRITIC CELLS WITH PLASMID DNA CONTAINING HIV-1C GENES: EFFECT OF TRANSFECTION METHODS ON ANTIGEN PROCESSING AND PRESENTATION TO T LYMPHOCYTES

FABIAN FIFF



TRANSFECTION OF BABOON DENDRITIC CELLS WITH PLASMID DNA CONTAINING HIV-1C GENES: EFFECT OF TRANSFECTION METHODS ON ANTIGEN PROCESSING AND PRESENTATION TO T LYMPHOCYTES

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Thesis presented in partial fulfillment of the requirements for the degree of Master of Sciences in Medical Sciences (Medical Virology) at the Faculty of Health Sciences, University of Stellenbosch.

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DECEMBER 2005

DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my original work and that I have not previously submitted it, in its entirety or in part, at any university for a degree.

Signature: ____________________ Date: ____________________

SUMMARY

There is an urgent need for a safe, effective, affordable human immunodeficiency virus type 1 (HIV-1) vaccine that induces both cellular and humoral immunity. A popular strategy for vaccine design is the use of plasmid DNA encoding HIV-1 genes for priming vaccinations followed by either viral vector or recombinant protein boosting. DNA-based vaccines are attractive because they are safe, easily administered and can induce both cellular and humoral immune responses. In order for DNA vaccination to induce a potent immune response it is necessary for plasmid-encoded genes to be targeted to dendritic cells (DCs) as these are the key antigen presenting cells in natural HIV infection.

The immunogenicity of all potential vaccine candidates needs to be assessed in animal models prior to entry into human trials. Nonhuman primates are the best alternative to humans for assessment of vaccine immunogenicity and protective efficacy. In order to clearly understand how DNA vaccines interact with DCs, suitable *in vitro* DC culture systems for nonhuman primates need to be developed.

This study investigated the culture and characterisation of chacma baboon DCs *in vitro*, and was the first to assess the effect of various transfection methods on baboon DC maturation and function. The study also evaluated the efficacy of a candidate HIV-1 subtype C DNA vaccine at the level of baboon DC transfection, gene transcription and antigen presentation.

Generation of immature DCs (iDCs) in the presence of interleukin-4 (IL-4) and granulocyte-macrophage colony stimulating factor (GM-CSF) was accompanied by a loss in the monocyte marker CD14. Expression of the markers CD80 and CD83 was observed on a minority of iDCs, whereas CD86 was expressed on almost all iDCs. Following maturation, all these markers were expressed on an increased number of cells, a pattern of marker expression and upregulation that

is similar to that observed in both human and macaque DCs. Transfection of baboon DCs by passive pulsing, lipofection and electroporation was evaluated and compared in several ways. Transfection efficiency, cytotoxicity, the effect of the transfection on DC maturation and subsequent presentation of plasmid-encoded antigen to memory T lymphocytes was examined.

Baboon DCs lipofected with pDNA efficiently took up HIV-1 subtype C plasmid DNA, transcribed plasmid-encoded genes into mRNA, translated the mRNA into protein, processed the protein and presented peptide antigens to antigen-specific memory T cells. The other methods of transfection were less effective than lipofection due to either decreased transfection efficiency or increased cell cytotoxicity. However, neither lipofection nor passive pulsing in any way negatively impacted on DC marker, CD83, or costimulatory molecule, CD80 and CD86, upregulation. Both methods were found to be as effective as a standard cytokine maturation cocktail in inducing DC maturation. Transfected DCs were also found to be more potent inducers of allogeneic T cell stimulation than their untransfected counterparts, which would appear to indicate enhanced major histocompatibility complex (MHC) expression concurrent with DC maturation marker expression. Lipofection with candidate HIV-1 subtype C vaccine plasmid DNA constructs led to antigen-specific expansion of autologous memory T cells, a finding which indicates the effective expression of plasmid-encoded HIV genes in baboon DCs.

This study highlights the functional activity of *in vitro* generated baboon DCs and provides the groundwork for future studies addressing targeting of plasmid DNA to DCs and enhancement of expression of plasmid-encoded antigens in DCs. A more detailed evaluation of baboon DC interaction with simian immunodeficiency viruses/chimeric simian human immunodeficiency viruses (SIVs/SHIVs) may also reveal how the course of infection in this primate differs from that seen in the macaque or chimpanzee and also how it relates to HIV-1 infection in humans.

OPSOMMING

Daar is `n dringende behoefte aan `n veilige, effektiewe en bekostigbare menslike immuniteitsgebreksvirus tipe 1 (MIV-1) entstof wat sowel sellulêre asook humorale immuniteit induseer. `n Algemene strategie vir entstof ontwerp is die gebruik van plasmied DNS gekodeerde MIV-1 gene vir primêre inenting, gevolg deur `n virale vektor of rekombinante proteïen versterking. `n DNS-gebaseerde entstof is `n aantreklike alternatief omdat dit veilig en maklik toedienbaar is en beide sellulêre en humorale immuniteit aanwakker. Vir DNS entstowwe om `n kragtige immuunreaksie te induseer, is dit noodsaaklik dat plasmied-gekodeerde gene na dendritiese selle (DSe) geteiken word aangesien dit die belangrikste antigeen presenterende selle in natuurlike MIV infeksie is.

Die immunogenesiteit van alle potensiële entstowwe moet eers in diermodelle vasgestel word voordat proewe in mense kan plaasvind. Nie-menslike primate is die beste alternatief vir die assessering van entstof immunitieit en doeltreffende beskerming. Om duidelik die wisselwerking tussen DNS-entstowwe en DSe te begryp, moet geskikte *in vitro* DS kultuursisteme vir nie-menslike primate ontwikkel word.

Hierdie studie ondersoek die kweking en karakterisering van chacma bobbejaan DSe *in vitro* en was ook die eerste om die effek van verskeie transfeksie metodes op bobbejaan DS maturasie en funksie te bepaal. Hierdie studie het ook die doeltreffendheid van `n potensiële MIV-1 subtipe C DNA-entstof op die vlak van bobbejaan transfeksie, geen transkripisie en antigeen presentering geëvauleer

`n Verlies aan uitdrukking van die monosiet CD14 merker is waargeneem met die kweking van onvolwasse DS (oDSe) in die teenwoordigheid van interleukin-4 (IL-4) en Granulosiet-Makrofaag Kolonie Stimulerende Faktor (GM-KSF). Die uitdrukking van die CD80 en CD83 merkers in oDSe was laer as die van merker

CD86. Na maturasie word al die merkers in `n verhoogde hoeveelheid selle uitgedruk, `n patron wat soortgelyk is aan die merkeruitdrukking en opregulasie soos waargeneem met mens en macaque DSe. Die transfeksie van bobbejaan DSe deur passiewe pulsering, lipofeksie en elektroporasie is deur verskeie maniere geevalueer en vergelyk. Die effektiwiteit van transfeksie, sitotoksisiteit, die effek van transfeksie op DS maturasie en die daaropvolgende presenteering van plasmied gekodeerde antigeen aan geheue T limfosiete, is ondersoek.

Die opname van MIV-1 subtipe C plasmied DNA deur lipofekteerde bobbejaan DSe was doeltreffend, asook die transkripsie van plasmied-gekodeerde gene na boodskapper RNS (bRNS), die translasie van bRNS na proteïene, die prossessering van die proteïene en die presenteering van peptied antigene aan antigeen spesifieke geheue T selle. Ander metodes van transfeksie was minder effekief as lipofeksie as gevolg van verlaagde transfeksie doeltreffentheid of verhoogde selsitotoksisiteit. Die opregulasie van DS merker, CD83, en die mede-stimulatoriese molekules, CD80 en CD86, is geensins negatief beïnvloed deur lipofeksie of passiewe pulsering nie. Beide hierdie metodes is net so effektief as `n standaard sitokien maturasie mengsel vir die induksie van DS maturasie. Daar is gevind dat getransfekteerde DSe kragtiger induseerders van allogeniese T sel stimulasie is as hulle ongetransformeerde teenstanders. Dit dui op verhoogde MHC uitdrukking en val saam met DS maturasie merker uitdrukking. Lipofeksie met potensiële MIV-1 subtipe C entstof plasmied DNS konstrukte het gelei tot die antigeen spesifieke uitbreiding van outologiese geheue T selle wat dui op die effektiewe uitdrukking van plasmied-gekodeerde MIV gene in bobbejaan DSe.

Hierdie studie beklemtoon die funksionele aktiwiteit van *in vitro* gegenereerde bobbejaan DSe en verskaf die grondslag vir toekomstige studies wat die teikening van plasmied DNS na DSe en die verhoging van die uitdrukking van plasmied-gekodeerde antigene in DSe kan aanspreek. `n Meer gedetaileerde evaluasie van die interaksie tussen bobbejaan DSe en simian

immuniteitsgebreksvirusse/chimeriese menslike immuniteitsgebreksvirusse (SIVe/SMIVe) kan ook openbaar hoe die verloop van infeksie in hierdie primaat verskil van dié van macaque of sjimpansee, asook hoe dit verband hou met MIV-1 infeksie in die mens.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude towards my enormously supportive promoter, Dr R H Glashoff for his insight, unceasing guidance, positive criticism and never closing his door throughout my M.Sc. Thank you for fruitful discussions, for teaching me the importance of scientific independence, encouragement and constructive criticism for further personal development.

I thank my co-promoter, Dr W Liebrich, for sharing his knowledge of science, positive criticism and guidance and for devoting his time.

I thank, Florette K Treurnicht for assisting me with molecular aspects of the study and technical support.

I thank, Dalene de Swardt for technical assistance.

Dr Susan Barnett from Chiron Corporation, Emeryville, USA for supplying the plasmid constructs pCMVLinkgp140dV2.TV1 and pCMKm2GagCPol.BW used in the study.

All friends in the department sharing daily life at the lab/office thank you for pleasant times in and outside the lab, especially for many laughs.

I wish to thank, my great love and best friend for support, patience, love, encouragement and understanding.

My parents for their continued support and understanding.

The crew at the Medical Research council (MRC) animal center in Delft for taking care of the baboons, thank you.

The Poliomyelitis Research Foundation and the South African AIDS Vaccine Initiative (SAAVI) for funding the study.

Being defeated is often a temporary condition. Giving up is what makes it permanent (*Anonymous*).

List of Abbreviations

Ab(s)	Antibody/Antibodies
Ag(s)	Antigen(s)
AIDS	Acquired Immunodeficiency Syndrome
ATV	Active Trypsin Versine
CCR5	Chemokine Receptor 5
CD	Cluster of Differentiation
cDNA	Copy DNA
CLA(s)	Lymphocyte-associated Antigen(s)
CLP(s)	Common Lymphoid Progenitor(s)
CLIP	Class II-associated invariant-chain
cpm	Counts per minute
CMP(s)	Common Myeloid Progenitor(s)
CMV	Cytomegalovirus
CRD	Carbohydrate Recognition Domain
CTL	Cytotoxic T lymphocyte
CXCR4	A chemokine receptor of the CXC family
DC(s)	Dendritic Cell(s)
DC-SIGN	Dendritic Cell-Specific ICAM-Grabbing Non-Integrin
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic Acid
dsRNA	Double-Stranded Ribonucleic Acid
EDTA	Ethylene diamine tetra acetic Acid
ELISA	Enzyme-linked immunosorbent assay
env	Envelope gene
Env	Envelope protein
ER	Endoplasmic Reticulum
FACS	Fluorescence-activated cell sorter/sorting
FBS	Fetal Bovine Serum
FcεRI/II	Fc Epsilon Receptor I and II (receptors for IgE)

FcγR(s)	Fc Gamma Receptor(s) (receptors for IgG)
gag	Group antigen gene
Gag	Group antigen protein
GM-CSF	Granulocyte macrophage-colony stimulating factor
gp	Glycoprotein
HSC(s)	Haematopoietic Stem Cell(s)
HIV	Human Immunodeficiency Virus
HLA	Human leukocyte antigen
ICAM	Intracellular Adhesion Molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
kb	Kilobases
Lag(s)	Langerhans-cell-associated antigen(s)
LAMP	Liposome-associated Membrane Protein
LB	Luria Bertani
LC(s)	Langerhans Cell(s)
LFA	Lymphocyte function associate antigen
LPS	Lipopolysaccharide
mAb(s)	Monoclonal antibody(s)
MHC	Major histocompatibility
MIV	Menslike Immuniteitsgebreksvirus
MoDC(s)	Monocyte derived dendritic cell(s)
Mr	Molecular weight
mRNA	Messenger Ribonucleic acid
MVA	Modified Vaccinia Ankara
nAb(s)	Neutralising antibody(s)
OD	Optical density
PBMC(s)	Peripheral Blood Mononuclear cell(s)
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction

pDC	Plasmacytoid dendritic cell
pDNA	Plasmid DNA
PGE	Prostaglandin E
PLG	Poly lactide-co-glycolide
pol	Polymerase gene
Pol	Polymerase protein
PP	Passive pulsing
psi	pound per square inch
rh	Recombinant human
RNA	Ribonucleic Acid
rpm	Revolutions per minute
RT	Reverse transcriptase
SD	Standard deviation
SHIV	Simian Human Immunodeficiency Virus
SIV	Simian Immunodeficiency Virus
SMIV	Chimeriese Menslike Immuniteitsgebreksvirusse
TGF	Transforming growth Factor
Th1	T helper cell type 1
Th2	T helper cell type 2
TLR(s)	Toll-like receptor(s)
TNF	Tumor necrosis factor
IU	International Unit
V	Volts

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CHAPTER 1
INTRODUCTION

1.1. Dendritic Cells

1.1.1. Origin and Differentiation of Dendritic cells (DCs)

Dendritic cells (DCs) are bone marrow-derived leukocytes that function as professional antigen presenting cells (APCs) (Pope, 2003; Loré, 2004; Rinaldo and Piazza, 2004). DCs express high levels of major histocompatibility complex molecules (MHC class I and II), important for antigen presentation, as well as accessory molecules that mediate T cell binding and co-stimulation (Freudenthal and Steinman, 1990; O'Doherty *et al.,* 1993; Perruccio *et al.,* 2004).

The first description of DCs was in the mid 1970s by Steinman and colleagues, who observed a subpopulation of cells with a striking shape in the spleens of mice (Steinman and Cohn, 1973; Steinman *et al.,* 1974; Steinman *et al.,* 1975). Our understanding of these cells has increased exponentially since the development of *in vitro* culture systems and clarification of marker expression (Loré, 2004). DCs are a family of APCs that patrol all tissues of the body including the epidermis of the skin, where they are termed Langerhans cells (LCs), and mucosal surfaces of the respiratory, urogenital and gastro-intestinal tracts. They are also present within the interstitial spaces of solid organs, such as the heart and kidney, but are absent from some "immunologically privileged" sites, such as the brain and testes (Steinman, 1991; Cella *et al.,* 1997). The study of DCs has long been hampered by their scarcity *in vivo* and by the lack of a specific cell marker universally expressed by all members of the DC family (Banchereau and Steinman, 1998; Paluka and Banchereau, 1999).

In humans, three distinct DC subsets have been identified (Figure 1.1) namely; Langerhans cells, interstitial DCs (also known as dermal DCs) and plasmacytoid DCs (Pope, 2003; Valladeau and Saeland, 2005). LCs and interstitial DCs form part of the myeloid DC lineage. LCs, identified by expression of CD1a, Birbeck granules and langerin (Valladeau *et al.,* 2000;

Hoshino *et al.,* 2005), are localised in the basal and suprabasal layers of the epidermis (Valladeau and Saeland, 2005). Interstitial DCs are identified by the expression of CD14, CD68 and the coagulation factor XIIIa and are found in the dermis and most organs including the lungs and heart (Holt, 1993; Nestle *et al.,* 1998; Shortman and Liu, 2002). Plasmacytoid DCs (pDCs) are $CD4^+$, $CD11c^-$, $CD13^-$, $CD33^-$ and $CD123^+$ and are present in both blood and lymphoid organs including the thymus (Grouard *et al.,* 1997; Res *et al.,* 1999). Plasmacytoid DCs are characterised by a unique phenotype and possess the ability to secrete large amounts of type I interferons (IFNs) - α and β upon viral stimulation (Siegal *et al.,* 1999; Asselin-Paturel and Trinchieri, 2005; Barchet *et al.,* 2005). Unlike LCs and interstitial DCs, pDCs require interleukin 3 (IL-3) for their differentiation and are derived from a $CD11c^-$ blood precursor that has low expression of GM-CSF receptor. These cells lack the myeloid markers CD14, CD13, and CD33, they also lack mannose receptors but do express high levels of CD123 (Cella *et al.,* 1999; Kohrgruber *et al.,* 1999; Barchet *et al.,* 2005). Just like LCs and interstitial DCs, pDCs have the ability to activate CD4 and CD8 naïve T cells and secrete IL-12 upon CD40L activation (Kohrgruber *et al.,* 1999; Cella *et al.,* 2000;).

Although the majority of work on DCs has focused on human and murine models, similar DC subsets have also been isolated, identified and characterised from analogous locations in primates. Most of the research on nonhuman primate DCs has been conducted in rhesus macaques (*Macaca mulatta*), and Langerhans cells, interstitial DCs and plasmacytoid DCs have been described in this species (Pope *et al.,* 1997; Ignatius *et al.,* 1998; Pope, 1998; Frank and Pope, 2001; Loré, 2004).

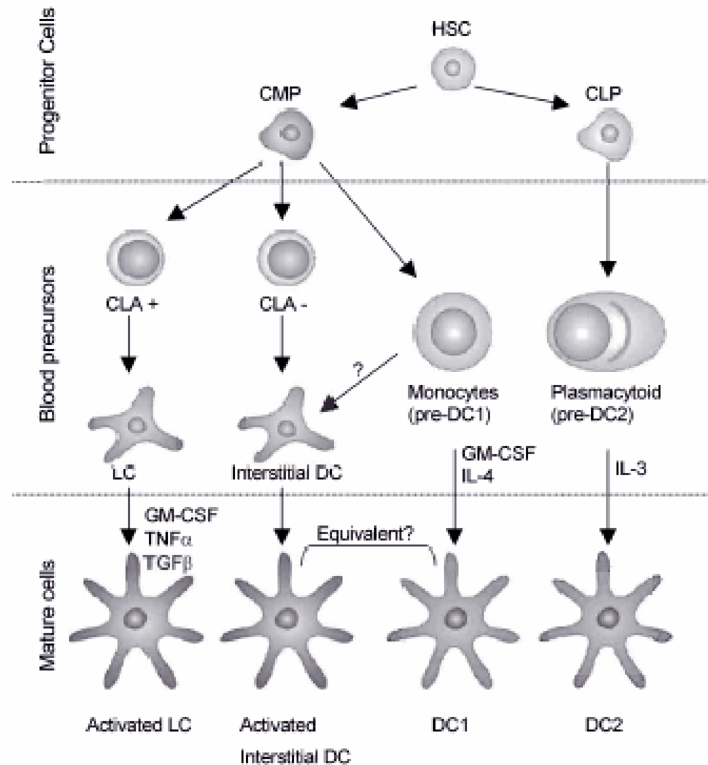


Figure 1.1. DC subsets and lineages. Myeloid progenitors differentiate into CLA+ or CLA- precursors or monocytes. CLA+ precursors yield Langerhans cells in response to GM-CSF, TNF-α and TGF-β. CLA - precursors yield interstitial DCs. Monocytes may develop into both interstitial DCs and/or mature DCs (DC1). Lymphoid progenitors differentiate into plasmacytoid DCs which develop into mature DCs (DC2) in response to IL-3. Abbreviations: CLA - Cutaneous Lymphocyte-associated Antigen; LC - Langerhans cells; HSC - Haematopoietic stem cell; CLP - Common Lymphoid Progenitor; CMP - Common Myeloid Progenitor.
Adapted from Shortman and Liu, 2002.

Different precursor-cell types have been used as starting points for the generation of human DCs *in vitro*. The earliest precursor known is the $CD34^+$ haematopoietic stem cell (HSC) isolated from bone marrow or umbilical cord blood (Caux *et al.,* 1996; Shortman and Liu, 2002). During haematopoiesis HSCs differentiate into common lymphoid progenitors (CLP) or common myeloid progenitors (CMP) in the bone marrow (Figure 1.1).

Culturing CMPs in the presence of GM-CSF and TNF-α leads to two types of intermediate precursor and two separate pathways of DC development (Caux *et al.,* 1996; Caux *et al.,* 1997). The intermediates along one of these pathways express the cutaneous lymphocyte-associated antigen (CLA) and CD11c (Strunk *et al.,* 1997). In the presence of exogenous stimulus, these

intermediates lead to the generation of LCs, a process dependent on the presence of TGF-β (Strobl *et al.,* 1996; Borkowski *et al.,* 1996; Valladeau and Saeland, 2005). The second CMP-pathway leads to DCs resembling interstitial DCs (Ito *et al.,* 1999). These cells lack LC specific Birbeck granules, Langerhans-cell-associated antigens (Lag) (Kashihara *et al.,* 1986; Hubert *et al.,* 2005), langerin (Valladeau *et al.,* 2000) and E-cadherin (Caux *et al.,* 1997; Dubois *et al.,* 1999). The intermediates along this pathway lack CLA but do express the myeloid differentiation antigen CD14 and resemble blood monocytes in many respects. These two types of DC display different phenotypes and functions (Caux *et al.,* 1997). Interstitial DCs, but not LCs, have the ability to take up large amounts of antigen by the mannose receptors and to produce IL-10, which may contribute to naïve B cell activation and IgM production (Caux *et al.,* 1997; Dubois *et al.,* 1999). LCs on the other hand are specialised APCs that reside in the epidermis with unique migratory ability. They monitor the epidermal microenvironment by taking up antigen and transport it from the epidermis to regional lymph nodes, where they can initiate systemic immune responses.

Besides Langerhans and interstitial DCs, CMPs also give rise to monocytes (or pre-DC1s) whereas CLPs give rise to plasmacytoid cells (pre-DC2s) (Liu *et al.,* 2001) (Figure 1.1). The pre-DC1 (monocytes) and pre-DC2 (plasmacytoid DCs) cells express different sets of pattern-recognition receptors (Kadowaki *et al.,* 2001) and show corresponding differences in reactivity to different microbial products. Pre-DC1, but not pre-DC2, express mannose receptors and CD1 molecules. Pre-DC1 differentiate into immature myeloid DC1 in culture with GM-CSF and IL-4 (Sallusto and Lanzavecchia, 1994; Bender *et al.,* 1996; Romani *et al.,* 1996; Lehner *et al.,* 2005). Pre-DC2, on the other hand, differentiate into immature DCs (iDCs) in culture with IL-3. Several lines of evidence suggest that pre-DC2 are of lymphoid origin (Liu *et al.,* 2001). They can be derived from the earliest T cell precursors within the thymus (Shortman, 2000; Barchet *et al.,* 2005) and in humans, pre-DC2 also lack expression of the myeloid antigens CD11b, CD11c, CD13, CD33, and mannose receptors but do express the lymphoid markers CD2,

CD5 and CD7. Upon stimulation, DC1 produce large amounts of IL-12 and induce strong T helper cell type 1 (Th1) and cytotoxic T lymphocyte (CTL) responses (Kalinski *et al.,* 1999; Tanaka *et al.,* 2000; Loré, 2004), while DC2 produce low amounts of IL-12 and induce T helper cell type 2 (Th2) responses or the generation of IL-10 producing $CD8^+$ T suppressor cells (Ito *et al.,* 1999; Rissoan *et al.,* 1999). Unlike IL-3 and CD40 ligand-induced DC2, which promote Th2 responses, viral induced DC2 promote helper T cells to produce both IFN-α/β and IL-10 (Rissoan *et al.,* 1999). Pre-DC2 represent the key effector cells in the early anti-viral innate immune response (Liu *et al.,* 2001; Barchet *et al.,* 2005). Pre-DC2 are also sometimes referred to as interferon producing cells (IPCs).

DCs generated from CLA^+ precursors in the presence of TGF-β resemble LCs while those derived from CLA^- precursors and human monocytes are most similar to interstitial DCs. DCs derived from plasmacytoid DC precursors may be equivalent to $CD123^+$ DCs described in tissues. As different subsets of DC express different Toll-like receptors (TLRs), certain classes of microbes that are recognised by particular TLR, activate distinct DC subsets. For example, CpG containing oligonucleotides bind to and signal via TLR9. Expression of TLR9 is restricted to plasmacytoid DCs in humans (Barton and Medzhitov, 2002; Shortman and Liu, 2002).

1.1.2. DC maturation, antigen processing and presentation

Since the first description by Steinman and Cohn in 1973 of the plasmacytoid DC as a cell type isolated from lymphoid tissues of mice, it has become clear that cells of this lineage are widely distributed throughout lymphoid and most non-lymphoid tissues of all mammalian species studied (Steinman and Cohn, 1973; Shortman and Liu, 2002; Loré, 2004). DCs are able to engulf a wide variety of antigens (Ags) and subsequently present processed peptide antigens in the context of major histocompatibility complex (MHC) class I and II molecules to induce an antigen-specific immune response (Banchereau and Steinman, 1998; Wilson and Villadangos, 2005). MHC class I presents

peptides predominantly to $CD8^+$ T lymphocytes and MHC class II to $CD4^+$ T lymphocytes (Banchereau and Steinman, 1998). These responses are critical for defence against infections and also tumours. In some instances, DCs can also generate regulatory T cell function and are important for the control of immunopathological phenomena and transplant rejection (Steptoe and Thomson, 1996; Ludewig *et al.,* 1999; Quaratino *et al.,* 2000).

DCs exist in two major functionally and phenotypically distinct stages, immature and mature, as illustrated in Figures 1.1 and 1.2. (Banchereau and Steinman, 1998; Larsson, 2005). Immature DCs have a strong capability to capture antigen by three distinct mechanisms: 1) macropinocytosis, a process in which large amounts of extracellular fluid are taken up non-specifically in single vesicles; 2) adsorptive endocytosis, mediated by binding to clathrin-coated pits and 3) phagocytosis, a process leading to ingestion of particles by attachment to receptors and subsequent engulfment (Lanzavecchia, 1996; Albert *et al.,* 1998; Banchereau and Steinman, 1998; de Baey and Lanzavecchia, 2000; Loré, 2004). Receptors involved in antigen binding and uptake include the phagocytic Fc gamma receptors (FcγRs), CD32 and CD64 (Rossi and Young, 2005); the high- and low–affinity IgE receptors FcεRI and FcεRII (CD23) (Regnault *et al.,* 1999; Holloway *et al.,* 2001); the complement receptors CD11b and CD11c (Julia *et al.,* 2002); a C type lectin mannan binding receptor (DEC205) (Lu *et al.,* 2001; Rossi and Young, 2005) and the scavenger receptor pair for apoptotic cells namely αvβ5 and CD36 (Fadok *et al.,* 1998). During maturation, as endocytosis decreases, these receptors are usually down-regulated (Banchereau and Steinman, 1998). The process of maturation including enhanced antigen uptake potential, antigen presentation, enhanced expression of co-stimulatory molecules and increased immunostimulatory capacity have also been described in primate DCs in response to microbial products (Frank and Pope, 2001; Loré, 2004).

Upon infection of the host with pathogens, a variety of soluble factors are produced by phagocytes and other cells involved in innate immune responses. Two important classes of soluble factors are the chemokines,

which promote recruitment of DC precursors to sites of infection, and cytokines (TNF-α, IL-1, IL-6, IL-10, TGF-β) and prostaglandins, which promote DC activation and trigger DC maturation (Shibuya *et al.,* 1998; Brunner *et al.,* 2000; Lyakh *et al.,* 2000; Pope, 2003). In addition, other microbial products have also been shown to regulate DC maturation (Figure 1.2). These include lipopolysaccharides (LPS) from gram-negative organisms (Cella *et al.,* 1997; Rescigno *et al.,* 1999), bacterial DNA (Akbari *et al.,* 1999; Steinman and Pope, 2002) and double-stranded RNA (dsRNA) (Cella *et al.,* 1999; Steinman and Pope, 2002), all of which regulate DC maturation via TLR engagement (Rossi and Young, 2005). During the transition or maturation process the immature DC undergoes phenotypic and functional changes which results in the loss of endocytic activity, upregulation of adhesion and costimulatory molecules (CD40, CD58, CD80/B7.1 and CD86/B7.2) and redistribution of MHC class II molecules (Sallusto *et al.,* 1995; Pope, 2003). DCs also modify their profile of chemokine receptor expression that facilitates homing to lymphoid organs (Banchereau and Steinman, 1998; Frank and Pope, 2002). Chemokine receptors expressed by DCs are indicated in Table1.1.

Maturation also entails profound changes in DC morphology (e.g. the development of “dendrites”), cytoskeleton reorganisation, loss of adhesive structures, surface expression of several integrin and chemokine receptors, which are involved in mediating migration from peripheral tissues to secondary lymphoid organs, and enhanced cellular motility (Winzler *et al.,* 1997). MHC class II molecules also accumulate in late endosomal compartments and lysosomes in immature DCs, while in mature DCs class II molecules accumulate at the cell surface (Howarth and Elliot, 2004).

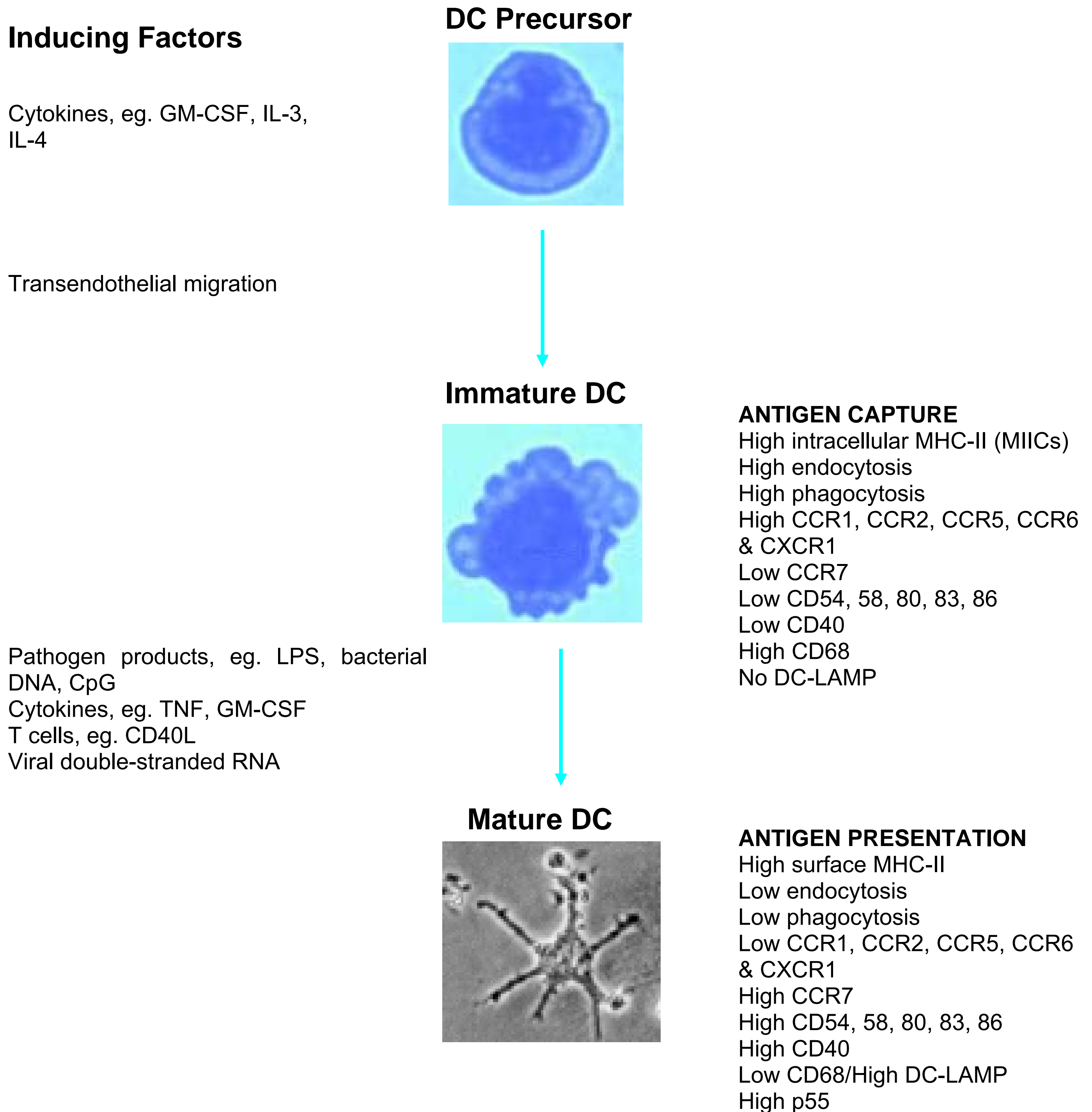


Figure 1.2. Maturation of dendritic cells (DCs). The left side of the figure indicates the factors inducing progression from one stage to another. Properties of DCs at the various stages of maturation are indicated on the right side of the figure. (GM-CSF, granulocyte/macrophage colony-stimulating factor; IL, interleukin; LPS, lipopolysaccharide; TNF, tumor necrosis factor; dsRNA, double-stranded RNA; IFN, interferon; MHC-II, major histocompatibility complex II; MIIC, MHCII-rich compartment; LAMP, lysosome-associated membrane protein).

