Dendritic Cell Regulation of HIV-1 Latency in CD4$^+$ T-cell

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1. Thesis abstract

Combination antiretroviral therapy (cART) is able to control HIV-1 replication and prevent acquired immune deficiency syndrome (AIDS), however does not provide a cure. The major barrier to HIV-1 cure is the persistence of long-lived, latently infected resting CD4$^+$ T-cells. We developed an *in vitro* dendritic cell (DC)-T-cell latency model, which showed that myeloid (m)DC induced post-integration latent infection in non-proliferating, resting and proliferating CD4$^+$ T-cells. Gene expression profiles indicated that mDC could regulate the establishment of latent infection in non-proliferating and proliferating CD4$^+$ T-cells through interaction between adhesion molecules, co-stimulatory molecules and immune checkpoints. On the contrary, pDC-T-cell interactions led to the inhibition of latent infection in co-cultured resting CD4$^+$ T-cells and death of proliferated CD4$^+$ T-cells. Gene expression profiles suggested that inhibition is mediated through type-I and III interferons. Investigation of the mechanisms of mDC mediated establishment of latent infection and pDC mediated inhibition of latent infection, may provide novel elimination strategies for HIV-1 cure.

Monash University

Declaration for thesis based or partially based on conjointly published or unpublished work

2. General Declaration

In accordance with Monash University Doctorate Regulation 17.2 Doctor of Philosophy and Research Master's regulations the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes 1 original paper published in peer-reviewed journal and 3 unpublished publications. The core theme of the thesis is DC mediate regulation of latency in $CD4^+$ T-cells. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Department of Infectious Diseases under the supervision of Professor Sharon Lewin and Associate Professor Paul Cameron.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of chapters 2-3 and 5 my contribution to the work involved the following:

Nitasha Kumar completed experiments in chapter 2 with training from Vanessa Evans, Candida da Fonseca Pereira, Paula Ellenberg and Paul U Cameron. Nitasha Kumar completed experiments in chapter 3 with assistance from Karey Cheong, and training from Candida da Fonseca Pereira, Jenny Anderson and Ajantha Solomon. Chapter 3 bioinformatics alignment and analysis was completed in collaboration with David Powell. Nitasha Kumar completed analysis of wet lab experiments from chapters 2, 3, and 4. Paul U Cameron, Sharon Lewin, Vanessa Evans and Renee van der Sluis assisted with ideas and experiment design in all chapters. Nitasha Kumar completed writing of thesis and manuscripts with assistance from co-authors.

Thesis chapter	Publication title	Publication status	Nature and extent of candidate's contribution
2	Myeloid dendritic cells induce HIV-1 latency in non-proliferating CD4$^+$ T-cells	Published	Second Author
3	The role of antigen presenting cells in the induction of HIV-1 latency in resting CD4$^+$ T-cells	Submitted	First Author
4	Myeloid DC induce latency in proliferating CD4$^+$ T-cells	Manuscript in preparation	First Author
5	Potential genes that mediate inhibition of latent infection in non-proliferating CD4$^+$ T-cells.	Manuscript in preparation	Second author

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Signed: …………………………………………………………

Date: ·08/06/2015·…………………

3. Preface

This thesis was completed under the supervision of Associate Professor Paul U Cameron and Professor Sharon Lewin within the HIV and HBV Immune-Pathogenesis Laboratory, Department of Infectious Diseases, Monash University at the Alfred Hospital and Burnet Institute, Melbourne, Australia. This thesis was also co-supervised by Dr Vanessa Evans from the same laboratory.

Others have also contributed to the work presented in this thesis. Dr Vanessa Evans performed transwell and supernatant swap experiments (Department of Infectious Diseases, Monash University, Australia). Dr Suha Saleh and Dr Vanessa Evans completed the experiments to quantify integrated HIV-1 DNA using PCR (Department of Infectious Diseases, Monash University, Australia). Miss Karey Cheong assisted with isolation of antigen presenting cells. The Australian Genome Research Facility (AGRF) at the Walter and Eliza Hall Institute (WEHI) in Melbourne, Australia. RNA-seq alignment was completed by Dr David Powell who also assisted with gene expression analysis (Monash Bioinformatics Platform, Monash University, Clayton, Australia).

We thank Damian Purcell (University of Melbourne, Parkville, Australia) for providing us with the CCR5-EGFP-reporter virus. We thank Geza Paukovics, Michael Thomson, Jeanne Le Masurrier and Phil Donaldson (Alfred Medical Research and Educational Precinct (AMREP) Flow Cytometry Core Facility, Melbourne, Australia) for flow cytometric cell sorting. We thank the Australian Gene Research Facility Ltd (AGRF, Melbourne, Australia) for the guidance and support in processing of samples for RNA-seq. We thank Andrew Harman (Westmead Millennium Institute for Medical Research, Sydney University, Sydney, Australia) for access to microarray data. We thank Jingling Zhou for assistance with elutriation protocol and Ajantha Solomon for assistance with RNA isolation techniques. I thank the O'Keefe laboratory (Department of Biomedicine, Burnet Institute, Melbourne, Australia) for

lending us cytokines and antibodies. I thank Renee van der Sluis for support in preparation of the Thesis.

5. Acknowledgements

6. List of abbreviations

AICD	Activation induced cell death
AIDS	Acquired immune deficiency syndrome
APC	Antigen presenting cell
APOBEC	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like
ARV	Anti-retroviral
Bcl-2	B-cell lymphoma 2
BM	Bone marrow
BTLA	B- and T-lymphocyte attenuator
Ca^{2+}	Calcium
cART	Combination antiretroviral therapy
CDP	Common DC progenitor
CFB-1	Complement factor B-1
CLEC-7A	C-type lectin domain family 7 member A
cMoP	Common monocyte progenitor
CNS	Central nervous system
CTL	Cytotoxic T-cell
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DC	Dendritic cell
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DDC	Dermal dendritic cell
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
EC	Endothelial cell
EGFP	Enhanced green fluorescent protein
ESCRT	Endosomal sorting complexes required for transport
FBS	Foetal bovine serum
FC	Fold change
FDC	Follicular DC
FDR	False discovery rate
Gal9	Galectin-9
GALT	GIT-associated lymphoid tissue
GCPR	G-coupled protein receptor
GEO	Gene omnibus
GIT	Gastrointestinal tract
GM-CSF	Granulocyte-macrophage colony-stimulating factor
gp	Glycoprotein
HAND	HIV associated neurocognitive disorder
HAVR2	Hepatitis A virus cellular receptor 2
HIV-1	Human immunodeficiency virus-1
HIV-2	Human immunodeficiency virus-2
HPC	Haemopoetic progenitor cells
HVEM	Herpesvirus entry mediator
IC	Immune checkpoint
ICAM	Intracellular adhesion molecule-1

ICB	Immune checkpoint blocker
ICOS	Inducible T-cell Co-stimulator
IFN	Interferon
IgG	Immunoglobulin
IL-2	Interleukin-2
IL-7	Interleukin-7
IN	Integrase
IS	Immune synapse
LAG-3	Lymphocyte-activation gene 3
LC	Langerhans cell
LFA-1	Lymphocyte function-associated antigen-1
LILR	Leukocyte-associated immunoglobulin like receptor
LN	Lymph node
LRA	Latency reversing agents
LTR	Long terminal repeats
mDC	Myeloid dendritic cell
MDDC	Monocyte-derived-DC
MDM	Monocyte derived macrophage
MHC	Major histocompatibility complex
MIP-1/2α	Macrophage inflammatory protein-1/2a
MLR	Mixed leukocyte reaction
MOI	Multiplicity of infection
Mono	Monocyte
mRNA	Messenger RNA
ND	Not done
NFAT	Nuclear factor of activated T-cells
NFκB	Nuclear factor kappa B
NHP	Non-human primate model
NP	Non-polarised
NR	Not reported
P	Protease
PAMPs	Pattern associated molecular protein
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PD-1	Programmed death receptor-1
PD-L2	Programed death receptor ligand 1/2
pDC	Plasmacytoid dendritic cell
PHA	Phytohaemagglutinin
RNA	Ribonucleic acid
RT	Reverse transcriptase
RTE	Recent thymic emigrants
SAMHD1	SAM domain and HD domain-containing protein 1
SDF-1	Stromal cell derived factor-1
SEB	Staphyloccocal enterotoxin B
SIGLEC	Sialic acid-binding immunoglobulin-type lectin
siRNA	Small interfering RNA

SIV	Simian immunodeficiency virus
SLAN	6-sulfo LacNAc
Sp1	Specificity protein 1
ssRNA	Single stranded RNA
TCID50	Tissue culture infectious dose
T_{CM}	Central memory T-cell
TcR	T-cell receptor
T_{EM}	Effector memory T-cell
TF	Transcription factor
TFH	Follicular helper cell
TGFβ	Transforming growth factor beta
Tim-3	T-cell immunoglobulin mucin-3
TLR	Toll like receptor
T_{NA}	Naïve T-cell
TNF	Tumour necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
T_{REG}	Regulatory T-cell
T_{SC}	T--stem cell
T_{TM}	Transitional memory T-cell
VOA	Viral outgrowth assay

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Evans VA, Kumar N, Filali A, Procopio FA, Yegorov O, Goulet JP, Saleh S, Haddad E, Pereira CdF, Ellenberg P, Sekaly RP, Cameron PU, Lewin SR. (2013) Myeloid dendritic Cells Induce HIV-1 Latency in Non-proliferating CD4$^+$ T-cells. PLoS Pathogen 9(12).

Khoury G, Kumar N, Solomon A, Lu H, Wightman F, Cameron PU, Lewin SR. (2015) Naïve CD4$^+$ T-cells are Resident to Both HIV-1 and HIV-2 Infection *In Vitro*.
Manuscript in preparation.

Anderson, JL., Mota, T., Evans, VA., Kumar, NA., Rezaei, SD., Cheong, K,. Saleh, S., Solomon, A., Wightman, F., Cameron, PU., Lewin, SR (2015) Establishing HIV Latency in Resting CD4$^+$ T-cells *In Vitro* Using the Chemokine CCL19: Effects of Donor Susceptibility and Viral Inoculum
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Kumar, NA., Cheong, K., Pereira, CdF., Anderson, J., Evans, VA., Lewin, SR., Cameron, PU. (2015) The role of Antigen Presenting Cells in the Induction of HIV-1 Latency in Resting CD4$^+$ T-cells.
Retrovirology, 11(12).

Kumar, NA., van de Sluis RM., Mota, T., Evans, VA., Lewin, SR., Cameron, PU. (2016) Dendritic cell interactions lead to an inducible HIV-1 reservoir in proliferating CD4$^+$ T-cells.
Manuscript in preparation.

van de Sluis RM., Kumar, NA., Mota, T., Evans, VA., Lewin, SR., Cameron, PU. (2016). Role of type-I and III interferons in the inhibition of latent infection.
Manuscript in preparation

1. Chapter 1

Literature review

1.0. Chapter 1. Literature Review

1.1. Introduction

Human Immunodeficiency Virus type 1 (HIV-1) remains a major global burden with an estimated 35 million people currently infected worldwide (The Global Report, UNAIDS, 2013). Although globally, the number of HIV-1 related deaths has decreased from 1.7 million in 2005 to 1.5 million in 2013 (The Global Report, UNAIDS, 2013), the number of new HIV-1 infections continues to be high at 1.8 million per year (The Global Report UNAIDS, 2013). While Australia's contribution to the global HIV-1 burden is less than 5%, with 26,800 (range 24500-30900) people living with HIV-1, HIV-1 infection rates in Australia have increased from 724 new infections in 1999 to 1236 new infections in 2013 (McDonald et al., 2014). Since the discovery of effective combination antiretroviral therapy (cART) the morbidity and mortality of HIV-1-infected patients on treatment has dramatically decreased (Ho et al., 1995; McManus et al., 2012; Perelson et al., 1997). Patients on cART have lower HIV-1 transmission rates and increased quality of life (Loutfy et al., 2013; Supervie et al., 2014; Venturini et al., 2014). However, currently cART alone is unable to provide a cure for HIV-1, making lifelong cART necessary. Despite the availability of several resources, both globally and within Australia, the burden of HIV-1 infections remains high. Therefore, the development of a cure for HIV-1 remains an important goal to alleviate the global burden of HIV-1.

The major barrier to HIV-1 cure is the persistence of latently infected CD4^{+} T-cells (Chun et al., 1995; Finzi et al., 1997). Latent infection occurs when the HIV-1 genome has integrated into the host cell genome without expression of virus (Aiyar et al., 1996; Brown et al., 1987). Latently infected resting CD4^{+} T-cells have a long half-life of approximately 44 months, and their natural decay *in vivo* is estimated to take more than 60 years (Archin et al., 2012; Finzi et al., 1999; Siliciano et al., 2003). Previously, the study of latent infection has been limited to cell lines, which fail to represent latent infection *in vivo*. However,

recent advances in the development of *in vitro* latency models using primary cells (Bosque and Planelles, 2009; Evans et al., 2011; Marini et al., 2008; Sahu et al., 2009; Saleh et al., 2007; Shen et al., 2013; Yang et al., 2009b), animal models (Brooks et al., 2001; Dinoso et al., 2009a; Marsden et al., 2012; North et al., 2010) and more sensitive techniques for detection of low levels of latent infection (Chomont et al., 2009; Josefsson et al., 2013a; Lewin et al., 1999) have furthered our understanding of the characteristics of HIV-1 persistence (Deeks et al., 2012; Hakre et al., 2012; Spina et al., 2013). By further defining the latent reservoir *in vivo* we hope to identify new targets to eliminate HIV-1 latency and ultimately develop a cure for HIV-1 to allow patients to stop long-term cART.

The major pool of latently infected cells consists of resting central memory CD4$^+$ T-cells (Brenchley et al., 2004; Chomont et al., 2009), which reside in high concentrations in lymphoid tissues and the gastrointestinal tract (GIT; Chun et al., 1997, 2008; Dinoso et al., 2009a; North et al., 2010; Yukl et al., 2010). Cell interactions in the *in vivo* tissue environment have been shown to be important in the spread of HIV-1 infection (Murooka et al., 2012; Sigal et al., 2011), and the establishment and maintenance of latent infection (Eckstein et al., 2001; Kreisberg et al., 2006). Along with a high concentration of resting CD4$^+$ T-cells, lymphoid tissue also have a high concentration of residential and circulating DC. DC-T-cell interactions in the lymph node (LN) lead to regulation of T-cell activation by providing potent activation stimuli (T-cell activation) or dampening of the immune response (activation of immune checkpoints (IC)). In this thesis we hypothesised that DC-T-cell interactions control the establishment and maintenance of latent HIV-1 infection in CD4$^+$ T-cells.

1.2. **HIV-1 Virology**

HIV-1 is a retrovirus that belongs to the lentivirinae genus, like simian immunodeficiency virus (SIV). Both viruses are known to cause slow and

persistent infections by infection and depletion of CD4$^+$ T-cells in humans and primates respectively (Chiu et al., 1985; Gifford, 2012).

1.2.1.   HIV-1 Genome and Structure

HIV-1 is a small, enveloped, single stranded RNA (ssRNA) virus with a relatively large 9.7 kB genome, consisting of 9 genes flanked by long-terminal repeat sequences (LTR) at the 5' and 3' ends (Figure 1.1; Turner and Summers, 1999). The envelope consists of a host derived plasma membrane bilayer studded with viral glycoproteins (gp), gp120 and gp41, and host proteins including MHC class I, ubiquitin and integrin, captured during budding from host cells (Figure 1.1A; Arthur et al., 1992; Barré-Sinoussi et al., 1983). The internal plasma membrane is lined with matrix proteins that protect the conical capsid core, which encapsulates 2 copies of genomic RNA, along with viral enzymes; reverse transcriptase (RT), integrase (IN), protease (P), and accessory proteins (Nef, Vpu, Vpr, Vif) important for viral infectivity (Turner and Summers, 1999) (Figure 1.1).

Figure 1.1 HIV-1 Virus and Gene Structure.

HIV-1 is a small, enveloped, single stranded RNA (ssRNA) virus **A.** HIV-1 has a lipid bilayer envelope with expression of viral glycoproteins, gp120 and gp41, and cellular proteins. Matrix proteins line the inside of the lipid bilayer. A conical shaped capsid, within the matrix, contains the single-stranded RNA genome, accessory proteins (Vif, Vpr, Vpu, Nef) and vital enzymes (integrase [I], reverse transcriptase [RT], protease [P]). Image courtesy of Dr. David Iser. **B.** The HIV-1 genome is 9.7kB, consisting of Gag, Pol, Env, and accessory proteins and flanked by long-terminal repeat sequences (LTR) at the 5' and 3' ends.
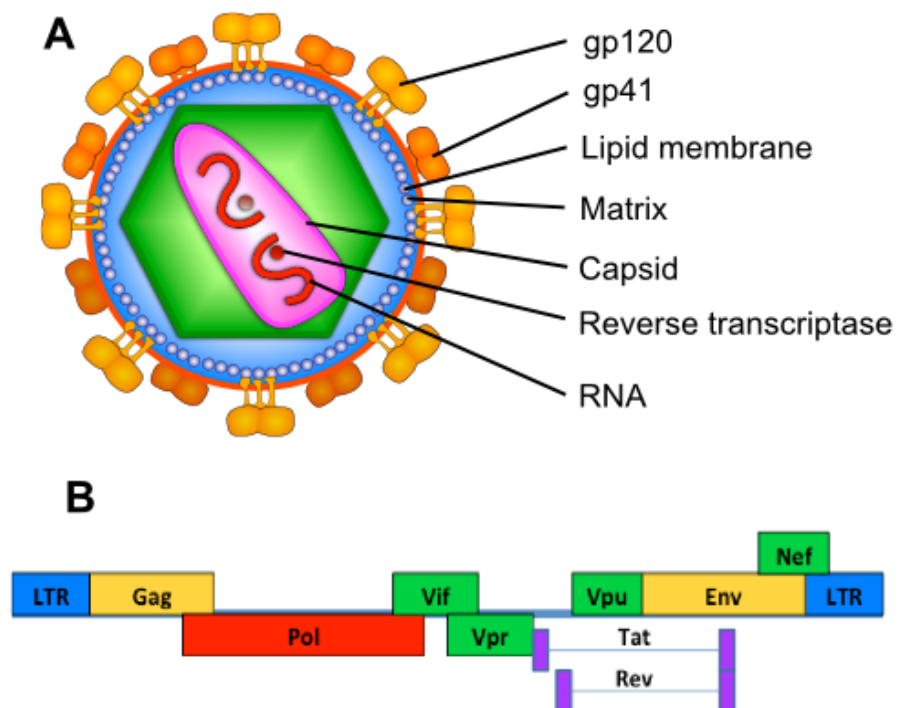
1.2.2. Viral Lifecycle

HIV-1 entry into host cells is initiated by gp120 binding to CD4, expressed on the surface of target cells including, CD4$^+$ T-cells, monocytes, macrophages and DC (Figure 1.2A; Dalgleish et al., 1984; Klatzmann et al., 1984; Oberlin et al., 1996; Bleul et al., 1997; Platt et al., 1998; Lee et al., 1999). CD4 binding to gp120 causes a conformational change, which facilitates gp120 interaction with the chemokine co-receptor CCR5 or CXCR4, leading to further conformational changes and exposure of gp41 (Feng et al., 1996; Massanella et al., 2009; Huang et al., 2012; Dobrowsky et al., 2013; Reviewed in Gorry and Ancuta, 2011). Gp41 mediates membrane fusion and release of the viral capsid into the cytosol where capsid un-coating, reverse transcription and nuclear import occur simultaneously (Figure 1.2B; Reviewed in Ambrose and Aiken, 2014).

The pre-integration complex (PIC) is formed in the nucleus to facilitate viral integration. The PIC is made up of proviral DNA, integrase (IN), viral matrix proteins, RT antigens and host proteins, including lens epithelium-derived growth factor (LEDGF)/p75 (Figure 1.2C; Bukrinsky et al., 1993). Integrase-associated host nuclear trafficking proteins, histones and DNA repair enzymes then mediate integration site selection, where LEDGF/p75 tethers the PIC to host DNA (Reviewed in Craigie and Bushman, 2012). Subsequently, IN mediates DNA strand joining and binding of viral and host DNA strands (Brown et al., 1987; Norton and Coffin, 1987; Fujiwara, 1988; Bushman and Miller, 1997; Ciuffi et al., 2005; Emiliani et al., 2005). If integration fails to occur, the un-integrated linear provirus is susceptible to degradation or may undergo circularisation leading to the development of one- or two-LTR circles, which represents a dead end for the virus (Figure 1.2C; Zack et al., 1990; Bukrinsky et al., 1991; Farnet and Haseltine, 1991).

Following integration, host transcription factors (TF), including nuclear-factor-Kappa-B (NFκB), nuclear factor of activated T-cells (NFAT) and specificity protein 1 (Sp1) can bind to the HIV-1 LTR, and promote initiation of viral

transcription (Jones et al., 1986; Kawakami et al., 1988; Kinoshita et al., 1997). The absence of one or more of these TF can prevent transcription, leading to stable, integrated, long-lived and "silent" or latent infection (Chun et al., 1995; Kinoshita et al., 1997). When host TF are present, viral transcription is initiated, leading to the production of Tat (trans-activator of transcription), which works in two ways (Nabel and Baltimore, 1987). First, Tat binds to an RNA stem-loop structure within the 5' LTR, Tar, which facilitates elongation of viral transcription (Dingwall et al., 1990; Parent et al., 2005). Second, Tat recruits the host cell proteins p-TEFb, CDK9 and cyclin 1, leading to RNA polymerase II (RNA pol II) binding and subsequent the production of short and long viral mRNAs (Nekhai et al., 2014; Zhou et al., 2000; Reviewed in Kilareski et al., 2009).

Resultant gag, gag-pol and genomic mRNA transcripts can be spliced (Michael et al., 1991; Purcell and Martin, 1993), and exposed to host or virus derived regulatory post-transcriptional defences including small interfering (si)RNA and micro (mi)RNA (Reviewed in Harwig et al., 2015). siRNA and miRNA interaction with viral transcripts promotes latent infection by blocking translation and targeting viral mRNAs for degradation (Bennasser et al., 2005; Klase et al., 2007; Omoto et al., 2004; Schopman et al., 2012). siRNA and miRNA can also promote latency by inhibition of the Tat-TAR interaction (Ouellet et al., 2008). For successful transcription, mRNA species and full length genome are transported to the cytosol, by the viral protein Rev, for translation and virion assembly (Henderson and Percipalle, 1997; Reviewed in Karn and Stoltzfus, 2012).

Concerted assembly of viral proteins and genomic RNA at the plasma membrane is mediated by Gag and host chaperone proteins (Frankel and Young, 1998; Kleiman et al., 2010; Moore et al., 2009; Ono and Freed, 2001). Once a virion is formed, the host endosomal sorting complexes required for transport (ESCRT) pathway mediates viral budding (Carlson and Hurley, 2012). Upon virion release protease is activated, which facilitates cleavage of

gag-pol, leading to formation of a mature and infectious virion, which can further infect other CD4$^+$ expressing cells (Swanstrom and Wills, 1997).

Figure 1.2 HIV Life Cycle.

A. Virus enters the cell via gp120 binding to CD4 and chemokine receptor CXCR4 or CCR5. Subsequent conformational change allows gp41-mediated fusion of virus and host membrane. **B.** In the cytoplasm, capsid uncoating, viral RNA reverse transcription and nuclear import occur simultaneously to delivery proviral DNA to the nucleus. **C.** Viral cDNA is integrated within the host genome via integrase and host proteins. In the absence of required host proteins, viral cDNA becomes circularized and degraded, representing a dead end for the virus. In the absence of transcription signals the virus remains indefinitely latent. **D.** In the presence of transcription factors, like in activated CD4$^+$ T-cells, viral transcription of virus begins. **E.** mRNA transcripts are transported to the cytoplasm for translation and assembly at the plasma membrane surface, for subsequent budding and maturation of new virions. Anti-retroviral therapy works by blocking various steps of the life cycle, indicated with the red blunt end line (Adapted from Coiras et al., 2009).
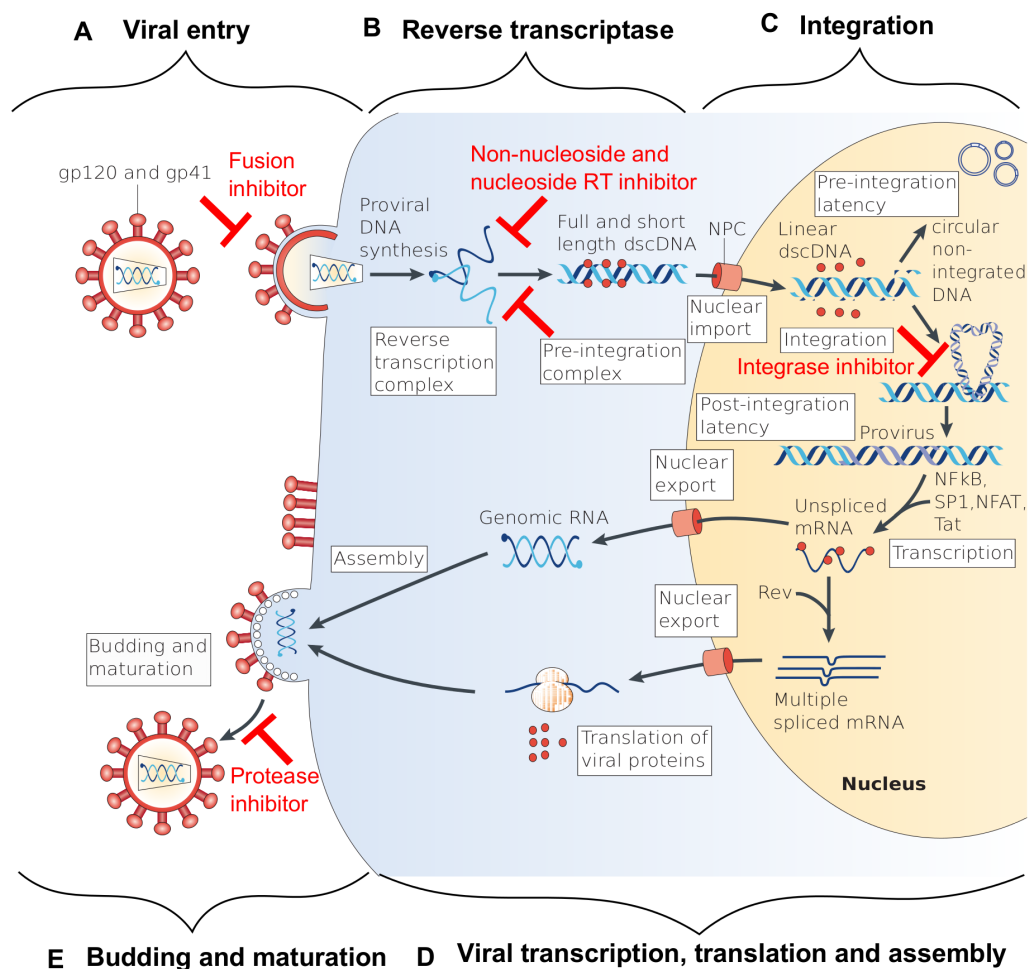
1.3. **HIV-1 Pathogenesis**

In Australia, HIV-1 is most commonly transmitted by sexual contact, and less frequently by injecting drug use (Maartens et al., 2014; McDonald et al., 2014). Therefore, HIV-1 enters the body via mucosal (vaginal) and epidermal surfaces (rectal, circulatory system), where it infects local CD4 expressing cells (Dalgleish et al., 1984; Klatzmann et al., 1984; Zhang et al., 1999a; Hladik et al., 2007; Berges et al., 2008). Infected CD4^{+} T-cells cause local infection and can carry the virus systemically upon migration (Murooka et al., 2012). Antigen presenting cells (APC) including, mucosal DC, macrophages, Langerhans cells (LC), circulating (blood) DC and monocytes, can also be infected with HIV-1 at mucosal and epithelial surfaces, as they express low levels of CD4 and CCR5 (Cameron et al., 2007; Cunningham et al., 2000; Hussain and Lehner, 1995; Turville et al., 2001). The additional expression of pathogen-associated molecular patterns (PAMPs) allows these APC to bind and carry HIV-1 to the local draining lymph nodes (LN), where virus gains access to higher concentrations of circulating CD4^{+} T-cells within the T-cell zones (Hurtrel et al., 1994; Reece et al., 1998; Turville et al., 2004). This is called *trans*-infection and will be discussed further in section 1.8.

Disease progression as a result of HIV-1 infection is a continual balance between HIV-1 dissemination, destruction of the immune system, and the immune system's ability to overcome infection. Infection of activated CD4^{+} T-cells leads to high levels of *de novo* virus production, and local and systemic spread of infection to CD4^{+} cells via migratory infected CD4^{+} T-cells and virus carrying DC (Ammassari et al., 2014; Cameron et al., 1992; McKellar et al., 2013; Murooka et al., 2012; Pope et al., 1995). During acute infection, plasma HIV-1 RNA is high and CD4^{+} T-cells counts are low. After an initial or acute infection phase, the host immune system controls virus expression to varying extents, thereby reducing the spread of infection (Deeks et al., 2004). Viral control by expansion of HIV-1-specific T-cells slows infection and eventually leads to a chronic phase, where patients remain asymptomatic, with stable HIV-1 RNA and slowly declining CD4^{+} T-cell counts (Pantaleo et al., 1993;

Graziosi et al., 1993; Michael et al., 1992; Reviewed in Palmer et al., 2011). Over time the virus slowly escapes immune control, leading to increasing plasma HIV-1 RNA and declining blood CD4 counts. In turn, low $CD4^+$ T-cell numbers leads to increased risk of opportunistic infection, malignancy and eventually death (Miranda et al., 2014; Reviewed in Okoye and Picker, 2013; and Maartens et al., 2014).

Low $CD4^+$ T-cell counts (below 200 cells/mm^3) or the development of the first opportunistic infection leads to the diagnoses of acquired immunodeficiency syndrome (AIDS; Barré-Sinoussi et al., 1983; Chermann et al., 1983; Gabillard et al.; Miranda et al., 2014). Progression to AIDS is attributed to $CD4^+$ T-cell depletion via cytopathic effects of the virus, including syncytia formation (X4 viruses only), rapid turnover of T-cells due to chronic immune activation, *de novo* virus production and induction of apoptosis by viral accessory proteins, Vpr and Vif (Hazenberg et al., 2000; Sakai et al., 2006; Ward et al., 2009; Arokium et al., 2009; Zhao et al., 2011; Reviewed in Okoye and Picker, 2013). Chronic immune activation also leads to activation of apoptosis in uninfected, bystander activated and quiescent $CD4^+$ T-cells, and their subsequent depletion (Doitsh et al., 2014; Lelièvre et al., 2004; Monroe et al., 2014). The severity of $CD4^+$ T-cell loss is enhanced by further destruction of primary and secondary lymphoid tissue (lymph nodes and thymus) preventing regeneration of $CD4^+$ T-cells and thereby facilitating rapid progression to AIDS (Alòs et al., 2005; Bonyhadi et al., 1993; Dion et al., 2007; Doitsh et al., 2010; Reviewed in Zeng et al., 2012).

1.4. **HIV-1 Treatment**

1.4.1. **Combination Anti-Retroviral Therapy**

The introduction of combination anti-retroviral therapy (cART), a combination of 3 or more antiretroviral drugs, has resulted in a significant reduction in HIV-1 related morbidity and mortality rates (Gulick et al., 1997; Hammer et al., 1997). The several classes of anti-retrovirals inhibit essential steps in the viral

life cycle, and have transformed HIV-1 into a chronic infection (Figure 1.2). There are over 25 antiretroviral drugs available today that target different steps in the HIV-1 lifecycle, shown in Figure 1.2 (Alkhatib et al., 1996; Ho et al., 1995; Markowitz et al., 1995, 1998; Saravolatz et al., 1996; Stefanidou et al., 2012; Reviewed in Geretti and Tsakiroglou, 2014). In the majority of patients, the initiation of cART leads to a rapid, two-stage, decay in HIV-1 RNA in plasma, elimination of infected cells and a subsequent restoration of CD4$^+$ T-cells, but residual immune defects persist in multiple tissue sites including the lymph node (LN), thymus and gastrointestinal tract (GIT; Table 1.1; Autran et al., 1997; Ho et al., 1995; Perelson et al., 1996, 1997; Vrisekoop et al., 2008).

While on cART, many patients can achieve and maintain plasma HIV-1 RNA beneath the limit of detection of commercial assays, usually less than 20 copies/mL and life expectancy is comparable to those of uninfected individuals (Figure 1.3; Lohse et al., 2007; Palmer et al., 2003; Chun et al., 2010; Sabin, 2012; Zheng et al., 2013). However, within weeks of cART cessation, plasma HIV-1 RNA rebounds to a similar levels seen prior to cART initiation, regardless of the time on therapy, drug regimen or time of treatment initiation (Figure 1.3; Chun et al., 1995, 2000; Finzi et al., 1997; Furtado et al., 1999; Siliciano et al., 2003; Lewin et al., 2008). These observations indicate that long-lived forms of the virus can persist throughout treatment. Therefore, patients must remain on cART for life, which despite its benefits, has been associated with cardiovascular disease, osteoporosis (bone disorders), cognitive impairment, stigma, discrimination and overall decreased quality of life compared to uninfected individuals (Bellasi et al., 2013; Guaraldi et al., 2009; Leeansyah et al., 2013; Pfefferbaum et al., 2014; Reviewed in Desai and Landay, 2010). Finding a cure for HIV-1 that allows cessation of antiviral therapy, a functional cure, or completely eliminates virus *in vivo*, a sterilising cure, remains a major scientific priority with the overall goal to improve the quality of life of HIV-1-infected individuals and reduce costs associated with life long care (Trono et al., 2010).

Figure 1.3 Dynamics of Plasma Virus Levels in Combination Anti-Retroviral Treated Patients.

Plasma HIV-1 RNA (pink line) is elevated during acute infection and is associated with CD4$^+$ T-cell depletion (blue line). Upon combination anti-retroviral therapy (cART) initiation plasma HIV-1 RNA decreases rapidly and becomes undetectable, CD4$^+$ T-cell counts rise but rarely reach levels seen in uninfected people. When cART is stopped, virus rapidly rebounds, leading to an increase in plasma HIV-1 RNA and depletion of CD4$^+$ T-cells. A functional cure would allow for a patient to cease cART but the virus would remain at a low but detectable level. A sterilising cure would mean complete elimination of the virus from all blood and tissue sites (Adapted from Lint et al., 2013).
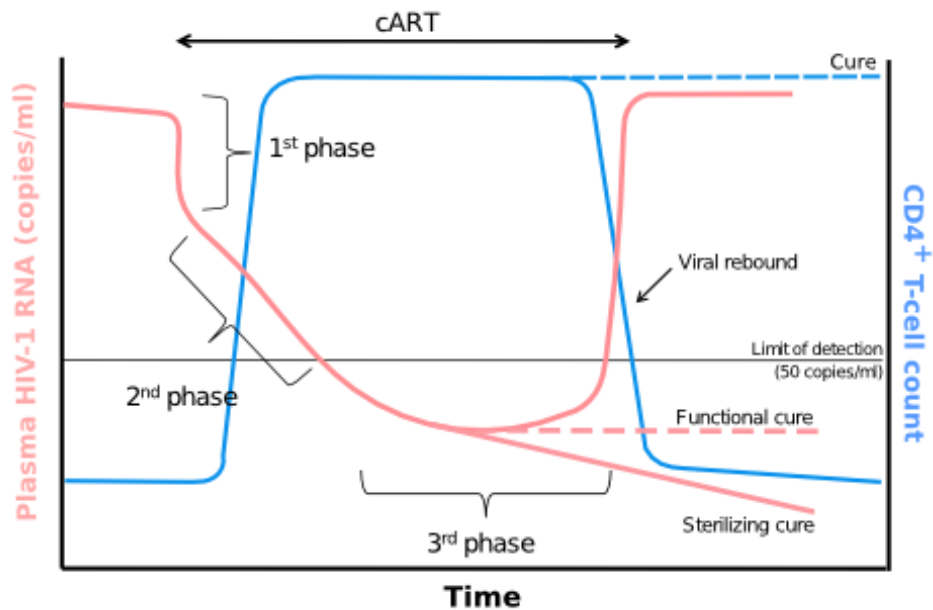
1.5. HIV-1 Persistence

Rebound of plasma HIV-1 RNA upon cART cessation is clear evidence that HIV-1 persists in patients despite cART. HIV-1 persistence is hypothesised to be multifactorial, including ongoing low-level replication in patients on cART and the establishment and maintenance of cellular and anatomical reservoirs. Low-level viremia is defined as the detection of cell free virions in patients on cART, however, the importance of low-level viremia in HIV-1 persistence remains unclear (Havlir et al., 2003; Izopet et al., 2002; Mavigner et al., 2009; Shiu et al., 2009). There are 2 hypothesised causes of low-level viremia; first, low-level virus replication and second, "leaky" latent reservoirs.

1.5.1.   Low-level Residual Replication

Ongoing, low-level viral replication in the setting of cART could be a consequence of low drug penetration in tissue including LN, GIT and vaginal tissue (Mavigner et al., 2009; Trezza and Kashuba, 2014; Fletcher et al., 2014). Suboptimal cART concentrations may allow for viral replication, viral escape, transmission and ongoing opportunities for the establishment of latent infection. To block low-level viremia and improve peripheral tissue drug penetration cART intensification was tested by the addition of an extra antiretroviral drug, including raltegravir (integrase inhibitor), T20 (fusion inhibitor) and protease inhibitors (Reviewed in Wiesch and Lunzen, 2011). In every cART intensification study to date, the level of viral RNA in plasma or DNA in CD4$^+$ T-cells did not change.(Buzón et al., 2010; Dinoso et al., 2009b; Gandhi et al., 2010; Hatano et al., 2010; McMahon et al., 2010; Ramratnam et al., 2004; Vallejo et al., 2012; Yukl et al., 2010). However in the studies of raltegravir which measured a bi-product of viral replication, 2LTR circles, there was a transient increase in 2LTR circles in approximately one third of patients within the first 2 weeks of intensification (Buzón et al., 2010; Hatano et al., 2010). This finding suggests that low-level replication does indeed occur, at least in a subset of patients on cART but the site and source of residual virus replication remains unknown.

It is possible that residual virus replication occurs in cells other than CD4$^+$ T-cells, such as macrophages, which have different cell-drug interactions and viral kinetics. In support of this hypothesis, one small study found that the virus sequence from patient plasma who have ceased cART, differed to virus found in circulating T-cells, but further studies are needed to confirm this (Sahu et al., 2009). Alternatively, residual virus replication may originate in specific T-cell subsets or tissue environments such as in T follicular helper (T$_{FH}$) cells in the B-cell follicles of LN (Fukazawa et al., 2015).

1.5.2. **Leaky Latent Reservoirs**

Latently infected cells may "leak" or produce small amounts of virions, resulting in low-level viremia but not cell death of latently infected T-cell. To determine the source of viremia in patients on cART, sequencing and phylogenetic analysis of low-level viremia was conducted by several groups. There was no longitudinal viral evolution or compartmentalisation to a cell type or tissue, suggesting that low-level viremia has a common source that remains stable over time (Anderson et al., 2011; Palmer et al., 2008; Sahu et al., 2009). The stability of latent infection would support the hypothesis that low-level viremia is indeed from latently infected cells. However, to understand the relationship between low-level viremia and latent infection further, we must first understand the cells that are latently infected and their dynamics.

1.5.3. **Cellular HIV-1 Reservoirs**

The major cellular reservoir for latent HIV-1 infection is central memory (T$_{CM}$) CD4$^+$ T-cells, however latency is also detected in transitional (T$_{TM}$) and effector memory (T$_{EM}$) CD4$^+$ T-cells, naïve CD4$^+$ T-cells (T$_{NA}$) and other minor CD4$^+$ T-cell populations, such as T$_{REG}$, T-memory stem cells (T$_{SC}$) and T$_{FH}$ (Table 1.1; Brenchley et al., 2004; Chomont et al., 2009; Chun et al., 1995; Finzi et al., 1997; Wightman et al., 2010). T$_N$ harbour the least latent infection

compared to other memory T-cell populations, therefore their contribution to the latent reservoir is small (Buzón et al., 2014; Josefsson et al., 2013a; Rönsholt et al., 2012). While T_{NA} are long lived, upon T_{NA} activation they become effector T-cells, produce virus and most likely die (Table 1.1). T_{CM} harbour the greatest proportion of latent infection *in vivo* and are the most long-lived memory compartment compared to T_{TM} and T_{EM}, which may explain why T_{CM} contribute the most to long lived latent infection (Brenchley et al., 2004; Chomont et al., 2009; McKinstry et al., 2008). T_{TM} were the next CD4$^+$ T-cell subpopulations to harbour the most latent infection, these cells are enroute to becoming either T_{CM} or T_{EM} (Table 1.1; Chomont et al., 2009). T_{EM} from the blood, harbour less latency than T_{TM} from the blood but more than T_{NA}. T_{EM} from the blood differ from T_{CM} in that they circulate between the spleen and non-lymphoid organs via blood and return to spleen via lymphatics (Gehad et al., 2012; Sallusto and Lanzavecchia, 1994). Less latent infection in T_{EM} from the blood suggests that T_{EM} fail to receive the same signals as T_{CM} to promote latent infection, there could include exposure to LN environment. T_{EM} from tissue contain as much latent HIV-1 DNA as T_{CM} from the blood (Yukl et al., 2013). Therefore, memory cells in tissue environment like T_{CM} and T_{EM} have a high contribution to the latent reservoir. Most studies are focused on eliminating latent infection in T_{CM} and T_{EM} as decay in this cellular reservoir is slowest, they contribute the most to the latent reservoir, and represents the major barrier to HIV-1 cure.

Figure 1.4. Life Cycle of the CD4$^+$ T-cell.

Naïve T-cells (T$_{NA}$) exit the thymus and circulate through the periphery. Upon exposure to foreign antigen they differentiate into effector T-cells (T$_{EFFECTOR}$) that produce a Th1, Th2 or Th17 response. Upon antigenic clearance, most effector T-cells undergo apoptosis via activation induced cell death (AICD) mechanisms. A small proportion of effector T-cells differentiate into transitional memory cells (T$_{TM}$), which further differentiate into effector memory (T$_{EM}$) and central memory T-cells (T$_{CM}$). T-memory stem cells (T$_{SC}$) are a new population of progenitor T-cells found in circulation, they are hypothesised to arise directly from either T$_{NA}$ or are differentiated from T$_{CM}$. Follicular helper T-cells (T$_{FH}$), which support B-cell function in the germinal centres of the lymph node, are directly differentiated form T$_{NA}$. Regulatory T-cells (T$_{REG}$) can be differentiated from T$_{NA}$ or memory CD4$^+$ T-cells under specific stimuli. Solid lines represent known pathways and dotted lines represent hypothesised pathways.
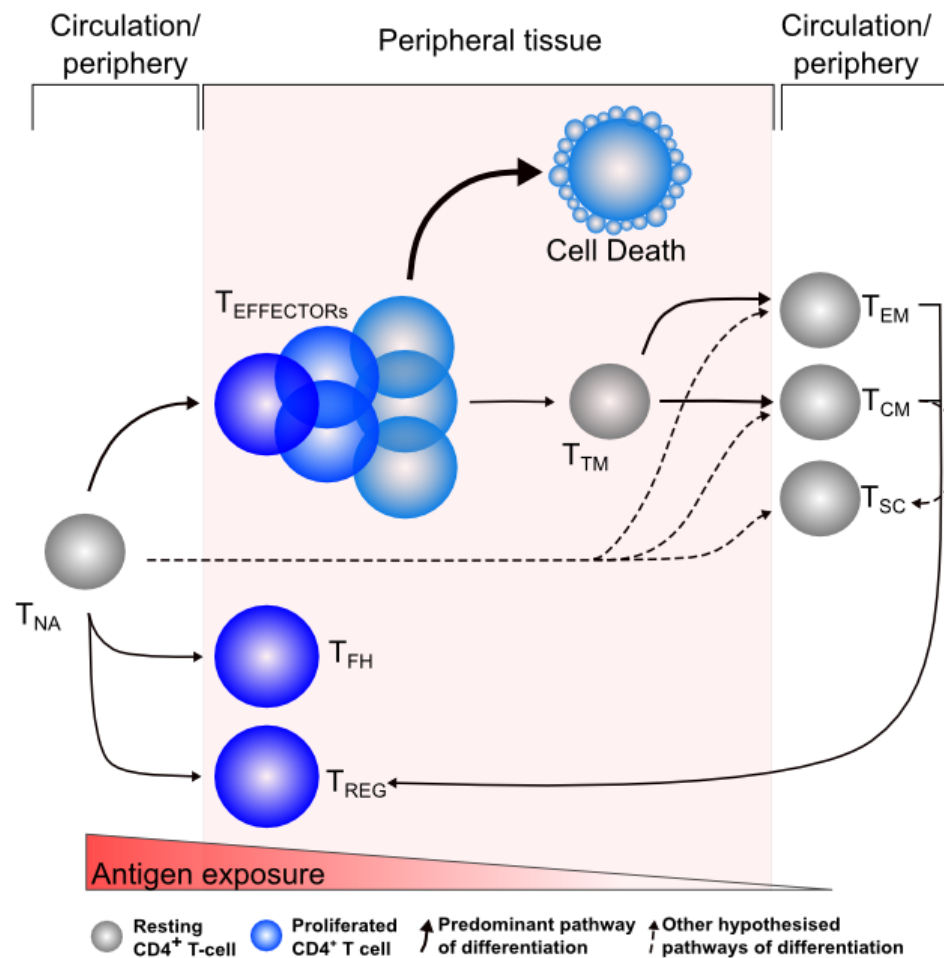
1.5.4. Anatomical Reservoirs of HIV-1

Cellular reservoirs of HIV-1 latency exist at anatomical locations; these anatomical locations are referred to as anatomical reservoirs of HIV-1 latency. Anatomical reservoirs with the highest concentrations of latently infected cells include the LN, GIT and the central nervous system (CNS). The existence of specific cellular reservoirs at each site, with unique characteristics, suggests that anatomical reservoirs possess factors that promote the seeding of latent infection in specific cell populations, and/or maintain latent infection. Additionally, the anatomical reservoirs at the LN and GIT may simply exist because CD4$^+$ T-cells circulate through these tissues during normal cell function.

The Lymph Node

The LN is the most important anatomical reservoir of HIV-1 latency because it has the highest concentration of T-cells with latent infection, that is latently infected T$_{CM}$ (Table 1.1; Chomont et al., 2009; Chun et al., 1998). Both human and non-human primate (NHP) studies, using nested PCR, have shown that despite cART, high concentrations of HIV-1 DNA can be detected in resting CD4$^+$ T-cells from LN compared to those in the blood (summarised in Table 1.1; Chun et al., 1997; Dinoso et al., 2009a; Chomont et al., 2009; North et al., 2010; Josefsson et al., 2013a; Wong et al., 1997). HIV-1 DNA detected in the LN was found in CD4$^+$ T$_{CM}$ and T$_{FH}$ and could contribute to low-level viremia (Table 1.1; Chomont et al., 2009; Josefsson et al., 2013a; Perreau et al., 2013). T-cells circulating through the LN are exposed to cell interactions from endothelial cells (EC) and DC in the T-cell zones, and a rich chemokine environment. According to *in vitro* data, resting T-cell interactions with DC, EC and chemokines provides sufficient signalling to promote latent infection, demonstrating that the LN environment is important for HIV-1 persistence (Kreisberg et al., 2006; Saleh et al., 2007; Shen et al., 2013; Evans et al., 2013). Additional detection of HIV-1 RNA in LN of treated patients suggests that there remains some productive infection or low-level viremia, which could originate from resting CD4$^+$ T-cells that are becoming activated, like T$_{FH}$, virus

bound to DC or follicular DC (FDC; Chomont et al., 2009; Petrovas et al., 2012; Perreau et al., 2013; Josefsson et al., 2013a; Fukazawa et al., 2015).

FDC are a unique cell of the LN that maintains the structure of germinal centres, where B-cells are primed. In HIV-1 infection, FDC have been shown to sequester or hide high amounts of HIV-1 virions prior to cART initiation (Hlavacek et al., 2000; Bordon, 2013; Schacker et al., 2000; Schmitz et al., 1994). Sequestered virus binds to FDC via C-type lectins and is protected from detection by HIV-1 specific immune cells including; DC, cytotoxic T-lymphocytes (CTL), monocyte or macrophage and $CD4^+$ T-cell. Sequestered viruses are also shielded from cART by remaining between the dendrites of the FDC. Therefore FDC can harbour high concentrations of infectious HIV-1 virions without direct infection of the FDC itself. Upon cART initiation, the majority of FDC bound virus is cleared within 6 months (Alòs et al., 2005; Cavert et al., 1997; Smith et al., 2001; Zhang et al., 1999b). However, in the event of suboptimal cART, free infectious virus may remain sequestered to the FDC. In this way FDC are important in HIV-1 persistence as they can promote viral transmission of sequestered virus to surrounding T-cells, that is T_{FH}, thereby increasing infection of T-cells in the LN (Thacker et al., 2009; Zhou et al., 2011). Additionally, FDC bound virus is hypothesised to retain infectivity for longer than cell-free virus, up to 9 months, and these viruses represent greater genetic diversity compared to blood, thereby forming an archive of viruses present in the LN (Keele et al., 2008). Together this data shows that the FDC represent a hub within the LN where virus binds, can be retained and transmitted to T-cells, thereby promoting HIV-1 persistence and spread.

Gastrointestinal Tract (GIT) Associated Lymphoid Tissue (GALT)
The GIT associated lymphoid tissue (GALT) represents the mucosal associated lymphoid tissue that form the Peyer's patches and the draining mesenteric lymph nodes. The GIT and GALT have high levels of $CD4^+$ expressing cells that can be infected by HIV-1, including $CD4^+$ T-cells, monocytes and DC. Th17 T-cells represent a high proportion of $CD4^+$ T-cells in the GIT, as they

mediate defence against bacteria and fungus via promoting production of antibacterial defensins, recruitment of neutrophils and proliferation of epithelial cells at the mucosal surface (Reviewed in Symons et al., 2012). In HIV-1 infection all CD4$^+$ T-cells are depleted, including Th17 T-cells (Brenchley et al., 2008; Kim et al., 2013). Loss of Th17 T-cells drives microbial translocation or "leaky gut syndrome" where bacteria are able to cross the epithelial layer in sites where epithelial cell are compromised, and cause activation of monocytes, DC and macrophages via LPS and other bacterial products (Brenchley et al., 2006; Favre et al., 2010). cART partially restores Th17 T-cell numbers and function, however microbial translocation is never fully resolved and represents a continual source of inflammation, leading to chronic immune activation (Kim et al., 2013; Macal et al., 2008; Mehandru et al., 2006; Ross et al., 2009).

GIT is a unique HIV-1 reservoir as chronic immune activation provides a constant flow of activated T-cells. Activated T-cells from the GIT are characterised by high HLA-DR and CD38 expression and can support productive HIV-1 infection (Jain et al., 2013; Kitchen et al., 2011). CD4$^+$ resting T-cells of the GIT are characterised by expression of high levels of CCR5, CXCR3 and CCR6, which mediate tissue migration (Reviewed in Brucklacher-Waldert et al., 2014). High levels of HIV-1 DNA are detected in bulk cells and resting T-cells from the GIT compared to blood (Table 1.1; Chun et al., 2008, 2010; Yukl et al., 2010, 2013). Chemokine receptor expression memory T-cells, can be latently infected therefore promoting viral persistence in the GIT. Together these data show that the GIT, with the constant flow of activated and resting CD4$^+$ T-cells and chronic immune activation provides the ideal reservoir for HIV-1 persistence. Like LN, the GIT environment is likely important for both the establishment and maintenance of latently infected cells.

Central Nervous System
HIV-1 infection of the CNS can occur in some patients and leads to HIV-associated-neurocognitive disorder (HAND; Desplats et al., 2013). Microglia

cells and macrophages are hypothesised to be the main cell types infected with HIV-1 in the brain as they express low levels of CD4 and CCR5 (Albright et al., 1999; Bagasra et al., 1996; Cosenza et al., 2002; Wiley et al., 1986; Williams et al., 2013; Yang et al., 2009a). Despite some ART being able to penetrate the blood brain barrier, studies of SIV-infected NHP on cART have detected HIV-1 DNA in brain tissue, suggesting that latent infection can also occur in the CNS (Clements et al., 2005; North et al., 2010). In cART treated patients with viral suppression, some patients still have evidence of neuro-inflammation and experience accelerated aging of brain structures, showing again that there is an urgent need to develop a cure for HIV-1 (Anthony et al., 2005; Pfefferbaum et al., 2014).

Together, data from all anatomical reservoirs of HIV-1 infection suggest that low-level viremia, chronic immune activation and, importantly, establishment and maintenance of HIV-1 latency are important in HIV-1 persistence in tissue sites. The development of a successful cure for HIV-1 we must address all persistent viral reservoirs.

Table 1.1. Measurement of Latent Infection in Anatomical and Cellular Reservoirs in HIV-1 Infected Patients.

Reference	Tissue	Cell population used (purity>95%)	% LI **=% of total latent cells	Method of virus quantification	Patients	Years on cART
(Chun et al., 1995)	PBMC	$T_{resting}$	0.33	Nested PCR	HIV-1+	No cART
(Wong et al., 1997)	PBMC	PBMC	1-5 copies/100 ng	Gag PCR	HIV-1+ on cART	1
	LN	Bulk cells	4.1-8.7 copies /100ng			
(Chun et al., 1997)	PBMC	$T_{resting}$	0.0001-0.0081	Nested PCR, $T_{resting}$ activation	HIV-1+ on cART	7.4
	LN	$T_{resting}$	0.0005			
	LN	Macs	0.0054	Nested PCR		
(Finzi et al., 1997)	PBMC	$T_{resting}$	0.00002-0.00162	VOA	HIV-1+ on cART	>2
		Activated T-cells	0.002-0.0002			
(Chun et al., 1998)	PBMC	$T_{resting}$	0.00016-0.020	Nested PCR	HIV-1+ on cART	0.01 (1week) -1.4
(Brenchley et al., 2004)	PBMC	T_{NA}	0.003	Nested PCR	HIV-1+ on cART	NR
		T_{CM}	0.03			
(Chun et al., 2008)	PBMC	$T_{resting}$	0.1083	Nested PCR	HIV-1+ on cART	8.4
	GALT	PBMC (-CD8)	0.4887			
(Chomont et al., 2009)	PBMC	$T_{resting}$	0-1.02	Nested PCR	HIV-1+ on cART	3.7
		T_{CM}	51.7**			
		T_{NA}	1.9**			
		T_{CM} and T_{EM}	46.6**			
		$T_{resting}$	34.3**			
		$T_{resting}$ and T_{EM}	13.9**			
	LN	T_{CM}	0.01			
		T_{TM}	0.01			
	GIT	Bulk cells	0-1.02			
(Chun et al., 2010)	PBMC	$T_{resting}$	0.0076	Nested PCR, HIC	HIV-1+ on cART	7-10
	GALT	PBMC (-CD8)	0.0089	Nested PCR		
(Yukl et al., 2010)	PBMC	$T_{resting}$	0.3	Nested PCR	HIV-1+ on cART	>1
	Duodenum	Bulk cells	0.84			
	Ileum	Bulk cells	1.95			
	Colon	Bulk cells	1.89			

	Rectum	Bulk cells	2.73			
(Josefsson et al., 2013a)	LN	T$_{CM}$	0.37-0.46	SCA	HIV-1+ on cART	0.7-11.9
		T$_{NA}$	0.04			
	PBMC	T$_{CM}$	0.12-0.15			
(Perreau et al., 2013)	LN	CXCR5-PD-1-	0.17	Gag PCR	HIV-1+ on cART	1.4
		CXCR5-PD-1+	1			
		T$_{CM}$	1.15			
		T$_{FH}$	1.4			
(Yukl et al., 2013)	PBMC	Non-T	0.01	Nested PCR	HIV-1+ on cART	12.5
		T$_{resting}$	1			
		T$_{TM}$	1			
		T$_{EM}$	1			
	Ileum	Non-T	0.1	Nested PCR		
		T$_{resting}$	1.1			
		T$_{TM}$	1			
		T$_{EM}$	1.7			
	Rectum	Non-T	0.15	Nested PCR		
		T$_{resting}$	1.5			
		T$_{TM}$	1			
		T$_{EM}$	1.5			
(Yukl et al., 2014)	Rectum	T$_{resting}$	0.01-0.8	Nested PCR	HIV-1+ on cART	NR
		Non-T	0.0005-0.011			
(Buzón et al., 2014)	PBMC	T$_{NA}$	15-16	VOA	Short-long term cART	Baseline-8 years
		T$_{SCM}$	14-24			
		T$_{CM}$	20-22			
		T$_{EM}$	35-26			
		T$_{TD}$	13-15			

Peripheral blood mononuclear cells (PBMC), lymph node (LN), polymerase chain reaction (PCR), viral outgrowth assay (VOA), high input co-culture assay (HIC), single copy assay (SCA), naïve T-cells (T$_{NA}$), central memory T-cells (T$_{CM}$), effector memory T-cells (T$_{EM}$), transitional memory T-cells (T$_{TM}$), terminally differentiated (T$_{TD}$), T-memory stem cells (T$_{SC}$), bulk resting T-cells (T$_{resting}$), combination anti-retroviral therapy (cART), macrophages (macs), not reported (NR).

1.6. **Establishment and Maintenance of HIV-1 Latency in CD4$^+$ T-cells**

The establishment and maintenance of long-lived, latent infection of CD4$^+$ T-cells is the main reason HIV-1 cannot be cured by cART. Latent infection is established as early as 1 week following HIV-1 infection and despite changes in plasma HIV-1 RNA and CD4$^+$ T-cell reconstitution, there is little viral evolution of latent virus *in vivo* (Ananworanich et al., 2012; Chun et al., 1998; Sáez-Cirión et al., 2013; Siliciano et al., 2003). The establishment of latency refers to the processes involved in facilitating infection of resting CD4$^+$ T-cells and allowing for integration without the expression of virus. Maintenance of latency refers to the processes involved in silencing the integrated virus and promoting survival of the latently infected CD4$^+$ T-cell, without the expression of virus. HIV-1 latency in long-lived resting CD4$^+$ T-cells may be established by 2 pathways; by direct infection of resting T-cells, referred to as pre-activation latency, or by infection of activated cells that subsequently revert to resting cells, referred to as post-activation latency (Figure 1.5).

Figure 1.5. Pathways to Establish HIV-1 Latency.

A. Infection of an activated CD4$^+$ T-cell leads to virus production and cell death. Some activated T-cells can revert to a resting state retaining the integrated provirus thus becoming latently infected. This pathway of latency establishment is referred to as post-activation latency. Alternatively, resting CD4$^+$ T-cells can be directly infected with HIV-1, leading to latent infection. This pathway of latency establishment is referred to as pre-activation latency. **B.** Virus can efficiently enter, reverse transcribe and integrate into activated T-cells. However to maintain latency activated T-cells need an active "negative" signal to suppress viral expression, and become latently infected via the post-activation pathway. **C.** The establishment of pre-activation latency, via direct infection of resting CD4$^+$ T-cells, may follow 2 pathways; either as a result of the absence of signalling where the resting phenotype of the CD4$^+$ T-cell is maintained leading to inhibition of viral expression. Alternatively, low-level signalling events may facilitate active positive signalling that facilitates early steps in the virus life cycle, but the resting state of the cells fails to allow virus expression.
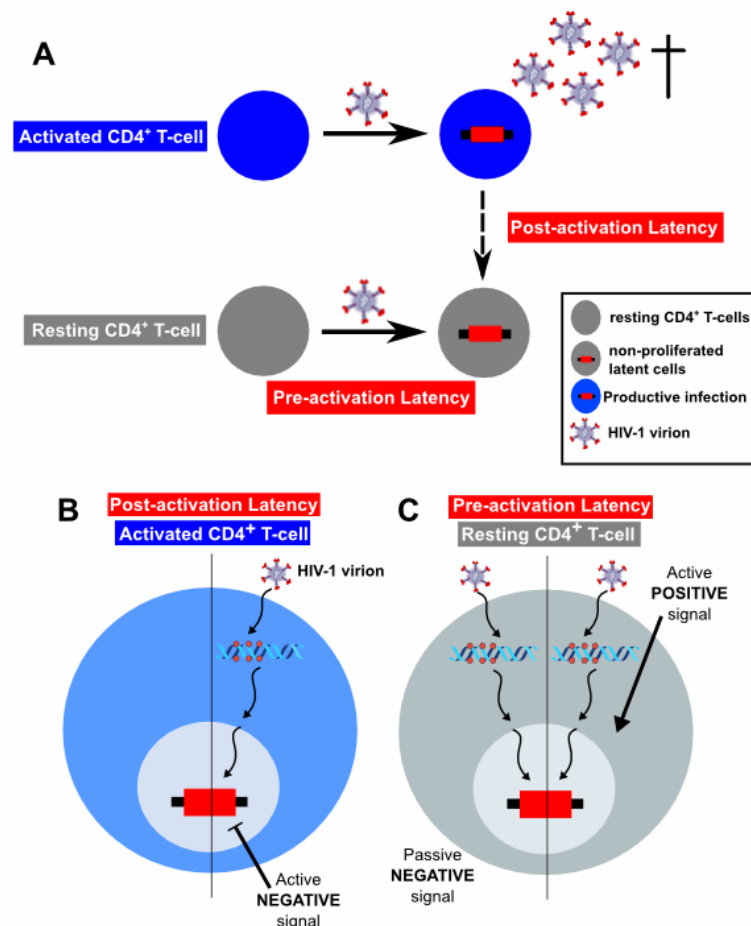
1.6.1. **Establishment of Post-Activation Latency**

Post-activation latency occurs when an activated, productively infected CD4^{+} T-cell receives signals to revert to resting memory state (Figure 1.5A, B). Whether the signals that drive reversion to a resting state originate from the normal process of generating memory cells during an immune reaction, or if they are a result of viral infection remains unknown. The observation that latent infection is mostly found in memory CD4^{+} T-cells supports the post-activation latency model (Chomont et al., 2009; Chun et al., 1995, 1998, 2008; Finzi et al., 1999; Siliciano et al., 2003). However, the model of post-activation latency fails to describe all the *in vivo* and *ex vivo* observations from latently infected resting T-cells and productively infected activated T-cells. Firstly, activated T-cells infected with HIV-1 *in vitro* have a high rate of cell death via cytopathic effects of the virus or bystander mechanisms (Sakai et al., 2006; Arokium et al., 2009; Doitsh et al., 2014; Lelièvre et al., 2004). Therefore not many activated, productively infected CD4^{+} T-cells survive. This discrepancy suggests that a difference exists *in vivo* between an activated cell that reverts to resting state with integrated virus and one that progresses to productive infection. Secondly, in the normal progression of the immune response the effector T-cell population contracts to the resting memory population upon removal of antigenic stimulation (Harbertson et al., 2002; Hu et al., 2001; McKinstry et al., 2007), activation induced cell death (AICD) and response to IFN-γ and IL-2 (Figure 1.5; Reviewed in McKinstry et al., 2010). However in HIV-1 infection antigenic removal fails to occur and AICD is ongoing. Therefore, the model of post-activation latency suggests that that activated CD4^{+} T-cells receive external stimuli that promote reversion to resting state without antigenic removal, which may not be immunologically possible. Additionally, given that reversion of effector CD4^{+} T-cells to resting state takes up to 2 weeks and the establishment of latent infection occurs within 1 week of HIV-1 infection, the post-activation model of latency establishment alone is unlikely, another additional mechanism for latency establishment must occur (Ananworanich et al., 2012; Pepper and Jenkins, 2011; Sáez-Cirión et al., 2013).

It is hypothesised that cells that revert to a resting state receive additional signals for survival (Figure 1.5B). The immune checkpoint (IC), PD-1, is highly expressed on latently infected cells *ex vivo* and on latently infected cells generated *in vitro* (Chomont et al., 2009; Iglesias-Ussel et al., 2013). Together these data suggesting that IC are involved in promoting latent infection in the model of post-activation latency or have an alternative role in pre-activation latency, however the specific mechanism remains unclear. IC may be involved in the long-term maintenance of HIV-1 latency *in vivo*; therefore its upregulation on latently infected cells provides the ability for inhibition of viral expression during reversion of active T-cell to resting state and maybe for the lifetime of the patient. Therefore involvement of IC in the establishment of post-activation latency is likely, and the theme of several current investigations. Together these observations suggest that the establishment of post-activation latency needs active signalling, where external stimuli that promote T-cell survival and inhibit virus expression also mediates long-term establishment and maintenance of HIV-1 latency.

1.6.2. Establishment of Pre-Activation Latency

The model of pre-activation latency is the more controversial model of latency establishment because resting CD4$^+$ T-cells alone were initially found to be resistant to HIV-1 infection *in vitro*. Resistance of resting T-cells to HIV-1 infection is mediated by low deoxynucleotide triphosphates (dNTP) concentrations in the cytosol, lack of host machinery availabile in the cytosol and nucleus to facilitate HIV-1 reverse transcription, nuclear import and integration (Zack et al., 1990, 1992). Therefore the early steps of viral life cycle in resting T-cells are slow and inefficient. Detection of latent infection in CD4$^+$ T$_{NA}$ *in vivo* was an important finding in defining the mechanisms of the establishment of HIV-1 latency (Brenchley et al., 2004). T$_{NA}$ are antigen naïve and have not been activated, therefore cannot have followed the post-activation latency model. Latent infection of T$_{NA}$ *in vivo* and *ex vivo* indicates that HIV-1 is able to directly infect and establish latent infection in T$_{NA}$, without productive infection (Brenchley et al., 2004; Chomont et al., 2009; Eckstein et

al., 2001; Kreisberg et al., 2006). Together these data show that the pre-activation model of latency establishment does occur *in vivo*. It is most likely that latent infection is established via both pre and post-activation latency simultaneously, via different mechanisms in T-cells that are in different stages of life cycle. Together both pathways contribute to the total pool of latently infected cells, in currently unknown proportions.

Compared to the post-activation model of latency, the pathways to establish pre-activation latency require different conditions in the establishment of infection. The establishment and maintenance of latency in T-cells via the pre-activation latency model requires viral entry at early stages of T-cell life cycle, T_{NA}, or late stages of their life cycle as T_{CM} or T_{EM}. It's hypothesised that pre-activation latency can result from two different mechanisms in resting CD4$^+$ T-cells (Figure 1.5C). First, as a result of enhancement of viral entry, reverse transcription and integration by activation of specific signalling cascades that can activate the required host proteins to overcome blocks in viral life cycle present in resting T-cells, and induce successful viral integration. Second, the passive suppression of viral expression in latently infected T-cells by processes that maintain the resting cell phenotype, thereby blocking virus expression. Passive suppression of viral expression in the pre-activation model of latency will likely limit host transcription factors and transcriptional machinery availability for viral transcription and subsequent translation, thereby promoting latent infection.

The similarities between the maintenance of T_{NA} and T_{CM} niches and pathways of activation suggests that establishment of pre-activation latency occurs during common events in the T_{NA} and T_{CM} life cycle. For example, both cells migrate through lymphoid tissue including LN, are homeostatically maintained and interact closely with APC (Reviewed in Gasper et al., 2014). *In vitro* experiments show that treatment of resting T-cells with chemokines that promote LN migration, CCL19, can facilitate the establishment of HIV-1 latency in memory but not naïve T-cells (Saleh et al., 2007). Similarly, other

signals that cause resting T-cell migration like SDF-CXCR4 interactions, also used by gp120, can promote viral entry (Yoder et al., 2008). These studies show that activation of the actin cytoskeleton facilitates faster kinetics of viral entry, reverse transcription and nuclear import thereby resulting in more successful viral integration (Cameron et al., 2010; Yoder et al., 2011; Yu et al., 2009). The lack of transcription activity in the resting CD4$^+$ T-cell prevents any virus expression. Hence, migration of resting T-cells to LN tissue could facilitate the establishment of pre-activation latency.

Our group and Shen et al have shown that resting T-cell interaction with DC and endothelial cells (EC) leads to the establishment of pre-activation latency, however the mechanisms of latency establishment remains unknown (Evans et al., 2013; Shen et al., 2013). DC and EC represent two cell types that resting CD4$^+$ T-cell interact with enroute to and within the LN. EC mediate T-cell migration and DC mediate T-cell activation (DC mediated T-cell activation discussed in 1.7.1; Berg et al., 2002; Tay et al., 2004). CD4$^+$ T-cells can interact with DC and other APC via several mechanisms that can lead to activation of intracellular signalling and may facilitate pre-activation latency, including cell-adhesion, T-cell receptor (TcR) mediated activation, co-stimulatory signals, negative regulation and signalling via soluble factors. Although two models of latency establishment exist, pre and post-activation latency, there are likely shared pathways of latency establishment and maintenance.

1.6.3. **Mechanisms of Latency Maintenance in CD4$^+$ T-cells**

Understanding the establishment of HIV-1 latency is important in the development of strategies to prevent and reverse latency establishment, however the major barrier to HIV-1 cure remains the maintenance of latently infected cells. Long-term analysis of latently infected cells shows that latent infection is either maintained as a stable population life-long or that the rate of

decay and establishment of new latent infection is similar thereby maintaining the magnitude of the latent reservoir (Blankson et al., 2000; Josefsson et al., 2013b; Perelson et al., 1997).

Maintenance of latency in activated CD4^{+} T-cells may eventually become independent of the initial viral suppression as the activated T-cell transitions into a resting state. "It is hypothesised that cells that revert to a resting state receive additional signals for survival (Figure 1.5B). Immune checkpoints (IC), are markers that when ligated lead to the dampening of T-cell activation. Programmed death receptor -1 (PD-1; CD279) is an IC highly expressed on T-follicular helper cells, is normally upregulated upon CD4+ T-cell activation, and binds to ligands PD-L1/PD-L2 (CD274/CD273; Che et al., 2012; Coull et al., 2000; Parry et al., 2005; Tyagi and Karn, 2007). Despite relatively low expression on resting CD4+ T-cells, PD-1 is found to be more highly expressed on latently infected resting CD4+ T-cells ex vivo and in vitro compared to non-latently infected CD4+ T-cells (Chomont et al., 2009; Iglesias-Ussel et al., 2013). These data suggest that IC may represent a biomarker for latently infected resting cells or be involved in promoting latent infection in the model of post-activation latency. Latent infection may occur by inhibition of T-cell activation in resting CD4+ T-cells upon PD-1 ligation, and thereby inhibition of virus expression. However the specific role and mechanism of PD-1's involvement in latent infection is yet to be determined.

Maintenance of latency in resting CD4^{+} T-cells may be passive process from the point of initial entry onwards and simply reflect lack of transcriptional activity in the resting cells. The natural lack in host factors necessary for completion of viral life cycle can act as a block in the viral life cycle itself, and maintain latent infection within resting CD4^{+} T-cells. The block in viral life cycle, or promotion of latency, in resting CD4^{+} T-cells could be a result of three possible pathways: 1. lack of essential host factors for virus production; 2. inaccessibility of HIV-1 DNA in coiled chromatin structures (Bartholomeeusen et al., 2013; Ramakrishnan et al., 2015; Reuse et al., 2009)

and; 3. survival of resting T-cells (Bosque and Planelles, 2009; Marini et al., 2008; Sahu et al., 2006; Tyagi and Karn, 2007; Yang et al., 2009b). The preservation of the resting T-cell phenotype results in the lack of active host transcription factors including, nuclear factor κ-B (NFκB), nuclear factor of activated T-cells (NFAT), specificity proteins-1 (SP-1), activator protein-1 (AP-1) and host transcriptional machinery P-TEFb, CDK9 and cyclin-1, which fail to initiate and sustain HIV-1 transcription (Mbonye and Karn, 2014; Taube and Peterlin, 2013). Additionally, the lack of host cell transcriptional machinery also leads to low levels of Tat production, which is necessary for successful transcription initiation (section 1.2.2; Um et al., 2012; Yukl et al., 2009).

Differences in gene expression between activated and resting CD4$^+$ T-cells lead to different integration sites in both populations (Brady et al., 2009; Mitchell et al., 2004; Wang et al., 2007). Viral integration is known to occur in sites of active gene expression. In the post-activation model of latency, it is possible that virus has integrated into sites of active gene expression, which are supressed during the resting state. Therefore, the provirus itself is unavailable for transcription due to epigenetic silencing where genomic DNA is coiled around histones into nucleosomes, which form the chromatin structures and is unavailable for transcription (Pearson et al., 2008; Gallastegui et al., 2011). Recent data has shown that a proportion of latently infected cells *in vivo* harbour provirus that is not expressed on primary stimulation of T-cells, however can be detected by PCR techniques as full length, replication competent virus, referred to as non-inducible intact virus (Ho et al., 2013). It is possible that latently infected cells established by the post-activation pathway harbour non-inducible virus while T-cells from the pre-activation model have virus integrated into genes that maintain resting state, and are more readily expressed, however, to date this has not been explored.

1.6.4. *In Vitro* Models of HIV-1 Latency

In vitro models of latent infection using primary T-cells are important for identifying the mechanisms of how HIV-1 latency is established and reversed. This is difficult to do using *ex vivo* latently infected cells from HIV-1-infected patients on cART as these cells are infrequent *in vivo* (Finzi et al., 1999; Perelson et al., 1997). Cell line models of latency do not accurately model latent infection *in vivo* because they are proliferating and are usually clonal, so unlike natural infection, there is only a single site of integration.

There are several models of post-activation latency. Generally, in the post-activation model of latency, CD4^{+} T-cells are activated *in vitro*, infected and allowed to rest. These models generate varying frequencies of latently infected cells that have low expression of activation markers and limited proliferation (summarised in Table 1.2; Bosque and Planelles, 2009; Marini et al., 2008; Sahu et al., 2006; Tyagi et al., 2010; Yang et al., 2009b). There are some limitations to post-activation latency models. First, in the Tyagi et al., Yang et al. and Bosque et al. models, mutated viruses are used, preventing multiple rounds of infection and viral transcription that are important in the establishment and maintenance of latent infection *in vivo* (Bosque and Planelles, 2009; Yang et al., 2009b; Tyagi et al., 2010; Bonczkowski et al., 2014). Second, all models of post-activation latency fail to take into consideration non-TcR signals that can lead to the establishment of latency and signals that actively suppress transcription leading to latency, like IC. Third, many of these models require prolonged culture periods and therefore are not feasible for any high throughput studies (Bosque and Planelles, 2009; Marini et al., 2008; Sahu et al., 2006; Tyagi and Karn, 2007; Yang et al., 2009b).

