A systems analysis of dendritic cell interactions with HIV-1 during cell-cell transmission: a focus on cytokines, chemokines and receptors

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Summary

The propagation of HIV-1 is driven by mechanisms of innate immune evasion, a phenomenon observed in dendritic cells (DCs) and their subsets, one of the earliest cell types likely to encounter HIV-1 during acute infection. Infected DCs are capable of highly effective viral transfer to target CD4+ T-cells across the virological synapse (VS), a specialised virus-induced cell junction, which enables rapid viral dissemination and accelerates disease progression. Previous studies have implicated a major role for cytokines and chemokines in the infection of DC subsets, though their functions are yet to be fully characterised.

Integrative, data-driven approaches to disease biology have become central to understanding systems-level processes. In this study, we used high-throughput RNAi screening techniques using the On-Target SMARTpool cytokine/chemokine siRNA library of 319 genes to screen for the differential effects on HIV-1 viral transfer from monocyte-derived DCs to CD4+ T-cells.

Our screen highlights a potent restrictive role for the cytokine-mediator protein, Macrophage Migration Inhibitory Factor (MIF) during HIV-1 *trans*-infection. The activities of MIF were validated using three key loss-of-function assays including genetic downregulation, neutralisation by biologics and pharmacological inhibition in *trans*- to both SupT1 cell lines and autologous CD4+ T-cells.

Further investigations found that MIF regulates autophagy in MDDC which has been previously been described as a protective mechanism against infection by HIV-1. Loss of MIF was associated with impaired LC3+ autophagosome formation, leading to intracellular accumulation of virus and enhanced capacity for cell-cell transfer. These findings bridge an important gap between the cytokine network and autophagy which will inform therapeutic strategies against HIV-1 infection and transmission.

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Abbreviations

4-IPP	4-Iodo-6-phenylpyrimidine
Ab	Antibody
AIDS	Acquired immunodeficiency syndrome
AMPK	AMP activated protein kinase
APC	Antigen-presenting cell
ATG	Autophagy-related
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle medium
CA	HIV-1 Capsid
CD	Cluster of differentiation
CQ	Chloroquine
DC	Dendritic cell
DC-SIGN	DC-specific intracellular adhesion molecule-3-grabbing non-integrin
DMSO	Dimethylsulphoxide
Env	HIV-1 envelopse
ER	Endoplasmic reticulum
FR	Far Red CellTrace dye
Gag	HIV-1 group specific antigen
GO	Gene Ontology
HDF	Host dependency factor
HIV	Human immundeficiency virus
HT (HTS)	High-throughput (screening)
IFN	Interferon
IL	Interleukin
IMDM	Iscove's modified Dulbecco's medium

LAP	LC3-associated phagocytosis
MACS	Magnetic-activated cell sorting
MAD	Median absolute deviation
MAP1LC3 (LC3)	Microtubule-associated proteins 1A/1B light chain 3B
nAb	Neutralising antibody
MDDC	Monocyte-derived dendritic cell
MIF	Macrophage migratory inhibitory factor
mTOR	Mammalian target of rapamycin
p24	HIV-1 p24 capsid protein antigen
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
Pol	HIV-1 polymerase
PRR	Pattern-recognition receptor
R5	CCR5-tropic HIV-1
Rap	Rapamycin
RPMI	Roswell Park Memorial Institute medium
RNA	Ribonucleic acid
RNAi	RNA interference
RT	Reverse Transcriptase
SD	Standard deviation
SE (SEM)	Standard error (of the mean)
siRNA	Small interfering ribonucleic acid
TGF	Transforming growth factor
TNF(SF)	Tumour necrosis factor (superfamily)
TWEAK	TNF-Related WEAK Inducer Of Apoptosis
VCC	Virus-containing compartment
X4	CXCR4-tropic HIV-1

Chapter 1

General introduction

1.1 HIV/AIDS: The global pandemic

The HIV/AIDS pandemic is nearly in its fourth decade, which is estimated to have claimed 32 million lives to date (UNAIDS, 2019). Currently, there are 36.7 million people who live with HIV worldwide, which has resulted in a total of 1.7 million new cases of infection and responsible for 770,000 AIDS-related deaths in 2018 alone (UNAIDS, 2019). Among many economically developed countries, HIV/AIDS can be effectively managed by antiretroviral treatment (ART), however ensuring that these therapies are accessed remains a key public health challenge. Global ART coverage has made significant progress over the current decade, where 62% of all HIV-infected individuals are now accessing treatment (UNAIDS, 2019). However, there is still room for improvement and an urgent need for further development of new, effective and accessible therapies to achieve greater ART coverage.

In the UK, it is estimated that 101,600 are currently living with HIV, which is most prevalent among gay, bisexual men and other men who have sex with men (MSM). Results from the Department of Health and Social Care suggest that the number of people living with HIV and the rate of new HIV acquisitions have been in steady decline over the past 4-5 years. But despite the efforts to provide combination prevention measures against transmission and encouraging

the uptake of HIV testing, the proportion of infected individuals unaware of their status has remained high over a number of years with 13% of people being undiagnosed in 2016 (Kirwan et al., 2014, 2016). Increasing uptake of HIV testing and the awareness of infection status are therefore critical components in the national strategy to ending the UK epidemic. Without effective policies, health surveillance and pre-exposure prophylaxis, the potential of wide-spread HIV transmission remains a hidden and significant threat to public health.

1.1.1 Clinical stages of HIV-1 infection

HIV/AIDS is characterised by a chronic decline in T-helper CD4+ T cells, the primary target cell in which HIV-1 preferentially binds and replicates within. The loss of effector CD4+ T-cells manifests into an inability to stimulate an effective immunogenic response capable of eradicating pathogens, leading to an array of late-complications. Acute HIV-1 infection presents with flu-like symptoms within two weeks post-infection and is often in parallel with rapid and extensive CD4+ cell depletion. Seroconversion (the period in which anti-HIV antibodies develop and becomes detectable) is typically between 2-8 weeks post-infection, where 70% of individuals will show symptoms of acute infection. A period of clinical latency occurs where, although asymptomatic, individuals enter a stage of chronic infection associated with gradual depletion of CD4 cells over an average course of 10 years. Infection subsequently progresses into AIDS, defined by a CD4 count of 200 cells/mm^3 or less, where individuals become vulnerable to potentially fatal opportunistic infections.

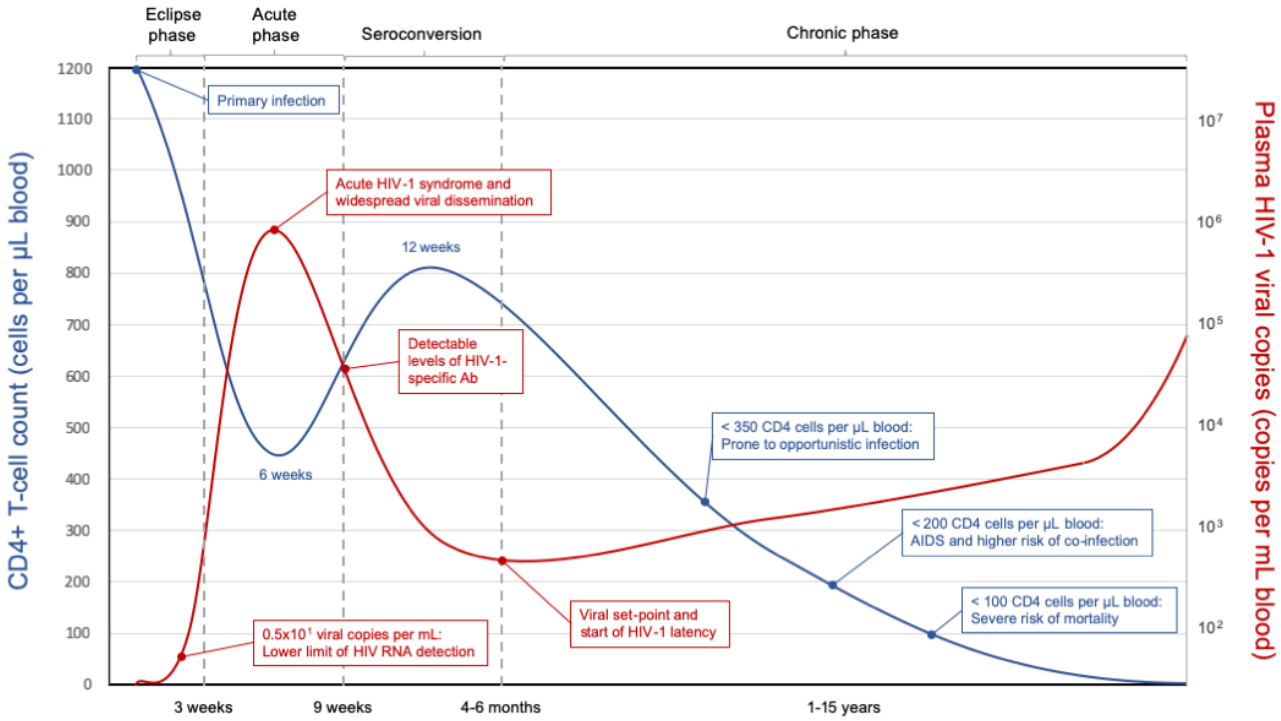

Figure 1.1: Clinical stages of HIV-1 infection. Following initial exposure at the mucosa and viral dissemination to lymphoid tissues, during the eclipse phase, HIV-1 RNA levels increase exponentially and this results in rapid, widespread CD4+ T-cell depletion. At this point, a viral reservoir is already established. Acute phase infection presents with flu-like symptoms with peak viraemia and CD4 count at a 6-week low post-infection. Cell-mediated immunity and seroconversion, the generation of HIV-specific antibodies, shortly follows and suppresses viraemia efficiently, although not entirely. Residual HIV-1 remains latent within the viral reservoir, reflected by the viral set-point. Over the course of several years, progressive HIV-1 reactivation leads to a steady decline in CD4 count and the heightened risk of HIV-associated immunodeficiencies, opportunistic infection and AIDS. Adapted from (Deeks et al., 2015)

In rare cases, some patients are able to maintain immune responses against infection and control viraemia and can remain asymptomatic in the absence of treatment. These individuals are often referred to as viral controllers, who maintain low level viraemia, or elite controllers who maintain viraemia below the lower limit of clinical detection and together represent approximately 1% of the general population (Cockerham and Hatano, 2015). Both typically fall within the group of long-term non-progressors who achieve long term clinical and immunological stability defined by high level CD4+ count for more than 10 years post-infection (Palacios et al., 2012).

1.1.2 Management of HIV/AIDS

Previous NICE guidelines regulating clinical practice in the UK, recommended the use of rapid point-of-care tests (POCT) and third-generation testing as a first-line HIV-1 diagnostic in the UK. POCT tests provide results within minutes by capillary (finger-prick) sampling to test for the presence of HIV-specific antibodies, however these tests are less specific and sensitive with false positive readings that occurs once in every 142 tests that are performed. In light of this, government recommendations suggest that all positive results are confirmed in clinical laboratories using an Enzyme Linked Immunosorbent Assay (ELISA) based approaches, which are more effective at detecting low concentrations of anti-HIV-1 antibodies. Since 2016, new guidelines were issued by Public Health England (PHE) with the aim of improving HIV-1 testing uptake in primary and secondary care. A faster fifth-generation HIV-1 test is now available, which reduces the negative-window of detection from 6 weeks in older generation tests to 11–14 days post-infection: it is a double sandwich-ELISA platform for the detection of both anti-HIV-1 antibodies and presence of serum HIV-1 p24 core antigen protein, a well established biomarker of disease progression (Alexander, 2016; NICE, 2016).

Pre-exposure prophylaxis with emtricitabine with tenofovir disoproxil (Truvada) along with safer sex practices are recommended among high risk individuals by the British Association for Sexual Health and HIV. However in confirmed primary HIV-1 infection, current NICE guidelines suggest HIV-1 specialist referral and to offer immediate combination antiretroviral therpay (cART). In addition, cART should only be initiated when the infected individual feels ready to do so as non-adherence risks the development of drug resistance in individuals where cART is started too early. However, trial data strongly recommends rapid initiation of treatment which can stabilise CD4+ cell count and significantly reduce viral load and set-point (Violari et al., 2008; Group, 2015). Treatment regimens are dependent on certain considerations including CD4+ count, clinical symptoms, co-morbidities and possibility of adverse reactions. Combination anti-retroviral therapy is usually initiated with two nucleoside reverse transcriptase inhibitors (NRTI) and either: a non-nucleoside reverse transcriptase (NNRTI), an integrase or

protease inhibitors. In specific cases, entry and fusion inhibitors including the CCR5-antagonist Maraviroc® may be prescribed conditional to tropism confirmation (Churchill et al., 2016; NICE, 2016).

Whilst there is no cure for HIV-1 infection, the current strategies aim to encourage early uptake of cART, preventing uncontrolled viral replication and maintaining suppression over the patient's lifetime. Advances in therapeutics and management has seen marked efficacy in extending the number quality-adjusted life years (QALY), allowing infected individuals to lead near-normal life expectancies during uninterrupted cART. But despite being effectively managed across many western societies, access to affordable treatment is limited among less economically developed countries. In 2014, UNAIDS announced an ambitious target to end the AIDS pandemic by 2020, creating the 90-90-90 continuum of care which aims for 90% of those living with HIV-1 to be diagnosed, 90% of which to be on ART treatment and a further 90% of that population to have viral suppression below the limit of detection. At the time of publication, no single country had met these criteria (UNAIDS, 2014). However, the latest global data now shows that the UK, along with several other individual countries, have now met the 90-90-90 targets (Nash et al., 2018). But while significant progress has been made over the past 5 years, the global rate of new HIV-1 acquisitions still remains high, showing little signs of improvement and continues to present a key barrier to success. With just one year to go before the 2020 deadline, it is clear that the UNAIDS target is attainable but whether it will be achieved, particularly in less economically developed countries and among key populations, will depend on global preventative strategies against HIV-1 transmission.

1.2 HIV-1 structure and genome

HIV-1 is a RNA retrovirus comprising of a 9.7kb genome encoding a total of fifteen viral proteins, read across three open reading frames (ORF). The HIV-1 genome can be broadly classified into three distinct areas of function which encode structural, regulatory and accessory

proteins. Structural genes consist mostly of three key viral genes: Gag (group specific antigen), Pol (DNA polymerase) and Env (envelope) which are conserved across all retroviral species. These genes make up 60% of the proteins encoded by the virus while the remaining 40% is comprised of non-structural viral accessory proteins Vif, Nef, Vpu, Vpr, Rev and Tat which are involved in enhancing virulence, replication and immune evasion (section 1.5.2).

The viral genome is protected within the viral capsid (CA), a cone-shaped protein shell, which facilitates the delivery of viral RNA into host cells. A fully mature CA is built from a lattice of p24 proteins which fold and link to form ring domains containing either five or six units. A single CA contains approximately 250 hexamer proteins and exactly 12 pentamers with 7 located at the head of and 5 at the tail of the cone (Pornillos et al., 2009). More recently, X-ray crystallography studies of CA hexamers found that they function as dynamic pores to allow import of deoxynucleoside triphosphates (dNTP) to facilitate reverse transcription before uncoating (Jacques et al., 2016).

The viral envelope is a trimeric glycoprotein comprising gp120 and gp41 subunits which protrude from the outer membrane of the virus containing five conserved domains and five variable loop domains. During productive infection, entry into the host cell is mediated via interaction between gp120 and the target receptor CD4 and co-receptor (CCR5 or CXCR4) (Kwong et al., 1998), though other attachment factors are known to exist as described in 1.2. The localisation as a surface glycoprotein and roles in cell fusion and entry make env the primary rational target for vaccine design and development, however HIV-1 vaccines have faced many obstacles over high mutation rates and lack of vaccine immunogenicity (Wang et al., 2006). Currently, the RV144 trial is the only successful vaccine that provided modest protection against HIV-1 acquisition (Rerks-Ngarm et al., 2009).

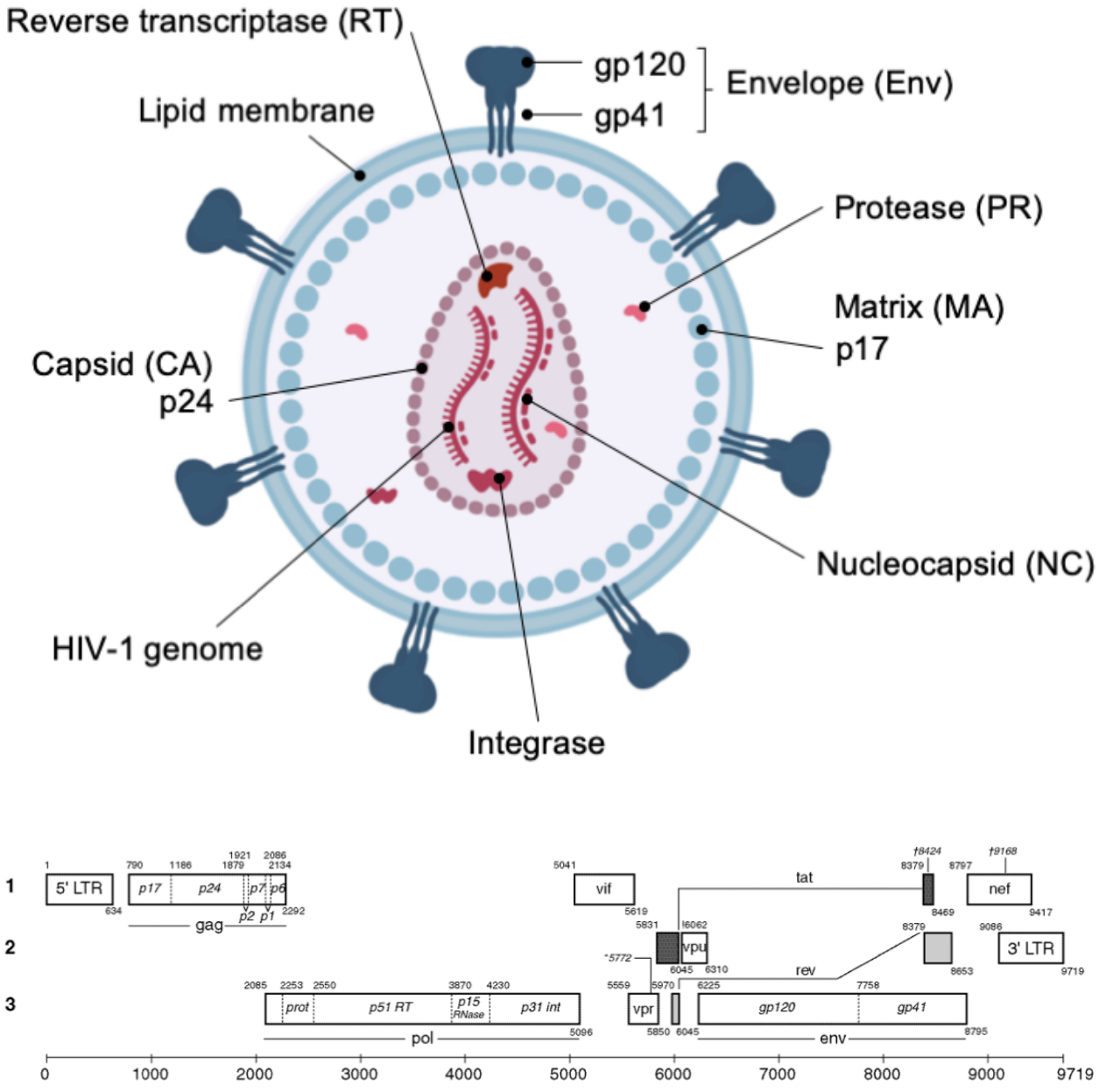

Figure 1.2: Structure and genome of HIV-1. A) As part of the *Retroviridae* family, HIV-1 are enveloped particles with its RNA contained within an inner capsid. B) Nine genes make up the HIV-1 genome: Gag, Pol and Env encode the structural polyprotein components of the virion; tat and rev are key regulators of transcription; vpu, vpr, vif and nef are the accessory proteins involved in immune evasion.

ORFs are continuous sequence regions between start/stop codons and multiple reading frames are biologically significant in enabling the relatively small HIV-1 genome to produce multiple

viral proteins from a single mRNA strand – a feature particularly important in the production of the gag-pol fusion protein, which is translated through a process termed -1 ribosomal frameshifting at the point of partial overlap between two coding regions. The ratio of gag:gag-pol is tightly regulated in the HIV-1 life cycle, resulting in a 20:1 (or 5%) production of gag-pol (Shehu-Xhilaga et al., 2001). Given that pol can only be produced as a fusion protein with gag, translating ribosomes must therefore be capable of switching reading frames. The ribosome frameshift site is therefore significant in this process and is comprised of two elements: the slippery sequence (UUUUUUA) and a *cis*-coupled RNA stem-loop. As a ribosome reaches the slippery sequence, 5% of ribosomes slip backward by a single nucleotide whilst the RNA stem-loop unwinds and allows the translation to continue along the pol reading frame (Hung et al., 1998; Biswas et al., 2004). The regulated control of HIV-1 gag/gag-pol production is important for optimal incorporation of viral enzymes required for efficient viral infectivity and replication (Dulude et al., 2006)

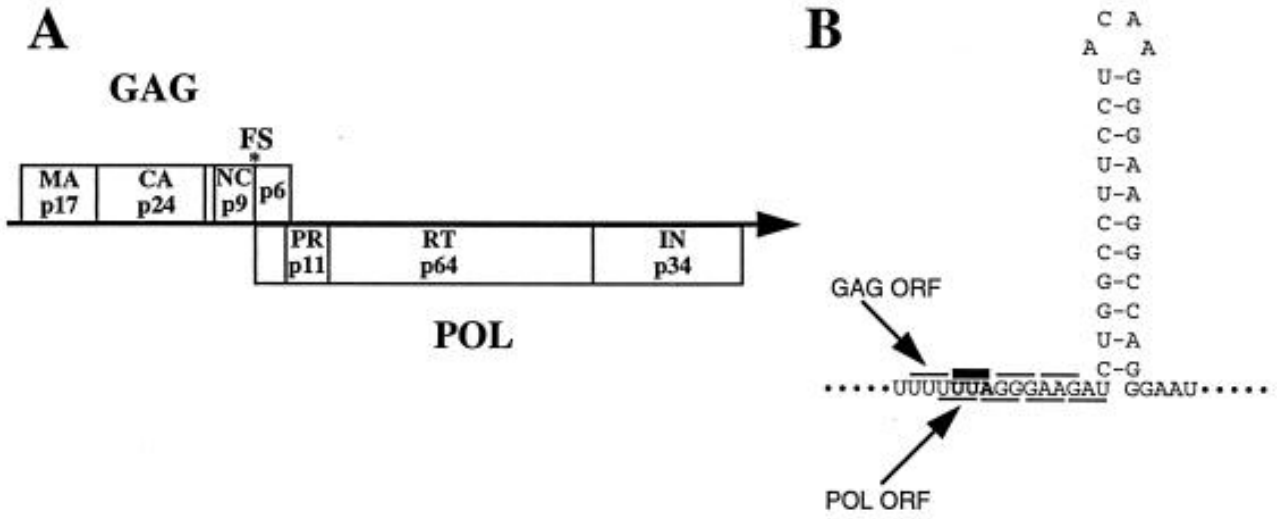

Figure 1.3: Programmed -1 ribosomal frameshift. A) An illustration of the HIV-1 frameshift site and overlapping gag and pol open reading frames (ORF). B) The UUUUUUA slippery sequeunce and RNA stem-loop which stimulates the -1 frameshift

1.3 HIV-1 life cycle

Attachment, fusion and entry — The first stage of productive life cycle describes the attachment, fusion and entry into the host cell which is primarily mediated by initial gp120-CD4 interaction. When virus comes into close association with the host membrane, target CD4 receptor binds to gp120 causing conformational rearrangement in the variable loop domains V1 and V2 to allow co-receptor engagement with the gp120 V3 loop (Tamamis and Floudas, 2013). Co-receptor utilisation is used to define the tropism of the HIV-1 strain with CCR5-utilising viruses termed R5 HIV-1, CXCR4-utilising as X4 HIV-1 and lastly R5X4 HIV-1 which have dual tropism. Upon engagement with the corresponding co-receptor, further structural rearrangement of the Env gp120 occurs which exposes the gp41 trimer to the target cell membrane. Viral gp41 contains a highly hydrophobic fusion peptide which inserts into the host cell membrane to tether the virus to the host. Following attachment, formation of a six-helix bundle (6HB) fusion complex occurs, which leads to pore formation in the host membrane and the delivery of the viral capsid into cytosol (Chan et al., 1997; Melikyan, 2008; Wilen et al., 2012).

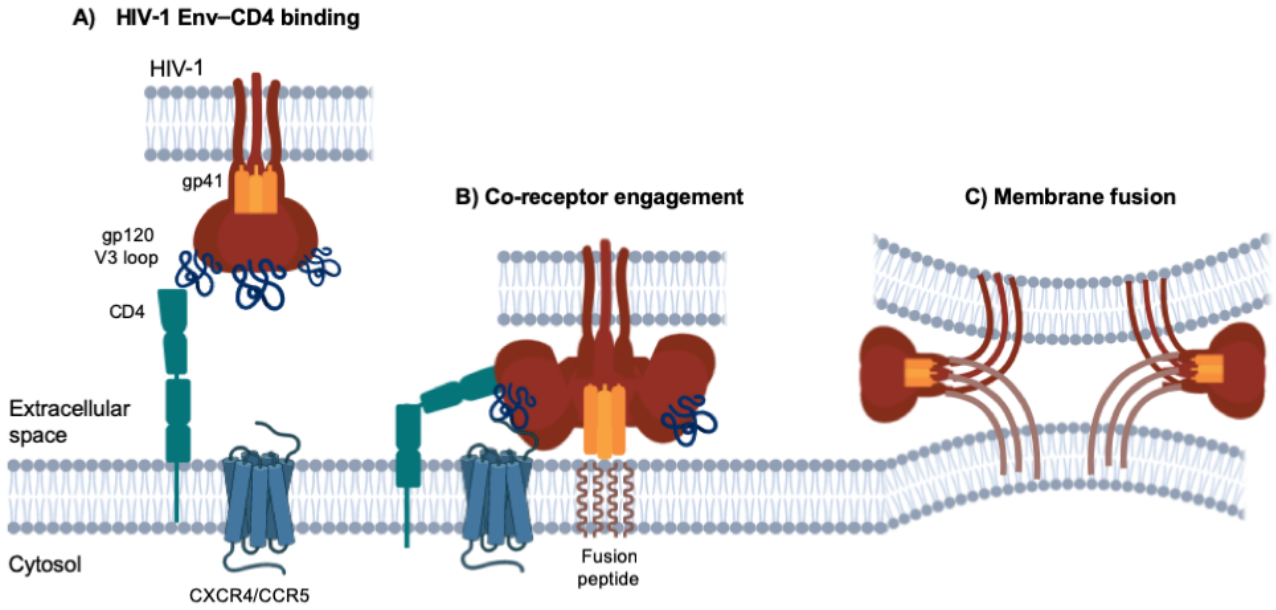

Figure 1.4: HIV-1 attachment and fusion with the host membrane. A) Target CD4 interacts with the HIV-1 Env gp120 V3 variable loop domain. B) Conformational rearrangement of the Env trimer allows engagement of the co-receptor with gp120 and exposes gp41 fusion peptides which insert into the host membrane. C) Multiple Env proteins tether viral and host membranes leading to fusion and successful viral entry. Adapted from (Wilen et al., 2012)

Naturally, viral entry receptors were identified as important therapeutic targets in the development of HIV-1 antiretrovirals. As previously mentioned, co-receptor inhibition has been one of several strategies and Maraviroc^{TM} is currently authorised for EU use as a CCR5 antagonist, effectively suppressing R5 HIV-1 RNA levels and maintaining CD4+ T-cell survival (Gulick et al., 2008). However attempts to develop inhibitors for the more pathogenic X4-tropic strain have been less successful and there are currently no authorised CXCR4 antagonists available for use in HIV-1 infection. Early *in vitro* findings showed that treatment with the *de facto* CXCR4 substrate, CXCL12, effectively reduces infection by X4 HIV-1 (Oberlin et al., 1996), but despite this CXCR4 antagonists could not address primary infections by R5-tropic strains which account for the majority of cases. AMD3100 (now plerixafor) was one of the earliest small molecule compounds against CXCR4 which showed anti-viral potency *in vitro*, suppressing viral replication, production and preventing syncytium formation (De Clercq et al., 1994). However phase 1 clinical trials highlighted issues of poor oral bioavailability and development

was subequently discountinued (Hendrix et al., 2000; Hatse et al., 2002), though CXCR4 antagonists continue to be of interest in chronic infections and prevention of disease progression. Another strategy against viral entry is to block fusion. Currently, the NHS recommends enfuvirtide as the only authorised fusion inhibitor for indicated use in patients unresponsive to other antiretroviral combinations.

Reverse Transcription — Following the fusion and release of viral contents into the host, CA uncoating and reverse transcription occur to convert single-stranded viral RNA into double-stranded DNA facilitated by the virally encoded reverse transcriptase (RT) enzyme. However, the stage at which reverse transcription and uncoating occurs is still debated. While most findings previously supported a model where reverse transcription follows capsid uncoating, there is enough evidence to suggest that RT occurs within the viral core and that intact CA is capable of reaching the nuclear membrane (Arhel et al., 2007) in order to prevent premature uncoating and immune detection by innate sensing (Rasaiyaah et al., 2013). In addition, recent structural studies of the HIV-1 hexamer protein revealed that they are capable of acting as dynamic pores to control the influx of host-cytosolic nucleotides for reverse-transcription as it traverses the cytoplasmic compartment (Jacques et al., 2016).

RT inhibitors represent one of the first line of anti-retrovirals developed against HIV-1 infection and come in two major drug classes: nucleoside/nucleotide analogue (NRTI) and non-nucleoside (NNRTI) inhibitors, which block reverse transcription by inhibiting RT either competitively as a substrate or by indirect mechanisms, respectively. However, NNRTI treatment is not effective against infection by HIV-2 due to structural difference in the NNRTI binding sites compared with HIV-1 RT and studies suggest that changing RT conformation could serve as a potential mechanism for drug resistance (De Clercq, 1998; Ren et al., 2002).

Import and integration — Like many retroviral species, HIV-1 relies on integration of the viral cDNA into the host genome for continued viral production or latent infection. Following CA uncoating, formation of a reverse transcription complex (RTC) containing viral proteins (MA, CA, NC, IN, Vpr and tRNA primers) occurs at close proximity to the nuclear membrane

(Charneau et al., 1994) and matures into a pre-integration complex (PIC). Import into the nucleus is mediated by Vpr-dependent interaction with the nuclear pore complex (NPC) (Popov et al., 1998) allowing passage of the PIC into the nucleus (Arhel et al., 2007; Ocwieja et al., 2011). During integration, the host DNA is cleaved and the 3' end of viral DNA strands are simultaneously transferred to the host 5' end in a single-step *trans*-esterification reaction (Engelman et al., 1991).

The viral integrase enzyme (IN), encoded by HIV-1 Pol and incorporated into the PIC, has a number of roles including PIC stablisation and as a catalyst of 3'-processing and strand transfer of viral cDNA as it inserts into the host genome. There are currently three licensed integrase inhibitors available: dolutegravir, elvitegravir and raltegravir which bind to the IN active site blocking strand transfer, integration and viral propagation.

Replication — After successful integration, the HIV-1 genome can fully exploit the transcriptional and translational machinery of its host to produce new viral proteins. At this stage, the HIV-1 accessory proteins Tat and Rev significantly enhance the efficiency of virus replication and are required for transcriptional elongation and export of viral transcripts, respectively. (Malim et al., 1989; Engelman and Cherepanov, 2012). Pharmacological therapies become much more limited beyond post-integration infection, which marks entry into the late-stage of viral life cycle. However, since Rev-mediated mRNA transport was found to be required during replication and detectable at the early stages of replication, it was proposed as a suitable target for antiviral therapy (Reddy et al., 1999).

Assembly, budding and release — After mRNA export and translation of viral proteins in cytoplasm, newly synthesised Gag/Gag-Pol polyproteins bind viral genomic RNA and are trafficked to the assembly point at the plasma membrane where Gag is anchored via the MA-containing domain, multimerises to form a lattice layer within the budding virion and incorporates Env into the outer membrane. The trafficking pathway regulating the recruitment of viral proteins to the plasma membrane is still yet to be defined, although it is known that the endosomal sorting complex required for transport (ESCRT) pathway is exploited by the Gag

p6 domain at the late stages of budding and release through a multi-step cleavage process (Göttlinger et al., 1991; Freed, 2015). Immediately following fission of the viral and host membranes, the HIV-1 maturation process occurs, driven by protease (PR) which cleaves Gag/Gag-Pol to produce the viral proteins and is followed by distinct structural changes and formation of the conical viral capsid, visible under electron microscopy (Li et al., 2000; Zhao et al., 2013).

Protease inhibitors against HIV-1 PR were first authorised in 1995 and has since been used as a integral component of combined therapy and there are currently seven protease inhibitors for licensed use on the NHS. However, long-term treatment with protease inhibitors are often associated with high levels of adverse reactions including metabolic syndrome and new approaches to drug design are still required to reduce off-target binding (Lv et al., 2015). Although there is currently no drug target to block the budding and release of immature virions, investigations into host antiviral activities using microarray screening by Neil and colleagues (Neil et al., 2008) led to the identification of tetherin as an IFN-inducible type II transmembrane protein which blocks release by anchoring virus to the plasma membrane (section 1.5.1).

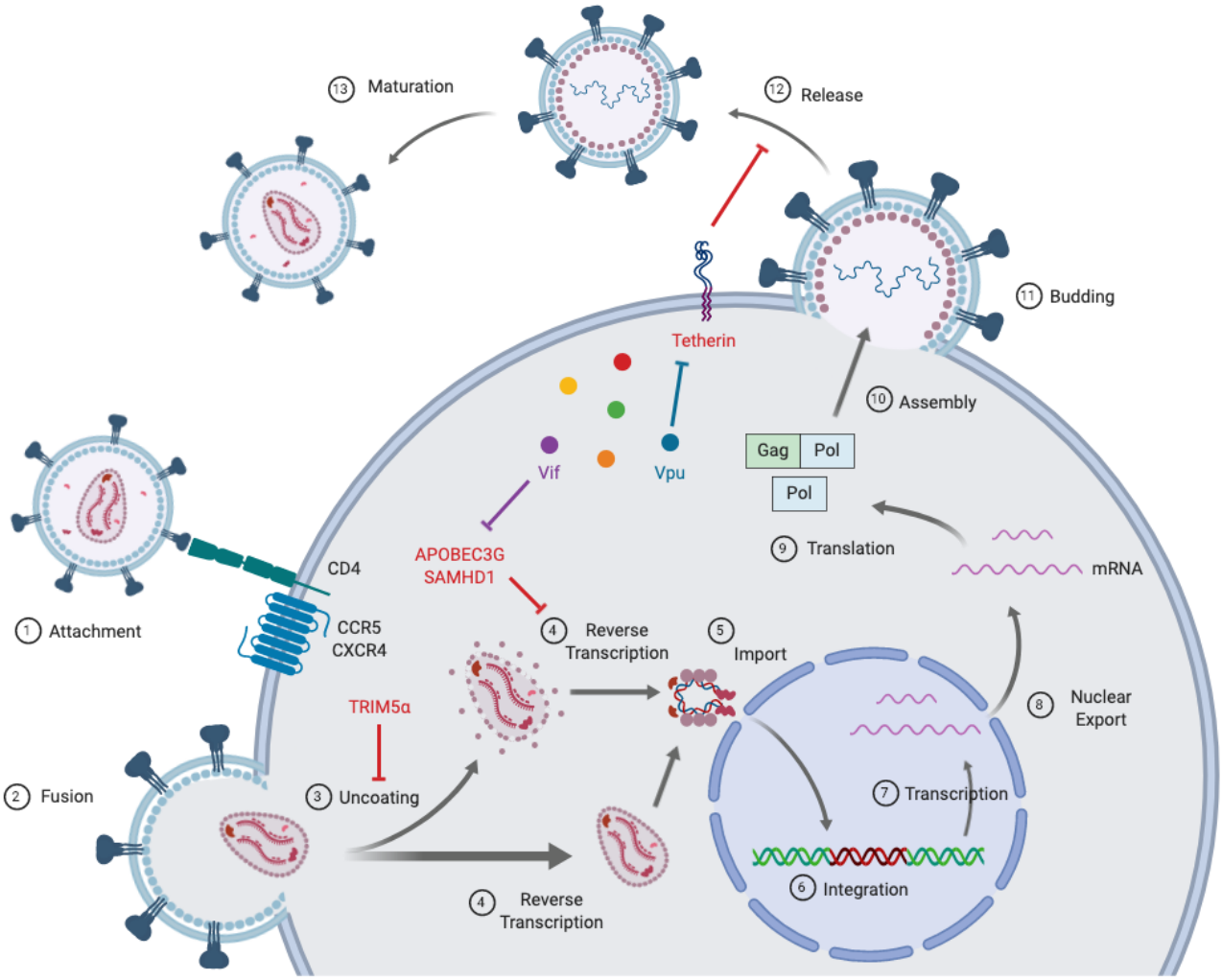

Figure 1.5: HIV-1 life cycle. 1–2) Attachment and fusion of HIV-1 with the CD4/CCR5/CXCR4-expressing target cell. 3–4) Release of capsid into cytosol leads to uncoating for reverse transcription to occur, although this stage is currently debated. Reverse transcription alternatively imports dNTPs through dynamic pores within the CA and fully intact perinuclear CA has been observed. 5) Following uncoating, the pre-integration complex forms at the nuclear pore and is imported into the nucleus. 6–8) HIV-1 integrates into the host genome, enabling transcription of viral mRNA which is exported to cytosol. 9–10) Translation of viral mRNA leads to production of HIV-1 structural polyproteins which are transported to the cell membrane for assembly. Translation of viral proteins helps further suppress host responses. 11–13) After assembly, the virus buds from the host, to release an immature virion which matures for the subsequent round of infection. Adapted from (Engelman and Cherepanov, 2012)

1.4 The innate immune system and HIV-1 infection

The human immune system is under constant challenge by microbial infection and is crucial for maintaining host protection against the external environment. Human immunity has a complex architecture built from two cellular subsystems: innate and adaptive immunity, each providing coordinated responses to preserve short and long-term protection against invasive pathogens.

Innate immunity represents one of the first lines of protection (after physical barriers such as the skin and mucus) against infection by pathogens and typically considered the most primitive and evolutionarily conserved of the two. The innate immune system provides broad, non-specific defence and occurs rapidly from the outset of pathogen detection. Mechanisms of innate defence may include anatomical barriers (skin, mucus membranes, gastric acid), the complement system, leukocyte activation (dendritic cells, macrophages, NK cells, mast cells and others), inflammation and priming of adaptive immunity (Janeway et al., 1996).

Adaptive immunity, on the other hand, refers to a specialised system of immunity which is antigen-specific and crucially features immunological memory against recognised pathogens. Adaptive immunity is initiated following exposure, recognition and presentation of antigenic epitopes to circulating T- and B-lymphocytes, which become activated to produce both cell-mediated and humoral responses, respectively. Conventional T-cells of the adaptive immune system have a specialised T-cell receptor (TCR) which exhibits a diverse immune repertoire and come in four major groups consisting of helper CD4+, cytotoxic CD8+, memory T-cells and regulatory T-cells, each diverging into specialised subsets with unique effector function. Briefly, CD4+ T-cells are activated by MHC-II expressing APCs such as DCs to help promote and enhance immune responses through rapid clonal expansion and the release of a broad range of cytokines to help guide activation, differentiation and proliferation of other T-cell and B-cell populations. CD8+ T-cells are mostly responsible for exerting cytolytic activities against infection by intracellular pathogens and are primed through APC MHC-I cross-presentation of antigens. Memory T-cells are a long-lived subset involved in providing "immunological mem-

ory" which facilitates rapid expansion of effector cells upon re-exposure to a specific antigen. Lastly, regulatory T-cells are homeostatic subsets which promote "immunological tolerance" by blocking cytokine production, cell activation and proliferation to prevent excessive immune reaction and autoimmunity. The adaptive immunity is therefore highly-specific and is shaped by cells of the innate immune system. Unlike the innate system which responds to infection within hours, the adaptive response is slower acting and takes between 4–5 days for clonal expansion of antigen-specific lymphocytes to be complete (Janeway Jr et al., 2001). Although the unique feature of immunological memory is a hallmark of adaptive immunity which provides sustained protection after infection or vaccination.

During acute HIV-1 infection, virus exploits cellular components of the innate immune system to subvert detection for effective propagation to and widespread destruction of target CD4+ T-cells. Adaptive responses that are primed by the infection are "too little and too late" and fail to fully neutralise virus, leaving a residual viral compartment termed the "viral reservoir" which are latent and provide long-term viral survival and replication during the course of chronic infection. In this section we explore these mechanisms further and describe the successful (and unsuccessful) immunological events that arise in response to HIV-1 at the first stages of infection.

1.4.1 Dendritic cell biology and their subsets

First described by Nobel laureate Ralph M Steinman in 1974 (Steinman and Cohn, 1974), dendritic cells (DCs) are specialised antigen presenting cell (APC) which form an important interface between the innate and adaptive immune systems (Banchereau and Steinman, 1998; Piguet and Steinman, 2007). Together with their diverse subsets – including Langerhans cells (LC) – DCs have widespread distribution and are mostly present in skin and mucosa-associated lymphoid tissues (MALT), where they are responsible for the maintenance of the immune microenvironment at mucosal surfaces. The majority of tissue-resident DCs are typically immature, have highly active membrane dynamics and constantly sample their surrounding environment

in search of antigens through a process termed fluid-phase endocytosis (Norbury, 2006).

DCs express a diverse range of surface receptors which aid in the recognition of pathogenic material and soluble antigens. C-type lectin receptors (CLRs) are a large family of surface receptors widely expressed by DCs and detect a broad range of ligands (carbohydrate, non-carbohydrate, lipid and protein) and function as PRRs which signal to help shape DC immune function through internalisation, degradation and presentation (Geijtenbeek and Gringhuis, 2009; Dambuza and Brown, 2015) (described in section 1.5). Following antigenic recognition, DCs undergo a complex maturation process needed to support effective function as APCs. Many models of maturation support a two-step process where *in vivo* DCs firstly mature phenotypically, upregulating surface expression of co-stimulatory molecules MHC-II, CD40, CD80, CD86 and CCR7, although lack functional cytokine secretion. This is followed by a second step of full functional maturation which occurs at draining lymph nodes during T-cell interaction and induces pro-inflammatory cytokine release including IL-1β, IL-6, IL-12 and TNF-α (Hackstein and Thomson, 2004). However, this model has since been revised and it is suggested that DCs enter an intermediate phase termed "semi-mature" DCs which show tolerogenic activities mediated by CD4+ regulatory T-cell interaction and low level production of the anti-inflammatory cytokine IL-10 (Lutz and Schuler, 2002). CCR7+ DCs migrate from the lamina propria to secondary lymphoid sites along chemotactic CCL19 and CCL21 gradients produced by endothelial venules. (Ohl et al., 2004). Lymph nodes are highly populated with B-cells (folicular) and CD4+/CD8+ T-cells (paracortical); once migrating DCs arrive at these sites, they can present peptides through two main mechanisms using major histocompatibility complex (MHC) class I and II proteins.

DCs present antigens to CD4+ T-cells at draining lymph nodes through peptide-bound MHC class II (pMHC-II), which is exclusively expressed among professional APCs (Itano and Jenkins, 2003; Itano et al., 2003). MHC-II is a protein heterodimer first synthesised in the ER, where it exists bound to the invariant chain (CD74). MHC-II-CD74 complex is trafficked through the Golgi apparatus toward the antigen-procesing compartment, where CD74 dissociates to

allow peptide-groove binding and recycling to cell surface for antigen presentation (Roche and Furuta, 2015). During cellular interaction, pMHC-II binds the T-cell receptor (TCR) with high affinity, although additional binding of CD4 to MHC-II is required to achieve a 100-fold higher antigen sensitivity (Janeway et al., 1996; Neefjes et al., 2011).

On the other hand, DCs may cross-present antigen peptides via MHC-I expressed on all nucleated cells to directly prime CD8+ T-cells and cytolytic effector response. Similar to MHC-II, synthesis of MHC-I occurs within the ER and is inactivated by chaperone proteins calnexin, calreticulin and ERp57. However the pathways of intracellular peptide loading differ and can occur through two main routes: cytosolic or vacuolar pathways. The former, follows a path where ingested antigens are exported and processed within cytosol before being transported into the ER for MHC-I loading via TAP transport proteins. The latter, described a mechanism where antigen peptide loading occurs immediately within the phagosome following pathogen degradation, however the origins of MHC-I is still unclear and whether it is exported from the ER prior to loading or recycled from the cell surface is debated (Joffre et al., 2012).

DCs exhibit broad diversity and a number of subsets have been characterised, each with specific tissue distributions and functions which has been largely used to categorise DCs based on their anatomical localisation, morphology and haemopoetic origins. DCs can be broadly grouped into several major classifications including classical (myeloid), plasmacytoid, skin-associated and monocyte-derived subsets, although these are by no means exhaustive.

Myeloid or "classical" – The first main branch of subsets are myeloid DCs which originate from bone-marrow-derived common myeloid progenitors (CMP) and are further subdivided into two types of classical DC, cDC1 and cDC2. The first class of classical DC are typically CD8α, CD1c, CD103 and CD141-expressing and mostly recognised for their high capacity of MHC-I cross-presentation to CD8+ T-cells (Pooley et al., 2001; Allan et al., 2003; Jongbloed et al., 2010). In humans, the majority of cDC1 are distributed in blood but are also found in non-lymphoid tissues and migrate to local draining lymph nodes following activation (Collin and Bigley, 2018). On the contrary, type 2 classical DCs are distinguished by CD11b+ expression

and shape adaptive immunity through priming of CD4 T-cells to promote Th2 and Th17 effector responses during viral, fungal and helminth infection (Minoda et al., 2017). Type 2 cDC typically lack CD103, although its expression is occasionally tissue-dependent and CD103 may be used to distinguish between multiple CD11b+ cDC subsets (Merad et al., 2013; Cancel et al., 2019).

Plasmacytoid DC – Plasmacytoid DCs (pDC) belong to a separate lineage of DCs originating from the common lymphoid progenitor (CLP) and produce high levels of type 1 interferons (IFN) by TLR7 and TLR9 in response to viral infection (Ito et al., 2005). Like cDCs, the core functions and phenotypes of pDCs are mostly conserved and retain their capacity as presenting cells, albeit less efficiently due to lower phagocytic activity (Dalgaard et al., 2005). More recently, some pDC-like DCs were found to originate from common DC progenitors (CGP) most often associated with pre-cDC generation, suggesting alternative developmental pathways for pDC production (Manz, 2018). However unlike cDC subsets, pDCs exhibit distinct morphological differences including a rounded cell body and well-developed ER resembling plasma cell morphology. In addition, the antigen-presentation pathways in pDC are not shared by cDC; MHC-II generation and peptide loading is sustained throughout pDC activation and has higher rates of turnover suggesting that pDC do not retain long-lived pMHC-II complexes (Villadangos and Young, 2008).

Skin-resident DCs – In human skin, DC populations are heterogeneous and several subclasses of tissue-resident DCs have been identified, although the main subsets are CD1c+ dDC, CD141^{high} dermal DC (dDC) and LC. In the dermis, dDC generally assume cDC1 and cDC2-like phenotypes although occasional exceptions in receptor expression phenotypes exist. CD141^{high} dDCs resemble the dermal cDC1 subset and represent a rare minority population in human skin. Dermal cDC1 have CD141^{high} expression and, similar to non-lymphoid CD103+ cDC, are distinguished for their ability to efficiently perform cross-presentation to CD8+ T-cells (Haniffa et al., 2012). On the other hand, CD1c+ dDC correspond with the cDC2 classification and constitute the large majority of the human dDC population. Unlike cDC1, dermal cDC2 can

also be distinguished by CD11b+ CXC3CR1+ expression and some dermal subsets have been shown to express CD14 (Kashem et al., 2017). Lastly, LCs are a unique and specialised DC subset, which are found exclusively in the epidermis where they are spatially distributed and extend dendrites to scavenge antigens across large areas in the epidermis and through epithelial linings to sample from the external environment (Hladik et al., 2007; Romani et al., 2010). Unlike most dDC, LCs are not highly motile, have homogenous phenotypic expression with no further subsets and are distinguished by characteristic high level expression of the CLR langerin within Birbeck granules. Curiously, LCs are the only self-renewing DC subset which undergo mitotic division at steady-state to maintain cell number independently of precursor cells (Merad et al., 2008).

Historically, a distinct subset of dDC were described by CD14 expression, although this subset has been recently dismissed from DC classification as a blood monocyte derivative and found to be homologous with murine CD11b+CD64+ monocyte-derived macrophage populations (McGovern et al., 2014). However, monocytes can still give rise to monocyte-derived DCs (MDDC), which are still highly relevant in mucosal immunity (reviewed in subsection 3.1.1). An overview of major DC classifications, their receptor expression and tissue distribution is described in table 1.1.

DC subset	**Markers**	**CLR expression**	**Distribution**
Myeloid cDC1	CD11b– CD11c+ CD103+ CD141+	CLEC4A+ CLEC9A+ DEC-205+	Blood Non-lymphoid tissues
Myeloid cDC2	CD1c+ CD11b+ CD11c+	CLEC4A+ DEC-205+	Blood Non-lymphoid tissues
Plasmacytoid DC (pDC)	CD11c– CD123+	CLEC4C+ CD304+	Blood
Langerhans cell (LC)	Langerin+ CD1a+ CD11c+	Langerin+ CLEC4A+ DEC205+	Stratified epidermis
Dermal 'cross-presenting' cDC1	CD1a+ CD11c+ CD14- CX3CR1-	DC-SIGN± CLEC4A+ DEC-205+ MR+	Dermis
Dermal 'conventional' cDC2	CD1c+ CD11b+ CD14± CX3CR1+		
Monocyte-derived DC (MDDC)	CD1a+ CD1c+ CD14– CD11c+	DC-SIGN+ DEC-205+ MR+	Dermis Blood (?)

Table 1.1: DC subsets, expression profile and anatomical distribution. Adapted from (Ahmed et al., 2015). MR, mannose receptor.

DC subset classification remains a complex field to delineate given the limited availability of markers and since it is yet unclear exactly how many subsets exist and the lines between DCs and other mononuclear phagocytes are still blurred. Current trends in classification are progressively turning toward transcriptomic profiling on both bulk and single-cell levels to gain

an unbiased global view of the DC landscape (Villani et al., 2017; Bertram et al., 2019).

RNA sequencing of DC populations has led to the identification of novel blood-DC subsets with both cDC- and pDC-like gene expression signatures (Villani et al., 2017) and epidermal CD11C+ DCs which resemble cDC2 both transcriptionally and morphologically, preferentially interact with HIV-1 via higher CCR5 expression and can transfer virus to CD4+ T-cells with greater efficiency than epidermal Langerhans cells as a result (Bertram et al., 2019).

1.4.2 Early-stage HIV-1 infection at mucosal surfaces

HIV-1 transmission among adults occurs almost exclusively through unprotected sexual contact with an infected individual; therefore, the mucosal surfaces (mucosa) which line the rectovaginal tracts form the first physical barrier of entry for infectious viral pathogens. The anatomical structure of the mucosa is comprised of multiple layers including the epithelial membrane which overlay fibrous extracellular matrix (basement membrane) and loose connective tissue (lamina propria). Underneath lies the submucosa containing dense irregular connective tissue containing networks of blood and lymphatic vessels, which are highly significant for the egress of lymphocytes into the epithelial and subepithelial layers needed to support mucosal immunity at local sites.

While the mucosal barrier was originally considered to be impenetrable by most pathogens, HIV-1 can in fact cross the epithelial lining at the rectovaginal tract through several modes. Firstly, tissue injury and loss of integrity at epithelial linings can allow direct passage of virus into the submucosa where they are encountered by subepithelial DCs. Secondly, LCs can emigrate from the submucosa into the epidermis and mucosal epithelia where they reside and extend membrane protrusions toward the epithelial surface where HIV-1 uptake and cell-mediated transfer can occur (described further in section 1.4.4). Lastly, HIV-1 is capable of exploiting the endosomal recycling pathways in epithelial cells. Despite their absence of CD4 and low-level co-receptor expression, HIV-1 can be internalised by columnar epithelial cells allowing passage via transcytosis and bypassing the epithelium (Kinlock et al., 2014).

Transmission of HIV-1 occurs through *cis*- and *trans*-infection pathways, which are associated with the uptake of cell-free virus or the cross-presentation of virus in *trans* by HIV-1 bearing cells, respectively. During *cis*-infection at the submucosa, cell-free HIV-1 directly interacts with the CD4 receptor on target cells to gain entry into the cytosol and achieve productive infection by integrating into the host gnome. The *de novo* production and release of new progeny virions from the host propagates the dissemination of HIV-1, allowing infection in multiple rounds. This pathway is opposed to that of *trans*-infection, a highly efficient mode of transmission occurring at the cell-cell junction, where HIV-1 and other retroviruses including HTLV-1 (Shimauchi et al., 2019) and MLV (Sewald et al., 2015) can exploit this contact for transmission. In DCs, the capture and uptake of HIV-1 typically follows a pathway of non-productive infection, allowing virus to be trafficked through the cell and – through an as-yet uncharacterised cellular mechanism – for cell-to-cell transmission across a highly efficient virological synapse (described further in sections 1.4.3–1.4.4).

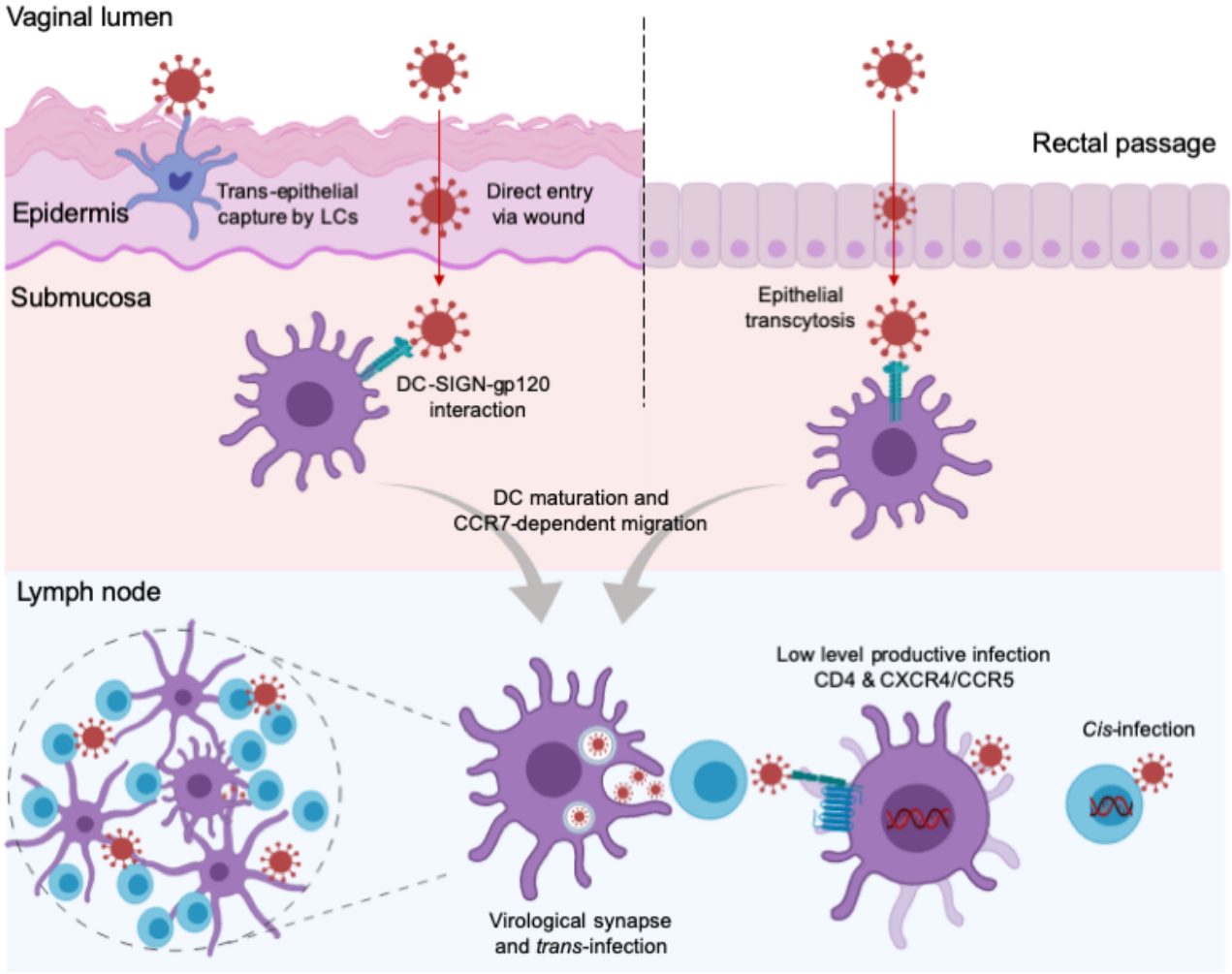

Figure 1.6: HIV-1 infection at mucosal surfaces. HIV-1 accesses the epidermis and submucosa through a number of mechanisms including direct passage during tissue injury, trans-epithelial capture by resident LCs occuring in vaginal transmission, and epithelial transcytosis by columnar epithelia at the anorectal tract. HIV-1 encounters and internalised within DCs, which exploits the migratory functions of DCs for tissue penetration into secondary lymphoid tissues, highly populated with CD4+ target cells for widespread viral dissemination. Adapted from (Wu and KewalRamani, 2006; Piguet and Steinman, 2007).