Table 1.1 Expression of chemokine receptors by dendritic cells[a]

Receptor	Ligands
Immature DC	
CCR1	MIP-1α, RANTES, MCP-3, MIP-5
CCR2	MCPs
CCR4	TARC, MDC
CCR5	MIP-1α, MIP-1β, RANTES
CCR6	MIP-3α
CXCR1	IL-8
CXCR4	SDF-1
Mature DC	
CCR7	MIP-3β, SLC (6 Ckine)

[a]Abbreviations: DC, dendritic cell; MIP, macrophage inflammatory protein; RANTES, regulated on activation normal T expressed and secreted; MCP, monocyte chemoattractant protein; TARC, thymus and activation-regulated chemokines; MDC, monophage derived chemokines; SDF, stromal derived factor; IL, interleukin; SLC, secondary lymphoid-tissue chemokine

The increased capacity to generate functional peptide-MHC complexes may result in these complexes reaching the cell surface partly associated with CD86 (Inaba *et al.,* 2000; Turley *et al.,* 2000). In contrast, MHC class I molecules do not accumulate in the lysosomes, but are up-regulated upon maturation, possibly reaching the surface in part together with class II molecules (Turley *et al.,* 2000).

Antigen processing and presentation are processes that occur within a cell that result in fragmentation of proteins into peptides, association of the peptides with MHC molecules, and expression of the peptide-MHC molecules at the cell surface where they can be recognised by the T cell receptor on a T cell. Three major antigen presentation pathways have been described in DCs, the exogenous, endogenous, and alternative presentation pathways. Endogenous antigens are presented by MHC class I molecules present on most somatic cell types, including DCs. Exogenous antigens are generally presented by MHC class II molecules present on specialised APCs, including

DCs. Recent work has identified an alternative pathway by which DCs are able to process extracellular antigens for presentation by MHC class I molecules (Yewdell *et al.,* 1999; Howarth and Elliot, 2004; Trombetta *et al.,* 2005). This process has been termed cross-presentation and the resulting immune response is termed cross-priming (Figure 1.3). Two different mechanisms have been identified for the processing of exogenous antigens by the alternative MHC class I pathway, a TAP-dependent pathway and a TAP-independent pathway (Morel *et al.,* 2000; Ackerman and Cresswell*,* 2004). Antigens presented by MHC class I molecules can activate $CD8^+$ T cells whereas antigens associated with MHC class II molecules are presented to $CD4^+$ T cells. The importance of cross presentation lies in the fact that endogenous antigens can be presented to CD8 T cells, resulting in a CTL response (Castellino and Germain, 1995; Shi *et al.,* 2000; Lizee *et al.,* 2003).

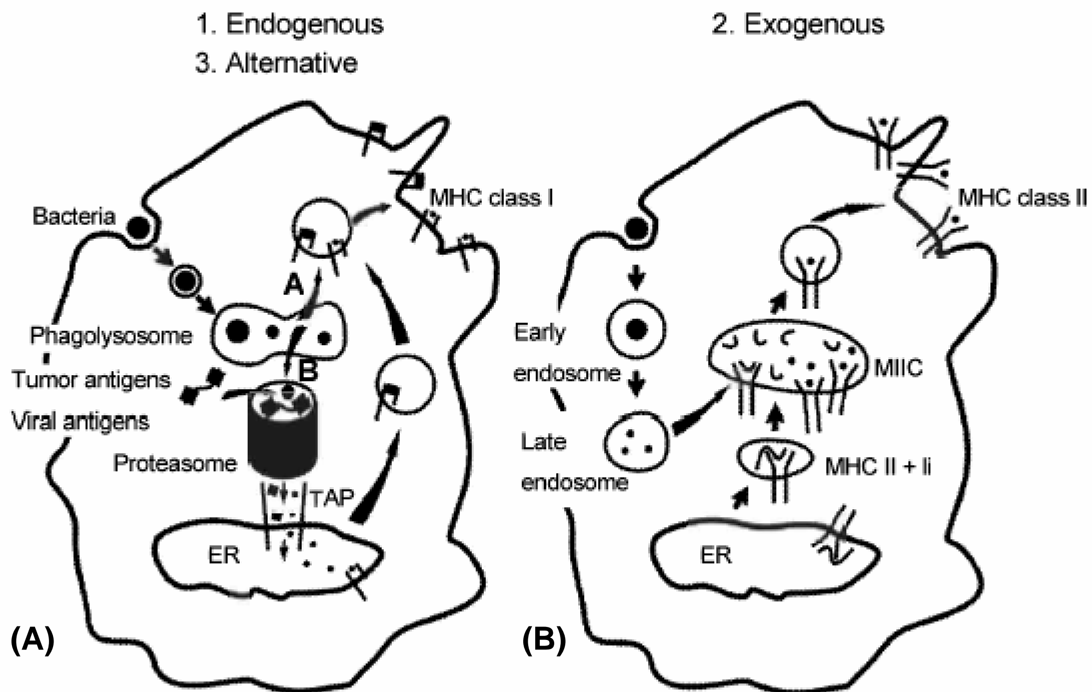


Figure 1.3. The three antigen presentation pathways operative in DCs. Presentation of antigen by MHC-1 is illustrated in (A) whereas MHC-II presentation of exogenous antigen is illustrated in (B). ER: Endoplasmic Reticulum; TAP: Transporter Associated with Antigen Processing; Ii: Invariant Chain; MIIC: MHC class II rich compartments. Adapted from Banchereau *et al.,* 2000.

An additional DC antigen presentation pathway involving CD 1 molecules has also been described. CD1 molecules are a family of non-polymorphic histocompatibility antigens associated with β2m, as are MHC class I molecules (Brenner and Porcelli, 1997; Shinkai and Locksley, 2000; Brigl and Brenner, 2004). However, antigen processing and presentation by CD1 differs from that described for MHC class I and class II. Antigens presented by CD1 are not peptides but rather microbial lipids and glycolipids of both endogenous and exogenous origin, which require uptake and intracellular processing by DCs (Porcelli and Modlin, 1999; Sugita *et al.,* 2004; Hava *et al.,* 2005).

1.1.3. DC-Pathogen interactions

The majority of DCs in peripheral tissues exhibit an immature phenotype. After contact with microbial products or proinflammatory cytokines, immature DCs convert to mature DCs and this process drives their migration first to lymphatic vessels and then to the draining lymph nodes (Loré, 2004; Larsson, 2005).

DCs in the draining lymph nodes are capable of delivering different types of signals depending on the microorganisms that they have encountered. It is generally acknowledged that certain maturation stimuli will differentiate DCs into subtypes that favour the development of either Th1 or Th2 cells *in vitro* (Wan and Bramson, 2001). In general, DCs infected by viruses, intracellular pathogens or yeasts produce IL-12 and skew T cells toward Th1 differentiation (Reis e Sousa, 2001; Ito *et al.,* 2002; Jankovic *et al.,* 2002). Conversely, DCs having encountered extracellular pathogens, including parasites or fungal hyphae, will induce Th2 response via an unknown mechanism (Reis e Sousa, 2001; Rescigno, 2002; Perruccio *et al.,* 2004). DCs can release IL-10 and activate regulatory/suppressor T cells after encounter with certain pathogens e.g. *Bordetella pertussis* (McGuirk *et al.,* 2002). Finally, DCs can also activate B cells in response to viruses and certain bacteria e.g. *Streptococcus pneumonia*, presumably as a consequence of activation by type I IFNs and promote immunoglobulin isotype switching (Le Bon *et al.,* 2001).

There is emerging evidence that two different classes of receptors are important for the interaction of DCs with microorganisms, those involved in internalisation and those involved in DC activation. The best characterised receptors involved in activation are the Toll-like receptors (TLRs) (Hemmi *et al.,* 2000; Kaisho and Akira, 2001). They are a family of innate immune-recognition receptors that recognise molecular patterns associated with microbial pathogens (PAMPs) and induce antimicrobial immune responses. TLRs are expressed on multiple cell types including DCs, macrophages and epithelial cells and they cooperate to transduce cellular activation and cytokine production signals by activating members of NF-κB transcription

factor family (Kaisho and Akira, 2001). *In vitro* addition of double-stranded RNA (dsRNA) or CpG DNA to DCs has been shown to promote their activation via TLR3 (Alexopoulou *et al.,* 2001) or TLR9 (Hemmi *et al.,* 2000), respectively. Infection of immature human DCs with viruses e.g. influenza virus (dsRNA) induces DC activation and stimulates IL-12 production, driving T cell polarisation toward the production of Th1-type cytokines (Cella *et al.,* 1999).

Immune evasion strategies by many pathogens are aimed at impairing recognition of infected cells or providing resistance to immune effector mechanisms (Tortorella *et al.* 2000; Maksymowych and Kane, 2000). DC-pathogen interactions may lead to immune evasion by different mechanisms. Infection of immature DCs by influenza virus, for example, can lead to apoptotic death of the DCs (Plotnicky-Gilquin *et al.,* 2001). Other viruses can also impair the acquisition and processing of antigens by DCs. A more effective mechanism by which pathogens may inhibit the functions of immature DCs is via interference with DC maturation, as has been shown in the case of herpes simplex virus type 1 (HSV-1), vaccinia viruses and human T lymphotropic virus type I (HTLV-1) (Engelmayer *et al.,* 1999; Salio *et al.,* 1999; Makino *et al.,* 2000; Hirata *et al.,* 2001; Prechtel *et al.,* 2005). Another strategy is inhibition of migration from sites of antigen capture into the T cell areas of lymphoid tissues. For example, in DCs infected with HSV-1, upregulation of CCR7 is blocked, preventing the responsiveness to chemokines that direct DC migration (Salio *et al.,* 1999; Prechtel *et al.,* 2005). In the case of human immunodeficiency virus type 1 (HIV-1), infected monoctye-derived DCs have been reported to have a reduced ability to stimulate $CD4^{+}$ T cell proliferation and produce IL-2 (Kawamura *et al.,* 2003; Fantuzzi *et al.,* 2004).

1.2. HIV

1.2.1. HIV origin, classification and structure

Human immunodeficiency virus type 1 (HIV-1) is a member of the genus *Lentivirinae* within the family *Retroviridae*. HIV-1 was first isolated and characterised in 1984 (Gallo *et al.,* 1984). Three groups of HIV-1 have subsequently been described and classified as follows: M (major), N (new) and O (outlying), with group M being responsible for most infections worldwide. Within group M there are nine distinct viral subtypes namely: A-D, F-H, J and K (Spira *et al.,* 2003). The HIV-1 genome consists of two single-stranded RNA molecules per virion, each approximately 9 kilobases in length (Greene, 1991). The HIV genome encodes three structural genes, a feature common to all retroviruses. These are: *gag* (group antigen), encoding the precursor for virion capsid proteins; *pol* (polymerase), which encodes the precursor for the three essential enzymes (protease, reverse transcriptase with Rnase H and integrase) and *env* (envelope), encoding for the precursor of envelope glycoprotein (Greene, 1991; Levy, 1993; Tang *et al.,* 1997). Two overlapping exons encode the genes for transcriptional transactivation (*tat*) and regulator of viral expression (*rev*), these small non-virion proteins enhance viral replication (Vaishnav and Wong-Staal, 1991). In addition HIV also carries additional genes, called “accessory genes”; *vif* (viral infectivity factor), *vpu* (viral protein unique), *vpr* (viral protein) and a repressor gene *nef* (negative factor). Unlike other viruses in the lentivirus family, HIV-1 has no *onc* gene (Tang *et al.,* 1997). The HIV genome is illustrated in Figure 1.4. In 1986, a second type of HIV was discovered in West Africa, namely: HIV-2 (De Cock *et al.*, 1993; Santiago *et al.,* 2005). HIV-2 is similar to a simian immunodeficiency virus (SIV) found in sooty mangabeys (SIV_{sm}). HIV-2 and SIV_{sm} share an identical genome structure, including an accessory protein termed *vpx,* that is not found in HIV-1 and other primate lentiviruses (Khamsri *et al.,* 2005)

SIVs that infect different African nonhuman primate species have also been identified (Ndongmo *et al.,* 2004). These viruses belong to the lentivirus subfamily of retroviruses and are genomically similar to HIV (Courgnaud *et al.,* 2002). Chimeric simian human immunodeficiency viruses (SHIVs) have also

been designed by replacing the native *env* gene of SIV with the HIV-1 *env, rev, tat* and *vpu* genes. These viruses were designed to mirror HIV-1 disease in macaques and for use in challenge studies (Reinmann *et al.,* 1999; Cayabyab *et al.,* 2004).

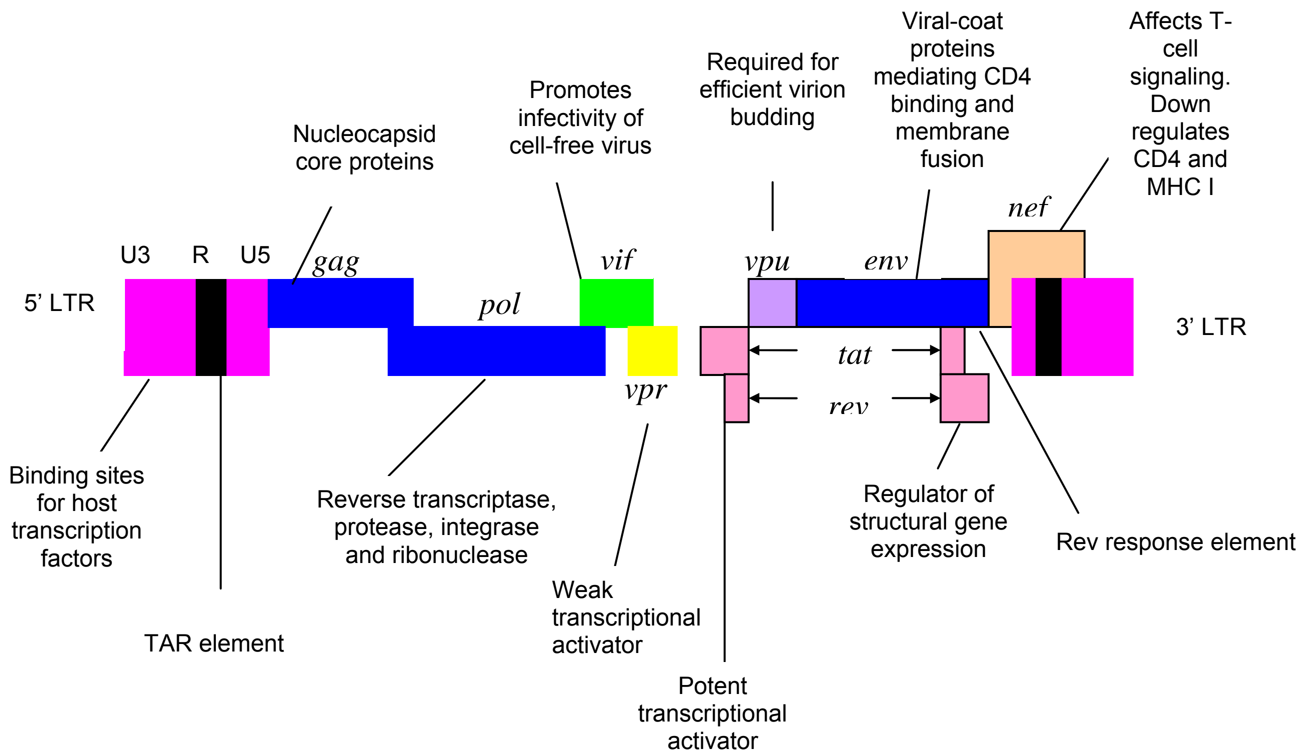


Figure 1.4. Genomic Structure of HIV-1. All nine known HIV-1 genes are shown in this schematic diagram, with recognised primary functions also indicated. The 5' and 3' long terminal repeats (LTRs) containing regulatory sequences for recognition by various host transcription factors are also depicted. Note that the *tat* and *rev* genes consist of more than a single encoding region. The *tar* (transactivation response element) and the *rev* response element are also indicated.
Source: Adapted from Greene, 1991

1.2.2. HIV-1 Pathogenesis

The course of HIV-1 infection can be divided into three different stages. The first stage is acute primary HIV-1 infection and is characterised by being asymptomatic or manifests as a mononucleosis-like illness lasting between 1-3 weeks (Levy, 1993; Derdeyn and Silvestri, 2005). During this stage the plasma viral load generally peaks and is associated with a sharp drop in $CD4^+$ T cells followed by a gradual decline in viral load, after which it remains relatively stable (Figure 1.5) (Levy, 1993; Sabin *et al.,* 2000). HIV primarily targets $CD4^+$ T cells for infection and destruction (Derdeyn and Silvestri, 2005). The second stage is clinical latency and is illustrated by a period of clinical but not necessarily virological latency. Reports have documented the continuous expression of virus during this stage that can be controlled by the immune system (Ho *et al.,* 1995). After the clinical latent stage, which can last for a number of years, the final stage namely, acquired immunodeficiency syndrome (AIDS) is reached. This stage is characterised by a further decline in the number of $CD4^+$ cells and an increase in viral load (Figure 1.5). Inevitably a stage is reached where immunodeficiency results in the development of opportunistic infections (e.g. oral thrush, herpes zoster and *Pneumocystis carinii* pneumonia) and other pathological conditions e.g. Kaposi's sarcoma. At this stage the immunocapacity of the host is poor (Wahren *et al.,* 1986; Hazenburg *et al.,* 2000; Derdeyn and Silvestri, 2005).

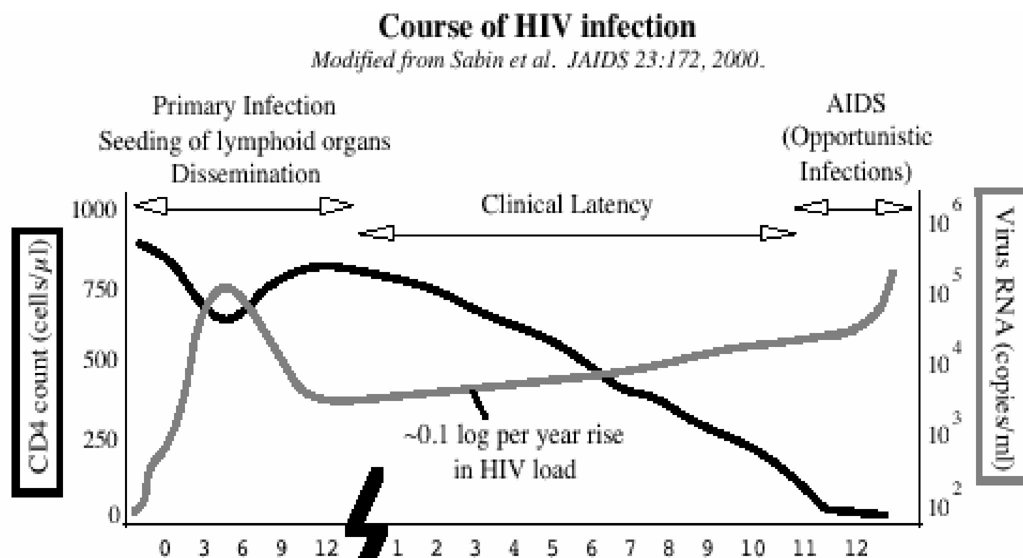


Figure 1.5. Stages of HIV disease progression and changes in viral load and $CD4^+$ T cells during HIV infection.
Source: www.urmc.rochester.edu/smd/mbi/grad2/pdf/gr01hiv2.pdf

1.2.3. Immune response to HIV

1.2.3.1. Humoral immune response

The strongest antibody responses are induced against the env (gp120 & gp41) and gag (p24 & p17) proteins. Most antibodies (Abs) generated during natural HIV-1 infection bind but do not neutralise virus well and are probably of limited efficacy in controlling the virus (Fënyo *et al.,* 1996; Wyatt *et al.,* 1998; Richman *et al.,* 2003). The antibody recognition of antigenic HIV-1 envelope epitopes is hindered by several factors. These include extensive variation in the amino acid sequence of the envelope proteins, conformational differences and masking of epitopes by other protein domains (variable loops) and carbohydrate groups (Wyatt and Sodroski, 1998; Moore *et al.,* 2001; Nara *et al.,* 2005). Neutralising antibodies (nAbs) may be a component of the initial control of HIV-1 replication. However as HIV-1 variants emerge over time in the host due to the high mutation rate, neutralising capacity may be lost, as evidenced by new variants frequently not being neutralised by autologous sera (Albert *et al.,* 1990; Moog *et al.,* 1997; Paranjape, 2005). During infection, disassembled envelope glycoproteins elicit most of the Abs directed to these viral components (Parren *et al.,* 1997). This results in Abs that are unable to recognise the functional envelope glycoprotein complex (Burton *et al.,* 2004). The fact that infection is already established when nAbs emerge, appears to indicate that humoral immunity is not highly effective (Wyatt and Sodroski, 1998; Letvin, 2005). HIV-1 specific Abs have even been shown to enhance infection under certain conditions e.g. enhancing uptake via Fc receptors (Matsuda *et al.,* 1989) and binding of complement (Robinson *et al.,* 1988).

Although antibody responses appear to be inefficient, their importance is highlighted by passive transfer of nAb-mediated protection. Transfer of potent neutralising antibodies results in protection against HIV-1 and SHIV infection in primate models (Baba *et al.,* 2000; Nishimura *et al.,* 2003; Veazey *et al.,* 2003; Ferrantelli *et al.,* 2004). Inducing Abs by vaccination of monkeys or humans has not, however, to date resulted in development of potent nAb responses (Letvin, 2005).

1.2.3.2. Cellular immune response

$CD8^+$ cytotoxic T lymphocytes (CTLs) appear to be important in containing the spread of HIV-1 in infected individuals (Letvin, 1998; Letvin *et al.,* 2002). The replication of HIV-1 in $CD4^+$ T-cells can be inhibited by autologous $CD8^+$ CTLs (Letvin, 2005), through mechanisms which probably involve both lysis and the release of chemokines (i.e., MIP-α, MIP-β and RANTES), cytokines and other inhibitory factors (Yang *et al.,* 1997; Chun *et al.,* 2001; Letvin, 2005). The viral containment in early HIV-1 infection coincides in time with the emergence of a virus specific CTL response (Pantaleo *et al.,* 1994). In chronically HIV-1 infected individuals, a high CTL frequency is correlated with the maintenance of low viral load and stable clinical status (Ogg *et al.,* 1998; Letvin *et al.,* 2002). Furthermore, studies have shown that HIV-1 specific CTL responses are maintained throughout the asymptomatic stage but decline with disease progression (Paranjape, 2005). Despite vigorous CTL responses, virus is not eradicated from infected persons (Collins, 2003). This implies that the T-cells (both T-helper cells and CTLs) of HIV-1 infected persons may be dysfunctional in some way (Brander and Walker, 1999). Although CTL escape has been observed, this is probably not the major mechanism of viral persistence.

In summary since natural immunity fails to eradicate the virus from the body, a vaccination strategy that will prime the immune system to respond more swiftly and which will elicit potent and vigorous humoral and cellular immune responses is desperately needed (Hanke, 2001; Letvin *et al.,* 2002; Collins, 2003).

1.2.3.3. DCs, HIV and the immune response

Human and primate DCs are key players during the transmission of the HIV-1 and SIV to $CD4^+$ T lymphocytes (Wu *et al.,* 2002). In their immature state, DCs reside and sample foreign antigens at peripheral sites (Banchereau and Steinman, 1998; Larsson, 2005). The most common route for infection with HIV-1 worldwide is through sexual contact via oral, rectal and/or vaginal mucosae (Ayehunie *et al.,* 1997; Pope, 2003; Excler, 2005). DCs residing at

these sites play a pivotal role in HIV-1/SIV capture and dissemination. DCs also appear to serve as reservoirs for HIV/SIV *in vivo* (Hu *et al.,* 1998) and they shuttle these viruses from the periphery into the lymphoid tissues (Loré, 2004; Larsson, 2005).

In their immature state, DCs express co-receptors essential for HIV-1 entry namely CD4 and the chemokine receptor CCR5 (David *et al.,* 2001, Larsson, 2005). At this stage, the costimulatory molecules; CD40, CD80 and CD86; are absent (Granelli-Piperno *et al.,* 1998; Rinaldo and Piazza, 2004). Due to CCR5 expression immature DCs readily bind macrophage (M)-tropic HIV-1 strains (also termed R5 viruses), which are the most readily transmitted virus type and are most prevalent early in infection (David *et al.,* 2001; Gross *et al.,* 2003). In contrast, it has been shown that iDCs lack expression of CXCR4 and resist infection by T-lymphotropic (T-tropic) isolates (or X4 virus) of HIV-1 (Granelli-Piperno *et al.,* 1998; Rinaldo and Piazza, 2004) that infects T cells and predominates at the late stages of HIV infection (Gross *et al.,* 2003).

1.2.3.3.1. HIV replication in DCs and DC-T cell conjugates

Studies have shown that purified DCs alone cannot support productive infection by HIV. Interaction of DCs with syngeneic $CD4^{+}$T cells does, however, lead to vigorous viral replication, a finding implicating DC-T cell interaction in viral replication (Pope *et al.,* 1995; Granelli-Piperno *et al.,* 1998; Larsson, 2005).

There have been discrepant reports on the susceptibility of DCs to HIV-1 infection (Rinaldo and Piazza, 2004; Larsson, 2005). This contradiction may partly be due to different methodologies of DC culture resulting in the generation of cells with different phenotypic characteristics. In addition, it has recently been illustrated that the infectability of DCs could be related to the state of maturation and the level of the relevant chemokine receptor expression (Granelli-Piperno *et al.,* 1996; Blauvelt *et al.,* 1997; Zaitseva *et al.,* 1997; Reece *et al.,* 1998). The developmental stage of DCs can thus affect their interaction with HIV-1 and the extent to which M- and T-tropic virus can

replicate (Compton *et al.,* 1996; Warren *et al.,* 1997). Mature DCs seem unable to support replication of HIV-1, while iDCs have been shown to be productively infected with M-tropic strains (Larsson, 2005). In mature DCs the block in replication has been suggested to be at the level of reverse transcription (Granelli-Piperno *et al.,* 1996; Granelli-Piperno *et al.,* 1999). Conversely, a recent study has shown that iDCs cultured in the presence of GM-CSF and IL-4 successfully complete reverse transcription of viral RNA. These iDCs in turn can produce viral particles that easily infect activated T cells (Granelli-Piperno *et al.,* 1998). Even though mature DCs do not produce infectious virus, they are still able to transmit viral particles to T cells (Weissman *et al.,* 1995). Furthermore, Granelli-Piperno and colleagues have observed that both M- and T-tropic HIV-1 particles readily adhere to and enter DCs (Granelli-Piperno *et al.,* 1999). The exact mechanism whereby this occurred was revealed upon the identification of DC-SIGN (Dendritic cell-specific ICAM-3-grabbing non-integrin), a protein that interacts with HIV-1 (Geijtenbeek *et al.,* 2000a).

DC-SIGN, also known as CD209, is a type II integral membrane protein, belonging to the C-type lectin family expressed by both immature and mature DCs (Geijtenbeek *et al.,* 2000b; Turville *et al.,* 2004). DC-SIGN contains a short cytoplasmic N-terminal, a “neck” region comprising of seven and a half tandem repeats and a C-terminal lectin or carbohydrate-recognition domain (CRD) (Pohlmann *et al.,* 2001a). The structure of DC-SIGN is illustrated in Figure 1.6.

Intercellular adhesion molecule 2 (ICAM-2) and ICAM-3 have been identified as the natural ligands of DC-SIGN. DC-SIGN-ICAM-2 interactions mediate DC transmigration into vascular and lymphoid endothelium (Nobile *et al.,* 2003; Arrighi *et al.,* 2004). The DC-SIGN-ICAM-3 interaction allows for the transient DC-T cell clustering necessary for T cell activation (Geijtenbeek *et al.,* 2000b; Nobile *et al.,* 2003; Arrighi *et al.,* 2004). DC-SIGN mediates binding of primary M-, T- and dual tropic (R5X4) HIV strains (i.e. use both CCR5 and CXCR4 coreceptors) as well as HIV-2 and SIV strains to the T cell

surface, indicating that this molecule may function as a universal attachment factor for primate lentiviruses (Geijtenbeek *et al.,* 2000a; Pohlmann *et al.,* 2001b; Princen and Schols, 2005). DC-SIGN does not allow the virus to enter or infect the DC (Geijtenbeek and van Kooyk 2003). Instead, it retains the attached virus in a stable, infectious state for several days, which permits the DC to migrate from the mucosal to local lymph nodes where it then facilitates HIV infection of permissive $CD4^+$ T cells in *trans* (Geijtenbeek *et al.,* 2000b; Nobile *et al.,* 2003; Lekkerkerker *et al.,* 2004). These $CD4^+$ T cells also express the chemokine coreceptor CCR5 (Derdeyn and Silvestri, 2005). The virus therefore exploits DC migration by binding to DC-SIGN on the surface of the iDC via gp120, hitch-hiking on the migrating DC to its target, the $CD4^+$ T cell (Rowland-Jones, 1999). On interaction between DCs and $CD4^+$ T cells the HIV-1 virus is placed in close proximity to the T-cell, facilitating cell entry via CD4 and CCR5 (Teleshova *et al.,* 2003; Larsson, 2005).

The exact mechanism by which bound virions are transported from the periphery to the lymphoid organs by DCs is poorly understood. Presently, it still has to be elucidated whether infectious viral particles remain bound to the cell surface or if virions are internalised into intracellular vesicles (Blauvelt *et al.,* 1997; Granelli-Piperno *et al.,* 1998; Geijtenbeek *et al.,* 2000a; Pohlmann *et al.,* 2001b). One very pertinent question is how HIV remains DC-SIGN-bound, enters endosomal and lysosomal compartments, yet resists endosomal degradation (Turville *et al.,* 2004). DCs possess rapid recycling pathways in the superficial part of their cytoplasm (Thery and Amigorena, 2001), which may explain how HIV avoids degradation. It has also been demonstrated that DC-SIGN-bound HIV is rapidly internalised into low pH non-lysosomal compartments and that this internalisation is necessary for the virus to retain its competence and infect target cells (Kwon *et al.,* 2002). Recently, it has been reported that when DCs are HIV infected, Nef inhibits the endocytosis of DC-SIGN (Sol-Foulon *et al.,* 2002; Nobile *et al.,* 2003). This increased presence of DC-SIGN caused by Nef results in enhanced binding of HIV to DCs and also enhanced exposure to T cells, which in turn may ultimately increase viral transmission (Nobile *et al.,* 2003; Larsson, 2005).

1.3. Primate models of HIV-1 infection

1.3.1. Primates used in HIV research

Animal models of AIDS have been invaluable in assessing the efficacy of experimental prophylactic and therapeutic vaccines and/or drug regimens (Letvin, 2005). They have aided in developing our understanding of the immune responses associated with a clinically healthy state and disease progression (Nathanson *et al.,* 1999). Nonhuman primates particularly have served as excellent models for human disease because of their close phylogenetic relationship to humans. In addition they exhibit remarkable similarities to humans in almost every aspect of their anatomy and physiology. Non-human primates preferentially used in HIV-1 vaccine research are the chimpanzee (*Pan troglydytes*), the macaque monkey including cynomologus (*Macaca fascicularis*), rhesus (*Macaca mulatta*) and pig-tailed (*Macaca nemestrina*) varieties, the African green monkey (*Cercopithecus aethiops*) and the baboon (*Papio cynocephalus*) (to date predominantly HIV-2 research) (Kennedy *et al.,* 1997; Leung *et al.,* 2004; ten Haaft *et al.,* 2004; Ellenberger *et al.,* 2005; Goldstein *et al.,* 2005).