Similarly, there are several models of pre-activation latency *in vitro*. Swiggard et al was the first to show that resting CD4^{+} T-cells could be latently infected following spinoculation of resting CD4^{+} T-cells with replication competent virus (Swiggard et al., 2005). Latency was established in this model via cytoskeletal changes induced by gp-120 binding to CXCR4 and spinoculation

itself (Balabanian et al., 2004; Guo et al., 2011). Using *ex vivo* tissue blocks Eckstein et al. and Kriesberg et al. showed that the LN environment can induce latent infection in naïve T-cells (Eckstein et al., 2001; Kreisberg et al., 2006). Based on these findings, our lab generated *in vitro* models to investigate the importance of different LN derived signals including chemokines (Saleh et al., 2007; Cameron et al., 2010) and DC-T cell interactions (Evans et al., 2013), and found that both could induce pre-activation latency in resting CD4$^+$ T-cells. Additionally, Shen et al. and Spina et al. both showed that interactions between resting CD4$^+$ T-cells and EC or effector T-cells can also lead to induction of latency in resting CD4$^+$ T-cells (Shen et al., 2013; Spina et al., 2013). Together, these models generate physiologically relevant latently infected cells and show that tissue and lymphoid environments are important in the establishment of HIV-1 latency.

An important aspect of both *ex vivo and in vivo* studies of latent infection is accurate techniques to quantitate latent infection. Quantification of integrated HIV-1 DNA using PCR or induction of virus expression is commonly used (Butler et al., 2001). Nested PCR techniques measure all integrated virus, including mutated, misaligned and virus with deletions. Some of these integrants may not be replication competent and therefore wont contribute to the reservoir of infectious virus that leads to viral rebound once cART is stopped (Ho et al., 2013). Additionally, nested PCR techniques differ between labs in primers and controls used, adding further variables (Chomont et al., 2009; Lewin et al., 2008; O'Doherty et al., 2002). Detection of integrated HIV-1 in nested PCR techniques is based on the detection of LTR close to genomic Alu repeat elements, however if the LTR has integrated far from the Alu repeat element, the integration event will not be detected (Butler et al., 2001). Therefore, in addition to just measuring integrated virus, viral reactivation methods should also be used to quantify latent.

Detection of latency by viral reactivation more accurately quantifies replication competent and inducible virus, however based on findings from Ho et al., a

single round of reactivation is not enough to induce expression of all integrated virus (Ho et al., 2013; Laird et al., 2013). Furthermore, Ho et al found that even following a second round of T-cell activation, 11% of intact, inducible virus was not expressed. Additionally, the method used to measure virus expression following reactivation can also add variability to quantifying latent infection. Virus expression can be measured as an increase in the reverse transcriptase (RT) enzyme or proteins, p24 and gag, or virion by RT-PCR (Marini et al., 2008; Pace et al., 2012, 2013; Saleh et al., 2007, 2011). An increase in RT enzyme, p24 and gag or viral RNA in supernatant will not identify the number of cells producing virus, as many proteins particles and copies of RNA are produced per cell. Furthermore, this may not necessarily correlate with virion production. Measurement of EGFP under the control of the Nef promoter, as we have used in our lab, will quantify expression of *de novo* protein production per cell (Evans et al., 2013). In other models, amplification of virus released following reactivation, using feeder peripheral blood mononuclear cells (PBMC) is used (Evans et al., 2013). This technique will therefore only detect true infectious virus and in addition will enhance the sensitivity to detect small amounts of infectious virus, but the addition of feeder cells makes this method qualitative but not quantitative. Despite the limitations of these *in vitro* models of latency, these models have broadened our understanding of latent infection and possible mechanisms for establishment and maintenance of latency.

Table 1.2. Summary of Pre- and Post-Activation *In Vitro* Latency Models.

Central memory T-cells (T_{CM}), naïve T-cells (T_{NA}), effector memory T-cells (T_{EM}), effector T-cells ($T_{EFFECTORs}$), resting T-cells ($T_{resting}$), Complement factor B-1 (CFB-1), interleukin (IL), monocyte derived dendritic cells (MDDC), multiplicity of infection (MOI), dendritic cells (DC), not reported (NR), non-polarised (NP), reverse transcriptase (RT)-assay, phorbol 12-myristate 13-acetate (PMA), Ionomycin (I), ** = 1 activated, infected T-cell to 4 resting T-cells, # = specific volume of viral supernatant that were equivalent to a given number of virus producing 293T cells (used to grow virus).

	Reference	T-cell Subset	Additional T-cell Survival Signal?	Initial T-cell Stimulant	HIV Virus/Vector — Co-receptor	HIV Virus/Vector	MOI	Productive Infection (%)	Method of Latency Reactivation	Time to Complete Model (days)
Post-Activated Latency	Sahu, 2006	T_{CM}	H80 cell line	Anti-CD3/CD28 + IL-2 (40U/ml)	X4	Replication competent	1-10	~80	Anti-CD3/CD28	60
	Tyagi, 2007	T_{CM}	H80 cell line	Anti-CD3/CD28 + IL-2 (20U/ml)	X4	Δgag/Δtat/Δnef	NR	~85	Anti-CD3/CD28	30-60
	Marini, 2008	T_{CM}	IL-7 (1ng/ml) + IL-2 (25U/ml)	MDDC	X4	Replication competent	0.002	5-10	Anti-CD3/CD28 + MDDC	~60
	Bosque, 2009	T_{CM}/T_{EM}	IL-2 (30U/ml)	Anti-CD3/CD28, Spinoculation	X4	Δenv	50	3 NP 14.5 Th1 3 Th2	Anti-CD3/CD28 & nested PCR	30
	Yang, 2009	T_{EM}	Bcl-2 transduction and IL-7 (20ng/mL)	Anti-CD3/CD28 + IL-2 (100U/ml)	X4	Δgag/Δ vif/vpr/vpu/env	<0.01	5-10	Anti-CD3/CD28 or PMA	~120
Pre-Activated Latency	Swiggard, 2005	$T_{NA}/T_{CM}/T_{EM}$	IL-7 (20ng/mL)	Spinoculation	X4	Replication competent	22-150	0.3	Anti-CD3/CD28 & nested PCR	<14
	Saleh, 2007	T_{CM}, T_{NA}, $T_{resting}$	IL-2 (2U/ml)	Chemokines (CCL19)	X4	Replication competent	1	RT-assay	RT-assay (day 10) & nested PCR	<14
	Evans, 2013	T_{CM}, T_{NA}, $T_{resting}$	IL-2 (2U/ml)	Primary DC	R5	Replication competent	0.5	5	Anti-CD3/CD28 & nested PCR	14
	Shen, 2013	$T_{resting}$	None	Endothelial Cells (HUVEC)	X4	Δenv/replication competent	#	12-14	PMA/I	<14
	Spina 2013	T_{NA}/T_{CM} /T_{EM}, T_E	IL-2 (5U/ml)	Productively infected T_E	X4	Replication competent	Unknown **	NR	Anti-CD3/CD28 & nested PCR	<9

1.7. **A Brief Introduction to Dendritic Dells.**

DC are in high concentration in LN where they control T-cell activation by direct interactions and secretion of soluble factors (Lukas et al., 1996; Steinman and Cohn, 1973; Steinman et al., 1983; Wendland et al., 2011). The LN is also a site of high frequency of latently infected resting CD4$^+$ T-cells, so we investigated the role of DC and their ability to control CD4$^+$ T-cells in latent infection. DC were first identified as unique cells of the immune system in 1973 in work started by Ralph Steinman (Steinman and Cohn, 1973). DC are now classified as APC because they have an extraordinary capacity to induce, sustain, and regulate the immune responses (Schmitz et al., 2005). DC are able to control T-cell activation via secretion of soluble factors and direct cell interaction molecules, including pro-inflammatory and regulatory cytokines, chemokines, adhesion molecules, co-stimulatory molecules and TcR-MHC-II interactions. To understand the interactions between DC and T-cells that play a role in regulation of HIV-1 persistence we must first understand DC function and DC interaction with HIV-1.

1.7.1. **Life Cycle of the Dendritic Cell**

DC Ontology

Several DC subsets exist in the periphery and populate peripheral tissue sites, however they can be derived from either bone marrow (BM) resident progenitor cells (hematopoietic progenitor cells (HPC)), and referred to as **circulatory DC** from tissue resident progenitor cells and referred to as **tissue resident DC** (Bogunovic et al., 2006; Haniffa et al., 2009; Ivanovs et al., 2011). Both tissue resident and circulatory DC share the same lifecycle; they scan the local environment for pathogens in an immature state and upon maturation, migrate to local draining LN. To understand the similarities between DC subsets and monocytes, it is critical to understand DC precursors, and the cellular origin, development and life cycle of DC and monocytes.

Recent advances in fate mapping techniques have facilitated the study of DC and monocyte ontology in mouse models and findings have now been tested and aligned with human DC progenitor cells and DC subpopulations (Figure 1.6; Reviewed in Becher et al., 2014). Tissue resident DC and monocytes have yolk sac origin and populate peripheral tissue during early embryogenesis (mouse, day 8.5-9.0), while DC and monocytes with feotal liver origin populate the bone marrow and circulate to tissue in adult life (Ginhoux et al., 2010; Hoeffel et al., 2012). The DC that arise from the yolk sac origin and include Langerhans cells (LC) and dermal DC (DDC), kupffer cells from the liver, alveolar macrophages from the lungs, macrophages from the heart, microglial cells from the brain and tissue resident lymphoid DC in LN, spleen and thymus (Doulatov et al., 2010; Ginhoux et al., 2010; Yona et al., 2013). Circulating DC and monocytes are derived from foetal liver HPC which are formed 1.5-2 days later (mouse, D11.0) and populate bone marrow as HPC (Chen et al., 2013; Klimchenko et al., 2011; Zhang et al., 2000). From HPC, DC differentiate into DC progenitor cell (CDP), which then differentiate into pre-classical DC (pre-cDC) and are further differentiated into mDC subpopulations, $CD1c^+$ (mouse $CD11b^+$) and $CD141^+$ (mouse $CD103^+$), or pDC ($CD123^+$; Figure 1.6; Liu et al., 2009; Reviewed in Dutertre et al., 2014).

Monocytes differ from this pathway of differentiation. Monocytes branch from HPC to become the common monocyte progenitor cell (cMoP), which differentiate into $CD14^+$ or $CD14^{lo}CD16^{hi}$ monocyte subpopulations (Figure 1.6; Reviewed in Ginhoux and Jung, 2014). These studies show that, in mice, both tissue resident and bone marrow HPC can differentiate into several DC and monocyte subpopulations that may share function. By identification of characteristic markers the function of specific DC and monocyte subpopulations can be determined in both tissue and circulation.

Figure 1.6. Ontology and Nomenclature of Antigen Presenting Cell Subsets.

DC and monocytes can be of embryonic yolk sac origin or foetal liver hematopoietic progenitor cell (HPC) origin. Yolk sac HPC reside in tissue and differentiate into tissue resident DC or monocytes. Foetal liver HPC populate the bone marrow and differentiate into circulating DC and monocytes. Nomenclature, suggested by authors, first defines cell ontology, cell type then cell location (Adapted from Guilliams et al., 2014).
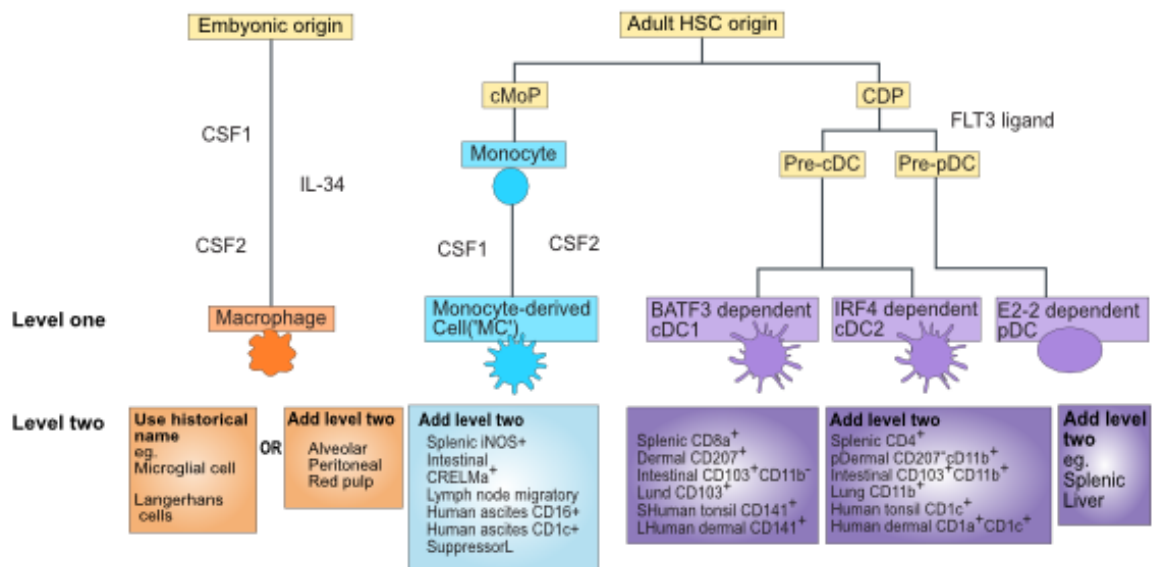
Immature DC

Once fully differentiated from HPC, DC exist in an immature state in peripheral tissue where they actively sample the environment for foreign antigens by endocytosis, pinocytosis, adsorption and detection of extracellular antigens (Delamarre et al., 2005; Lee et al., 2007; Diken et al., 2011; Nakamura et al., 2014). Immature DC detect antigen via the expression of a variety of pathogen-associated molecular pattern (PAMP) molecules, including toll-like receptors (TLRs) and C-type lectins, which are expressed on the DC surface membrane or intracellular membrane organelles (endosomes, lysosomes; summarised in Table 1.3; Harman et al., 2013; Blander and Medzhitov, 2006; Piccioli et al., 2007; Reviewed in Lundberg et al., 2014). Once an antigen is recognised by a PAMP, it is internalised and prepared for loading on the MHC-II molecule (Inaba et al., 1998, 2000; Platt et al., 2010). An MHC-II containing vesicles from the endoplasmic reticulum combines with the endosome/lysosome, and the immunoprotease degraded antigens are loaded onto MHC-II molecules, which then migrate to the plasma membrane for expression (Paul and Neefjes, 2013; Pierre et al., 1997; Rouette et al., 2014; Trombetta et al., 2003; Turley et al., 2000). DC are unique in that they can cross present antigen, which is when foreign antigen in loaded onto MHC-I molecules. MHC-II molecules usually only express intracellular antigens. Cross presentation occurs when endosomes carrying foreign antigen combine with MHC-I containing vesicles from the endoplasmic reticulum, thereby allowing foreign antigens to be loaded onto the MHC-I molecules and expressed on the DC surface (Guermonprez et al., 2003; Nair-Gupta et al., 2014; Sabado et al., 2007; Chatterjee et al., 2012; Delamarre et al., 2003). Consequently, the same antigen is expressed on both MHC-II and MHC-I molecules allowing presentation and arming of both CD4^{+} and CD8^{+} T-cells respectively (Burgdorf et al., 2007; Vander Lugt et al., 2014). Once the DC detects foreign antigen, the DC becomes activated and DC maturation begins (Chow et al., 2002).

Mature DC

Upon maturation there is a change in DC function from antigen uptake to antigen presentation that is reflected in DC phenotype and gene changes, measured by changes in protein and gene expression profiles (Diken et al., 2011; Naour et al., 2001; Ryan et al., 2004). During maturation, the ability for antigen uptake and immunproteosome expression are downregulated while expression of MHC-II molecules, co-stimulation molecules and cell-adhesion molecules are upregulated (Bax et al., 2007; Dannull et al., 2007). Mature DC migrate to LN via downregulation of CCR6 which mediates migration to peripheral tissue, and upregulation of CCR7 which allows DC to migrate toward the local draining LN via a gradient of CCL19 and/or CCL21 (Lukas et al., 1996; Carramolino et al., 1999; Penna et al., 2001; Marsland et al., 2005; Britschgi et al., 2010). Mature DC enter the LN by lymphatic high endothelial vessels (HEV) or the afferent lymphatic vessels and migrate to the T-cell zones where they can interact with many T-cells (de la Rosa et al., 2003; Wendland et al., 2011).

Mature DC have increased potential to interact with and activate CD4$^+$ T-cells in the LN T-cell zones via the expression of MHC-II, adhesion molecules (ICAMs, integrin, lymphocyte function associated antigens (LFA), selectins, galectins), co-stimulatory molecules (CD80, CD86, CD40) and increased the capacity for secretion of cytokines (summarised in Table 1.3). DC-T-cell interaction is initiated by interaction of adhesion molecules like ICAM-1 (CD54) and LFA-1 (CD18 and CD11a), which mediated close cell proximity (Kis-Toth and Tsokos, 2014; Ning et al., 2013; Rodriguez-Plata et al., 2013). Close cell proximity allows binding of MHC-II with TcR (CD3) and opportunity for antigen recognition (de la Fuente et al., 2005; Katakai et al., 2013; Mandl et al., 2012; Vroomans et al., 2012). Upon T-cell recognition of MHC-II loaded antigen, immune synapse (IS) formation is initiated via formation of lipid rafts which mediate efficient intracellular signal transduction (Jarrossay et al., 2001; Luft et al., 2002; Mackey et al., 1998; Meyer zum Bueschenfelde et al., 2004). Concentrated signalling between DC and T-cell

allows antigen specific and rapid immune activation. DC also secrete soluble factors, which include chemokines and cytokines that promote attraction and interaction with T-cells, and regulate T-cell activation respectively (Ito et al., 2005; Jarrossay et al., 2001; Kassianos et al., 2012; Piccioli et al., 2007; Piqueras et al., 2006; Wan et al., 2013). All DC follow this life cycle and only live for 4-9 days, after which they undergo controlled apoptosis. Immature DC are re-generated from HPC or tissue resident progenitor cells and continue surveillance of the periphery (Kamath et al., 2002).

Table 1.3. Summary of Surface Expression Molecules Expressed and Response to Stimulation on Dendritic Cells and Monocytes.

| | Marker | | Blood DC | | | | *In vitro* | Mono-cytes | | |
	Marker names	CD classification	pDC	CD1c+ mDC	CD141+ mDC	SLAN+ DC	MDDC	CD14+	CD16+	Reference
Characteristic DC/monocyte Markers	BDCA 1	CD1c	-	++++ +	-	-	+	-	-	(Bigley et al., 2010; Dzionek et al., 2000; Grassi et al., 1998; MacDonald et al., 2002)
	BDCA 2, CLEC4C	CD303	++++	-	-	-	-	NR	-	(Bigley et al., 2010; Dzionek et al., 2000; Harman et al., 2013)
	BDCA 3	CD141	-	-	++++	-	-	NR	-	(Bigley et al., 2010; Dzionek et al., 2000; Haniffa et al., 2013; Jongbloed et al., 2010)
	BDCA 4	CD304	+++++	-	-	-	-	NR	-	(Bigley et al., 2010; Dzionek et al., 2000)
		CD123	+++++	+	-	-	-	-	-	(Bigley et al., 2010; Dzionek et al., 2000; Harman et al., 2013; Jongbloed et al., 2010; MacDonald et al., 2002)
	M-DC8	SLAN	-	-	-	++++	-	-	-	(de Baey et al., 2001; MacDonald et al., 2002; Schäkel et al., 1999, 2002)
		CD16	-	-	-	++++	-	-	+++ ++	(Bigley et al., 2010; Flinsenberg et al., 2012; Schäkel et al., 2006)
		CD14	-	+/-	-	+/-	-/+	+++ ++	++	(Grassi et al., 1998; Schäkel et al., 2002)
	HLA-DR, MHC-II		+++++ ↑	++++ + ↑	++++ +	++++ + ↑	++++	+++ +	+++ +	(Bigley et al., 2010; Dzionek et al., 2000; Grassi et al., 1998; MacDonald et al., 2002; Zheng et al., 2014)
Adhesion		CD11c	-	++++ +	+++	++++ +	+++	+++ ++	+++ +	(Bigley et al., 2010; Dzionek et al., 2000; Jongbloed et al., 2010; MacDonald et al., 2002; Tanaka et al., 1999)
		CD33	+	+++	+++	++	++	+++	+++	(Dzionek et al., 2000; Garnache-Ottou et al., 2005)

		Marker								Reference
		CD11b	-	++	NR	++	+++	+++	++	(Ammon et al., 2000; Dzionek et al., 2000; Grassi et al., 1998; Gutzmer et al., 2006; Schäkel et al., 2006; Tanaka et al., 1999)
		CD2	+*	+	-	-	+	+	NR	(Dzionek et al., 2000; MacDonald et al., 2002)
		CD11a	+++	+	NR	NR	+	++++	++++	(Ammon et al., 2000; Dzionek et al., 2000; Hänsel et al., 2012; Tanaka et al., 1999)
Activation/co-stimulatory	BB1, CD28LG	CD80	- ↑	- ↑	-	++ ↑	+	-	+	(Dzionek et al., 2000; Harman et al., 2013; MacDonald et al., 2002; Zheng et al., 2014)
	B70, CD28lG2	CD86	++ ↑	++ ↑	++	+++ ↑	++ ↑	+	++	(Dzionek et al., 2000; Harman et al., 2013; MacDonald et al., 2002; Said et al., 2015; Zheng et al., 2014)
	BL11, HB15	CD83	+	+	-/+	+ ↑	+ ↑	++	NR	(Dzionek et al., 2000; Harman et al., 2013; Said et al., 2015)
	TNFRSF5, CDW40	CD40L	+ ↑	+ ↑	+++	+ ↑	+++	+	NR	(Dzionek et al., 2000; Harman et al., 2013)
	Selectin E,ELAM	CD62E	-	+	NR	-	-	-	NR	(Harman et al., 2013)
	Selectin L, LAM1	CD62L	+++++	+++	+++	++	-	+++	+	(Dzionek et al., 2000; Harman et al., 2013; MacDonald et al., 2002)
	Selectin P, LECAM3	CD62	++++	+	NR	++	+	++		(Harman et al., 2013)
HIV receptors		CD4	+++	+++ ↓	+	+	++	++++	++++	(Ellery et al., 2007; Lee et al., 1999; MacDonald et al., 2002; Penna et al., 2001; Tanaka et al., 1999)
	CXCR4	CD184	+	+	NR	-	+	++	++	(Ellery et al., 2007; Lee et al., 1999, 2011)
	CCR5	CD194	++++	+++	+++	++++	+	++	++	(Ellery et al., 2007; Lee et al., 1999, 2011)
C-type lectin	CLEC2C	CD69	+++++	++++	NR	+++	+++	+++	NR	(Dzionek et al., 2000; Harman et al., 2013)
	CLEC4A, DCIR	CD367	+	++	NR	+	+++	+	NR	(Harman et al., 2013)**
	CLEC4E, MINCLE		-	-	NR	-	-	+	NR	(Harman et al., 2013)

Name	CD								Reference
CLEC4F, KCLR		-	++++	NR	++++ +	++++	+	NR	(Harman et al., 2013)
CLEC4G, LSECtin		-	-	NR	-	++	NR	NR	(Harman et al., 2013)
CLEC4K, langrin	CD207	-	-	-	-	-	-	NR	(Harman et al., 2013; MacDonald et al., 2002)
CLEC4L, DC-SIGN	CD209	- ↓	-	NR	-	+++ ↓	-	NR	(Harman et al., 2013)
CLEC4M, LSIGN	CD299	+	++	NR	++	++ ↓	+	NR	(Harman et al., 2013)
CLEC6A, Dectin-2		-	++	NR	+	++	++	NR	(Harman et al., 2013)
CLEC7A, Dectin-1	CD369	-	+	NR	+++	+++	+++	NR	(Harman et al., 2013)
CLEC9A, DNGR-1	CD370	-	++++/-	NR	+/-	+	+/-	NR	(Harman et al., 2013)
CLEC13B, DCE205	CD205	+++	+++	++++	+++	++	+++	NR	(Harman et al., 2013; MacDonald et al., 2002)
CLEC13D, MMR, MCR1	CD206	-	-	-	-	++++ +	-	NR	(Harman et al., 2013; MacDonald et al., 2002)
TLR — TLR1	CD281	++++ ↓	++ ↓	+++	+++	++++ ↓	+++	NR	(Harman et al., 2013; Iwasaki and Medzhitov, 2004; Rozis et al., 2008)
TLR2	CD282	-	++ ↓	++	+++	++ ↓	+++	+++	(Belge et al., 2002; Harman et al., 2013; Iwasaki and Medzhitov, 2004; Rozis et al., 2008)
TLR3	CD283	-	+++ ↓	+++	+	+ ↓	++	NR	(Harman et al., 2013; Iwasaki and Medzhitov, 2004; Rozis et al., 2008)
TLR4, ARMD10	CD284	-	++ ↓	-	+++	++++ ↓	+++	+++	(Belge et al., 2002; Harman et al., 2013; Iwasaki and Medzhitov, 2004; Rozis et al., 2008)
TLR5, SLEB1	CD285	-	++ ↓	-	+	-	+	NR	(Harman et al., 2013; Iwasaki and Medzhitov, 2004; Rozis et al., 2008)
TLR6	CD286	+++ ↓	+++ ↓	++	++	+++ ↓	++	NR	(Harman et al., 2013; Iwasaki and Medzhitov, 2004; Rozis et al., 2008)
TLR7	CD287	+++++ + ↓	++ ↓	-	+++	-	+++	NR	(Harman et al., 2013; Iwasaki and Medzhitov, 2004; Rozis et al., 2008)

TLR	CD	pDC	CD1c+ mDC	CD141+ mDC	SLAN+ DC	MDDC	CD14+	CD16+	References
TLR8	CD288	-	+++ ↓	-	++++ +	++++ ↓	+++ ++	NR	(Harman et al., 2013; Iwasaki and Medzhitov, 2004; Rozis et al., 2008)
TLR9	CD289	+++++ + ↓	+++ ↓	-	-	-	+	NR	(Harman et al., 2013; Iwasaki and Medzhitov, 2004; Rozis et al., 2008)
TLR10	CD290	+ ↓	+ ↓	++	+	++	-	NR	(Harman et al., 2013; Iwasaki and Medzhitov, 2004; Rozis et al., 2008)

	Stimulant	Response	pDC	CD1c+ mDC	CD141+ mDC	SLAN+ DC	MDDC	CD14+	CD16+	References
Response to activation		T-cell activation	++	+++	+++	+++	+++	++	+	(MacDonald et al., 2002)
	Mixed (poly-IC, LPS,)	Secretion of soluble factors	INF-α, IFN-λ, CCL3, CCL4	IL-12, IFN-β, TGF-β, IL-10, IL-1, IL-6, IL-8, TNF-α, IL-23 IL-17	IL-12, IL-10, IFN-γ	IL-12, IL-23 TNF-α	TNF-α, IL-6, IL-8, IL-10, IL-12 IL-17	NR	NR	(de Baey et al., 2001; Günther et al., 2012; Haniffa et al., 2012; Hänsel et al., 2012; Heil et al., 2004; Hontelez et al., 2012; Jarrossay et al., 2001; Kassianos et al., 2012; Lee et al., 2007; Liu et al., 2009; Manuzak et al., 2012; Nakamura et al., 2014; Nizzoli et al., 2013; Piccioli et al., 2007; Piqueras et al., 2006; Rozis et al., 2008; Said et al., 2015; Sancho et al., 2009; Schäkel et al., 2006; Stopak et al., 2007)
		T-cell activation	++++	+++	NR	++++	++	+++ +	++	(Cameron et al., 2007; Dutertre et al., 2012; Wilson et al., 2014; Xu et al., 2014)
	HIV-1	Secretion of soluble factors	IFN-α	IL-12, IFN-β, TGF-β, IL-10, IL-1, IL-6, IL-8, TNF-α, IL-17 IL-15 ↓	NR	TNF-α ↓	IL-12, IFN-β, IL-10, IL-1, IL-6, IL-8, TNF-α, IL-15 ↓	NR	NR	(Buisson et al., 2009; Colpitts et al., 2013; Dutertre et al., 2012; d'Ettorre et al., 2004; Garg et al., 2015)

+/++ Represents low expression, +++ represents intermediate expression and ++++/+++++ represents high expression by Mean Fluorescence Intensity (MFI). SLAN$^+$ DC data is only included when CD16$^+$ monocyte are depleted, and CD16$^+$ monocyte data is only included when SLAN$^+$ DC are depleted. Arrow up (↑) indicates that expression of marker is increased upon DC or monocyte activation and arrow down (↓) indicates that expression of marker is decreased upon DC or monocyte activation. * Indicates a subpopulation. NR = not reported.

1.7.2.   Dendritic Cell Subpopulations

Since the discovery of the DC, a complex network of DC subpopulations in different anatomical locations, with an array of functions have been identified. Each of the DC subpopulations express a unique pattern of PAMPs while in immature state, and a unique cytokine secretion profile, patterns of IC and adhesion molecules when mature (summarised in Table 1.3). Given that HIV is a human disease and does not infect mice, here we only discuss human DC subpopulations, unless otherwise specified. The study of the different DC subpopulations enhances our understanding of DC function and we use this data to differentiate between HIV-1 specific and non-specific functions that may be important in HIV-1 persistence.

Two main subpopulations of DC exist in the blood of all animals, plasmacytoid DC (pDC) and myeloid DC (mDC; O'Doherty et al., 1994). mDC make up 0.6% of PBMC while pDC make up approximately 0.5% of PBMC in humans (Cella et al., 1999; MacDonald et al., 2002; Siegal et al., 1999). There are three subpopulations of mDC, classified by $CD1c^+$, $CD141^+$ and $SLAN^+$ (6-sulfo LacNAc+) DC in humans. $CD1c^+$ mDC, also referred to as the classical DC (cDC), are the most abundant mDC subpopulation in the circulation (0.24% PBMC) making them the most well defined human DC subset, however they are also found in all tissue sites (MacDonald et al., 2002; Siedlar et al., 2000). $CD141^+$ (BDCA-3) mDC are a rare subpopulation of mDC in circulation (0.036% of human PBMC; (Autissier et al., 2010; Bachem et al., 2010; Haniffa et al., 2012; Jongbloed et al., 2010; Lundberg et al., 2014), however according to mouse studies, they exist at higher concentrations in tissues, such as tonsils and spleen (up to 0.08% of isolated cells; Ginhoux et al., 2009). $SLAN^+$ DC, also referred to as $MDC8^+$ or $CD16^+$ DC, are also abundant in circulation (0.65% of human PBMC). Historically $SLAN^+$ DC have been classified within the $CD16^+$ monocytes. In Table 1.3, we include data from $SLAN^+$ DC that are depleted of $CD16^+$ monocytes and $CD16^+$ monocytes that are depleted of $SLAN^+$ DC (Schäkel et al., 1999, 2002). The function of all three mDC subpopulations are similar; they are able to potently activate $CD4^+$ T-cells but

small changes in PAMP expression profiles allow detection of a different range of pathogens.

mDC can form close T-cell interactions and are potent T-cell activators (Bhardwaj et al., 1993; MacDonald et al., 2002). They have a multi-faceted role, with the ability to induce a strong pro-inflammatory response to TLR-3 (poly:IC) stimulation and a regulatory response to TLR-4 (LPS) stimulation (Kassianos et al., 2012; Lowes et al., 2005). In humans, CD141^{+} mDC differ from CD1c^{+} mDC in their lack of TLR 4, 5 and 8 expression, and they are able to cross present more efficiently (Flinsenberg et al., 2012; Jongbloed et al., 2010; Meixlsperger et al., 2013; Nizzoli et al., 2013). The role of CD141^{+} DC remains controversial, but similar to CD1c^{+} DC they are able to secrete IL-12, IL-10 and IFN-γ in response to poly:IC (TLR-3) and up-regulate PD-L1 expression in humans (Table 1.3; Gupta et al., 2013; Meixlsperger et al., 2013; Nizzoli et al., 2013). CD1c^{+} DC and CD141^{+} DC can form long dendritic processes which mediate DC interaction with many T-cells, while SLAN^{+} DC form shorter dendrite projections (Dzionek et al., 2000; Schäkel et al., 1999). SLAN[+] DC can also potently activate T-cells and form close contacts with the secretion of high amounts of IL-12, IL-23 and TNF-α *in vitro* (de Baey et al., 2003; Günther et al., 2012; Hänsel et al., 2012; Schmitz et al., 2005). SLAN expressing DC are yet to be identified however due to high potential to secrete pro-inflammatory cytokines SLAN[+] DC have been likened to mouse inflammatory DC, which migrate to tissue and exert a local inflammation response (Hänsel et al., 2012). mDC can also dampen T-cell activation by expression of IC and their ligands (PD-L1/2, CTLA-4, herpes virus entry mediator (HVEM), galectin 9 (Gal9), inducible co-stimulator ligand (ICOSL), Tim-3 (Breton et al., 2009; Che et al., 2012; Kuipers et al., 2006).

Like mDC, pDC are also found in the spleen, thymus and lymph nodes, and they can be both tissue derived and circulatory (Cella et al., 1999; Colonna et al., 2004; Grouard et al., 1997). pDC are specialised type-I IFN secreting cells, which upon stimulation can secrete up to 1000 times more type-I IFN than any

other immune cells (Jongbloed et al., 2010; Siegal et al., 1999; Takeda et al., 2003). They are able to secrete IFN-α and pro-inflammatory cytokines IL-6, IL-8, TNF- α, CXCL10 and MIP1-α (CCL3) and MIP1-β (CCL4) thereby producing a strong anti-viral response (Matsui et al., 2009; Rissoan et al., 1999; Siegal et al., 1999). pDC differ from mDC in expression of PAMPs with expression of only TLR-7 and 9. Other differences are summarised in Table 1.3.

1.7.3.   **Monocytes and Dendritic Cells**

Monocytes are precursor cells for macrophages and DC in the circulation, which upon stimulation can differentiate into different cell types (Ginhoux et al., 2006; Hashimoto et al., 2013; Jakubzick et al., 2008, 2013). Upon inflammation, and exposure to GM-CSF, monocytes differentiate into macrophages, *in vitro* these are called monocyte-derived-macrophages (MDM) (Smith et al., 1998; Delneste et al., 2003; Way et al., 2009). Upon exposure to GMCSF and IL-4, monocytes can differentiate into monocyte-derived-DC (MDDC; Romani et al., 1994; Sallusto and Lanzavecchia, 1994). MDDC are often used *in vitro* to model primary mDC interactions with other cells or pathogens as they are easier to work with *in*. MDDC have similar expression profile of MHC-I and II, co-stimulatory and adhesion molecules, including mDC specific markers and low expression of monocyte markers (Table 1.3; Caux et al., 1996; Mallon et al., 1999; Romani et al., 1994). Compared to mDC, MDDC have a similar milieu of cytokine and chemokine secretion profiles, ability to activate and interact with T-cells, however they are less efficient at T-cell activation (Bhardwaj et al., 1992; Buisson and Triebel, 2005; Sallusto and Lanzavecchia, 1994; Turville et al., 2004). MDDC do also differ from primary mDC in expression of DC-SIGN, MMR, Dec-205, CD62L, TLR-3 and TLR-7, and secretion of soluble factors in response to poly-IC, LPS, and HIV-1 stimulation (Summarised in Table 1.3). Additionally, MDDC gene expression does not cluster with primary isolated mDC, rather they cluster with primary LC, making MDDC a useful tool to study skin DC but not for blood or tissue mDC (Harman et al., 2013). Monocytes can also be differentiated into

monocyte derived langerhans cells (MDL) by treatment with GM-CSF, TGF-β, and TNF-α, however these differ in function compared to mDC, primary LC and MDDC (Chung et al., 2013; Peiser et al., 2004; Rozis et al., 2008; Said et al., 2015). Our interest in DC is their function in the LN therefore we focus our attention on data from primary DC from blood.

1.8. **Dendritic Cells Interactions with HIV-1**

Cells that are common to mucosal and epithelial surfaces, blood and LN and are also important in the establishment and maintenance of latency, these include include T-cells, DC and monocytes. Given that DC regulate T-cell activation and these pathways are important in latency establishment and maintenance, we hypothesise that DC play a key role in the regulation of latency. First we must understand the way that DC interact with HIV-1. Given that HIV-1 infects humans, all data presented here is from the study of human DC, unless otherwise specified.

1.8.1. **Dendritic cells as vehicles for HIV-1 infection**

Cis-infection

Immature DC express low levels of CD4, CCR5 and CXCR4 therefore can be infected with HIV-1 (Cameron et al., 2007; Fong et al., 2002; Groot et al., 2006; Lee et al., 1999; Smed-Sorensen et al., 2005; Zaitseva et al., 1997). HIV-1 infection of a DC is known as *cis*-infection, where virus enters the DC and completes the full viral life cycle, resulting in *de novo* virion production (Figure 1.7A). *Cis*-infection of DC is generally inefficient due to low cytoplasmic nucleotide pools and low proliferative capacity of the DC, translating to poor reverse transcription and nuclear import (Cameron et al., 1992; Cunningham et al., 2000; Fong et al., 2002; Schmidt et al., 2004). Additionally, detection of viral ssRNA in endosomes by TLR-7, 8 or 9 (expressed in pDC only) leads to DC activation and expression of viral restriction factors APOBEC3G and SAMHD1 which leads to blockade of

productive infection of DC (Beignon et al., 2005; Ito et al., 2005; Mohanram et al., 2013; Uchijima et al., 2005).

APOBEC3G can be expressed in mDC, pDC, monocytes and macrophages as well as activated and resting T-cells where expression leads to hyper-mutation during viral reverse transcription (Chiu et al., 2005; Peng et al., 2007; Stopak et al., 2007; Wang et al., 2008). However, APOBEC3G can be counteract by viral protein Vif, facilitating successful completion of viral life cycle (Biasin et al., 2007; Bishop et al., 2004). Another restriction factor is SAMHD1 which regulates the availability of dNTP's in APC as a means to maintain resting phonotype, limit proliferation and protect against invading retrovirus' (Hrecka et al., 2011; Laguette et al., 2011; Lahouassa et al., 2012). SAMHD1 is highly expressed in mDC and macrophages, rendering them less susceptible to HIV-1 infection, however, viral protein Vpx in HIV-2, not HIV-1, targets SAMHD1 for proteosomal degradation and facilitates completion of viral life cycle (Berger et al., 2011; Goldstone et al., 2011).

Trans-infection

gp120 expressed on HIV-1 also binds receptors from the C-type-lectin family expressed on DC that do not result in viral entry but do allow the DC to carry the virus on its surface, this is called *trans*-infection (Figure 1.7B; Cavrois et al., 2007; Yu et al., 2008). Once virus is bound to C-type-lectin receptors, it can be carried with the DC during migration to local draining LN and interact with high concentrations of T-cells present in T-cell zones. C-type lectins on the surface of mDC that can bind virus include DC-SIGN (Cassol et al., 2012; Geijtenbeek et al., 2000), mannose receptor (Nguyen and Hildreth, 2003), DCIR (Jin et al., 2014; Lambert et al., 2008, 2011), syndecan-3 (de Witte et al., 2007) and SIGLEC-1 (Izquierdo-Useros et al., 2012; Puryear et al., 2013; Turville et al., 2001). By carrying the virus from a peripheral site of infection to a site high in HIV-1 target cells, DC act like a "trojan horse" and expose uninfected CD4^{+} T-cells to HIV-1 infection (Reviewed in Cavrois et al., 2008).

HIV-1 Activates pDC

Expression of TLR-7 and 9 on pDC, facilitate pDC activation by HIV-1 and results in potent type-I IFN and TNF-α response, bystander mDC activation and inhibition of HIV-1 infection in CD4$^+$ T-cells (Figure 1.7C; Beignon et al., 2005; Fonteneau et al., 2004; Groot et al., 2006; Heil et al., 2004; Kader et al., 2013; O'Brien et al., 2011; Royle et al., 2014). Despite the protective mechanisms in preventing HIV-1 infection of CD4$^+$ T-cells, pDC are hypothesised to propagate CD4$^+$ T-cell depletion during acute HIV-1 infection. *In vivo* type-I IFN promotes T-cell activation and enhances T-cell death via AIDC pathways and activation of TNF-related apoptosis-inducing ligand (TRAIL), thereby promoting progression toward chronic HIV-1 infection and AIDS (Hardy et al., 2007; Herbeuval et al., 2006; Stary et al., 2009). HIV-infected patients who do not develop symptomatic acute infection, HIV-1 elite controllers (subjects who naturally control HIV-1 replication to low levels or <50 copies/ml) or long-term non-progresses, have lower expression of TRAIL on pDC which authors hypothesis to contribute to lower T-cell death and slower progression to AIDS (Barblu et al., 2012). Furthermore, depletion of pDC in a humanised mouse model led to higher plasma HIV-1 RNA but less T-cell depletion and slower progression to chronic infection (Li et al., 2014). Modulation of pDC activation may be a strategy to protect CD4$^+$ T-cells and enhance an HIV-1-specific immune response, thereby allowing control of HIV-1 replication and pathogenesis, however prolonged activation of these pathways can lead to disease progression.

Role of mDC in HIV-1 Infection

mDC express high levels of several C-type lectins and therefore can mediate *trans*-infection of T-cells. They do not express TLR-7 or 9 and only express low level of TLR-8, therefore are poorly activated by HIV-1 (Table 1.3) (Dong et al., 2007; Granelli-Piperno et al., 2006; Hsieh et al., 2003). When mDC are activated either by bacterial products or HIV-1; they secrete the pro-inflammatory cytokines IL-6, TNF-a and IL-12 that activate CD4$^+$ T-cells and NK cells; IL-10 which down regulates T-cell activation; and secretion of

CCL17 and CCL22 to attract T-cells (Buisson et al., 2009; Dutertre et al., 2014; Granelli-Piperno et al., 2004). Taken together, mDC interactions with HIV-1 promote T-cell interaction, spread of virus and immune activation. mDC could therefore promote disease progression by chronic immune activation.

DC Mediated Transfer of Infection to T-cells

Both *cis* and *trans*-infection of DC allow efficient and explosive transfer of HIV-1 infection to DC-interacting CD4^{+} T-cells (Figure 1.7A, B; Barat et al., 2009; Cameron et al., 1992; Groot et al., 2006; Holl et al., 2010; Lore et al., 2005; Pope et al., 1995; Smed-Sorensen et al., 2005; Turville et al., 2004; Vanham et al., 2000). Increased efficiency of T-cell infection in the presence of *trans*-infected DC is believed to be multifactorial; enrichment of target CD4^{+} T-cells close to DC, increased DC induced T-cell activation thereby increasing the number of activated T-cells for HIV-1 infection and shielding virus from neutralizing antibodies and/or cART, similar to FDC in the germinal centres (Chen et al., 2007; Duncan et al., 2013; Hübner et al., 2009; Martin et al., 2010; Massanella et al., 2009). Upon pathogen detection, the DC usually forms an immune synapse to enhance T-cell activation. During *cis*-infection, virus can hijack immune synapses to become viral synapses and enhance viral infection of target cells. Using this synapse, virus can be directly transferred to CD4^{+} T-cells via vesicles or just close proximity (Ghez et al., 2006; Hübner et al., 2009; Chen et al., 2007; Gelman and Nguyen, 2010). Viral synapses use cellular machinery of the immunological synapse to promote fluid movement of virus between an infected and non-infected cell, including enriched lipid rafts and ICAM/LFA interactions (Hioe et al., 2011; Jolly et al., 2007; Martin et al., 2010; McDonald et al., 2003). Together viral synapses, close DC-T-cell interactions and DC mediated activation of CD4^{+} T-cells results in efficient transfer of productive infection to CD4^{+} T-cells (Lore et al., 2005; Sigal et al., 2011).

DC Infection In Vivo

In untreated HIV-1 infection, DC in blood are found to be infected but at low frequency (Benlahrech et al., 2011; Dillon et al., 2011; Donaghy et al., 2003; Fernandez et al., 2008; Khokhlova et al., 2014; Lehmann et al., 2008; Sabado et al., 2010; Tilton et al., 2008). However, the number of mDC and pDC in LN of HIV-1 infected patients compared to uninfected is higher, suggesting that DC are redistributed to or retained in LN during HIV-1 replication, not depleted by virus infection (Biancotto et al., 2007; Lehmann et al., 2010). Upon cART initiation, the frequency and function of both mDC and pDC normalise (Finke et al., 2004; Sabado et al., 2010). Neither pDC nor mDC are thought to play a direct role in HIV-1 persistence on cART because DC are not easily infected by HIV-1 and they only have a life span of 4-9 days (Kamath et al., 2002).

Figure 1.7. Dendritic Cell Interactions with HIV-1.

DC interaction with HIV-1 leads to *cis* or *trans*-infection, or antigen presentation. **A.** *Cis*-infection occurs when virus binds to CD4 and CCR5, leading to viral entry and completion of the viral life cycle within the DC, resulting in production of *de novo* virions. **B.** *Trans*-infection occurs when virus binding to C-type lectins allows the virus to be carried by the DC and infect CD4^{+} T-cells that are interacting with the DC. **C.** Antigen presentation occurs when PAMP bound virus leads to viral internalisation, DC activation and subsequent CD4^{+} T-cell activation.
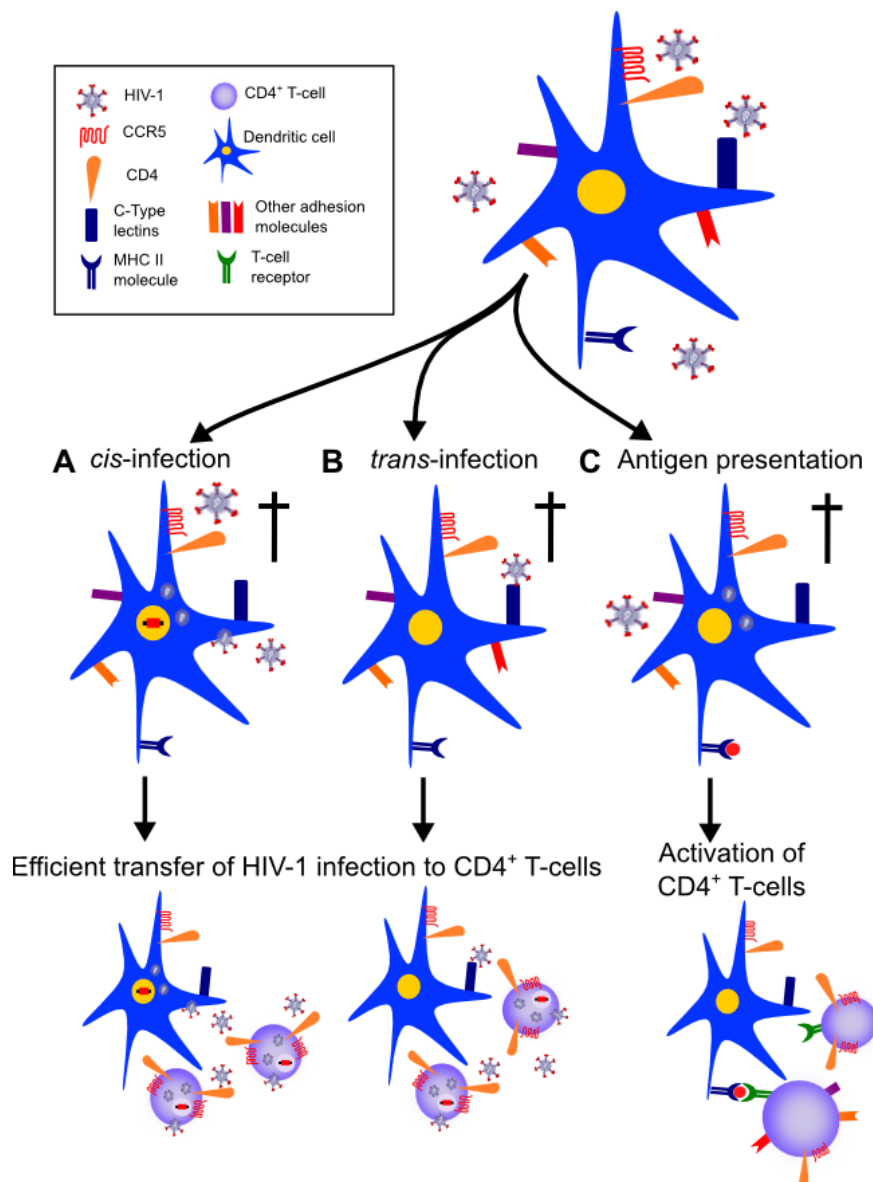
1.8.2. **Role for Dendritic Cells in HIV-1 Persistence**

In the LN, DC control T-cell activation via direct interaction with T-cells and secretion of soluble factors. *In vitro* latency models have shown that TcR signalling (Bosque and Planelles, 2009), activation of actin dynamics (Cameron et al., 2010; Guo et al., 2011; Yoder et al., 2008), and cell-cell interactions (Evans et al., 2013; Shen et al., 2013; Spina et al., 2013) can facilitate the establishment of pre- and post-activation latency. Maintenance of latency is thought to occur by a combined effect of latently infected cells receiving survival signals (Bosque et al., 2011), homeostatic proliferation signals (Chomont et al., 2009), transcriptional control to maintain a resting phenotype (Abrahams et al., 2009; Argyropoulos et al., 2004; Kauder et al., 2009; Sahu et al., 2006; Tyagi and Karn, 2007) and inhibition of the immune response via IC expression (Breton et al., 2009; Che et al., 2012; Kuipers et al., 2006). DC can mediate all of these interactions.

DC Interactions with CD4$^+$ T-cells

DC can interact with CD4$^+$ T-cells in several ways, namely, cognate and non-cognate interactions, and secretion of soluble factors. T-cell activation involves TcR and MHC-II interaction with positive signals from the co-stimulatory molecule CD28, this is also defined as a cognate interaction. Cognate interaction between an mDC or pDC and a CD4$^+$ T-cell leads to secretion of cytokines, cytoskeletal changes in the T-cell, and changes in calcium (Ca^{2+}) flux, resulting in T-cell activation (Boise et al., 1995; Hochweller et al., 2010; Neve-Oz et al., 2015; Seya et al., 2001; Suh et al., 2004).

In the absence of cognate interactions, DC and T-cell interaction still occurs and this is referred to as non-cognate interactions, but does not result in T-cell activation. Non-cognate signalling between DC-T-cell, results in interactions between adhesion molecules and co-stimulatory molecules (Berg et al., 2002; De Riva et al., 2007). The activation of non-cognate interactions can lead to migration, polarisation, enhance T-cell survival and activation of cellular apoptosis (Choi et al., 2005). Non cognate interactions can also lead to the

transfer of productive HIV-1 infection, however less efficiently than in the presence of cognate interactions (Vanham et al., 2000). The effect on non-cognate interactions in the setting of latent HIV-1 infection has not been investigated, however the observations that actin dynamics (Cameron et al., 2010; Guo et al., 2011; Yoder et al., 2008) and cell-cell interactions (Evans et al., 2013; Shen et al., 2013; Spina et al., 2013) can facilitate latent infection leads to the hypothesis that non-cognate interactions maybe one pathway responsible for the establishment of HIV-1 latency.

Finally, DC can also interact with CD4$^+$ T-cells via IC, which lead to the inhibition of T-cell activation in the setting of both cognate and non-cognate interactions (Fife and Pauken, 2011; Said et al., 2010). Known IC expressed on T-cells include PD-1, CTLA-4 (CD152), LAG-3 (CD223) and BTLA-4 (CD272), which can bind to their respective receptors on DC including, PD-L1/L2, CD80/CD86, galectin-9, MHC class II and Tim-3 (HVEM, CD270; Jones et al., 2008; Kuipers et al., 2006; Pentcheva-Hoang et al., 2009; Walunas et al., 1994; Wolchok and Saenger, 2008; Zhu et al., 2005). We also propose that interactions between IC and their ligands are likely to play a major role in either the establishment of maintenance of latency and that DC are key to these interactions.

DC Provide Homeostatic Control of T-cells
DC can control T-cell homeostasis by presentation of self-peptide on MHC class II presentation and cytokine signalling (Ge et al., 2002; Mayer et al., 2012; Wendland et al., 2011). IL-15 and IL-7 are two cytokines that control homeostasis, however only IL-15 is secreted by DC which leads to homeostatic proliferation and cell survival of CD4$^+$ T-cells (Colpitts et al., 2013; Kassianos et al., 2012; Khaled and Durum, 2003).

The IL-7/IL-15 signalling pathway has been investigated in the induction of latency by Yang et al, who transduced resting CD4$^+$ T-cell with intracellular protein Bcl-2, a known inhibitor of apoptosis (2000; Yang et al., 2009b). Bcl-2 maintains cell survival and a resting state in T-cells in response to IL-7 and IL-

15 (Vaux et al., 1988). Bosque et al. further showed that IL-7 induced cell proliferation in the absence of virus expression, leading to the maintenance and expansion of latently infected cells *in vitro* (Bosque et al., 2011). Furthermore, phylogenetic analysis of integrated latent virus over time shows that there is no viral evolution, suggesting that once latency is established, maintenance of latency either involves *de novo* viral production and further seeding of new latently infected cells or that latently infected cells may arise from a common source, via homeostatic proliferation or clonal expansion (Chomont et al., 2009; Josefsson et al., 2013b). This was recently demonstrated through the detection of frequent sites of integration in latently infected cells from HIV-1-infected subjects on cART, consistent with clonal expansion *in vivo* (Cohn et al., 2015; Maldarelli et al., 2014; Wagner et al., 2014). Together these data show that homeostatic proliferation and cell survival signals can expand the latent population and are likely critical for maintenance of latently infected cells.

1.8.3.   *In Vitro* DC-T-cell Latency Model

We recently developed an *in vitro* DC-T-cell latency model using flow cytometry (FCM) to separate proliferated and non-proliferated T-cells, and productively infected and uninfected T-cells, thereby giving us the ability to determine the differences in HIV-1 infection outcome upon cognate and non-cognate interactions. Furthermore, FCM also allows us to study the role of specific receptors and markers of HIV-1 latent infection. For example, we showed that elimination of CD69 expressing T-cells still leads to latent infection indicating that pathways to establish latency exist besides T-cell activation (Evans et al., 2013). Using a similar method, we can interrogate the role of negative regulation using specific receptors or a family of receptors. Using the DC-T-cell model we can determine the role of DC subpopulations in establishment of pre- and post-activation latency, maintenance of latent infection and then identify the relevance of specific signalling pathways.

1.9. Aims and Hypothesis

1.9.1.   Specific Aims

We hypothesise that mechanisms used by DC to control T-cell activation are also active in the control of latent infection of CD4$^+$ T-cells. Evidence of a role for DC in the control of latent infection is limited, however the observation of a high frequency of latently infected resting CD4$^+$ T-cells in the LN, where mature DC are also in high concentration supports our hypothesis. Specifically we believe that non-cognate interaction between mDC and resting CD4$^+$ T-cells lead to the establishment of HIV-1 latency in non-proliferating (pre-activation) and proliferating (post-activation) CD4$^+$ T-cells.

In this thesis we will:

1. Study the ability of DC to induce latent infection in non-proliferating CD4$^+$ T-cells (pre-activation latency), investigate which DC subpopulations induce latency in resting CD4$^+$ T-cell subpopulations, which specific DC-T-cell interactions are involved and the role of different viral titres on the establishment of pre-activation latency.
2. Use RNA-seq to understand the differential capacity of several different APC subpopulations to establish and maintain pre-activation latency and identify the relevant specific signalling pathways.
3. Use the *in vitro* DC-T-cell latency model to explore the establishment of latent infection in proliferating CD4$^+$ T-cells (post-activation latency).
4. Use RNA-seq to identify signalling pathways that may facilitate inhibition of latency.

1.9.2.   Significance

Using the in vitro DC-T-cell model we aim to characterise the mechanism of how latency is established and maintained following non-cognate interactions between DC and T-cells. The research presented here highlights possible specific intracellular pathways that can be used as drug targets to prevent the establishment of latency upon HIV-1 infection or to activate latently infected

cells in patients who are already infected and on cART. This study adds to the understanding of the mechanism of establishment and maintenance of HIV-1 latency that is required to develop a cure strategy to eliminate HIV-1 latency.

2. Chapter 2

Myeloid DC Induce Latency in Resting Memory CD4^{+} T-Cells

Monash University

Declaration for Thesis Chapter Two

Nitasha Kumar

In the case of Chapter Two, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Experiments	95%
Analysis	95%
Writing	100%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Dr. Vanessa Evans	2 experiments, Figure 2.6	
Miss Gabriella Khoury	Assistance with naïve T-cell isolation	5%

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	■■■■■■■■■	**Date:** 08/06/2015

Main Supervisor's Signature	■■■■■■■■■	ate 08/06/2015

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

2.0. **Chapter 2: Myeloid DC induce latency in resting memory CD4$^+$ T-cells**

2.1. **Abstract**

Background: Latently infected resting CD4$^+$ T-cells are the major barrier to the eradication of HIV-1 infection. Given that latently infected resting CD4$^+$ T-cells are enriched in lymphoid tissue compared to blood, we hypothesised that interactions between dendritic cells (DC) and resting CD4$^+$ T-cells are critical for the establishment and maintenance of HIV-1 latency.

Methods: Resting CD4$^+$ T-cells labeled with eFluor670 were cultured alone or with syngeneic DC for 24 hours prior to infection with a CCR5-tropic, EGFP-reporter virus. Non-proliferating (eFluor670^{hi}), non-productively-infected (EGFP$^-$) CD4$^+$ T-cells were sorted on day 5 post-infection. Latent infection was stimulated and amplified by co-culture of sorted eFluor670^{hi} T-cells with mitogen (PHA) and IL-2 stimulated PBMC.

Results: Latent infection in non-proliferating CD4$^+$ T-cells co-cultured with myeloid (m)DC was significantly increased compared T-cells cultured alone. Increase in latent infection was consistent with both R5 and X4 viruses at an MOI of 0.5 and 0.01-0.05 respectively. Latent infection was not induced in non-proliferating CD4$^+$ T-cells co-cultured with plasmacytoid (p)DC. Latent infection was preferentially induced in non-proliferating memory (CD45RO$^+$) CD4$^+$ T-cells, and not naïve (CD45RO$^-$) CD4$^+$ T-cells. When mDC-T-cell contact was prevented, by culture of mDC within a transwell above the resting CD4$^+$ T-cells, the frequency of latent infection in non-proliferating CD4$^+$ T-cells was significantly reduced. Treating the mDC-T-cell co-cultures with antibodies to the chemokines CCL19 and CXCL10 (shown to facilitate latent infection in resting CD4$^+$ T-cells) and the chemokine receptor CXCR3 led to no change in the frequency of latent infection in non-proliferating CD4$^+$ T-cells. However, treatment of resting CD4$^+$ T-cells prior to co-culture with the

neutralising antibody to adhesion molecule LFA-1 led to a small but significant decrease in latent infection of non-proliferating memory CD4$^+$ T-cells.

Conclusions: mDC play a key role in the establishment of HIV-1 latency in resting memory CD4$^+$ T-cells. Our results suggest that mDC induced latent infection is likely to be mediated via close proximity of mDC and CD4$^+$ T-cells.

2.2. Introduction

Despite combination antiretroviral therapy, HIV-1 cannot be cured due to the establishment and persistence of latent infection in resting memory CD4$^+$ T-cells (Chun et al. 1995; Coffin 1995; Finzi et al. 1997; Siliciano et al. 2003; Reveiwed in Katlama et al. 2013). Latent infection is difficult to study due to the low frequency of latently infected cells *in vivo* and the lack of physiologically relevant *in vitro* latency models. We have recently developed a novel *in vitro* latency model where co-culture of dendritic cells (DC) with autologous resting CD4$^+$ T-cells can induce latent infection in non-proliferating CD4$^+$ T-cells (DC-T-cell model; Evans et al. 2013).

In vivo studies in both human and primates have shown that the lymph nodes (LN) have a high concentration of latent infection in resting CD4$^+$ T-cells (Dinoso et al. 2009; Eckstein et al. 2001; Horiike et al. 2012; Kreisberg et al. 2006; North et al. 2010). Our lab has previously shown that incubation of CD4$^+$ T-cells with chemokines that mediate LN homing, CCL19 and CCL21, can facilitate latent infection (Cameron et al. 2010; Saleh et al. 2007). Together these findings suggest that the lymphoid environment is important for the establishment and maintenance of latent infection *in vivo*.

In the current study we used the novel DC-T-cell model to investigate the importance of cell contact and soluble factors in the establishment of mDC-induced HIV-1 latency non-proliferating memory CD4$^+$ T-cells. The DC-T-cell model is the first to use a co-culture system with primary DC to mimic the interactions that occur in the T-cell zones of the lymph node. We label resting CD4$^+$ T-cells from the blood with cytoplasmic dye, eFluor670, and co-culture these with primary mDC and pDC. The cytoplasmic dye allows tracking of T-cell activation and the EGFP reported virus used allows identification of productive HIV-1 infection. Finally, activation of sorted, non-productively infected cells from co-cultures allows clear identification of a latently infected

population of CD4$^+$ T-cells. We hypothesise that DC-T-cell contact, which exists in the LN, plays a role in the establishment of latent infection *in vivo*.

2.3. **Results**

2.3.1.   **mDC induced latency in resting CD4$^+$ T-cells**

We had previously shown that co-culture of bulk DC (Lineage$^-$, HLA-DR$^+$ cells) were able to induce latent infection in non-proliferating CD4$^+$ T-cells (Evans 2010, PhD Thesis). To determine which DC subsets were able to induce latent infection in resting CD4$^+$ T-cells, mDC (HLA-DR$^+$, CD11c$^+$) and pDC (HLA-DR$^+$, CD123$^+$) were sorted from healthy donors as described in Figure 2.1A, B.

Measurements of DC maturation markers showed that sorted mDC had a mature phenotype due to high levels of CD83 expression, intermediate expression of CD86 and low expression of CD80 (Figure 2.1C). Sorted pDC had low CD80 and CD86 expression, consistent with literature which describes no CD80 or CD86 expression on pDC, even upon pDC maturation (Dzionek et al. 2000; MacDonald et al. 2002). CD83 was not measured on pDC, as we were unable to source the appropriate antibody (non-PE).

NK cells were not removed from DC preps in the initial depletion cocktail on the autoMACS, which included CD3, CD19 and gly-A. Immature NK cells express low-intermediate levels of CD16 and high levels of CD56, while mature NK cells express high levels of CD16 (Seillet et al. 2015; Romee et al. 2013; Poli et al. 2009). Any contaminants in the sorted DC cells were not CD16 positive, however, we did not test for CD56. Contaminants in the FACSAria sorted DC subsets were always less than 3%, therefore we do not expect contaminants to alter results from these experiments, however, this should be tested in future experiments.

Figure 2.1. Isolation of Resting CD4^{+} T-Cells and Dendritic Cells.

A. Resting CD4^{+} T-cells were isolated from freshly isolated peripheral blood mononuclear cells (PBMC) using a hybridoma cocktail. Only cells of purity greater than 98% were used, measured by flow cytometry. In experiments where T-cell subsets were isolated, bulk resting CD4^{+} T-cells were stained with CD45RA and further sorted on the FACSAria for naïve (CD45RA^{+}) and memory (CD45RA^{-}). **B.** Dendritic cells (DC) were enriched from PBMC by depletion of lineage positive cells (CD19, CD3, CD11b, GlyA) using a hybridoma cocktail. The enriched DC population was then stained with HLA-DR, CD11c and CD123 and further sorted on the FACSAria into myeloid DC (mDC) and plasmacytoid DC (pDC). **C.** Freshly isolated DC subsets were then stained with antibodies to the co-stimulatory markers CD80 and CD86, maturation marker CD83 and HIV-1 co-receptor CCR5.
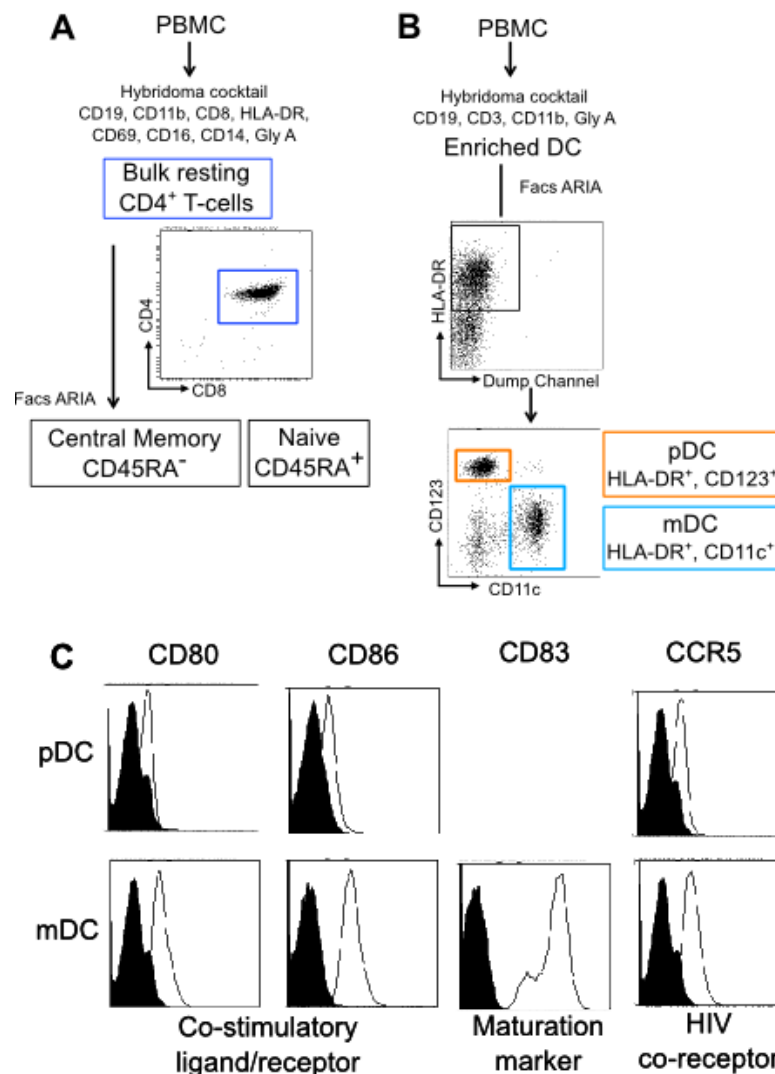
Resting T-cells were labeled with proliferation dye eFluor670 or SNARF, then cultured alone or in combination with mDC or pDC at a ratio of 10 T-cells:1 DC (Figure 2.2A). After 24 hours, the cultures were pulsed for 2 hours with NL(AD8)Δ*nef*EGFP (Figure 2.2A). In some experiments staphylococcal enterotoxin B (SEB) stimulation was used to enhance DC-T-cell interaction as SEB stabilises MHC class II (MHC-II) and T-cell receptor (TcR) interaction by linking the TcR-Vβ chain with MHC-II. The number of EGFP$^+$ cells was quantified 5 days post-infection as a measure of productive infection in total DC-T-cell cultures (Figure 2.3A). mDC were more efficient at induction of productive infection in resting CD4$^+$ T-cells compared to pDC, however both pDC and mDC co-cultures had significantly more productive infection compared to T-cells alone ($p<0.05$, Figure 2.3A). The difference in productive infection induced by mDC and pDC was eliminated with the use of SEB (Figure 2.3B).

Latency was quantified by addition of phytohaemagglutinin (PHA) and IL-2 activated feeder PBMC to sorted non-proliferating (EGFP$^-$eFluor670^{hi}) CD4$^+$ T-cells (Figure 2.2B). After 5 days, EGFP expression was measured in PBMC as a surrogate marker of latent infection. We showed that latent infection was induced in the presence of mDC and not pDC ($p<0.05$, Figure 2.3C). In the presence of SEB, where there was enhanced DC-T-cell interaction, there was no increase in the amount of latent infection in non-proliferating CD4$^+$ T-cells that had been co-cultured with either mDC or pDC (Figure 2.3D). Post-integration latency in non-proliferating CD4$^+$ T-cells that had been co-cultured with mDC was confirmed using nested Alu-LTR nested PCR (Figure 2.3E, F; Butler et al. 2001).

Latent infection was also determined by the direct activation of sorted, non-proliferating (eFluor670^{hi}EGFP$^-$) CD4$^+$ T-cells with anti-CD3/CD28 in the presence of IL-7 (Figure 2.2C). The addition of PHA/IL-2 activated feeder PBMC can introduce variables such as different feeder PBMC donors, thaw of PBMC for each use and lot-to-lot variation of PHA. Anti-CD3/CD28 is

technically more consistent with the only variation being the in the infected donor cells themselves. There was no difference in detection of latent infection in resting CD4$^+$ T-cells when cultured with PHA/IL-2 activated feeder cells or direct anti-CD3/CD28 stimulation. Overall these results clearly demonstrate that mDC-T-cell co-culture facilitates the establishment of latent infection in non-proliferating CD4$^+$ T-cells

Figure 2.2. *In Vitro* DC-T-Cell Latency Model.

A. Resting CD4$^+$ T-cells were labeled with the cytoplasmic dye SNARF or eFluor670 to track proliferation as the intensity of the dye halves with each cellular division. T-cells were cultured alone or with a DC subpopulation, myeloid (m)DC or plasmacytoid (p)DC, at a 10:1 ratio for 24 hours before a 2-hour pulse with NL(AD8)Δ*nef*-EGFP reporter virus. Five days post infection, non-proliferating (eFluor670^{hi}), non-infected (EGFP$^-$) CD4$^+$ cells were sorted on the FACSAria. EGFP+ cells were quantified at day 5 as a measure of productive T-cell infection. **B.** Sorted, non-proliferating CD4$^+$ T-cells (SNARF^{hi}EGFP$^-$) were added to phytohaemagglutinin (PHA)/IL-2 activated PBMC, and cultured for a further 5 days to amplify latent signal. Representative facsplots of the number of EGFP expressing feeder cells at day 5 are shown, which represent latent infection. **C.** Alternatively, sorted non-proliferating CD4$^+$ T-cells were directly activated with anti-CD3/CD28 with IL-7 and integrase inhibitor (L8 or raltegravir) for 3 days as a direct measure of post-integration latency. Representative facsplots are shown. In the presence of integrase inhibitor, non-integrated forms of viral DNA are blocked from integration and expression, and there are no subsequent rounds of T-cell infection. As a control, sorted non-proliferating CD4$^+$ T-cells were also left unstimulated for 3 days in the presence of integrase inhibitor to measure unstimluated virus production.

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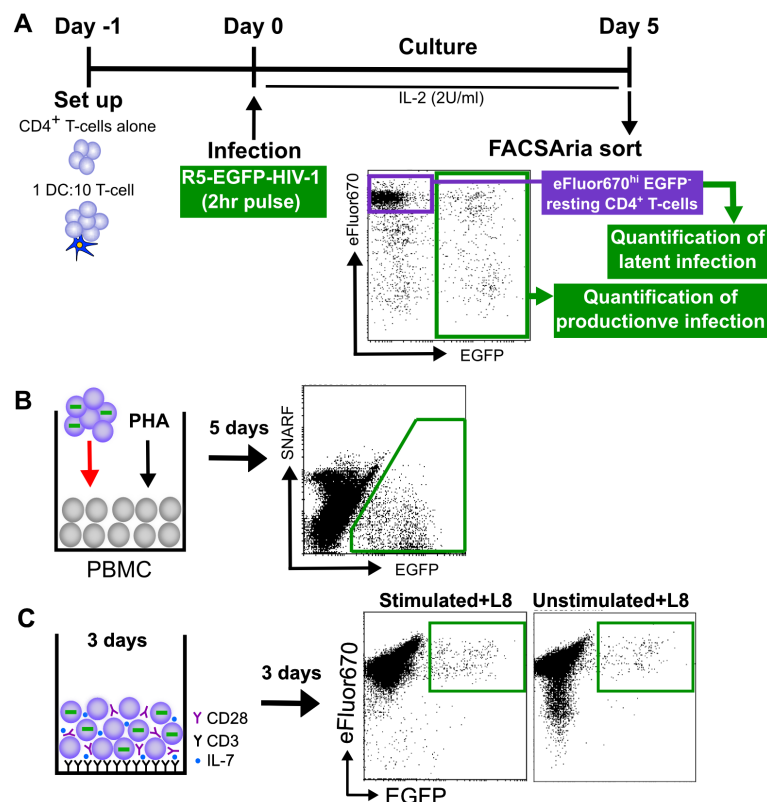
Figure 2.3. mDC Induce Latency in Non-Proliferating CD4$^+$ T-Cell Latency.

eFluor670 labeled resting CD4$^+$ T-cells were co-cultured with pDC or mDC at a ratio of 10:1, then infected for 2 hours with CCR5-EGFP reporter virus. **A&B.** Productive infection in total unstimulated (n=10) and SEB treated (n=2) cultures at day 5 post infection. **C&D.** Stimulation of sorted, non-proliferating (eFlour670^{hi}EGFP$^-$) CD4$^+$ T-cells showed an increase in EGFP expression, consistent with latent HIV-1 infection in resting CD4$^+$ T-cells. **E&F.** Integration latency was confirmed using nested Alu-LTR PCR (limit of detection 300 copies per million cells; n=3-4). Median is represented where n>5 and mean where n<3, dotted lines represented limit of detection. Significance was determined using paired students T-test where n>3 or <5 and Wilcoxon signed-ranked test where n≥5; *=p<0.05, **=p<0.005, ***=p<0.0005.
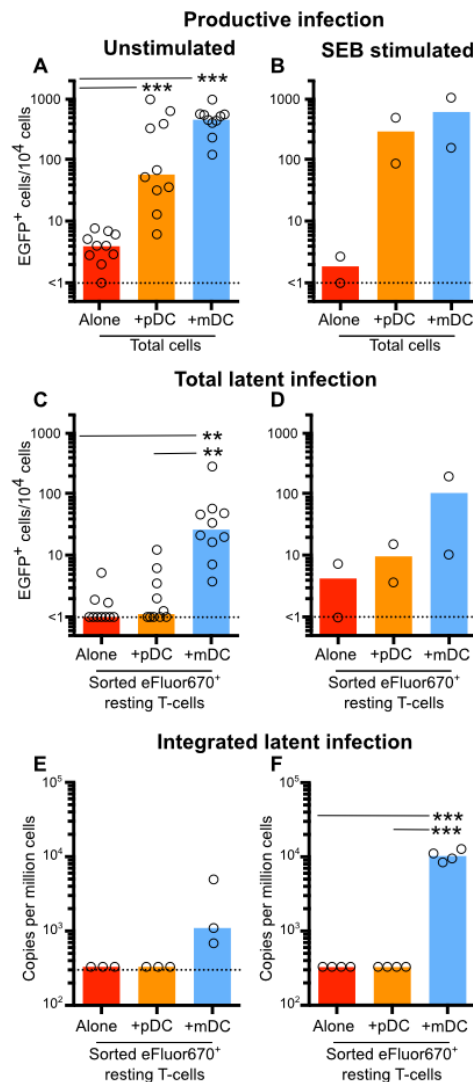
2.3.2. Titration of latent infection in the mDC-T-cell model with a R5 and X4 using virus.