1.4.3 Dendritic cell interactions with HIV-1

Due to their distribution throughout the upper layers of the mucosa, DCs and their subsets represent some of the earliest immune cell targets for HIV-1 infection. Historically, classical

mechanisms of infection centred around a model of entry by cell-free virus with permissive CD4+ target cells and propagation through successive life cycles. However, closer investigation into the molecular interactions in DCs found that HIV-1 can be internalised independently of its target receptor CD4. Instead, uptake is mediated through binding of the CLR, DC-specific ICAM-grabbing non-integrin (DC-SIGN) which is constitutively expressed in DCs with the highest expression in immature compared to mature populations. DC-SIGN exhibits multiple functions as an antigen receptor (Engering et al., 2002) and as a cellular adhesion molecule binding ICAM-2/ICAM-3 on resting T-cell populations to mediate cell-cell interactions and trans-endothelial migration (Geijtenbeek et al., 2000b).

Crucially, DC-SIGN accounts for 90% of all receptor-mediated HIV-1 interactions in DCs, binding at HIV-1 gp120 Env at N-linked glycosylation sites and represents an important target receptor for HIV-1 pathogenesis (Curtis et al., 1992; Geijtenbeek et al., 2000b; Feinberg et al., 2001; Arrighi et al., 2004). As an antigen receptor, DC-SIGN is closely linked with endosomal internalisation and degradation pathways (Engering et al., 2002). However, while the majority of virus that binds DC-SIGN is rapidly degraded, a small proportion of intact virus evades degradation, is retained within non-lysosomal tetraspanin-rich compartments and can remain infectious for more than 5 days post-exposure (Geijtenbeek et al., 2000a; Garcia et al., 2005). Additionally, uptake in DCs may occur independently of receptors all together. HIV-1 exploits the DC actin cytoskeleton and is captured within membrane-continuous invaginated pockets termed virus-containing compartments (VCC) (Lehmann et al., 2011), allowing virus to be sequestered from the extracellular environment to evade Ab neutralisation.

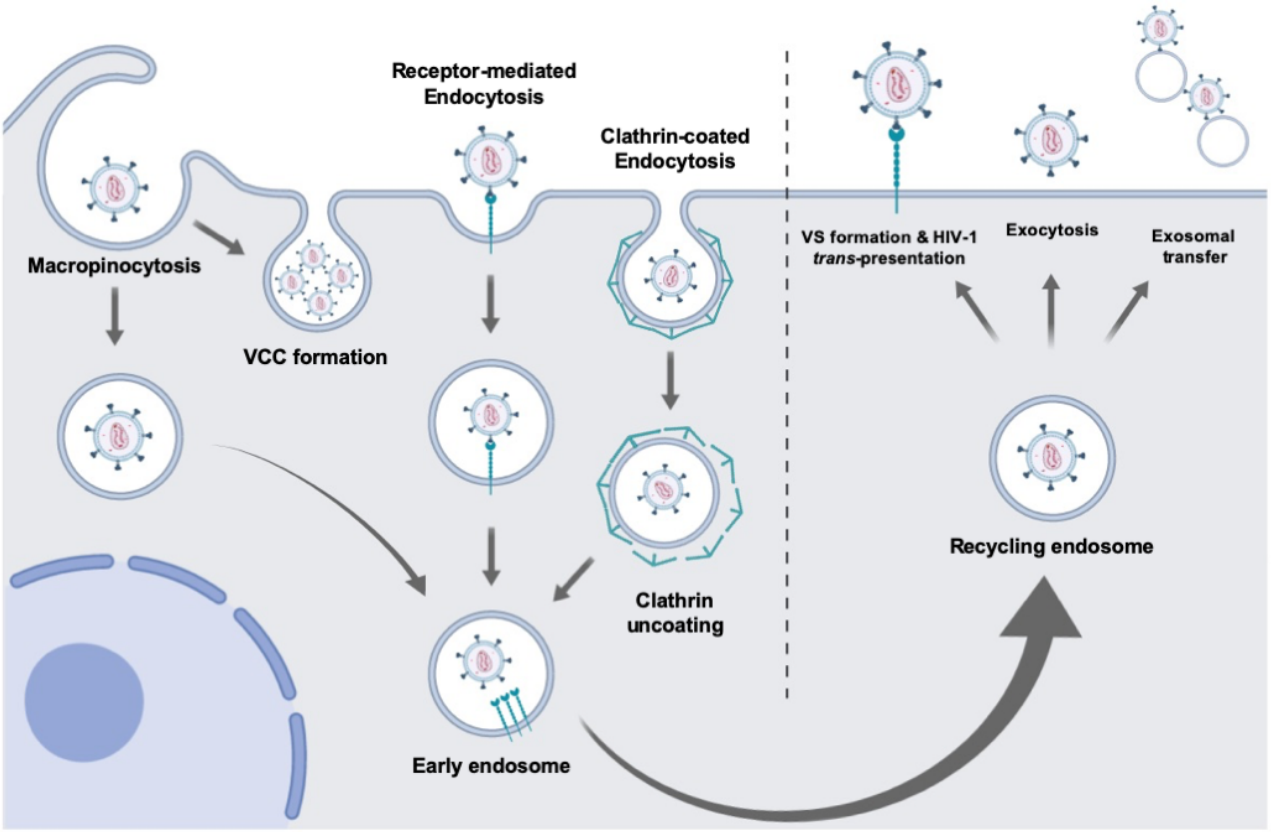

Figure 1.7: Mechanisms of HIV-1 interactions with DCs. HIV-1 is captured and internalised through distinct pathways. Macropinocytosis can capture large quantities of virus, using large membrane sheets to sequester virus within virus-containing compartments. Receptor-dependent (DC-SIGN) and independent (Clathrin) mechanisms internalise virus into central compartments resembling early endosomes. Virus can be trafficked back toward the plasma membrane through recycling endosomes for *trans-infection* using exocytosis and by exosome-associated transfer. Cell-cell association and virological synapse formation causes polarisation of virus to the zone of contact for highly efficient viral transfer and presentation of DC-SIGN-associated HIV-1 in *trans*.

1.4.4 HIV-1 *trans*-infection across the virological synapse

As HIV-1-bearing DCs migrate from mucosal tissue to local draining lymph nodes highly populated with permissive T-cells, where DCs present virus to target CD4+ cells in *trans* across the virological synapse (VS) which is estimated to be between 100-18,000 times more efficient than infection by cell-free virus (Dimitrov et al., 1993). A synapse is defined as a "point of contact" between two cells; in an immunological context, there are two types of junction exist

that form between APCs and effector cells: the immunological and virological synapses, which each have distinct functions and molecular arrangements.

The immunological synapse describes specialised contact structures that arise between APCs and T-lymphocytes, characterised by T-cell receptor (TCR) engagement of a peptide-loaded MHC-II complex, which activates effector function and clonal expansion (Grakoui et al., 1999). Additional molecules including CD4, protein kinases and adhesion molecules ICAM-1-LFA-1/Talin which are recruited to stabilise the cell junction, while TCR engagement induces T-cell migratory arrest to promote full activation (Dustin et al., 1997). In addition, ICAM-1-LFA-1 binding promotes clustering of peptide-MHC-II complexes in mature DC and is thought to enriching antigen-presentation and efficient effector cell priming, though this is not observed in immature DCs.

The earliest characterisation of the VS was in T-cell-to-T-cell transmission of Human T-lymphotropic virus type 1 (HTLV-1) (Bangham, 2003), which described polarisation of concentrated virus to the zone of contact via the microtubule organizing centre (MTOC) and remains a defining feature of the VS. However, the exact mechanisms of how HIV-1 is trafficked from virus-containing compartments in DCs to the VS during *trans*-infection is still not known. While the VS displays many similarities with the immunological synapse, VS formation and *trans*-infection occurs independently of CD3/TCR-pMHC-II binding at the DC-T-cell interface and transfer is predominantly driven by ICAM-1-LFA-1/Talin interaction. At the VS, HIV-1 may be presented directly by DC-SIGN and other attachment factors to the post-synaptic target cell in *trans* (McDonald et al., 2003; Piguet and Sattentau, 2004; Arrighi et al., 2004; Jolly and Sattentau, 2004; Levy et al., 2009a).

The remarkable efficiency of the VS is in part attributed to viral exploitation of the highly dynamic DC actin cytoskeleton, which wraps large membrane sheets over target cells (Lehmann et al., 2011). HIV-1 viral proteins can induce actin remodelling in DCs through activation of cell-cycle protein Cdc42, producing membrane extensions to facilitate cell-cell spread (Nikolic et al., 2011). Similarly, non-infected cells extend stable CD4-rich filopodia which insert into the

VCC forming a bridge or 'viral cytoneme' for rapid viral transfer (Sherer et al., 2007; Felts et al., 2010). Cell-cell HIV-1 transmission therefore poses a significant obstacle to researchers and particularly challenges the development of immunotherapies including virus-specific broadly-neutralising antibodies which are unable to neutralise HIV-1 at cell junctions (Burton and Mascola, 2015).

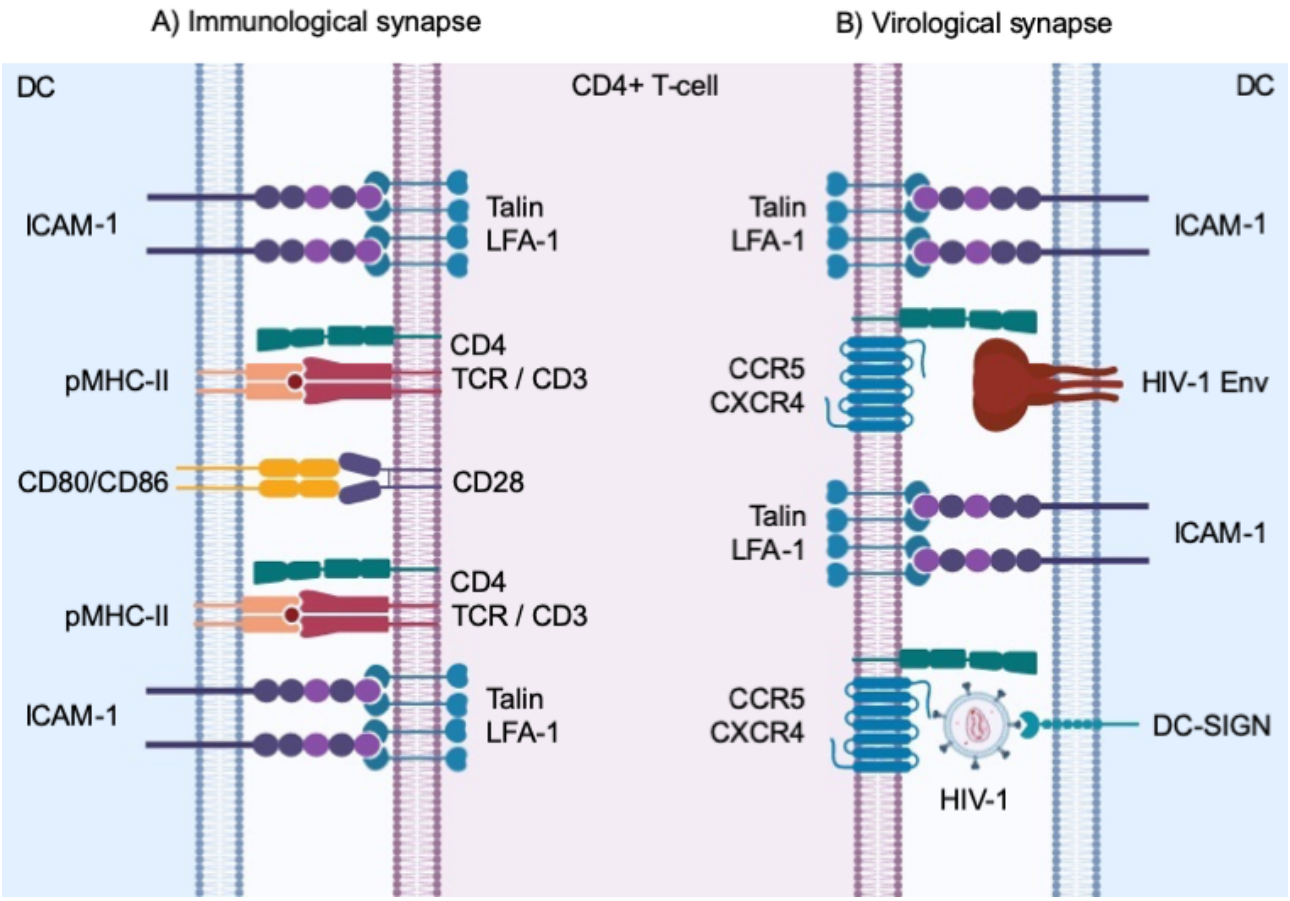

Figure 1.8: The immunological and virological synapse. A) The immunological synapse represents a mechanism for antigen-presentation by DCs to CD4+ T-cells required for priming of adaptive immunity. B) During contact between an infected or HIV-1-bearing DC and the target cell, HIV-1 exploits the binding of high affinity adhesions molecules ICAM-1/LFA-1-Talin for Env ligation with CD4/CCR5/CXCR4. Adapted from (Piguet and Sattentau, 2004).

1.5 Innate sensing of HIV-1 by dendritic cells

Innate sensing is a primitive component of human immunity preceding the evolution of adaptive immunity and is responsible for the initial detection of pathogens and activation of host defence mechanisms. DCs express a vast range of membrane-associated pattern-recognition receptors (PRRs) including DC-SIGN, siglec-1, DCIR, mannose receptors and toll-like receptors (TLR) which are recognise pathogen associated molecular patterns (PAMP) and damage-associated molecular patterns (DAMP) during phagocytic engulfment of pathogens. PRRs are also present in cytosol including cGAS/STING, NOD-like receptors (NLR), RIG-I-like receptors (RLR), which sense intracellular PAMPS, viral ssRNA, dsRNA and DNA (Medzhitov and Janeway Jr, 1998; Rustagi and Gale, 2014). The cGAS/STING pathway is a potent mechanism of cytosolic sensing which occurs after viral entry and release of viral nucleic acids. Sensors cGAS and IFI16 bind to viral RNA to initiate production of the secondary messenger cGAMP, recruiting the STING adaptor protein and downstream induction of NF-κB and release of pro-inflammatory cytokines, type 1 interferon (IFNα/β) release and IRF3-dependent induction of host restriction factors (described in 1.5.1). IFNα/β signalling primes neighbouring cells and typically results in signal transduction via the JAK-STAT pathway, a complex cascade mediated by interferon regulatory factors (IRFs), controlling the activation of interferon-stimulated genes (ISG) which amplify PAMP sensitivity and further release of type 1 IFNs. (Schoggins and Rice, 2011; Schneider et al., 2014; Altfeld and Gale Jr, 2015). The induction of innate signalling by PRR is crucial in the control of HIV-1 dissemination. DCs are key mediators of immune responses during early-stage infection; successful PAMP recognition leads to the induction of several anti-viral mechanisms MHC presentation of HIV-1 peptides, enhanced co-stimulatory capacity, pro-inflammatory and anti-viral cytokine production, which primes Th1 differentiation and HIV-specific cytotoxic CD8+ T-cell responses (Luban, 2012). Despite this, HIV-1 virus evades recognition and degradation within DCs and remains concealed from the innate immune system. Rapid viral replication and immune evasion typically outweigh viral clearance and virus-specific responses are typically "too late and too little" (Reynolds et al., 2005).

1.5.1 Host restriction factors

Viruses are obligate intracellular pathogens, hijacking the cell to propagate itself through the host's normal cellular machinery. However, mammalian cells have evolved to assume mechanisms of intrinsic innate immunity with potent inhibitory effects aimed to disrupt various stages of viral life cycle. These inhibitory proteins, termed 'restriction factors' (RF), offer front-line anti-viral activity at the earliest stages of infection and may even precede innate immunity (Bieniasz, 2004; Malim and Bieniasz, 2012).

Some of the founding evidence for host restriction factors began in mouse models of murine leukaemia virus infection by Best and colleagues, who showed that homozygous cells encoding one of the two Fv1 gene alleles (Fv1n and Fv1b) had strain-specific susceptibilities which when fused, formed a heterozygous cell resistant to both strains (Best et al., 1996). This particular finding gives evidence of a dominant restriction phenotype which is lacking in susceptible cells. Likewise, if a permissive phenotype was observed after fusion, this would suggest non-permissive cells lacked a co-factor for infection which permissive cells express. Since then a body of evidence began to grow, supporting the idea of restriction against RNA retroviruses including HIV-1 and similar cell-fusion assays followed including the trans-complementation strategy adopted by Simon and colleagues (Simon et al., 1998) used to identify factors involved in regulating viral restriction. The assay fused a HIV-$1^{\Delta env}$ cell with one expressing the complementing HIV-1 Env glycoprotein, creating a heterokaryon capable of producing new progeny virus. Culturing the virus-containing supernatants with a reporter cell could detect changes in infectivity brought by changes to parental cell genotypes. Together, these studies have helped characterise the early mechanisms of host restriction and identified roles for HIV-1 accessory proteins in immune evasion and as targets for therapeutic intervention and is described further in section 1.5.2 (Madani and Kabat, 1998; Varthakavi et al., 2003).

In a review of HIV-1 restriction biology, Malim and Bieniasz (Malim and Bieniasz, 2012) set out a series of cardinal features defining RFs including: constitutive and IFN-inducible expression, cell-autonomous mechanisms of restriction, species-specific protection, potential for regulation

by viral accessory proteins and utilisation of the ubiquitin/proteosome system.

1.5.2 Host dependency factors and immune evasion

One of the key aspects of viral evolution is the ability to evade the immune system and retroviral species circumvent the host immunity through a number of mechanisms. The survival of HIV-1 as a species is in part owed to its high rate of mutation (Cuevas et al., 2015), to generate a genetic diversity needed to escape neutralisation by circulating HIV-specific Abs, cytolytic CD8+ T-cell responses and build drug-resistance. However, HIV-1 has evolved to escape strong inhibition by intrinsic RFs; viral regulatory and accessory proteins (Rev, Tat, Nef, Vpu, Vif and Vpr) constitute almost half of all genes within the HIV-1 genome and, although are not strictly required for viral replication, are dedicated to ensure escape and survival from strong innate and adaptive immune pressure. Accessory proteins mostly achieve this through direct engagement of host RFs and are often linked with the ubiquitin-proteosomal system which mark them for degradation (Malim and Emerman, 2008) which has been shown in APOBEC3G-Vif (Yu et al., 2003) and tetherin-Vpu (Neil et al., 2008) interactions. Viral proteins may also modulate the cell surface expression of receptors and molecules. Vpu and Nef are responsible for the downregulation and degradation of surface CD4 and MHC-I, which may be necessary for avoiding immune recognition and preventing receptor incorporation during virion assembly and aggregation of circulating virus (Chen et al., 1996; Piguet et al., 1998, 1999; Cohen et al., 1999; Piguet et al., 2000). Table 1.2 describes a collection of cellular RFs, their mechanisms and HIV-1 viral proteins which inhibit their effect.

Restriction factor	Mechanism of action	Inhibitory protein
APOBEC3G	Hypermutates viral DNA by cytidine deamination preventing integration (Harris et al., 2003). Binds HIV-1 RT to block replication (Mangeat et al., 2003).	HIV-1 Vif (Sheehy et al., 2003)
TRIM5α	Targets HIV-1 CA to prevent uncoating, blocks nuclear translocation and labels for degradation (Stremlau et al., 2004; Sayah et al., 2004).	?
SAMHD1	Depletes deoxynucloside triphosphates (dNTP) pools to preventing reverse transcription (Laguette et al., 2011).	HIV-2/SIV Vpx (Hrecka et al., 2011; Laguette et al., 2011)
Tetherin	Blocks budding and release of progeny virions by anchoring viral particles to the cell membrane (Neil et al., 2008).	HIV-1 Vpu, Nef, HIV-2 Env (Neil et al., 2008; Sauter et al., 2009)
Schlafen 11	Binds to viral tRNA to prevent translation of viral peptides (Li et al., 2012).	?
MX2	GTPase which suppresses HIV-1 replication, import and integration. Also acts to target viral CA uncoating by interaction with CypA (Goujon et al., 2013; Kane et al., 2013).	?
IFITM	Transmembrane proteins which restrict HIV by inhibiting virus entry by interfering with viral fusion. IFITM can also colocalize with HIV-1 proteins Env and Gag which can incorporate into new viral particles to limit HIV-1 entry into new target cells (Lu et al., 2011).	?

Table 1.2: HIV-1 cellular restriction factors, their mechanisms and viral proteins which inhibit their action.

The substantial advances in RF biology have led to the identification of new factors which inhibit viral replication and a better understanding of the mechanisms involved in disrupting HIV-1 life cycle progression. However, the co-evolution between host and virus has resulted in an evolutionary arms race where HIV-1 is continuously adapting to surpass the pressures of selection (Duggal and Emerman, 2012). This relationship is well documented in non-human primate studies of Bioko Island Old World monkeys which, though phylogeographic analyses, were found to have been infected with SIV for at least 32,000 years, allowing the natural adaptation between host and virus (Worobey et al., 2010). Interestingly, SIV infection in African non-human primates is non-pathogenic, however, SIV infection in non-African primates (such as Asian or Indian rhesus macaques) leads to the development of simian AIDS, similar to immunodeficiencies observed in HIV-1 infected humans, as a direct consequence of the lack of co-evolution and adaptation. The regulatory landscape around host restriction is still not fully understood and there are currently strong suggestions that many more cellular RFs may exist than currently identified. Although the therapeutic potential of RFs is yet to be realised in the clinic, the discovery of new factors and regulators of intrinsic immunity could inform the development of existing antiviral drug compounds and set the stage for novel therapeutic strategies against infection.

1.6 The role of cytokines and chemokines in acute HIV-1 infection

Cytokines are small signalling peptides ($\sim$ 5-20 kDa) secreted by cells which regulate many aspects of the haematopoetic and immune systems. In addition, inflammation is controlled largely by extracellular mediators including cytokines and are therefore broadly categorised into two groups depending on their role in the immune response as either pro-inflammatory or anti-inflammatory cytokines. Chemotactic cytokines or 'chemokines' represent another major cytokine subgroup, which are best recognised for their functions in cell migration. There are

four chemokine subfamilies that exist which reflect the structural configuration of cysteine residues and include: XC, CC, CXC and CX3C chemokines. Chemokine receptors belong to a large family of 7-transmembrane G-protein coupled receptors (GPCR) which are highly promiscuous constituting 18 receptors which collectively bind 50 chemokine ligands (Baggiolini et al., 1997).

1.6.1 Cytokine signalling at the DC-T-cell junction

In DCs, cytokine production plays an important part in antigen presentation and priming the adaptive immune response by CD4+/CD8+ T-cells. Generally, cytokine secretion in DCs typically follows maturation and the types of cytokines produced are subset-dependent. At basal levels immature DCs are poorly immunogenic and lack the ability to produce a cytokine response until a fully functional maturation state is achieved, where DCs may assume either inflammatory or tolerogenic properties. Maturation into inflammatory DCs is characterised by the release of IL-1β, IL-6, IL-12, IL-15, IL-23, CCL2, CCL5, CCL19, CCL21 and TNF-α (Palucka and Banchereau, 2013; Chistiakov et al., 2014), while tolerogenic DCs are predominantly IL-10 and TGF-β secreting (Rutella et al., 2006) and are responsible for maintaining immune homeostasis at the mucosa and limiting persistent inflammation.

The T-cell cytokine profile is highly dependent on effector cell differentiation and have unique signatures used to determine their subset. At steady state, naive CD4+ T-cells require IL-2, IL-7 and IL-15 for homeostatic proliferation and survival (Lodolce et al., 1998; Tan et al., 2001). At the DC-T-cell junction, TCR interaction with pMHC-II by DCs drives naive CD4+ T-cell polarisation into one of several Th effector subsets, depending on the receptor signalling and cytokine cues to best control the presented pathogen species. T-cell subsets are now well characterised beyond Th1 and Th2 to include Th9, Th17, T follicular helper (Tfh) and T regulatory (Treg) populations which have functions in response to all types of pathogens and summarised in table 1.3 (DuPage and Bluestone, 2016).

Receptor/cytokine ineraction & sensing	**Subset differentiation**	**Cytokine production**	**Receptor expression**
Strong TCR signal, IL-2, IL-12, IFN-γ	Th1 (Anti-viral)	↑ IFN-γ	↑ CXCR3 ↑ CCR5
Weak TCR signal, IL-2, IL-4	Th2 (Anti-parasitic)	↑IL-4 ↑IL-5 ↑IL-13	↑CCR4 ↑CCR8
TCR signal, IL-2, IL-4, TGF-β	Th9 (Anti-parasitic)	↑IL-9	↑CCR3 ↑CCR6 ↑CXCR3
TCR signal, IL-6, IL-21, IL-23, TGF-β	Th17 (Anti-fungal)	↑IL-17 ↑IL-22	↑CCR6
Strong TCR/ICOS signal IL-6, IL-12	Tfh (Anti-microbial)	↑ IL-21	↑CXCR5, ↑CD40L
TCR/CD28 signal, IL-2, TGF-β	Treg (Regulatory)	↑IL-10 ↑TGF-β	↑PD1 ↑CLTA4

Table 1.3: CD4+ T-cell effector cell differentiation pathways as a result of DC-T-cell priming. Adapted from (DuPage and Bluestone, 2016).

Together, both cytokines and chemokines are central in the resolution of infection acting through a number of mechanisms including the promotion of inflammation, leukocyte trafficking, induction of lymphocyte differentiation, priming of anti-viral host defence and effector cell function by type-1 interferons. However, the cytokine network is remarkably complex and is susceptible to dysregulation which gives rise to disease states characterised by chronic inflammation and tissue remodelling.

1.6.2 Dysregulation of the cytokine network by HIV-1

The chemokine receptors CCR5 and CXCR4 are particularly relevant in HIV-1 infection since their identification as co-receptors which are directly exploited for viral entry into permissive cells (Oberlin et al., 1996; Choe et al., 1996; Feng et al., 1996; Berger et al., 1999). HIV-1 viral proteins can also elicit indirect modulation of the immune system at both acute and chronic stages of infection. In DCs, which internalise virus through CD4-independent mechanisms and avoid productive infection, HIV-1 drastically alters DC function to evade innate sensing, delay and dampen HIV-specific adaptive immunity (Gringhuis et al., 2010; Miller and Bhardwaj, 2013; Manches et al., 2014; Nasi et al., 2017).

Although there are mixed reports on the affects of HIV-1 on DC function, several studies have suggested that infected DCs may not achieve full maturation(Granelli-Piperno et al., 2004; Hertoghs et al., 2015), have enhanced IL-10 production (Alter et al., 2010) and reduced IL-12 production which is HIV-1 gp120/Vpr-mediated and required for priming of Th1 anti-viral responses (Fantuzzi et al., 2004; Majumder et al., 2005). HIV-1-induced cytokine dysregulation was also shown by Maggi and colleagues (Maggi et al., 1994) in HIV-1 infected CD4+ T-cell clones, which was found to promote class switching of Th1 cells toward a Th0 phenotype. In addition, HIV-1 replication was preferential within T-cell clones with Th2-type and Th0-type cytokine producing phenotypes. Th2 cells are predominantly associated with release of IL-4, IL-5, IL-6, IL-10 and IL-13 and contribute to antibody-mediated immunity and allergy response. Unlike Th1, Th2 are not capable of IFN-γ driven anti-viral immunity and would likely explain their predisposition to HIV-1 infection.

1.7 Project rationale

DCs are one of the first cell types to encounter HIV-1 at mucosal surfaces and represent an ideal model cell type for understanding infection at the earliest events. DC to CD4+ T-cell HIV-1 mucosal transmission is an essential component of early viral propagation, but has not been extensively studied so far, as opposed to cell-free viral transmission between T-cells. There is clear evidence that cell-associated viral transmission via the VS is up to 18,000 times more efficient than cell-free viral transmission for retroviruses such as HIV-1 and human T-cell leukaemia virus type 1 (HTLV-1). Our group has previously demonstrated that Langerhan's cells (LC), a subset of DCs, have a natural resistance to infection with HIV-1 compared to DCs that this is attributed to post-entry restriction by TGF-β cytokine signalling (Czubala et al., 2016). Although a handful of restriction factors have been identified in DCs, more factors are yet to be discovered. In agreement with previous findings within our group, we hypothesise that cytokines and chemokines have a significant role in host-virus interaction and propose a RNA interference (RNAi) screen for the discovery of novel targets affecting viral transfer.

This study will enable the application of a systems approach for the first time to dissect the interactions between HIV-1, DCs and its cytokine receptors which are known to contribute to early dissemination of HIV-1 during mucosal transmission. The aim of this thesis is to investigate the role of cytokines, chemokines, their receptors and regulators on cell-cell HIV-1 transmission from dendritic cells to CD4+ T-cells. The identification of novel cytokine/chemokine targets will inform development of therapeutic strategies against the early events of infection.

1.8 Thesis aims

This study aims to develop the first systematic analysis of cytokine receptors during cell-to-cell viral transmission of HIV-1. DC-to-T-cell transmission of HIV-1 allows the virus to circumvent exposure to neutralising antibodies and represents a major obstacle to anti-retroviral therapy and vaccine development to prevent new HIV-1 acquisitions and recurrent transmission.

We aim to delineate cytokine and chemokine factors regulating DC-to-T-cell transmission of HIV-1 by establishing an *in vitro* cell model of early-stage transmission and identify proof-of-principle targets to disrupt *trans*-infection. We will perform a global siRNA screen of cytokines, chemokines and their receptors regulated during DC-mediated HIV-1 transfer to T-cells and identify potential candidates, which will be studied and tested by a systematic validation of pathways and targets using established assays in virology and immunology. This new approach will identify all cytokine receptors involved in cell-to-cell transmission of HIV and generate new potential therapeutic targets to disrupt the early events of HIV-1 transmission.

Our principle objectives are:

- To establish an *in vitro* model of cell-cell transfer of HIV-1 from DC to CD4+ T-cells.
- To identify novel candidates using high-throughput siRNA screening to genetically down-regulate 319 genes in MDDC targeting cytokine and chemokine function.
- To systematically validate candidate genes and identify novel targets that disrupt viral DC-T cells HIV-1 transmission.

Chapter 2

Materials and methods

2.1 Cell and tissue culture

2.1.1 Buffers

- Pre-MACS separation buffer — 1X PBS (phosphate-buffered saline, Sigma-Aldrich), 2% v/v FCS (Foetal Calf Serum).
- MACS buffer — 1X PBS (Gibco^{TM} Life Technologies), 0.5% w/v BSA (Bovine Serum Albumin, Sigma-Aldrich), 0.5M EDTA (Gibco^{TM} Life Technologies). Sterile filtered using a 0.22μm SteriCup® (Sigma-Aldrich) filtration system.
- CD4+ culture medium — RPMI 1640 (Gibco^{TM} Life Technologies); 10% FCS; 1X Sodium Pyruvate (Gibco^{TM} Life Technologies); 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco^{TM} Life Technologies).
- CD14+ PBMC/MDDC culture medium — Iscove's Modified Dulbecco's Medium (IMDM Life Technologies); 10% FCS; 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco^{TM} Life Technologies).
- HEK293T/TZM-bl culture medium — Dulbecco's Modified Eagle Medium (DMEM Life

Technologies); 1X Sodium Pyruvate (Gibco™ Life Technologies); 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco™ Life Technologies).

- GHOST (3) culture medium — High glucose DMEM; 90% v/v FCS, 10% 500 μg/mL G418; 100 μg/mL hygromycin; 100 U/mL penicillin and 100μg/mL streptomycin (Gibco™ Life Technologies); 1 μg/mL puromycin (Hi-5 only) (Mörner et al., 1999).

2.1.2 HEK293T cell line

HEK293T cells were maintained at 40-80% confluency in 20 mL DMEM in a T175 culture flask. For routine maintenance, cells were harvested by incubation with trypsin-EDTA to allow cells to detach. Cells were harvested, counted and re-seeded at 3x10^5 cells per flask.

2.1.3 TZM-bl cell line

TZM-bl cells (Cat 8129) were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH from Dr. John C. Kappes, and Dr. Xiaoyun Wu. TZM-bl cells were cultured under similar conditions to HEK293T cells as previously described. No antibiotic selection processes were required. The TZM-bl cell line is stably transfected with the firefly luciferase and β-galactosidase genes. Transcriptional control of the Luc gene is mediated by the HIV long terminal repeat (LTR) region (Montefiori, 2009).

2.1.4 GHOST/GHOST-R5 cell lines

GHOST (3) parental (CXCR4+ CCR5+) and GHOST (3) Hi-5 (CCR5+) are a human osteosarcoma-dervied adherent cell line and were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH from Dr. Vineet N. KewalRamani and Dr. Dan R. Littman (Mörner et al., 1999; Vödrös et al., 2001). GHOST (3) cells were cultured as previously described in T125 culture flasks and split at 90% confluence in a 1:5 ratio using trypsin-EDTA. The parental

cell line is puromycin-sensitive and was used to select for GHOST CCR5+ expressing cultures only.

2.1.5 SupT1 cell line

SupT1 cells were provided by the NIH AIDS Reagent program. The CD4+ CD3- SupT1 cell line is a T-lymphoblastic leukaemia derived cell line and highly susceptible to HIV-1 infection due to high CD4 expression. SupT1 were cultured in 30mL complete RPMI in a T75 culture flask at 37 °C at 5% CO_2 and split at a 1:5 ratio every 2-3 days depending on culture density (not exceeding $5x10^6$/mL).

2.2 Primary cell isolations

2.2.1 PBMC (Peripheral Blood Mononuclear Cell) isolation

PBMCs were collected from anonymous healthy donor buffy coats (Welsh Blood Service) by Ficoll-Paque® (GE Healthcare) density separation. Approximately 50 mL of concentrated peripheral blood was diluted up to 120 mL final volume using pre-MACS seperation buffer. For gradient seperation, we used SepMate50 (STEMCELL Technologies) seperation tubes, filling the lower chamber with 15mL of Ficoll-Paque® (GE Healthcare) and the upper chamber with 30mL of diluted blood. Tubes were spun at 700 xG (with brake) for 10 minutes and the enriched supernatant was collected and washed twice with pre-MACS buffer to remove traces of Ficoll-Paque® (GE Healthcare). A cell count was determined using a Countess II FL Automated Cell Counter (Thermo Fisher Scientific) with 0.4% Trypan Blue staining solution (Gibco™ Life Technologies).

2.2.2 CD14+ monocyte isolation

PBMC were equilibrated in chilled MACS buffer and resuspended to 80 μL per 10x^7 PBMC. 10 μL per 10x^7 PBMC of CD14+ magnetic isolation beads (Miltenyi Biotech, 130-050-201) were added, gently mixed and incubated at 4°C for 15 minutes. After incubation, CD14+ cells were isolated using column filtration by LS positive selection column (Miltenyi Biotech) or using an AutoMACS cell separator (Miltenyi Biotech). Cells were washed once in 1X PBS and centrifuged at 300 xG for 5 minutes before counting using a Bright-Line haemocytometer (Hasser Scientific, Harsham) and re-suspension to a required volume of culture media.

2.2.3 CD4+ T-cell isolation

For CD4+ T-cell isolation, PBMC were equilibrated in chilled MACS buffer and resuspended to 80 μL per 10x^7 PBMC. 20 μL per 10x^7 PBMC of CD4+ magnetic isolation beads (Miltenyi Biotech, 130-050-201) were added, gently mixed and incubated at 4°C for 15 minutes. CD4+ cells were isolated using column filtration by LS positive selection column (Miltenyi Biotech) or using an AutoMACS cell separator (Miltenyi Biotech). Cells were washed once in 1X PBS and centrifuged at 300xG for 5 minutes before counting using a Bright-Line^{TM} haemocytometer (Hasser Scientific, Harsham) and re-suspension to a concentration of 1x10^6 cells/mL in complete RPMI. CD4+ enriched cells were maintained using 50U/mL IL-2 after isolation and every 2 days following. Depending on the metabolic activity, a complete media change may be required by centrifuging at 300 xG for 5 minutes and resuspending in culture media with IL-2.

2.2.4 MDDC generation from CD14+ PBMC

To generate MDDC, CD14+ enriched PBMCs were seeded into 6-well plates at a density of 2x10^6/well. Cells were differentiated over a 6 day period by culturing in 3mL of complete IMDM per well supplemented with 500 U/mL GM-CSF, IL-4 and 50 μM β2-mercaptoethanol. For maintenance, 1mL of culture supernatant was extracted and replaced with 1 mL of IMDM

from a master-mix containing 500 U/mL GM-CSF and IL-4 every 2 days. After differentiation, MDDC were sampled and analysed by flow cytometry for the expression of cell-specific markers prior to use in experiments.

2.3 Virus production

2.3.1 Buffers

0.5 M CaCl2 — Prepared by dissolving 36.75 g of CaCl2 (SigmaUltra C5080) in 500 mL of distilled H2O and it was stored at -90°C until use.

2X HBS — Prepared by mixing 16.36 g NaCl (SigmaUltra S7653) (final 0.28 M), 11.9 g HEPES (SigmaUltra H7523) (final 0.05 M), and 0.213 g anhydrous Na2HPO4 (SigmaUltra S7907) (1.5 mM final) in 1000 mL distilled H2O. Final solution was adjusted to pH 7.00 with NaOH solution. Solution was stored at -80°C until use.

HEPES H2O — Prepared by adding 125 μL of 1 M HEPES (Gibco-BRL. Ref 15630-056) (final 2.5 mM) to 50mL of distilled H2O. HEPES H2O was stored at 4°C until use.

2.3.2 Virus strains

2.3.2.1 NL4-3 HIV-1

The NL4-3 construct encodes full-length CXCR4-utilising HIV-1 and was obtained through the NIH AIDS Reagent Program (Adachi et al., 1986). Plasmid pNL4-3 provided by Trono D, EPFL, Lausanne.

2.3.2.2 R9 HIV-1

The R9 virus is a lab adapted full-length HIV-1 clone containing NL4-3-derived gag, pol and env and HXB2 5' and 3' LTR sequences. R9 virus is CXCR4-utilising and fully replicative (Smith et al., 2004).Plasmid pR9 provided by Trono D, EPFL, Lausanne.

2.3.2.3 R8-BaL HIV-1

R8-BaL is a lab adapted construct derived from an R9 plasmid but with a substituted CCR5-utilising BaL envelope sequence. R8-BaL encodes full-length HIV-1 virus and is fully replicative (Guyader et al., 2002). Plasmid pR8-Bal provided by Trono D, EPFL, Lausanne.

2.3.2.4 F522Y HIV-1ΔEnv fusion mutant

Based on the NL4-3 HIV-1 strain, the F522Y construct mutant encodes for fusion-defective HIV-1 created by point mutation of the HIV-1 gp41 Env amino acid sequence (Bergeron et al., 1992).

2.3.3 HEK293T transfection and virus production protocol

HEK293T cells were seeded into 6xT175 tissue flasks at 8x10^5 cells/mL in final volume of 15 mL DMEM and cultured until 70-80% confluency. A transfection mix was prepared by mixing 90 μg plasmid DNA with HEPES buffered dH2O (2.5 mM) to a final volume of 750 μL per flask. After 5 minutes incubation at room temperature, DNA-HEPES mix was added to 750 μL $CaCl_2$ per flask and the combined mix was added drop-by-drop into a tube containing 1.5 mL of 2xHBS per flask while continuously vortexing at a low speed. Following a 30 minute incubation at room temperature, 3 mL of transfection mix was added equally to growing HEK293T cells in a tissue culture flask using a transfer Pasteur pipette (VWR International). Cells were then incubated at 37°C for 6 hours. Afterwards, all medium from flask was carefully removed without

disturbing the HEK293T monolayer and cells were washed once with sterile 1X PBS. 15 mL of DMEM was added to each flask of HEK293T and cells were left for 48 hours at 37°C. Culture supernatants were collected, filtered using 0.45 μm sterile millex® GP filter (Millipore Ireland Ltd.) and overlaid on top of 20% sucrose (Sigma-Aldrich) gradient in Beckman ultracentrifuge conical tubes (Beckman Coulter). The gradient was centrifuged in ultracentrifuge at 10,000 xG, 4°C for 90 minutes. The supernatant and sucrose were then aspirated using VACUSAFE™ Vacuum aspiration system (INTEGRA Biosciences), avoiding the viral pellet. Tubes were inverted for 10 minutes and viral particles were resuspended in 300 μL DMEM per tube. The pellet in medium was left at room temperature for 20 minutes and resuspended viral particles were aliquoted into sterile o-ringed screw tubes (Fisher Scientific). Viral preparations were stored until use at -80°C.

2.3.4 Virus quantification

Viral titers were quantified using Lenti-X™ p24 rapid titre ELISA kit (Clontech Laboratories). Virus-containing supernatants were lysed in Triton X-100 (BDH Limited) at 1:10 ratio. A serial dilution of lysed samples were prepared using DMEM culture medium at concentrations ranging between 1:1000 – 1:100000. Dilutions of 0 – 200 pg/ml HIV-1 Gag p24 standard curve were prepared by diluting the p24 control (manufacturer supplied) in complete DMEM culture medium. For the p24 ELISA assay 20μL of lysis buffer was aliquoted into each well and 200μL p24 standard curve dilutions and virus production samples were aliquoted into appropriately labelled duplicate wells and incubated at 37 ($\pm$1)°C for 60 ($\pm$5) minutes. Wells were aspirated and the plate was washed with 1X wash buffer before 100μL of biotin-conjugated anti-p24 detector antibody was added to each well and incubated at 37 ($\pm$1)°C for 60 ($\pm$5) minutes. The plate was washed again and 100 μL of streptavidin-HRP conjugate was added into each well and incubated at room temperature for 30 ($\pm$5) minutes. A final wash was performed and 100 μL of substrate solution added to all wells. After a 20 ($\pm$2) minute incubation at room temperature, the reaction was stopped by addition of 100μL of stop solution to each well.

The absorbance values of each well were measured using a microtitre plate reader (Fluostar Optima) set at 450 nm wavelength and blanked on negative control wells. The standard curve was constructed based on the values acquired for a p24 control dilution, which allowed the calculation of p24 content in virus samples.

For functional quantification in the HIV-1 transfer assay, we titrated virus on cis-infection of SupT1 cells. 200,000 cells/well were seeded into a 96-well microplate and spinoculated for 2.5 hours with incrementing volumes of X4 or R5 tropic HIV-1. After spinoculation, cells were cultured for 24 hours before p24 intracellular staining and analysis by flow cytometry. Samples with 100% positivity would be referenced at 1 MOI.

2.3.5 Quantification by fluorescence reporter cells

The GHOST reporter cell line provides the optimum platform for the quantification of viral titers, the GFP reporter construct gives an indication of viral integration into the host genome. The proportion of productively infected cells can be easily distinguished and the viral titer can be calculated as in equation 2.1. GHOST GFP-reporter cells were seeded at a density of 2x10^5 cells/well and infected using a serial dilution of R5-tropic HIV-1_{R8-Bal} starting at 1 μL of virus. Cells were spinoculated at 1,200 xG for 2.5 hours at 37°C, washed and cultured for 48 hours. Cells were fixed for 20 minutes using 4% paraformaldehyde (PFA) and analysed using flow cytometry. Quantification and MOI was calculated using the method as described in equation 2.2. Given the variability in viral titers between productions and within batches, establishing the multiplicity of infection (MOI) provides a means of standardising viral batches. MOI is defined as the ratio of infectious agents to cells in a culture, based on the Poisson distribution.

$$\text{Viral titer (IU}/\mu\text{L)} = \text{Cell count} \times \text{Infected cells (\%)} \times \text{Dilution factor} \tag{2.1}$$

$$\text{MOI} = \frac{\text{Virus titer} \times \text{Volume of virus}}{\text{Number of cells in infection}} \tag{2.2}$$

2.4 *In vitro* infection assays

2.4.1 Buffers

- PFA fixing buffer — Paraformaldehyde (PFA) (Fisher Scientific) was prepared by diluting PFA stock solution in H_2O to achieve a final 4% (v/v) concentration.

2.4.2 Luciferase infection assay

TZM-bl cells were seeded in a 96-well flat-bottom plate at 10,000 cells/well at day -1 in 100 μL of medium and cultured overnight at 37°C. At day 0, cells were incubated with chemokines for 1 hour. Cells were infected with 3 ng of R8-Bal for 3 hours, washed and cultured in 100μL chemokine containing medium for 48 hours. At day 2, 100 μL SteadyGlo® reagent was added to wells and allowed 5 minutes to lyse cells before being read on a luminometer at 37°C.

2.4.3 HIV-1 infection assay

All viral infections assays were performed using full-length replicative X4 or R5-tropic HIV-1 in a BSL-3 laboratory. Harvested cells were reseeded into a U-bottom 96-well plate (Thermo Fisher Scientific) at a density of 1x10^5-2x10^5 cells/well in 200 μL of DMEM. For spinoculation, 30 ng p24 Gag of R9, NL4-3 or R8Bal HIV-1 added to sample wells and centrifuges at 1,200 xG for 2 hours at 37°C.

2.4.4 HIV-1 transfer assay

MDDCs were harvested and seeded in a 96-well U-bottom plate at 100,000 cells/well and spinoculated (1,200 xG, 2hrs, 37°C) with 10 - 30 ng X4/R5 HIV-1 (equivalent to 1 MOI). After infection, cells were washed 1X PBS and co-cultured with 100,000 CellTrace^{TM} FarRed-labelled SupT1 cells/well for 48 hours in 200 μL complete RPMI medium (10% FCS, pen/strep, glutamate, sodium pyruvate). After co-culture, cells were fixed in 4% PFA for 30 minutes at room temperature.

2.4.5 MDDC stimulation with recombinant cytokines

MDDC were harvested and seeded in a 96-well U-bottom plate at 100,000 cells/well and cultured for overnight (16 hours) in cytokine-containing culture medium prior to spinoculation. After washing unbound virus, recombinant cytokines were replaced in the culture medium for the duration of co-culture. Candidate cytokines and concentrations are described below.

Protein	**Manufacturer**	**Concentration**	**Reference**
r4-1BBL (TNFSF9)	Peprotech	10–100 ng/mL	(Habib-Agahi et al., 2007)
rCD70 (TNFSF7)	Peprotech	1–10 ng/mL	(Wolf et al., 2002)
rIL-6	Peprotech	5–10 ng/mL	(Giorgetti et al., 2009)
rIL-7	Peprotech	20 ng/mL	(You et al., 2012)
rIL-8	Peprotech	50–150 ng/mL	(Nilsson et al., 1999; Yu et al., 2011)
rMIF	Peprotech	10–100 ng/mL	(Bernhagen et al., 1994)
rTWEAK (TNFSF12)	Peprotech	100 ng/mL	(Sims et al., 2012)

Table 2.1: Table of human recombinant cytokines

2.4.6 MDDC neutralisation assay

MDDC were harvested and seeded in a 96-well U-bottom plate at 100,000 cells/well and cultured for overnight (16 hours) in nAb-containing culture medium prior to spinoculation. After washing unbound virus, nAb were replaced in the culture medium for the duration of co-culture. Candidate nAb and concentrations are described below.

Target	**Manufacturer**	**Concentration**	**Reference**
α4-1BB (TNFRSF9)	R&D Bioscience	1 μg/mL	Optimised
αCD70 (TNFSF7)	BioLegend	μg/mL	No data
αIL-6	BioLegend	1 μg/mL	(Wyant et al., 1999)
αIL-7R	BioLegend	μg/mL	No data
αIL-8	R&D Biosciences	1 μg/mL	Optimised
αMIF	BioLegend	1 μg/mL	(Zhou et al., 2009)
αTWEAK (TNFSF12)	BioLegend	5 μg/mL	(Nakayama et al., 2003)

Table 2.2: Table of neutralising antibodies (nAb)

2.4.7 Preparation of category 3 infectious samples

BSL-3 regulations require that HIV-1 infected samples are fixed or lysed *in situ*, sealed and disinfected using 70% IMS to neutralise infectious viral particles before removal from the laboratory to areas of lower-level biosafety containment. For cell fixing, sample plates were washed in 1X PBS and 200 μL 4% PFA buffer was added per well and incubated at room temperature for 20 minutes. For cell lysis, samples were washed an incubated in lysis buffer for 30 minutes at 4°C as detailed in section 2.7.1.

2.5 RNA interference

2.5.1 Buffers and reagents

- HiPerfect® transfection reagent (QIAGEN).
- Serum-free IMDM media (ThermoFisher Scientific) without supplements.

- ON-TARGETplus SMARTpool siRNA (GE Dharmacon) — Human cytokine and chemokine library; siGAPDH; siMIF; siIL-8; siTNFSF7; siTNFSF9; siTNFSF12.

2.5.2 Human cytokine and chemokine screen library design

The Human Cytokine and Chemokine siRNA library manufactured by GE Dharmacon contained 319 individual wells of pooled lyophilised siRNA each containing 4 sequences of siRNA. The library was divided across 4x96-well plates using a layout shown in figure 2.1.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Empty	1	2	3	4	5	6	7	8	9	10	Empty
B	Empty	11	12	13	14	15	16	17	18	19	20	Empty
C	Empty	21	22	23	24	25	26	27	28	29	30	Empty
D	Empty	31	32	33	34	35	36	37	38	39	40	Empty
E	Empty	41	42	43	44	45	46	47	48	49	50	Empty
F	Empty	51	52	53	54	55	56	57	58	59	60	Empty
G	Empty	61	62	63	64	65	66	67	68	69	70	Empty
H	Empty	71	72	73	74	75	76	77	78	79	80	Empty

Figure 2.1: GE Dharmacon Human Cytokine Receptor RNAi library plate layout. The first plate containing siRNA pools 1–80 is illustrated.

2.5.3 RNA interference protocol using siRNA

Genetic downregulation of MDDC was performed using siRNA from the GE Dharmacon ON-Target pooled siRNA. A single reaction was optimised to 3 μL siRNA which was pipetted directly into a U-bottomed 96-well plate and 47 μL of a master-mix containing 2 μL HiPerfect® transfection reagent (Qiagen) and 45 μL serum-free IMDM per well with an additional 10% allowance to avoid volumetric error. The siRNA-lipid reaction was incubated for 15 minutes at room temperature to allow assembly and stabilisation. Meanwhile, MDDC were harvested and resuspended to a density of 100,000 cells per 150 μL complete IMDM culture media and

were reverse transfected by addition to the reaction mix before culture at 7°C. To achieve maximal protein knock-down, MDDC were transfected again after 24 hours of culture. A forward transfection was performed, in which a separate siRNA-lipid reaction was created in a blank 96-well plate as previously described. 50 μL of culture supernatant was carefully removed from transfected cells and replaced with fresh transfection mixture. MDDC were allowed to rest in culture for 48 hours at 37°C to ensure optimal mRNA degradation.

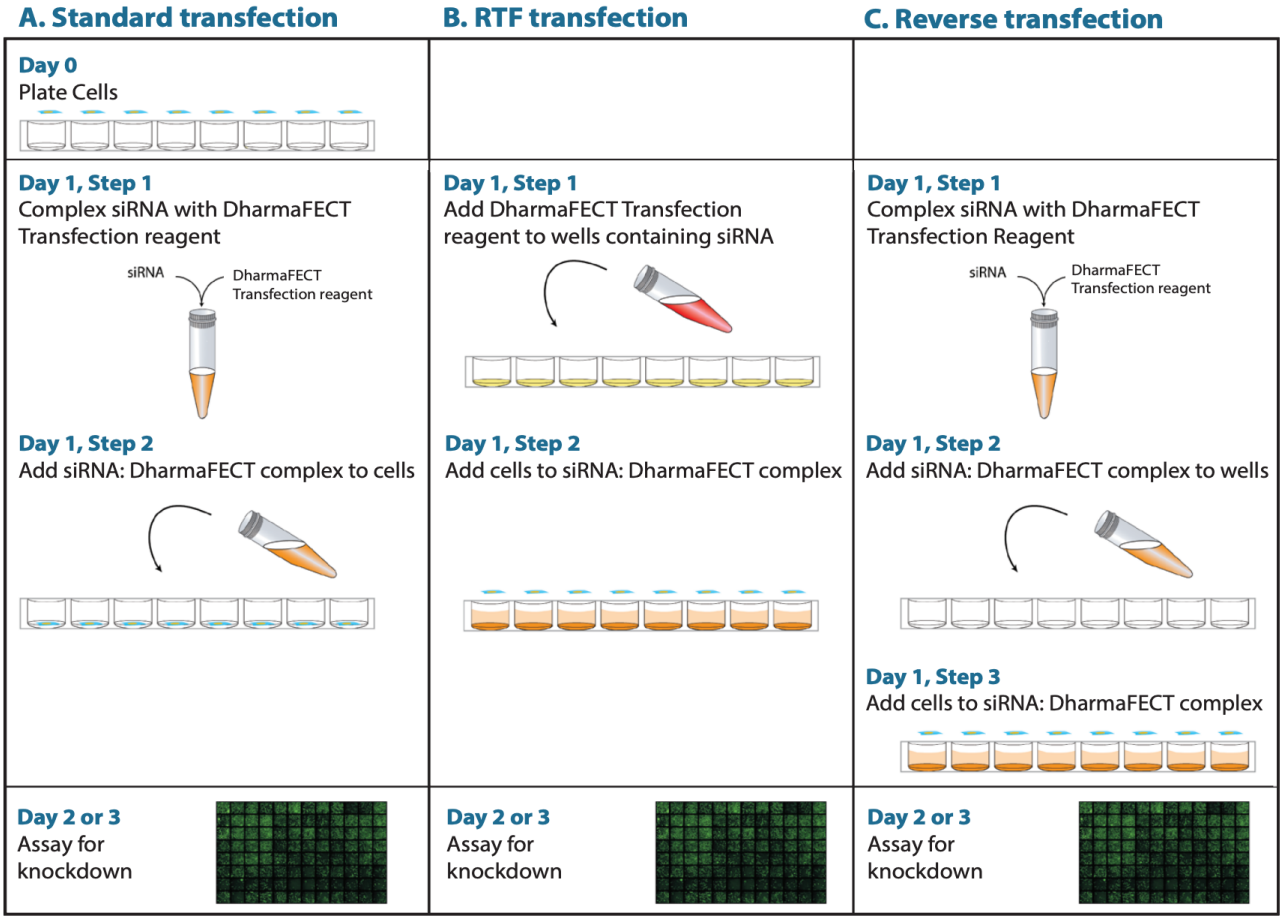

Figure 2.2: A) Standard (forward) transfection B) Reverse Transfection Format (RTF) and C) Reverse transfection methods. Two round transfection in MDDC was performed using a combination of RTF and forward transfection protocols.

2.6 Flow cytometry

2.6.1 Buffers

- FACS buffer — A 10X stock solution of 1% BSA, 0.025% sodium azide was diluted 1:10 using 1X dPBS for extracellular surface staining.
- 10X Phosflow® perm/wash buffer (BD Biosciences) — Diluted 1:10 using 1X PBS for permeabilisation, intracellular staining and washing.