1.3.2. Primates and vaccine immunogenicity studies

It has been reported that infections by primate lentiviruses occurs solely amongst African monkeys and chimpanzees in their natural habitats (Kennedy *et al.,* 1997; Hirsch, 2004; Bontrop and Watkins, 2005). Investigators have highlighted the absence of fatal disease in certain species of monkey infected with SIV and have demonstrated disease to occur only after transmission to another species (Hirsch, 2004). Evidence suggests that macaques (*Macaca mulatta*), the most popular and universal of all nonhuman primate models in HIV research, are not the natural host of simian immunodeficiency virus that infects macaques (SIV_{mac}) (Seman *et al.,* 2000; Hirsch, 2004; Bontrop and Watkins, 2005). Rather, it is believed that captive macaques acquired SIV, via horizontal transmission, from sooty mangabeys through fighting and biting whilst being co-housed in outdoor corrals (Gardner, 1996). As in human

AIDS, disease in macaques is associated with weight loss, deterioration of $CD4^+$ lymphocyte levels, opportunistic infections and lymphadenopathy (Daniel *et al.,* 1985; Benveniste *et al.,* 1988).

The SIV_{mac} macaque model has significantly contributed to our understanding of HIV-1 biology, by defining the mechanisms of pathogenesis, viral transmission (Sodora *et al.,* 1998; Vodros and Fenyo, 2004) and also in the assessment of antiviral drug efficacy (Überla *et al.,* 1995; Van Rompay *et al.,* 2002). Furthermore, the SIV_{mac} macaque model has also addressed questions regarding the dosage, timing and the effects of antiviral therapy on disease progression (Überla *et al.,* 1995; Van Rompay *et al.,* 1998). Various groups have demonstrated that immune responses in infected macaques resemble those in humans infected with HIV-1. In both instances, cell mediated immune responses suppress viral replication whilst the appearance of neutralising antibodies does not inhibit viral replication (Letvin *et al.,* 1993; Johnson, 2002; Kumar *et al.,* 2002). Javaherian and colleagues suggest that the principal neutralising domains of SIV_{mac} are conformational rather than linear as in HIV-1 (Javaherian *et al.,* 1992). This finding would appear to limit the utility of this model for evaluating passive immunisation and HIV-1 envelope based vaccine strategies. A significant disadvantage of the SIV_{mac} macaque model is the rapid disease course following infection with SIV, a pattern very different to HIV infection in humans (Benveniste *et al.,* 1988; Joag, 2000).

Chimpanzees (*Pan troglodytes*) have been extensively used as a model to examine novel vaccine and therapeutic strategies for a number of human pathogens including HIV-1 (Girard *et al.,* 1991; Fultz *et al.,* 1992; Kennedy *et al.,* 1997). The attractiveness of this model lies in the fact, that it is the only other species besides humans that is vulnerable to infection by HIV-1 (Bontrop and Watkins, 2005). After exposure of the animal to viral inoculum, there is a seroconversion phase whereby the virus establishes a long-term persistent infection, concomitantly inducing cellular and humoral immune responses. Virus can also be isolated from peripheral blood lymphocytes of infected animals (Mwaengo and Novembre, 1998; Novembre *et al.,* 2001).

Despite the advantages, research using this model suffers from two main drawbacks; firstly, chimpanzees exposed to live virus seldom develop full-blown AIDS (Novembre *et al.,* 2001), and secondly, due to their endangered species status and their reputation as an highly intelligent animal species, the use of chimpanzees is controversial (Novembre *et al.,* 1997). Research with this species is closely monitored by animal rights groups (Joag, 2000). The availability of chimpanzees per research group is limited. Therefore due to the small number of animals normally used, these experiments often raise questions regarding statistical significance of results (Kennedy *et al.,* 1997; Joag, 2000). The cost of this species is also a prohibitive factor for most research groups (Novembre *et al.,* 1997).

Chimeric simian human immunodeficiency viruses (SHIVs) were originally designed to better mimic HIV-1 disease in macaques (Reinmann *et al.,* 1999; Cayabyab *et al.,* 2004). SHIV chimeras are created by replacing the native *env* gene of SIV with the HIV-1 *env, rev, tat* and *vpu* genes. This allows for evaluation of HIV envelope vaccines directly in an animal model (Reinmann *et al.,* 1999; Kuwata *et al.,* 2002; Si *et al.,* 2004). Initial SHIVs were avirulent, as infected macaques remained asymptomatic (Sakuragi *et al.,* 1992). In order to enhance the virulence of SHIVs, *in vivo* animal-to-animal passage in macaques has been performed (Reinmann *et al.,* 1996; Endo *et al.,* 2000; Sadjadpour *et al.,* 2004). Utilising this approach, several pathogenic SHIVs have been generated (Joag *et al.,* 1996; Reinmann *et al.,* 1996; Shibata *et al.,* 1997; Lu *et al.,* 1998; Luciw *et al.,* 1999; Sadjadpour *et al.,* 2004). SHIVs have been shown to establish infection by transmission routes known to occur during natural HIV-1 infection of humans such as intravenous, intravaginal, intrarectal and oral routes. The aforementioned studies also revealed that infection with pathogenic SHIV shared common features with HIV-1 infection in humans, most importantly a profound state of immunodeficiency characterised by a precipitous loss in peripheral $CD4^+$ T cells (Joag *et al.,* 1996; Reinmann *et al.,* 1996; Lu *et al.,* 1998; Luciw *et al.,* 1999; Sadjadpour *et al.,* 2004; Si *et al.,* 2004). However, the kinetics of SHIV-induced lymphocyte loss differed significantly from that observed in either SIV or HIV-1 infection, raising questions as to the validity of this model system (Letvin, 1998;

Nishimura *et al.,* 2004). Interestingly, all the pathogenic SHIVs used in these studies utilise the CXCR4 coreceptor or are dual-tropic (Cayabyab *et al.,* 2004; Princen and Schols 2005). This differs from HIV-1 infection, where the infecting viruses are predominantly CCR5-tropic. The disease induced by these X4 and R5X4 SHIVs is similar to late-stage HIV-1 infection (Koot *et al.,* 1993; Cayabyab *et al.,* 2004). A study conducted by Klinger and associates demonstrated that while constructing a subtype E SHIV the infectivity properties of the original virus was changed. A resultant novel SHIV containing a subtype E envelope was found to infect both human and baboon PBMCs but not rhesus macaque PBMCs, despite the origins of the SIV backbone (Klinger *et al.,* 1998).

1.3.2.1. Baboons as a model for vaccine immunogenicity studies

Baboons (*Papio* species) are not extensively used in first world countries and are not easily obtainable there, resulting in minimal published work on the model. To date no work on baboon DCs has been published and the current study is thus the first to examine baboon DCs in detail. Recent viral persistence and pathogenesis studies have shown that baboons may represent an attractive alternate animal model for the study of HIV pathogenesis. Infection of baboons with HIV-2 results in chronic viral infection that progresses over a number of years to a final stage of onset of AIDS (Barnett *et al.,* 1994; Locher *et al.,* 1998a). End stage disease in these animals is associated with a wasting syndrome, Kaposi's sarcoma-like lesions and a decline in $CD4^+$ T cells (Barnett *et al.,* 1994; Locher *et al.,* 1998b). Baboons do not develop SIV-induced AIDS. Due to the development of chronic viral infection, it is hoped that this model can enhance our understanding of the immuno-biology of viral latency, clinical stages of disease, virus infection of lymphatic tissue and HIV transmission (Benveniste *et al.,* 1988; Locher *et al.,* 2003; Locher *et al.,* 2004).

Baboons are an appealing alternative animal model for the study of AIDS pathogenesis and particularly vaccine induced immunogenicity (Leung *et al.,* 2004). This is especially pertinent in an African setting. Baboons have been

shown to thrive in captivity, are readily available and unlike chimpanzees, they are not endangered (Rogers and Hixson, 1997; Locher *et al.,* 2003). Additionally, because of their size, greater volumes of blood can be collected than from most other species. This aids in detailed assessment of cellular immune responses. A further advantage of baboons is that they are not natural host of herpes B virus. This results in safer handling conditions and also minimises risks for researchers utilising animal blood products (Kennedy *et al.,* 1997). Finally, baboons are phylogenetically, physiologically, anatomically and genetically more closely related to humans than macaques (Hainsey *et al.,* 1993; Kennedy *et al.,* 1997; Rogers and Hixson, 1997; Harewood *et al.,* 1999; Locher *et al.,* 2003). In contrast to monkeys, baboons and humans have four immunoglobulin G (IgG) subclasses, thus making them more useful for the preclinical evaluation of vaccines (Damian *et al.,* 1971; Shearer *et al.,* 1999; Locher *et al.,* 2004).

1.4. Vaccines

1.4.1. Introduction to vaccines

The first vaccine can be attributed to Edward Jenner, who inoculated a healthy boy with cowpox in 1796. This "variolation" proved to protect the boy form repeated subsequent challenge with virulent smallpox (http://www.sc.edu/library/spcoll/nathist/jenner2.html; http://www.zephyrus.co.uk/edwardjenner.html). Subsequently viral vaccines based on attenuated live virus or killed whole virions have been produced (reviewed in Plotkin, 2005). More recent strategies include the isolation of immunogenic parts of viruses to produce subunit vaccines based on viral proteins or peptides. Viral vectors for the delivery of genes of other unrelated viruses have been studied extensively in recent years, and offer an attractive alternative approach (Plotkin, 2003; Plotkin, 2005; Excler, 2005; Quintana-Vazquez *et al.,* 2005). DNA vaccination is the focus of many groups at present. DNA plasmids containing viral genes have also been demonstrated to induce good immune responses (Imami *et al.,* 2004; Rosati *et al.,* 2005).

1.4.2. HIV-1 vaccines

The quest for an HIV-1 vaccine began soon after the discovery of the virus. In the early 1980's optimistic researchers predicted the availability of a vaccine within 10 years (reviewed in Hanke, 2001). At the time, scientists applied the traditional vaccine approaches, namely, killed-whole inactivated virus vaccines and live attenuated virus vaccines. At this stage there was incomplete understanding of which immune responses were essential for preventing HIV replication. These conventional approaches were soon abolished when researchers discovered that they were unsafe and ineffective (Hanke, 2001; Letvin, 2002; Letvin, 2005).

Early HIV vaccine efforts focused on generating Abs against the envelope protein of the virus: gp160 (fusion of gp120 and gp41) or gp120 (Berman *et al.,* 1988; Redfield *et al.,* 1991). It was demonstrated that soluble gp120 was able to induce nAbs in small animals and in non-human primates (Robey *et al.,* 1986; Haigwood *et al.,* 1992; McKeating *et al.,* 1993). Further, envelope-based vaccine approaches were also shown to protect chimpanzees from HIV following challenge with homologous virus (Berman *et al.,* 1988; Berman *et al.,* 1990). Safety and immunogenicity studies carried out with subunit gp120 were limited to homologous, laboratory-adapted HIV strains and not primary isolates (Belshe *et al.,* 1994; Kahn *et al.,* 1994; Salmon-Ceron *et al.,* 1999; McElrath *et al.,* 2000; Graham, 2002; Paranjape, 2005). Attempts to induce neutralising antibodies against primary isolates of HIV has proven to be remarkably difficult and remains one the field's most challenging problems (Graham, 2002; Burton *et al.,* 2004).

Unfortunately, broad-spectrum neutralisation epitopes on the envelope complex appear to be rare and poorly immunogenic. To date only five human monoclonal antibodies capable of efficient, broad spectrum, cross-clade neutralisation of primary isolates have been identified, namely b12, 2G12, 2F5, Z13 and 4E10 (Zwick *et al.,* 2001; Dorgham *et al.,* 2005). Two of these monoclonal antibodies (b12, 2G12) bind to distinct epitopes on gp120, whereas the remaining three (2F5, Z13 & 4E10) recognise a region of gp41 close to the transmembrane region. Antibodies with similar binding

characteristics are rarely seen in infected individuals and are seldom isolated (Zwick *et al.,* 2001; Dorgham *et al.,* 2005). Many studies have shown that envelope based vaccine modalities fail to induce significant cytotoxic T cell responses against HIV (McElrath *et al.,* 2000; Letvin, 2005) and concomitantly fail to elicit neutralising antibodies to primary HIV isolates (Burton *et al.,* 2004). Additionally, these vaccine strategies fail to protect macaques from challenge with chimeric SHIVs (Stott *et al.,*1998; Kumar *et al.,* 2000).

More recent vaccine approaches have focused on inducing cellular immunity with the aid of viral vectors delivering HIV-1 genes. Live attenuated viral vectors encoding and expressing the gene of interest, are attractive in that they are able to infect cells and produce the antigen of interest for processing and presentation through the MHC class I presentation pathway (Letvin *et al.,* 2002). Thus an immune response to the gene insert as well as the recombinant organism is generated (Letvin, 2005). HIV genes have been inserted into such diverse vectors as avian and mammalian poxviruses (Lee *et al.,* 2004; Harrer *et al.,* 2005), replication-defective adenoviruses (Shiver and Emini, 2004), alphaviruses (Quintana-Vazquez *et al.,* 2005) rhabdoviruses (Tan *et al.,* 2005a), herpseviruses (Murphy *et al.,* 2000), adeno-associated virus (Chikhlikar *et al.,* 2004) and picornaviruses (Fultz *et al.,* 2003). The uniqueness in each of these vector systems lies in the size of the vaccine insert that it can carry, the host pro-inflammatory responses stimulated and the immune-evasion strategies of the vector (Murphy *et al.,* 2000; Fultz *et al.,* 2003; Lee *et al.,* 2004; Harrer *et al.,* 2005; Quintana-Vazquez *et al.,* 2005; Tan *et al.,* 2005a). An approach of this type offers the possibility of inducing the long-lasting, potent immunity that is achieved during infection with a live organism without the drawback of having to deliver attenuated, potentially pathogenic HIV (Graham, 2002).

1.5. DNA vaccines

Wolf and co-workers were the first to show that non-replicating DNA expression vectors could be taken up by muscle cells when injected intramuscularly (i.m.) without the use of a delivery vehicle (Wolf *et al.,* 1990). Shortly, thereafter, it was demonstrated that when mice were inoculated with

plasmids encoding a foreign gene by the particle bombardment method, antibodies were elicited against the plasmid encoded antigen, thus DNA immunisation could induce a humoral response (Tang *et al.,* 1992). Mice immunised intramuscularly with plasmids encoding the influenza nucleoprotein developed protection from subsequent challenge with a heterologous influenza strain. Both nucleoprotein-specific cytotoxic T-lymphocyte (CTL) responses and high-titer antibody were detected (Ulmer *et al.,* 1993).

DNA vaccines are able to induce broader cross-reactive immune responses than protein-based vaccines (Wahren and Brytting, 1997). A significant advantage of DNA vaccines is that they mimic some of the aspects of natural infection in host cells. Transfection of APCs with plasmid DNA results in transcription of the DNA into mRNA which then encodes proteins. These proteins can then be processed as antigen. DNA vaccines simultaneously engage both the MHC-I and MHC-II pathways for antigen presentation of endogenous or secreted exogenous protein antigens thus allowing for the elicitation of $CD8^+$ and $CD4^+$ T cell responses (Huygen, 2005). HIV specific DNA vaccines have been evaluated for safety, tolerability and immunogenecity in non-human primates and humans, with several reports showing the generation of protective immune responses (Boyer *et al.,* 1999; Lockey *et al.,* 2000; MacGregor *et al.,* 2000; Smith *et al.,* 2004; MacGregor *et al.,* 2005).

The DNA plasmid vaccine vector is bacterium-derived and besides being equipped with the antigen-encoding sequences, it contains a bacterial origin of replication that allows plasmid replication after introduction into a bacterial cell and a prokaryotic selectable marker gene which confers a means of eliminating all cells lacking the plasmid (Wahren and Brytting, 1997; Liljeqvist and Ståhl, 1999; Donnelly *et al.,* 2003).

It is important to design the plasmid for optimal expression in eukaryotic cells. Therefore strong eukaryotic transcription regulatory elements (e.g., promoter and enhancer sequences) are needed for this purpose. Virally derived

promoters from cytomegalovirus (CMV) or simian virus 40, have been shown to provide good gene expression. To achieve stabilisation of mRNA transcripts, polyadenylation sequences (Poly-A) can be incorporated into the plasmid DNA construct (Gurunathan *et al.,* 2000; Doria-Rose and Haigwood, 2003; Garmory *et al.,* 2003). Translation initiation can also be optimised by incorporation of a translation initiation region (An *et al.,* 2000).

Immunisation with DNA vaccines offers several advantages of other vaccine modalities. Unlike most conventional inactivated vaccines, DNA vaccines induce both cell-mediated and antibody-based immune responses (Thalhamer *et al.,* 2001; Leung *et al.,* 2004; Rosati *et al.,* 2005). Many studies have shown that immunisation with plasmid DNA encoding HIV-1 antigens have the ability to shift the immune response from a T-helper type (Th2) to a Th1 response (Ugen *et al.,* 1997; Muthumani *et al.,* 2002; Excler, 2005). DNA vaccines are easy to manipulate and combination vaccine constructs are readily made because all the components can be purified by the same procedure (Wahren and Brytting, 1997; Gurunathan *et al.,* 2000; Thalhamer *et al.,* 2001). This method of producing the protein antigen of interest directly in host cells will more likely result in the encoded protein antigen being folded in its native conformation, correctly glycosylated and with normal post-translation modifications as would occur during a natural infection (Otero *et al.,* 2004). This would generally favour the generation of relevant neutralising antibodies and concomitantly would facilitate the induction of cellular immune responses (Wahren and Brytting, 1997; Davis and McCluskie, 1999). The same vector can also be used for subsequent immunisation since no immune response is elicited against the vector itself, as in the case of vaccinia and other live viral vectors (Liljeqvist and Ståhl, 1999; Farina *et al.,* 2001). In addition, unlike live vaccines, DNA vaccines are unable to revert into virulence (Otten *et al.,* 2005).

Genetic vaccines are relatively inexpensive and easy to manufacture on a large scale, are more temperature stable than traditional vaccines and thus boast a longer shelf life. Collectively all these attributes of DNA vaccines impact significantly on cost for developing countries, as it eliminates the need

for cold chain storage and thereby aids in its mobility (Davis and McCluskie, 1999; Gurunathan *et al.,* 2000; Huygen, 2005).

Although DNA vaccines are potent inducers of T cell responses, they appear less efficient in priming antibody responses than immunisation with recombinant protein in adjuvant. This has been observed in mice and non-human primates (Barnett *et al.,* 1997; Otten *et al.,* 2005). A possible explanation for this may be that extremely small amounts of protein antigen is produced within the cells of the plasmid inoculated host. Reporter gene studies have demonstrated that only picogram to nanogram amounts of protein are expressed (Wolf *et al.,* 1990; Manthorpe *et al.,* 1993; Huygen, 2005). Such protein amounts would not be sufficient to induce a good immune response if they were to be given in subunit vaccine form. In contrast, studies have shown that very small amounts of antigen, when presented in the context of MHC by antigen presenting cells is sufficient to elicit T-cell responses (Fields *et al.,* 1998). Two factors are especially important for priming of T-cell responses by DNA vaccines, namely, the type of cells involved and the potential effects of immunostimulatory motifs within bacterial plasmid DNA. Following intramuscular delivery of plasmid DNA, the bulk of the gene product is produced by non-antigen presenting cells such as myocytes and cells of the skin (Huygen, 2005). These cells do not directly prime T-cell responses. For optimal T cell priming professional bone marrow derived antigen presenting cells are required (Herrera *et al.,* 2002; Larregina *et al.,* 2004). Antigen presenting cells can either be transfected directly by the DNA vaccine or they can take up antigen synthesised by another transfected cell or they can take up components of transfected cells which have apoptosed or been damaged by inflammatory processes and the innate immune response. This latter process is termed cross-priming (Giri *et al.,* 2004). Evidence now exists for both direct production of antigen by antigen presenting cells and cross-priming following DNA vaccination (Ulmer *et al.,* 1996; Mincheff *et al.,* 2001; Giri *et al.,* 2004; Huyden, 2005).

DNA vaccines are efficient at inducing broad-based immune responses in small animal models (Hinkula *et al.,* 1997; Larregina *et al.,* 2004). However,

the immune responses induced in primates are of a lower magnitude or more variable (Plotkin, 2003; Coban *et al.,* 2005). Also immunisation of primates requires much larger amounts of DNA to induce efficient immune responses (Leung *et al.,* 2004). Several immunisations are usually required, and boosting with recombinant proteins or viral vectors is usually performed (Putkonen *et al.,* 1998; Imami *et al.,* 2004; Villamide-Herrera *et al.,* 2004; Wang *et al.,* 2004).

One of the major drawbacks of DNA vaccines is that the immune response generated against certain antigens (e.g. HIV envelope and malaria proteins) wanes over time, thus producing suboptimal protection. Therefore booster regimens with either recombinant protein or utilising virus vectors have been introduced (Lee *et al.,* 2004; Excler, 2005; Plotkin, 2005; Wang *et al.,* 2005a).

In future it will be important to improve the efficacy of DNA vaccines, thus making them more effective in smaller doses. The vector itself can be optimised to express higher levels of protein, by using optimal promoters, enhancers, poly (A) signals and translation initiation signals (Davis and McCluskie, 1999; Gurunathan *et al.,* 2000; Garmory *et al.,* 2003). The foreign genes can also be codon-optimised to achieve higher levels of protein expression (Andre *et al.,* 1998; zur Megede *et al.,* 2000; Garmory *et al.,* 2003).

Delivery and uptake of DNA to the cytoplasm is a barrier to efficient transfection of cells *in vivo* (Davis and McCluskie, 1999). DNA can be coated onto gold particles and delivered epidermally by a gene gun to enhance delivery to APCs (Haynes *et al.,* 1996; Porgador *et al.,* 1998; Donnelly *et al.,* 2003). Uptake can also be enhanced by *in vivo* electroporation, which has been demonstrated to significantly increase DNA delivery and DNA vaccine potency (Widera *et al.,* 2000; Donnelly *et al.,* 2003; Giri *et al.,* 2004). Adsorption of DNA onto cationic microparticles (e.g. PLG) may also result in improved delivery of DNA to APCs (Otten *et al.,* 2005). The micro-environment in which the antigen is presented to the immune system can itself be changed to enhance and direct the immune response (Gurunathan *et*

al., 2000). Recently it has been shown that rhesus monkeys vaccinated with cytokine-augmented DNA were able to control viremia and prevent development clinical AIDS after challenge (Barouch *et al.,* 2000; Imami *et al.,* 2004). In the study by Barouch and colleagues, an IL-2/Ig fusion protein was used to enhance DNA mediated immune responses. The cytokine IL-12 can also be used to activate the Th1 subset of $CD4^+$ T-cells and suppress the Th2 subset (Giri *et al.,* 2004). Co-delivery of cytokines or cytokine genes can also be used to recruit and mature DCs, for example with GM-CSF (Conry *et al.,* 1996; Donnelly *et al.,* 2003).

The inclusion of bacterial derived CpG immunostimulatory DNA sequences, co-stimulatory molecule-encoding plasmids (Donnelly *et al.,* 2003; Giri *et al.,* 2004), or the use of self-replicating genetic vaccines (Karlsson *et al.,* 2004) have been reported to enhance DNA vaccine efficacy. In addition, use of heterologous prime-boost regimens and multiple site delivery of DNA vaccines have been implicated in further positively influencing DNA vaccine efficacy (Davis and McCluskie, 1999; Plotkin, 2005).

To date, many challenges have hampered the search for a potential effective prophylactic HIV-1 vaccine. More than two decades after HIV was first described an ideal vaccine candidate has still not emerged. The infection of nonhuman primate models with SIV, SHIV and HIV-1 has significantly contributed to our understanding of AIDS disease and pathogenesis and concomitantly provided important information for vaccine development. Many new emerging HIV vaccine candidates are based on a heterologous DNA prime plus viral vector boost approach. Although this approach shows promising results in the $SHIV_{89.6P}$ model it was unable to protect animals from infection (Barouch *et al.,* 2001; Mooij *et al.,* 2004; Wang *et al.,* 2004). Therefore, ways to improve vaccine delivery and induce broader immune responses are urgently needed. The major focus of the current study was to develop an *in vitro* system whereby plasmid DNA vaccine construct delivery to DCs could be evaluated. This is the first study outlining the culture, characterisation, maturation and transfection of baboon DCs as an *in vitro*

model for assessing vaccine efficacy at a plasmid-DC-T cell level. The usefulness of this model lies in the fact that potential future candidate vaccines can be initially tested *in vitro* prior to evaluation *in vivo.* Importantly, delivery and gene expression of DNA constructs, as well as antigen presenting capacity following vaccination can be optimised *in vitro* prior to using expensive nonhuman primates to test immunogenicity.

1.6. Aims of the study

The aim of this study was to establish and evaluate a baboon *in vitro* DC culture system, to characterise baboon DC maturation and to assess this model as readout for efficacy of HIV-1 subtype C DNA vaccine delivery.

In order to realise this goal, the following objectives had to be achieved:

- Optimise successful culture and maturation of monocyte-derived baboon DCs.
- Assess the efficiency of transfection of baboon monocyte-derived DCs using different modes of plasmid DNA delivery (passive pulsing, lipofection, and electroporation).
- Evaluate the ability of transfected DCs to present peptide to antigen-specific $CD4^+$ and $CD8^+$ T lymphocytes.

CHAPTER 2
MATERIALS AND METHODS

2.1. Animals, Human donors and Peripheral blood mononuclear cell (PBMC) isolation

Five healthy, young adult male and female chacma baboons *Papio cynocepahalus* subspecies *ursinus* were used in this study and were housed individually at the MRC Delft Animal Centre (Brentwood Park, Driftsands, RSA). The animals were monitored clinically on a daily basis for a minimum of 6 weeks during an adaptation to captivity and quarantine period. During this period the animals were screened for tuberculosis three times using the intradermal tuberculin skin test. The initial tests were performed six weeks apart during the adaptation period and the last was performed prior to the initiation of vaccine studies. The animals were subsequently divided into two groups; an unvaccinated group (UV), consisting of animals: 472, 564, 565 (table 2.1.) and a vaccinated group (V), consisting of animals: 517 and 522 (section 2.1.1.). The animals were cared for in compliance with the South African National Code for Animal Use in Research, Education, Diagnosis and Testing of Drugs and Related Substances, promulgated by the Department of Agriculture in 1990. (Ethics approval # 2002/CO90).

Prior to commencement of any experiments using baboon peripheral blood, all animals from both groups were screened and tested negative on an *env* based PCR for both SIV and HIV-2 and for antibodies to HIV-1, verified by means of an enzyme-linked immunosorbent assay (ELISA) (ABBOTT, Wiesbaden, Germany). A summary of characteristics of the animals used is presented in Table 2.1.

2.1.1. Vaccinated Animals

The vaccinated animals of Group V (517 and 522), see table 2.1., were part of a separate vaccine immunogenicity study under the supervision of Dr Richard

Glashoff. These animals were used specifically to test HIV-1 subtype C antigen-specific memory responses following vaccination with either naked or poly lactic-co-glycolide (PLG) microparticle-formulated DNA. Prior to each vaccination, the animals of this group were physically restrained in crush back cages. They were immobilised by intramuscular administration of Ketamine Hydrochloride (Anaket-V^R Kyron Laboratories, Benrose, RSA) at a dosage of 10 mg/kg body mass. The two animals of Group V received 3 priming vaccinations at 4 week intervals with 5 mg each of either naked (animal 517) or PLG microparticle-formulated (animal 522) plasmid DNA constructs (pCMVKm2GagPolBW and pCMVLinkgp140dV2TV1) (see section 2.4.2.). A combination vaccination (5 mg of each plasmid DNA construct in either naked or PLG-formulated form plus 100 μg of MF59 adjuvanted recombinant gp140dV2 protein) was given to baboons from the naked (animal 517) or PLG microparticle-formulated (animal 522) groups 40 weeks after the last prime. A second boost (100 μg of MF59 adjuvanted recombinant gp140dV2) was given to both animals in Group V 13 weeks after the first boost. Both vaccinated animals displayed antigen specific cellular immune responses to Gag, Pol and Env peptide pools as evidenced by detection of intracellular IFN-γ following *in vitro* peptide stimulation of PBMC (Glashoff *et al.,* manuscript in preparation). The animals also displayed antigen-(peptide-) specific memory T cell responses as detected by lymphoproliferation assays (LPAs). The Env-specific stimulation indices (SIs) of baboon 517 at weeks 67 and 73 were reported to increase from 4.5 to 6.8, respectively. Gag-specific SIs increased from 1.2 to 1.5 at weeks 67 and 73, respectively. The Env-specific SIs in baboon 522 increased from 1.2 to 2.9 at the aforementioned time points, whereas Gag-specific SIs increased from 3.1 to 5.9. The heparinised blood samples for the current study was collected at weeks 95 and 98, 34 weeks after the second boost (100 μg of MF59 adjuvanted recombinant gp140dV2).

Table 2.1. Characteristics of study animals.

Baboon	Sex	Weight[a]	Group
472	Male	16.1	UV
517	Male	9.9	V-A
522	Male	10.7	V-B
564	Female	8.4	UV
565	Female	8.2	UV

a: Baboon weight in kilograms at commencement of the study.
V-A: Vaccinated with naked pDNA, boosted with recombinant gp140 protein; V-B: Vaccinated with PLG microparticle-formulated pDNA, boosted with recombinant gp140 protein; UV - Control unvaccinated animals.

2.1.2. Human donors

Peripheral blood samples were collected from healthy normal adult volunteers. All donors were confirmed to be HIV-1 antibody negative by means of an enzyme-linked immunosorbent assay (ELISA) (ABBOTT, Wiesbaden, Germany). Additional donor information is listed in Table 2.2. In certain experiments plasma from a confirmed HIV positive individual (R214) was used.

Table 2.2. Characteristics of human donors used in optimisation experiments.

Donor	Age	Sex
1	33	Male
2	48	Female
3	34	Female
4	42	Female

2.1.3. Peripheral blood mononuclear cell (PBMC) isolation

Fifty millilitres (50 ml) of baboon peripheral blood was collected in heparinised tubes (BD Vacutainer, Plymouth, UK). Identical volumes of blood were collected from human donors. PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation as previously described (Villamide-Herrera *et al.,* 2004). Briefly, 15 ml Histopaque®-1077 (Sigma, Steinheim, Germany) was centrifuged into the lower chamber of 50 ml Accuspin tubes (Sigma, Steinheim, Germany) at 800 *x g* for 30 seconds. Heparinised fresh whole blood was then pipetted into the upper chamber of each tube. PBS was then added to make up a final volume of 50 ml. Following centrifugation at 800 *x g* for 15 minutes, the mononuclear cells were harvested with a pipette and washed three times in 25 ml of magnesium and calcium free phosphate buffered saline (PBS) (Gibco, Invitrogen Corporation, UK). Cells were pelleted between washes by centrifugation for 10 minutes at 325 *x g* after the first wash and then 275 *x g* for the final 2 washes. After the final wash the cell pellet was resuspended in AR-10 media (50% AIM-V, 50% RPMI 1640 (Gibco, Invitrogen Corporation, UK), 10% heat inactivated FBS (Delta Bioproducts, Kempton Park, RSA), supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate and 50 μg/ml of gentamicin (Gibco, Invitrogen Corporation, UK). A 50 μl aliquot of cells was pipetted into a micro-centrifuge tube and mixed with an equal volume of Turks stain (0.02% crystal violet, 7% glacial acetic acid in water). The cells were then counted with the aid of a haemocytometer and a light microscope (Nikon ECLIPSE, Tokyo, Japan).

2.2 Monocyte-derived DC culture

2.2.1. Monocyte purification by magnetic bead selection

Immature dendritic cells (iDCs) were generated from $CD14^+$ monocytes following their isolation from both human and baboon PBMCs.