To determine if induction of latent infection followed the same pattern with both CCR5 (R5) and CXCR4 (X4) viruses, we titrated both CCR5 (NL(AD8)Δ*nef*EGFP) and CXCR4 (NL4.3EGFP; *nef* competent) using virus in the *in vitro* mDC-T-cell latency model. Latent infection was measured by activation of non-proliferating CD4$^+$ T-cells using anti-CD3/CD28 and IL-7 with or without the addition of integrase inhibitor (L8) (Figure 2.2C). In the absence of L8, both pre and post-integrated virus is measured. Upon T-cell activation pre-integration complexes quickly become integrated and lead to expression of viral proteins, which in our system leads to EGFP expression. Addition of integrase inhibitor blocks integration of non-integrated DNA, and therefore measures only post-integration latency.

In order to compare the infectivity of R5 and X4 virus in establishment of latent infection, we cultured T-cells alone and infected with MOI concentrations ranging from 0.1-50 infectious particles (IP)/ml of R5 virus. High MOI concentrations (MOI>5) resulted in high levels of productive infection in T-cells alone, while total latent infection (pre- and post-integration latency) was only detected at an MOI of 5.0 and above (defined as >10 EGFP$^+$ cells/10,000 viable cells; Figure 2.4A, B). Post-integration latency was not observed at any of the MOIs tested (up to MOI 10) in T-cells alone, indicating that viral integration had not been established (Figure 2.4C). mDC-T-cell co-cultures had higher levels of productive infection in all MOI tested compared to T-cells cultured alone (MOI 0.1-50, Figure 2.4A, B). mDC co-cultured non-proliferating CD4$^+$ T-cells (MOI 0.1-50) also had high levels of post-integration latent infection at all MOI tested (Figure 2.4C). Together, these results indicated that *in vitro,* high CCR5 viral titers were able to facilitate only pre-integration latency in CD4$^+$ T-cells alone, however post-integration latency was established in the presence of mDC at a wide range of viral titres.

Next we infected T-cells alone or in the presence of mDC with X4 using virus at multiple MOI concentrations ranging from an MOI of 0.0005 to 2.0. T-cells cultured alone had detectable productive infection at an MOI of 0.01 and above (Figure 2.4D). While latent infection was stochastic at low MOIs and detectable above an MOI of 0.5 (MOI of 0.0005-0.05; Figure 2.4E). Stochastic latent infection is most likely attributed to high variability of CXCR4 expression on resting CD4$^+$ T-cells between donors. Detection post-integration latency in X4 infection in non-proliferating CD4$^+$ T-cells culture alone above an MOI of 0.5 (Figure 2.4E), was accompanied with virus production in unstimulated, sorted CD4$^+$ T-cells (virus production defined as >10 EGFP$^+$ cells/10,000 viable cells, Figure 2.4F). Expression of viral proteins, including EGFP, during unstimulated post-sort culture indicates that these CD4$^+$ T-cells are not latently infected, but productively infected. These observations indicate that X4 using virus can readily infect non-proliferating CD4$^+$ T-cells at low viral titers, however many of these cells progress to virus production not latent infection. Difference between virus expression from unstimulated and stimulated culture of non-proliferating CD4$^+$ T-cells shows that some inducible virus remains between an MOI of 0.05 and 2.0 in T-cells cultured alone (integrated latency). Together, these data indicate that there is a small population of non-proliferating CD4$^+$ T-cells containing integrated virus that may contribute to the latent reservoir, and some non-proliferating CD4$^+$ T-cells are in fact productively infected (Figure 2.4G).

Similar to R5 infection, latent infection was established in mDC co-cultured non-proliferating CD4$^+$ T-cells upon infection with X4 virus at all MOI concentrations. All mDC co-cultures infected with X4 virus had high levels of productive infection, and detectable total and post-integration latent infection (MOI > 0.0001; Figure 2.4E, F, G). The MOI of X4 virus necessary to induce latent infection in mDC co-cultured non-proliferating CD4$^+$ T-cells was much lower than the MOI required for R5 latent infection. For example, X4 infection at an MOI 0.01 resulted in 33-175 latently infected cells (per 10,000 viable cells; Figure 2.4B), whereas upon R5 infection at an MOI of 0.1, 10 fold higher

resulted in 15-30 latently infected cells (per 10,000 viable cells; Figure 2.4E). Virus production from unstimluated non-proliferating CD4$^+$ T-cells was high after MOI of 0.01 (>10 EGFP$^+$ cells/10,000 viable cells) in X4 infection, indicating productive infection. Therefore MOI of 0.01 would be ideal for any further experiments with X4 virus in the DC-T-cell *in vitro* latency model.

Lastly, infection with both X4 and R5 viruses of resting T-cells in the presence of mDC resulted in a plateau of the number of latently infected cells (X4 virus MOI>0.5, R5 virus MOI 0.1-10; Figure 2.4B, E, G). These data suggest that there may be a finite population of non-proliferating CD4$^+$ T-cells that can be infected in the presence of mDC following infection by both R5 and X4 viruses.

Figure 2.4. The effect of MOI changes on the establishment of latent infection in the presence of mDC.

eFluor670 labeled resting CD4$^+$ T-cells cultured alone (red) and with mDC (blue) were infected with increasing concentrations of R5 and X4 using virus, n=2-4. **A&D.** R5 and X4 productive infection was measured at day 5 post-infection by quantification of EGFP$^+$ cells. **B&E.** Post-integration latent infection was measured in sorted, non-proliferating (eFluor670^{hi}, EGFP$^-$) CD4$^+$ T-cells using anti-CD3/CD28+IL-7+IL-2 stimulation in the presence of integrase inhibitor, L8. **C.** Latency in R5 infection was amplified using anti-CD3/CD28+IL-7 and IL-2 stimulation in the absence of L8, allowing multiple rounds of infection. **F.** Unstimulated virus production, in the presence of L8, was measured upon X4 infection in sorted, non-proliferating (eFluor670^{hi}, EGFP$^-$) CD4$^+$ T-cells. **G.** Total post-integration latency upon X4 infection was quantified by subtraction of unstimulated virus production from stimulated virus production, indicative of integrated latent infection. Columns represent median, symbols represent results from individual experiments and different donors, dotted line indicates limit of detection. Significance was determined using paired students T-test where n≥3; *=p<0.05, **=p<0.005, ***=p<0.0005.

Figure 2.4. The effect of MOI changes on the establishment of latent infection in the presence of mDC.
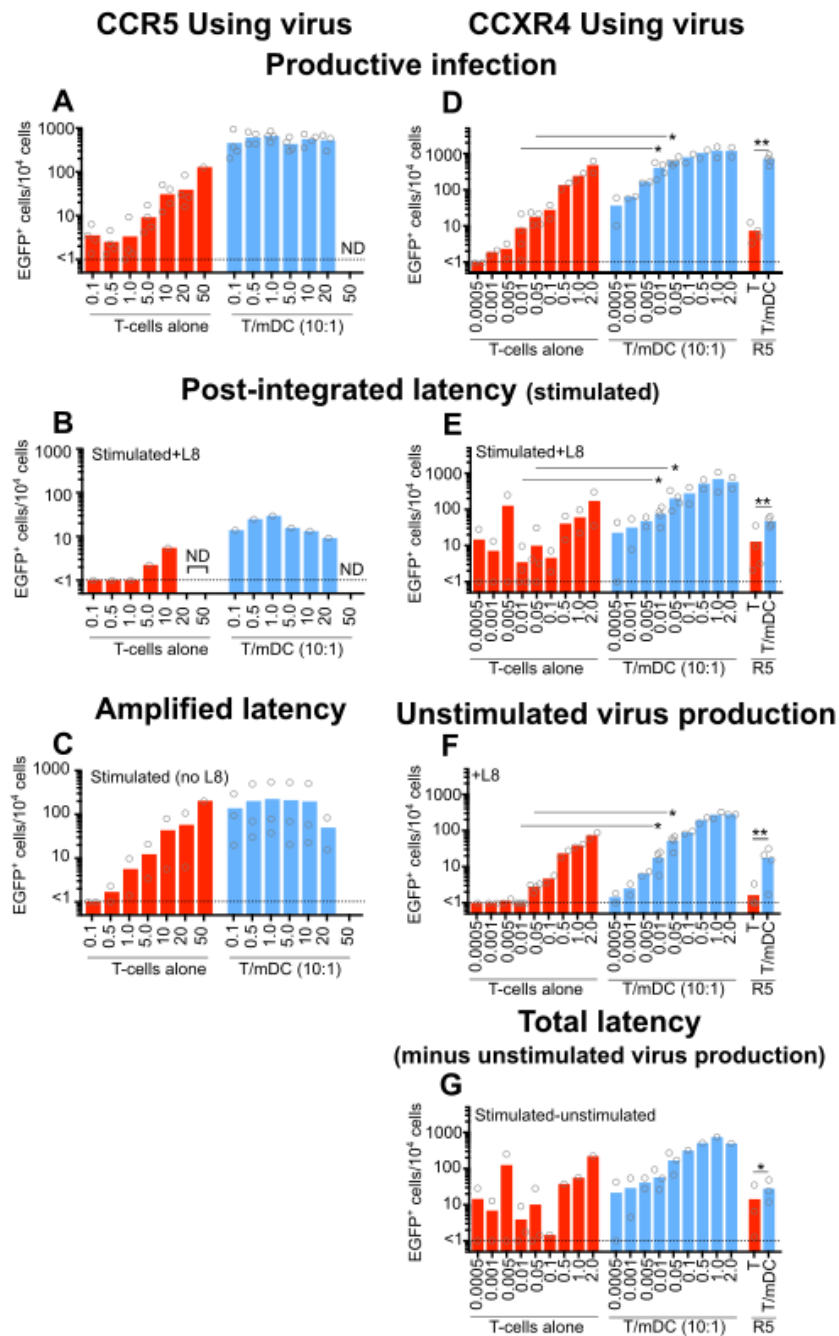
2.3.3. mDC induce latency in memory but not naïve CD4$^+$ T-cells

To determine whether mDC were able to induce latency in naïve or memory CD4$^+$ T cells, we sorted bulk resting T-cells into memory (CD45RA$^-$) and naïve (CD45RA$^+$) populations (purity >98%; Figure 2.1A). Cells were then cultured and infected as shown in Figure 2.2A. mDC and pDC co-culture led to an increase in productive infection in all T-cell subpopulations compared to T-cells infected alone (bulk T cells p=0.009, memory T-cells p=0.01; Figure 2.5A). Latent infection, measured as EGFP expression following the addition of PHA/IL-2 activated feeder PBMC, was only induced in memory CD4$^+$ T-cells (Figure 2.5B). Latent infection could not be established in naïve T-cells following infection of either naïve T-cells cultured alone, with pDC or mDC.

Figure 2.5. Central Memory CD4^{+} T-Cells are Susceptible to Myeloid Dendritic Cells Induced Latent Infection.

A. Bulk resting, central memory and naïve CD4^{+} T-cells were cultured alone (red), co-cultured with pDC (orange) or mDC (blue) and infected with EGFP reporter virus. The number of EGFP+, productively infected cells is shown (n=4-5). **B.** Latent infected was quantified following stimulation of non-proliferating (eFluor670^{hi}EGFP^{-}) CD4^{+} T-cells using phytohaemagglutinin (PHA) activated feeder PBMC (n=3-5). Columns represent median, symbols represent results from individual experiments and different donors, dotted line represented limit of detection. Significance was determined using paired students T-test where n is between 3-5; *=p<0.05, **=p<0.005, ***=p<0.0005.
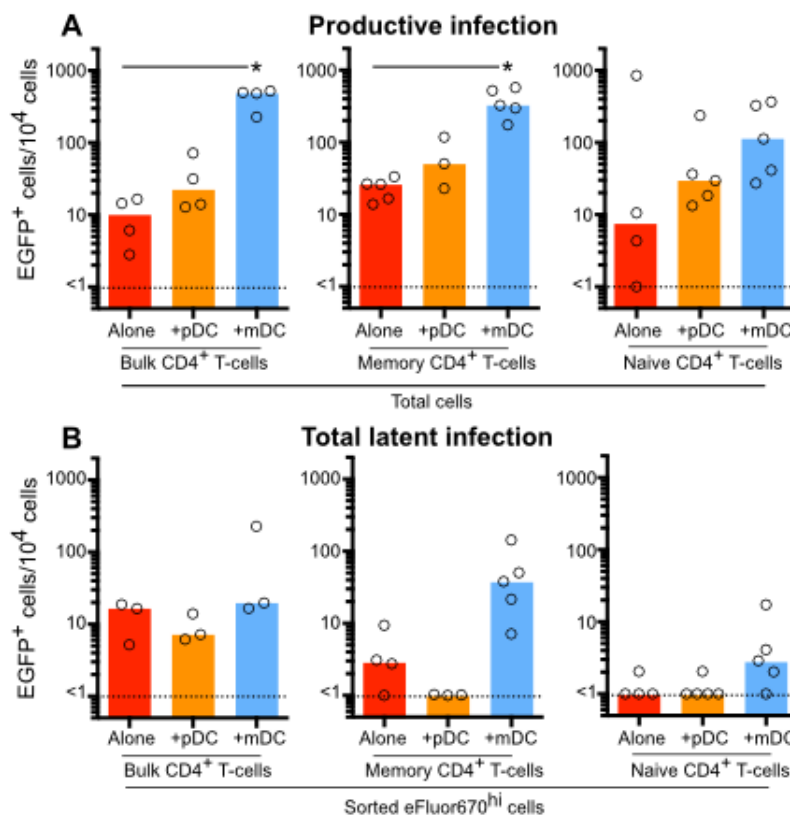
2.3.4. Myeloid Dendritic Cells-induced T-Cell Latency is not Mediated by a Known Soluble Factor.

Upon mDC co-culture with CD4$^+$ T-cells several DC-T cell contacts are established, these lead to increased DC-T-cell proximity, cognate interactions (MHC-class-II and TcR) and secretion of multiple soluble factors. All these interactions could be involved in the induction of HIV-1 latency within mDC-T-cell co-cultures. To determine the importance of mDC derived soluble factors and mDC-T-cell contact in mDC-T-cell co-cultures, mDC were cultured within transwells above the resting CD4$^+$ T-cells. Transwells allow only mDC derived soluble factor and T-cell-to-T-cell interactions in the co-culture system. In the presence of the transwell, mDC were unable to induce latent infection, as measured by quantification of EGFP following the addition of PHA/IL-2 activated feeder PBMC (p=0.06, Figure 2.6A). It was possible that the soluble factors secreted by the mDC were not high enough in concentration around the T-cells due to a very short half-life. Therefore to confirm a role for mDC derived soluble factors, supernatants from mDC-T-cell co-cultures were added to CD4$^+$ T-cells cultured alone, and then these supernatant treated cells were infected with EGFP virus. mDC derived soluble factors did not lead to the establishment of latent infection (Figure 2.6B).

Figure 2.6. Soluble Factors are not Essential in Myeloid Dendritic Cells Induced Resting T-Cell Latency.

A. eFluor670 labeled resting CD4^{+} T-cells were cultured alone (red) or with blood mDC (blue). Virus was added to CD4^{+} T-cells alone, CD4^{+} T-cells cultured with mDC (light blue) and CD4^{+} T-cells cultures with mDC within a 0.4mm membrane transwell (dark Blue; n=5). Latent HIV-1 infection was quantified at day 5 post-infection by measuring EGFP following stimulation with phytohaemagglutinin (PHA) activated feeder PBMC (n=5). **B.** CD4^{+} T-cells were cultured alone (red) or with blood mDC (blue) and infected. Following 24 hours, supernatant from infected mDC-T-cell co-cultures was added to uninfected CD4^{+} T-cells (dark blue), which were then infected (n=3). Columns represent median, symbols represent results from individual experiments and different donors and dotted line represents the limit of detection. Significance was determined using a student T-test where n<5 and a Wilcoxon signed-ranked test where n≥5. ***=p<0.05. Dr Vanessa Evans generated this data.
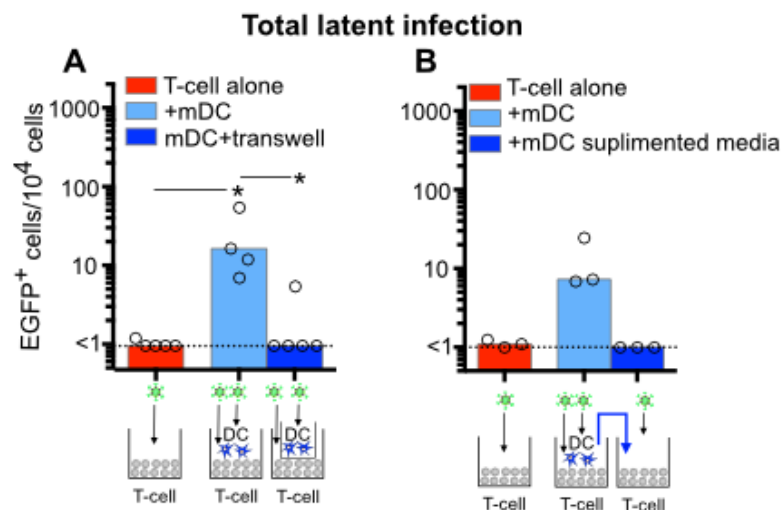
Our lab had previously demonstrated a role for chemokines in the induction of latency, therefore next we explored if these chemokines were required for mDC induced latency (Saleh et al. 2007; Cameron et al. 2010). Neutralising antibodies to the chemokines CCL19, CXCL10 and chemokine receptor CXCR3 (receptor for CXCL9, CXCL10 and CXCL11) were examined, as each chemokine had been shown to induce latency following culture with resting CD4$^+$ T-cells (Cameron et al. 2010). Neutralizing antibody to these specific chemokines was first tested and titrated using a migration assay (Figure 2.7A,B, C). Neutralizing antibody against the chemokines was then added at the time of mDC-T-cell co-culture and re-added post-infection, therefore chemokine signalling was inhibited for the duration of culture period. There was no change in productive (Figure 2.7D-F) or latent infection (Figure 2.7H-J) following addition of these antibodies, either alone or in combination (Figure 2.7G, K). Together, these experiments suggested that physical contact between mDC and T-cells is more important for mDC-induced latent infection.

Figure 2.7. Dendritic Cell-derived chemokines are not important in mDC induced latency in non-proliferating CD4$^+$ T-cells.

A,B&C. A migration assay where T-cells from an upper well migrate to lower well in the presence of chemokine and neutralizing anti-bodies was performed to confirm activity of the antibody. Total or memory CD4$^+$ T-cells were added to the top chamber of a 3μm pore migration well and chemokines (CCL19, CXCL10 or CXCL9) were added to the bottom chamber (n=4 and 2 respectively). T-cell cultured without chemokine treatment or with only CCL19 are shown in light blue, T-cells treated with CCL19 and IgG antibody are shown in blue and T-cells treated with CCL19 and neutralising antibody are shown in dark blue. After 6-24 hours the percentage of migrated cells was determined. **D-K.** CD4$^+$ memory T-cells were cultured alone (red) or with mDC (light blue), mDC-memory CD4$^+$ T-cell co-cultures were treated with either anti-IgG (blue) or neutralizing antibody to CCL19, CXCL10 and chemokine receptor CXCR3, alone or in combination (dark blue). Antibody was added at the beginning of co-culture and replenished post-infection. **D-G** Productive infection was quantified at day 5 by measurement of EGFP+ cells (n=6) and **H-K** latency was quantified by measurement of EGFP expression following stimulation of sorted, non-proliferating (eFluor670^{hi}EGFP$^-$) CD4$^+$ T-cells (n=6). Columns represent the median, symbols represent results from individual experiments and different donors and dotted line represents the limit of detection. Significance was determined using a student T-test where n<5 and a Wilcoxon signed-ranked test where n≥5. *=p<0.05, **=p<0.005, ***=p<0.0005.

Figure 2.7: Dendritic Cell-derived chemokines are not important in mDC induced latency in non-proliferating CD4$^+$ T-cells.
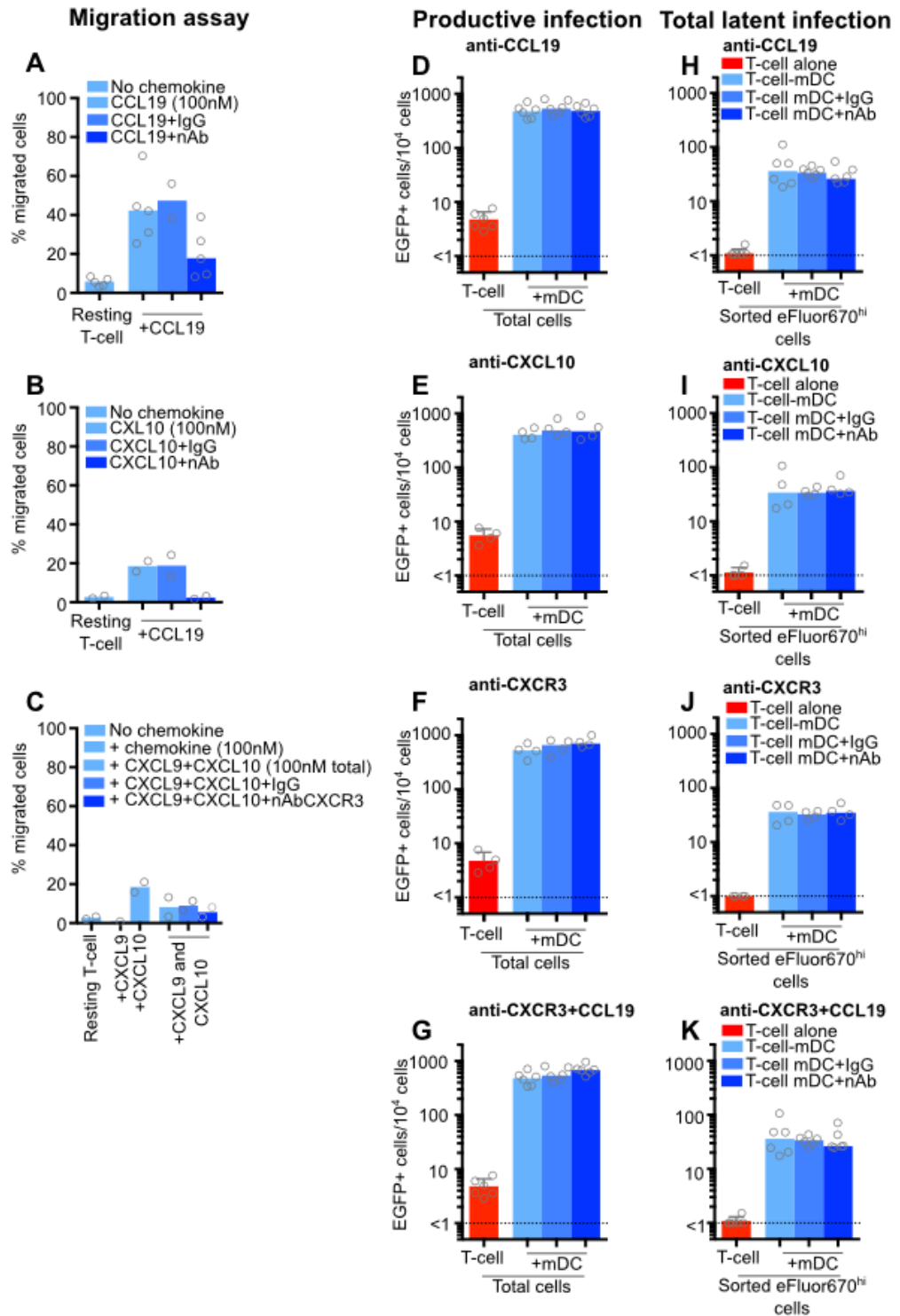
2.3.5. Myeloid Dendritic Ccell-T-cell Contact is Important in mDC Induced Latency

To test the role of cell contact in the mDC-T-cell latency model, cell adhesion was inhibited by neutralization of CD18. CD18 dimerises with CD11a to form lymphocyte function-associated antigen-1 (LFA-1) and interacts with intracellular adhesion molecule (ICAM)-1 or 3 (Fawcett et al. 1992; Stent et al. 1994; Verma et al. 2012). Using microscopy we demonstrated a reduction, but not elimination, of cell clusters in mDC-T-cell co-cultures incubated with anti-CD18 (Figure 2.8A). Co-staining with a second, conjugated anti-CD18 antibody of the same clone showed inhibition of CD18 staining, consistent with successful neutralization (Figure 2.8B, C). Upon CD18 neutralisation in mDC-T-cell co-cultures we found no decrease in productive infection (Figure 2.8D), however there was a small but significant decrease in the number of latently infected cells ($p > 0.01$, Figure 2.8E). Therefore latency was inhibited but not eliminated in the presence of anti-CD18. This was likely because despite LFA-1 inhibition, mDC express many other receptors that can maintain DC-T-cell contact including ICAM-1, 2 and 3 (Herman et al. 2012; de la Fuente et al. 2014). Collectively these results show that cell-cell contact in mDC-induced latency is important.

Figure 2.8. Reduced Cell Contact Partially Inhibited mDC Induced Latency in Resting CD4$^+$ T-cells.

eFluor670 labeled resting CD4$^+$ T-cells were cultured alone (red), with mDC (light blue) and treated with neutralizing anti-CD18 anti-body (dark blue) to block cell interactions in the DC-T-cell *in vitro* model. **A.** Representative, bright-field images of T-cells and mDC-T-cell co-cultures following the addition of anti-CD18. **B.** To quantify neutralization by the anti-CD18 antibody we used a secondary, PE conjugated anti-body of the same clone and **C.** quantified CD18-PE expression in the presence and absence of neutralizing anti-CD18 on CD4$^+$ T-cells cultured alone (red), in the presence of mDC alone (light blue), treated with IgG (blue) and anti-CD18 (dark blue) at day 5 post-infection. **D.** Productive infection was measured at day 5 post-infection **E.** Latency was measured on sorted non-proliferating CD4$^+$ T-cells by quantification of EGFP following stimulation with phytohaemagglutinin (PHA) activated feeder PBMC. Columns represent median, symbols represent results from individual experiments and donors and dotted line represents the limit of detection. Significance was determined using a student T-test where n<5 and a Wilcoxon signed-ranked test where n≥5. *=p<0.05, **=p<0.005, ***=p<0.0005.
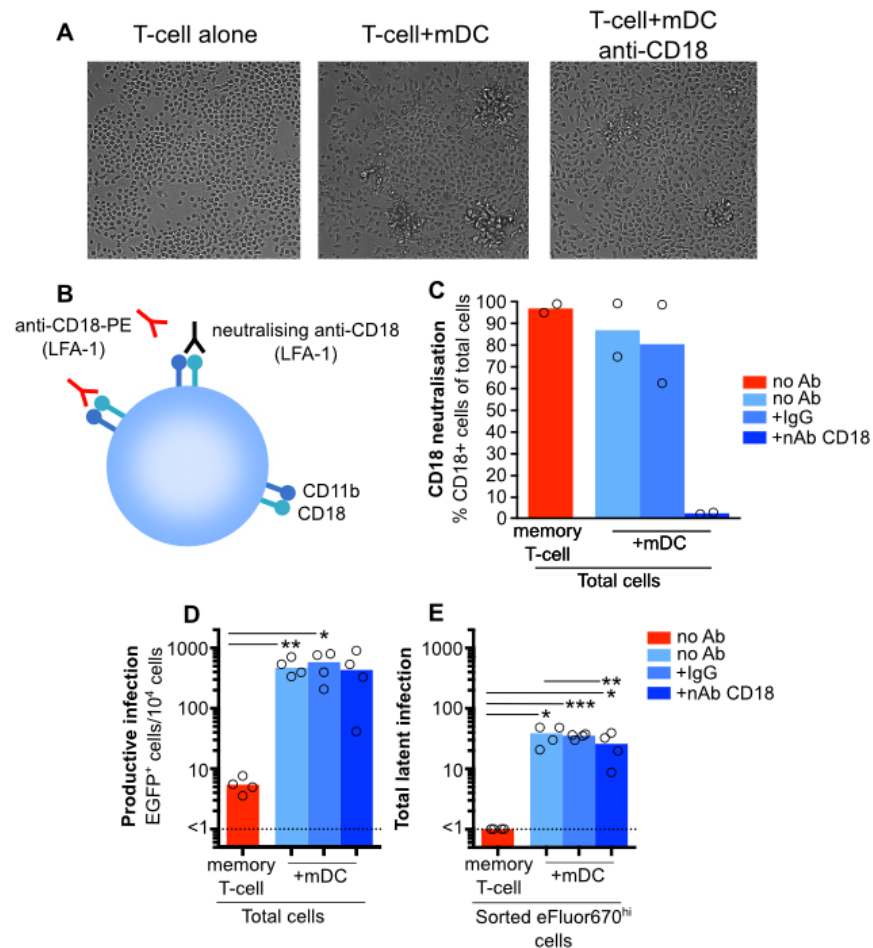
2.4. **Discussion:**

HIV-1 latency is difficult to study due to low numbers of latently infected cells *in vivo* and, until recently, the lack of physiologically relevant *in vitro* models. Here we describe a physiologically relevant model using primary DC and resting CD4$^+$ T-cells that mimic DC-CD4$^+$ T-cell interactions in the LN. Specifically we demonstrate that mDC are able to induce latent infection in central memory CD4$^+$ T-cells with both R5 and X4 using viruses. Furthermore, we show that cellular interactions are important in the induction of latency by mDC.

2.4.1. **Myeloid Dendritic Cell Induced Latency is Mediated by Cellular Interactions.**

Induction of latent infection by mDC and not pDC suggests that either mDC provide a stimulus that leads to latent infection, which are absent in pDC or that pDC are able to inhibit latency. Here we tested the former showing that soluble factors, including chemokines previously shown to be important in the induction of latency in resting CD4$^+$ T-cells, were not involved in mDC induced latent infection (Cameron et al. 2010). Despite our extensive testing it may still be possible that the soluble factor, or a combination of soluble factors may be involved in latency induction, but the specific factor was not evaluated in our experiments.

DC can mediate T-cell interaction by cellular adhesion molecules (ICAM-1 [CD54], ICAM-3 [CD50], CD2; Reviewed in Long 2011; Verma et al. 2012), cognate interactions (MHC-class-II-TcR interactions; Weissman et al. 2000; Chen et al. 2012), immune checkpoint blockers (ICB; PD-1 [CD279], TIM-3 [CD366], CTLA [CD152]; Day et al. 2006; Del Rio et al. 2008) or co-stimulators of T-cell activation (CD28, CD40; Hargreaves et al. 2004; Gao et al. 2013; Reviewed in Chen & Flies 2013). We show that neutralization of adhesion molecule, LFA-1, on T-cells led to a small but significant reduction of latency, indicating the importance of cell proximity of mDC and T-cells to

the induction of latency. It is possible that latency failed to be completely eliminated with neutralization of CD18 as there was other cell interactions involved in DC-T-cell contact, including other adhesion molecules (integrins; Schürpf & Springer 2011), C-type lectin domain (CLEC) family of proteins (Huysamen et al. 2009; Reviewed in Plato et al. 2013; Lundberg et al. 2014), sialic acid-binding immunoglobulin-type lectins (SIGLEC) family of proteins (Nguyen et al. 2006), co-stimulatory molecules (Scholler et al. 2001), MHC-I, MHC-II and other lipid presentation molecules (CD1c, CD1d; Brutkiewicz 2006; Facciotti et al. 2011; Yu & Milstein 1989). Recently, the adhesion molecule CD2 was found to be highly expressed on latently infected cells *in vivo* and *in vitro* (Iglesias-Ussel et al. 2013), suggesting that these molecules are important in the regulation of latent infection. The role of cellular interactions in the transfer of productive infection is well known, with the expression of ICAM-1 and 3 positively correlated with increased transfer of infection (Barat et al. 2004; Wang et al. 2009), formation of virological synapses (Jolly et al. 2007; Martin et al. 2010), efficient cell-cell spread (Turville et al. 2004; Groot et al. 2006; Sigal et al. 2011) and viral dissemination (Murooka et al. 2012). The role of adhesion molecules in latent infection remains unclear; however together these data build a picture that cell interaction is important in establishment of latent infection.

The interactions between mDC and non-proliferating CD4$^+$ T-cells, via LFA-1 or CD2, could lead to activation of common signalling pathways that facilitate efficient infection of resting CD4$^+$ T-cells by several mechanisms. These include enhancing speed and efficiency of reverse transcription and nuclear import, as occurs with virus induced CXCR4 signalling (Yoder et al. 2008), stabilisation of protein-protein interactions required for completion of the viral life cycle as described for JNK and Pin1 with HIV-1 integrase following activation of the PKC pathway (Manganaro et al. 2010), opening of chromatin complexes to allow integrase and pre-integration complex access to host DNA (Zack et al. 1990), expression of a novel factor which blocks productive

infection or promotes latent infection in resting T-cells, or by a novel mechanism. Further work to define these pathways is described in Chapter 3.0.

CD18 can combine with the integrins CD11a, b, c or d to bind to the ICAM family of adhesion molecules. Specifically, CD18 and CD1a combine to form LFA-1, which binds ICAM-1 (Reviewed in Shimizu 2003). In the *in vitro* DC-T-cell latency model, mDC to T-cell signalling leads to the induction of latency in resting memory CD4$^+$ T-cells. We hypothesise that the induction of latency is mediated by signalling events that either actively allow the integration of virus or passively inhibit viral expression, thereby leading to latent infection. While blockade of CD18 on DC will block DC-T-cell interaction, it will allow CD18 expressed on the CD4$^+$ T-cells to couple with other integrins on the T-cell and mediate cell adhesion and signalling via other receptors. On the contrary, blockade of CD18 on the T-cell, as we have done, blocks the ability of CD18 to combine with other integrins, thereby preventing cell adhesion and signalling through the CD18 molecule. In the event that downstream pathways of the CD18 interaction are important in the induction of latency, latency will be reduced, as shown in Figure 2.8. Therefore, blockade of CD18, and other receptors are more important on T-cells rather than the mDC as they provide a direct measure of function of CD18 in the events that lead to latency induction.

2.4.2. Viral Entry and Viral Titers

Virus mediated activation of co-receptors also leads to enhanced viral infection. Other groups have shown that with increased viral input and availability of CXCR4 on activated and resting T-cells (Balabanian et al. 2004; Guo et al. 2011), or CCR5 on myeloid derived dendritic cells (MDDC; Weissman et al. 1997; Platt et al. 1998), leads to more efficient HIV-1 entry and infection (Davis et al. 1997; McDonald et al. 2003; Reviewed in Wu & Yoder 2009). Our results add to these observations showing that pre-integration latency was increased in resting CD4$^+$ T-cells that were exposed to high viral titres. However, the establishment of post-integration latency

required additional signalling, here by the mDC and as we have previously described through the chemokine treatment with CCL19 (Saleh et al. 2007).

We show that even at low MOIs, mDC were able to potently induce latent infection in resting CD4$^+$ T-cells, the specific pathway involved through mDC-T-cell interactions that led to latent infection remains unclear. We have previously shown that direct DC infection, *cis*-infection, is not important in establishment of latency in the mDC-T-cell model (Evans et al. 2013). *Trans*-infection occurs when HIV-1 is carried on the outside of the DC without infection of the DC itself, via HIV-1 interaction with C-type lectins like DC-SIGN, mannose receptor (MMR) and the CLEC family of proteins, allowing close interaction between HIV-1 virions and uninfected T-cells (Geijtenbeek et al. 2000; Turville et al. 2002). It is possible that HIV-1 virions can stick to the mDC, mediating small concentrations of virus to contact with large proportions of CD4$^+$ T-cell in the background of DC interaction, thereby facilitating viral entry and latent infection at all MOI concentrations.

2.4.3.   **Subpopulations of Susceptible T-Cells**

We showed that resting central memory CD4$^+$ T-cells, and not naive CD4$^+$ T-cells were permissive to latent infection by R5 virus, similar to findings in other models of latent infection (Swiggard et al. 2005; Saleh et al. 2007; Shen et al. 2013; Dai et al. 2009). We do not expect this to be different with X4 virus as both central memory and naïve T-cells express similar amounts of both CCR5 and CXCR4 (Bleul et al. 1997; Lee et al. 1999). Increased *in vitro* susceptibility of central memory CD4$^+$ T-cells has previously been attributed to the increased receptiveness to activation stimuli of central memory CD4$^+$ T-cells compared to naïve T-cells (Dai et al. 2009). Together this data suggest that pathways involved in T-cell activation are important in latency establishment.

2.4.4. Comparison of DC-T-Cell Latency with Other Models of Latency

In vitro models of HIV-1 latency allow the study of specific mechanisms of latency establishment. The DC-T-cell latency model is unique in that we use primary DC and resting CD4^{+} T-cells thereby mimicking physiologically relevant interactions that occur in LN and the periphery and, we use replication competent virus where multiple rounds of infection can occur (Bosque & Planelles 2009; Jordan et al. 2003; Sahu et al. 2006; Tyagi & Karn 2007; Yang et al. 2009). Like other studies, we explored physiologically relevant signalling events, like DC interaction, endothelial cell interaction and MHC-II (anti-CD3/CD28) stimulation (Bosque & Planelles 2009; Marini et al. 2008; Shen et al. 2013). These *In vitro* models mimic the complexity of *in vivo* cell interactions, which clearly differ from cell lines and mutated viruses. In the DC-T-cell latency model, we were only able to establish a low frequency of latent infection, 1-0.1%, compared other models of direct resting T-cell latent infection of 4-5% (Swiggard et al. 2005) and 2-20% established in activated T-cells reverted to a resting state (Bosque & Planelles 2009; Marini et al. 2008; Sahu et al. 2006; Tyagi & Karn 2007; Yang et al. 2009). Although this model is not technically easy compared to others, it provides an optimal screening tool for potential latency eliminating therapies in a physiologically relevant setting, for future cure studies.

2.4.5. Using EGFP as a Measure of Replication Competent Virus

In the mDC-T-cell latency model only replication competent latent virus was measured, that is virus that had successfully integrated and was able to produce new virions. Based on observations in HIV-1-infected patients on cART by Ho et al., latent reservoir contain a significant proportion of non-inducible virus. These viruses have intact sequences, and could be re-activated upon repeated stimulation (Ho et al. 2013). Future work in this model could use Alu-LTR to quantify total integrated DNA and then determine the proportion of inducible latency that could be stimulated to express EGFP.

2.4.6.   **Conclusion and significance**

The ability of mDC to induce efficient latent infection of resting CD4$^+$ T-cells could explain the rapid, early establishment of latently infected cells in sites such as lymphoid tissue. Here we showed that blood derived mDC are able to induce latent infection in non-proliferated CD4$^+$ T-cells and, this was mediated by DC-T-cell contact. Ongoing transfer of virus from mDC to resting T-cells in tissue sites may be one pathway to replenish or maintain latently infected cells in patients on cART.

2.5. **Materials and methods**

2.5.1. **Isolation of T-cell Subsets and Dendritic Cells Subsets**

Human PBMC were isolated from buffy coats obtained from the Australian Red Cross by ficoll-paque density gradient (GE Healthcare, Chalfont St. Giles, United Kingdom). An aliquot of PBMC was used for resting CD4$^+$ T-cell isolation as previously described (Evans et al. 2013; Saleh et al. 2007; Cameron et al. 2010). Briefly, cells were incubated with hybridoma cocktail containing anti-CD11b, CD8, CD14, CD16, CD19, HLA-DR, CD69 and Gly-A, then labeled with IgG beads (Miltenyi, Biotech, Bergisch Gladbach, Germany) and isolated using magnetic bead depletion on an AutoMACS (Miltenyi; Figure 2.1A). Only cells with a purity of >98% were used, with routine checks of CD69 and HLA-DR contaminants (Figure 2.1A). Isolated CD4$^+$ resting T-cells were stained with cytoplasmic dye eFluor670 (eBiosciences, San Diego, CA) according to manufacturer's protocol. Briefly, serum was washed from cells before incubating at 37°C with 5μM eFluor670. Cells were then washed thoroughly with media.

DC were isolated as previously described (Evans et al. 2013). Briefly, PBMC were stained with hybridoma cocktail consisting of anti-CD19, CD3, CD11b and Gly A, then labeled with IgG microbeads (Miltenyi) and depleted on the AutoMACS (Miltenyi). The enriched DC population was stained with HLA-DR, CD11c and CD123 (BD Biosciences) for further sorting by flow cytometry (BD FACSAria) for mDC (HLA-DR$^+$, CD11c$^+$, CD123$^-$) and pDC (HLA-DR$^+$, CD11c$^-$, CD123$^+$; Figure 2.1B). Cells with purity ≥95% were used.

2.5.2. **Preparation of Viruses**

In most experiments CCR5 (R5) virus was used where AD8 envelope was inserted into the HIV-1 NL4.3 backbone with an EGFP insertion in the *nef* open reading frame at position 75 (NL(AD8)Δ*nef*EGFP; (Evans et al. 2013), kindly provided by Damian Purcell from the University of Melbourne (Melbourne, Australia). Where CXCR4 (X4) virus was used, EGFP was inserted with an IRES element for co-expression with *nef* (NL4.3EGFP),

kindly provided by Takuya Yamamoto from the National Institute of Infectious Diseases (Shinjuku, Japan; (Yamamoto et al. 2009).Viral stocks were generated by FuGene6 (Promega, Madison, WI) transfection of 293T cells as previously described (Evans et al. 2013; Saleh et al. 2007). DC-T-cell co-cultured cells were infected at a multiplicity of infection (MOI) of 0.5, unless otherwise stated. MOI was determined on PHA activated human PBMC using the Reed and Muench method, also called the tissue culture infectious dose (TCID50; Reed & Muench 1938). To ensure consistency of MOI between X4 and R5 viruses, and limit batch-to-batch variation, all TCID50 experiments, were performed along side a reference virus.

2.5.3.   Co-culture and Infection

eFluor670 labeled resting CD4$^+$ T-cells were cultured alone or with syngeneic mDC or pDC at a ratio of 10 T-cells:1 DC in IL-2 (2U/mL, Roche Diagnostics) supplemented media (Figure 2.2). Cells were infected with NL(AD8)Δ*nef*EGFP reporter virus for 2 hours, unless stated otherwise. At day 5 post-infection, all co-cultured cells were sorted by flow cytometery (FACSAria, BD Biosciences) for non-proliferating (eFluor670^{hi}), non-productively infected (EGFP$^-$) cells. At the time of sort, total cells in co-culture were also analysed for productive infection, measured by EGFP expression (Figure 2.2A).

2.5.4.   Quantification of Latent Infection

Latent infection was detected from 1×10^5 sorted eFluor670^{hi}EGFP$^-$ cells by addition of phytohaemagglutinin (PHA) and IL-2 (10U/ml, Roche) activated allogeneic feeder PBMC at a ratio of 1 sorted T-cell: 5 feeder PBMC for 5 days. The number of EGFP$^+$ feeder cells was used as a surrogate measure for latent infection in the sorted resting CD4$^+$ T-cells (Figure 2.2B). Total and post-integration latent infection was directly measured by activation of 1×10^5 sorted eFluor670^{hi}EGFP$^-$ CD4$^+$ T-cells with immobilized anti-CD3 (7ug/mL; Beckman Coulter), in RF10 media supplemented with CD28 (7 ug/mL, BD Biosciences), IL-7 (50ng/mL; Sigma, St Louis, MO) and with (post-integration latency) or without (total latency: pre- and post-integration latency) integrase

inhibitor L8 (1μM/mL, Merck) for 3 days (Figure 2.2C). Latency was measure in all cells by quantification of EGFP expression using by flow cytometry (FACS Calibur; BD Biosciences).

2.5.5. **Measurement of surface markers for cellular activation and subpopulations**

Expression of various surface markers was determined using specific antibodies listed in Table 2.1. Cells were stained in a total volume of 100ul with previously titrated volume of antibody for 25-30 min, on ice (4°C). Cells were then washed and fixed with 100ul of 1% formaldehyde. Samples were analysed on a flow cytometer, either FACS Calibur or the LSR-II (BD Biosciences), and data was analysed on Weasel software (WEHI, Melbourne, Australia).

Table 2.1. Measured surface markers

	Clone	Function	Source	References
CD25-PE	2A3	Early T-cell activation maker and IL-2 receptor	BD Biosciences	(Schiott et al. 2004; Reviewed in Shipkova & Wieland 2012)
CD69-FITC	L78		BD Biosciences	
HLA-DR-APC-Cy7	L243	MHC class II molecule and late marker of T-cell activation	BD Biosciences	(Bains et al. 2003; MacDonald et al. 2002)
CD80-FITC	B7-1	Co-stimulatory marker expressed by DC	eBioscience	(MacDonald et al. 2002)
CD86-APC	IT2.2		BioLegend	
CD83-PE	HB15e	Marker of DC maturation	BioLegend	
CCR5	CTC5	Chemokine receptor, HIV Co-receptor	BD Biosciences	(Bleul et al. 1997, p.5)
SLAN-FITC	DD-1	Specific DC subset maker on $CD16^+$ DC	Miltenyi, Biotech, Bergisch Gladbach, Germany	(Schäkel et al. 1999, p.8; Schäkel et al. 2002; Siedlar et al. 2000, p.8)
CD141-VioBlue	AD5-14H12	Specific DC subset marker	Miltenyi, Biotech, Bergisch Gladbach, Germany	(Dzionek et al. 2000)
CD11c-APC	B-146	Adhesion molecule	BD Biosciences	(Osugi et al. 2002)
CD123-PE	9F5	Specific DC subset marker	BD Biosciences	(Dzionek et al. 2000)
CD1c-APC	AD5-8E3	Lipid presentation molecule, specific DC subset marker	Miltenyi, Biotech, Bergisch Gladbach, Germany	(Piccioli et al. 2007)

2.5.6.   Transwell Experiments

A culture insert with 0.4um pores (transwell; BD, Franklin, NJ, USA) was used to physically separate mDC and resting CD4$^+$ T-cells where mDC were cultured above the resting T-cells. Following 24 hours of culture, cells were infected as described previously in section 2.5.3 and experiment was continued with mDC in the culture insert.

2.5.7.   Supernatant Transfer Experiments

Swapping supernatant from infected mDC-T-cell cultures to uninfected resting CD4$^+$ T-cells tested the role of DC-derived soluble factors. Supernatant from uninfected mDC-T-cell cultures were added to the resting T-cell culture's daily to replenish DC-derived soluble factors.

2.5.8.   Imaging of DC-T-Cell Co-cultures

mDC-T-cell co-cultures were treated with neutralizing anti-CD18 antibody to limit cell proximity. Cultures were infected for 5 days as previously described in section 2.5.3. Prior to culture sort, 5 representative images were taken in brightfield (X10; DeltaVision Deconvolution Microscope, with EMCCD camera; Applied Precision) and annotated on ImageJ software (NIH, Bethesda, Washington DC, USA).

2.5.9.   Migration Assay

A migration assay was used to titrate anti-chemokine (CCL19, CXCL10) and anti-chemokine receptor (CXCR3) antibodies. One million resting T-cells were added to the top chamber of a migration plate in 200uL media (24 well, upper chamber containing polyester filter membrane of 3um pore size; Costar®, Corning Incorporated, Corning, New York). Chemokines, CCL19 (100nM; R&D), CXCL10 (300nM; R&D) and CXCL9 (300nM; R&D) alone or in combination (total 300nM chemokine) were added directly to the lower chamber of a migration plate with a final volume of 700ul. Neutralizing antibodies against chemokines were added to the bottom chamber (anti-CCL19, anti-CXCL10; R&D), while chemokine receptor anti-CXCR3 antibody (R&D) was pre-incubated with resting memory CD4$^+$ T-cells for 10

minutes prior to addition to the top chamber. The assay plate was incubated for 6-24 hours at $37^{o}C$ in 5% CO^{2}. Cells that had migrated to the lower chamber were quantified at 6, 12 and 24 hours using a haemocytometer with a bright field microscope (Olympus CKX31, Olympus, Japan). Total migrated cells were calculated as a percentage of the amount of total cells added to the upper chamber of the migration plate.

2.5.10. Statistical Analysis

Differences between experimental conditions were analysed using Wilcoxon signed rank test (n≥5) or paired student T-test (n<5) on Graphpad Prism (version 6). P-values of <0.05 were considered significant.

Disclaimer of author contributions:

Most of the data presented here has been published in PLoS Pathogens with Dr Vanessa A Evans (VE) as first author (Section 8.1; Evans et al. 2013). The work presented here is my contribution to this paper. Figure 2.1 was adapted from figures produced by VE. Figure 2.3A-D is a summary of my experiments to replicate the data initially generated by VE. Figure 2.3E-F and Figure 2.6 is experiments completed by VE.

3.   Chapter 3

The Role of Antigen Presenting Cells in the Induction of HIV-1 Latency in Resting CD4$^+$ T-Cells

Kumar, NA., Cheong, K., Powell DR., Pereira, CdF., Anderson, J., Evans, VA., Lewin, SR., Cameron, PU. (2015) The role of Antigen Presenting Cells in the Induction of HIV-1 Latency in Resting CD4+ T-cells. Retrovirology 2015:12(76)

Monash University

Declaration for Thesis Chapter Three

Nitasha Kumar

In the case of Chapter Three, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Experiments	95%
Analysis	90%
Writing	90%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Miss Karey Cheung	Assistance with APC isolations	
Dr David Powell	Bioinformatics analysis	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	■■■■■■■	Date: 08/06/2015

Main Supervisor's Signature	■■■■■■■	Date 08/06/2015

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

3.0. Chapter 3: The Role of Antigen Presenting Cells in the Induction of HIV-1 Latency in Resting CD4$^+$ T-Cells

3.1. Abstract

Background: Combination antiretroviral therapy (cART) is able to control HIV-1 viral replication, however long-lived latent infection in resting memory CD4$^+$ T-cells persist. The mechanisms for establishment and maintenance of latent infection in resting memory CD4$^+$ T-cells remain unclear. Previously we have shown that HIV-1 infection of resting CD4$^+$ T-cells co-cultured with CD11c$^+$ myeloid dendritic cells (mDC) produced a population of non-proliferating T-cells with latent infection. Here we asked whether different antigen presenting cells (APC), including subpopulations of DC and monocytes, were able to induce post-integration latent infection in non-proliferating CD4$^+$ T-cells, and examined potential cell interactions that may be involved using RNA-seq.

Results: mDC (CD1c$^+$), SLAN$^+$ DC and CD14$^+$ monocytes were most efficient in stimulating proliferation of CD4$^+$ T-cells during syngeneic culture and in generating post-integration latent infection in non-proliferating CD4$^+$ T-cells following HIV-1 infection of APC-T-cell co-cultures. In comparison, plasmacytoid DC (pDC) and B-cells did not induce latent infection following APC-T-cell co-cultures. We compared the RNA expression profiles of APC subpopulations that could and could not induce latency in the non-proliferating CD4$^+$ T-cells. Gene expression analysis, comparing the mDC, SLAN$^+$ DC and CD14$^+$ monocyte subpopulations to pDC identified 53 upregulated genes that encode proteins expressed on the plasma membrane that could signal to CD4$^+$ T-cells via cell interactions (32 genes), immune checkpoints (IC; 5 genes), T-cell activation (9 genes), regulation of apoptosis (5 genes), antigen presentation (1 gene) and through unknown ligands (1 gene).

Conclusions: APC subpopulations from the myeloid lineage, specifically CD1c$^+$ mDC, SLAN$^+$ mDC and CD14$^+$ monocytes, were able to efficiently induce post-integration HIV-1 latency in non-proliferating CD4$^+$ T-cells *in vitro*. Inhibition of key pathways involved in mDC-T-cell interactions and HIV-1 latency may provide novel targets to eliminate HIV-1 latency.

3.2. Introduction

Despite the successes of cART in the reduction of morbidity and mortality world wide, HIV-1 is able to persist in resting CD4^{+} T-cells (Chun et al. 1995; Finzi et al. 1997). Viral persistence as latent infection occurs when viral DNA is integrated within the host genome and remains transcriptionally silent. Latent infection of resting CD4^{+} T-cells therefore represents the major barrier to HIV-1 eradication and requires patients to remain on lifelong cART to maintain HIV suppression

It remains unclear how latency is established in resting CD4^{+} T-cells *in vivo*. Initial *in vitro* studies showed that direct HIV-1 infection of resting CD4^{+} T-cells isolated from peripheral blood was inefficient and integration rarely occurred due to incomplete reverse transcription, reduced nuclear import of the viral DNA and/or limited integration within the host genome (Stevenson et al. 1990; Zack et al. 1990; Zack et al. 1992). However, *in vitro* latent infection can occur following the reversion of a HIV-1 infected, activated CD4^{+} T-cell to a resting state (Bosque & Planelles 2009; Marini et al. 2008; Sahu et al. 2006; Yang et al. 2009). Alternatively, latent infection can also occur following the direct infection of a resting CD4^{+} T-cell exposed to high viral titers and spinoculation (Pace et al. 2012; Swiggard et al. 2005), chemokines (Saleh et al. 2007) or co-culture with other cell types, such as myeloid dendritic cells (mDC) and endothelial cells (EC) (Evans et al. 2013; Shen et al. 2013).

As professional APCs, DC interact with HIV-1 during initial infection at vaginal and rectal mucosa sites and in blood. Langerhans cells (LC), and dermal (D)DC at mucosa and, bone marrow derived classical or myeloid (m)DC and plasmacytoid (p)DC in blood are able to interact with T-cells, but their role in the establishment and maintenance of HIV-1 latency remains unclear (Hladik et al. 2007; Hussain & Lehner 1995; Zaitseva et al. 1997). Blood derived mDC subpopulations, CD141^{+} and CD1c^{+}, differ from tissue DC however both are found as resident cells in tissue (lymph node (LN), spleen, lungs, skin) and, as more mature cells, circulating through the

lymphatics to the LN (Cerovic et al. 2012; Chu et al. 2012; Ginhoux et al. 2009; Yu et al. 2013). SLAN$^+$ DC represent a subpopulation of monocytic cells with increased potential to secrete pro-inflammatory cytokines and develop a DC phenotype, however precise residence remains unknown (Hänsel et al. 2012; Qu et al. 2004). CD14$^+$ monocytes represent DC and macrophage precursors in blood (Reviewed in Ginhoux & Jung 2014), and were also tested for their ability to establish latent infection in resting CD4$^+$ T-cells.

We have previously developed an *in vitro* latency model demonstrating that CD11c$^+$ myeloid dendritic cells (mDC) induce post-integration latency in non-proliferating memory CD4$^+$ T-cells. Here we demonstrate that in addition to the mDC subsets (CD1c$^+$, SLAN$^+$ and CD141$^+$), CD14$^+$ monocytes were also able to induce post-integration HIV-1 latency in non-proliferating CD4$^+$ T-cells. In comparison, T-cells co-cultured with pDC and B-cells were inefficient in the induction of latency. Using RNA-seq and Illumina gene expression microarrays, we also identified potential mediators expressed on APC that could induce latency in the non-proliferating CD4$^+$ T-cells during APC-T cell interactions.

3.3. Results

3.3.1. Monocytes are Able to Induce Latency in Resting CD4^{+} T-Cells

We have previously reported that mDC, but not pDC, are able to efficiently induce post-integration, latent infection in resting CD4^{+} T-cells using an *in vitro* DC-T-cell co-culture model (Evans et al. 2013). However, mDC and their subpopulations represent only a small proportion of peripheral blood mononuclear cells (PBMC) compared to monocytes, which represent a precursor to some DC and macrophage subpopulations. Therefore we compared monocytes and mDC isolated from healthy donors for their ability to induce latency in resting CD4^{+} T-cells (Figure 3.1). eFluor670 labeled resting CD4^{+} T-cells were cultured alone, with CD11c^{+} mDC or bulk monocytes and infected with an R5 tropic virus that expresses green fluorescent protein (EGFP). Similar to mDC, monocytes were able to induce productive infection in CD4^{+} T-cells, as measured by total EGFP expression at day 5 post-infection (Figure 3.1B). At day 5 post-infection, sorted non-proliferating (eFluor670^{hi}EGFP^{-}) CD4^{+} T-cells were cultured with phytohemagglutinin (PHA)-stimulated feeder PBMC, where the number of EGFP^{+} cells was quantified by flow cytometry as a surrogate marker of inducible latent infection. Along with mDC, CD14^{+} monocytes were also able to significantly increase the induction of latent infection in non-proliferating cells (p>0.05; Figure 3.1C).

Figure 3.1.Monocyte Induced Latency in Non-Proliferating CD4$^+$ T-Cells.

A. Resting CD4$^+$ T-cells were labeled with eFluor670 and cultured alone or with bulk myeloid (m)DC or bulk monocytes (mono) at a ratio of 10:1. Following 24 hours of culture, APC-T-cell co-cultures were infected with NL(AD8)Δnef-EGFP. At day 5 post-infection **B.** productive infection was determined by quantification of EGFP in total cells and; **C.** non-proliferating, non-productively infected (eFluor670^{hi}EGFP$^-$) cells were sorted using flow cytometry. Sorted non-proliferating (eFluor670^{hi}EGFP$^-$) CD4$^+$ T-cells were stimulated with phytohaemagglutinin (PHA) stimulated feeder PBMC for 5 days and EGFP quantified as a measure of reactivated HIV-1 latency. Columns represent the median of the log transformed values, open circles represent individual donors, Dotted lines represents the limit of detection. *p≤0.05, **p≤0.005 as determined by paired students T-test.
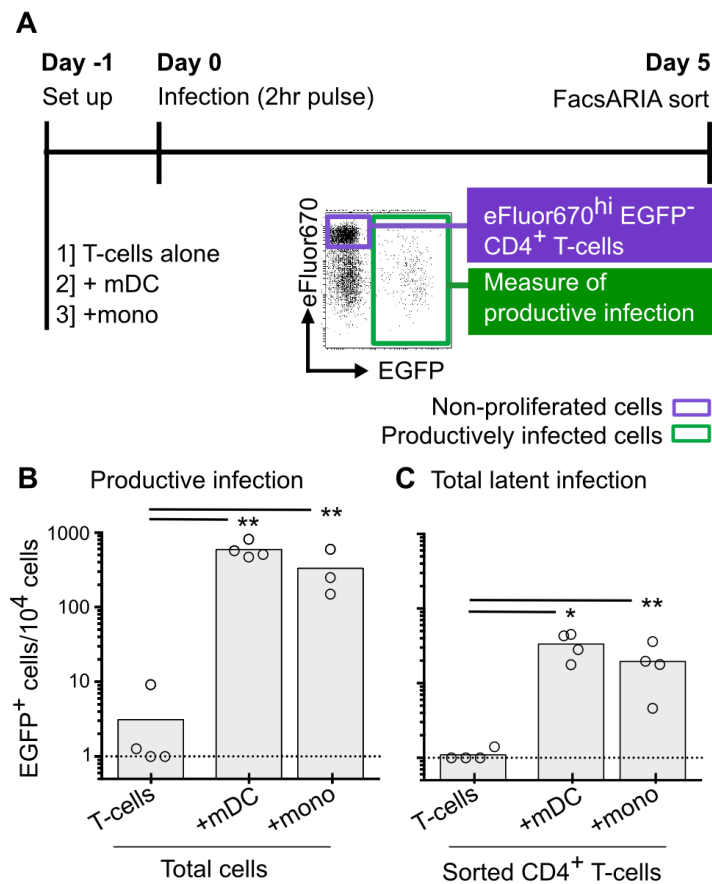
3.3.2. Isolation of Functional Antigen Presenting Cells.

As we were able to show induction of latency in non-proliferating CD4$^+$ T-cells following co-culture with bulk monocytes and mDC, we next aimed to compared the latency inducing potential of the different monocyte and mDC subpopulations. Monocytes were sorted into CD14$^+$ and CD14^{lo}CD16$^+$ cells and mDC were sorted into CD1c$^+$, CD141$^+$ and SLAN$^+$ mDC (Figure 3.2A), B-cells and pDC were also isolated by flow cytometric sorting (Figure 3.2A). The final purity for all sorted APC subpopulations was >90%, as determined post-sort by expression of specific known surface markers for the various subpopulations (MacDonald et al. 2002; Nagasawa 2006; Rissoan et al. 2002; Schäkel et al. 1998; Ziegler-Heitbrock et al. 2010). The APC subpopulations were examined using brightfield microscopy after culture (Figure 3.2B, C). The mDC and monocyte subpopulations were characterized with the formation of dendritic processes and membrane ruffles (Figure 3.2B, C). Comparatively, pDC and B-cells had less processes or ruffles (Figure 3.2B; Geissmann et al. 2003; O'Doherty et al. 1993; Rissoan et al. 2002; Schäkel et al. 1998; Steinman & Cohn 1973)).

APC function was tested in a syngeneic mixed leukocyte reaction (MLR) using the proliferation dye eFluor670 to measure proliferation of resting CD4$^+$ T-cells. In the absence of mitogen stimulation, the relative potency of the various APC to induce T-cell proliferation at a ratio of 1 APC:10, 100 or 1000 CD4$^+$ T-cells is shown (Figure 3.3A). CD1c$^+$ DC were the most potent in activating resting CD4$^+$ T-cells, while pDC and CD141$^+$ DC were least potent. The use of superantigen staphylococcal enterotoxin B (SEB) at low dose in the MLR increased the capacity of APC to induce T-cell proliferation. T-cell proliferation following co-culture and SEB treatment was highest with CD1c$^+$ DC and lowest with B-cells (Figure 3.3B), confirming previous observations by others (MacDonald et al. 2002). B-cells had a similar stimulatory capacity with and without superantigen (1.0% and 1.3% proliferated CD4$^+$ T-cells

respectively). Finally, there was a dose response of CD4$^+$ T-cell proliferation with decreasing APC:T-cell ratio (1:10-1000). Together, these data confirm that all the APC subpopulations isolated remained functional in the co-cultures used for infection.

Figure 3.2. Isolation of Antigen Presenting Cells.

A. Peripheral blood mononuclear cells (PBMC) were elutriated into three fractions: small lymphocytes, large lymphocytes and a monocyte/DC fraction. Resting CD4$^+$ T-cells were isolated from the small lymphocyte fraction by negative selection using magnetic beads. Bulk B-cells were isolated from a mixture of the small and large lymphocyte fractions using positive magnetic bead selection for CD19. Bulk DC subpopulations were positively selected on the basis of expression of CD1c, CD141, SLAN and CD123 from the DC/monocyte fraction using magnetic bead selection. The positive "DC enriched" (DC) population was then sorted by flow cytometry into the four DC populations (purity >95%). The negative "DC depleted" (mono) fraction was labeled with the monocyte markers CD14 and CD16, positively selected using magnetic beads and sorted by flow cytometry into CD14$^+$ and CD14^{lo}CD16^{hi} subsets (purity >90%). **B, C**. Representative flow cytometry dot plots and brightfield images show the purity and morphology of the sorted APC subpopulations, respectively. The scale bars represent 20μm, images were annotated using ImageJ software.

Figure 3.2 Isolation of Antigen Presenting Cells
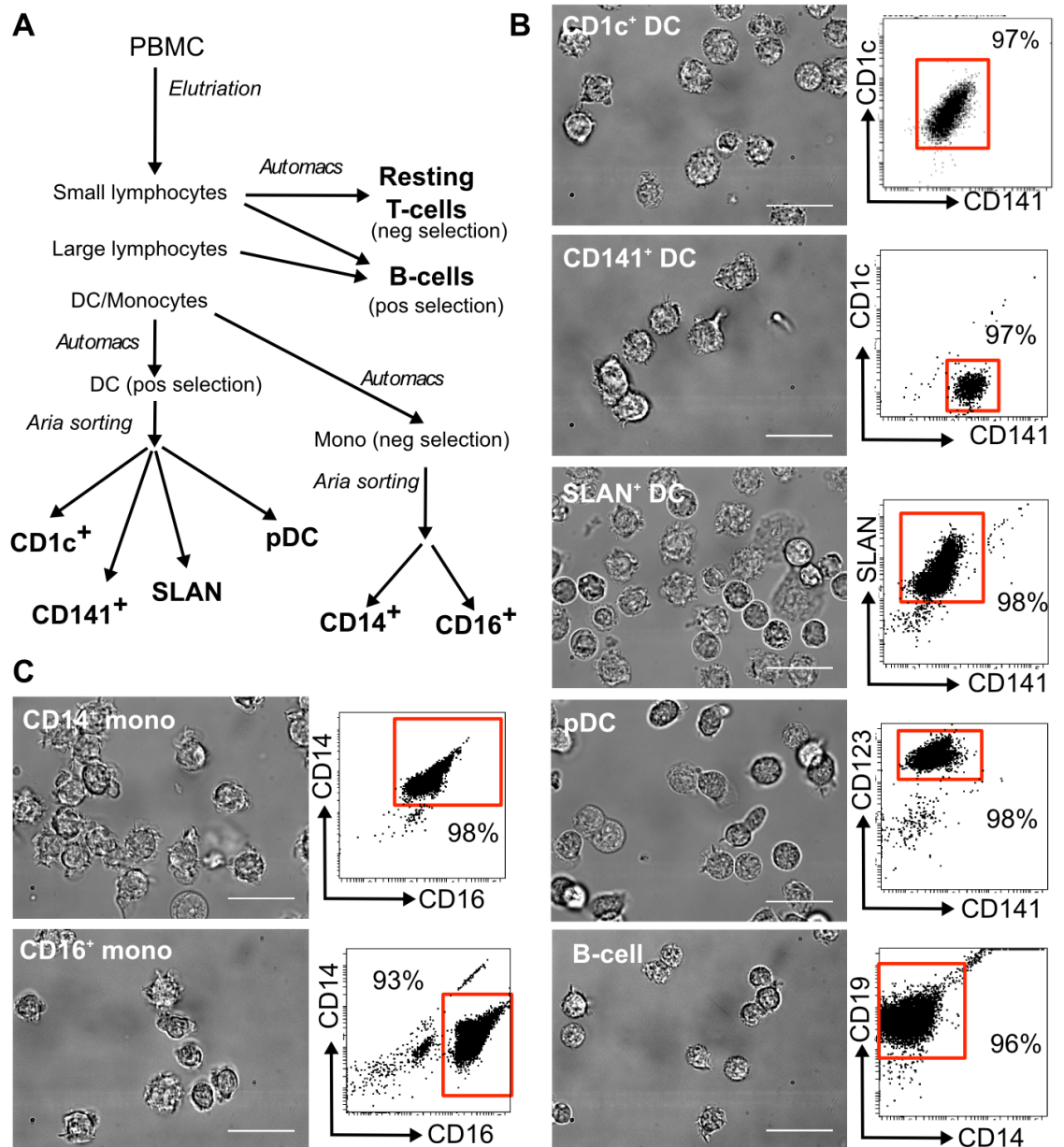
3.3.3. T-Cell Stimulation by Antigen Presenting Cell Subpopulations in HIV-1 Infected Co-cultures

We then measured T-cell proliferation following co-culture with different APC 3 days post-infection with HIV-1 R5-EGFP reporter virus. The pattern of APC potency in induction of CD4$^+$ T-cell proliferation in the presence of HIV-1 was similar to uninfected, syngeneic, co-cultures (Figure 3.3C, A), where proliferation of CD4$^+$ T-cells was highest with CD1c$^+$ DC and lowest with pDC. These experiments demonstrate that HIV-1 infection did not independently alter APC or CD4$^+$ T-cell function with respect to T-cell proliferation.

Figure 3.3. Resting CD4$^+$ T-Cell Stimulation Following Co-culture with Antigen Presenting Cells.

Isolated resting CD4$^+$ T-cells were labeled with the proliferation dye eFluor670 and either co-cultured alone or co-cultured with one of seven antigen presenting cell (APC) subpopulations, including B-cells; monocyte subpopulations CD14^{hi} and CD14^{lo}CD16^{hi}; DC subpopulations- plasmacytoid (p)DC and myeloid (m)DC subpopulations CD1c$^+$, CD141$^+$ and SLAN$^+$. CD4$^+$ T-cells were cultured with APC at a ratio of log 1 (10:1), 2 (100:1) or 3 (1000:1). The percentage of eFluor670^{lo}, a measurement of CD4$^+$ T-cell proliferation, was determined following 5 days of culture in the **(A)** absence (syngeneic) or **(B)** presence of staphylococcal enterotoxin B (SEB). **C.** eFluor670 labeled, resting CD4$^+$ T-cells were cultured alone, or with APC subpopulations at a ratio of 10:1 and infected with NL(AD8)Δ*nef*-EGFP. At day 3 post-infection, CD4$^+$ eFluor670^{lo} T-cells was measured. Columns represent the median, open circles represent individual donors. Where sample numbers are not the same, there was not enough APC to set up the culture. *p$\leq$0.05, as determined by Wilcoxon matched pairs signed rank test.
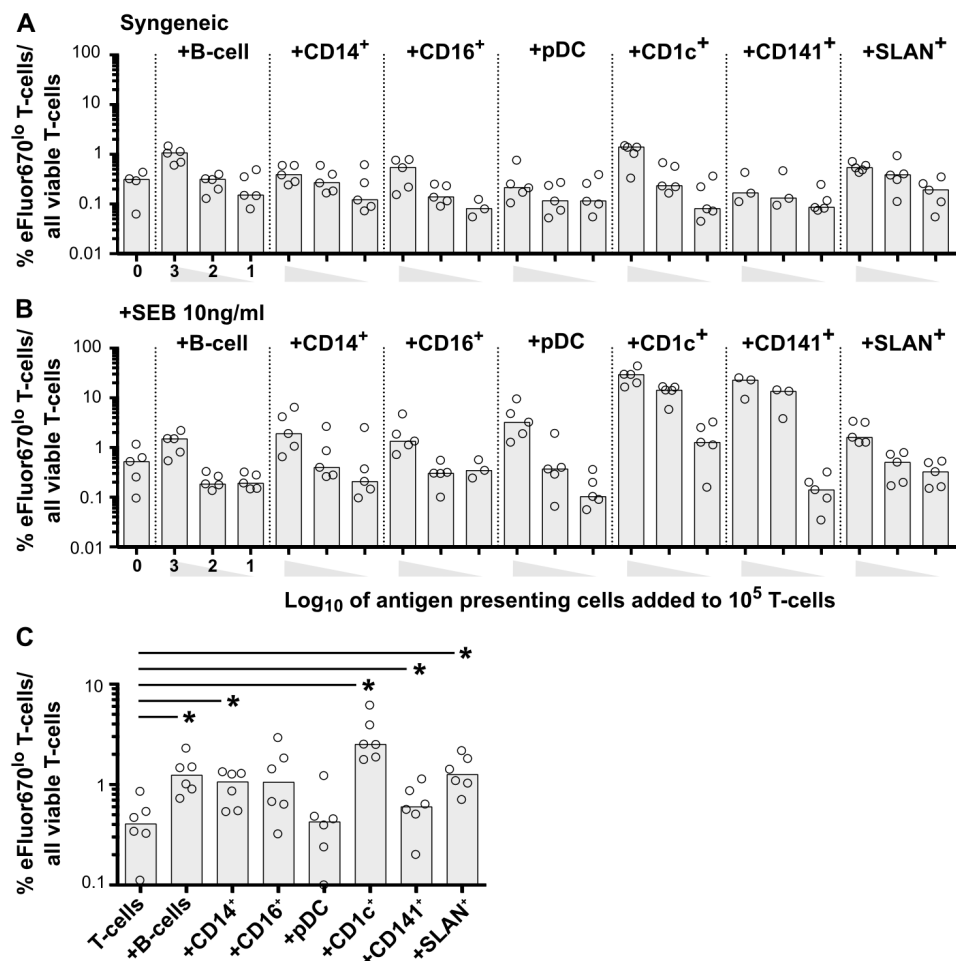
3.3.4. Several Antigen Presenting Cell Subpopulations Enhanced Productive Infection of Resting CD4$^+$ T-Cells.

We tested the ability of APC subpopulations to induce both productive and latent infection in resting CD4$^+$ T-cells when cultured alone or co-cultured with one of the seven sorted APC subpopulations. Five days following infection EGFP expression was quantified by flow cytometry as a measure of productive infection (Figure 3.4A). We observed a significant increase in productive infection following HIV-1 infection of all APC co-cultured T-cells compared to resting CD4$^+$ T-cells cultured alone (p=0.03 for all APC co-cultures; Figure 3.4B).

3.3.5. Different Antigen Presenting Cell Subpopulations can Effectively Induce Latent Infection in Non-Proliferating CD4$^+$ T-Cells

Five days following infection, non-proliferating (eFluor670^{hi}EGFP$^-$) CD4$^+$ T-cells were sorted from the APC-T-cell co-cultures to quantify latent infection (Figure 3.4A). The sorted CD4$^+$ T-cells were directly stimulated with anti-CD3/CD28 and IL-7 (Figure 3.4C) in the presence and absence of an integrase inhibitor, L8, and EGFP quantified by flow cytometry to measure inducible latent infection. Total latent infection (-L8; pre- and post-integrated latency) was significantly increased in non-proliferating CD4$^+$ T-cells co-cultured with all mDC subpopulations, CD14$^+$ monocytes and B-cells, when compared to CD4$^+$ T-cells cultured alone (p=0.03; Figure 3.4D). In comparison, total latent infection following co-culture with CD14^{lo}CD16$^+$ monocytes that were depleted of SLAN+ DC was highly variable and not significantly different to T-cells cultured alone. As previously shown, latent infection was not found in pDC co-cultures (p=0.03 compared to mDC co-cultures; Figure 3.4D).