2.6.2 Immunostaining antibodies

Target protein	**Channel/λ**	**Manufacturer**	**Catalogue no.**
AnnexinV	FITC	Biolegend	640914
CellTrace™	FarRed	Life Technologies	C34564
DAPI	358/461 nm	Life Technologies	D1306
LC3A/B	GFP	Enzo	ENZ-51031-K200
LIVE/DEAD® Aqua viability dye	367/526 nm	Life Technologies	L34965
Propidium Iodide (PI)	493/636 nm	Biolegend	640914
TO-PRO-3®	642/661 nm	Life Technologies	T3605

Table 2.3: List of immunostaining dyes for flow cytometry

Target protein	**Conjugate**	**Manufacturer**	**Catalogue no.**
2° goat α-rabbit	AlexaFluor-647	Life Technologies	A27040
CD4	FITC	BD	555748
CD5	APC	BD	555355
CD11b		BD	562793
CD11c	APC-Cy7	Biolegend	337218
CD45Ra	FITC	BD	556626
CD45Ro	PE	BD	555492
CD80	PE	BD	557227
CD83	PerCP-Cy5.5	BD	561129
CD86	APC	BD	555660
DC-SIGN	APC	BD	551545
GAPDH	Unconjugated	Abcam	ab9485
HLA-DR	APC	BD	560896
LC3A	PE	Abcam	ab211691
LC3A/B	Unconjugated	Sigma-Aldrich	L8918
LC3B	Unconjugated	Abcam	ab48394
MIF	Unconjugated	Abcam	ab7207
HIV-1 Gag p24	Unconjugated	Abcam	ab9071
HIV-1 Gag p24	FITC	Beckman-Coulter	6604665
HIV-1 Gag p24	PE	Beckman-Coulter	6604667

Table 2.4: List of immunostaining antibodies for flow cytometry

2.6.3 Extracellular surface staining

Cell surface staining was performed by creating a master-mix of required antibodies in FACS buffer at 1:100 dilution ratio per antibody. Cells were washed once in FACS buffer and transferred to FACS tubes (Gosselin) prior to staining using 100 μL of prepared staining mix per 1x10^5 cells. Extracellular staining was performed over 30 minutes at 4°C protected from light. Tubes were washed using 20X staining volume (∼2–3 mL) FACS buffer and centrifuged at 500 xG for 5 minutes, supernatant discarded and cells were resuspended in the remaining residual volume. An optional post-staining fixation step using 1% PFA for 15 minutes at room temperature may be performed at this point to retain antibody binding during short-term storage at 4°C.

2.6.4 Cell viability and intracellular staining

To determine cellular viability, a fixable LIVE/DEAD aqua dye (ThermoFisher Scientific) was diluted 1:1000 in 1X PBS. Samples were washed once in PBS and stained for 15 minutes at room temperature, protected from light and washed. Viability staining strictly cannot be performed following permeabilisation or fixation since the dye will readily bind intracellular amines only available after disruption to membrane integrity.

Intracellular staining was performed using a similar protocol to extracellular staining protocols detailed previously. A 1X Perm/Wash buffer diluted from a 10X PhosFlow™ (BD Biosciences) was used instead of FACS buffer as a staining medium. After staining, cells were washed once in 2–3 mL 1X Perm/Wash followed by a second wash using 2–3 mL FACS buffer and resuspension in residual volume.

2.6.5 Flow cytometry acquisition and analysis

Acquisition was performed on BD Canto™ II (BD Biosciences) flow cytometer. Isotype/unstained control samples were set up for each experiment and used to calibrate channel detector

voltages. For multi-colour flow cytometry, single-stained controls were used to generate a compensation matrix to account for spectral overlap. At least 5000 events were recorded per sample and exported Flow Cytometry Standard (FCS) data was analysed using FlowJo software package (FlowJo Enterprise). Gating strategies are described per experiment using SSC-A/FSC-A and FSC-H/FSC-A for cell size and cell doublet discrimination.

2.7 Immunoblotting

2.7.1 Buffers

- Lysis buffer — 20 mM Tris (pH 7.5) (Fisher); 150 mM NaCl (Fisher); 1% Tergitol-type NP-40 (Sigma-Aldrich); 1 mM MgCl2; 1 mM EGTA (Sigma-Aldrich); 1 mM NaVO4; 10 mM Na4P2O7; 1% n-dodecyl-β-D-Maltoside (Sigma-Aldrich) and 1X Protease Inhibitor (Roche).
- Running buffer — 1X MOPS SDS running buffer diluted from 20X stock (ThermoFisher Scientific).
- Transfer buffer — 1X NuPAGE^{TM} buffer diluted from 20X stock (ThermoFisher Scientific); 20% methanol (Fisher Scientific).
- Wash buffer — 1X PBS (Gibco^{TM} Life Technologies); 0.05% Tween-20.
- Blocking buffer — 1X PBS (Gibco^{TM} Life Technologies); 0.05% Tween-20; 1% bovine serum albumin (BSA).

2.7.2 Cell lysis and sample preparation

Cells were washed with 1X PBS, centrifuged at 300 xG for 5 minutes and all excess supernatant removed. 20μL of NP-40 lysis buffer was added to each well and cells were lysed for 20 minutes on ice. After lysis, samples were collected into 1.5mL eppendorf tubes and centrifuged at 15,000

xG for 20 minutes at 4°C. Supernatants were transferred to fresh tubes and quantified by NanoDrop at 280 nm absorbance to give an approximate concentration for SDS-PAGE. After quantification, samples were reduced in a NuPage sample reducing agent (10X, Invitrogen) and NuPage LDS sample buffer (4X, Invitrogen) mix and denatured by heating at 80°C for 10 minutes in an analogue electric heat block. Prepared samples were subsequently used for SDS-PAGE or transferred to -30°C for long-term storage.

2.7.3 Immunoblot protocol

4-12% bis-tris gels were hand-cast using Novex mini-cassette (Novex Life Technologies). High-range Amersham™ Rainbow™ markers (GE Healthcare Life Sciences) and SuperSignal™ molecular weight protein ladders were used for reference. 30 μg of samples were loaded into each lane and the SDS gel was run in X-Cell SureLock® running system (Invitrogen, Novex® Mini-cell) in MOPS running buffer at 150 V for 60 minutes using a Power350 system (Fisher Scientific). Proteins were transferred from the gel into 0.45 μm Nitrocellulose Blotting Membrane (Amersham™ Hybond ECL, GE Healthcare Life Sciences) using Mini ProteanII™ transfer system (Bio-Rad) in Pierce® Western Blotting Transfer Buffer. Transfer was performed at 100V for 60 minutes and transfer efficiency was confirmed using 0.4% Ponceau red solution stain. After transfer the membrane was blocked in 5% BSA for 30 minutes prior to overnight incubation with primary antibody at 4°C.

2.7.4 Immunoblot detection and quantification

After overnight primary antibody incubation using α-GAPDH or α-Actin, the nitrocellulose membrane was incubated for 1 hour at room temperature with a relevant secondary HRP-conjugated antibody. Following several washers, bands were detected using high performance chemiluminescence film (Hyperfilm™ ECL, GE Healthcare Amersham) using SuperSignal West Pico Solution (Thermo Scientific) in AGFA SRX 101A (Konica). An Unstained Standard

Mark12™ Ladder was used for molecular weight identification. Where required antibodies were removed from membranes using Restore™ Western Blot Stripping Buffer (Thermo Scientific) by 5–15 minutes treatment at room temperature. Before re-use of the membrane it was blocked on the rocking platform with 5% BSA for 30–40 minutes. Band intensity was analysed using image processing and analysis software Java ImageJ 1.48 (National Institute of Health).

2.8 Confocal immunofluorescence microscopy

2.8.1 Buffers and materials

- BioCoat™ poly-D-lysine coated 12 mm diameter glass coverslips (Corning®).
- Glycine buffer — 1X PBS (Gibco™ Life Technologies); 0.1 M glycine (Sigma-Aldrich).
- Permeabilisation/blocking buffer — 1X PBS (Gibco™ Life Technologies); 0.2% BSA (Sigma-Aldrich); 0.05% Saponin (Sigma-Aldrich); 1% human IgG.
- Permeabilisation/wash buffer — 1X PBS (Gibco™ Life Technologies); 0.2% BSA (Sigma-Aldrich); 0.05% Saponin (Sigma-Aldrich).
- VECTASHIELD® Antifade Mounting Media (Vector Laboratories).
- ZEISS LSM 880 scanning confocal microscope — 405 nm, 488 nm, 647 nm exictation lasers with Windows 10 PC running ZEN Blue/ZEN black (ZEISS) acquisition software.

2.8.2 Immunostaining protocol

12mm diameter BioCoat™ poly-D-lysine coated coverslips (Corning®) were placed into a 12-well plate and washed once with 1X PBS. Cells were taken out of culture and seeded at a density of 50,000 cells/coverslip in 50 μL, made up to a total volume of 300 μL and incubated for 1 hour at 37°C allow natural adherence of cells to the coverslip. Cells were then fixed at 1%

PFA (uninfected) or 4% PFA (HIV-1 infected) for 30 minutes and washed in PBS and stored at 4°C overnight for immunostaining.

Coverslips were then washed once with PBS and quenched in 300 μL glycine buffer for 20 minutes at room temperature and subsequently blocked for 30 minutes at room temperature with human IgG. Coverslips were washed in permeabilisation buffer and stained within the plate using an antibody mix in a total volume of 50 μL for 30 minutes at room temperature protected from light and washed in 3 times over 5 minutes in PBS whilst maintaining gentle agitation on a plate-shaker. A nuclear counterstain (DAPI or TOPRO-3) was used at a 1:1000 or 1:800 dilution respectively for 10 minutes. After washing the counterstain, coverslips were mounted onto microscope slides using VECTASHIELD and allowed 1 hour at 4°C protected from light before analysis.

2.8.3 Image acquisition

Imaging was performed on a Zeiss LSM 880 scanning confocal microscope using 405 nm, 488 nm, 647 nm lasers. A smart setup with best-signal configuration was used for acquisition with a pinhole aperture set to 1 AU. Laser powers were calibrated at 0.1–0.2% at 600-800 W gain and remained consistent between experimental replicates. After acquisition, image files were processed and exported using ZEN blue for analysis.

2.8.4 Image quantification

Image quantification was performed using Fiji (Fiji Is Just ImageJ) open-source distribution of ImageJ. Analyses were performed in batch using scripted macros written using the java-based ImJ macro language. Multi-channel confocal microscopy images were loaded in batch and pre-processed to split channels, enhance contrast and Gaussian blur for iterative particle analysis using the in-built ImageJ feature. Summary statistics for each image were generated detailing count, minimum, maximum and average size of autophagic spots and the total nuclei

count for normalisation. After batch analysis, summary statistics were exported and analysed in R/RStudio for further analysis and visualisation. An average spot count per cell variable was calculated and used a measure of autophagy induction. Full details of batch quantification methods can be found in Appendix A.2.

2.9 Bioinformatics

2.9.1 Functional enrichment analysis and gene ontology

The siRNA screen candidate list was converted into corresponding EntrezIDs using the DAVID gene ID conversion tool and uploaded as a list into DAVID for functional annotation. Enrichment analysis was used to correlate genes of interest with relevant GO terms using a modified Fisher's exact test of association. The results yield several annotation categories highlighting significantly enriched biological processes (BP), cellular compartments (CC) and molecular functions (MF).

2.9.2 Network analysis and visualisation

Enriched data was exported from DAVID and imported into Cytoscape V3.3.0. Annotations were visualised using EnrichmentMap (Bader Lab), a gene-network plug-in and an enrichment network was generated.

2.10 Statistical analyses

All statistical analyses and data visualisations were performed using R-3.3.0 and RStudio. Statistical significance is as described in figure legends with *, ** and *** representing p-values (p) of < 0.05, < 0.01 and < 0.001, respectively. Paired tests were performed using the Wilcoxon-paired ranked sums test unless otherwise specified. Multiple comparisons were performed using

Kruskal-Wallis with Dunn post hoc analysis with the dunn.test R package (Alexis Dinno). Principal components analysis (PCA) and hierarchial clustering analysis were performed using the PCA and HCPC (Hierarchical Clustering on Principal Components) function from the FactoMineR package. A full list of all R packages used is described below.

Package name	**Lead author**	**Version**
dunn.test	Alexis Dinno	1.3.5
dplyr	Hadley Wickham	0.8.2
FactoMineR	Francois Husson	1.42
ggplot2	Hadley Wickham	3.2.0
pROC	Xavier Robin	1.15.0
stringr	Hadley Wickham	1.4.0
reshape2	Hadley Wickham	1.4.3
Tidyverse	RStudio Development	1.2.1

Table 2.5: List of R packages used for statistical analyses

2.11 Ethics statement

All human tissues and associated data were handled in accordance with Human Tissues Act 2004. Healthy donor buffy coats provided by the Welsh Blood Service were anonymised and no patient identifiable material was received. Written informed consent for the use of human blood products in research was obtained from donors and all samples and procedures were approved by the local research ethics committee at Cardiff University.

Chapter 3

Establishing an *in vitro* cell model of DC–T-cell HIV-1 *trans*-infection

3.1 Background

This chapter explores the immunological properties of dendritic cells (DC) during HIV-1 infection and establish an *in vitro* model of cell-to-cell *trans*-infection to CD4+ T-cells. In addition, it aims to optimise methods of genetic downregulation in DCs in preparation for a high-throughput RNA interference (RNAi) screen for the identification of novel factors involved in the cellular transmission of HIV-1.

3.1.1 The monocyte-derived dendritic cell model

Tissue resident DCs and their subsets are naturally present in the dermis and are highly populated in the vaginal and rectal tracts making them some of the earliest targets for HIV-1 during infection at mucosal surfaces. Most dermal DCs (dDC) phenotypically resemble immature myeloid DC and develop from circulating CD14+ monocytes or bone marrow-derived CD34+ progenitor cells (pre-cDC) extravasated from the capillary bed into the dermis which differenti-

ate into CD14+ CD1a+ dDC subsets (Ahmed et al., 2015). Dermal DCs continuously sample the submucosa in search of pathogens such as viruses and bacteria in the extracellular space which are internalised into DCs by phagocytosis, macropinocytosis or by cell-surface receptor endocytosis. Following internalisation, antigenic stimulation initiates a complex maturation pathway in DC resulting in terminal differentiation by markedly upregulating co-stimulatory and chemotactic molecules to enable effective functions as professional APCs. Phenotypically mature DCs gain CCR7 expression and migrate from the dermis to local draining lymph nodes, highly populated with CD4+ T-cells where an adaptive immune response can be raised. Isolated dDC have been widely characterised as part of *ex vivo* human skin immune cell models previously developed within our group and have been useful in helping understand how DCs respond to infection and their potential roles as targets for vaccine delivery. However, these models face several practical limitations including the access and availability of patient samples, tissue quality and various ethical issues such as study approval and licensing which are barriers for many groups interested in studying these cells *in situ*. Therefore, it is unsurprising that techniques were developed to fabricate a cellular system which mimics the behaviour of infiltrating progenitors and their development into tissue-resident DC. Early work by Sallusto and Lanzavecchia (Sallusto and Lanzavecchia, 1994) first demonstrated that monocytes can differentiate into DCs *in vitro* in the presence of IL-4 and GM-CSF, giving rise to monocyte-derived dendritic cell (MDDC) cell systems, which have since been used over the last two decades and are now well defined showing both morphological and functional resemblance to DC found *in vivo* (Shortman and Liu, 2002; Leon et al., 2005). Monocytes can be easily isolated from peripheral blood; gradient centrifugation of whole blood using a high-density separation medium (such as Ficoll-Paque solution is commonly used to enhance purity and reduce platelet contamination (Repnik et al., 2003)) produces three visibly distinct fractions consisting of plasma, buffy coat (mononuclear cells, B-cells, T-cells, NK cells) and a denser fraction containing granulocytes and erythrocytes. After careful extraction of the peripheral blood mononuclear cells (PBMC)-containing buffy coat from in-between both layers, magnetic-activated cell sorting (MACS) is used to enhance the isolation purity of a target population. MACS microbeads are specific

antibodies conjugated with 50nm magnetic nanoparticles which either positively or negatively label cells for separation. Anti-CD14 microbeads are used to positvely label monocyte populations from PBMC making up 10-12% of the fraction and are passed through magnetic columns which retain microbead-labelled cells while unlabelled cells flow through the column. After several washing steps, the column can be eluted to produce a highly-purified CD14+ monocyte population for culture and differentiation into DCs. For the purposes of this research, the MDDC cellular model is a valid system for replicating the early events of HIV-1 infection and as a platform for the identification of new target genes involved in viral transfer from DC to CD4+ T-cells. However, further validation of the cellular model will be required to test primary DC subsets from blood and tissue explant models such as the vaginal mucosa and the foreskin.

3.2 Results

3.2.1 The roles of chemokines in mediating HIV-1 *cis*-infection

Chemokine receptor blocking agents, such as the CCR5 antagonist Maraviroc, have been used as a strategy against HIV-1 entry for over a decade and shown to be effective and well tolerated in large multi-centre clinical trials (Gulick et al., 2008). As part of a collaborative investigation with Bernhard Moser and colleagues into the novel discovery of CXCL12-CXCL14 chemokine synergy during in their interaction with CXCR4, we further explored the role of these chemokines in the regulation of viral infection through HIV-1 co-receptor modulation (Collins et al., 2017).

We used the engineered HeLa-derived TZM-bl reporter cell line expressing CD4, CXCR4 and CCR5 co-receptors with integrated reporter genes for firefly luciferase and β-galactosidase under transcriptional control of the HIV-1 long-terminal repeat (LTR). In a single-round infection assay using X4 HIV-1, we demonstrated that CXCL12 treatment induced a strong inhibition (Figure 3.1A) shown by the luminescence reduction in a dose-dependent manner with an ap-

proximate IC50 of 1 nM in agreement with similar reports (Balabanian et al., 2005). Since CXCL14 was found to induce conformational change of the CXCR4 upon binding we hypothesised that this could competitively inhibit gp120-CXCR4 interaction and block viral entry of X4 HIV-1. Conversely, CXCL14 treatment in fact greatly enhanced infection by more than 3-fold at 10-1000 nM doses. Further to this, concomitant treatment with CXCL12 only partially blocked infection but diminished infection with increasing treatment concentrations at 0.1nM and 1nM, suggesting that CXCL14 did not act synergistically with CXCL12 (Figure 3.1B). Since TZM-bl are co-expressing CXCR4 and CCR5, to assess CXCL14 potency to alter the entry and integration of R5- tropic HIV-1 virus we pre-treated cells with either CXCL12 or CXCL14 and infected with the R8-Bal HIV-1 strain in the same assay (Figure 3.1C). As expected, CXCL12 showed no inhibitory potential against R5 HIV-1. However, CXCL14 treatment at 1000 nM showed a similar 3-fold increase in productive infection. This observation of CXCR4-dependent modulation of R5 HIV-1 infection was both unusual and interesting. To explore this further, we used the CD4 and CCR5 expressing GHOST-R5 cell line stably transfected with a GFP reporter gene driven by the HIV LTR as an indicator of HIV-1 entry and integration (Vödrös et al., 2001). After pre-treatment with 0-1000 nM concentrations of CXCL14 and infection with R5 HIV-1, no differences were observed which suggests that augmentation of infection by CXCL14 is a CXCR4-dependent process (Figure 3.1D). Together, these results support a critical role for chemokines in the control of HIV-1 *cis*-infection. However, their functions in *trans*- are yet to be defined and warrant further investigation.

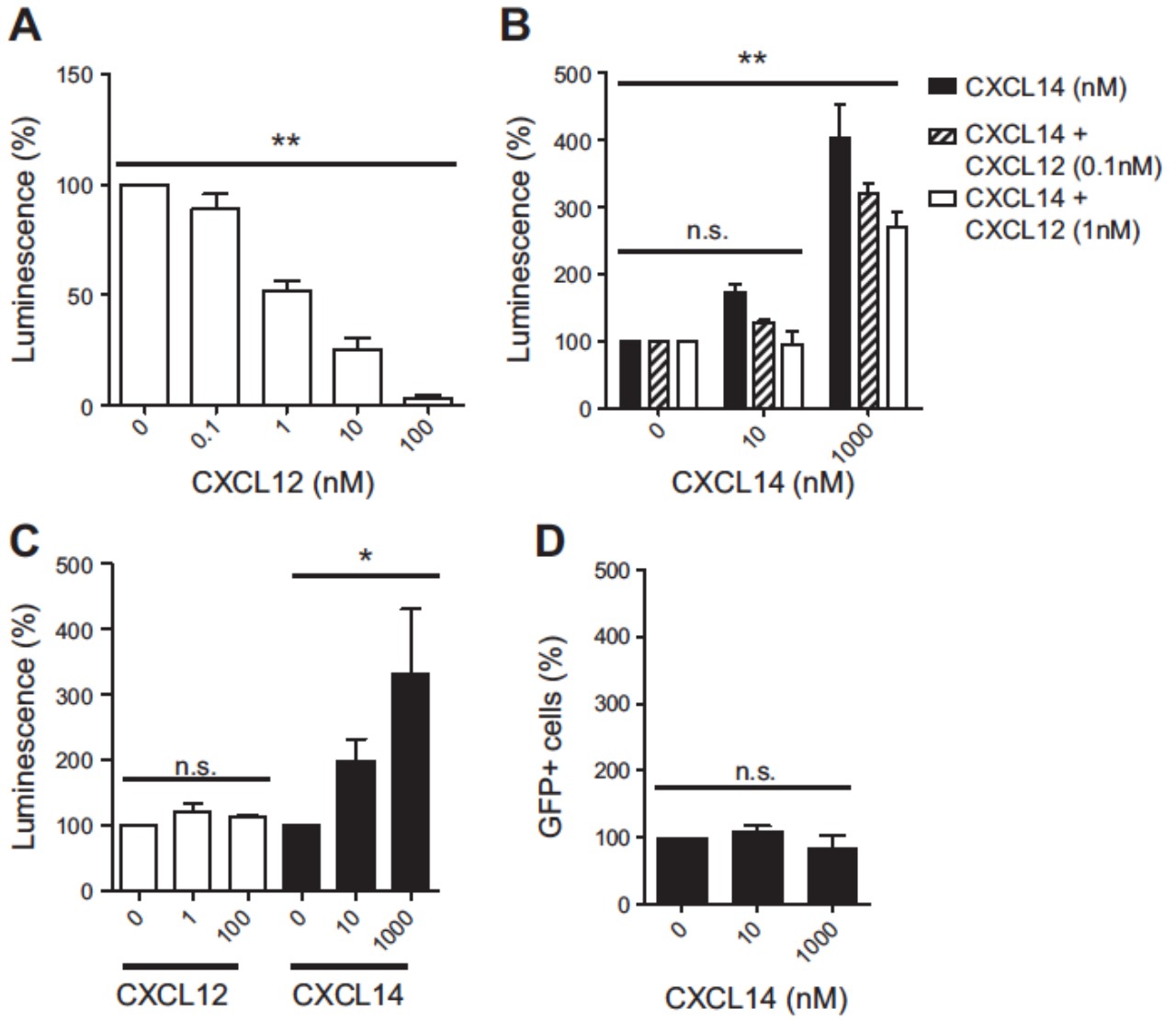

Figure 3.1: The role of CXCL12/CXCL14 stimulation on HIV-1 *cis*-infection. A) X4-tropic HIV-1 viral entry into TZM-bl cells expressing CD4, CXCR4 and CCR5 during incremental CXCL12 treatment. TZM-bl cells contain a luciferase reporter gene construct under transcriptional control of the HIV-1 long-terminal repeat (LTR). All data are normalised to infection in the absence of CXCL12 treatment. B) Entry of X4 HIV-1 into TZM-bl cells during combination treatment of 0–1000 nM CXCL14 (black bars) or CXCL14 in combination with 0.1 nM CXCL12 (dashed bars) or 1 nM CXCL12 (open bars). C) Viral entry of R5 HIV-1 into TZM-bl cells in the presence of 0–100 nM CXCL12 and 0–1000 nM CXCL14. 100% luminescence corresponds to luciferase reporter activity in the absence of chemokines. D) Viral entry of R5 HIV-1 into GHOST-R5 GFP reporter cells coexpressing CD4 and CCR5, but not CXCR4. All reported data are mean + SEM of 3 independent experiments. Statistical analyses performed using 1-way ANOVA with Bonferroni post-hoc. NS non-significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

3.2.2 Establishing a DC-to-T-cell HIV-1 *trans*-infection assay

We isolated and infected MDDCs using either F522Y (fusion-defective control virus) or a full-length, replicative, X4-tropic HIV-1 and co-cultured with the SupT1 CD4+ T-lymphoblastic cell line as a readout for viral transfer (detailed in Figure 3.2A). Although R5 HIV-1 viruses are typically most prevalent viral strain during new acquisitions, we investigated the role of X4 HIV-1 which are less prone to productive infection in DCs and are trafficked through endosomal pathways relevant for VS formation. In our assay we used a high-speed spinoculation (spin-infection) protocol, in which cells and virus are centrifuged at 1,200xG for 2 hours. Previous reports have suggested that these methods enhance infection through increased virus binding, which is often rate-limiting in most virus-pulsing type experiments (O'Doherty et al., 2000) and therefore important in maximising HIV-1 capture by DCs without using excessive viral titres which could saturate the cell system. We used the fusion-defective F522Y HIV-1 mutant as a negative control for viral transfer to SupT1 or forced entry into MDDC. Spinoculation is generally well tolerated as no significant levels of MDDC cell death were observed. After extensive washing steps to remove unadsorbed virus from cell surface, SupT1 were labelled using a CellTrace^{TM}FarRed membrane dye and added to culture at a 1:1 density for 48 hours. Whilst such dyes are typically used in cell proliferation assays, we were able to use this to easily distinguish cell types during analysis. After culture, cells were stained using a viability dye and fixed in 4% PFA prior to intracellular p24 staining and analysis by flow cytometry to selectively stain for the presence of HIV-1 capsid protein in both donor and recipient cells during transfer. Gating methods for HIV-1 transfer assays are described (Figure 3.2B). SupT1 viability staining suggests that up to 10% cell death can occur, though this would be expected since *in vitro* data suggests that X4 HIV-1 is more cytopathic than R5 strains (Grivel and Margolis, 1999; Penn et al., 1999). Viral transfer from MDDC typically follows a two-phase kinetic, which firstly increases over time in a linear phase before reaching a critical mass where transfer increases exponentially driven in part by low-level productive infection observed between 48–72 hours post-infection (Turville et al., 2004). Therefore, our assay was limited to a 48 hour co-culture

period to minimise levels of *cis*-infection. In summary, these methods are a suitable model for investigating HIV-1 *trans*-infection from MDDC by flow cytometry. Using a cell tracing dye to distinguish cell types, we are able to easily observe the viability and p24 staining in both DC and SupT1 to gain broader insight into cell-cell interaction.

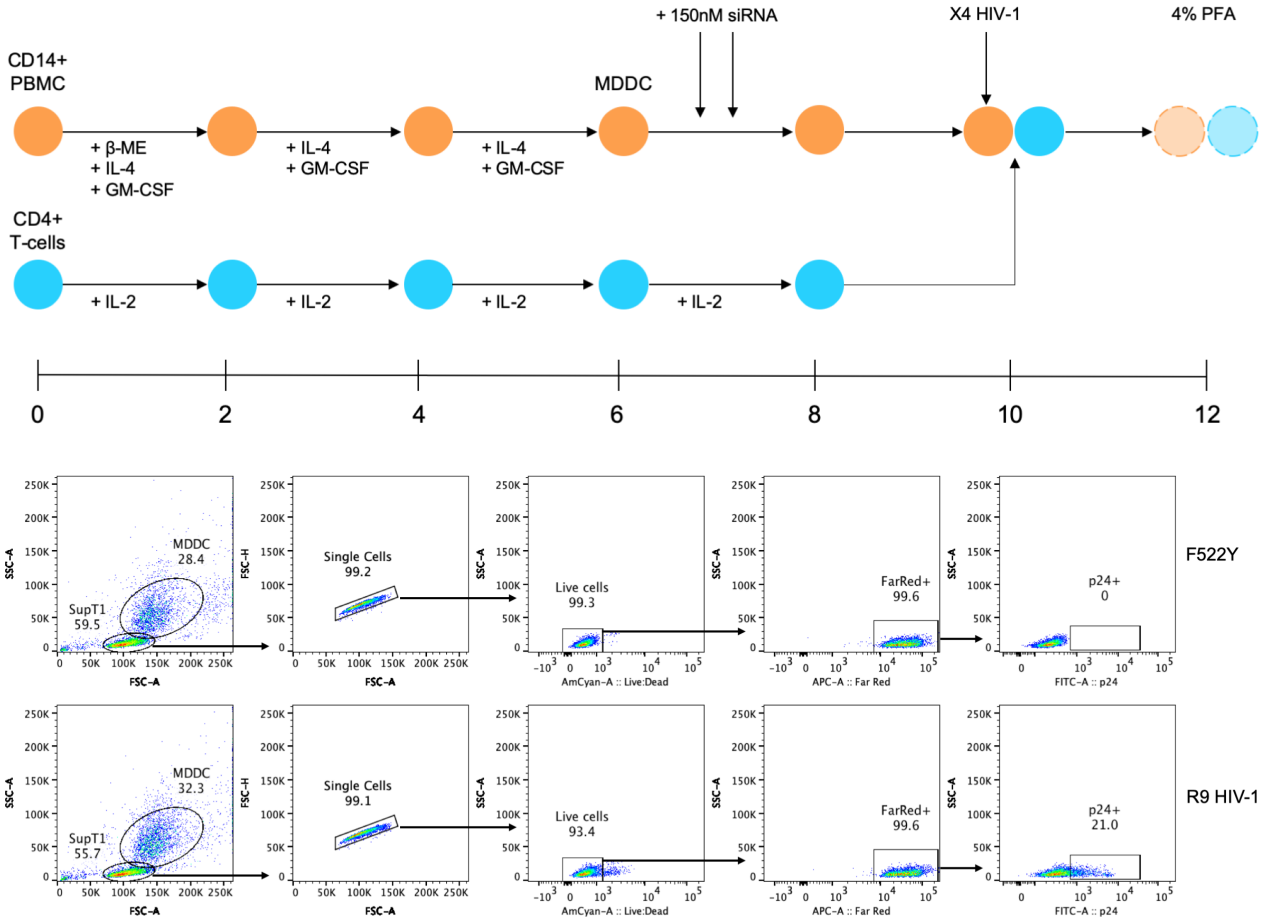

Figure 3.2: HIV-1 transfer assay. (A) Overview of experimental procedures describing differentiation protocol for CD14+ PBMC into MDDC using 500U/mL IL-4, GM-CSF and 50U/mL β-mercaptoethanol and maintenance of CD4+ T-cells using and 50U/mL IL-2.

3.2.3 Optimisation of genetic downregulation in MDDC

In preparation for a large scale high-throughput siRNA screen for cytokine and chemokine genes involved in cellular HIV-1 transmission, we first aimed to optimise gene silencing and establish protein knockdown efficiency in MDDC using siRNA. Following *in vitro* differentia-

tion over 7 days using IL-4/GM-CSF, genetic downregulation was performed using two rounds of 150nM siRNA 24 hours apart as detailed in section 2.7.3. MDDC were treated with media and transfection reagent as mock controls or transfected with non-target or siGAPDH. GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) is a 37kDa metabolic enzyme involved in the sixth stage of glycolysis involved in the breakdown glyceraldehyde-3-phosphate into 1,3-bisphosphoglycerate. Due to the high, stable and constitutive nature of its expression, GAPDH was used as a control in RNAi/protein quantification and was used as a target housekeeping gene in order to optimise the efficiency of downregulation compared to non-target.

Figure 3.3A shows a representative western blot of GAPDH expression following lysis of treated cells. Densitometry analysis of cell lysates across 3 donors showed an average -40.86% (SD=27.42, SEM=15.82, $p<0.05$) change in relative protein expression compared to non-target siRNA, which in keeping with previous findings within our lab that typical RNAi experiment in MDDC achieves an average 50% transfection efficiency (Figure 3.3B). This is an important consideration as this will naturally introduce variability into any gene silencing assays and poor transfection efficiencies could potentially mask the true effect size of target siRNA. These results show that significant levels of protein knockdown can be achieved using 150 nM siRNA in MDDC.

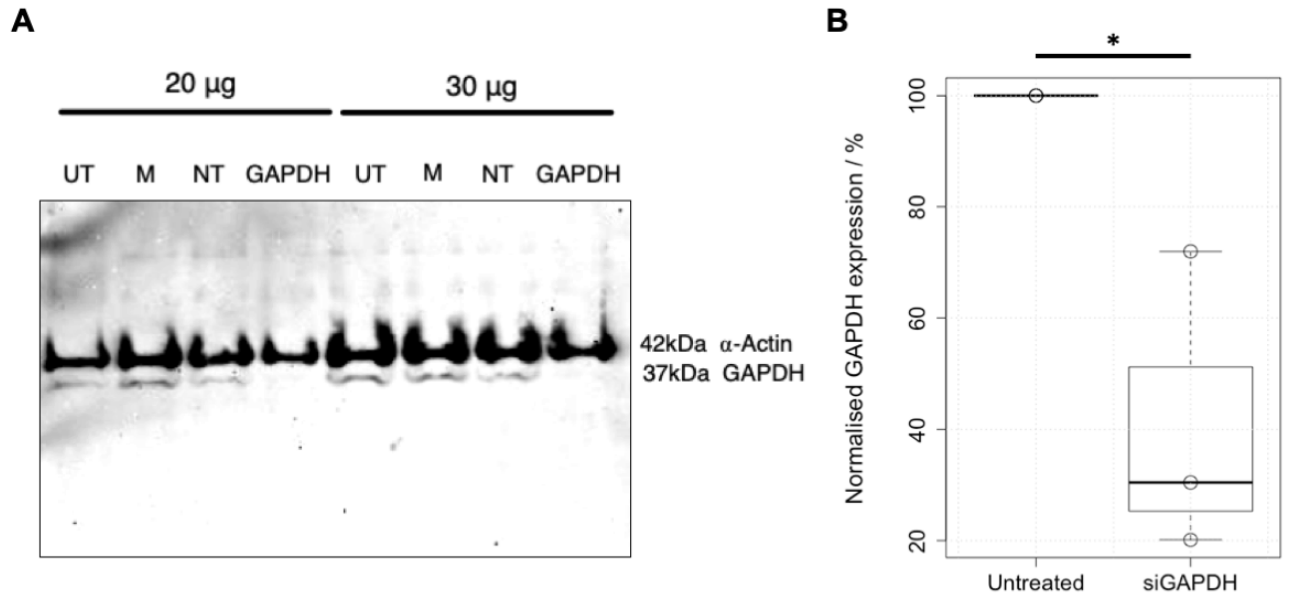

Figure 3.3: A) Protein knockdown efficiency of GAPDH in MDDC lysates. Lanes represent untreated (UT), HiPerFect mock treated (M), non-target siRNA transfected (NT) and GAPDH siRNA treated MDDC. B) GAPDH relative expression and knockdown determined by densitometry. Wilcoxon ranked sums paired test,* $p<0.05$, ** $p<0.01$, *** $p<0.001$. Data representative of 3 independent experiments.

3.2.4 Transfection reagents may influence viral transfer from DCs

To test for any non-specific changes in viral transfer of silenced MDDC, we spinoculated MDDC-siRNA controls and co-cultured with SupT1 cells in accordance with the HIV-1 transfer assay protocol detailed in section 2.4.4. Downregulation of GAPDH had no substantial effect on levels of viral transfer compared to transfection with non-target siRNA suggesting that MDDC maintain their functional characteristics. However, a noticeable increase in transfer was observed following treatment with the transfection reagent alone compared to mock-transfection controls increasing transfer 6-fold. When MDDC were matured using 1 μ/mL LPS prior to co-culture, this effect became much more pronounced resulting in an 8-fold increase in transfer between untreated and HiPerFect treated controls. LPS maturation led to an overall average 5.5-fold increase compared to non-LPS MDDC (Figure 3.4).

Off-target effects are a notoriously common occurrence in RNAi-based studies, however by

properly addressing these issues they can be mitigated. There are multiple types of off-target effect which can appear at various stages including mRNA degradation, inhibition of translation or induction of an interferon responses (Jackson and Linsley, 2010). Older generation transfection reagents have typically been associated with technical problems including cytotoxicity, lack of reproducibility and low transfection efficiency. Our method uses a mixture of cationic and neutral lipids needed for liposomal delivery of siRNA which is purposely designed to address these issues. However observation that viral transfer among HiPerFect treated MDDC is markedly increased compared to untreated MDDC highlights a potential caveat within our study. Since mature DC are known to be more effective during trans-infection than immature DC, it is plausible that toxicity-induced maturation may be a likely cause for these observations. In addition, siRNA have been reported to be toxic to cells and increases the risk of false-positive results (Fedorov et al., 2006), however cell viability among non-LPS transfected samples remained largely unchanged. Despite this, all samples within the screen will be exposed to the same concentrations of transfection reagent. The screen readout is therefore be based on relative change of viral transfer from sample to non-target siRNA and is not invalidated by these findings.

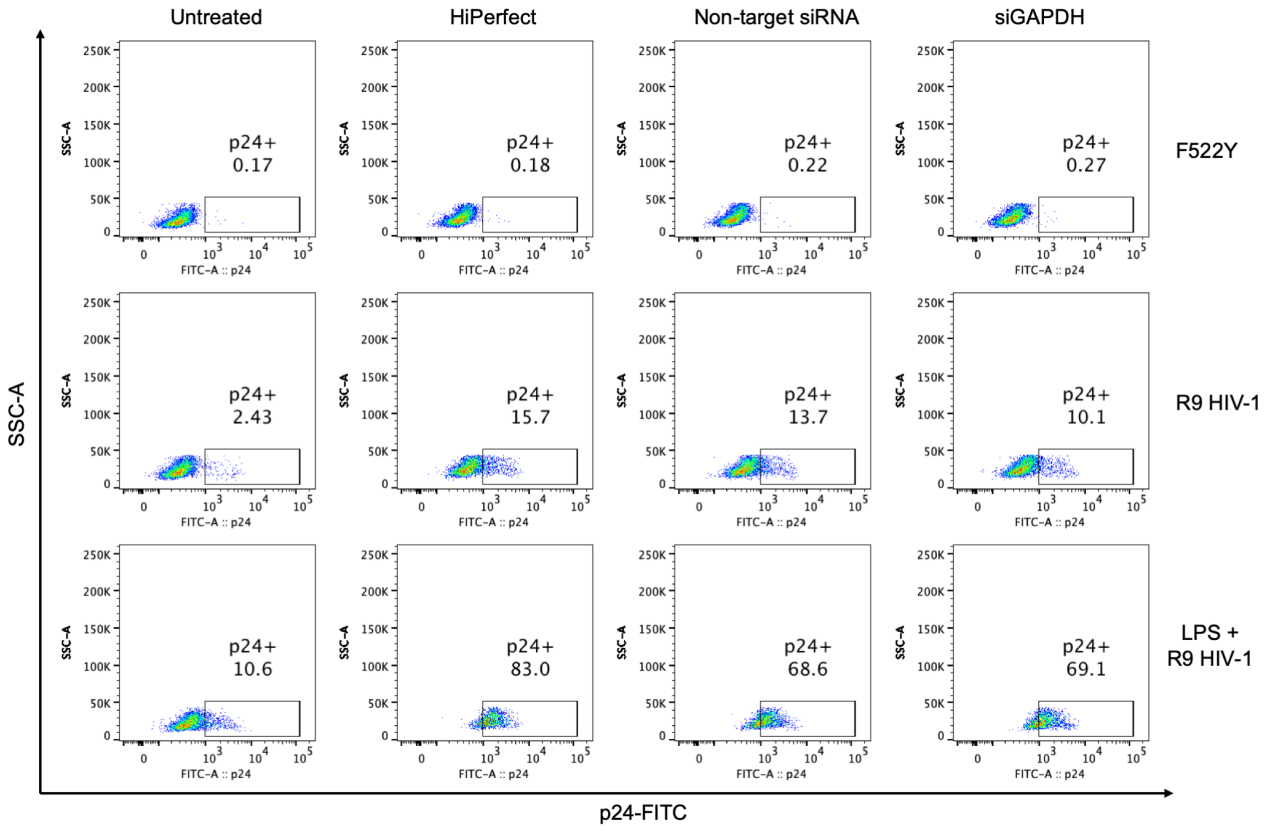

Figure 3.4: Optimisation of siRNA transfection and reagents during HIV-1 transfer assay to SuPT1 T-cells. MDDC (±LPS maturation) were either untreated, treated with HiPerFect transfection reagent only or transfected with non-target/GAPDH siRNA before infection with X4 HIV-1 and co-culture with SupT1 recipient cells.

3.2.5 Transfection with siRNA may induce partial DC maturation

To assess phenotypic changes to MDDC induced by our transfection protocol, we stained for an array of related DC maturation and activation markers. Of these we stained for surface expression of lineage markers DC-SIGN, CD11b and CD11c which are all highly expressed in MDDC and are frequently used to quality control cells following differentiation from CD14+ monocytes. In addition, we stained for co-stimulatory antigens CD80, CD83 and CD86 (Figure 3.5). We transfected MDDC with GAPDH siRNA, non-target siRNA or tested treatments with HiPerFect transfection reagent or transfection media only and analysed phenotypic changes compared to MDDC stimulated with common TLR agonists LPS, PolyI:C and CL075.

CD11b-CD11c double positive expression ranged from 85.9%-95.1% across all samples suggesting no substantial change to DC lineage. After gating on this population we observed a modest increase in CD86 expression from 73.9% positivity in untreated MDDC to 91.1%, 90.0% in HiPerFect-treated and siGAPDH MDDC respectively which was comparable with LPS (94.2%), Poly(I:C) (96.5%) with exception to CL075 (73.2%). DC-SIGN expression was unchanged between samples (88.4%-96.3% expression) further suggesting that transfection had no effect on DC lineage. CD83 is not expressed in immature MDDC but is markedly upregulated during maturation and activation. In untreated MDDC, 4.59% of cells were CD83-positive which increased to 18.9% and 19.5% positivity for HiPerFect-treated and siGAPDH transfected samples respectively. LPS-matured MDDC were 52.2% positive for CD83 with only modest changes in expression observed in MDDC treated with CL075 or Poly(I:C). These data suggest that our methods may induce some degree of maturation during transfection, though this was not considered substantial and would continue to assume a mixed DC population.

DCs form the first-line of defence against infection and prime secondary responses by the adaptive immune system through antigen-presentation, driven by dynamic maturation and activation processes. Our results found modest increases in both CD86 and CD83 in mock and transfected MDDC. CD86 is highly expressed on APCs and is required for T-cell activation via interaction with and is typically used as a measure of maturation, since DCs typically inherit antigen-presenting capabilities following a response to external stimuli such as pathogen recognition. During differentiation from CD14 enriched PBMC to MDDC, we would expect steady-state CD86 expression between 70%-90% which naturally reflects their biological functions in cross-priming. CD83 is perhaps the best characterised and most sensitive marker for fully matured, immunogenic DCs (Prechtel and Steinkasserer, 2007). Our results showed that upregulated CD83 expression was driven mostly by exposure to HiPerFect. Lipid complexes are known to induce cytotoxicity during transfection (Breunig et al., 2007) and may therefore promote maturation through induction of a stress response. No changes in expression were observed between HiPerFect and siGAPDH MDDC, suggesting that siRNA does not induce phenotypic changes. While siRNA are usually well tolerated, there is a possibility of endosomal

TLR7/8 activation if siRNA fails to be delivered into cytosol, which could in itself could alter the dynamics of viral transfer. In summary our data suggest modest changes to DC phenotype though these differences were not deemed substantial enough to justify isolated investigation of MDDC at different states of maturation (immature vs mature). Studying a single homogenous DC population would be technically challenging in practice as our cell model assumes a mixed population of DCs. Immature cells would require cell sorting which in itself could induce maturation.

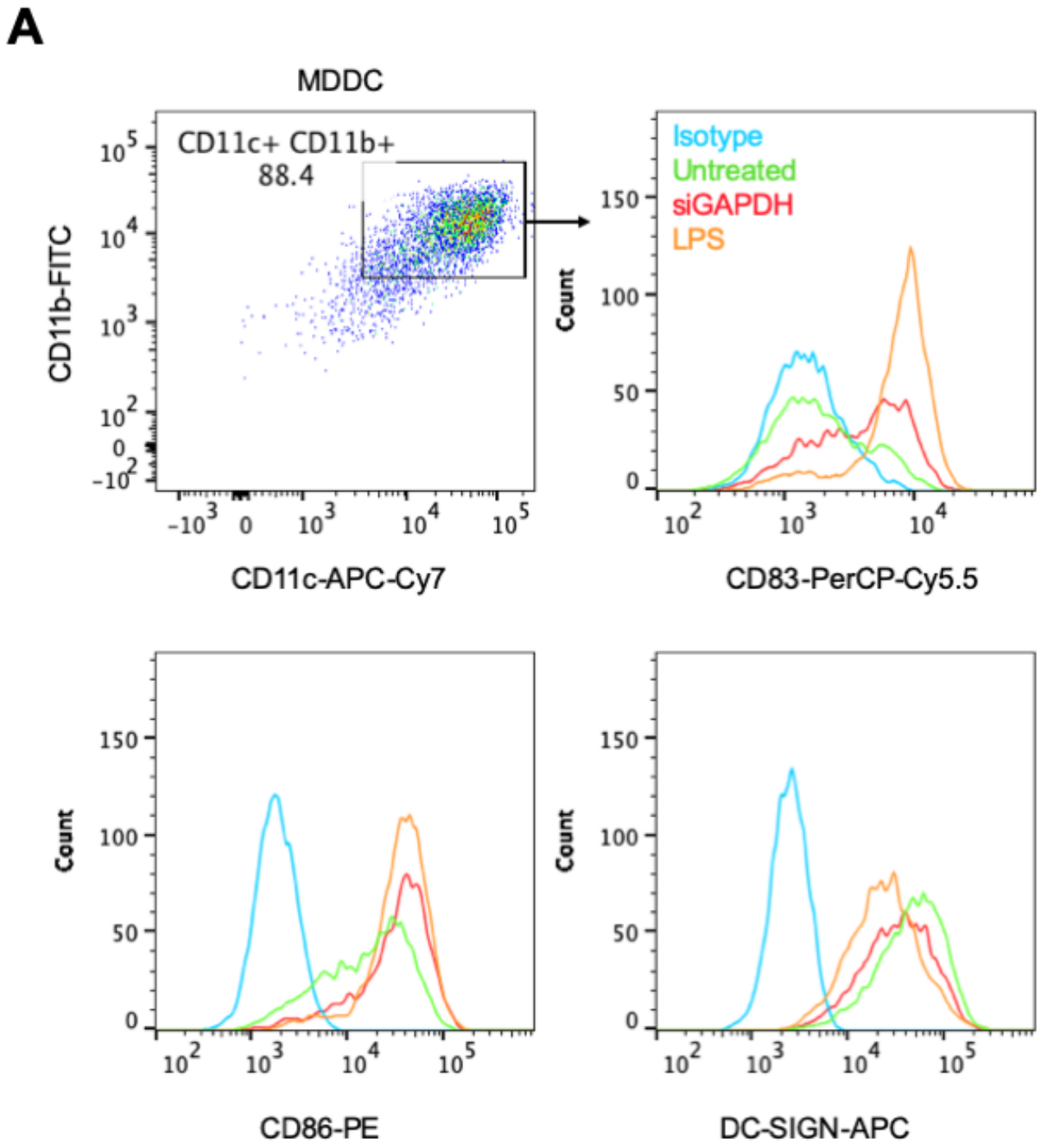

Figure 3.5: MDDC maturation phenotype and activation following two rounds of transfection with 150nM non-target siRNA in comparison to LPS-induced maturation at 1 μg/mL 24 hours prior to analysis.

3.3 Discussion

3.3.1 The role of CC- and CXC-chemokines in the modulation of HIV-1 entry

It is clear that cytokine and chemokine signalling plays a pivotal role in the regulation of immune processes guiding cell differentiation, migration, activation and importantly the cellular responses to infection. The discovery that HIV-1 exploits chemokine receptors CXCR4 and CCR5 for entry and that the CCR5Δ32 mutation conveys resistance to infection founded new insights into the viral life cycle and informed the design of novel antiviral therapeutics including the CCR5 antagonist Maraviroc. Such studies would suggest that long term protection against new infections could be best achieved by blocking host proteins and receptors rather than targetting viral proteins which are known to subvert the immune system (Oberlin et al., 1996; Huang et al., 1996; Gulick et al., 2008; Hardy et al., 2010). In agreement, we were able to confirm that blockade of CXCR4 by its *de facto* substrate CXCL12 can effectively reduce levels of infection by X4 HIV-1 in TZM-bl. Further to this, our studies also investigated a new role for CXCL14 in HIV-1 infection. CXCL14 is known to synergise with CXCL12 and act as a positive allosteric modulator, capable of altering the structural conformation of CXCR4 to enhance interaction with its primary ligand (Collins et al., 2017). Contrary to our initial hypothesis, CXCL14 treatment substantially enhanced infection by X4 HIV-1. These findings suggest that CXCL14-CXCR4 interaction leads to conformational changes that are preferential for binding and entry of HIV-gp120. Given the trimeric structure of the HIV-1 Env and the ability for CD4 to oligomerise at the cell surface, a likely scenario of virus attachment may involve multiple CD4-gp120 interactions, creating a stable tether between virus and the target cell, increasing the likelihood of co-receptor engagement (Sakihama et al., 1995; Singer et al., 2001). Together with growing evidence that suggests oligomerisation is required for effective signalling through chemokine receptors (Hauser et al., 2016), CXCL14 may facilitate this process and enhance co-receptor recruitment though further work would be required to

understand this mechanism fully. Interestingly, the ability for CXCL14 to enhance infection was observed across both X4 and R5-tropic strains of HIV-1 in cells that co-express both receptors. However, infection of CCR5+ GHOST cells did not show any further enhancement suggesting that CXCL14 acts in a CXCR4-dependent manner. Since CD4/CXCR4/CCR5 oligomers have been found to inhibit binding with gp120 (Martinez-Munoz et al., 2014) in CXCR4+ CCR5+ cells, CXCL14-induced conformational changes to CXCR4 could prevent its oligomerisation with CD4/CCR5 and enhance the interaction of R5-tropic HIV-1 (Martinez-Munoz et al., 2014; Collins et al., 2017). Additionally, since CXCL14 is a chemoattractant for CD14+ DC precursors and constitutively expressed at high levels in epithelial cells (Schaerli et al., 2005), it is likely that CXCL14 could alter the susceptibility to infection at mucosal surfaces and facilitate uptake by resident DCs.

3.3.2 Review of the MDDC cell model

DCs and their subsets represent some of the earliest known targets for HIV-1 and have critical roles in determining the outcome of acute infection. The MDDC cell model offers many advantages over other systems using DCs derived from ex vivo human skin which mostly address issues of practical feasibility as skin tissue is often difficult to source and dependent on surgery dates, ethical approval and informed consent. PBMC are easily obtained in high number through venipuncture and can be sorted using MACS to produce a highly-purified 10-12% yield of CD14+ monocytes for differentiation into MDDC. Magnetic separation allows for effective sorting without the need FACS, which could predispose cells to unfavourable conditions for long periods outside of culture. In addition, such methods may expose cells to contaminants (such as particulates, debris and secreted proteins) which sterile single-use magnetic columns seek to avoid. Concentrated donor buffy coats was used and could provide as much as 10^8 CD14+ monocytes per sample, allowing the generation of higher volumes of DCs needed for larger-scale experiments. An interesting characteristic of monocytes is their ability to develop into a number of cell types depending on their culture conditions and exposure to certain cytokine

signals. While GM-CSF/IL-4 remains the most conventional method for MDDC generation, alternative differentiation protocols have previously been described for the study of specific DC subgroups such as GM-CSF/IL-15 based protocols to produce DCs with properties similar to LCs (Mohamadzadeh et al., 2001), or culture with GM-CSF/TNF-α producing inflammatory DC that are known to polarise Th1/Th17 T-cell responses (Iwamoto et al., 2007). More recent findings have shown that human monocytes themselves exist in 3 major groups: classical (CD14+CD16-), intermediate (CD14+CD16+) and non-classical (CD14-CD16+) subsets, changing the common perspective as a developmental intermediary cell to one with more significant effector function (Ziegler-Heitbrock and Hofer, 2013). Though interestingly, in the presence of GM-CSF/IL-4 they have been shown to differentiate into DCs irrespective of their subset (Geissmann et al., 2003, 2008; Liu and Nussenzweig, 2010). Using our methods, MDDC are differentiated into a mixed population of immature and mature cells which resemble blood-derived myeloid DC subsets and DC-SIGN+ dermal DCs together with the loss of CD14 expression during differentiation, which distinguishes MDDC from inflammatory DC subsets (Hespel and Moser, 2012; Mildner and Jung, 2014). Together, the MDDC cell model is therefore a viable solution to addressing limitations associated with isolating primary DCs.

However despite the practicality and convenience that the MDDC cell model can offer, it is still challenged with various limitations. Firstly and simply, *in vitro* models are not fully representative of what is observed *in vivo* - tissue-resident dermal DCs are likely to have undergone significantly more complex differentiation processes and been exposed to a range of environmental signals from within the tissue, which would explain the generation of multiple DC subsets that have been previously observed in skin tissue explants. Secondly, although the MDDC cell model partially addresses the issue of subset heterogeneity, it does not account for donor-dependent variabilities. Several factors including the responsiveness to cytokines, prior antigenic exposure/stimulation and ratio between immature vs mature DCs can differ between donors. One strategy for studying a single population would be to induce maturation with LPS, however this has been previously shown to alter the susceptibility to infection and our results show this drastically changes the dynamics of viral transfer in our system. Immature

DCs are more receptive to infection but less efficient at transferring virus, on the other hand matured DCs are not as easily infected but are highly efficient at transferring virus.

3.3.3 MDDC interactions with HIV-1

In the context of HIV-1 cis-infection, the levels of productive infection in MDDC and their subsets remain relatively low as a result of their low surface expression of CD4/CXCR4 and the presence of host restriction factors which block viral life cycle events. Our results showed that only several percent of MDDCs exposed to X4 HIV-1 expressed intracellular gag-p24. Although MDDC are generally considered non-permissive to productive infection by X4 HIV-1, our results show they are more susceptible to infection by R5-tropic strains. This is in agreement with clinical observations that the majority primary infections are predominantly of R5 origin due to co-receptor availability at the mucosal sites, which later switch to an X4 tropism in approximately 50% of patients over the course of disease (Regoes and Bonhoeffer, 2005). Therefore, R5-tropic (historically termed M-tropic or Macrophage-tropic) HIV-1 is perhaps most representative of early stage infections, however we chose to continue optimisation using X4-tropic HIV-1 for our *in vitro* models in order to minimise levels of *cis*-infection, focussing more specifically on trafficking pathways associated with *trans*-infection (Garcia et al., 2005).

Several mechanisms of HIV-1 entry into DCs via the *trans*-infection pathway have now been characterised. HIV-1 exploits alternative attachment factors for *trans*-infection and viral capture occurs mainly through the C-type lectin receptor DC-SIGN, which is highly expressed on MDDC cell surface and makes them an attractive model for studying early host-virus interactions (Curtis et al., 1992). HIV-1 gp120 binds DC-SIGN with high affinity resulting in rapid virus internalisation by endocytosis which is mostly degraded following delivery to lysosomes. However a small proportion (5-10%) of virus remains intact, evading degradation and is trafficked through endosomal sorting pathways to accumulate within tetraspanin-enriched virus-containing compartments (VCC) resembling late endosomes (Garcia et al., 2008). From

here, the VCC is polarised to the zone of contact during cell-cell association for transfer across the VS. While receptor-mediated endocytosis by DC-SIGN continues to be the most well established mode of entry, attributing for the majority of internalised virus, there is now substantial evidence supporting receptor-independent entry mechanisms including clathrin-mediated endocytosis (Vendeville et al., 2004; Daecke et al., 2005; Cambi et al., 2009), clathrin-independent (CLIC/GEEC) endocytosis (Mayor et al., 2014) and macropinocytosis (Wang et al., 2008). Our assay is limited in the inability to distinguish which endocytic pathway HIV-1 utilises for DC entry, though regardless of entry mechanism the evidence would suggest that viral trafficking pathways converge at the VCC during viral accumulation prior to transfer. One exception to this would be emerging models of uptake in mature DCs. Developments in 3D electron microscopy have recently shed light on VCC formation showing that mature DCs can capture and retain HIV-1 into invaginated pockets continuous with the plasma membrane and during cell-cell contact, the target cell is able to extends CD4-rich filopodia into these compartments for *trans*-infection (Izquierdo-Useros et al., 2011; Felts et al., 2010). Although debated, these invaginations are considered to be accessible by exogenous reagents and antibodies, however tight association at the DC-T-cell junction may prevent this. Nevertheless, we were unable to observe p24 signal generation in MDDC following spinoculation with the F522Y fusion-mutant, suggesting that all unbound virus was washed off leaving only actively internalised virus available for transfer.

HIV-1 transfer at the DC-T-cell contact has been well established as the most efficient mode of transmission and estimated at 1,000-18,000 times more efficient than infection by cell-free virus (Chen et al., 2007; Martin et al., 2010; Lehmann et al., 2011). Our experimental methods centre around well defined *in vitro* model of *trans*-infection from HIV-1 spinoculated MDDC to co-cultured CD4+ target cells across a highly efficient VS (Arrighi et al., 2004; Garcia et al., 2005). In preparation for a large-scale viral transfer screen, we optimised our assay using the T-cell lymphoblastic cell line, SupT1 as a recipient cell for trans-infection. SupT1 are frequently used in HIV-1 infection assays due to the high levels of CD4 surface expression and are highly permissive to infection by both X4 and R5 tropic strains of HIV-1. In addition, SupT1 are

CD3-TCR negative and have low surface expression of heparin sulphate proteoglycans that are typically involved in cell adhesion processes and previously been reported to interact with HIV-1 env as an attachment factor (Jones et al., 2005; Vongchan and Linhardt, 2007; Herold et al., 2014). As a homogenous immortalised cell line, the SupT1 were beneficial in maintaining consistent transfer readouts and by limiting the number of primary cell types in our assay we reduce the risk of introducing any further variability to our results.