$CD14^+$ monocytes were separated by positive selection using the MiniMACs high-gradient magnetic sorting system (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. Briefly, 1 x 10^7 PBMCs were resuspended in 80 μl of column equilibration buffer (PBS pH 7.2, supplemented with 0.5 % bovine serum albumin (BSA) (Amersham Pharmacia Biotek, UK) and 2 mM ethylene diamine tetra-acetic acid (EDTA). Twenty microlitres of appropriate CD14 microbead preparation (human or non-human primate anti-CD14) was added to every 1 x 10^7 PBMCs and incubated at 4°C for 15 minutes. After incubation, the cell-bead mixture was washed with the appropriate volume of column equilibration buffer, centrifuged at 300 *x g* for 10 minutes and resuspended in 500 μl of fresh column equilibration buffer. Magnetic bead-labelled cells were pipetted into the MiniMACs magnetic separation (MS+) column which was attached to the magnet. The $CD14^-$ fraction was allowed to elute by gravity flow. After washing the column through three times with 500 μl column equilibration buffer, the $CD14^+$ labelled and positively enriched cells were eluted from MS+ columns by removal of columns from the magnetic device and flushing with column equilibration buffer with the aid of a plunger. Finally, the cells were centrifuged at 300 *x g* for 10 minutes to rid them of column equilibration buffer. The purity of the $CD14^+$ monocyte fraction was determined by flow cytometry (see section 2.2.4.).

The $CD14^-$ cell fraction was used for isolating $CD4^+$ and $CD8^+$ cell fractions for the mixed lymphocyte reaction (MLR) and/or antigen-specific LPAs, discussed in sections 2.12. and 2.13., respectively.

2.2.2 DC culture

The $CD14^+$ monocyte fraction was seeded into 6-well tissue culture plates (Corning Incorporated, NY, USA) at 1 x 10^6 cells/ml in 3 ml of DC culture medium supplemented with 300 μl of immature DC (iDC) cytokine cocktail. DC culture medium consisted of RPMI 1640 (Gibco, Invitrogen Corporation, UK) containing 2mM L-glutamine, 10 mM HEPES, supplemented with 50 μM 2-mercaptoethanol (Sigma, St Louis, MO, USA), 50 μg/ml gentamicin (Gibco, Invitrogen Corporation, UK) and 10% heat inactivated Fetal Bovine Serum (FBS) (Delta Bioproducts, Kempton Park, RSA). Optimised iDC cytokine cocktail consisted of 1000 IU/ml recombinant human granulocyte-macrophage colony stimulating factor (rhGM-CSF) and 1000 IU/ml recombinant human interleukin-4 (rhIL-4) (R & D Systems, Minneapolis, MN, USA). Varying concentrations of rhGM-CSF and rhIL-4 were used in attempts to optimise culture conditions. The $CD14^+$ monocyte fraction was subsequently cultured for six to seven days in an Autoflow CO_2 water-jacketed incubator at 37°C containing a humidified atmosphere and 5% CO_2. Cytokines were replenished every other day (on days 2, 4 and 6) by removing 300 μl of the medium and adding back 300 μl fresh medium with 1X cytokines. On day seven, maturation was induced by the addition of maturation cocktail consisting of 20 ng/ml recombinant human interleukin-6 (rhIL-6), 10 ng/ml recombinant human interleukin-1β (rhIL-1β), 10ng/ml recombinant human tumor necrosis factor alpha (rhTNF-α) (R & D Systems, Minneapolis, MN, USA) and 10^{-6}M (1μg/ml) prostaglandin E_2 (PGE_2) (Sigma, St Louis, MO, USA). The cells were cultured for a further three days. Following culture cells were harvested by gently rinsing the individual wells three times with PBS with the aid of a graduated pipette. The cells were pelleted by centrifugation at 300 *x g* for 5 minutes and prepared for phenotypic analysis. Cell differentiation was monitored daily by light microscopy.

2.2.3. CD4$^+$ and CD8$^+$ T cell selection and cryopreservation

CD4$^+$ and CD8$^+$ cells were separated from fresh PBMC preparations by positive selection using the MiniMACs high-gradient magnetic sorting system according to the manufacturers protocol. The principal is as described for CD14$^+$ cell purification (section 2.2.1.), with anti-CD14 coupled beads replaced by anti-CD4 and anti-CD8 beads. After selection, the purified cell populations were counted following staining with trypan blue (Sigma, Steinheim, Germany) and centrifuged at 300 *x g* for 10 minutes at 4°C and supernatant decanted. The cell pellet was resuspended at 20 x 10^6 cells/ml in 1 ml cell culture freezing-medium DMSO (Gibco, Invitrogen Corporation, UK) and transferred to sterile screw cap cryogenic vials and stored at -70°C until needed.

2.2.4. Surface staining and immunophenotypic analysis of cells by flow cytometry

The expression of surface markers on PBMCs, CD14$^+$ monocytes, iDCs and mDCs was determined by flow cytometric analysis of cells stained with relevant monoclonal antibodies (BD Biosciences, San Diego, CA, USA). A summary of the antibodies used in the current study is provided in Table 2.3.

Cells were washed twice with PBS and adjusted to a concentration of 1 x 10^6 cells/ml. Cells (500 μl or 0.5 x 10^6 cells) were incubated in the dark at 4°C in the wells of a U-shape 96 well plate (CELLSTAR, Greiner Bio-One, Frickenhausen, Germany) with 25 μl of the appropriate monoclonal antibody (mAb) or mAb cocktail for 25 minutes. For CD14$^+$ staining, 5 μl of antibody plus 20 μl staining buffer was added to the cells. Dendritic cell antibody cocktails consisted of three monoclonal antibodies (CD80, 83, 86), each with a different fluorescent marker (FITC, PE and APC). Cocktails were prepared by adding 5 μl of each monoclonal antibody to 10 μl of staining buffer (PBS supplemented with 0.25 % BSA). All solutions and antibodies were kept cold by working on ice.

Table 2.3. Monoclonal antibodies used for cell staining.

Antibody	Clone	Isotype	Colour[a]
CD14	M5E2	Mouse IgG_{2a},κ	FITC
CD80	L307.4	Mouse IgG_1,κ	R-PE
CD80	L307.4	Mouse IgG_1,κ	FITC
CD83	HB15e	Mouse IgG_1,κ	R-PE
CD83	HB15e	Mouse IgG_1,κ	APC
CD86	FUN1	Mouse IgG_1,κ	R-PE

R-PE: R-Phycoerythrin;
FITC: Flourescein isothiocyanate; APC: Allophycocyanin

Following staining the cells were centrifuged at 200 *x* g for 5 minutes and washed with 100 μl of staining buffer. Thereafter, the cells were resuspended in 150 μl of staining buffer and transferred to 5 ml Falcon tubes (BD Labware, Franklin Lakes, NJ, USA) containing 350 μl staining buffer. The cells were stored at 4°C in the dark until analysed on the FACSCalibur (BD Biosciences, San Diego, CA, USA). Non-reactive isotype-matched antibodies were also prepared in the same way and used as controls. Flow cytometric data was acquired and analysed using CellQuest software (BD, San Jose, CA, USA). Cells were gated on forward and side scatter and a total of 10 000 events were usually acquired.

2.2.5. Microscopic analysis and photography

$CD14^+$ monocyte differentiation into DCs was monitored morphologically according to Lohmann et al. on a daily basis using a Nikon ECLIPSE TS100 inverted light microscope (Lohmann *et al.,* 2000). Digital photographs were taken at various intervals throughout the culture period to monitor changes in cellular morphology using a Nikon COOLPIX4500 Digital camera (both microscope and camera from Nikon Corporation, Tokyo, Japan). All photographs were taken at a magnification of 200X.

2.3. HeLa cell culture

HeLa cells, an adherent immortalised epithelial cell line, were used to standardise transfection procedures. These cells were originally obtained from the American Type Culture Collection (ATCC), USA, ATCC number CCL-2. The cells were cultured and maintained in Minimum Essential Medium Eagle (MEM) (Gibco, Invitrogen Corporation, UK) supplemented with 10% heat inactivated Fetal Bovine Serum (Delta Bioproducts, Kempton Park, RSA) and 50 μg/ml gentamicin (Gibco, Invitrogen Corporation, UK).

The cultures were maintained in an Autoflow CO_2 water-jacketed incubator (NuAire Incorporated, Plymouth, MN, USA) at 37°C containing a humidified atmosphere and 5% CO_2. Cells were cultured in sterile 75 cm^2 tissue culture flasks with 0.2 μm vented caps (Corning Incorporated, NY, USA). The media was changed every 3-4 days and cells were sub-cultured once a week. Cells were sub-cultured by trypsinising with a 10% active trypsin versine (ATV) solution (135 mM NaCl, 5 mM KCl, 5.6 mM Glucose, 7 mM $NaHCO_3$, 0.5% Trypsin, 0.5 mM EDTA) to the culture flask and incubating the cells for 5-10 minutes at room temperature or until the cell monolayer lifted off the surface of the vessel. The cell suspension was then centrifuged at 600 *x g* for 5 minutes and the supernatant was removed and discarded. The cells were then resuspended in MEM at 3 x 10^5 and placed into 75 cm^2 disposable sterile flasks with 0.2 μm vented caps and maintained as described above.

2.4. Plasmid DNA constructs

2.4.1. pSV-β-Galactosidase control vector

The pSV-β-Galactosidase control vector (Promega, Madison, WI, USA) was used for the optimisation of transfection procedures. The principle of the pSV-β-Galactosidase reporter assay system is based on the detection of β-Galactosidase enzyme activity in lysates prepared from cells transfected with the β-Galactosidase reporter plasmid vector. The addition of the substrate ONPG (*o*-

nitrophenyl-β-D-galactopyranoside) allows for the β-Galactosidase activity to be measured. β-Galactosidase hydrolyses the colourless substrate to *o*-nitrophenol, which is yellow. The extent of colour change can be determined by measuring absorbance at 420 nm. In the current study, the 6820 bp mammalian control vector served to monitor and evaluate the efficacy of various transfection techniques. The vector has an SV40 promoter and enhancer region that drives transcription of the bacterial *lacZ* gene, which is translated into the β-Galactosidase enzyme. The vector contains an ampicillin resistance gene. In *Escherichia coli* (*E. coli*), the vector is able to express β-Galactosidase due to the presence of a *gpt* promoter located upstream of the *lacZ* gene. β-Galactosidase reporter enzyme activity is assayed directly in cell extracts following treatment with the β-Galactosidase Enzyme Assay System with Reporter Lysis Buffer (Promega, Madison, WI, USA).

2.4.2. Plasmid vectors containing HIV-1 subtype C genes used in vaccine study

Two codon-optimised constructs were used in both the transformation and transfection procedures. These constructs were the same used in the baboon vaccine immunogenicity study outlined in section 2.1.1. The details of the constructs are as follows:

1. An *env* construct (pCMVLinkgp140dV2.TV1) (GenBank accession #: AX456004; a gift from Susan Barnett, Chiron Corporation) based on the HIV-1 subtype C Tygerberg Virology 1 (TV1) isolate, that encodes a V2-deleted gp140, codon optimised *env* gene ~ 1986 bp in length. The gp140dV2 insert contains a signal leader sequence and was generated from native TV1 gp160 following the induction of a V2-deletion. The gp140dV2 *env* gene was subsequently cloned into the *Eco*RI and *Xho*I restriction sites of the eukaryotic expression vector pCMVLink (Susan Barnett, personal communication, Lian *et al.*, *in press*). This vector

(Chiron Corporation, Emeryville, CA, USA) contains a CMV major immediate-early (IE1) promoter, an intron A which acts as an enhancer for the expression of protein, a bGH poly adenylation signal for termination and an SV40 origin and a kanamycin resistance gene (Chapman *et al.,* 1999).

2. A *gagpol* construct (pCMVKm2GagCPol.BW) (a gift from Susan Barnett, Chiron Corporation), based on the HIV-1 subtype C Botswana (BW) isolate that encodes a ~ 1500 bp codon optimised *gagpol* gene cassette. The gene cassette was modified by removing the frameshift region to allow the expression of the *gag* and *pol* genes in frame (zur Megede *et al.,* 2003). The protease and integrase genes were inactivated. The *gagpol* gene cassette was cloned into the *Eco*RI and *Sal* I restriction sites of the pCMVKm2 vector containing the CMV immediate-early enhancer-promoter and the bGH terminator and a kanamycin resistance gene (zur Megede *et al.,* 2000).

2.5. Transformation reactions

One shot TOP10 competent bacterial cells (Invitrogen Corporation, Carlsbad, CA, USA) with a genotype of: [F$^-$ *mrc*A Δ(*mrr-hsd*RMS-*mrc*BC) φ80*lacZ*ΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G] were transformed with 10 ng/μl of the respective plasmid construct DNA mixtures. A positive pUC18 ligation reaction from the competent cell kit together with a cell only reaction, served as positive and negative control reactions respectively. Transformation of the TOP10 cells with plasmids (pSV and vaccine constructs) (sections 2.4.1. and 2.4.2.) was performed according to manufacturers instructions from the TOP®:XL PCR Cloning kit (Invitrogen Corporation, Carlsbad, CA, USA). Fifty microlitres of the transformed bacterial cells were plated onto Luria-Bertani (LB) agar plates (10 g/L bacto-tryptone, 5 g/L bacto-yeast extract, 10 g/L NaCl, 15 g/L bacto-agar) (Hispanlab, SA) containing 50 mg/ml of kanamycin (Gibco, Invitrogen Corporation, UK). The plates were

incubated at 37°C overnight. After incubation the plates were screened for recombinant colonies.

2.6. Screening for recombinant colonies

Colonies from the LB agar plates were inoculated into 2 ml LB media (10g/L bacto-tryptone, 5 g/L bacto-yeast extract, 10 g/L NaCl) (Hispanlab, SA) containing kanamycin (Gibco, Invitrogen Corporation, UK) and incubated on a shaking incubator at 225 rpm at 37°C overnight. DNA was isolated using to the alkaline lysis method according to the protocol of Sambrook et al. (Sambrook *et al.,* 1989). Plasmid DNA was separated and visualised by gel electrophoresis on 0.6% agarose gels. To confirm that the isolated plasmid DNA contained the inserts, plasmids were digested with restriction enzymes for 2 hours (h) at 37°C in a waterbath. Digested plasmid DNA was separated and visualised by gel electrophoresis on a 0.6% agarose gel.

The pCMVLinkgp140dV2.TV1 plasmid DNA was digested with the *EcoRI* and *XhoI* (Pomega, Madison, WI, USA) restriction enzymes in Buffer H (90 mM Tris-HCl, 10 mM $MgCl_2$, 50 mM NaCl, pH 7.5). The pCMKm2GagCPol.BW plasmid DNA was digested with the *SalI* and *EcoRI* (Pomega, Madison, WI, USA) restriction enzymes in Buffer H.

2.7. Preparation of endotoxin free plasmid DNA for transfections

Prior to transfection, endotoxins were removed from plasmid DNA preparations. The QIAfilter™ plasmid Midi kit (QIAGEN, Hilden, Germany) was used for the isolation of endotoxin-free plasmid DNA. The DNA concentration and purity was determined as described below.

Following confirmation of presence of the modified *env (gp140dV2)* and *gagpol* genes high quality endotoxin-free DNA was prepared from bacteria using the

QIAfilter™ plasmid Midi kit (QIAGEN, Hilden, Germany) according to the manufacturers instructions. Briefly, bacterial cells were harvested by centrifugation at 6000 *x g* for 15 minutes at 4°C. The bacterial pellet was then vigorously vortexed and cells were lysed by resuspension in buffer P1. Four millitres of buffer P2 was then added and the suspension incubated at room temperature for 5 minutes. Following incubation, 4 ml of buffer P3 was added and the lysate was gently mixed, transferred into the barrel of a QIAfilter Cartridge and incubated at room temperature for 10 minutes. During this incubation period a QIAGEN-tip 100 was equilibrated by applying 4 ml of buffer QBT and allowing the column to empty by gravity flow. The cell lysate was then passed through the QIAfilter Cartridge with the aid of a plunger into the equilibrated QIAGEN-tip. After the cleared lysate had passed through the QIAGEN-tip by gravity flow, the tip was flushed twice with 10 ml of QC buffer. Plasmid DNA was then eluted with 5 ml of buffer QF. The eluted plasmid DNA was precipitated by the addition of 3.5 ml of isopropanol, mixed and centrifuged for 1 hour at 15 000 *x g*. Following centrifugation the supernatant was decanted and the DNA pellet was washed by centrifuging with 2 ml of 70% ethanol at 15 000 *x g* for 10 minutes. The DNA pellet was then allowed to air-dry and was redissolved in 120 μl of TE buffer. The concentration and purity of the isolated plasmid DNA was determined using the following calculation (Sambrook *et al.,* 1989):

DNA concentration = (OD at 260 nm ÷ 20) x dilution factor

DNA purity = OD at 260 nm ÷OD at 280 nm

Optical density (OD) values were measured using a spectrophotometer. Glycerol stocks were prepared from all positively transformed cells by mixing 300 μl of sterile glycerol and 700 μl of bacterial culture (Sambrook *et al.,* 1989) in sterile screw cap cryogenic vials. These were then stored at -70°C.

2.8. Transfections

2.8.1. Transfection techniques

Four different transfection techniques were evaluated in this study: passive pulsing (2.8.2.1.); lipid-mediated transfection (2.8.2.2.), cationic liposomal transfection (2.8.2.3.), and electroporation (2.8.2.4.). All techniques were used to transfect both HeLa and dendritic cells.

2.8.2.1. Passive pulsing

The day before transfection HeLa cells were trypsinised and seeded at 2 x 10^5 in a total volume of 2 ml in 6-well tissue culture plates. The cells were allowed to adhere by overnight incubation at 37°C in a water-jacketed incubator. The following day the 10 μg of pDNA was added to the serum containing media in which HeLa cells were cultured. The cultures containing the pDNA were returned to the water-jacketed incubator at 37°C for 72 hours.

After 6 days in culture, DCs were harvested by centrifugation at 300 *x g* for 10 minutes at 4°C and resuspended in Opti-MEM (Gibco, Invitrogen Corporation, UK) medium at a concentration of 3 x 10^6 cells/ml. Ten micrograms of endotoxin free plasmid DNA was added to 3 x 10^6 cells and the mixture was incubated in a 37°C water bath for 3 hours. Following incubation, the cells were washed twice with PBS, centrifuged at 300 *x g* for 10 minutes at 4°C and resuspended in DC culture media containing 1000 IU/ml rhGM-CSF and 1000 IU/ml rhIL-4. The cells were then returned to the incubator for 12-16 hours at 37°C. After incubation maturation cocktail was added and the cells were returned to the incubator for a further 48 hours under the described conditions.

2.8.2.2. Lipid-mediated transfection (FuGene 6)

The FuGene 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN, USA) is a propriety blend of lipids and other components that

have been filter-sterilised and stabilised in 80 % ethanol. This reagent was used according to the instructions provided in the reagents kit protocol. A range of DNA concentrations was used namely; 1 μg, 2 μg and 5 μg of endotoxin free plasmid DNA per 3 ml culture media. Various ratios of FuGene 6 transfection reagent to DNA were assessed before deciding on an optimal ratio. The ratio of FuGene 6 reagent to DNA was finally optimised to 6:1 in reactions for both HeLa and dendritic cells.

The day before transfection HeLa cells were trypsinised, seeded in 6-well tissue culture plates and allowed to adhere by overnight incubation as described in section 2.8.2.1. The following day the HeLa cells were transfected with the appropriate FuGene 6 reagent to DNA ratio in the presence of serum and returned to the incubator for 72 hours. The FuGene 6 reagent is not cytotoxic and therefore did not have to be removed.

On day 6 of culture, dendritic cells were transfected as described above in the presence of serum and antibiotics. Following a 3-4 hour incubation period, in an Autoflow CO_2 water-jacketed incubator at 37°C containing a humidified atmosphere and 5% CO_2, 300 μl of DC culture media containing 1000 IU/ml rhGM-CSF and 1000 IU/ml rhIL-4 was added and the cells were incubated overnight. The following day, maturation cocktail was added and the cells incubated a further 72 hours as described above.

2.8.2.3. Cationic Liposomal-mediated transfection (Lipofectamine™ 2000)

The Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, CA, USA) is a cationic liposomal reagent. This reagent was used according to the instructions provided in the reagents kit protocol. The ratios of Lipofectamine™ 2000 to DNA (μl:μg) tested were 2:1, 3:1 and 6:1. The day before transfection HeLa cells were trypsinised and seeded at 2×10^6 cells/well in a total volume of 2 ml serum containing MEM in 6-well tissue culture plates. The cells were allowed to adhere

by overnight incubation at 37°C in a water-jacketed incubator. HeLa cells were 90-95% confluent at the time of transfection as specified by the manufacturer. Importantly, transfection was performed in a total volume of 2 ml in the absence of both antibiotics and serum. DNA was diluted into 50 μl of Opti-MEM® Reduced Serum Medium (Gibco, Invitrogen Corporation, UK). The Lipofectamine™ 2000 was also mixed into 50 μl of Opti-MEM® Reduced Serum Medium and allowed to incubate at room temperature for 5 minutes. Following the 5 minute incubation period, the diluted DNA and the diluted Lipofectamine™ 2000 reagent were combined, mixed and incubated at room temperature for an additional 20 minutes. After the incubation, 100 μl of the Lipofectamine™ 2000-DNA complex was added to each well and mixed by rocking the plates back and forth. The cells were then incubated at 37°C in a CO_2 incubator for 4 hours after which 2 ml was replaced with antibiotic and serum containing-medium. The cells were returned to the incubator for 72 hours until assayed.

Dendritic cells were seeded at 3×10^6 cells/well prior to transfection in 6-well plates. They were also transfected using Opti-MEM® Reduced Serum Medium in the absence of serum and antibiotics as described above. Four hours after the addition of the Lipofectamine™ 2000-DNA complexes, the cells were washed twice with PBS by centrifugation at 300 *x g* for 10 minutes at 4°C and resuspended in DC culture media containing 1000 IU/ml rhGM-CSF and 1000 IU/ml rhIL-4. The maturation procedure was then followed as described in section 2.8.2.1.

2.8.2.4. Electroporation

Following trypsinisation, HeLa cells were washed twice with PBS by centrifugation at 300 *x g* for 10 minutes and resuspended in serum free MEM at 2×10^7/ml. Five hundred microlitres of the cell suspension was transferred into a 4MM cuvette (BIO-RAD Laboratories, Hercules, CA, USA) and mixed with 1, 2 or 5 μg endotoxin free plasmid DNA. The cuvette containing the cell-DNA mixture

was then placed into the electroporation chamber of the Gene Pulser® II Electroporation System (BIO-RAD Laboratories, Hercules, CA, USA) with the Capacitance Extender PLUS module (BIO-RAD Laboratories, Hercules, CA, USA). The following voltages (250 V, 300 V) were tested with each of the respective capacitance settings (300 μF, 400 μF, 300 μF). Optimal electroporation was observed at the following physical parameters: voltage of 250 V, capacitance of 300 μF. These conditions were used in all subsequent electroporation procedures. Immediately after electroporation the cells were transferred into 3 ml warm serum containing MEM and incubated at 37°C in a humidified incubator with a 5% CO_2 atmosphere for 72 hours.

On day 6 of culture, iDCs were harvested by gently dislodging them from the bottom of a 6-well plate with a sterile disposable cell scraper (Costar Corporation, Cambridge, MA, USA). The harvested cells were washed twice with PBS by centrifugation at 300 *x g* for 10 minutes and resuspended in DC culture media to a final concentration of 2×10^7 cells/ml. Thereafter 500 μl of the cell suspension was mixed with 10 μg endotoxin free plasmid DNA and electroporated in a 4MM cuvette (BIO-RAD Laboratories, Hercules, CA, USA) using the Gene Pulser® II Electroporation System (BIO-RAD Laboratories, Hercules, CA, USA) with the Capacitance Extender PLUS module (BIO-RAD Laboratories, Hercules, CA, USA). The physical parameters were identical to those used for HeLa cells. After electroporation the cells were immediately transferred into 3 ml warm complete DC culture medium containing iDC cocktail, in one well of a 6-well plate and incubated for 12-16 hours at 37°C in a humidified incubator with a 5% CO_2 atmosphere. After incubation maturation cocktail was added and the cells were returned to the incubator for a further 48 hours under the described conditions.

2.9. Cytoplasmic RNA extractions and DNase digestion

Cytoplasmic RNA was extracted from transfected cells in order to monitor transfection efficiency by RT-PCR. Cytoplasmic RNA was extracted from fresh

cells following the termination of culture using the RNeasy Mini kit (QIAGEN, Hilden, Germany) according to the manufacturers specifications. Briefly, HeLa cells were harvested by directly trypsinising them in the 6-well culture dish, the suspension was then pipetted into a micro-centrifuge tube. The trypsinisation and washing procedure was carried out with PBS as described in sections 2.3 and 2.8.2.4. The cells were then pelleted and the supernatant aspirated with the aid of a pipette. The cell pellet was loosened by flicking the micro-centrifuge tube and cells were then resuspended in 175 μl of cold buffer RLN (50 mM Tris·Cl, 140 mM NaCl, 1.5 mM $MgCl_2$, 0.5% (v/v) Nonidet P-40 (1.06 g/ml), 1000 IU/ml Rnase inhibitor (Promega, Madison, WI, USA), 1mM DTT) to cause lysis of the cell membrane. The suspension was incubated on ice for 5 minutes. The lysate was then centrifuged for 2 minutes at 4°C at 300 *x g*. Thereafter the supernatant was transferred to a new centrifuge tube and the pellet discarded. Six hundred microlitres of buffer RLT was then pipetted into the new micro-centrifuge tube containing the supernatant. After vigorously vortexing the mixture, 430 μl of pure ethanol (96-100%) was added to the homogenised lysate. The aforementioned lysate was thoroughly mixed by pipetting, after which 700 μl of the sample was applied to an RNeasy mini column, placed in a collection tube and centrifuged at 8000 *x g* for 15 seconds at room temperature. The RNeasy mini column was then washed by applying 700 μl of buffer RW1 and centrifuging at 8000 *x g* for 15 seconds. This centrifugation step and all subsequent ones were carried out at room temperature. The RNeasy column was transferred into a new 2 ml collection tube and washed twice with 500 μl of buffer RPE by centrifuging for 15 seconds at 8000 *x g*. The elution of the RNA was achieved by transferring the RNeasy column into a new 1.5 ml collection tube, 30 μl of RNase-free water was then directly pipetted onto the RNeasy silica-gel membrane and centrifuged at 8000 *x g* for 1 minute. RNA samples were stored at - 20°C in small aliquots until DNase digestion and RT-PCR.

The same method was used for DCs except that they were directly pipetted into the micro-centrifuge tubes and the wells were rinsed twice and aspirated with

PBS in order to remove all remaining cells. They were then washed as described in section 2.8.2.4. and RNA extraction was performed as outlined above.

Extracted Cytoplasmic RNA was treated with DNases prior to RT-PCR using the RQ1 Rnase-Free DNase kit (Promega, Madison, WI, USA) according to the manufacturers instructions. Eight microlitres of the aforementioned isolated RNA was pipetted into a sterile micro-centrifuge tube. One microlitre of RQ1 RNase-free DNase 10X reaction buffer and 1 u/μg of RNA-free DNase was also added. The final volume of the reaction mixture was made up to 10 μl by adding nuclease-free water and incubated at 37°C for 30 minutes in a waterbath. In order to terminate the reaction 1 μl of RQ1 DNase Stop solution was added. The DNase was inactivated by incubating the micro-centrifuge tube containing the RNA at 65°C for 10 minutes on a heating block.

2.10. Evaluation of Transfection techniques (β-Gal expression and RT-PCR)

Transfection efficiency when using the β-Gal vector was assessed by determining enzyme activity. In the case of the vaccine plasmid construct, RT-PCR was used to determine levels of plasmid DNA expression in transfected cells.

2.10.1. β-Gal expression

The levels of β-Galactosidase expression in the transfected cells was determined by using the β-Galactosidase Enzyme Assay System with reporter Lysis Buffer (Promega, Madison, WI, USA) according to the manufacturers instructions. Growth medium was removed from the HeLa cells by aspiration with the aid of a pipette. The cells were washed twice with PBS directly in the 6-well plates. After washing, the cells were incubated for 15 minutes at room temperature in the

presence of 900 μl 1X Reporter Lysis Buffer (RLB). The plate was rocked several times during this process to ensure complete coverage of the cells. The DCs were harvested by rinsing the wells of the tissue culture plate at least three times with PBS and then pipetting the cells into 15 ml conical tubes. The DCs were then pelleted by centrifugation at 300 *x g* for 5 minutes at 4°C. The pellet was dislodged by gently flicking the tube and then 900 μl of 1X RLB was added. To ensure that the cells were in constant contact with 1X RLB during a 15 minutes room temperature incubation period, the 15 ml conical tube was occasionally swirled. Following incubation the HeLa cells were scraped off the surface of the plate, the dish was tilted and the cell lysate was pipetted into a micro-centrifuge tube and placed on ice. The tubes containing the cell lysate was vortexed for 15 seconds, then centrifuged at 13 000 *x g* for 2 minutes at 4°C and finally the supernatant was transferred to a new micro-centrifuge tube. The DCs were already in suspension at this stage, therefore they were simply vortexed in the 15 ml conical tubes, and an identical procedure was followed as described for HeLa cells.

For the β-Galactosidase Assay a 2:1 dilution of the lysate to 1X RLB was prepared in a fresh micro-centrifuge tube (100 μl lysate plus 50 μl 1X RLB). Assay 2X buffer (150 μl) was added to each tube and the samples were briefly vortexed. The reactions were then incubated at 37°C for 30 minutes or until a faint yellow colour developed. DC samples were incubated overnight at 37°C. All reactions were stopped by the addition of 500 μl of 1 M Sodium Carbonate and then vortexing. One hundred microlitres of sample was pipetted into a cuvette and absorbance was then read at 420 nm using a spectrophotometer. The spectrophotometer was zeroed using the 1 M Sodium Carbonate stop solution.

2.10.2. RT-PCR

For the study plasmids, pCMVLinkgp140dV2.TV1 and pCMKm2GagCPol.BW, RT-PCR was used to amplify mRNA. RT-PCR was carried out using the Access RT-PCR System (Promega, Madison, WI, USA) according to the manufacturer's recommendations. For *gagpol* the reaction mixture contained 10 μl RNA, 0.2 mM of each nucleotide, 0.8 μM of each primer *Gag A* (aga gaa cca agg gga agt ga) and *Gag B* (act cta aag ggt tcc ttt gg) (Engelbrecht and van Rensburg, 1995), 0.1 U/μl AMV reverse transcriptase enzyme, 0.1 U/μl *Tfl* DNA Polymerase, 1.5 mM $MgCl_2$ and 10X buffer in a 50 μl reaction.

Amplification cycles were carried out using the 9700 PCR machine (Applied bio-systems, Foster City, CA, USA) as follows: An extension at 48°C for 45 minutes, the reverse transcriptase enzyme was inactivated by heating at 94°C for two minutes, followed by 40 cycles of denaturing at 94°C for one minute, primer annealing at 40°C for one minute and elongation at 68°C for one minute and thirty seconds and a final extension at 68°C for seven minutes. This reaction rendered a ~ 300 bp product.

A nested PCR was also performed on the *Gag* RT-PCR product as the prenested product did not give good band resolution when run on an agarose gel. The nested reaction rendered a ~ 190 bp fragment with good band resolution. This was done using 2 μl for HeLa cells or 10 μl for DCs of the prenested amplified product. The primers used in this reaction were *Gag C* (cat agc agg aac tac tag ta) and *Gag D* (tcc ttg tct tat gtc cag aa) (Engelbrecht and van Rensburg, 1995) respectively, at 0.8 μM each per reaction. The final volume per reaction was 100 μl, of which 2.5 mM $MgCl_2$, 2.5 mM dNTPs and 1 U of *Taq* DNA polymerase (Promega, Madison, WI, USA). PCR conditions were as follows: 40 cycles were performed (one minute at 94°C, one minute at 40°C and ninety seconds at 72°C) with an initial denaturation of two minutes at 94°C and a final extension of ten

minutes at 72°C. The PCR products were visualised using agarose gel electrophoresis (section 2.11.).

Alternatively, for the mRNA isolated from cells transfected with the pCMVLinkgp140dV2.TV1 plasmid, the *pCMVF* (agt ctg agc agt act cgt tg) and *pCMVR* (gct ggc aac tag aag gca ca) primers were designed and utilised in the RT-PCR protocol. This reaction rendered a ~ 200 bp fragment. All cycling conditions remained identical, with the exception that the optimal annealing temperature was 52°C.