We also quantified post-integration latent infection by stimulating T-cells with anti CD3/CD28 and IL-7 stimulation in the presence of L8 (Figure 3.4C). The integrase inhibitor L8 prevented integration of pre-integrated complex and inhibited secondary rounds of infection. Following infection of CD4$^+$ T-cells cultured alone or co-cultured with each APC subpopulation, post-integration latency followed a similar pattern to that observed for total latency, but at a lower frequency (Figure 3.4D, E). Post-integration latency was significantly increased in CD4$^+$ T-cells following co-culture with mDC subpopulations CD1c$^+$ and SLAN$^+$, and CD14$^+$ monocytes (p=0.03, 0.02 and 0.01, respectively; Figure 3.4E). Post-integration latency induced by CD141$^+$ DC was elevated, similar to what was induced by other mDC subsets, but this did not reach statistical significance. In comparison, HIV-1 infection of T-cells co-cultured with CD16$^+$ monocytes, B-cells and pDC was similar to infection of CD4$^+$ T cells alone. Together these data show that only CD1c$^+$mDC, SLAN$^+$ DC and CD14$^+$ monocytes were able to establish post-integration latent infection in non-proliferating CD4$^+$ T-cells, while B-cells and CD141$^+$ mDC were able to establish pre-integration latent infection. CD14^{lo}CD16^{hi} monocytes, like pDC were unable to establish either pre or post-integration latency.

Next, we looked for a correlation between productive infection and post-integration latency following infection of T-cells co-cultured with the different APC subpopulations (Figure 3.4F). Overall, we found a weak correlation between productive and latent infection (Spearman's r=0.12; p=0.02), which supports our previous findings (Evans et al. 2013). However, the induction of productive infection does not inevitably lead to post-integration latency in resting CD4$^+$ T-cells, as observed following co-culture with CD14^{lo}CD16^{hi} monocytes, B-cells and pDC. We conclude that APC able to establish both productive and latent infection likely share common functional characteristics, which favour the establishment and maintenance of latent infection in non-prolifertating CD4$^+$ T-cells.

Figure 3.4. Productive and Latent Infection in Resting T-Cells Co-cultured with Antigen Presenting Cell Subsets.

A. Representative dot plot of CD4$^+$ T-cells co-cultured with antigen presenting cell (APC) subpopulations on day 5 post infection with NL(AD8)Δ*nef*-EGFP. Non-proliferating (eFluor670^{hi}), non-productively infected (EGFP$^-$) T-cells were sorted 5 days following infection. **B.** EGFP expression in the total cell cultures on day 5 was used as a measure of productive infection **C.** Total and post-integrated latency were quantified by flow cytometry of EGFP expression following stimulation of sorted eFluor670^{hi}EGFP$^-$ cells with anti-CD3/CD28 and IL-7. Representative dot plots of EGFP expression following stimulation of eFluor670^{hi}EGFP$^-$ sorted CD4$^+$ T-cells in the absence (-L8 plot; pre-a dn post-integrated latency) and presence (+L8 plot; post-integration latency) of the integrase inhibitor L8. **D.** The frequency of total latent infection and **E.** post-integration latency in resting CD4$^+$ T-cells following co-culture with the different APC subpopulations. **F.** Correlation of the frequency of productive infection and post-integrated latency (+L8) in each APC-T-cell co-culture. Each condition is identified by a different color and/or symbol. For all other panels, columns represent the median, open circles represent results from individual donors and dotted lines represent the limit of detection. Significant differences between conditions was measured by Wilcoxon matched pairs signed rank test where *p≤0.05.

A eFluor670hi EGFP- CD4+ T-cells

EGFP+ cells: Productive infection

C anti-CD3/CD28 + IL-7

-L8   +L8

[1] Amplification of latent signal. TOTAL LATENCY

[2] Quantifation of latently infected cells. POST-INTEGRATED LATENCY

F Correlation of productive to latent infection

+ L8

- T Alone
- T/B-cell
- T/pDC
- T/CD14+ Mono
- T/CD16+ Mono
- T/CD1c+
- T/CD141+
- T/SLAN+

Latent infection (EGFP+ cells/10,000 viable cells)

Productive infection (EGFP+ cells/10,000 viable cells)

B Productive Infection

EGFP+ cells/10^4 cells

D Total Latency

EGFP+ cells/10^4 cells

E Post-integration Latency

EGFP+ cells/10^4 cells

T-cells +B-cell +CD14 +CD16 +pDC +CD1c +CD141 +SLAN

Monocytes   DCs

3.3.6. Differential Gene Expression of Cell-Surface Expressed Molecules on Antigen Preseting Cell using RNA-Seq

We next used RNA-seq to compare the expression of genes involved in T-cell interactions in APC subpopulations that significantly induce latency (CD1c^{+} DC, SLAN^{+} DC and CD14^{+} monocytes) compared to pDC, the APC subpopulation that could not induce latency. Low cell viability after culture and infection limited isolation of APC after HIV-1 infection and co-culture, so freshly isolated APC subpopulations were used for analysis of differential gene expression (Ahn et al. 2002; Huang et al. 2001; Verdijk et al. 2004). We have shown that cell contact is important in mDC-induced latency (Evans et al. 2013), therefore we specifically selected genes encoding proteins that mediate mDC-T-cell interactions, and were expressed on cell membrane compartments at the cell surface, and on intracellular vesicles such as endosomes and exosomes.

In APC subpopulations that induced latency compared to APC that didn't significantly induce latency, we found 754 upregulated genes (fold change ≥2, p-value<0.01; Figure 3.5A). Analysis for expression in cellular compartment (GeneCodis ; http://genecodis.cnb.csic.es), identified 285 known genes expressed in: membrane, plasma membrane, integral to membrane and cell junction (Figure 3.5A; Carmona-Saez et al. 2007; Nogales-Cadenas et al. 2009; Tabas-Madrid et al. 2012). Of these, 53 protein-encoding genes that could establish cell contact with CD4^{+} T-cells and potentially induce T-cell signalling were selected (Figure 3.5; Table 3.1; Appendix table 8.1). Functionally these genes included; cellular adhesion (31 genes), antigen presentation (1 gene), T-cell activation (9 genes), immune checkpoints (IC; 6 genes), regulation of apoptosis (5 genes), and an unknown protein (1 gene). We further analysed the role of each gene in HIV-1 infection of DC and CD4^{+} T-cells using PubMed search for the interactions between DC and T-cells, and potential roles in the establishment of HIV-1 latency.

We performed the same comparisons between selected APC subpopulations using our previously published microarray data (Harman et al. 2013) and found 27 genes that could potentially induce T-cell signalling (Appendix figure 8.1; Appendix table 8.2; Harman et al. 2013). Five of these genes were common between microarray and RNA-seq analyses, seven genes shared protein family and thirteen genes fell outside of significance (cut off of p<0.01), often with inconsistent replicates (n=3). The common genes included *C-type lectin domain family 7 member A* (*CLEC-7A*), *endoglin, intracellular adhesion molecule 3* (*ICAM-3*), *sialic acid-binding immunoglobulin-type lectins* (*SIGLEC*)-*10* and *CD1d*. CD1d is involved in lipid antigen presentation to T-cells, while the other 4 surface expressed proteins are involved in cellular adhesion (Kelly et al. 2013). The common protein families included the SIGLEC family, CLEC family, leukocyte associated immunoglobulin like receptor (LILRA) family, G-Protein coupled receptor (GCPR) family and the tumor necrosis factor (TNF) receptor superfamily.

Figure 3.5. Comparison of Gene Expression Between Latency Inducing and Non-Inducing Antigen Presenting Cell Subpopulations using RNA-Seq.

A. Genes differentially upregulated in latency inducing APC subpopulations (CD1c$^+$ mDC, SLAN$^+$ mDC and CD14$^+$ monocytes) compared with the non-latency inducing pDC, were selected (fold change $\geq$2, p<0.01). Using GeneCodis, these 754 selected genes were categorized according to cellular compartment expression. **B.** Encoded proteins expressed on APC surface and membrane compartments were further analysed for ability to signal to T-cells and involvement in HIV-1 infection. A representative heat map is shown for genes that are able to signal to T-cells, which have a >2 fold differential gene expression between latency inducing (CD1c$^+$ mDC, SLAN$^+$ mDC and CD14$^+$ monocytes) and non-inducing pDC. Results for each APC subpopulations tested in 3 independent donors is shown. CD1c$^+$ refers to CD1c$^+$ mDC, CD14$^+$ refers to CD14$^+$ monocytes and SLAN$^+$ refers to SLAN$^+$ DC.

A

CD14⁺ Monocytes

pDC

SLAN

754 genes

CD1c⁺

Total=754 genes

- Plasma membrane (149)
- Membrane (127)
- Cell Junction (7)
- Integral to membrane (2)
- Non-plasma membrane (469)

B

log2 Reads per Million difference from row mean

-4 -2 0 2 4

row mean

pDC   pDC   pDC   CD1c⁺ CD1c⁺ CD1c⁺ SLAN⁺ SLAN⁺ SLAN⁺ CD14⁺ CD14⁺ CD14⁺

TMEM2
CLEC1A
LILRA6
SIGLEC9
NFAM1
PVR
CLEC7A
GPR133
SDC3
SECTM1
TIAM1
SGPL1
LRRC25
SIGLEC10
MSR1
CD101
CD48
CD86
CD33
CEACAM4
SIGLEC7
EVI2A
CSF3R
VSIG2
MEGF9
IL15RA
PLXDC2
SIGLEC14
NETO2
OSCAR
TSPAN17
VIPR1
EFHD2
CRTAM
ICAM3
LGALS1
CLEC4G
ACPP
LRRC8C
HAVCR2
ADAM15
MPP7
TNFRSF10D
ESAM
TNFRSF10A
SYNJ2BP
CD83
EFNB1

0   5

Table 3.1. Effects on HIV-1 Infection of Genes Differentially Expressed by Latency Inducing and Non-Inducing Antigen Presenting Cell Subpopulations using RNA-Seq.

The functional category shown were determined by the description from the DAVID (david.abcc.ncifcrf.gov/) and GeneCards (genecards.org/) databases.

Gene name	Gene symbol	Function					
		Antigen Presentation	Apoptosis regulation	Cell proximity presentation	Immune checkpoint blocker	T-cell activation	Unknown
Number of genes expressed in each category		1	5	32	5	9	1
CD1d molecule	*CD1d*	-*,**					
Lectin, galactoside-binding, soluble, 1	LGALS1		+				
Vasoactive intestinal peptide receptor 1	VIPR1		+*				
EF-hand domain family, member D2	EFHD2		-				
Tumor necrosis factor receptor superfamily, member 10a	TNFRSF10A		+				
Tumor necrosis factor receptor superfamily, member 10d, decoy with truncated death domain	TNFRSF10D		+				
Acid phosphatase, prostate	ACPP			+			
ADAM metallopeptidase domain 15	ADAM15			+			
Integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)	CD18			+*,**			
Carcinoembryonic antigen-related cell adhesion molecule 4	CEACAM4			+			
C-type lectin domain family 4, member G	CLEC4G			-*,**			
C-type lectin domain family 7, member A	*CLEC7A*			+*			
Cytotoxic and regulatory T cell molecule	CRTAM			-**			
Colony stimulating factor 3 receptor (granulocyte)	CSF3R			+*			
Ephrin-B1	EFNB1			-			
Endoglin	*END*			+*			
Endothelial cell adhesion molecule	ESAM			+			
G protein-coupled receptor 133	GPR133			+			
Intercellular adhesion molecule 3	*ICAM3*			+*			
Leucine rich repeat containing 8 family, member C	LRRC8C			+			
Multiple EGF-like-domains 9	MEGF9			+			
Membrane protein, palmitoylated 7 (MAGUK p55 subfamily member 7)	MPP7			+			
Macrophage scavenger receptor 1	MSR1			+			

Description	Symbol						
Osteoclast associated, immunoglobulin-like receptor	OSCAR			+			
Plexin domain containing 2	PLXDC2			+			
Syndecan 3	SDC3			+*			
CD33 molecule	CD33			-	-		
Sphingosine-1-phosphate lyase 1	SGPL1			+	+		
Sialic acid binding Ig-like lectin 10	*SIGLEC10*			-*	-		
Sialic acid binding Ig-like lectin 7	SIGLEC7			+**	+		
Sialic acid binding Ig-like lectin 9	SIGLEC9			-	-		
Synaptojanin 2 binding protein	SYNJ2BP			+	+		
T-cell lymphoma invasion and metastasis 1	TIAM1			+*	+		
Transmembrane protein 2	TMEM2			+	+		
Tetraspanin 17	TSPAN17			-	-		
C-type lectin domain family 1, member A	CLEC1A			+		+	
Sialic acid binding Ig-like lectin 14	SIGLEC14			+		+	
CD101 molecule	CD101				-		
CD52 molecule	CD52		-		-		
Hepatitis A virus cellular receptor 2	HAVR2 /Tim-3				-**		
Leukocyte immunoglobulin-like receptor, subfamily A (with TM domain), member 6	LILRA6				-		
Poliovirus receptor	PVR					+*	
Neuropilin (NRP) and tolloid (TLL)-like 2	NETO2					+	
CD48 molecule	CD48					0**	
Interleukin 15 receptor, alpha	IL15RA					-**	
Leucine rich repeat containing 25	LRRC25					+	
NFAT activating protein with ITAM motif 1	NFAM1					+	
Secreted and transmembrane 1	SECTM1					+	
V-set and immunoglobulin domain containing 2	VSIG2					+	
CD300e molecule	CD300e					+	
CD83 molecule	CD83					0**	
CD86 molecule	CD86					0**	
Ecotropic viral integration site 2A	EVI2A						+

+ = Hypothesised increased latent infection, - = hypothesised inhibition of virus expression, 0 = undefined. Genes that were common to the RNA-seq and microarray generated gene lists are in *italics* and **bold**, * represent a role in HIV-1 infection of either DC (*) or T-cell (**). [1]

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[1] Data in Table 3.1 is presented in detail in Appendix table 8.1.

3.4. Discussion:

Latently infected cells are infrequent in HIV-1-infected patients on cART, and therefore robust *in vitro* models are needed to better understand the establishment and maintenance of latent infection. We have now shown that multiple APC, in addition to mDC, are able to induce HIV-1 latency in non-proliferating CD4$^+$ T-cells (Evans et al. 2013). We have also shown that the myeloid lineage cells capable of producing latent T-cell infection include subpopulations of blood derived mDC; CD1c$^+$, SLAN$^+$ DC and CD14$^+$ monocytes, and confirmed that pDC are distinct in not generating latent infection. We have used RNA-seq analysis to define genes differentially expressed between APC subpopulations that could (CD1c$^+$, SLAN$^+$, CD14$^+$ monocytes) and could not induce latent infection (pDC). We identified genes mediating cell adhesion, T-cell activation, immune checkpoints and regulation of apoptosis as important pathways upregulated in the APC that are able to induce latent infection.

Our results show that multiple blood derived mDC subsets can induce latent infection in non-proliferating CD4$^+$ T-cells, suggesting that this observation may extend to other DC and myeloid lineage cells, such as LC and dermal DC (dDC) though they may have different ontogeny (Haniffa et al. 2012; McGovern et al. 2014). It is likely that mDC and monocyte derived cells in lymphoid tissue, skin, mucosal surfaces, gastrointestinal tract (GIT) and sites of inflammation would allow seeding of CD4$^+$ T-cell viral reservoirs early following infection or during ART in tissue sites where cART penetration may not be optimal (Trezza & Kashuba 2014). Further testing the tissue resident APC in the DC-T-cell model would be interesting to confirm their function similarities to blood derived APC.

We observed a trend between the ability of the different APC to induce latent infection and efficient T-cell proliferation. This suggests that there may be a common mechanism for the induction of T-cell proliferation and the induction

of latent infection, even in non-proliferating cells, perhaps through a bystander mechanism. Efficient T-cell proliferation is favoured by the formation of an immunological synapse (Revy et al. 2001; Hochweller et al. 2010) with cellular proximity (Jolly et al. 2007), major-histocompatibility complex (MHC)-T-cell receptor (TcR) interaction and co-stimulation (Seya et al. 2001; Fedde Groot et al. 2006; Sabado et al. 2007; Sanchez-Lockhart et al. 2014). ICAM-1-leukocyte function-associated antigen (LFA)-1 interactions can facilitate induction of latent infection (Evans et al. 2013) or reaction of latency (van der Sluis et al. 2013) and in other models of *in vitro* latency, CD2, a molecule that binds to LFA-3, was increased latently infected cells (Iglesias-Ussel et al. 2013). A large portion (60%) of cell surface expressed genes identified in the gene expression analysis mediates cellular proximity and cell adhesion, compared to any other function. Taken together these data suggest that cell adhesion is important in the induction of latency. However, identification of a single specific adhesion molecule critical for induction of latent infection is likely limited by functional redundancy in mediators of APC-T-cells interactions. To confirm a role for adhesion factors whole protein families of adhesion molecules may have to be inhibited.

Compared to the other DCs, the pDC were least efficient for T-cell proliferation and induced significantly less productive and latent infection compared to mDC. The differences between mDC and pDC in induction of productive infection (Cella et al. 1999; F. Groot et al. 2006) and suppression of virus production has been observed previously (Evans et al. 2013). We have also previously shown that pDC were unable to induce HIV-1 latency, and that there was a more substantial suppressive effect on latency establishment compared to productive infection (Evans et al. 2013, p.013). We and others have shown multiple differences between mDC and pDC that may reduce the ability of pDC to establish close interactions with T-cells (Dzionek et al. 2000; Harman et al. 2013; MacDonald et al. 2002; O'Doherty et al. 1994), which in combination with increased type-I IFN secretion from pDC may inhibit the capacity of pDC to establish latency in CD4^{+} T-cells.

The ability of B-cells to induce latent infection in non-proliferating CD4$^+$ T-cells was also tested in this study as B-cells express MHC-II, circulate through LN, and have been reported to transfer HIV-1 infection to T-cells (Rappocciolo et al. 2006). Induction of latency occurred only at low levels, suggesting that B-cells lack factors that facilitate efficient induction of latency.

Comparison of APC subpopulations that could and could not induce latent infection in non-proliferating CD4$^+$ T-cells identified several factors that may be important in the establishment of latency, including cell adhesion, immune checkpoints (IC), T-cell co-stimulation, antigen presentation and regulation of apoptosis. The IC, programmed death receptor (PD)-1, is proposed to play a role in the establishment and/or maintenance of HIV-1 latency (Porichis & Kaufmann 2012; Chomont et al. 2009). Engagement of IC, led to reduced T-cell activation by inhibition of signalling cascades, as well as physical inhibition of the formation of lipid rafts and cellular interactions (Parry et al. 2005). In this study, we observed an up-regulation of the IC; *CD101, T-cell immunoglobulin mucin-3* (*Tim-3, HAVR2*), *leukocyte immunoglobulin-like receptor member 6* (*LILR6*) and *CD52*, on latency inducing APC subpopulations when compared to pDC. IC expression may be important for the establishment of HIV-1 latency in this model, but further work is required to confirm this. We are pursuing the investigation further by measurement of a series of IC and their ligands; PD-1-PD-L1/L2, CTLA-4-CD80/CD86, Tim-3-Gal-9, BTLA-HVEM, on CD4+ T-cells and APC respectively. We plan to block their function using neutralising anti-bodies alone and in combination to confirm a role for IC in the establishment of latency.

Additionally, we identified differential expression of the SIGLEC family of proteins between APC subpopulations that could and could not induce latent infection. We specifically found *SIGLEC 5, 7, 9, 10* and *14* to be upregulated on latency inducing APC. From this family, SIGLEC 3, 5-11 have all been implicated in the inhibition of T-cell activation (Whitney et al. 2001; Avril et

al. 2004; Ikehara et al. 2004). SIGLEC 5 has been shown to inhibit T-cell activation in chimpanzees, where blockade of SIGLEC 5 led to increased T-cell activation and transfection of SIGLEC 5 into SIGLEC negative cells reduced T-cell activation (Bibollet-Ruche et al. 2008; Nguyen et al. 2006; Soto et al. 2013; Ikehara et al. 2004). SIGLEC 10 is hypothesized to have similar function in inhibition of T-cell activation (Chen et al. 2009; Bandala-Sanchez et al. 2013). Together these data suggest that SIGLEC 5 or 10 binding to its ligand on the CD4$^+$ T-cell may reduce T-cell activation, reduce productive infection and potentially promote latent infection. This is a novel association but further work will be required to explore any direct effects of SIGLEC proteins and the establishment of latency. Given the lack of neutralising antibodies against the SIGLEC family of proteins siRNA could be used to block SIGLEC 5 and 10 function. Alternatively, similar to the study by Soto et al., we could express SIGLEC 5 and 10 in B-cells to test if this specific interaction allows B-cell to induce latency in non-proliferating CD4$^+$ T-cells.

In summary, this study has established that multiple myeloid lineage APC subpopulations can facilitate latent infection in resting CD4$^+$ T-cells. Particularly important is the observation that CD14$^+$ monocytes can induce latent infection in resting CD4$^+$ T-cells. The use of CD14$^+$ monocytes in this model will greatly enhance the ease and availability of antigen presenting cells for this assay. In addition, through a comparative analysis of APC populations, we have identified new pathways that may potentially be involved in the establishment and/or maintenance of HIV-1 latency.

3.5. Materials and Methods

3.5.1. Isolation and Preparation of Resting CD4$^+$ T-cells and B-cells

The use of blood samples from normal donors for this study was approved by the Alfred Hospital HREC 156/11 and Monash University CF11/1888 research and ethics committees. Donors were recruited by the Red Cross Blood Transfusion Service as normal blood donors and consented to the use of blood products without identifiers for research. PBMC were isolated by Ficoll-Paque density gradient centrifugation (GE Healthcare, Chalfont St. Giles, United Kingdom) from healthy buffy coats obtained from the Australian Red Cross. PBMC were further separated into three populations by counter-current elutriation using Beckman J-6 M/E centrifuge equipped with a JE 5.0 rotor (Beckman Coulter, Pasedena, CA, USA; Grosse et al. 2012). The three fractions were isolated at rates of 12 (small lymphocytes), 16 (large lymphocytes) and 20 (DC/Monocytes fractions) ml/min (Figure 3.1). Resting CD4$^+$ T-cells, negative for the activation markers CD69 and HLA-DR, were sorted from the "small lymphocyte" fraction, as previously described (Evans et al. 2013; Section 2.5.1), with a purity always >98%. B-cells were isolated with a purity of ≥90% from the "small and large lymphocyte" fractions using positive magnetic bead selection on an AUTOMacs (Miltenyi) with anti-CD19$^+$ hybridoma (clone FMC63) and anti-IgG microbeads (Miltenyi, Bergisch Gladbach, Germany).

3.5.2. Isolation and preparation of DC and monocytes

The DC/monocyte fraction was first stained with antibodies specific for the DC subsets, including CD1c-APC (clone AD5-8E3; Miltenyi), CD141-VioBlue (clone AD5-14H12; Miltenyi), CD123-PE (clone 9F5; BD Bioscience, Franklin Lakes, NJ, USA) and SLAN-FITC (clone DD-1; Miltenyi), and labeled with anti-IgG beads (Miltenyi). DC were then isolated using an AutoMACS (Miltenyi) into positive and negative fractions. The positive fraction (DC enriched) was further sorted into four DC subsets: CD1c$^+$ mDC,

SLAN$^+$ DC, CD141$^+$ mDC and CD123$^+$ pDC, using a FACSAria (BD Biosciences). The negative fraction (DC depleted/mono) was stained with anti-CD14-FITC (clone M5E2; BD Biosciences) and anti-CD16-PE (clone B73.1; BD Biosciences) antibodies, labeled with IgG beads (Miltenyi) and a positive selection performed using an AutoMACS (Miltenyi) to obtain a bulk monocyte population. These cells were further sorted to obtain the CD14$^+$CD16$^-$ (CD14$^+$) and CD16$^+$CD14^{lo} (CD16$^+$) monocyte subsets using a FACSAria. Cell populations with a purity ≥90% were used, as determined by flow cytometry (LSR II or FACSAria; BD Bioscience).

3.5.3. Imaging Antigen Presenting Cell Subpopulations

After isolation, each antigen presenting cell (APC) subpopulation was cultured in RF10 media (RPMI 1640; Life Technologies, Carlsbad, CA), supplemented with 10% foetal bovine serum (FBS; Interpath, Heidelberg, Australia), Penicillin-Streptomycin-Glutamine (PSG; Life Technologies) for 1-2 hours at 37°C in glass-bottom imaging plates (µ-slide, ibidi, Martinsried, Germany). Ten representative images were captured on a CCD camera through a 10x0.3 NA lens on a Olympus IX51 microscope and annotated using ImageJ software.

3.5.4. Viral Plasmids, Virus Preparation and Infection

In all experiments, we used HIV-1 NL4.3 plasmid backbone with an AD8 envelope and EGFP inserted in the *nef* open reading frame at position 75 (NL(AD8)Δ*nef*EGFP; Section 2.5.2; Evans et al. 2013), kindly provided by Damian Purcell, University of Melbourne (Melbourne, Australia). Viral stocks were generated by FuGene (Promega, Madison, WI, USA) transfection of 293T cells as previously described (Section 2.5.2; Evans et al. 2013). Cells were infected at an MOI of 0.5, as determined by limiting dilution in PHA-stimulated PBMC using the Reed and Muench method (described in section 2.5.2; Reed & Muench 1938).

3.5.5. Syngeneic Mixed Leukocyte Reactions

Resting CD4$^+$ T-cells were labeled with the cytoplasmic dye eFluor670 and co-cultured with decreasing concentrations of each APC subpopulation; log 1

(10:1), 2 (100:1) and 3 (1000:1), in the absence (syngeneic) or presence of superantigen SEB (10ng/mL; Sigma). At day 5, cells were harvested and labeled with antibody against CD3 (V450, BD Bioscience). Cells were analysed by flow for T-cells that proliferated and therefore expressed low levels of eFluor670.

3.5.6. *In Vitro* Latency Model

Resting CD4$^+$ T-cells were labeled with the cytoplasmic dye eFluor670 and cultured alone or with one of seven sorted syngeneic APC subpopulations at a ratio of 10:1 for 24 hours in IL-2 (2U/mL, Roche Diagnostics, Basel, Switzerland) supplemented RF10 media. APC subpopulations included monocyte subpopulations (CD14$^+$CD16$^-$ and CD14^{lo}CD16$^+$), DC subpopulations (pDC, CD1c$^+$, CD141$^+$ and SLAN$^+$), and B-cells. Co-cultures were then infected with NL(AD8)Δ*nef*EGFP for 2 hours, after which time excess virus was washed away and cells were cultured for an additional 5 days. In order to compare APC stimulatory capacity between APC-T-cell co-cultures, at day 3 post-infection cells were stained with anti-CD3-V450 (clone UCHT1 ;BD Biosciences) to differentiate between T-cell and APC, and the proportion of proliferated (eFluor670^{lo}) CD4$^+$ T-cells were determined. At day 5 post-infection, productive infection was determined by EGFP expression and non-proliferating, non-productively infected (eFluor670^{hi} EGFP$^-$) CD4$^+$ T-cells were sorted using a FACSAria.

3.5.7. Reactivation of Latency from Non-Proliferating T-Cells

Latent infection in the sorted, non-proliferating CD4$^+$ T-cells (eFluor670^{hi}EGFP$^-$) was determined by comparing stimulated with unstimulated T-cells sorted from APC-T-cell co-cultures (control). 1x10^5 sorted CD4$^+$ T-cells was stimulated with immobilized anti-CD3 (7ug/ml; Beckman Coulter), in RF10 media supplemented with CD28 (5ug/mL; BD Biosciences), IL-7 (50ng/mL; Sigma, St Louis, MO), IL-2 (5U/mL; Roche),

with (post-integrated latency) or without (total latency: pre- and post-integrated latency) integrase inhibitor L8 (1μM; Merck, White House Station, NJ). Cells were harvested after 72 hours of stimulation and EGFP expression was quantified on the FacsCalibur (BD Biosciences).

In some experiments PHA (10ug/mL) and IL-2 (10U/mL) stimulated PBMC were used to activate T-cells to measure inducible virus replication form latently infected cells, as described previously (Evans et al. 2013).

3.5.8. Cell Preparation for Next Generation Sequencing and Generation of Gene Lists

APC subpopulations from 3 donors were sorted as described above to obtain mDC subpopulations CD1c$^+$ and SLAN$^+$ mDC, CD14$^+$ monocytes and pDC, which were immediately stored in RLT buffer (Qiagen, Limburg, Netherlands). Total RNA was isolated from low cell number samples (<500,000 cells) using QiagenALL prep micro kits (Qiagen), while RNA from samples with >500,000 cells were isolated using Qiagen RNeasy mini kits (Qiagen), according to the manufacturer's instructions. Total RNA content varied from 270.0-1879.7ng.

The Australian Gene Research Facility Ltd (AGRF, Melbourne, Australia) prepared cDNA libraries, which were multiplexed on the Illumina HiSeq 2000 (Illumina, San Diego, CA). For gene expression analysis, single reads were selected with 20 million reads of 50bp read size gathered. The RNA-Seq reads were aligned to the human reference hg19 using the TopHat2 aligner (Kim et al. 2013, p.2; Langmead et al. 2009) and quantified using htseq-count (Anders et al. 2015). Differential expression was calculated using Voom/Limma (Law et al. 2014) and visualization performed using Degust (http://victorian-bioinformatics-consortium.github.io/degust/; Powell 2014b) and Vennt (http://drpowell.github.io/vennt/; Powell 2014a). Genes with fewer than 10 reads across every sample were removed from the analysis.

APC subpopulations were categorised as latency-inducing and latency-non-inducing subsets. Using a fold change of greater than 2 and false discovery rate (FDR) of 0.01, we identified 754 genes that were significantly upregulated in latency inducing populations (CD1c$^+$ mDC, CD14$^+$ monocytes, SLAN$^+$ DC) compared to latency non-inducing populations (pDC; Figure 3.4). As direct cell contact is required for the establishment of mDC induced latency, only proteins implicated in cell contact were selected in RNA-seq expression profiles using the GeneCodis database (http://genecodis.cnb.csic.es). We identified 285 genes from the initial list that encode for proteins known to be expressed on the plasma-membrane, membrane, integral to the plasma-membrane/membrane and cell junctions (Carmona-Saez et al. 2007; Nogales-Cadenas et al. 2009; Tabas-Madrid et al. 2012). Finally, we manually curated this list to identify 53 genes known to be involved in T-cell signalling (Table 3.1 and Appendix table 8.1). RNA-seq data is available through Gene Omnibus (GEO), serial number GSE70106.

For comparison, we performed the same analysis using our previously published microarray data using the same APC subpopulations (Harman et al. 2013). Microarray data was kindly provided by Andrew Harman, Westmead Millennium Institute for Medical Research, Sydney University, Sydney (Harman et al. 2013). The RNA extraction, labeling, hybridization, data processing, and analysis procedures used by Harman et al are described previously for the cDNA gene array (Harman et al. 2006) and Illumina arrays (Harman et al. 2009). Hybridization and data processing was performed by AGRF using sentrix human 6 v2 expression chips (Illumina).

3.5.9. Statistical analysis

Differences between experimental conditions were analysed using Wilcoxon matched pairs signed rank test (n≥5) or paired Student T-test (n<5) on GraphPad Prism (Version 6). P-values ≤0.05 were considered significant. Differentially expressed RNA-seq and microarray genes were found to be significant using ANOVA (Harman et al. 2013).

Acknowledgements and contributions to manuscript.

Author contributions

NK carried out cell isolations and infection experiments with the assistance of KC. CdfP and JA assisted with imaging experiments NK performed RNA isolation. DRP performed most of the bioinformatics analysis for RNA-seq data and with NK generated and analysed genes lists. Microarray analysis was completed by PUC and NK. PUC, NK and SRL conceived the study, and participated in its design and coordination. Manuscript was prepared by PUC, NK, VE and JA. All authors read and approved the final manuscript.

4. Chapter 4

Myeloid DC Induce Latency in Proliferating CD4^{+} T-Cells

Manuscript in preparation

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4.0. Chapter 4: Myeloid DC Induce Latency in Proliferating CD4$^+$ T-Cells

4.1. Abstract

Background: Currently two hypotheses exist for the mechanisms of the establishment of HIV-1 latency *in vivo:* direct infection of resting CD4$^+$ T-cells (pre-activation latency) or reversion of an infected, activated CD4$^+$ T-cell to a resting state (post-activation latency). We have previously shown that latency is established through direct infection of non-proliferating CD4$^+$ T-cells during co-culture with myeloid dendritic cells (mDC). In this study we aimed to determine if HIV-1 latency was also induced in proliferating CD4$^+$ T-cells, using our *in vitro* DC-T-cell co-culture model.

Methods: Resting CD4$^+$ T-cells were stained with a cytoplasmic dye (eFluor670) and co-cultured with mDC, plasmacytoid DC (pDC) or monocytes (CD14$^+$) in the presence of staphylococcal enterotoxin B (SEB). Five days post infection, non-productively-infected, non-proliferating (eFluor670^{hi}EGFP$^-$, pre-activation latency) and proliferating (eFluor670^{lo}EGFP$^-$, post-activation latency) CD4$^+$ T-cells were sorted. Latency was quantified in both sorted populations by measuring EGFP expression following 3 days of stimulation with anti-CD3/CD28 and IL-7 (day 5 latent infection). Some of these cells were also cultured with IL-7, T-20 (a HIV-1 fusion inhibitor) and L8 (a HIV-1 integrase inhibitor) for 7 days, and latency was again quantified (day 12 latent infection).

Results: Following infection of CD4$^+$ T-cell co-cultured with mDC or monocyte, proliferating CD4$^+$ T-cells (eFluor670^{lo}) had significantly more latent infection on day 5 post-infection compared with non-proliferating (eFluor670^{hi}EGFP$^-$) CD4$^+$ T-cells (p<0.01). At day 12, latent infection in non-proliferating cells remained similar to day 5. Latent infection in proliferating CD4$^+$ T-cells co-cultured with mDC had decreased by 39 fold between day 5 and 12, but remained detectable. On day 12 proliferating CD4$^+$ T-cells from

co-cultures with pDC had low viability, and therefore latent infection could not be quantified.

Conclusion: HIV-1 latency can be established in both non-proliferating and proliferating CD4$^+$ T-cells during *in vitro* co-culture with mDC and monocytes. These results demonstrate that both pre- and post-activation latency can be established in the DC-T-cell *in vitro* model. Together these results suggests that HIV-1 expression is not directly linked to activation of CD4$^+$ T cells and that activated CD4$^+$ T-cell do contribute to the latent reservoir.

4.2. Introduction

Combination antiretroviral therapy (cART) has revolutionised the treatment of HIV-1 but has to be administered lifelong (Ho et al. 1995; El-Sadr et al. 2006; Palmer et al. 2008). The major barrier to HIV-1 cure is the persistence of long-lived, latently infected, resting CD4^{+} T-cells (Chun et al. 1995; Chun et al. 1998; Siliciano et al. 2003; Finzi et al. 1997). Better understanding of the mechanisms that lead to the establishment and maintenance of latent infection is critical to develop novel strategies to eliminate latent infection and eventually cure HIV-1 infection.

In vitro latency models suggest that latent infection can be established via the pre-activation (direct infection of non-proliferating CD4^{+} T-cells) or post-activation pathways (reversion of an activated, infected CD4^{+} T-cell to a resting state). Pre-activation latency can be established *in vitro* through direct infection of non-proliferating memory CD4^{+} T-cells using spinoculation (Swiggard et al. 2005), chemokine treatment (Saleh et al. 2007), co-culture with myeloid dendritic cells (mDC; Evans et al. 2013) or endothelial cells (EC; Shen et al. 2013). *In vitro*, post-activation latency has been demonstrated following the reversion of infected activated CD4^{+} T-cells to a resting state (Bosque & Planelles 2009; Marini et al. 2008; Sahu et al. 2006; Tyagi & Karn 2007). Additionally, in a different model systems, following infection of activated CD4^{+} T-cells, a subset of T-cells have been shown to contain inducible virus, before reversion to a resting state (van der Sluis et al. 2013). In this study, we show that mDC and monocytes can induce latency in non-proliferating and proliferating CD4^{+} T-cells *in vitro*. We show that latent infection was established in proliferating CD4^{+} T-cells and a small proportion of these cells could be maintained in culture for up to 12 days *in vitro*. Additionally, we show that pDC can only induce short-lived latency in proliferating CD4^{+} T-cells.

4.3. Results

4.3.1.   Inducible Virus in Proliferating CD4$^+$ T-Cells

Previously we demonstrated the establishment of pre-activation HIV-1 latency in non-proliferating CD4$^+$ T-cells following co-culture with syngeneic mDC and monocytes (results section 3.3.5; Evans et al. 2013). To determine whether mDC or pDC could establish latency in proliferating T-cells we performed experiments where eFluor670 labeled resting T-cells were cultured alone, with mDC or pDC as previously described Chapter 3.0 (Section 3.5.5). Following infection with a CCR5-EGFP reporter virus uninfected non-proliferating CD4$^+$ T-cells and proliferating CD4$^+$ T-cells were sorted (Figure 4.1A; Evans et al. 2013). Five days post-infection, productive infection (EGFP+) was high in T-cells cultured with either mDC and pDC compared to T-cells cultured alone, as previously described (Figure 4.1C; Evans et al. 2013). Latent infection was determined by quantification of EGFP using flow cytometry following stimulation of sorted CD4$^+$ T-cells with phytohaemagglutinin (PHA)/IL-2 activated peripheral blood mononuclear cells (PBMC). Following HIV-1 infection of both mDC and pDC-T-cell co-cultures, proliferating CD4$^+$ T-cells had more latent infection compared to the non-proliferating CD4$^+$ T-cells (Figure 4.1D).

Figure 4.1 Latent Infection in Proliferating CD4$^+$ T-Cells.

A. eFluor670 labeled resting CD4$^+$ T-cells were cultured alone (red), with mDC (blue) or pDC (orange) at a ratio of 10:1. After 24 hours, cultures were pulsed for 2 hours with a CCR5-tropic EGFP reported virus. **B.** 5 days post-infection, we used flow cytometry to sort for uninfected, non-proliferating (eFluor670^{hi}EGFP$^-$) and proliferating cells (eFluor670^{lo}EGFP$^-$). **C.** At day 5, EGFP expression was used to quantify productive infection in total cell cultures (n=2). **D.** Latency was measured as EGFP expression following stimulation of non-proliferating and proliferating CD4$^+$ T-cells with the addition of phytohaemagglutinin (PHA) and IL-2 activated feeder PBMC (n=2). Columns represent median, symbols represent results from individual donors and dotted line represents the limit of detection.
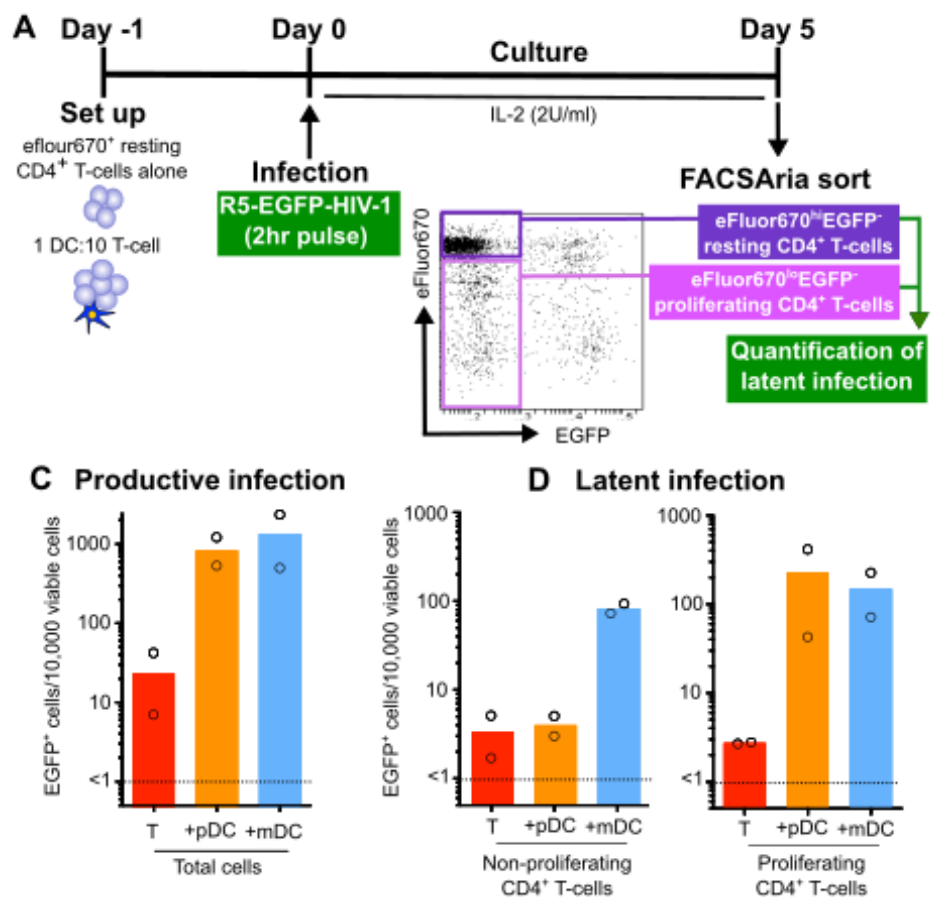
4.3.2. Staphylococcal Enterotoxin B Specific CD4^{+} T-Cell Activation

To enhance the number of proliferating CD4^{+} T-cells, all co-cultures were treated with the superantigen staphylococcal enterotoxin B (SEB), which mimics an antigen specific reaction by stabilising the interaction between major histocompatibility complex (MHC)-II and T-cell receptor (TcR), leading to T-cell activation and proliferation (Bhardwaj et al. 1992). An advantage of SEB-induced T-cell proliferation is that not all T-cells have SEB-specific TcR allowing a proportion of T-cells to remain resting.

A syngeneic mixed leukocyte reaction (MLR) reaction was used to determine the optimal SEB concentration where only a subset of T-cells that are SEB-specific were activated (Figure 4.2A, B). Resting CD4^{+} T-cells were cultured alone, with mDC or pDC, in the absence or presence of increasing concentrations of SEB (0.0001-1000ng/mL). CD4^{+} T-cell proliferation was measured at day 5 as the number of proliferating cells (eFluor670^{lo/-} cells) per total CD3^{+}CD4^{+} T-cells using flow cytometry. At day five, a maximum of 62-75% of CD4^{+} T-cells in mDC and pDC co-cultures had proliferated using 10ng/ml of SEB (Figure 4.2A, B) and this concentration of SEB was used in all subsequent experiments.

Figure 4.2. Proportion of proliferating CD4$^+$ T-cells following titration with staphylococcal enterotoxin B.

The optimal concentration of staphylococcal enterotoxin B (SEB) to increase the proportion of proliferated CD4$^+$ T-cells was determined in T-cell-DC co-cultures. eFluor670 labeled CD4$^+$ T-cells were co-cultured with **A.** mDC (T/mDC) and **B.** pDC (T/pDC) at a ratio of 10:1 and with increasing concentrations of SEB (n=4). Resting CD4$^+$ T-cells cultured with SEB were used as a control (T). The solid line represents the median of 4 individual experiments. Symbols represent results from individual donors.
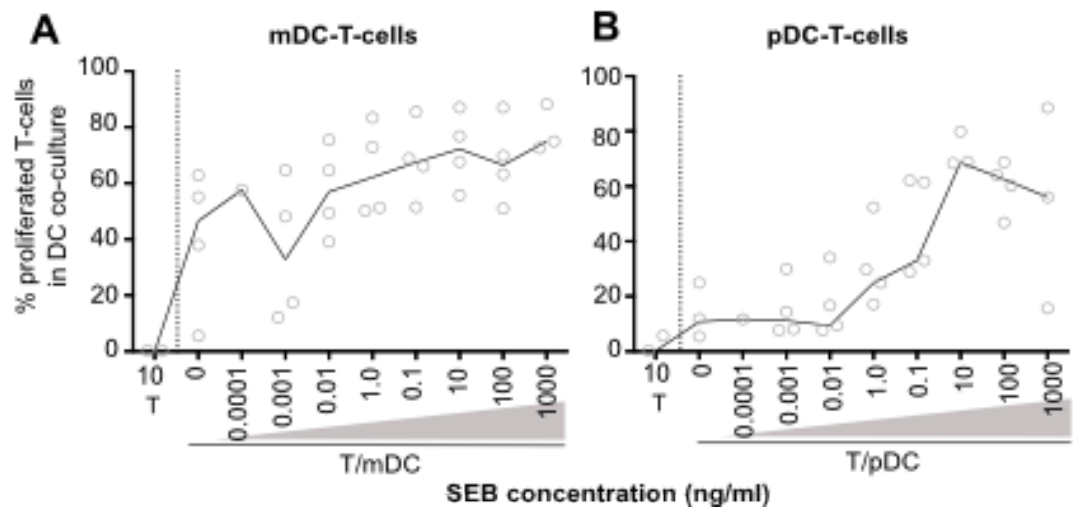
4.3.3. Latent Infection in Proliferating CD4$^+$ T-Cells at Day 5 Post-Infection.

To investigate whether latent infection could be detected in proliferating CD4$^+$ T-cells, eFluor670-labeled resting CD4$^+$ T-cells were cultured alone and co-cultured with mDC, pDC or monocytes in the presence of SEB (Figure 4.3A). Monocytes were included because compared to DC they are easier to isolate from PBMC due to their abundance and are also able to induce latency in non-proliferating CD4$^+$ T-cells (Section 3.3.1). After 24 hours, co-cultures were infected with CCR5-EGFP reporter virus for 2 hours (Figure 4.3A). At day 5 post-infection T-cells co-cultured with mDC, pDC and monocytes had significantly higher levels of productive infection compared to T-cells cultured alone (Figure 4.3B), as previously shown (Section 3.3.4). However, even with the super-antigen SEB, T-cells co-cultured with pDC had significantly less productive infection compared to T-cells cultured with either mDC or monocytes (mean EGFP+ cells/10,000 viable cells following co-culture with pDC, mDC and monocytes was 300, 1200 and 1100 respectively; Figure 4.3B).

To investigate the establishment of HIV-1 latency in non-proliferating and proliferating CD4$^+$ T-cells, co-cultures were sorted into non-proliferating and non-productively infected (eFluor670^{hi}EGFP$^-$) and proliferating and non-productively infected (eFluor670^{lo}EGFP$^-$) CD4$^+$ T-cells using flow cytometry (Figure 4.3A). To exclude DC and monocytes, as well as activated T-cells, from the proliferating T-cell (eFlour670^{lo}) populations, cell cultures were stained with antibodies specific for CD3 and HLA-DR. DC and monocytes express high levels of HLA-DR, and CD4+ T-cells upregulate HLA-DR expression upon activation, therefore, we were able to sort unactivated CD3+HLA-DR- T-cells using these markers (Figure 4.3A). Additionally, DC-T-cell doublets were further excluded using side scatter height and width to gate on single cells. To quantify latency in the sorted populations, the number of EGFP positive cells was determined after 3 days of culture with or without anti-CD3/CD28 and IL-7 in the presence of the integrase inhibitor L8 or

raltegravir (post-integration latency; Figure 4.3C, D). The frequency of unstimulated virus production (unstimulated+L8) was subtracted from the frequency of stimulated virus (stimulated+L8; Figure 4.3D) to determine post-integration latency. As previously reported, post-integration latency was only observed in non-proliferating CD4$^+$ T-cells following co-culture with mDC (p=0.02) and not pDC (p>0.05; Figure 4.4E; also shown in sections 2.3.1 and 3.3.5; Evans et al. 2013). Non-proliferating CD4$^+$ T-cells co-cultured with monocytes did not have a significant increase in level of post-integration latency after subtraction of virus production from unstimulated cells (Figure 4.3D, E).

Proliferating CD4$^+$ T-cells co-cultured with mDC, pDC and monocytes had significantly more post-integration latency on day 5 post-infection compared to T-cells cultured alone (p= 0.004, 0.04, and 0.02 respectively; Figure 4.3D, E). Post-integration latency in proliferating CD4$^+$ T-cells was greater in magnitude compared to post-integration latency in non-proliferating CD4$^+$ T-cells of the same cultures. This data indicates that proliferating CD4$^+$ T-cells can harbor latent HIV-1 infection.

Figure 4.3. Detection of Post-Integration Latent Infection in Proliferating CD4$^+$ T-Cells on Day 5 Post-Infection.

A. eFlour670 labeled resting CD4$^+$ T-cells were cultured alone (red), with mDC (blue), pDC (orange) or monocytes (green) at a ratio of 10:1. After 24 hours, co-cultures were infected with CCR5-EGFP reporter virus. On day 5 post-infection, non-productively infected, non-proliferating (eFluor670^{hi}EGF$^-$) and proliferating (eFluor670^{lo}EGFP$^-$) CD4$^+$ T-cells were sorted by flow cytometry. Non-proliferating and proliferating cells that were not infected were selected from viable cells based on eFluor670 and EGFP expression, DC were eliminated by selection of T-cells that were CD3$^+$HLA-DR$^-$. **B.** EGFP expression was measured at day 5 post-infection to quantify productive infection in total cells cultures. **C.** Latent infection was measured in the sorted T-cells by stimulation with anti-CD3/CD28 in the presence of IL-7, IL-2 and integrase inhibitor, L8. Representative dot plots of EGFP positive cells with or without 3 days of anti-CD3/D28 stimulation are shown **D.** T-cells were cultured for 3 days without stimulation and presence of IL-2 and L8 to measure unstimulated virus production. **E.** Post-integration latent infection was calculated by subtracting the number of EGFP+ cells following culture without stimulation (unstimulated+L8) from the number of EGFP+ cells following stimulation with anti CD3/CD28 (stimulated+L8). Columns represent median, symbols represent results from individual donors and dotted lines respresent the limit of detection. Significance was measured by Paired Students T-test where n<5 or Wilcoxon signed-rank test where n≥5, *p≤0.05, **p≤0.005, ***p≤0.0005. ND means not done.

Figure 4.3. Detection of Post-Integration Latent Infection in Proliferating CD4$^+$ T-Cells on Day 5 Post-Infection.
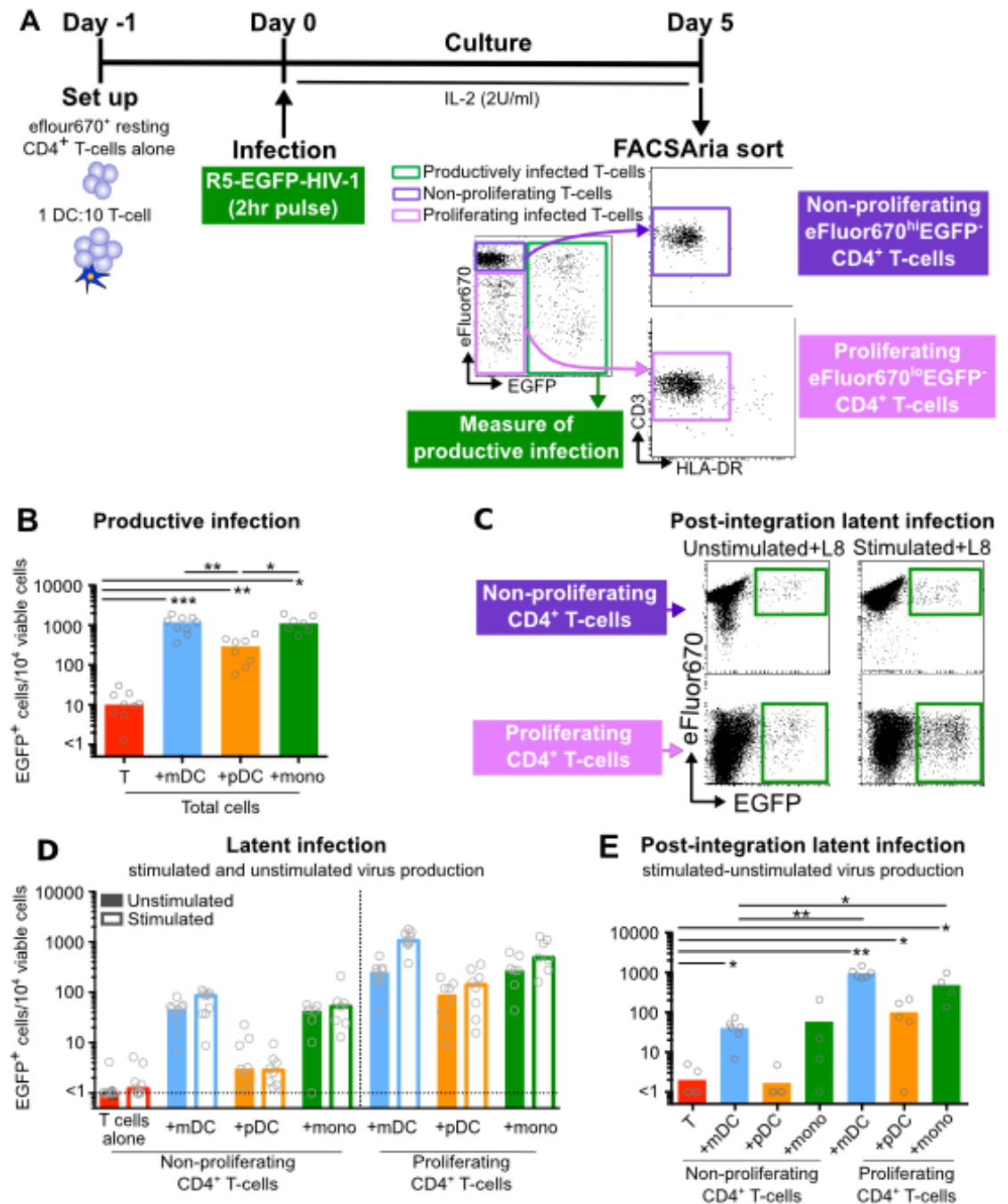
4.3.4. Expression of Activation Markers in Proliferating CD4$^+$ T-Cells

To determine whether T-cells express cellular activation markers at the time of cell sort the expression of early activation markers CD69 and CD25, and the immune checkpoints (IC); T-cell immunoglobulin and mucin domain 3 (Tim-3) were determined on sorted CD4$^+$ T-cells (Figure 4.4). HLA-DR, a late activation marker, was not used to measure T-cell activation as this marker was used to identify and exclude DC. HLA-DR is expressed on activated T-cells also but at an intermediate level compared to APC. Expression of CD25 and CD69 on non-proliferating CD4$^+$ T-cells following co-culture with mDC, pDC and monocytes was significantly higher than T-cells alone, but expression between the different cultures was not significantly different (expression of T cells following co-culture with mDC, pDC or monocytes relative to T-cells alone for CD69: $p<0.001$ for all; and CD25: $p<0.001$, <0.001, <0.001 respectively; Figure 4.4). Expression of CD69 and CD25 was significantly higher in proliferating CD4$^+$ T-cells compared to non-proliferating CD4$^+$ T-cells (all comparisons $p <0.0001$; Figure 4.4).

Tim-3 expression increases following T-cell activation and facilitates suppression of an immune response (Jones et al. 2008; Mujib et al. 2012). Non-proliferating CD4$^+$ T-cells from all cultures had low Tim-3 expression (Figure 4.4H). As expected, all proliferating CD4$^+$ T-cells had significantly higher expression of Tim-3, which was not significantly different between the different co-culture conditions (Figure 4.4H).

Figure 4.4. Expression of Activation Markers on Proliferating CD4$^+$ T-Cells.

eFlour670 labeled resting CD4$^+$ T-cells were cultured alone (red), with mDC (blue), pDC (orange) or monocytes (green), after 24hrs co-cultures were infected with EGFP reporter virus. **A.** CD4$^+$ T-cell proliferation was measured by quantification of eFluor670$^{lo/-}$ cells. Expression of activation markers **B.** CD69, **C.** CD25 and **D.** Immune checkpoint(IC), Tim-3, was measured on sorted non-proliferating (eFlour670^{hi}EGFP$^-$) and proliferating (eFluor670^{lo}EGFP$^-$) CD4$^+$ T-cells. Columns represent median, symbols represent results from individual donors and dotted line represents the limit of detection. Significance was measured by Paired Students T-test where n<5 or Wilcoxon signed-rank test where n≥5, *p≤0.05, **p≤0.005, ***p≤0.0005.
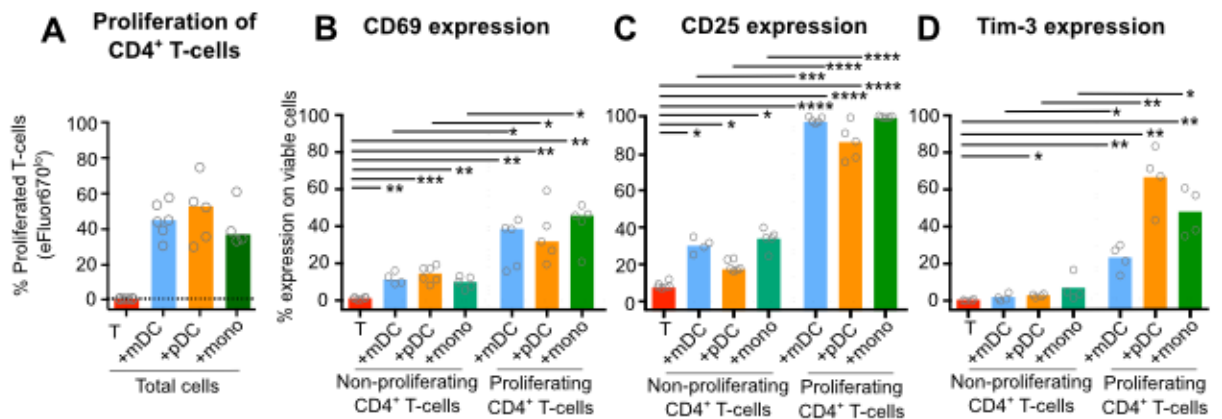
4.3.5. Post-integration Latency in Non-Proliferating and Proliferating CD4$^+$ T-Cells is Stable *In Vitro*

To determine whether post-integration latent infection in this *in vitro* system was stable, five days following infection of DC-T-cell co-cultures, non-proliferating and proliferating CD4$^+$ T-cells were sorted and further cultured for 7 days (up to day 12 post-infection). Cells were cultured with IL-7 (1ng/mL) and IL-2 (10U/mL) to maintain cell viability, HIV-1 fusion inhibitor T-20 and HIV-1 integrase inhibitor L8 to prevent a spreading infection (Figure 4.5A). Cell viability remained high, above 60% for all conditions except pDC co-cultures. Non-proliferating CD4$^+$ T-cells that had been co-cultured with pDC had high viability until day 12, however proliferating T-cells from the same co-cultures had high levels of cell death (cell viability <20%; Figure 4.5B, C). Latency was not quantified in these cells.

EGFP expression infection at day 12 in the non-proliferating and proliferating CD4$^+$ T-cells that had been co-cultured with mDC, pDC and monocytes was low compared to day 5 (Figure 4.5E compared to D; <10 EGFP+ cells/10,000 viable cells). Post-integration latency at day 12 was quantified as described for day 5 (Figure 4.3D). Comparison of post-integration latency at day 5 and day 12 showed that there was an overall decrease in latent infection from both non-proliferating and proliferating CD4$^+$ T-cells, however, the pattern of latency remained the same (Figure 4.5F, G). The decrease in post-integration latency from day 5 to day 12 was far less pronounced in the non-proliferating compared to proliferating CD4$^+$ T-cells that had been co-cultured with mDC (fold change decrease of 2.45 compared to 39.4 respectively; Figure 4.5F, G). The non-proliferating and proliferating CD4$^+$ T-cells that had been co-cultured with monocytes also had a decrease in post-integration latency; however, the fold decrease was much smaller (fold change decreases of 7.4 compared to 8.0 respectively; Figure 4.5F, G). These data demonstrate that mDC were able to induced latent infection in both non-proliferating and proliferating CD4$^+$ T-

cells using our *in vitro* DC-T-cell model, and that latency was stably integrated.

Figure 4.5. Post-Integration Latency in Non-Proliferating and Proliferating CD4$^+$ T-Cells is Stable.

A. eFlour670 labeled resting CD4$^+$ T-cells were cultured alone (red), with mDC (blue), pDC (orange) or monocytes (green) and, after 24hrs co-cultures were infected with EGFP reporter virus. At day 5, cell cultures were sorted into non-proliferating (eFlour670^{hi}EGFP$^-$) and proliferating (eFluor670^{lo} EGFP$^-$) CD4$^+$ T-cells populations. A subset of the sorted cells were cultured for a further 7 days in IL-2, IL-7, the HIV-1 fusion inhibitor T-20 and the HIV-1 integrase inhibitor L8 to measure the stability of latent infection. Cell culture viability of total cell cultures was quantified using forward-side-scatter on flow cytometry at **B.** day 5 and **C.** day 12. EGFP was quantified at **D.** day 5 and **E.** day 12 post-infection as a measure of productive infection. Latent infection was measured by quantification of EGFP expression following stimulation with anti-CD3/CD28 and IL-7. **F.** Post-integration latency was calculated by subtraction of the number of EGFP+ cells following culture without stimulation, from the number of EGFP+ cells with stimulation. **G.** At day 12, latent infection was measured again. Columns represent median, symbols represent results from individual donors and dotted lines represent the limit of detection. Significance was measured by paired students T-test where n<5 and Wilcoxon signed-rank test where n≥5, *p≤0.05, **p≤0.005, ***p≤0.0005. ND means not done.

Figure 4.5. Post-Integration Latency in Non-Proliferating and Proliferating CD4+ T-Cells is Stable.
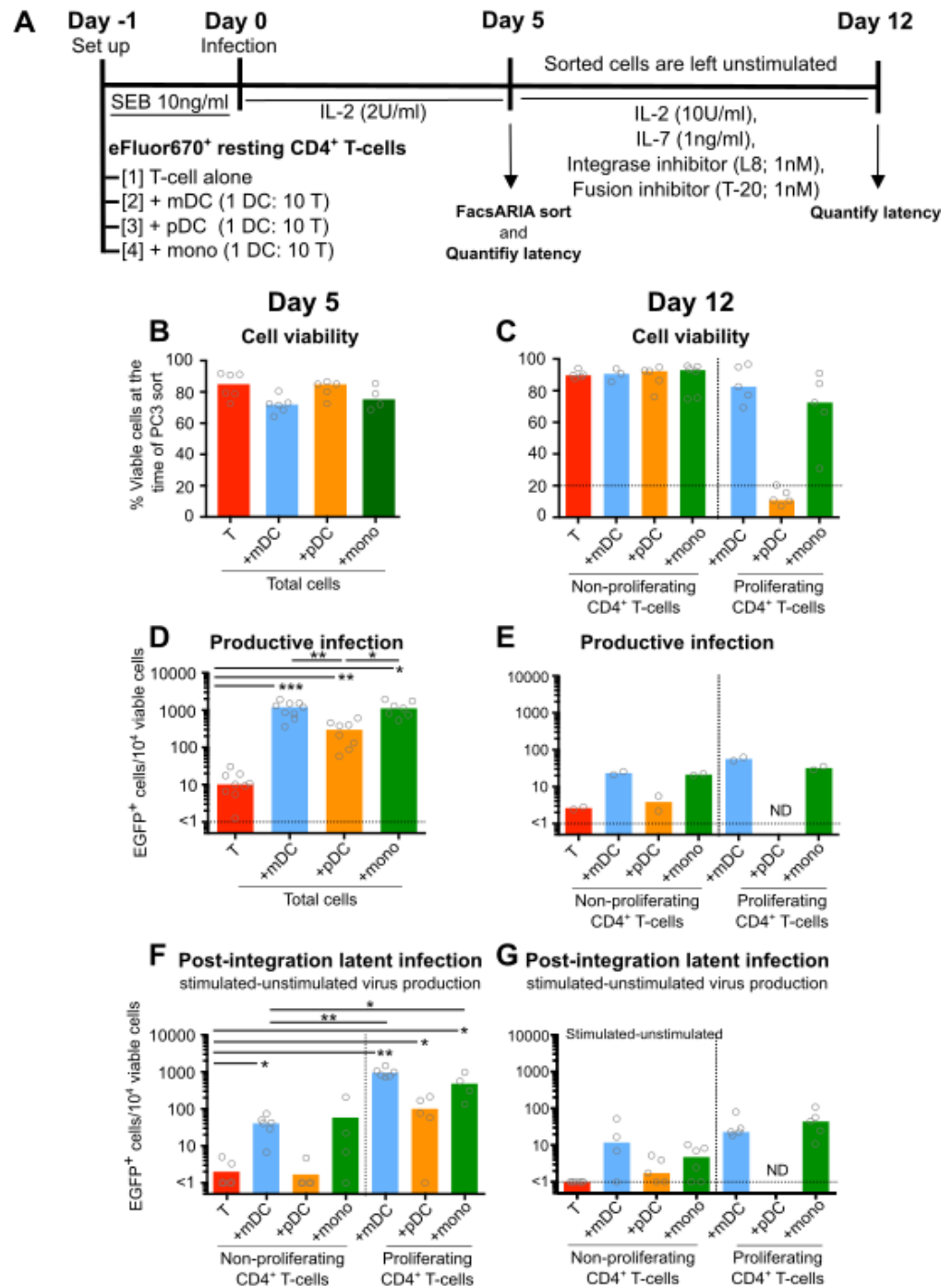
4.3.6. Establishment of Post-Integration Latent Infection Following Prolonged DC-T-Cell Co-cultures

To determine if the presence of DC or monocytes altered the establishment of latency in long-term DC-T-cell co-culture we extended co-culture conditions to day 11 and day 15. eFluor670 labeled resting CD4$^+$ T-cells were cultured either alone, or with mDC, pDC and monocytes and infected with EGFP-reporter virus at 24 hours. Cell cultures were sorted at day 5, 11 and 15 when cell viability, productive infection and latent infection was measured (Figure 4.6A). Cell viability was measured according to forward and side scatter of total cell cultures. Cell viability remained high in all cell cultures until day 15, except for T-cells that had been co-cultured with pDC, where viability fell below 50% (Figure 4.6B-D). Productive infection at each time point remained similar in CD4$^+$ T-cells that had been co-cultured with mDC, pDC and monocytes at day 5, 11 and 15 (Figure 4.6E-G). However, productive infection in T-cells cultured alone increased over time (Figure 4.6E-G). Post-integration latency in non-proliferating and proliferating CD4$^+$ T-cells co-cultured with mDC decreased from day 5 to 15 (Figure 4.6H-J), similar to the day 12 data described above (Figure 4.5D, E). Similarly, post-integration latency in proliferating CD4$^+$ T-cells that had been co-cultured with pDC decreased with time (Figure 4.6H-J).

Figure 4.6. Establishment of Latency During Prolonged DC-T-Cells Co-culture.

A. eFluor670 labeled CD4$^+$ T-cells were cultured alone (red), with myeloid (m)DC (blue), plasmacytoid (p)DC (orange) or monocytes (mono; green) at a ratio of 10:1 and infected with CCR5-EGFP reporter virus. Non-productively infected (EGFP$^-$), non-proliferating (eFluor670^{hi}) and proliferating (eFluor670^{lo}) T-cells were sorted on day 5, 11 and 15 post-infection. **B, C, D.** Cell culture viability was measured each day post-infection using flow cytometry. **E, F, G.** Productive infection was measured by quantification of EGFP in total cell cultures. **H, I, J.** Post-integration latent infection was measured from non-proliferating and proliferating CD4$^+$ T-cells by subtraction of the number of EGFP cells in culture without stimulation, from the number of EGFP cells following stimulation. Columns represent median, symbols represent results from different donors and dotted line represents the limit of detection. Significance was measured by paired students T-test where n≤5 *p≤0.05, **p≤0.005, ***p≤0.0005. ND means not done.

Figure 4.6. Establishment of Latency During Prolonged DC-T-Cells Co-culture.
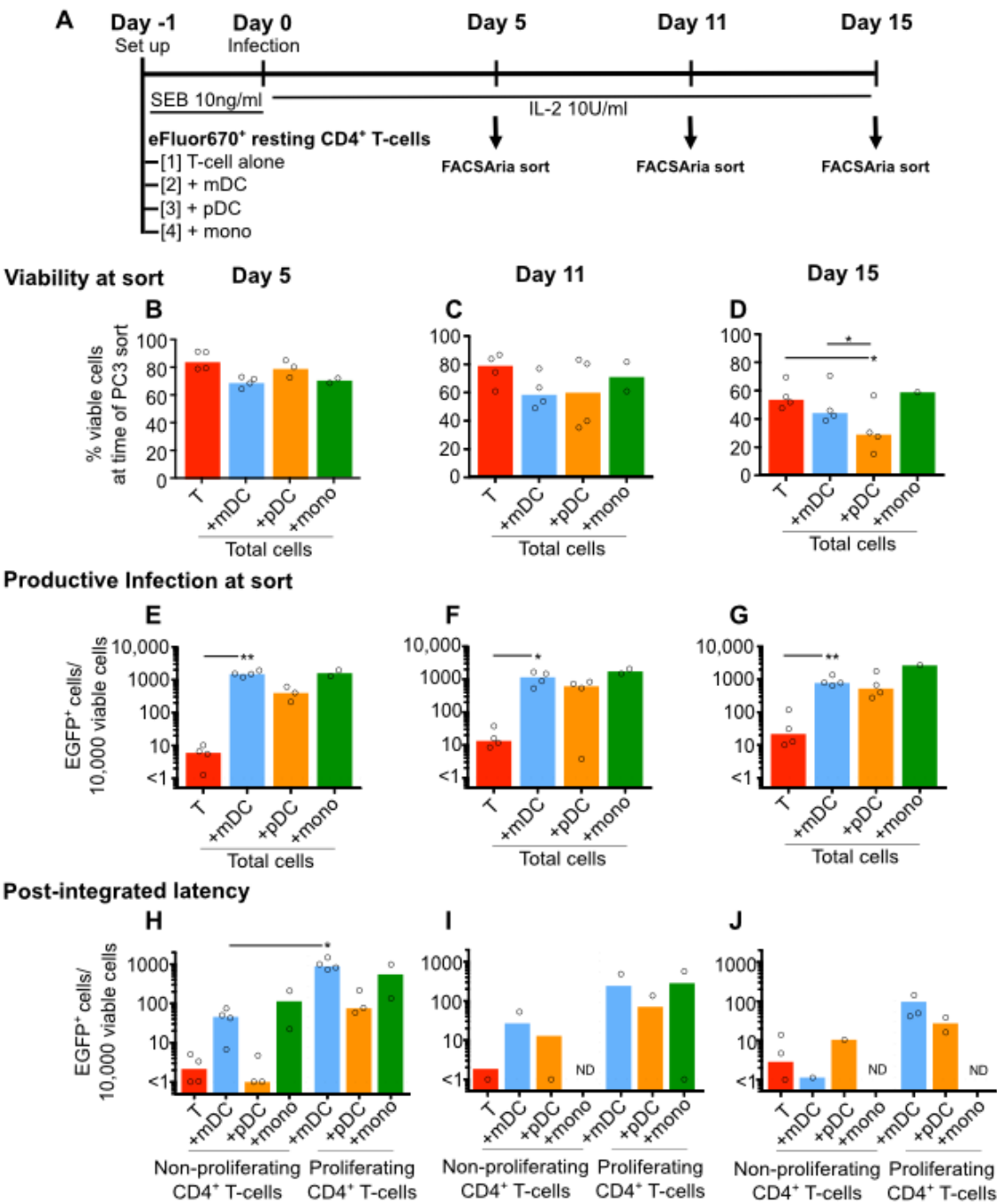
4.3.7. Proliferating CD4$^+$ T-Cells Remain CCR7$^+$ and CD27$^+$

We next determined the phenotype of freshly isolated resting CD4$^+$ T-cells, non-proliferating and proliferating CD4$^+$ T-cells that had been cultured alone, with mDC, pDC or monocytes at day 5 and 12 following infection. To quantify the proportion of naïve T-cells, the percentage of CD45RO$^-$ cells was determined (Figure 4.7A). Naïve CD4$^+$ T-cells made up approximately half the non-proliferating cell population and remained stable during 12 days of culture (Figure 4.7B, C). As expected naïve cells were rare in the proliferating T-cell population.

To quantify memory cell populations CD45RO$^+$ cells were further phenotyped based on CCR7 and CD27 expression. The majority of CD45RO expressing cells, in both non-proliferating and proliferating T-cells populations, was of central memory phenotype and remained stable through to day 12 (80-90%; Figure 4.7E, F). The remaining CD45RO expressing cells were of transitional memory and effector cell phenotype and remained a small fraction of the proliferating T-cell population through to day 12 (<5%; Figure 4.7).

Figure 4.7. T-Cell Subset Phenotype of Non-Proliferating and Proliferating CD4$^+$ T-Cells.