In contrast to the roles of chemokines and their receptors as host dependency factors (HDF) as previously discussed, there is growing evidence suggesting that the cytokine-chemokine network is implicated in the innate control and restriction infection (Katsikis et al., 2011; Arnold et al., 2015; Czubala et al., 2016). However, the role of cytokines/chemokines regulating mechanisms of HIV-1 *trans*-infection has remained largely unexplored. The cytokine-driven pro-inflammatory events that occur during acute infection are of particular importance in the generation of a hostile environment to prevent viral dissemination. Of note, the type 1 interferon (IFN-I) response is considered the hallmark anti-viral effector module against early HIV-1 infection which is released in large quantities by plasmacytoid DCs (Isaacs and Lindenmann, 1957; Isaacs et al., 1957; Siegal et al., 1999; Beignon et al., 2005).

In addition, IFN-I signalling is required for DC maturation *in vivo* and can metabolically reprogramme DCs to support the energy needs for antigen-presentation, motility and cell survival (Minton, 2014). It is now well established that DC maturation state can influence both *cis-/trans*-infection of HIV-1 (Granelli-Piperno et al., 1998; Bakri et al., 2001) and is therefore an important consideration for our cell model. While immature DCs preferentially replicate R5 HIV-1 (Granelli-Piperno et al., 1998), mature DCs are less prone to productive infection since maturation downregulates the surface expression of CCR5 and shuts down endocytic pathways such as macropinocytosis, blocking the entry and internalisation of virus (Garrett et al., 2000; Cavrois et al., 2006). However, these factors only partly contribute to the overal restriction observed in mature DCs. A study by Bakri *et al.* (Bakri et al., 2001) delineated the effects of maturation on each stage of replication and found that DC maturation induced

a post-integration block of HIV-1 replication and although the exact mechanisms still remain unclear, potential negative regulatory events of HIV-1 LTR transcription have been proposed. Consistent with previous reports (Geijtenbeek et al., 2000a; McDonald et al., 2003), our results demonstrate that *trans*-infection by mature DCs across the VS is more efficient than immature DCs. These results are not surprising given the specialist APC functions that DCs assume during maturation, allowing DCs to form large actin-containing membrane extensions around target CD4+ T-cells to maximise cell-surface contact and achieve effective viral transfer (Lehmann et al., 2011). In addition, the intracellular trafficking of HIV-1 to the VCC during *trans*-infection differs between immature and mature DCs which may impact successful transfer. Current data suggests that internalised HIV-1 localises in scattered peripheral vesicles in immature DC but are trafficked to larger perinuclear compartments following maturation (Frank et al., 2002; Garcia et al., 2005).

While DCs typically upregulate maturation markers in response to stimuli such as PAMP recognition, pro-inflammatory cytokines or stress, the results of our siRNA optimisation experiments showed that exposure to transfection reagents alone was enough to induce marginal levels of maturation, enhancing CD83 and CD86 expression. Our methods used a first-generation delivery system based on electrostatic interaction between cationic (conventional) liposomes and largely anionic cellular membranes. Although modern transfection reagents are developed for use in low volumes to minimise cytotoxicity, previous reports suggest that the physiochemical properties of cationic liposomes and liposomal-mediated delivery may still induce DC maturation (Quer et al., 2012; Soema et al., 2015). However cationic lipids fail to enhance NF-κB expression or pro-inflammatory cytokine release (Vangasseri et al., 2006) suggesting that transfected DCs only achieve partial phenotypic maturation. This may in part explain the difficulty of transfecting primary cells with dynamic endo-lysosomal function, since DC maturation regulates lysosomal acidification, enhancing proteolysis through induction of the vacuolar proton pump on the lysosome membrane (Trombetta et al., 2003). Lysosomal pH in mature DC was approximately pH 4.5 in contrast to pH 5.4 in immature DC (Trombetta et al., 2003; McCurley and Mellman, 2010) which could facilitate siRNA degradation. Interestingly while most

viral infections can trigger DC maturation, there is evidence to suggest that HIV-1 subverts this mechanism by dysregulating the cytokine profile to prevent recognition and restriction, although this is still debated (Granelli-Piperno et al., 2004; Weissman et al., 2000; Hertoghs et al., 2015).

3.4 Concluding remarks

In summary, this chapter highlights the importance of cytokines and chemokines in the control of HIV-1 life cycle, adding to building evidence supporting the rationale of this project. Optimisation of an *in vitro* model of *trans*-infection from MDDC to CD4+ T-cells is a critical step in this project. Moreover, this chapter found that transfection lead to functional changes in the cell system suggesting that transfection reagents may induce partial DC maturation and potentially impacting the dynamics of viral transfer. The cytokine milieu that arises during infection at mucosal surfaces is both complex and difficult to dissect given the diversity of cell types and functions that exist at these sites. Despite efforts to better understand the cytokine network that arises in response to acute HIV-1 infection, their role has only been partially addressed and is yet to be elucidated during *trans*-infection. Therefore, these results warrant further investigation of cytokines and chemokines in the regulation HIV-1 infection in *trans* and our cell model can provide a stable platform for high-throughput screening for novel candidates.

Chapter 4

A high throughput screen for HIV-1 restriction and host-dependency factors in dendritic cells

4.1 Background

The optimisation of an *in vitro* cell-to-cell transmission model from MDDC to CD4+ T-cells serves an important platform to identify novel factors regulating the mechanisms of *trans*-infection. This chapter aims to cover the whole process of high-throughput RNA interference including the design and development of an analytics workflow and statistical approaches for hit selection. The candidates generated from this screen are discussed, detailing their roles in DC biology and interactions with HIV-1 to explore potential avenues for downstream validation.

4.1.1 Principles of RNA interference and strategies for research

RNA carries genetic information required for direct synthesis of specific proteins. Non-coding tRNA was first discovered during the mid 1960s and was initially overlooked until the identification of new RNA classes more than a decade later. The importance of non-coding RNA was soon realised as key regulators of gene transcription, constituting 60% of total non-coding RNA (Delpu et al., 2016) and enabling research in a bid fully understand the regulatory network controlling gene expression. Inclusive of the classes of non-coding RNA, are small-interfering RNA (siRNA) and micro RNA (miRNA), found to exist in both plant and animal cells and naturally regulate gene expression through targeted disruption of mRNA translation. In 1998, Fire and Mello first characterised the mechanisms of RNA interference (RNAi) for post-translational gene silencing in nematodes, which was later followed by findings that exogenous siRNA was able to recapitulate this knockdown in mammalian cells, opening a new avenue for drug discovery and targeted therapies (Fire et al., 1998; Elbashir et al., 2001). It has since been a powerful tool for understanding molecular mechanisms of disease and investigating host-pathogen interactions, particularly during infection by RNA retroviruses such as HIV-1. Our laboratory was one of the first to successfully down-regulate gene expression in DCs using siRNA which we have been able to use to explore mechanisms regulating cell-cell transmission of HIV-1 (Arrighi et al., 2004; Blanchet et al., 2010). Genetically silencing by siRNA has been historically performed using a $Ca_3(PO_4)_2$-based transfection protocol, which temporarily permeabilises cell membranes to allow the movement of dsRNA into mammalian cells (Graham and van der Eb, 1973). However in practice, these methods were often prone to pH and temperature changes, produced cytotoxic effects and associated with higher rates of off-target effects. More recently, cationic-lipid transfection has been a more popular method of gene delivery which is generally well tolerated and maintains optimal delivery when using low concentrations of siRNA. Lipid-based delivery involves electrostatic interaction and complex formation between liposomes and nucleic acids and are subsequently endocytosed by the host cell. In order to be released into cytosol, siRNA must undergo endosomal escape where it can elicit it's effect. Failure to escape the endosome may

lead to activation of RNA sensors TLR7/8 and unwanted immune response. (van den Boorn et al., 2011). Electroporation may also be used to create pores in cells through disruption of the phospholipid bilayer and maximise the delivery efficiency. Double stranded (duplex) siRNA can either be delivered direct into the cytosol or generated via cleavage of long double stranded RNA (dsRNA) by the dicer enzyme. The duplex is then bound by the RNA-interference silencing complex (RISC), a multifunctional cytoplasmic protein which unwinds double stranded siRNA and cleaves the sense (passenger) strand using the endonuclease Argonaute 2 (Ago2). The antisense (guide) strand remains bound to activated RISC, which selectively binds and cleaves complementary target mRNA (Whitehead et al., 2009). After cleavage, siRNA-RISC is recycled to cause repeat degradation of additional target mRNA (Hutvágner and Zamore, 2002). A full schematic of RNA interference by siRNA is detailed in Figure 4.1. Off-target effects are particularly common among gene silencing assays and are an important consideration when using RNAi. These effects can induce expression across multiple genes in response to transfection. The number of off-target effects can be reduced through several means, including careful siRNA design, pooling multiple sequences and bioinformatic applications to predict the contribution of off-target genes to an observed phenotype (Riba et al., 2017).

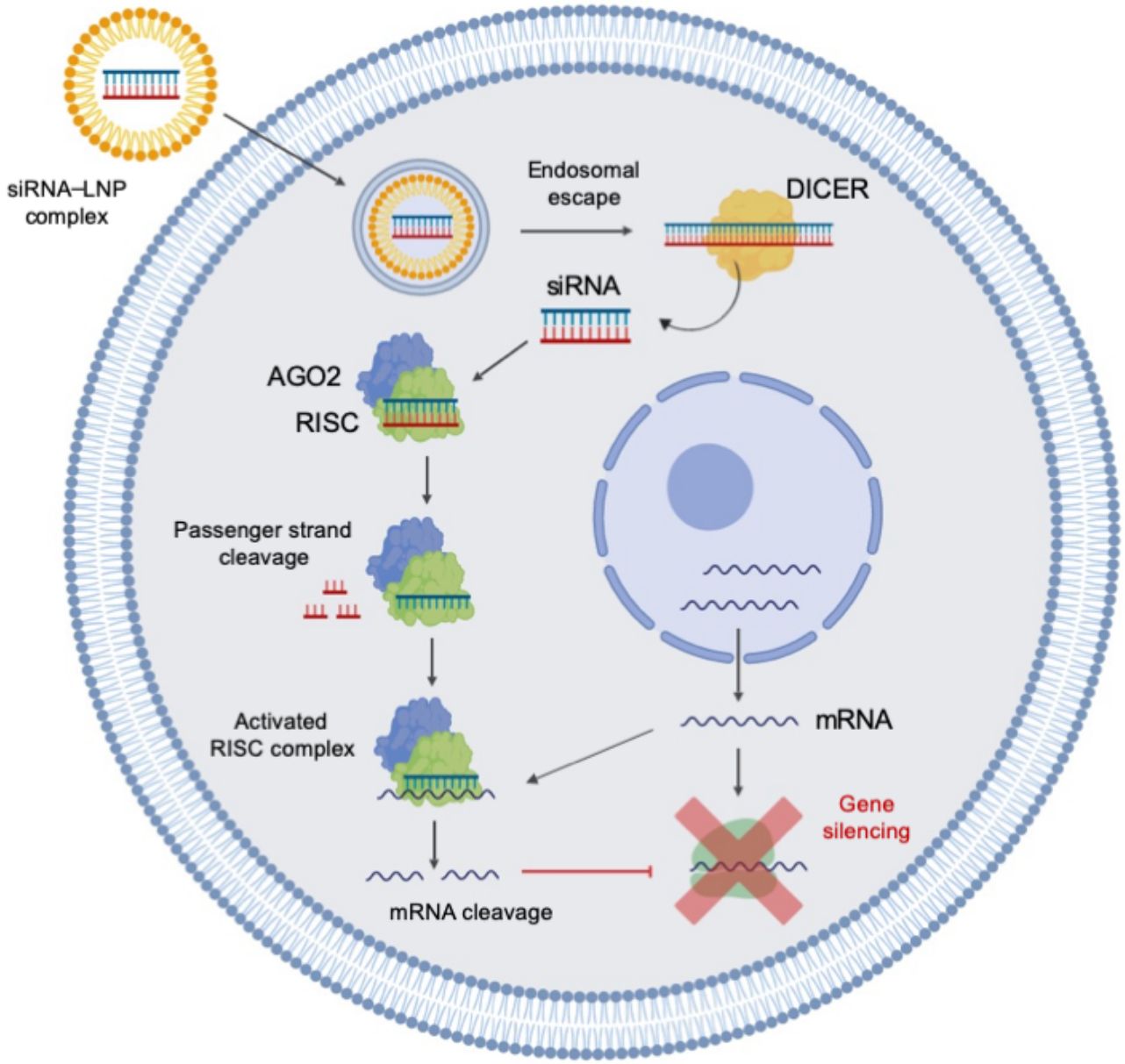

Figure 4.1: Schematic representation of siRNA mechanisms of gene silencing. Complex formation between exogenous duplex RNA and liposomal nanoparticles facilitates endosomal uptake. After endosomal escape and delivery into cytosol, siRNA duplex associates with endonuclease Argonaute 2 (Ago2) and RNA-interference silencing complex (RISC) resulting in the cleavage of the passenger siRNA strand to form the activated RISC complex with the guide strand. Complementary binding with target mRNA causes cleavage preventing translation.

Today, siRNAs are made synthetically and in large quantities suitable for high-throughput screening (HTS). Over time RNAi-based HTS has improved, becoming more effective, specific, with better delivery vehicles and has since continued to prove a powerful tool for functional

genomic studies. RNAi screen design is particularly important for answering the correct biological question and should be carefully considered. Genome-wide screens aim to identify all possible regulators involved in general biological processes and are most successful when paired with predictive data for potential regulatory proteins. This was demonstrated by Müller and colleagues who used genome-wide RNAi HTS in Drosophilla to successfully identify previously uncharacterised JAK/STAT signalling components (Muller et al., 2005). However, these approaches are often very costly given the need for liquid handling automation running an excess of 100 plates for approximately 20,000 mammalian genes. On the other hand, many groups opt for a targeted approach with an RNAi library limited to target a specific pathway for a more focussed study. Targeted studies are particularly beneficial when a known pathway is broadly implicated in a disease model containing previously characterised proteins with unknown molecular functions (Sharma and Rao, 2009; Park et al., 2017). In August 2018, Onpattro (Patisiran) was FDA approved as the first-ever siRNA therapy for the treatment of familial transthyretin amyloidosis with polyneuropathy in adults. However many view this with cautious optimism as siRNA-based therapeutics are still challenged by pharmacokinetic issues including delivery, potency and clearance. However, the clinical realisation of RNAi therapeutics marks an important translational milestone and beginning of a new era of gene therapy.

4.2 Results

4.2.1 Development of statistical and computational methods for screen analysis

Researchers are facing new challenges of data management and analysis brought about by the big-data era and careful experimental design is of particular importance when approaching a high throughput screen to ensure consistency and reproducibility of the data generated. Flow cytometry is a high throughput technology, capable of analysing fluorescence intensity at a single-cell level on a large amount of cells and generating highly dimensional data for each

sample. Taken together, large scale flow cytometry screens require careful analysis in order to generate accurate insight and in light of this, we developed an analytics pipeline in the open-source statistical programming language R to provide rapid analysis of high-content data.

R is an object-orientated programming language and software environment originally created at the University of Auckland, New Zealand in 1995, specifically designed to address issues of lack of functionality and incompatibility among existing software (Ihaka and Gentleman, 1996; Thieme, 2018). It is now a widely-used tool among statisticians and bioinformaticians, featuring in-built statistical functions from standard tests for significance (t-tests, wilcoxon tests) to more advanced statistical modelling (linear/non-linear regression) and machine-learning techniques (classification and clustering). The success of R is attributed to its open-source availability and wide user base; R is a library-based platform which offers a range of packages available on CRAN (The Comprehensive R Archive Network) which are user-developed and peer-reviewed within the R-community, further extending its capabilities in statistical programming. It therefore provides an excellent environment to perform rapid analysis of large datasets and is an ideal tool to support high-throughput screening. Figure 4.2 details a typical workflow for high-throughput screening, applicable to both siRNA or shRNA screens.

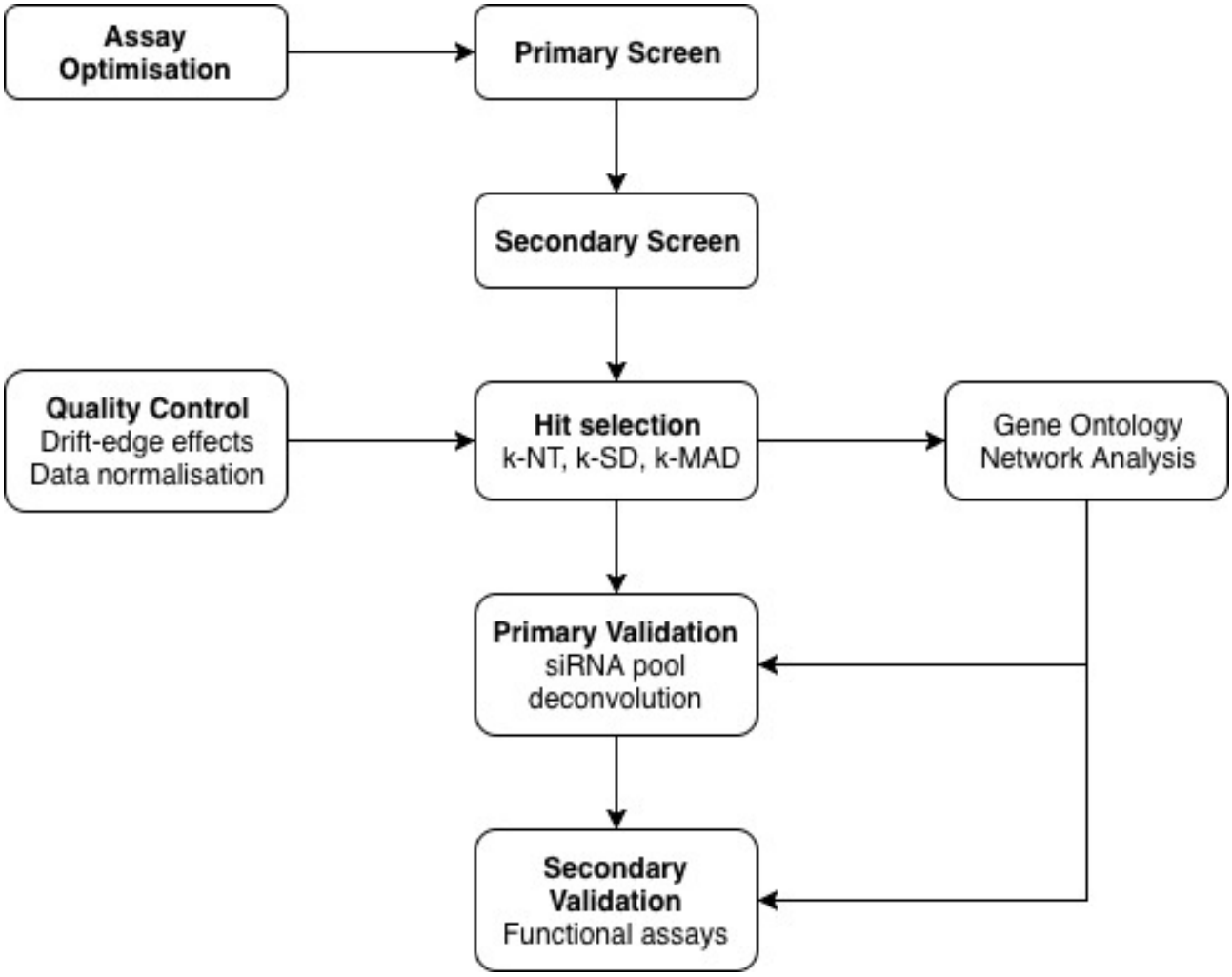

Figure 4.2: RNA interference screen workflow describing screening approach, quality control, hit selection and validation stages. Adapted from (Sharma and Rao, 2009).

Primary & secondary screen

As discussed in the previous chapter, initial assay optimisation plays a critical part of high-throughput screening ensuring reproducible and consistent readouts. But despite preliminary adjustments, systematic errors may still be introduced through a variety of means. Therefore, given the high incidence of off-target effects in RNAi-based approaches, secondary screens are strictly necessary to minimise the generation of false positive results which may be misleading for downstream analysis.

Quality control

For genome-wide screens with a much higher throughput, quality measurements are often re-

quired to ensure that samples qualifying for analysis are sufficiently sensitive to discriminate positive hits from negative background and may include the Signal-to-Noise ratio, Z'-score and the Strictly Standardised Mean Difference (SSMD) (Zhang et al., 1999; Zhang, 2007). While the use of quality metrics can be helpful in assessing the statistical rigour of samples, it may not always be appropriate for the experimental design. For instance, calculation of the Z'-score and SSMD require both positive and negative controls to determine the readout range, however since no positive control exists where DC-mediated HIV-1 transfer is 100% efficient, our capacity for statistically-informed quality control is limited.

Visualisation of plate uniformity can be used to assess drift-edge effects (discussed in 4.2.3) which may have confounding impact on the quality of raw data. Automated microfluidics systems for screens with higher throughput, may offer real-time visualisation during acquisition to allow for adjustments but alternatively, raw data readout may be analysed *post hoc* for evidence of errors. In addition, careful plate design has been studied extensively and a number of strategies have been proposed to allow the best distribution of samples/controls and to reduce edge effects (Malo et al., 2006; Zhang, 2008; Zhang et al., 2008).

Data normalisation & hit selection

Several methods have been proposed for normalisation and hit selection in high-throughput screening (Sharma and Rao, 2009; Birmingham et al., 2009), of which we will address three key methods and strategies for hit identification using normalisation based on: 1) non-target siRNA 2) z-score and 3) median absolute deviation (MAD). Hit selection criteria are determined by the normalisation method used and candidates are typically identified if they exceed a given threshold and is detailed further in section 4.2.2.

Candidate validation

Validation processes aim to refine and characterise the candidate gene set by eliminating possibilities of false discovery and ensuring the generation of reproducible leads. Assays typically complement the original experimental methods used during HTS, but test the biological significance of leads under set conditions. In RNAi screens using pooled siRNA sequences, de-

convolution is often required to determine the individual significance of each phenotype per target sequence; where the phenotype is recapitulated in at least 2 of 4 individual siRNA within the pool, the target can be classed as a true positive and progressed to secondary validation and further functional analyses. Since the rise of high-throughput experiments, there has been a growing need for analytical techniques which can generate biological insight from big data. Tertiary analysis using database mining strategies and techniques in network biology therefore aim to support the characterisation of candidate genes and describe their molecular interactions.

4.2.2 Hit selection strategies

4.2.2.1 Non-target siRNA normalisation

Our first approach normalises the raw CellTrace+ p24+ transfer readout against a standard negative control for the respective donor. Each sample is paired with a non-target siRNA transfer with no known target sequence. Normalisation against mock-transfected samples allows us to calculate a relative percentage change, providing a baseline level of viral transfer in our system described in Equation 4.1. It is worth noting, given the nature of this interference screen, that increased viral transfer in the absence of a gene suggests that the gene restricts transfer in its natural state of expression. Likewise, genes that fall below our lower threshold are considered as permissive candidates to trans-infection.

Based on preliminary optimisations of viral transfer by transfected MDDC, we allowed a $\pm 30\%$ change in our readout to account for the assay variability and used this to set our lower threshold for permissive candidates. Since our research interests are focussed mostly on the identification of novel restriction factors, we used a more stringent $+50\%$ threshold to increase our effect size with the aim of refining a candidate list to those with the most prominent phenotype whilst controlling for false positive results. Such methods of normalisation against internal biological controls are widely accepted and used by Littmann and colleagues in a recent shRNA screen

for membrane/cytoskeletal genes using a similar system (Ménager and Littman, 2016).

$$x_{nt.norm} = \frac{x_i - x_{nt.siRNA}}{x_i} \times 100 \tag{4.1}$$

4.2.2.2 Z-score normalisation

Our second statistical approach involves use of the z-score (standard score) as a way of normalising our data. The z-score is widely used in machine learning and is typically a most preferred choice for normalisation and hit identification of screen data due to ease of calculation (Equation 4.2). This method subtracts the dataset mean from each observation and divides this value by the standard deviation of the dataset. The resulting output is a standardised dataset with a central zero mean and indicates how many SD an observation lies from this point. Incorporation of sample variance is a particular advantage to this method over normalisation against an biology control, however it is important to note the samples themselves act as a *de facto* internal control (Birmingham et al., 2009) and bears less functional relevance compared to non-target siRNA normalisation.

The hit identification component of this method sets a threshold of standard deviations (k-SD) from the central mean. Thresholds of SD are typically set quite high; Sharma and Rao define a weak hit as an observation $> 2-3$ SD from the mean, a moderate hit as $> 3-5$ SD and a strong hit as > 5 SD (Sharma and Rao, 2009). However since many siRNA screens are performed in cell lines with effective transfection efficiencies and a straight-forward readout, based on our preliminary experiments we felt that these thresholds were potentially too stringent for the complexity of our cell model and could mask many, if not all, of our hits. To resolve this, we set a k-SD that would capture approximately 95% of our data and would identify potential candidates accordingly.

$$z = \frac{x_i - \mu}{\sigma} \tag{4.2}$$

4.2.2.3 Median absolute deviation normalisation

As our final method of normalisation, we used the median absolute deviation (MAD) described in Equation 4.3. Similar to the mean-based z-score, MAD uses the median as a measure of central tendency which is less susceptible to change by extreme values in the dataset. Donoho and Huber describe this phenomenon in terms of a 'breakdown point', defined as "*the smallest amount of contamination that may cause an estimator to take on arbitrarily large aberrant values*" (Donoho and Huber, 1983). For instance, when a single value within a dataset becomes infinite, so too does the mean of all observations. However, the median remains unchanged and becomes aberrant only when 50% of values are infinite (Leys et al., 2013) making the MAD a more efficient measure of scale than the standard deviation in practice where small errors will occur in observation and measurement. As a result, MAD is a robust development on k-SD hit selection, regarded as a preferential method for identifying outliers irrespective of sample size and sensitive to weaker hits whilst capable of effectively controlling false positive results (Birmingham et al., 2009; Chung et al., 2008).

$$\text{MAD} = median_i(|x_i - median(x)|) \tag{4.3}$$

4.2.3 Human cytokine/chemokine siRNA screen

After assay optimisation and design of an analytics workflow, we have established a platform to begin screening for novel factors involved in *trans*-infection to CD4+ T-cells. The current consensus is that many more factors with restrictive and permissive effects on HIV-1 *trans*-infection exist in primary cells such as DCs than currently identified. To address this, we used high-throughput siRNA screening of 319 cytokine, chemokine and related genes in our model of cell-cell transmission to reveal these potential candidates. The siRNA library we chose contained 319 pools of 4 siRNA sequences per gene, targeting a list of human genes with known roles in cytokine/chemokine function and signalling. The whole assay was spread out in 4x96-

well V-bottom plate format at 5nmol lyophilised siRNA per well allowing us to run 2 complete screens using 150nM transfections. Because of the low number of MDDCs recovered from each blood sample, we were unable to perform a whole screen in a single donor. We divided the siRNA library to screen a single donor per plate per week, which would be most appropriate for our groups weekly turnover of donor PBMC. In total, the screen (including replicate) was performed across 8 healthy donor PBMC. For the first round of transfection we used 150nm siRNA and reverse-transfected differentiated MDDC (by complexing the siRNA in the plate prior to the addition of cells to the mix). After 24 hours, a second round of transfection was done using a fast-forward transfection protocol (due to the natural adherence of MDDC to the well) and cultured for 48 hours to ensure maximal protein knockdown. Following downregulation, we spinoculated silenced MDDC (siMDDC) with X4-tropic HIV-1 before washing and co-culture with a CellTrace-labelled SupT1 $CD4^+$ T-cell line at a 1:1 ratio. We then stained cells using a viability dye, fixed cells in 4% PFA and stained for intracellular p24. Flow cytometry analysis of our samples would provide four key parameters including proportion of live cells and the intracellular p24 content for both MDDC and SupT1 target cells.

As mentioned previously, some HTS technologies offer real-time analysis of screen data which is particularly beneficial for early detection and correction of systematic errors that may occur mid-screen. Drift and edge effects are common issues in HTS; drift patterns refer to systematic volume error in which a liquid handling robots or manual air displacement pipettes produce repeated inaccuracies. Such errors are likely to affect cell seeding, siRNA transfections and culture conditions which could influence readout. Edge effects refer to the variability on the outer-wells of the microplate, which may be influenced by environmental conditions such as increased evaporation and uneven heat-exchange which may occur during prolonged incubation periods. Acceptable drift-edge affects are typically within $< 20\%$ of mid-max signal intensity (Iversen et al., 2012). Whilst avoiding drift effects are more difficult to avoid, measures can be easily taken to prevent edge effects. In our siRNA screen, MDDC were seeded away from the plate edge and 200 μL 1X PBS was added to surrounding empty wells to minimise. Although our experimental setup was not able to provide any quality control in real-time, we were able to

visualise plate uniformity following acquisition for any evidence of experimental error (Figure 4.3). Visualisation of raw transfer data across all plates from primary and secondary screens do not suggest that edge effects have any effect on screen quality. Likewise, no evidence of drift effects were observed.

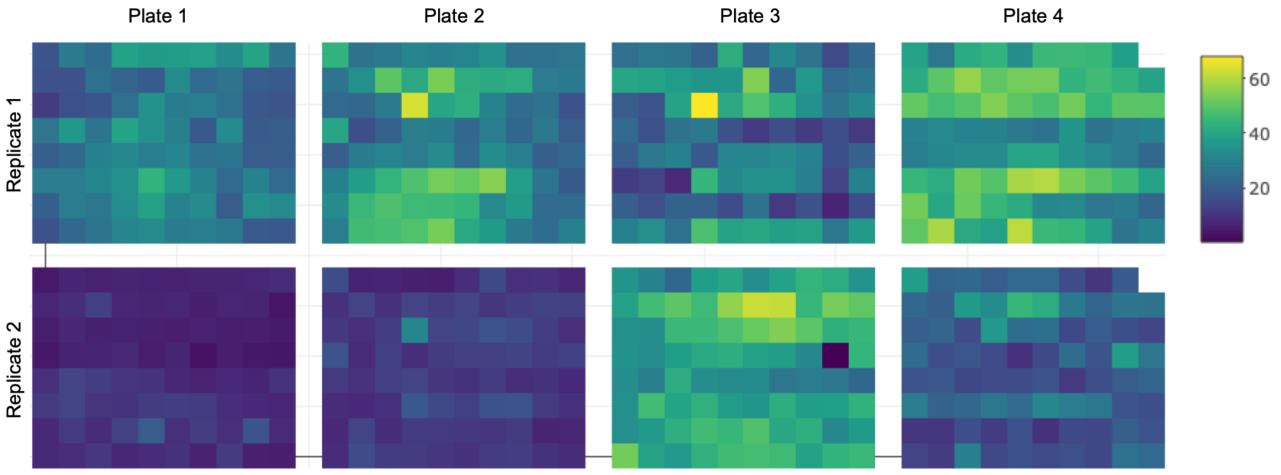

Figure 4.3: Quality control of plate-wise data for both primary and secondary screens. Colour intensities represent raw transfer values for the visual assessment of drift and edge effects.

4.2.3.1 HIV-1 transfer correlates with MDDC infection but lacks reproducibility between screen replicates

We used Pearson's correlation coefficient to test the relationship between cis-infection and viral transfer. Unsurprisingly, correlation of raw p24+ MDDC against p24+ SupT1 cells from both screens showed a very strong and significant positive association ($\rho = 0.80$, $p < 0.001$) with an approximate 1:4 ratio of infected DC:SupT1 (Figure 4.4A). While this result was expected, this observation shows consistent and proportional levels of viral transfer and supports reproducible kinetics of our cell model during RNAi.

When assessing screen reproducibility between replicate screens, individual plate-wise correlation of raw p24 transfer replicates showed that although the gradients of fitted lines are generally in agreement with our reference, the data are weakly associated and the distribution appears to cluster more towards the second replicate. When taken overall, we observed a very

weak and near-zero association ($\rho = 0.07$, p = 0.20) suggesting a loss of reproducibility within our experimental readout (Figure 4.4B). Again, these results may not be surprising since both replicates were performed across multiple donors. Given the complexity of the cell model, we would expect some degree of variation between replicate samples; it is likely that viral transfer is susceptible to many donor-dependent factors which cannot always be controlled for including viral uptake by MDDC, any cellular responses to infection, siRNA knockdown efficiency and target sequence specificity. When these data are normalised as previously described, reproducibility appears to become more consistent but continue to suggest that higher levels of transfer in the first screen replicate. However, MAD normalisation did not show any significant association, though this is likely a technical issue of absolute value transformation of negative samples and does not reflect screen quality (data not shown).

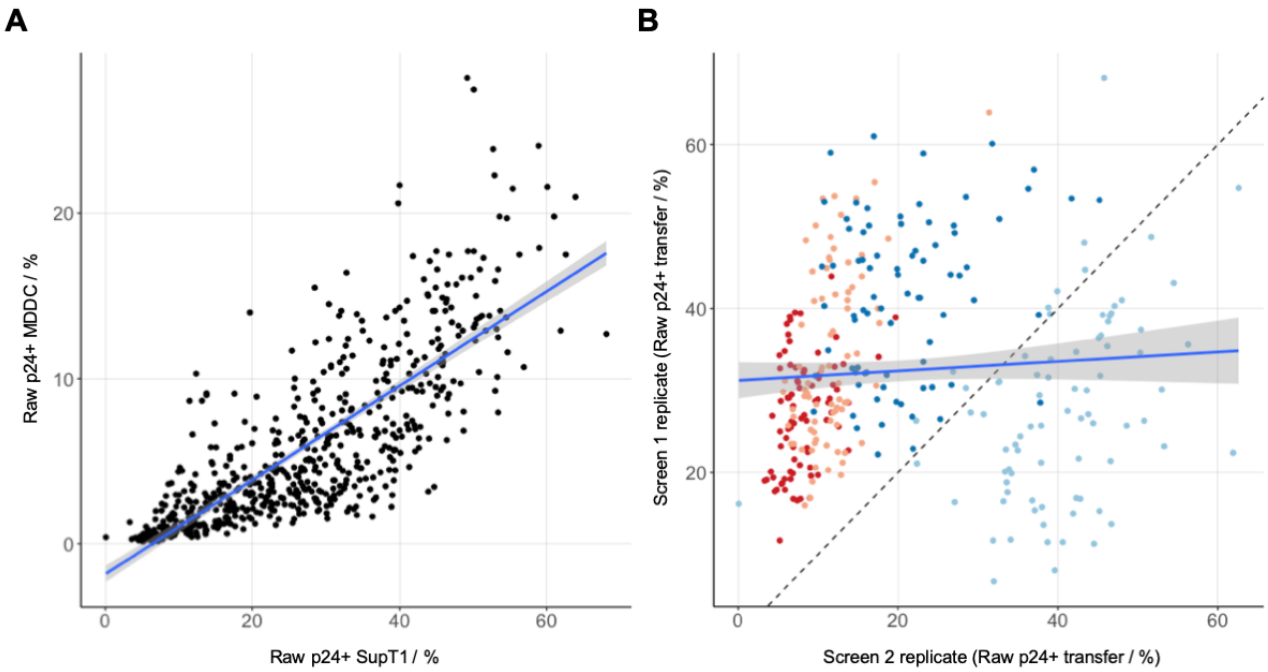

Figure 4.4: Correlation between A) p24+ MDDC vs. p24+ SupT1 for all screen samples and replicate correlation plots for B) plate-wise raw p24 transfer. A linear regression is fitted (blue) with standard error confidence interval (grey). Dashed black line represents perfect positive association.

4.2.3.2 Candidate identification using hit selection strategies

Our first approach normalises data to a sample transfected with non-target control siRNA (Figure 4.5), an approach previously used to analyse changes in viral transfer by RNAi (Ménager and Littman, 2016). As our screen was spread across a total of 8 donors, we normalised all target samples against the non-target siRNA control for the corresponding donor. For hit identification, we set our threshold to allow a ± 30% range, accounting for the maximum variability of the assay and used this as our lower cut-off to identify permissive candidate genes. For our upper threshold, we used a +50% change in viral transfer. Given our interests in restriction factors, this strategy would provide a more stringent cut-off and generate a refined candidate list, selecting candidates with an adequate effect size. The average viral transfer of both screens ranged from -62.12% to +216.06% (SD = 35.23; SEM = 1.97) and 84 hits (28 restrictive + 56 permissive) shown in Table 4.1. While this method is a popular choice and has previously been used during viral transfer, we found that this method was particularly susceptible to the effects of donor variability. Unexpectedly, the second half of the screen (wells 192-319) showed a significant amount of positive skew likely caused by an anomalous result in the non-target siRNA control which these data are normalised to. However, non-target controls were performed in triplicate and the average used for normalisation to minimise the influence of outliers. While non-target siRNA transfected MDDC reflect a true biological control, our results clearly show it is not without limitation. In summary, this method generated a total of 84 candidates which is not practically feasible for validation and subsequently aimed for a more robust method of detection.

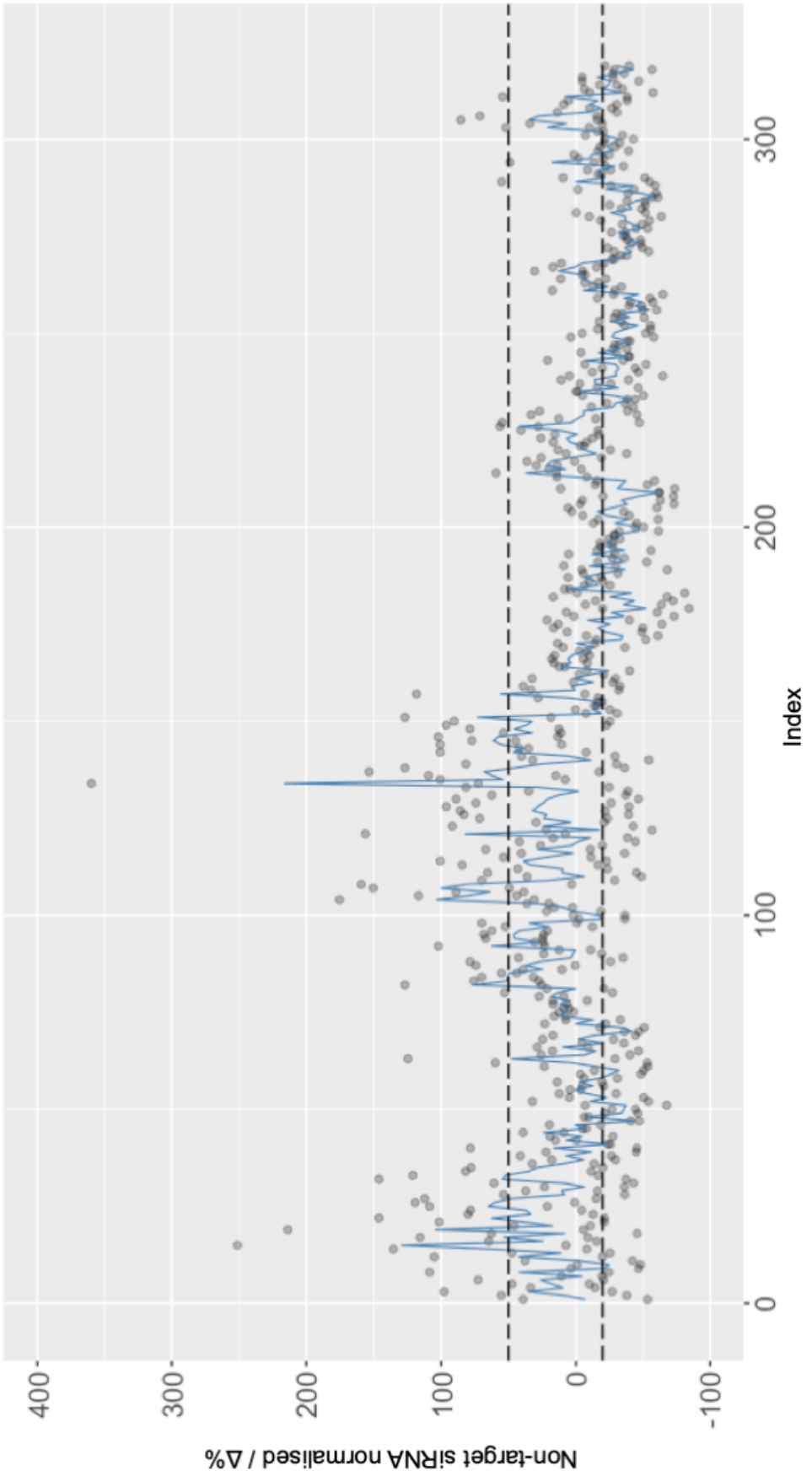

Figure 4.5: Non-target siRNA normalised screen data. Blue trace represents average between two replicates. Black lines represent upper and lower cut-off thresholds at +50% and -30% respectively.

Well index	Gene symbol	Accession	ΔNT siRNA / %
134	MIF	NM_002415	+216.06427
15	CCNC	NM_001013399	+129.30860
19	CCNF	NM_001761	+103.96219
104	IL6	NM_000600	+103.05077
107	IL7R	NM_002185	+99.91323
121	INCENP	NM_020238	+82.06811
108	IL8	NM_000584	+81.10418
105	IL6R	NM_181359	+80.37791
82	IFNA16	NM_002173	+76.19481
151	PF4V1	NM_002620	+72.68761
...	...	...	...
285	IL21	NM_021803	-58.95888
256	IL1F6 (IL36A)	NM_014440	-55.05793
179	CCL23	NM_145898	-52.15511
286	CRLF2	NM_001012288	-49.39390
277	ERBB2IP (ERBIN)	NM_001006600	-49.28893

Table 4.1: Screen hits for non-target normalised hit selection strategy. Highlighted candidates have no known HIV-1 protein-protein interactions reported by the NCBI HIV-1 interaction database (Fu et al., 2008).

To address this issue of donor variability, we compared the result to our model using z-score normalisation approach. The results of this normalisation strategy adequately resolved the issue of skew and variability by using the dataset as its own control. Average Z-score ranged between -1.58 and +4.12 SD and resulted in 15 hits (14 restrictive + 1 permissive) detailed in Table 4.2. Due to the complexity of the cell model and assay readout, we were unable to observe hits within the moderate-high ranges ($> \pm 3$ SD) previously seen in the literature. Instead, we set thresholds that would capture 95% of observations within our entire dataset to create our candidate list, giving us a z-score cut-off of $\pm$ 1.5 SD from the central mean.

Figure 4.6: Z-normalised siRNA normalised screen data. Blue trace represents average between two replicates. Black lines represent upper and lower k-SD cut-off thresholds at ± 1.5 SD from the central mean.

Well index	Gene symbol	Accession	Z-score
134	MIF	NM_002415	4.115235
15	CCNC	NM_001013399	2.758457
226	SCYE1	NM_004757	2.412832
305	FLJ40432	NM_152523	2.202188
214	TNFSF12	NM_003809	2.083017
19	CCNF	NM_001761	2.068142
306	CKLFSF8	NM_178868	1.981031
303	CKLFSF4	NM_178818	1.845356
25	TNFSF7	NM_001252	1.799038
107	IL7R	NM_002185	1.772827
294	NEK9	NM_033116	1.684316
104	IL6	NM_000600	1.680051
266	FZR1	NM_016263	1.661685
216	TNFSF9	NM_003811	1.555056
217	SOCS2	NM_003877	1.462522
...	...	...	...
70	GRN	NM_001012479	-1.319441
179	CCL23	NM_145898	-1.359828
47	CSF2RB	NM_000395	-1.369452
285	IL21	NM_021803	-1.445318
51	CX3CR1	NM_001337	-1.580058

Table 4.2: Screen hits for Z-score normalised hit selection strategy. Highlighted candidates have no known HIV-1 protein-protein interactions reported by the NCBI HIV-1 interaction database (Fu et al., 2008).

Since the strategy for high-throughput screening candidate selection is founded on the ability to detect outliers from a sample population, it is important to minimise the effect these outliers have on the hit selection model. The presence of outliers in a particular plate will inflate the SD of that sample population resulting in a more stringent selection threshold. To address this we used the k-MAD threshold as a robust development on k-SD with greater sensitivity for weaker hits (Figure 4.7). As MAD is a ranked measure, it is less likely to be affected by the presence of a strong outliers and should have improved selection capable of rescuing false negative data. Using this method, we defined a hit based on a threshold of +1.5 MAD, corresponding to the 95% CI of the data. MAD normalised screen data ranged from +0.03 to +4.21 deviations and generated a total of 15 hits (14 restrictive + 1 permissive) shown in Table 4.3.

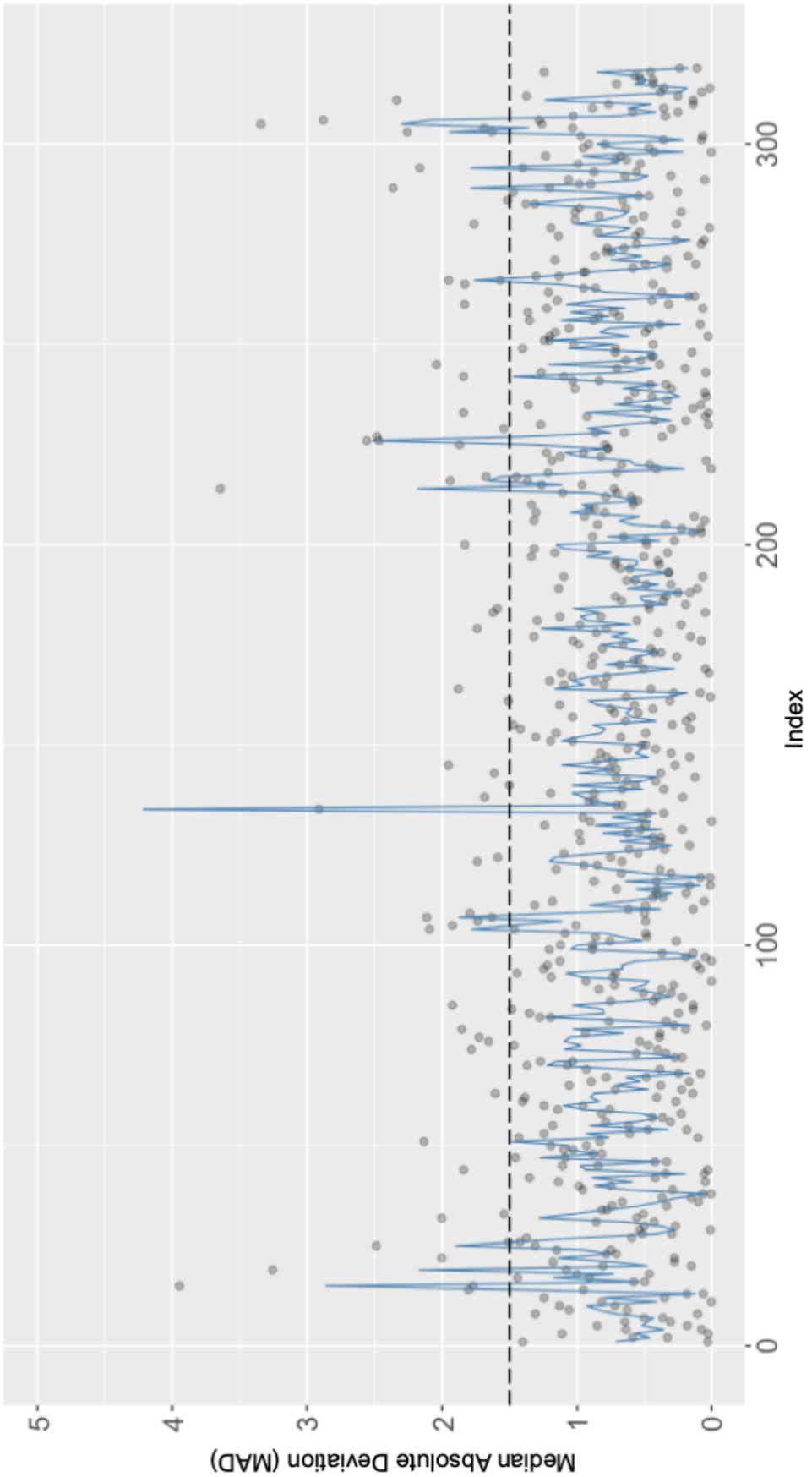

Figure 4.7: Median Absolute Deviation (MAD) normalised screen data. Blue trace represents average between two replicates. Black line represents k-MAD cut-off threshold at +1.5 MAD reflecting 95% of captured data.

Well index	Gene symbol	Accession	MAD-score
134	MIF	NM_002415	4.213798
15	CCNC	NM_001013399	2.857020
226	SCYE1	NM_004757	2.511395
305	FLJ40432	NM_152523	2.300752
214	TNFSF12	NM_003809	2.181580
19	CCNF	NM_001761	2.166705
306	CKLFSF8	NM_178868	2.079595
303	CKLFSF4	NM_178818	1.943919
25	TNFSF7	NM_001252	1.897602
107	IL7R	NM_002185	1.871390
294	NEK9	NM_033116	1.782879
104	IL6	NM_000600	1.778615
266	FZR1	NM_016263	1.760248
216	TNFSF9	NM_003811	1.653620
217	SOCS2	NM_003877	1.561086
51	CX3CR1	NM_001337	1.481494
242	CXCL13	NM_006419	1.469520
26	CDC2	NM_033379	1.4656339
105	IL6R	NM_181359	1.464938
227	CER1	NM_005454	1.423043
14	CCNB1	NM_031966	1.377199

Table 4.3: Screen hits for median absolute deviation (MAD) normalised hit selection strategy. Highlighted candidates have no known HIV-1 protein-protein interactions reported by the NCBI HIV-1 interaction database (Fu et al., 2008).

Figure 4.8 shows a venn diagram of 104 total hits across all hit selection strategies. Of the 84 candidates identified by non-target siRNA selection constituting 80.8% all hits, 6 candidates (5.8%) were shared with z-score and MAD models. A total of 9 candidates (8.7%) were exclusive to Z-score/MAD models. Interestingly, no candidates were unique to z-score hit selection.

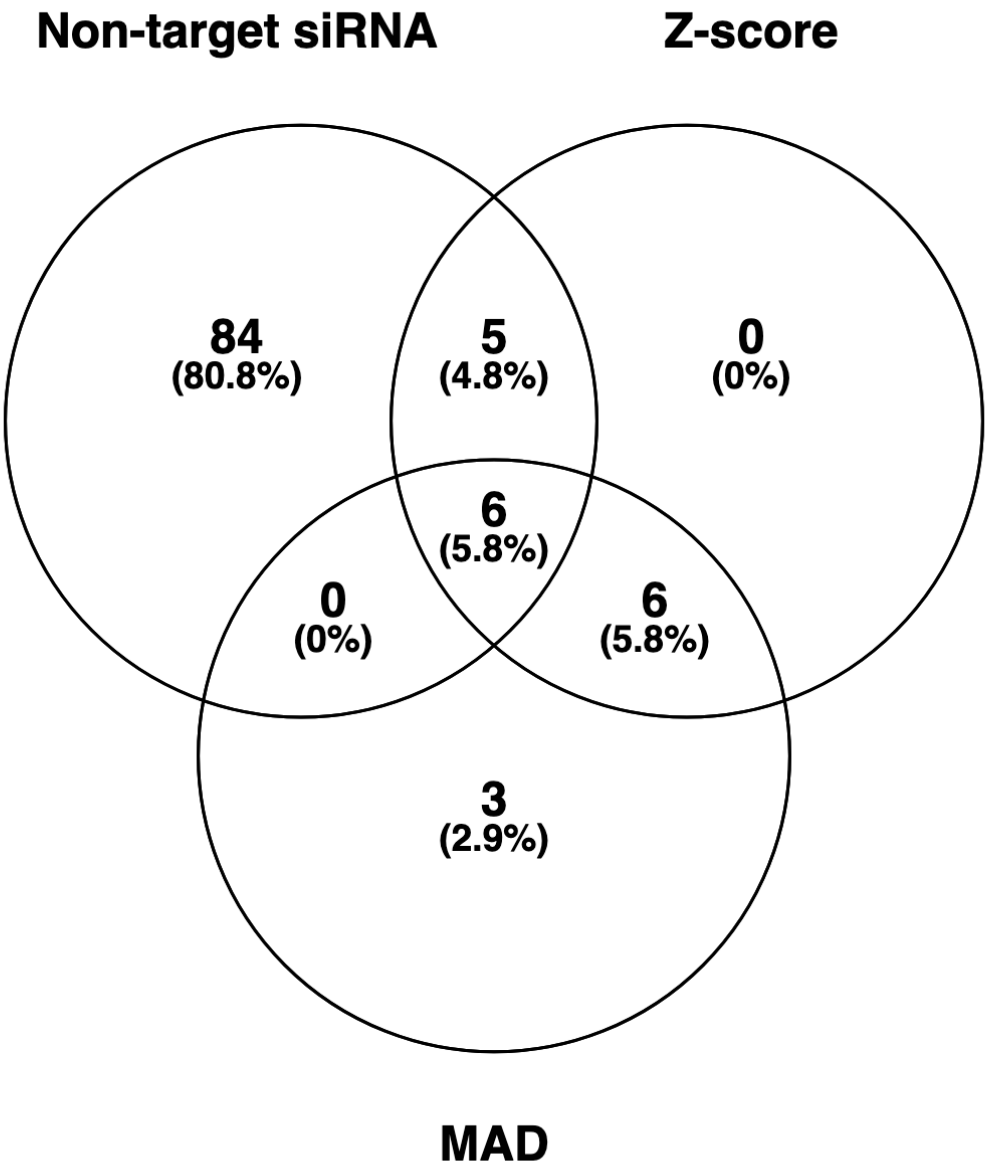

Figure 4.8: Venn diagram of hit selection models detailing the number of candidates (percentage of total candidates) for each intersect of non-target siRNA, k-SD and k-MAD hit selection strategies.

4.2.3.3 ROC curve analysis identifies MAD as the highest performing model

To evaluate the performance of our three statistical models, we used receiver operating characteristic (ROC) curves to predict the sensitivity (probability of detection) against fall-out (false positive rate) shown in Figure 4.9. ROC is a statistical method frequently used in diagnostics to evaluate the performance of binary classification models. In a clinical setting, these are typically used to determine case vs control (disease vs healthy), however in our system we used ROC curves to predict the accuracy of discriminating a hit vs non-hit as a method of diagnostic decision-making. ROC curves are based on confusion matrices which give an indication of the rates of true positive (TP), true negative (TN), false positive (FP) and false negative (FN) events. The area under the curve (AUC) is used in ROC curves as the general measure of predictive performance and reflects the ability of the model to distinguish classifications (Metz, 1978). The greater the AUC, the greater the sensitivity and specificity of the classifier; a perfect model would have an AUC of 1, whilst the least performing model would have an AUC of 0.5, suggesting that the model has no capacity to discriminate between classes (has equal performance as random chance, i.e. flipping a coin). As the percentage change from non-target siRNA transfer is already a validated method of hit selection, we used this as our gold-standard and compared the AUC of each statistical model relative to this. ROC curve analysis of non-target siRNA gave a baseline performance of 0.702; we found that MAD-normalised data had the highest sensitivity and specificity with 0.962 AUC compared and 0.888 AUC for z-score normalisation approaches (Figure 4.9A).