2.11. Agarose Gel electrophoresis

A 10 μl aliquot of the PCR product was added to 2 μl of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll) and mixed. The mixture was then loaded into a slot of a 16 well 2% agarose mini-gel submerged in TAE buffer (0.04 M Tris acetate, 0.001 M EDTA), containing 0.5 μg/ml ethidium bromide. The 1 kb DNA marker (Promega, Madison, WI, USA) was used as a size marker. The dimensions of the mini-gel electrophoresis apparatus were 10 cm X 10 cm and gels were run at a constant voltage of 5 Volts/cm. The PCR products were visualised under ultraviolet light and photographed with the Syngene GeneGenius (Synoptics Ltd, UK). Non-specific bands were identified as bands other than ~ 200 bp or ~ 190bp in size.

2.12. Mixed lymphocyte reaction (MLR)

The mixed lymphocyte reaction (MLR) was used to monitor the antigen presenting potential of both transfected and untransfected baboon DCs. Both transfected and untransfected DCs were mixed with T cells from HLA mismatched donors and the effect of transfection on the immunostimulatory potential of DCs was evaluated by measuring T cell proliferation.

Following the transfection of DCs and the addition of maturation cocktail on the seventh day, the cells were allowed to rest overnight in the 6-well plates. On day 8 DCs were counted and reseeded into U-bottomed 96-well plates at the following concentrations; 2 x 10^4, 5 x 10^3, and 2 x 10^3 cells/well (for final T:DC ratios of 100:1, 40:1 and 10:1) and were allowed to adjust to the new conditions by overnight incubation in an Autoflow CO_2 water-jacketed incubator at 37°C containing a humidified atmosphere and 5% CO_2. Simultaneously, the purified $CD4^+$ and $CD8^+$ cells (section 2.2.3.) were thawed. Cells were thawed by placing cryogenic vials in a 37°C waterbath, 1 ml of AR-10 medium was then pipetted dropwise into the cryovials. The cells were subsequently transferred to 15 ml conical tubes where an additional 8 ml of AR-10 medium was added. The cells were washed twice by centrifugation at 200 *x g* for 10 minutes at 20°C. Following centrifugation the cell pellet was resuspended in 1 ml of AR-10 medium and counted using trypan blue as described in section 2.1.3. The cells were then resuspended in AR-10 medium at 10 x 10^6 cells/ml and placed in disposable sterile 6-well tissue culture plates and incubated overnight at 37°C in a water-jacketed incubator. The following day $CD4^+$ and $CD8^+$ T cells were mixed at a ratio of 1:1 and added to the DCs in U-bottomed 96-well plates at 2 x 10^5 cells/well, giving final ratios of 100:1, 40:1 and 10:1, and returned to the incubator for 5 days. The day prior to harvesting, the cells were pulsed with 10 μl/well (100μCi) tritiated [*methyl*-^{3}H] thymidine (Amersham Biosciences limited, UK) and incubated for a further 16 hours.

The cells were harvested using a Tomtec HARVESTER 96® by placing the printed 90 x 120 mm glass fibre filter mat (Perkin Elmer, WALLAC, Finland) on the filter head of the harvester. First the filter mat was pre-wet with distilled water using an empty plate. The head pressure on the reagent containers was set to 3.0 psi. Thereafter plates containing the cells pulsed with radioactive tritiated [*methyl*-^{3}H] thymidine were harvested using a preset pulse wash program comprising of five repetitive wash/aspirate cycles. The cells were harvested at a flow rate of 0.5 ml/well/second during which approximately 150 μl of water is

added and aspirated per well every 0.3 seconds. The printed glass fibre mat containing the radioactively pulsed cells was then allowed to dry overnight at room temperature. Filter mats were then placed in plastic sample bags (Perkin Elmer, WALLAC, Finland). Prior to heat-sealing, 4 ml of Betaplate scintillant (Perkin Elmer, WALLAC, Finland) was added and evenly distributed on the glass fibre filter mat using a roller. The bag with the filter mat was then heat sealed with the aid of a Heat sealer (Perkin Elmer, WALLAC, Finland). The amount of tritiated [*methyl*-^{3}H] thymidine incorporated during the last 16 hours of T cell proliferation was determined using the liquid scintillation counter (Microbeta, Perkin Elmer, WALLAC, Finland). Data was expressed as counts per minute (cpm) per well.

2.13. Antigen specific lymphoproliferation

To test the ability of transfected cells to present antigen to memory T cells, transfected baboon DCs were co-cultured with autologous matched T cells from vaccinated animals shown to possess a memory T cell response to HIV antigens (section 2.1.1.). The protocol was similar to that described for MLR (section 2.12.). The only difference being that autologous $CD4^+$ and $CD8^+$ memory T cells were added to the transfected DCs. T:DC ratios were the same as for MLR. Maximal proliferation of memory T cells was attained by adding a superpool of HIV-1 subtype C Env peptides (NICD, Johannesburg, South Africa) to the DCs prior to the addition of T cells. The peptide superpool was added at a final total peptide concentration of 1 mg/ml. The Env superpool consisted of 10 sub-pools containing a total of 114 15-20mer peptides overlapping by 10 amino acids. The Env peptides were generated from the sequence data of HIV-1 subtype C isolate Du179, covering the whole of gp160. The Env sequence of the selected isolate was similar to the HIV-1 subtype C Env consensus sequence at the time of manufacture (2001). Incubation conditions, addition of tritiated thymidine, harvesting and counting were all as described in section 2.12.

CHAPTER 3
RESULTS

3.1. DC Culture and Characterisation

DCs were cultured from $CD14^+$ enriched monocyte fractions obtained from fresh PBMC preparations from both human and baboon donors. The conditions for the culture of DCs were optimised by monitoring DC maturation-associated marker expression by flow cytometry.

3.1.1. Assessment of Purity of $CD14^+$ Monocytes

Both the freshly purified PBMC population and the $CD14^+$ enriched cell fractions of humans and baboons were assessed for relative CD14 marker expression by flow cytometry. Matched isotype control antibody staining was used to determine relative marker expression. Based on the data from 3 human donors and 3 baboons, approximately 10-15% of the total PBMC fraction of both species was $CD14^+$ positive. A typical flow cytometry dot plot of CD14 staining of PBMCs from a single baboon is illustrated in Figure 3.1.A. The $CD14^+$ sub-population in each of 3 human donors represented 11%, 13% and 15% of the total PBMCs, respectively. In baboons these values were 10%, 12% and 14%.

Following purification with a magnetic bead sorting system, the enriched human and baboon cells were again labeled with anti-CD14 immunoflourescent antibody. Isotype matched control samples were prepared in parallel. Figure 3.1.B shows that the purity of an enriched baboon population was >98%. Both human and baboon CD14-enriched populations were consistently >95% $CD14^+$ following enrichment.

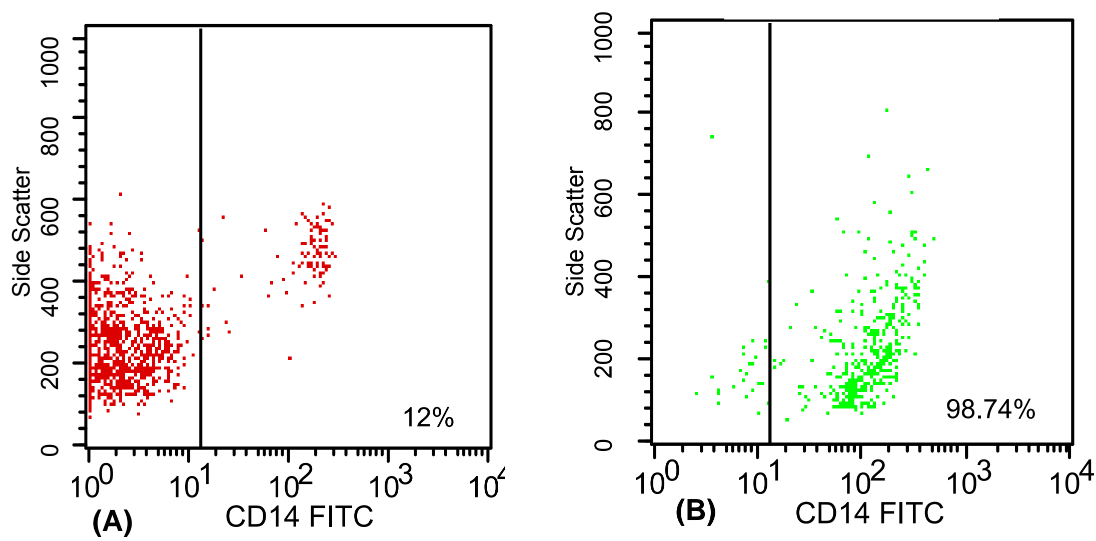


Figure 3.1. Typical dot plots depicting phenotypic analysis of (A) freshly isolated baboon PBMCs and (B) enriched baboon CD14$^+$ monocytes following staining with anti-CD14 FITC immunoflourescent monoclonal antibody. Numbers in the lower right of each plot indicate the percentage CD14-expressing cells. The data were generated from baboon #472.

3.1.2 Optimisation of *in vitro* generation of baboon monocyte-derived DCs

In order to establish optimal baboon monocyte-derived DC culture conditions, human CD14$^+$ monocytes were cultured *in vitro* in parallel with baboon CD14$^+$ monocytes. Baboon culture conditions were optimised by varying the concentrations of rhIL-4 and rhGM-CSF in tissue culture. Successful generation of baboon DCs was evaluated by monitoring the levels of maturation marker (CD80, CD83 and CD86) expression by flow cytometry.

To generate immature dendritic cells (iDCs), baboon monocytes were initially cultured at a density of 3 x 10^6 cells/ 3ml in six-well plates in culture medium supplemented with rhGM-CSF and rhIL-4. Initial concentrations of rhGM-CSF and rhIL-4 were based on those described to give optimal iDC development (based on CD80, 83 and 86 expression) in humans (Romani *et al.,* 1996) and macaques (Mehlhop *et al.,* 2002). Baboon iDC culture conditions were then optimised by varying the concentrations of rhGM-CSF and rhIL-4 in the initial

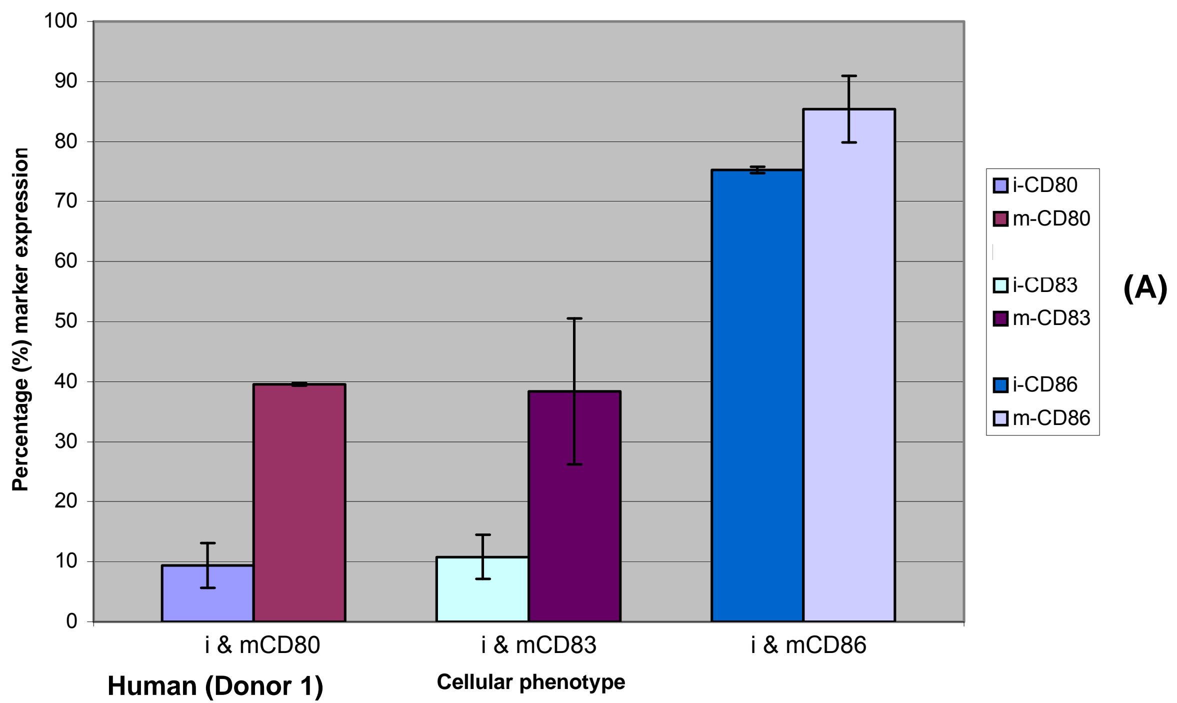
cultures. The combinations used included: 800 IU/ml GM-CSF and 500 IU/ml IL-4 (Sallusto *et al.,* 1994), 1000 IU/ml GM-CSF and 100 IU/ml IL-4 (Messmer *et al.,* 2002) and 1000 IU/ml GM-CSF and 1000 IU/ml IL-4 (Ignatius *et al.,* 1998). Generally poor upregulation of the iDC markers occurred at 800 IU/ml GM-CSF plus 500 IU/ml IL-4 and 1000 IU/ml GM-CSF plus 100 IU/ml IL-4 on baboon cells. Good optimal marker expression was observed at 1000 IU/ml GM-CSF plus 1000 IU/ml IL-4 in both human and baboon DCs. These data are illustrated in Figure 3.2.A and B.

Following culture at optimised conditions, a minority of immature baboon DCs stained positive for CD80 (mean 25% of iDCs in animal 472, 11% in animal 564). Low levels of expression of CD83 were also observed (mean 9% of cells in animal 472, 2% in animal 564), however a strong majority of cells expressed CD86 (mean 89% of cells in animal 472, 82% in animal 564). Patterns of marker expression in human donors were similar to those in baboons. In donor 1 for example, 9% of iDCs were $CD80^+$, 11% $CD83^+$ and 75% $CD86^+$. These data are illustrated in Figure 3.2.

Terminal differentiation of iDCs to mature DCs (mDCs) was induced by the addition of maturation cocktail composed of rhIL-6, rhIL-1β, rhTNF-α, $PGE_{2;}$ as described in the Materials and Methods section 2.2.2. A protocol developed for macaque DCs with minor adjustments was used as a guide for optimisation of baboon DC maturation (Mehlhop *et al.,* 2002). Optimal concentrations for baboon DC maturation based on CD80, 83 and 86 expression, were 20 ng/ml rhIL-6, 10 ng/ml rhIL-β, 10ng/ml rhTNF-α and 10^{-6}M (1μg/ml) PGE_2. Following maturation, cells were harvested and their surface expression of the 3 DC markers was evaluated by flow cytometry. Baboon mDCs were characterised by increased levels of CD80, CD83 and CD86 expression (mean positivity of 51%, 46% and 95%, respectively in animal 472) (Figure 3.2.B). Similar levels of increased staining for all markers was observed in human mDCs. In donor 1, marker expression was as follows: 40% $CD80^+$, 38% $CD83^+$ and 85% $CD86^+$.

These data are illustrated in Figure 3.2.A and B. There was no major difference in levels of marker expression between humans and baboons. Thus, both the baboon iDCs and mDCs were phenotypically similar to their human DC counterparts.

CD14 expression was observed to be minimal to absent on both human and baboon cells by day 2 of cell culture. CD14 expression was found to be absent on both immature and mature human DCs. Baboon iDCs and mDCs were similarly found to lack CD14 expression, as illustrated in Figure 3.4.A and B.



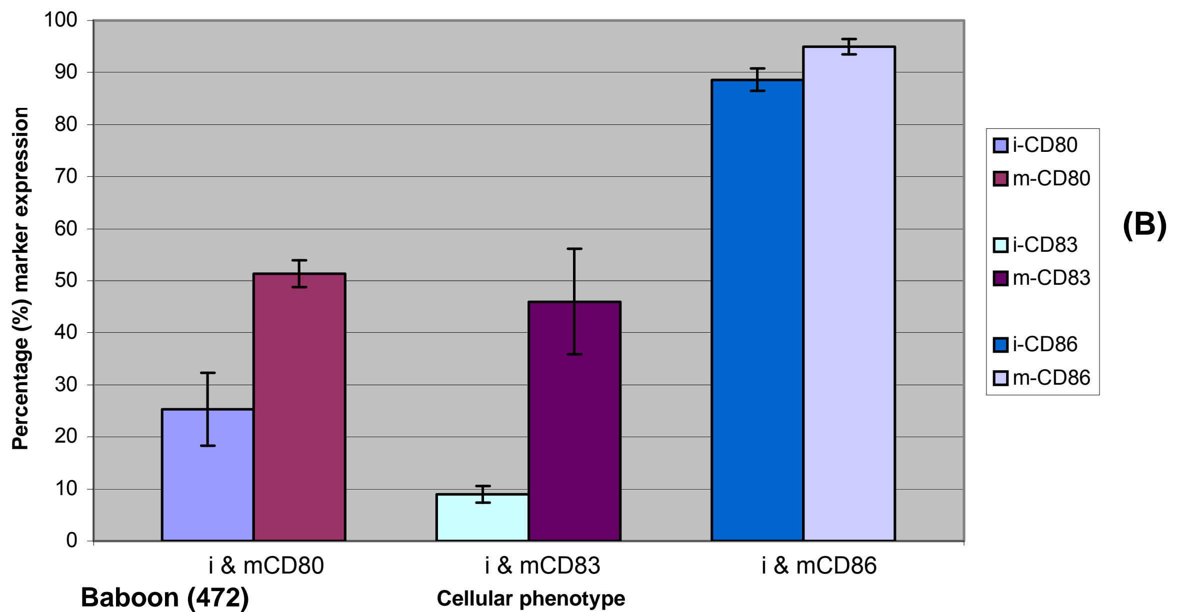


Figure 3.2. Comparison of surface expression of different markers on (A) human and (B) baboon monocyte-derived DC following culture optimisation. The y-axis depicts the percentage of cells expressing the respective cell surface markers and the x-axis shows the cell surface markers investigated (CD80, CD83 and CD86). Both immature (iDC) and mature DC (mDC) expression is shown for each marker. In each doublet, the first bar indicates the iDC and the second the mDC. The data represents the mean of two independent experiments with standard deviation (SD) indicated by error bars. n=2

3.1.3. Morphology of freshly isolated CD14$^+$ and GM-CSF/IL-4 cultured iDCs

CD14$^+$ baboon monocytes comprised of a homogenous cell population of nonadherent, uniformly sized, round shaped cells, lacking detectable cytoplasmic processes (Figure 3.3.A). After stimulation with GM-CSF and IL-4, iDC precursors showed a tendency for aggregate formation (Figure 3.3.B) in liquid culture. Cytoplasmic processes or veils (cellular extensions) were apparent from day 4 on the surfaces of the DC aggregates (Figure 3.3.C and D) (Lohmann *et al.,* 2000; Loré, 2004).

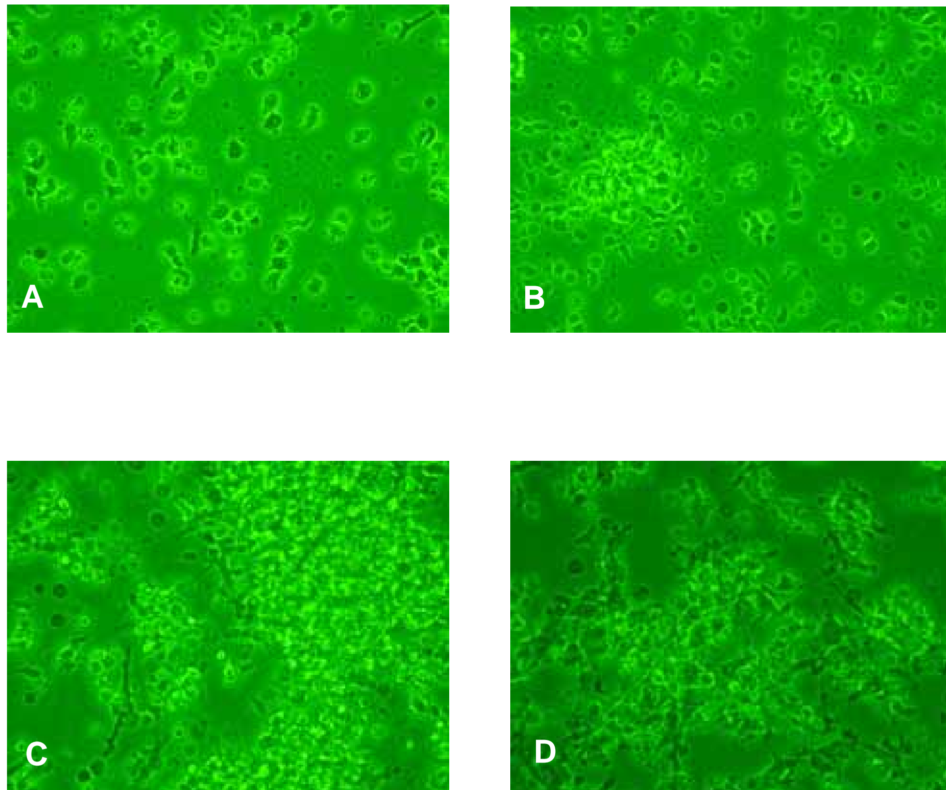


Figure 3.3. Morphology of isolated baboon cells in culture. (A) Purified adherent CD14$^+$ monocytes 2 hours after CD14 enrichment used for initiation of DCs. **(B)** After 2 days in culture with GM-CSF and IL-4, monocytes start clustering into aggregates typical of DC precursors **(C)** Day 4, the clustering pattern becomes more prominent, DC precursors became large with ruffled, stellate (star-like) membranes and are still adherent **(D)** Day 6 cells have the characteristic veiled iDC cell morphology and clustered aggregates are still visible (all magnifications at 200X).

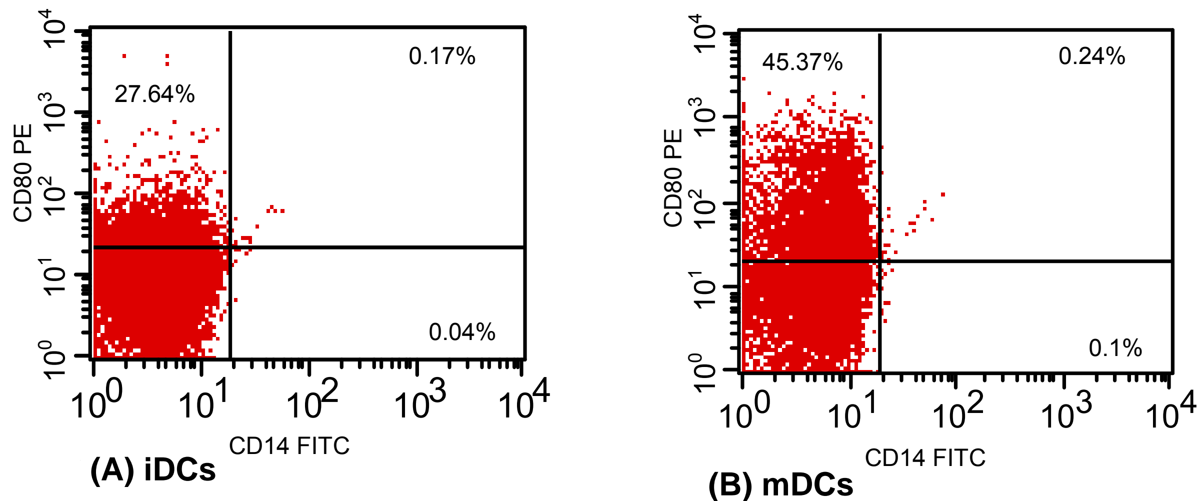


Figure 3.4. Flow cytometric dot plots depicting the phenotypic change with regard to CD14 and CD80 expression on immature (iDCs) and mature (mDCs) baboon DCs (baboon #564), **(A)** day 6 iDCs **(B)** day 9 mDCs following staining with anti-CD14 and anti-CD80 immunoflourescent monoclonal antibody.

3.2. Plasmid DNA Preparation and Screening

3.2.1. Bacterial Transformation and Recombinant Screening

Before commencement of mammalian cell transfection sufficient amounts of the necessary plasmid DNA had to be generated. This was done by transforming competent bacterial cells with the respective plasmids to be used in transfection experiments (i.e. the pSV-β-gal reporter plasmid and the two vaccine constructs, pCMVKm2gagpolBW and pCMVLinkgp140dV2TV1) and isolating the endotoxin free pDNA described in Materials and Methods section 2.7.

Small-scale prepared plasmid DNA was digested with restriction enzymes and visualized on a 0.8% agarose gels to verify insert presence. In Figure 3.5. an agarose gel showing pCMVLink plasmid DNA digested with the *Eco*RI and *Xho*I restriction enzymes is shown. Positive clones were identified by plasmid DNA

showing the excision of an approximately 1986 bp fragment, which correlates with the size of the codon-optimised *env* gene. One clone was selected to produce sufficient plasmid DNA for the transfection experiments.

Figure 3.6. shows the pCMVKm2 plasmid DNA digested with Sal*I* and Eco*RI*. Positive clones were identified by plasmid DNA showing the excision of an approximately 1500 bp fragment, which correlates with the size of the *gagpol* insert. Figure 3.7. shows the pSV plasmid DNA digested with Hind *III* and Pst *I* restriction enzymes. Positive clones were identified by plasmid DNA showing the excision of an approximately 3579 bp fragment.

All selected clones used in transfection experiments were screened by performing agarose gel electrophoresis to confirm the presence of the insert prior to transfection.

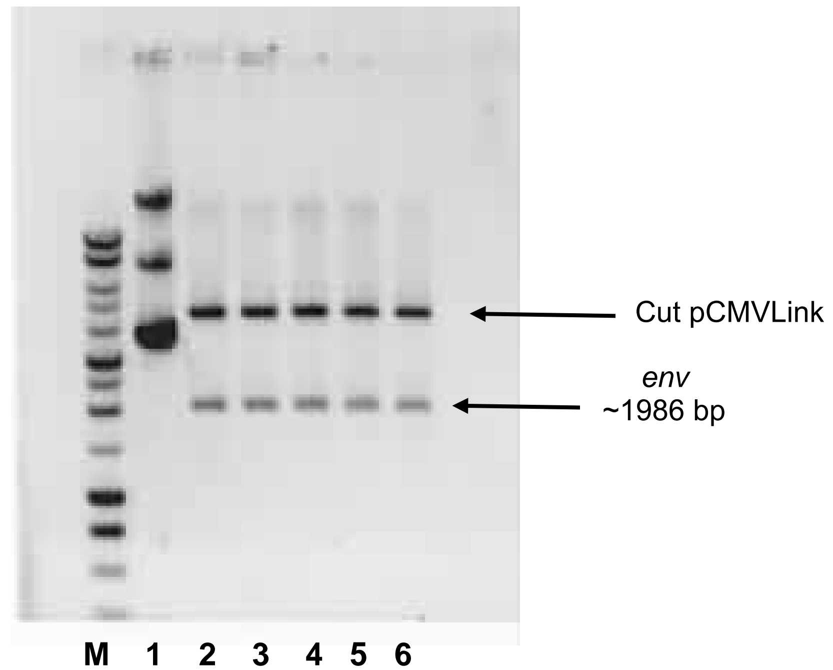


Figure 3.5. Photograph of an ethidium bromide stained 0.8% agarose gel showing the *Eco*RI and *Xho*I digested pCMVLinkgp140 plasmid DNA. M 1 kb DNA ladder. Lane **1** undigested vector, the 3 bands indicate supercoiled, circular and linear plasmid DNA. Lanes **2-6** DNA where the *env* (gp140) insert was excised from the vector. The excised fragments are approximately 1986 bp in length, which correlates with the length of the insert.

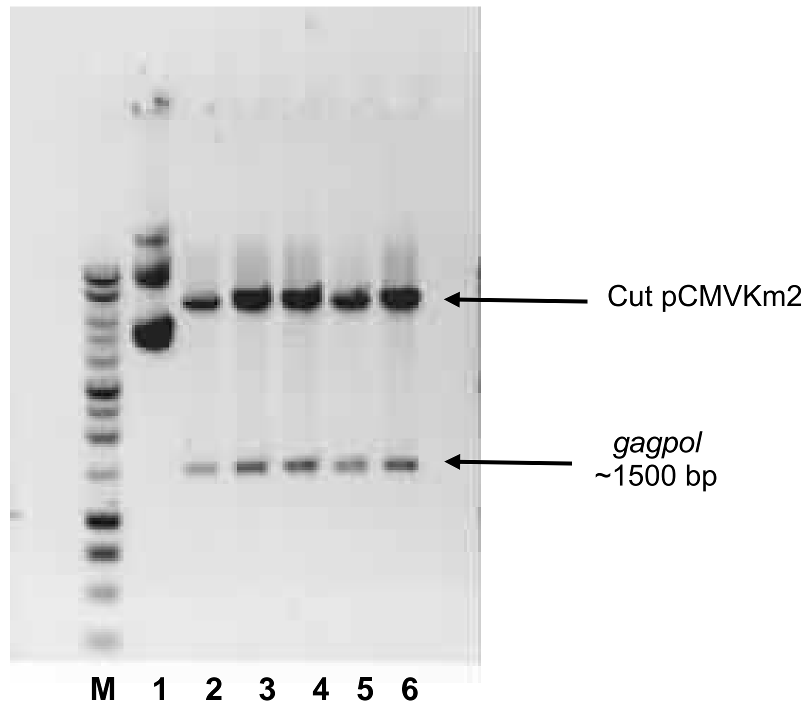


Figure 3.6. Photograph of an ethidium bromide stained 0.8% agarose gel showing the *Eco*RI and *Sal* I digested pCMVKm2gagpol plasmid DNA. M 1 kb DNA ladder. Lane **1** undigested vector, the 3 bands indicate supercoiled, circular and linear plasmid DNA. Lanes **2-6** DNA where the *gagpol* insert was excised from the vector. The excised fragments are approximately 1500 bp in length, which correlates with the length of the insert.

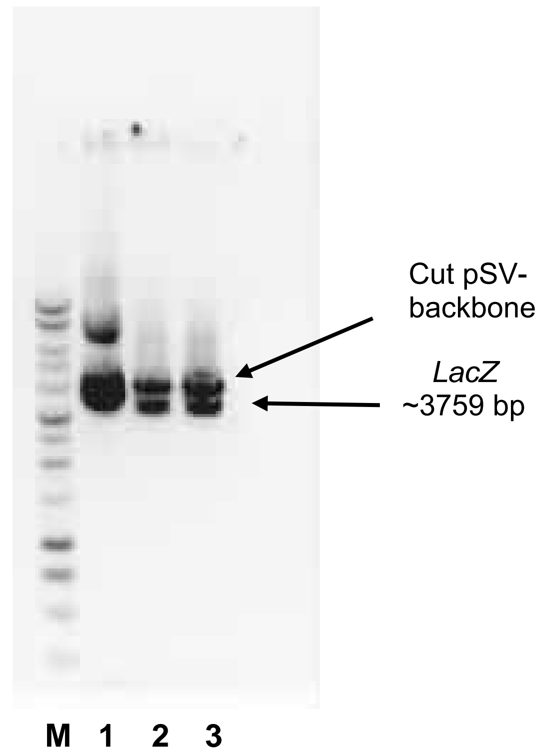


Figure 3.7. Photograph of an ethidium bromide stained 0.8% agarose gel showing the *Hind* III and *Pst* I digested pSV plasmid DNA. M 1 kb DNA ladder. Lane **1** indicates undigested vector, the 3 bands indicate supercoiled, circular and linear plasmid DNA. Lanes **2-3** DNA that has been treated with the aforementioned restriction enzymes. The excised fragments are approximately 3759 bp in length.

3.3. Optimisation of Transfection in HeLa cells and DCs with plasmid DNA

3.3.1. Optimisation of Transfection in HeLa cells

Prior to optimimisation of transfection in DCs, HeLa cells were used to evaluate transfection methods using both the pSV reporter plasmid and the two vaccine plasmid constructs (Materials and Methods*,* section 2.4.).

Due to difficulties associated with culturing and transfecting DCs, particularly the long culturing periods involved and the expense of the growth factors used, the three transfection methods (passive pulsing, lipofection and electroporation) were initially optimised and evaluated in HeLa cells. The pSV-β-Galactosidase reporter assay system was used to establish optimal transfection efficiency for all the transfection methods studied.