The phenotype of proliferating and non-proliferating T-cells following culture of T-cells alone, or co-culture with mDC, pDC or monocytes was determined at day 5 and 12 post-infection using antibodies to CD45RO, CCR7 and CD27. **A.** Representative dot plot of total viable T-cells gated on CD45RO$^+$ and CD45RO$^-$ is shown **B.** 5 days and **C.** 12 days after infection. **D.** Representative dot plot of CD45RO$^+$ cells further phenotyped based on CCR7 and CD27 expression is shown **E.** 5 days and **F.** 12 days after infection; Columns represent median with inter-quartile-range (IQR; n=6).
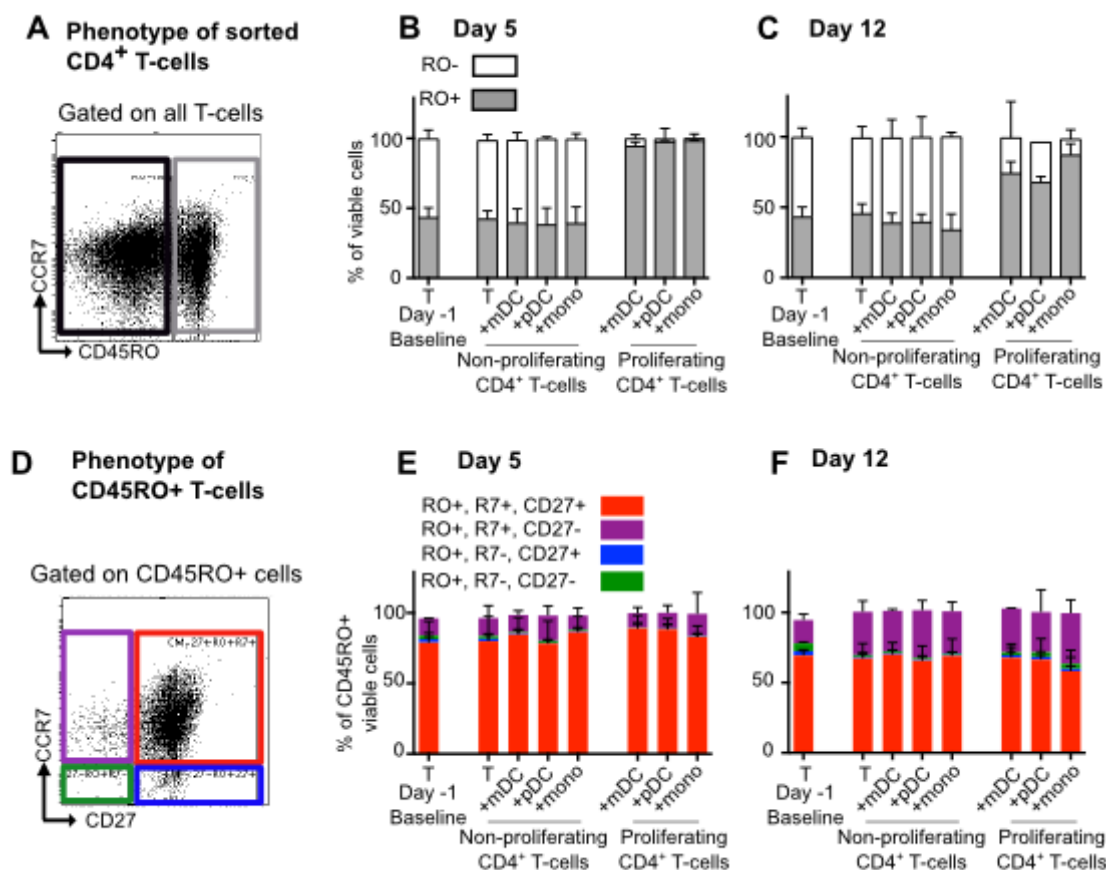
4.4. Discussion

Previously we showed that HIV-1 latency could be established following infection of non-proliferating CD4$^+$ T-cells that were co-cultured with mDC (section 2.3.1; Evans et al. 2013) and monocytes (Section 3.3.1 and 3.3.5). Here, we extend on these findings to demonstrate the successful establishment of latency in both proliferating and non-proliferating CD4$^+$ T-cells following co-culture with mDC and monocytes. Additionally, we found that latency established in non-proliferating and proliferating cells was stable, with detection of post-integration latency after 7 days of additional cell culture.

4.4.1. Dendritic Cell can Facilitate the Establishment of Post Integration Latent Infection in Proliferating CD4$^+$ T-Cells

Upon exposure to HIV-1 and in the presence of mDC and pDC, activated T-cells become productively infected (Groot et al. 2006). DC can interact with T-cell via cognate interactions, major histocompatibility complex (MHC)-II-T-cell receptor (TcR), leading to T-cell activation, therefore more efficient HIV-1 infection (Delon et al. 1998; Revy et al. 2001; Mempel et al. 2004). Proliferating CD4$^+$ T-cells found in our DC-T-cell culture were indeed activated as they expressed the early activation markers CD69 and CD25. Here we show that the interaction between T-cells with mDC and pDC gave rise to a population of productively infected T-cells and non-infected but activated T-cells. We are the second group to show latent infection of activated T-cells. Previously van der Sluis et al. showed that T-cells activated with anti-CD3/CD28 expressed inducible virus upon reactivation with MDDC and primary mDC (van der Sluis et al. 2013). As well as showing the importance of activated T-cells as a reservoir of latency this study highlights the versatile function of DC.

Expression of CD25 on proliferating CD4$^+$ T-cells that have interacted with DC may also indicate induction of T-regs. This should be determined in future

experiments by measurement of FoxP3 (Coquet et al. 2013; Yu et al. 2011; Reviewed in Kornete & Piccirillo 2012). Given that the freshly isolated resting CD4^{+} T-cells were CD25 and CD69 negative, we expect that any T-regs generated were differentiated as a results of mDC or pDC interactions.

Ongoing studies in our lab are to determine the role of IC in latency induction. To this end we have measured the IC; PD-1, Tim-3, ICOS, CTLA-4, Lag-3, BTLA and TIGIT on non-proliferating CD4^{+} T-cells co-cultured with mDC, pDC and monocytes and their respective ligands on the APC; PD-L1/L2, Gal-9, ICOSL, CD80/CD86, HLA-DR, HVEM, PVR/PVR-L2 (Evans, manuscript in preparation; van der Sluis, unpublished work). To determine if the interaction between IC and their ligands is important in latency induction by mDC and monocytes, expression of IC on non-proliferating and proliferating CD4^{+} T-cells will be correlated with latent infection. If IC interactions are important for the induction of latency in non-proliferating or proliferating CD4^{+} T-cells we expect that there will be strong correlation between expression of specific IC and latent infection, as shown in *in vivo* and *ex vivo* with PD-1 (Chomont et al. 2009; Iglesias-Ussel et al. 2013). Following this, expression of IC that correlate with latent infection will be blocked *in vitro*, however we do not expect to see complete inhibition of latency but reduction in the magnitude of latency as there are several IC that will lead to redundancy in signalling via IC and their ligands. Currently there is no published data on the correlation between latency and IC other than PD-1.

Productively infected CD4^{+} T-cells usually die due to cytopathic effects of the virus (Sakai et al. 2006; Arokium et al. 2009; Ward et al. 2009; Zhao et al. 2011), detection by the host immune response (Hazenberg et al. 2000; Doitsh et al. 2014; Reviewed in Okoye & Picker 2013) or bystander effects (Lelièvre et al. 2004; Monroe et al. 2014). The population of proliferating CD4^{+} T-cells that contained post-integration latency may evade cytopathic effects due to the lack of viral protein expression and/or expression of immune checkpoints (IC) such as Tim-3 (CD366), expressed on proliferated CD4^{+} T-cells. Using IC such

as Tim-3, CTLA-4 (CD152), PD-1 (CD276) expressed on T-cells, DC are also able to regulate T-cell activation via inhibitory signalling that blocks T-cell activation (Parry et al. 2005) and are known to be active in HIV-1 infection (Kassu et al. 2010, p.201; Shankar et al. 2011; Che et al. 2012; Leitner et al. 2013). Negative T-cell regulation is achieved by ligation of IC expressed on T-cells, PD-1, CTLA-4 or Tim-3, by ligands on DC or monocytes like, PD-L1/2 (Boasso et al. 2008; Mott et al. 2014) , CTLA-4 (Krummel & Allison 1995; Wolchok & Saenger 2008) and Tim-3 (Mujib et al. 2012). Negative regulation via IC on proliferating T-cells with post-integration latency, may block T-cell activation thereby promoting T-cell survival and allowing reversion of the infected T-cell to memory state, thus become part of the long-lived latent reservoir.

Absence of viral transcription and protein expression from proliferating CD4$^+$ T-cells with post-integration latency could be mediated by negative regulation of T-cell activation via IC. We hypothesise that IC are involved in blocking T-cell activation and virus expression in 2 ways. First, signalling by IC leads to disruption of intracellular signalling cascades, for example CTLA-4-CD80/CD86 or PD-1-PD-L1 (CD274) interaction, thereby blocking T-cell activation and early events in the viral life cycle (Chemnitz et al. 2004; Parry et al. 2005; Yokosuka et al. 2012). These processes may be involved in establishment of latency in non-proliferating CD4$^+$ T-cells. Second, IC may block transcriptional activity, thereby blocking virus transcription and production of *de novo* virions, as shown in CTLA-4-CD80/CD86 binding (Reviewed in Larsson et al. 2013). This mechanism may contribute to latency in proliferating T-cells, i.e. the cell is activated yet the virus remains latent. In previous chapters we have identified negative regulator encoding genes *CD101, Tim-3 (also known as HAVR2)*, *leukocyte immunoglobulin-like receptor member 6* (*LILR6*) and *CD52* to be highly expressed on mDC. Additionally, others have also shown that PD-1 is upregulated on latently infected cells *in vitro* (Iglesias-Ussel et al. 2013) and in CD4$^+$ T-cells from HIV-1-infected patients on cART *ex vivo* (Chomont et al. 2009). Together,

these data support a potential role for IC in the regulation of T-cell activation and latency *in vivo* and in our DC-T-cell model. However, further investigation is needed to identify the specific protein interactions and signalling cascades involved in blocking HIV-1 expression via IC in proliferating and non-proliferating T-cells.

4.4.2. Loss of Latent Virus During Prolonged Unstimulated Cell Culture

In HIV-1-infected patients on cART, latent infection is detected in resting CD4$^+$ T-cells and is stable over the life time of the patient (Siliciano et al. 2003). Here we have generated post-integration latency in proliferating CD4$^+$ T-cells where with prolonged *in vitro* culture, we observed a significant decline in post-integration latency in proliferating (eFluor670^{lo}EGFP$^-$) CD4$^+$ T-cells from mDC co-culture and a further 7 days of culture in the absence of additional stimulation. The decrease in latently infected proliferating T-cells could be multifactorial, including: [1] progression of some latently infected proliferating CD4$^+$ T-cells to virus expression and therefore moving to the EGFP+ population as productive infection or [2] cell death due to proliferation and T-cell activation.

Proliferating cells are likely activated, and therefore possess all factors necessary to produce *de novo* virions (Brooks et al. 2003; Zack et al. 1990). *In vitro*, with a decline in latent infection, we would expect to see an increase in productive infection. We showed that there was some *de novo* expression of EGFP, or virus production throughout the 7-day culture period in the absence of additional stimulation. Therefore it is possible that some of the latently infected cells progressed to productive infection. Alternatively, it is possible that upon sorting of non-proliferating and proliferating T-cells from the DC co-cultures, the factors that maintained latent infection have been removed, for example ligation of IC which may have blocked expression of virus, were no longer active. To counteract this we used the prolonged culture of cells with

the initial DC population. However DC in prolonged *in vitro* DC-T-cell cultures will not survive beyond 5 days, therefore DC death will act to withdraw ligation of IC and survival signals. We proposed that the mDC provided proliferating CD4$^+$ T-cells with signals to suppress viral expression thereby promoting the establishment of post-activation latency.

Upon activation, naïve and central memory T-cells expand into effector T-cells of which only a small proportion of effector T-cells revert to a resting state (Sallusto et al. 1999; Dai et al. 2009). The remaining effector cells, approximately 95%, undergo apoptosis, a process that maybe active in the *in vitro* DC-T-cell culture system and responsible for the loss of latently infected proliferating CD4$^+$ T-cells (Pepper & Jenkins 2011). Apoptosis of effector T-cells can be mediated by negative regulation of the immune response via natural killer cells (NK; Sallusto et al. 1999), regulatory T-cells (T$_{REG}$; Pandiyan et al. 2007), absence of antigenic stimulation (Ottina et al. 2013) or IFN-γ secretions (Li et al. 2007). We would expect that proliferating T-cells from infected, DC co-cultures may die within 10 days of stimulation via lack of antigen stimulation and exposure to IFN-γ.

In vivo homeostasis of resting naive and memory T-cells is regulated by cytokines IL-2, IL-7 and IL-15 (Jaleco et al. 2003; Reviewed in Castillo & Schluns 2012). However, to stimulate maximal virus production from latently infected cells high concentrations of IL-7 (50ng/mL) have been used (Scripture-Adams et al. 2002; Wang et al. 2005, p.-7). The maintenance of resting memory cell populations requires only low concentration of IL-7 (1ng/mL; Bosque et al. 2011). In this model of DC-T-cell co-cultures, we used a low concentration of IL-7 (1ng/ml) together with IL-2 (10U/ml) to maintain cell viability over extended culture periods. However, the combination of IL-7 and IL-2 has also been shown to lead to the preservation and expansion of latently infected cells (Bosque et al. 2011; Chomont et al. 2009). Despite using minimal concentrations of IL-7 and IL-2 in this study it is possible that these

cytokine contributed to the preservation and/or expansion of latently infected cells.

Proliferating and non-proliferating CD4+ T-cells show high expression of CD27 (Figure 4.7). The ligand to CD27 is CD70, which is expressed on activated DC, B-cells and T-cells (Brugnoni et al. 1997; Reviewed in Denoeud & Moser 2011). CD27 and CD70 belong to the TNF family of receptors and is believed to behave like a co-stimulatory molecule, which promotes the survival of pathogen specific T-cells via activation of the IL-2 gene, and is also involved in DC and EC mediated differentiation of T-regs (Coquet et al. 2013; DeBarros et al. 2011; McKinstry et al. 2014). Expression of CD70 on mDC would support the hypothesis that activation of cell survival pathways are involved in the establishment of HIV-1 latency and should be looked at in future experiments.

4.4.3.   pDC Induced Cell Death of Proliferated CD4$^+$ T-Cells.

Despite the lack of IL-3 to pDC co-cultures, which can enhance pDC survival *in vitro* (Grouard et al. 1997; Demoulin et al. 2012), we believe that the increased cell death of the sorted proliferating T-cells that were co-cultured with pDC is a specific pDC response to HIV infection. Activated pDC are known to promote T-cell activation and cell death of co-cultured cells via cognate interactions and stimulation of ISG (Chawla-Sarkar et al. 2003, p.-; Stawowczyk et al. 2011; Schoggins et al. 2011). Activation of T-cells leads to T-cell proliferation and AICD thereby causing death of activated and bystander T-cells, and activation of ISGs in T-cells leads to expression of TRAIL ligands thereby increasing susceptibility to apoptosis (Lelièvre et al. 2004; Li et al. 2014; Stary et al. 2009; Serei et al. 2012). The activation of apoptosis can be measured by activation of mediators of apoptosis including caspase 3, 8 and Annexin V, which are downstream of Fas-FasL and TRAIL-TRAIL-RI/RII (DR4/DR5) interactions (Lin et al. 2006; Li et al. 2007). Furthermore to confirm the mechanism of cell death we can measure the expression of TRAIL

and its ligands TRAIL ligands by flow, qPCR or RNA-seq. Using RNA-seq of pDC co-cultured proliferating CD4$^+$ T-cells we can also obtain additional information about the activation of other networks active in proliferating T-cells co-cultured with pDC, including activation of ISG and other networks involved in apoptosis. Comparative analysis of RNA-seq expression profiles from proliferated CD4$^+$ T-cells from mDC co-culture to pDC co-cultures may also shed light on novel mechanisms of inhibition or elimination of latent infection.

4.4.4. Conclusion and Significance

This is the first *in vitro* latency model to show that latency can be establishment in non-proliferating (pre-activation latency) and proliferating CD4$^+$ T-cells (post-activation latency) by DC and that these mechanisms can occur simultaneously and the second study to show latent infection in activated CD4$^+$ T-cells. We measured latent infection by quantification of EGFP expression following a TCR-mediated activation stimulus. However, it is possible that some viruses were not activated with this stimulus and therefore we may have under-estimated the frequency of latent infection in this co-culture system. Non-inducible proviruses have recently been described to be present in CD4$^+$ T-cells from HIV-1-infected patients on cART (Ho et al. 2013). These are full-length viruses, without mutations and are not expressed following activation of T-cells with mitogen or TCR stimulation. To fully determine whether there were non-inducible proviruses in latently infected CD4$^+$ T-cells from the DC-T-cell model, further work should be done using Alu LTR PCR and/or sequencing to directly quantify integrated virus in the non-proliferating and proliferating CD4$^+$ T-cells.

Here we show that latency can be established in non-proliferating and proliferating CD4$^+$ T-cells in the presence of mDC. Unlike latency in non-proliferating T-cells, active suppression of virus by mDC may play a role in the induction of latency in proliferating CD4$^+$ T-cells. Whether latently infected proliferating cells can revert into resting cells and thereby potentially

contribute to the long-lived latent reservoir remains to be investigated. Post-activation latency in T-cells co-cultured with pDC was established but high levels of cell death were observed. Further investigation of the mechanism leading to cell death in this setting, which we propose may be regulated by type-I IFN, may lead to new strategies to eliminate latent infection *in vivo*.

4.5. Materials and Methods

4.5.1. Isolation of T-cells, Dendritic Cells Subpopulations and Monocytes

Resting CD4$^+$ T-cells, mDC (HLA-DR$^+$CD11c$^+$CD123$^-$) and pDC (HLA-DR$^+$CD11c$^-$CD123$^+$) were isolated from healthy donor PBMC (Australian Red Cross) as described in section 2.5.1. Isolated resting CD4$^+$ T-cells were stained with proliferation dye eFluor670 (eBiosciences) according to manufacturer's protocol. Bulk monocytes (CD14$^+$) were isolated from syngeneic donors using positive selection of CD14 on PBMC using the autoMACS (Miltenyi Biotech). Only DC subpopulations and monocytes with a purity ≥95% and resting CD4$^+$ T-cells with a purity of >98% were used.

4.5.2. Preparation of Viruses

CCR5-EGFP reporter virus (NL(AD8)Δ*nef*EGFP) was used in all experiments (as described in section 2.5.2 and Evans et al. 2013). All cells were infected with an MOI of 0.5 as determined by the Reed and Muench method (Reed & Muench 1938).

4.5.3. Latency in Proliferating CD4$^+$ T-Cells from the *In Vitro* Dendritic Cell-Induced T-Cell Latency Model

In preliminary experiments (Figure 4.1A), eFluor670 labeled resting CD4$^+$ T-cells were cultured alone, or with mDC or pDC as previously described (Evans et al. 2013). To test for establishment of latency in proliferating CD4$^+$ T-cells, total cells were sorted based on eFluor670^{lo} and EGFP$^-$ expression. Latent infection in proliferating CD4$^+$ T-cells was measured by stimulation with PHA activated feeder PBMC, as described previously (Evans et al. 2013).

4.5.4. Induction of T-Cell Proliferation Using Staphylococcal Enterotoxin B in a Mixed Leukocyte Reaction.

To maximized the number of proliferating CD4$^+$ T-cells in DC-T-cell co-cultures SEB was used at a concentration where only SEB-specific TcR would interact and induce T-cell activation. In order to determine the optimal concentration of SEB to use in these experiments, eFluor670 labeled resting CD4$^+$ T-cells were cultured alone, with syngeneic mDC and pDC at a ratio of 10:1 (T-cell:DC) in IL-2 (2U/mL, Roche Diagnostics) and increasing concentrations of SEB (0, 0.0001, 0.001, 0.01, 0.1, 1.0, 10, 100, 1000 ng/mL, Sigma). 5 days after co-culture, cells were harvested and labeled with CD3 for flow cytometry analysis on the FACSCalibur (BD Biosciences). The number of eFluor670$^{lo/-}$ T-cells was analysed using weasel software (WEHI, Melbourne, Australia; Figure 4.3A), and used as a measure of T-cell activation. 10ng/mL of SEB was used for all subsequence experiments.

4.5.5. The *In Vitro* DC-T-cell Latency Model Optimised for Latency in Proliferated T-Cells

To measure latency in proliferated T-cells, eFluor670 labeled resting CD4$^+$ T-cells were cultured alone, with syngeneic mDC, pDC or monocytes at a ratio of 10:1 (T-cell:DC) for 24 hours in the presence of IL-2 (2U/mL, Roche diagnostics) and SEB (10 ng/mL, Sigma). CD4$^+$ T-cells co-cultured with mDC, pDC and monocytes were infected with NL(AD8)Δ*nef*EGFP reporter virus and cultured for a further 5 days in IL-2 (2U/mL) supplemented media, SEB was not re-added post-infection. Productive infection was measured at day 5 post-infection by quantification of EGFP expression (Figure 4.3B). Cultures were then sorted using a FACSAria (BD Biosciences) for CD4$^+$ T-cells that were non-productively infected (EGFP$^-$), and either non-proliferating (eFluor670^{hi}EGFP$^-$) or proliferating (eFluor670^{lo}EGFP$^-$). DC and monocytes were excluded from the eFluor670^{lo} cells by further gating for HLA-DR$^-$CD3$^+$ T-cells (Figure 4.3B).

4.5.6.   Quantification of Latency

Latent infection was determined following activation of 1×10^5 sorted CD4$^+$ T-cells (eFluor670^{hi}EGFP$^-$ or eFluor670^{lo}EGFP$^-$) with immobilized anti-CD3 (7ug/ml; Beckman Coulter), in RF10 media supplemented with soluble CD28 (7ug/ml; BD Biosciences) and IL-7 (50ng/ml; Sigma, St Louis, MO). In order to distinguish between pre and post-integration latency, cells were activated in the presence (post-integration latency only) or absence (total latency: pre and post-integration latency) of integrase inhibitor, either L8 (1mM; Merck) or raltegravir (1μM; NIH AIDS reagent program, Cat#11680). L8 and raltegravir was titrated in PHA or SEB activated PBMC that were infected with CCR5-EGFP reporter virus at an MOI of 0.5. The concentration of L8 and raltegravir that completely blocked productive infection was used in experiments to measure latent infection. Sorted cells were harvested 3 days after stimulation and analysed for activation of virus by quantification of EGFP expression on the FACSCalibur (BD Biosciences). Unstimulated virus production from the sorted T-cells (eFluor670^{hi}EGFP$^-$ and eFluor670^{lo}EGFP$^-$) was also measured by culture of T-cells without stimulation, but with IL-2 (10U/ml) and L8 (1mM) or raltegravir (1μM) for 72 hours (unstimulated+L8; Figure 4.3C). To calculate post-integration latency in proliferating CD4$^+$ T-cells unstimulated virus production (unstimulated+L8) was subtracted from stimulated post-integration virus (stimulated+L8).

4.5.7.   Stability of Latent Infection in
Unstimulated Culture of Sorted CD4$^+$ T-Cells

To measure the stability of latent infection in non proliferating and proliferating CD4$^+$ T-cells, $>5 \times 10^5$ sorted T-cells (eFluor670^{hi}EGFP$^-$ or eFluor670^{lo}EGFP$^-$) were cultured without stimulation for 7 days in RF10 media supplemented with IL-2 (10U/mL), IL-7 (1ng/mL), the fusion inhibitor T-20 (1ug/mL, NIH reagent program, Cat# 9845) and either L8 (1mM) or raltegravir (1μM). After 7 days (day 12), the cells were counted, viability determined using trypan blue and T-cells were then stimulated to determine total and post-integrated latent infection, as described above.

4.5.8. Flow Cytometry

Surface expression markers were stained using specific antibodies detailed in Table 4.1. Antibodies Used for Flow Cytometry Analysis. Cells were stained as described in section 2.5.5. Samples were analysed on either a FACSCalibur or LSR II (BD Biosciences) and data analysed using Weasel software (version 2.7;WEHI).

Table 4.1. Antibodies Used for Flow Cytometry Analysis

Antibody used	Clone	Function	Source	Reference
CD14-FITC	M5E2	Monocyte marker, Fc-gamma receptor	BD Bioscience	(Thomas & Lipsky 1994; Siedlar et al. 2000; Mandl et al. 2014)
CD11c-APC/V450	B-146	mDC marker, adhesion molecule	BD Bioscience	(Osugi et al. 2002)
CD123	9F5	pDC marker	BD Bioscience	(Dzionek et al. 2000)
HLA-DR-APC-Cy7/PerCP	L243	MHC II, DC marker	BD Bioscience	(Bains et al. 2003; MacDonald et al. 2002)
CD69-FITC	L78	Early activation marker	BD Bioscience	(Schiott et al. 2004; Reviewed in Shipkova & Wieland 2012)
CD25-PE	2A3	Early activation marker	BD Bioscience	
CD3-V450	UCHT1	TcR, T-cell marker	BD Phamingen	(Schiott et al. 2004; Okada et al. 2008)
CD45RO-ECD	UCHL1	Marker of T-cell phenotype, memory and activated cells	Beckman Coulter	
CCR7-PE-Cy7	3D12	Marker of memory, T-cell chemotaxis to lymph node	BD Bioscience	
CD27-PE	L128	Co-stimulatory molecule, activated T-cells	BD Bioscience	
Tim-3-PE	F38-2E2	Negative regulator	BioLegend	

4.5.9. Statistical Analysis

Differences between experimental conditions was first analysed using ANOVA to confirm that differences between conditions were significant. Each condition was then analysed using Wilcoxon matched pairs signed rank test (n≥5) or paired student T-test (n<5). Graphpad Prism (version 6) was used for all statistical tests. Statistical analysis was not performed on data where n<3. P-values of <0.05 were considered significant. Bonferroni correction was not used.

5. Chapter 5

Identification of Genes Important in pDC Mediated Inhibition of HIV-1 Latency in Resting CD4^{+} T-cells

Monash University

Declaration for Thesis Chapter Five

Nitasha Kumar

In the case of Chapter Five, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Experiments	100%
Analysis	90%
Writing	100%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Dr David Powell	Bioinformatics analysis	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	███████████	Date: 30/04/2015

Main Supervisor's Signature		Date

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

5.0. Chapter 5: Identification of Genes Important in pDC Mediated Inhibition of HIV-1 Latency in Resting CD4⁺ T-Cells

5.1. Abstract:

Background: Previously we have shown that CD4$^+$ T-cells co-cultured with a range of antigen presenting cells (APC) enhances the establishment of HIV-1 latency in both proliferating and non-proliferating T-cells. In contrast, resting CD4$^+$ T-cells co-cultured with plasmacytoid DC (pDC) suppressed the establishment of latency and increased death of proliferating CD4$^+$ T-cells. To identify proteins involved in pDC mediated inhibition of latency in non-proliferating CD4$^+$ T-cells and cell death of proliferating CD4$^+$ T-cells we compared RNA expression profiles of pDC with latency inducing APC subpopulations, CD1c$^+$ mDC, SLAN$^+$ mDC and CD14$^+$ monocytes.

Results: Comparison of RNA-seq gene expression profiles in freshly isolated pDC, with latency inducing APC subpopulations (CD1c$^+$ mDC, SLAN$^+$ mDC and CD14$^+$ monocyte) identified 754 differentially expressed genes. Of these, 65 protein encoding genes were expressed on the plasma membrane, with the potential to signal to CD4$^+$ T-cells. Comparison of genes differentially upregulated in latency inducing APC compared to pDC (Chapter 3) with genes differentially upregulated in pDC compared to APC (current chapter 5), shown here, shows that function of encoded proteins in the 3 gene lists were similar, and not unique to pDC. To identify a specific factor unique to pDC that could mediate inhibition of latent infection and CD4$^+$ T-cell death we examined the effects of pDC activation on gene expression. We compared microarray gene expression profiles of HIV-1 treated pDC to freshly isolated pDC that was previously published. There was differential upregulation of 24 genes, of which 14 mediated cell contact and 10 were soluble factors. Analysis of differentially upregulated genes in pDC treated with HIV-1 suggested that both cell contact and soluble factors, including antigen presentation and ligands for TRAIL, may be important in latency inhibition and induction of cell death.

Conclusions: Freshly isolated pDC, mDC and monocytes do not differ in gene expression profiles, therefore no unique genes were identified as important in inhibition of latency or activation of T-cell death. However upon treatment with HIV-1, pDC differentially upregulate antiviral factors that may control HIV-1 latent infection. The pathways involved in the inhibition of latent infection and induction of cell death by pDC should be explored to identify novel strategies to eliminate HIV-1 infection.

5.2. Introduction

Latent infection in resting CD4$^+$ T-cells remains the major barrier to HIV-1 cure however, the role of cellular interactions and soluble factors derived from DC in the establishment of T-cell latency *in vivo* is uncertain. Using the *in vitro* DC-T-cell model we have shown that upon pDC co-culture, direct latent infection of non-proliferating CD4$^+$ T-cells is inhibited (Evans et al. 2013) and latent infection in proliferating T-cells is short lived (Section 4.3.5). To further to identify genes that maybe important in inhibition of HIV-1 latency we used previously generated RNA-seq data (Section 3.3.6), and microarray data from HIV-1 treated pDC (Thomas et al. 2014)

Like mDC, pDC also circulate in the periphery in an immature state and upon interaction with HIV-1, pDC can become activated and undergo maturation (Groot et al. 2006). HIV-1 ssRNA and/or un-methylated DNA is able to stimulate TLR-7 and -9 expressed in pDC, which leads to pDC activation and a potent antiviral response (Gurney et al. 2004; Ito et al. 2005; Chaperot et al. 2006). Upon activation, pDC produce large amounts of type-I interferon's (IFN-I); including IFN-α, IFN-β and IFN-ω (Cella et al. 1999; Ito et al. 2002; Gurney et al. 2004; O'Brien et al. 2011; Thomas et al. 2014), pro-inflammatory cytokines; including IL-6, IL-1, IL-8 (also known as CXCL8; Jarrossay et al. 2001; Ito et al. 2005; Piqueras et al. 2006). Secretion of these cytokines and pDC activation leads to recruitment of accessory cells, including natural killer (NK) cells and regulatory T-cells (T$_{REGS}$; Romagnani et al. 2005; Manches et al. 2008). These processes inhibit productive HIV-1 infection in both cell lines and primary CD4$^+$ T-cells by up-regulation of interferon stimulated genes (ISG) including the host antiviral factors apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC3) family, Tripartite motif-containing (TRIM) family and sterile alpha motif and histidine-aspartate domain-containing protein 1 (SAMHD1; Chiu et al. 2005; Barr et al. 2008; Goldstone et al. 2011; Mubil et al. 2011; Pillai et al. 2012). ISG also include activation of proteins responsible for T-cell apoptosis, including TNF-related

apoptosis inducing ligand (TRAIL; Stent et al. 2002; Chaperot et al. 2006; Hardy et al. 2007; Stary et al. 2009; Serei et al. 2012).

The role of pDC and type-I IFN in HIV-1 infected patients on cART remains unclear. During chronic HIV-1 infection *in vivo*, CD4$^+$ T-cell depletion is suggested to be mediated by type-I IFN and ISG activation (Barblu et al. 2012; Reviewed in Cha et al. 2014), however one study showed that CD4$^+$ T-cell death may not be TRAIL related (Chehimi et al. 2010). Studies in HIV-1-infected humanized mice (Li et al. 2014) and SIV-infected rhesus macaques (Sandler et al. 2014) have shown that pDC depletion led to increased CD4$^+$ T-cell survival, higher viral loads in plasma and slower disease progression. Together these studies suggest that pDC or pDC responses are important in HIV-1-related disease progression, however the benefit of pDC activation in disease progression and persistence remains unclear.

In the current study we specifically aimed to identify genes differentially expressed in pDC that may mediated inhibition of HIV-1 latency in non-proliferating CD4$^+$ T-cells and death of proliferating CD4$^+$ T-cells. Using RNA-seq, we demonstrated that genes differentially upregulated in freshly isolated pDC compared to latency inducing APC were not responsible for inhibition of latency and CD4$^+$ T-cell death in the *in vitro* DC-T-cell model. However, using published microarray expression data, we show that pDC treated with HIV-1 differentially upregulated genes involved in the anti-viral response that may mediate inhibition of latency in non-proliferating T-cell and T-cell death in proliferating CD4$^+$ T-cells. We propose that inhibition of latent infection is mediated by HIV-1 treated pDC, which potently secrete type-I IFNs and upregulate TNF receptor superfamily, including ligands to TRAIL, which mediate T-cell death.

5.3. Results

5.3.1. Differentially Upregulated Gene Expression in pDC Compared to Latency Inducing Antigen Presenting Cell Using RNA-Seq.

To identify genes involved in the inhibition of latent infection in non-proliferating CD4$^+$ T-cells and induction of cell death in proliferating CD4$^+$ T-cells, we compared gene expression profiles of freshly isolated pDC, with latency inducing APC subsets (CD1c$^+$ DC, SLAN$^+$ DC, CD14$^+$ monocytes). Given that we have not yet determined whether inhibition of latency in the presence of pDC is attributed to a soluble factor or cell interactions, we included genes that encoded proteins that were expressed on the pDC surface, on intracellular membrane organelles like vesicles, and can be secreted in our analysis (Table 5.1). Using GeneCodis we determined the compartment of cellular expression and specifically selected all membrane organelles and secreted factors, including the compartments of; the plasma membrane, cytoplasmic membranes, cell junction and extracellular spaces (Table 5.1; http://genecodis.cnb.csic.es; Carmona-Saez et al. 2007; Nogales-Cadenas et al. 2009; Tabas-Madrid et al. 2012).

Comparison of RNA-seq derived gene expression in freshly isolated pDC with latency inducing APC, revealed differential expression of 924 genes (p=0.01, fold change [FC]=2;

Figure 5.1A). These genes were then selected based on their expression in specific cell compartments using GeneCodis and are listed in **Table 5.1**. We manually curated these lists using online databases; Pubmed, NCBI, GeneCards, for genes that could specifically interact via cell-cell contact or soluble factors, and signal to CD4$^+$ T-cells. This analysis identified 65 protein encoding genes that were able to mediate cell proximity (35 genes), apoptosis regulation (3 genes), negative regulation of T-cell activation (7 genes), T-cell activation (9 genes) and soluble factor signalling (11 genes;

8.0   Figure 5.1 and **Chapter 5 Supplementary Information**

Table 5.1. Selection of Cell Compartments from the GeneCodis Database.

Using GeneCodis, cellular compartment expression of genes differentially expressed between freshly isolated pDC, and latency inducing APC (mDC and monocytes) were analysed. To include genes encoding proteins that maybe involved in pDC-T-cell interaction via cell contact or soluble factors, genes expressed in the plasma membrane, membrane, cell junction and extracellular spaces were selected. For RNA-seq analysis freshly isolated APC subpopulations were used. To account for changes in gene expression due to DC maturation, we also selected genes expressed in cytoplasmic vesicles.

GeneCodis Cell Compartment Classification	Reason for Selection
Integral to membrane	To detect genes that encode proteins and are expressed on the cell surface likely to allow interaction between APC and CD4$^+$ T-cells.
Membrane	
Plasma membrane	
External side of plasma membrane	
Integral to plasma membrane	
Membrane fraction	
Cell surface	
Cytoplasmic vesicles	To capture any secreted proteins held in cytoplasmic vesicles.
Cell junction	To capture any proteins which directly interacted with CD4$^+$ T-cells.
Synapse	
Cell-cell junction	
Cell-cell adherence junction	
Extracellular region	To capture any secreted proteins.
Extracellular space	
Extracellular matrix	

Figure 5.1. Differentially Upregulated RNA-Seq Gene Expression Profile in pDC Compared with Latency Inducing Antigen Presenting Cell Subpopulations.

A. Genes that were differentially upregulated in pDC compared to latency inducing APC subsets (CD1c$^+$ mDC, SLAN$^+$ mDC and CD14$^+$ monocytes) were selected (p=0.01, fold change (FC)=2). Using GeneCodis, 924 genes were filtered according to cellular compartment expression. Genes encoding proteins expressed in the plasma membrane, membrane, cell junction and extracellular spaces were selected and further analysed for their ability to signal to CD4$^+$ T-cells **B.** Heat map showing short listed, differentially upregulated genes expressed in pDC by >2 fold compared to CD1c$^+$ mDC, SLAN$^+$ mDC and CD14$^+$ monocytes, and can signal to CD4$^+$ T-cells. The functional category of genes differentially upregulated on pDC was determined by gene description on the DAVID (david.abcc.ncifcrf.gov/) and GeneCards (genecards.org/) databases. Grey boxes represent function. Where a gene was hypothesized to fall into two categories, two boxes are shown in grey. Nuclear factor kappa beta (NFkB); Glycosylphosphatidylinisotol (GPI); Mouse mammary tumor virus (MMTV); epidermal growth factor (EGF)

Figure 5.1. Differentially Upregulated RNA-Seq Gene Expression Profile in pDC Compared with Latency Inducing Antigen Presenting Cell Subpopulations
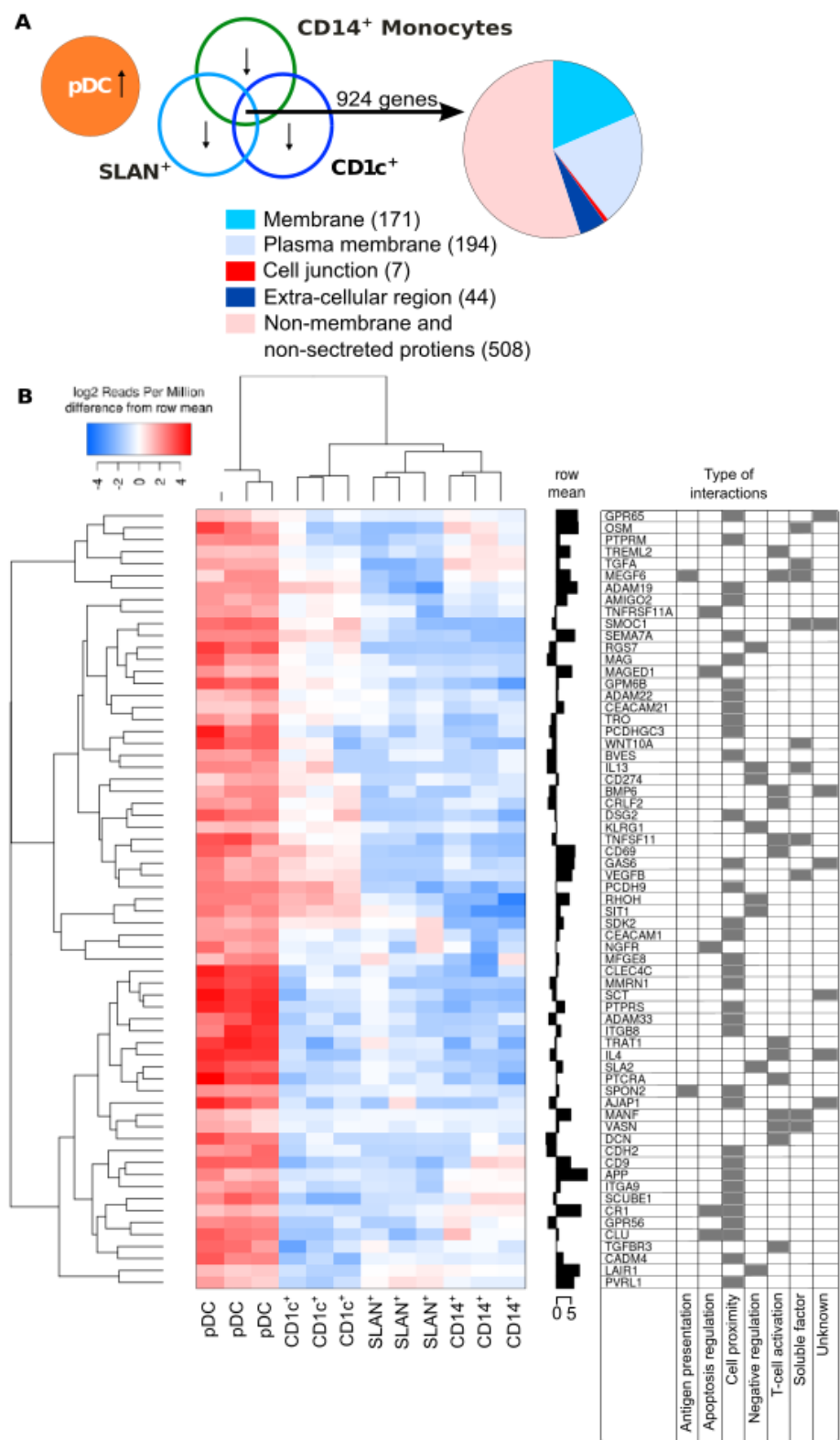
5.3.2. Comparison of Genes Upregulated in pDC Compared to Latency Inducing Antigen Presenting Cell (mDC and monocytes).

Type-I IFN are known to be secreted by pDC upon interaction with HIV-1 but were not identified in the shortlist of differentially expressed genes identified in the comparison of RNA-seq data derived from freshly isolated pDC and latency inducing APC (Figure 3.5; Cella et al. 1999; Ito et al. 2002; Gurney et al. 2004; O'Brien et al. 2011; Thomas et al. 2014). Therefore, we compared gene function of shortlisted genes that were differentially upregulated in freshly isolated pDC and freshly isolated latency inducing APC (Table 3.1 compared to Table 5.2). We found that differentially upregulated genes in freshly isolated pDC (Table 5.2) were similar in function to those differentially expressed in CD1c$^+$ DC, SLAN$^+$ DC and CD14$^+$ monocytes (Table 3.1). In Chapter 0, we hypothesized that these gene functions, including apoptosis regulation, cell proximity, negative regulation, synapse formation and T-cell activation, were important in the establishment of latency (Discussed in section 3.4). However given the similarities in gene function between the two gene lists, independently generated, we hypothesise that genes important in inhibition of latency is expressed in pDC upon exposure to HIV-1, within our culture system. Our gene lists are representative of immature pDC, therefore to identify genes that may be specifically involved in inhibition of HIV-1 latency, we also examined HIV-1 treated pDC.

Table 5.2. Comparison of Gene Function in Differentially Upregulated Genes in Freshly Isolated pDC and Latency Inducing Antigen Presenting Cells (mDC and monocytes).

Using RNA-seq, genes that were differentially upregulated in latency inducing APC (mDC and monocytes) compared to pDC were shortlisted. Using the DAVID, GeneCards and national centre for biotechnology information (NCBI) databases, genes that could interact with and induce signalling in CD4$^+$ T-cells were selected (Establishment of latency list, Chapter 3). Similarly using RNA-seq, genes that were differentially upregulated in pDC compared to latency inducing APC were shortlisted. Using the online databases genes that could interact with CD4$^+$ T-cells via cell surface expressed proteins or soluble factors, and induce signalling were selected (inhibition of latency, Chapter 5).

RNA-Seq list:	Chapter 3 Establishment of Latency	Chapter 5 Inhibition of Latency
Function	Genes Upregulated in mDC and Monocytes Compared to pDC	Genes Upregulated in pDC Compared to mDC and Monocytes
Antigen presentation	1	0
Apoptosis regulation	5	3
Cell proximity	31	35
Negative regulation	5	7
Synapse formation	1	0
T-cell activation	9	9
Unknown	1	1
Secreted	Excluded	11
Total genes	**53**	**65**

5.3.3. Expression of Genes in HIV-1 Treated pDC Using Previously Published Illumina Microarray Data

The similarities identified using RNA-seq in gene function between genes differentially upregulated in freshly isolated pDC compared to latency inducing APC and, genes upregulated in freshly isolated latency inducing APC compared to pDC, suggested that factors that mediated inhibition of latency may be derived from pDC treated with HIV-1. Additionally we know that activated pDC are the predominant type-I IFN secreting cell and that type-I IFN are potent inhibitors of productive HIV-1 infection. Type-I IFN was not found to be differentially upregulated in freshly isolated pDC. Thomas et al compared microarray gene expression profiles of pDC in response to treatment with multiple viruses, including Influenza, Hepatitis C virus (HCV), HIV-III (R5) and HIV-BaL (X4; Thomas et al. 2014). In order to identify factors in HIV-1 treated pDC that mediate inhibition of latent infection in $CD4^+$ T-cells, we went on to analyse microarray data from this published study.

Using microarray data, we generated 2 genes lists by comparison untreated pDC to HIV-IIIB or HIV-BaL treated pDC. These were then filtered for protein encoding genes that were expressed on plasma membrane or secreted, and could specifically signal to $CD4^+$ T-cells, as described with our RNA-seq gene expression analysis in section 5.3.1. We found 370 and 121 genes differentially upregulated in HIV-IIIB and HIV-BaL treated pDC respectively, with 117 of these genes commonly upregulated (p=0.01, Figure 5.2A). Only 4 genes were uniquely expressed in HIV-BaL treated pDC and none of these were expressed on cell surface or secreted, therefore further analysis was continued with differentially upregulated in HIV-IIIB treated pDC only. Of the 370 genes differentially upregulated in HIV-IIIB treated pDC, we found 138 genes expressed on the pDC membrane or secreted using GeneCodis (http://genecodis.cnb.csic.es), of which 16 genes mediated cell contact and 25

genes encoded secreted soluble factors (Figure 5.2; Carmona-Saez et al. 2007; Nogales-Cadenas et al. 2009; Tabas-Madrid et al. 2012). Protein-encoding genes that could establish CD4^{+} T-cell contact and signal to CD4^{+} T-cells included the functions of: T-cell activation (8 genes), cell adhesion (4 genes) and unknown function (4 genes). Soluble factor-encoding genes that could signal to CD4^{+} T-cells included cytokines (17 genes), chemokines (6 genes), apoptosis regulation (2 genes) and growth factor (1 gene). The cytokine encoding genes included IFN-α (alpha; 13 different subtypes), IFN-λ (lamda) 1 and 2, IFN-ω (omega), tumor necrosis factor (TNF), TNF-receptor superfamily member 10 (TNFSF10) and granzyme (Figure 5.2). Genes that were unknown have predicted gene function and protein structure based on other species. The ability of an encoded protein to establish T-cell contact was determined by searching the literature for these predicted functions to determine importance of the gene in our study. Of genes important in signalling to CD4^{+} T-cells, 8 of 117 genes were commonly differentially upregulated by both HIV-IIIB and HIV-BaL treated pDC. These included CD83, CXCL10, IFNα10, IFNα17, IFNα21, transmembrane protein 50B (TMEM50B), TNFSF10 and tumor necrosis factor (ligand) superfamily member 10 and 13B (TNFSF10 and TNFSF 13B; Figure 5.2).

To eliminate genes expressed commonly among APC that induce latency analysed, we compared genes differentially upregulated in HIV-1 treated pDC from microarray analysis with RNA-seq data from untreated pDC and CD1c^{+} mDC, SLAN^{+} DC, CD14^{+} monocytes (Appendix figure 8.3). This comparison showed that of genes that established cell contact, only FAT tumor suppressor homolog 3 (FAT3) and transmembrane protein 52 (TMEM52) were specifically expressed by HIV-1 treated pDC (Appendix figure 8.3A). Amongst genes that encoded soluble factors, all IFN-α isotypes, IFN-λ, IFN-ω and the growth factor bone morphogenetic protein 2 (BMP2) were differentially upregulated in HIV-1 treated pDC compared to freshly isolated pDC and RNA-seq data of CD1c Additionally, to date these genes have not been found to be expressed on CD1c^{+} mDC, SLAN^{+} DC and CD14^{+}

monocytes. Genes that have previously been shown to be upregulated on myeloid derived dendritic cells (MDDC) by gene expression analysis and were differentially upregulated following HIV-IIIB treatment of pDC included CD69, CD83, CD48, CXCL10 and IL8 (Figure 5.2; Table 3.1; Harman et al. 2013). Of these, CXCL10 and IL8 were upregulated on MDDC upon exposure to HIV-1 (Harman et al. 2006; Singh 2010). Genes commonly expressed by both HIV-1 treated pDC and mDC in RNA-seq or microarray data were excluded from further analysis, as these genes are not specifically involved in inhibition of latency.

Figure 5.2. Microarray Analysis of Genes Expressed in HIV-1 Activated pDC Using Illumina Microarray.

A. To identify genes differentially upregulated on activated pDC we compared published microarray data from freshly isolated pDC and pDC treated with HIV-III and HIV-BaL (Thomas et al. 2014). Using GeneCodis, we identified 370 and 121 differentially upregulated genes following treatment of pDC with HIV-III and HIV-BaL, respectively. These were then filtered according to cell compartment expression. Protein encoding genes expressed in the plasma membrane, membrane, cell junction and extracellular spaces were selected and further analysed for the ability to interact with and signal to CD4$^+$ T-cells. Heat maps show shortlisted **B.** membrane bound genes and **C.** soluble factor encoding genes expressed by HIV-IIIB treated pDC compared to freshly isolated pDC. The table describes the functional category of genes differentially upregulated on HIV-1 treated pDC as determined by the description on the DAVID (david.abcc.ncifcrf.gov/) and GeneCards (genecards.org/) databases, grey boxes represent function. Where a gene is hypothesized to fall into two categories, two boxes are shown in grey. Genes in **bold** were commonly upregulated in HIV-IIIB an HIV-BaL treated pDC. *Indicates that the gene was expressed on immature or unactivated mDC or MDDC and **indicates that the gene was found to be expressed on MDDC exposed to HIV-1. References include [[1]**Table 3.1**]([2]Harman et al. 2006, [3]2013; [4]Singh 2010). Major histocompatibility complex (MHC); transmembrane (TM); immunoreceptor tyrosine-based inhibition (ITIM); coxsackie virus and adenovirus receptor (CXADR); FAT atypical cadherin (FAT); death receptor 5 (DR5).

Figure 5.2. Microarray Analysis of Genes Expressed in HIV-1 Activated pDC Using Illumina Microarray
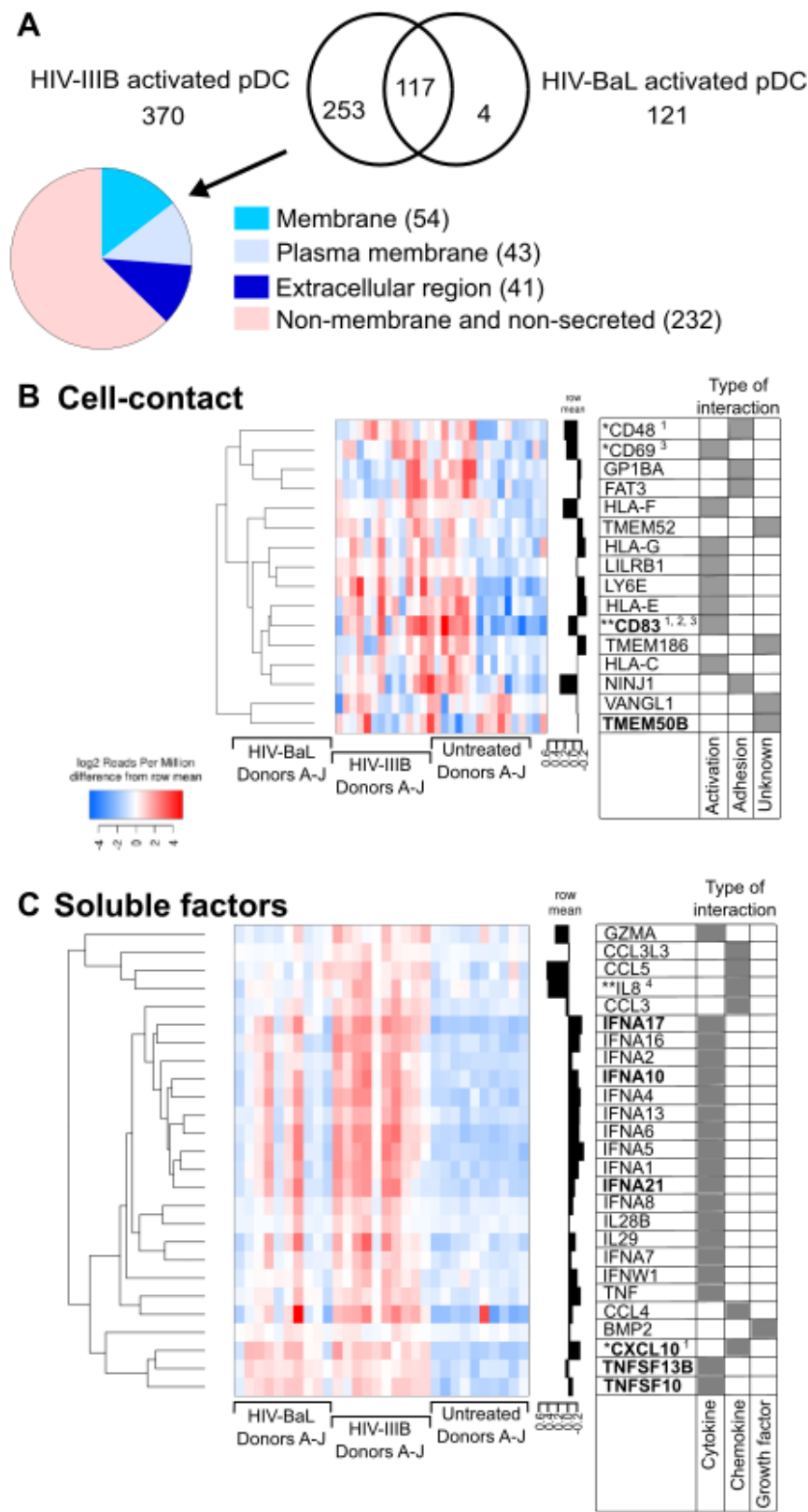
5.4. Discussion:

We have previously shown that mDC and monocytes mediated the establishment of latent infection, while pDC were shown to inhibited latent infection in non-proliferating CD4$^+$ T-cells (Section 2.3.1; Evans et al. 2013) and mediate cell death of proliferated T-cells (Section 4.3.5 and 4.3.6). To identify genes that may be important in the inhibition of latency and T-cell death we first compared RNA-seq gene expression analysis between freshly isolated pDC compared to latency inducing APC. We found 65 differentially upregulated genes in pDC compared to latency inducing APC that were similar in gene function when compared to previously generated gene list (Table 3.1), which compared differentially upregulated genes in latency inducing APC to pDC. Given that we did not find a unique factor that may be involved in latency inhibition and T-cell death, we concluded that latency inhibition and T-cell death was mediated by pDC in response to either T-cell co-culture or treatment with HIV-1. Despite limited gene expression analysis in the literature of primary pDC and mDC, we found 1 study that measured gene expression using microarray in primary pDC treated with HIV-IIIB and HIV-BaL (Thomas et al. 2014). Using this microarray data previously published by Thomas et al., we shortlisted 42 differentially upregulated genes from HIV-IIIB treated pDC compared to untreated pDC. Genes were selected based on the ability to interact with the CD4$^+$ T-cells via surface expressed proteins or secreted soluble factors. Of the 42 identified genes, 17 genes encoded for proteins that mediated cell interactions and 25 genes encoded soluble factors that can mediate anti-viral effects.

The function of each of the 754 genes was determined by a literature search using pubmed, gene cards and NCBI, any genes that could specifically interact via cell contact or soluble factors, and signal to T-cells, were shortlisted. A final list of 27 genes is presented. In this analysis, we were specifically looking for genes that could be involved in mediating inhibition of the establishment of latent infection, therefore, any of the 27 genes shortlisted that were also expressed by mDC were excluded, in bold and with asterisks, resulting in a

final shortlist of 20 genes (Figure 5.2). Given that many (8) of the genes were different IFN-alpha isotypes, we selected 1 representative isotype further shortening the list to 13 genes. Future experiments will measure the expression of these 13 soluble factors in the pDC-T-cell co-cultures, before blockade of their interaction with their respective receptors expressed on CD4$^+$ T-cells.

To accurately measure gene changes of pDC and mDC in the *in vitro* DC-T-cell model, T-cell co-culture, HIV-1 treatment and DC infection must be considered. Currently there is limited, published gene expression analysis, including RNA-seq and microarray, of primary mDC, monocytes and pDC. This data is difficult to acquire for two main reasons. Firstly, mDC and pDC are rare in blood and tissue, and high concentrations of unspliced and total RNA were originally required for accurate gene expression analysis (Schena et al. 1995; Lashkari et al. 1997; Marioni et al. 2008; Chu and Corey 2012). High-throughput sequencing techniques are now more sensitive, such that only 200ng of RNA are required to perform RNA-seq gene expression analysis on the desired sample (Islam et al. 2014). The tissue DC will be more activated than the blood precursors and, therefore, may be functionally different in terms of IC induction on T-cells. However, in unpublished data we have observed a similar enrichment of latent infection of non-proliferating T-cells expressing IC following co-culture with mDC, in particular PD-1, compared to ex vivo results from HIV-infected individuals (Chomont et al. 2009). Second, mDC and pDC are sensitive to *in vitro* manipulation and without exogenous cytokine treatment these cells quickly die, leading to inaccurate sequencing data. Monocytes remain more viable *in vitro*, however their adherent nature makes them difficult to obtain after culture on plastic culture plates. Ideally, to identify factors important in establishment of latent infection by mDC and inhibition of latent infection by pDC we would compare APC that were freshly isolated, treated with HIV-1, co-cultured with T-cells in the absence and presence of HIV-1 treatment. Due to size of the experiment, cost of RNA-seq and inefficiency to process the data, this was not feasible. Instead we started with RNA-seq analysis of freshly isolate APC, which lead to identification of

genes important in the establishment of latency, however did not identify genes important in inhibition of latency. To identify genes that may be important in the inhibition of latency we compared previously published data from freshly isolated and HIV-1 treated pDC. In future studies, we propose a comparison of HIV-1 treated pDC with HIV-1 treated mDC and/or monocytes, and test our findings *in vitro* to confirm gene expression analysis findings.

5.4.1.   Type-I IFN is Differentially Upregulated in pDC Treated with HIV-1.

pDC are the most potent type-I IFN secreting cells in the immune system and secrete high amounts of IFN-α in response to HIV-1 infection *in vitro* (Cella et al. 1999; Jarrossay et al. 2001; Ito et al. 2002; Piqueras et al. 2006). Additionally, during acute and chronic HIV-1 infection IFN-α is detected at high levels in plasma (Herbeuval et al. 2006; Lehmann et al. 2009; Hardy et al. 2013). We therefore examined gene expression for factors expressed in pDC treated with HIV-1 and found that of the 25 genes encoding soluble factors, 17 can mediate anti-viral effects including induction of ISG and cell death in the local environment.

Detection of IFN-α isotypes in gene expression analysis of activated pDC was expected, additionally we show here that IFN-ω, another type-I IFN, and IFN-λ1 and 3, which are type-III IFN, and are also secreted by pDC in response to HIV-IIIB treatment. Although the role of type-I IFN in HIV-1 disease remains controversial, we hypothesis that IFNα, IFN-ω, IFN-λ1 (IL-29) and 3 (IL-28B) may play a role in the inhibition of latent infection. Both IFN-λ and IFN-ω possess potent anti-viral effects and inhibit HIV-1 replication *in vitro* in T-cells and macrophages (Künzi and Pitha 1996; Hou et al. 2009; Wang et al. 2015). IFN-ω inhibits HIV-1 replication just as potently as IFN-α by inhibition of proviral synthesis, inhibition of viral protein expression and sustained expression of ISG, including anti-viral proteins Mx1 and 2, thereby providing long lasting protection of cells *in vitro* (Künzi and Pitha 1996). IFN-λ1 and 3

mediate T-cell protection via blockade of post-transcriptional events and *in vitro* upregulation of viral restriction factors MxA, OAS and PKR (Tian et al. 2012). However a protective role for IFN-λ against HIV-1 infection *in vivo* is controversial (Rallon et al. 2011; Tian et al. 2012). To confirm the activity of type-I and III IFN we could sequence T-cell RNA from pDC co-culture and measure for upregulation of specific ISG. This would confirm activity of IFN in pDC-T-cell culture system and guide us to the specific pathways involved in inhibition of latency.

The cellular source of each of these type-I and III IFN are incompletely defined. IFN-λ has been shown to be secreted by endothelial cells in the central nervous system and mediates protection of macrophages against HIV-1 infection (Li et al. 2013). Thomas et al. data builds on evidence that IFN-α is secreted by pDC in HIV-1 infected patients (Beignon et al. 2005; Lehmann et al. 2010; Thomas et al. 2014) and shows that IFN-ω and IFN-λ1 and 3 *in vivo*, may also be derived from pDC. Detection of IFN-ω and IFN-λ1 in our in vitro culture system would directly pDC as a source and build on our current understanding of pDC function.

5.4.2.   pDC Mediate Cell Death by Induction of Apoptosis

CD4^{+} T-cell depletion in acute HIV-1 infection is proposed to be mediated by type-I IFN, thereby leading to disease progression (Soumelis et al. 2001; Lehmann et al. 2008; Tilton et al. 2008; Kader et al. 2013). Our *in vitro* data shows that proliferating CD4^{+} T-cells but not non-proliferating CD4^{+} T-cells from pDC co-cultures, undergo cell death both after isolation from pDC and during prolonged pDC co-culture (Section 4.3.5 and 4.3.6). In HIV-1 infection, pDC can induce CD4^{+} T-cell death in 2 ways. First by the secretion of type-I IFN which in turn upregulates ISG that mediate apoptosis (Rosebeck and Leaman 2008; Lu and Liao 2011; Stawowczyk et al. 2011). Second, by the

expression death receptors that bind to TNF-Related Apoptosis-Inducing Ligand (TRAIL; Herbeuval et al. 2006; Hardy et al. 2007; Stary et al. 2009).

Here we show that TNFSF10, a ligand for TRAIL, is upregulated in both HIV-IIIB and HIV-BaL treated pDC, and can therefore mediate TRAIL induced T-cell death. However, recent reports have suggested that pDC derived TRAIL, when binding with its ligand on T-cells from HIV-1 infected patients *ex vivo,* failed to induce cell death (Chehimi et al. 2010). Chehimi et al. suggests that pDC do induce cell death however via the stimulation of other accessory cells like NK cells. NK cells mediate apoptosis by secretion of granzymes, upregulation of antibody dependent cell cytotoxicity receptors including CD16, and detection of decreased MHC class-I molecules on the T-cell surface (Alter et al. 2011; Reviewed in Vivier et al. 2011). Our analysis of microarray data from HIV-1 treated pDC shows upregulation of MHC class-I alleles C, E, F, G, which can prime a CTL response (Hervas-Stubbs et al. 2014), resulting in perforin/granzym and Fas-Fas-ligand (FasL) mediated apoptosis of target cells (Milstein et al. 2010). Our data does support a role of pDC in recruitment of accessory cells to induce death of HIV-1 infected T-cells. However, our *in vitro* model, does not include other accessory cells so there must be an alternate mechanism for pDC induced cell death of proliferated CD4$^+$ T-cells in the *in vitro* DC-T-cell latency model.

Type-I IFN upregulates over 900 ISGs including fas-fasL and TNF. TNF was found to be differentially upregulated in HIV-IIIB treated pDC in microarray analysis and both TNF and Fas are known to be upregulated *in vivo* in HIV-infected subjects (Dockrell et al. 1980; Aukrust et al. 1994; Badley et al. 1998; Demarchi et al. 1999; Hosaka et al. 2000; Petrovas et al. 2004). Upregulation of Fas-FasL in CD4$^+$ T-cells increases their sensitivity to cell death, irrespective of HIV-1 infection (Dyrhol-Riise et al. 2001; Mueller et al. 2001; Silvestris et al. 2001), and further treatment with IFN-α or IFN-β leads to activation of apoptosis via upregulation of the pro-apoptotic protein Bak (Fraietta et al. 2013). TNF treatment of T-cells leads to secretion of other pro-

inflammatory chemokines and cytokines (IL-6, IL-8, IL-18), cell proliferation and activation of cell death (Reviewed in Kumar et al. 2013). TNF mediated cell death is induced via activation of the caspase pathway (Hsu et al. 1995, 1996; Zheng et al. 1995). Both TNF and Fas-fasL could mediate cell death in our culture system and can be tested my measuring expression of caspase activation, fas expression or intracellular Bak expression.

5.4.3.   Conclusions and Significance

RNS-seq gene expression profiles suggest that genes expressed in freshly isolated pDC do not mediate latency inhibition and T-cell death in the *in vitro* DC-T-cell latency model. Moreover, using data published by Thomas et al., our analysis suggest that type-I and III IFN and the death receptors like TRAIL may be important factors involved in the inhibition of latent infection and induction of cell death in CD4$^+$ T-cells. Further investigation is necessary to confirm these primary findings by detection of type-I and III IFNs in the *in vitro* culture system and measurement of TNF receptor family expression on pDC and co-cultured T-cells compared cells from the mDC co-culture. The literature indicates that pDC are important in regulating productive HIV-1 infection and disease progression, therefore the potential role pDC play in latent infection should be investigated further. Here we provide the basis of further *in vitro* investigation.

5.5. Materials and Methods:

5.5.1. Cell Preparation and Generation of Gene Expression Profiles using RNA-Seq

RNA-seq data described in chapter 3 was used in this analysis. Briefly, freshly isolated APC subpopulations that were able to induce latency (CD1c$^+$ mDC, SLAN$^+$ mDC and CD14$^+$ monocytes) and pDC (Section 3.5.2) were isolated and total RNA extracted for RNA-seq analysis (Section 3.5.8).

5.5.2. Generation of Differentially Upregulated Gene in pDC from RNA-Seq

In **Chapter 3**, APC subsets were categorized as latency-inducing and latency-non-inducing subsets based on results from measurement of latent infection within the *in vitro* DC-T-cell latency model (Section 3.3.5). In that study, genes were selected using a fold change (FC) of 2 and false discovery rate (FDR) of 0.01. Using the same conditions, an FDR of 0.01 and FC of 2, comparison of pDC to latency inducing APC subpopulations (CD1c$^+$ and SLAN$^+$ mDC and CD14$^+$ monocytes) and identified 924 genes (

Figure 5.1A). Using the GeneCodis database (http://genecodis.cnb.csic.es) the cellular compartment expression was determined and only genes expressed on plasma-membrane, membrane, cell junctions and extracellular spaces were selected (Carmona-Saez et al. 2007; Nogales-Cadenas et al. 2009; Tabas-Madrid et al. 2012). The final list of 416 genes was manually filtered to select genes encoding surface expressed proteins or proteins that were soluble factors, and could induce signaling in a CD4^{+} T-cell using database tools DAVID bioinformatics resources 6.7 (http://david.abcc.ncifcrf.gov) and GeneCards (http://www.genecards.org). Filtering resulted in a gene list of 69 genes, listed in

Figure 5.1 and **Appendix table 8.4**. RNA-seq data is available through Gene omnibus (GEO), serial number GSE70106.

5.5.3. Generation of Gene List for Genes Differentially Upregulated on pDC During Culture and Activation

Published microarray data, accession number GSE59837 (Thomas et al. 2014), of pDC treated with HIV-IIIB, HIV-BaL and untreated pDC was compared and used to identify differentially upregulated genes in HIV-1 treated pDC. Genes lists were acquired from GenOmnibus (http://www.ncbi.nlm.nih.gov/geo/) and differential expression was visualised using online tools Degust (Powell 2014b) (http://victorian-bioinformatics-consortium.github.io/degust/) and Vennt (Powell 2014a) (http://drpowell.github.io/vennt/). Genes with fewer than 10 reads across every sample were removed from the analysis.

HIV-IIIB and HIV-BaL treated pDC (6 hours) were compared to untreated controls, with a false discovery rate (FDR) of 0.01. 307 and 121 genes were differentially expressed respectively, 117 of these genes were common to HIV-IIIB and HIV-BaL treated pDC, leaving only 4 genes unique to HIV-BaL treated pDC (Figure 5.2). Given that these genes did not encode proteins that were expressed at the pDC membrane or secreted by the pDC further analysis was completed on genes differentially unpregulated in the HIV-IIIB gene list only. Similar to filtering of the RNA-seq lists, GeneCodis was used to identify cell compartment expression of microarray gene lists and database tools DAVID bioinformatics resources 6.7 (http://david.abcc.ncifcrf.gov) and GeneCards (http://www.genecards.org) were used to filter the gene list further. 138 genes were either expressed at the pDC membrane or secreted by the pDC. These were manually curated for potential to mediate cell contact, soluble factor interactions and ability to signal to CD4^{+} T-cells. The resultant gene shortlist had 42 genes (Figure 5.2 and Appendix table 8.5).

6. Chapter 6

Integrated Discussion

6.0. Integrated Discussion

6.1. HIV-1 Latency in Resting and Activated CD4$^+$ T-Cells

The major barrier to a cure for HIV infection is the persistence of long-lived latent infection in resting CD4$^+$ T-cells (Chun et al. 1995; Finzi et al. 1997; Siliciano et al. 2003). Following cessation of combination anti-retroviral therapy (cART), virus rapidly rebounds, usually within 2-3 weeks (Oxenius et al. 2002; Wit et al. 2005; El-Sadr et al. 2006; Chun et al. 2010; Hamlyn et al. 2012). Latently infected resting memory CD4$^+$ T-cells are found at highest frequency in lymphoid organs, specifically the lymph node (LN), where dendritic cells (DC) mediate potent T-cell activation by antigenic stimulation (Chun et al. 1997; Chomont et al. 2009; Josefsson et al. 2013a; Wong et al. 1997).

In this thesis, we have used an novel *in vitro* model of latent infection to examine the role of DC subpopulations, myeloid (m)DC and plasmacytoid (p)DC, in the establishment of HIV-1 latent infection in non-proliferating and proliferating CD4$^+$ T cells. This *in vitro* model in novel because it uses primary resting CD4$^+$ T-cells and DC subpopulations, in a unique co-culture system, combined with specific sorting of non-proliferating and proliferating CD4$^+$ T-cells, and the ability to measure both pre- and post-integrated latency.

6.2. Cellular Interactions Between mDC and CD4$^+$ T-Cells

Using a physiologically relevant *in vitro* model, previously developed in our lab, we have shown that non-proliferating and proliferating CD4$^+$ T-cells co-cultured with mDC subpopulations (CD1$^+$ and SLAN$^+$) and CD14$^+$ monocytes, isolated from healthy donors, led to the establishment of HIV-1 latency (Section 2.3.1 and 3.3.5; Section 4.3.3). Although latency in resting and activated CD4$^+$ T-cells has been modelled and investigated previously, the DC-

induced T-cell model of latency is the first to assess the establishment of latent infection in both non-proliferating and proliferating CD4$^+$ T-cells simultaneously (Chapter 4).

In vivo and in our *in vitro* model, mDC are able to interact with CD4$^+$ T-cells in several ways, including cognate, non-cognate and interaction through soluble factors. Each interaction may contribute to different outcomes. Cognate interactions occur between the T-cell receptor (TcR) and major histocompatibility complex (MHC) class-II (MHC-II) presenting foreign antigen, which leads to T-cell activation (Lafferty and Woolnough 1977; Veillette et al. 1989). However, for successful T-cell activation, additional interactions between co-stimulatory molecules, including CD28, CD40 and Inducible T-cell Co-stimulator (ICOS), as well as adhesion molecules, including ICAM 1, 2 and 3 are also necessary (Pagès et al. 1994; Boise et al. 1995; Radvanyi et al. 1996; Viola and Lanzavecchia 1996; Appleman et al. 2000; Suh et al. 2004; So et al. 2008). Non-cognate interactions are interactions that do not require the MHC-II and TcR, and include interactions between adhesion molecules, co-stimulatory molecules and immune checkpoints (IC), which alone, lead to cell migration and cell survival. Finally, soluble factors, secreted by DC, interact with T-cells that express the respective receptors. Soluble factors secreted by DC include IL-6, TNF-α, IL-23 and IL-12, which promote T-cell activation (Piccioli et al. 2007), IL-10, which dampens T-cell activation (Alter et al. 2010; Kassianos et al. 2012), and the chemokines CCL19 and CCL21 (Piqueras et al. 2006).

6.2.1.   Establishment of pre-activation latency

We propose that mDC are able to induce latent infection in resting CD4$^+$ T-cells through a passive process, where there is entry, reverse transcription and integration but no viral expression or completion of the virus life cycle. In this thesis, using RNA-seq gene expression analysis (Section 3.3.6), we have identified a number of cell adhesion molecules, co-stimulation molecules and

the interaction between immune checkpoints (IC) that may be important in mDC-induced latent infection of proliferating and non-proliferating T-cells (Figure 6.1). We hypothesise that signalling between adhesion molecules and co-stimulatory molecules actively mediates latency in non-proliferating T-cells by facilitating early events in the viral life cycle, including viral entry, reverse transcription, nuclear import and integration. Interactions between adhesion molecules can cause cellular polarization, thereby inducing cytoskeleton changes (Figure 6.1; Harburger and Calderwood 2009). Changes in the cytoskeleton, initiated by chemokines and spinoculation, have already been shown to induce latency in our lab and by others (Swiggard et al. 2005; Saleh et al. 2007; Yoder et al. 2011). Therefore, in mDC-induced latency of non-proliferating CD4^{+} T-cells, latency could be induced due to activation of cytoskeletal changes or via a novel mechanism.

Figure 6.1. Proposed Model of mDC-Mediated Control of HIV-1 Latency.

A. HIV-1 infection of resting CD4$^+$ T-cells is inefficient, and productive and latent infection is not established. **B.** Direct latent infection of non-proliferating or resting CD4$^+$ T-cells is referred to as pre-activation latency. Pre-activation latency induced by mDC is controlled through the interaction of adhesion and co-stimulatory (co-stim) molecules. The role of T-cell receptor (TcR) activation, and subsequent signaling, in pre-activation latency remains unclear, but it is unlikely to play a major role in the establishment of latency via the pre-activation pathway. Low expression of immune checkpoints (IC) on resting CD4$^+$ T-cells suggests that IC may play a minor role in the establishment of pre-activation latency. **C.** Post-activation latency is the infection of an activated CD4$^+$ T-cell that reverts to a resting state. Virus entry in activated CD4$^+$ T-cells is initiated by cognate interactions involving TcR mediated T-cell activation. This pathway of latency establishment may be controlled via the interaction of IC, which blocks viral expression.