Cross-validation is important for establishing stable statistical models to improve their performance and reliability, whilst resampling the data for more unbiased training. To do this, we performed a leave-one-out cross-validation (LOOCV) which iteratively removes a single observation from the sample data for validation by recalculating the predicted outcome. This process is repeated such that every observation is used once and a new ROC curve is produced to assess how accurately these models perform. Following cross-validation our results showed slight performance reductions across all models; performance of the gold-standard dropped modestly

by -3.98% to 0.674. Z-score normalisation and hit selection decreased -3.38% to 0.858 ($p < 0.05$), while the MAD model remained the strongest performing down -5.41% to an AUC of 0.910 ($p < 0.001$). Testing of both ROC curves against the gold-standard suggest statistically significant improvements in sensitivity and specificity, suggesting these approaches have higher performance in hit identification (Figure 4.9B). These findings are in agreement with the surrounding literature which supports MAD as a the most sensitive and specific method for the identification of outliers. Based on this we are able to refine our candidate list and report a total of 15 candidates genes involved in the regulation of HIV-1 transfer.

```
model1=glm(samp$hit.cut ~ samp$p24.supt1 + samp$dead.supt1 + samp$p24.mddc
    ↪ + samp$dead.mddc,
data=samp,
family="binomial")
summary(model1)
predict(model1, type="response")
roc.cut=roc(samp$hit.cut, predict(model1, type="response"))
coords(roc.cut, x="best")
plot(roc.cut);
prediction.loo = as.vector(samp$hit.cut)
for(i in 1:length(samp$hit.cut)){
        train = samp[-i,]
        test = samp[i,]
        model1 = glm(hit.cut ~ p24.supt1 + dead.supt1 + p24.mddc + dead.
            ↪ mddc, data = train, family = "binomial")
        prediction.loo[i] = predict(model1, newdata = test, type = "
            ↪ response")
}
roc.cut = roc(samp$hit.cut, prediction.loo)
```

Listing 4.1: ROC curve analysis and LOOCV

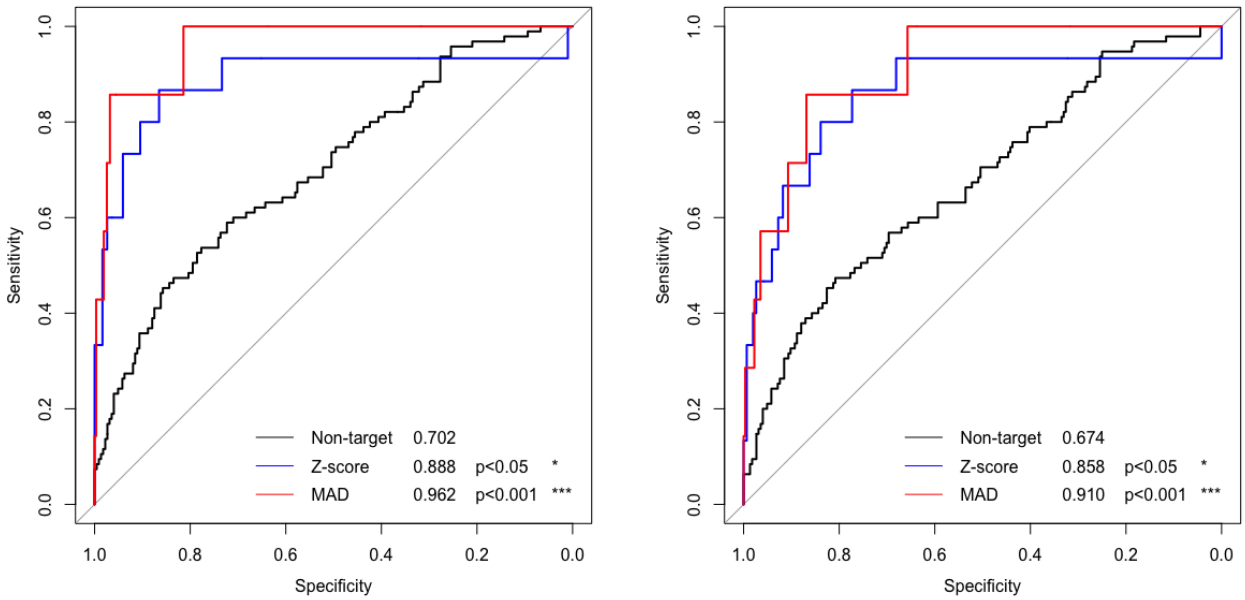

Figure 4.9: Receiver operating characteristic (ROC) curves to evaluate the performance (sensitivity vs specificity) of our three statistical models at predicting the probability of a hit. A) ROC curve analysis and B) ROC with 'leave-one-out' cross-validation (LOOCV). A perfect model would have an AUC 1.0 and becomes progressively less accurate with decreasing AUC until an AUC 0.5 which would suggest a model with equal performance as random chance. ROC curve analysis and LOOCV methodology developed together with Samuel Touchard (Statistician, BPM group, Cardiff University). DeLong's comparison of two ROC curves, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

To investigate our candidate genes for any similarities, we used principal components analysis (PCA), a dimensionality reduction technique, together with hierarchical clustering based on raw screen data. Clustering analysis did not suggest any distinct patterns. However, CD70 shared similar localisation with cyclin candidates *CCNC* and *CCNF*.

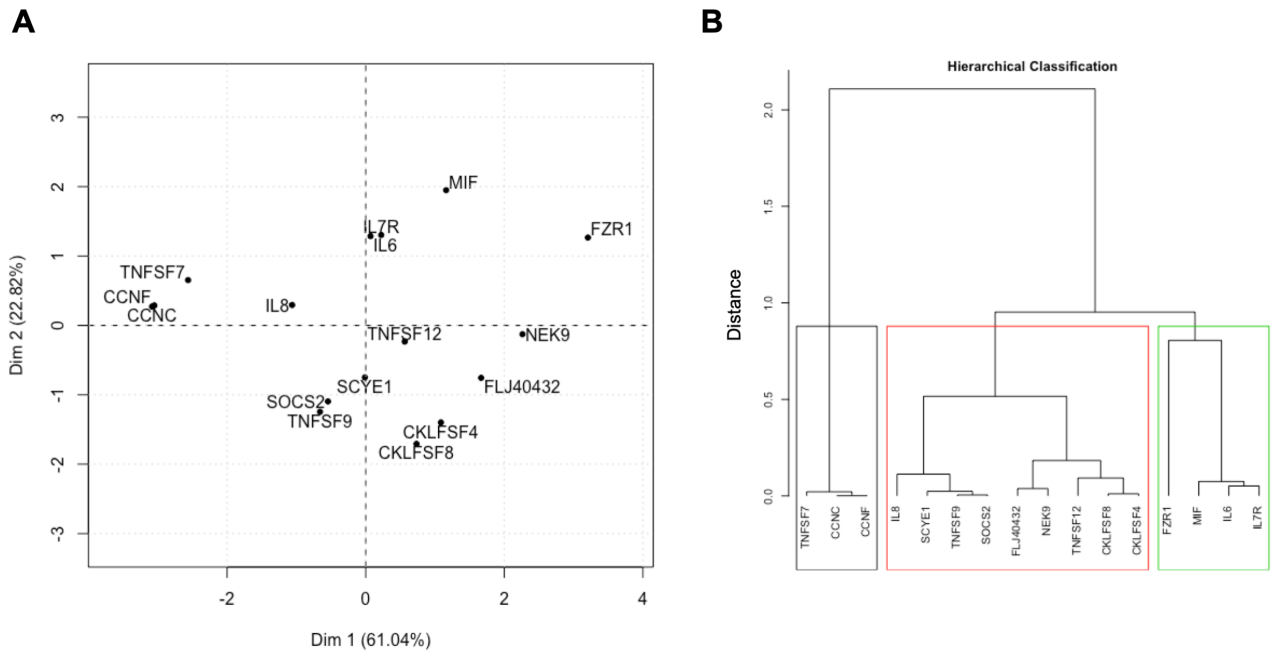

Figure 4.10: A) Principal components analysis (PCA) and B) hierarchical clustering of k-MAD gene candidates.

4.2.4 Gene ontology and network analysis

The Gene Ontology (GO) Consortium, founded in 1998 by three genomic research groups was formed with the goal of developing "an up-to-date, comprehensive, computational model of biological systems, from the molecular level to larger pathways, cellular and organism-level systems". The GO knowledgebase is a centralised public database (available at `http://geneontology.org/`) consisting of an excess of 4 million annotations supported by over 150,000 publications and ontologies are divided across three key domains: molecular functions, biological process and cellular component. These three categories represent broad biochemical activities, the contribution of a gene or gene product to a biological objective and the localisation of the gene, respectively. Ontologies are built up from a collection of GO terms containing meta-data detailing unique term identifiers, definitions, citations, a list of involved genes/gene products and relationships with other GO terms. DAVID (database for annotation, visualization and integrated discovery) is a bioinformatics tool developed by the Laboratory of Immunopathogenesis and Bioinformatics at National Cancer Institute at Frederick used to

condense large lists of genes into associated terms and is a powerful resource for extracting meaningful results in order to understand biological processes and pathways (Huang et al., 2007). DAVID functional annotation employs the Expression Analysis Systematic Explorer (EASE) score, a modified Fisher's Exact test, to statistically calculate over-expression of a gene set by describing the significance of association between two independent groups. As an example using the human genome background containing approximately 30,000 genes, the term for "GO:0019089 transmission of virus" contains 271 annotated genes. If a gene list containing 12 of 319 tested genes belong to this term, then the test seeks to determine whether the association of 12/319 genes is more than random chance compared to 271/30000. A p-value greater than the significance level would suggest that the association with a term was by random chance and vice versa. We performed an enrichment analysis using the candidates from k-MAD hit selection to generate a series of significant GO term associations in order to gain some insight into underlying functions and common biological pathways.

Enrichment of molecular functions and cellular components only revealed few and broad GO term associations mostly relating to cytokine and receptor activities at the cell surface and extracellular space. Our analysis identified a significant enrichment for TNF and TNFSF receptor binding, however continue to lack specific activities on the molecular level. Biological processes returned 137 enriched GO term annotations, of which the highest 25 were displayed (Figure 4.11). These terms provide a greater insight into the contribution of our gene-set to larger processes and pathways. Several themes appeared in our analysis, chiefly processes involving the positive regulation of cellular activation, proliferation and differentiation. Interestingly several cell death related processes were functionally enriched, including GO terms for apoptotic process and programmed cell death containing TNF superfamily candidates. As a well-characterised mediator of cell death, these findings support protective roles for TNF-induced apoptosis during HIV-1 infection.

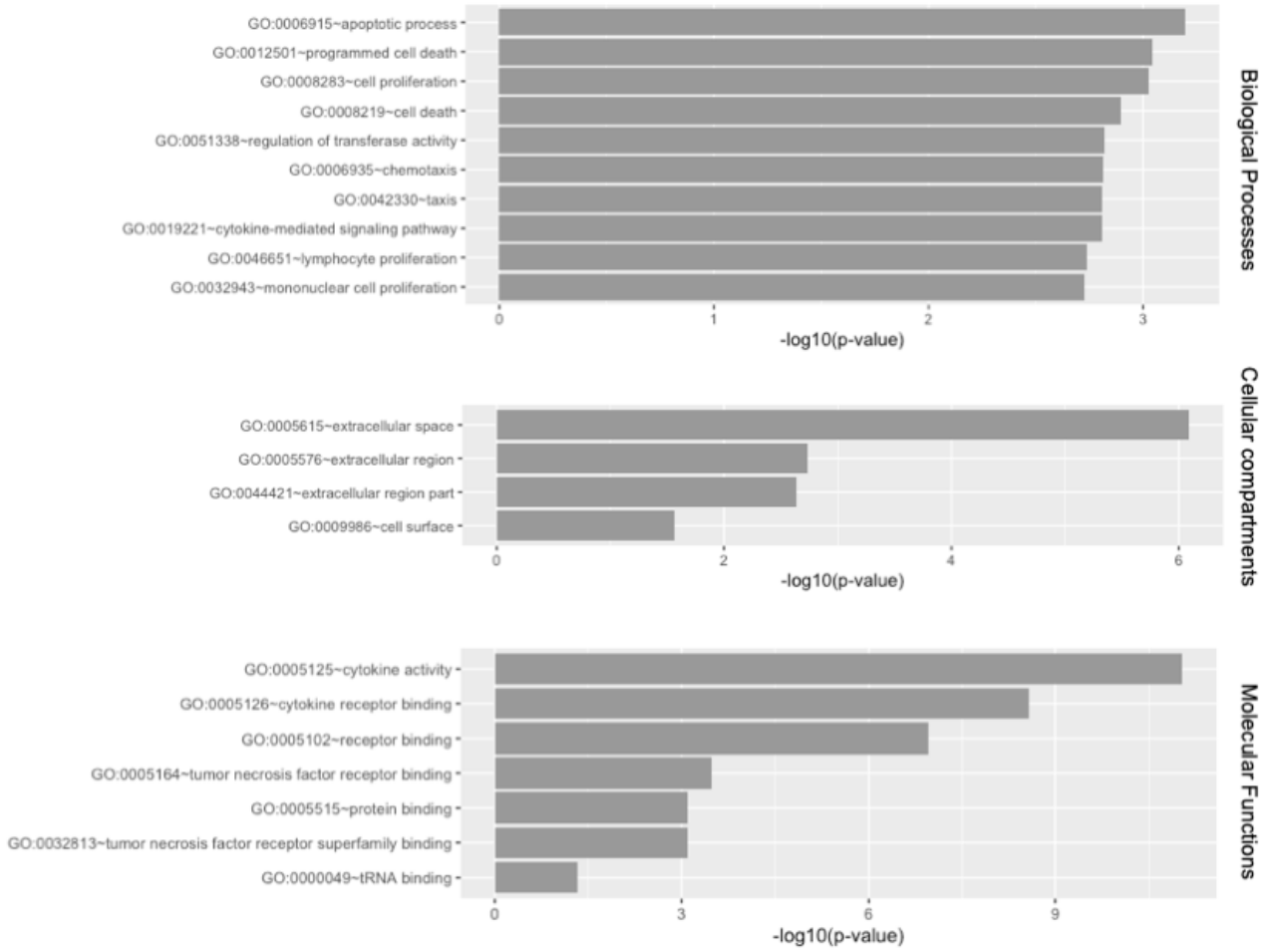

Figure 4.11: Gene set enrichment of k-MAD candidates using DAVID functional annotation grouped into molecular function, biological process and cellular component semantics. All GO term annotations are significant to $p < 0.05$.

Gene-set enrichments can be further visualised in networks which can give an insight into common overlap representing similarities between nodes. Gene-sets are subsequently grouped together based on similarity scores to improve interpretation. We imported the results from DAVID enrichment into the EnrichmentMap (Bader lab) Cytoscape plug-in and visualised the molecular functions of all genes within the siRNA library (blue) and overlaid enrichments for identified candidates (green). Similarly, given the broad nature of our enrichments, network analysis did not return any obvious similarities other than TNF/TNFSF related activity as previously observed. Though interestingly, no gene-set overlap similarities were observed with

terms for IL-6 receptor binding or cyclin-dependent protein serine/threonine activity despite being part of the candidate list. Networks for biological processes were also analysed however, GO terms did not meet FDR Q-value cut-off and were not included in the visualisation (data not shown).

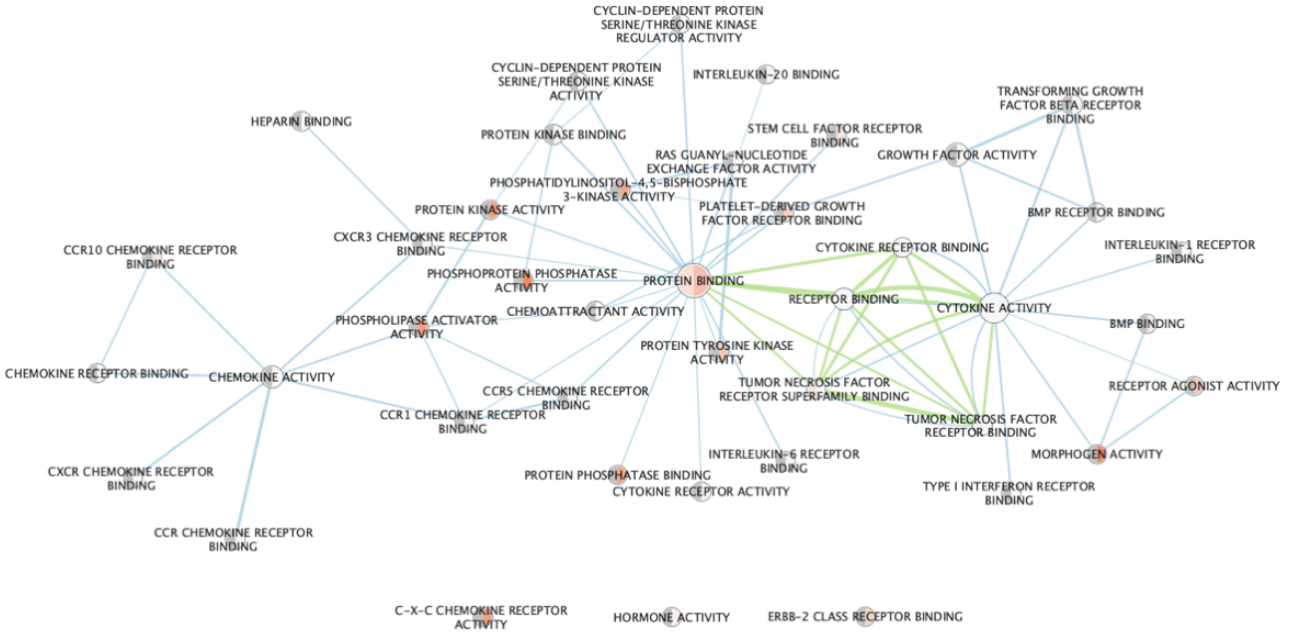

Figure 4.12: Gene ontology (GO) network of human cytokine and chemokine molecular function. Nodes represent enriched annotations. Edge width represents overlap similarity coefficients between nodes. Blue edges represent similarities from all screen genes, whilst green edges represent enriched similarities from k-MAD screen candidates.

GO and ontological analyses have the potential to provide powerful enrichment for long lists of genes, allowing biologists to draw meaningful understanding from complex datasets. Whilst these methods are recognised as the *de facto* gold-standard for secondary analysis of high-throughput screen data, they are not without limitation. One of the major obstacles for GO lies within the knowledgebase itself; since the vast majority of annotations are curated computationally based on existing literature, well-established processes and findings are likely to have higher quality annotations than emerging annotations which can introduce bias to the statistical enrichment. In addition, the quality of an enrichment output is dependent on the quality of the data input (Tipney and Hunter, 2010). While GO is still a valid tool for analy-

sis of our data, it is best optimised for the enrichment of expression microarray data and the analysis of larger gene sets. Despite the long-standing success of enrichment tools in helping investigators overcome issues of interpreting long gene lists, GO on this occasion did not offer informative analysis of candidate genes or any common pathways they are involved in. Pathway analysis of our candidate gene list could have guided downstream validation including a better characterisation of the biological and molecular mechanisms underlying the restriction of HIV-1 *trans*-infection.

4.3 Discussion

The post-genomic era has dramatically shaped the drug discovery process historically driven by medicinal chemistry to one with increasing co-operation with molecular biologists and bioinformaticians. In contrast to early phenotypic screening approaches, large-scale discovery screens are now target-based, founded by biological hypotheses and focus on the identification of individual gene targets with the belief that every target has the potential to be disease-modifying. Target-based screens informed by systems biology approaches and network analyses have been advantageous in improving clinical efficacy and reducing toxicity of candidates - two key causes of attrition within drug discovery (Hopkins, 2008).

Currently, there are a number of global siRNA screens exist which have investigated novel genes regulating HIV-1 infection. Genome-wide screens have had substantial success in the identification of host-dependency (Zhou et al., 2008) and restriction factors (Neil et al., 2008). However, many of these have been performed on immortalised cell lines which do not fully represent the biological events that occur *in situ*. Therefore, primary cells (in particular, DCs) are highly relevant for investigating novel targets that can be readily translated into therapeutic development. In addition, our previously reported data supports a role for cytokines and chemokines in the restriction of HIV-1 and this high-throughput screen for novel cytokines during DC-mediated HIV-1 *trans*-infection is the first of its kind (Czubala et al., 2016; Collins

et al., 2017).

Despite the advantages working with primary cells can offer, it is not without limitation. DC yield from donor peripheral blood is often limited; we were unable to perform individual screens using single donors and opted to divide the library for transfection across multiple donors. However, appropriate statistical techniques and data handling methods can be used to mitigate these issues. Although normalisation against non-target siRNA transfected controls were still prone to the effects of variation, normalisation strategies using the z-score and MAD effectively resolved these problems in how to analyse screen data. Comparison of hit selection strategies confirmed that the k-MAD model had the highest sensitivity for hit classification and was used to generate a final candidate gene list. Our results highlight several key candidates including three major protein families including the cyclin, tumour necrosis factor and interleukin superfamilies as factors involved in regulating HIV-1 transfer from DCs. Below we discuss these candidates further and explore and their relevance in DC biology.

Restrictive candidates

4.3.1 Macrophage Migratory Inhibitory Factor

Macrophage migration inhibitory factor (MIF) was identified as the strongest antiviral candidate within our screen, which when downregulated in MDDC resulted in a striking increase (+216.06%, +4.12 SD, +4.21 MAD) in viral transfer to CD4+ SupT1. MIF is a 12.3 kDa pro-inflammatory protein discovered in 1966 by David and Bloom during delayed-type (type IV) hypersensitivity studies (Bloom and Bennett, 1966; David, 1966). MIF was originally isolated from T-cell culture supernatant and described for its inhibition of the random movement of macrophages, making it one of the earliest cytokines to be discovered. However, the molecular functions of MIF were not fully explored until several decades after its discovery when human MIF cDNA was first cloned in 1989 (Weiser et al., 1989). Whilst MIF was originally considered a T-cell secreted cytokine, MIF is constitutively expressed with ubiquitous tissue distribution. Monocytes, macrophages and DCs in particular are predominant sources of local MIF release (Calandra et al., 1994; Bernhagen et al., 1996; Popa et al., 2006) but is also secreted systemically from anterior pituitary cells in response to LPS, suggesting hormone-like function and links with the endocrine system (Bernhagen et al., 1993). Moreover, the release of MIF is unlike that of typical cytokines and secreted proteins due to the absence of a signal sequence. MIF therefore does not translocate to the ER and is secreted through a regulated non-canonical pathway which is unaffected by brefeldin A or monensin (Bernhagen et al., 1994; Flieger et al., 2003).

The identification of a primary receptor for MIF had eluded investigators for almost 40 years, until Leng and colleagues (Leng et al., 2003) identified CD74, the type II transmembrane HLA-DR-associated invariant chain, as a high-affinity receptor for MIF though the lack of an intracellular signalling domain suggested the association with additional signalling proteins. MIF-CD74 signal transduction was soon reported to be CD44-dependent, which forms a receptor tyrosine kinase-like protein complex required for ERK1/ERK2 kinase phosphorylation (Shi

et al., 2006; Bernhagen et al., 2007; Gore et al., 2008). In addition, CXC chemokine receptors CXCR2/CXCR4 have also been reported as non-cognate receptors for MIF, which can further associate with CD74 for functional signalling (Bernhagen et al., 2007). It is therefore suggested that MIF can exhibit chemokine-like function and is involved in cell recruitment during inflammation.

X-ray crystallography studies continue to reveal unusual structural properties of MIF that are atypical of most conventional cytokines, which have shown that MIF can exist in monomeric, dimeric and homotrimeric structural arrangements (Sun et al., 1996). The latter containing an uncharacteristic central solvent channel, described as two funnels which empty into a central space (Sun et al., 1996), though its functional significance is yet to be established. The MIF homotrimer shares structural homology with microbial enzymes including members of the tautomerase family and can exhibit various catalytic activities. MIF engages CD74 at its tautomerase active site, which raised questions about whether its enzymatic activities are required for its biological function, however neither small-molecule inhibitors or point mutation of catalytic site residues had any affect on CD74 interaction or activation (Pantouris et al., 2015).

Today, MIF is known to be responsible for mediating a broad range of effects with pivotal roles in the regulation of innate immunity, driving downstream release of pro-inflammatory cytokines TNF-α, IFN-γ, IL-1, IL-6, IL-8 (Calandra et al., 1994), matrix metalloproteinases (Onodera et al., 2000), nitric oxide (Bernhagen et al., 1994) and prostaglandin E (Mitchell et al., 2002). In the context of infection, a MIF-mediated pro-inflammatory environment is important in maintaining protection against low-level infection by gram-negative bacteria and endotoxins (Calandra et al., 1998). However, high levels of MIF have been found to be harmful, resulting in excessive immune reaction, further potentiated in the presence of LPS and is associated with a spectrum of autoimmune and inflammatory diseases including rheumatoid arthritis, respiratory distress syndrome and severe sepsis. In experimental models of lethal endotoxic shock, r*Mif* significantly enhanced mortality rates, while *Mif*-deficient mice were protected against over-

whelming inflammatory responses when challenged by a high-dose bacterial insult(Bernhagen et al., 1993; Calandra et al., 1998). These findings implicate a role for MIF in maintaining a fine balance between the resolution of infection and bacterial pathogenesis.

Stress-induced glucocorticoid hormone secretion along the hypothalamic-pituitary axis producing strong immunosuppresive effects and is the basis of many therapeutic agents widely used in the control of chronic inflammation. An interesting and unique characteristic of MIF is its ability to counter-regulate glucocorticoid action, overriding anti-inflammatory pressure (Calandra et al., 1995), retaining its capacity for T-cell activation and induction of pro-inflammatory cytokine release. It has therefore been suggested that MIF and glucocorticoids work in conjunction to regulate mechanisms of innate and adaptive immunity, where locally sourced MIF acts to sustain inflammatory events at sites of infection which are otherwise dampened by systemic glucocorticoid release. In summary, MIF is a key regulator of many immune processes which can mediate host protection against invasive extracellular and intracellular pathogens. However, MIF has a structure-function relationship that is atypical of conventional cytokines and the, as yet uncharacterised, catalytic activities of MIF could have involvement in the regulatory mechanisms of HIV-1 *trans*-infection. Further work is required to validate these findings and explore new mechanisms of MIF-dependent control of HIV-1 transfer.

4.3.2 Cyclins

Our screen had identified cyclin C (+129.31%, +2.76 SD, 2.86 MAD) and cyclin F (+103.96%, +2.07 SD, 2.17 MAD) as a potential restrictive candidates of HIV-1 transfer. The cyclin protein family members are key regulators of cell cycle allowing cells to transition through phase checkpoints via activation of cyclin-dependent kinases (CDK). Cell cycle is particularly relevant during the innate and adaptive immune response, often triggered in response to stimulation by cytokines and transcription factors which prompt cell growth and differentiation (Guttridge et al., 1999).

4.3.2.1 Cyclin C

The mid-late G1 phase protein, encoded by the *CCNC* gene, was first discovered along with several other cyclin members during cDNA screening experiments in yeast (Lew et al., 1991). Cyclin C was originally characterised for its ability to promote exit from quiescent cell cycle arrest, now known to be dependent on CDK3 association (Ren and Rollins, 2004). But for many years, CDK8 was regarded as the putative protein kinase for cyclin C. However, this association is not involved in cell cycle regulation and recent studies have revealed more intricate roles for G1 cyclin-CDK complexes (Liu et al., 2017; Arand and Sage, 2017).

Though little is known about the interaction cyclin C has during HIV-1 infection, it has associations with GO term annotations for HIV life cycle. Cujec and colleagues (Cujec et al., 1997) were one of the few and first to explore the relationship between cyclin-CDKs and HIV-Tat, the viral accessory protein required for transcriptional elongation. The role for cyclin C-CDK8 soon became apparent, following studies that showed cyclin C-CDK8 complexes phosphorylate the carboxyl-terminal domain of the RNA polymerase II holoenzyme during the transactivation of HIV-1 LTR and enhancing its activity (Parada and Roeder, 1996; Rickert et al., 1996; Cujec et al., 1997; Edwards et al., 1998).

More recently cyclin T1, a member of the cyclin C subfamily, was also identified as having an important role in HIV-1 transcription. Cyclin T1 binds to CDK9-containing regions mutiprotein complexes (TAK/pTEFb), phosphorylating the carboxy-terminal domain of the largest RNA polymerase II subunit to promoting elongation and transcription of viral genes post-integration (Ghose et al., 2001). Similarly HIV-Tat has affinity for cyclin T1, binding to create a stable complex which in turn can recruit cyclin T1 to HIV-1 transactivation response element, an RNA stem-loop region and binding site on the viral promoter, acting as a cofactor for binding by HIV-1 Tat (Wei et al., 1998; Nilson and Price, 2011). Together these findings support a permissive roles for cyclin C and its subfamily members in facilitating *trans*-activation and transcription of viral genes. However, this is in disagreement with our observations that cyclin C downregulation significantly enhances HIV-1 transfer. One possible explanation could be that

altered RNA polymerase II regulation by cyclin C-CDK8 may influence the transcriptional activity in DCs, changing protein expression, cytokine profile and host-response to virus. Since cyclin C-CDK8 complexes have been reported to regulate IL-10 expression in both human and murine DCs during innate immune responses (Johannessen et al., 2017), it is plausible that the pleiotropic anti-inflammatory cytokine also may be implicated in host response and cell-cell transfer. However, there are discrepancies in the literature between the protective and pathogenic roles of IL-10 during HIV-1 infection.

As a pleiotropic anti-inflammatory cytokine, recognised for its production by tolerogenic DCs and its ability to suppress Th1 differentiation, elevated IL-10 production may suppress viral clearance and enable propagation through a lack of inflammatory pressure. Several studies have correlated elevated serum levels of IL-10 with disease progression which continues to increase over time (Stylianou et al., 1999; Brockman et al., 2009). Interestingly, IL-10 was found to enhance viral entry and production of X4 HIV-1 in DCs, but was blocked in macrophages (Ancuta et al., 2001). Although not selected as a hit, IL-10 showed moderate permissive phenotype in our screen (-6.423404%, -1.000227 SD, 1.314054 MAD). On the contrary, there is early evidence suggesting that IL-10 is involved in the suppression of HIV-1 replication *in vivo* (Fauci, 1996) and that genetic polymorphisms of the *IL10* promoter, decreasing its production, in fact accelerates disease progression (Shin et al., 2000). Further work is required to fully understand the molecular functions of IL-10 during HIV-1 infection.

4.3.2.2 Cyclin F

Cyclin F (CCNF) is a G2 cyclin and the founding member of the F-box protein family, characterised by the presence of a 40–50 aa long F-box motif. As a cell cycle regulator, cyclin F is detected during early events similar to the expression patterns of Cyclin A, being first detectable at S-phase and peaking during G2-phase, suggesting roles in DNA replication and G2/M transition (Bai et al., 1994). However, unlike the majority of conventional cyclins, CDK binding partners have yet to be identified and are not required for cell cycle regulation. Instead,

the F-box motif of cyclin F binds Skp1 of the Skp1–cullin-F-box E3 ubiquitin ligase complexes to function as a substrate-binding region during proteosomal degradation (Bai et al., 1994; Cardozo and Pagano, 2004; D'Angiolella et al., 2013). Other F-box proteins, including bTrCP (F-BOX/WD repeat-containing protein 1A), have previously been implicated in HIV-1 pathogenesis (Margottin et al., 1998). HIV-Vpu was found to interact with bTrCP, a key element in the ubiquitination pathway responsible for the selective degradation of CD4, a hallmark mechanism of immune evasion by HIV-1. Vpu in the ER of infected cells complexes with CD4 cytoplasmic tails, recruiting bTrCP into the complex and subsequently linking Skp1 and the rest of the ubiquitin ligase machinery, marking it for proteolysis (Margottin et al., 1998).

The ubiquitin-proteosomal system (UPS) is now widely recognised in the regulation of viral restriction (Seissler et al., 2017); the UPS may directly restrict viruses through degradation. On the contrary, it is also exploited by viral proteins to degrade cellular restriction factors to facilitate viral replication (Yu et al., 2003; Neil et al., 2008) and is involved in conjugation of HIV-Gag at the cell membrane which is essential for budding and release of HIV-1 (Sette et al., 2013).

Our findings support roles for cyclin C and cyclin F in the restriction of HIV-1 *trans*-infection. It is plausible that these candidates may be involved in the control of viral life cycle progression through interference of viral proteins required for replication or the maintenance of host restriction factor expression which may utilise the UPS for proteolysis. Alternatively since some viruses have previously been known to produce proteins with structural homology to cyclin family proteins and modulate cell cycle and apoptotic events (Swanton et al., 1997; Ojala et al., 2000), it is possible that cyclin C and cyclin F may prevent viral exploitation of cyclin-CDK interactions.

4.3.3 Interleukins

The interleukins are a cytokine superfamily, produced and released predominantly by the CD3+ CD4+ lymphocyte compartment and involved in systemic immune modulation and inflamma-

tory process. The results from our screen highlight three interleukins IL-6 (+103.05%, +1.68 SD, 1.78 MAD), IL-7R (+99.91%, +1.77 SD, 1.87 MAD) and IL-8 (+81.10%, +1.05 SD, 1.15 MAD) as promising candidates with restrictive properties against HIV-1 transfer from DC to SupT1 CD4+ T-cells.

4.3.3.1 IL-6

Interleukin-6 (IL-6) is a 26 kDa pleiotropic cytokine representing one of 33 identified members of the interleukin family which is generally pro-inflammatory in nature and is released by a wide range of cell types, particularly at sites of injury and inflammation. Originally considered a B-cell stimulating gene, IL-6 has been found to exert pleiotropic effects on other lymphocyte tissues including DCs and T-cell to regulate their survival, recruitment and differentiation. As an acute phase response cytokine, IL-6 has widespread roles in innate immunity through stimulating the release of multiple positive acute phase proteins (C-reactive protein, serum amyloid A, fibrinogen and others) by hepatocytes and regulating coagulation through megakaryocyte maturation and platelet formation; these responses are common indicators of inflammation severity often tested for in clinical laboratories (Ishibashi et al., 1989; Tanaka et al., 2014).

IL-6R, the primary receptor complex for IL-6 exists in both membrane-bound and the proteolytically cleaved soluble forms which gives rise to two distinct signalling models, referred to as classical signalling and *trans*-signalling, respectively. Since IL-6R alone does not convey functional signalling, transduction is mediated by the gp130 (CD130) transmembrane glycoprotein which promotes the recruitment and activation of STAT1 and STAT3. Therefore, IL-6 signalling is not limited to target cells that express IL-6R but also to those that express gp130 (Hunter and Jones, 2015). There is now a sufficient evidence to suggest a differential effect for IL-6 classical and *trans*-signalling cascades in health and disease. Classical IL-6 signalling has typically been observed in controlling the regulatory and homeostatic processes of immunity, whereas *trans*-signalling has established links with tissue inflammation and effector cell activity, though these functions are often tissue-specific and context-dependent (Jones, 2005; Hunter

and Jones, 2015). The balance between STAT1/STAT3 phosphorylation is now well recognised for its ability to guide CD4+ T-cell differentiation and effector function (Teague et al., 2000; Jones et al., 2010; Twohig et al., 2019). In addition, DCs have been recently highlighted as a relevant cellular source of IL-6 and are able to cross-present IL-6 via DC-bound IL-6Rα for the generation and maintenance of Th17 cells (Heink et al., 2017). Therefore, it is certainly plausible that DC-mediated IL-6 *trans*-presentation may be involved in mediating cellular responses within our cell model.

There are currently a number of studies describing IL-6 production in response to viral infection, earning its historic name IFN-β2 (Becker et al., 1991; Dienz et al., 2012). In the context of HIV-1 infection, circulating levels of IL-6 and sIL-6R are markedly elevated in infected individuals, continue to increase with disease progression and remain higher even during viral suppression (Breen et al., 1990; Honda et al., 1992). Enhanced IL-6 production is reportedly induced by HIV-1 gp120 Env and the viral accessory proteins HIV-1 Tat and Nef (Ambrosino et al., 1997; Shah et al., 2011; Armah et al., 2012; Liu and Kumar, 2015), though this is debated since IL-6 expression did not appear to correlate with HIV-1 RNA levels (Shive et al., 2012). It is now well established that HIV-1 infection subverts immunity through dysregulation of the cytokine network, but whether IL-6 overproduction contributes to viral pathogenesis is still unclear. Therefore protective roles for IL-6 cannot be ruled out, since it is responsible for promoting virus-specific memory T-cell responses (Longhi et al., 2008) and may have roles in priming adaptive immunity through stimulating HIV-specific IgG production by B-cells.

These findings highlight a previously unexplored area for investigation into the role of IL-6 in DCs during HIV-1 transmission.

4.3.3.2 IL-7R

Interleukin-7 receptor (IL-7R) is an 80 kDa heterodimer made from two major subunits IL-7Rα (CD127) and common-γ chain receptor (γc) which dimerise to form a functional signalling complex for high affinity IL-7 interaction (Al-Rawi et al., 2003). IL-7 belongs to a distinct

class of γc chain-utilising cytokine family, sharing common signalling cascades with IL-2, IL-4, IL-9, IL-15 and IL-21. Cytokines within this family have all been identified as key regulators of immune development and mutations in the xq13.1 locus X-chromosome containing the γc gene leads to X-linked severe combined immunodeficiency (SCID) (Noguchi et al., 1993).

IL-7R signalling is indispensable for T-cell development at the thymus and maintains the survival of circulating T-cells (Peschon et al., 1994). Like many other cytokine receptors IL-7R also exists in soluble form (sIL-7R) which is thought to be a product of alternative splicing rather than membrane shedding. The functional significance of sIL-7R is still not clear, though a study by Lundstrom and colleagues (Lundström et al., 2013) using human CD4+ and CD8+ T-cells have shown that sIL-7R binds IL-7 to compete with cell-associated IL-7R, extending the bioavailability of IL-7 and enhancing T-cell survival.

In light of this, the therapeutic value of IL-7/IL-7R was promptly realised in HIV/AIDS to prevent progressive CD4+ loss, which yielded successful results in phase I/IIA trials for idiopathic CD4+ lymphocytopenia and HIV-1 infection (Levy et al., 2009b, 2012; Sheikh et al., 2016). However, more recent findings are in disagreement suggesting IL-7 had no overall impact on HIV-1 RNA levels (Sereti et al., 2009). In addition, they implicated IL-7 in low-level reactivation of viral reservoirs and can phosphorylate SAMHD1 in CD4+ T-cells, increasing their susceptibility to infection, contributing to post-integration latency and increasing viral reservoir through proliferation of latently-infected cells (Katlama et al., 2016; Coiras et al., 2016). IL-7 signalling in DCs in known to downregulate MHC-II expression *in vivo* as a mechanism to prevent uncontrolled T-cell proliferation (Guimond et al., 2009). Therefore, IL-7 may act differentially in DCs to prevent the expansion of the viral reservoir through homeostatic proliferation.

Our results support a protective properties for IL-7 signalling in HIV-1 infection and indicate that IL-7R, but not the IL-7Rα subunit, is required for restriction of HIV-1 transfer. These findings may support previously unknown roles for IL-7 in DC-mediated immunity against viral infection; selective targeting of IL-7R on DCs could inform current strategies which have failed

to fully address issues of viral persistence.

4.3.3.3 IL-8

IL-8 (CXCL8) is an 11kDa pro-inflammatory cytokine and a member of the C-X-C chemokine family. It is typically secreted by the sensors of the innate immune system—mostly by macrophages, monocytes and dendritic cells— but is also known to be secreted from epithelial, endothelial and smooth muscle cells. The human *CXCL8* gene contains a promoter regions for NF-κB and AP-1 transcription factor binding and is required for IL-8 mRNA synthesis in response to direct or indirect stimulation by IL-1 and TNF-α. IL-8 production is tightly regulated by several protein-kinase cascades including NF-κB, MAPK/ERK and SAPK/JNK signalling pathways. While NF-κB exists in an inactive state and bound to IKBa/b in cytoplasm, during stress the phosphorylation of IKB results in translocation to the nucleus, binding the *CXCL8* promoter to induce transcription by the RelA p65 homodimer subunits (Baeuerle and Baltimore, 1996; Krause et al., 1998; Holtmann et al., 1999; Hoffmann et al., 2002; Jundi and Greene, 2015). Interestingly, *CXCL8* shares synergist transcriptional activation with *IL6* and are frequently produced in tandem (Matsusaka et al., 1993).

Inducible cytokines typically have short mRNA half-lives ($<$ 30 mins) due to AU-rich elements (ARE) in the 3'UTR, which are targets of rapid decay by RNA binding proteins. However, it is reported that modulation of ARE can lead to mRNA stabilisation of IL-8 transcripts for sustained expression over 15 hours (Mahmoud et al., 2014). The translation of human IL-8 mRNA leads to the synthesis of two cytokine isoforms, IL-8 72 aa and the more abundant IL-8 77 aa, which are produced from monocyte and epithelial origins respectively (Gimbrone et al., 1989; Skov et al., 2008). Though there are minimal functional differences between 72 aa and 77 aa isoforms in neutrophil chemotaxis and accumulation *in vivo*, there is in vitro data suggesting that 72 aa monocyte-derived IL-8 is capable of binding with greater affinity, inducing higher Ca2+ flux and is a more potent stimulator of neutrophil inflammatory responses (Nourshargh et al., 1992). Though the expression of IL-8 is low during unstimulated basal conditions due

to transcriptional repression of the promoter region, it plays a critical role as a mediator of inflammation and imbalance in its expression/release is often implicated in many inflammatory diseases, particularly those linked with neutrophil infiltration such as cystic fibrosis. The G-protein coupled receptors, CXCR1 and CXCR2 are the two primary receptors for IL-8 which are abundantly expressed on neutrophil cell surfaces, making them the primary target for IL-8 signalling. Interestingly, CXCR1 binds exclusively to IL-8 whilst CXCR2 exhibits affinity for other cytokines including growth-related protein-α (GROα), neutrophil-activating peptide-2, and epithelial-derived neutrophil attractant-78 (Schraufstatter et al., 2001).

DCs respond readily to IL-8 and can in turn be secreted in low amounts at basal levels, which is markedly enhanced during maturation and activation (Caux et al., 1994; Barrientos et al., 2014). As DCs express both CXCR1 and CXCR2 (Sallusto et al., 1998), autocrine and paracrine signalling could be possible, which may alter DC function and its interaction with CD4+ T-cells.

IL-8 is highly expressed at mucosal surfaces and in particular by epithelial cells and lung airway tissues, reflecting its function as an early mediator of migration and inflammation. Therefore, it is plausible that IL-8 secretion at sites of infection are likely to regulate the functions of tissue-resident DCs. In addition to our screen which has suggested strong protective roles for IL-8 against HIV-1 *trans*-infection, IL-8 is clearly relevant in our cell model. Together with the previously identified roles for IL-6, there is compelling evidence to warrant further investigation of these candidates within our system (Prodger et al., 2016).

4.3.4 Tumour Necrosis Factor Superfamily

Members of the tumour necrosis factor superfamily (TNFSF) have important biological functions in many aspects of immunity. The human cytokine siRNA library contained gene targets for all TNFSF members and their associated receptors; data from our screen suggests protective roles in DC-mediated HIV-1 *trans*-infection for CD70/TNFSF7 (+64.91%, +1.80 SD, 1.90 MAD), 4-1BBL/TNFSF9 (+21.56%, +1.56 SD, 1.65 MAD) and TWEAK/TNFSF12 (+36.87%,

+2.08 SD, 2.18 MAD). Here we discuss the the functions of each candidate with respect to DC biology.

4.3.4.1 CD70 (TNFSF7)

CD70 (TNFSF7) is a type II transmembrane protein, one of 20 superfamily members and expressed on the surface of most APCs including DCs but also on nearly all T-cell subsets and activated B-cells. As a co-stimulatory ligand, the expression of CD70 is mostly restricted to primed lymphocytes and is tightly regulated to maintain optimal effector cell homeostasis. The expression of CD70 is elevated through pro-inflammatory cytokines including (IL-1α, IL-12 and TNFs) and is lost during signalling by anti-inflammatory cytokines IL-4 and IL-10 (Lens et al., 1997; Arens et al., 2001). In DCs, CD70 expression is also induced through CD40 or TLR stimulation (Tesselaar et al., 2003b; Sanchez et al., 2007) and is transported to intracellular compartments shared with MHC-II. During T-cell contact, it is thought that CD70 polarises toward the immunological synapse using similar mechanisms to MHC class II in order to provide efficient and timely signalling that paralleling antigen recognition (Keller et al., 2007). TNFRSF7 (CD27) is a 55-kDa transmembrane receptor and the only known target for CD70, found predominantly on the CD4/CD8 T-cells, NK cells and mature B-cells. Interaction with CD27 drives multiple T-cell and B-cell processes including survival, proliferation and activation.

CD70-CD27 engagement results in the recruitment of the adaptor proteins TRAF2 and TRAF5 to the intracellular C-terminal domain and downstream activation of NF-KB and c-Jun N-terminal kinases (JNK) (Yamamoto et al., 1998; Akiba et al., 1998). In contrast, CD27 signalling can induce apoptotic pathways by the intracellular protein SIVA during viral infection and oxidative stress, which can signal in a CD70-independent manner when overexpressed in vitro (Prasad et al., 1997; Henke et al., 2000; Spinicelli et al., 2002; Cao et al., 2012). CD70 signalling leads to downregulation of CD27 surface expression and an increase in the release of its 32-kDa soluble form (Hintzen et al., 1994). The generation of soluble CD27 (sCD27) occurs

through proteolytic cleavage of membrane-bound CD27 by metalloproteinases or alternative splicing of the receptor protein (Loenen et al., 1992; Kato et al., 2007; Huang et al., 2013). More recently, CD70 has become an attractive target in for cancer immunotherapy due to its overexpression in tumour cells and roles in promoting growth through induction of Treg cells and subsequent decrease in tumour-specific T-cell response (Jacobs et al., 2015). CD70 overexpression is likely to lead increased sCD27 release, which has previously been observed in B-cell malignancies and are associated with an increased relative risk of developing AIDS-associated non-hodgkins lyphoma (Van Oers et al., 1993; Widney et al., 1999).

CD70-CD27 interaction has a unique feature of reverse signalling through the ligand-side which is shared among several cytokine/hormone families including TNFSF, interleukins, ephrins, semaphorins and Notch-associated ligands (Ross et al., 2001). CD27+ T-cells have previously been shown to enhance Ig-production in activated B-cells expressing CD70, which supports bidirectional signalling on the ligand-expressing cell (Bowman et al., 1994). It is likely that reverse signalling could enhance effector functions in both cells.

Our RNAi screen identifies CD70 as a restrictive candidate against HIV-1 trans-infection by DCs. Some groups have reported modest levels of CD70 expression in immature MDDC, which only becomes apparent during maturation, through activation of TLR or by CD40L co-stimulation (Boursalian et al., 2009; Arimoto-Miyamoto et al., 2010; Van Deusen et al., 2010). In our cell model, the activation of endosomal TLR8 by HIV-1 Env together with CD40L+ CD4+ T-cell co-culture should be sufficient in enhancing CD70 expression in MDDC, though this is yet to be fully determined.

4.3.4.2 4-1BBL (TNFSF9)

4-1BBL (TNFSF9; CD137L) is a type 2 transmembrane protein and ligand for the dimeric 50–55kDa receptor 4-1BB, which interact to provide crucial costimulatory signals at the APC-T-cell interface (Pollok et al., 1993). The expression profile of both receptor and ligand has remained complex and unclear since their first discovery - early cDNA clonal studies first characterised

4-1BB expression in T-cells and later found to be present in many activated non-T immune cells, with exception to DCs in which the expression is constitutive (Goodwin et al., 1993; Futagawa et al., 2002; Wilcox et al., 2002). In isolated T-cell cultures, 4-1BB is expressed to a greater extent in CD8+ than CD4+ T-cells and preferentially expands CD8+ T-cells, enhancing cytolytic effector function and IFNγ production (Shuford et al., 1997; Wen et al., 2002). But despite its predominant expression in CD8, 4-1BB has shown to promote survival and release of IL-2 and IL-4 (Gramaglia et al., 2000; Cannons et al., 2001). In DCs, 4-1BB functions as a receptor for activation which when bound, promotes cell survival, release of IL-6/IL-12 and augmentation of virus-specific Th1 effector and CD8 cytolytic responses (Futagawa et al., 2002; Choi et al., 2009; Zhang et al., 2010; Vinay and Kwon, 2011). 4-1BB signalling in DC upregulates the expression of maturation markers CD80 and CD86, which is unusual considering it is widely reported that STAT3 activation blocks DC maturation (Cheng et al., 2003; Park et al., 2004).

Interestingly, in addition to 4-1BB receptor expression, both human and murine DC from monocytic or other haematopoetic precursors express low levels of the ligand 4-1BBL which is enhanced under LPS-mediated maturation (Futagawa et al., 2002; Laderach et al., 2003; Lee et al., 2003). As DCs express both receptor and ligand, it is thought that 4-1BB-4-1BBL autocrine signalling or at a DC-DC junction may help drive maturation and cytokine production required for priming of CD4 or CD8 T-cell responses. As previously discussed, reverse signalling is a feature conserved across many of the TNF superfamily members with important functions in the amplification of adaptive immune responses. Signalling through 4-1BBL during DC-T-cell contact may therefore drive DC maturation to further enhance 4-1BBL expression and Th1-inducing cytokine production (Kim et al., 2002; Wilcox et al., 2002; Laderach et al., 2003).

4.3.4.3 TWEAK (TNFSF12)

TWEAK, or TNF-related weak inducer of apoptosis, is the twelfth member of the TNF cytokine superfamily first characterised in 1997 (Chicheportiche et al., 1997). It exists as either a 30 kDa

type II transmembrane form (mTWEAK) required for local cell-cell signalling or cleaved to form an 18 kDa soluble secreted cytokine (sTWEAK), which is characteristic of 'alarm signalling' found in TNF member cytokines (Chicheportiche et al., 1997; Kawakita et al., 2004). TWEAK has as wide expression profile which includes peripheral blood monocytes, macrophages and dendritic cells upon stimulation with IFNγ or phorbol myristate acetate (PMA) (Nakayama et al., 2000; Maecker et al., 2005) and has important roles in regulating innate immunity and priming of adaptive immune responses. TWEAK exhibits multifunctional biological activities by signalling through its main receptor Fn14 (also TWEAKR, TNFRSF12) though there are also reports of CD163 binding in monocytes/macrophages (Bover et al., 2007; Akahori et al., 2015) and initial studies suggesting DR3 affinity which have since been refuted (Marsters et al., 1998; Migone et al., 2002). TWEAK-Fn14 interaction leads to receptor trimerisation at the cell surface, signal transduction and regulation of cellular proliferation, survival, differentiation, death and migration in a cell-type dependent manner. While Fn14 expression is typically low in healthy tissue, it is upregulated during injury, oxidative stress, inflammation or tumourigenesis. In addition to high tumour expression, TWEAK-Fn14 signalling has been linked with promotion of angiogenesis and has become an attractive therapeutic target in oncology (Lynch et al., 1999). The Fn14 cytoplasmic tail contains TRAF binding domains which are required for the canonical and non-canonical induction of NF-κB and triggering of early inflammatory events including the synthesis and release of pro-inflammatory molecules such as TNF-α, IL-6, IL-8 and CCL5 (Chicheportiche et al., 1997; Winkles, 2008; Roos et al., 2010). TWEAK activates NF-κB primarily through the canonical pathway, with greater activation potency by mTWEAK than sTWEAK and both variants being poor inducers of non-canonical NF-κB activation (Roos et al., 2010). However, these findings are disputed in vivo, which support anti-inflammatory roles for TWEAK and suggest that TWEAK in fact attenuates the transition to adaptive immunity through blockade of STAT-1 signalling and IFN-γ and IL-12 production (Maecker et al., 2005).

As its name suggests, TWEAK is an apoptosis-inducing ligand which has reported activity in many human tumour cell lines and in human PBMCs, which can be augmented in the presence

of IFN-γ (Kaplan et al., 2002; Nakayama et al., 2002). Interestingly, Fn14 does not contain a death receptor so apoptotic events are likely driven by indirect effects of TWEAK signalling, though the exact mechanisms have long been debated. Two main apoptotic pathways have been characterised to date. The extrinsic pathway activates apoptosis through signal transduction by death receptors including TNFR members. Death receptor interaction leads to the formation of a Fas-associated death domain (FADD)-containing DISC (death-inducing signalling complex) at the cytoplasmic tail, which in turn recruits and activates effector caspases 8 and 3 to induce cell death (Chinnaiyan et al., 1995; Medema et al., 1997; Danial and Korsmeyer, 2004). In contrast, intrinsic cell death is driven by release of mitochondrial-derived intracellular cytochrome c, required for APAF1/caspase-9 apoptosome formation and activation of effector caspase-3 (Danial and Korsmeyer, 2004; Green and Kroemer, 2004; Fulda and Debatin, 2006). More recent findings by Ashkenazi (Ikner and Ashkenazi, 2011) suggest that TWEAK induces apoptosis using both pathways and suggests that TWEAK-Fn14 interaction recruits TRAF2 and cIAP1(cellular inhibitor of apoptosis protein 1) in the absence of death domain adaptors typically found in DISC. Instead, TWEAK signalling promotes autocrine TNF-α signalling via NF-κB to promote intrinsic cell death via RIP1-FADD-caspase-8 complex assembly.

Permissive candidates

4.3.5 CX3CR1

CX3CR1 was identified as the only permissive screen candidate by all hit selection models, which when downregulated by siRNA in MDDC, resulted in a reduction of viral transfer in *trans* to CD4+ T-cells (-37.40%, -1.58 SD, 1.48 MAD). These findings implicate that naturally expressed CX3CR1 in DCs would facilitate HIV-1 transfer. Also known as the Fractalkine receptor, CX3CR1 is a G-protein coupled receptor expressed on monocytes, DCs, NK cells, endothelial cells and microglia. As a chemokine receptor, CX3CR1 is involved in the migration and adhesion of leukocytes through interaction with its primary ligand fractalkine CX3CL1,

together forming the only receptor-ligand pair of the C-X3-C chemokine class (Imai et al., 1997; Jung et al., 2000). Like all chemokine receptors, CX3CR1 has a 7-helix transmembrane (7-TM) domain receptor architecture, which signal through G-protein linked signal transduction to provide chemotactic function through cytoskeletal reorganisation. Although interestingly, the functions of CX3CR1 as an adhesion molecule does not utilise these mechanisms (Imai et al., 1997).

Clinical studies investigating the relationship between CX3CR1 and HIV-1 pathophysiology have been a source of much controversy since the report by Faure et al., which suggested that homozygosity of the M280 allelic variant of CX3CR1 is associated with increased risk of infection and rapid progression to AIDS (Faure et al., 2000). These findings however were soon challenged by a number of groups showing discrepant results and scrutinised the initial study for lack of statistical power, minimal effect size and uncontrolled cohort composition (McDermott et al., 2000; Roman et al., 2001; Kwa et al., 2003). But despite this, *in vitro* studies continue to support an, albeit modest, degree of CX3CR1 affinity and co-receptor utilisation by HIV-1 Env and is associated with enhanced viral fusion and greater disease progression (Rucker et al., 1997; Combadiere et al., 1998; Garin et al., 2003). While these data offer minimal support for roles of CX3CR1 as a cofactor in HIV-1 pathogenesis, the underlying cause of these discrepancies should continue to be investigated.

CX3CL1 is constitutively expressed within the central nervous system and forms a key communication link between neuronal cells and CX3CR1-expressing microglia required for the modulation of neuroinflammation (Harrison et al., 1998). Currently there are mixed reports on the protective and detrimental functions of CX3CR1 during neuropathology. While abrogated CX3CL1/CX3CR1 interaction was shown to induce a protective inflammatory environment in some *in vivo* murine models of ischaemic injury (Tang et al., 2014), it has also been linked with prolonged microglial activation and sustained release of pro-inflammatory agents (Duan et al., 2014). Disruption of the ligand-receptor interaction by HIV-1 Tat has been described which could be responsible for driving impaired microglial function and accelerated development of

HIV-1-associated neurological disorders (Duan et al., 2014).

In DCs, CX3CR1 expression is typical in myeloid DC lineages and is maintained throughout all stages of development, in addition, has been previously reported in MDDC generated using similar methods of *in vitro* differentiation (Łyszkiewicz et al., 2011; Johnson and Jackson, 2013). CX3CR1 functions as an important receptor for immunosurveillance in the lamina propria. Intestinal DCs sample the intestinal lumen through *trans*-epithelial dendrites using a CX3CR1-dependent mechanism, which when lost, impairs phagocytic function and uptake of pathogenic bacteria (Niess et al., 2005). Clearly, CX3CR1 has diverse roles in leukocyte development and in supporting immune function. Given the affinity for HIV-1 Env, it is certainly plausible that CX3CR1 is responsible for facilitating productive infection of MDDC, although the impact of viral tropism on binding and fusion is not yet clear and should be tested in R5 and X4R5 strains. Our results from the siRNA screen identified CX3CR1 as our only permissive candidate which when downregulated, disrupts the ability of viral transfer. Our observations found that siCX3CR1 MDDC intracellular p24 content is lower than the matched-donor non-target controls and within the lower 5% quantile of screen samples, which support the hypothesis that the loss of CX3CR1 may be responsible for diminished *cis*-infection and lower likelihood of viral transfer across the VS. Taken together, it is plausible that CX3CR1 may be exploited by HIV-1 in MDDC for viral entry. Further investigation into CX3CR1 blocking strategies during acute infection could inform the development of a new line of HIV-1 entry inhibitors.

4.4 Concluding remarks

The results of our high-throughput RNAi screen generated a total of 15 restrictive candidates. Here, we have identified MIF as our strongest and most robust candidate, showing a potent restrictive phenotype against HIV-1 *trans*-infection from DC to SupT1 CD4+ T-cells. MIF has multi-faceted roles which are involved in many aspects of both innate and adaptive immunity.