3.3.1.1. Passive pulsing (PP) optimisation

In order to simulate conditions often encountered in *in vivo* vaccination studies, namely direct injection of pDNA into individuals, efficiency of passive pulsing (PP) of HeLa cells with pDNA was examined *in vitro* (Materials and Methods, section 2.8.2.1.).

HeLa cells were passively pulsed with 10 μg of pSV-β-Galactosidase pDNA and assayed with the pSV-β-Galactosidase reporter assay system 72 hours post transfection. It was found that the level of β-Galactosidase enzyme activity in the passively pulsed cells was not greater than that expressed by the untransfected control cells. The OD readings in the mock transfected control cells and the passively pulsed cells were almost identical (0.49 and 0.55 respectively). Passive pulsing is therefore not an efficient means of introducing of plasmid DNA into cells.

3.3.1.2. Lipofection optimisation

Prior to lipofection, cells were plated into 6-well plates as described in Materials and Methods, section 2.8.2.1. The cells reached a confluence of 70-80% the day after plating and were lipofected/transfected with the FuGene6 reagent and the pSV-β-Galactosidase plasmid DNA. The efficiency of the transfection method was evaluated by monitoring of optical density readings. Transfections were optimised by altering the transfection reagent to DNA ratios to 3:1, 3:2, 6:1 and 6:2 as described by the manufacturer. Different numbers of cells were used in certain experiments.

Optical density readings taken 72 hours post transfection indicated that transfection by the lipofection resulted in detection of β-Galactosidase activity greater than that observed in mock transfected cells (Figure 3.8.). This transfection method therefore successfully delivered plasmid DNA to HeLa cells. Increasing plasmid DNA concentration from 1μg to 2μg increased the OD

readings at both ratios tested. Further increasing the DNA concentration to 5μg did not additionally increase OD readings. The best transfection efficiency was observed when using 2 μg of DNA, at a ratio of 6:1 which saw an OD reading increase from 0.3 in mock transfected cells to 0.88 in transfected cells. Importantly, incubation with this modified lipid reagent at the optimal ratio showed minimal toxicity in HeLa cells, as indicated by viability, which remained >85% as measured by trypan blue staining.

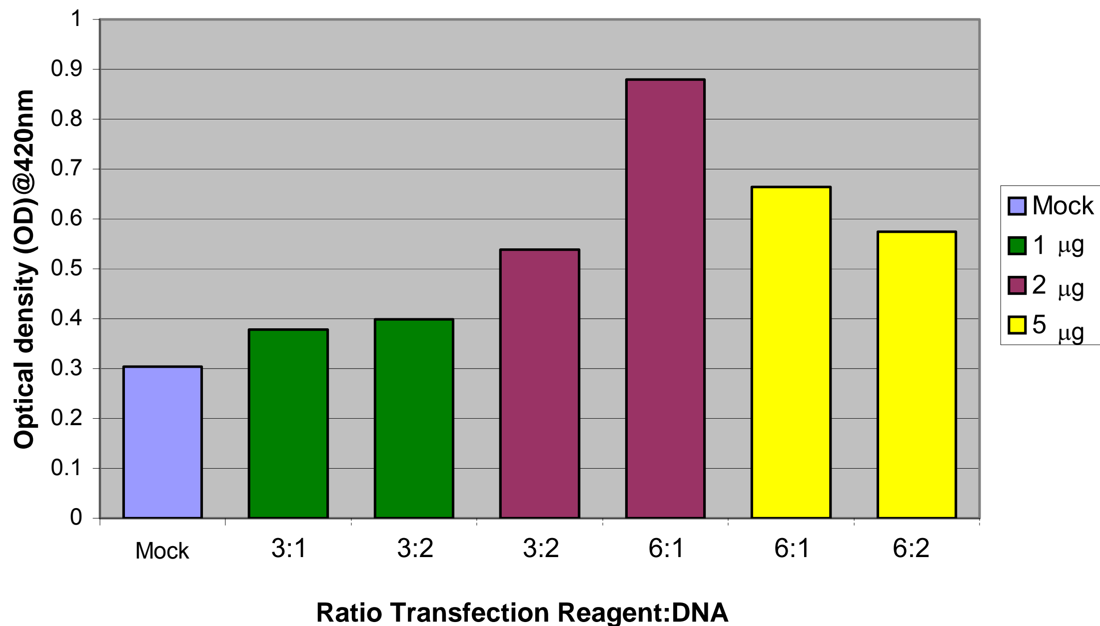


Figure 3.8. Transfection efficiency of HeLa cells using the FuGene6 lipofection reagent and the pSV-reporter plasmid. The ratio of reagent to DNA is shown on the x-axis. The optical density was measured at 420 nm, indicated on the y-axis. A total of 2×10^5 cells were used in all experiments. The DNA concentration is indicated by the legend – Mock no DNA; 1μg indicated by green; 2 μg by red and 5 μg by yellow. Data are representative of a single experiment.

The efficiency of transfection of another commercial liposomal transfection reagent (Lipofectamine™ 2000, Invitrogen, Carlsbad, CA, USA) was compared with the FuGene 6 reagent in HeLa cells using the pSV-reporter construct. The

results of this experiment are indicated in Figure 3.9. In this set of experiments a total of 3 x 10^6 cells was used as opposed to 2 x 10^5 cells as recommended by manufacturer illustrated in Figure 3.8. This accounts for certain differences in absolute OD values The cationic liposomal reagent (Lipofectamine™ 2000) produced the best transfection results at a reagent to DNA ratio of 3:1, with 1 μg of DNA (OD of 1.05 as compared with 0.25 in mock transfected control). Use of modified lipid reagent (FuGene 6), resulted in expression of more than twice as much β-Galactosidase activity compared to Lipofectamine at the optimised conditions i.e. a ratio of 6:1 with 2 μg DNA (OD values of 2.41 and 0.97, respectively). Furthermore, incubation with the liposomal reagent resulted in some cellular toxicity at certain concentrations as determined by staining with trypan blue. Transfection using both reagents resulted in highly reproducible results as evidenced in Figure 3.9., where standard deviation between repeat experiments is indicated. Transfection of HeLa cells with pDNA in combination with the modified lipid reagents (FuGene6) resulted in efficient transfection of cells with minimal toxicity. Due to the superiority of FuGene6 over Lipofectamine 2000, the former reagent was used in all subsequent lipid-carrier mediated transfection experiments.

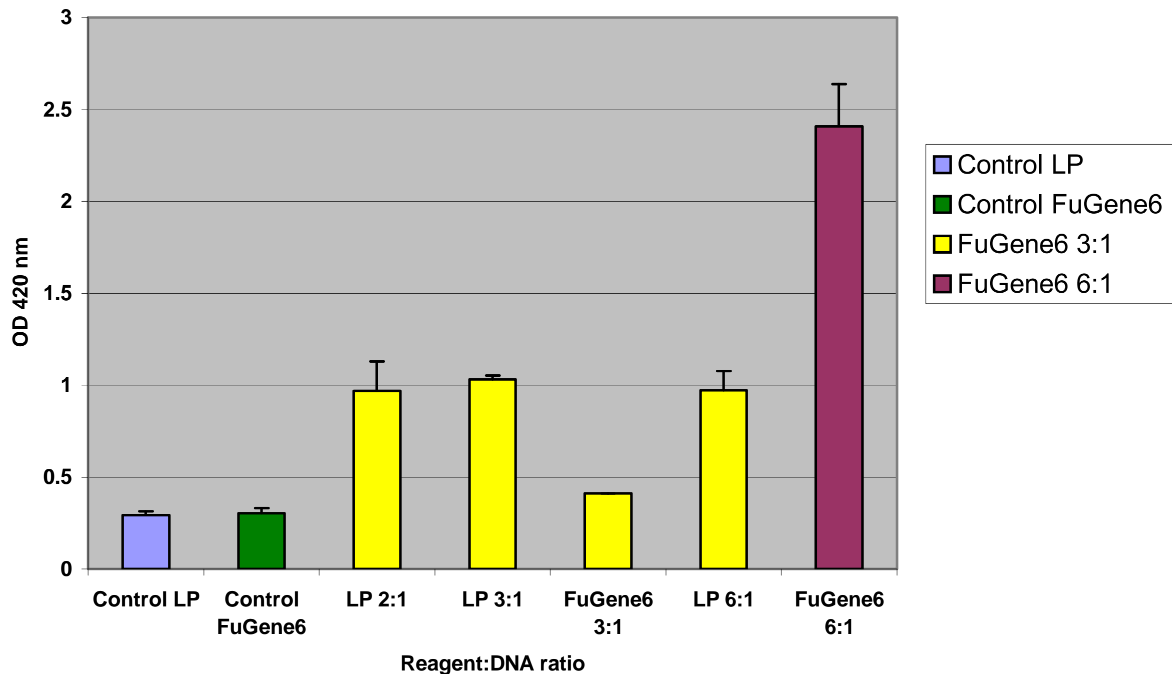


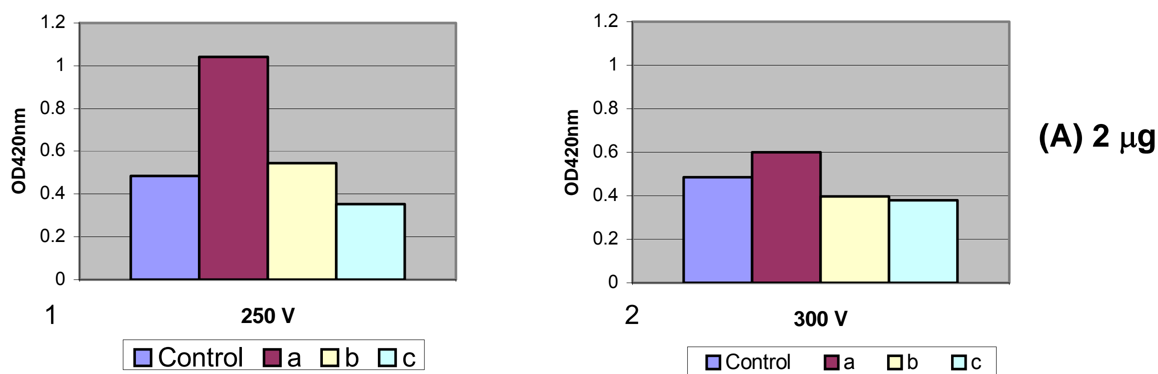
Figure 3.9. Transfection optimisation efficiency of Lipofectamine™ 2000 as compared to FuGene6. The ratio of reagent to DNA is shown on the x-axis. The optical density was measured at 420 nm as indicated on the y-axis. On the x-axis reagent and reagent to DNA ratios are indicated. A total of 3×10^6 cells were used in all experiments. The DNA concentration is indicated by the legend – yellow = 1 μg; red = 2 μg. Data are representative of the means of two experiments with the standard deviation (SD) indicated by the error bars. LP, Lipofectamine™ 2000

3.3.1.3. Electroporation optimisation

Electroporation conditions were optimised by assessing the effect of different voltages (250V and 300V), and capacitances (300, 400 and 500 μF) on transfection efficiency when using different concentrations of DNA (2 and 5 μg). The cells were prepared and transfected as described in Materials and Methods, section 2.8.2.4.

Increasing capacitance and voltage reduced transfection efficiency as illustrated in Figure 3.10. Increasing voltages was detrimental to the cells and resulted in

cell death as revealed by trypan blue viability staining. Electroporation at 300V reduced cell viability to approximately 60% as compared to approximately 70% at 250V. Increasing capacitance values had a similar effect on the cells. Increasing the voltages from 250V to 300V at a constant low capacitance (300 μF) when using 5 μg pDNA resulted in a reduction in transfection efficiency (OD = 0.45, lower than mock transfected control and approximately 50% reduction in OD compared to 250V). Substantial β-Galactosidase expression was always obtained in HeLa cells electroporated at lower voltages of 250 V at a low capacitance of 300 μF with both 2 μg pDNA (OD = 1.06) and 5 μg pDNA (OD = 0.81). The optimal electroporation conditions for HeLa cells were determined to be the following: (1) cell density of 2 x 10^7/ ml; (2) voltage of 250 V; (3) capacitance of 300 μF and (4) pulse times between 8 and 22ms. These optimised conditions were used in subsequent electroporation transfection experiments.



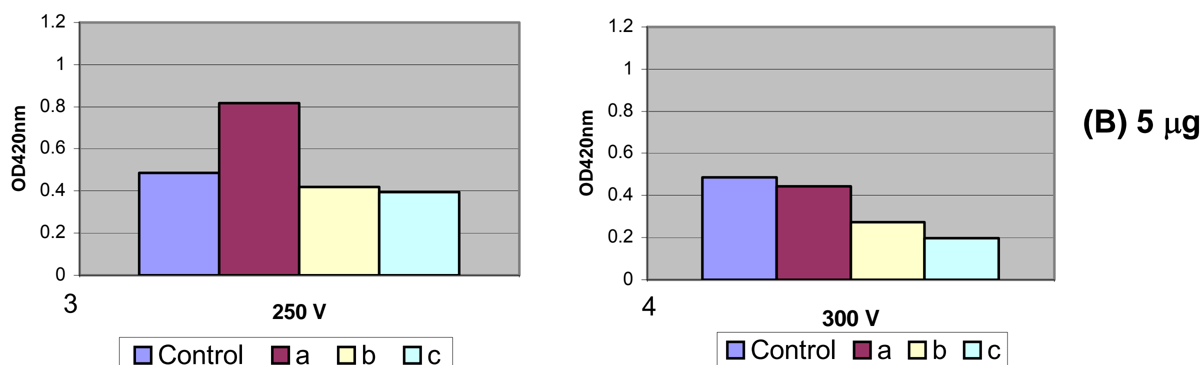


Figure 3.10. Optimisation of electroporation in HeLa cells using the pSV-β-Galactosidase Enzyme Assay System. **A** and **B** depicts transfections performed with 2 and 5 μg of reporter plasmid DNA respectively. The graphs on the left indicate transfection voltages of 250 V and the graphs on the right 300 V. Within each graph – capacitances are indicated as follows: **a**, 300 μF; **b**, 400 μF; **c**, 500 μF. The optical density was measured at 420 nm on the y-axis. Data represent a single experiment.

3.3.2. Transfection of HeLa cells with the *gagpol* and *env* vaccine plasmid DNA constructs

3.3.2.1. Passive pulsing

HeLa cells were not passively with the vaccine study plasmids because of the low transfection efficiency observed with the β-Galactosidase reporter gene (see section 3.3.1.1.).

3.3.2.2. Lipofection

Once optimal transfection conditions for the lipofection method were established in HeLa cells using the reporter pDNA (pSV-β-Gal), described in section 3.3.1.2., cells were transfected with the vaccine study plasmids, pCMVLinkgp140dV2.TV1 or pCMKm2GagCPol.BW, under the same conditions. Expression of transfected pDNA in HeLa cells was monitored by RT-PCR amplification of mRNA. Primers specific for each of the respective plasmid constructs were used (*gagpol* and *env*) following the RT-PCR protocol as described in the Materials and Methods section 2.10.2. Following transfection with FuGene 6 at optimal ratio, good mRNA expression levels were observed as evidenced by the strong bands produced by the RT-PCR products in the agarose gels. Transfection with both constructs was successful.

Figure 3.11. illustrates the banding patterns observed following RT-PCR of the *gagpol* construct. A positive HIV patient control was included as verification. In lanes 2 (lipofected product) and 4 (positive control) strong amplification of the correct gene fragment of 190 bp was observed. Weak amplification was observed with the electroporated product. In Figure 3.12. amplification of the 200 bp *env* gene fragment was demonstrated with the lipofected product, the positive control as well as the electroporated product, see below.

3.3.2.3. Electroporation

Electroporation of HeLa cells with vaccine plasmid constructs at optimised conditions (with 2 μg of pDNA, cell density of 2 x 10^7/ ml, at 250 V and 300 μF) resulted in expression of mRNA, as illustrated in Figures 3.11. and 3.12., The expression of electroporated plasmid DNA at the mRNA level was at lower levels than in lipofected cells (especially evident in the *gagpol* construct, Figure 3.11.).

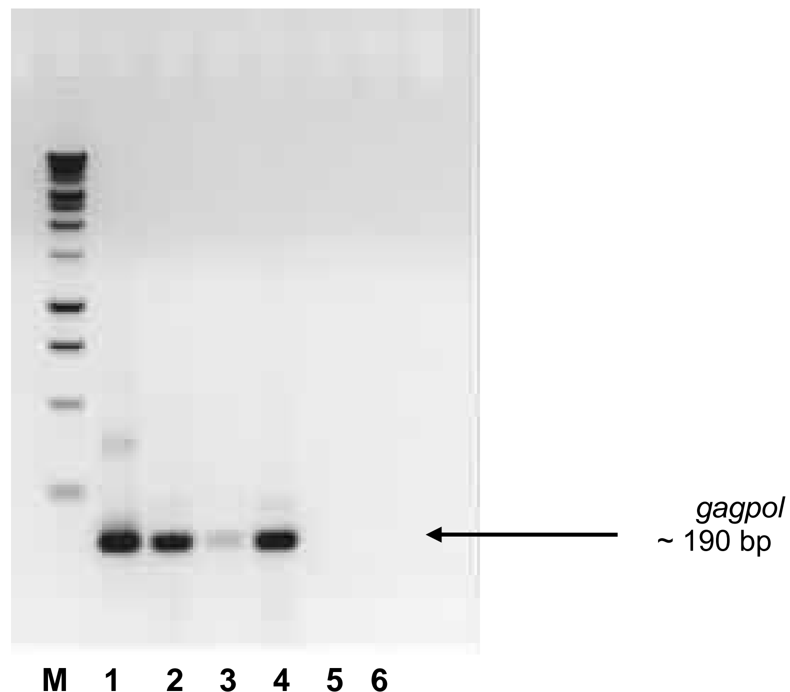


Figure 3.11. Photograph of an ethidium-bromide stained 2% agarose gel showing pCMKm2GagCPol.BW mRNA expression in HeLa cells. RNA was extracted from HeLa cells transfected in different ways and from untreated control cells. RT-PCR was performed as described in Materials and Methods, section 2.10.2. During agarose-gel electrophoresis, a *gagpol*-specific PCR product of 190 bp was detected in the HeLa preparations transfected with the plasmid DNA. **M** 1 kb DNA ladder. Lanes: **1,** unDNase treated RNA product; **2,** lipofection product; **3,** electroporated product; **4,** positive control (R214); **5,** negative control; **6,** reagent control.

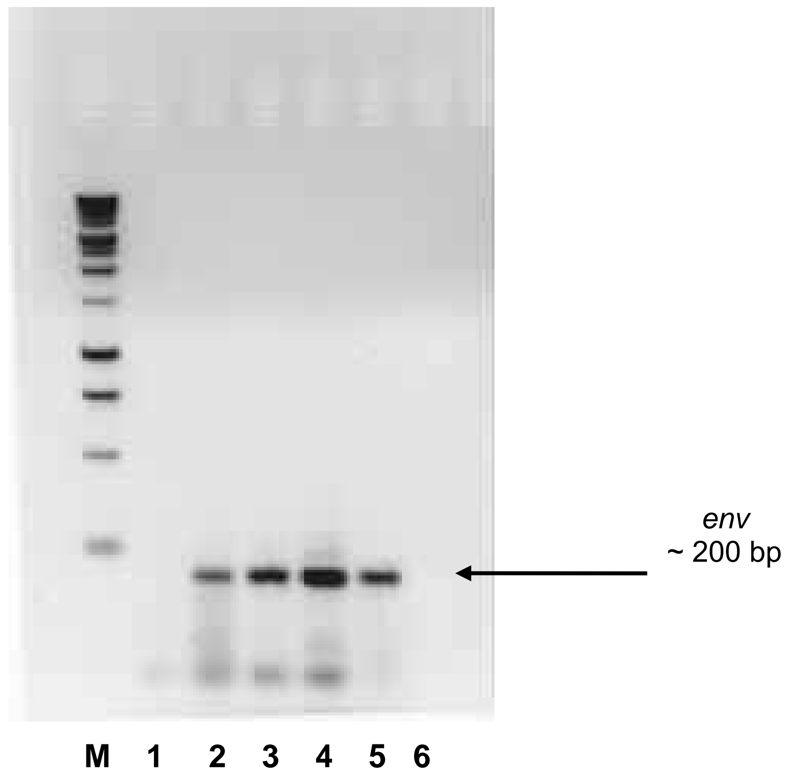


Figure 3.12. Photograph of an ethidium-bromide stained 2% agarose gel showing the expression of pCMVLinkgp140dV2.TV1 mRNA in HeLa cells. RNA was extracted from transfected HeLa cells and an untreated negative control. Afterward, RT-PCR was performed as described in Materials and Methods, section 2.10.2. During agarose-gel electrophoresis, an *env*-specific PCR product of 200 bp was detected in the HeLa preparation transfected with the plasmid DNA. **M** 1 kb DNA ladder. Lanes: **1,** negative control; **2,** electroporated product; **3,** lipofection product; **4,** positive control (R214); **5,** unDNase treated RNA product; **6,** reagent control.

3.3.3. Evaluation of β-Galactosidase expression in transfected DCs

3.3.3.1. Optimisation of passive pulsing in DCs

Passive pulsing of baboon DCs with pSV-β-Gal pDNA did not result in any detectable β-Gal expression above background when assayed with the pSV-β-Galactosidase reporter assay system. This finding was the same as that when passively pulsing HeLa cells (section 3.3.1.1.). PP conditions in baboon DCs were optimised by assessing the effect of various DNA concentrations on DC marker expression. Immature baboon DCs were incubated with 5, 10 and 20 μg of pSV-β-Gal pDNA at 37°C for 3 hours as described in Materials and Methods section 2.8.2.1. Following passive pulsing, the cells were washed with PBS and resuspended in DC culture media containing iDC cocktail. Increased levels of marker expression were observed following pulsing with pSV-β-Gal pDNA at various concentrations (Figure 3.13.). Optimal enhancement of DC marker expression was observed at 10 μg DNA, indicated in Figure 3.13.

3.3.3.2. Lipofection

Following the determination of optimal lipofection conditions for HeLa cells, the optimised conditions were then adapted for transfecting baboon immature (day 6) DCs. Initial transfection experiments were carried out using the pSV-β-Galactosidase control vector in conjunction with the β-Galactosidase enzyme reporter assay system. This allowed for an evaluation of DC transfection efficiency prior to use of vaccine plasmid constructs and RT-PCR of mRNA products.

Immature DCs were generated from $CD14^+$ enriched PBMCs in the presence of rhGM-CSF and rhIL-4 as described in the Materials and Methods section 2.2.2. On day 6 of culture, DCs were lipofected using the modified lipid reagent, FuGene6, with the β-Galactosidase pDNA and after a culture of 12-16h, to allow for protein expression and processing, a cytokine cocktail consisting of rhIL-6,

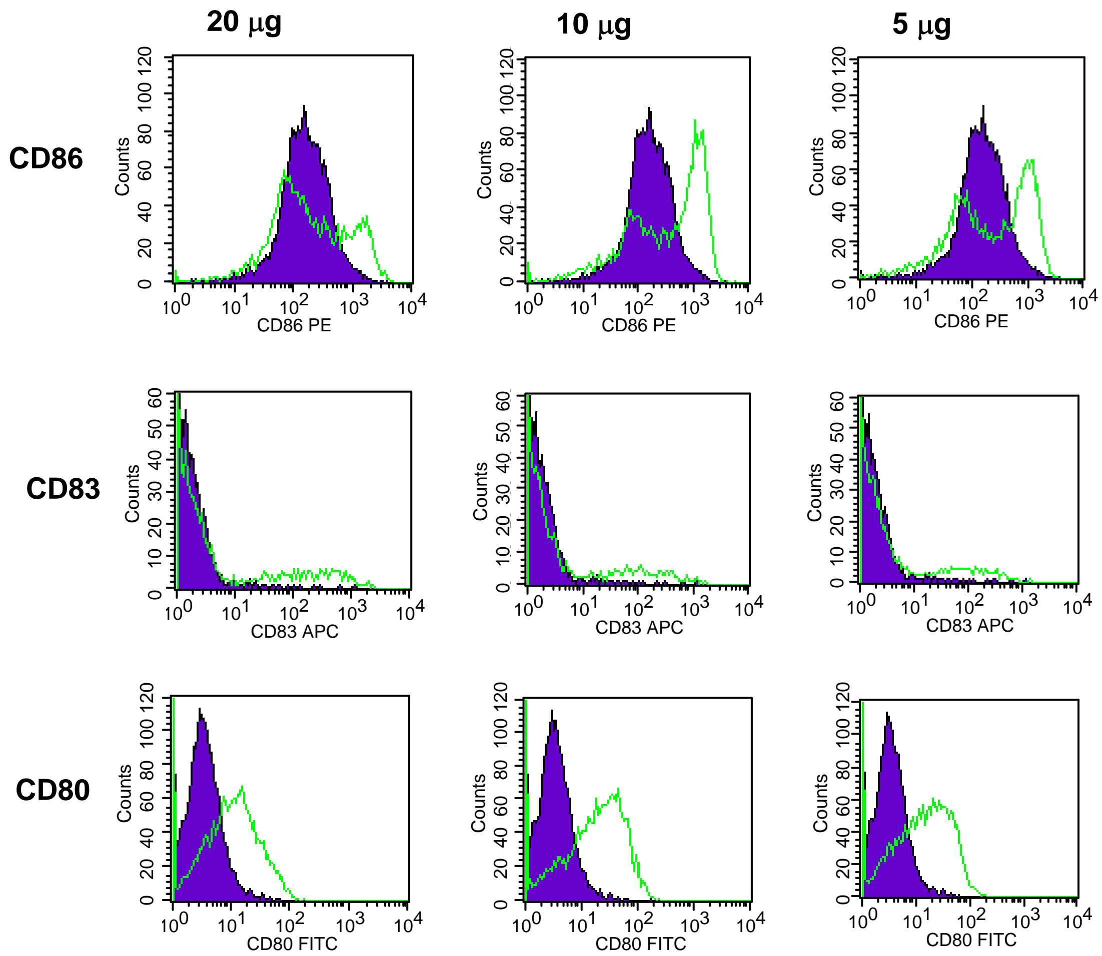


Figure 3.13. Flow cytometric analysis of baboon dendritic cell marker expression before and after passive pulsing with pSV-β-Gal pDNA. Increases in cell surface markers CD80, CD83 and CD86 following passive pulsing were observed. Marker expression before PP is indicated by the filled area, marker expression after PP is indicated by the line. The data illustrate the findings from baboon 472 and is representative of one experiment.

rhIL-1β, rhTNF-α, PGE_2 was added to induce the final DC maturation. After an additional 48h, the transgene expression was monitored by spectrophotometry and flow cytometry was used to monitor cell maturation-associated marker expression. Optimal conditions established in HeLa cells, i.e. 2 μg of plasmid DNA at a transfection reagent (FuGene6) to DNA ratio of 6:1 were applied to DC transfection. These conditions led to efficient transfection with low cell death as determined by monitoring of cell viability by staining with trypan blue. Cell viability remained consistently >85%.

Immature baboon DCs were successfully transfected using FuGene 6 optimised conditions. In both animals tested (564 and 565), expression of β-Gal increased after transfection (Figure 3.14.). Analysis of β-Galactosidase expression after lipofection revealed that the level of expression following transfection with the β-Galactosidase was different for the cells from each animal tested under the same transfection conditions (mean OD = 0.41 for 564 compared to mean OD = 0.28 for 565) for three independent experiments. The level of upregulation of β-Gal expression in the different animals as compared to control was similar (increases of 0.15 and 0.11, respectively).

The increase in OD in baboon DCs after transfection was lower than that observed in HeLa cells. For animal 564 and 565 (Figure 3.14.) the OD increased from 0.25 and 0.17 in mock transfected cells to 0.41 and 0.28 in β-Gal transfected cells, respectively. In HeLa cells however, the change in OD increased from 0.49 to 2.41 following transfection with the FuGene 6 transfection reagent as illustrated in Figure 3.9. under the same conditions.

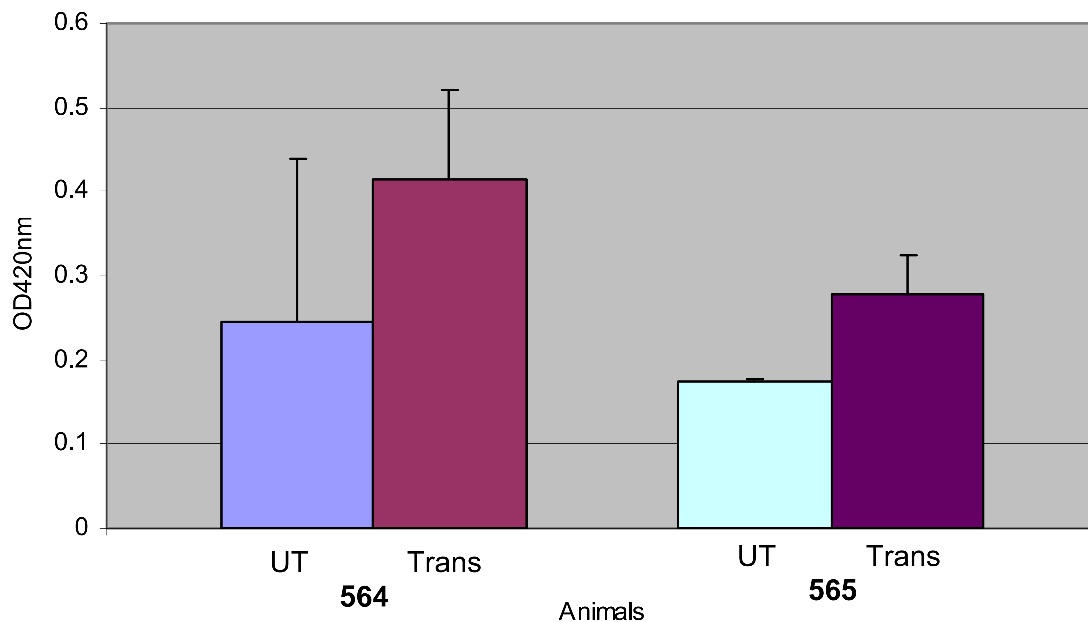


Figure 3.14. Level of β-Galactosidase expression in baboon DCs transfected with the pSV-β-Gal pDNA using the FuGene6 reagent. The level of β-Galactosidase expression in 3 x 10^6 DCs is indicated. Optical density is indicated on the y-axis. Results are representative of three independent experiments. SD is indicated by error bars. UT, untransfected; Trans, transfected.

Concurrent with transfection monitoring by β-Gal expression, cellular phenotype was monitored by FACS analysis. Figure 3.15. illustrates that the maturation markers CD80 and CD83 were significantly upregulated in the transfected DCs compared to the mature untransfected DCs. CD80 expression in animal 564 increased from 28% to 72%, whereas CD83 expression increased from 6% to 37%. Compared to marker upregulation in untransfected mDCs undergoing maturation (Figure 3.2.) it is apparent that transfection did not impair maturation. On the contrary, in the case of CD80 expression, it appears that transfection enhanced normal upregulation induced by maturation factors. CD86 was not examined in these experiments due to insufficient cell numbers.

Lipofection using the cationic lipid reagent, Lipofectamine^{TM} 2000, as optimised in HeLa cells, was also evaluated in baboon DCs. This method was found not to be effective for gene transfer into DCs and led to excessive cell death. Following transfection with the Lipofectamine^{TM} 2000 reagent, it was found that >70% of the cells had died as determined by trypan blue staining. Due to the toxic effect of Lipofectamine^{TM} 2000 on baboon DCs, as a comparison human DCs were examined with the same reagent. The Lipofectamine reagent was also found to be toxic to human DCs (>65% of cells died). Therefore this reagent was not used in further experiments.

The effect of transfection on baboon DC morphology was also monitored microscopically on a daily basis (Figure 3.16.). Both untransfected and transfected DCs showed the same stable morphology with characteristic nonadherent-round shaped cell body indicative of DC maturation and the initiation of migration to lymphoid tissue which occurs *in vivo* (Arthur *et al.,* 1997; Banchereau and Steinman, 1998; Loré, 2004). Day 7 iDCs displayed typical iDC characteristics, the cells were adherent, had long prominent extensions (pseudopodia) and retained their clustering pattern. Transfected day 8 and 9 cells were observed to be loosely adherent to completely non-adherent and had lost their stellate appearance (Figure 3.16. C and D). However, lipofection did not appear to affect DC development negatively when compared to untransfected mDCs.