A Infection of resting CD4+ T-cells

B Pre-activation latency

Adhesion molecules
Co-stim
TcR
IC

C Post-activation latency

Adhesion molecules
Co-stim
TcR
IC

Resting CD4+ T-cell
Non-proliferated latent T cell
Proliferated T cell
HIV-1 virion
Promotion of pathway
Inhibition of pathway
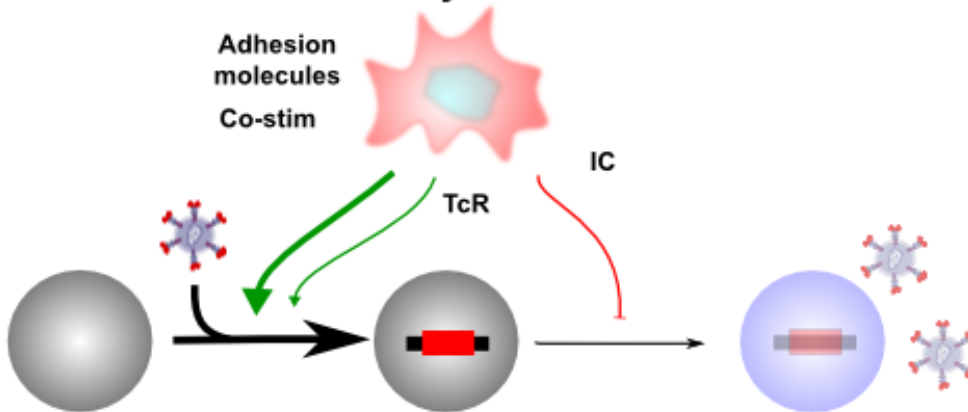
A novel mechanism leading to the establishment of latency in non-proliferating cells may involve co-stimulation or IC mediated signalling. DC efficiently activate T-cells via cognate interactions, including those between MHC-II-TcR, co-stimulation and adhesion molecules. In combination with MHC-II-TcR interactions, ligation of the co-stimulatory molecule CD28, with its ligands CD80 and CD86 on DC, induces a positive signal that can activate the T-cell. However, CD28 stimulation alone with CD80 and CD86 also leads to increased expression of cell survival proteins, such as Bcl-x_L, and the secretion of IL-2 (Boise et al. 1995; Radvanyi et al. 1996; Appleman et al. 2000). Ligation of co-stimulatory molecules including CD28, CD40L and ICOS, results in the activation of the phosphoinositide 3-kinase (Pi3K) and phospholipase Cγ (PLCγ) pathways, which leads to the activation of Nuclear Factor Kappa-β (NF$\kappa\beta$) via the Mitogen-Activated Protein Kinase (MAPK), Akt and c-Jun N-terminal kinase (JNK)/p38 pathways (Figure 6.2B; Thompson et al. 1992; August and Dupont 1996; Van Parijs et al. 1996; Khoshnan et al. 2000; Burr et al. 2001; Kane and Weiss 2003). In the presence of mDC, these may be some potential pathways that facilitate the efficient early steps of HIV-1 replication leading to integration in a resting T-cell.

In activated CD4$^+$ T-cells, the interaction of activated JNK with the HIV-1 enzyme integrase (IN) leads to more efficient viral integration. Activated JNK interacts with IN and promotes IN interaction with the host cell cycle regulator, peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 (Pin-1; Manganaro et al. 2010). Resting CD4$^+$ T-cells do not express JNK (Manganaro et al. 2010), therefore Pin-1 is not activated and cannot stabilize IN and promote integration. JNK is active in activated CD4$^+$ T-cells, as part of the MAPK family (Shen et al. 2001), and in response to cellular stress signals, such as reactive oxygen species (ROS; Kamata et al., 2005), tumor necrosis factor (TNF)-α (Kyriakis et al. 1994), heat shock (Han et al. 2000), osmotic stress (HuangFu et al. 2006) and ultra-violet (UV) radiation (Devary et al. 1991). In tumor metastasis JNK plays a role in actin remodeling (Rudrapatna et al. 2014) and cell interactions (Carrozzino et al. 2009; Lee et al. 2009). JNK also

mediates other T-cell functions, including cell survival (Yu et al. 2004), proliferation (Sabapathy et al. 2004) and apoptosis (Hu et al. 1999). Involvement of JNK in cell proliferation, cell survival, actin remodelling and cell interactions suggests that JNK may be activated in non-proliferating CD4$^+$ T-cells following non-cognate interactions with DC.

Like JNK, Pin-1 is important in several cellular processes (Esnault et al. 2007), but is also involved in the pathogenesis of several viruses. During Human T-lymphotropic virus (HTLV) the viral oncoprotein Tax binds Pin-1 and together they promote NFκB activation (Peloponese et al. 2009). During Epstein Barr virus (EBV) infection Pin-1 regulates viral DNA expression, where upon Pin-1 knockdown viral DNA expression was supressed (Narita et al. 2013). Pin-1 is also active during the anti-viral response, where Pin-1 negatively regulates interferon regulatory factor 3 (IRF3; Saitoh et al., 2006), and down-regulates APOBEC3G in HIV-1 infection (Watashi et al. 2008). Together these studies show that Pin-1 plays a regulatory role in cellular gene expression, suggesting that Pin-1 activation may promote viral expression and inhibit latency.

Further investigation is necessary to determine if JNK and Pin-1 are active in non-proliferating CD4$^+$ T-cells within the DC-T-cell model. Direct measurement of JNK and Pin-1, either using specific antibodies or a Chromatin Immuno-precipitation (CHiP) assay for Pin-1-IN interaction in non-proliferating CD4$^+$ T-cells, is difficult for two reasons. First, generating high numbers of latently infected cells, where JNK and Pin-1 are expressed and detectable, will be difficult using the DC-T-cell model, as this model only generates 10 to 1000 cells that are latently infected. Second, we have not yet determined the time at which integration is maximal in this model and we believe that latency may be continually established during several rounds of infection. The use of inhibitors against JNK or Pin-1 in the mDC-T-cell co-culture system is also difficult as addition of drugs to the co-culture will block JNK and Pin-1 in the DC as well as the CD4$^+$ T-cell, and blocking the activity

of JNK and Pin-1 in the DC will alter DC function and possibly their ability to induce latency in the T-cells.

Non-cognate interactions also encompass interactions between IC and their ligand, and lead to the activation of similar intracellular pathways as co-stimulatory molecule interactions, including activation of PI3K, Akt, MAPK and JNK (Figure 6.2). The IC, PD-1, has been detected on latently infected cells *ex vivo* (Chomont et al. 2009) and *in vitro* (Iglesias-Ussel et al. 2013). Preliminary unpublished data from our lab, using our *in vitro* DC-T cell model of latency, has shown that despite low level expression of the IC, PD-1, CTLA-4, BTLA and Tim-3 (Figure 4.4), latent HIV-1 infection is enriched in in non-proliferating $CD4^+$ T-cells that express these IC markers (van der Sluis, unpublished). While HIV-1 latent infection is enriched in proliferating $CD4^+$ T-cells that express PD-1 or TIGIT (van der Sluis, unpublished). Low expression of IC on non-proliferating $CD4^+$ T-cells may indicate that IC only play a minor role in the establishment and maintenance of latent infection via the pre-activation latency pathway (Figure 6.1). In a study from our lab, a single patient case study showed that treatment with a CTLA-4 antibody, Ipilimumab, led to an increase in cell associated HIV-1 RNA, a decrease in plasma virus (measured by Single Copy Assay; SCA) and increased memory and effector $CD4^+$ T-cells (from 610 to 900 cells/ul; Wightman et al. 2015). Despite these improvements, there was no change in levels of cell associated HIV-1-DNA. However, given the small fraction of the HIV-1 DNA reservoir that represents replication competent virus, it is possible that a change in replication competent virus may not be detected by just measuring HIV-1 DNA levels. Together these data indicate that IC markers do play a role in the maintenance of HIV-1 latency, but it is unclear if this is by direct or indirect effects on latently infected cells.

Non-proliferating $CD4^+$ T-cells express adhesion and co-stimulation molecules more highly than IC. Therefore, we suggest that adhesion and co-stimulation molecule interactions may play a more prominent role in the induction of

latency via the pre-activation pathway. Several adhesion and co-stimulatory molecules are expressed on resting memory CD4$^+$ T-cells, including CD27, CD28, ICAM-1, ICAM-3 (Reviewed in Gasper et al. 2014; Martín-Cófreces et al. 2014). As there is redundancy among these molecules, testing the involvement of a single molecule is difficult using blocking antibodies, therefore, future experiments exploring the role of these interactions in latency may be better done by detecting activation of downstream signalling molecules."

Given the importance of Gp120 and CXCR4 interaction in induction of latency. Gp120 expressing liposomes could be used to investigate if virus-mDC interaction is important in the induction of T-cell latency. While gp120 on the liposome would mediate binding to the mDC, and the mDC could internalise some liposomes via phagocytosis, this is relatively inefficient in mDC and many liposomes would be stuck to the outside of the mDC (Reece et al. 2001). However, gp120 has already been shown to activate signalling events in both T-cells and DC via interaction with CD4, CXCR4 or CCR5 and ICAM (Davis et al. 1997; Balabanian et al. 2004; Cicala et al. 2006; Yoder et al. 2008; Hioe et al. 2011; Hayasaka et al. 2015), and can lead to latent infection (Yu et al. 2009). Liposomes could be better used in place of the mDC to test the function of specific receptor-ligand interactions that we believe to be important in the induction of latency. Liposomes expressing specific ligands to bind receptors of the resting T-cell would allow isolated activation of specific receptor-ligand interactions, such as CD18/CD11a with ICAM-1, PD-1 with PD-L1/PD-L2, CTLA-4 with CD80/CD86, and other receptor-ligand pairing found to be important by gene expression analysis in chapter 3.

During APC and T-cell interaction a process called Trogocytosis may occur, this is the transfer of APC membrane to the interacting lymphocyte (Zhang et al. 2008). The role of trogocytosis in immune function remains unclear, but leads to enhanced CD8 and CD4 T-cell responses (Bourbié-Vaudaine et al. 2006; Riond et al. 2007). If the DC was expressing trans-membrane proteins

that enhanced T-cell activation, such as MHC-II, CD80 and CD86, then both T-cell and DC activation would be enhanced. However, if the DC was expressing trans-membrane proteins that can dampen the immune response, such as PD-L1/L2 and Gal-9, both T-cell and DC activation would be reduced. Additionally, transferred trans-membrane proteins may include chemokine or cytokine receptors that are not normally highly expressed on T-cells, such as CXCR3 and CCR5, which could lead to enhanced intracellular activity and enhanced latency induction. However, all these changes to T-cell function would be transient, as a result of the internalisation and recycling of this membrane.

Figure 6.2. Intracellular Pathways Activated in CD4$^+$ T-Cells Upon mDC Interactions.

Positive co-stimulation. Following cognate interactions between DC and T-cells, T-cell activation is initiated. Cognate interactions involve the ligation of TcR (green) with MHC Class-II, and CD28 (orange) with CD80 or CD86. Other co-stimulatory molecules can also be involved, including CD40 (pink) and ICOS (blue). Intracellular activation pathways in black lines indicate pathways activated by TcR activation, while pathways activated by CD28, CD40 or ICOS are in orange, pink and blue, respectively. **Negative regulation.** During cognate interactions, immune checkpoint (IC) markers are also expressed on T-cells. The co-stimulatory molecule CD28 competes with CTLA-4, also expressed on a T-cell, for binding with CD80 and CD86. Interaction of IC leads to negative regulation of T-cell activation, via activation of pathways shown in red, dotted lines.
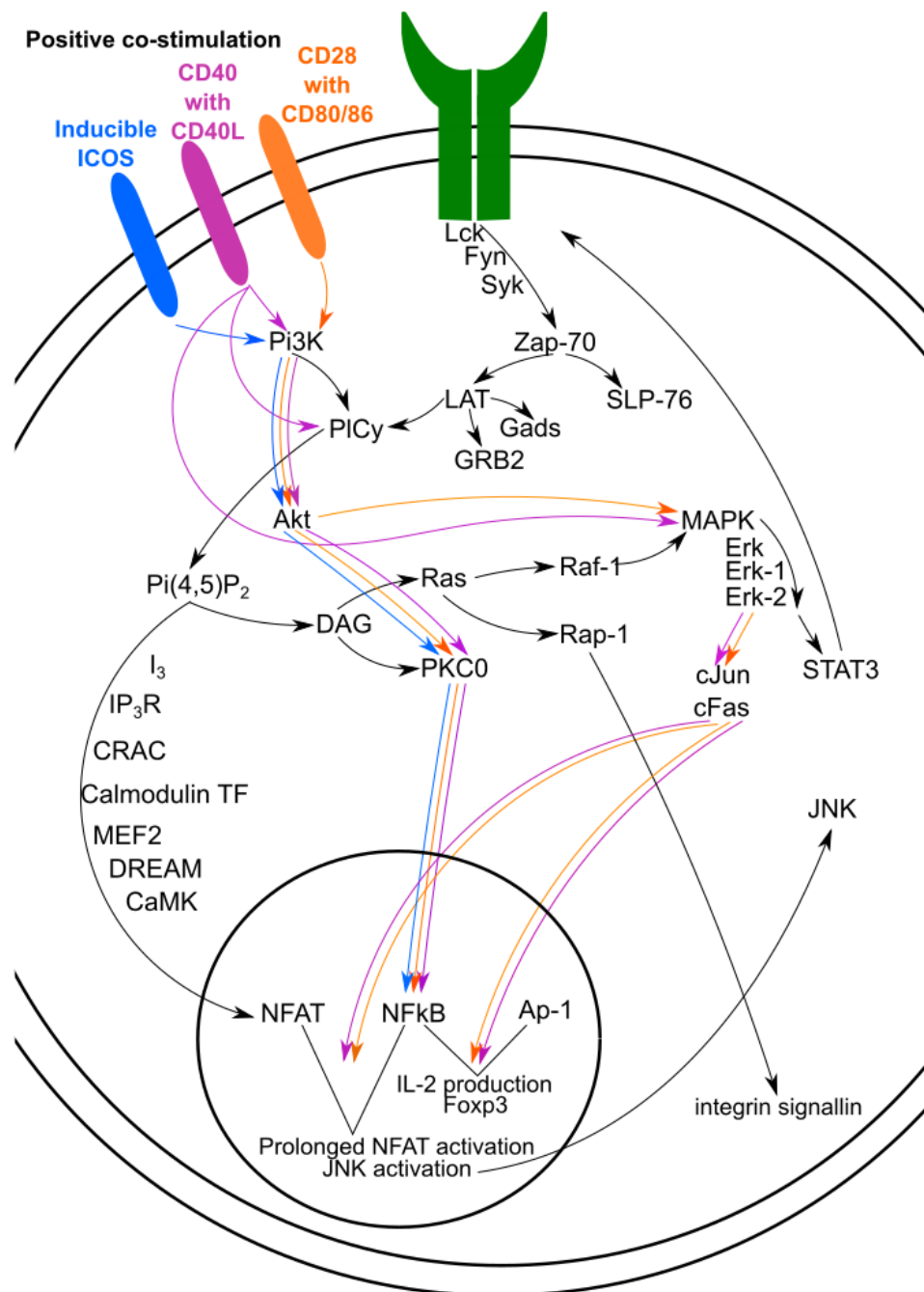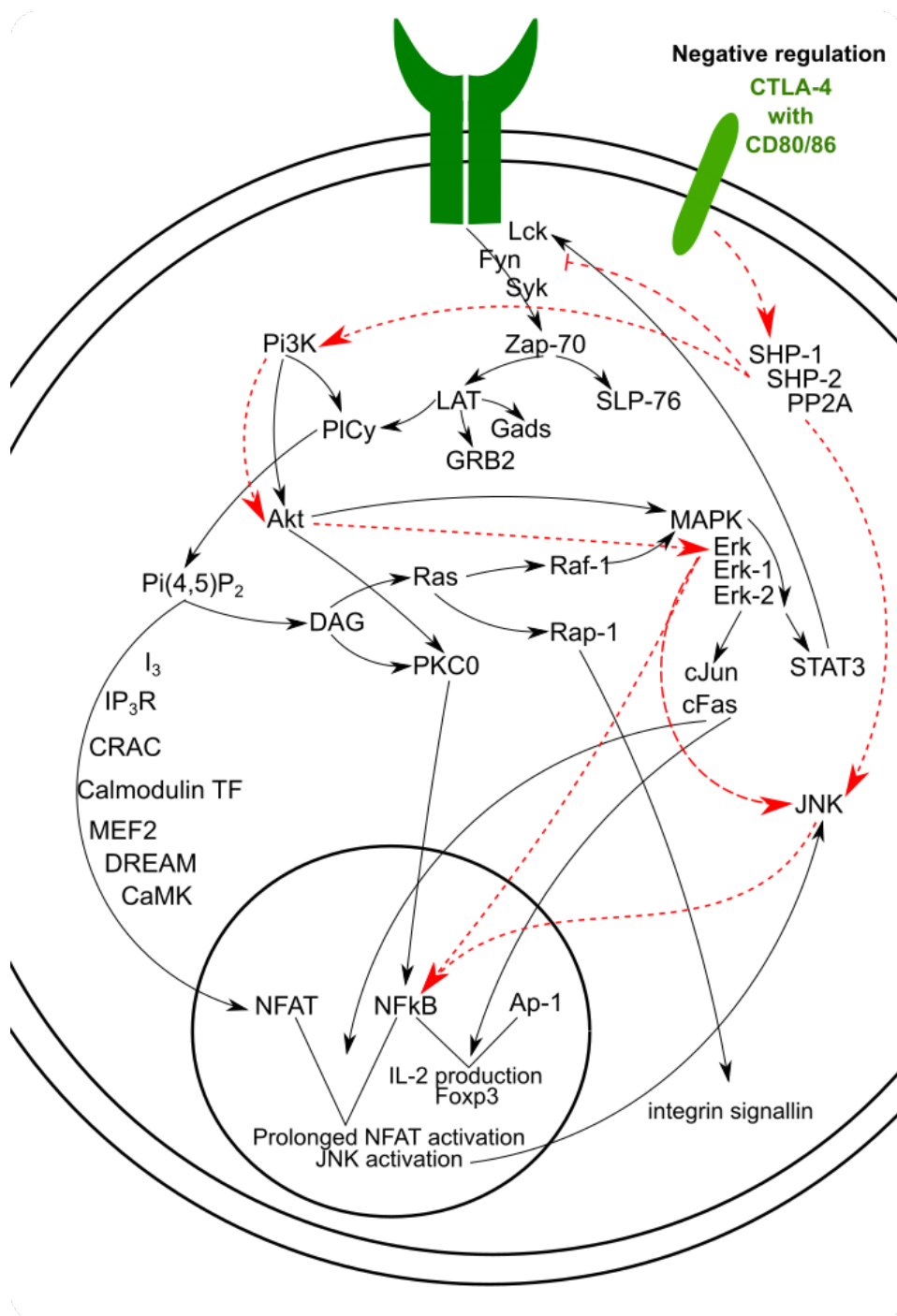
6.2.2. Establishment of Post-Activation Latency

In this thesis, using our DC-T-cell model we have demonstrated, that infected, proliferating CD4$^+$ T-cells could revert to a resting state, forming a long-lived stable latent reservoir (Section 4.3.5). It is likely that mDC-induced latency in proliferating CD4$^+$ T-cells is established via an alternative pathway to mDC-induced latency in non-proliferating CD4$^+$ T-cells (Figure 6.1). We propose that, in these proliferating CD4$^+$ T-cells, latency is established by active suppression of viral expression as a result of interactions between the mDC and T-cells.

Upon activation, CD4$^+$ T-cells proliferate and express activation markers, including CD69 and CD25, as reported in Figure 4.4. Unlike resting CD4$^+$ T-cells, activated and proliferating CD4$^+$ T-cells express high levels of ICs, including Tim-3 (Figure 4.4), PD-1 (CD276), CTLA-4 (CD152) and BTLA (CD272; Evans and van der Sluis et al., unpublished data). Therefore, mDC mediated ligation of IC expressed on T-cells, could lead to suppression of T-cell activation and suppression of virus transcription (Pentcheva-Hoang et al. 2004, 2007; Bashirova et al. 2014). Inhibition of virus expression by IC could be mediated via the disruption of TcR signal transducers, including leukocyte-specific tyrosine kinase (Lck), proto-oncogene tyrosine-protein kinase (fyn) and spleen tyrosine kinase (syk) by Src-homology 2 domain (SHP)-1/2 and phosphatase protein phosphate 2A (PP2A; Figure 6.2B; Chemnitz et al., 2004; Clayton et al., 2014; Sathish et al., 2001; Yokosuka et al., 2012). Alternatively, IC mediated inhibition of virus could also occur via the down-regulation of Nuclear Factor of Activated T-cells (NFAT) and activator protein-1 (AP-1), two transcription factors necessary for HIV-1 transcription (Che et al. 2012; Lee et al. 2012). Additionally, inhibition of viral transcription in proliferating CD4$^+$ T-cells could prevent cytopathic effects of the virus, further providing an opportunity for infected, proliferating CD4$^+$ T-cells to survive despite HIV-1 infection.

Inhibition of mechanisms that block virus expression in activated, infected CD4$^+$ T-cells or in latently infected resting CD4$^+$ T-cells may allow virus

expression and induce cytopathic effects of both the virus and host immune response. This hypothesis has been tested in *in vitro* and *in vivo* in latently infected resting CD4$^+$ T-cells using latency reversing agents (LRA), including histone deacetylase inhibitors (HDACi; Archin et al. 2009, 2012; Contreras et al. 2009; Elliott et al. 2014; Rasmussen et al. 2014). However, based on both *in vitro* and *in vivo* data, the number of cells that express virus following treatment with HDACi is only a small proportion compared to activation *in vitro* with a mitogen only (Archin et al. 2014; Cillo et al. 2014; Elliott et al. 2014). Additionally, virus expression upon treatment with LRA alone does not kill the latently infected cells, an additional "kill" signal is needed (Shan et al. 2012). Finding a strategy to "shock and kill" a latently infected cell is one of several strategies being tested to eliminate latency (further discussed below in section 6.4.1).

6.3. pDC Derived Type-I Interferons and HIV-1 Latency

Previously, we have shown that co-culture of CD4$^+$ T-cells with pDC does not lead to the establishment of HIV-1 latency in non-proliferating CD4$^+$ T-cells (Evans et al. 2013). In section 4.3.3, we show that pDC were able to induce latent infection in proliferating CD4$^+$ T-cells, however, these cells quickly died. Together, these observations suggest that pDC may be unable to provide the signals necessary to induce latency and are able to block the establishment of latency, or can mediate activation of apoptotic pathways, or a combination of both, or via a novel mechanism we have not yet considered. These pathways may occur either via direct pDC-T-cell interaction or via type-I IFN secretion. Whatever the mechanism, these data suggest that pDC may have an important role in preventing HIV-1 latency and HIV-1 persistence.

6.3.1. pDC Mediate Inhibition of Latent Infection

pDC interact with CD4$^+$ T-cells in a similar way to mDC, that is, via cell interactions, including cognate and non-cognate, as well as through soluble factors. We have previously observed that pDC can inhibit latency in non-

proliferating CD4$^+$ T-cells within mDC-T-cell co-cultures (Evans et al. 2013). This suggests that pDC, unlike mDC, possess an additional factor that leads to the inhibition of latency. We have recently shown, that pDC lose their inhibitory effect following irradiation (van der Sluis, unpublished data). Irradiation of the pDC eliminates their ability to both secrete soluble factors and facilitate outside-in signalling. Given that pDC secrete type-I IFN in response to HIV-1 infection *in vitro* (Cella et al. 1999; Fonteneau et al. 2004; Gurney et al. 2004; Beignon et al. 2005; Groot et al. 2006; Cavaleiro et al. 2009; O'Brien et al. 2011; Royle et al. 2014) and *in vivo* (Lehmann et al. 2008, 2010), and our analysis of HIV-1 treated pDC microarrays shows that IFN-α, IFN-λ and IFN-ω were specifically upregulated in HIV-1 treated pDC (section 5.3.3; Thomas et al., 2014). Therefore, we propose that INF-α, IFN-λ and IFN-ω, may mediate a potent anti-viral response leading to the inhibition of latency.

As well as inhibition of productive HIV-1 infection, induction of cell death and immune activation, INF-α induces expression of interferon-stimulated genes (ISG) in T-cells, including anti-viral restriction factors. These factors may also be important in establishing HIV-1 latency. Anti-viral restriction factors that are up-regulated by IFN-α include APOBE3G, SAMHD1, MX2, tetherin, RNA-associated early stage anti-viral factor (REAF), SLFN11 and the TRIM family of proteins (Asaoka et al. 2005; Carthagena et al. 2009; Uchil et al. 2013; Marno et al. 2014). Restriction factors that act early in the viral life cycle and may block the establishment of latent infection in non-proliferating CD4$^+$ T-cells include APOBEC3G, which induces hypermutation during reverse transcription (Sheehy et al. 2002; Harris et al. 2003; Lecossier et al. 2003; Mangeat et al. 2003), SAMHD1, which depletes available dNTP to prevent reverse transcription (Berger et al. 2011; Goldstone et al. 2011), MX2, which interferes with capsid uncoating (Goujon et al. 2013; Liu et al. 2013; Matreyek et al. 2014), REAF, which inhibits reverse transcription (Marno et al. 2014) and TRIM26 and TRIM31, which block viral entry and reverse transcription (Reviewed in Turrini et al. 2014). SAMHD1 and APOBEC are expressed in resting T-cells (Chiu et al. 2005; Baldauf et al. 2012), and at low levels in

activated and proliferating T-cells (Chauveau et al. 2015; Ruffin et al. 2015). Treatment of T-cells and monocytes with the HIV-2 derived viral protein, Vpx, promotes SAMHD1 degradation and facilitates infection of total (memory and naive) resting T-cells (Hrecka et al. 2011; Laguette et al. 2011). When total resting CD4$^+$ T-cells were infected with HIV-2, which express Vpx, productive infection was also induced (Chauveau et al. 2015). However upon Vpx treatment of naïve T-cells and subsequent infection with HIV-1, naive CD4$^+$ T-cells did not become permissive to productive infection (Khoury et al., manuscript in preparation). Therefore, expression of unknown restriction factors that act early in the viral life cycle could block both productive and latent infection both in non-proliferating and activated T-cells.

Expression of restriction factors in CD4$^+$ T-cells exposed to type-I IFNs does not explain how proliferating CD4$^+$ T-cells become latently infected. Type-I IFN also induce expression of restriction factors that act later in the viral life cycle, after viral integration, that could inhibit HIV-1 transcription and facilitate establishment of latent infection in proliferating CD4$^+$ T-cells. Restriction factors that act late in the viral life cycle include tetherin, also known as BST2, which prevents budding of new virions (Neil et al. 2008; Andrew et al. 2009), SLFN11, which alters tRNA during viral transcription to prevent HIV-1 transcription (Li et al. 2012), and TRIM19, TRIM22 and TRIM26, which block transcription, viral assembly and budding (Reviewed in Turrini et al. 2014). To test whether these restriction factors are important in establishment of latency in proliferating CD4$^+$ T-cells, they can be measured at day 5 post-infection in our DC-T-cell model of latency using RT-PCR, or if antibodies are available using flow cytometry.

6.3.2.   Type-I IFN in Reversal of Latent Infection

Although our data shows that pDC can inhibit establishment of latency, the role of type-I IFN in HIV-1 infection to block productive infection, stimulate a

potent anti-viral response and more recently, activate latency, has been studied intensely in non-human primate models. pDC have a potent type-I IFN response to acute HIV-1 infection, and given that they block latency in non-proliferating T-cells and promote cell death of proliferating CD4$^+$ T-cells, type-I IFN is currently being considered as a strategy to eliminate latency. IFN-α treatment has been tested *in vivo*, in non-human primate (NHP) models and clinical trials in the absence of cART. These studies showed that exogenous type-I IFN failed to reduce HIV-1 DNA *in vivo*, however, it did increase HIV-1 RNA expression (Asmuth et al. 2010; Manion et al. 2012; Mexas et al. 2012; Azzoni et al. 2013; Sandler et al. 2014). Studies of type-I IFN treatment in HIV-infected patients on cART also show a reduction in either HIV-1 antigen (p24) or HIV-1 RNA (Goujard et al. 2012; Boué et al. 2011; Angel et al. 2009; Haas et al. 2000), but no difference in disease progression (Tavel et al. 2010). However, type-I IFN production in chronic infection leads to enhanced cell death and chronic inflammation, therefore, administration of type-I IFN for HIV-1 elimination strategies should be carefully monitored (Sandler et al. 2014). One study that treated HIV-1 and HCV co-infected patients with IFN-α, alongside cART, showed a reduction in HIV-1 DNA, however, there was no sustained increase in CD4$^+$ T-cell counts (Sun et al. 2014). Despite the addition of type-I IFN at different stages of HIV-1 infection and both on and off cART regiments, no long-term benefit has yet been shown for *in vivo* IFN- α treatment. Further investigation needs to be completed in order to understand the mechanism of pDC-mediated inhibition of latent infection and the induction of cell death in the *in vitro* DC-T-cell latency model.

6.4. DC and HIV-1 Persistence

DC provide a highly regulated and efficient response to pathogens. In HIV-1 infection, DC can mediate the induction of restriction factors (Mohanram et al. 2013; Marno et al. 2014; Royle et al. 2014) and enhance the type-I IFN response to block productive infection (Cella et al. 1999; Fonteneau et al. 2004; Gurney et al. 2004; Beignon et al. 2005; Groot et al. 2006; Hardy et al. 2007; Cavaleiro et al. 2009; O'Brien et al. 2011; Khokhlova et al. 2014).

However, mDC and pDC also induce T-cell activation, which creates more target cells susceptible to HIV-1 infection, promoting T-cell infection and viral spread. In the setting of latent infection, we show here that blood derived mDC are able to induce the establishment of HIV-1 latency, while other investigators have shown that blood derived mDC can activate latency *in vitro* (van der Sluis et al. 2013, 2015). Together these studies highlight the diverse role of DC.

Our data shows that mDC and other myeloid lineage cells can induce latent infection *in vitro*. Reservoirs of latency *in vivo* include lymph node (LN), the GIT and the GALT, where a high frequency of latently infected cells are detected (Kreisberg et al. 2006; Dinoso et al. 2009; North et al. 2010; Yukl et al. 2013). At these sites, residential and circulating CD1c^{+} and CD141^{+} mDC could be responsible for the establishment and maintenance of latency. At epithelial or mucosal sites, mDC subpopulations also exist, including CD1c^{+} and CD141^{+} mDC, Langerhans cells (LC) and dermal DC (DDC; Ginhoux et al., 2007; Kanitakis et al., 2011; Klechevsky et al., 2008; Lenz et al., 1993; Romani et al., 1989; Wang et al., 2014). Given the phenotypic and functional similarities between LC, DDC, monocytes and mDC, and their common myeloid lineage (Harman et al. 2013), we hypothesise that LC and DDC can also induce latent infection. Early skin explant studies from HIV-1-infected patients off treatment show that skin explants harbor HIV-1 that is different to the circulating virus (Sala et al. 1994). In another study, Boy et al. found that 10 out of 35 cART naïve HIV-1 infected patients with high viral loads (32000-670,000 copies/ml plasma) had HIV-1 DNA in mucosal cells from the tongue, buccal mucosa (cheek lining) and gingival (gum) tissue, however no HIV-DNA was detected in LC or deep EC (Boy et al. 2009). More recent studies from patients on cART have shown that memory CD4^{+} T-cells from adipose tissue have detectable HIV-1 DNA (Couturier et al. 2015). Together these studies suggest that skin, adipose and mucosal surfaces may represent isolated viral reservoirs, different to peripheral sites that can harbor HIV-1 DNA infection. Further investigation in cART treated patients is necessary to

determine whether these anatomical sites are viral reservoirs that contribute to HIV-1 persistence.

LC express langerin, a C-type lectin, which is known to bind, internalize and degrade HIV-1 virions, thereby preventing LC infection (de Witte et al. 2007; Nasr et al. 2014). Langerin mediated internalization and degradation of HIV-1 virions can decrease local viral load and prevent cis-infection of LC. However, langerin does not block trans-infection of CD4$^+$ T-cells by LC (Nasr et al. 2014), and LC *cis* and *trans*-infection can be enhanced using TLR 1 and 2 activation of the LC (de Jong et al. 2008). Given that *trans*-infection is still efficient between LC and CD4$^+$ T-cells, we hypothesise that latent infection is established upon LC and T-cell interactions. This hypothesis is supported in mDC-T-cell experiments where T-cells were infected prior to co-culture with mDC, yet latent infection was still established (Evans et al. 2013).

6.4.1.   Persistence of HIV-1 Reservoirs

HIV-1 DNA in Myeloid Cells

mDC and other myeloid cells, such as monocytes and macrophages, are unlikely to be long-lived viral reservoirs, as the life span of these cells *in vivo* is short: 6-9 days for DC (Kamath et al. 2002); and 30 days for the monocytes (Yona et al. 2013). However, Yukl et al. recently showed that myeloid cells from rectal biopsies contain HIV-1 DNA (Yukl et al. 2014), and several earlier *in vitro* studies detected HIV-1 DNA in macrophages (Zalar et al. 2010; Josefsson et al. 2013b). These data suggest a direct role for myeloid lineage cells in the persistence of HIV-1.

Recent studies into DC and monocyte ontology suggest that tissue sites have resident DC and monocyte progenitor cells, which can produce progeny DC and monocytes indefinitely (Bogunovic et al. 2006; Ginhoux et al. 2010; Haniffa et al. 2009; Hoeffel et al. 2012; Ivanovs et al. 2011; Reviewed in Guilliams et al. 2014). If progenitor cells are infected, progeny cells may also

harbor HIV-1 DNA and potentially produce *de novo* virions under appropriate conditions of activation, similar to CD34+ hematopoietic progenitor cells (HPC) from bone marrow (Carter et al., 2010). Taken together with our observations, this data suggests that myeloid progenitor cells, with or without integrated HIV-1 infection, may be able to induce latent infection in local tissue environments. Therefore, DC and their progenitor cells should be considered in HIV-1 cure strategies.

6.4.2. DC in HIV-1 Cure Strategies

DC, Total Body Irradiation and Stem Cell Transplantation
The only documented case of HIV-1 cure to date followed total body irradiation (TBI) and allogeneic stem cell transplantation with stem cells from a donor who was delta CCR5 homozygous (Hütter et al. 2009). Stem cell transplantation is a strategy used to treat patients who require treatment of haematological malignancy and has been used in HIV-1-infected patients with coincidental lymphoma or leukaemia. The aim of TBI is to eliminate all recipient immune cells in preparation for donor immune cells via bone marrow transplantation. It has been shown in mice, that TBI can not eliminate macrophages from epithelia (Haniffa et al. 2009). An extension to this finding would be that as well as macrophages, precursor cells to macrophages, including monocytes and monocyte progenitor cells, are not fully eliminated.

Following TBI in HIV-1-infected subjects, prior to stem cell transplantation, it is possible that persistent recipient APC could contribute to viral rebound. In two other HIV-1-infected subjects on cART, who received TBI and stem cell transplantation from a donor with wild type CCR5, prolonged remission off cART was achieved. However, 2 and 4 months after cessation of cART, HIV-1 RNA rebounded in plasma (Henrich et al. 2013). The source of virus in these patients remains unclear, but may potentially have come from a long-lived latently infected CD4^{+} T-cell or possibly HIV-1-infected myeloid progenitor cells that could promote both productive and latent infection in neighbouring

T-cells. Further studies are necessary to confirm whether cells of the myeloid lineage or myeloid progenitor cells contribute to viral rebound off cART.

DC in "Shock and Kill"

Although we have shown that mDC induce latent infection in both non-proliferating and proliferating CD4$^+$ T-cells, others have shown that mDC can re-activate latent infection (van der Sluis et al. 2013). These opposing observations suggest that a fine balance must exist between mDC-induced latent infection and virus production. This balance could potentially be manipulated to activate latency, as described in section 6.2 (Reviewed in Deeks et al. 2012; and Archin and Margolis 2014).

van der Sluis et al. showed that compared to anti-CD3/CD28, IL-2 and Ionomycin, the CD1$^+$ mDC subpopulation efficiently reactivated latent infection in a post-activation latency model (van der Sluis et al. 2013, 2015). Using neutralizing antibodies, the authors went on to show that mDC-mediated reactivation of latency involved cell-cell interactions that could be inhibited by blocking the adhesion molecules Intercellular Adhesion Molecule (ICAM)-1 and -2. Based on our observations, ICAM-1 was also important in establishing latency in non-proliferating CD4$^+$ T-cells (Section 2.3.5; Evans et al., 2013). One caveat with the study by van der Sluis et al. was that inducible virus was measured in CD4$^+$ T-cells that were activated and proliferating. These cells would therefore have a lower threshold of T-cell activation and/or virus expression. This study suggests that mDC may represent a potent strategy to activate latent infection.

pDC are hypothesized to potentiate disease progression in HIV-1-infected subjects and type-I IFN are known to promote T-cell death and chronic immune activation. In our model, we saw similar levels of productive infection in T-cells that were co-cultured with either pDC or mDC (Section 3.3.4). Additionally, we observed high levels of cell death in activated T-cells following co-culture with pDC (Section 4.3.5), indicating that cell death

pathways activated by pDC via type-I IFN secretion may be active in our model (Herbeuval et al. 2006; Hardy et al. 2007; Lehmann et al. 2010; Dillon et al. 2011). Harnessing specific functions activated by type-I IFN that may eliminate latently infected cells or promote induction of cell death in HIV-1 cure strategies may be a fruitful investigation. Using further *in vitro* work, the specific signalling pathways important in latency re-activation (shock) and induction of apoptosis (kill) can be studied, and novel factors isolated, to develop specific agonistic or antagonist drugs.

6.5. Concluding Remarks

This thesis has demonstrated the versatility of the *in vitro* DC-T-cell latency model, showing that latent infection can be established by mDC in both non-proliferating and proliferating CD4$^+$ T-cells. Using a combination of cell culture manipulation and gene analysis, the data presented suggests that non-cognate interactions, including adhesion and co-stimulatory molecules, maybe important in the establishment of latency in non-proliferating CD4$^+$ T-cells. Similarly, our data suggest that IC maybe important in establishment of latency in proliferating CD4$^+$ T-cells, however further studies are needed to confirm these observations. This thesis has also built on previous work performed in the lab and enhanced our understanding of DC in establishment of latent HIV-1 infection. We have clearly shown that DC do play an important role in regulating the latent reservoir. Further studies to understand the specific mechanisms of latency establishment in non-proliferating and proliferating CD4$^+$ T-cells will lead to a better understanding of the role of DC in HIV-1 persistence. The results presented in this thesis provide a starting point to further understand and manipulate the interaction between the DC and CD4$^+$ T-cells to one day discover a long-term cure for HIV-1 infection.

7. Chapter 7

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8. Chapter 8

Appendix

8.1 Appendix

8.2 Chapter 2. Publication

Evans VA, <u>Kumar N</u>, Filali A, Procopio FA, Yegorov O, Goulet J-P, et al. Myeloid Dendritic Cells Induce HIV-1 Latency in Non-proliferating CD4$^+$ T Cells. *PLoS Pathog.* 2013 Dec; 9(12):e1003799–813.

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Myeloid Dendritic Cells Induce HIV-1 Latency in Non-proliferating CD4+ T Cells

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Abstract

Latently infected resting CD4+ T cells are a major barrier to HIV cure. Understanding how latency is established, maintained and reversed is critical to identifying novel strategies to eliminate latently infected cells. We demonstrate here that co-culture of resting CD4+ T cells and syngeneic myeloid dendritic cells (mDC) can dramatically increase the frequency of HIV DNA integration and latent HIV infection in non-proliferating memory, but not naïve, CD4+ T cells. Latency was eliminated when cell-to-cell contact was prevented in the mDC-T cell co-cultures and reduced when clustering was minimised in the mDC-T cell co-cultures. Supernatants from infected mDC-T cell co-cultures did not facilitate the establishment of latency, consistent with cell-cell contact and not a soluble factor being critical for mediating latent infection of resting CD4+ T cells. Gene expression in non-proliferating CD4+ T cells, enriched for latent infection, showed significant changes in the expression of genes involved in cellular activation and interferon regulated pathways, including the down-regulation of genes controlling both NF-κB and cell cycle. We conclude that mDC play a key role in the establishment of HIV latency in resting memory CD4+ T cells, which is predominantly mediated through signalling during DC-T cell contact.

Introduction

Antiretroviral therapy (ART) for the treatment of HIV has led to a substantial reduction in morbidity and mortality; however, ART cannot cure HIV and life-long treatment is required. This is directly due to the persistence of long-lived latently infected cellular reservoirs, that include microglia, astrocytes, macrophages and naïve T cells [1–4], however, resting memory CD4+ T cells [5–7], are considered to be the major contributors. Latently infected resting CD4+ T cells are found in blood and tissue sites, including lymphoid tissue and the gastrointestinal tract [7–10]. The frequency of latently infected cells is up to ten times higher in tissue than in blood in HIV-infected patients or SIV-infected macaques on suppressive ART [8,10].

It is unclear how latency is established *in vivo*. However, *in vitro*, latency can be established following survival of an activated CD4+ T cell that returns to a resting state carrying integrated virus [5,11–13]. Alternatively, latency has also been established following direct infection of resting cells in the presence of chemokines or following spinoculation [14–19]. Dendritic cells (DC) are found throughout the body and interact closely with resting CD4+ T cells within lymphoid tissues. Therefore, given the high frequency of latently infected cells in lymphoid tissue, we hypothesised that latency in resting CD4+ T cells may result from interactions with DC as CD4+ T cells recirculate through lymphoid tissue. Using a novel model of resting CD4+ T cells co-cultured with primary DC, we demonstrate that myeloid DC (mDC) induce post-integration latency in resting memory CD4+ T cells, which required close DC-T cell contact.

Results

Myeloid DC promote HIV latency in non-proliferating CD4+ T cells

Resting CD4+ T cells and syngeneic DC (including the two major blood DC subpopulations, plasmacytoid (pDC) and myeloid (mDC) DC) were sorted from the blood of healthy donors (Fig. S1). Seminaphtharhodafluor-1 (SNARF)-labelled resting CD4+ T cells were cultured either alone or co-cultured with DC at a DC: T cell ratio of 1: 10. Following 24 hours of culture, cells were infected with a CCR5-tropic enhanced green fluorescent protein (EGFP)-reporter virus, NL(AD8)-nef/EGFP (multiplicity of infection, MOI

Author Summary

Current antiretroviral drugs significantly prolong life and reduce morbidity but are unable to cure HIV. While on treatment, the virus is able to hide in resting memory T cells in a silent or "latent" form. These latently infected cells are rare and thus are hard to study using blood from HIV-infected individuals on treatment. Therefore, it is very important to have laboratory models that can closely mimic what is going on in the body. We have developed a novel model of HIV latency in the laboratory. Using this model we have shown that the presence of dendritic cells, an important type of immune cell that can regulate T cell activation, at the time of infection allows for the infection of resting T cells and the establishment of latency. We have demonstrated that this is predominantly mediated by direct cell-to-cell interactions. Further exploration of the mechanisms behind HIV latency could lead to new ways to treat and possibly eradicate HIV.

0.5), and cultured for 5 days (Fig. 1 A). Cells were then analysed for expression of EGFP by flow cytometry to quantify productive infection (Fig. 1 B).

In the DC-CD4$^+$ T cell co-cultures, we detected a spreading productive infection with the number of infected cells 5 days post-infection significantly greater (median (IQR) = 40 (31, 150) EGFP$^+$ cells/10^4 cells; n = 5) compared to CD4$^+$ T cells cultured alone (1.5 (1, 2.5) EGFP$^+$ cells/10^4 cells, p = 0.03; Fig. 1 C). These results were consistent with previous work demonstrating enhanced productive infection of CD4$^+$ T cells in the presence of DC [20,21].

At day 5 post-infection, non-proliferating (SNARF^{hi}) CD4$^+$ T cells that were not productively infected (EGFP$^-$) were sorted (purity was always >99%). Latent virus was quantified in the SNARF^{hi}EGFP$^-$ CD4$^+$ T cells upon stimulation with phytohaemagglutinin (PHA) in the presence of feeder peripheral blood mononuclear cells (PBMC) following a further 5 days of culture (Fig. 1 B). The number of EGFP$^+$ cells following stimulation was, therefore, a surrogate measure for the number of latently infected cells in the SNARF^{hi}EGFP$^-$ CD4$^+$ T cells. When SNARF^{hi}EGFP$^-$ CD4$^+$ T cells were sorted from cultures infected in the absence of DC, few latently infected cells were detected (2 (1, 8.5) EGFP$^+$ cells/10^4 cells; n = 5). In contrast, when SNARF^{hi}EGFP$^-$ CD4$^+$ T cells were sorted from the DC-T cell co-cultures a significant increase in the number of latently infected cells was observed (41 (28, 73) EGFP$^+$ cells/10^4 cells; p = 0.03; n = 5; Fig. 1 D). Furthermore, when infections were performed in the presence of the protease inhibitor indinavir, there was no significant difference in the number of latently infected cells, as measured by EGFP expression following co-culture of SNARF^{hi}EGFP$^-$ with PHA and feeder PBMC (Fig. 1 E). This confirms that a productive, spreading infection was not required to establish latency. Together, these results demonstrate that DC facilitate latent HIV infection in non-proliferating CD4$^+$ T cells.

We next asked whether DC-T cell co-culture had activated the SNARF^{hi} CD4$^+$ T cells, which allowed for HIV entry. Sorted SNARF^{hi}EGFP$^-$ CD4$^+$ T cells that were co-cultured with DC for 5 days showed signs of early activation with increased expression of CD69 (1.5% (0.1, 2.1); p = 0.02; n = 4; Fig. 1 F). However, these cells did not express either HLA-DR or Ki67 (Fig. 1 F). As expected, resting CD4$^+$ T cells that were cultured alone did not express any of the activation markers. These results confirmed that the sorted SNARF^{hi}EGFP$^-$ cells were non-proliferating, partially activated CD4$^+$ T cells.

To determine whether mDC or pDC were facilitating latency in resting CD4$^+$ T cells, we next co-cultured sorted mDC and pDC with SNARF-labelled resting CD4$^+$ T cells for 24 hours prior to infection, and experiments were performed as described above. While productive infection was enhanced in both the mDC and pDC-T cell co-cultures (Fig. 2 A), latent infection was only identified in the SNARF^{hi}EGFP$^-$ CD4$^+$ T cells that had been co-cultured with mDC (33 (19, 51) EGFP$^+$ cells/10^4 cells; n = 5) following re-stimulation with PHA and feeder PBMC (Fig. 2 B) or following direct activation with anti-CD3/CD28, together with IL-7 and the integrase inhibitor L8, which allowed the detection of post-integration latency (Fig. 2 C). To further confirm that post-integration latency was established in resting CD4$^+$ T cells co-cultured with mDC, we used a real time PCR assay to quantify integrated HIV DNA. Integrated HIV DNA was present in SNARF^{hi}EGFP$^-$ CD4$^+$ T cells sorted from the mDC co-cultures (1100 (686, 4960) HIV DNA copies/10^6 cells, n = 3; Fig. 2 D), but not in the CD4$^+$ T cells sorted from the pDC co-cultures or the CD4$^+$ T cells cultured alone (both <330 HIV DNA copies/10^6 cells). Similar results were observed with a nef competent EGFP-reporter virus (Fig. 2 E), demonstrating that the establishment of mDC-induced latency was not dependent on Nef.

Unlike experiments that utilised R5 EGFP HIV, when experiments were performed with an X4 EGFP reporter virus, latent infection was detected in the resting CD4$^+$ T cells cultured alone (87 (51, 155) EGFP$^+$ cells/10^4 cells; n = 4; Fig. 2 F). However, latency was still significantly enhanced in the non-proliferating CD4$^+$ T cells in the presence of mDC (468 (213, 621) EGFP$^+$ cells/10^4 cells) when compared to the T cells cultured alone.

In some experiments we added a low dose of Staphylococcus enterotoxin B (SEB; 10 ng/mL) to the mDC-T cell co-cultures to enhance productive infection and increase cognate interactions between mDC and T cells. In the presence of SEB we observed a significant increase in the level of productive infection; however, there was no difference in the level of latent infection (Fig. 2 G). Finally, we cultured mDC and T cells together at ratios ranging from 1:10 to 1:100 to determine the minimum interaction required between mDC and T cells to induce latency. We found that latency could still be established at a ratio of DC: T cells as low as 1:100 (Fig. 2 H).

Taken together, these results demonstrated that in vitro mDC and not pDC facilitated post-integration latency in non-proliferating CD4$^+$ T cells.

Myeloid DC induce latency in memory CD4$^+$ T cells

We have previously shown that memory CD4$^+$ T cells and not naïve CD4$^+$ T cells are susceptible to latent infection following chemokine exposure [14]. To determine whether mDC-induced T cell latency occurred in memory or naïve CD4$^+$ T cells, we separated the SNARF^{hi}EGFP$^-$ CD4$^+$ T cells into CD45RO$^+$ (memory) and CD45RO$^-$ (naïve) fractions prior to culture with feeder PBMC. In these experiments, latent infection was detected at significantly higher levels in the CD45RO$^+$ memory CD4$^+$ T cell fraction (146 (14, 197) EGFP+ cells/10^4 cells; Fig. 2 I).

Depletion of CD69 expressing cells has no effect on DC-induced latency

A proportion of non-proliferating SNARF^{hi}EGFP$^-$ cells sorted from DC-T cell co-cultures (Fig. 1 F and 3 A) expressed CD69. Therefore, in order to exclude the possibility that we were only detecting infection of the cells showing early signs of activation, we depleted CD69$^+$ cells from the SNARF^{hi}EGFP$^-$ T cells at day 5
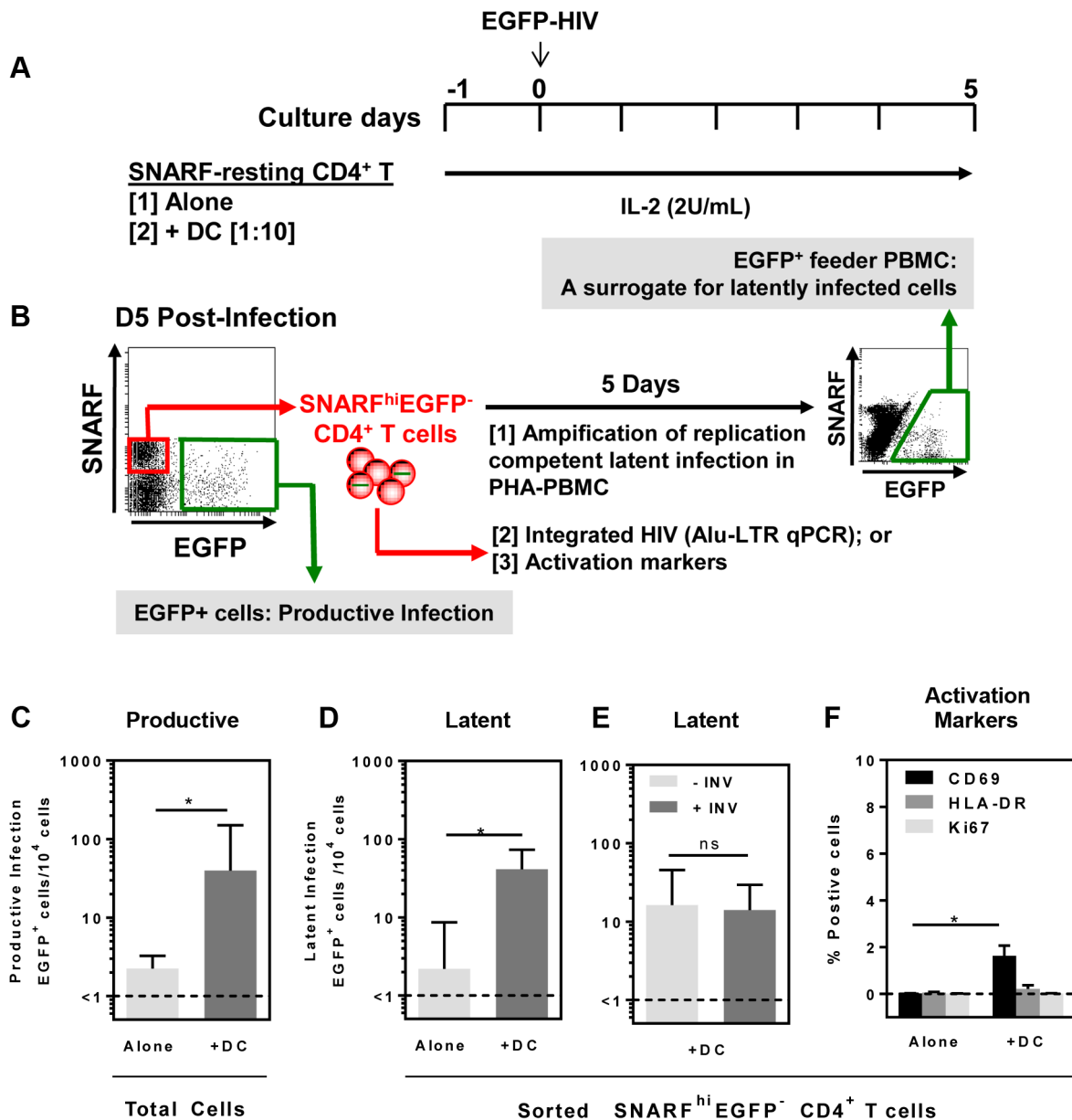
Figure 1. DC-induced latency in resting CD4^{+} T cells. (**A**) Resting CD4^{+} T cells were isolated from the blood of healthy donors and labelled with the proliferation dye SNARF, which decreases in intensity following each round of cell division allowing identification of non-proliferating cells. SNARF-labelled resting CD4^{+} T cells were cultured either alone or with syngeneic blood DC. Following 24 hours of culture, cells were infected with NL(AD8)-nef/EGFP at an MOI of 0.5. All culture media was supplemented with IL-2 (2 U/mL). (**B**) At day 5 post-infection, the number of productively infected (EGFP^{+}) cells was determined and the non-proliferating (SNARF^{hi}) cells that were not productively infected (EGFP[−]) were sorted. The sorted SNARF[hi]EGFP^{−} cells were stimulated with PHA/IL-2 in the presence of PBMC and cultured for 5 days to amplify any replication competent latent infection. (**C**) Productive infection and (**D**) latent infection following infection of T cells cultured alone (light grey) or in the presence of DC (grey) is shown. (**E**) Latent infection in the presence of DC cultured with (grey) or without (light grey) 0.1 µM Indinavir. (**F**) Expression of the early (CD69; black) and late (HLA-DR; grey) surface activation markers and the intracellular proliferation marker Ki67 (light grey) was quantified by flow cytometry on sorted SNARF^{hi}EGFP^{+} CD4^{+} T cells following HIV infection of T cells cultured alone or in the presence of DC. The lower limit of detection of each assay is represented by a dotted line. Columns represent the median of 5 independent experiments and error bars indicate the interquartile range. *$P<0.05$ (Wilcoxon signed-rank test).

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post-infection prior to co-culture with PHA and feeder PBMC. We found no significant difference in the level of latency following depletion of the CD69^{+} cells (Fig. 3 B). CD69 expression can be transient, therefore, to confirm that we had not missed cells that expressed CD69, which had then been down-regulated; we measured the expression of CD69 over time following co-culture

with mDC and infection with HIV. We demonstrated that CD69 expression peaked at day 2 and remained elevated out to day 5 post-infection (data not shown). These results demonstrate that the subpopulation of CD4^{+} T cells that were partially activated and expressing CD69 were not preferentially latently infected following mDC-T cell co-culture.
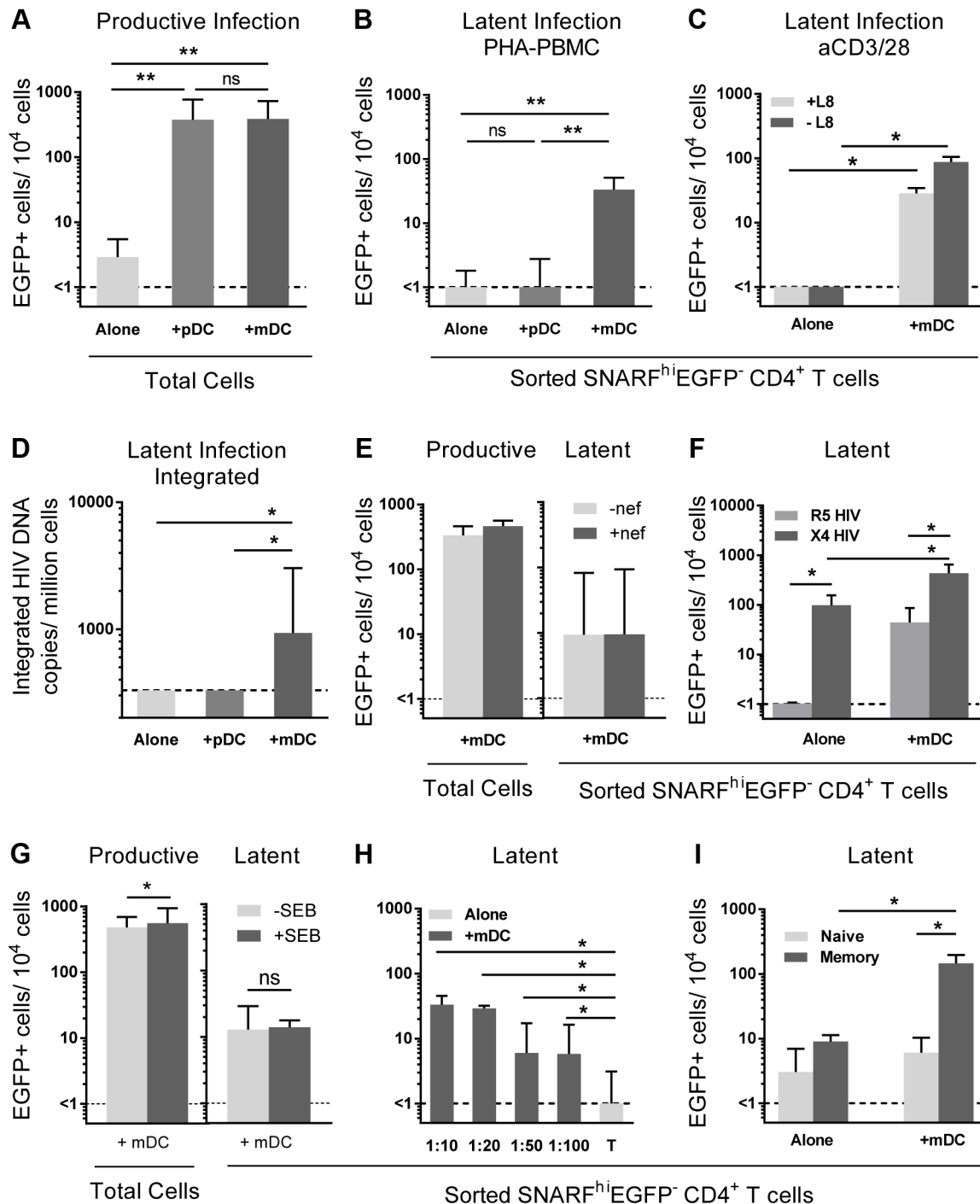
Figure 2. Myeloid DC induce post-integration latency in non-proliferating memory CD4$^+$ T cells. SNARF-labelled resting CD4$^+$ T cells were cultured alone (light grey) or with syngeneic plasmacytoid (pDC; grey) or myeloid DC (mDC; dark grey). (**A**) Productive infection (EGFP$^+$ cells) was determined by flow cytometry on day 5 post-infection. (**B**) Latent infection was quantified in SNARF^{hi}EGFP$^-$ cells following either addition of PHA-activated PBMC, n = 5; or (**C**) direct activation with anti-CD3/CD28 in the presence or absence of the integrase inhibitor L8. (**D**) Integrated HIV DNA was quantified in the sorted SNARF^{hi}EGFP$^-$ T cells by Alu-LTR real-time PCR, n = 3. (**E**) Productive and latent infection was determined in SNARF^{hi}EGFP$^-$ CD4$^+$ T cells from mDC-T cell co-cultures following infection with nef-deficient (-nef) or nef-competent EGFP HIV. (**F**) Latent infection was determined in sorted SNARF^{hi}EGFP$^-$ CD4$^+$ T cells, cultured alone or with mDC, following activation with PHA-PBMC. (**G**) Productive and latent infection was determined in SNARF^{hi}EGFP$^-$ CD4$^+$ T cells from mDC-T cell co-cultures with and without Staphylococcus Enterotoxin B (SEB), n = 4. (**H**) SNARF-labelled resting CD4$^+$ T cells were cultured alone (light grey) or with syngeneic mDC (grey) at decreasing DC:T cell ratios and latent infection quantified in sorted SNARF^{hi}EGFP$^-$ T cells following addition of PHA-activated PBMC, n = 5. (**I**) Resting CD4$^+$ T cells were cultured either alone or in the presence of mDC. At day 5 post-infection, SNARF^{hi}EGFP$^-$ cells were sorted into naïve (light grey) or memory (grey) CD4$^+$ T cells and latent infection quantified, n = 5. The lower limit of detection is represented by a dotted line. Columns represent the median of 3–7 donors and error bars indicate the interquartile range. *$P < 0.05$; **$P < 0.01$; ns, not significant (Wilcoxon signed-rank test).
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HIV infection of mDC-T cell co-cultures leads to an increase in chemokine and cytokine expression

To determine why mDC and not pDC led to the establishment of latency, we compared cytokine levels in pDC-T cell and mDC-T cell co-cultures 5 days following HIV infection using bead arrays for known DC-secreted cytokines. Supernatants collected from HIV-infected mDC-T cell co-cultures compared to the HIV-infected pDC-T cell co-cultures had significantly increased expression of IL-6 (p = 0.002), IL-10 (p = 0.01) and CXCL9 (p = 0.002; Fig. 4 A). TNF-alpha was also up-regulated in the mDC-T cell compared to pDC-T cell co-cultures but the difference was not statistically significant (Fig. 4 A). As expected, IFN-alpha was detected at high levels in the pDC-T cell co-cultures but not in the mDC-T cell co-cultures (Fig. 4 A; p = 0.01), and latency was not established in pDC-T cell co-cultures even in the presence of neutralising antibodies to IFN-alpha (Fig. 4 B). Interestingly, when equal numbers of pDC and mDC were added to resting CD4+ T cells latency was reduced when compared to T cells cultured only with mDC (Fig. 4 C). This suggests that while pDC themselves do not induce latency, they are able to inhibit the establishment of latency mediated by mDC.

Blocking Abs to IL-6, IL-10R, CXCR3 and CCL19 have no effect on DC-induced latency

To determine whether the soluble factors that were differentially expressed in mDC-T cell co-cultures compared to pDC-T cell co-cultures were contributing to the establishment of mDC-induced latency, neutralising antibodies (nAb), to either the soluble factor or its receptor, were added to the T cells prior to co-culture with DC and the addition of HIV. Specific nAbs or an anti-IgG control were added to eFluor670 (alternative proliferation dye to SNARF)-labelled resting memory (CD45RO+) CD4+ T cells prior to co-culture with mDC, and again following infection, and latency determined as described in Fig. 1 (Fig. 4 D).

When nAbs to IL-6, the IL-10 receptor (IL-10R) or CXCR3 (CXCL9 and CXCL10 receptor) were added to the mDC-T cell co-cultures, no significant decrease in the number of latently infected CD4+ T cells was observed when compared to cultures where control anti-IgG was added. As we had previously shown

that the chemokine CCL19 can condition resting CD4+ T cells allowing for enhanced entry and integration of HIV [14], we also added anti-CCL19, either alone or in combination with anti-CXCR3, to the mDC-T cell co-cultures. However, we did not detect a significant decrease in the number of latently infected cells. The activity of these nAbs was confirmed by their ability to block STAT3 signalling (aIL-6, aIL-10R) or chemokine induced migration (aCXCR3, aCCL19; Fig. S2).

DC-induced latency requires close proximity of DC and CD4+ T cells

To determine whether DC-T cell contact was required to establish latency in resting CD4+ T cells, we co-cultured mDC and resting CD4+ T cells separated by a 0.4 µm membrane transwell. Following 24 hours of culture, HIV was added to the mDC in the upper chamber and the CD4+ T cells in the lower chamber. Without mDC-T cell contact, the establishment of latency was significantly inhibited (<1 EGFP+ cell/10^4 cells) when compared to co-cultures without membranes (18 (10, 85) EGFP+ cell/10^4 cells; n = 5; p = 0.03; Fig. 4 E).

To further elucidate whether soluble factors other than those previously inhibited were involved in DC-induced latency, we added supernatant from infected mDC-T cell co-cultures to uninfected resting CD4+ T cells and then infected these cells with EGFP-HIV. Media changes were performed daily using supernatant from infected mDC-T cell co-cultures. Under these conditions the resting CD4+ T cells would be exposed to any soluble factors and free viral particles present in the mDC-T cell co-cultures but would not have any contact with the mDC. Latency was not detected in these cultures (Fig. 4 F), providing further evidence that DC-T cell contact, and not a soluble factor, was required for the establishment of DC-induced latency.

Direct DC-T cell signalling can occur following interactions between several cell surface receptors. In particular, interactions between lymphocyte function associated antigen-1 (LFA-1; composed of CD11a and CD18) on T cells and intercellular adhesion molecule 1 (ICAM-1) on DC are involved in DC-T cell adhesion [22] and subsequent T cell activation via formation of an immunological synapse [23]. To inhibit DC-T cell clustering, we used blocking antibodies to CD18 (10–20 µg/mL). Blocking of
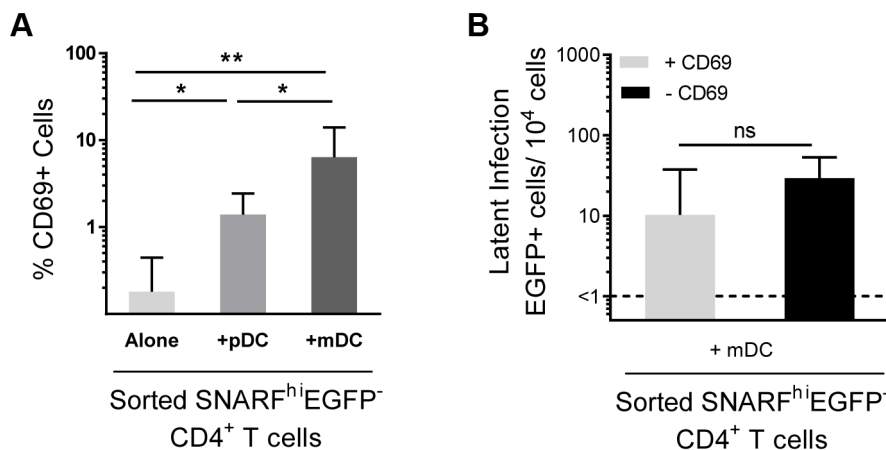
Figure 3. Depletion of CD69+ cells has no effect on mDC-induced latency. (A) Expression of CD69 was determined in sorted SNARF^hiEGFP− CD4+ T cells, n = 6. (B) Latent infection was determined for SNARF^hiEGFP− CD4+ T cells sorted from mDC-T cell co-cultures that either contained (+CD69, light grey) or were depleted of CD69-expressing cells (−CD69, black), n = 4. The lower limit of detection is represented by a dotted line. Columns represent the median of 4–6 donors and error bars indicate the interquartile range. *P<0.05; **P<0.01; ns, not significant (Wilcoxon signed-rank test).
doi:10.1371/journal.ppat.1003799.g003
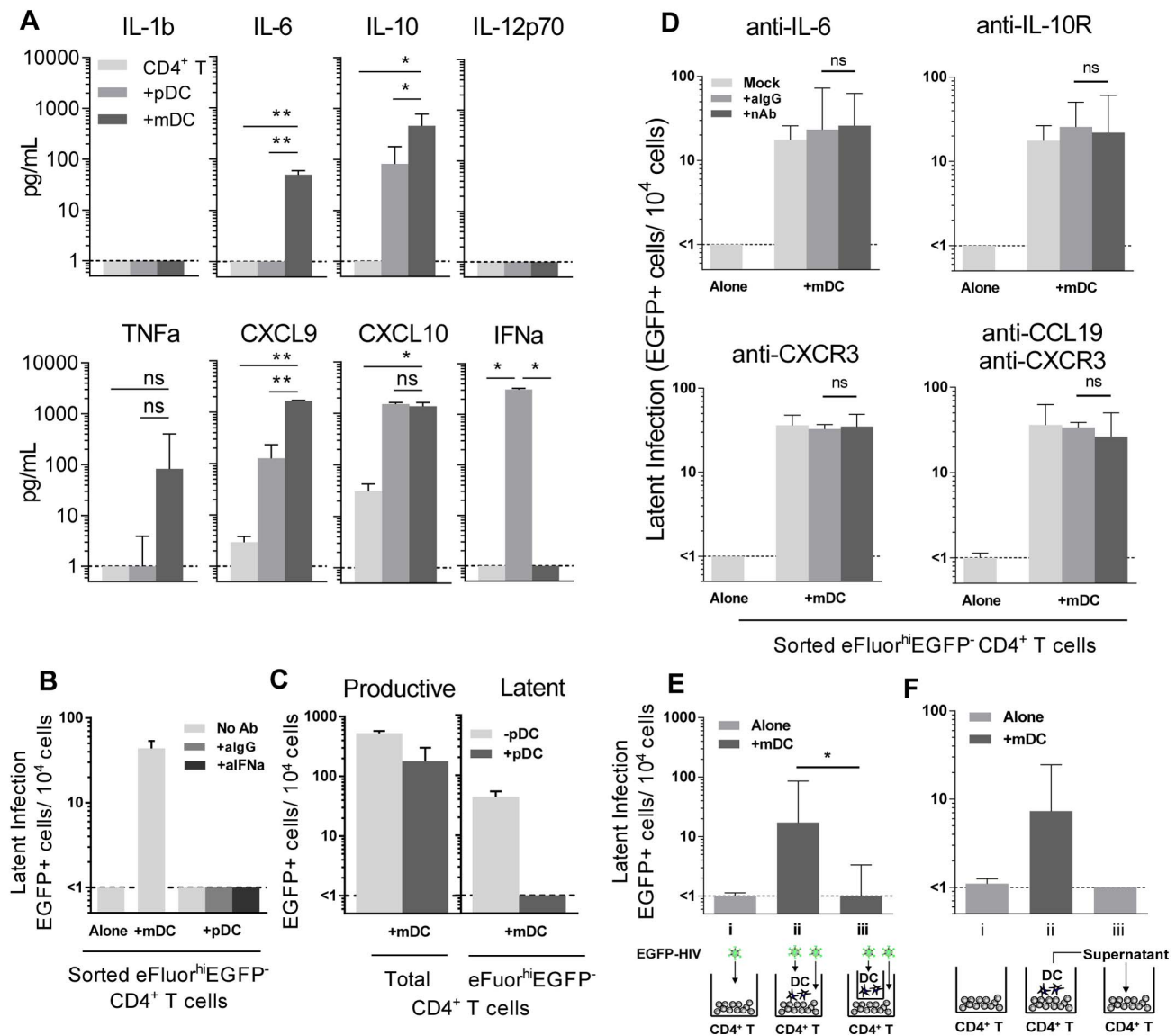
Figure 4. Soluble factors in DC-induced HIV latency. (**A**) Resting CD4$^+$ T cells were cultured alone (light grey) or with sorted pDC (grey) or mDC (dark grey). At day 5 post-infection, cytokines and chemokines were quantified in culture supernatants using cytometric bead arrays, n = 3. *$P<0.05$; **$P<0.01$ (paired t-test). (**B**) Latent infection was quantified in eFluor670^{hi}EGFP$^-$ resting CD4$^+$ T cells that were cultured either alone or with sorted pDC in the presence of media alone (light grey), anti-IgG (grey) or anti-IFN-alpha (dark grey) following stimulation with anti-CD3/CD28 in the presence of L8, n = 5. (**C**) Resting CD4$^+$ T cells were co-cultured with mDC with (grey) or without (light grey) the addition of equal numbers of pDC. Productive infection was determined at day 5 post-infection. Latent infection was determined in sorted eFluor670^{hi}EGFP$^-$ CD4$^+$ T cells following stimulation with anti-CD3/CD28 in the presence of L8, n = 5. (**D**) Latent infection was quantified in eFluor670^{hi}EGFP$^-$ resting memory CD4$^+$ T cells that were cultured either alone or with sorted mDC in the presence of media alone (light grey), anti-IgG (grey) or neutralising antibodies (dark grey) to IL-6, IL-10-receptor, CXCR3 or CCL19, n = 5. (**E**) eFluor670-labelled resting CD4$^+$ T cells were cultured either alone (light grey) or with blood mDC (dark grey). Virus was added to (**i**) CD4$^+$ T cells cultured alone; (**ii**) CD4$^+$ T cells co-cultured with mDC; (**iii**) CD4$^+$ T cells cultured with mDC in the presence of a 0.4 μm membrane transwell and latency determined at day 5 post-infection, n = 5. (**F**) eFluor670-labelled resting CD4$^+$ T cells were cultured either (**i**) alone (light grey) or (**ii**) with blood mDC (dark grey) and infected. (**iii**) Following 24 hours, supernatant from infected mDC-T cell co-cultures was added to uninfected resting CD4$^+$ T cells and these cells were then infected, n = 3. Columns represent the median of 3–5 donors and error bars indicate the interquartile range. *$P<0.05$; **$P<0.01$ (Wilcoxon signed-rank test).
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CD18 significantly inhibited, but did not eliminate, DC-T cell clustering, as observed by microscopy (data not shown). Following incubation with anti-CD18, there was no effect on the number of productively infected cells, however, we observed a significant decrease in the number of latently infected cells from the mDC-T cell co-cultures (20 (8, 34) latently infected cells/10^4 cells), when compared to cells cultured without anti-CD18 (25 (17, 48) latently infected cells/10^4 cells; p = 0.03) or in the presence of control anti-

IgG (32 (22, 36) latently infected cells/10^4 cells; n = 6; p = 0.03; Fig. 5 A). However, when resting CD4$^+$ T cells were stimulated with soluble ICAM-1 and anti-IgG there was no increase in latency observed (Fig. 5 B), suggesting that ICAM-LFA signalling alone does not induce latency in this model system. Furthermore, in the presence of anti-CD18 the number of latently infected cells from the mDC-T cell co-cultures remained greater than the CD4$^+$ T cells cultured alone (<1 latently infected cell/10^4 viable cells;

p = 0.01) suggesting that the effect of anti-CD18 was most likely due to the partial decrease in clustering/DC-T cell contact.

In order to determine whether mDC transfer of HIV was involved in the establishment of latency we performed experiments where we added virus to resting CD4+ T cells, washed off virus and added uninfected mDC to the CD4+ T cells (Fig. 5 C). Under these conditions we were still able to detect latency in the non-proliferating CD4+ T cells. Together, these results indicate that cell-cell contact plays a role in DC-induced T cell latency but that the mDC were not required to be infected and then transfer HIV to the resting CD4+ T cells.