As a pro-inflammatory cytokine-mediator, there is strong evidence to suggest that MIF may be involved in the generation of a restrictive inflammatory response against HIV-1 infection, which may prevent viral transfer at the virological synapse between DCs and CD4+ T-cells.

Our results also highlight the relevance of TNF superfamily members as restrictive candidates against HIV-1 transmission from DCs to CD4+ T-cells. Results from the siRNA screen identify CD70 (TNFSF7), 4-1BBL (TNFSF9) and TWEAK (TNFSF12) as promising candidates with diverse biological functions, offering unique insights into viral restriction. TNFs are recognised for their ability to induce immune responses, particularly at the DC-T-cell interface, responsible for driving proliferation, differentiation, inflammation and apoptosis. Together with our interleukin candidates (IL-6, IL-7R and IL-8) and the cytokine-mediator MIF, we hypothesise several mechanisms by which viral restriction may occur including the induction of pro-inflammatory responses (via NF-κB, IL-6 and IL-8) and the release of Th1 cytokines (IL-12, IFN-γ, TNF-α), both of which are well-defined restrictors of HIV-1 infection. Inflammation is capable of initiating DC maturation and T-cell differentiation and providing HIV-1 specific effector function observed in HIV-1 elite controllers. However, inflammation is not always protective. HIV-1 exploits chronic immune activation and inflammation as a mechanism of viral propagation and immune evasion; therefore while TNF-focussed strategies against HIV-1 may prove effective in acute infection, it also has the potential to accelerate disease progression in the context of chronic infection. In addition, TNF superfamily members can induce apoptosis through intrinsic and extrinsic pathways. Mechanisms of programmed cell death in DCs may play protective roles in the control of acute viral infection by disrupting viral life cycle and preventing the production and release of new progeny virus. However, it appears that HIV-1 has evolved to use these mechanisms to its advantage. Apoptosis is characterised by compartmentalisation and formation of apoptotic bodies. During infection, viruses may purposely induce apoptosis so that viral particles and progeny virus are packaged within membrane-bound compartments, subverting the immune system and ready for uptake by neighbouring cells (Roulston et al., 1999). More recently, the connection between apoptosis and autophagy has been unveiled, linking many apoptotic proteins with the autophagic machinery (Gump and Thorburn, 2011).

We previously described the relationship between our TNF superfamily candidates CD70, 4-1BBL and TWEAK and the induction of apoptotic cell death and interestingly, reports of dual function in autophagy regulation have emerged. For instance, CD27 has GO term association with apoptosis and autophagy, TWEAK has been reported to co-ordinate muscular atrophy through regulation of autophagy proteins, the UPS and caspase activity (Bhatnagar et al., 2012) and lastly there are a number of reports linking MIF with enhanced autophagic activity (Chuang et al., 2012; Xu et al., 2016).

Our screen library also targeted the cyclin protein family, which have central roles in the regulation of cell cycle progression, cell growth and survival. During productive infection by HIV-1, integration of viral DNA into the host genome results in the wide-spread dysfunction of many lymphocytes including DCs and there is evidence suggesting that cyclins be involved in these processes. Our screen identified cyclin C and F as protective candidates against viral transfer, but the current literature offers little support for these findings with several groups observing permissive functions of these candidates in HIV-1 pathogenesis. Cyclins have been previously reported to facilitate HIV-1 life cycle through enhanced transcriptional activity, immune evasion and contribution to dysregulation of the cytokine network. However, these observations were made in the context of productive infection which rarely occurs in DCs due to low level CD4 expression. Since DCs are able to capture and internalise HIV-1 through a number of mechanisms, alternate functions of cyclin-CDK interactions may convey protective immunity during these processes, though these roles beyond cell cycle remain largely unexplored. Nevertheless, these observations open a potentially interesting avenue of research linking cell cycle and immune regulation.

In summary, we have identified candidates from several protein/cytokine families as potential restrictors of HIV-1 *trans*-infection in DCs, which must be further tested to validate the authenticity of their biological functions in our system. A new hypothesis should be proposed detailing a potential mechanism of action to generate proof-of-principle targets with therapeutic value. MIF showed striking phenotype in our siRNA screen; as an upstream regulator of several

other screen candidates, it is possible that a pro-inflammatory MIF-TNF-IL-6/IL-8 axis may be involved in promoting antiviral restriction in DCs and curtailing cell-cell transmission. Interesting links between our candidates and the induction of apoptotic and autophagic machinery raise questions about whether these mechanisms are used to disrupt HIV-1 life cycle. Indeed, previous findings from our laboratory suggest this is the case, showing that the inhibition of autophagy leads to enhanced cell-cell transmission of HIV-1 (Blanchet et al., 2010; Chauhan et al., 2015; Ribeiro et al., 2016). It is plausible that novel cytokine signalling networks may exist which regulate this process and should be investigated further.

Chapter 5

Validation of siRNA screen candidates

5.1 Background

With a refined candidate list chosen through our model selection strategy, this chapter aims to validate these candidates using functional assays to gain reproducibility and to explore underlying mechanisms for the generation of proof-of-principle targets.

5.1.1 Molecular mechanisms of macroautophagy

Deriving from the Greek *auto-* and *-phagein*, or "self-eating", autophagy (macroautophagy) is a cellular mechanism of intracellular clearance, needed to maintain a homeostatic internal environment through lysosomal degradation and recycling of cytoplasmic components and organelles (Mizushima, 2007). Autophagy has been observed for over 50 years and has recently been 'rediscovered' with implications in infectious disease, cancer and neuropathology, sparking global scientific interest. Though despite being first described in mammalian cells during the late 1960s (Deter et al., 1967), the molecular understanding of autophagy was not known until the late 1990s.

Early studies were based on morphological observations and much of the current knowledge is

grounded by studies in yeast which have identified several autophagy-related (Atg) genes. One specific example of note is a pivotal experiment by Yoshinori Ohsumi in 1992, which demonstrated that disruption of the normal degradation processes in protease-deficient *S. cerevisiae* led to the accumulation of autophagosomes, double-membrane vesicles containing content for degradation (Takeshige et al., 1992). Ohsumi's findings paved the way for further identification of Atg genes underlying autophagic mechanisms which ultimately won the 2016 Nobel Prize in Medicine. Mammalian homologues of Atg genes have since been identified and used to characterise models of autophagy in human cells.

Autophagy is best described in a scenario of metabolic starvation, whereby the induction of autophagy acts to resolve energy deficit through recycling of internal components as a source of nutrients to maintain the homeostatic environment. Whilst it was initially thought that autophagy is a generic bulk process, evidence has been gathered to support the notion that autophagy can be selective, identifying roles for the ubiquitin/p62/NBR1 axis in targeted degradation (Kirkin et al., 2009). Selective autophagy therefore has important functions beyond cell survival and serves as a mechanism of innate immune defence against intracellular pathogens and even has roles in modulating macrophage phagocytosis (Sanjuan et al., 2007).

There are now several types of autophagic processes described, named accordingly with their target of degradation. Here we focus on macroautophagy which relates specifically to the recycling of macromolecules, though autophagy may also refer to microauthophagy, mitophagy (mitochondrial), ribophagy (ribosomal), aggrephagy (protein aggregates) and lipophagy (lipid). The process of (macro)autophagy is broadly comprised of several sequential steps: initiation of Atg protein complexes, elongation of the isolation membrane, sequestration of cytoplasmic cargo within autophagosomes, fusion with lysosomes, degradation and recycling of macromolecules (Figure 5.1).

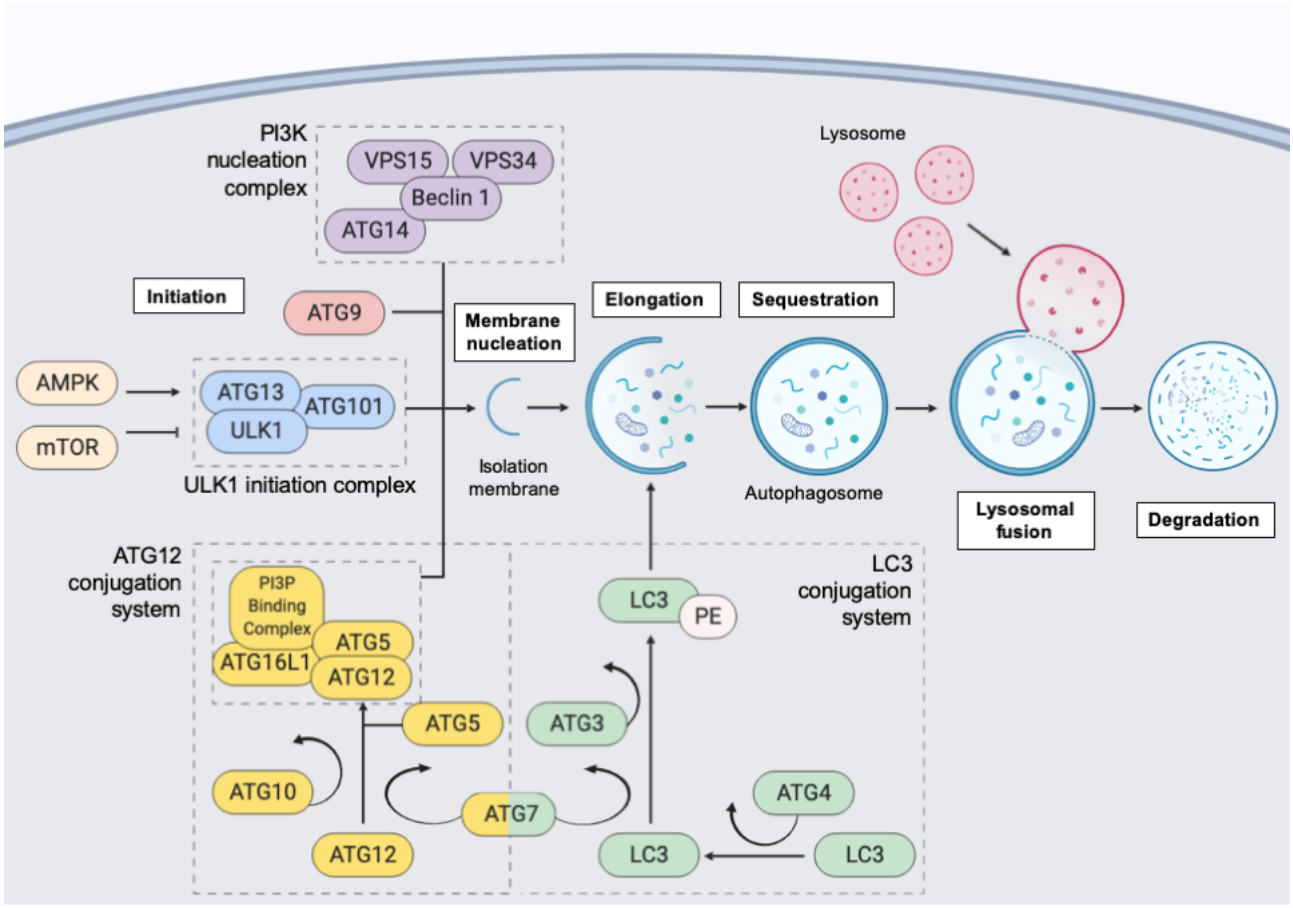

Figure 5.1: Stages of canonical macroautophagy. Initiation is controlled by upstream regulators mTOR and AMPK which govern formation of the ULK1 initiation complex. Membrane nucleation and elongation is driven by three major systems of PI3K nucleation and ATG12/LC3 conjugation in addition to ATG9 which facilitates membrane delivery. Cytoplasmic cargo is sequestered within the autophagosome, which fuses with lysosomes for degradation of its contents and recycling of nutrients and macromolecules. The fate of the autophagolysosome is not clear, but may be recycled into new lysosomes. Adapted from (Hansen et al., 2018). PE, phosphatidylethanolamine.

Initiation — Initiation of autophagy is driven by the Ulk1 complex, which is dependent on input from two key intracellular sensors of metabolism: mTOR/mTORC1 and AMPK which regulate its assembly. At basal levels, autophagy is usually repressed and regulated by the mammalian target of rapamycin (mTOR) and controlled upstream by PI3K-Akt and MAPK signalling pathways which facilitate mTOR complex 1 (mTORC1) formation (Jung et al., 2010). During optimal metabolic conditions, mTORC1 activity maintains Ulk1 Ser 757 phosphoryla-

tion to prevent initiation of autophagy (Kim et al., 2011). However under glucose and amino acid starvation, mTORC1 is blocked by AMP activated protein kinase (AMPK) which alleviates Ser 757 phosphorylation and promotes ULK1 complex formation to promote induction of autophagy (Hardie, 2007; Nicklin et al., 2009; Lee et al., 2010b; Kim et al., 2011; Hansen et al., 2018).

Biogenesis and elongation — Active ULK1 initiation complexes promote the formation of the (pre-autophagosomal) isolation membrane at the ER surface. This process is coupled with three additional pathways involving: 1) ATG14-dependent PI3K nucleation complex recruitment to the ER (Matsunaga et al., 2010; Hamasaki et al., 2013). 2) Generation of a phosphatidylinositol-3-phosphate (PI3P) pool supporting formation and maturation of autophagosomes (Singh and Cuervo, 2012). 3) The trafficking of ATG9-containing vesicles to the ER, which is responsible for the delivery of membrane during phagophore elongation (Mack et al., 2012).

Sequestration — As the phagophore expands, the LC3-modifier and ATG12-conjugation systems work in conjunction to incorporate cargo receptors which can selectively bind and sequester target proteins for degradation. Microtubule-associated proteins 1A/1B light chain 3 (LC3) once synthesised, is cleaved into the cytosolic form LC3-I by ATG4 and subsequently conjugated with phosphatidylethanolamine (PE) to form LC3-II for incorporation into the phagophore membrane via ATG12. LC3-II contains a LC3-interacting region (LIR) motif, responsible for binding selective target proteins (Behrends et al., 2010).

Fusion and degradation — Once the phagophore fully encloses target cargo, autophagosome formation is fully complete and is ready for fusion with lysosomes. ATG14, in addition to its role in membrane nucleation, serves important roles during fusion to create autophagolysosomes (Diao et al., 2015). Although the exact mechanisms are not yet fully understood, ATG14 is recruited to mature autophagosomes where it tethers to the outer membrane and promotes lysosomal fusion to form the autolysosome or autophagolysosome (Diao et al., 2015; Liu et al., 2015). During degradation, the contents as well as the inner membrane of the autolysosome

are broken down by hydrolases and exported back to cytosol. Beyond this, the fate of the autolysosome is not clear, although the most widely accepted model suggests that they continue to function as lysosomes for further fusion with endosomes or autophagosomes (Eskelinen and Saftig, 2009).

5.2 Results

5.2.1 Follow-up validation of siRNA screen candidates

After review of the surrounding literature around screen candidates, we excluded potential off-target hits that are irrelevant to DC function and cytokine activity. We finalised our candidate list to three members of the TNF superfamily: CD70 (TNFSF7), 4-1BBL (TNFSF9), TWEAK (TNFSF12), IL-6, IL-7R and MIF as key restrictive proteins in HIV-1 transfer. Despite falling short of threshold, we continued to include IL-8 throughout validation due to its nature as a downstream product of MIF/TNF signalling. IL-8 signalling has previously been implicated in mucosal HIV-1 acquisitions (Prodger et al., 2016) and by validating this in the context of *trans*-infection, IL-8 could help inform the development of potential restrictive mechanisms by DCs. Several follow-up experiments were performed however no conclusive evidence was observed, although our data suggested that of the whole candidate list, MIF, 4-1BBL and IL-8 showed the highest levels of restriction and consistency and were investigated further. A list of some key experiments are detailed below:

- Broad range neutralisation by overnight treatment with candidate neutralising antibody (nAb) during HIV-1 transfer – primary validation to further eliminate off-target candidates or those with weak effect.
- Phenotypic analysis of candidate nAb on MDDC – test for evidence of candidate-driven changes to DC maturation as a mechanism of HIV-1 restriction.
- Stimulation of MDDC using candidate recombinant cytokines during HIV-1 transfer – test

for restrictive properties during protein over-expression as opposed to loss-of-function.

5.2.2 Optimisation of αMIF, α4-1BB and αIL-8 nAb dose-ranges during *trans*-infection of SupT1 and autologous CD4+ T-cells

Given the role of MIF as an upstream mediator of IL-8 and TNF secretion, we decided to focus validation on these three candidates. Our previous data support 4-1BBL/4-1BB as our strongest TNF related candidate and so limited our study to these three proteins. We next investigated dose-dependent effects of nAb treatment on viral transfer at a range of 1 ng/mL – 10 μg/mL. To make the cell system more representative of DC-T-cell interactions *in situ*, we re-optimised our transfer assay to include primary autologous CD4+ T-cells as our recipient cell and compared these results to that of SupT1 CD4+ T-cell lymphoblastic cell line.

Our results showed that viral transfer in *trans* from DCs to SupT1 in the presence of αMIF, αIL-8 and α4-1BB was proportional and dose-dependent showing maximum effect at 10μg/mL for all candidate nAb. Neutralisation by αMIF showed the greatest increase of viral transfer by 4-fold (45.6% transfer) from isotype treated controls (11.53% transfer) whilst αIL-8 and α4-1BB both showed 2-fold increases (21.9% and 21.4% respectively) (Figure 5.2).

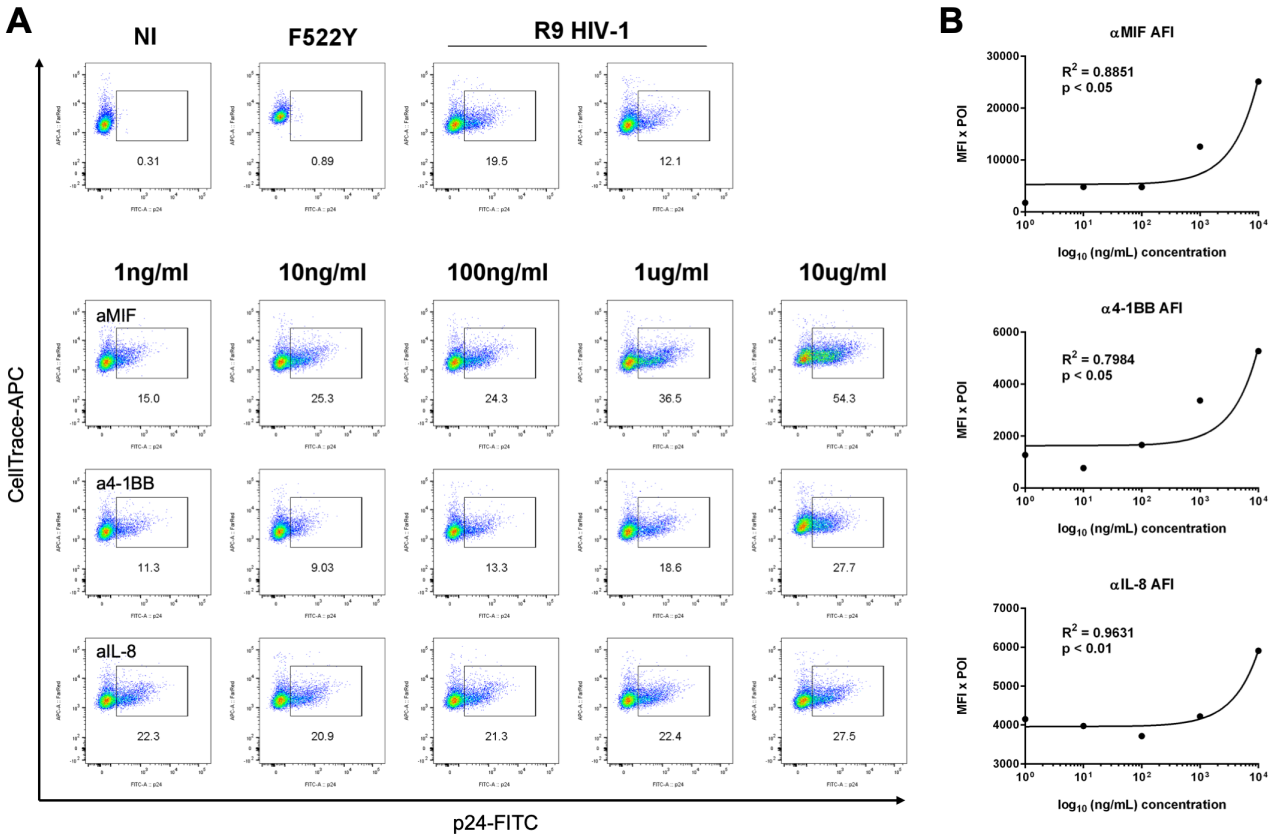

Figure 5.2: Titration of candidate neutralising antibodies (nAb) on HIV-1 transfer. A) Titration of αMIF, α4-1BB and αIL-8 nAb treatments during viral transfer from MDDC to SupT1 cells. B) Correlations of average fluorescence intensity calculated using median fluorescence intensity (MFI) x percentage of infection (POI) against nAb concentration.

Neutralising antibody titrations during DC-to-CD4+ T-cell transfer showed a loss of dose-dependent effect with a maximal response at 1μg/mL concentrations across all candidates which begins to decrease at 10μg/mL nAb concentrations. Both αMIF and α4-1BB showed increases in transfer at 1μg/mL (26.4% and 29.4% transfer respectively) from isotype control (11.4%) however, neutralisation by αIL-8 showed only modest increase (18.6%) in *trans* to autologous CD4+ T-cells (Figure 5.3).

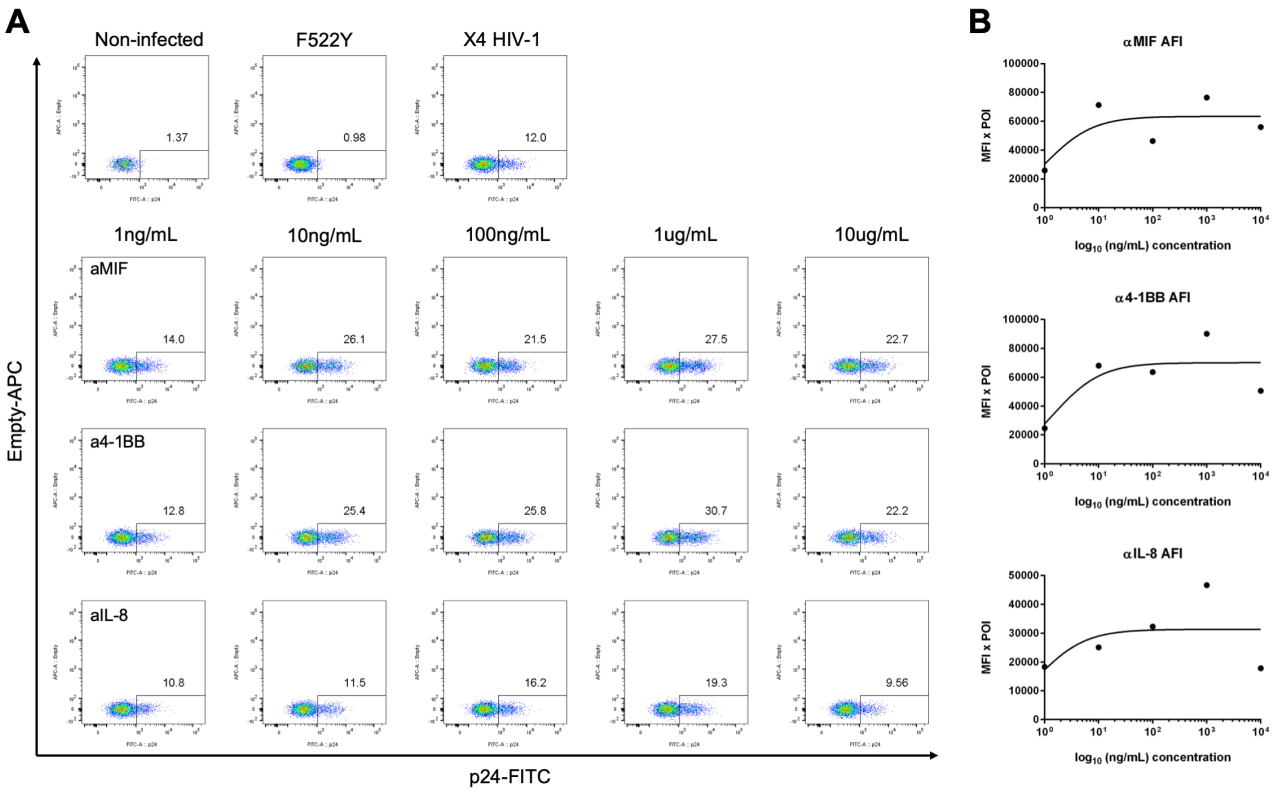

Figure 5.3: Titration of candidate neutralising antibodies on HIV-1 transfer to autologous CD4+ T-cells. A) Titration of neutralising anitbody (nAb) treatments during viral transfer from MDDC to autologous CD4+ T-cells. B) Correlations of average fluorecence intensity calculated using median fluorecence intensity (MFI) x percentage of infection (POI) against nAb concentration.

The use of autologous CD4+ T-cells as a HIV-1 recipient substantially improves the validity of our cell model since we are able to address a key caveat that SupT1 cells are not biologically representative since they are neither present in normal human tissues nor involved in the early stages of HIV-1 infection. With this in mind, our data continues to support MIF as a promising candidate with roles in regulating viral transfer to CD4+ T-cells. Anti-4-1BB treatment has been consistent throughout neutralisation experiments although showing signs of variability between assays. Interestingly, α4-1BB treatment appeared to have a greater impact on transfer to primary CD4+ T-cells than SupT1. Engagement of 4-1BB in T-cells provides co-stimulatory signals required for activation, differentiation and effector cell function which can occur in primary cells, but not SupT1 and could therefore explain this disparity. Lastly,

IL-8 neutralisation confirms a role for the pro-inflammatory cytokine in effective restriction of viral transfer to SupT1 but to a lesser extent to autologous CD4+ T-cells. From a technical perspective, primary T-cells are less receptive to infection compared with SupT1 given its very high surface CD4 expression. Together these data support the likely involvement of a MIF-TNF-IL-8 axis in the control of viral transfer from MDDC to CD4+ T-cells in *trans*.

5.2.3 Repeat siRNA validation of refined screen candidates using autologous CD4+ T-cells

Following the optimisation of a cell system of HIV-1 *trans*-infection from DC to autologous CD4+ T-cells, we next repeated our viral transfer assay during siRNA knockdown of refined candidates MIF, IL-8 and 4-1BBL in MDDC (Figure 5.4). After normalisation of target to non-target siRNA for the respective donor, we were able to reproduce restrictive phenotypes in 2 of 3 candidates. In siMIF MDDC, viral transfer continued to be upregulated 1.233-fold increase (SEM = 0.027, $p < 0.01$) with respect to non-target siRNA. Despite its low-level effect during nAb optimisation, siIL-8 downregulation statistically enhanced viral transfer by 1.114-fold (SEM = 0.025, $p < 0.05$). Interestingly, no significant changes were observed during 4-1BBL downregulation (1.0713-fold, SEM = 0.072, $p = 0.368$) during viral transfer.

Based on the evidence so far, we have been able to characterise restrictive roles for MIF, IL-8 and 4-1BB/4-1BBL during transfer to SupT1. However, when moving to a system using primary cells only, we fail to observe strong supporting evidence for IL-8 and 4-1BBL in the restriction of HIV-1 *trans*-infection. At this point we should acknowledge our limitations in the validation of 4-1BBL since neutralisation data for the ligand is lacking.

While neutralisation of its cognate receptor should theoretically block any activities of 4-1BBL, we cannot eliminate possibility of 4-1BB-independent bystander effects of 4-1BBL. In addition, since RNAi and nAb treatments only provide transient protein knockdown in these assays, knockout experiments may be required in order to achieve stronger phenotypes. We found

that MIF has consistently shown restrictive properties throughout the stages of validation and decided to focus future studies on this candidate alone until further evidence for the involvement of IL-8 and 4-1BBL emerges. Whilst we still firmly believe that a MIF-TNF-IL-8 axis could still be implicated in the regulation of viral transfer from MDDC, understanding this relationship on a mechanistic level could prove challenging, especially given the complex nature of 4-1BB/4-1BBL interaction and a weaker IL-8 phenotype in our experimental system.

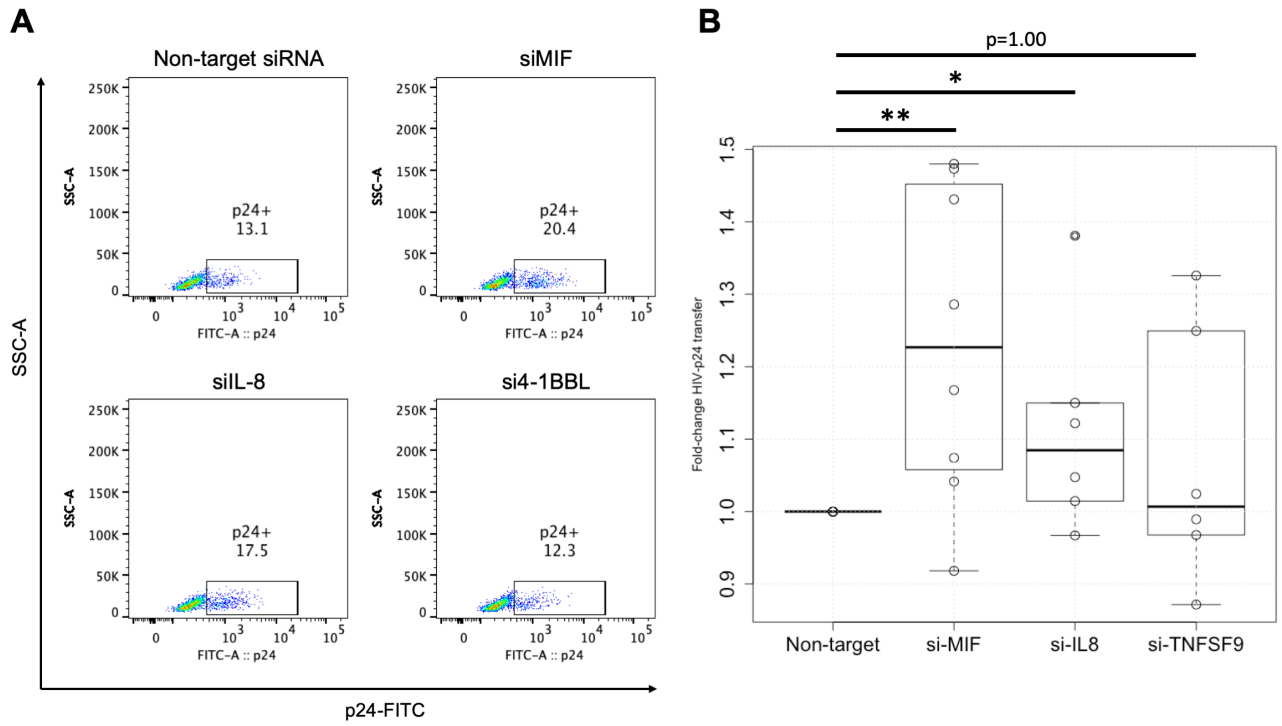

Figure 5.4: RNA interference (RNAi) of refined candidates MIF, IL-8 and 4-1BBL/TNFSF9 during viral transfer to autologous CD4+ T-cells. A) Representative flow cytometry plots. B) Aggregated data of fold-change viral transfer relative to non-target siRNA transfected control MDDC. Wilcoxon ranked sums paired test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data representative of 3 independent experiments.

5.2.4 MIF is highly expressed in MDDC and is a suitable target for RNAi

MIF has been reported to be produced in many lymphocytes, however its expression in MDDC has not previously been established. To ensure the validity of MIF as a candidate for RNAi we

used flow cytometry to determine its expression in our cell model. Since MIF, unlike conventional cytokines, is constitutively expressed and stored in large intracellular pools (Calandra et al., 1994), we were able to permeabilise MDDC and easily stain for its intracellular expression. We transfected MDDC with either non-target or MIF siRNA and compared the expression of MIF relative to isotype controls (Figure 5.5A). Our data confirm that MIF is highly expressed and present on average in 84.47% (SEM = 1.11) of MDDC with a MFI of 1002.89 (SEM = 29.18) compared to 114.71 (SEM = 3.67, $p < 0.001$) in isotype controls (Figure 5.5B). Transfection with MIF siRNA decreased the MFI to 659.78 (SEM = 22.77, $p < 0.01$). Relative to non-target siRNA, this decrease resulted in -34.63% (SEM = 0.68, $p < 0.001$) average expression change with a maximum knockdown efficiency of -43.70% (Figure 5.5C).

Our results are indicative of the constitutive intracellular expression of MIF in MDDC, however these data do not reflect the amount of secreted MIF or its intracellular localisation. Future experiments could include pre-treatment with protein transport inhibitors such as brefeldin A to enhance any staining signal lost through MIF release. However, the release of preformed MIF utilises an ER-independent secretory pathway known to be unaffected by protein transport blockers (Flieger et al., 2003). The efficiency of protein knockdown was slightly lower than what we would have expected based on pre-screen optimisations, but is generally in line with previous findings within our lab (Arrighi et al., 2004). Despite this, these findings would suggest that with further enhancement of knockdown, a more potent restrictive phenotype could be observed.

It is possible that with further siRNA optimisation, use of lentiviral-based shRNA or CRISPR-Cas9 genome editing, a more efficient, stable and longer-term gene knockdown or complete knock-out could be achieved. Full suppression of the cellular actions of MIF could lead to the generation of a more potent enhancement of HIV-1 *trans*-infection during gene downregulation. Taken together, these data confirm the high expression of MIF in MDDC and support its suitability as a target for RNAi.

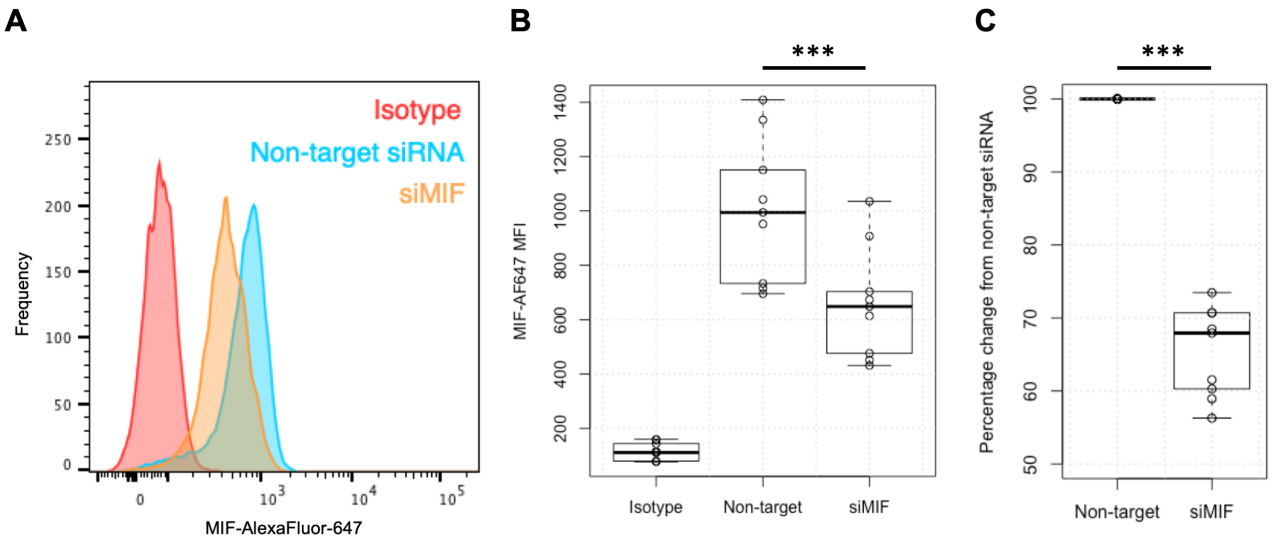

Figure 5.5: MIF expression in MDDC. A) Representative histogram of MIF expression in non-target and siMIF transfected MDDC. B) Collective MIF median fluorescence intensities. C) Relative percentage change in MIF expression from non-target siRNA transfected MDDC. Statistical significance determined by Wilcoxon ranked sums paired test,* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data representative of 3 independent experiments.

5.2.5 MIF neutralisation enhances transfer in *trans* from MDDC to SupT1 and autologous CD4+ T-cells

To test the reproducibility of αMIF neutralisation during DC-to-T-cell *trans*-infection and to confirm these effects on a statistical level, we repeated viral transfer assays during antibody neutralisation in *trans* to autologous CD4+ T-cells and in SupT1 for comparison. In line with our previous findings, αMIF nAb treatment of MDDC-SupT1 transfer show high levels of enhanced viral transfer in both raw and normalised FR+ p24+ readout. Mean raw transfer in isotype-treated controls was 14.83% (SEM = 0.71), increasing on average to 25.85% (SEM = 0.96, $p < 0.05$) during αMIF neutralisation. Following normalisation, this equates to a mean 1.87-fold (SEM = 0.081, $p < 0.001$) increase from baseline viral transfer (Figure 5.6A). In *trans* to autologous T-cells during αMIF treatment, viral transfer was enhanced to 26.09% (SEM = 5.50, $p < 0.05$) from isotype controls (18.95%, SEM = 5.57), resulting in a 1.79-fold ($p < 0.05$) increase in transfer following normalisation (Figure 5.6B).

Here we show that the loss of MIF by antibody neutralisation leads to a strong enhancement of viral transfer from MDDC to SupT1, suggesting that naturally expressed levels of MIF are required for the restriction of HIV-1 transfer. These findings validate the restrictive properties of MIF as previously observed during genetic downregulation with siRNA. In addition, MIF also demonstrates a modest, albeit significant, protection during viral transfer from MDDC to primary autologous CD4+ T-cells. It is not surprising that the potency of MIF-restriction is lower in primary CD4+ T-cells considering that, unlike SupT1, autologous cells may adopt functional antiviral states during DC-CD4 and CD4-CD4 interactions. Alternatively, since MIF is constitutively expressed and released from intracellular pools, it is possible that αMIF nAb may not neutralise these compartments entirely, which could mask any loss-of-function and be a cause for intra-assay variation. Although we are confident these findings are a product of technical challenges, we cannot eliminate the possibility of non-specific antibody binding or the presence of Fc-FcR interaction by autologous CD4 T-cells, which may impair the efficacy of neutralisation or create any undesired functional responses. Our assays did not include the use of any Fc-receptor blocking agents which may help address this, though theses agents are typically reserved exclusively for immunostaining optimisation. Despite early findings suggesting expression of FcγR, FcαR, FcμR and FcϵR on CD4+ T-cells, more recent studies suggest otherwise (Sandor and Lynch, 1993; Takai, 2002; Nimmerjahn and Ravetch, 2008) and this area continues to remain open for debate. In summary, these findings continue to support the role of MIF as a potential restriction factor against HIV-1 *trans*-infection from MDDC. Neutralisation using αMIF nAb recapitulates our previous observations during viral transfer to both SupT1 and autologous CD4+ T-cells. By observing the protective functions of MIF in a system of viral transfer between primary cells, this provides a strong rationale for further investigation of MIF functions in an *ex vivo* environment.

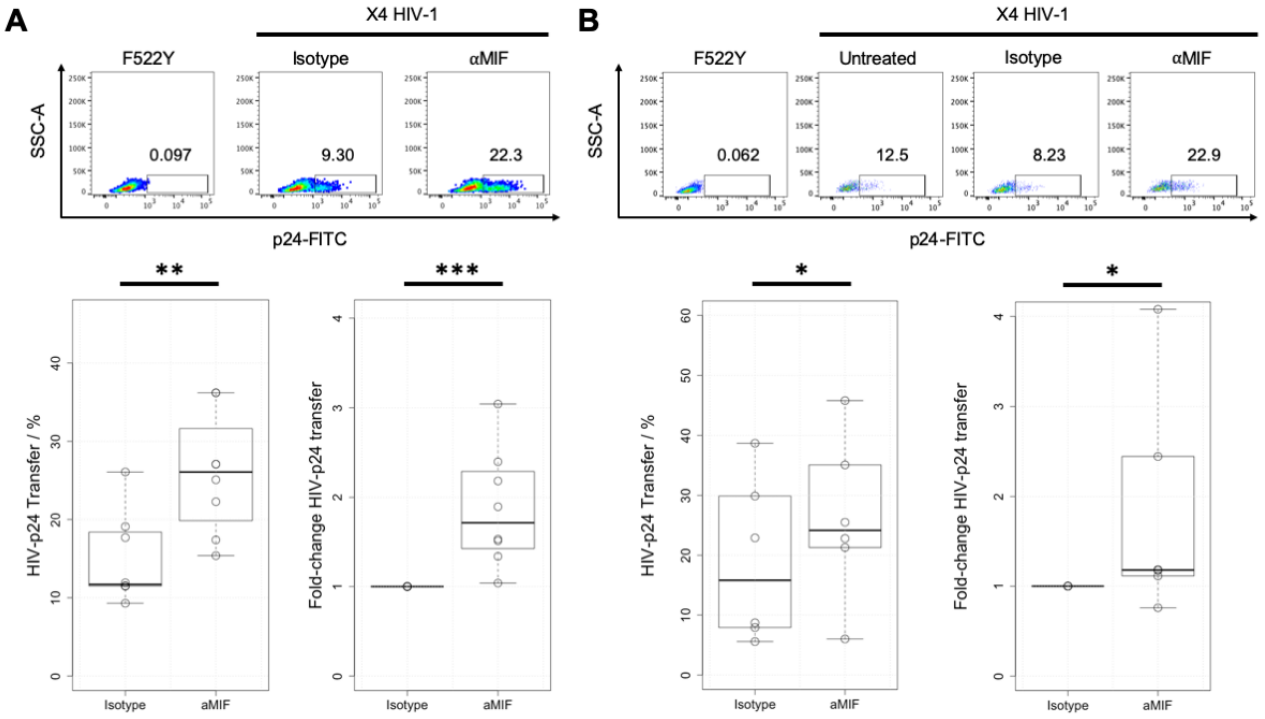

Figure 5.6: MIF neutralisation during HIV-1 transfer to A) SupT1 T-lymphoblastic cells and B) autologous CD4+ T-cells using 1μg/mL αMIF neutralising antibodies (nAb). Wilcoxon ranked sums paired test,* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data representative of 3 independent experiments in A and 2 independent experiments in B.

5.2.6 Chemical inhibition of MIF enhances viral transfer to autologous CD4 T-cells

To address the issues of siRNA knockdown efficiency and variability during neutralisation by nAb as previously described, we used 4-IPP (4-Iodo-6-phenylpyrimidine), a MIF-selective small molecule inhibitor to further validate the role of MIF in HIV-1 transfer. 4-IPP acts by irreversibly binding to N-terminal proline groups on the MIF homotrimer, modifying its protein conformation and blocking CD74/CXCR1/CXCR2-mediated signalling. Due to its low 282.08 Da molecular weight, 4-IPP is cell permeable and is capable of antagonising MIF within intracellular compartments. MDDC were pre-treated overnight (16 hours) with a 50μ M 4-IPP dose was used which has been previously reported to deliver maximal inhibition while maintaining minimal cytotoxicity (Winner et al., 2008; Yaddanapudi et al., 2013). After pre-treatment a

transfer assay was performed as previously described using X4 HIV-1 and 4-IPP was maintained during co-culture for 48 hours before fixation and staining. Raw HIV-1 transfer from DC to T-cells showed large variation between treatments due to donor-variability with a mean percentage transfer of 4-IPP-treated MDDC of 24.66% (SEM = 1.63, p = 0.136) from a mock-treated baseline of 18.69% (SEM = 1.65). However, donor-matched normalisation of 4-IPP vs DMSO treatments showed an average 1.55-fold (SEM = 0.058, $p < 0.001$) increase in transfer to autologous CD4 T-cells (Figure 5.7B). No significant change in cell viability was observed (data not shown).

Since its initial discovery as a key regulator of innate immunity and roles in disease pathogenesis, MIF has become an attractive target for therapeutic development (Riedemann et al., 2003). However, while many studies characterising the in vitro and in vivo effects of MIF are largely supported by use of MIF-specific antibodies, some regard this a difficult approach to delineating mechanisms of its activity (Al-Abed et al., 2005). As a result, a variety of MIF-selective inhibitors have since been synthesised and are now commercially available including ISO-1, often considered as the canonical MIF inhibitor. More recently, 4-IPP has succeeded ISO-1 as a more potent inhibitor of MIF activity (Winner et al., 2008). By using 4-IPP in our assay we are able to ensure a consistent inhibition of MIF at the extracellular space and within intracellular compartments in which MIF is stored. Similar to our previous findings during MIF neutralisation, inhibition by 4-IPP continues to support a restrictive role for MIF in viral transfer. In summary, we have identified MIF as a restrictive candidate and have validated its role in *trans*-infection using three key loss-of-function assays: genetic downregulation by siRNA, neutralisation by nAb biologics and lastly by small-molecule chemical inhibitors.

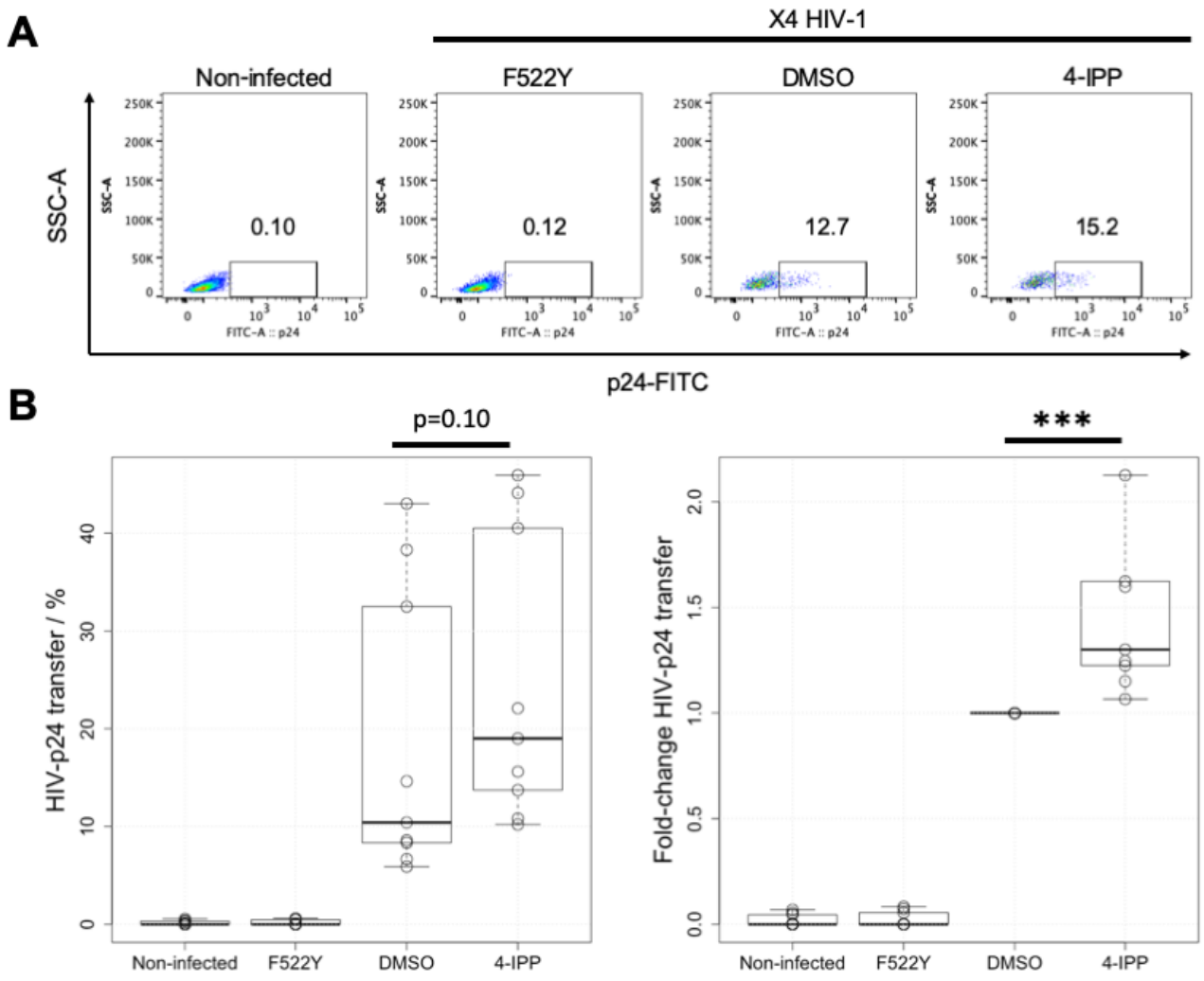

Figure 5.7: Chemical inhibition of MIF by 4-IPP. A) Representative flow cytometry viral transfer readout data of DMSO vs 4-IPP treated MDDC in *trans* to autologous CD4+ T-cells. B) Collective data for raw and donor-normalised fold-change viral transfer. Wilcoxon ranked sums paired test,* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data representative of 3 independent experiments.

5.2.7 Loss of autophagy as a mechanism for MIF-regulated HIV-1 transmission

After searching published literature surrounding the mechanisms of MIF in HIV-1 restriction, we found several reports of MIF involvement in the regulation of autophagy (Chuang et al., 2012; Xu et al., 2016). We were particularly interested in exploring this relationship linking the

cytokine network with autophagic machinery since previous findings within our lab established autophagy as a crucial mechanism of host defence against HIV-1 by MDDC (Blanchet et al., 2010; Chauhan et al., 2015). To test whether MIF is directly involved in regulation of autophagy in MDDC, we treated uninfected cells with 4-IPP and stained for the autophagosome marker LC3α/β isoforms for changes in relative expression. We used two well-known activators of autophagy rapamycin and chloroquine, acting on the initiation and lysosomal fusion stages respectively to maximise the formation and retention of autophagosomes for investigation.

Optimisation of the autophagy-inducing agents were analysed by confocal microscopy to visualise the structure of the autophagic compartment in uninfected MDDC (Figure 5.8A). Our data suggested low levels of autophagy at basal levels, while single-dose rapamycin/chloroquine overnight pre-treatments were able to generate modest induction and strong retention of LC3+ structures, respectively. When used in combination, co-treatment showed strong signal generation, although fluorescence was diffuse, had low signal:noise ratio and did not resemble other samples, which may suggest technical errors during the immunostaining process. One source of diffuse staining could be due to low dye-specificity, since the fluorescent dye used is specific to target multiple LC3 isoforms (Marx, 2015). Pre-conjugated LC3α/β exists in a cytosolic form which could explain any background-like fluorescence, although its localisation in cytosol has previously been described as mostly nuclear or perinuclear.

To quantify changes to the autophagic compartment during MIF inhibition, we pre-treated MDDC overnight with rapamycin/chloroquine in the presence or absence of 4-IPP. After normalisation against the z-score, we observed a strong induction of autophagy to $z = 1.040$ (SEM $= 0.052$, $p < 0.001$) during rapamycin/chloroquine co-treatment against mock-treated MDDC ($z = -0.672$, SEM $= 0.040$). Further addition of 4-IPP to maximally stimulated MDDC was able to significantly reduce LC3 expression ($z = 0.572$, SEM $= 0.045$, $p < 0.05$), suggesting that the induction of LC3+ autophagic structures may be regulated through MIF-dependent processes (Figure 5.8B). However, whilst our quantitative data suggests a role for MIF as an inducer of autophagy, we were unable to effectively resolve autophagosome structures during

confocal microscopy. We therefore hypothesised that non-specific labelling of the dye may be a contributing factor to the lack of resolution and that alternative methods of autophagosome detection using antibody-based approaches should be investigated.

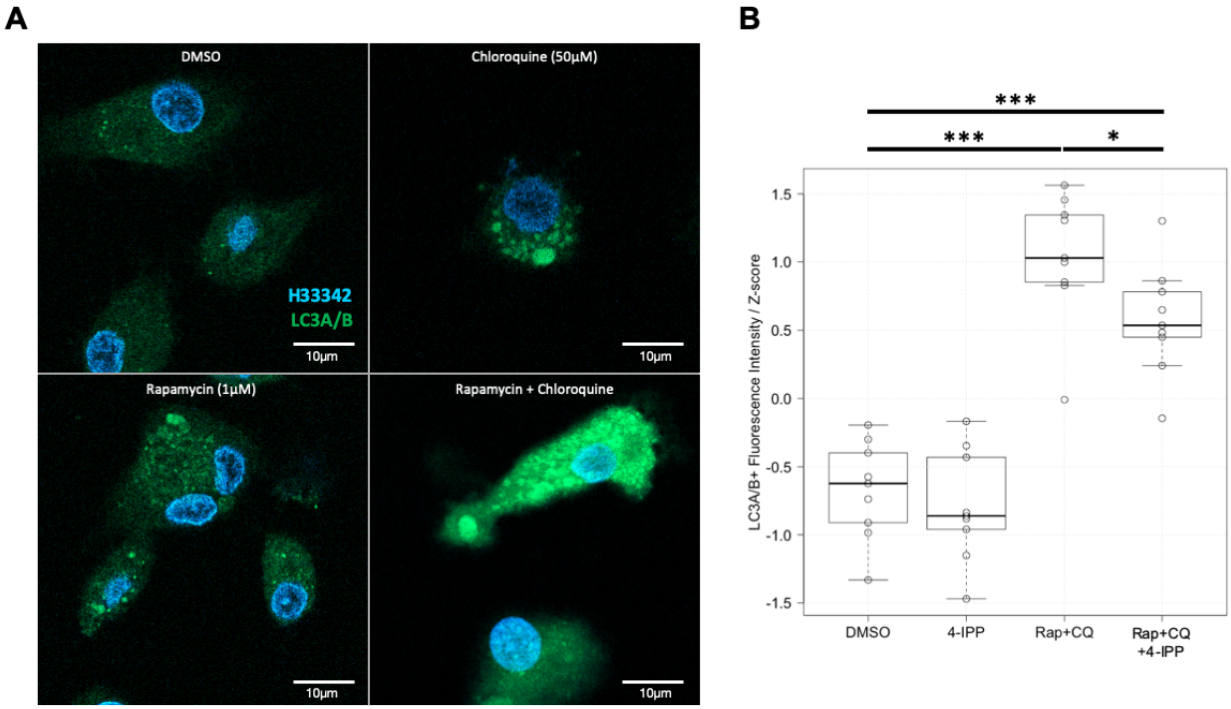

Figure 5.8: Estimation of the autophagic compartment in uninfected MIF-deficient MDDC. A) Optimisation of autophagy control treatments by confocal microscopy. Scale bar represents 10μm. Representative images of 5 fields of view in a single donor. B) Collective microplate fluorescence data for the detection of LC3α/β autophagic compartment in DMSO, rapamycin/chloroquine or 4-IPP treated uninfected MDDC. Data representative of 3 independent experiments. Wilcoxon ranked sums paired test,* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

5.2.8 Confocal microscopy and batch quantification of MIF-dependent autophagosome formation

To visualise the inhibitory effect of 4-IPP during autophagic induction, we used confocal microscopy and analysed the changes in the formation of LC3+ punctae using antibody immunostaining instead of fluorescence dyes. We stimulated autophagy in uninfected MDDC with combined rapamycin and chloroquine treatment overnight (16 hours) in the presence or

absence of 50μM 4-IPP. As expected, basal levels of autophagy were low in DMSO treated MDDC suggesting optimal culture conditions and 4-IPP treatment had no effect on LC3B expression. Unusually, we noticed perinuclear background staining across all samples resembling endoplasmic reticulum (ER) or Golgi apparatus which was not previously detected and present in all samples. However, this may not be surprising since the ER-Golgi intermediate compartment (ERGIC) has been identified as a membrane source for autophagosome biogenesis (Ge et al., 2013). Combined treatment with rapamycin/chloroquine results in a strong induction of autophagosome formation with punctate LC3B staining equally distributed throughout the cell cytoplasm. Interestingly, the addition of 4-IPP to rapamycin/chloroquine treated MDDC showed a considerable visual reduction in LC3B staining comparable to mock-treated controls suggesting impaired autophagosome formation in the absence of MIF (Figure 5.9A). Using ImageJ in-built functions, we created macros to perform iterative quantification across an archive of microscopy images through pre-processing and using the particle analysis feature to generate summary statistics including number, size of autophagic spots and nuclei count. Further details of batch analysis macros can be found in appendix A.2. At basal levels, image quantification across two donors (5 fields of view per donor) detected an average 5.086 (SEM = 0.222) and 6.809 (SEM = 0.226) spots per cell in DMSO and 4-IPP treated MDDC, respectively. During concomitant rapamycin/chloroquine treatment, we observe an increase in average spot per cell of 13.499 (SEM = 0.670, $p < 0.001$). Interestingly, the addition of 4-IPP counteracted spot formation by rapamycin/chloroquine to 6.385 average spots (SEM = 0.163, $p < 0.01$), reducing in levels of autophagy statistically comparable to mock DMSO treatment (Figure 5.9B).