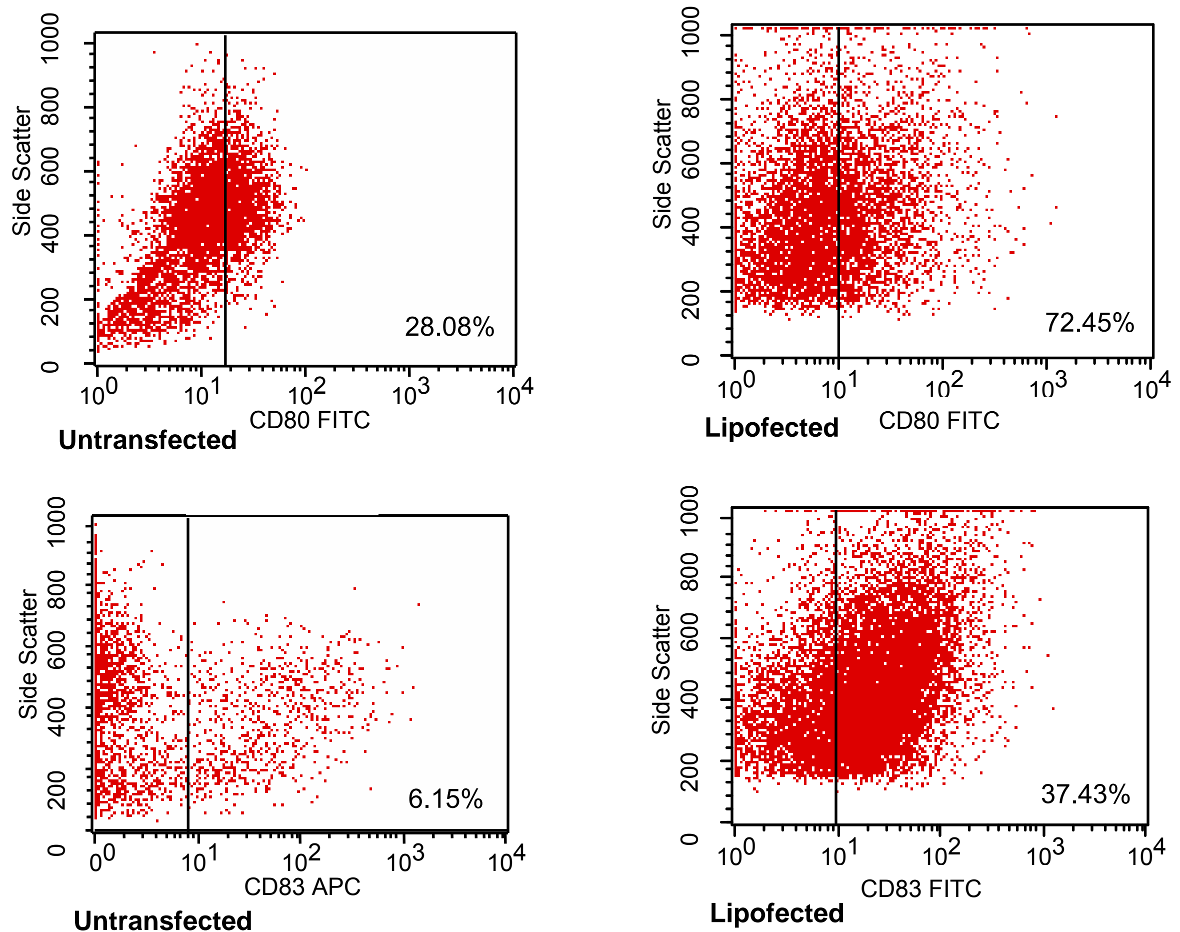


Figure 3.15. Flow cytometric analysis comparing the level of DC maturation marker (CD80 and CD83) expression between mature untransfected and mature β-Gal transfected baboon DCs using the FuGene6 reagent. Percentage of cells positive for the respective markers are indicated in right corners. Isotype controls were used to set negative marker positions for both CD80 and CD83 in untransfected and transfected cells. Results illustrate a single experiment representative of 3 independent experiments from the same animal (baboon #564).

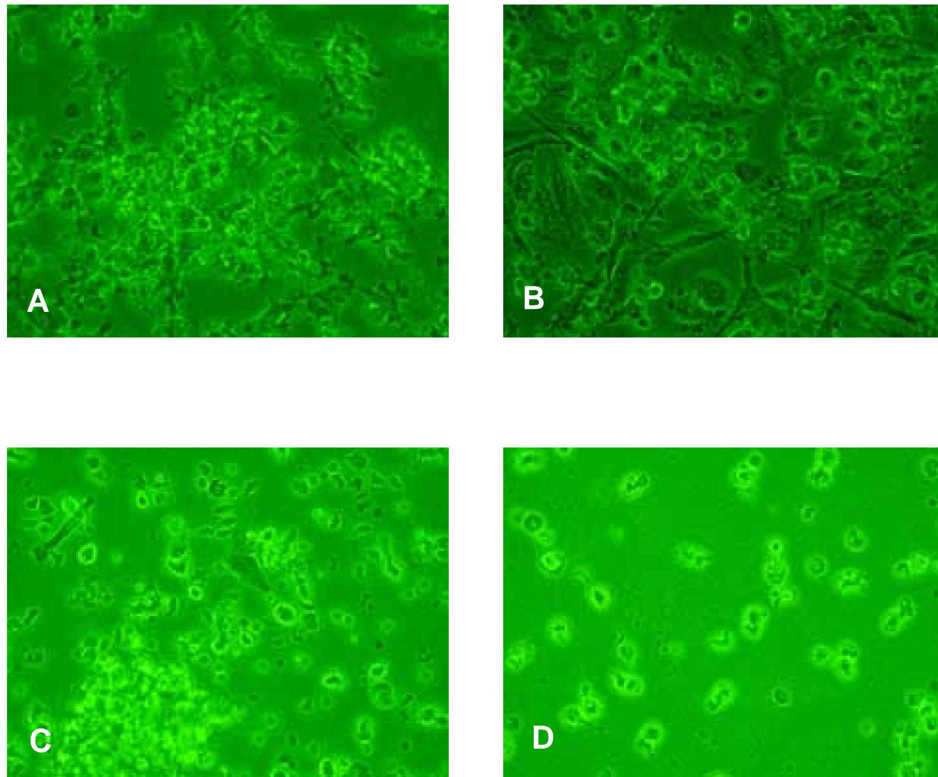


Figure 3.16. Comparative morphological analysis of immature and mature FuGene6 lipofected baboon DCs. **(A)** Day 6 cells (untransfected) have the characteristic veiled iDC morphology and still retain the clustered aggregates **(B)** Day 7 untransfected iDCs, start loosing their clustering pattern and the large stellate (star-like) appearance becomes more prominent. At this stage DCs are still adherent **(C)** Day 8, pSV-β-Gal FuGene 6 lipofected DCs cultured in DC maturation cocktail loose their pseudopodia and are irregularly shaped. The clustering pattern is almost completely absent **(D)** Day 9 lipofected mDCs have lost their stellate appearance and clustering pattern and are non-adherent (all magnifications at 200X).

In comparing lipofection and passive pulsing as means of introducing pDNA into DCs, lipofection appears to be the superior method. When comparing PP and lipofection with regard to DC maturation marker expression, both methods had some effect, but lipofection had the most dramatic effect in most instances. For example, animal 472 CD83 positivity increased from 1% to 7% in the presence of maturation cocktail. PP increased CD83 expression to 12% and lipofection increased it by a further 3% (to 15%). The trend is similar for the other markers examined (i.e. CD80 and CD86) on all the animals studied. However, a minor variation was observed for animal 565 in both Figures 3.19. and 3.20. during the lipofection procudure. Cell viability assessment by trypan blue indicated that PP was slightly less stressful to the cells (>88% viable vs. >80% viable following lipofection). However, since PP did not result in expression of plasmid DNA in target cells, this method was not useful in assessing antigen specific and T cell stimulation (see below).

3.3.3.3. Electroporation

Electroporation of baboon MoDCs was carried out as described for HeLa cells, however this method of transfection was found to be unsuccessful. Various voltages and capacitances were tested and examined as in HeLa cells (section 3.3.1.3.), but all resulted in high cellular mortality Staining with trypan blue indicated that >80% of baboon DCs were dead.

3.3.4. Expression of study plasmids at the mRNA level in DCs

3.3.4.1. Passive pulsing

RT-PCR was not performed on baboon DCs because no β-Gal expression above background was observed during the optimisation experiments (in both HeLa cells and DCs). Thus efficient transfection with the vaccine constructs was unlikely.

3.3.4.2. Lipofection

Having established optimal lipofection conditions using FuGene 6 and reporter pDNA, iDCs were transfected with HIV-1 vaccine pDNA constructs encoding genes of interest. The efficacy of transfection was measured by RT-PCR of transcribed mRNA encoding the *gagpol* or *env* genes, Materials and Methods, section 2.10.2.

Incubation of DCs with 2 μg pDNA resulted in successful transcription of RNA, as evidenced by the amplification of the respective gene products from transfected DCs illustrated in Figures 3.17. and 3.18. RT-PCR resulted in amplification of a 190 bp and 200 bp product for the respective constructs. Amplification of the *gagpol* construct was less efficient than the *env* construct. This would appear to indicate less efficient transfection with the *gagpol* construct, possibly due to it being a fusion construct of two genes as opposed to the modified single gene of the *env* construct. Taken together, these results indicate that transfected vaccine construct pDNAs are expressed at the mRNA level. Baboon DCs could thus successfully be transfected *in vitro* with HIV-1 vaccine pDNA and this transfection led to transcription of mRNA.

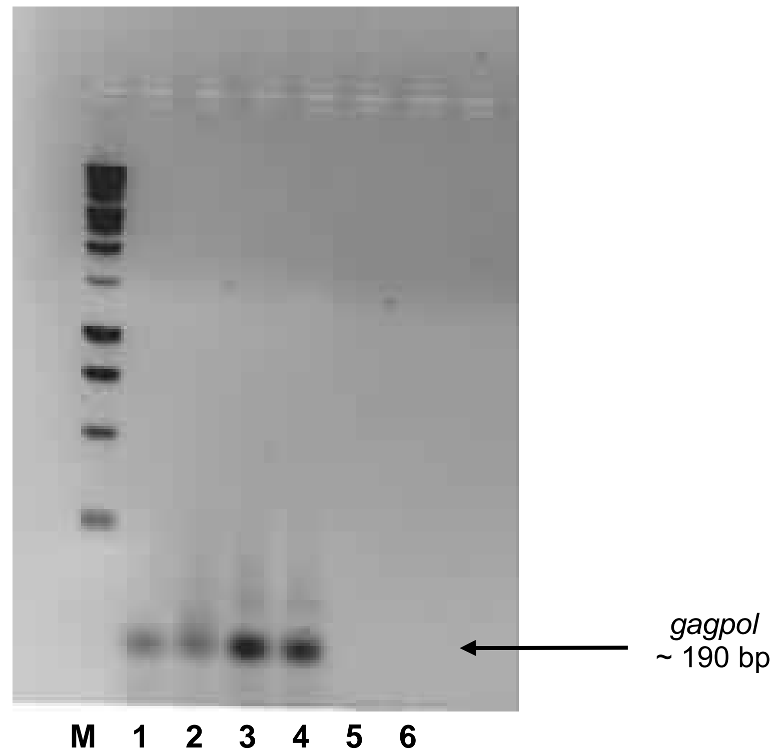


Figure 3.17. Expression of the *gagpol* plasmid DNA insert in baboon DCs following lipofection-mediated gene transfer (baboon #565). RNA was extracted from both untransfected and transfected DCs and pooled. RT-PCR was performed as described in Materials and Methods, section 2.10.2. During agarose-gel electrophoresis, a *gagpol*-specific PCR product of 190 bp was detected in the DC preparation transfected with the plasmid DNA. As an internal positive control for RNA preparation, RNA was extracted from HeLa cells that had undergone similar treatment and analysed in parallel. **M** 1 kb DNA ladder. Lanes: **1-2** DNA from DCs; **3,** positive control (R214); **4,** HeLa control; **5,** reagent control; **6,** negative control.

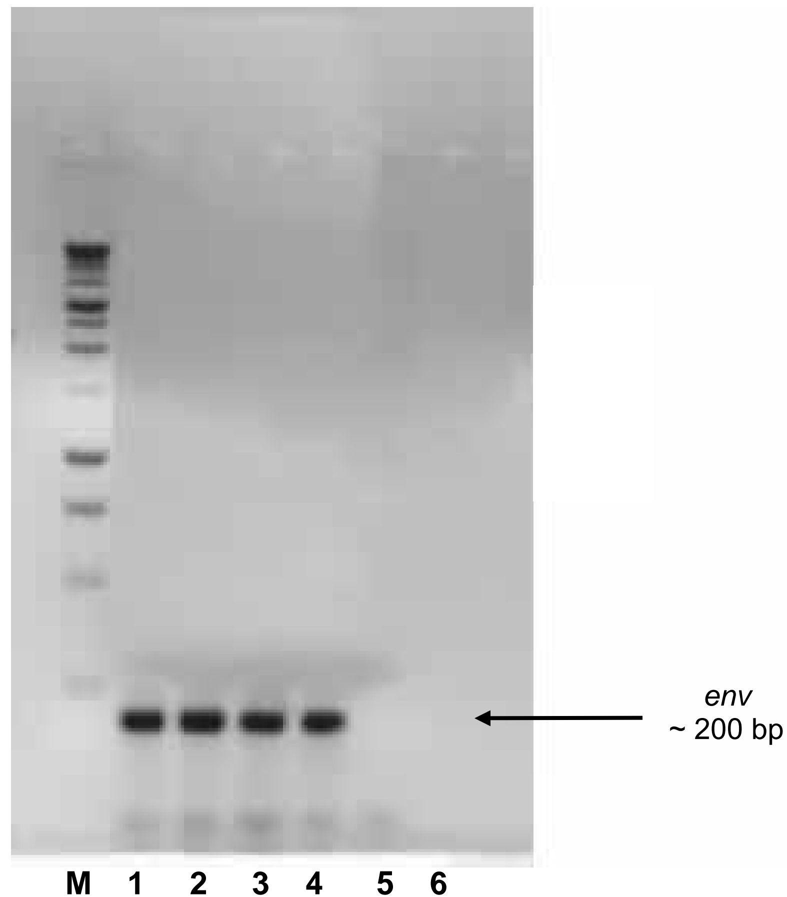


Figure 3.18. Expression of the *env* plasmid DNA insert in baboon DCs following lipofection-mediated gene transfer (baboon #564). RNA was extracted from both untransfected and transfected DCs and pooled. RT-PCR was performed as described in Materials and Methods, section 2.10.2. During agarose-gel electrophoresis, a *env* specific PCR product of 200 bp was detected in the DC preparation transfected with the plasmid DNA. As an internal positive control for RNA preparation, RNA was extracted from HeLa cells that had undergone similar treatment and analysed in parallel. **M** 1 kb DNA ladder. Lanes: **1-2** DNA from DCs; **3,** positive control (R214); **4,** HeLa control; **5,** reagent control; **6,** negative control.

3.3.4.3. Electroporation

RT-PCR was not performed using this method because of its harsh nature and detrimental effect on cells (described in section 3.3.3.3.).

3.3.5. Effect of transfection on DC maturation

The effect of the respective transfection methods using the two vaccine plasmid constructs (*gagpol* or *env*) on DC maturation was evaluated by flow cytometry 72 hours after PP or lipofection. The markers examined were CD80, CD83 and CD86. The electroporation method was excluded for reasons described in section 3.3.3.3.

The response of baboon DCs to the different transfection methods (passive pulsing, lipofection) was compared to standard growth factor-induced maturation (Figures 3.19. and 3.20.). Immature DCs were cultured in the presence of GM-CSF and IL-4 as described in Materials and Methods, section 2.2.2. The iDCs were transfected by either lipofection or PP with the *gagpol* and *env* constructs and matured by the addition of maturation cocktail. Seventy-two hours post stimulation, the DCs were harvested and their cell surface immunophenotype evaluated.

In both Figures 3.19. A-C and 3.20. A-C, representing the *gagpol* and *env* plasmid constructs respectively, variation was observed between the animals for each of the specific markers tested. It was demonstrated that CD80, CD83 and CD86 expression always increased on all mature DC populations compared to iDCs. Transfection with the *env* construct, whether by PP or lipofection, tended to enhance marker upregulation. For example, in animal 564, Figure 3.20., CD80 expression was increased from 14% to 18% in the presence of maturation cocktail. PP further increased CD80 expression to 20%, whereas lipofection increased it yet further to 25%. Differences in marker upregulation following transfection with the β-galactosidase pSV DNA and the candidate vaccine plasmid constructs were observed. In lipofection experiments conducted with pSV vector (Figure 3.15.) an increase of 44% in the CD80 marker expression was observed (from 28% on immature untransfected DCs to 72% on mature lipofected DCs). Figure 3.20., on the other hand, illustrates an 11% increase in the same marker following transfection. A similar trend was seen in the other

animals and with another marker, CD83. In contrast to the *env* construct (Figure 3.20), where transfection generally enhanced marker expression, cells transfected with the *gagpol* construct showed some variability with regard to marker expression (Figure 3.19.). Marker expression was not generally significantly different in transfected DCs as compared to the normal mature DCs for CD83 and CD86. In the case of CD80 the pattern was different. For example animal 472 displayed strong CD80 upregulation following PP, but not lipofection. Animal 565 showed CD80 downregulation following lipofection, but not PP. Furthermore, it was observed that the overall extent of DC maturation following transfection varied between the different animals (B472, B564, B565), whereas in the same animal in repeated experiments it remained relatively constant. Transfection did not therefore inhibit DC maturation as evidenced by absence of any major down-regulation of maturation marker expression following transfection as compared to normal mDCs

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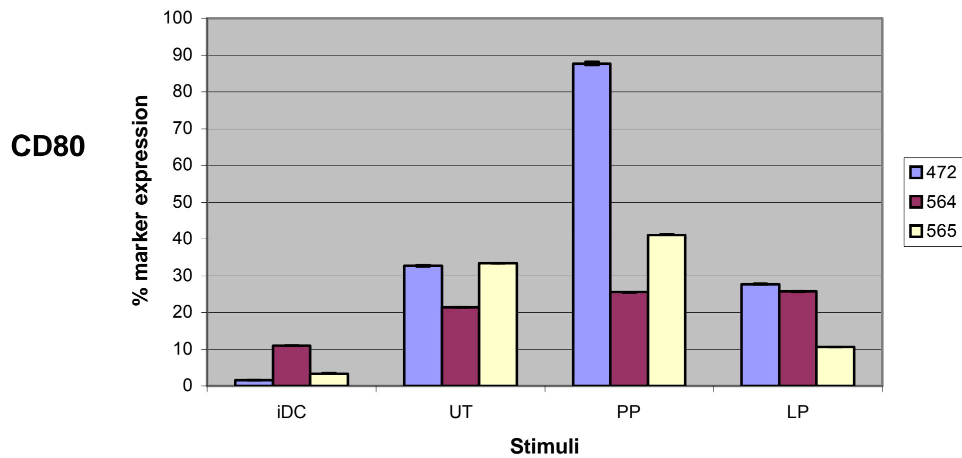
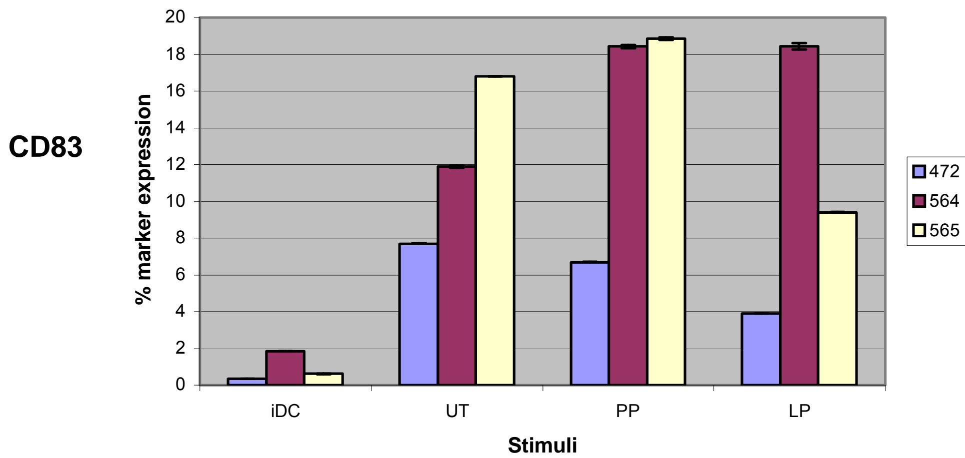




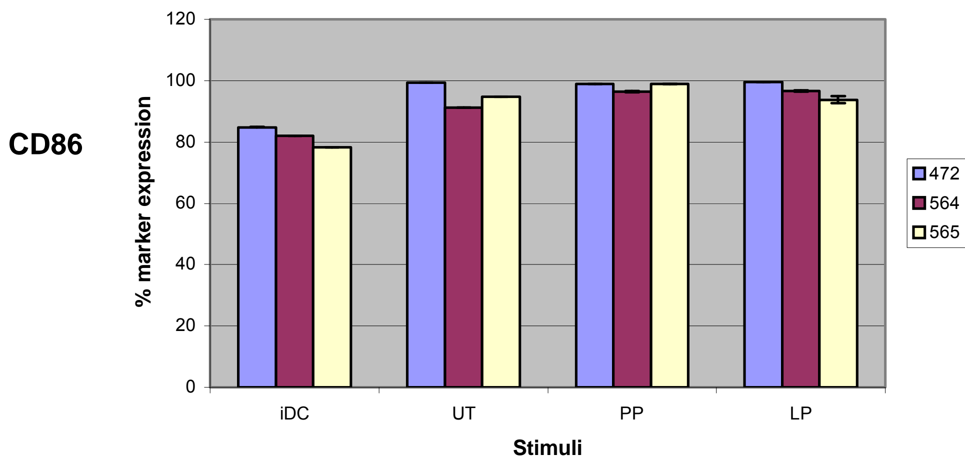
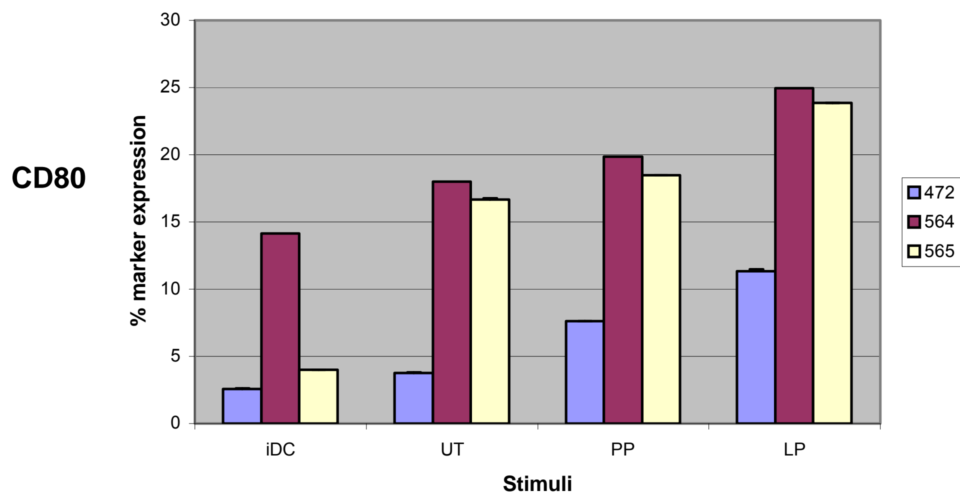
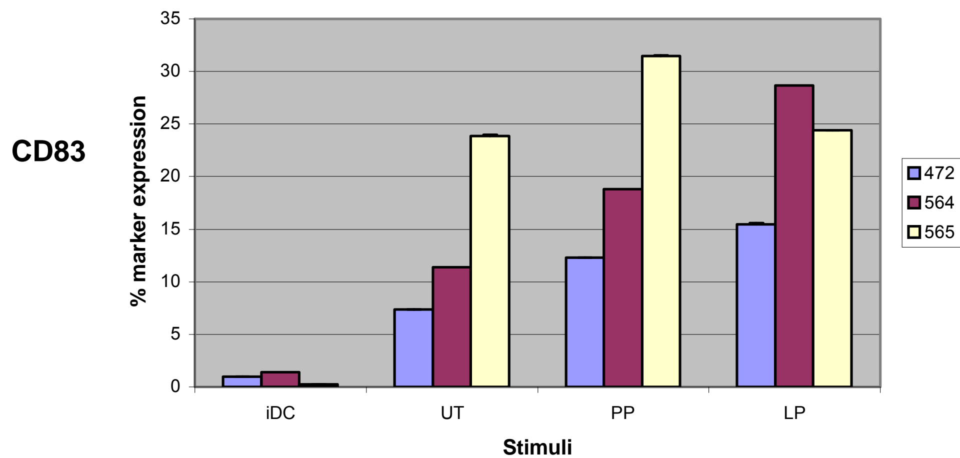


Figure 3.19. Comparative phenotypic analysis of the reaction of monocyte-derived baboon DCs transfected with the *gagpol* plasmid construct to culture induced maturation of DCs. Immature DCs were cultured in medium or stimulated to mature for the last 3 days of culture by the respective transfection methods indicated on the x-axis Cell phenotype was monitored by FACS analysis. The percentage marker expression for each of the respective markers are indicated on each of the respective graphs. The results are representative of at least 3 total experiments with the SD indicated by the error bars. iDC, immature DCs; UT, untransfected; PP, passive pulsing; LP, Lipofection.





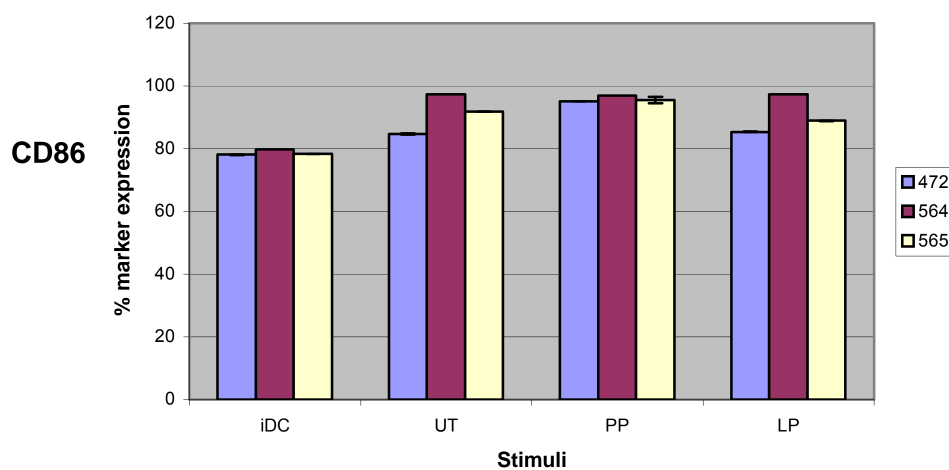


Figure 3.20. Comparative phenotypic analysis of the reaction of monocyte-derived baboon DCs to *env* plasmid construct to culture induced maturation of DCs. Immature DCs were cultured in medium or stimulated to mature for the last 3 days of culture by the respective transfection methods indicated on the x-axis. The effect of transfection on DC phenotype was monitored by flow cytometry. The percentage marker expression for each of the respective markers are indicated on each of the respective graphs. The results are representative of at least 3 total experiments with the SD indicated by the error bars. iDC, immature DCs; UT, untransfected; PP, passive pulsing; LP, Lipofection.

3.4. Effect of transfection on allostimulatory capacity of baboon DCs

Having demonstrated that transfection of baboon DCs with the vaccine plasmid DNA constructs resulted in expression of the relevant genes at the mRNA level, and that transfection did not adversely affect DC maturation processes, further investigation into DC antigen-presenting potential was examined at a functional level using the mixed lymphocyte reaction (MLR). The MLR serves as an indirect functional readout of DC expression of MHC and co-stimulatory markers. The degree of MHC mismatch is related to the extent of proliferation induced. The MLR has been used extensively in DC studies as a readout of antigen-presenting potential (Strobel *et al.,* 2000; Peretti *et al.,* 2005). The relative change in allostimulatory potential of DCs following transfection is likely to be linked primarily to changes in MHC expression, since allogeneic T cells respond to foreign MHC.

Passively pulsed and lipofected DCs were cocultured with a 1:1 mixture of purified allogeneic $CD4^+$ and $CD8^+$ T cells at different T:DC ratios (Materials and Methods, section 2.12.) in comparison to untransfected controls (Figure 3.21.). Untransfected, PP and lipofected matured DCs were compared for their ability to stimulate allogeneic naïve T cells. Figure 3.21. shows that vaccine construct pDNA lipofected DCs were better stimulators of T cell proliferation than their passively pulsed and untransfected counterparts. Different combinations of DC and T cells were used (472 DC vs. 564 and 565 T cells and also 564DC vs 565 and 472 T cells) but the resulting allostimulatory patterns were similar. Lipofection with the *gagpol* construct always resulted in enhanced allostimulation as compared to PP. Lipofection was more effective in DC transfection (section 3.3.3.2.), and also more efficient in enhancing maturation (section 3.3.5.). Thus lipofection was an efficient method of introducing foreign genetic material into DCs and for upregulating expression of surface molecules. Even low numbers of lipofected DCs 2 x 10^3 (ratio of T cells:DC of 100:1) were able to induce strong T cell proliferation. Lipofection of DCs with pDNA showed strongest stimulatory capacity at a T:DC of 10:1 as expected, since at this ratio T cells have easier

access to DCs. It is interesting that PP, which was unable to successfully deliver foreign DNA to target cells, also enhanced allostimulatory capacity.