Multiple genes are differentially expressed in latently infected, non-proliferating CD4+ T cells co-cultured with DC

To determine the effect of DC on gene transcription in latently infected resting CD4+ T cells, SNARF-labelled resting CD4+ T cells, from four independent donors, were cultured either alone or with syngeneic bulk blood DC at a 1:10 ratio for 24 hours prior to infection with NL(AD8)-nef/EGFP. In these experiments, we included IL-7 (10 ng/mL) in all cultures to increase cell survival of the resting cells and infections were performed at an MOI of 5 to ensure a high frequency of latently infected cells. Mock infections were performed in parallel with media alone. Non-proliferating (SNARF^{hi}) CD4+ T cells that were not productively infected (EGFP$^-$) were sorted 5 days post-infection and lysed for either the detection of HIV DNA by real-time PCR or RNA for microarray studies (Fig. 6 A).

Infection was confirmed in the resting CD4+ T cells following co-culture with DC, in 4 independent experiments, by detection of HIV DNA (3×10^4 (7.4×10^3, 5.7×10^5) copies/10^6 cells; Fig. 6 B). Changes in gene expression were quantified in the sorted SNARF^{hi}EGFP$^-$ CD4+ T cells using Illumina oligonucleotide microarrays. To identify genes expressed in DC-induced latency, we compared the expression profiles of non-proliferating, latently infected CD4+ T cells (HIV T (+DC)) to mock infected CD4+ T cells (Mock T (+DC)) that had been co-cultured with DC. In order to control for the effect of virus or DC alone, we first subtracted the gene expression profiles of control cells, which were T cells that

had been cultured alone that were either uninfected (Mock T) or exposed only to virus (HIV T).

A scatter plot (Fig. 7 A), representing the common (genes that fall on the diagonal) and differentially expressed genes (genes that fall off the diagonal) from this comparison, highlighted the significant differences in gene expression between latently infected cells and controls (r = 0.77). Additionally, this plot showed that several of the genes that discriminate latently infected cells from control cells were genes downstream of type I interferons, including interferon-induced protein with tetratricopeptide repeats 1 (IFIT-1), interferon alpha-inducible protein 27 (IFI27), and 2′-5′-oligoadenylate synthetase 1 (OAS1).

Inhibition of cell cycle entry in latently infected, non-proliferating CD4+ T cells

Heatmaps of the top 100 genes (Fig. S3) confirmed the de novo induction of genes encompassing several biological and metabolic pathways in T cells exposed both to DC and virus. These included transcripts of the Interferon pathway, genes involved in the regulation of cell cycle entry and mitosis, as well as receptor and effector molecules of cell survival and apoptosis.

Network analysis (Fig. 7 B and C) showed that two major pathways were regulated in T cells following co-culture with DC exposed to HIV. Exposure of CD4+ T cells to HIV and DC led to the up-regulation of genes downstream of type I interferon by nucleotide sensors. Figure 7 B confirms the wide-ranging impact of the up-regulation of the type I Interferon pathway, as several molecules with antiviral activity were up-regulated, including ISG15 ubiquitin-like modifier (ISG15), and DEAD box polypeptide 58 (DDX58; also known as RIG-I). Genes involved in actin polymerisation, and the organisation of microtubules, were also up-regulated as a consequence of interferon pathway up-regulation. Additionally, the interferon pathway intercepted with the mammalian target of rapamycin complex 2 (mTORC2) pathway, which plays an important role in autophagy and T cell survival [24] (Fig. 7 B; Table S1).

Network analysis confirmed the negative impact of exposure of CD4+ T cells to DC and virus on the NF-κB pathway as well as several cellular metabolic pathways (fatty oxidation and glucose
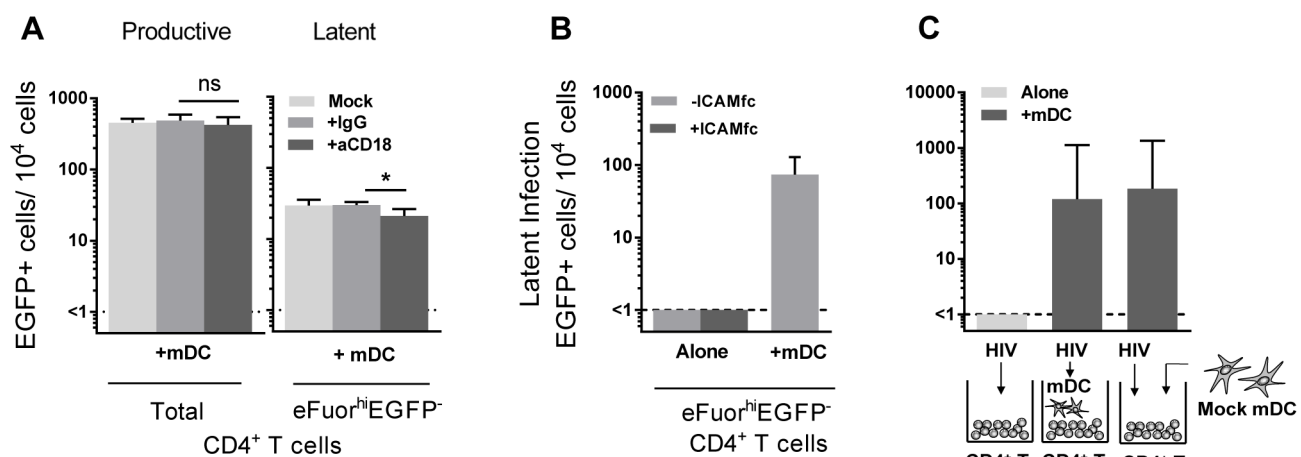
Figure 5. DC-T cell interactions. (**A**) Productive and latent infection was quantified in eFluor670-labelled resting memory CD4+ T cells that were cultured either alone or with sorted mDC in the presence of media alone (light grey), anti-IgG (grey) or anti-CD18 (dark grey) prior to infection, n = 5. (**B**) Latent infection was determined in eFluor670-labelled resting CD4+ T cells that were cultured alone, with mDC or alternatively with soluble ICAM-1-fc and anti-IgG-fc, n = 2. (**C**) Latent infection was determined in sorted eFluor^{hi}EGFP$^-$ CD4+ T cells following stimulation with anti-CD3/CD28, that were cultured alone or with mDC that were added prior to infection or post-infection, n = 5. Columns represent the median of 5 experiments and error bars the interquartile range. *P<0.05; ns, not significant (Wilcoxon signed-rank test).
doi:10.1371/journal.ppat.1003799.g005
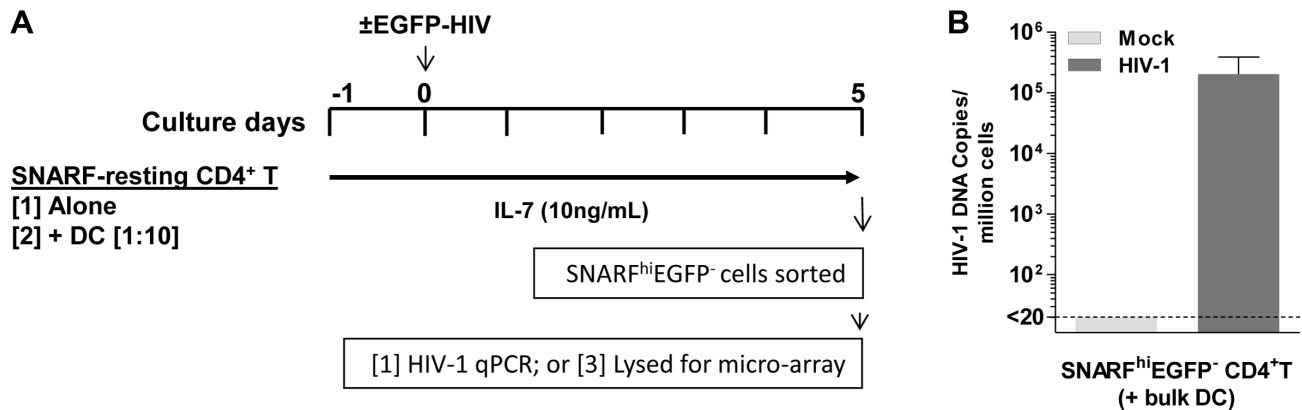
Figure 6. Acquisition of latently infected cells for microarray analysis. (**A**) SNARF-labelled resting CD4+ T cells were cultured with or without syngeneic blood DC. Following 24 hours of culture, cells were mock-infected with media alone (control) or infected with NL(AD8)-nef/EGFP (MOI of 5). Cells were cultured with IL-7 (10 ng/mL) throughout the culture period. On day 5 post-infection, SNARFhiEGFP−CD4+ T cells were sorted and lysed for either microarray or (**B**) qPCR, where total HIV DNA was quantified.
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metabolism) regulated by peroxisome proliferator-activated receptor gamma (PPARG; Fig. 7 C). Inhibition of the NF-κB transcriptional network, which plays a significant role in HIV transcription, led to the down-regulation of protein kinase C alpha (PRKCA). This observation confirms the quiescence of these cells and may also be a step towards the induction of HIV latency. Triggering of a transcriptional program leading to T cell quiescence was confirmed by the increased expression of Kruppel-like factor 6 (KLF6), a gene with anti-proliferative functions [25,26], as well as activating transcription factor 3 (ATF3) that has been recently been shown to negatively regulate activating protein 1 (AP-1)-mediated HIV transcription. Additionally, we observed a down-regulation of several molecules involved in DNA replication such as members of the minichromosome maintenance (MCM) complex (MCM4, MCM5, and MCM10) and the aurora kinase (Fig. 7 C). Pathways controlling pyrimidine and purine synthesis were also expressed at lower levels in cells exposed to virus and DC highlighting a reduced availability of nucleotides for cell division (Table S1). The down-regulation of NF-κB resulted in the decreased expression of several molecules that play a critical role in T cell survival, including CD27 (TNFRSF7), baculoviral IAP repeat containing 5 (BIRC5/ survivin) and tumor necrosis factor receptor superfamily, member 6b (TNFRSF6B/DCR3), a decoy receptor that inhibits Fas ligand and LIGHT-mediated signalling [27]. In order to confirm the differential expression of genes in these different populations of cells, we used a highly quantitative PCR approach and showed a strong correlation between gene expression data measured by either gene array or PCR (Fig. 7 D and Table S2).

Taken together, results of transcriptional profiling highlighted the impact of two major transcriptional nodes in the inhibition of viral replication and the induction of latency. The up-regulation of Type I Interferons and their downstream target genes could trigger several genes endowed with antiviral activities and would also impact cell proliferation, survival and metabolic processes. Concomitantly, the down-regulation of NF-κB will lead to T cell quiescence and decreased levels of activation, both of which are required for HIV replication.

Discussion

The study of latently infected resting CD4+ T cells *ex vivo* from HIV-infected patients on ART is greatly limited by the low frequency of latently infected cells and the lack of a distinctive surface marker to distinguish latently infected from uninfected cells. Here we demonstrate that latency can be efficiently established via direct infection of non-proliferating CD4+ T cells in the presence of DC. Using primary blood DC and resting CD4+ T cells we have demonstrated that: [1] co-culture of resting memory CD4+ T cells with DC can establish latent infection; [2] mDC but not pDC mediate this effect; [3] close cell-cell proximity is required between DC and T cells; and [4] multiple cell cycle genes were altered in non-proliferating CD4+ T cells, containing latently infected cells. These novel findings provide a potential pathway for the establishment and maintenance of latent infection in resting CD4+ T cells that recapitulates the likely events within lymphoid tissues in HIV-infected patients *in vivo*.

Previous studies have explored the ability of DC to enhance productive HIV infection within DC-CD4+ T cell co-cultures [28–31]; however, we are the first to present data demonstrating the ability of specific subpopulations of DC to induce latency in resting CD4+ T cells in these co-cultures. Using this model we clearly demonstrated that following co-culture of mDC with resting memory CD4+ T cells, post-integration latency was established. This was demonstrated by inducible virus (established both in the presence and absence of indinavir) and detectable integrated HIV DNA in T cells cultured with mDC but not those cultured alone following infection with an R5 EGFP reporter virus. While integrated R5 HIV DNA was only detected following co-culture with mDC in our model of latency, it was similar to that previously reported for resting CD4+ T cells infected in isolation with a wild type X4 NL4.3 virus [32]. Resting CD4+ T cells express very low levels of CCR5 in contrast to expressing very high levels of CXCR4. Additionally, we saw significantly higher levels of latency (~100 fold) in our T cells cultured alone when we used an X4 EGFP reporter virus compared to an R5 EGFP virus.

Unlike memory CD4+ T cells, we were unable to detect latency in naïve CD4+ T cells following mDC co-culture. It is possible that the differential establishment of latency in resting naïve and memory T cells was due to differences in their cortical actin density and actin dynamics as previously suggested by others [33]. While mDC induced T cell latency in this model, pDC did not. Interestingly, pDC played an active inhibitory role in establishing latency, when co-cultures were performed with equal numbers of pDC and mDC (Fig. 4 C). One potential explanation for the difference between co-cultures of bulk DC
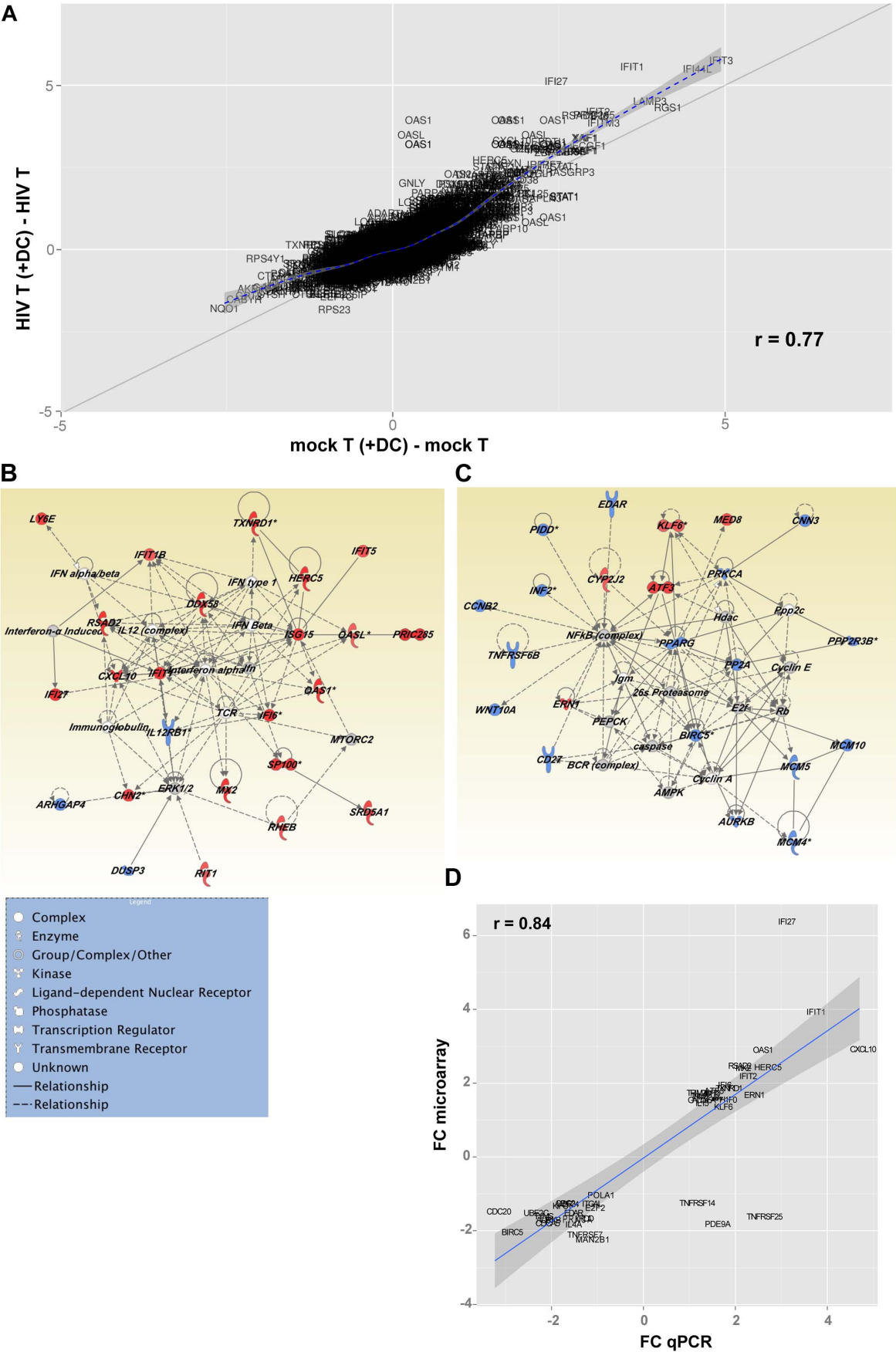
Figure 7. Changes in gene expression in DC-induced latently infected CD4$^+$ T cells. (**A**) Fold change scatterplot comparing gene expression between HIV T (+DC) (CD4$^+$ T cells cultured with DC and HIV), and Mock T (+DC) (CD4$^+$ T cells cultured with only DC) relative to their controls, HIV T (CD4$^+$ T cells cultured with HIV) and Mock T (CD4$^+$ T cells cultured in media alone) respectively. The solid line indicates absolute 2-fold change. (**B** and **C**) Top two gene interaction networks as ranked by Ingenuity Pathway Analysis. The networks were built from the list of differentially expressed genes induced by HIV T (+DC), relative to Mock T (+DC) after subtracting HIV T and Mock T from each group respectively. Genes highlighted in red were up-regulated and those in blue were down-regulated. The different node shapes indicate genes in different functional categories according to the legend. The interactions between the different nodes are shown as solid (direct interaction) or dashed (indirect interaction) lines (edges). (**D**) Fold change in gene expression values for selected genes from the Illumina BeadArrays plotted against Real-Time PCR (qPCR) deltaCt values for each target gene. PCR targets were mapped to BeadArray probes by matching the official gene symbols.
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and T cells (where latency was established) and DC-T cell co-cultures containing purified equal numbers of pDC and mDC (where latency was inhibited) could potentially be that the number of pDC present in the bulk DC (roughly 1 pDC to 3 mDC) was too low to inhibit latency. How pDC actively suppress the establishment of latency is unknown, but it does not appear to be mediated by IFN-alpha.

Establishment of mDC-induced latency was not dependent on DC-T cell transfer of HIV, as latency was still detected when T cells were infected in isolation and uninfected mDC added only after virus had been washed off. Nor was it dependent on the amount of virus replication, because while only mDC were able to induce latent infection, similar levels of productive infection were observed in both the pDC and mDC co-cultured CD4$^+$ T cells (Fig. 2 A). Furthermore, we found that addition of SEB to the culture model enhanced productive infection but did not increase latent infection (Fig. 2 G). Together, these data provide evidence that the establishment of latency in the non-proliferating CD4$^+$ T cells when co-cultured with mDC was not simply due to higher viral exposure in these cultures.

These results differ from a previous study that also looked at direct infection of resting CD4$^+$ T cells, which concluded that DC had no effect on the integration levels of R5 or X4 virus in either naïve or memory CD4$^+$ T cells [34]. However, in this study, although primary DC were used (defined as BDCA-1$^+$ and BDCA-4$^+$ cells), total DC were present at a frequency of only 0.89% and therefore the frequency of mDC may have been too low to demonstrate an effect of mDC on the infection of resting CD4$^+$ T cells. Additionally, contrary to our data, a recent study has reported the ability of monocyte derived DC (MDDC) to activate latent infection in T cells [35]. A key difference was that latency in this study was unusually established in proliferating CD4$^+$ T cells and not non-proliferating T cells as in our study. Furthermore, MDDC, as opposed to primary DC, were utilised in this study. MDDC have multiple significant functional and lineage differences to primary DC as we have recently demonstrated using detailed sorting and gene expression analyses [36].

In this study we utilised total blood CD11c$^+$ mDC, which consist of at least three different subsets, a major SLAN (6-sulfo LacNAc$^+$), an intermediate CD1c$^+$ (BDCA-1) and a minor CD141$^+$ (BDCA-3) population, each with different functional properties [37–40]. However, it is currently unclear whether one or more of these subsets is responsible for inducing latency in resting CD4$^+$ T cells.

We have previously demonstrated that multiple chemokines, including CCL19, CXCL9 and CXCL10, can condition resting CD4$^+$ T cells allowing for the establishment of HIV latency [14,17]. However, blocking CCL19 and CXCR3, the receptor for CXCL9, 10 and 11, had a minimal impact on DC-induced latency (Fig. 4 D). While it is possible that there may be involvement of chemokines other than those inhibited, given that latency was not detected in resting CD4$^+$ T cells infected in the presence of infected mDC-T cell culture supernatants, this is unlikely (Fig. 4 F). Rather, our data supports an essential role for direct DC-T cell

interactions or DC-T cell signalling as mDC-induced latency was prevented when the mDC were cultured in transwells above the resting CD4$^+$ T cells (Fig. 4 E). Unlike our previous work that was performed in the absence of productive infection [14], latency following DC-T cell co-culture was established in the presence of productive infection, which may more accurately mimic the establishment of latency in acute infection *in vivo*. Therefore, in the presence of productive infection it is possible that there are alternative pathways that lead to the establishment and maintenance of latency in resting CD4$^+$ T cells.

Interactions between ICAM-1, found on DC, and LFA-1, found on T cells, strengthen DC-T cell adhesion and play a key role in the formation of the immunological synapse [41]. We have shown that clustering, facilitated by ICAM-1-LFA-1 interactions, contributed to DC-induced T cell latency, as latency was significantly reduced, but not eliminated, when blocking antibodies to CD18/LFA-1 were added to the DC-T cell co-cultures (Fig. 5 A). However, interactions between ICAM-1 and LFA-1 alone were not sufficient to induce T cell latency in the absence of mDC (Fig. 5 B), therefore, the reduction in latency observed in the presence of anti-CD18 was most likely due to the reduction in DC-T cell clustering rather than specific LFA-ICAM signalling events. As there are numerous other molecules involved in DC-T cell clustering, such as LFA-3 and CD2, additional signalling pathways should be explored as potential mediators of DC-induced HIV latency. Interestingly, a recent paper has demonstrated enrichment of latency in CD2 expressing T cells from HIV-infected patients on ART [42].

Transcriptional profiling experiments served to highlight changes in cellular gene expression in resting non-proliferating CD4$^+$ T cells that contained latently infected CD4$^+$ T cells. We showed significant differences in gene expression between resting CD4$^+$ T cells from HIV and mock infected DC-T cell cultures. However, it is important to note that, while all cells within our "latent" cell population were exposed to virus, only a proportion were actually infected (median of 3%). It is possible that some of the observed differences in gene expression may be due to uninfected cells that were exposed to HIV but not infected. This may include the genes downstream of type I interferons as our *in vitro* experiments have shown that pDC, the major producers of type I interferons, were not involved in the induction of T cell latency. Therefore, it is possible that type I interferons are necessary but alone are not sufficient to induce HIV latency. We have demonstrated significant differential expression of genes involved in cell cycle, in particular those associated with cell cycle arrest (Fig. 7 C). During DC-T cell interactions in the presence of HIV, differential expression of co-stimulatory and negative regulatory factors determines the fate of the interacting CD4$^+$ T cell [43]. These interactions can result in active suppression of T cell cycle and as a result may inhibit post-integration steps in viral replication and promote the establishment of latency. Indeed, in HIV-infected patients on ART, HIV DNA is found at higher frequencies in CD4$^+$ T cells expressing the negative regulator PD-1 [44].

Latency has also been shown to be triggered by the absence of certain transcriptional machinery in resting CD4$^+$ T cells, such as NF-κB [45] and nuclear factor of activated T (NFAT) [11,46]. In DC-induced latently infected CD4$^+$ T cells we observed the suppression of multiple genes associated with the activation of NF-κB (Table S1), including protein kinase C alpha, PRKCA, which also plays a role in the activation of NFAT [47,48]. Therefore, it is possible that the global suppression of genes associated with the activation of NF-κB and/or NFAT may also contribute to the maintenance of latency in DC- T cell co-cultures by preventing progression to productive infection in cells that contain integrated HIV. However, while this data provides insights into genes that may potentially be important for both the establishment and maintenance of latency, it will be important to conduct gene knockdown experiments within our model in order to determine the specific role of individual genes in establishing and maintaining mDC-induced T cell latency.

In summary, this study has demonstrated a novel pathway for the establishment of latency in resting memory CD4$^+$ T cells that was dependent on close proximity to mDC. Efficient infection of resting CD4$^+$ T cells in close contact with mDC and HIV could explain the rapid early establishment of the latent HIV reservoir. Additionally, if infectious virus persists in tissues such as lymph node in patients on ART, mDC may facilitate ongoing infection of resting T cells leading to replenishment of the reservoir.

Materials and Methods

Isolation of cellular subsets

PBMC were isolated from buffy coats obtained from the Australian Red Cross Blood Service (Melbourne, Australia). Resting CD4$^+$ T cells were negatively selected using magnetic cell sorting and a cocktail of antibodies to CD8, CD11b, CD16, HLA-DR, CD19 and CD69, as previously described [17,49]. Sorted cells were routinely negative for CD69, CD25 and HLA-DR (Fig. S1 A). In some experiments bulk resting CD4$^+$ T cells were further sorted into CD45RA$^+$ naïve and CD45RA$^-$ memory CD4$^+$ T cells using phycoerythrin (PE)-labelled antibody to CD45RA and a FACSAria (BD Biosciences). DC were isolated from blood as previously described [50]. Briefly, DC were enriched using magnetic bead depletion and antibodies to CD3, CD11b and CD19. Enriched cells were then sorted using a FACSAria (BD Biosciences) to obtain a bulk cocktail$^-$ HLA-DR$^+$ DC population, HLA-DR$^+$CD11c$^+$ mDC or HLA-DR$^+$CD123$^+$ pDC. The purity of sorted cells was always >98% (Fig. S1 B).

Plasmids, virus production and infection

In all experiments except where noted we used an NL4-3 virus with EGFP inserted into the *nef* open reading frame at amino acid position 75 at the aKpnI (Acc651) site with a CCR5-tropic (AD8) envelope (NL(AD8)-nef/EGFP), alternatively we used this virus with a CXCR4-tropic (NL4-3) envelope (NL4-3-nef/EGFP; both kindly provided by Damian Purcell, University of Melbourne, Melbourne, Australia). In one set of experiments we used a Nef-competent EGFP reporter virus, kindly provided by Yasuko Tsunetsugu-Yokota (National Institute of Infectious Diseases, Tokyo, Japan) [51]. HIV stocks were generated by FuGene (Promega, Madison, WI) transfection of 293T cells as previously described [49,50]. Cells were infected at 37°C for 2 hours at an MOI of 0.5 or 5, as determined by limiting dilution using the Reed and Muench method [52], followed by a wash step to remove unbound virus.

In vitro HIV latency model

Resting CD4$^+$ T cells were labelled with proliferation dye, either SNARF (10 μM; Invitrogen) or eFluor®670 (5 μM; eBiosciences, San Diego, CA), according to the manufacturer's instructions. SNARF/eFluor670-labelled resting CD4$^+$ T cells were cultured in media supplemented with IL-2 (2 U/mL; Roche Diagnostics) for 24 hours, with or without syngeneic bulk DC or sorted DC subsets (DC: T cell ratio of 1:10), in the presence or absence of SEB (10 ng/mL; Sigma). Cells were then infected using an EGFP-reporter virus and cultured for a further 5 days (Fig. 1 A). In some experiments, cells were cultured with and without the protease inhibitor Indinavir (0.1 μM final) for 30 minutes at 37°C prior to infection. At day 5 post-infection, cells were analysed by flow cytometry for productive infection by detecting EGFP$^+$ cells. Subsequently, the non-proliferating (SNARF^{hi}/eFluor670^{hi}) CD4$^+$ T cells that were not productively infected (EGFP$^-$) were sorted using a FACSAria (BD Biosciences). In order to amplify any latent infection, the sorted CD4$^+$ T cells were stimulated with PHA (10 ug/mL)/IL-2 (10 U/mL) in the presence of PBMC and cultured for a further 5 days. The number of EGFP$^+$ cells following re-stimulation was used as a surrogate measure for the number of latently infected, non-proliferating CD4$^+$ T cells in the original cultures (Fig. 1 B). In some experiments, we also stimulated the sorted SNARF^{hi}/eFluor670^{hi}EGFP$^-$ CD4$^+$ T cells directly with plate bound anti-CD3 (Beckman Coulter; 5 μg/mL) and soluble anti-CD28 (BD; 5 μg/mL). Flat bottomed 96 well plates were coated with anti-CD3 (5 μg/mL) for 3 hours at 37°C. Unbound antibody was then removed and 1×10^5 SNARF^{hi}/eFluor670^{hi}EGFP$^-$ CD4$^+$ T cells were plated per well in 200 μL of media containing soluble anti-CD28 (5 μg/mL), IL-7 (50 ng/mL) and the integrase inhibitor, L8 (1 μM final). The number of EGFP$^+$ cells was determined following 72 hours of culture. As a control for the activity of the integrase inhibitor L8, we cultured SEB-stimulated PBMC with and without L8 for 30 minutes prior to infection, and productive infection was determined at day 5 post-infection (Fig. S2).

Phenotyping

For phenotypic analysis of the CD4$^+$ T cells before culture, we stained sorted resting CD4$^+$ T cells with CD69-FITC, CD25-PE and HLA-DR-perCP (BD Bioscience) on ice for 25 minutes. To determine whether co-culture with DC had altered the activation state of the resting CD4$^+$ T cells, in some experiments the sorted SNARF^{hi}EGFP$^-$ CD4$^+$ T cells were labelled with either CD69-FITC or HLA-DR-FITC (BD Biosciences). Intracellular staining was also performed on the sorted SNARF^{hi}EGFP$^-$ CD4$^+$ T cells to detect expression of the cell cycle marker Ki67. Cells were permeabilised with 500 μL of 1× FACS Permeabilising Buffer (BD Biosciences) in the dark at room temperature for 10 minutes, washed once with FACS wash and incubated with Ki67-FITC (5 μL/10^5 sorted CD4$^+$ T cells; Dako) for 45 minutes on ice. Following incubation, cells were washed twice with FACS wash and resuspended in 1% FACS fix. We performed analyses on a FACSCalibur (BD Biosciences) and results were analysed using Weasel software (Walter and Elisa Hall Institute, Melbourne, Australia).

Bead arrays

Cytokine bead arrays (eBioscience) were used according to the manufacturer's directions to determine the concentration of IL-1-beta, IL-6, IL-10, IL-12p70, TNF-alpha, CXCL9 and CXCL10 in the cell cultures.

Inhibition of soluble factors/surface receptors

In some experiments, nAbs to CD18 (10 µg/mL (prior to infection) and 20 µg/mL (post-infection); clone 7E4; Beckman Coulter), CCL19 (25 µg/mL), CXCR3 (20 µg/ml), IFN-alpha (5 µg/mL) or control IgG (R&D Systems, Minneapolis, MN); IL-6 or IgG1 (10 ug/mL; BioLegend, San Diego, CA); IL-10R or IgG2 (10 ug/mL; Biolegend) were used. In these experiments, both the DC and the resting CD4$^+$ T cells were pre-incubated with nAbs for 15 minutes on ice prior to culture. The nAbs were added again to the co-cultures following infection. Neutralising activity of anti-CCL19 (25 µg/mL) and anti-CXCR3 (20 µg/ml) was confirmed using a chemokine-induced migration assay. Resting CD4$^+$ T cells were added to the top chamber of a 3 µM pore transwell migration plate (Sigma) and either CCL19 (100 nM) or CXCL10 (300 nM) was added to the bottom chamber. In experiments using anti-CXCR3, cells were treated with nAb for 15 minutes at 37°C and washed off prior to chemokine treatment. In comparison, in experiments using anti-CCL19, nAb was added together with chemokine to the bottom chamber. Migrated cells in the bottom chamber were then counted in duplicate at 20 hours post addition of chemokine. Anti-IL-6 and anti-IL-10R were used at neutralising concentrations previously described [53,54]. As positive controls for these nAbs we demonstrated that 10 µg/mL of anti-IL-6 and anti-IL10R or 5 µg/mL of anti-IFN-alpha efficiently blocked IL-6 (100 ng/mL), IL-10 (50 ng/mL) or IFN-alpha (50 ng/mL) mediated STAT3 phosphorylation respectively (Fig. S2).

ICAM-fc stimulation

In order to determine the role of ICAM-1 and LFA-1 interactions in mDC-induced latency, resting CD4$^+$ T cells were cultured alone or with 10 ug/mL of ICAM-1fc together with 6 µg/mL of anti-IgG-fc (both from R&D Systems) for 24 hours prior to infection and maintained post-infection.

Transwell/transfer experiments

DC were cultured with resting CD4$^+$ T cells in the presence and absence of 0.4 µm cell culture inserts (BD, Franklin Lakes, NJ) with DC in the top chamber and resting CD4$^+$ T cells in the lower chamber. Following 24 hours of culture, both the DC and the CD4$^+$ T cells were infected as described above. In other experiments, we added supernatant from infected mDC-T cell co-cultures to uninfected resting CD4$^+$ T cells and then infected these cells. Media changes were performed daily using supernatant from infected mDC-T cell co-cultures. To determine the role of DC-T cell transfer, resting CD4$^+$ T cells were infected in the absence of mDC and uninfected mDC were added back to the T cells only after virus had been washed off.

Microarrays

SNARF^{hi}EGFP$^-$ CD4$^+$ T cells cultured with DC, in the presence (latently infected) or absence (mock-infected) of HIV, were sorted 5 days following infection with NL(AD8)-nef/EGFP. In these experiments, all culture media was supplemented with 10 ng/mL of IL-7 (Sigma) instead of IL-2, in order to increase cell survival of resting cells, and infections were performed at an MOI of 5 to ensure high numbers of latently infected cells.

Microarrays were performed as previously described [55]. Briefly, cells were lysed and RNA extracted (Qiagen, Valencia, CA), amplified (Ambion Applied Biosystems, Austin, TX) and hybridised to an Illumina Human-Ref8 (v3) BeadChip (Illumina, San Diego, CA). Beadchips were scanned using an Illumina BeadStation 500GX scanner and Illumina BeadStudio (version 3) software (Ilumina). Illumina probe data was exported from BeadStudio as raw data and screened for quality. Samples failing chip visual inspection and control examination were removed. Gene expression data was analysed using Bioconductor (http://bioconductor.org/) [56], an open-source software library for the analyses of genomic data based on R, a language and environment for statistical computing and graphics (www.r-project.org). The R software package was used for pre-processing, first to filter out genes with intensities below background in all samples, then to minimum-replace (a surrogate-replacement policy) values below background using the mean background value of the built-in Illumina probe controls as an alternative to background subtraction (which may introduce negative values) to reduce "over inflated" expression ratios determined in subsequent steps, and finally quantile-normalise the gene probes intensities. Genes were then filtered by intensity and by variance filters to allow a reduction in the number of tests and a corresponding increase in power of the differential gene expression analysis. The resulting matrix showing filtered genes as rows and samples as columns was log$_2$ transformed and used as input for linear modelling using Bioconductor's *limma* package, which estimates the fold-change between two predefined groups by fitting a linear model and using an empirical Bayes method to moderate standard errors of the estimated log-fold changes for expression values from each gene. *P* values from the resulting comparison were adjusted for multiple testing according to the method of Benjamini and Hochberg [57]. This method controls the false discovery rate, which was set to 0.05 in this analysis. Microarray data is available through the National Center for Biotechnology Information Gene Expression Omnibus (GEO), series accession number pending.

Functional microarray analysis and network generation

Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, www.ingenuity.com) was used to identify canonical signalling pathways and networks associated with the expression profiles of the non-proliferating CD4$^+$ T cells cultured with DC in the presence (HIV T (+DC)) or absence (Mock T (+DC)) of HIV. Differentially expressed Illumina Probe IDs were imported into the Ingenuity software and mapped to the Gene Symbol from Ingenuity knowledge database. The significance of the association between the dataset and the canonical pathway was measured in two ways: 1) A ratio of the number of genes from the dataset that map to the pathway divided by the total number of genes that map to the canonical pathway; 2) Over-representation Fisher's exact test was used to calculate a p-value determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone. The pathways were ranked by −log p-value. This score was used as the cut-off for identifying significant canonical pathways (p value<0.05). IPA's networks are built from direct or indirect physical, transcriptional, and enzymatic interactions between the mapped genes (focus genes). Two genes are considered to be connected if there is a path in the network between them. Ingenuity's approach is based on a multi-stage, heuristic algorithm that iteratively constructs networks that greedily optimize for both interconnectivity and number of Focus Genes under the constraint of a maximal network size. Each individual IPA network has a maximum of 35 focus genes and is assigned a significance score (based on *P* value) representing the likelihood that the focus genes within the network are found there by random chance.

Real-time PCR analysis

As previously described, full length viral DNA was quantified using primers specific for the HIV-1 long terminal repeat (LTR) and Gag [58], and integrated HIV-1 DNA was quantified using a

nested Alu-LTR real-time PCR [15,59,60]. Results were normalised for total input DNA as determined by real-time PCR for the CCR5 gene [61]. The correlation between gene arrays and real-time PCR was performed using a Spearman correlation test.

Microarray expression data were validated in two donors by reverse transcriptase real-time PCR (RT-qPCR), as previously described [62]. Briefly, SNARF^{hi}EGFP$^-$ CD4$^+$ T cells were lysed for RNA extraction and DNAse treatment (Qiagen, RNAeasy mini kit). cDNA was generated using CellsDirect qRT-PCR mix (Invitrogen). After reverse transcription all target genes were pre-amplified (18 cycles) using Taqman primers (Roche Probe library) specific for the transcripts of interest, which were also used for quantification. qPCR were performed on a Roche Light Cycler 348II and analysed according to the $\Delta\Delta$ct method.

Statistical analysis

In all experiments, Wilcoxon signed-rank or student paired t tests (for n<5) were performed for comparisons between populations using Graphpad Prism 5.0 software. P values of less than 0.05 were considered significant. Statistical analyses for microarray data were performed with program R, according to the method of Benjamini and Hochberg [57].

Supporting Information

Figure S1   Gating strategy and phenotype of isolated cell populations. (**A**) Resting CD4$^+$ T cells were isolated from PBMC from the blood of healthy donors using antibodies to CD8, CD11b, CD14, CD16, CD19, CD69 and HLA DR and magnetic bead depletion. Purity was always greater than 98% and the sorted CD4$^+$ T cells were negative for the activation markers CD69, CD25 and HLA-DR. (**B**) Syngeneic blood dendritic cells (DC) were enriched using magnetic bead depletion and antibodies to CD3, CD11b and CD19. Enriched cells were then sorted using a FACSAria into an HLA-DR$^+$ DC population or further sorted into HLA-DR$^+$CD11c$^+$ myeloid DC (mDC) or HLA-DR$^+$CD123$^+$ plasmacytoid DC (pDC). The purity of sorted cells was always >98%. (**C**) Phenotypic analysis of sorted pDC and mDC before culture.
(PDF)

Figure S2   Drug and nAb controls. (**A**) SEB-stimulated PBMC were cultured with or without 1 µM L8 for 30 minutes prior to infection with NL(AD8)-nef/EGFP. Productive infection (EGFP$^+$ cells) was determined at day 5 post-infection. (**B**) Neutralising activity of anti-CCL19 (25 µg/mL) was confirmed using a chemokine-induced migration assay. (**C**) Neutralising

activity of anti-IL-10R (10 µg/mL), anti-IL-6 (10 µg/ml) and anti-IFN-alpha (5 µg/mL) was confirmed by their ability to efficiently blocked IL-6 (100 ng/mL), IL-10 (50 ng/mL) or IFN-alpha (50 ng/mL) mediated STAT3 phosphorylation respectively.
(PDF)

Figure S3   Top differentially expressed genes. Supervised clustering heatmap of the top differentially expressed genes resulting from comparing HIV T (+DC) and Mock T (+DC) samples after subtracting HIV T (CD4$^+$ T cells cultured with HIV) and Mock T (CD4$^+$ T cells cultured in media alone) from each group respectively. Genes were selected as differentially expressed based on Fold Change ($\geq$1.5 fold up or down-regulation) and a p-value<0.05, following a moderate t test as implemented in the LIMMA package. The scale shows the level of gene expression where red and blue correspond to up and down-regulation respectively.
(PDF)

Table S1   Significant pathways. Significant pathways differentially expressed in HIV (+DC) relative to Mock T (+DC) after the subtraction of HIV T and Mock T respectively. Gene symbols are colour coded indicating either up-regulation (red) or down-regulation (blue). © 2000–2013 Ingenuity Systems, Inc. All rights reserved.
(PDF)

Table S2   RT-PCR validated genes. Fold change obtained from either gene-array or RT-PCR representing the change in expression level for each gene in HIV T (+DC) relative to Mock T (+DC) after the subtraction of HIV T and Mock T respectively.
(PDF)

Author Contributions

Conceived and designed the experiments: VAE PUC SRL RPS. Performed the experiments: VAE NK SS CdFP PCE FAP OY. Analyzed the data: VAE JPG AF EKH RPS SRL PUC. Contributed reagents/materials/analysis tools: SRL PUC RPS. Wrote the paper: VAE SRL RPS.

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8.3   Chapter 3. Publication

Kumar, NA., Cheong, K., Pereira, CdF., Anderson, J., Evans, VA., Lewin, SR., Cameron, PU. (2015) The role of Antigen Presenting Cells in the Induction of HIV-1 Latency in Resting CD4+ T-cells. Retrovirology 2014:12(76)

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The role of antigen presenting cells in the induction of HIV-1 latency in resting CD4+ T-cells

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Abstract

Background: Combination antiretroviral therapy (cART) is able to control HIV-1 viral replication, however long-lived latent infection in resting memory CD4+ T-cells persist. The mechanisms for establishment and maintenance of latent infection in resting memory CD4+ T-cells remain unclear. Previously we have shown that HIV-1 infection of resting CD4+ T-cells co-cultured with CD11c+ myeloid dendritic cells (mDC) produced a population of non-proliferating T-cells with latent infection. Here we asked whether different antigen presenting cells (APC), including subpopulations of DC and monocytes, were able to induce post-integration latent infection in resting CD4+ T-cells, and examined potential cell interactions that may be involved using RNA-seq.

Results: mDC (CD1c+), SLAN+ DC and CD14+ monocytes were most efficient in stimulating proliferation of CD4+ T-cells during syngeneic culture and in generating post-integration latent infection in non-proliferating CD4+ T-cells following HIV-1 infection of APC-T cell co-cultures. In comparison, plasmacytoid DC (pDC) and B-cells did not induce latent infection in APC-T-cell co-cultures. We compared the RNA expression profiles of APC subpopulations that could and could not induce latency in non-proliferating CD4+ T-cells. Gene expression analysis, comparing the CD1c+ mDC, SLAN+ DC and CD14+ monocyte subpopulations to pDC identified 53 upregulated genes that encode proteins expressed on the plasma membrane that could signal to CD4+ T-cells via cell–cell interactions (32 genes), immune checkpoints (IC) (5 genes), T-cell activation (9 genes), regulation of apoptosis (5 genes), antigen presentation (1 gene) and through unknown ligands (1 gene).

Conclusions: APC subpopulations from the myeloid lineage, specifically mDC subpopulations and CD14+ monocytes, were able to efficiently induce post-integration HIV-1 latency in non-proliferating CD4+ T-cells in vitro. Inhibition of key pathways involved in mDC-T-cell interactions and HIV-1 latency may provide novel targets to eliminate HIV-1 latency.

Keywords: Dendritic cells, Monocytes, B-cells, HIV Latency, Resting CD4+ T-cells, Antigen presenting cells, APC, Viral reservoir, Latency induction, Post-integration latency

Background

Despite the successes of cART in the reduction of morbidity and mortality world wide, treatment is required life long. HIV-1 persists in individuals on cART in resting CD4+ T-cells as latent infection [1–3]. Latency occurs when viral DNA is integrated within the host genome and remains transcriptionally silent. Latent infection of resting CD4+ T-cells therefore represents the major barrier to HIV-1.

It remains unclear how latency is established in resting CD4+ T-cells in vivo. Initial studies in vitro, showed that direct HIV-1 infection of resting CD4+ T-cells isolated from peripheral blood was inefficient and integration rarely occurred due to incomplete reverse transcription,

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reduced nuclear import of the viral DNA and/or limited integration within the host genome [4–6]. However, in vitro latent infection can occur following the reversion of a HIV-1 infected, activated CD4$^+$ T-cell to a resting state [7–10]. Alternatively, latent infection can also occur following the direct infection of a resting CD4$^+$ T-cell exposed to high viral titers and spinoculation [11, 12], chemokines [13] or co-culture with other cell types [14, 15].

As professional APCs, DC interact with HIV-1 during initial infection at vaginal and rectal mucosa sites and in blood. Langerhan cells (LC) and dermal (D)DC at mucosa and, bone marrow derived classical or myeloid (m)DC and plasmacytoid (p)DC in blood are able to interact with T-cells, but their role in the establishment and maintenance of HIV-1 latency remain unclear [16–18]. Blood derived mDC subpopulations differ from tissue DC. CD141$^+$ and CD1c$^+$ mDC are both found as resident cells in tissue (lymph node (LN), spleen, lungs), skin and, as more mature cells, circulating through the lymphatics to the LN [19–22]. SLAN$^+$ DC represent a subpopulation of monocytic cells with increased potential to secrete pro-inflammatory cytokines and develop a DC phenotype, however precise residence remains unknown [23, 24]. CD14$^+$ monocytes represent DC and macrophage precursors in blood [Reviewed in 25], and were also tested for their ability to establish latent infection in resting CD4$^+$ T-cells.

We have previously developed an in vitro co-culture model demonstrating that CD11c$^+$ myeloid dendritic cells (mDC) induce post-integration latency in non-proliferating memory CD4$^+$ T-cells. Here we demonstrate that in addition to the mDC subsets (CD1c$^+$, SLAN$^+$ and CD141$^+$), CD14$^+$ monocytes were also able to induce post-integration HIV-1 latency in non-proliferating CD4$^+$ T-cells. In comparison, T-cells co-cultured with pDC and B-cells were inefficient in the induction of latency. Using RNA-seq and Illumina gene expression microarrays, we also identified potential mediators of latent infection expressed by APC that could induce latency in the non-proliferating CD4$^+$ T-cells during APC-T cell interactions.

Results

Monocytes are able to induce latency in resting CD4$^+$ T-cells

We have previously reported that mDC, but not pDC, are able to efficiently induce post-integration latent infection in resting CD4$^+$ T-cells using an in vitro DC-T-cell co-culture model [14]. However, mDC and their subpopulations represent only a small proportion of peripheral blood mononuclear cells (PBMC) compared to monocytes, which represent a precursor to some DC

and macrophage subpopulations. Therefore we compared monocytes and mDC isolated from healthy donors for their ability to induce latent infection in resting CD4$^+$ T-cells (Fig. 1). eFluor670 labeled resting CD4$^+$ T-cells were cultured alone, with CD11c$^+$ mDC or bulk monocytes and infected with an R5 using virus that expresses enhanced green fluorescent protein (EGFP). Similar to mDC, monocytes were able to induce productive infection in CD4$^+$ T-cells, as measured by total EGFP expression at day 5 post-infection (Fig. 1b). At day 5 post-infection non-proliferating (eFluor670^{hi}EGFP$^-$) CD4$^+$ T-cells were sorted and cultured with phytohemagglutinin (PHA)-stimulated feeder peripheral blood mononuclear cells (PBMC), where the number of EGFP$^+$ cells was quantified by flow cytometry as a surrogate marker of inducible latent infection. CD14$^+$ monocytes were also able to significantly increase the induction of latent infection in non-proliferating cells (p > 0.05; Fig. 1c).

Isolation of functional APC

Given that we were able to show induction of latency in non-proliferating CD4$^+$ T-cells following co-culture with both bulk monocytes and mDC, we next compared the latency inducing potential of the different monocyte and mDC subpopulations. Monocytes were sorted into CD14$^+$ and CD14^{lo}CD16$^+$ (CD16$^+$) cells and mDC were sorted into CD1c$^+$, CD141$^+$ and SLAN$^+$ DC, B-cells and pDC were also isolated by sorting (Fig. 2a). The final purity for all sorted APC subpopulations was >90 %, as determined post-sort by expression of specific known surface markers for the various subpopulations [26–30]. The APC subpopulations were examined using brightfield microscopy after culture (Fig. 2b, c). The mDC and monocyte subpopulations were characterized with the formation of both long and short dendritic processes (Fig. 2b, c) Comparatively, pDC and B-cells had few processes or ruffles (Fig. 2b, c; [28, 29, 31–33]).

APC function was tested in a syngeneic mixed leukocyte reaction (MLR) using the proliferation dye eFluor670 to measure proliferation of resting CD4$^+$ T-cells. In the absence of mitogen stimulation, the relative potency of the various APC to induce T-cell proliferation at a ratio of 1 APC:10 CD4$^+$ T-cells is shown (Fig. 3a). CD1c$^+$ DC were the most potent at activating resting CD4$^+$ T-cells, while pDC and CD141$^+$ DC were least potent. The use of superantigen staphylococcal enterotoxin B (SEB) at low dose in the MLR had a modest effect on enhancing the capacity of APC to induce T-cell proliferation. T-cell proliferation following co-culture and SEB treatment was highest with CD1c$^+$ DC and lowest with B-cells (Fig. 3b), confirming previous observations by others [26]. B-cells had a similar stimulatory capacity with and without superantigen (1.0 and 1.3 % proliferated CD4$^+$ T-cells).

Fig. 1 Monocyte induced latency in non-proliferating CD4+ T-cells. **a** Resting CD4+ T-cells were labeled with eFluor670 and cultured alone, with bulk myeloid (m)DC or bulk monocytes (mono) at a ratio of 10:1. Following 24 h of culture, APC-T-cell co-cultures were infected with NL(AD8)Δnef-EGFP. **b** At day 5 post-infection productive infection was determined by quantification of EGFP in total cells and, non-proliferating, non-productively infected (eFluor670hiEGFP−) cells were sorted using flow cytometry and reactivated to determine frequency of latent infection. **c** Sorted non-proliferating (eFluor670hiEGFP−) CD4+ T-cells were stimulated with phytohaemagglutinin (PHA) stimulated feeder PBMC for 5 days and EGFP quantified as a measure of HIV-1 latency. *Columns* represent the median of the log transformed values, *open circles* represent individual donors. *p ≤ 0.05, **p ≤ 0.005 as determined by paired students t-test

Finally, there was a dose response of CD4+ T-cell proliferation with decreasing APC:T-cell ratio (1:10–1000). Together, these data confirm that all the APC subpopulations isolated remained functional in the co-cultures used for infection.

T-cell stimulation by APC subpopulations in HIV-1 infected co-cultures

We then measured T-cell proliferation following co-culture with different APC subpopulations at 3 days following HIV-1 infection. The pattern of APC potency in induction of CD4+ T-cell proliferation in the presence of HIV-1 was similar to uninfected, sygeneic, co-cultures (Fig. 3c), where proliferation of CD4+ T-cells was

highest with CD1c+ DC and lowest with pDC. These experiments demonstrate that HIV-1 infection did not independently alter APC or T-cell function with respect to T-cell proliferation.

Several APC subpopulations enhanced productive infection of resting CD4+ T-cells

We tested the ability of APC subpopulations to induce both productive and latent infection in resting CD4+ T-cells when cultured alone or co-cultured with one of the seven sorted APC subpopulations (Fig. 2a). Five days following infection, EGFP expression was quantified by flow cytometry as a measure of productive infection (Fig. 4a). We observed a significant increase in productive infection following HIV-1 infection in all APC co-cultured with T-cells compared to resting CD4+ T-cells cultured alone (p = 0.03 for all APC co-cultures; Fig. 4b).

Different APC subpopulations can effectively induce latent infection in non-proliferating CD4+ T-cells

Five days following infection, non-proliferating (eFluor670hiEGFP−) CD4+ T-cells were sorted from the APC-T-cell co-cultures to quantify latent infection (Fig. 4a). The sorted CD4+ T-cells were directly stimulated with anti-CD3/CD28 and IL-7 (Fig. 4c) in the presence and absence of an integrase inhibitor, L8. EGFP was quantified by flow cytometry as a measure of inducible latent infection. Total latent infection (no L8) was significantly increased in non-proliferating CD4+ T-cells co-cultured with all mDC subpopulations, CD14+ monocytes and B-cells, when compared to CD4+ T-cells cultured alone (p = 0.03; Fig. 4d). In comparison, total latent infection following co-culture with CD14loCD16+ monocytes, that were depleted of SLAN+ DC, was highly variable and not significantly different to T-cells cultured alone. As previously shown, latent infection was not found in T-cells co-cultured with pDC (p = 0.03 compared to mDC co-cultures; Fig. 4d).

We also quantified post-integration latent infection by stimulating T-cells with anti CD3/CD28 and IL-7 stimulation in the presence of L8. (Fig. 4c). The integrase inhibitor, L8, prevented any progression of pre-integration complexes to integration and inhibited secondary rounds of infection. Following infection of CD4+ T-cells co-cultured with each APC subpopulation, post-integration latency followed a similar pattern to that observed for total latency, but at a lower frequency (Fig. 4d, e). Post-integration latency was significantly increased in CD4+ T-cells following co-culture with mDC subpopulations CD1c+ and SLAN+, and CD14+ monocytes (p = 0.03, 0.02 and 0.01, respectively; Fig. 4e). Post-integration latency induced by CD141+ DC was elevated, similar to what was induced by other mDC subsets, but this did

Fig. 2 Isolation of antigen presenting cells. **a** Peripheral blood mononuclear cells (PBMC) were elutriated into three fractions: small lymphocytes, large lymphocytes and a monocyte/DC fraction. Resting CD4$^+$ T-cells were isolated from the small lymphocyte fraction by negative selection using magnetic beads. Bulk B-cells were isolated from a mixture of the small and large lymphocyte fractions using positive magnetic bead selection for CD19. Bulk DC subpopulations were positively selected on the basis of expression of CD1c, CD141, SLAN and CD123 from the DC/monocyte fraction using magnetic bead selection. The positive "DC enriched" (DC) population was then sorted by flow cytometry into the four DC populations (purity >95 %). The negative "DC depleted" (mono) fraction was labeled with the monocyte markers CD14 and CD16, positively selected using magnetic beads and further sorted by flow cytometry into CD14$^+$ and CD14^{lo}CD16^{hi} subsets (purity >90 %). **b, c** Representative *dot plots* and brightfield images show the purity and morphology of the sorted APC subpopulations, respectively. The *scale bars* represent 20 μm, images were annotated using ImageJ software

not reach statistical significance. In comparison, HIV-1 infection of T-cells co-cultured with SLAN DC depleted CD14$^-$ CD16$^+$ monocytes, B-cells and pDC was similar to infection of CD4$^+$ T cells alone. Together these data show that only CD1c$^+$mDC, SLAN$^+$ DC and CD14$^+$ monocytes were able to establish post-integration latent infection in non-proliferating CD4$^+$ T-cells, while B-cells and CD141$^+$ mDC were able to establish pre-integration latent infection. CD14^{lo}CD16^{hi} SLAN$^-$ monocytes, like pDC, were unable to establish either pre or post-integration latency.

Next, we looked for a correlation between productive infection and post-integration latency following infection of T-cells co-cultured with different APC (Fig. 4f). Overall, we found a weak correlation between productive and latent infection (Spearman's r = 0.12; p = 0.02), which supports our previous findings [14]. However, the induction of productive infection does not inevitably lead to post-integration latency in resting CD4$^+$ T-cells, as observed following co-culture with CD14^{lo}CD16^{hi} monocytes, B-cells and pDC. We conclude that cells able to establish both productive and latent infection likely share
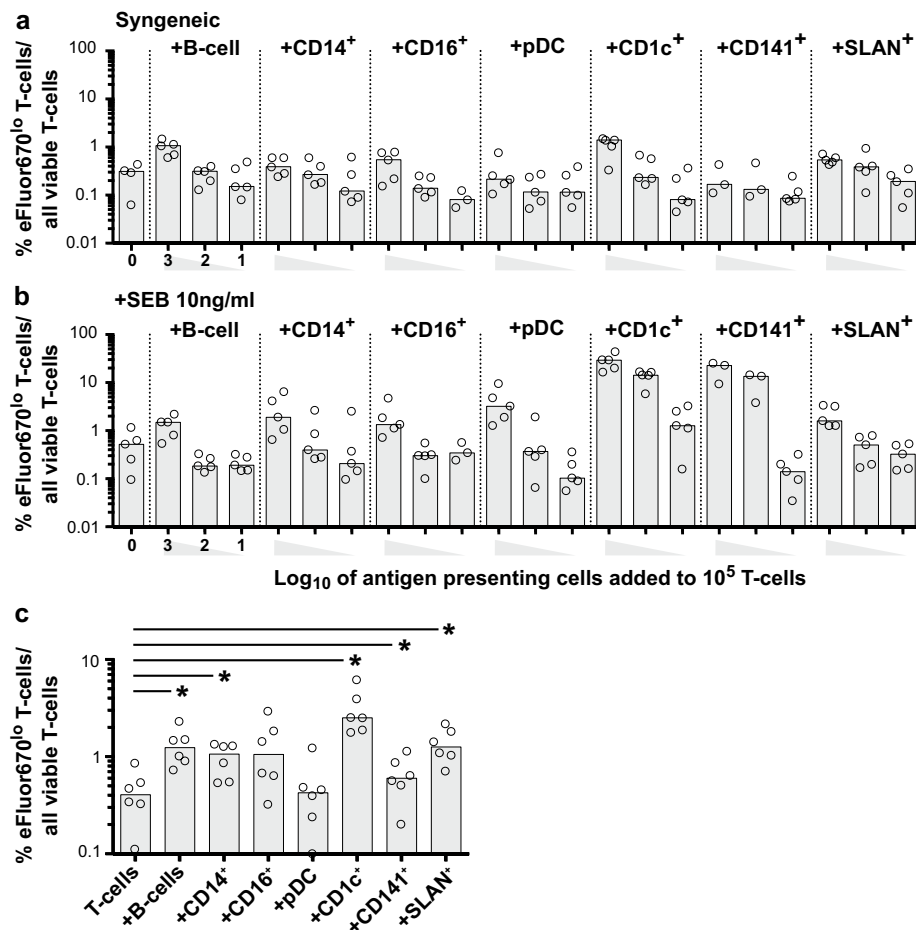
Fig. 3 Resting CD4^{+} T-cell stimulation following co-culture with antigen presenting cells. Resting CD4^{+} T-cells were labeled with the proliferation dye eFluor670 and co-cultured with one of seven antigen presenting cell (APC) subpopulations, including B-cells; monocyte subpopulations-CD14^{hi} and CD14^{lo}CD16^{hi}; DC subpopulations- plasmacytoid (p)DC and myeloid (m)DC subpopulations—CD1c^{+}, CD141^{+} and SLAN^{+}, at a ratio of log 1 (10:1), 2 (100:1) or 3 (1000:1) T-cells : APC. T-cell stimulation was measured by quantification of the percentage of eFluor670^{lo} CD4^{+} T-cells from APC-T-cell co-cultures following 5 days of culture in the **a** absence (syngeneic) or **b** presence of staphylococcal enterotoxin B (SEB). **c** eFluor670 labeled, resting CD4^{+} T-cells were cultured alone, or with APC subpopulations at a ratio of 10:1 and infected with NL(AD8)Δnef-EGFP. At day 3 post-infection, CD4^{+} eFluor670^{lo} T-cells were measured. Columns represent the median, open circles represent individual donors, *p ≤ 0.05, as determined by Wilcoxon matched pairs signed rank test

common functional characteristics, which favour the establishment and maintenance of latent infection.

Differential gene expression of cell-surface expressed molecules on APC

We next used RNA-seq to compare gene expression for genes involved in T-cell interactions with APC subpopulations that induce latency (CD1c^{+} DC, SLAN^{+} DC and CD14^{+} monocytes) compared to APC that could not (pDC). Due to difficulties isolating APC from T-cell co-cultures and HIV-1 infection, gene expression analysis was performed on freshly isolated APC subpopulations [34–36]. Component analysis showed clustering of the

SLAN DC and CD14 monocytes and separate clusters of pDC and mDC (Additional file 1: Figure S1). Given that we have previously shown that cell contact is important in mDC-induced latency [14], we specifically selected genes encoding proteins that mediate mDC-T-cell interactions, including those in cell membrane compartments at the cell surface, and in intracellular vesicles such as endosomes and compartments giving rise to exosomes.

In APC subpopulations that induced post-integration HIV-1 latency compared to APC that didn't induce latency, we found 754 differentially upregulated genes (fold change ≥2, p-value <0.01; Fig. 5a). Analysis for expression in cellular compartment (GeneCodis; http://
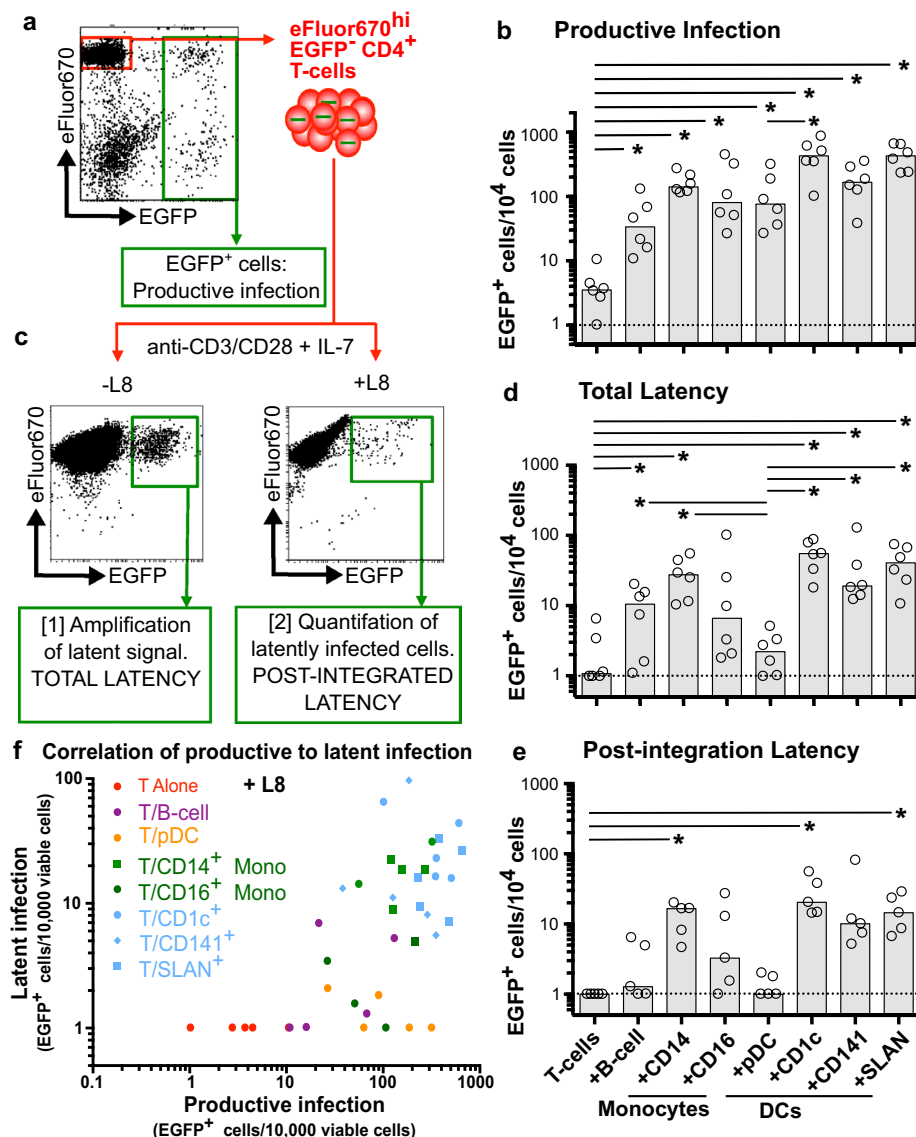
Fig. 4 Productive and latent infection in resting T-cells co-cultured with antigen presenting cell subsets. **a** Representative dot plot of CD4+ T-cells co-cultured with antigen presenting cell (APC) subpopulations day 5 post infection with NL(AD8)Δnef-EGFP. Non-proliferating (eFluor670hi), non-productively infected (EGFP−) T-cells were sorted 5 days following infection. **b** EGFP expression in the total cell cultures at day 5 post-infection was used as a measure of productive infection. **c** Total and post-integrated latency was stimulated from eFluor670hiEGFP− cells with anti-CD3/CD28 and IL-7 for 3 days and EGFP expression was quantified using flow cytometry. Representative *dot plots* show EGFP expression following stimulation of eFluor670hiEGFP− sorted CD4+ T-cells in the absence (-L8 plot; total latency) and presence (+L8 plot; post integration latency) of the integrase inhibitor L8. **d** The frequency of total and **e** post-integration latent infection in resting CD4+ T-cells following co-culture with APC subpopulations. **f** Correlation of the frequency of productive infection and post-integrated latency (+L8) in each APC-T-cell co-culture. Each condition is identified by a *different color* and/or *symbol*. For all other panels, columns represent the median and open circles represent results from individual donors. Significant differences between conditions was measured by Wilcoxon matched pairs signed rank test where *p ≤ 0.05

genecodis.cnb.csic.es), identified 285 known genes expressed in: membrane, plasma membrane, integral to membrane, integral to plasma membrane and cell junction ([37–39]; Fig. 5a). Of these, 53 protein-encoding genes that could establish cell contact with CD4+ T-cells and potentially induce T-cell signaling were selected

(Table 1, Additional file 2: Table S1). Functionally these genes included; cellular adhesion (32 genes), antigen presentation (1 gene), T-cell activation (9 genes), immune checkpoints (5 genes), regulation of apoptosis (5 gene), and an unknown protein (1 genes). We further analysed the role of each gene in HIV-1 infection of DC and

Fig. 5 Comparison of gene expression between latency inducing and non-inducing antigen presenting cell subpopulations using RNA-seq. **a** Gene expression profiles common to the latency inducing APC subpopulations (CD1c⁺ mDC, SLAN⁺ mDC and CD14⁺ monocytes) compared with non-latency inducing APC (pDC) were selected (fold change ≥2, p < 0.01). Using GeneCodis, these 754 genes were categorised according to cellular compartment expression. **b** Encoded proteins expressed on APC surface and membrane compartments were further analysed for ability to signal to T-cells and involvement in HIV-1 infection. Representative heat map with >twofold differential gene expression of genes able to signal to T-cells, which are differentially expressed between latency inducing (CD1c⁺ mDC, SLAN⁺ mDC and CD14⁺ monocytes) and non-inducing APC subpopulations (pDC)

CD4⁺ T-cells using a PubMed search for the interactions between DC and T-cells, and potential roles in the establishment of HIV-1 latency (Additional file 2: Table S1).

We performed the same comparisons between selected APC subpopulations using our previously published microarray data [40] and found 27 genes that could potentially induce T-cell signalling (Additional file 4: Figure S2; Additional file 5: Table S2; [40]). Five of these genes were common between microarray and RNAseq analyses, seven genes shared protein family and thirteen genes fell outside of significance (cut off of p < 0.01), often with inconsistent replicates (n = 3). The common genes included *C-type lectin domain family 7 member A* (*CLEC-7A*), *endoglin, intracellular adhesion molecule 3* (*ICAM-3*), *sialic acid-binding immunoglobulin-type lectins* (*SIGLEC*)-*10* and *CD1d*. CD1d is involved in lipid antigen presentation to T-cells, while the other 4 surface expressed proteins are involved in cellular adhesion [41]. The common protein families included the SIGLEC family, CLEC family, leukocyte associated immunoglobulin like receptor (LILRA) family, G-Protein coupled receptor (GCPR) family and the tumor necrosis factor (TNF) receptor superfamily.

Discussion

Latently infected cells are infrequent in HIV-1-infected patients on cART, and therefore robust in vitro models are needed to better understand the establishment and maintenance of latent infection. We have now shown that multiple APCs, in addition to mDC, are able to induce HIV-1 latency in non-proliferating CD4⁺ T cells [14]. Here, we have shown that the myeloid lineage cells capable of producing latent T-cell infection include subpopulations of blood derived mDC; CD1c⁺, SLAN⁺ DC and CD14⁺ monocytes, and confirmed that pDC are distinct in not generating latent infection. We have used RNA-seq analysis to define genes differentially expressed between APC subpopulations that could (CD1c⁺, SLAN⁺, CD14⁺ monocytes) and could not induce latent infection (pDC), and identified genes mediating cell adhesion, T-cell activation, immune checkpoints (IC) and regulation of apoptosis as important pathways differentially upregulated in the APC that are able to induce latent infection.

Our results show that multiple blood derived mDC subsets can induce latent infection in non-proliferating CD4⁺ T-cells, suggesting that this observation may extend to other DC and myeloid lineage cells, such as LC and dermal DC (DDC) though they may have different ontogeny [42–44]. It is likely that mDC and monocyte lineage cells in lymphoid tissue, skin, mucosal surfaces, gastrointestinal tract (GIT) and sites of inflammation could allow seeding of CD4⁺ T-cell viral reservoirs early following infection or during ART in tissue sites were cART penetration may not be optimal [45].

We observed a trend between the ability of the different APCs to induce latent infection and efficient T-cell proliferation. This suggests that there may be a common mechanism for the induction of T-cell proliferation and induction of latent infection, even in non-proliferating cells, perhaps through a bystander mechanism. Efficient

Table 1 Effects on HIV infection of genes differentially expressed by latency inducing and non-inducing antigen presenting cell subpopulations using RNA-seq

Gene name	Gene symbol	Function					
		Antigen presentation	Apoptosis regulation	Cell proximity presentation	Immune checkpoint blocker	T-cell activation	Unknown
Number of genes expressed in each category		1	5	32	5	9	1
CD1d molecule	*CD1d*	−*,**					
Lectin, galactoside-binding, soluble, 1	LGALS1		+				
Vasoactive intestinal peptide receptor 1	VIPR1		+*				
EF-hand domain family, member D2	EFHD2		−				
Tumor necrosis factor receptor superfamily, member 10a	TNFRSF10A		+				
Tumor necrosis factor receptor superfamily, member 10d, decoy with truncated death domain	TNFRSF10D		+				
Acid phosphatase, prostate	ACPP			+			
ADAM metal-lopeptidase domain 15	ADAM15			+			
Integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)	CD18			+*,***			
Carcinoembryonic antigen-related cell adhesion molecule 4	CEACAM4			+			
C-type lectin domain family 4, member G	CLEC4G			−*,***			
C-type lectin domain family 7, member A	*CLEC7A*			+*			
Cytotoxic and regulatory T cell molecule	CRTAM			−**			
Colony stimulating factor 3 receptor (granulocyte)	CSF3R			+*			
Ephrin-B1	EFNB1			−			
Endoglin	*END*			+*			
Endothelial cell adhesion molecule	ESAM			+			

Table 1 continued

Gene name	Gene symbol	Function					
		Antigen presentation	Apoptosis regulation	Cell proximity presentation	Immune checkpoint blocker	T-cell activation	Unknown
G protein-coupled receptor 133	GPR133			+			
Intercellular adhesion molecule 3	*ICAM3*			+[*]			
Leucine rich repeat containing 8 family, member C	LRRC8C			+			
Multiple EGF-like-domains 9	MEGF9			+			
Membrane protein, palmitoylated 7 (MAGUK p55 subfamily member 7)	MPP7			+			
Macrophage scavenger receptor 1	MSR1			+			
Osteoclast associated, immunoglobulin-like receptor	OSCAR			+			
Plexin domain containing 2	PLXDC2			+			
Syndecan 3	SDC3			+[*]			
CD33 molecule	CD33			−	−		
Sphingosine-1-phosphate lyase 1	SGPL1			+	+		
Sialic acid binding Ig-like lectin 10	*SIGLEC10*			−[*]	−		
Sialic acid binding Ig-like lectin 7	SIGLEC7			+[**]	+		
Sialic acid binding Ig-like lectin 9	SIGLEC9			−	−		
Synaptojanin 2 binding protein	SYNJ2BP			+	+		
T-cell lymphoma invasion and metastasis 1	TIAM1			+[*]	+		
Transmembrane protein 2	TMEM2			+	+		
Tetraspanin 17	TSPAN17			−	−		
C-type lectin domain family 1, member A	CLEC1A			+		+	
Sialic acid binding Ig-like lectin 14	SIGLEC14			+		+	
CD101 molecule	CD101				−		
CD52 molecule	CD52		−		−		
Hepatitis A virus cellular receptor 2	HAVR2/Tim-3				−[**]		

Table 1 continued

Gene name	Gene symbol	Function					
		Antigen presentation	Apoptosis regulation	Cell proximity presentation	Immune checkpoint blocker	T-cell activation	Unknown
Leukocyte immunoglobulin-like receptor, subfamily A (with TM domain), member 6	LILRA6				−		
Poliovirus receptor	PVR				+[*]		
Neuropilin (NRP) and tolloid (TLL)-like 2	NETO2				+		
CD48 molecule	CD48					0[**]	
Interleukin 15 receptor, alpha	IL15RA					−[**]	
Leucine rich repeat containing 25	LRRC25					+	
NFAT activating protein with ITAM motif 1	NFAM1					+	
Secreted and transmembrane 1	SECTM1					+	
V-set and immunoglobulin domain containing 2	VSIG2					+	
CD300e molecule	CD300e					+	
CD83 molecule	CD83					0[**]	
CD86 molecule	CD86					0[**]	
Ecotropic viral integration site 2A	EVI2A						+

The functional category shown were determined by the description from the DAVID (http://david.abcc.ncifcrf.gov/) and GeneCards (http://genecards.org/) databases

+, increased latent infection; −, inhibition of virus expression, 0, undefined. Genes that were common to the RNA-seq and microarray generated gene lists are in italics.
* represent a role in HIV infection of either DC (*) or T-cell (**). Data in Table 1 is presented with additional detail and references in Additional file 3: Supplementary references

T-cell proliferation is favoured by the formation of an immunological synapse [46, 47] with cellular proximity [48], major-histocompatibility complex (MHC)-T-cell receptor (TcR) interaction and co-stimulation [49–52]. ICAM-1 interaction with leukocyte function-associated antigen (LFA)-1 can facilitate induction of latent infection in the DC-T-cell model [14], while in other models of in vitro latency CD2 expression, a molecule that binds to LFA-3, was increased on latently infected cells [53]. A large portion (60 %) of genes identified in the gene expression analysis mediates cellular proximity and cell adhesion. Taken together these data suggest that cell adhesion/contact is important in the induction of latency. However, identification of a single specific adhesion molecule critical for the induction of latent infection is likely limited by functional redundancy in mediators of APC-T-cells interactions.