Taken together, our data support the notion that LC3B+ autophagosome formation is a MIF-dependent process in MDDC. However, whether the loss of MIF is associated with failure to induce formation or enhanced clearance of autophagosomes is yet to be determined. As previously mentioned, our lab has described protective roles for autophagy against HIV-1 infection in MDDC by enhancing pathogen degradation, antigen recognition and presentation (Blanchet et al., 2010). These findings contribute to a growing body of evidence suggesting that cytokines are critical mediators of autophagy regulation (Harris, 2011).

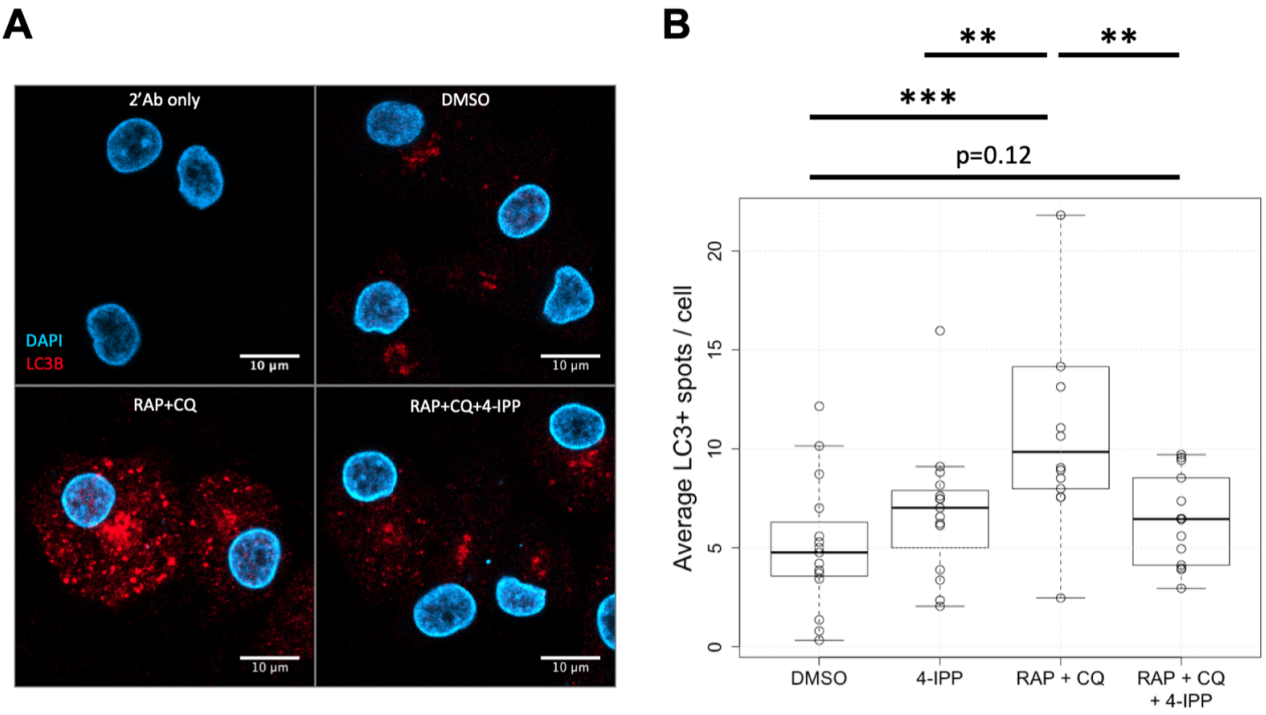

Figure 5.9: Confocal microscopy of LC3β+ autophagosome expression. A) Uninfected MDDC pre-treated with DMSO or rapamycin/chloroquine ± 4-IPP. Scale bar represents 10μm. B) Batch quantification of average number of LC3+ spots per cell. Dunn's test of multiple comparisons using rank sums, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data representative of 2 independent experiments.

5.2.9 Inhibition of MIF is associated with intracellular accumulation of HIV-1

Having characterised a role for MIF in enhancing autophagosome formation in MDDC in the absence of infection, we next investigated this relationship during HIV-1 *cis*-infection to determine whether the loss of autophagosome formation is linked with impaired intracellular clearance of virus by autophagy. Here, we pre-treated MDDC with DMSO, 50 μM 4-IPP or 1μM rapamycin overnight (16 hours) before challenging with R5-tropic HIV-1 spinoculation, fixing immediately after infection and analysing intracellular LC3A and HIV-1 p24 by flow cytometry (Figure 5.10A). Since infection with X4 HIV-1 is generally trafficked through pathways associated with non-productive infection, we used an R5-tropic HIV-1 strain to maximise productive infection

of MDDC and investigate the clearance of cytosolic p24 by canonical autophagy.

Compared to DMSO treated MDDC, our results showed that 4-IPP treatment was able to reduce LC3 expression in line with our prior results and was associated with enhanced intracellular HIV-1 p24-gag staining in 4-IPP treated MDDC (Figure 5.10B). Rapamycin treatment only showed modest upregulation of LC3 with respect to DMSO-treated MDDC. However, increased LC3 expression showed trends of decreasing p24-gag expression from DMSO (2091 MFI, SEM = 48.192) during rapamycin treatment (1991 MFI, SEM = 15.692), which would be in agreement with previous observations suggesting that LC3-dependent mechanisms are required for the clearance of intracellular virus Blanchet et al. (2010). In addition, we observed higher levels of cell death ranging between 20–30% compared to previous infection assays using X4 HIV-1.

Our findings may suggest that loss of MIF by 4-IPP results in loss of LC3 autophagosomes leading to intracellular HIV-1 accumulation due to impaired clearance. Such a hypothesis would be in agreement with previous findings by Blanchet *et al.* and support the notion that impaired or exhaustion of autophagy leads to DCs bearing greater quantities of intact HIV-1 p24-gag which are likely to be more successful of *trans*-infection to CD4+ T-cells. However, in the same study HIV-1 has also been shown to subvert this mechanism of host defence through HIV-1 Env-mediated activation of mTOR resulting in autophagy exhaustion. Downregulation occurs rapidly and loss of LC3-II could be observed as early as 1 hour although this does not reach maximal effect until 10 hours post-infection and sustained up to 20 hours after exposure (Blanchet et al., 2010). Based on these data, we suggest that MIF positively regulates the initiation of autophagosome formation necessary for the degradation of internalised virus.

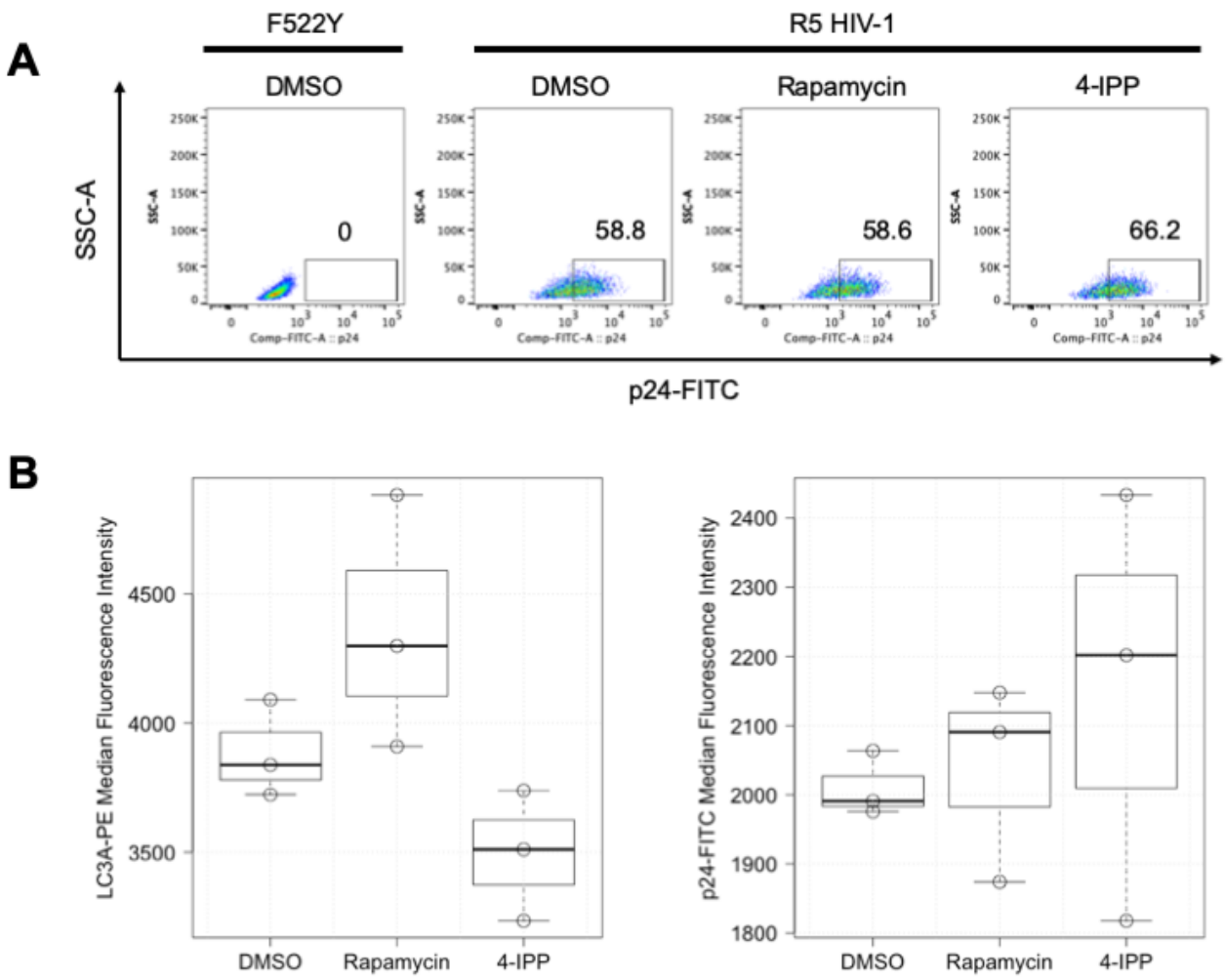

Figure 5.10: Relationship between loss of autophagy and intracellular p24 accumulation. A) Representative flow cytometry data of R5 HIV-1 *cis*-infected MDDC pre-treated overnight with DMSO, rapamycin (1 μM) or 4-IPP (50 μM). B) Collective LC3A and p24 median fluorescence intensity values across 3 replicate samples in 1 donor. Preliminary data, not tested for significance.

5.3 Discussion

It is clear that cytokines and chemokines orchestrate many biological processes that are critical for fine-tuning innate and adaptive immune responses by invasive pathogens. During acute HIV-1 infection, the cytokine network is dysregulated which provides virus a window of opportunity to disseminate before a delayed immune response can be initiated. Dysregulation continues

throughout the course of disease into chronic infection, where persistent immune activation and inflammation contributes to CD4+ T-cell depletion and disease progression (Paiardini and Müller-Trutwin, 2013). Intrinsic immunity by restriction factors is an effective mechanism of host defence against intracellular pathogens; since many of these factors are driven by cytokines including type I IFNs, there is a great scope for further identification of novel factors which enhance viral restriction.

Here we have validated a selection of antiviral genes and progressively refined our candidate list to MIF, 4-1BBL and IL-8. Downstream validation assays are intended to test the reproducibility of gene candidates in experimental conditions which closely reflect those of the original siRNA screen. Our library contained pools of 4 siRNA sequences per gene which is typical in most large-scale knockdown studies, increasing the likelihood of on-target specificity and reducing potential false positives hits. Therefore, in order to establish active sequences involved in the assay pool, deconvolution is typically used to identify the proportion of functional siRNA and pools containing >50% target knockdown can be progressed as valid candidates. Since our library is targeted to secretory proteins, we were able to bypass primary validation using pool deconvolution, to mimic loss-of-function using nAb and chemical inhibitors in place of siRNA.

In our validation assays, we explored the role of candidate genes in *trans*-infection from DCs to autologous CD4+ T-cells. Repeat siRNA trials of refined candidates showed that downregulation of MIF/IL-8, but not 4-1BBL, was able to recapitulate restrictive properties observed in transfer to SupT1. It is likely that signalling through the T-cell receptor (TCR) in autologous CD4+ T-cells may be involved in this discrepancy since the TCR is able to cross-link the 4-1BB receptor in T-cells, which signal synergistically to enhance IFN-γ/IL-2 production and cell survival/proliferation independently of CD28 (Wen et al., 2002; Nam et al., 2005). As a cell line not expressing the CD3-TCR, SupT1 lack the ability to mount functional responses and, in addition to the over-expression of CD4 which is 62% higher than peripheral blood lymphocytes (Cortés et al., 2002), are more permissive to infection than autologous cells which is reflected

in our changes to the cell model. Similar findings were observed during αMIF neutralisation, which although showed strong restrictive potential of HIV-1 from DC to SupT1, was only modest in autologous CD4+ T-cells. However, we proposed that nAb may be effective only against secreted proteins and may not neutralise the intracellular compartments where preformed MIF is stored. Using a membrane-permeable MIF-selective small-molecule inhibitor, we were able to address this issue to confirm that MIF conveys antiviral protection in *trans*- from DC to SupT1 and primary autologous CD4+ T-cells .

Autophagy is an evolutionarily conserved feature of cellular homeostasis with crucial functions in health and disease. While autophagy was first considered to be initiated solely by the intracellular metabolic sensors AMPK and mTOR, emerging findings now support a significant overlap between autophagy and the cytokine network. Several cytokines have been identified including IFN-γ, TNF-α, IL-1α/β, IL-2, IL-6 and TGF-β which enhance autophagy and negatively regulated by IL-4, IL-10 and IL-13 (Harris, 2011, 2013). Our results establish a common link between MIF and autophagy, adding to growing evidence which suggests roles for MIF in the regulation of autophagic machinery through the promotion of autophagosome formation. However, the molecular mechanisms underpinning its functions are not known and whether MIF interacts directly with autophagy regulators or indirectly via MIF-products remains to be determined. In line with this, one study by Xu and colleagues (Xu et al., 2016) found that MIF directly regulates the activity of RhoA GTPases and Rho-associated coiled-coil-containing protein kinase 1 (ROCK1) signalling cascades and are required for the formation of autophagosomes under metabolic stress (Gurkar et al., 2013). ROCK1 and other serine/threonine protein kinases are important in the regulation of actin dynamics, subcellular organisation of organelles and cell polarity, which are well recognised in facilitating the formation and transport of autophagosomes and its cargo (Kast and Dominguez, 2017). In addition, several studies have now identified mitochondrial reactive-oxygen species (ROS) as a product of MIF-CD74 interaction and are also required for autophagosome formation (Chuang et al., 2012; Lee et al., 2016) through binding with the autophagy-related protease Atg4 regulating the LC3 conjugation pathway (Azad et al., 2009).

While our results did not fully explore the molecular interactions of MIF signal transduction, our findings are in agreement with the existing literature which strongly suggest that autophagosome formation is a MIF-dependent process (Xu et al., 2016). Here we used two methods for autophagosome detection in DCs, firstly using fluorescence plate-reader assays and secondly by immunofluorescence confocal microscopy to investigate MIF-dependent regulation of autophagic machinery. We used a validated CYTO-ID^{TM} autophagy detection kit (Enzo) containing a proprietary GFP-labelled dye specific for autophagosome formation. Whilst microplate-reader assays offer many advantages for rapid analysis, they are still faced with a number of technical challenges which can introduce intra-assay variabilities. 1) Maintaining consistent cell seeding densities between samples is one source but was addressed using a homogenised master mix of cells was used. 2) In semi-adherent cell types such as DCs, cellular attachment is a factor which is more difficult to control and was assumed to be consistent. 3) Drug toxicity may impact cell viability and autofluorescence risks being detected in the GFP channel, however flow cytometry data suggests that the drug treatments used had minimal affect on DC viability. 4) Lastly, LC3-GFP signal generation is dye-based and known to be affected by exocytosis and transmembrane leakage (Heusinkveld and Westerink, 2011). DCs have highly active vesicular transport compartments, reflected by their functions as APCs and are likely subject to these issues.

The hallmark feature of autophagy induction is the formation of LC3+ autophagosomes, which are characterised by punctate staining patterns distributed evenly within the cytoplasm. While measuring fluorescence may give an indication of total protein, it does not generate insight into morphological changes and protein localisation which defines autophagy and therefore used confocal microscopy to address these technical issues. However, when using the CYTO-ID^{TM} detection kit we were unable to effectively resolve these structures and overall experienced poor signal generation with diffuse and irregular staining patterns which became overexposed in positive controls. After optimising our immunostaining protocol using αLC3B, we were able to clearly resolve the structure of LC3+ autophagosomes. High resolution confocal micrographs are well suited for automated image quantification, which allowed us to develop ImageJ macro

scripts (detailed in appendix A.2) to batch process MDDC (nuclei) count, LC3 spot count and size per field of view (FOV) and calculate the average number of autophagic spots in per cell. In our results, we clearly demonstrate that MIF is a central component in autophagy, which when blocked fails to promote the formation of autophagosomes. In the majority of cells analysed, we were able to detect a perinuclear aggregation of LC3B resembling the ER and Golgi apparatus. Particle analysis functions allow size discrimination of particles and was able to exclude these organelle and larger particles from spot count analysis. Together, these techniques demonstrate an important application of batch image quantification in the measure of autophagy.

Using the techniques outlined above, we found that the inhibition of MIF by 4-IPP was able to completely block the formation of LC3+ puncta under pharmacological induction of autophagy. Since it has already been established that autophagy contributes to host defence in infectious disease and pathogen endocytosis (Blanchet et al., 2010; Chauhan et al., 2015; Deretic et al., 2015), we therefore hypothesised that MIF could be a central element in the innate immune response against HIV-1. Currently there is no known connection between MIF-mediated autophagy and HIV-1 infection; therefore, we investigated whether the loss of MIF could alter the intracellular accumulation of virus during *cis*-infection. Indeed, our preliminary findings supported the hypothesis that in the absence of MIF, autophagosome formation in DCs is impaired and exhaustion leads to an inability to clear internalised virus. Intracellular HIV-1 subsequently accumulates and provides DCs with a greater capacity for successful *trans*-infection during co-culture with target CD4+ T-cells.

5.4 Concluding remarks

In summary, this chapter has covered the process of candidate validation which aims to test the reproducibility of screen candidates. Here we eliminated weaker hits and refined our candidate list to MIF, TNF and IL-8, highlighting a potential axis which could act to restrict viral transfer. As our strongest candidate, we focused our efforts on understanding the role of

MIF and retested this candidate using three loss-of-function assays: 1) Genetic downregulation by siRNA. 2) Neutralisation by antibodies. 3) Pharmacological inhibition by small-molecule compounds. MIF was able to consistently reproduce a restrictive phenotype of HIV-1 infection from DC to CD4+ T cells among all assays.

We investigated this further to understand the cellular mechanisms of MIF-mediated HIV-1 restriction. Indeed, our findings supported an essential role for MIF in the formation of autophagosomes which are required for the clearance of intracellular pathogens. Together, our findings demonstrate a clear protective role for MIF during early-stage HIV-1 infection through the regulation of autophagic machinery. However further work is needed to elucidate the signalling events which link MIF and upstream inducers of autophagy.

Chapter 6

General discussion

The aims of this thesis are to investigate the role of the cytokine and chemokine network in the control of HIV-1 *trans*-infection from DCs to CD4+ T-cells using a high-throughput RNAi screen. In this chapter we will discuss HIV-1 screening as a strategy for target identification, the MIF-TNF-IL-8 axis identified in our study and the mechanisms of MIF-dependent autophagosome formation in the restriction of HIV-1 in DCs.

6.1 High-throughput screening approaches for the identification of novel HIV-1 targets

With the development of high-throughput technologies, generated datasets are becoming larger which poses a challenging obstacle for researchers in translating readouts into meaningful insights. Functional genomics screening has led to remarkable discoveries among many areas of research and can be performed on a genome-wide scale or targeted to specific pathways or functions depending on the nature of the biological question. Genome-wide screens target $\sim$ 21,000 proteins coded by the human genome and are effective for identifying a network of all possible regulators within a particular biological process (Sharma and Rao, 2009). Currently a handful of genome-wide screens on HIV-1 infection have been performed (Brass et al., 2008;

König et al., 2008; Zhou et al., 2008; Yeung et al., 2009; Park et al., 2017) although there is a surprisingly low level of overlap in target hits observed between screens. This is likely to be due to several factors of experiments design including different cell types (HeLa vs HEK293T), screening technologies used (siRNA, shRNA, CRISPR, cDNA), different assay readouts (fluorescence reporter, antibody staining, microscopy) and culture conditions (HIV-1 strain, MOI, incubation period, post-infection culture). One limitation of genome level interference screens is that they are often restricted to use in immortalised cell lines, which although are robust in their ability to withstand toxicity and homogenous phenotype, do not provide a representative cell type for modelling of biological functions. In contrast, arrayed pathway-targeted screening approaches aim to identify specific proteins that have not previously been characterised within a known biological mechanism or pathway. Systematic screens have practical advantages over whole-genome screens for reasons of cost, greater flexibility (more readout options and multiplexing) and efficiency. Several targeted screens have successfully led to the identification of many important anti-viral genes and restriction factors including APOBEC3G and tetherin (Jarmuz et al., 2002; Neil et al., 2008; Lee et al., 2010c; Ménager and Littman, 2016). In terms of RNAi screening, there is currently no targeted screen for cytokine and chemokine function on HIV-1 and remains a novel area for further investigation, particularly during *trans*-infection from DC to CD4+ T-cells.

RNA interference and controlled gene expression methods have made expeditious development in the past couple of decades which have seen production of large-scale knockdown/knockout screens using siRNA, short-hairpin RNA (shRNA) and now genome-wide CRISPR/Cas9 editing. In practice, there are several considerations that should be made in deciding upon the right platform to best suit the experimental design and resource constraints.

Since siRNA and shRNA exist as distinct molecules, they therefore have different mechanisms of action of RNAi. The delivery of exogenous siRNA is relatively straightforward, relying on liposome-associated uptake (occasionally using electroporation) by endosomes for direct entry into cytosol, where siRNA-RISC-AGO2 complex can form. On the other hand, shRNA are

naturally synthesised within the nucleus before export into cytosol where they are processed to become functional. Exogenous shRNA are therefore virally encoded and require vector-based delivery which must first gain entry into the nucleus in order for transcription of pre-shRNA to take place (Cejka et al., 2006). Theoretically, the mechanisms of action for siRNA and shRNA converge at the point of AGO2-RISC complex formation and are assumed identical beyond this point, although both methods have unique limitations associated with their delivery, processing and target specificity (Rao et al., 2009).

Using siRNA, effective knockdown in cell lines can be achieved at low nanomolar concentrations, which peaks between 48–72 hours after treatment and can be suppressed in 5–7 days in actively dividing cells which is well within the time-frame of our experimental assays. However, the delivery of nucleic acids to primary cells such as DCs with highly active endolysosomal machinery is notoriously challenging. Since siRNA loading is approximately 10 times as inefficient and are highly degraded on entry compared with shRNA (Cejka et al., 2006), DCs therefore require multiple transfections of high concentration siRNA which has potential to drive cellular responses. Our results have already highlighted several pitfalls of using siRNA as a platform for RNAi, suggesting that DCs undergo stress-induced semi-maturation, which varies between donors and alters the dynamics of viral capture and transfer in our system. However, with careful optimisation and use of widely accepted data handling techniques, we are able to mitigate or avoid entirely off-target effects induced by siRNA.

Viruses including retrovirus, herpes simplex virus, adenovirus and adeno-associated viruses have been widely studied in gene therapy and as a vectors for stable transduction of shRNA which have yielded high levels of loading and transient gene expression. But despite this, they are not entirely exempt from immunosurveillance or off-target effects and just as viruses have evolved to successfully infect cells, so too has host immunity against intracellular pathogens. Species-specific immune responses to viral vectors have been reported including TLR9 DNA sensing, inflammatory cytokine production (type I/II IFNs, IL-1β, IL-6, IL-12), initiation of complement cascades and activation of DCs, pDC and NK cells which limits the delivery of

nucleic acids both *in vitro* and *in vivo* (Robbins and Ghivizzani, 1998; Thomas et al., 2003; Nayak and Herzog, 2010).

In DCs, HIV-1 transfer is best studied using RNAi or genetic knockout since drug compound screening may elicit non-specific effects (Lehmann et al., 2011). Based on practicality we believe a pathway targetted siRNA screen was the ideal platform for the discovery of novel cytokines and chemokines. Taken together, the use of shRNA is perhaps better suited for follow-up experiments rather than for RNAi screening, since they are more stable during transfection and provide longer-term gene knockdown which is not strictly necessary given the short 48 hour time frame of the assay co-culture. Genome editing is now becoming widely practised among loss-of-function studies; the CRISPR-Cas9 system selectivity targets gene-encoding DNA sites via Cas9 nucleases in complex with CRISPR/*trans*-activating RNA. Following DNA recognition and cleavage, double strand break repair machinery acts to rejoin strands using non-homologous end joining or homology directed repair pathways, leading to indels or specific mutations/knock-ins to disrupt the target gene sequence, respectively. Whilst genome-editing results in the complete ablation of expression on the RNA and protein level, there are several limitations which prevent its widespread use. Firstly, genome-editing is still not void of off-target effects since Cas9 has been reported to cut at the wrong cleavage sites. In addition, primary cells still remain highly resistant to the transfection of foreign nucleic acids, particularly in terminally differentiated DCs. However, new approaches for efficient delivery in primary cells using virus-like particles are now being developed for the efficient delivery of Cas9-sgRNAs to primary cells, which could have promising application within our cell system (Mangeot et al., 2019).

6.2 Review of the MIF-TNF-IL-8 axis in HIV-1 *trans*-infection

Results from our screen and validation revealed compelling evidence for the role of MIF, TNFSF and IL-8 in the restriction of HIV-1 *trans*-infection. Here, we propose a mechanism for these

candidates in the regulation of innate immunity against infection.

6.2.1 MIF

MIF is a multi-faceted, pleiotropic cytokine-mediator with a broad range of functions across many areas of innate and adaptive immunity. Our findings demonstrate a potent suppressive role for MIF in the restriction of HIV-1 *trans*-infection, although little is known about its function in response to viral infection. Several reports have already established an association between MIF and infection by a broad range of viral species, suggesting both protective and detrimental roles in viral pathogenesis. For instance, MIF has been shown to contribute to the pathophysiology of dengue virus infections *in vivo*, suggesting that MIF alters vascular permeability allowing virus to penetrate physiological blood barriers and access tissue compartments that may otherwise be immunologically privileged (Assunção-Miranda et al., 2010; Chuang et al., 2011). On the other hand, MIF has shown to induce protective functions in cytomegalovirus infection, demonstrating deficient production and impaired lymphocyte proliferative activity in infants with congenital CMV infection (Gehrz and Leonard, 1985).

In the context of HIV-1, elevated plasma levels of MIF have previously been reported in infected individuals, driven in part by virally-enhanced MIF release by PBMC cultures (Regis et al., 2010). The authors describe HIV-1 transcriptional promotion at the LTR by MIF in PBMC and CD4+ T-cells, although the study did not describe its role within innate immune cells and lacked an isolated study in monocyte, macrophage and DC cultures which are mostly non-permissive to infection. Since these cells have distinct and contrasting roles to lymphocytes in human immunity, it is highly likely MIF may have differential functions in the response to HIV-1.

For several decades, the primary MIF receptor had eluded researchers; prior to the identification of CD74 by Leng and colleagues (Leng et al., 2003), MIF was most often associated with its ability to regulate cell mobility through the utilisation of CXC chemokine receptor CXCR2 due to its remarkable structural homology with IL-8 (CXCL8) (Bernhagen et al., 2007). More

recently, MIF the roles of allosteric binding and partial agonism of CXCR4 have been further characterised, although little to no effect on HIV-1 entry as a result of low affinity binding with the co-receptor have been observed (Rajasekaran et al., 2016). Given that MIF modulates and signals through chemokine receptors and shown to block the random migration of macrophages *in vitro* and *in vivo*, it is plausible that MIF may also alter DC migration, which may be necessary for the movement toward target cells and the synapse formation required for HIV-1 transfer. Although the role of MIF in DC migration was not tested in our hands, it is apparent that signalling through its cognate receptor CD74 is a key factor in the regulation of cell migration. In a study by Faure-André and colleagues (Faure-André et al., 2008) linking the antigen-presenting machinery with migration, the authors found that CD74-deficient DCs showed faster and more uniform patterns of migration, compared to DCs over-expressing CD74 which had slower migration than wild-type DC.

MIF can regulate cytokine expression in DCs through a number of pathways including TLR4, ERK, MAPK and JNK resulting in enhanced production of TNF, IL-1, IL-6 and IL-8 production (Calandra and Roger, 2003; Murakami et al., 2002) which are known to restrict HIV-1 infection through the generation of an antiviral pro-inflammatory environment. However, a further role MIF has recently been described in inflammasome assembly, suggesting that the release of IL-1α, IL-1β and regulation of IL-18 by MIF is not mediated through transcriptional changes but instead through activation of NLRP3, a key component of the inflammasome complex (Lang et al., 2018). Inflammasomes are cytoslic PAMP/DAMP sensors which trigger highly pro-inflammatory signalling leading to caspase-1-dependent pyroptotic termination of the host cell. Assembly of the inflammasome is a two-step process which is first primed by a sensing signal from PRRs such as TLRs or TNFRs to initiate pro-IL-1β and NLRP3 transcription. The priming step licences inflammasome formation which occurs following NLRP3 activation in response to by viral RNA, bacteria or fungal PAMPS and endogenous DAMPs (Swanson et al., 2019). Activated NLRP3 further activates caspase-1 to drive oligomerisation and complex formation to initiate cytokine release and pyroptosis.

Interestingly in a cohort study of inflammasome activation in MDDC derived from healthy and HIV-1 infected individuals, inflammasome observation was only observed in the control group and not MDDC from HIV-1 infected patients, suggesting that this could represent an early mechanisms of defence through DC activation and priming T-cell effector function (Pontillo et al., 2012b). Additionally, identification of inflammasome gene polymorphisms for *NLRP1*, *NLRP3*, *NLRC4*, *CARD8*, *CASP1* and *IL1B* were associated with HIV-1 acquisition (Pontillo et al., 2012a). On the contrary, there are conflicting reports that HIV-1 can activate NLRP3 via TLR8 sensing in monocytes and macrophages (Chattergoon et al., 2014), although as an endosomal sensor of viral ssRNA, it does not reflect the mechanisms of non-productive infection that occur in DCs. The discrepancy between DCs between patient groups in the former study by Pontillo and colleagues (Pontillo et al., 2012b) could suggest that HIV-1 exhausts or subverts inflammasome activation following established infection. It is possible that MDDC exposed to HIV-1 may lead to MIF-dependent NLRP3 activation and restriction of virus. In the context of *trans*-infection, the loss of MIF may ablate the NLRP3 sensing machinery, enabling evasion of inflammasome activation, enhanced cell survival and thus promoting viral transfer. However, the molecular interactions between MIF and NLRP3 are still not clear; further investigation into immune escape of NLRP3 activation by HIV-1 and whether MIF is involved in blocking evasion could be of considerable interest for the development of MIF-based therapeutics.

6.2.2 TNFSF: CD70/4-1BBL/TWEAK

CD70 (TNFSF7) – CD70 was detected as a candidate in our final selection, showing moderate restrictive potential during viral transfer to SupT1 cells, but showed high levels of variability during downstream validation. However, CD70-expressing DCs have a central role in orchestrating the transition between the innate and adaptive immune systems and in determining the immunogenicity of effector cells. In the context of viral infection, mixed roles for CD70-CD27 signalling in HIV-1 pathogenesis have been described. During acute HIV-1 infection, the inflammatory and antigen-rich environment causes CD70 expression to increase in CD4+/CD8+

T-cells (Wolthers et al., 1996). The upregulation of CD70 and interaction with CD27 may contribute to the mechanisms of T-cell homeostasis against rapid CD4+ loss and rising viral load. However, T-cells from HIV-1 infected individuals lose CD27 expression (Meyaard et al., 1995) and plasma levels of sCD27 are associated with viral load and negatively correlate with CD4 T-cell count, which continue to show paralleled kinetics during effective management by and interruption of ART (Messele et al., 2001; Milito et al., 2002). In agreement with this, recombinant sCD27 has been reported to block CD70-mediated signalling and inhibit T-cell proliferation *in vitro* (Agematsu et al., 1994; Matter et al., 2006). It is therefore possible that CD27 cleavage may be an evasion strategy required for interruption of normal CD70-CD27 signalling and progressive CD4+ T-cell depletion. While is unclear if sCD27 is capable of reverse signalling during CD70 interaction, sCD27 may be involved in hyperactivation of the immune system and contribution to T-cell exhaustion and depletion. Such interaction has been shown to further enhance CD70 expression in T-cells (Huang et al., 2013), supporting a positive feedback loop of persistent immune activation. In addition, chronic immune activation is associated with disease progression as a result of continuous differentiation and activation of the naive T-cell pool. (Hazenberg et al., 2003). Studies of CD70 overexpression in transgenic mice appear to show an immune profile resembling hallmark features of HIV-1 immunodeficiency including high T-cell turnover, exhaustion, loss of naive CD4+/CD8+ T-cell pools (Tesselaar et al., 2003a). In our system, MDDC lacking function CD70-expression may not be able to efficiently prime CD4+ T-cells toward anti-viral Th1 effector states, which could explain an increase in viral transfer. However, SupT1 cells are not known to become activated or assume effector cell functions, suggesting that CD70 may restrict HIV-1 through receptor-independent mechanisms in the MDDC. Given that CD70 is involve in the licensing of DC immunogenicity and contributes to the pro-inflammatory environment generated by DCs (Van Deusen et al., 2010), it is possible that these mechanisms may help restrict HIV-1 *trans*-infection upon capture by the MDDC via the PRR DC-SIGN.

It is well-known that DCs and their subsets are crucial in mediating antiviral immunity (Steinman and Hemmi, 2006). Important roles for CD70 in cross-priming of CD8+ T-cell responses by

DCs and LCs have emerged, highlighting its protective roles against viral infections and also in the generation of DC-led tumour-specific immunity (Bullock and Yagita, 2005; Ballesteros-Tato et al., 2010; van der Aar et al., 2011; Polak et al., 2012). The majority of HIV-specific CD8+ T-cell populations are of a CD27 expressing memory phenotype, which lose CD27 expression during further differentiation into an effector phenotype (Lalvani et al., 1997; Rosenberg et al., 1997; Ogg et al., 1999; van Baarle et al., 2002a). Unlike CD27+ memory T-cells, CD27- effector memory CD8+ T-cells gain cytolytic activity during differentiation through the expression of perforin and granzyme A. However, failure to differentiate and persistent CD27 expression in HIV-specific cells is associated with a lack of perforin expression in CD8+ T-cells (Appay et al., 2000, 2002). In addition to the induction of T-cell immunity, (resting) DCs are also tolerogenic and can induce peripheral CD8+ T-cell tolerance through PD-1 and CTLA-4 signalling leading to anergy and deletion (Belz et al., 2002; Probst et al., 2003, 2005). In models of transgenic CD70 expression in murine resting DCs, CD27-CD70 interaction was able to prime virus-specific responses in CD8+ T-cells overriding PD-1/CTLA-4 tolerogenic signals (Keller et al., 2008). This preferential priming could contribute to the maintenance of virus-specific CD8+ T-cell pool required to control infection. In support of this, findings by Van Baarle and colleagues suggest that HIV-1 elite controllers (long-term infected and asymptomatic) have a higher CD27- HIV-specific CD8+ T-cell compartments compared to HIV-1 infected individuals who progressed to AIDS (van Baarle et al., 2002b,a), suggesting a protective role for CD27 signalling and receptor shedding in CD8+ T-cells. Given that the intensity and onset of HIV-specific CD8+ T-cell responses during acute HIV-1 infection are pivotal in establishing viral set point (Ndhlovu et al., 2015), there is clear evidence for CD70-CD27 interaction and CD8+ T-cell differentiation in the control of acute HIV-1 infection.

Perhaps more relevant to our cellular model, DC-CD4+ interactions utilise CD70/CD27 signalling at the immunological synapse, necessary for cell cycle entry and proliferation of CD4+ T-cells. Naive CD4+ T-cells from HIV-1 infected individuals fail to adequately upregulate surface expression of costimulatory molecules CD27/CD28, and undergo cell cycle arrest as a result (Luciano et al., 2007). Since CD27 expression is driven by TCR engagement (Hintzen

et al., 1993), DCs can prime CD4+ T-cells triggering differentiation into IFN-γ producing Th1 effector cells through both CD27 and sensitisation to Th1-inducing cytokines including IL-12 (Van Oosterwijk et al., 2007). The Th1 effector response is a restrictor of HIV-1 infection and imbalances in this response are associated with progression to AIDS (Clerici and Shearer, 1993, 1994). However, despite the ability for Th1 responses to produce effective antiviral control during early stage infection, CD70-CD27 signalling is offset by detrimental effects to the humoural response by B-cells (Nolte and van Lier, 2006).

While CD27 has predominantly been associated with T-cells, its expression on B-cells is up-regulated following antigen stimulation and is used as a marker of memory B-cell and plasma cell populations (Agematsu et al., 2000). During HIV-1 infection, CD27+ memory B-cell count is significantly reduced accompanied with plasmacytosis. While the loss of CD27 may be attributed mostly to receptor cleavage, *in vitro* studies suggest that B-cells may not be the main cellular source of sCD27 (Widney et al., 1999). Instead, abnormal differentiation of B-cells into plasma cells is a likely mechanism which is in accordance with observations of hypergammaglobulinemia (increased serum Ig levels) in infected individuals (Nagase et al., 2001). B-cells are required for the maintenance of the splenic marginal zone and overexpression of CD70 in transgenic mice led to chronic CD27 activation, B-cell depletion and ultimately destruction of secondary lymphoid tissues (Arens et al., 2001; Nolte et al., 2004). Similar observations have been found in post-mortem spleens of HIV-1 infected individuals, which showed progressive histological changes with advanced HIV-1 infection (Wilkins et al., 2003). In addition, IFN-γ produced by Th1 effector cells has directly impacts the development of B-cell progenitors and block IL-7 mediated functions leading to induction of apoptotic pathways (Grawunder et al., 1993; Garvy and Riley, 1994).

4-1BBL (TNFSF9) – Our screen data highlighted 4-1BBL as another restrictive candidate against HIV-1 transfer to SupT1 cells. Downstream validation assays suggested a moderate to strong phenotype. Neutralisation of its primary receptor 4-1BB showed a dose-dependent increase in viral transfer from DC to SupT1, also acting in similar fashion in *trans* to au-

tologous CD4+ T-cells. However, siRNA knockdown of 4-1BB during HIV-1 transfer from DCs to autologous CD4+ T-cells were not conclusive; it is possible this may be due to donor variability or other technical errors. Despite this, our data is in agreement with the existing literature suggesting that 4-1BBL/4-1BB interaction is an important feature in DC biology and clearly demonstrates some capacity for restriction against HIV-1 transfer in our experimental system.

Currently, there is mixed evidence for 4-1BBL and 4-1BB in the protection against HIV-1 pathogenesis. 4-1BB expression was reported to be decreased in CD4+ T-cells from chronically infected HIV-1+ individuals, which is associated with T-cell exhaustion, loss of proliferation, altered cytokine production and enhanced viral replication (Kassu et al., 2009). Since 4-1BB is required for CD8+ cross-presentation, signalling can contribute alongside CD70-CD27 interaction to the efficient generation of perforin/granzyme A expressing CD8+ effector memory cells required for the control of HIV-1 infection (Bukczynski et al., 2004). However this has been disputed *in vivo*. A study by Vinay and colleagues found that while 4-1BB is capable of stimulating both CD4+ and CD8+ responses, a CD4-suppressing response is elicited by activated CD8 T-cells, resulting in a loss of CD4 effector function and deletion mediated by IFN-γ, TNF-α, TGF-β and IDO1 (indoleamine 2,3-dioxygenase) (Choi et al., 2006; Ménoret et al., 2006; Vinay et al., 2006; Niu et al., 2007). It is therefore plausible that the protective properties of 4-1BB/4-1BBL signalling during early infection may be outweighed with disease progression and may in fact be harmful in chronic HIV-1 infection by indirectly facilitating CD4+ T-cell depletion.

An important consideration to make in these studies is the activation profile of DCs given the highly plastic nature of monocytes and their derivatives in response to certain stimuli. DC maturation is known to have differential effect on both *cis*- and *trans*-infection to CD4+ T-cells, where immature DC are more susceptible to infection but less effective at transfer and mature DC are less receptive to infection but transfer with greater efficiency (Jolly and Sattentau, 2004). In our hands, we found preliminary evidence of 4-1BB/4-1BBL signalling in the partial

maturation of DCs which led to upregulation of certain markers but remained CD83 negative suggesting that treated DCs were not fully functionally matured.

4-1BB has been well characterised in T-cell populations buts its role in DCs is unclear. Contrary to previous reports that support a co-stimulatory role for 4-1BB in DCs (Choi et al., 2009), our findings in fact show enhanced expression of costimulatory molecules CD86 and HLA-DR during neutralisation by α4-1BB. Crucially, the 4B4-1 clone of α4-1BB used in these assays is non-stimulatory and does not induce signalling by cross-linking (Salih et al., 2000). Signalling through 4-1BBL has been previously described in tissue inflammation, where 4-1BB-expressing epithelial cells bind 4-1BBL+ monocytes, driving local inflammation and supporting differentiation into either IL-12-producing DC or M1 macrophages (Kwajah and Schwarz, 2010). This mechanism is thought to play a key role in the amplification of a vicious inflammatory cycle, further recruiting inflammatory mediators (Kwon, 2015).

Taken together, several important functions of 4-1BB/4-1BBL signalling in DCs have been described including the induction of DC maturation, cell survival and promotion of CD4+/CD8+ T-celll mediated immunity. However, the direct antiviral effects of 4-1BB/4-1BBL in DCs remain largely unexplored; since DCs are reported to express both ligand and receptor, which may self-signal, it remains difficult to delineate the effect of each especially in the context of mixed cell cultures. However, the potent anti-viral effects of 4-1BBL/4-1BB should not be dismissed and further investigation is required to understand these mechanisms of HIV-1 suppression. Interestingly, our findings suggest that 4-1BBL expression in MDDC is required for HIV-1 restriction to SupT1, but not in *trans* to autologous CD4+ T-cells.

TWEAK (TNFSF12) – TWEAK was the strongest TNF-related candidate in our final model of hit selection, showing a moderately strong restriction of viral transfer from DCs to SupT1 cells. However, validation using αTWEAK nAb neutralisation showed variable results in *trans*. TWEAK neutralisation was not tested in a system of viral transfer from DC to autologous CD4+ T-cells, but could nevertheless be interesting for further investigation. TWEAK is expressed on DCs and has multifaceted roles in the regulation of inflammation and cell death.

Taken together, the molecular functions of TWEAK during the early interactions between HIV-1 and DCs may have a substantial impact on the innate immune response to infection and the outcome of viral transfer.

The role of TWEAK in HIV-1 infection has not been extensively studied, though its role in apoptotic cell death has often been thought to contribute toward progressive CD4+ T-cell loss (Lichtner et al., 2004). Over the current decade, attention has focused on the scavenging and decoy TWEAK receptor, CD163, which has come to light as a novel marker of HIV-1 related immune activation in monocyte/macrophage populations (Burdo et al., 2011; Tippett et al., 2011; Knudsen et al., 2016) and may also have important roles during DC-HIV-1 interaction. CD163 exists in two forms: firstly, membrane-associated CD163 is a endocytic scavenging receptor typically expressed in M2 macrophages and generally considered as an anti-inflammatory receptor based on protective inflammatory resolution in studies of atherosclerotic lesions (Boyle et al., 2009). Secondly, CD163 may exist in soluble form (sCD163) following cleavage of its membrane-bound form, where it is able to interact with soluble TWEAK (sTWEAK) to promote resolution of inflammation and regulation of tissue regeneration (Akahori et al., 2015). Clinical studies by Beltrán and colleagues found lower plasma levels of sTWEAK and elevated sCD163 in untreated HIV-1 infected patients (Beltran et al., 2014). Plasma levels of sCD163 in HIV-1 infected individuals on effective ART are significantly reduced but do not fully return to basal levels in healthy controls. In contrast, sTWEAK expression is unchanged by ART (Beltran et al., 2014). While it is yet to be determined whether TWEAK is primarily secreted or membrane-anchored in MDDC, these findings are in agreement with our observations that the absence of TWEAK is linked with dissemination of HIV-1 and when silenced, leads to a higher rate of viral transfer to CD4 T-cells. These findings of elevated sCD163 during HIV-1 infection may also have a role within our system. Since CD163 is expressed in low levels on monocyte-derived cells including MDDC and MDLC (Maniecki et al., 2006), the inflammatory-mediated shedding of CD163 into its soluble form may bind sTWEAK to antagonise its biological functions. In addition, one study reports greater susceptibility to HIV-1 infection in monocyte-derived macrophages with enhanced CD163 expression, shedding light on a possible

interaction between the two (Tuluc et al., 2014). Although the association between CD163 and viral entry is unclear, it is possible that HIV-1 may be internalised using CD163-utilising scavenging machinery and that sTWEAK-CD163 binding may block facilitated entry.

Together, it is possible that sTWEAK may act to restrict viral transfer by 1) promoting a pro-inflammatory environment through induction of cytokines or 2) driving apoptotic pathways to terminate HIV-bearing DC or productively infected CD4+ T-cells limiting viral transfer and replication. The former, as previously discussed, is particularly important in maintaining the antiviral response against early infection. Inflammation primes CD4+ Th1 cells to secrete antiviral cytokines including TNF-α, IFN-γ and IL-2 which sustain the cytotoxic activities of effector CD8+ T-cells. The latter raises several questions regarding HIV-1 pathophysiology and strategies for maintaining viral persistence. Wide-spread CD4+ T-cell apoptosis is a hallmark feature of chronic HIV-1 infection and also extends to DCs and their subpopulations which are substantially diminished with the progression of infection (Sabado et al., 2010). Dysregulation of both innate and adaptive immune systems is one of many successful immune evasion mechanisms by HIV-1 and the loss of an effective response again virus through progressive lymphocyte destruction is likely to support viral dissemination. But despite this, the exact mechanisms underpinning cell death and why HIV-1 should want to promote death are unclear and controversial. Findings by Doitsh et al. recently challenged the view that CD4+ T-cell depletion was mostly attributed to apoptosis, suggesting that loss of activated permissive CD4+ T-cells by HIV-1 represented only 5% of cell death. Instead, the authors suggest that the remaining 95% of death occurs in non-permissive CD4+ T-cells initiated through a caspase-1 dependent pytoptotic pathway by abortive HIV-1 infection, which drives the extensive loss of bystander CD4+ T-cells (Doitsh et al., 2010, 2014). Pyroptosis is an inflammatory programmed cell death mechanism, characterised by the swelling, lysis and release of pro-inflammatory cytokines; it has been shown to be effective in resolving infection by several bacterial strains (Man et al., 2017). However, it's role in HIV-1 infection is not protective; sustained pyroptosis leads to chronic inflammation and persistent immune activation known to exhaust the cellular response against HIV-1 and potentially promote the maintenance of a latent viral reservoir

(Aberg, 2012; Doitsh et al., 2014).

Nevertheless, apoptosis is a form of programmed cell death which has essential protective roles in normal physiology, tissue homeostasis and in response to injury by oxidative stress, DNA damage, auto-immunity and pathological insult (Elmore, 2007). Several studies support protective roles of apoptosis in viral infections and suggest that apoptosis may either act directly to inhibit viral propagation or by promoting viral clearance through apoptosis-dependent phagocytosis by neighbouring macrophages (Fujimoto et al., 2000; Stenfeldt et al., 2017). In our cell model, TWEAK may therefore help control the initial spread of virus by induction of apoptotic pathways in DCs and limiting the capacity of for cell-cell transmission. Our screen did not detect any significant change in cell death in siTWEAK MDDC as determined by fixable viability dye staining, but the relationship between viral transfer and apoptosis should be further elucidated in conjunction with an Annexin V protocol to distinguish early/late apoptotic cells. If MDDC express CD163, it is possible that sTWEAK may interfere with scavenging mechanisms and block CD163-facilitated entry of HIV-1. Together these findings suggest that CD27-dependant CD4+ T-cell priming by DCs can generate highly effective Th1 responses against early stage HIV-1 infection.

Shared signalling of CD70/4-1BBL/TWEAK – All TNF superfamily members are common activators of NF-κB signalling, which have essential roles in mediating DC survival, maturation and the regulation of their homeostatic/tolerogenic functions (O'Dea et al., 2007) and the loss of signalling is often associated with immune dysfunction (Silke and Brink, 2010). During the early interactions between HIV-1 and DCs, TNF-mediated NF-κB activation may provide protective mechanisms against viral propagation. In support of this, the host restriction factor tetherin/BST-2, which senses intracellular HIV-1 activity, has been widely reported to phosphorylate NF-κB and is thought to contribute to the pro-inflammatory response by the host cell upon recognition (Tokarev et al., 2013). The loss of TNF-mediated NF-κB signalling during siRNA or nAb treatment in our experiments may likely impede on the ability for MDDC to generate a hostile inflammatory environment against HIV-1, thus promoting *trans*-infection.

However, NF-κB is an attractive target for many viruses, including HIV-1, which co-opts host regulation of NF-κB to promote viral transcription in productively infected cells containing the HIV-1 provirus (Berkhout et al., 1990) and is thought to be involved in the maintenance of the latent viral reservoir in memory T-cell subsets via chromatin remodelling (Williams et al., 2006; Chan and Greene, 2011). These data suggest that NF-κB may have multifacted roles in the regulation of HIV-1 life cycle, which may or may not be controlled by TNF superfamily cytokines. Further to this, TNF cytokines including 4-1BBL/4-1BB and CD70/CD27 can activate PI3K-Akt signalling in T-cells to promote clonal expansion, differentiation and the enhanced release of pro-inflammatory cytokines (So and Croft, 2013), which may have a key role in determining the outcomes of HIV-1 *trans*-infection at the DC-CD4+ T-cell junction. Both candidates share the TNF receptor associated factor, TRAF2, a critical mediator in the assembly of the TNFR-signalosome (Zapata et al., 2018), which can further potentiate NF-κB activities. Our findings suggested that MDDCs pre-treated with nAb for each TNF superfamily candidate were unable to reproduce a consistent restrictive phenotype observed in the siRNA screen. Since these candidates share common signalling pathways, it is possible that neutralisation studies using multiple nAb may elicit a strong knockdown of the antiviral response to HIV-1 in MDDC and should be tested to determine the effect this has on *trans*-infection to autologous CD4+ T-cells.

6.2.3 IL-8

Although our results have shown that IL-8 fell marginally short of our final hit selection threshold, we were still able to observe protective roles in DC-mediated HIV-1 transfer. The nature of IL-8 as a downstream product of MIF signalling provided a sound rationale to investigate this candidate further and we were able to demonstrate restrictive properties during viral transfer from DC to both SupT1 and autologous CD4+ T-cells in *trans* using both siRNA and αIL-8 nAb. Our data strongly support an antiviral role for IL-8 in DCs during early interactions with HIV-1.

The role for IL-8 in HIV-1 infection was first described in the late 90s with cohort studies finding elevated IL-8 levels in peripheral blood of HIV-1 infected patients (Matsumoto et al., 1993). Since then, a growing body of evidence has emerged to support the notion of IL-8 as a *de facto* contributor to HIV-1 pathogenesis (Denis and Ghadirian, 1994; Esser et al., 1996; Lafrenie et al., 1997; Lane et al., 2001). But despite efforts made to elucidate the mechanisms of IL-8 signalling during infection, it still remains unclear and many studies are in disagreement, with some suggesting minimal, if any, effect on HIV-1 replication (Mackewicz et al., 1994; Capobianchi et al., 1998; Mackewicz et al., 1994; Nagira et al., 1999).

Interestingly, it is now becoming clearer that HIV-1 viral proteins have a wider role in exploiting IL-8 signalling mechanisms and its production by DCs. For instance, HIV-1 Nef can induce functional phenotypes in DCs promoting maturation and increasing IL-8 production at both mRNA and protein levels, which recruits and promotes activation of the uninfected CD4+ T-cell compartment (Quaranta et al., 2002). Nef-induced IL-8 production may therefore serve as an alternative mechanism of viral propagation. In addition, several studies have also found HIV-1 Tat to induce the transcription of IL-8 production in CD3/CD28-stimulated T-cells, macrophages and astrocytes (Ott et al., 1998; Kutsch et al., 2000; Lane et al., 2001). Since CD4+/CD8+ T-cells have been found to produce IL-8 and express CXCR1/CXCR2, it is possible that HIV-1 may exploit self-stimulation through autocrine signalling and perhaps potentially making target cells more receptive to infection. As the cellular export of both HIV-1 Tat and Nef from infected cells is highly active, circulating Tat and Nef could be internalised by neighbouring cells into cytosol where it can signal and stimulate nearby T-cells and can differentially regulate the apoptotic mediator Bcl-2 forwidespread bystander CD4+ T-cell apoptosis (Zhang et al., 2002; Lenassi et al., 2010). Another notable viral protein able to exploit IL-8 function is the HIV-1 matrix protein p17, which similar to HIV-1 Tat and Nef, is continuously exported by infected cells into the extraceullar space. But despite a lack of amino acid sequence similarities with IL-8, p17 can directly interact with CXCR1 to induce IL-8-like signalling properties via Rho/ROCK to enhance cell adhesion and migration in monocytes and pDC (Giagulli et al., 2012; Fiorentini et al., 2008).

IL-8 was recently identified as a key correlate for HIV-1 acquisition by Prodger and colleagues who reported a positive association of IL-8 levels in human penile coronal sulcus swabs with HIV-1 acquisition in Ugandan men (Prodger et al., 2016). The authors hypothesise that epithelial-derived IL-8 secretion recruits CD15+ neutrophils, activating them and in turn secreting Th17 and Th1 recruiting cytokines MIP-3α/MCP-1 and MCP-1/IP-10, respectively and increasing the target cell pool at mucosal tissues. T-cell recruitment gives rise to two positive feedback loops: Th17 recruitment promotes further IL-8 secretion via IL-17 and Th1 derived IFN-γ contributing to neutrophil cytokine secretion (Prodger et al., 2016).

Despite the discordant findings with our results, we were able to observe protective roles for IL-8 in an isolated study on DCs and it is certainly plausible that IL-8 may act in a context-dependent manner to restrict the early events of HIV-1 propagation. As a pro-inflammatory chemokine, IL-8 may be responsible for supporting the generation of a hostile antiviral environment against HIV-1. In monocyte cultures infected with vpx-containing HIV-1, immune escape of the potent restriction factor SAMHD1 was offset by stimulation of TLR7/8 through a post-entry mechanism that occurs at reverse transcription and is associated with 100-fold increase of pro-inflammatory cytokines including IL-8 and involved in the protection of bystander cells through type-I IFN release (Nian et al., 2012; Hofmann et al., 2016). However whilst TLR7 is widely accepted as a strong inhibitor of HIV-1 replication, the roles for TLR8 in sensing and restriction are debated, suggesting that TLR8 signalling is exploited by virus for productive infection (Gringhuis et al., 2010). Given that our MDDC model is derived from peripheral blood monocytes, these mechanisms of restriction are still likely to be relevant in our system, although the extent of antiviral activity may vary across DC subsets with different haematopoietic origins. In future experiments, exploring the role of IL-8 in *ex vivo* DC tissue models may help provide greater insight into subset-specific effects during HIV-1 restriction. Tissue explant studies of mucosal DCs during infection may highlight context-dependent roles for IL-8, which may convey protection at the earliest stages of infection but is later offset by the harmful recruitment and chronic activation of other immune cells, favouring HIV-1 survival.

IL-8 is produced at low levels by DC at immature steady state which is markedly enhanced following maturation and express both CXCR1 and CXCR2 suggesting that DCs are capable of autocrine signalling to regulate its migratory functions (Sozzani et al., 1997; Feijoó et al., 2005). Moreover, CXCR1/CXCR2 signalling events lead to the activation of Rho-family GTPases which are key regulators of actin nucleation, cytoskeleton organisation and rearrangement (Waugh and Wilson, 2008), which are crucially exploited by HIV-1 for survival and dissemination in DCs (Ospina Stella and Turville, 2018). It has been previously suggested that the cortical actin network acts as a barrier to viral infection at the cell surface (Marsh and Bron, 1997) and that disruption to the actin skeleton leads to increased HIV-1 infectivity and complements the roles of Nef-mediated actin remodelling for enhanced delivery of HIV-1 capsid through cortical actin structures (Campbell et al., 2004). Therefore, IL-8 signalling may be a key factor for maintaining the integrity of the MDDC cytoskeleton through sustained actin polymerisation, which could be protective against productive infection in MDDCs and would lead to a dampened ability for cell-cell *trans*-infection with SupT1 and autologous CD4+ T-cells.