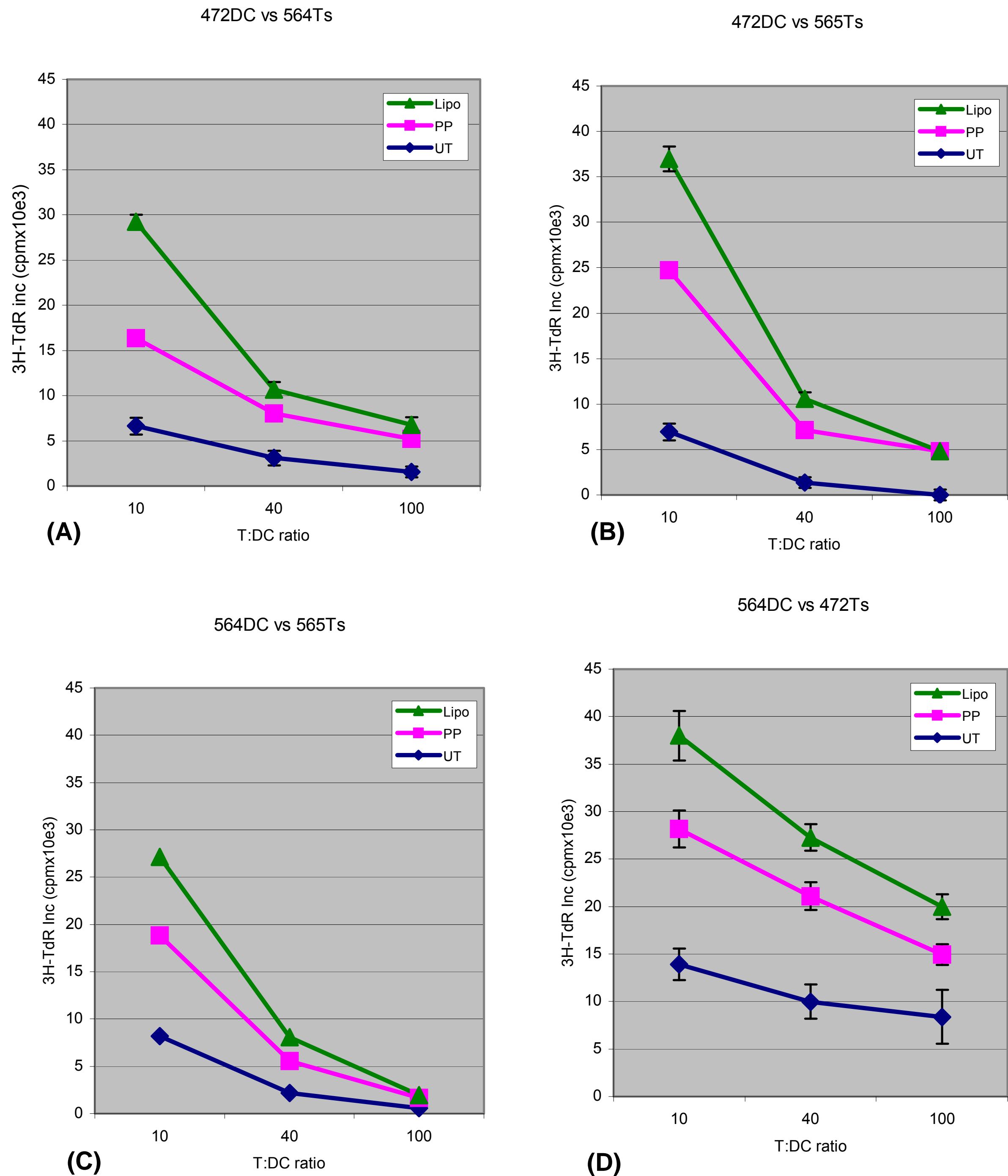


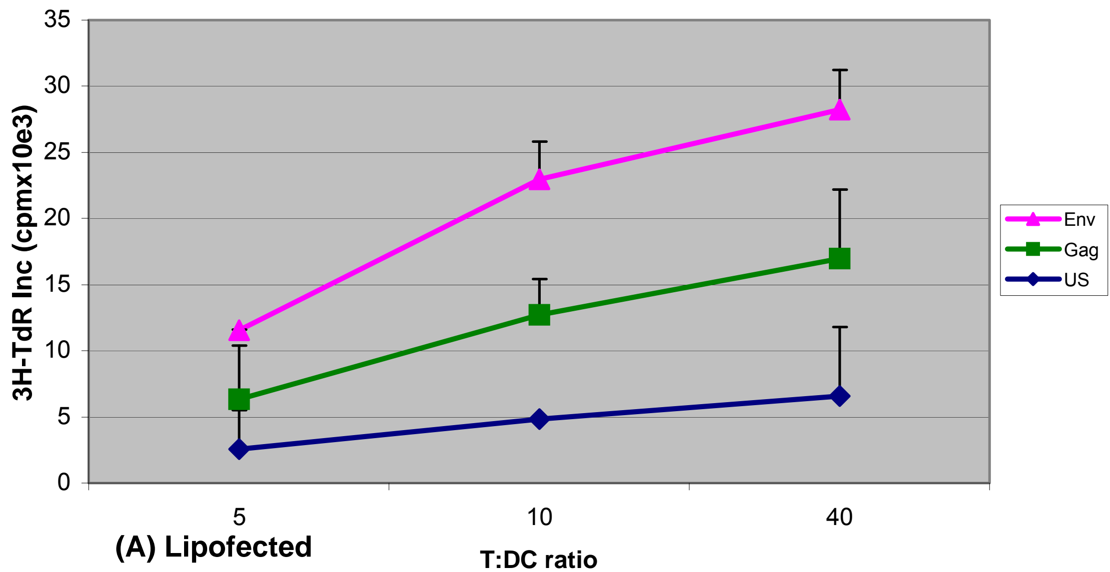
Figure 3.21. Allostimulatory capacity of transfected DCs. Immature DCs were transfected with pDNA encoding the *gagpol* construct using PP or lipofection. T cells (2×10^5 cells/well) cultured with three different concentrations of allogeneic DC (2×10^4, 5×10^3, 2×10^3 cells/well) for T/DC ratios of 10:1, 40:1 and 100:1. After 5 days, T cell proliferation was quantified by [^{3}H] thymidine incorporation. The mean ± SD of triplicates are shown. Data are means of triplicate cultures in one representative out of three. **(A)** 472DC vs 564Ts **(B)** 472DC vs 565Ts **(C)** 564DC vs 565Ts **(D)** 564DC vs 472Ts. The counts for T cells alone was deducted from data represented above.

3.5. Assessment of DC Ag presentation to memory T cells following transfection with vaccine plasmid DNA

In the previous set of experiments, the functional properties of transfected DCs were evaluated by assessing their potential to stimulate T cells in an MLR. These data indicated that transfection appeared to enhance maturation and also enhanced antigen-presenting potential of DCs. In order to study antigen-specific expansion of memory T cells by transfected DCs, antigen-specific T cell proliferation in vaccinated baboons was assessed. These animals had been demonstrated to possess memory T cell responses to gag, pol and env antigens (section 2.1.1., R. Glashoff, manuscript in preparation).

Here the stimulatory capacity of transfected DCs on autologous antigen specific memory T cells was assessed in a modified baboon DC lymphoproliferation assay (LPA). DCs were lipofected with the respective study plasmids and cocultured with a combination of autologous $CD4^+$ and $CD8^+$ memory T cells obtained from vaccinated baboons (Figure 3.22.A). In addition, in certain experiments untransfected DCs were loaded with a superpool of HIV env peptides, to demonstrate the maximal response induced in this system (Figure 3.22.B). Figure 3.22.A illustrates that lipofected DCs induced stronger expansion of memory T cells than did unstimulated (US) DCs. Loading of DCs with peptides also resulted in strong T cell proliferation comparable to transfected DCs. Interestingly, lipofection of DCs with pDNA showed best stimulatory capacity at higher T:DC ratios, a pattern different to that observed in the allostimulation experiments. Peptide-loading resulted in a pattern more similar to the allostimulation. It is possible that the low level of antigen expression in transfected cells necessitated closer contact of T cells for optimal proliferation. At T:DC ratios of 5:1 it is possible that memory T cells did not come into contact with transfected DCs as easily or efficiently as at ratios of 40:1. In peptide-loading experiments it is likely that all DCs expressed peptide bound to MHC, thus even at lower T:DC ratios proliferation could occur.

The fact that transfected baboon DCs could induce proliferation of memory T cells indicates that successful transfection not only leads to DNA transcription but also translation of plasmid-associated genes into protein and subsequent antigen processing and presentation in the context of MHC class I and II molecules. At T:DC ratios of 40:1, *env* transfected DCs were more efficient in stimulating memory T cell responses than peptide-loaded DCs. Baboon DCs cultured *in vitro* are thus an effective model for evaluating plasmid DNA-DC interactions as they display patterns of maturation and antigen presentation .



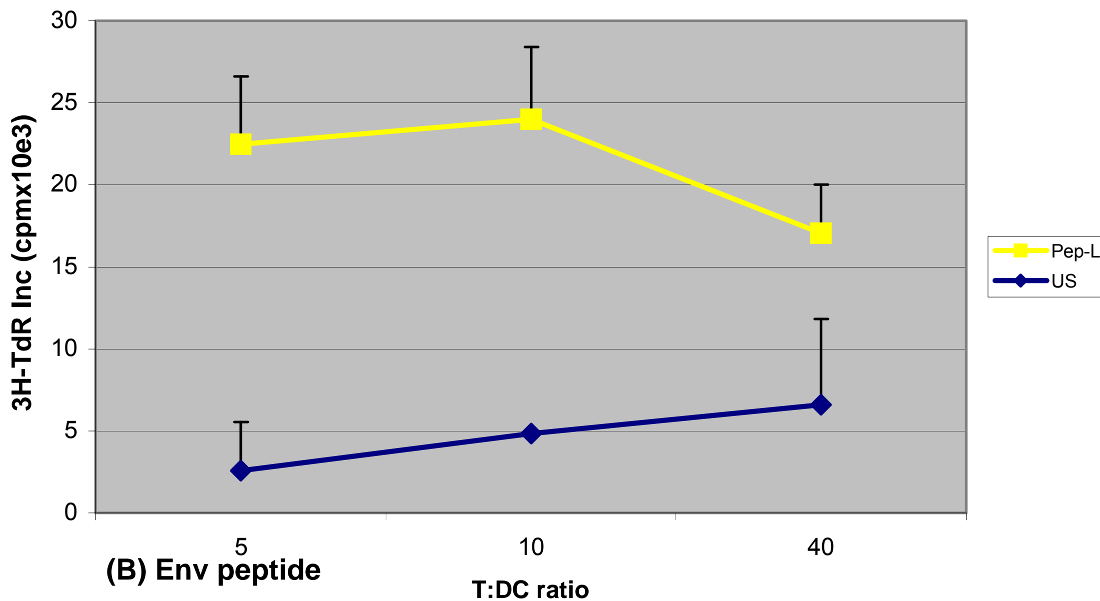


Figure 3.22. Antigen-specific proliferative capacity of transfected DCs. Immature DCs were lipofected with pDNA encoding the *gagpol* or *env* constructs respectively. Simultaneously, iDCs were passively pulsed with the env POPs and matured. T cells (2 x 10^5 cells/well) cultured with three different concentrations of autologous DCs at T/DC ratios of 5:1, 10:1 and 40:1. After 5 days, T cell proliferation was quantified by [^{3}H] thymidine incorporation. **(A)** the Ag-presenting ability of DCs lipofected with the *gagpol* or *env* constructs were compared to that of unstimulated (US) DCs **(B)** the Ag-presenting potential of peptide-loaded (Pep-L) DCs compared to US DCs. The mean ± SD of triplicates are shown. Data are means of triplicate cultures in one representative out of three. The counts for T cells alone was deducted from data represented above. Data are from baboon 522. Similar data were observed in animal 517. US DCs in A and B are from the same data set.

CHAPTER 4
DISCUSSION

4.1. Introduction

Nonhuman primates exhibit substantial immunologic similarities to humans. Comparative cross-species studies of DCs are important in contributing to understanding DC immunobiology and their potential for clinical application (Ardavin *et al.,* 2001). At present, much of human DC biology has been uncovered by studying *in vitro*-propagated, blood monocyte-derived DCs, generated using GM-CSF and IL-4. Using similar protocols studies have been able to generate monocyte-derived DCs (MoDCs) from several nonhuman primate species including chimpanzees (*Pan troglodytes*) (Barrat-Boyes *et al.,* 1997), cynomolgus monkeys (*Macaca fascicularis*) (Soderlund *et al.,* 2000) and rhesus macaques (*Macaca mulatta*) (O'Doherty *et al.,* 1997). Despite some recent advances, nonhuman primate DC biology generally lags behind what has been achieved in human DC biology. The characterisation of nonhuman primate DCs and DC subsets and the ability to manipulate their production *in vitro* are important steps toward the therapeutic application of DCs in clinically relevant models. An understanding of nonhuman primate DC biology and an investigation of the interaction of these DCs with SIVs is essential for future evaluations of vaccine immunogenicity and vaccine-induced protection from viral challenge. Use of a novel nonhuman primate species such as the baboon (*Papio cynocephalus*) for assessing vaccine immunogenicity requires concurrent advancement in basic research around SIV pathogenesis, including DC pathobiology. The pivotal role of DCs in retroviral pathogenesis and also in induction of vaccine-mediated immunity was a motivation for the current study, which assessed DC-plasmid DNA vaccine interactions in the baboon.

4.2. Baboon DC Culture and Characterisation

The current study is the first detailing the establishment of an *in vitro* chacma baboon monocyte derived DC culture system, including morphological and

phenotypical characterisation. Existing protocols describing $CD14^+$ monocyte enrichment, for both humans and macaques, were adapted for use in the baboon system (Ignatius *et al.,* 2000; Frank *et al.,* 2002). A cytokine strategy was employed to drive the differentiation of baboon $CD14^+$ monocytes into i- and mDCs as previously documented for human and rhesus macaque DC culture systems (Romani *et al.,* 1996; Melhop *et al.,* 2002). GM-CSF and IL-4 stimulation resulted in the differentiation of baboon monocytes into iDCs. Published reports phenotypically identify human iDCs as expressing moderate levels of MHC and costimulatory molecules and lacking CD14 expression (Sallusto and Lanzavecchia, 1994; Romani *et al.,* 1996; Steinman and Banchereau, 1998). Rhesus macaque DCs on the other hand continue to express CD14 as both immature and mature DCs (O'Doherty *et al.,* 1997; Barratt-Boyes *et al.,* 2000). In the macaque model CD14 downregulation cannot therefore be used as an indicator of iDC generation. Baboon iDCs were characterised in the current study by the absence of CD14, low CD80 and CD83 expression, and high CD86 expression. These expression patterns are similar to those observed in previously published studies describing human DCs (Steinman and Banchereau, 1998; Ponsaerts *et al*., 2002), and also to parallel investigations of human DC maturation in the present study.

In the current study immature baboon DCs were matured by exposure to maturation cocktail (rhIL-6, rhIL-1β, rhTNF-α, PGE_2). A coordinate series of changes have been reported to mark maturation of DCs including downregulation of phagocytic potential (macropinocytosis), expression of activation markers, changes in chemokine receptor expression and costimulatory molecule expression (Romani *et al.,* 1996; Rossi and Young, 2005). For the purpose of the current study DC maturation was monitored by the acquisition of the following properties: typical veiled morphology and nonadherence in culture, upregulation of costimulatory molecules (CD80 and CD86) and of the DC-restricted molecule CD83 (Zhou and Tedder, 1995; Romani *et al.,* 1996). The maturation of both human and baboon DCs was accompanied by the upregulation of CD80, CD83

and CD86, and changes in cellular morphology. Several studies have demonstrated similar patterns of maturation in both human and macaque DCs (Melhop *et al.,* 2002; Ponsaerts *et al.,* 2002). Cytokine-generated macaque and human DCs have been reported to be large, stellate (star-like) and non-adherent upon maturation (O'Doherty *et al.,* 1997; Lohmann *et al.,* 2000), phenomena also observed in the baboon DCs.

4.3. Comparison of Transfection methods

Given the central role of DCs in both the innate and adaptive immune systems, genetic manipulation of DCs is a promising therapeutic strategy and an important aspect of vaccine design. Targeting HIV-1 DNA vaccines to DCs is essential if generation of both $CD4^+$ and $CD8^+$ anti-viral responses are to be invoked. Attempts to enhance targeting of DCs and DC uptake and expression of plasmid DNA constructs must also assess the effect of the vector itself and the delivery method on the phenotype and function of the DC (Giri *et al.,* 2004; Rossi and Young, 2005). Any detrimental effects on DC maturation would minimize vaccine effectiveness in that sub-optimal antigen processing and presentation would occur. In addition to efficient DNA delivery into DCs, both transcription of pDNA-encoded genes into mRNA and translation into protein is essential for optimal antigen-presentation and also for subsequent generation of both effector and memory T cells. Determining the effects of transfection on DC maturation has been investigated *in vitro* by several groups. Studies that have been conducted in human DCs have shown that it is relatively difficult to transfect DCs by all of the standard *in vitro* transfection techniques such as electroporation, PP and lipofection (Arthur *et al.,* 1997; Kalady *et al.,* 2002; Larregina *et al.,* 2004).

The present study investigated the effect of several different transfection methods on baboon DCs. The methods assessed included passive pulsing, lipofection (lipid-mediated lipofection (FuGene 6) and cationic liposomal-mediated lipofection (Lipofectamine™ 2000)) and electroporation. Most studies evaluating transfection efficiency utilise a marker for evaluation of transfection

efficiency. Popular markers include enhanced green fluorescent protein (EGFP) (Mu *et al.,* 2003) and green fluorescent protein (GFP) (Lundqvist *et al.,* 2005), both of which indicate the extent of plasmid DNA uptake. The luciferase reporter gene system (Larregina *et al.,* 2004) indicates the level of gene expression by detection of plasmid DNA-encoded enzyme activity. The current study utilised an analogous system, the β-Galactosidase reporter gene system, where plasmid DNA-encoded enzyme activity was monitored as a marker of transfection efficiency. In addition to transfection efficiency, the three transfection methods employed were also compared on the basis of DC cytotoxicity, effect on maturation and also reproducibility.

An important component of this study was an evaluation of delivery of candidate HIV-1 subtype C vaccine constructs to baboon DCs. These constructs (pCMVLinkgp140dV2.TV1 and pCMVKm2GagCPol.BW) have been shown to induce antigen-specific cellular and humoral immune responses in baboons following vaccination (Glashoff *et al.,* manuscript in preparation). Detection of expression of vaccine plasmid DNA-encoded gene products following baboon DC transfection would provide an added level of proof of efficacy of the constructs in inducing immunity in this species, and was thus an important thrust of this project.

In the current study, DC transfection was evaluated by four basic criteria: transfection efficiency, maturation, cytotoxicity and reproducibility. In order to simulate conditions often encountered in *in vivo* vaccination studies, namely direct injection of pDNA into individuals (usually into muscle tissue), the efficiency of passive pulsing (PP) of baboon DCs with pDNA was examined *in vitro*. To date studies conducted on human DCs using the PP method with either DNA or mRNA on iDCs have yielded unsatisfactory results both at the level of transfection efficiency as evaluated by reporter gene expression, and the levels of DC maturation marker (CD80, CD80 and CD86) upregulation. Several groups have reported that EGFP reporter product was undetectable when immature

human DCs were passively pulsed using mRNA (van Tendeloo *et al.,* 2001; Meirvenne *et al.,* 2002). These studies might have been unsuccessful due to a lack of serious attempts at optimisation. It is unknown whether the concentration of the plasmid DNA added or the cell density was optimal in these studies. Although most reports have emphasized the inefficiency of PP, Nair and colleagues demonstrated that PP with mRNA was able to prime tumor-specific CTL *in vitro* (Nair *et al.,* 1998).

Although in the present study immature baboon DCs were not transfectable by PP it was demonstrated that this method induced baboon DC maturation and resulted in minimal cellular mortality. The lack of successful transfection was evidenced by the fact that there was no detectable β-Galactosidase expression in the iDCs, suggesting poor uptake of plasmid DNA into the cell cytoplasm as reported in earlier studies conducted on human DCs (Van Tendeloo *et al.,* 2001; Kalady *et al.,* 2002;). The problems associated with PP is that it does not direct gene delivery to the target cell, there is low uptake across the plasma membrane as there is no carrier (e.g. lipids) and there is no protection from endogenous nucleases following uptake which is mostly by endocytosis (Zhdanov *et al.,* 2002; Roth and Sundaram, 2004). Although transfection efficiency was poor, PP did not in any way inhibit DC cellular differentiation and morphology. Exposure of iDCs to plasmid DNA in its naked form resulted in induction of maturation accompanied by normal maturation-associated morphological changes. The finding that poor transfection was still associated with changes indicative of DC maturation may be related to foreign DNA stimulation of DCs via pattern recognition receptors (e.g. Toll-like receptors, TLRs) (Barton and Medzhitov, 2002). Plasmid DNA plays an important role as an adjuvant for inducing DC maturation through pattern recognition, a phenomenon also observed in other studies (Spies *et al.,* 2003; Pulendran, 2004). The fact that PP also enhanced allostimulation of T cells, indicates that plasmid DNA-induced maturation also effects function of the DCs in a non-antigen-specific manner indicative of innate immune responses.

In this study lipofection of baboon immature monocyte-derived DCs was performed using either a modified-lipid reagent (FuGene 6) or a cationic lipid formulation (Lipofectamine^{TM} 2000). The FuGene 6 transfection reagent is an optimised blend of lipids and other propriety compounds for transfection of DNA into cells that produces higher transfection efficiencies. Lipofectamine 2000 is a cationic lipid formulation designed to enhance transfection via charge assisted targeting of lipid-mediated plasmid DNA delivery.

Lipofection of baboon DCs with pDNA (both pSV-β-Gal and vaccine study plasmids) using the FuGene6 reagent was successful. DCs were efficiently transfected and plasmid DNA-encoded genes were expressed as evidenced by β-Galactosidase enzyme detection and vaccine pDNA-encoded gene expression at the mRNA level. The good transfection levels were accompanied by upregulation of DC maturation marker expression and characteristic morphological changes. These results are in line with other published data for both humans and macaques (Pecher *et al.,* 2001; Melhop *et al.,* 2002).

Strobel and associates tested two modified-lipid reagents, Superfect (QIAGEN, Hilden, Germany) and FuGene 6 for transfection of human MoDCs with GFP pDNA. They demonstrated extremely low transfection efficiencies equal to or below background using both reagents (Strobel *et al*., 2000). The lack of success using FuGene 6 in their study as compared to the present study may be related to species differences, DCs being at different stages of maturation when transfected, and the inclusion of a washing step 1 hour post transfection in the former study. The good transfection levels observed in the current study may also be due to baboon DCs being more resilient to the transfection reagent (as compared to human DCs) or they may have an enhanced ability for the uptake of lipid-DNA complexes. This method was not toxic to the cells and was accompanied by enhanced maturation marker expression post transfection. The levels of maturation marker enhancement following FuGene 6 transfection were similar to those observed following unsuccessful PP. This would appear to

indicate that maturation is induced by the presence of foreign DNA rather than transfection itself. The changes in maturation marker expression following lipomediated transfection were consistent in individual animals, even though absolute marker expression levels varied between animals, a phenomenon noted in earlier studies (Pecher *et al.,* 2001). The differences in marker expression may be explained by genetic differences directly influencing the level of marker expression (Michou *et al.,* 1997; Lefesvre *et al.,* 2003).

Besides the effectiveness of the lipofection method, this technique has an additional advantage in that Lipid-DNA complexes are easily prepared and the procedure can be performed rapidly. This also contributes to the consistency of results. The promising data generated by FuGene 6 lipofection would appear to encourage the use of lipid-based carrier materials for the delivery of vaccine antigens *in vivo*. It must however be remembered that *in vitro* assessment of transfection efficiency of DCs is not strictly analogous to the *in vivo* situation. Delivery of plasmid DNA intramuscularly results in transfection predominantly of somatic cells (myocytes) rather than DCs (Huygen, 2005). DCs would appear to capture antigen expressed by myocytes or other cells or via uptake of apoptotic cells destroyed by localised inflammatory responses and innate immunity. Despite this, lipid-mediated delivery of vaccine antigens to DCs is effective and for the purposes of *in vitro* screening of vaccine gene expression this procedure can be recommended.

In contrast to the FuGene 6 data, in most human DC studies where lipofection was performed with pDNA using cationic reagents, varying levels of success have been reported (Arthur *et al.,* 1997; Kalady *et al.,* 2001; Lundqvist *et al.,* 2002). These studies have reported low reporter gene expression when using pDNA to transfect MoDCs. In addition, significant reagent toxicity on the DCs has also been reported. Several studies conducted on human DCs have reported poor transfection efficiency and/or no transfection using the cationic lipid reagent, Lipofectamine^{TM} 2000 (Van Tendeloo *et al.,* 1998; Pecher *et al.,* 2001).

The weak transfection efficiency is most likely due to the cell-type dependency of cationic lipid reagents. Certain types of primary cultured cells, such as neurons, DCs and endothelial cells remain recalcitrant to traditional transfection methods including cationic lipids. Studies have attributed this to a number of biological processes involved in these methods, such as the mechanism of endocytosis, which is strongly cell-type dependent (Zabner *et al.,* 1995; Rémy-Kristensen *et al.,* 2001). In addition, physiochemical parameters of complex formulation, such as lipoplex size, charge and DNA/vector dissociation have also been shown to influence transfection efficiency. The current study found that transfection of baboon DCs with the cationic lipid reagent, Lipofectamine™ 2000, resulted in extensive cell death, these findings are thus in accordance with the published literature for human DCs (Van Tendeloo *et al.,* 1998; Pecher *et al.,* 2001).

In general, electroporation has been reported to result in efficient gene delivery to many cell types. The efficiency of gene transfer by electroporation is influenced by several physical factors (pulse duration and electric field strength) as well as biological factors (DNA concentration and conformation, cell size) (Gehl, 2003). In contrast to lipofection, electroporation generally utilises large amounts of DNA (between 10-20 μg). Several studies evaluating electroporation in human DCs have emphasised that this method is laborious and time consuming. The process entails the removal of cells from culture plates, transferring them to cuvettes, electroporating and returning cells to culture. These requirements add stress to an already very delicate cell type. Furthermore there is conflicting data regarding the cell viability and the levels of reporter gene expression in human DCs following the electroporation procedure. Studies performed on human DCs appear to indicate that using mRNA generally is less harmful to cells (greater cell viability after transfection) and results in better transfection efficiencies than using DNA (Kalady *et al.,* 2002; Lundqvist *et al.,* 2002; Strobel *et al.,* 2000). Van Tendeloo and co-workers conducted experiments comparing the level of pDNA-encoded GFP expression following electroporation in three different subsets of DCs: $CD34^+$ progenitor cell DCs (PC-DCs), Langerhans cells (PC-LCs) and

monocyte-derived DCs (Mo-DCs). The highest transfection efficiency was observed in PC-LC, moderate transfection was observed in PC-DC and the lowest efficiency was obtained in MoDC. They also found that electroporation significantly lowered MoDC viability (Van Tendeloo *et al.,* 1998). In the current study it was found that unlike PP and lipofection, electroporation of baboon MoDCs resulted in extremely high cellular mortality, >85%, a finding in agreement with Van Tendeloo et al. (Van Tendeloo *et al.,* 1998).

Due to reports that traditional transfection methods (PP, lipofection and electroporation) result in low transfection efficiencies and transgene expression (Arthur *et al.,* 1997; Kalady *et al.,* 2002; Larregina *et al.,* 2004), certain investigators have switched to using viral vectors for DNA delivery to DCs. Accumulating evidence has demonstrated that transfection of human DCs with viral vectors resulted in high transfection efficiency (Strobel *et al.,* 2000; Lundqvist *et al.,* 2002). This is probably related to viral targeting to specific cell types. A drawback of viral vectors is that along with the gene of interest, viral proteins and genes that are highly immunogenic are also delivered (Zoltick *et al.,* 2001; Larregina *et al.,* 2004). In addition, some studies have shown that the interaction of certain viral vectors interferes with the main functions of DCs in both humans and macaques (Humrich and Jenne, 2003; Peretti *et al.,* 2004). In contrast all traditional transfection methods have the advantage of not inducing irrelevant or unrelated antigen-specific immune responses. Viral vectors were not investigated in the current study.

In summary, the present study showed that lipofection (FuGene 6) was the superior method for delivering pDNA into baboon MoDCs.

4.4. Transfection of Baboon DCs with Vaccine study plasmids and MLR responses

Lipofection was demonstrated to successfully shuttle pDNA across the plasma membrane into target DCs. Vaccine plasmid-encoded genes were then expressed (as mRNA and protein), indicating that following delivery pDNA survived the potentially hostile intracellular environment and was transcribed. Having demonstrated that transfection of baboon DCs with the vaccine plasmid DNA constructs resulted in expression of the relevant genes at the mRNA level and that transfection did not adversely affect DC maturation processes, further investigation into DC antigen-presenting potential was examined at a functional level using the mixed lymphocyte reaction (MLR). Futhermore, the fate of the transcribed mRNA was examined by determining whether mRNA was translated into protein and successfully presented to memory T lymphocytes.

The MLR serves as an indirect functional readout of DC expression of MHC as the degree of MHC mismatch is related to the extent of proliferation induced. The MLR has been used extensively in DC studies as a readout of antigen-presenting potential (Strobel *et al.,* 2000; Peretti *et al.,* 2005). Any enhancement of antigen presenting potential following transfection is evidenced by increased proliferation. In the current study, increased allostimulatory capacity was observed in transfected baboon DCs, with lipofected DCs displaying optimal enhancement, This was not linked to enhanced co-stimulatory marker expression since both PP and lipofection had similar effects on expression of these markers. The MLR data would thus appear to indicate that lipofected DCs expressed higher levels of MHC I and II associated with relevant antigen. The relative contribution of the MHC molecules themselves or the expression of foreign vaccine plasmid-encoded antigen could not be determined in this system, but was investigated in antigen-specific assays assessing memory cell expansion.

4.5. HIV Ag presentation by vaccine construct-transfected Baboon DCs

The stimulatory capacity of transfected DCs to expand autologous memory T cells was assessed in modified baboon DC lymphoproliferation assays (LPAs). DCs were lipofected with the respective study plasmids and cocultured with a combination of autologous $CD4^+$ and $CD8^+$ memory T cells obtained from vaccinated baboons. In an attempt to demonstrate the maximal response induced in the modified LPA, untransfected baboon DCs were also pulsed with a superpool of HIV env peptides in a separate series of experiments. Loading of DCs with peptide fragments (15-20mers) should result in effective expansion of memory cells as the peptides interact directly with MHC molecules on the DCs (Howarth and Elliot, 2004). The level of antigen presentation by transfected DCs would in all probability be less efficient as it requires successful transcription, translation, processing and presentation of antigens encoded from plasmid DNA. Both lipofected and peptide-loaded untransfected baboon DCs were shown to have the ability to similarly activate $CD4^+$ and $CD8^+$ memory T cell responses. Interestingly the transfected DCs were generally more efficient in stimulating memory T cells than peptide-loaded DCs, a result which indicates the potent immunostimulatory capacity of successfully transfected DCs. The contrasting responses of peptide-loaded versus lipofected DCs at different T:DC ratios may be explained by the relatively low level of transfection and subsequent antigen presentation in transfected DCs. At T:DC ratios of 5:1 it is possible that memory T cells did not come into contact with a successfully transfected DC presenting appropriate antigen, whereas at ratios of 40:1 this could occur. In peptide-loading experiments, the majority of DCs would be presenting antigen, so even at low T:DC ratios stimulation could occur (Coutsinos *et al.,* 2005; Wang *et al.,* 2005b).

The fact that transfected baboon DCs could induce proliferation of memory T cells indicates that successful transfection not only leads to DNA transcription but also translation of plasmid-associated genes into protein and subsequent antigen processing and presentation. Baboon DCs cultured *in vitro* are thus an effective

model for evaluating plasmid DNA-DC interactions as they display patterns of maturation and potent antigen presentation capabilities similar to those observed in DCs from other species (Melhop *et al.,* 2002; Messmer *et al.,* 2002; Gabriela *et al.,* 2005).

4.6. Conclusion

This is the first study to investigate the culture and characterisation of chacma baboon monocyte-derived DCs *in vitro*. Major similarities between human and baboon DCs cultured and matured *in vitro* were observed – both on a morphological level and with regard to DC marker expression. The effect of transfection of baboon DCs with both reporter gene expressing plasmid DNA and HIV-1 subtype C gene expressing vaccine plasmid DNA constructs was examined using different modes of DNA delivery. Lipofection using the FuGene 6 reagent was found to be the optimal method for successful delivery of pDNA to baboon MoDCs. Following transfection, baboon DCs underwent a maturation process and were also shown to effectively express vaccine construct-encoded genes and to expand antigen-specific memory T cells. The level of memory T cell expansion by transfected DCs was similar to that induced by HIV-1 peptide-loaded DCs. This finding indicated that successfully transfected DCs can efficiently process and present antigen. This study highlights the functional integrity of *in vitro* generated baboon DCs and it is likely that future studies addressing targeting of HIV-1 vaccine plasmid DNA to DCs and enhancement of expression of plasmid-encoded antigens in DCs can be performed using this culture system. In addition, studies investigating the interaction of SIVs with baboon DCs will aid in comparative nonhuman primate studies of SIV pathogenesis and by extension will contribute to our understanding of HIV-1 pathogenesis in humans.

4.8 Conclusion

This is the first study to investigate the culture and characterisation of baboon monocyte-derived DCs *in vitro*. Major similarities between human and baboon DCs cultured and matured *in vitro* were observed – both on a morphological level and with regard to DC marker expression. The effect of transfection of baboon DCs with both reporter gene expressing plasmid DNA and HIV-1 subtype C gene expressing vaccine plasmid DNA constructs was examined using different modes of DNA delivery. Lipofection using the FuGene 6 reagent was found to be the optimal method for successful delivery of pDNA to baboon MoDCs. Following transfection, baboon DCs underwent a maturation process and were also shown to effectively express vaccine construct-encoded genes and to expand antigen-specific memory T cells. The level of memory T cell expansion by transfected DCs was similar to that induced by HIV-1 peptide-loaded DCs. This finding indicated that successfully transfected DCs can efficiently process and present antigen. This study highlights the functional integrity of *in vitro* generated baboon DCs and it is likely that future studies addressing targeting of HIV-1 vaccine plasmid DNA to DCs and enhancement of expression of plasmid-encoded antigens in DCs can be performed using this culture system. In addition, studies investigation the interaction of SIVs with baboon DCs will aid in comparative nonhuman primate studies of SIV pathogenesis and by extension will contribute to our understanding of HIV-1 pathogenesis in humans.

CHAPTER 5

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