Compared to the other DCs, the pDC were least efficient for T-cell proliferation and induced significantly less productive and latent infection compared to mDC. The differences between mDC and pDC in induction of productive infection [54, 55] and suppression of virus production has been observed previously [14]. We have also previously shown that pDC were unable to induce HIV-1 latency, and that there was a more substantial suppressive effect on the establishment of latency compared to productive infection. We and others have shown multiple differences between mDC and pDC that may reduce the ability of pDC to establish close interactions with T-cells [26, 40, 56, 57], which in combination with increased type-I IFN secretion from pDC may inhibit the capacity of pDC to establish latency in T-cells.

The ability of B-cells to induce latent infection in non-proliferating CD4+ T-cells was also tested in this study as

B-cells express MHC-II, circulate through LN, and have been reported to transfer HIV-1 infection to T-cells [58]. Induction of latency occurred only at low level and was only in the form of pre-integration latency suggesting that B-cells lack factors that facilitate efficient induction of post-integration latency.

Comparison of APC subpopulations that could and could not induce latent infection in non-proliferating CD4^{+} T-cells identified several functions that may be important in the establishment of latency, including cell adhesion, IC, T-cell co-stimulation, antigen presentation and regulation of apoptosis. The IC, programmed death receptor (PD)-1, is proposed to play a role in the establishment and/or maintenance of HIV-1 latency [59, 60]. Engagement of ICs, led to reduced T-cell activation by inhibition of signaling cascades, as well as physical inhibition of the formation of lipid rafts and cellular interactions [61]. In this study, we observed an up-regulation of the ICs; *CD101, T-cell immunoglobulin mucin-3* (*Tim-3, HAVR2*), *leukocyte immunoglobulin-like receptor member 6* (*LILR6*) and *CD52*, on latency inducing APC subpopulations when compared to pDC. IC expression may be important for the establishment of HIV-1 latency in this model, but further work is required to confirm this.

Additionally, we identified differential expression of the SIGLEC family of proteins between APC subpopulations that could and could not induce latent infection. We specifically found *SIGLEC 5, 7, 9, 10* and *14* to be upregulated on latency inducing APCs. From this family, SIGLEC 3, 5-11 have all been implicated in the inhibition of T-cell activation [62–64]. SIGLEC 5 has been shown to inhibit T-cell activation in chimpanzees, where blockade of SIGLEC 5 led to increased T-cell activation, and transfection of SIGLEC 5 into SIGLEC negative cells reduced T-cell activation [64–67]. SIGLEC 10 is hypothesized to have similar function in inhibition of T-cell activation [68, 69]. Together these data suggest that SIGLEC 5 or 10 binding to its ligand on the CD4^{+} T-cell may reduce T-cell activation, reduce productive infection and potentially promote latent infection. This is a novel association but further work will be required to explore any direct effects of SIGLEC proteins and the establishment of latency.

Conclusion

This study has established that multiple myeloid lineage APC subpopulations can facilitate latent infection in resting CD4^{+} T-cells. Particularly important is the observation that CD14^{+} monocytes can induce latent infection in resting CD4^{+} T-cells. The use of CD14^{+} monocytes will greatly enhance the utility of this model. In addition, through a comparative analysis of APC populations,

we have identified new pathways that may potentially be involved in the establishment and/or maintenance of HIV-1 latency. Inhibition of key pathways involved in mDC-T-cell interactions and HIV-1 latency may provide novel targets to eliminate HIV-1 latency.

Methods

Isolation and preparation of resting CD4^{+} T-cells and B-cells

PBMC were isolated by Ficoll-Paque density gradient centrifugation (GE Healthcare, Chalfont St. Giles, UK) from healthy buffy coats obtained from the Australian Red Cross. PBMC were further separated into three populations by counter-current elutriation using Beckman J-6M/E centrifuge equipped with a JE 5.0 rotor (Beckman Coulter, Pasedena, CA, USA; [70]). The three fractions were isolated at rates of 12 (small lymphocytes), 16 (large lymphocytes) and 20 (DC/Monocytes fractions) ml/min. Resting CD4^{+} T-cells, negative for the activation markers CD69 and HLA-DR, were sorted from the "small lymphocyte" fraction, as previously described [14], with a purity always >98 %. B-cells were isolated with a purity of ≥90 % from the "small and large lymphocyte" fractions using positive magnetic bead selection on an autoMACS (Miltenyi) using anti-CD19^{+} hybridoma (clone FMC63) and anti-IgG microbeads (Miltenyi, Bergisch Gladbach, Germany).

Isolation of DC and monocytes

The remaining elutriated fraction, containing the larger cells (20 ml/min), was used to isolate DC and monocytes. The large cell fraction was first stained with antibodies specific for the DC subsets, which included CD1c-APC (Miltenyi), CD141-VioBlue (Miltenyi), CD123-PE (BD BioSciences, Franklin Lakes, NJ, USA) and SLAN-FITC (Miltenyi), and labeled with anti-IgG beads (Miltenyi). DC were then isolated using an AutoMACS (Miltenyi) into positive and negative fractions. The positive fraction (DC enriched) was further sorted into four DC subsets: CD1c^{+} mDC, SLAN^{+} DC, CD141^{+} mDC and CD123^{+} pDC, using a FACSAria (BD BioSciences). The negative fraction (DC depleted/mono) was stained with anti-CD14-FITC and anti-CD16-PE (BD Biosciences) antibodies, labeled with IgG beads (Miltenyi) and a positive selection performed using an AutoMACS (Miltenyi) to obtain a bulk monocyte population. These cells were further sorted to obtain the CD14^{+}CD16^{−} (CD14^{+}) and CD16^{+}CD14^{lo} (CD16^{+}) monocyte subsets using a FACSAria. Cell populations with a purity ≥90 % were used, as determined by flow cytometry (LSR II or FACSAria; BD Bioscience). In the event of low yields of some APC subpopulation, the experiment was continued without that population. In these experiments the missing data was

omitted from the plots and therefore not every donor has data shown for all conditions tested.

Imaging antigen presenting cell subpopulations

After isolation, each antigen presenting cell (APC) subpopulation was cultured in RF10 media (RPMI 1640; Life Technologies, Carlsbad, CA, USA), supplemented with 10 % fetal bovine serum (FBS; Interpath, Heidelberg, Australia), Penicillin–Streptomycin-Glutamine (PSG; Life Technologies) for 1–2 h at 37 °C in glass-bottom imaging plates (μ-slide, ibidi, Martinsried, Germany). Ten representative images were captured on a CCD camera through a 10 × 0.3 NA lens on a Olympus IX51 microscope and annotated with ImageJ software.

Syngeneic mixed leukocyte reactions

Resting CD4$^+$ T-cells were labeled with eFluor670 and co-cultured with decreasing concentrations of each APC subpopulation; log 1 (10:1), 2 (100:1) and 3 (1000:1), in the absence (syngeneic) or presence of superantigen SEB (10 ng/mL; Sigma). At day 5, cells were harvested and labeled with antibody against CD3 (V450, BD Bioscience). Cells were analysed by flow for T-cells that proliferated and therefore expressed low levels of eFluor670.

Viral plasmids, virus preparation and infection

In all experiments, we used HIV-1 NL4.3 plasmid backbone with an AD8 envelope and EGFP inserted in the *nef* open reading frame at position 75 (NL(AD8)Δ*nef*EGFP) [14], kindly provided by Damian Purcell, University of Melbourne (Melbourne, Australia). Viral stocks were generated by FuGene (Promega, Madison, WI, USA) transfection of 293T cells as previously described [14]. Cells were infected at an MOI of 0.5, as determined by limiting dilution in PHA-stimulated PBMC using the Reed and Muench method [71].

In vitro latency model

Resting CD4$^+$ T-cells were labeled with the proliferation dye eFluor670 and cultured alone or with one of seven sorted syngeneic APC subpopulations at a ratio of 10:1 for 24 h in IL-2 (2U/mL, Roche Diagnostics, Basel, Switzerland) supplemented RF10 media. APC included monocyte subpopulations (CD14$^+$CD16$^-$ and CD14^{lo}CD16$^+$), DC subpopulations (pDC, CD1c$^+$, CD141$^+$ and SLAN$^+$), and B-cells. Co-cultures were then infected with NL(AD8)Δ*nef*EGFP for 2 h, after which time excess virus was washed away and cells were cultured for an additional 5 days. In order to compare APC stimulatory capacity between APC-T-cell co-cultures, at day 3 post-infection, cells were stained with anti-CD3-V450 (BD Biosciences) to differentiate between T-cell and APC, and the proportion of proliferated (eFluor670^{lo})

CD4$^+$ T-cells were determined. Day 3 was used because this is when productive infection reached is maximum and remained high until day 5 (unpublished data). Additional APC-T-cell ratio's were not used due to low APC yields. At day 5 post-infection, productive infection was determined by EGFP expression and non-proliferating, non-productively infected (eFluor670^{hi} EGFP$^-$) CD4$^+$ T-cells were sorted using a FACSAria.

Reactivation of latency from resting T-cells

Latent infection in the sorted, non-proliferating CD4$^+$ T-cells (eFluor670^{hi}EGFP$^-$) was determined by comparison of stimulated with un-stimulated T-cells sorted from APC-T-cell co-cultures (control). 1x10^5 sorted CD4$^+$ T-cells were stimulated with immobilized anti-CD3 (7 μg/ml; Beckman Coulter), in RF10 media supplemented with CD28 (5 μg/mL; BD Biosciences), IL-7 (50 ng/mL; Sigma, St Louis, MO, USA), IL-2 (5U/mL; Roche), with (post-integrated latency) or without (total latency: pre- and post-integrated latency) integrase inhibitor L8 (1 μM; Merck, White House Station, NJ, USA). The concentration of L8 was determined previously by titration of L8 in phytohaemagglutinin (PHA; 10 μg/mL) activated PBMC infected with R5-EGFP virus at an MOI of 0.5, same concentration usedin co-cultures, and showed productive infection was completely blocked at 1 μM. This concentration used for all subsequent experiments. Cells were harvested after 72 h of stimulation and EGFP expression was quantified on the FacsCalibur (BD BioSciences).

In some experiments PHA (10ug/mL) and IL-2 (10 U/mL) stimulated feeder PBMC were used to activate T-cells as a measure of inducing virus replication form latency, as described previously [14].

Cell preparation for next generation sequencing and generation of gene lists

APC from 3 donors were sorted as described above to obtain mDC subpopulations CD1c$^+$, SLAN$^+$, CD14$^+$ monocytes and pDC which were immediately stored in RLT buffer (Qiagen, Limburg, The Netherlands). Total RNA was isolated from low cell number samples (<500,000 cells) using Qiagen ALL prep micro kits (Qiagen), while RNA from samples with >500,000 cells were isolated using Qiagen RNA easy mini kits (Qiagen), according to the manufacturer's instructions. Total RNA content varied from 270.0 to 1879.7 ng.

The Australian Gene Research Facility Ltd (AGRF, Melbourne, Australia) prepared cDNA libraries, which were multiplexed on the Illumina HiSeq 2000 (Illumina, San Diago, CA, USA). For gene expression analysis, single reads were selected with 20 million reads of 50 bp read size gathered. The RNA-seq reads were aligned to the

human reference hg19 using the TopHat2 aligner [72, 73] and quantified using htseq-count [74]. Mapping rates for RNA seq are shown (Additional file 6: Table S3). Differential expression was calculated using Voom/Limma [75] and visualization performed using Degust [76] (http://victorian-bioinformatics-consortium.github.io/degust/) and Vennt [77] (http://drpowell.github.io/vennt/). Genes with fewer than 10 reads across every sample were removed from the analysis.

APC subsets were categorized as latency-inducing and latency-non-inducing subsets. Using a fold change of greater than 2 and false discovery rate (FDR) of 0.01, we identified 754 genes that were significantly upregulated in latency inducing populations (CD1c$^+$ mDC, CD14$^+$ monocytes, SLAN$^+$ DC) compared to latency non-inducing populations (pDC; Fig. 4). As direct cell contact is required for the establishment of mDC induced latency, only protein encoding genes from APC implicated in cell contact were selected using the GeneCodis database (http://genecodis.cnb.csic.es). We identified 285 genes from the initial list that encode for proteins known to be expressed on the plasma-membrane, membrane, integral to the plasma-membrane/membrane and cell junctions [37–39]. Finally, we manually curated this list to identify 53 genes known to be involved in T-cell signaling (Table 1; Additional file 1: Table S1). RNA-seq data is available through Gene Omnibus (GEO), serial number GSE70106.

As a comparison, we performed a similar analysis using our previously published microarray data using the same APC subpopulations [40]. Microarray data was kindly provided by Andrew Harman, Westmead Millennium Institute for Medical Research, Sydney University, Sydney [40]. The RNA extraction, labeling, hybridization, data processing, and analysis procedures used by Harman et al. are described previously for the cDNA gene array [78] and Illumina arrays [79]. Hybridization and data processing was performed by AGRF using sentrix human 6 v2 expression chips (Illumina).

Ethics approval

The use of blood samples from normal donors for this study was approved by the Alfred Hospital (HREC 156/11) and Monash University (CF11/1888) Human Research and Ethics Committees. Donors were recruited by the Red Cross Blood Transfusion Service as normal blood donors and all provided written informed consent for the use of their blood products for the research.

Statistical analysis

Differences between experimental conditions were analyzed using Wilcoxon matched pairs signed rank test ($n \geq 5$) or paired student T-test ($n < 5$) on GraphPad Prism (Version 6). P-values ≤ 0.05 were considered significant.

Differentially expressed RNA-seq and microarray genes were found to be significant using ANOVA [40].

Additional files

Additional file 1: Figure S1. Multidimensional scaling (MDS) of sequenced APC subpopulations. RNA sequences were measured according to two dimensions, 1 (x-axis) and 2 (y-axis). Each dot represents an antigen presenting cell (APC) subpopulation sequence, as labeled, n = 3. Clustering of dots is indicative of similar gene expression profiles.

Additional file 2: Table S1. Comparison of gene expression between latency inducing and non-inducing antigen presenting cell subpopulations using RNA-seq. Using the bioinformatics databases DAVID [80], GeneCards and GeneCodis, cell compartment gene function was determine of each gene. Genes expressed on antigen presenting cell (APC)-surface with the ability to signal to T-cells were shortlisted and their role in HIV-1 infection and DC-T-cell interaction was further determined using PubMed. Genes that were common to the RNA-seq and microarray generated gene lists are in *italics* and underlined in Table 1. Acronyms used: intracellular adhesion molecules (ICAM), C-type lectin (CLEC), immunoglobulin (Ig), T-cell immune-receptor with Ig and tyrosine-based inhibition motif (ITIM) domains (TIGIT), DNAX accessory molecule-1 (DNAM-1), cytotoxic and regulatory T-cell molecule (CRTAM), junction adhesion molecules (JAMs), blood brain barrier (BBB), leukocyte function antigen (LFA), dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), galectin-1 (Gal-1), cysteine-dependent aspartate-directed proteases (caspase), factor for adipocyte differentiation 158 (FAD158), extracellular matrix (ECM), lymph node (LN), nuclear factor-kappa-B (NFkB), vascular endothelial growth factor (VEGF), major histocompatibility complex (MHC), T-cell receptor (TcR), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), TYRO protein tyrosine kinase-binding protein (TYROBP), interleukin (IL-), nuclear factor of activated T-cells (NFAT), vasoactive intestinal polypeptide receptor 1 (VIPR1), T-cell immunoglobulin mucin-3 (Tim-3), monocyte derived dendritic cells (MDDC), natural killer cells (NK), human T-lymphotropic virus (HTLV-1), NFAT activating protein with immune-receptor tyrosine-based activation motif (NFAM1, CNAIP), cortical thymocyte-like protein (CTH, CTXC), B-cell receptor (BcR), HIV-associated neurocognitive disorder (HAND), antibody dependent cellular cytotoxicity (ADCC), Fc receptor (FcR), scavenger receptor class-A-1 (SRA-1), epidermal growth factor like domain, multiple/protein 5/9 (EGFL5).

Additional file 3: Supplementary references to Table 1. Included is the literature documenting the association of specific genes found in this study and changes in HIV infection and expression.

Additional file 4: Figure S2. Differential gene expression assessed by microarray analysis between latency inducing and non-inducing antigen presenting cells. **A**. Microarray gene expression profiles of antigen presenting cell (APC) subpopulations that could induce latency (CD1c$^+$, SLAN$^+$, CD14$^+$ monocytes) in non-proliferating CD4$^+$ T-cells were compared with APC subpopulations that could not induce latency (plasmacytoid (p)DC). Genes that were expressed in all 3 latency inducing APC subpopulations, CD1c$^+$, SLAN$^+$, CD14$^+$ monocytes, were categorized as candidate 3, genes expressed in only 2 APC subpopulations were categorized as candidate 2 and genes expressed only in 1 APC subpopulation were categorized as candidate 1. **B.** Using the bioinformatics databases DAVID, GeneCards and GeneCodis, Candidate 2 and 3 gene lists were analyzed for cellular compartment and function. Genes expressed on the APC cell surface, with the ability to signal to T-cells were shortlisted. **C.** Heat map shows differentially expressed genes with $\geq$ twofold differences between latency inducing APC subpopulations (CD14$^+$ monocytes, CD1c$^+$ mDC and SLAN$^+$ mDC) and non-latency inducing (pDC).

Additional file 5: Table S2. Comparison of gene expression between latency inducing and non-inducing antigen presenting cell subpopulations using microarray. Using the bioinformatics databases DAVID, GeneCards and GeneCodis, gene expression compartment and function was determined. Genes expressed on the antigen presenting cell (APC)-surface with the ability to signal to T-cells were shortlisted.

Additional file 6: Table S3. Sequence mapping rates in RNA-seq.

Abbreviations

APC: antigen presenting cell; cART: combination antiretroviral therapy; CLEC-7A: C-type lectin domain family 7 member A; DC: dendritic cells; DDC: dermal dendritic cell; EGFP: emerald-green fluorescent protein; FBS: fetal bovine serum; FDR: false discovery rate; FC: fold change; GCPR: G-coupled protein receptor; GEO: gene Omnibus; GIT: gastrointestinal tract; HAVR2: Hepatitis A virus cellular receptor 2; HIV-1: human immunodeficiency virus; ICAM: intracellular adhesion molecule 3; ICB: immune checkpoint blocker; IL-7: interluekin-7; IL-2: interluekin-2; LC: langerhan cell; LFA: leukocyte function associated antigen; LILR: leukocyte-associated immunoglobulin like receptor; LN: lymph node; mDC: myeloid dendritic cell; MHC: major histocompatibility complex; MLR: mixed leukocyte reaction; Mono: monocyte; PBMC: peripheral blood mononuclear cells; PD-1: programmed death receptor-1; pDC: plasmacytoid dendritic cell; PHA: phytohemagglutinin; SEB: staphylococcal enterotoxin B; SIGLEC: sialic acid-binding immunoglobulin-type lectins; SLAN: 6-sulfo Lac-NAc; Tim-3: T-cell immunoglobulin mucin-3; TNF: tumor necrosis factor.

Authors' contributions

NK carried out cell isolations and infection experiments with the assistance of KC. CdfP and JA assisted with imaging experiments NK performed RNA isolation. DRP performed most of the bioinformatics analysis for RNA-seq data and with NK generated and analysed genes lists. Microarray analysis was completed by PUC and NK. PUC, NK and SRL conceived the study, and participated in its design and coordination. Manuscript was prepared by PUC, NK, VE and JA. All authors read and approved the final manuscript.

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Compliance with ethical guidelines

Competing interests

The authors declare that they have no competing interests.

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8.4 Chapter 3 Supplementary Information

Appendix figure 8.1 Differential Gene Expression Assessed by Microarray Analysis Between Latency Inducing and Non-Inducing Antigen Presenting Cells.

A. Microarray gene expression profiles of antigen presenting cell (APC) subpopulations that could induce latency (CD1c$^+$, SLAN$^+$, CD14$^+$ monocytes) in non-proliferating CD4$^+$ T-cells were compared with APC subpopulations that could not induce latency (plasmacytoid (p)DC). Genes that were expressed in all 3 latency inducing APC subpopulations, CD1c$^+$, SLAN$^+$, CD14$^+$ monocytes, were categorized as candidate 3, genes expressed in only 2 APC subpopulations were categorized as candidate 2 and genes expressed only in 1 APC subpopulation were categorized as candidate 1. **B.** Using the bioinformatics databases DAVID, GeneCards and GeneCodis, Candidate 2 and 3 gene lists were analyzed for cellular compartment and function. Genes expressed on the APC cell surface, with the ability to signal to T-cells were shortlisted. **C.** Heat map shows differentially expressed genes with ≥2 fold differences between latency inducing APC subpopulations (CD14$^+$ monocytes, CD1c$^+$ mDC and SLAN$^+$ mDC) and non-latency inducing (pDC). Results for each APC subpopulation tested in 3 independent donors is shown. CD1c$^+$ refers to CD1c$^+$ mDC, CD14$^+$ refers to CD14$^+$ monocytes and SLAN$^+$ refers to SLAN$^+$ DC.

Total genes = 1381

☐ Candidate 3 (129)
▨ Candidate 2 (204)
☐ Candidate 1 (1048)

Total genes = 123

■ Secreted Proteins (3)
■ Non-channel (24)
■ Channel
■ Nuclear
■ Transcription Factors
■ Cytosolic
■ Intracellular membrane
■ Underfined
■ Protien domains
☐ Enzyme

Appendix figure 8.2 Multidimensional Scaling (MDS) of Sequenced Antigen Presentation Cell Subpopulations.

RNA sequences were measured according to two dimensions, 1 (x-axis) and 2 (y-axis). Each dot represents an antigen presenting cell (APC) subpopulation sequence, as labeled (n=3). Clustering of dots is indicative of similar gene expression profiles. pDC refers to plasmacytoid DC, $CD1c^+$ refers to $CD1c^+$ mDC, $CD14^+$ refers to $CD14^+$ monocytes and $SLAN^+$ refers to $SLAN^+$ DC.
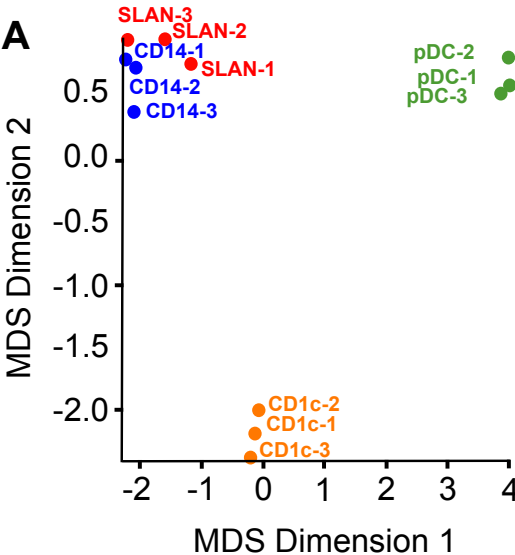
Appendix table 8.1 Comparison of Gene Expression Between Latency Inducing and Non-Inducing Antigen Presenting Cell Subpopulations Using RNA-Seq.

Using the bioinformatics databases DAVID, GeneCards and GeneCodis, cell compartment and gene function was determine for each gene. Genes expressed on antigen presenting cell (APC)-surface with the ability to signal to T-cells were shortlisted and their role in HIV-1 infection and DC-T-cell interaction was further determined using PubMed. Genes that were common to the RNA-seq and microarray generated gene lists are in *italics* and underlined.

Acronyms used: intracellular adhesion molecules (ICAM), C-type lectin (CLEC), immunoglobulin (Ig), T-cell immune-receptor with Ig and tyrosine-based inhibition motif (ITIM) domains (TIGIT), DNAX accessory molecule-1 (DNAM-1), cytotoxic and regulatory T-cell molecule (CRTAM), junction adhesion molecules (JAMs), blood brain barrier (BBB), leukocyte function antigen (LFA), dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), galectin-1 (Gal-1), cysteine-dependent aspartate-directed proteases (caspase), factor for adipocyte differentiation 158 (FAD158), extracellular matrix (ECM), lymph node (LN), nuclear factor-kappa-B (NFkB), vascular endothelial growth factor (VEGF), major histocompatibility complex (MHC), T-cell receptor (TcR), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), TYRO protein tyrosine kinase-binding protein (TYROBP), interleukin (IL-), nuclear factor of activated T-cells (NFAT), vasoactive intestinal polypeptide receptor 1 (VIPR1), T-cell immunoglobulin mucin-3 (Tim-3), monocyte derived dendritic cells (MDDC), natural killer cells (NK), human T-lymphotropic virus (HTLV-1), NFAT activating protein with immune-receptor tyrosine-based activation motif (NFAM1, CNAIP), cortical thymocyte-like protein (CTH, CTXC), B-cell receptor (BcR), HIV-associated neurocognitive disorder (HAND), antibody dependent cellular

cytotoxicity (ADCC), Fc receptor (FcR), scavenger receptor class-A-1 (SRA-1), epidermal growth factor like domain, multiple/protein 5/9 (EGFL5).

Gene	Gene Symbol	Other Names	Ligand	Gene Expression Platform	Function	Short Description	Role in HIV on DC?	Role in HIV on T-cell?	Affect on DC-T-Cell Interaction?
Acid phosphatase, prostate	ACPP	ACP3, 5'-NT, ACP-3		RNA-seq	Cell proximity	Found to be important in HIV-1 attachment and promotion of viral-target cell proximity[1]			Promotes virus and cell proximity
ADAM metallopeptidase domain 15	ADAM15	MDC15		RNA-seq	Cell proximity	May be involved in cell-surface proteolysis, cell adhesion or intracellular protein maturation			Control cell proximity
Integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)	CD18	CD18, ITGB2		RNA-seq	Cell proximity	Integrin αL/β2 is a receptor for ICAM1, ICAM2, ICAM3 and ICAM4	Enhances transmission of virus	Increased virus binding and interaction, leading to increased viral entry	Enhanced DC-T-cell interaction, enhance T-cell infection
CD33 molecule	CD33	p67, SIGLEC3	a-2,3 or a-3,6 sialic	RNA-seq	Cell proximity/ Negative	Mediates cell adhesion and inhibition of T-cell			Inhibition of immune response

		acids.			regulation	activation			
Carcinoembryonic antigen-related cell adhesion molecule 4	CEACAM4	NCA, CGM7	hypothesised: Annexin A2 and PTPN11.	RNA-seq	Cell proximity	Mediates cell adhesion			Promotes virus and cell proximity
C-type lectin domain family 1, member A	CLEC1A			RNA-seq	Cell proximity/ T-cell activation	CLEC family shares common protein fold, has diverse functions, including cell adhesion, cell signalling, glycoprotein turnover, and roles in inflammation and immune response [2,3]			Promote HIV-1 infection
C-type lectin domain family 4, member G	CLEC4G	CD299 (Ly9), ICAM3 (L-SIGN)		RNA-seq	Cell proximity	Paralog of Langerin (CD209), also only expressed on langerhan cells. Facilitates antigen uptake and presentation pathways	Langerin is protective against HIV-1 due to detection	Not expressed on T-cells	Can avert HIV-1 infection

C-type lectin domain family 7, member A	CLEC7A, dectin-1	BGR, CANDF4, DECTIN 1, CLECSF12		Both	Cell proximity	Recognises glutans of fungi and plants, regulates IL-10. Leads to a Th17 response from T-cells (NFkB involved)	Enhanced HIV-1 infection	CLEC4G may be similar [4]	DC secretion of IL-10 and Th17 will enhance T-cell HIV-1 infection
Cytotoxic and regulatory T cell molecule	CRTAM	CD355	CADM1, PVRL3, CADM3, CRTAM, Necl2, Necl proteins.	RNA-seq	Cell proximity	Promotes NK cell, CD8$^+$ T-cell cytotoxicity and type-I IFN secretion via initiating close cellular contact [5]. May also be induced in cellular migration. Part of Ig superfamily with TIGIT, CD96, DNAM-1	Gene expression decreased in HIV-1 infected patients [6]		Can limit HIV-1 spread by killing infected cell or enhance HIV-1 infection due to increased cell proximity
Colony stimulating factor 3	CSF3R	CD114, GCSFR	CFND, CFNS, EFB1,	RNA-seq	Cell proximity	Receptor for granulocyte colony-stimulating	Can interact with		Enhances DC and T-cell infection [8]

receptor (granulocyte)		EFL3, EPLG2, Elk-L, LERK2				factor (CSF3). May also function in cell adhesion or antigen recognition events.	HIV-1 [7]	Regulation of T-cell activation via immune synapses could also lead to regulation of viral synapses
Ephrin-B1	EFNB1			RNA-seq	Cell proximity	Regulates axonal projections and possibly inflammation [9]		Promotes infection of cells
Endoglin	END	ORW1, CD105, HHT1	TNFR3 (high affinity), TNFR2 (low affinity)	Both	Cell proximity	Mediated cell adhesion	Increased expressio n in pregnant women infected with HIV-1 May disregula te featal	

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Name	Gene	Aliases	Binding partners	Method	Category	Function	Role in HIV-1
Endothelial cell adhesion molecule	ESAM		JAMs	RNA-seq	Cell proximity	Involved in maintaining BBB [11]. Plays a role in forming tight junctions	Enhance DC-T-cell contact and enhance HIV-1 infection
G protein-coupled receptor 133	GPR133	PGR25	ECM	RNA-seq	Cell proximity	An orphan receptor, mediates cell adhesion surrounding environments	Enhances T-cell infection via cell mediated virus transfer
<u>Intercellular adhesion molecule 3</u>	<u>ICAM3</u>	CD50, CDW50, ICAM-R	LFA-1, Integrin-aD and Integrin-b2, DC-SIGN	Both	Cell proximity	Mediates cellular adhesion and protein signalling [12] Enhances HIV-1 infection in surrounding cells.	Promotes infection of cells
Lectin, galactoside-binding, soluble, 1	LGALS1	Gal-1, beta-galactoside binding protien 14kDa	CD2, CD3, CD4, CD7, CD43 and CD45.	RNA-seq	Apoptosis regulation	Ligation with CD7 leads to caspase and cytochrome independent cell death [13]. Leads to IL-10 secretion from activated T-cells. Also thought to negatively	Promote viral adsorption by cross linking APC-T-cell interaction [14]

Name	Symbol	Aliases	Method		Function		Implication
Leucine rich repeat containing 8 family, member C	LRRC8C	FAD158, AD158	RNA-seq	Cell proximity	regulate cell growth	May be involved in cell adhesion, cellular trafficking and hormone receptor interactions	Enhanced viral entry
Multiple EGF-like-domains 9	MEGF9	EGFL5	RNA-seq	Cell proximity		EGF domains are involved in cell rolling and tissue migration. Functions as a guidance or signalling molecule	Could enhance DC-T-cell interaction and lead to T-cell signalling thereby, enhancing HIV-1 infection
Membrane protein, palmitoylated 7 (MAGUK p55 subfamily member 7)	MPP7		RNA-seq	Cell proximity		Found in synapses, adherans junctions and tight junctions, acts as adaptor protein, and is required for cell polarity [15]	Helps in the formation of DC-T-cell contact, and synapse formation. May lead to

Name	Symbol	Aliases	Method	Evidence	Function	HIV-1 relevance
						enhanced HIV-1 entry
Macrophage scavenger receptor 1	MSR1	SCARA 1, CD204, SRA1	RNA-seq	Cell proximity	Scavenger receptors mediate endocytosis and adhesion to surfaces and cells	Enhances DC-T-cell interaction, leading to greater viral entry.
Osteoclast associated, immunoglobul in-like receptor	OSCAR	PIGR3, PIgR-3	RNA-seq	Cell proximity	Member of leukocyte receptor complex family, family can mediate monocyte adhesion	Enhance DC-T-cell interaction, leading to greater viral entry
Plexin domain containing 2	PLXDC2	TEM7R semaphorins (5A)	RNA-seq	Cell proximity	Promotes axonal growth	Helps in the formation of DC-T-cell contact, and synapse formation. May lead to enhanced HIV-1 entry
Syndecan 3	SDC3	SDCN, SYND3, N-	RNA-seq	Cell proximity	Controls cell shape via actin cytoskeleton, may	SDC3 binds and	Enhanced virus transfer between and

syndecan						act as a growth factor receptor, or adhesion to ECM and cells. Enhanced by Nef[16]	protects HIV-1 virion, thereby preserving infectivity[17,18]	increased T-cell infection
Sphingosine-1-phosphate lyase 1	SGPL1	SPL1, S1PL, hSPL		RNA-seq	Cell proximity/ Negative regulation	Mediates T- and B-cell migration out of LN in mice[19]. Also may decrease migration of DC[20]	May lead to increased viral entry and infection by promoting cell proximity
Sialic acid binding Ig-like lectin 10	SIGLEC 10	PRO940, SLG2	a-2,3 or a-3,6 sialic acids.	Both	Cell proximity/ Negative regulation	May acts as an inhibitory receptor by blocking signal transduction via SH2 domains in the cytoplasm, by similarity to Siglec5. Can also act as an adhesion molecule	Inhibition of immune response and T-cell infection. By sequence similarity to Siglec-5[21] can protect T-cells from HIV-1 infection

Name	Symbol	Aliases	Binding	Method	Role	Description	Outcome
Sialic acid binding Ig-like lectin 14	SIGLEC 14		a-2,3 or a-3,6 sialic acids.	RNA-seq	Cell proximity/ T-cell activation	Acts as an activation receptor DAP12. Can also act as an adhesion molecule (similarity to Siglec-5, 10; [22])	Enhanced T-cell activation and productive infection
Sialic acid binding Ig-like lectin 7	SIGLEC 7	QA79, AIRM1, CD328, D-siglec, SIGLEC P2, SIGLEC 19P, p75/AIRM1	a-2,3 or a-3,6 sialic acids.	RNA-seq	Cell proximity/ Negative regulation	Part of the CD33 SIGLEC family, thus could mediate inhibitory effects like Siglec-5	Negative regulation of T-cell activation
Sialic acid binding Ig-like lectin 9	SIGLEC 9	CD329, FOAP-9, OBBP-LIKE	a-2,3 or a-3,6 sialic acids.	RNA-seq	Cell-cell proximity/ Negative regulation	Part of the CD33 SIGLEC family, thus could mediate inhibitory effects like Siglec-5	Negative regulation of T-cell activation
Synaptojanin 2 binding protein	SYNJ2BP	ARIP2, FLJ1127 1, FLJ4197		RNA-seq	Cell proximity/ Negative regulation	Involved in dendrite guiding by promoting delta-notch signalling [23]	May initiate DC-T-cell contact

	3, OMP25, activin receptor interacti ng protein 5				Regulates cell adhesions, polarity, migrations, invasion, metastasis and carcinogenesis.	Used by Nef to downregulate MHC-I [24]	May initiate DC-T-cell contact.
T-cell lymphoma invasion and metastasis 1	TIAM1		RNA-seq	Cell proximity/ Negative regulation	Connects extracellular signals to cytoskeletal activities by modulation of RHO-like protein activity		
Transmembra ne protein 2	TMEM2	ECM	RNA-seq	Cell proximity/ Negative regulation	Influences ECM		Promote virus and cell proximity
Tetraspanin 17	TSPAN1	FBX23,	RNA-seq	Cell proximity/ Negative regulation	Play's a role in the		Can regulate

	7 FBXO23 TM4SF17				proximity/ Negative regulation	regulation of cell development, activation, growth and motility. Specific function of TSPAN17 remains unknown.			T-cell activation, therefore promote T-cell infection
CD1d molecule	CD1d	CD1DR3, CD1A	TcR	Both	Antigen presentation	Presents lipid antigens to T-cells via interaction with TcR.	Downregulated by Vpu and Nef in infected DC [25,26]	CD1d expressing NK-T have impaired PD-1 expression in chronic HIV-1 infected patients [27]	Maintains DC-T-cell interaction and promote T-cell infection
EF-hand domain family, member D2	EFHD2			RNA-seq	Apoptosis regulator	Regulation of apoptosis and maturation of B-cells. May also negatively regulate NFkB			May inhibit viral expression by downregulation of NFkB in T-cell,

Tumor necrosis factor receptor superfamily, member 10A	TNFRSF 10A	DR4, APO2, CD261, TRAILR 1, TRAILR -1	TRAIL (TNFRS F10)	RNA-seq	Apoptosis regulator	Mediates apoptosis via TRAIL pathway.	Enhance survival of T-cells that interact with DC therefore promote latency
Tumor necrosis factor receptor superfamily, member 10D, decoy with truncated death domain	TNFRSF 10D	DCR2, CD264, TRUND D, TRAILR 4, TRAILR- R4		RNA-seq	Apoptosis regulator	Protects against TRAIL mediated apoptosis. Truncated signalling domain.	Enhance survival of T-cells
Vasoactive intestinal peptide receptor 1	VIPR1	HVR1, RDC1, VIRG, VIPR, VIRG, VAPC1, VPAC1, VPAC1		RNA-seq	Apoptosis regulator	Decreases immune activation and apoptosis by decreasing expression of Fas ligand [28]. Constitutively expressed.	Ligation on macroph age leads to enhance HIV-1 infection Promote infection of T-cells.

		R, VIP-R-1, VPCAP1 R, PACAP-R2, PACAP-R-2				[29]. No role in prevention of integration [30]	
CD101 molecule	CD101	V7, IGSF2, EW1-101	CD3	RNA-seq	Negative regulator	Inhibitor of T-cell proliferation via CD3. Is able to block NFAT	Inhibition of cell proximity, can protect cells from HIV-1 transfer or dampen immune response
CD52 molecule	CD52	CAMPA TH-1 antigen, CDW52, HE5	Siglec-10 [31]	RNA-seq	Negative regulator	Inhibits cell adhesion	Blocks T-cell activation
Hepatitis A virus cellular receptor 2	HAVCR 2	Tim-3, TIMD-3	Possibly Galectin-9,	RNA-seq	Negative regulator	Tim-3 is a negative regulator, dampens the immune response by	Could downregulate T-cell activation, Increased upon exposure to HIV-1

						decreasing T-cell activation.	loaded MDDC and in HIV-1+ individuals [32,33] thus decrease virus expression and promote latency.
Leukocyte immunoglobul in-like receptor, subfamily A (with TM domain), member 6	LILRA6	ILT5, ILT8, CD85b, ILT-8, LILRB3, LILRB6	MHC-I	RNA-seq	Negative regulator	Negatively regulates the inflammatory response, by blocking MHC-I binding [34,35]	Could negatively regulate T-cell activation and block response to HIV-1
Poliovirus receptor	PVR	PVS, HVED, CD155, NECL5, TAGE4, Necl-5	DNAM-1 (CD226), CD96	RNA-seq	Negative regulator	Involved in formation of immunological synapse. Can also bing TIGIT [36]. Expressed on NK cells and CD8$^+$ T-cells [37]	Nef downreg ulates PVR [38], but Vpr upregulat es PVR in Jurkat T-cells [39] Could negatively regulate T-cell activation and block response to HIV-1
Neuropilin (NRP) and tolloid (TLL)-	NETO2	NP1, NRP, BDCA4,	VEGF, semorphi ns	RNA-seq	Cell proximity	Important in synapse formation, receptor for HTLV-	Enhance DC-T-cell interaction,

Molecule	Gene	Aliases	Receptor/Ligand	Method	Function	Mechanism	HIV expression	Outcome
like 2		CD304, VEGF165R					1 [40,41]	leading to greater viral entry
CD300e molecule	CD300e	CLM2, CLM-2, IREM2, PIgR2, IREM-2, PIgR-2, CD300LE, CMRF35-A5	TYROBP	RNA-seq	T-cell Activation	Involved in T-cell activation		Promote T-cell activation and further HIV-1 infection
CD48 molecule	CD48	BCM1, BLAST, hCD48, mCD48, BLAST1	CD2, TcR	RNA-seq	T-cell Activation	Mediates bidirectional stimulatory signalling [42]	Downregulated on in HIV-1 infected patients [43]	Promotes T-cell activation, therefore T-cell infection
CD83 molecule	CD83	SLAMF2, MEM-102	CTLA-4, CD28	RNA-seq	T-cell Activation	Immuno-regulatory (bi-directional) co-stimulatory signalling	Upregulated in HIV-1 infected	Promotes T-cell activation, therefore T-

Gene	Symbol	Alias	Interacting	Method	Pathway	Description	Mechanism	HIV relevance
						molecule, marks DC maturation	patients (along with CD86) [44]	cell infection
CD86 molecule	CD86	B70, B7-2, B7.2, LAB72	CD28, CTLA-4	RNA-seq	T-cell Activation	Co-stimulatory molecule	Positive correlation with HIV-1 production and DC activation [45–47]	Promotes T-cell activation, therefore T-cell infection
Interleukin 15 receptor, alpha	IL15RA			RNA-seq	T-cell Activation	IL-15Ra and IL-15 complex can signal to CD8⁺ T-cells to promote cell survival and improve T-cell function [48,49]	Ligation leads to increased memory cell survival or T-cell activation.	Could increase DC-T-cell interaction and promote T-cell survival, thus lead to persistence of HIV-1 infected cell
Leucine rich repeat	LRRC25	Monocytes and		RNA-seq	T-cell Activation	May be involved in the activation of		May lead to T-cell infection

Description	Symbol	Alt name		Method	Category	Function	Effect
containing 25	plasmacytoid-activated protien (MAPA)					cells of innate and acquired immunity Down-regulated in CD40-activated MDDC [50]	activation and thus enhance productive infection
NFAT activating protein with ITAM motif 1 [Source:HGNC Symbol;Acc:2 9872]	NFAM1	CNAIP		RNA-seq	T-cell Activation	Regulates intracellular gene promoters. Can associate with BcR and regulate B-cell development, may act as a co-stimulator	May stabilise DC-T-cell binding and enhance viral entry
Secreted and transmembrane 1	SECTM1	K12 protien	CD7	RNA-seq	T-cell Activation	Co-stimulatory molecule for T-cell activation that acts synergistically with CD28	Enhances T-cell activation and leads to increased in infection.
V-set and immunoglobul in domain containing 2	VSIG2	CTH, CTXL		RNA-seq	T-cell Activation	Has an Ig domain with the potential to signal	Promote DC-T-cell signalling therefore infection
Ecotropic viral integration site	EV12A	EVDA, EVI2,		RNA-seq	Unknown	Has transmembrane	Enhance DC-T-cell contact

2A	EVI-2A					signalling receptor activity. May work in complex with other surface expressed proteins.		and therefore, HIV-1 infection.

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Appendix table 8.2 Comparison of gene expression between latency inducing and non-inducing antigen presenting cell subpopulations using microarray.

Using the bioinformatics databases DAVID, GeneCards and GeneCodis, gene expression compartment and function was determined. Genes expressed on the antigen presenting cell (APC)-surface with the ability to signal to T-cells were shortlisted.

Gene	Gene Symbol	Other names	Function	Short description
Ficolin (collagen/fibrinogen domain containing) 1	*FCN1*	*ficolin-1, ficolin-A , ficolin-alpha, FCNM, M-ficolin*	Cell proximity	Binds N-glycosylated carbohydrates on the cell surface.
Lymphocyte antigen 6 complex, locus H	LY6H	NMLY6	Cell proximity	Unknown, may have function in central nervous system and immune system. Possibly function within synapse formations.
C-mer proto-oncogene tyrosine kinase	MERTK	MER, RP38, c-mer	Cell proximity	Inhibition leads to decrease in colony formation and apoptosis.
Synapsin 1	SYN1	SYNI; SYN1a; SYN1b	Cell proximity	Expressed in axonal synapses, regulates axonagenesis and synaptogenesis. May be involved in HAND. Plays a role in T-cell activation.
Killer cell lectin-like receptor subfamily G, member 2	KLRG2	CLEC15B	Cell proximity or PAMP	Part of CLEC family

	Symbol	Aliases	Function	Description
Tumor necrosis factor receptor superfamily, member 10B	TNFRSF10B	DR5, CD262, KILLER, TRICK2, ZTNFR9, TRAILR2, TRICK2A, TRAIL-R2, KILLER/	Apoptosis regulator	Receptor for the cytotoxic ligand TNFSF10/TRAIL. Mediates apoptosis via activation of NFkB
G protein-coupled bile acid receptor 1	GPBAR1	BG37, TGR5, M-BAR, GPCR19, GPR131	Negative regulation	Binds to bile acids
Leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 3	LILRB3	HL9, ILT5, LIR3, PIRB, CD85A, ILT-5, LIR-3, LILRA6	Negative regulator	Interacts with MHC-I and can form a dimer with LIR7 or Fc epsilon RI. May lead to inhibition of functions like histamine release
Sialic acid binding Ig-like lectin 5	SIGLEC5	CD170, OBBP2, CD33L2, OB-BP2	Negative regulator	Acts as an inhibitory receptor by blocking signal transduction via SH2 domains in the cytoplasm, which dephosphorylates signalling molecules. Can also act as an adhesion molecule
Signal-regulatory protein beta 1	SIRPB1	CD172b, SIRP-BETA-1	Negative regulator	Involved in inhibition of TLR signalling, also involved in synapse formation [51]. Activates myeloid cells via DAP-12 signal induction [52]
Transmembrane protein 176A	*TMEM176A*	*GS188, HCA112*	*Negative regulator*	With TMEM176B is able to inhibit DC maturation [53]
Leukocyte immunoglobulin-like receptor, subfamily A (with TM domain), member 2	LILRA2	ILT1, LIR7, CD85H, LIR-7	T-cell activation	May act as a MHC-I receptor

Leukocyte immunoglobulin-like receptor, subfamily A (without TM domain), member 3	LILRA3	HM31, HM43, ILT6, LIR4, CD85E, ILT-6, LIR-4	T-cell activation	Interacts with MHC-I can be secreted [54]
SMAD family member 7	SMAD7	CRCS3; MADH7; MADH8	T-cell activation	Part of TGF family of receptors. Able to induce proliferation of macrophages in pancrease of mice [55]. Inhibits a plasticity of T-cells [56]
V-set and transmembrane domain containing 1	VSTM1	SIRL1, SIRL-1	T-cell activation	Able to induce IL-17 secretion from T-cells [57]
Complement component 2	C2	ARMD14, C3/C5 convertase, CO2,	T-cell activation	Part of the complement system, triggers the activation of inflammation.
CD79b molecule, immunoglobulin-associated beta	CD79B	B29, IGB, AGM6	T-cell activation	Binds with BcR to interact with MHC-I.
Dysferlin, limb girdle muscular dystrophy 2B (autosomal recessive)	DYSF	FER1L1, Fer-1-like protein 1	T-cell activation	Transmembrane protein that mediated membrane repair of muscle cells [58]. Hypothesised to be involved in inflammation.
Fc fragment of IgG, low affinity IIa, receptor (CD32)	FCGR2A	FCG2, FCGR2A, IGFR2, Fc-gamma-RIIa (FcGR, fcRIIa)	T-cell activation	FcR on DC, can mediate binding to lymphocytes [59]. Can mediate ADDC by NK cells [60]
Immunoglobulin superfamily, member 6	IGSF6	Down-Regulated By Activation (DORA)	T-cell activation	Downregulated on DC upon CD40L ligation, however also expressed in germinal centres. May function in T-cell activation as a co-receptor.
G protein-coupled receptor 35	GPR35		Unknown	Orphan receptor - part of G-couple protein receptor family.
Transmembrane protein 89	TMEM89		Unknown	Unknown

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Appendix table 8.3. Sequence mapping rates in RNA-seq

Sample	Donor #	FastQC Pre-Trim Total Sequences	FastQC Post-Trim Total Sequences	Number of reads	Reads with unique alignments	Reads with multiple alignments	Reads which could not be aligned	Mapped	Mapping Rate	Mapped Pairs	Unpaired Reads	Intragenic Rate	Exonic Rate	Mapped Unique	Unique Rate of Mapped	Mapped Unique Rate of Total	Duplication Rate of Mapped
				TopHat Alignment Stats				RNA-SeQC									
CD14+ Mono	28	16,098,307	15,919,565	15,919,217	14,271,638	927,317	720,610	15,198,955	0.9547552	0	15,919,217	0.9537282	0.8002105	8,490,814	0.5586446	0.5333688	0.4413554
CD1c+ DC	28	16,376,979	16,191,429	16,191,021	14,245,365	1,082,729	863,335	15,328,094	0.9467034	0	16,191,021	0.9438672	0.74515784	9,201,003	0.6002705	0.56827813	0.3997295
SLAN+ DC	28	17,321,109	17,129,834	17,129,401	15,439,064	942,841	747,929	16,381,905	0.9563619	0	17,129,401	0.96140814	0.8188368	8,838,751	0.53954 35	0.51599884	0.46045646
pDC	28	16,680,031	16,492,002	16,491,609	14,933,859	781,496	776,647	15,715,355	0.9529304	0	16,491,609	0.94824165	0.7175076	10,210,965	0.64974445	0.61916125	0.35025552
CD14+ Mono	97	15,137,197	14,956,310	14,955,989	13,221,361	950,952	783,997	14,172,313	0.9476012	0	14,955,989	0.9477913	0.8134096	7,747,995	0.5466994	0.518053	0.4533006
CD1c+ DC	97	16,161,213	15,977,082	15,976,672	14,116,173	993,175	867,734	15,109,348	0.9457131	0	15,976,672	0.95963657	0.8340455	8,027,079	0.53126574	0.50242496	0.46873426

SLAN+ DC	97	16,821.214	16,623.903	16,623.602	14,916,494	911,057	796,352	15,827,551	0.9521132	0	16,623,602	0.9653766	0.856287	8,122,098	0.513162	0.48858833	0.48683798
pDC	97	16,123.951	15,929.850	15,929.491	14,345,044	617,502	967,304	14,962,546	0.93929845	0	15,929,491	0.95930386	0.70304954	9,907,554	0.66215694	0.621963	0.33784303
CD14+ Mono	36	17,386.950	17,182.229	17,181.821	15,359,048	958,789	864,392	16,317,837	0.94971526	0	17,181,821	0.9566783	0.8431474	8,279,566	0.5073936	0.48187944	0.4926064
pDC	36																
CD1c+ DC	36	15,903.116	15,722.608	15,722.251	14,055,533	887,738	779,337	14,943,271	0.95045364	0	15,722,251	0.9639394	0.8342171	8,051,598	0.53881097	0.5121148	0.46118906
SLAN+ DC	36	14,952.422	14,783.727	14,783.313	13,409,543	731,311	642,873	14,140,854	0.9565416	0	14,783,313	0.9660527	0.83764434	7,752,659	0.54824543	0.5244196	0.45175454
pDC	36	15,301.623	15,114.571	15,114.352	13,171,175	1,235,778	707,618	14,406,953	0.9531969	0	15,114,352	0.9459112	0.90125823	5,982,978	0.41528407	0.39584747	0.58471596

8.5 Chapter 5 Supplementary Information

Appendix table 8.4. Genes Upregulated in Latency Non-Inducing Cells, pDC, Measured by RNA-Seq.

Using the bioinformatics databases DAVID, GeneCards and GeneCodis, gene expression compartment and function was determined. Genes expressed on pDC cell-surface with the ability to signal to T-cells were shortlisted.

Gene name	Gene symbol	Type of interaction	Other names	Description
ADAM metallopeptidase domain 19	ADAM19	Adhesion		Membrane anchor protein, demonstrated to be an active metalloproteinase, which may be involved in normal physiological processes such as cell migration, cell adhesion, cell-matrix interactions and signal transduction.
ADAM metallopeptidase domain 22	ADAM22	Adhesion		Membrane anchor protein that has been implicated in cell-cell and cell-matrix interactions.
ADAM metallopeptidase domain 33	ADAM33	Adhesion		Membrane anchor protein that has been implicated in cell-cell and cell-matrix interactions.
Adhesion molecule with Ig-like domain 2	AMIGO2	Adhesion		May be involved in cell-cell interactions and intracellular signal transduction.
Amyloid beta (A4) precursor protein	APP	Adhesion	AD1, CVAP, ABETA, PN2, ABPP	A cell surface receptor, which mediates neurite growth, neuronal adhesion and axonogenesis, also involved in cell mobility, transcription regulations. Couples to apoptosis-inducing pathways by inhibition of G(o) alpha ATPase activity (By similarity).
Blood vessel epicardial substance	Bves	Adhesion		Involved in cell-adhesion, formation of tight junctions, vesicular transport and cell shape.

Name	Gene symbol	Function	Aliases	Description
C-type lectin domain family 4, member C	CLEC4C	Adhesion	DCIR, LLIR, DDB27, CLECSF6,	Involved in antigen-capturing, may mediate inhibition of IFN-alpha/beta expression from plasmacytoid dendritic cells. Members of this family share a common protein fold and have diverse functions, such as cell adhesion, cell-cell signalling, glycoprotein turnover, and roles in inflammation and immune response.
Cadherin 2, type 1, N-cadherin (neuronal)	CDH2	Adhesion	CDHN, NCAD, CD325	Calcium dependent cell adhesion proteins, which preferentially interacts with themselves in a homophilic manner in connecting cells.
Carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein)	CEACAM1	Adhesion		Cell adhesion molecule detected on leukocytes, epithelia, and endothelia that mediates differentiation and arrangement of tissue structure, angiogenesis, apoptosis, tumour suppression, metastasis and the modulation of innate and adaptive immune responses.
Carcinoembryonic antigen-related cell adhesion molecule 21	CEACAM21	Adhesion	CEACAM3	Belongs to CEA family therefore might be able to establish cell-cell adhesion.
CD9 molecule	CD9	Adhesion	MIC3, MRP-1, BTCC-1, DRAP-27, TSPAN-29	Tetraspanin Involved in platelet activation and aggregation. Regulates paranodal junction formation. Involved in cell adhesion, cell motility and tumour metastasis.
Cell adhesion molecule 4	CADM4	Adhesion		Involved in the cell-cell adhesion. Has calcium and magnesium independent cell-cell adhesion activity.
Desmoglein 2	DSG2	Adhesion		Component of intercellular desmosome junction that is involved in the interaction of plaque proteins and intermediate filaments mediating cell adhesion.
G protein-coupled receptor 56	GPR56	Adhesion	BFPP, BPPR, TM7LN4, TM7XN1	Could be involved in cell interactions.
Glycoprotein M6B	GPM6B	Adhesion	M6B	Proteolipid protein family members are expressed in most brain regions and are thought to be involved in cellular housekeeping functions, such as membrane trafficking and cell communications [61,62]. Role in HIV infection of expressing cell.

Name	Symbol	Category	Aliases	Description
Integrin, alpha 9	ITGA9	Adhesion	RLC, ITGA4L, ALPHA-RLC	Integrin alpha-9/beta-1 is a receptor for VCAM1, cytotactin and osteopontin. VCAM1 is expressed on T-cells
Integrin, beta 8	ITGB8	Adhesion		Integrin alpha-V/beta-8 is a receptor for fibronectin, which non-covalently binds to an alpha subunit to form a heterodimeric integrin complex
Milk fat globule-EGF factor 8 protein	MFGE8	Adhesion	BA46, HMFG, MFGM, SED1, hP47, EDIL1, MFG-E8, SPAG10	MFGE8 may function as a cell adhesion protein to connect smooth muscle to elastic fibre in arteries
Multimerin 1	MMRN1	Adhesion		Multimerin is a factor V/V-alpha binding protein and may function as a carrier protein for platelet factor V. May have functions as an extracellular matrix or adhesive protein
Myelin associated glycoprotein	MAG	Adhesion	GMA, S-MAG, SIGLEC4A, SIGLEC-4A	Adhesion molecule that mediates sialic-acid dependent cell interactions between neuronal and myelinating cells. Preferentially binds to alpha-2,3-linked sialic acid.
Poliovirus receptor-related 1 (herpesvirus entry mediator C)	PVRL1	Adhesion		Ca2+ independent adhesion protein that plays a role in the organization of adherens junctions and tight junctions in epithelial and endothelial cells. Acts as a receptor for glycoprotein D (gD) of herpes simplex viruses 1 and 2 (HSV-1, HSV-2), and pseudorabies virus (PRV) and mediates viral entry into epithelial and neuronal cells
Protocadherin 9	PCDH9	Adhesion		Potential calcium-dependent cell-adhesion protein
Protocadherin gamma subfamily C, 3	PCDHGC3	Adhesion	PC43, PCDH2	These neural cadherin-like cell adhesion proteins most likely play a critical role in the establishment and function of specific cell-cell connections in the brain
Semaphorin 7A, GPI membrane anchor (John Milton Hagen blood group)	SEMA7A	Adhesion	JMH, CD108, SEMAL, SEMAK1	The protein encoded by this gene binds to cell surfaces through a glycosylphosphatidylinositol (GPI) linkage. May be involved in immune-modulatory and neuronal processes

Name	Symbol	Process	Aliases	Function
Sidekick homolog 2 (chicken)	SDK2	Adhesion		Cell adhesion protein that guides axonal terminals to specific synapses in developing neurons. Function remains unclear
Signal peptide, CUB domain, EGF-like 1	SCUBE1	Adhesion		Is a member of the SCUBE family. Could function as an adhesive molecule.
Trophinin	TRO	Adhesion		Could be involved with bystin and tastin in a cell adhesion molecule complex that mediates an initial attachment of the blastocyst to uterine epithelial cells. Directly responsible for homophilic cell adhesion.
Protein tyrosine phosphatase, receptor type, M	PTPRM	Adhesion		Involved in cell adhesion through homophilic interactions. May play a key role in signal transduction and growth control.
Protein tyrosine phosphatase, receptor type, S	Ptprs	Adhesion	PTPSIGMA	Is a member of the PTP family, which are known to be signalling molecules that regulate cell growth, differentiation, mitotic cycle, and oncogenic transformation. Mice studies suggest that this PTP may be involved in cell interaction, primary axonogenesis and axon guidance during embryogenesis.
Clusterin	CLU	Adhesion, apoptosis regulation	DAG protein, TRPM-2, SGP-2, CLI	Implicated in a number of biological processes, including lipid transport, membrane recycling, cell adhesion, programmed cell death and complement cascade.
Complement component (3b/4b) receptor 1 (Knops blood group)	CR1	Adhesion, apoptosis regulation		Mediates cellular binding of particles and immune complexes that have activated complement.
Spondin 2, extracellular matrix protein	SPON2	Adhesion, pathogen recognition	DIL1, MINDIN, M-SPONDIN	Cell adhesion protein that promote adhesion and outgrowth of hippocampal embryonic neurons, functions as an opsonin for macrophage phagocytosis, essential in the initiation of the innate immune response and is a unique pattern-recognition molecule for microbial pathogens.
Melanoma antigen family D, 1	MAGED1	Apoptosis		Demonstrated to be involved in the p75 neurotrophin receptor mediated programmed cell death pathway.
Nerve growth factor receptor	NGFR	Apoptosis	p75, LNGFR, p75NTR, p75NGFR, Tnfrsf16	Low affinity receptor that can bind to NGF, BDNF, NT-3, and NT-4. Can mediate cell survival and cell death of neural cells.

Tumor necrosis factor receptor superfamily, member 11a, NFKB activator	TNFRSF11A	Apoptosis		Receptor for TNFSF11/RANKL/TRANCE/OPGL; essential for RANKL-mediated osteoclastogenesis. Regulates interactions between T-cells and dendritic cells.
Killer cell lectin-like receptor subfamily G, member 1	KLRG1	Negative regulation		Inhibitory role on natural killer (NK) cells and T-cell functions. Binds E-cadherin/CDH1, N-cadherin/CDH2 and R-cadherin/CDH4, part of KLR family.
Leukocyte-associated immunoglobulin-like receptor 1	LAIR1	Negative regulation		Plays a constitutive negative regulatory role on cytolytic function of natural killer (NK) cells, B-cells and T-cells. Down regulates IL-2 and IFN-gamma from CD4+ T-cells, but induces TGF-beta secretion. Also Inhibits differentiation of peripheral blood precursors towards dendritic cells.
Ras homolog gene family, member H	RHOH	Negative regulation	TTF; ARHH	It functions as a negative regulator of cell growth and survival.
Src-like-adaptor 2	SLA2	Negative regulation		Adapter protein, which negatively regulates TcR signalling. Inhibits TcR induced activation of NFAT, may act by linking signalling proteins such as ZAP70 with CBL, leading to CBL dependent degradation of signalling proteins.
CD274 molecule	CD274	Negative regulation	PD-L1	Involved in the co-stimulatory signal, essential for T-cell proliferation and production of IL-10 and IFN-gamma. Interaction with PD-1 inhibits T-cell proliferation and cytokine production.
Signaling threshold regulating transmembrane adaptor 1	SIT1	Negative regulation	SIT, SIT-R	Negatively regulates TcR mediated signalling in T-cells. Involved in positive selection of T-cells.
Regulator of G-protein signaling 7	RGS7	Negative regulation, intracellular activity		Inhibits signal transduction by increasing the GTPase activity of G protein alpha subunits thereby driving them into their inactive form. Activity on G(o)-alpha is specifically enhanced by the RGS6/GNG5 dimer.
Wingless-type MMTV integration site family, member 10A	WNT10A	Secreted, development		Member of the WNT gene family, these proteins have been implicated in oncogenesis and in several developmental processes, including regulation of cell fate and patterning during embryogenesis.

Name	Abbr.	Category	Aliases	Description
Vascular endothelial growth factor B	VEGFb	Secreted, growth factor		Growth factor for endothelial cells. Binds heparin and neuropilin-1, neuropilin-1 binding is regulated by proteolysis.
Oncostatin M	OSM	Secreted, growth regulator		Growth regulator, which inhibits the proliferation of a number of tumour cell lines. It regulates cytokine production, including IL-6, G-CSF and GM-CSF from endothelial cells.
Transforming growth factor, alpha	TGFa	Secreted, T-cell activation		TGF-alpha is able to bind to the EGF receptor and to act synergistically with TGF-beta, mediates inflammation.
Tumor necrosis factor (ligand) superfamily, member 11	TNFSF11	Secreted, T-cell activation		Cytokine that binds to TNFRSF11B/OPG and to TNFRSF11A/RANK. Augments the ability of dendritic cells to stimulate naïve T-cell proliferation. May be an important regulator of interactions between T-cells and dendritic cells.
Vasorin	VASN	Secreted, T-cell activation		May act as an inhibitor of TGF-beta signalling.
SPARC related modular calcium binding 1	SMOC1	Secreted, unknown		Encodes a multi-domain secreted protein that may have a critical role in ocular and limb development. Unclear function
Secretin	SCT	Secreted		Secretin is a hormone that regulates water homeostasis throughout the body, and influences the environment of the duodenum by regulating secretions in the stomach and pancreas.
Multiple EGF-like-domains 6	MEGF6	Secretory, adhesion, T-cell activation	EGFL6, W80, MAEG	This superfamily is characterized by the presence of EGF-like repeats and are often involved in the regulation of cell cycle, proliferation developmental processes and adhesion
Interleukin 13	IL-13	Secretory, negative regulation		Inhibits inflammatory cytokine production, synergizes with IL-2 in regulating interferon-gamma synthesis. May be critical in regulating inflammatory and immune responses.
Interleukin 4	IL-4	Secretory, T-cell activation		Participates in several activation processes. It is a co-stimulator of DNA-synthesis. Promotes Th2 response from T-cells
Mesencephalic astrocyte-derived neurotrophic factor	MANF	Secretory, T-cell activation	ARP, ARMET	Reducing expression of this gene increases susceptibility to ER stress-induced death and results in cell proliferation.

Name	Symbol	Function	Alias	Description
CD69 molecule	CD69	T-cell activation		Involved in lymphocyte proliferation and functions as a signal transmitting receptor in lymphocytes, natural killer (NK) cells, and platelets. Ligands of CD69 have not yet been identified, can bind galectin 1 [63] and S1P1 [64]
Cytokine receptor-like factor 2	CRLF2	T-cell activation		Receptor for TSLP. Forms a functional complex with TSLP and IL7R, which is capable of stimulating cell proliferation through activation of STAT3 and STAT5. Also activates JAK2 (By similarity).
Decorin	DCN	T-cell activation		Involved in fibrogenesis, primary function of decorin involves regulation during the cell cycle
T-cell receptor associated transmembrane adaptor 1	TRAT1	T-cell activation		Stabilizes the TcR/CD3 complex at the surface of T-cells
Pre T-cell antigen receptor alpha	PTCRA	T-cell activation	PT-a	Is a single-pass type I membrane protein that is found in immature but not mature T-cells. Along with TcR-beta and CD3 complex, forms the pre-T-cell receptor complex, which regulates early T-cell development
Transforming growth factor, beta receptor III	TGFBR3	T-cell activation	BGCAN, betaglycan	Could be involved in capturing and retaining TGF-beta for presentation to the signalling receptors. Often functions as a co-receptor with other TGF-beta receptor superfamily members. Ectodomain shedding produces soluble TGFBR3, which may inhibit TGFB signalling
Triggering receptor expressed on myeloid cells-like 2	Treml2	T-cell activation	TREML2P	Acts as a counter receptor for CD276 and interaction with CD276 on T-cells enhances T-cell activation
Adherens junctions associated protein 1	AJAP1	Unknown, adhesion	MOT8, SHREW1	Plays a role in cell adhesion and cell migration.
Growth arrest-specific 6	GAS6	Unknown, adhesion	AXSF, AXLLG	Ligand for tyrosine-protein kinase receptors AXL, TYRO3 and MER whose signalling is implicated in cell growth and survival, cell adhesion and cell migration
G protein-coupled receptor 65	GPR65	Unknown, apoptosis	TDAG8	Receptor for the glycosphingolipid PSY and several related glycosphingolipids. May have a role in activation-induced cell death or differentiation of T-cells
Bone morphogenetic protein 6	BMP6	Unknown, secreted, development	VGR, VGR1	Secreted family of proteins that induce cartilage and bone formation part of the TGF-beta family.

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Appendix table 8.5. Genes Upregulated in Latency Non-Inducing Cells, pDC, Measured by Microarray.

Using the bioinformatics databases DAVID, GeneCards and GeneCodis, gene expression compartment and function was determined. Genes expressed on pDC cell-surface with the ability to signal to T-cells were shortlisted.

Gene	Gene Name	Other names	Type of interaction	Ligand	Description
CD69	CD69 molecule		Activation		Early activation marker in T-cells, also involved in egress of T-cells from LN, lymphocyte proliferation and functions as a signal transmitting receptor in lymphocytes.
CD83	CD83 molecule	BL11, HB15	Activation	Sialic acids	Upregulated on DC upon DC activation. Acts as a co-stimulator for T-cell activation. Part of the SIGLEC family [65]
HLA-C	Major histocompatibility complex, class I, C		Activation	TcR, BcR	MHC-I
HLA-E	Major histocompatibility complex, class I, E		Activation	TcR, BcR	MHC-I
HLA-F	Major histocompatibility complex, class I, F		Activation	TcR, BcR	MHC-I
HLA-G	Major histocompatibility complex, class I, G		Activation	TcR, BcR	MHC-I

			Activation?		
LILRB1	Leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 1		Inhibition	MHC-I	MHC-I receptor. Recognizes HLA-A, HLA-B, HLA-C and HLA-G alleles. MHC-I binding results in inhibition of target cell activation and down-regulation of the immune response. Engagement of LILRB1 present on natural killer cells or T-cells by class I MHC molecules protects the target cells from lysis.
LY6E	Lymphocyte antigen 6 complex, locus E	RIGE, SCA2, RIG-E, SCA-2, TSA-1	Activation/ Inhibition		Activated by IFN-I's, inhibition of TcR activation [66]
AMICA1	Adhesion molecule, interacts with CXADR antigen 1		Adhesion	CXADR	AMICA1-CXADR interaction leads to proliferation and production of cytokines and growth factors by T-cells, which in turn stimulate epithelial tissue repair. Enhances myeloid leukemia cell adhesion to endothelial cells. Upregulated on stimulation of cell. Signaling through PI3- and MAP kinases in gamma-delta T-cells.
CD48	CD48 molecule	BLAST-1, SLAMF-2	Adhesion	CD2	Facilitate interaction between activated lymphocytes, probably involved in regulating T-cell activation.
GP1BA	Glycoprotein Ib (platelet), alpha polypeptide		Adhesion	A1 domain of vWF	Adheres to platelets and ECM. Involved in platelet plug formation binding other platelet cells.
NINJ1	Ninjurin 1		Adhesion		Homophilic cell adhesion molecule that promotes axonal growth.
FAT3	FAT tumour suppressor homolog 3 (Drosophila)		Adhesion/activation		Member of the cadherin superfamily, likely functions as a cell adhesion molecule, controlling cell proliferation and playing an important role in cerebellum development.

GRAMD1A	GRAM domain containing 1A		Unknown	
TMEM186	Transmembrane protein 186		Unknown	
TMEM50B	Transmembrane protein 50B		Unknown	
TMEM52	Transmembrane protein 52		Unknown	
VANGL1	Vang-like 1 (van gogh, Drosophila)	LPP2, STB2, STBM2, KITENIN	Unknown	Part of tetraspanin family.
GZMA	Granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3)		Soluble factor - apoptosis	Necessary for target cell lysis in cell-mediated immune responses, involved in apoptosis. Cleaves after Lys or Arg.
TNFSF10	Tumour necrosis factor (ligand) superfamily, member 10		Soluble factor - apoptosis	TNFRSF10A/ TRAILR1, TNFRSF10B/ TRAILR2, TNFRSF10C/ TRAILR3, TNFRSF10D/ TRAILR4, TNFRSF11B/ OPG — Can activate cell death in target cell
CCL3	Chemokine (C-C motif) ligand 3		Soluble factor - Chemokine	CCR1, CCR4, CCR5 — One of the major HIV-suppressive factors produced by CD8+ T-cells. Inhibitor of HIV-1-infection by binding CCR5.

Symbol	Name	Alias	Type	Receptor(s)	Function
CCL3L3	Chemokine (C-C motif) ligand 3-like 3	CCL3L1	Soluble factor - Chemokine	CCR1, CCR3, CCR5	Inhibitor of HIV-1-infection by binding CCR5.
CCL4	Chemokine (C-C motif) ligand 4	MIP-1-beta	Soluble factor - Chemokine	CCR1, CCR5, CCR2	Inhibitor of HIV-1-infection by binding CCR5.
CCL5	Chemokine (C-C motif) ligand 5	RANTES	Soluble factor - Chemokine	CCR1, CCR3, CCR4, CCR5	Inhibitor of HIV-1-infection by binding CCR5.
CXCL10	Chemokine (C-X-C motif) ligand 10		Soluble factor - Chemokine	CXCR3	Attracts T-cells and monocytes.
IL8	Interleukin 8	CXCL8	Soluble factor - Chemokine	CXCR1, CXCR2	Promotes angiogenesis, involved in migration and phagocytosis during inflammation, induced by TLR stimulation.
TNFSF13B	Tumour necrosis factor (ligand) superfamily, member 13b	APRIL	Soluble factor - Cytokine	TNFRSF13B/ TACI, TNFRSF17/B CMA	involved in the stimulation of B-and T-cell function and the regulation of humoral immunity. Induced upon interferon-gamma stimulation.
TNF	Tumour necrosis factor		Soluble factor - Cytokine	TNFRSF1A/T NFR1, TNFRSF1B/T NFBR	Pro-inflammatory cytokine
IFNA1	Interferon, alpha 1		Soluble factor - Cytokine, anti-viral		Stimulates antiviral activities.
IFNA10	Interferon, alpha 10		Soluble factor - cytokine, anti-viral		Stimulates antiviral activities.
IFNA13	Interferon, alpha 13		Soluble factor - cytokine, anti-viral		Stimulates antiviral activities.
IFNA16	Interferon, alpha 16		Soluble factor - cytokine, anti-viral		Stimulates antiviral activities.
IFNA17	Interferon, alpha 17		Soluble factor - cytokine, anti-viral		Stimulates antiviral activities.

IFNA2	Interferon, alpha 2		Soluble factor - cytokine, anti-viral	Stimulates antiviral activities. Used as an anticancer drug for its anti-proliferative activity.
IFNA21	Interferon, alpha 21		Soluble factor - cytokine, anti-viral	Stimulates antiviral activities.
IFNA4	Interferon, alpha 4		Soluble factor - cytokine, anti-viral	Stimulates antiviral activities.
IFNA5	Interferon, alpha 5		Soluble factor - cytokine, anti-viral	Stimulates antiviral activities.
IFNA6	Interferon, alpha 6		Soluble factor - cytokine, anti-viral	Stimulates antiviral activities.
IFNA7	Interferon, alpha 7		Soluble factor - cytokine, anti-viral	Stimulates antiviral activities.
IFNA8	Interferon, alpha 8		Soluble factor - cytokine, anti-viral	Stimulates antiviral activities.
IFNW1	Interferon, omega 1		Soluble factor - cytokine, anti-viral	Inhibits proliferation of cell lines, related to IFN-a/b and IFN-y [67]. Induced by DNA and RNA viruses, including FIV [68]. Stimulates antiviral activities.
IL28B	Interleukin 28B (interferon, lambda 3)	IFN-lambda3	Soluble factor - cytokine, anti-viral	IL10RB, IL28RA	Stimulates antiviral activities. Up-regulates MHC class I antigen expression. Displays antitumor activity. Signals through the Jak-STAT pathway. Induced by viral infections or double stranded RNA.
IL29	Interleukin 29 (interferon, lambda 1)	IFN-lamda1	Soluble factor - cytokine, anti-viral	IL10RB, IL28RA	Stimulates antiviral activities. Up-regulates MHC class I antigen expression. Displays antitumor activity. Signals through the Jak-STAT pathway. Induced by viral infections or double stranded RNA.

| BMP2 | Bone morphogenetic protein 2 | Soluble factor - Growth factor (bone) | Induces cartilage and bone formation. Involved in the hedgehog pathway, TGF beta signalling pathway, and in cytokine-cytokine receptor interaction. |
| CARHSP1 | Calcium regulated heat stable protein 1, 24kDa | Soluble factor - regulated mRNA | Binds mRNA and regulates the stability of target mRNA |

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Appendix figure 8.3.Comparison of Genes Differentially Upregulated on HIV-1 Treated pDC with RNA-Seq Gene Expression on mDC and Monocytes.

Genes that were found to be differentially upregulated on HIV-IIIB treated pDC, using microarray were compared on RNA-seq data from myeloid lineage APC. RNA from freshly isolated myeloid cells, including CD1c^{+} mDC, SLAN^{+} DC, CD14^{+} monocytes, and pDC were isolated for RNA-seq analysis. Genes that had low expression were not included.