6.2.4 Model outline

Taken together, we outline a MIF-TNF-IL-8 axis which may restrict multiple stages of HIV-1 pathogenesis. Following early interaction between HIV-1 and DC surface PRRs induce the rapid release of MIF which signals through paracrine and autocrine modes to activate CD74 in complex with CXCR4/CD44. MIF contributes to the assembly and activation of the NLRP3 inflammasome, which is key upstream regulator of pro-inflammatory cytokine release and a central mediator of pyroptosis. Together, these functions may be important in the control of HIV-1 infection and could shape the early inflammatory responses to virus in DCs.

MIF is upstream of TNF production, which may have wider roles in restriction involving priming of the adaptive immune response against infection. Since several TNFSFR are capable of bidirectional signalling at the DC-T-cell junction, TNF receptor interaction may promote the

maturation of DCs, which leads to dampened endocytic activity and are less receptive to viral capture. Despite this, mDCs have heightened capacity for cell-cell transfer which we have previously demonstrated in *trans* to SupT1 CD4+ T-cells (figures 3.4 – 3.5). In contrast, TNF production may enhance the differentiation of effector cell subsets. At the DC-CD4+ T-cell synapse, TNF may prime HIV-specific functions and the polarisation of Th1 subset differentiation. Likewise, reverse signalling of TNFSF/TNFRSF may induce DC maturation and the priming of inflammatory responses.

IL-8 is another downstream product of MIF signalling which potentially contributes to the antiviral inflammatory response to infection. TLR7/8-dependent IL-8 production potently restricts HIV-1 in monocytes through an unknown SAMHD1-independent mechanism (Nian et al., 2012; Hofmann et al., 2016). In addition, IL-8 signalling via CXCR1/CXCR2 in DCs may have roles in actin polymerisation and organisation of the cortical actin network, which is a known barrier against entry of RNA viruses (Marsh and Bron, 1997; Campbell et al., 2004). A schematic of our proposed model of cytokine-driven immune control of infection by DCs is shown in figure 6.1.

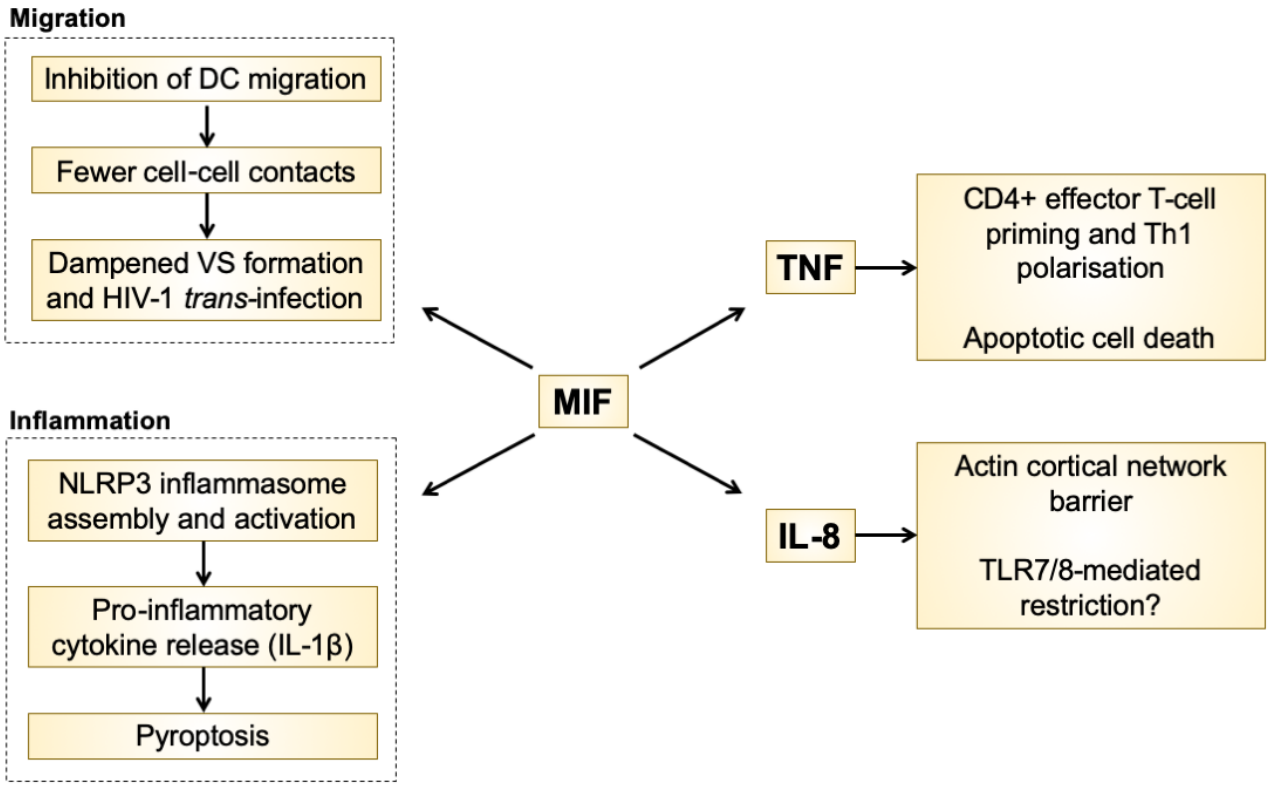

Figure 6.1: A proposed model of cytokine-driven HIV-1 restriction via MIF, TNF and IL-8 signalling functions

6.3 MIF restricts viral transfer by enhancing autophagosome formation

It is now clear that autophagy and degradation processes are central for cellular homeostasis. Autophagy is a key regulator of cell death and survival amid unfavourable conditions of stress and metabolic starvation. However, alterations and dysregulation of autophagic processes are linked with a plethora of disease states such as infections, cancers and neurodegenerative diseases. Crucially, autophagy has functions beyond homeostasis including the selective degradation of intracellular pathogens. Previous studies from our laboratory have demonstrated that autophagy selectively degrades HIV-1 in DCs and limits its infectious capacity by *trans*-infection of CD4+ cells (Blanchet et al., 2010); the methods from this publication have been

used to inform the experimental design of the autophagy research conducted in this thesis. Our findings of MIF-dependent autophagosome formation in DCs highlights a new mechanism of host restriction against HIV-1, linking this cytokine network with intrinsic immunity. Here we discuss some of most accepted experimental approaches to autophagy monitoring, common pitfalls and further investigate potential models of MIF signalling in the regulation of autophagy.

6.3.1 A review of experimental methods for autophagy detection

There are many ways in which autophagy can be interpreted (Lee and Klionsky, 2014), however there are several techniques available and they have been detailed extensively in Mizushima's methods in mammalian autophagy research (Mizushima et al., 2010). However, for many years, the experimental methods of quantifying autophagy have been limited by several technical challenges with many investigators in the field recognising that the "ability to monitor and measure autophagy is not as good as it should be" (Klionsky et al., 2012; Marx, 2015).

Autophagic flux, defined as the rate of autophagic degradation activity, reflects the process of LC3 autophagosome formation and its loss during lysosomal fusion and degradation. Autophagic flux is most conventionally detected by immunoblotting, which generates two protein bands between ∼18 and 16 kDa representing non-lipidated (LC3-I) and lipidated (LC3-II) forms, respectively. In the presence of lysosomal inhibitors, western blot approaches can be used to determine both LC3 turnover by comparing LC3-II density between untreated vs treated samples (LC3 turnover assay) and also the amount of autophagosomes by quantifying LC3-I:LC3-II conversion. However, despite being the most widely used autophagy marker, LC3 monitoring has several shortcomings and should be approached cautiously. For instance, detection by western blot has stringent sample preparation requirements to ensure complete protein extraction and detection. Since LC3-II is naturally hydrophobic, it is only partially soluble in PBS and lysis buffers containing 1% Triton X-100, NP40 and TWEEN 20, has stronger retention on PVDF membranes compared to nitrocellulose and is more susceptible to degradation

during repeat freeze-thaw cycles than its precursor LC3-I (Tanida et al., 2008; Klionsky et al., 2012). In addition, the ability to distinguish LC3 isoforms (LC3A, LC3B, LC3B2 and LC3C), which have distinct subcellular distributions and exhibit different rates of turnover depending on cell type and autophagy-induction conditions, has been a substantial challenge. The functional significance of LC3 isoforms is not currently well described, however of these LC3B is the most well recognised. Isoform-specific antibodies may therefore be necessary and should ideally be used for labelling, although LC3 antibody isoform cross-reactivty has been previously reported and should be considered (Martinet et al., 2013).

More recently, autophagy research has turned to novel, alternative approaches of autophagosome detection including the Cyto-ID detection kit containing a cationic amphilic dye which selectively stains for autophagic structures whilst minimising lysosomal signal (Chan et al., 2012; Oeste et al., 2013; Guo et al., 2015). Another advantage is that Cyto-ID can be used to analyse live cell cultures over time and in the presence of lysosomal inhibitors, can be used to estimate autophagic flux. Here we used Cyto-ID to test the role of MIF on the expression of LC3 as a marker for autophagosomes in DCs. Autophagy can be stimulated by rapamycin and chloroquine co-treatment, which induces autophagy initiation and retains autophagosomes by blocking lysosomal acidification respectively. In accordance with reports in the literature suggesting MIF-dependent functions in the regulation autophagosome formation, we were solely focused on investigating the dynamics of autophagy initiation, rather than autophagic flux. Since the modulation of autophagy is best studied using multiple detection methods, we used confocal microscopy to determine LC3 protein localisation to complement our data. Although Cyto-ID was able to provide an reliable bulk fluorescence readout for the estimation of the autophagic compartment by microplate assay, we found that it was unsuitable for analysis using fluorescence confocal microscopy and was unable to effectively resolve autophagic structures in high detail for image quantification. Using an LC3B-specific antibody for fluorescence microscopy was able to effectively resolve autophagosomes, however we were able to detect staining in non-autophagic structures as previously described. One caveat to our methods for LC3B autophagosome detection by fluorescence microscopy is the inability of our primary an-

tibodies to distinguish between LC3B-I and LC3B-II forms. Despite this, unlike its cytosolic precursor, LC3-II localises into punctate structures or 'spots' which are readily identifiable and easily quantified using computation. Since LC3 is cleaved from the outer membrane during lysosomal fusion and is not detectable by immunofluorescence, LC3 puncta can be assumed to be autophagosomes and counting the number of puncta per cell is an accepted readout for the rate of generation of autophagosomes, although it is not sufficient as an indicator of autophagic flux (Mizushima et al., 2010).

LC3 is however not entirely exclusive to autophagosome formation and is observed in non-canonical pathways of autophagy including LC3-associated phagocytosis (LAP). LAP shares common pathways with autophagy, utilising a subset of ATG genes or LAP-specific genes from canonical autophagy including (*ATG5, ATG7, BECN1, RUBCN and CYBB/NOX2*) (Zhou et al., 2019) to recruit LC3 to the phagosome to promote lysosomal fusion and phagosome maturation (Martinez et al., 2011). Unlike autophagosomes, LC3-associated phagosomes are larger and only have a single-membrane layer which is thought to enhance the efficiency of lysosomal degradation. LAP phagosomes are rich in PRRs such as TLR, NLR, RIG-I and work in tandem with the autophagic machinery to induce inflammatory responses and priming of adaptive immunity. In DCs, TLR-rich phagosomes activate ATGs during viral infection to trigger autophagy and can enhance antigen presentation *in vivo* (Lee et al., 2010a). The NLRs, NOD1 and NOD2, activate MAPK, NF-κB in response to bacterial entry to recruit ATG16-like 1 (ATG16L1) to the plasma membrane for the pre-initiation of autophagy (Travassos et al., 2010). In addition, while RIG-I directly suppresses the autophagic process, downstream activation by viral dsRNA can induce autophagy via STING localisation with ATG9a, p62 and LC3 (Saitoh et al., 2009; Burdette and Vance, 2013). The ability to distinguish between LAP and canonical autophagy is currently limited and may therefore be a potential confounding factor in the study of canonical pathways. Although Run domain Beclin-1-interacting and cysteine-rich domain-containing protein (Rubicon) has been identified as the first LAP-specific protein not involved in canonical autophagy, which functions as a molecular switch polarising autophagy toward LAP and is required for the LC3 recruitment to the phagosome (Martinez

et al., 2015; Boyle and Randow, 2015). The roles of LAP are particularly relevant in DC interactions with HIV-1 but have not been fully explored in our system. It is not clear whether MIF is required for the recruitment of LC3 to phagosomes but could facilitate the process by promoting LC3 conjugation.

Our findings show that bulk macroautophagy occurs in a MIF-dependent manner, suggesting that MIF is required for the formation of autophagosomes and degradation of internalised HIV-1. However since it is now well established that autophagy and intracellular clearance of virus occurs as a selective process (Blanchet et al., 2010), alternative markers for autophagy may be used to complement LC3. The p62 protein is the most well known substrate of selective autophagy which delivers poly-ubiquitinated cargo via the UPS to LC3-II via the LIR motif at the autophagosome, earmarking it for degradation (Liu et al., 2016). During autophagy, the degradation of p62 occurs as part of its natural cycle of turnover and its expression is inversely correlated with autophagic activity, which accumulates during shutdown of the autophagic machinery. Therefore, changes in p62 expression may be used as an estimator of autophagic flux detectable by immunoblot (Mizushima and Yoshimori, 2007). As we have previously observed a loss of autophagosome formation during MIF inhibition by 4-IPP, further investigation into the relationship between MIF and the regulation of selective autophagy substrates such as p62 is therefore warranted. Since our data suggest that MIF-deficient DCs have defective regulation of bulk autophagy, investigating the expression of p62 in 4-IPP treated DCs would determine whether intracellular HIV-1 accumulation occurs as a product of impaired selective autophagy.

6.3.2 MIF-dependent regulation of autophagy and HIV-1

Several studies have now reported MIF involvement in the regulation of autophagy, however little is known about the signalling events that are responsible for this mechanism. MIF is thought to signal through its cognate receptor CD74 which complex with CD44 to trigger sustained activation of several signalling pathways (Shi et al., 2006). MIF-CD74 binding leads to

sustained activation of ERK1/2 MAPK signalling (Mitchell et al., 1999) resulting in the induction of canonical autophagy via the autophagy/apoptotic reguators Beclin 1, Bcl-2/adenovirus E1B 19 kDa-interacting protein 3 (BNIP3) and enhanced LC3-I lipidation activity (Cagnol and Chambard, 2010). ERK1/2 activation by MIF is CXCR7-dependent (Alampour-Rajabi et al., 2015), although the signalling capacity of the CXCR4 'decoy receptor' remains controversial (Rajagopal et al., 2010). Since MIF is known to interact directly with BNIP3 which functions as an LC3-binding receptor (Hanna et al., 2012), it is plausible that the enzymatic properties of the MIF homotrimer may catalyse its interaction and enhance selective autophagy. JAB1 and Src-kinases have been identified as upstream regulators of ERK1/2 MAPK signalling by MIF-CD74 binding (Lue et al., 2006). However, interestingly, MIF exhibits non-classical intracellular signalling by directly co-localising with cytosolic JAB1 and initiating downstream induction of multiple ATG genes (*ULK1*, *BECN1* and *GABARAP*), NF-κB and regulation of the JNK pathway which may be involved in the process of autophagic cell death (Kleemann et al., 2000; Shimizu et al., 2010; Lue et al., 2011; Zhou et al., 2015). Conversely, MIF-CD74 signalling and JAB1 may also be responsible for governing the fate of the cell through PI3K/AKT signalling, which activates mTOR for the negative regulation autophagy and promotion of apoptotic and necrotic cell death (Lue et al., 2007; Wu et al., 2009). Taken together, it is clear that MIF predominantly acts at the early stages of autophagy initiation and nucleation. However, the extent to which intracrine, autocrine or paracrine modes of MIF signalling share the same molecular pathways and at which point these pathways converge during autophagy are still yet to be fully investigated.

Our findings suggest that in HIV-1 infected DCs, autophagy serves as a protective mechanism for intracellular viral clearance, by selective degradation or through non-canonical autophagy pathways such as LAP. However, it is also important to recognise that HIV-1 has evolved mechanisms to subvert degradation by autophagy (Leymarie et al., 2017) which may potentially complicate the cell system. As the control of autophagy is mediated by upstream inhibitory regulators including mTOR and S6K, these protein complexes repress the initiation stage of autophagy under normal conditions and have been found to be targets for immune evasion by

HIV-1 viral proteins. HIV-1 Env can evade degradation at the earliest stages of host-virus interaction through interaction with DC surface receptors to activate phosphorylation of upstream regulators. HIV-1 Env activation of mTOR and S6K inhibits the release of inhibitory pressure on autophagy initiation which ultimately leads to progressive autophagosome exhaustion and successful evasion of degradation (Blanchet et al., 2010). Nef appears to counter the intrinsic host defence through several mechanisms which ultimately functions to maintain viral loads and progression to disease. More recently, a new mechanism has been unveiled (Kyei et al., 2009), suggesting that Nef interferes with the autophagic machinery to block the maturation of autophagosomes and degradation by lysosomal fusion through direct interaction with Beclin 1 and inhibition of TFEB nuclear translocation (Campbell et al., 2015). Similar observations were found with Tat, which blocked fusion in neuronal cells (Fields et al., 2015). Additionally, Tat was found to intercept the initiation of autophagy through Src-Akt- and STAT-3-dependent mechanisms, reducing the LC3 autophagic compartment in monocytes and their derivatives (Van Grol et al., 2010). Vpu is well recognised as a counter-regulator of the restriction factor tetherin, although it has recently emerged that Vpu utilises LC3C for the displacement of tetherin from the HIV-1 budding site (Madjo et al., 2016). Together with ATG5 and Beclin 1 which are associated with non-canonical pathways of autophagy, the authors propose a model where Vpu can exploits LAP to degrade surface tetherin for enhanced HIV-1 release. However, tetherin is dispensable for Vpu-mediated enhancement of virion release (Miyagi et al., 2009), suggesting that alternative mechanisms of Vpu-driven immune evasion may exist. It is clear that HIV-1 subverts many aspects of innate and adaptive immunity including disruption of the cytokine network, but whether HIV-1 viral proteins can subvert autophagy through MIF dysregulation is still not clear and further investigation into the upstream control of MIF and changes to its expression at both RNA and protein level during infection are required.

Autophagy serves as a central homeostatic process necessary for cell survival and adaptation to adverse circumstances including physical, chemical and pathogenic insult. Together with reports that pathogens can escape intrinsic immunity, the autophagy process is therefore an attractive target for therapeutic intervention. Manipulation of the upstream signalling events

that modulate autophagy could help restore function caused by viral dysregulation. Since many autophagy-modulating agents including rapamycin, flubendazole and chloroquine and their derivatives are already authorised for clinical use, repurposing these drugs to fit the biological context of early stage HIV-1 infection at the mucosa could be a viable strategy against viral dissemination. Several small-molecule screens in search of novel autophagy-modifying agents have already been performed including by our laboratory (Chauhan et al., 2015), which have yielded promising results for new antiviral drug candidates and hold significant potential for translation into clinical practice. However, maintaining an effective therapeutic index, which balances drug efficacy against toxicity, has typically presented a major challenge for autophagy-modifying agents and must be taken into consideration throughout future stages of development (Rubinsztein et al., 2012; Klionsky and Thorburn, 2014). Our findings therefore offer a solution based on the rapidly expanding field of cytokine biologics, suggesting that recombinant MIF may have therapeutic promise as a disease-modifying regulator of autophagy. However, a better understanding of MIF cell signalling events, protein-protein interactions and its as-of-yet uncharacterised enzymatic activity is crucial for cytokine-focussed strategies against acute infection. A schematic representation of MIF functions in autophagy and its role in the control of HIV-1 infection is detailed in figure 6.2.

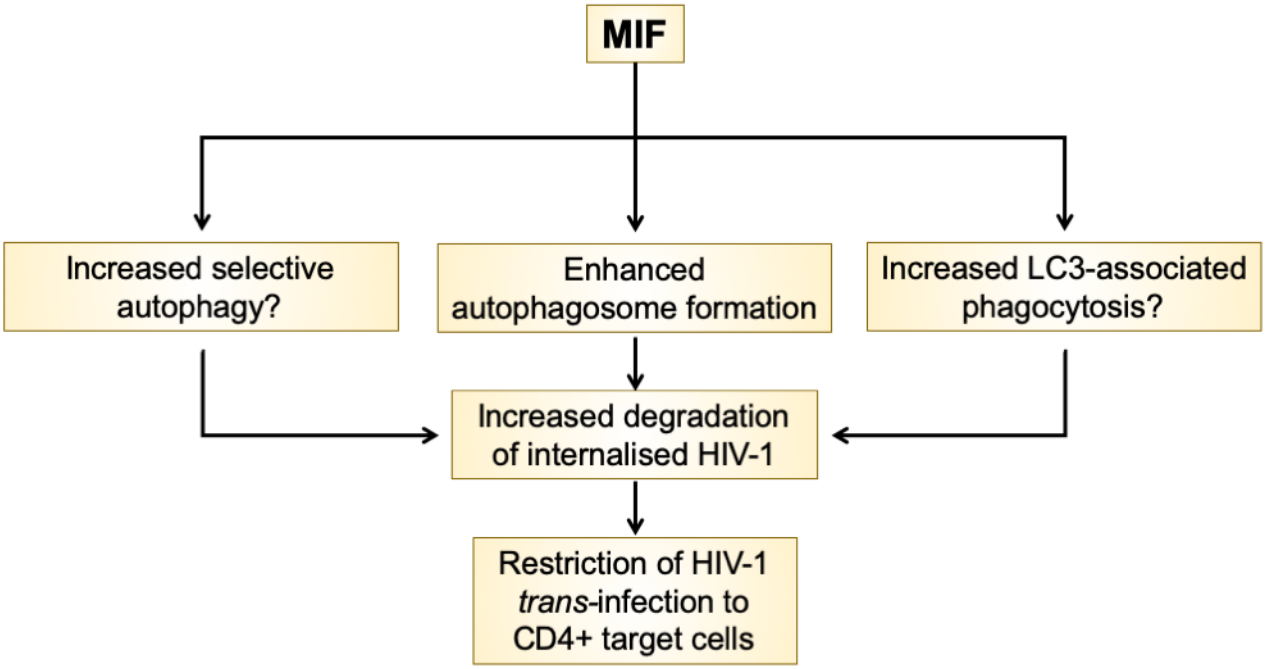

Figure 6.2: MIF, autophagy and the restriction of HIV-1 *trans*-infection from DCs to CD4+ T-cells.

6.4 Summary and future directions

Antiretroviral therapies have made substantial advances over the past couple of decades, which have had tremendous impact on prolonging the life expectancy of many HIV-infected individuals. However, despite the viral suppression, those on long-term treatment experience deteriorating qualities of life and have suffer secondary HIV/AIDS-related diseases and are still at risk of opportunistic infections. Therefore, new strategies to target the early stages of HIV-1 pathogenesis before latent infection can be established may be paramount to the success of anti-HIV therapies.

The relationship between virus and host has resulted in an evolutionary arms race between host restriction and immune evasion. Whilst a handful of cellular restriction factors have already been identified, it is thought that many more may exist than currently identified and the

field of restriction factor biology has grown rapidly in the search for novel candidates. Since cytokines are fundamental in determining the outcome of acute HIV-1 infection, dictating the magnitude and onset of HIV-specific immune responses and the viral set-point at chronic stages of infection, we proposed the hypothesis that cytokines may directly interfere with HIV-1 life cycle or regulate intrinsic host immunity for viral restriction in DCs.

The aim of this thesis was to produce the first systematic analysis of the cytokine network in DC-T-cell HIV-1 *trans*-infection to identify proof-of-principle targets to disrupt viral cell-cell transmission. The objectives were met as follows:

i. An *in vitro* cell system of HIV-1 *trans*-infection by monocyte-derived dendritic cells (MDDC) was established to model the early events of infection that occur at mucosal surfaces. The role of cytokines in HIV-1 infection, together with the immunological properties of MDDC and their interactions with CD4+ T-cells were explored further to understand the dynamics of cell-cell viral transfer ahead of a global RNAi screen.

ii. Following optimisation of MDDC genetic downregulation using siRNA, we performed a high-throughput RNAi screen for 319 genes targeting cytokine and chemokine function. In accordance with siRNA screens literature, we employed three statistical models for hit selection and identified a final candidate list containing genes with either restrictive (pro-host) or permissive (pro-viral) effects on HIV-1 transfer from DC to CD4+ T-cells.

iii. Systematic validation of candidate genes led to the identification of a restrictive MIF-TNF-IL-8 axis against HIV-1 transfer from DC to both SupT1 cell lines and autologous CD4+ T-cells. We describe a model where pro-inflammatory events in MDDC restrict HIV-1 dissemination through mechanisms including inflammasome activation, TNF-induced DC maturation, CD4+ Th1 subset priming and apoptosis.

iv. As our strongest restrictive candidate, further investigation into the mechanisms of MIF action found a common link between the cytokine network and the autophagic process. The role of autophagy as a mechanism of intrinsic host defence in DCs has previously been described within our group, however our findings add to immunomodulatory functions of

cytokines in autophagy showing that MIF is required for autophagosome formation and is in agreement with previous data that autophagy is required for the degradation of internalised virus and blocks viral dissemination in *trans*.

Our inquiry into novel HIV-1 restriction factors has led us to MIF, which satisfies many of the cardinal features for restriction factors set out by Malim and Bieniasz in their review of HIV-1 host-virus interaction (Malim and Bieniasz, 2012). MIF is germline-encoded, constitutively expressed and IFN-inducible (Wang and Goff, 2003; Feng et al., 2017); MIF acts through independent and cell-autonomous mechanisms to restrict HIV-1 using bulk autophagic activity and utilises the UPS in selective autophagy; MIF therefore mediates potent viral suppression in its natural state but the species-specific restriction is yet to be observed; MIF may be regulated by HIV-1 accessory proteins, although this is not yet clear; MIF shows hallmarks of cross-species evolutionary conservation (Panstruga et al., 2015), but the extent of host-virus co-evolution and positive genetic selection is unknown. Taken together, MIF may be eligible for status as a novel restriction factor, adding to our understanding of intrinsic immunity and to a growing list of antiviral host proteins.

Whilst the therapeutic exploitation of restriction factor biology has yet to surface, selectively targeting MIF to enhance its activity could prove an effective antiviral strategy against acute HIV-1 infection. The work presented in this thesis has described a number of proposed mechanisms for MIF-dependent restriction, although several questions regarding the molecular and immunological functions of MIF remain unanswered, which must first be addressed for future development and translation into clinical practice.

i. Do MIF-deficient MDDC have impaired NLRP3 inflammasome assembly and activation? If so, is HIV-1 infection and transfer enhanced as a result? Understanding the pro-inflammatory roles of MIF could be valuable for therapeutic design.

ii. Can the upstream molecular mechanisms of MIF expression and signalling be circumvented by HIV-1 as a means of autophagy dysreguation and immune escape? Since HIV-1 has co-evolved to evade immune pressure from restriction factors, mechanisms which manipulate

MIF interactions may exist.

iii. Are mechanisms of MIF-induced autophagy specific to canonical pathway induction or does MIF regulate non-canonical pathways of autophagy including LAP and selective autophagy? Investigating the role of MIF in selective forms of autophagy may guide research toward enhancing clearance of HIV-containing autophagic structures.

iv. Is MIF-dependent autophagosome formation observed in *ex vivo* DC? Validation of our findings in dermal DC subsets or other models of HIV-1 mucosal transmission using foreskin or vaginal tissues for instance could have therapeutic implication and potential for translation into clinical practice.

v. The earliest identifying feature of MIF is the migratory block in macrophages – is the same observed in DCs? Is HIV-1 transfer restricted due to lack of cell motility and inability to establish virological synapse (VS) formation at the DC-T-cell junctions? A better understanding of cytokine regulation of the cellular interactions at the DC-T-cell junction could uncover new mechanisms of VS formation and HIV-1 *trans*-infection.

The therapeutic value of cytokine biologics has now been realised, with many biological agents that modulate cytokine activity now authorised for clinical application in many immune-dysregulated and inflammatory diseases. Similarly, cytokine and chemokine research has shaped the landscape of HIV-1 antiretroviral therapy, but there is still much more to be done. As of 2016, HIV/AIDS remains the fourth highest cause of death among less economically developed countries with only 62% of HIV-infected individuals accessing antiretroviral therapy globally, falling significantly short of the UNAIDS 2020 target for a 90-90-90 continuum of care. Although HIV-1 can be effectively managed using ART, patients require lifelong treatment, suffer poor quality of life and are prone to HIV-associated co-morbidities. Therefore, new transmission-preventative approaches are required to end the global burden of disease caused by HIV/AIDS. Our work identifies MIF as a previously uncharacterised antiviral cytokine-mediator with protective functions against HIV-1 infection and transmission. MIF is highly expressed in DCs, one of the earliest immune cells to encounter HIV-1 during acute infection, and restricts viral

propagation through intracellular degradation by autophagy. These data add to a growing body of evidence within the literature, linking the cytokine network with autophagic process as a mechanism of host defence. A better understanding of the molecular mechanisms of MIF signalling in DCs, together with an understanding of host-virus interactions could help guide the development of new MIF-based therapeutics against acute HIV-1 infection. Further to this, therapeutic approaches which aim to enhance and sustain the expression and protective functions of MIF in DCs could form the basis of a viable vaccine strategy, addressing several key challenges currently facing vaccine developers. Together, these novel approaches to drug and vaccine design are of critical importance toward ending the HIV/AIDS pandemic and offer a realistic solution to addressing the unmet clinical need for accessible therapies among some of the most deprived societies.

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Appendix A

Supplementary material

A.1 HTS analytics pipeline developed in R

bin/sirna_io.R

```
### siRNA file i/o

# Import libraries ====
library(plyr)
library(dplyr)


# Import siRNA cytokine receptor library ====
cclib = read.csv("data/G-104000␣OTP␣Human␣Cytokine␣Receptors␣Lot␣060724.csv")
cclib = `colnames<-`(cclib, c("plate","well","catalog1","catalog2","genesymbol",
    ↪ "geneid", "accession", "ginumber"))
cclib = na.omit(cclib)

# Data import ====
import = function(path){
  x = read.csv(path, sep=",")
  x = `colnames<-`(x, c("sample","p24.supt1","dead.supt1", "p24.mddc", "dead.mddc"
      ↪ )); x=x[,1:5]
  ind = which(with(x, x$sample == "Mean" | x$sample == "SD"))
  if("Mean" %in% x$sample) x = x[-ind,] else x=x
}

raw1 = import("data/180117␣p1-1.csv")
raw2 = import("data/220117␣p1-2.csv")
raw3 = import("data/290117␣p1-3.csv")
raw4 = import("data/060217␣p1-4.csv")


# Subset and clean ====
clean = function(x, plateno, wellstart){
  x = filter(x, !grepl("Control_*", sample))
  n = length(x[,1])-1
  x$plateno = rep(plateno, n+1)
  x$wellindex = wellstart:(wellstart+n)
  x$genesymbol = as.character(unique(cclib$genesymbol)[wellstart:(wellstart+n)])
  x$accession = as.character(unique(cclib$accession)[wellstart:(wellstart+n)])
   return(x)
}

```

```
raw1.c = filter(raw1, grepl("Control_*", raw1$sample))
raw2.c = filter(raw2, grepl("Control_*", raw2$sample))
raw3.c = filter(raw3, grepl("Control_*", raw3$sample))
raw4.c = filter(raw4, grepl("Control_*", raw4$sample))

raw1 = clean(raw1,1,1)
raw2 = clean(raw2,2,81)
raw3 = clean(raw3,3,161)
raw4 = clean(raw4,4,241)

# Collate data
samp = rbind.fill(raw1,raw2,raw3, raw4)
samp.c = rbind.fill(raw1.c, raw2.c, raw3.c, raw4.c)


# Screen 2 ====
raw5 = import("data/010317␣p2-1.csv")
raw5.c = filter(raw5, grepl("Control_*", raw5$sample))
raw5 = clean(raw5,1,1)

raw6 = import("data/070317␣p2-2.csv")
raw6.c = filter(raw6, grepl("Control_*", raw6$sample))
raw6 = clean(raw6,2,81)

raw7 = import("data/130317␣p2-3.csv")
raw7.c = filter(raw7, grepl("Control_*", raw7$sample))
raw7 = clean(raw7,3,161)

raw8 = import("data/200317␣p2-4.csv")
raw8.c = filter(raw8, grepl("Control_*", raw8$sample))
raw8 = clean(raw8,4,241)

samp2 = rbind.fill(raw5, raw6, raw7, raw8)
samp2.c = rbind.fill(raw5.c, raw6.c, raw7.c, raw8.c)
```

bin/sirna_qc.R

```
### siRNA Quality Control

# Import libraries ====
#source("http://bioconductor.org/biocLite.R")

library(stats)
library(ggplot2)

# Import data
source("scripts/sirna_io.R")

# Raw data visualisation ====

ggplot(data = samp, aes(x=samp$wellindex, y=samp$p24.supt1))+
  ggtitle(label = "Screen␣1:␣Raw␣transfer")+
  geom_point()

ggplot(data = samp2, aes(x=samp2$wellindex, y=samp2$p24.supt1))+
  ggtitle(label = "Screen␣2:␣Raw␣transfer")+
  geom_point()


# Normalisation to non-target siRNA ====
raw1$nt.norm = ((raw1$p24.supt1/raw1.c[which(raw1.c$sample=="Control_NT.fcs"),"
    ↪ p24.supt1"])-1)*100
raw2$nt.norm = ((raw2$p24.supt1/raw2.c[which(raw2.c$sample=="Control_NT.fcs"),"
    ↪ p24.supt1"])-1)*100
raw3$nt.norm = ((raw3$p24.supt1/mean(raw3.c[grep("NT", raw3.c$sample),"p24.supt1"
```

```
    ↪ ]))-1)*100
raw4$nt.norm = ((raw4$p24.supt1/mean(raw4.c[grep("NT", raw4.c$sample),"p24.supt1"
    ↪ ]))-1)*100

raw5$nt.norm = ((raw5$p24.supt1/mean(raw5.c[grep("NT", raw5.c$sample),"p24.supt1"
    ↪ ]))-1)*100
raw6$nt.norm = ((raw6$p24.supt1/mean(raw6.c[grep("NT", raw6.c$sample),"p24.supt1"
    ↪ ]))-1)*100
raw7$nt.norm = ((raw7$p24.supt1/mean(raw7.c[grep("NT", raw7.c$sample),"p24.supt1"
    ↪ ]))-1)*100
raw8$nt.norm = ((raw8$p24.supt1/mean(raw8.c[grep("NT", raw8.c$sample),"p24.supt1"
    ↪ ]))-1)*100


ggplot(data = raw1, aes(x=raw1$wellindex, y=raw1$nt.norm[order(raw1$nt.norm)]))+
  ggtitle(label = "Plate␣1␣Non-target␣normalised␣transfer")+
  geom_bar(stat = "identity", width=0.5)+
  geom_hline(yintercept = c(-20,50))

ggplot(data = raw2, aes(x=raw2$wellindex, y=raw2$nt.norm[order(raw2$nt.norm)]))+
  ggtitle(label = "Plate␣2␣Non-target␣normalised␣transfer")+
  geom_bar(stat = "identity", width=0.5)+
  geom_hline(yintercept = c(-20,50))

ggplot(data = raw3, aes(x=raw3$wellindex, y=raw3$nt.norm[order(raw3$nt.norm)]))+
  ggtitle(label = "Plate␣3␣Non-target␣normalised␣transfer")+
  geom_bar(stat = "identity", width=0.5)+
  geom_hline(yintercept = c(-20,50))

ggplot(data = raw4, aes(x=raw4$wellindex, y=raw4$nt.norm[order(raw4$nt.norm)]))+
  ggtitle(label = "Plate␣4␣Non-target␣normalised␣transfer")+
  geom_bar(stat = "identity", width=0.5)+
  geom_hline(yintercept = c(-20,50))

ggplot(data = raw5, aes(x=raw5$wellindex, y=raw5$nt.norm[order(raw5$nt.norm)]))+
  ggtitle(label = "Plate␣5␣Non-target␣normalised␣transfer")+
  geom_bar(stat = "identity", width=0.5)+
  geom_hline(yintercept = c(-20,50))

ggplot(data = raw6, aes(x=raw6$wellindex, y=raw6$nt.norm[order(raw6$nt.norm)]))+
  ggtitle(label = "Plate␣6␣Non-target␣normalised␣transfer")+
  geom_bar(stat = "identity", width=0.5)+
  geom_hline(yintercept = c(-20,50))


samp$nt.norm = c(raw1$nt.norm, raw2$nt.norm, raw3$nt.norm, raw4$nt.norm)
samp2$nt.norm = c(raw5$nt.norm, raw6$nt.norm, raw7$nt.norm, raw8$nt.norm)

ggplot(data = samp, aes(x=samp$wellindex, y=samp$nt.norm[order(samp$nt.norm)]))+
  ggtitle(label = "Screen␣1␣Non-target␣normalised␣transfer")+
  geom_bar(stat = "identity", width=0.5)+
  geom_hline(yintercept = c(-20,50))

ggplot(data = samp2, aes(x=samp2$wellindex, y=samp2$nt.norm[order(samp2$nt.norm)
    ↪ ]))+
  ggtitle(label = "Screen␣2␣Non-target␣normalised␣transfer")+
  geom_bar(stat = "identity", width=0.5)+
  geom_hline(yintercept = c(-20,50))


# Normalisation: z-score (x-u/o) ====
raw1$z = scale(raw1$p24.supt1)
raw2$z = scale(raw2$p24.supt1)
raw3$z = scale(raw3$p24.supt1)
```

```
raw4$z = scale(raw4$p24.supt1)

raw5$z = scale(raw5$p24.supt1)
raw6$z = scale(raw6$p24.supt1)
raw7$z = scale(raw7$p24.supt1)
raw8$z = scale(raw8$p24.supt1)

samp = rbind.fill(raw1,raw2, raw3, raw4)
summary(samp$z)
samp = na.omit(samp)

samp2 = rbind.fill(raw5,raw6, raw7, raw8)
samp2 = na.omit(samp2)

# Quality metrics: Z-factor ====
neg.cont = samp.c[which(samp.c$sample=="Control_F522Y.fcs"),]
pos.cont = samp.c[grep("CD4", samp.c$sample),]

zfactor = function(pos, neg){
  sig1 = sd(pos[,2])
  sig2 = sd(neg[,2])
  mu1 = mean(pos[,2])
  mu2 = mean(neg[,2])
  z1=1-(3*sig1+3*sig2)/abs(mu1-mu2)
  return(z1)
}
zfactor(pos.cont, neg.cont)
zfactor(samp, neg.cont)

# Quality metrics: SSMD ====
ssmd = function(pos,neg){
  mu1 = median(pos[,2])
  mu2 = median(neg[,2])
  sig1 = var(pos[,2])
  sig2 = var(neg[,2])
  ssmd= (mu1-mu2)/sqrt(sig1 + sig2)
  return(ssmd)
}
ssmd(pos.cont, neg.cont)
ssmd(samp, neg.cont)
```

bin/sirna_hitselection.R

```
### siRNA hit selection

# Import libraries ====
#source("http://bioconductor.org/biocLite.R")
library(pROC)

# Import data ====
source("scripts/sirna_qc.R")

# Mean +/- k-SD ====
quantile(samp$z, c(0.05, 0.95), type = 4)

ggplot(data = samp, aes(x=samp$wellindex, y=samp$z))+
  geom_point()+
  ggtitle(label = "Screen␣1:␣k-standard␣deviations␣hit␣identification")+
  geom_hline(yintercept = c(-1.321557,1.67679))+
  geom_text(aes(label=ifelse(samp$z>1.67679,as.character(samp$genesymbol),'')),
      ↪ hjust=-0.1,vjust=-0.1)+
  geom_text(aes(label=ifelse(samp$z<(-1.321557),as.character(samp$genesymbol),''))
      ↪ ,hjust=-0.1,vjust=-0.1)
```

```
19
20 ggplot(data = samp2, aes(x=samp2$wellindex, y=samp2$z))+
21   geom_point()+
22   ggtitle(label = "Screen␣2:␣k-standard␣deviations␣hit␣identification")+
23   geom_hline(yintercept = c(-1.5,1.5))+
24   geom_text(aes(label=ifelse(samp2$z>1.5,as.character(samp2$genesymbol),'')),hjust
       ↪ =-0.1,vjust=-0.1)+
25   geom_text(aes(label=ifelse(samp2$z<(-1.5),as.character(samp2$genesymbol),'')),
       ↪ hjust=-0.1,vjust=-0.1)
26
27 # Median +/- k-MAD Hit Identification ====
28 median(samp$z)
29 samp$zmad = abs(samp$z- median(samp$z))
30
31 ggplot(data = samp, aes(x=samp$wellindex, y=samp$zmad, label=samp$genesymbol))+
32   ggtitle(label = "k-Median␣absolute␣deviation␣hit␣identification")+
33   geom_point()+
34   geom_hline(yintercept = 2)+
35   geom_text(aes(label=ifelse(samp$zmad>2,as.character(samp$genesymbol),'')),hjust
       ↪ =-0.1,vjust=-0.1)
36
37 # ROC curves ====
38 samp$hit.cut = ifelse(samp$nt.norm>50 | samp$nt.norm<(-20), 1, 0)
39 samp$hit.ksd = as.vector(ifelse(samp$z>1.5 | samp$z<(-1.5), 1, 0))
40 samp$hit.zmad = as.numeric(ifelse(samp$zmad>1.98, 1, 0))
41
42
43 model1=glm(samp$hit.cut ~ samp$p24.supt1 + samp$dead.supt1 + samp$p24.mddc + samp
       ↪ $dead.mddc,
44            data=samp,
45            family="binomial")
46 summary(model1)
47 predict(model1, type="response")
48 roc.cut=roc(samp$hit.cut, predict(model1, type="response"))
49 #roc(samp$hit.cut, runif(240,0,1), plot=T) #Coin flipping ROC curve
50 coords(roc.cut, x="best")
51
52
53 model2=glm(samp$hit.zmad ~ samp$p24.supt1 + samp$dead.supt1 + samp$p24.mddc +
       ↪ samp$dead.mddc,
54            data=samp,
55            family="binomial")
56 summary(model2)
57 predict(model2, type="response")
58 roc.zmad=roc(samp$hit.zmad, predict(model2, type="response"))
59
60
61 model3=glm(samp$hit.ksd ~ samp$p24.supt1 + samp$dead.supt1 + samp$p24.mddc + samp
       ↪ $dead.mddc,
62            data=samp,
63            family="binomial")
64 summary(model3)
65 predict(model3, type="response")
66 roc.ksd=roc(samp$hit.ksd, predict(model3, type="response"))
67
68
69 plot(roc.cut);plot(roc.zmad, add=T, col="red"); plot(roc.ksd, add=T, col="green")
70 legend(0.4,0.2, c("nt.cut␣0.7656", "zmad␣0.8056", "ksd␣0.699"), lty=1, col=c('
       ↪ black','red','green'), bty='n', cex=.75)
71
72 # ROC Cross-validation: 'leave-one-out' ====
73 # Model 1 (nt.norm cut-off)
74 plot(roc.cut);plot(roc.zmad, add=T, col="red"); plot(roc.ksd, add=T, col="green")
75
```

```
76  prediction.loo = as.vector(samp$hit.cut)
77
78  for(i in 1:length(samp$hit.cut)){
79    train = samp[-i,]
80    test = samp[i,]
81    model1 = glm(hit.cut ~ p24.supt1 + dead.supt1 + p24.mddc + dead.mddc, data =
          ↪ train, family = "binomial")
82    prediction.loo[i] = predict(model1, newdata = test, type = "response")
83  }
84
85  roc.cut = roc(samp$hit.cut, prediction.loo)
86
87
88  # Model 2 (zMAD)
89  prediction2.loo = as.vector(samp$hit.zmad)
90
91  for(i in 1:length(samp$hit.zmad)){
92    train = samp[-i,]
93    test = samp[i,]
94    model2 = glm(hit.zmad ~ p24.supt1 + dead.supt1 + p24.mddc + dead.mddc, data =
          ↪ train, family = "binomial")
95    prediction2.loo[i] = predict(model2, newdata = test, type = "response")
96  }
97  roc.zmad = roc(samp$hit.zmad, prediction2.loo)
98
99
100
101 # Model 3 (ksd)
102 prediction3.loo = as.vector(samp$hit.ksd)
103
104 for(i in 1:length(samp$hit.ksd)){
105   train = samp[-i,]
106   test = samp[i,]
107   model3 = glm(hit.ksd ~ p24.supt1 + dead.supt1 + p24.mddc + dead.mddc, data =
          ↪ train, family = "binomial")
108   prediction3.loo[i] = predict(model3, newdata = test, type = "response")
109 }
110 roc.ksd = roc(as.vector(samp$hit.ksd), prediction3.loo)
111
112 plot(roc.cut, add=T, col="blue")
113 plot(roc.zmad, add=T, col="blue")
114 plot(roc.ksd, add=T, col="blue")
115
116 plot(roc.cut, main="Cross␣validation:␣Leave-one-out");plot(roc.zmad, add=T, col="
        ↪ red"); plot(roc.ksd, add=T, col="green")
117 legend(0.4,0.2, c("nt.cut␣0.7504", "zmad␣0.6102", "ksd␣0.6293"), lty=1, col=c('
        ↪ black','red','green'), bty='n', cex=.75)
118
119
120 # Strictly standardised mean difference (SSMD) hit identification ====
121
122
123 # Hit confirmation ====
124 avg = matrix(c(samp$z, samp2$z), 319, 2)
125 avg = cbind(avg, rowMeans(avg[,1:2]))
126
127
128 samp2$z.avg = avg[,3]
129
130 ggplot(data = samp2, aes(x=samp2$wellindex, y=samp2$z.avg))+
131   geom_point()+
132   ggtitle(label = "Screen␣1&2␣z.avg:␣k-standard␣deviations␣hit␣identification")+
133   geom_hline(yintercept = c(-1.5,1.5))+
134   geom_text(aes(label=ifelse(samp2$z.avg>1.5,as.character(samp2$genesymbol),'')),
```

```
      ↪ hjust=-0.1,vjust=-0.1)+
135   geom_text(aes(label=ifelse(samp2$z.avg<(-1.5),as.character(samp2$genesymbol),'')
      ↪ ),hjust=-0.1,vjust=-0.1)+
136   geom_text(aes(label=ifelse(samp2$genesymbol=="IL8",as.character(samp2$genesymbol
      ↪ ),'')),hjust=-0.1,vjust=-0.1)
137
138 # Output ====
139 write.table(samp$accession[which(samp$nt.norm > 50)], "output/ntup.txt", quote=F,
      ↪  row.names = F, col.names = F)
140 write.table(samp$accession[which(samp$nt.norm <(-20))], "output/ntdown.txt",
      ↪ quote=F, row.names = F, col.names = F)
141 write.table(samp$accession[which(samp$z>1.5)], "output/ksdup.txt", quote=F, row.
      ↪ names = F, col.names = F)
142 write.table(samp$accession[which(samp$z<(-1.5))], "output/ksddown.txt", quote=F,
      ↪ row.names = F, col.names = F)
143 write.table(samp$accession[which(samp$zmad>1.98)], "output/kmad.txt", quote=F,
      ↪ row.names = F, col.names = F)
144
145 #cor1=cor(samp$p24.supt1[which(samp$plateno=="plate1")], samp$p24.supt1[which(
      ↪ samp$plateno=="plate2")])
146 #plot(samp$p24.supt1[which(samp$plateno=="plate1")], samp$p24.supt1[which(samp$
      ↪ plateno=="plate2")])
```

bin/sirna_clust.R

```
1  # siRNA data mining
2
3  # Import libraries ====
4  library(FactoMineR)
5  library(mixOmics)
6
7  # Import data ====
8  source("scripts/siRNA_hitselection.R")
9
10 # Hit classification
11 #samp2$hit = ifelse(samp2$z>1.5 | samp2$z<(-1.5), T,F)
12 #samp2$hit = ifelse(samp2$z.avg>1.5 | samp2$z.avg<(-1.5), T,F)
13 #samp2$hit = ifelse(scale(samp2$dead.mddc)>1.5 | scale(samp2$dead.mddc)<(-1.5), T
      ↪ ,F)
14 samp2$hit = ifelse(scale(samp2$p24.supt1)>1.5 | scale(samp2$p24.supt1)<(-1.5),
      ↪ 1,0)
15
16 z = scale(samp2$p24.supt1)
17
18 type.hits=factor(c("No","Low","High"))
19 for(i in 1:nrow(samp2)){
20   type.hits[i]="No"
21   if (z[i]<(-1.5)){
22     type.hits[i]="Low"
23   }
24   if (z[i]>1.5){
25     type.hits[i]="High"
26   }
27 }
28
29 # PCA ====
30 data.pca=samp2[,c(2:5)]
31 data.pca=cbind(data.pca,type.hits)
32
33 pca = PCA(data.pca, quali.sup = 5)
34 plot.PCA(pca,axes=c(1,2), habillage=5)
35 pca$eig
36 pca$var$coord
37 pca$var$contrib
```

```
38
39
40 # PCA without p24.supt1
41 data.pca=data.pca[,-1]
42
43 pca = PCA(data.pca, quali.sup = 4)
44 pca$eig
45 pca$var$coord
46 pca$var$contrib
47
48 plot.PCA(pca,axes=c(1,2), habillage=4)
49 clust=HCPC(pca, nb.clust=4)
50
51 # PLS-DA
52 plsda1=plsda(Y=data.pca$type.hits, X=samp2[,3:5])
53 plotIndiv(plsda1, ellipse=T)
```

A.2 Batch image processing for autophagic spot counting in dendritic cells

A.2.1 TIFF channel splitting macro

```
1         // Create batch .tiff files
2         wd = getDirectory("Choose␣a␣Directory");
3         list = getFileList(wd);
4
5         // Create channel directories
6         dir1 = wd + "T1" + File.separator;
7         File.makeDirectory(dir1);
8
9         dir2 = wd + "T2" + File.separator;
10        File.makeDirectory(dir2);
11
12        // Loop
13        for (i = 0; i < list.length; i++) {
14        setBatchMode(true);
15        name = File.getName(list[i]);
16        path = dir1 + name;
17        //print(path);
18
19        if (endsWith(name, "T1.tif")){
20        File.rename(wd + list[i], dir1 + File.separator + list[i]);
21        }
22        else {}
23        }
24
25        for (i = 0; i < list.length; i++) {
26        setBatchMode(true);
27        name = File.getName(list[i]);
28        path = dir1 + name;
29        //print(path);
30
31        if (endsWith(name, "T2.tif")){
32        File.rename(wd + list[i], dir2 + File.separator + list[i]);
33        }
34        else {}
35        }
```

A.2.2 Autophagic spot count macro

```
// Batch import files
setBatchMode(true)
run("Image␣Sequence...", "open=[]␣use");
//run("Images to Stack");
inputdir = getDirectory("image");

// Set scale and adjust image
run("Set␣Scale...", "distance=0");
run("8-bit");
run("Enhance␣Contrast...", "saturated=0.1␣normalize␣process_all␣use");
run("Subtract␣Background...", "rolling=10␣stack");
run("Gaussian␣Blur...", "sigma=1␣stack");

// Create binary and threshold
setOption("BlackBackground", false);
run("Make␣Binary", "method=MaxEntropy␣background=Dark␣calculate␣black");
setAutoThreshold("MaxEntropy␣dark");

// Analyse
run("Analyze␣Particles...", "size=10-600␣show=[Overlay␣Masks]␣include␣
    ↪ summarize␣stack");

// Export output files
selectWindow("Summary␣of␣T1");
saveAs("Measurements", inputdir + "/../Summary_spot.csv");

setBatchMode(false)
```

A.2.3 Nuclei counting macro

```
// Batch import files
setBatchMode(true);
run("Image␣Sequence...", "open=[/Users/james-mac/Documents/Confocal␣images
    ↪ /040918␣LC3/T2]␣use");
//run("Images to Stack");
inputdir = getDirectory("image");

// Process and count nuclei
run("Set␣Scale...", "distance=0");
run("8-bit");
run("Gaussian␣Blur...", "sigma=3␣stack");
setAutoThreshold("MaxEntropy␣dark");
//setOption("BlackBackground", false);
//run("Make Binary", "method=MaxEntropy background=Dark calculate black");
//run("Watershed");
run("Analyze␣Particles...", "size=2000-9999␣show=[Overlay␣Masks]␣include␣
    ↪ summarize␣stack");

// Export results
selectWindow("Summary␣of␣T2");
saveAs("Measurements", inputdir + "/../Summary_nuclear.csv");

setBatchMode(false);
```

A.2.4 Autophagic spot/nuclei quantification

bin/batch_confocal.R

```
library(tidyverse)
library(dunn.test)

#### 280818 Batch LC3 ####
{
  nucl <- read_csv("data/nucl.csv")
  spot <- read_csv("data/spot.csv")


  x1 = list(
    dmso = spot$Count[which(grepl("DMSO", spot$Slice))],
    ipp = spot$Count[which(grepl("^4IPP", spot$Slice))],
    rapcq = spot$Count[which(grepl("RAPCQ[^-]", spot$Slice))],
    rapcq_ipp = spot$Count[which(grepl("RAPCQ-4IPP", spot$Slice))]
  )


  x2 = list(
    dmso = nucl$Count[which(grepl("DMSO", nucl$Slice))],
    ipp = nucl$Count[which(grepl("^4IPP", nucl$Slice))],
    rapcq = nucl$Count[which(grepl("RAPCQ[^-]", nucl$Slice))],
    rapcq_ipp = nucl$Count[which(grepl("RAPCQ-4IPP", nucl$Slice))]
  )

  x3 = list(
    dmso = x1$dmso/mean(x2$dmso),
    ipp = x1$ipp/mean(x2$ipp),
    rapcq = x1$rapcq/mean(x2$rapcq),
    rapcq_ipp = x1$rapcq_ipp/mean(x2$rapcq_ipp)
  )


  boxplot(x1)
  boxplot(x3)

  dunn.test(x1)
  dunn.test(x3)
  t.test(x3$rapcq, x3$rapcq_ipp)
}


#### 040918 Batch LC3 ####
{
  nucl2 <- read_csv("data/nucl2.csv")
  spot2 <- read_csv("data/spot2.csv")

  nucl2$Slice = str_replace(nucl2$Slice, "RAPCQ4IPP", "RAPCQ-4IPP")
  spot2$Slice = str_replace(spot2$Slice, "RAPCQ4IPP", "RAPCQ-4IPP")

  x4 = list(
    dmso = spot2$Count[which(grepl("DMSO", spot2$Slice))],
    ipp = spot2$Count[which(grepl("^4IPP", spot2$Slice))],
    rapcq = spot2$Count[which(grepl("RAPCQ[^-]", spot2$Slice))],
    rapcq_ipp = spot2$Count[which(grepl("RAPCQ-4IPP", spot2$Slice))]
  );x4


  x5 = list(
    dmso = nucl2$Count[which(grepl("DMSO", nucl2$Slice))],
    ipp = nucl2$Count[which(grepl("^4IPP", nucl2$Slice))],
```

```
    rapcq = nucl2$Count[which(grepl("RAPCQ[^-]", nucl2$Slice))],
    rapcq_ipp = nucl2$Count[which(grepl("RAPCQ-4IPP", nucl2$Slice))]
  )

  x6 = list(
    dmso = x4$dmso/mean(x5$dmso),
    ipp = x4$ipp/mean(x5$ipp),
    rapcq = x4$rapcq/mean(x5$rapcq),
    rapcq_ipp = x4$rapcq_ipp/mean(x5$rapcq_ipp)
  )


  boxplot(x5)
  boxplot(x6)

  dunn.test(x5)
  dunn.test(x6)
  t.test(x6$rapcq, x6$rapcq_ipp)
}

#### Concat ####
xall_spotpercell = list(
  dmso = c(x3$dmso, x6$dmso),
  ipp = c(x3$ipp, x6$ipp),
  rapcq = c(x3$rapcq, x6$rapcq),
  rapcq_ipp = c(x3$rapcq_ipp, x6$rapcq_ipp)
)

mean(xall_spotpercell$rapcq_ipp)
sd(xall_spotpercell$rapcq_ipp)/length(xall_spotpercell$rapcq_ipp)

par(mar=c(4,5,2,2))
boxplot(xall_spotpercell, names = NA, ylab="Average␣LC3+␣spots␣/␣cell", cex.axis
    ↪ =1.5, cex.lab=2, outline = F)
mtext(c("DMSO", "4-IPP", "RAP␣+␣CQ", "RAP␣+␣CQ\n+␣4-IPP"), side = 1, line = c
    ↪ (1,1,1,2.5), at = c(1,2,3,4), cex=1.5)
stripchart(xall_spotpercell, add=T, vertical=T, pch=21, cex=1.5)
grid()
dunn.test(xall_spotpercell)
t.test(xall_spotpercell$rapcq, xall_spotpercell$rapcq_ipp)
```